Society for Theriogenology
Veterinarians Dedicated to Animal Reproduction

Clinical Theriogenology

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

ISSN: 2154-3968

Volume 8, Number 3
September 2016

Clinical Theriogenology

Clinical Theriogenology is indexed in the Global Health and CAB Abstracts databases.

Clinical Theriogenology
ISSN: 2154-3968

Annual subscriptions to *Clinical Theriogenology* are a benefit of membership for members of the Society for Theriogenology and are complimentary to libraries at colleges of veterinary medicine in the United States and Canada. Non-members may purchase subscriptions at the following rates by contacting the Society Office:

Individual subscriptions ;

Within the United States:	$75.00 USD per annum
Outside the United States:	$125.00 USD per annum

Institutional subscriptions:

Within the United States:	$225.00 USD per annum
Outside the United States:	$375.00 USD per annum

Single copies of manuscripts published in *Clinical Theriogenology* are available from the Society Office for a fee of $25.00 each payable by credit card.

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 (preferred) or on a CD compatible with Microsoft Word. The disk should be accompanied by a hard copy of the manuscript. Submit manuscripts to:

  R. S. Youngquist
  Editor, *Clinical Theriogenology*
  4435 Highway PP
  Columbia, MO 65202
  573-474-6737
  or to the following e-mail address:
  youngquistr@missouri.edu

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

The general format for scientific manuscripts is as follows:

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

  Examples:

  Journal article (single author)

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

  Journal article (more than three authors)

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

  Book (personal author)

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

  Book (edited, multi-author)

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

  Proceedings

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

- Units of measurement: Measurements should be listed in Système Internationale (SI) units with non-SI units listed in parentheses if they are needed for clarification.
- Abbreviations: Abbreviations other than standard units of measurement should not be used excessively but if necessary, the abbreviation must be defined at first use in the manuscript. Do not use abbreviations in the title or in the abstract. Abbreviations are used exclusively subsequent to being defined except at the beginning of a sentence.
- Tables: Data can be summarized in well-designed tables prepared with the table function of Microsoft Word. Each table should be on a separate page with the legend typed above the table. Tables are numbered consecutively as they appear in the text.

- Numerals: Spell out numbers one through ten and note 11 onward as numerals. Write out numbers if they appear as the first word in a sentence.
- Illustrations: Illustrations must not be embedded in the text. Figures are to be cited consecutively by number (Fig.1, Fig. 2, etc). All images and text are sent to the printer in digital format. Acceptable formats are .jpeg and .tiff. Minimum acceptable resolution is 300 pixels/inch for halftones and 1,200 pixels per inch for line art. Black and white illustrations are reproduced at no charge but the cost of including color plates will be billed to the author.
- Acknowledgements: Persons who make substantial contributions to the content of the article but who do not qualify as authors should be acknowledged. Authors must provide written permission from all persons who are acknowledged.
- Sources and manufacturers: Only generic names of drugs, chemicals, test kits, and equipment should be used in the text followed in parentheses by the tradename, supplier's name, and supplier's address (city, state [country if not in the United States]). For example: The cow was treated with 100 mcg gonadorelin hydrochloride im (Factrel™, Fort Dodge Animal Health, Ft. Dodge, IA).
- Conflict of Interest: Authors are required to disclose any conflicts of interest such as financial or personal relationships with people, organizations, or commercial interests that may influence or be perceived to influence their conclusions. Please see the conflict of interest statement of the International Committee of Medical Journal Editors (http://wwwcf.nlm.nih.gov/lstrc/lstrcform/med/interest_desc.cfm).
- Care and Use of Animals: Manuscripts reporting experimental data must include a statement that all procedures were approved by the Institutional Animal Care and Use Committee (or its equivalent in countries other than the United States). Please see the statement of human and animal rights of the International Committee of Medical Journal Editors (http://wwwcf.nlm.nih.gov/lstrc/lstrcform/med/human_desc.cfm).
- Manuscripts are initially reviewed by the editor and those that meet the requirements for publication are submitted to at least two reviewers. Authors are invited to suggest potential reviewers in their initial submission. Manuscripts are prepared for publication in the order in which they pass the peer review process

Outline for Case Reports and Case Series

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.

Society for Theriogenology
"Veterinarians dedicated to animal reproduction"
Officers and Directors 2015-2016

President
Dr. Mike Thompson
Holly Springs, MS

President-Elect
Dr. Peter Sheerin
New Freedom, PA

Vice President
Dr. Isaac Bott
Payson, UT

Immediate Past President
Dr Herris Maxwell
Auburn, AL

Secretary/Treasurer
Dr. Robyn Wilborn
Auburn University, AL

Directors
Dr. Arn Anderson
Bowie, TX

Dr Ernest Martinez
Lexington, KY

Dr. Joann Randall
Woodstock, IL

Dr. Colin Palmer
Saskatoon, SK

Dr. Jack Smith
Starkville, MS

Dr. Paul Mennick
Los Molinos, CA

Dr. Lisa Pearson
Pullman, WA

Dr. Jill Colloton
Edgar, WI

Dr. Charles Scoggin
Paris, KY

SFT Executive Office
761 Tiger Oak Drive
Pike Road, AL 36064-3063
334-395-4666

Executive Director
Dr. Charles Franz
Charles@franzmgt.com

Director of PR/Communications
Mrs. Linda Tynan
LTynan@franzmgt.com

Director of Meetings/Events
Mrs. Tara Beasley
Tara@franzmgt.com

Director of Accounting and Membership Management
Mrs. Linda Cargile
Linda@franzmgt.com

American College of Theriogenologists
Officers and Directors 2015-2016

President
Dr. Sara Lyle
Raleigh, NC

President-Elect
Dr. Ram Kasimanickam
Pullman, WA

Vice President
Dr. John Kastelic
Calgary, AB

Secretary
Dr. Reed Holyoak
Stillwater, OK

Treasurer
Dr. John Dascanio
Harrogate, TN

Immediate Past President
Dr. Barry Ball
Lexington, KY

Directors
Dr. Juan Samper
Langley, BC

Dr. Karen Wolfsdorf
Lexington, KY

Dr. Sherrie Clark
Blacksburg, VA

ACT Executive Office
761 Tiger Oak Drive
Pike Road, AL 36064-3063
334-395-4666

Executive Director
Dr. Charles Franz
Charles@franzmgt.com

Director of PR/Communications
Mrs. Linda Tynan
LTynan@franzmgt.com

Director of Meetings/Events
Mrs. Tara Beasley
Tara@franzmgt.com

Director of Accounting and Membership Management
Mrs. Linda Cargile
Linda@franzmgt.com

Future Theriogenology Conference Dates

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

The Conference Planning Committee is developing outstanding programs!

MAKE PLANS NOW TO JOIN US AT
THE FOLLOWING LOCATIONS:

2017
August 2-August 5
Fort Collins, Colorado

2018
August 1-August 4
Milwaukee, Wisconsin

Clinical Theriogenology
Contents
September 2016, Volume 8, Number 3

Species Abstracts

Small Animals

Food and Fiber Animals

Case Reports

Student Case Reports

The circuitous path of an academic theriogenologist
2016 Bartlett Address

Dickson D. Varner
Department of Large Animal Clinical Sciences; College of Veterinary Medicine and Biomedical Sciences; Texas A&M University, College Station, TX

How in the world did I get from there to here?

Folks, I wonder nearly every day how I reached this lot in life . . . that of an academic theriogenologist. Certainly the path to my current position was unpredicted by me and nothing short of incomprehensible to the loved ones that that offered guidance during my formative years as an impressionable youngster. I was born the son of a bonifide cowboy and cowgirl.

My father, Victor "Tex" Varner

My mother, Hope Carol Varner

My parents were rodeo producers that settled in the Ozarks of Missouri near the time of my birth to start up a Wild West show with a variety of other offerings. I reckon it was this very upbringing that inspired my fascination for animals, for I was exposed on a daily basis to an assortment of animals that most youth could only read about in books or visit at the zoo.

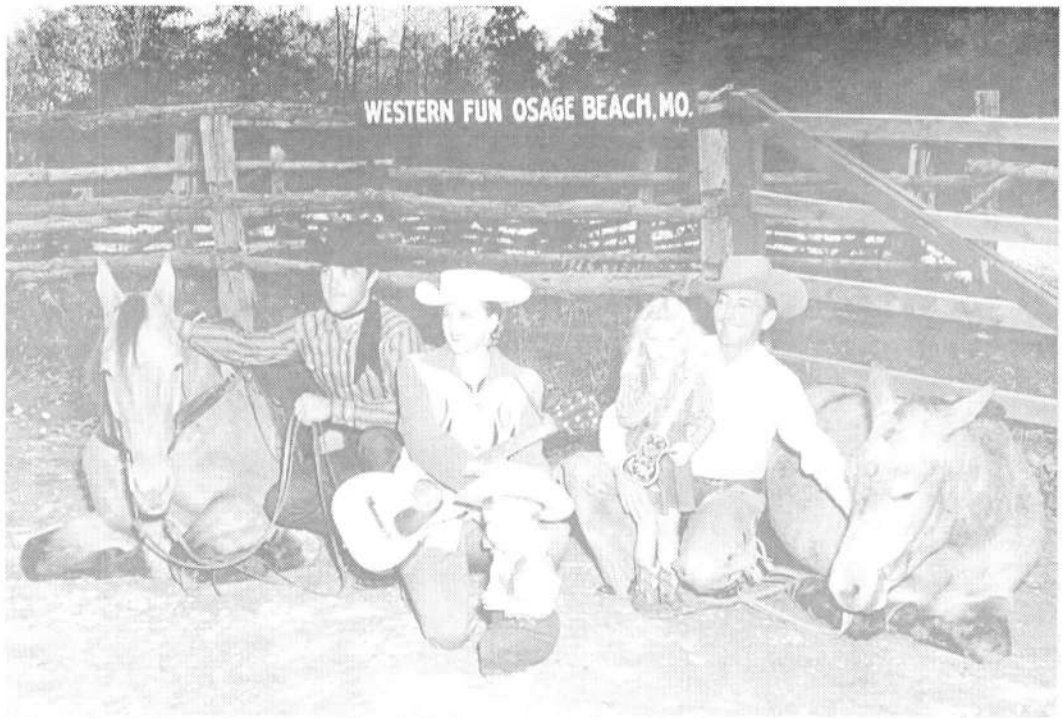
From the time I was an infant, my parents immersed me, and my two sisters, in animal related activities. As an infant, I spent much time in an Indian cradle board that was hung in a tree over the watering tank where the horses would water off after the trail rides.

Hope and Tex Varner with Dickson in Indian cradle board

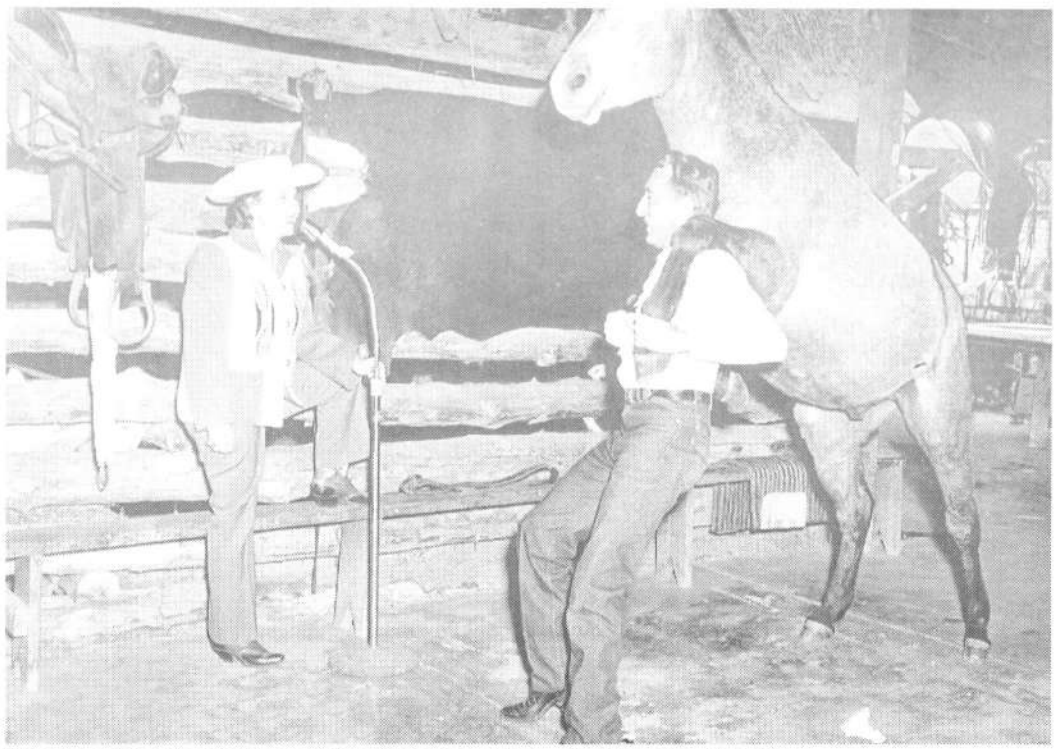
My first steed was a mule, named Harry the Educated Mule, after the President at the time, Harry S Truman.

Dickson on his first steed, Harry the Educated Mule, in the Missouri Ozarks.

My dad was an extraordinary horse trainer and he trained this mule to do tricks and incorporated the mule in the programs at various events.

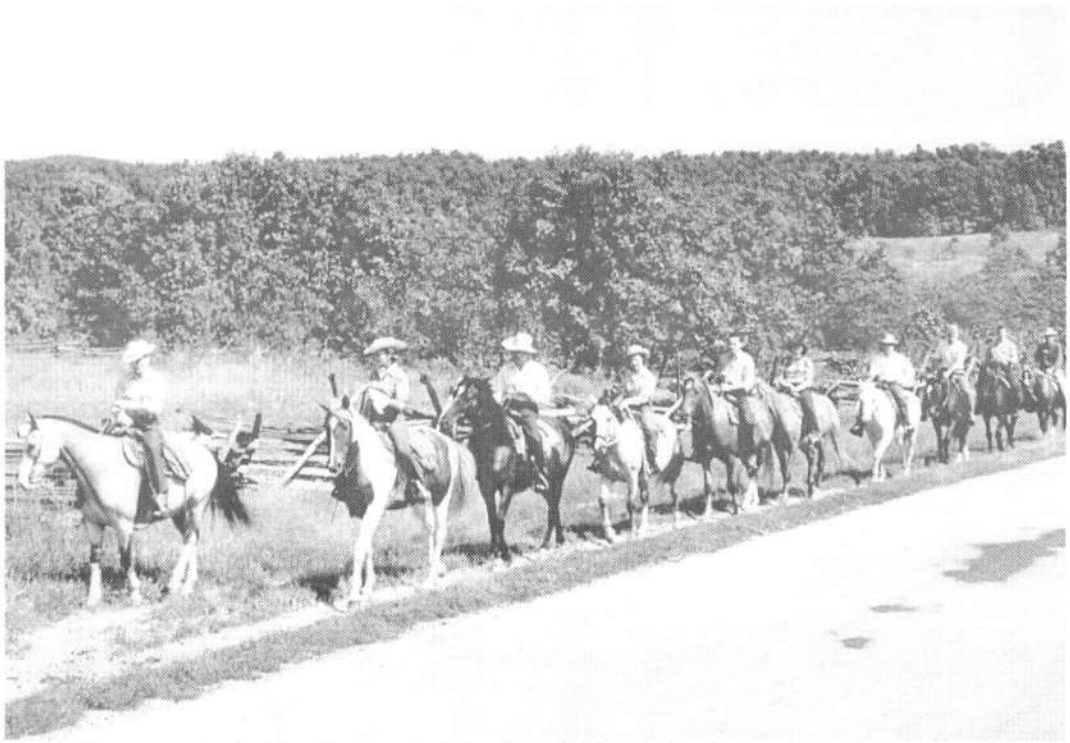


Mom, Dad, Gay, Dick, and Chuck Grimes at ranch party, Osage Beach, Missouri



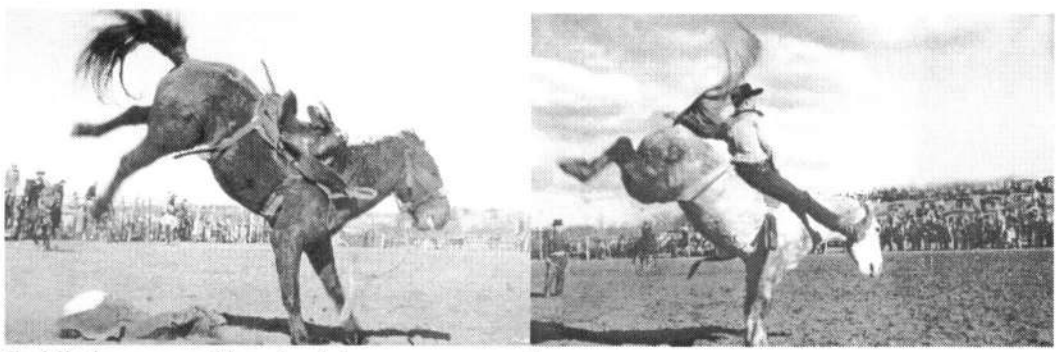
Mom and Dad with Harry The Educated Mule at a hayride function

When I became old enough to ride on my own, I was mounted on Harry the Educated Mule, riding with a bareback rigging while assisting with trail rides that were offered daily. We had up to 30 riders per trail ride and offered these trail rides hourly eight times daily.

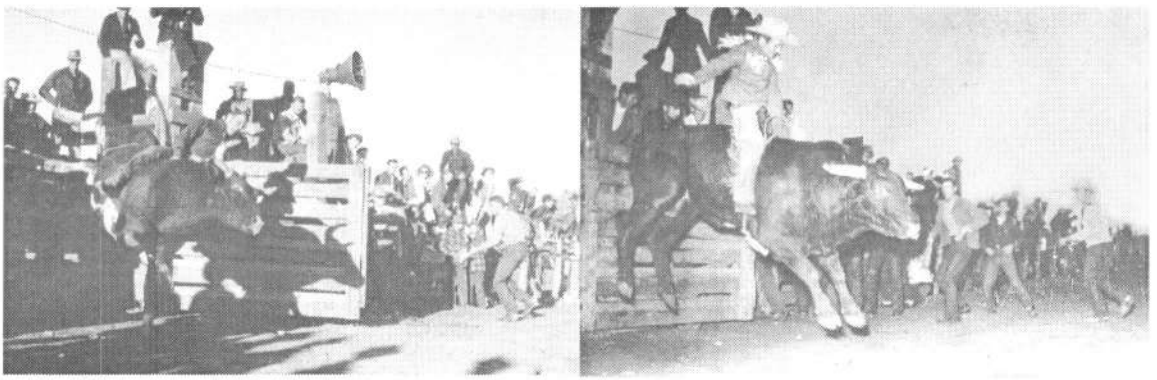


Trail riders coming in for dismount at the Ozark Homestead

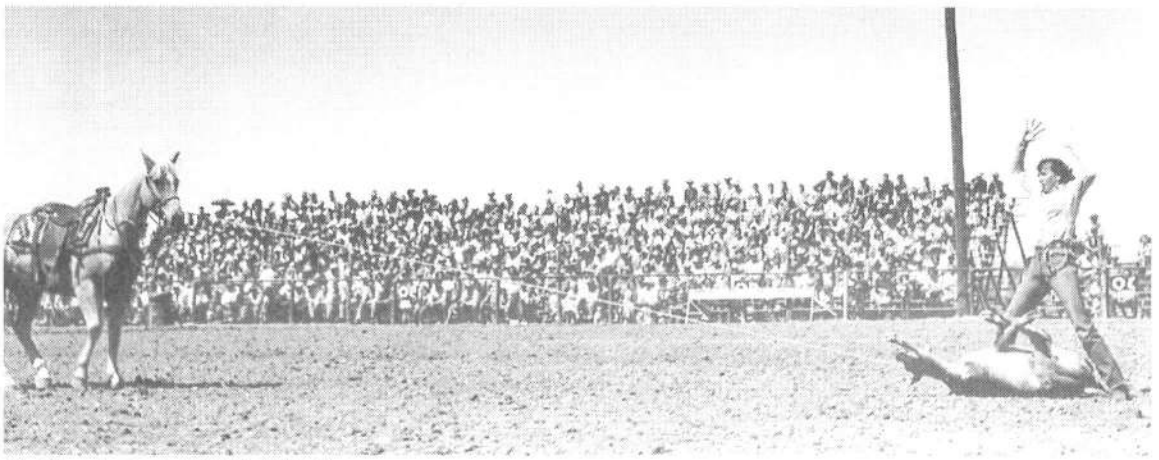
Our Wild West show, called the Ozark Stampede, was sight to behold! We had the usual rodeo events, with a focus on rough stock, because calf roping, team roping, and bull dogging (steer wrestling) were not as popular for the general public back in those those days. We even had a special national All-Girl Rodeo on one occasion.



Saddle broncs and bareback broncs were rank

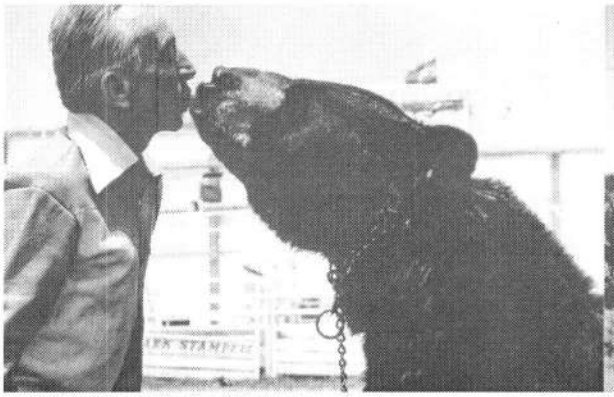


Bull riding was excitin'



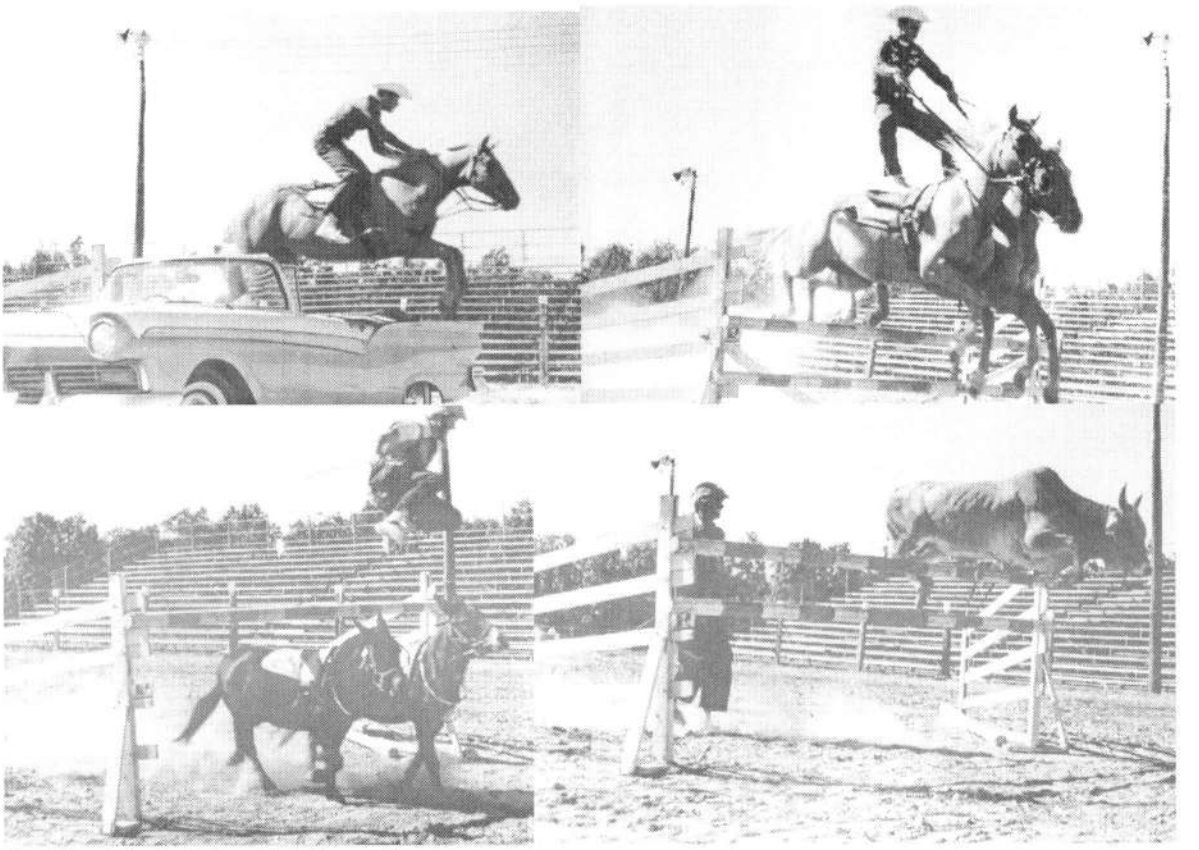
Calf roping at All-Girl Rodeo

What made the show so spectacular though, was the assortment of specialty acts. My parents partnered with a number of individuals to bring in acts ranging from wrestling bears, to high-wire acts, to jumping horses and mules, to trick horses, to trick roping, to trick riding, to chariot races, to quick draw artists, to whip acts.

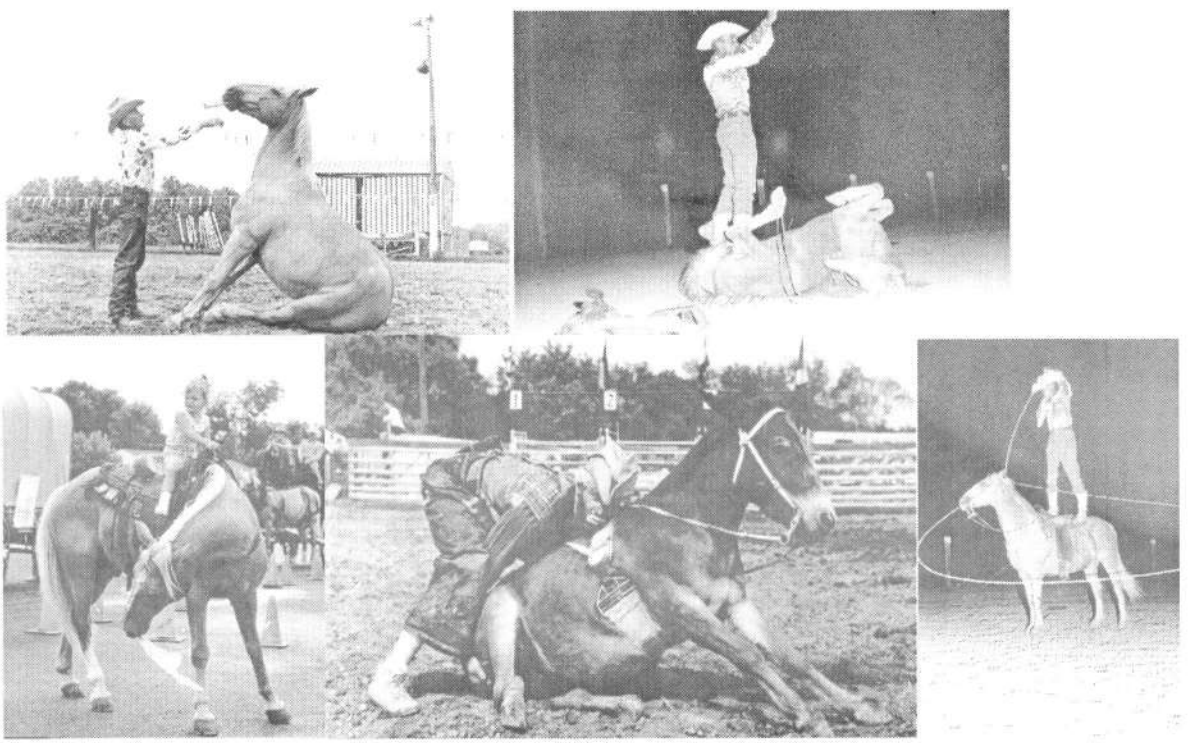


Dad with Vic The Bruiser

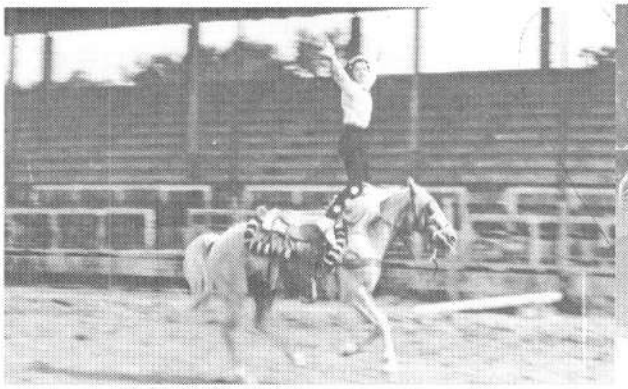
Triska High Wire Act



Jumping stock rehearsing for rodeo



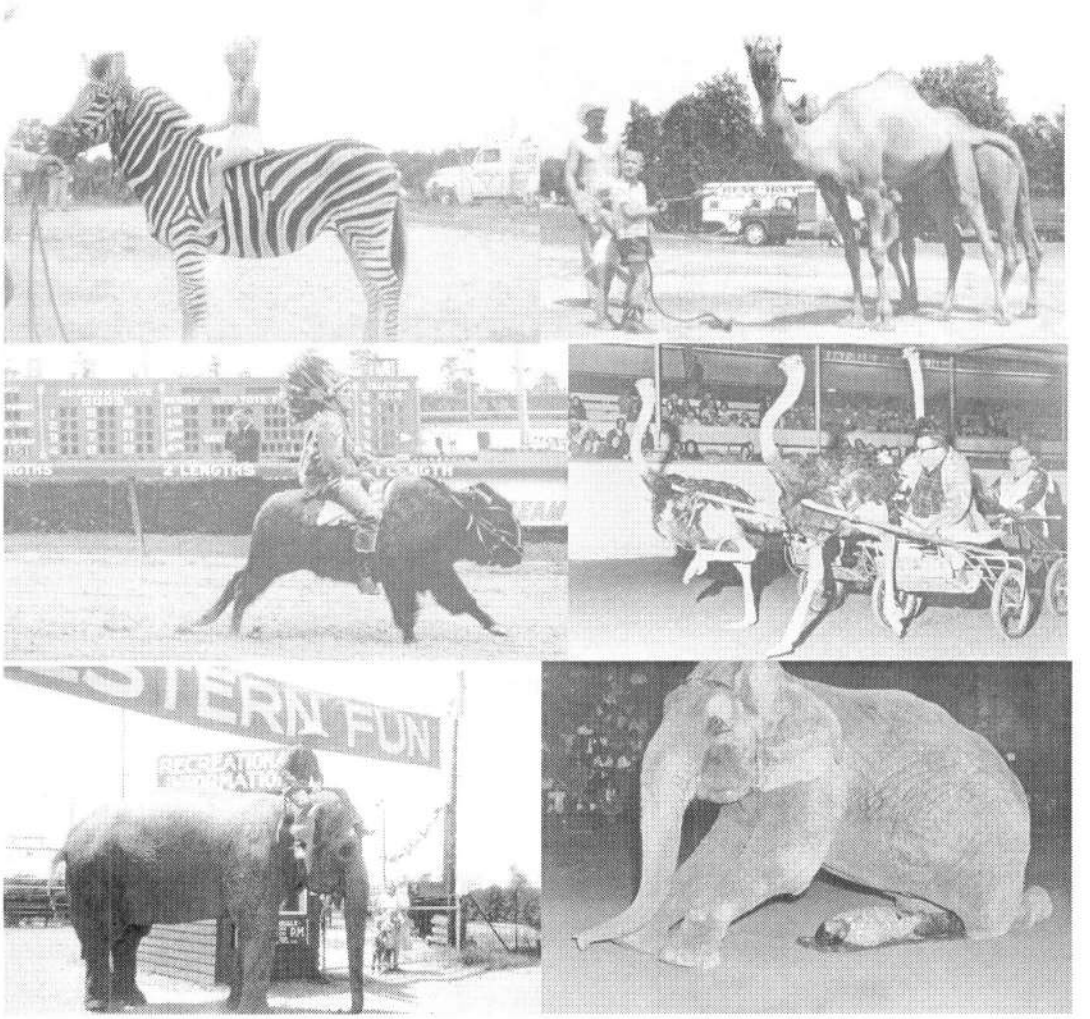
Trick horses and mules



Trick riding

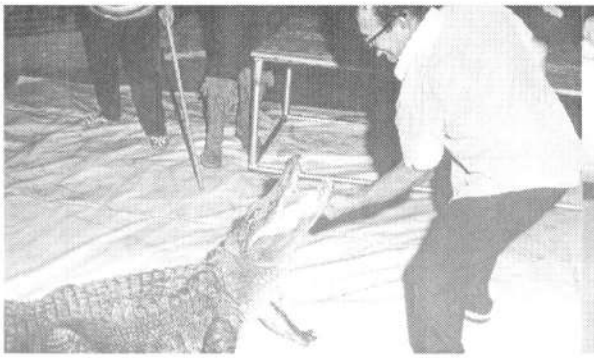
Trick roping

Zebras, elephants, camels, ostriches, llamas, buffalo, and chimpanzees also made their way into the performances.

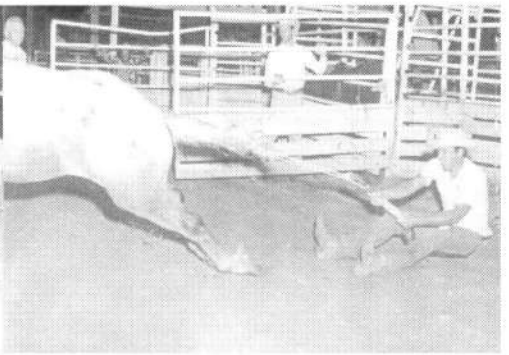


My sisters I even performed as the "Ropin' Rodeo Rascals." We had stage coach holdups, dog acts, wrestling alligators, wild animal races, and even "scoop shovel" races for the bucking horses that decided to quit bucking for one reason or another. Eventually, these horses made their way into the dude

string for trail rides, and might wind up carrying an 80-year-old grandmother or a 4-year-old child on a trail ride through the Ozarks.

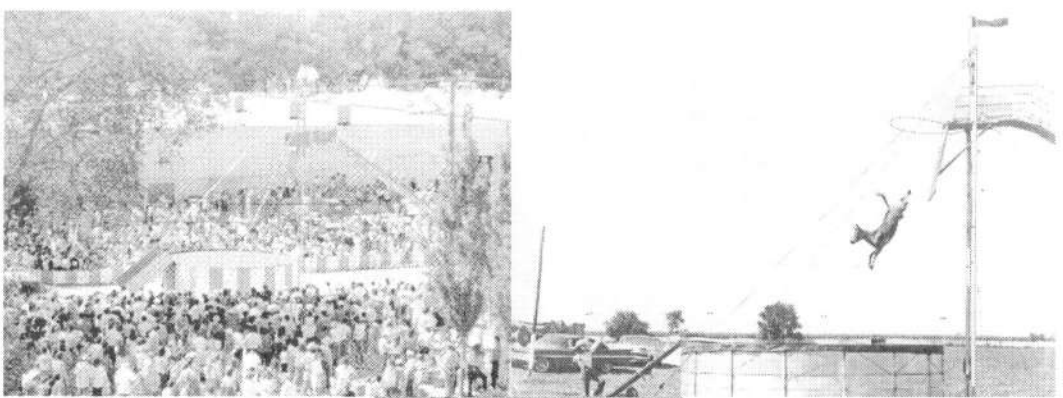


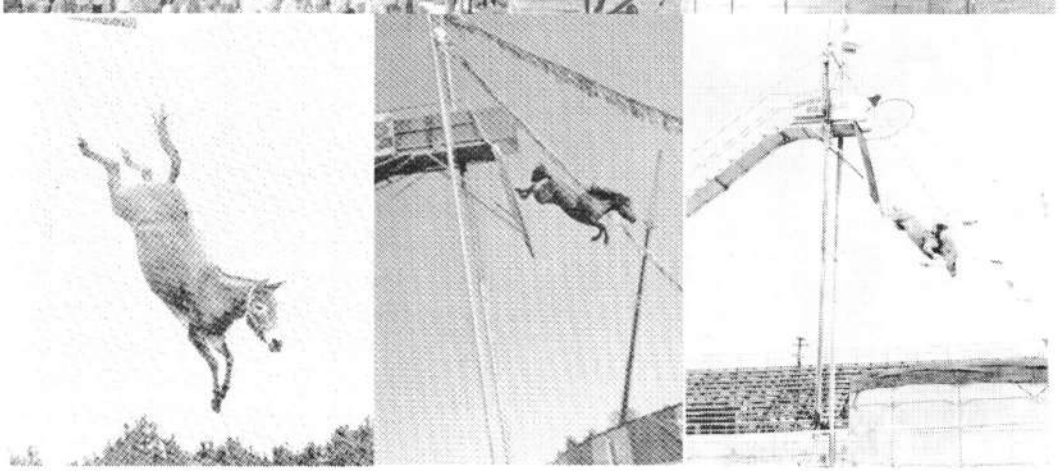
Wrestling alligator



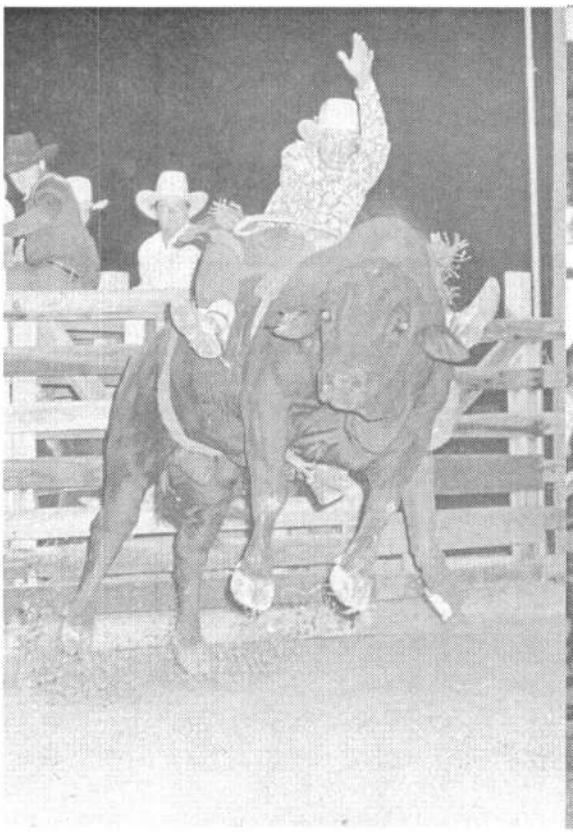
Practicing for scoop shovel race

Probably the most popular act was Jonny River's High Diving Mules. These critters would dive off a platform high in the air into a tank of water about 8 feet deep. We even had a monkey that would ride a diving pony!

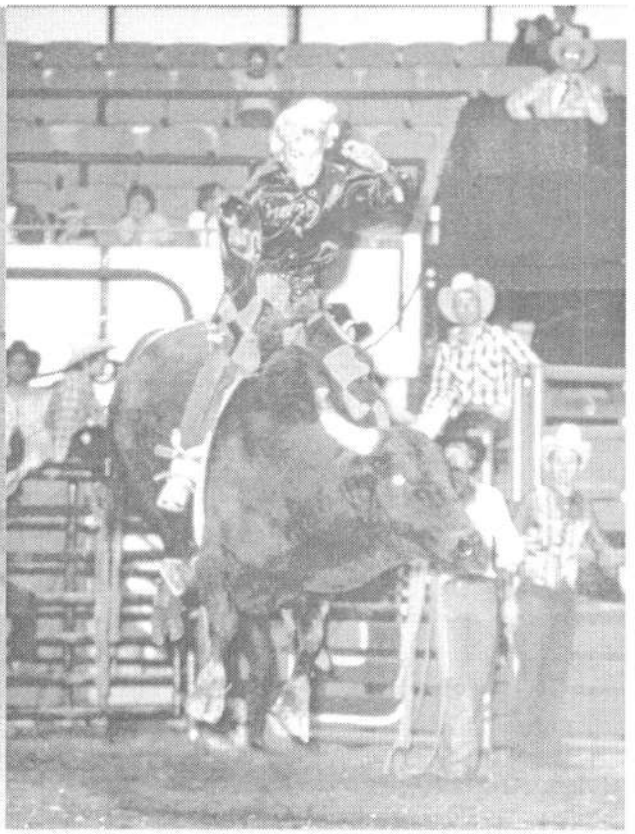




As I got older (about 10 years of age), my dad insisted that I start riding bulls. I told him that I wanted to rope calves, but he shook a rosined bull rope with a bell attached and said "this is the only rope you can make a living with." I made it through the first few bulls that he loaded in the chutes, and I eventually became hooked on this event. I rode these critters until the weekend before I entered veterinary medical school.



Ridin' "088" in Kansas



Ridin' "Honker" in Madison Square Garden

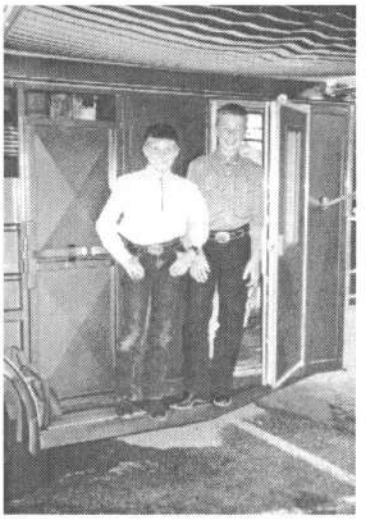
Looking back, I think my youth was quite incredible even though I didn't fully appreciate it until I became older and could reflect back on my youth. I am so thankful to my parents for providing me with such a unique and adrenaline-charged childhood.

Why I wound up in veterinary school, I can't rightly recollect. My dad did most of his own "veterinary work" - deworming horses with tobacco, suturing lacerations, and castrating horses. He even had a stomach tube in the barn that he passed into the stomach for administration of mineral oil and castor oil to the occasional horse that showed signs of colic. I remember traveling with my dad to the veterinary college at the University of Missouri when his favorite trick horse, Nugget, had a foul smelling nasal discharge. It was there that I met Dr. Joe McGinity for the first time. Being the showman he was, my dad could not pass up the opportunity to perform some tricks with Nugget, so he had the horse sit down and drink Coke out of a bottle for some of the faculty and students. It was a real hit, and my dad stayed in fairly close contact with Dr. McGinity.

As a third-year undergraduate student at the University of Missouri, I remember passing through the Student Union and spotting an informational booth regarding veterinary medicine. Well, my heart was set on just making the national finals of the Rodeo Cowboy Association in those days, but somehow I picked up an application form that was due the following Monday. I completed most of the form, but had to write a composition on why I wanted to be a veterinarian. Well, I had a rodeo in western Kansas that weekend, so I asked my sweet momma to help me with the letter. When I returned from the rodeo that Sunday, I read over the letter and it sounded so good that I don't think I changed a thing! In fact, Mom even included several informational brochures on the Ozark Stampede in the application packet. When it came time for my interview with the admissions committee, they pulled out the brochures and only asked me about the high-diving mules, ostrich races, wrestling bears and such! I reckon I owe my acceptance into veterinary school solely to my adoring mother!

Now realize that I had not even worked for a veterinarian prior to veterinary school. As such, I entered the professional curriculum with little idea of what to expect. Nonetheless, I enjoyed all facets of this educational experience. I was especially intrigued with the clinical years, particularly so with my rotations through theriogenology with Drs. Robert Youngquist, Ronnie Elmore, and C.J. "Bush" Bierschwal. It was this experience that prompted me to focus on animal reproduction following graduation. It was also at this time that met my eventual bride, Tricia Anne Wilcox ("Annie"). We married during her senior year of veterinary school and she has stuck by the side of this renegade for the last 38 years! Those of you that know Annie and me, also know that any of my accomplishments are due in large part due to the support and guidance of my beautiful bride! During this time we have begotten two fine young men, Victor and Zack, and now have four grandchildren.


Trish during courting years


Victor and Zack in 4-H

Following graduation, I hit the ground running as an intern at Castleton Farms, Lexington, Kentucky, where Dr. H. Steve Conboy was responsible for molding me into a worthwhile veterinarian. While he had his work cut out for him, I am so pleased that he took the time and effort to give me the opportunity to develop my skills as a veterinarian on a large broodmare farm. I stayed at Castleton Farms for another two years before heading for New Bolton Center to enter a residency program in animal reproduction, primarily under the tutelage of Drs. R.M. "Bob" Kenney, John P. Hurtgen, and eventually Terry L. Blanchard. My oh my, these were such enriching years . . . not only because of the direction and support received from my mentors, but also because I was surrounded by such bright, energetic, and kindly residents like J. Stanley "Stan" Brown, Katrin Hinrichs, and Charles C. "Charley" Love and graduate students like Sue McDonnell. Indeed, I consider the time spent at the University of Pennsylvania as one of the most exciting and inspiring periods in my professional career!

After four years at the University of Pennsylvania (as a resident and then a lecturer), I accepted a position at Texas A&M University in the Department of Large Animal Medicine and Surgery. Dr. Ron Elmore was Section Chief of Theriogenology at the time and was largely responsible for my relocation to Texas to embark on a career as an academic veterinarian. I have been here for the past 30 years, so you can see that I don't move around too much. My dreams became a reality when folks like Katrin Hinrichs, Terry Blanchard, Charley Love, and Steve Brinsko also moved to Texas A&M University. We aren't just colleagues, we are family . . . and I can't envision a more perfect scenario for a professional career!

Principles for survival in an academic world

Life at an academic institution can be both exhilarating and maddening. Life sometimes seems to be brimming with exasperating moments, regardless of one's vocation, but there is something about the

incessant demands for productivity in an academic setting that can wreak havoc with one's basic quest for both peace and fulfillment. So how does one deal with this conundrum in an effort to shift the equation in favor of exhilaration? After 30 years in a tenure-track position within a clinical department at a tier-one research institution, I think that I finally know the answer – surround yourself with brilliant minds that thoroughly enjoy the discipline, e.g., theriogenology; and build a family-like relationship. Enjoy each other's company at work and engage in extracurricular activities together on a regular basis. Over the course of your career, you will likely spend as many, or more, waking moments with your colleagues as with your immediate family, so do all possible to bring enrichment to these relationships. The end result – you will cherish each other, work as a team to accomplish your goals, and, importantly, you will enjoy coming to work each and every day!

Longevity in an academic veterinarian can be quite fleeting. Many of the obstacles that must be overcome in this "publish or perish" environment have taken their toll on many a fledgling academician. So what, in my view, are the critical tools for making a career in academic veterinary medicine? Well, below, I share my **ABCs of Guiding Principles for Survival in an Academic World**

Seek **A**ccountability

First and foremost, one should seek accountability for one's actions, and as an academic leader, to do the same for those under your care and guidance. The saying holds true that no matter how much you love your university, the University does not love you. While this concept sounds both glaring and unemotional, one must realize that the University must also be held accountable for its actions to those providing state resources or private donations for University functions. Bottom line: Git 'er dun!

Boost confidence

Most of us attended veterinary school to become practitioners. We had little exposure to critical thinking from a scientific perspective, and scientific writing, experimental methods, and p-values were not part of the curriculum. As such, many of us learned this through "on-the-job" training. Today is another era in academic veterinary medicine. Technologic advances are continuingly unfolding and expectations from administrators are unrelenting (see above). As such, we owe it to those that come in behind us to boost their confidence through group meetings to discuss experimental ideas and procedures, review written proposals and manuscripts prior to submission, and recommend further coursework that could strengthen their academic fiber.

Connect with others.

Generally, no one goes their way alone in academia. Collaborations are the key to success. As Will Rogers once pointed out, "No one is as ignorant as an educated man you talk to him about something that he ain't educated in." Well, that pretty well sums it up for an academician. We are encouraged to demonstrate more depth than breadth of focus in an effort to advance or professional discipline. We are now in the "-omics" era – genomics, toxicogenomics, proteomics, metabolomics, etc. We in the clinical departments oftentimes do not have formal education in areas such as these, so it is important for us to team up with basic scientists as collaborators, at our institution and at other instiitutions, to develop strategies for pursuing academic excellence.

Dream with the intention of making it a reality.

Dreams can become reality but this requires steadfast determination and regular meetings with those that share in those dreams. Remember the old adage, success is more dependent on perspiration than inspiration. This applies to any type of livelihood, and is critical to growth in a university setting.

Create an **E**dge over the competition.

While we like to think of other academic units as those containing colleagues rather than competitors, most of us in academia are probably cursed with a Type A personalities (i.e., ambitious, competitive, outgoing, and occasionally aggressive). As such, we want to be the best that we can be, and to be recognized by others for our ingenuity and productivity. In my view, the best way to create an edge in a discipline is to surround yourself with likeminded individuals that are willing to work diligently together to accomplish lofty goals.

Find your sweet spot and **F**ocus.

Remember, depth of research is more important than breadth of research in the eyes of an academic administrator. To that end an academic veterinarian should pick research and clinical areas that are particularly titillating, and focus scientific efforts to build upon the current knowledge base. I consider it very important for academic clinicians to continually incorporate their research findings in the clinical setting and to expose veterinary students to this strategy. It helps them to become more critical thinkers.

Identify areas of **G**rowth potential.

Clinical research is that which is directly applicable to the clinics and to the animal industry being served. To that end, stay abreast of the visions and projections within the animal industry that you ultimately serve, and immerse yourself within this industry. Become an active member of breed and industry organizations. In this way, you can structure your clinical and research programs such that they have the highest impact on areas of growth within the industry.

Harness and empower those with which you work.

The bottom line – encourage each and every one of your team members to be the best they can be. We all work best when we are given the role of leading an effort, as opposed to simply being directed by others to do a job. Empower everyone around you to be in charge of something. All forty mules in a forty-mule train are equally important in delivering the load.

Show **I**nitiative.

"Actions speak louder than words". Demonstrate by doing and others will follow. Bring excitement to the table. Positive outcomes rely not only on ability, but also on readiness to accomplish the task at hand.

Don't be **J**udgemental.

Each of us is built differently. We have different approaches to accomplishing a goal. We each have different ways of interacting with others. We have different ways of reacting to life's challenges. Indeed, we are all unique. Relish in the fact that we are all different, and remain cognizant that we are all a part of mankind and have a fundamental urge to accomplish good deeds.

Know the strengths and weaknesses of your people.

How often do we encounter those with no faults? Each of us has definable strengths and visible weaknesses. Each of us has unique capabilities and limitations that are sometimes not recognized by us, but are easily detectable by others. Work as a team to maximize the strengths of each and every person, and build a team with varied strengths, as opposed to one that is replete in a singular strength.

Realize that **L**eadership is a trust, not a right.

If placed in a position as a leader, lead by example, show compassion for all, and have the best interest of your employees first in mind at all turns. When one becomes dictatorial and leads from behind, accomplishments become few and far between, and chaos will evolve.

Make a positive difference to society and maximize those contributions.

We have accepted a position in academia to advance the care of animals and to be penetratingly involved with the betterment of society. Start your day by asking yourself, "How can I make this world a better place." That mindset will take you far toward maximizing your individual contributions to society.

Nothing can be achieved alone.

In nearly any facet of life, no one goes their way alone. For progress in academia, collaboration with others is particularly important. Engage with others, contemplate and massage common interests and make plans together to go forward as a team. Capitalize on diverse strengths.

Don't miss **O**pportunities (timing is everything).

Advancements are due in large part to captured opportunities. Understand the current needs of the animal industry in which you are engaged. Keep your eyes and ears open for new methodologies and research findings. Examine how advancements in the basic sciences may have application to the clinical arena. Get to know new personnel at your institution, and investigate ways in which they may provide valuable input to your area of interest.

Prioritize correctly.

As we all know, prioritization is a big key to success. What I will point out here is that family and outside interests should not be considered low on the priority list. Escape from work is important to revitalize the mind and invigorate passion in the workplace. Regularly enjoy the things in life that bring you relaxation. Engage in organized sports, go fishin', embark on camping trips, enjoy the solitude of biking or running outdoors, ride a horse (or mule), raise some cattle, play a musical instrument, or watch new movies . . . just do something that you enjoy immensely on a regular basis.

Avoid **Q**uarrelsome situations, but encourage debates.

Disagreements are a simple part of life, both at home and in the workplace. Disagreements can be healthy as they bring more intensive study to effect resolution. Don't shy from away from differences in thought. Meet to discuss divisive issues and focus on ideas and productive debates rather than personality traits. Put simply, work together. Speak up, but respect your colleagues. Resolve conflicts in a rational way.

Be more **R**elational than positional.

The phrase, a chain is no stronger than its weakest link, fits well in the academic community. Healthy relationships are vital to the strength of a group or organization. Institutions are replete with hierarchical structure and, in my mind, this can be problematic if not handled correctly. Leaders should focus on being more relational than positional – building upon the strengths of personnel and engaging all employees in a positive and supportive manner. A positional stance on matters can be disparaging to those with which you work and this can lead to ambivalence or even apathy. The result is highly destructive to both efficiency and output.

Step forward to meet challenges.

It may be important to back down from a fight, because the outcome is generally negative for all involved; however, never back away from a challenge. Challenges bring opportunities, and opportunities can bring growth and success to a program.

Encourage **T**eamwork.

I have addressed teamwork many times above, but must include it one more time. It is a term that deserves to "stand alone." We cannot singularly make progress to the same extent as that which involves interaction with colleagues. Multiple minds are always far brighter than any single mind. Embrace this concept and relish in the fruits that can be derived from its application to life.

Create **U**nification of thought.

A team approach is critical to innovations and progress, but the team-approach concepts requires regularly scheduled meetings of the minds to establish unification of thought before implementing strategies. Where possible, meet as a group prior to examining a clinical patient or executing a research project so that all have had an opportunity to inject their thoughts regarding approach to a case or experimental design.

Match **V**ision with **V**alues.

The term, vision, seems to be the buzz word for administrators nowadays. Vision is important for execution of missions and paths of focus, but vision must be paired with shared values to be effective. Dream dreams and envision the path to success, but also delve into the mechanisms by which to accomplish your goals. All decision making regarding implementation of strategies should be based with core values foremost in mind.

Walk the halls slowly.

How often do we pass someone in the hallways or on the sidewalks that is traveling fast with their head down when we pass by them? We live in a fast-paced society and, in academia, time management is a key component of efficiency and success. Nonetheless, we need to "stop and smell the roses" more often than not, to engage in pleasantries and conversations with those that we rarely communicate, to meet someone new each and every day. Life is fleeting. Take a deep breath, enjoy the companionship of others . . . maybe we can slow life down just a little bit.

Make sure that ideas pass the **X**-Ray test before implementation.

I incorporate this thought particularly as it relates to research endeavors. Each of us has thoughts regarding new research projects. We may have come up with an idea after reading a manuscript, or maybe something popped up out of a dream that we had the previous night. Whatever the origin of a new research thought, it is important to familiarize oneself with the related literature and meet in a group setting to deliberate the strengths and weakness of a project, understand the intended impact of the study, and massage experimental methods. The best way to begin an experiment is not in the laboratory, but in a closed room with a marker board to hash out all the details associated with the experiment. Don't begin the project until you are satisfied with the approach.

Visualize how **Y**ou will be defined at the end of your professional career.

All of us in academia want to be remembered for our accomplishments. Accomplishments can take various forms . . . number of publications, quality of publications, impact on trainees . . . the list goes on and on. Early in your career, select that which is most important to you and stay the course throughout your professional life. Remember, we are generally remembered for what we have done for others; not what we have done for ourselves.

Get in a **Z**one and be **Z**estful. Become feverish about that which you engage.

I had to end with a double-Z! To be productive, we can't be all things to all people. We have selected a profession in academia that provides us with the ability to specialize. So specialize in a discipline that brings you utter excitement (i.e., get in a zone). Become involved in an a specific area that makes you think "Wow!" every morning that you head to work (be zestful). Excitement begets contentment!

Conclusions

So there you have it – my background and bits of philosophy, all in one bundle. However, I don't know that I quality for discharging pearls of wisdom. Dr. Thomas Armstrong, author of "The 12 Stages of Life," proposes that it is not until one reaches 80+ years of age that he/she represents a source of wisdom! Therefore consider the above to simply be mentorship-related thoughts. I reckon my position all along was to simply play a minor role as part of a much greater whole . . . that of veterinary medicine, veterinary medical education, theriogenology, and most importantly, humanity.

I thank the Society of Theriogenology immensely for bestowing upon me such an admired and respected award. My most enduring friendships emanate from my interactions within the Society of Theriogenology and the American College of Theriogenologists. While I have many outside interests and affections, the discipline of theriogenology is my professional lifeblood, and the pulse of the Society and College beats within me each and every day!

Genetics, genomics and fertility

Joseph Dalton,[a] Dale Moore,[b] Thomas E. Spencer,[c] Peter J. Hansen,[d] John B. Cole,[e] Holly Neibergs[f]
[a]Department of Animal and Veterinary Science, University of Idaho, Caldwell, ID; [b]Department of Veterinary Clinical Sciences, Washington State University, Pullman, WA; [c]Division of Animal Sciences, University of Missouri, Columbia, MO; [d]Department of Animal Sciences, University of Florida, Gainesville, FL; [e]USDA-ARS, Animal Genomics and Improvement Laboratory, Beltsville, MD; [f]Department of Animal Sciences, Washington State University, Pullman, WA

Introduction

Early studies investigating the genetic material of life began with Gregor Mendel, who published his research results in 1866. Through the use of peas in crossbreeding experiments, Mendel reported the inheritance of characteristics or traits occurred via units (which would later be described as genes; Mendel, 1866).

In 1944, scientists at the Rockefeller Institute in New York (Avery, MacLeod, and MacCarty, 1944) reported that the genetic material was "DNA" or deoxyribonucleic acid. Watson and Crick, in 1953, described the structure of DNA as a double helix. Twenty-two years later, in 1975, Sanger and Coulson developed a method of DNA sequencing, that is, determining the sequence of the building blocks of DNA, referred to as nucleotides. A nucleotide consists of three components: a sugar molecule (deoxyribose in DNA), a phosphate group and a nitrogen-containing base. The nitrogenous bases include cytosine (C), guanine (G), adenine (A), and thymine (T). The double helix, similar to a spiral staircase, allows DNA the ability to store and transmit information. The bases link across the two strands of the double helix: cytosine (C) pairs with guanine (G), and adenine (A) pairs with thymine (T).

The genetic code describes the relationship between the bases (A, C, G, and T) along a single strand. The alphabet (A, C, G, and T) forms a codon - a group of three letters - that code for specific amino acids, e.g., "AGC" is the codon for the amino acid serine. A gene is a sequence of nucleotides along a DNA strand that spells out codons (in three-letter sequences) that direct the sequence of amino acids necessary to form a protein. Therefore, each gene combines the four bases in various orders to spell out three-letter codons that specify which amino acid is needed at every step in making a protein which determines the form and function of the organism.

Genome, genetics, genomics, and snps

What is the difference between genome, genetics, and genomics? Genome refers to the haploid genetic material of an organism. Genetics is focused on heredity and variation of organisms. In simpler terms, genetics focuses on the characteristics or traits that are passed from one generation to another. However, genomics is more focused on the molecular aspects of genetics: DNA sequencing, genetic mapping, and analysis of the complete genome of an organism, including organizing the results in databases.

When gametes are produced (sperm in the male and oocytes in the female) each gamete may not carry the exact same DNA sequence, i.e., a polymorphism (poly=many, morph=form) may occur which involves one of two or more variants of a particular DNA sequence. The most common polymorphism involves variation at a single nucleotide, or base pair. This variation is called a single nucleotide polymorphism, or SNP (pronounced "snip"), and may serve as a marker for a variety of genes. Scientists are currently studying how SNPs in the genome are associated with disease, production traits, and fertility in livestock.

The big picture: genomics, animals, and humans

The National Center for Biotechnology Information, National Library of Medicine contains an up-to-date list of genome information (www.ncbi.nlm.nih.gov/genome). The list is exhaustive, and includes the cat (*Felis catus*), dog (*Canis lupus familiaris*), horse (*Equus caballus*), and cattle (*Bos taurus and Bos indicus*).

Genomic analyses have been used to discover new information pertaining directly to the animal and (or) in relation to similar diseases in humans. For example, the domestic dog exhibits great diversity in body size. Sutter et al. (2007), following a genome-wide association analysis (GWAA), reported the discovery of a single IGF1 (insulin-like growth factor-1) SNP common to all small breeds, but nearly absent from giant breeds, thereby providing evidence the same causal sequence variant is a major contributor to body size. Further research provides evidence that approximately 50% of the variance in body size of dog breeds can be explained by seven markers, including GHR (growth hormone receptor), IGF1, and IGF1R (insulin-like growth factor-1 receptor) (Rimbault et al. 2013).

A GWAA also revealed a gene mutation (superoxide dismutase-1 or SOD1) in canine degenerative myelopathy that resembles amyotrophic lateral sclerosis in humans (Awano et al., 2009), while the gene ADAMTS20 has been identified as a risk variant for cleft lip and palate in both dogs and humans (Wolf et al., 2015). Thus, genomics can be used to identify SNPs associated with important or desirable traits in animals and humans.

Dairy cattle genomics and fertility

In 2009, after six years of research by 300 scientists from 25 countries, the genome of cattle was decoded (Elsik et al., 2009). The bovine genome has approximately 22,000 genes, of which 80% are the same as human genes.

The focus of this manuscript, and the research funded by USDA National Institute of Food and Agriculture (2013-68004-20365) discussed herein, is dairy cattle genomics and fertility. As described by Spencer et al., (2014) there is sufficient genetic variability within major cattle breeds for fertility traits that are complex and polygenic (poly=many, genic=genes). Therefore, genomic selection strategies will require many different markers developed from analysis of carefully phenotyped animal populations (Spencer et al., 2014). Genomic technologies are currently available to identify genes to improve dairy cattle fertility without negatively affecting milk yield. Ultimately, the goal of research into genomics and fertility is to increase dairy cow fertility, thereby increasing the sustainability of dairy enterprises.

Ample evidence exists that fertility of lactating dairy cows has declined (Lucy, 2001; Washburn et al., 2002). Lucy (2001) reported conception rates decreased from 55% in the 1950s (Casida, 1961) to 45% for cows that received artificial insemination (AI) following spontaneous estrus by the 1990's (Dransfield et al., 1998). Presynchronization during the voluntary waiting period, coupled with improvements to Ovsynch, have resulted in conception rates of 45% following timed AI (Brusveen et al., 2008), while use of double Ovsynch has resulted in conception rates >50% in primiparous, but not multiparous cows (Souza et al., 2008; Herlihy et al., 2012). Moeller et al. (2010), however, using data from 85 herds, 231,288 cows and 649,495 matings, reported that the upper 20th percentile of dairy herds achieved first service conception rate of greater than 38% for timed AI and AI upon detected estrus, providing evidence that many herds struggle with reproduction.

As cow fertility declined in recent years, average milk yield per lactation increased (Lucy, 2001; Washburn et al, 2002). A negative genetic correlation between milk yield and fertility exists in dairy cattle (VanRaden et al., 2004, Pritchard et al., 2013). The heritability of fertility traits is low (1-10%) (Sun et al., 2010) in comparison with milk yield which is considered to be moderately heritable (20-40%) (Hayes et al., 2010, Kemper and Goddard, 2012). Veerkamp and Beerda (2007) suggest selection on milk yield without concomitant selection for fertility is a major cause of the decline in cow fertility in spite of low heritabilities for reproductive traits. Lucy (2007) argued that poor fertility of dairy cattle involves many factors including anovulation, inadequate expression of estrus, irregular estrous cycles, and pregnancy loss.

To enhance the sustainability of dairy businesses, new management tools are needed to increase fertility of dairy cattle. Genomic selection has been successfully used by AI studs to screen potential sires and significantly decrease the generation interval of sires (Sattler, 2013; Schefers and Weigel, 2012). Buoyed by the success of genomic selection on the male side, coupled with continuing fertility challenges on the female side, researchers are investigating genomics and the potential to increase the fertility of lactating dairy cattle.

Research update: loci associated with fertility in Holstein heifers and cows

The objective of the first portion of our study, as described by Moraes et al. (2015), was to identify genomic loci (particular positions or locations) associated with fertility in nulliparous Holstein heifers. Breeding and health records of Holstein heifers (n= 2,333) were analyzed from a commercial heifer raising facility in Southwestern Idaho. Of these, 1,114 heifers were classified as highly fertile (conceived on first AI service) and 209 were identified as subfertile (did not conceive until after the fourth AI service or were culled due to failure to conceive). Blood samples were obtained from the fertility-classified heifers, and DNA was extracted from 497 high fertile and 209 subfertile heifers. The DNA was genotyped with the Illumina Bovine HD Genotyping BeadChip (Neogen, Lincoln, NE). After quality control, 581,918 SNPs, 468 highly fertile, and 188 subfertile heifers remained for analysis. Subsequently a GWAA was conducted and heritability estimate determined for identified SNPs.

Moraes et al. (2015) reported the GWAA identified two quantitative trait loci (QTL) with strong association with fertility, while 72 loci were identified with a moderate association with fertility. The heritability estimate for fertility in Holstein heifers was 0.56 (Moraes et al., 2015). Keuter et al. (2016) investigated breeding and health records of Holstein heifers (n=926) from the same facility as Moraes et al. (2015). Heifers received artificial insemination (AI) at observation of estrus and were subsequently classified into two groups: highly fertile (n=497; conceived on first AI service) and subfertile (n=429; did not conceive until after the fourth AI service or were culled due to failure to conceive). DNA was extracted from blood samples and genotyped as previously described by Moraes et al. (2015). After quality control, 590,904 SNPs, 466 highly fertile and 368 subfertile heifers remained for analysis. A GWAA was conducted and identified 153 SNPs representing 147 QTLs that were moderately associated and 34 SNPs representing 26 QTLs that were strongly associated with heifer fertility. Taken together, the results of Moraes et al. (2015) and Keuter et al. (2016) provide evidence that selection for fertility in dairy heifers is feasible and has the potential to improve fertility as the trait is moderately heritable and QTLs with large effects have been identified.

In a companion investigation, with the objective to identify genomic loci associated with fertility in primiparous Holstein lactating cows, samples have been collected for analysis in late 2016. Briefly, records will be used to classify primiparous Holstein lactating cows as highly fertile, subfertile, or infertile. Blood samples from highly fertile (n=500; pregnant as a result of first AI), subfertile (n=500; pregnant after fourth AI), and infertile (n=500; not pregnant after six or seven AI attempts and removed from herd) classified primiparous Holstein cows have been collected and samples from 2,000 unclassified primiparous Holstein cows will be collected for a validation study. Genomic DNA will be isolated and stored for genetic analyses. The cows used for fertility classification must have a normal reproductive tract, uncomplicated pregnancy, and no records of diseases (mastitis, retained placenta, metritis or uterine infection, milk fever, displaced abomasum, clinical lameness) preceding or after AI. Separate research, by another group of collaborating scientists, investigating genomics, animal health, and reproduction is also currently underway (Santos et al., 2015).

Research update: SNPs in genes associated with daughter pregnancy rate

Daughter pregnancy rate (DPR) involves use of days open (DO), which is computed from breeding dates for current cows and from calving interval for historical cows, which are subsequently transformed into a pregnancy rate. Bulls generally range between +3.0 and -3.0 in DPR. An increase of 1% in DPR corresponds to a decrease of 4 DO (VanRaden et al., 2004); therefore, daughters of the highest and lowest DPR sires differ by 24 DO per lactation. As DPR is correlated to fertility traits such as days to first service, conception rate, and pregnancy rate, dairy producers can expect daughters of higher DPR bulls to have improved fertility across management systems.

The heritability of DPR has been estimated at 4% (VanRaden et al., 2004); therefore, genetic selection for fertility has been hampered. Cochran et al. (2013) argued that identification of SNPs for specific genes involved in reproduction might improve reliability of genomic estimates for a low-heritability trait such as DPR. Briefly, semen from over 500 Holstein bulls of high (≥ 1.7) or low (≤ -2)

DPR was genotyped for 434 candidate SNPs. (The candidate approach focuses on specified genes of interest and phenotypes). As stated by Cochran et al. (2013), the goal of the investigation was to identify SNPs in candidate genes affecting reproduction, to ultimately explain genetic variation in DPR. An additional goal included the evaluation of SNPs for their relationship to other traits, such as milk, fat, and protein yield.

Cochran et al. (2013) reported a total of 40 SNPs associated with DPR. Additionally, 22 SNPs were associated with heifer conception rate, 33 with cow conception rate, 36 with productive life, 34 with net merit, 23 with milk yield, 19 with fat yield, 13 with fat percent, 19 with protein yield, 22 with protein percent, and 13 with somatic cell score (Cochran et al., 2013). Perhaps most exciting were the results that there were 29 SNPs associated with DPR that were not negatively associated with production traits.

Nevertheless, genetic markers in one study are often not predictive in other studies (Siontis et al., 2010; Ioannidis et al., 2011). Consequently, Ortega et al. (2016), in a follow-up investigation to Cochran et al. (2013), stated the objective was to evaluate SNPs in candidate genes previously associated with genetic merit for female fertility in Holstein bulls (Cochran et al. 2013) in a separate population of Holstein cows. Briefly, 69 SNPs in genes previously related to fertility and production traits for their relationship to DPR were evaluated in a separate population of Holstein cows grouped according to their predicted transmitting ability [< -1 (n=1,287) and > 1.5 (n= 1,036)] for DPR. Ortega et al. (2016) reported 29 SNPs associated with DPR, and of the SNPs reported to be associated with DPR by Cochran et al. (2013), 19 were significantly associated with DPR in the Ortega et al. (2016) study. For 15 of the 19 genes, Ortega et al. (2016) reported the beneficial allele was the same as that found by Cochran et al. (2013), providing evidence that many of the candidate gene SNPs found by Cochran et al. (2013) are likely to represent true causal variants.

Failure of many of the SNPs found by Cochran et al. (2013) to have a significant effect on DPR in the Ortega et al. (2016) investigation may be related to a variety of factors, including false positives, and the reality that reliabilities of the cow population used in the Ortega et al. (2016) study were lower than the reliabilities for the bull population used by Cochran et al. (2013).

An unfortunate characteristic of some GWAA and candidate gene studies is that associations between genotype and phenotype are not repeatable (Ioannidis et al. 2011). Ortega et al. (2016) provides evidence that many SNPs previously related to fertility traits in Holstein bulls (Cochran et al. 2013) had similar relationships in a separate population of cows. Furthermore, Ortega et al. (2016) assert that the inclusion of these genes in genetic evaluations can improve reliabilities of genomic estimates for fertility. These results, when taken together with the conclusions of Cochran et al. (2013) that numerous SNPs associated with DPR were not negatively associated with production traits, support the premise that it should be possible to select for DPR without compromising production.

Research update: SNPs in genes associated with days open

Previously, a candidate gene approach identified 51 SNPs associated with genetic merit for reproductive traits and 26 SNPs associated with genetic merit for production in dairy bulls. Ortega et al. (2015) evaluated the association of these 77 SNPs with days open for primiparous Holstein cows. Cows were grouped based on DPR: ≤ -1 (n = 1,220) and ≥ 1.5 (n = 1,053) and were housed on 11 farms in Florida and California. To evaluate phenotypes, records were retrieved from on-farm computers and combined with records from the national genetic evaluation system. Days open were lower for cows in the high DPR group as compared with the low DPR group (97.8 ± 2.6 d vs 163.0 ± 2.9 d). There were 6 SNPs with significant additive effects on days open. For example, days open for cows with 0, 1, or 2 copies of the minor allele for COQ9 (coenzyme Q9 which is involved in protein coding) averaged 139.4 ± 3.5, 134.3 ± 2.8, and 123.6 ± 3.5 d, respectively. (A minor allele is considered the least common member of a pair of genes occupying a specific spot on a chromosome that controls the same trait). Days open for cows with 0, 1, or 2 copies of the minor allele for FST (Follistatin: protein coding) averaged 124.9 ± 3.3, 134.8 ± 2.6 and 135.8 ± 4.4 d, respectively. Ortega et al. (2015) concluded: 1) SNPs in specific candidate genes are associated with phenotypic differences in days open for primiparous Holstein cows; and 2)

SNPs related to genetic and phenotypic estimates of fertility are likely to be informative markers for selection.

Conclusions

Genome-wide association assays are powerful tools being used by scientists to gain greater insight into the genetic make-up and potential of animals. Genomic testing and selection is underway on many dairies today. Producers are sampling animals while still young and employing a variety of management strategies to optimize use of their cattle. In fact, nearly 250,000 females were genotyped in 2014, with greater than 100,000 sampled before six months of age (Cole, 2015, unpublished). Promising research results provide evidence that: 1) there may be ample opportunity to make significant gains in Holstein heifer fertility using genomic selection; and 2) there are a large number of SNPs associated with DPR that are not negatively associated with production traits, perhaps allowing for selection for DPR without compromising production. Lastly, identification of genomic loci associated with fertility in primiparous Holstein lactating cows is underway, with results expected in late 2016-2017.

Acknowledgement

This project is supported by Agriculture and Food Research Initiative Competitive Grant no. 2013-68004-20365 from the USDA National Institute of Food and Agriculture.

References

Avery, O.T., C. M. MacLeod, and M. MacCarty. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J. Exp. Med. 79: 137-158. Available on-line at: http://scarc.library.oregonstate.edu/coll/pauling/dna/papers/avery-studies.html

Awano, T., G.S. Johnson, C.M. Wade, M.L. Katz, G.C. Johnson, J.F. Taylor, M. Perloski, T. Biagi, I. Baranowska, S. Long, P.A. March, N.J. Olby, G.D. Shelton, S. Khan, D.P. O'Brien, K. Lindblad-Toh, and J.R. Coates. 2009. Genome-wide association analysis reveals a SOD1 mutation in canine degenerative myelopathy that resembles amyotrophic lateral sclerosis. Proc. Nat'l. Acad. Sci. 106:8:2794-2799.

Brusveen, D.J., A.P. Cunha, C.D. Silva, P.M. Cunha, R.A. Sterry, E.P.B. Silva, J.N. Guenther, and M.C. Wiltbank. 2008. Altering the time of the second gonadotropin-releasing hormone injection and artificial insemination (AI) during Ovsynch affects pregnancies per AI in lactating dairy cows. J. Dairy Sci. 91:1044–1052.

Casida, L.E. 1961. Present status of the repeat-breeder cow problem. J. Dairy Sci. 44:2323–2329.

Cochran, S.D., J.B. Cole, D.J. Null, and P.J. Hansen. 2013. Discovery of single nucleotide polymorphisms in candidate genes associated with fertility and production traits in Holstein cattle. BMC Genet. 14:49.

Dransfield, M.B.G., R.L. Nebel, R.E. Pearson, and L.D. Warnick. 1998. Timing of insemination for dairy cows identified in estrus by radiotelemetric estrus detection system. J. Dairy Sci. 81:1874–1882.

Elsik, C.G., R.L. Tellam, and K.C. Worley. 2009. The genome sequence of taurine cattle: A window to ruminant biology and evolution. Science, 324:5926:522-528.

Hayes, B.J., J. Pryce, A.J. Chamberlain, P.J. Bowman, and M.E. Goddard. 2010. Genetic architecture of complex traits and accuracy of genomic prediction: coat colour, milk-fat percentage, and type in Holstein cattle as contrasting model traits. PLoS Genet. 6:e1001139.

Herlihy, M.M., J.O. Giordano, A.H. Souza, H. Ayres, R.M. Ferreira, A. Keskin, A.B. Nascimento, J.N. Guenther, J.M. Gaska, S.J. Kacuba, M.A. Crowe, S.T. Butler, and M.C. Wiltbank, 2012. Presynchronization with Double-Ovsynch improves fertility at first postpartum artificial insemination in lactating dairy cows. J. Dairy Sci. 95:12:7003–7014.

Ioannidis J.P.A., R. Tarone, and J.K. McLaughlin. 2011. The false–positive to false–negative ratio in epidemiologic studies. Epidemiology. 22:450-456.

Kemper, K.E., and M.E. Goddard. 2012. Understanding and predicting complex traits: knowledge from cattle. Hum. Mol. Genet. 21:R45-51.

Keuter, E.M., C.M. Seabury, M. Neupane, J.N. Kiser, J.G.N. Moraes, J. Dalton, G.W. Burns, T.E. Spencer, and H.L. Neibergs. 2016. Identification of Loci Associated with Fertility in US Holstein Heifers. J. Anim. Sci. (Submitted)

Lucy, M.C. 2001. Reproductive loss in high-producing dairy cattle: Where will it end? J. Dairy Sci. 84:1277-1293.

Lucy, M.C. 2007. Fertility in high-producing dairy cows: Reasons for decline and corrective strategies for sustainable improvement. Soc. Reprod. Fertil. Suppl. 64:237-254.

Mendel, G. J. 1866. Versuche über Pflanzen-Hybriden [Experiments Concerning Plant Hybrids]. In: Verhandlungen des naturforschenden Vereines in Brünn [Proceedings of the Natural History Society of Brünn] IV (1865): 3–47. English translation available on-line at: http://www.esp.org/foundations/genetics/classical/gm-65.pdf

Moeller, L.M., N.A. Michael, J.C. Dalton, and G.C. Lamb. 2010. Assessment of reproductive outcomes in United States Holstein dairies by geographic region. In: Proc. Amer. Assoc. Bov. Pract. Ann. Conf., Albuquerque, NM.

Moraes, J., J.C.Dalton, T.E. Spencer, J.N. Kiser, G.W. Burns, A. Wojtowicz, M. Neupane, and H.L. Neibergs. Identification of loci associated with fertility in Holstein heifers. J. Anim. Sci. 93:E. Suppl. s3:650.
Ortega, M.S., A.C. Denicol, D.J. Null, J.B. Cole, and P.J. Hansen. 2015. Single nucleotide polymorphisms in specific candidate genes are associated with phenotypic differences in days open for first lactation in Holstein cows. J. Dairy Sci. 98: E. Suppl. 2: 648.
Ortega, M.S., A.C. Denicol, J.B. Cole, D.J. Null, and P.J. Hansen. 2016. Use of single nucleotide polymorphisms in candidate genes associated with daughter pregnancy rate for prediction of genetic merit for reproduction in Holstein cows. Anim. Genetics doi: 10.1111/age.12420
Pritchard, T., M. Coffey, R. Mrode, and E. Wall. 2013. Genetic parameters for production, health, fertility and longevity traits in dairy cows. Animal 7:34-46.
Rimbault, M., H.C. Beale, J.J. Schoenebeck, B.C. Hoopes, J.J. Allen, P. Kilroy-Glynn, R.K. Wayne, N.B. Sutter, and E.A. Ostrander. 2013. Derived variants at six genes explain nearly half of size reduction in dog breeds. Genome Res. 23:1985–1995.
Sanger, F., and A.R. Coulson. 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J. Mol. Biol. 94:3:441-446.
Santos, J.E.P., P. Pinedo, G.M. Schuenemann, R.C. Bicalho, R.C. Chebel, K.N.A. Galvão, R.O. Gilbert, S. Rodrigez-Zas, G.J.M. Rosa, C. Seabury, J. Fetrow, and W.W. Thatcher. 2015. Improving fertility of dairy cows through genomic selection. In: Proc. Dairy Cattle Reproduction Council Annual Conference, Buffalo, NY, pp. 3-9.
Sattler, C.G. 2013. Progeny testing and genomics: Where are we and Where are we going? In: Proc. Dairy Cattle Reproduction Council Annual Conference, Indianapolis, IN, pp. 98-103.
Schefers, J.M., and K.A. Weigel. 2012. Genomic selection in dairy cattle: Integration of DNA testing into breeding programs. Anim. Frontiers 2:4-9.
Siontis K.C.M., N.A. Patsopoulos, and J.P.A. Ioannidis.2010. Replication of past candidate loci for common diseases and phenotypes in 100 genome-wide association studies. Euro. J. Human Genet. 18:832-837.
Souza, A.H., H. Ayres, R.M. Ferreira, and M.C. Wiltbank. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology 70:208–215.
Spencer, T.E., P.J. Hansen, J.B. Cole, J. Dalton, H. Neibergs. 2014. Genomic selection and reproductive efficiency in dairy cattle. In: Proc. Dairy Cattle Reproduction Council Annual Conference, Salt Lake City, UT, pp. 16-31.
Sun, C., P. Madsen, M.S. Lund, Y. Zhang, U.S. Nielsen, and G. Su. 2010. Improvement in genetic evaluation of female fertility in dairy cattle using multiple-trait models including milk production traits. J. Anim. Sci. 88:87.
Sutter, N.B., C.D. Bustamante, K. Chase, M. M. Gray, K. Zhao, L. Zhu, B. Padhukasahasram, E. Karlins, S. Davis, P.G. Jones, P. Quignon, G.S. Johnson, H.G. Parker, N. Fretwell, D.S. Mosher, D.F. Lawler, E. Satyaraj, M. Nordborg, K.G. Lark, R.K. Wayne, E.A. Ostrander. 2007. A single IGF1 allele is a major determinant of small size in dogs. Science. 316:112-115.
VanRaden, P.M., A.H. Sanders, M.E. Tooker, R.H. Miller, H.D. Norman, M.T. Kuhn, and G.R. Wiggans. 2004. Development of a national genetic evaluation for cow fertility. J. Dairy Sci. 87: 2285-2292.
Veerkamp, R.F., and B. Beerda. 2007. Genetics and genomics to improve fertility in high producing dairy cows. Theriogenology 68 (Suppl. 1):S266-S273.
Washburn, S.P., W.J. Silvia, C.H. Brown, B.T. McDaniel, and A.J. McAllister. 2002. Trends in reproductive performance in Southeastern Holstein and Jersey DHI herds. J. Dairy Sci. 85:244-251.
Watson J.D., and F.H.C. Crick. 1953. A Structure for Deoxyribose Nucleic Acid. Nature 171:737-738. Available on-line at: http://www.nature.com/nature/dna50/watsoncrick.pdf
Wolf, Z.T., H.A. Brand, J.R. Shaffer, E.J. Leslie, B. Arzi, C.E. Willet, T.C. Cox, T. McHenry, N. Narayan, E. Feingold, X. Wang, S. Sliskovic, N. Karmi, N. Safra, C. Sanchez, F.W.B. Deleyiannis, J.C. Murray, C.M. Wade, M.L. Marazita, and D.L. Bannasch. 2015. Genome-wide association studies in dogs and humans identify ADAMTS20 as a risk variant for cleft lip and palate. PLoS Genet 11(3): e1005059. doi:10.1371/journal.pgen.1005059

Neonatal care

Marthina L. Greer
Veterinary Village, Lomira, WI

Breeders and veterinarians share two great fears: the first and most devastating is the loss of a bitch "in whelp". The second is the loss of a litter or an overwhelming number of pups.

A 10% to 40% loss of pups is reported to be a "normal loss". This staggering figure is difficult to substantiate and sources do not always indicate if it includes loss from conception to 12 weeks of age or from birth to 12 weeks of age. Losses that exceed 10% warrant veterinary investigation. If an underlying cause can be determined, the remaining pups in that litter as well as pups in subsequent litters can be protected. Under the best circumstances, we must recognize that there will be unavoidable losses. Neonates are fragile; disorders that are mild in an adult or slight alterations in environment often have much more severe consequences in the neonate.

Teamwork with the breeder-client is essential. To develop the client-staff team, you can:

1. Coach your team to ask the "right" questions on the telephone.

Reason for appointment	Puppies that are sick, crying inconsolably, having vomiting, diarrhea or coughing, losing weight, dehydrated or dying.
How soon to schedule sppointment/**urgency**	Sick babies are always urgent enough to see the day the client calls, immediately if possible.
Request client to b**ring** with them to appointment	1. The entire litter 2. A way to keep them warm and safe in transit 3. The mother of the litter 4. Sheets of data on puppies or kittens weights, temps, vaccinations, worming, meds, diet fed etc 5. Always bring all the dead pups or kittens they have for examination and possible testing 6. Fresh fecal sample
To **ask** client before appointment	1. Weights of pups 2. Urine color of pups 3. Temperatures of pups
Special **instructions** client should know about their appointment	Keep the live pups or kittens warm in transit by using a heating pad, an ice chest lined and covered with a towel to prop the lid open. A thermometer to monitor temperature.

2. Teach your client how to monitor the whelping and pups in the early postpartum period. Objective measurements including rectal temperature, urine color, and weight gain are easily quantified and charted. Trends can alert you early on if there is a problem. Pups should be identified by a marking system or photography–avoid neckbands.

Normal puppy development from birth to six weeks

What is normal?	Week 1	Week 2	Weeks 3-4	Weeks 5-6
Temperature, rectal	96 - 98° F	96-99 ° F	100 ° F	100 - 101 ° F
Ambient temperature	75 to 80 ° F	70 to 80 ° F	70 to 75 ° F	65 to 75 ° F
Contact surface	90 to 95° F	85 to 90° F		
Heart rate & blood Pressure	200 to 240 beats per min	200 to 240 beats per min, sinus rhythm	160 to 200 beats per min, sinus rhythm	Varies with breed, sinus rhythm
Blood volume	75 ml/kg			
Lab values	PCV = to dam (42-48%) TP 5-6 gm/dl BUN 7-10 mg/dl Glucose 40-60 mg/dl	Total Protein and Albumin TP 4.1 Albumin lower at 2.1		PCV = 24% ALP - Increased up to 4000 IU/L due to bone growth Mild proteinuria normal up to 6 weeks of age Electrolytes Normal
Respiratory rate	15-35/min	15-35/min	15-25/min	15-25/min
Mucus membranes color/CRT	Pink to hyperemic if recently nursed	Pink/1 second	Pink/1 second	Pink/1 second
Urine color	Very pale yellow, <1.020	Very pale yellow, <1.020	Pale yellow	Pale to moderate yellow
Weight	May lose up to 10% in the first 3 days. Birth weight: Toy 100-200 gms; Large 400-500 gms; Giant 700 gms	Gaining 5 to 10% daily, many double birth weight by day 10. Calculate weight gain of 2 -4 gm/day/kg anticipated adult weight.	Calculate weight gain of 2 -4 gm/day/kg anticipated adult weight.	Calculate weight gain of 1 -4 gm/day/kg anticipated adult weight. Giant and large breed at faster rate than small breeds.
Activity	Sleeps & eats 90% of time, twitch while sleeping. Movement is a crawl. Nurses, snuggles with littermates.	Sleeps & eats 90% of time, twitch while sleeping. Begin to support themselves on their forelegs. Nurse aggressively, limited interaction with littermates	Beginning to stand and walk by day 21. Start to play with littermates and toys when eyes open. Can sit. More sturdy.	Walking, climbing, playing, may bark, begin to explore environment, mouthing. Normal postural reflexes.
Attitude	Quiet, cry infrequently.	Quiet	Quiet, more active	Start to develop "personalities",
Body tone & reflexes	Flexor dominance for first 4 days, then extensor. Righting, rooting, weak withdrawal.	Extensor dominance, righting, rooting, crossed extensor. Withdrawal developing.	Approaching normal for adult. Suckling reflex & crossed extensor disappears.	Normal adult
Vision and hearing	No vision but blink with bright light. Limited hearing.	None to limited vision and hearing, menace present but slow initially. Limited hearing,	Vision blurry, pupillary light reflex present within 24 hours of eyelids opening, respond to	Approaching full vision and hearing.

		waxy discharge.	sound. Startle reflex develops.	
Teeth	None	None	Deciduous incisors & canine erupt	Deciduous premolars erupt
Breeder's interaction	Assure pups are nursing, supplement if necessary. 1 to 2 times daily take and record temp, weight, urine & stool character. Stimulate urination and defecation if not done by mother. Start Early Neurologic Stimulation days 3-16.	Assure pups are nursing, supplement if necessary. Daily temp take and record, weight, urine & stool character. Continue early neurologic stimulation day 3-16	Continue to assure pups are thriving, begin to enrich environment by variation of toys, surfaces.	Continue to assure pups are thriving, continue to enrich environment. Lots of human interaction for socialization
Veterinary care	Assess & treat if not thriving, taildocks and dewclaws prior to 5th day if appropriate for breed.	Assess & treat if not thriving. Dispense pyrantel pamoate to use on day 14 after birth.	Assess & treat if not thriving. Dispense pyrantel pamoate to use on day 28 after birth.	Veterinary wellness visit – assess pups for any abnormalities to allow breeder to sell pup with full disclosure. First vaccinations (DAPPv) end of 7th week. Dispense pyrantel pamoate to use on day 42 after birth.
Food and water	Nursing only. If supplementing, 60 ml/lb/24 hours divided by 12, fed every 2 hours.	Nursing only. If supplementing, 70 ml/lb/24 hours divided by 8, fed every 3 hours.	Offer water, then gruel to start weaning. If supplementing, 90 ml/lb/24 hours divided by 6, fed every 4 hours.	Teething. Many pups weaned, on full food and water, some still nurse for social interaction.

(Adapted from Hoskins JD: Small animal pediatric medicine. Tufts Animal Expo; North Grafton, MA. 2002.)

Abnormal findings in the first two weeks

Many pups lose weight in the first 24 hours–but this should not exceed 10% of their total body weight. After the initial loss, weight gain should be 5 to 10% of their birth weight daily. Pups should gain 2 to 4 g/day/kg of anticipated adult weight. Many breeders want the birth weight to double in the first seven to ten days. Most pups should be receiving all of their fluid and nutritional needs by nursing. Careful monitoring of rectal temperature, weight gain, urine color, and overall well-being will indicate if some or all the pups require supplemental feeding.

Despite the varied disorders in newborns, their clinical signs may be so similar that they do not help distinguish the cause. The most obvious complaint is incessant crying or mewing. This grabs the attention of even the most inattentive owner and bitch, but may not identify the sickest pups. Sick puppies move very little, and make no or weak efforts to nurse. They do not twitch while sleeping. Sick pups are often separated in the whelping box from littermates, not cuddled in the group. They are not

gaining weight or worse, are losing. Body temperature is below 94 °F (this is a chilled pup who should not be fed milk until warmed to 96°F) or above 98°F (this is a fever in a neonate). If the rectal temperature of all pups is high, this may either indicate all the pups are sick or that the environmental temperature is too high. Urine color on a dry cotton ball is an obvious color of yellow. Milk may flow from the nostrils of pups with cleft palates.

Sick pups have a look and "feel" that can be difficult to describe. The general appearance is unthrifty and either overly active or overly still. When picked up, they feel limp and scrawny. Frequently, there is a history of prolonged labor. If a breeder says there is something wrong, but they can't describe it fully, believe them and have the litter come in for evaluation and treatment.

If a pup seems to be sick or abnormal, take a rectal temperature. If below 96°F, warm the pup inside the breeder's shirt or carefully with an external heat source. A heating pad covered with a towel, hot water bottle, or sock filled with heated white rice can be used, but care must be taken to avoid burning, overheating or warming too quickly. Diarrhea, nasal discharge, inflammation of the umbilicus, and/or sloughing of the toes and/or tail tips are early symptoms of illness in newborns.

Advanced in-home care–the breeder as the health care provider

Breeder skills and supplies

A well-educated breeder paired with a well-educated veterinary staff and a veterinarian willing to learn from their breeder-clients make a formidable team. Veterinarians and veterinary staff can learn as much from their breeder-clients as they can learn from us.

The most essential step in good record keeping is to be able to definitively identify each individual in a litter. This is useful for several reasons, including tracking health, weight gain, monitoring medical care, and identifying the purchaser.

For pups born at the veterinary hospital: there are several techniques that can be used for tracking individuals in the litter. With one system, sterile colored hand-towels are used to receive the neonates during a cesarean section. This allows birth order and each pup's immediate neonatal care to be more easily recorded following resuscitation. The towel color is translated into permanent marker or nail polish and the information is mapped onto a uterine diagram with one copy maintained in the hospital record and one copy provided to the owner. This information may become vital in monitoring an at-risk pup more carefully or establishing a diagnosis of illness in a newborn.

Basic equipment for clients and veterinary clinics to have for whelping:

1. Record keeping system.
2. Method to re-mark puppy's identification.
3. Room thermometer to track temperature at the puppy's surface.
4. Rectal thermometer, digital is ideal, to monitor the bitch's and pup's temperatures.
5. Scale to weigh pups. This should weigh in ounces or grams.
6. Cotton balls to check urine color of pups.
7. Feeding tube and appropriate syringes if supplementation is necessary.
8. Formula to feed, if supplementation is necessary.
9. Tincture of iodine to dip the umbilicus in at birth two and eight hours postpartum.
10. Chlorhexidine disinfectant solution to disinfect surfaces in the whelping and nursery areas
11. Bulb syringe and Dee Lee mucus trap.
12. Laundry basket or ice chest (do not seal with the lid and monitor temperature to prevent overheating or chilling) to keep pups in during daily evaluation; makes sorting who is done and who is not easier and faster.
13. Heating pads, rice bags, or Snuggle Safe^{R}.
14. Towels, blankets, tarps and flannel-backed vinyl table cloths to keep the pups and room sanitary.

Causes of neonatal mortality

There is no specific disorder known as "fading puppy syndrome". Early recognition and effective medical care can significantly reduce neonatal illness and death. This can be divided by age range into three time periods. First are problems that occurred in utero or during whelping (from pre-birth to two weeks of age); second are problems that occur early in the postpartum period (from two to five weeks of age); and third are those in the postweaning period (from five to 12 weeks of age). Common causes of neonatal illnesses include: dystocia, physiologic or nutritional causes, environmental causes including poor husbandry, genetic and congenital causes, trauma, teratogens and toxins, infectious diseases (viral and bacterial), parasites, and failure of passive immunity transfer.

Aiding lactation in the dam

Commonly, a bitch may require several days to lactate sufficiently to support a litter without assistance. The bitch may be allowed to eat food that is not ordinarily part of her normal diet. She must be fed a diet that contains carbohydrates to lactate well. Fluid therapy can be useful in improving lactation. Subcutaneous fluids can be administered at the hospital or by the clients at home.

Oxytocin can aid in milk letdown. Metoclopramide or domperidone can help increase lactation. Oxytocin and metoclopramide may be used simultaneously. The metoclopramide dosage is 0.2 to 0.4 mg/kg subcutaneously or by mouth (tablets or syrup) three times a day as indicated for up to seven days. Metoclopramide is contraindicated in any bitch with gastrointestinal hemorrhage, gastrointestinal obstruction or perforation, a hypersensitivity to the drug, or a seizure disorder.

Excellent maternal nutrition is essential to support pregnancy and lactation. A high quality commercial dog food – pregnancy, puppy and performance diets are currently recommended. She should be at an ideal body condition prior to pregnancy. During the first four weeks of pregnancy, no diet changes or increase in quantity is indicated. During the last five weeks of pregnancy, she will benefit from an increase of 20% to 50%, depending on the size of litter she is expected to have. No nutritional supplements are necessary if she is fed a commercially available diet for puppies, performance or pregnancy. Uncooked diets put the pups at risk of developing bacterial and parasitic conditions.

Neonatal examination, supportive care, symptoms, and diagnostic work-up

The importance of an office visit – assistance when they need it most; veterinary intervention can be the difference between life and death for the neonate. This needs to be offered on an emergency basis because sick pups can deteriorate quickly when they become ill.

Handling the newborn at the veterinary hospital should be done with great care. Two disinfected laundry baskets with a heat source and towel should be ready prior to the arrival of the client. The pups can be moved from one basket to the next as they are handled to prevent confusion regarding which pup has been examined and treated.

All staff should wash their hands thoroughly and wear examination gloves prior to touching the pups. The examination room should not have recently been used by a patient with a potentially contagious disease. A circulating water blanket or heating pad should be placed under a clean towel as an examination surface. Although the bitch should be present for an examination (to evaluate for a retained pup or placenta, metritis, mastitis, or other illness), she may be more comfortable in another room during examination and treatment of the pups.

The physical examination should be similar to that of any patient. Vital signs including rectal temperature, heart rate, respiratory rate, and mucus membrane color/capillary refill time should be taken. Urine color (including urine specific gravity) and stool character should be evaluated.

Remember, puppies can have most of the same diagnostics used on adults – blood chemistries, complete blood counts, ultrasound and radiographic imaging. Different normal values must be applied, and diagnostics and treatments must be modified, but they are just small dogs in many respects and their small size and immaturity should not put off the veterinarian as the diagnostic plan is developed. Many of the in-house veterinary chemistry and blood count analyzers now use such a small blood volume that nearly any size patient can be evaluated safely.

Supportive care or "treat for the treatable"

Neonatal and pediatric patients can be treated with the following protocol (unless there is a contraindication) until they return to a clinically normal state or a diagnosis can be confirmed, allowing for a specific protocol. These patients are fragile and deteriorate so quickly when ill that treatment should be initiated while diagnostics are pending. Sick neonates symptoms include constant crying, weakness, abdominal distention or pain, anorexia, poor weight gain, poor nursing, restlessness, and isolation. None of these symptoms are pathognomonic for the underlying cause. Assume the sick neonate has a treatable disease and initiate care until proven otherwise.

Summary of empirical treatment of the sick neonatal puppy
☐☐Identify the sick individual(s) and initiate diagnostics
☐☐1. Oxygen
☐☐2. Warm the hypothermic pups slowly until rectal temperature reaches 96°F IMPORTANT do not over heat pups
☐☐3. Provide nutritional support in the form of tube feeding formula or IV/PO/IO glucose
☐☐4. Fluid therapy PO/IO/SQ
☐☐5. Antibiotic therapy & topical treatment of the umbilicus
☐☐6. Plasma or serum administration
☐☐7. Transfuse
☐☐8. Vitamin K
☐☐9. Hygiene and elimination
☐☐Reintroduce normal diet – nursing, tube feeding, puppy food
☐☐Continue to carefully monitor with temp/weight/urine color twice daily
☐☐Consider hand-raising the pup(s) and using empirical antibiotics and supportive care on the entire litter if no source of infection can be found.

Oxygen. Hypoxia is common in the newborn. Intubation and ventilation may be necessary immediately following birth. Oxygen therapy can improve the status of any sick puppy. Pups born prematurely, that aspirate amniotic fluid or meconium, or that are traumatized or oxygen-deprived at birth, will benefit from supplemental oxygen therapy.

Supplemental oxygen can be administered by nasal cannula or placement of an IV catheter in the trachea, if only one pup is affected. There are many techniques for constructing an oxygen chamber. Oxygen concentrators can also be used to deliver a continuous supply of oxygen. The greatest limitation to oxygen use is restriction of the treatment group of pups from association with their dam and littermates.

Thermal support. Normal body temperature for newborns is 96 to 98°F, rising one degree a week until four weeks of age, when they can maintain their own body temperature.

A rectal and room thermometer must be available for monitoring the pups. Thermal support is frequently necessary. Great care is needed at the hospital, during transport, and at home to prevent chilling or overheating. The right combination of warmth and ventilation is essential. The ideal ambient temperature for a litter and the bitch is 70° F with a relative humidity of 55 to 65%. Ambient temperatures of 85 to 90° F are only necessary for orphaned or sick pups or those removed from the care of the bitch.

Newborn pups cannot maintain their own body temperature without an adequate ambient temperature along with the bitch and littermates for thermal support. A healthy newborn can only maintain a body temperature of 12° F above the ambient temperature. A sick newborn is even more vulnerable to hypothermia. The newborn's inability to shiver and lack of peripheral vasoconstriction leave them dependent on an external heat source for maintenance of a safe core temperature. This situation is exacerbated by their lack of body fat and a relatively large surface area allowing for heat loss.

Poor mothering skills, such as moving the pups out of the warm whelping area, pushing a sick pup aside, or excessive licking of the pups is a common cause of chilling, which leads to illness.

It is essential to avoid overheating puppies that are in a confined space with no ability to move away from the heat source. A range of temperatures and space should be provided which allows the pups to move closer to or away from the heat source. It is possible to kill pups in a short time by overheating and dehydration, and careful monitoring is essential. Heat sources include heating pads, heat lamps, heated microwave pads, heated rice bags, incubators, and hot water bottles.

Low body temperature (less than 96°F) is a very common and often life-threatening problem in the neonate. It should be suspected in any sick pup or pup that is unusually quiet or still. Bradycardia is often secondary to hypothermia, dropping the heart rate from 200 bpm to the 100 to 150 bpm range.

If pups are too warm, they are usually fussing/crying or overly active. To distinguish between the ambient temperature being too warm and a fever, all pup's temperatures should be taken rectally. If the ambient temperature is too high, all of the pups will usually have a rectal temperature over 100°F. If only a portion of the pups have rectal temperatures over 100°F, it is more likely that the hyperthermic pups are febrile and ill. Feeding should not be attempted, by nursing, bottle or tubing, until the rectal temperature can be maintained at 96°F.

Electric heating pads intended for humans can pose a risk of thermal burn or electrocution if the dam or pups chew through the cord. Many of the newer pads have an automatic shut-off. There are several safer alternatives to the use of a heat lamp. Human neonatal incubators or whelping nests with an electric heat source are safe and very effective. Circulating hot water blankets are also a safe and can be wrapped around the pups. Hot water bottles, SnuggleSafe^{R}, or bags/socks filled with rice or oats that can be heated in the microwave will temporarily warm the pups. Rice bags can serve as substitute littermates for singletons and can help support weak pups when they nurse. Water bottles that cool will actually draw heat from the puppies so must be monitored frequently.

A pup that becomes chilled rapidly will develop a life-threatening condition. A chilled pup does not nurse effectively. When the rectal temperature drops below 94°F, peristalsis slows. The pup is at risk of vomiting or backflow followed by aspiration pneumonia. When the rectal temperature plummets below 85°F, bacterial overgrowth occurs in the gut, often followed by sepsis, and the pup becomes hypoglycemic. If allowed to progress, the pup's rectal temperature will approach room temperature; the pup will not move, breathing becomes almost indistinguishable, the heart rate slows and cannot be palpated, and the pup appears dead.

Nutritional support including tube feeding

Symptoms of inadequate nutrition and hypoglycemia include crying, weakness and inability to nurse effectively. The most reliable technique for nutritional support is tube feeding. It assures that the volume necessary is delivered efficiently, even if the pup is too sick to nurse.

Prior to initiating nutritional support, it is important that the rectal temperature of the pup is 96°F or slightly higher and that it is well-hydrated. Gentle and slow warming of the pup should be initiated prior to feeding to prevent ileus. Most sick neonates are also hypoglycemic. Nutritional support early in the course of treatment is critical. Once warmed, assist the pup in nursing or tube feed. There are many reasons a pup may be under nourished and dehydrated. The underlying problem needs to be addressed (sick mother, sick puppy, too large a litter), but the first thing to do is warm and supplement the pup. Feeding a pup with a rectal temperature below 96°F will result in ileus and fermentation of the milk in the gut. This leads to aspiration and perpetuates the pup's distress. Tube feeding is recommended over bottle-feeding. Most breeders can be quickly trained to tube feed. Done correctly, it is a safe, fast and effective method. Supplemental feeding with an eyedropper or makeup sponge is dangerous and not recommended.

Low birth weight and failure to ingest colostrum has a strong correlation with failure to thrive. Pups that are 25% below the weight of the average pup in the litter are at increased risk of hypothermia, hypoglycemia, hypoxia, and septicemia. Pups that lose more than 10% of their birth weight in the first 24 hours are sick enough to seek veterinary attention. Treatment including supplemental feeding, fluid

therapy, supplemental heat, antibiotics, and possibly plasma infusion, and oxygen therapy should be initiated. Diagnostics may also be indicated.

Some pups requiring supplemental feeding have serious medical or genetic disorders. Most, however, are just off to a rough start. The least we can do is support them with the warmth and nutritional care they require. Most pups who start off poorly begin to thrive with a few feedings a day and a little extra care. Clients who are willing to invest the extra effort should be encouraged and educated to promote this. More pups are lost to starvation than to any other problem in the immediate postpartum period. Fear of tube feeding or lack of training should not be an excuse. If warming and feeding do not return the pup to normal, seek veterinary care.

The most effective way to tube feed a neonate is to pass a soft silicone feeding tube through the oral cavity into the stomach at each feeding. An NE tube can be placed and sutured into position for long-term nutritional support. Surgical placement of a pharyngostomy or esophagostomy may also be indicated for long term feeding. This can be done with a brief anesthetic period using gas induction and/or propofol induction and maintenance. Despite the tendency of some pups to gag on the tube when fed past the second week of life, as they mature, many pups see the feeding tube and become very excited and cooperative as the tube is passed making surgical placement unnecessary. Tube feeding will not reduce the pup's drive to suckle and stimulate the bitch to lactate. At birth or the first few days that follow, it is not possible to tell how the pup will develop. We should be very careful not to "discard" a pup for lack of nutritional support.

Puppies require 22 to 26 kcal per 100 gm of body weight for the first 12 weeks. This translates into 13 ml of formula per 100 grams of body weight per day per pup for the first seven days. This increases to 17 ml/100 gm for week two, 10 ml/ 100 gm body weight for week three, and 22 ml/100 gm body weight for week four. This should be divided into a minimum of four feedings per day.

Another easy to remember formula is based on the stomach capacity of the newborn, rather than a caloric intake. A neonate stomach can accommodate one ml = (one cc) of formula per one oz of body weight. However, the first several feedings should be approximately 30% lower than the calculated formula to allow for the stomach capacity to accommodate this volume. A smaller quantity should also be fed if he pup appears to have nursed. The stomach can be gently palpated prior to feeding to estimate fullness.

Puppy milk replacer formulas can be purchased commercially, made at home with a recipe, or goat's milk can be used. The commercially available formulas are preferred as their amino acid and fat/protein ratios most closely match bitch's milk. Pups fed homemade diets or goats milk often develop nutritional cataracts.

If a puppy is presented in a hypoglycemic crisis, an IV catheter should be placed in the jugular vein if possible (use the largest vein possible or the intraosseous (IO) route to reduce the likelihood of phlebitis). Then 1 to 2 ml/kg of 5% to 20% dextrose should be administered slowly IV. IF an IV or IO catheter cannot be placed, 50% dextrose, honey or corn syrup can be rubbed onto the gums and/or administered by stomach tube when the pup is warm enough (over 96°F). Alternatively, 2.5% dextrose and 0.45% NaCl can be given subcutaneously if the IV or IO route is unavailable. For patients that present in a coma or in shock, 20% dextrose administered IV or IO should be used, but with care.

Food should be offered as soon as the pup is alert enough to eat. Five percent dextrose can be continued IV. Prior to discharge from the hospital, the pup must be weaned off the dextrose and must be eating an appropriate diet to prevent relapse of the hypoglycemic episode.

Fluid support and methods of administration–IM, IO, IP, IV, PO, PR, and SQ

Dehydration may occur because of inadequate nursing or secondary to diarrhea and/or other illnesses. Neonates are more susceptible to dehydration because of a higher ratio of surface area to body mass and the inability of their kidneys to concentrate urine.

Hydration status can be assessed on tacky mucus membranes, history of loss and/or inadequate intake, urine with a specific gravity exceeding 1.020 or that is dark yellow in color. Hydration status of

the newborn and neonate cannot be assessed by skin turgor due to a lack of subcutaneous fat. Nearly all sick pups will benefit from fluid replacement therapy. Care should be taken to avoid over-hydration.

The fluids given by any of these routes should be warmed to body temperature (95° to 99°F or 35° to 37°C) before being administered. Neonatal fluid maintenance is 60 to 180 ml/kg of body weight per 24 hours.

If indicated, fluid loading can be initiated by using warmed fluids at a rate of 1 cc per 30 gm of body weight over five to ten minutes, continuing until the patient shows improved mucous membrane color. The fluid maintenance rate can then be increased to 30 to 50 cc/lb/24 hour period (60 to 100 cc/kg/24 hour period) based on deficit and ongoing loss. Potassium chloride can be supplemented via the fluids or orally (using KCL elixir) if the potassium is below 2.5 meq per L. The IV and IO routes are most effective for debilitated patients but are more costly, require hospitalization and separation from the dam and littermates and increased levels of veterinary skills.

IV fluids. The jugular vein is the largest, most accessible vein in the neonatal and pediatric patient. An IV catheter (20 g to 24 g, up to 1 inch in length) can be placed, with or without a cutdown, and sutured into place with a tape butterfly. If the skin is difficult to pierce without damaging the catheter, a 20 g needle can be used to nick the overlying skin allowing easier introduction of the catheter. The catheter can be supported with a light tape bandage, taking care to protect the patency of the catheter and the comfort of the puppy. A bandage that is too heavy and restrictive can be detrimental to the pup. In larger pups, other peripheral veins can be accessed. If the catheter is expected to be indwelling for over 12 hours, a sterile skin preparation with povidone iodine or chlorhexidine should be applied to minimize complications. To minimize clotting of the catheter if it is used intermittently, 0.5 cc of 50% dextrose can be injected into the catheter at the end of each injection cycle. Heparin should be avoided.

Fluids that can be administered via the IV route include 2.5% dextrose and 0.45% NaCl, Ringer's solution, normal saline, dextrose 5%, hypertonic fluids such as 50% dextrose and 7% saline, hetastarch, other colloidals, blood and blood products as well as drugs labeled for IV administration. Lactate cannot be metabolized by the neonate. It is better avoided if there is an alternative fluid source, but can be used if lactated Ringer's solution is the only fluid option available.

IO fluids. The intraosseous route allows rapid absorption of fluids or blood/blood products by administration directly into the vascular space. It is easier to place an IO needle than it is to cannulate a small vein on a neonatal patient. Equipment needs are simple – either a 22 gauge spinal needle or a hypodermic needle, size 20 to 25 gauge, materials for a sterile preparation, bandaging materials, and a routine IV administration setup with an extension set. The sites most commonly used are the trochanteric fossa of the femur, the tibial tuberosity, and the trochanteric fossa of the proximal humerus, taking care to avoid injuring the growth plates.

Palpation of the various sites is done to determine the location best suited for the patient. The site should be 1 cm distal to the trochanteric fossa of the femur or humerus, or 1 cm distal to the tibial tuberosity. The site is clipped and a sterile preparation is applied, taking care to avoid overuse of fluids that may cause chilling of the patient. A small bleb of lidocaine or bupivicaine can be administered if indicated by the patient's condition, taking care to use a minimal dose as neonates have a reduced tolerance for these drugs. A small nick is made in the skin with a scalpel blade. The limb should be stabilized with the free hand cupping the stifle or other joint distal to the insertion site, and the middle finger parallel to the long bone to aid in directing the needle into the lumen. A 22 g spinal needle or a hypodermic needle is selected to suit the size of the patient's intramedullary space. The needle is inserted by attaching an injection port to the hub to maintain sterility, then twisting the needle as it is advanced. If a hypodermic needle is used and the lumen is plugged at insertion, the first needle should be removed and a second smaller needle or IV stylet should be placed through the needle to dislodge the obstruction. The needle should feel securely seated in the bone when correctly placed. Tape the needle into place by a figure eight strip of one inch white tape around the hub of the needle, around the thigh on each side, and crossing over near the stifle. Place an antibiotic cream around the opening in the skin. Position folded gauze squares around the hub of the needle to support the needle and administration set. Tape the needle and administration set securely to the patient to avoid dislodging the needle or discomfort to the patient.

Complications from IO administration are rare. This technique does require removal of the pup from the other healthy littermates and dam.

Fluids that can be administered via the IO route include Ringer's solution, normal saline, 5% dextrose, hypertonic fluids such as 50% dextrose and 7% saline, hetastarch, other colloidals, blood and blood products ans drugs labeled for IV administration.

SQ fluids. Subcutaneous fluid administration is a very simple method of rehydrating a neonatal patient. The patient must be warm and have adequate peripheral circulation to pick up the fluids. This can often be done by the client at home. This route has the advantage of keeping the affected neonates with their littermates and dam. It is also more affordable for the client.

Not all fluids can be administered via the SQ route. Lactated Ringer's solution, normal saline, 2.5% Dextrose with 0.45% NaCl, serum and plasma and drugs labeled for subcutaneous administration can be given via this route. Five percent dextrose is not recommended for administration subcutaneously. Warm the fluids to body temperature and inject them into one or two sites in the interscapular space. Injection with a 20 gauge needle and appropriate sized syringe is easier than use of a bag of fluids with a venoset. The typical dose is approximately one cc per one oz of body weight per time of administration. Overhydration can be a serious complication. Rapid or labored respirations are a symptom of overhydration and indicate that fluid therapy rates must be adjusted.

PO fluids. Fluids can be administered by mouth or by feeding tube, if the gut is working. This means that the rectal temperature of the pup must be over 96°F, the gut is patent (stools have been passed) and there is no ongoing vomiting

If the pup is strong and has an adequate suckling response, a small "preemie" nipple and bottle or a small bottle (15 ml medi-nurser) designed to administer oral medication to infants can be used. Many pet nursers and human baby bottles are not well suited to this because the pups do not take the nipple well and the bottle is so large it is difficult to assess how much fluid the pup has taken.

Initially, a sick pup can be fed electrolytes (warmed lactated Ringer's solution) or a 50:50 mix of milk replacer, mother's milk extracted from the bitch, or goat's milk combined with warmed lactated Ringer's solution or other appropriate oral electrolyte solution. This can be administered by bottle or by feeding tube, but never by eyedropper as this is too likely to lead to aspiration. Alternatively, a 5% to 10% dextrose solution can be given by feeding tube at 0.25 ml per oz of body weight. There is a small but serious risk of aspiration or perforation of the gut when a tube is used for oral supplementation, but when done correctly, the risk is very small and the benefits are great.

Antibiotics

Antibiotics should be considered a first line of defense and started immediately for any sick or debilitated pup. This includes pups born distressed, with diarrhea, with meconium in the fetal fluids, or born after a protracted labor. They should be started at the first sign of illness. The decision to treat the affected pup only or the entire litter is a clinical decision and will vary from case to case. Antibiotics can be administered to neonates through the same routes that are labeled for an adult dog. Critically ill or septic pups should have antibiotics administered by injection, not by PO route.

Orally, or through a feeding tube, is a reliable route if the gut is working. The intraosseous route, which may be preferred if IV administration is not possible, can be substituted for any antibiotic labeled for IV administration. Intravenous injection into the umbilical vein can be useful if the use is indicated at birth. Subcutaneous injection can be used when the pup has adequate peripheral circulation. Intramuscular administration should be used with caution due to the small size and limited circulation of the target site. The intraperitoneal route is no longer considered appropriate for antibiotic administration.

Bacterial infections, as a primary cause of disease, as a secondary invader, and as sepsis are common in the neonate. Many of these bacteria in pups are are relatively antibiotic sensitive. This, and the immature metabolism of a neonate, make penicillin, amoxicillin, amoxicillin with clavulanic acid and cephalexin antibiotics of first choice. Ceftiofur can be used as an injectable for newborns that have had meconium in their fetal membranes.

Potentiated sulfonamides must be used with caution and only in the well-hydrated patient. This is an appropriate drug choice in a lactating bitch with metritis, mastitis, or other bacterial infections. Aminoglycosides are rarely indicated and should only be used based on culture and sensitivity results.

The fluoroquinolones are reported to cause defects in the formation of articular cartilage and they are contraindicated from eight weeks to beyond eight months of age, depending on the length of the growth phase in different breeds. Drugs in this class can probably be safely used up to four weeks, and to eight weeks of age if absolutely necessary, but only if based on culture and sensitivity results and if there is an adequate risk-benefit ratio (when articular cartilage damage is a better outcome than a fatal disease). This drug class can also be used in the lactating bitch with pups under four weeks of age, but only if necessary because there are other preferred antibiotics for this application. The tetracyclines should not be used in pups prior to eruption of all adult dentition due to alteration of the enamel color.

The umbilicus should be closely evaluated and treated by continued dipping with tincture of iodine. The umbilicus is a common source of infection in the neonate, causing fatal peritonitis.

The carbapenems (amoxicillin, penicillin, amoxicillin with clavulanic acid and cephalexin), macrolides (erythromycin and azithromycin) and lincosamines (lincomycin, clindamycin) are generally safe choices for use in the lactating bitch, if she is not sensitive to the products. Although many drugs cross into the milk, in no case should this be considered an adequate route of administration for antibiotics (or any drug) to the neonate.

Passive immunity– plasma or serum

Newborn pups are dependent on ingestion of colostrum in their first 24 hours of life to develop adequate passive immunity to carry them through their first few months of life. The passive immunity transmitted transplacentally in the dog (and cat) is insufficient protection.

Alternatively, blood can be collected (then harvest plasma or serum) from the dam or another dog from the same kennel. The commercially available product is harvested from health-screened dogs. Although there are reports that suggest using non-commercial colostrum, serum, or plasma harvested from facilities other than the breeder's kennel, this risks introducing non-endemic diseases. The product from the individual breeder's kennel has the advantage of offering passive protection to the newborns to diseases they will be exposed to in their own environment.

The dam can be used if she is physically able to withstand the associated blood loss. This is not recommended if she has just undergone a cesarean section or is otherwise debilitated. The candidate should be a resident of the breeder's kennel, have not previously had a blood transfusion or a pregnancy, be young and in peak health. A larger dog should be selected because she will be able to tolerate having a larger volume of blood withdrawn. The average puppy will require 45 to 60 ml of whole blood (16 cc of plasma or serum).

Administration of the plasma or serum. IgG antibodies are well absorbed when administered subcutaneously. They can also be given IO; IP administration poses significant risk and does not offer any benefits over SQ administration. Oral administration is inferior to SQ when comparing absorption rates of antibodies. The published dose for a puppy is 16 cc of plasma administered SQ aseptically in divided doses over 12 hours.

Kittens require smaller doses, 5 cc SQ three times in 12 hours, and show high levels of circulating antibodies within 12 hours of administration.

FRESH FROZEN PLASMA FOR NEONATAL PUPPIES

1. Keep all plasma frozen until use. Take care in handling plasma as it can easily become contaminated with bacteria.
2. To thaw, carefully warm the plasma to body temperature. Only warm the tubes that will be used at each administration – keep the remaining tubes in the freezer. This is best done by placing tube against the body or in a pocket for warming. Do not heat in warm water or microwave as this will denature/damage the proteins and render the product ineffective. Gently rock the tube during thawing; do not shake.
3. The dose is 5.4 cc per puppy three times over a 24 hour period, totaling 16.2 cc per puppy. If this can be administered in the first 24 hours after birth, it can be given orally with a feeding tube. After the pups are 24 hours old, it must be given by SQ or IO injection to be effective systemically.
4. Draw 5.4 cc of warmed plasma into a 6 cc syringe. Using a feeding tube (only if the pups are less than 24 hours old) or a 20 or 22 gauge needle (for pups over 24 hours old), inject the warmed plasma. If given SQ, hold the skin pinched to prevent outflow from the injection site. If given by feeding tube, carefully follow instructions for feeding tube administration.
5. Repeat two more times in the next 24 hours. Change to SQ injection if the pups have exceeded 24 hours of age before the doses are administered.

Transfusion. Anemia severe enough to merit a transfusion is rare in the puppy. Neonatal isoerythrolysis, seen in some breeds of kittens, is not a condition seen in the puppy

A pup with severe anemia or a hematocrit of less than 15%, and associated symptoms, may require a whole blood transfusion. Blood is administered at a rate of 10 ml/lb of body weight over 2 hours IV or IO. The whole blood should be collected with a citrated anticoagulant and administered with a standard Millipore blood filter. Administration of blood IP should be done as a last resort only.

Vitamin K injection. Any neonatal pup under four days of age showing symptoms of hemorrhage (internally or externally) should have an injection of Vitamin K1 administered at the rate of 0.25 to 2.5 mg SQ or IM once. Neonates are deficient in thrombin and may show signs of hemorrhage associated with sepsis, trauma, or other illness.

Basic hygiene and assistance with eliminations. Pups up to four weeks of age lack the ability to urinate or defecate without assistance. Every four hours, or after each feeding, the abdomen and rectal region of each pup should be wiped in a circular motion with warm wet cotton balls to stimulate elimination. If the pups appear soiled with feces or look greasy, they should be carefully washed to reduce contamination without chilling the pups.

In some cases, the pups will have a gas accumulation in the stomach and/or intestinal tract. Gas in the stomach can be relieved by passing a feeding tube and allowing the gas to escape. This type of tube can also be passed rectally if there is gas in the colon or if an enema would benefit the pup. If there is a frothy gas or the gas is lower in the intestines, pediatric simethicone can be administered orally.

Adjusting drug doses for the neonate

The dosage of most drugs needs to be adjusted for the neonate. There is no published information for most drugs and there are many physiologic differences between the neonate and the adult patient. The neonate has a relatively lower body fat and higher water content than the adult; the neonatal blood-brain-barrier is more highly permeable to drugs; the neonate has reduced albumin so has a lower protein binding of drugs; the neonate has reduced renal clearance of drugs; and the neonate has lower hepatic clearance and altered metabolism of drugs due to an immaturity of enzyme function. Even the site and type of administration in the neonate has an altered absorption rate compared to the adult; drugs administered by IM injection have a lower absorption rate and drugs administered by SQ injection and PO routes have an increased rate of absorption compared to adults. Routes of administration such as IO not used in adults are options in the neonate.

In general, drugs that are water-soluble should have the dose increased to compensate for the higher percentage of body water in the neonate. Drugs that are fat-soluble should have the dose decreased (up to 30% to 50%) to compensate for decreased clearance.

Each drug dosage should be researched and calculated based on the case. Many antibiotics have a wide margin of safety, and this needs to be considered when drugs are selected. The benefit-risk ratio of each drug should be carefully assessed and the breeder/owner should be included in this discussion. The Johns Hopkins formulary in *The Harriet Lane Handbook: A Manual for Pediatric House Officers* book is very useful and available used for a very affordable price. This focuses on drugs used in human pregnancy; the data can be extrapolated to veterinary use if necessary.

Prior to administering any drug to any patient, particularly a neonate, the following thought process is useful:

1. Is there a better alternative treatment? Do we need a drug at all? Do we need this drug?
2. Are the risks of treatments balanced by the benefits?
3. What do we know about this drug in this type of patient considering the physiology of the neonate?
4. Are there data we can use to compensate for lack of knowledge about this drug in this type of patient?
5. Do we have informed consent of the owner?
6. What parameters will we use to assess treatment success? Toxicity?
7. Should littermates also be treated?

Key drugs, dosages and indications

Key Drug	Indication	Dose Range (dose adjusted for neonates and pediatric patients)	Frequency	Route	Precautions	Reference	Margin of Safety
Acyclovir	Herpes	10 mg/kg As suspension	q 6 hrs until 3 weeks of age.	PO	Anecdotal only	Kampschmidt and others Unpublished observation	narrow
Amikacin	Gram negative Septicemia	Up to 20-25 mg/kg (with caution)	Q 36-48 hrs if under 6 weeks.	IV	May cause renal and ototoxicity. Reserve for suspected severe life-threatening gram negative infections. Monitor blood levels.	Plumb	narrow
Amoxicillin	Infection	6-22 mg/kg	q12 hr	PO		Various	wide
Amoxicillin/ Clavulanic Acid	Infection	12.5-25 mg/kg	q12 hr	PO		Lee	wide
Ampicillin	Infection	22 mg/kg	q 8 hr	IV		Lee	wide

Cefazolin	Infection	10-30 mg/kg	q 8 hr	SC or IO	Decrease dose if diminished renal function	Root Kustritz	wide
Cefotaxime	Infection	25-50 mg/kg	q 8 hr	SC or IO		Root Kustritz	wide
Cephalexin	Infection	10-30 mg/kg	q 8-12 hr	PO		Various	
Chloramphenicol	Mycoplasma Infection	22 mg/kg (Puppies only)	Q 8 hr (up to 7 days total)	PO	Reserve for CNS disease or cases where bacteria are resistant to other antibiotics. Use minimized due to human health risk when handled.	Poffenberger	narrow
Fenbendazole	Anthelmintic	50 mg/kg	Q 24 hr for 3 days	PO	5 days if treating for Giardia	Various	wide
Fluoroquinolones	Infection		5 to 20 mg/kg per day PO. 5 to 20 mg/kg q 12 hours IV for sepsis		To protect against cartilage damage, avoid use from 8 weeks to 8 months of age.	Various	narrow
Metronidazole	Giardia or anaerobic infection	30 mg/kg in pups at least 2 weeks of age.	q 24 hr for 7 to 10 days	PO	Neurotoxicity or "intoxication"	Plumb	Narrow
Pyrantel Pamoate	Parasites	5–10 mg/kg	Q 14 days starting at 14 days of age	PO		Various	Wide
Sulfadimethoxine	Coccidiosis (not labeled for this use)	50 mg/kg first dose, then 25 mg/kg	Q 24 hr for 5-10 days	PO	Avoid if under 4 to 5 weeks of age. Can cause renal precipitate if patient is not well hydrated. May cause KCS or thrombocytopenia.	Plumb	Narrow

Trimeth/Sulfa	Infection	30 mg/kg	q 24 hr	PO	Same as for sulfadimethoxine	Various	Narrow
Tylosin	Mycoplasma or GI Infection				Off label use		Wide
Vitamin K 1	Neonatal hemorrhage	0.01-0.1 mg total or 5 mg/kg	Once	SQ, IM	Use small gauge needle. May cause anaphylaxis by injection. PO is better absorbed.	Various	Narrow

(Kampschmidt K: Drug use in the neonatal pediatric small animal patient. Western Veterinary Conference.2006.)

Drugs to avoid in neonates and pediatric patients:

Drug name:	**Reason to avoid:**
Doxycycline and tetracyclines	Discoloration of teeth and alteration in bone development
Fluoroquinolones	Damage to cartilage in pups over 8 weeks and under 8 months.
Griseofulvin	Liver damage – diminished liver clearance
Ivermectin	When neonates, blood-brain barrier too permeable
Metronidazole	When neonates, blood-brain barrier too permeable
NSAIDS	In the neonate, renal function may be damaged
Long acting corticosteroids	

(Gelens H: Drug therapy in pediatric practice. Western Veterinary Conference, Las Vegas, 2003.)

Diagnostic testing

1. Blood tests: CBC, chemistry panel and electrolytes and titers.
2. Urinalysis: dipstick, specific gravity, organic acids to detect biochemical pathway errors at Metabolic Screening Laboratory, section of Medical Genetics of the Veterinary Hospital of the University of Pennsylvania.
3. Cultures: Viral cultures, PCRs and bacterial cultures.
4. EKG lead II
5. Radiographs and ultrasound:
6. Function testing: gastrointestinal function testing (tli), hepatic function tests (bile acids), renal clearance iohexol clearance test and urine protein creatinine ratio.
7. Postmortem examination: in house and at the reference laboratory.

Tube feeding directions

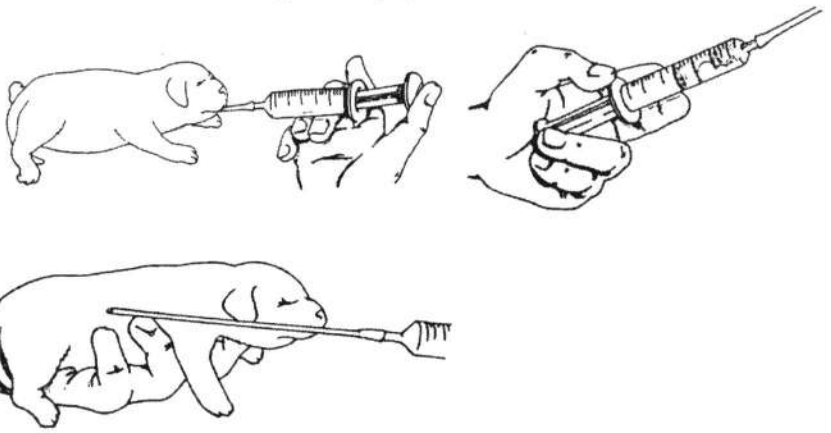
Materials

1. Goat's milk, (pasteurized is preferred), or commercial milk replacer
2. Feeding tube, silicon or red rubber feeding tube 8 to 14 French
3. Permanent marker
4. Syringe of appropriate size with catheter tip (10 or 60 cc)
5. Puppy scale
6. Rectal thermometer

Steps

1. Establish a well-lit warm location where you can hold the pup comfortably and all materials are within reach. Be attentive and do not rush.

2. Take the puppy's temperature rectally; do not feed unless the rectal temperature is between 96°F and 99°F. If the temperature is below 96°F, gently warm the pup before feeding.
3. On a safe surface, hold the pup with the neck extended. Hold the tapered end of the feeding tube even with the last rib of the largest pup to be fed. Lay the tube along the side of the pup, mark the tube even with the tip of the pup's nose



4. Fill the syringe with the calculated amount of formula or milk (20 cc/16 oz body weight or approximately 1 cc per ounce) plus 1 cc of air. Pre-warm the formula to body temperature in a warm water bath–avoid microwaving. Feedings should be administered every three to six hours as indicated by weight gain and hydration status.
5. Attach the syringe to the feeding tube.
6. With the pup fully awake, warm (over 96°F rectal temperature) lying horizontally on the chest, gently pass the tube over the center of the pups tongue, applying gentle pressure to slide the tube up to the mark. Keep the pup's chin below its ears and pass the tube along the left side of the throat to reduce the chance of mistakenly introducing the tube into the trachea instead of the esophagus. If resistance is met, remove the tube and start over.
7. If you are right handed, cup your left hand around the back of the pup's head and hold the tube between your index and middle finger to prevent it from moving out of the correct position while feeding. Reverse this if you are left handed.

8. BEFORE FEEDING, firmly pinch the pup on the foot or tail. If the pup vocalizes, the tube placement is correct and you can proceed with feeding. If the tube is mistakenly in the trachea, the pup will struggle but will not be able to make any sound – STOP IMMEDIATELY, REMOVE THE TUBE AND START THE PROCESS OVER.
9. With your right hand, depress the plunger on the syringe, not too quickly, delivering the calculated amount, stopping sooner should milk reflux out of the mouth or nose.
10. Flex the tube on itself to prevent milk from being aspirated in to the pup's airway. Repeat for each pup.
11. Wash syringe and tube with hot soapy water and allow to air dry until next feeding.
12. Stimulate the external anal and urinary orifices with a warm moistened cotton ball or washcloth to effect defecation and urination.

Remember the 5 p's of safe tube feeding:

1. premeasure the tube
2. pass with the chin down
3. pass along the left side
4. pinch to assure the pup can vocalize before feeding
5. prewarm the pup and formula before feeding

Deworming

Recommendations by the Center for Disease Control (CDC) and Companion Animal Parasite Council (CAPC) have updated worming protocols. The CAPC website, www.capcvet.org may be reviewed by the veterinarian and pet owner for most current recommendations.

Pups and their dams should receive their first deworming when the pups are two weeks of age. Repeat at four, six, and eight weeks, then place on monthly broad-spectrum heartworm anthelmintics that are effective against parasites with zoonotic potential.

Fecal examinations should be performed two to four times during the first year and repeated one to two times a year for adult pets. Use of fenbendazole during pregnancy will minimize transmission of roundworms and hookworms through the placenta and the milk.

It's time, past time, or is it? Managed whelping and cesarean section in the dog

C. Scott Bailey
College of Veterinary Medicine, North Carolina State University, Raleigh, NC

The most common reasons for estimation of gestational age or determination of fetal readiness for birth are the presentation of a bitch for timed cesarean section or presumed dystocia. Timed cesarean section may be desirable in bitches with known compromised pregnancy, such as evidence of puppy loss, presence of an obstructive vaginal mass or trauma. Alternately, they may be based on risk factors for dystocia, such as breed history of dystocia, pregnancy with one or two pups, history of failure to whelp in previous litters and breed predisposition to dystocia.[1] Techniques for estimation of gestational age and determination of fetal maturity have been described for all stages of gestation with varying degrees of accuracy and can be used singly or in combination for management of routine and high-risk pregnancy.

The easiest methods to estimate whelping dates involve breeding management. However, breeding dates provided by owners are notoriously unreliable. Parturition may occur anywhere from 57 to 72 days after a single observed breeding.[2] On the contrary, the easiest and most accurate way to predict whelping is to diagnose or estimate the time of luteinizing hormone (LH) surge. Eighty-seven percent of bitches whelp 64-66 days following the LH surge,[3-5] which can be diagnosed by repeated LH assay (every 12 hours, due to the short duration of the peak).[6] The luteinizing hormone peak may also be estimated by observing serum progesterone levels that achieve 2-3ng/ml and continue to rise thereafter, or by combining progesterone and LH assays.[5,6] Shortly after this period, vaginal cytology may be used to diagnose the onset of diestrus, occurring approximately 51-60 days before whelping (80% on day 57).[7,8]

Ultrasonographic determination of gestational age to predict whelping has been investigated extensively and several equations exist to calculate gestational age. However, results have been conflicting.[5,6,8,9] In the author's experience, ultrasonographic estimation of gestational in early or mid-gestation is not accurate enough to predict whelping or time a cesarean section in high-risk animals. A recent publication by Gropetti and co-workers suggests that this is likely due to the need for breed-specific parameters rather than ultrasonographic capability.[10] Nevertheless, ultrasonographic examination around day 30 after the LH surge is a reliable means of diagnosing pregnancy, evaluating gestational health or confirming gestational progression. A few parameters used by the author for approximate estimation of gestational age are listed below:

- The fetal heartbeat is visible at approximately 22-26 days.
- Limbuds, fetal movement and a fluid filled stomach may be seen on day 29, 30 and 33.
- Fetal length exceeds chorionic width at approximately day 42.

Likewise, determination of gestational age based on radiographic films has been described.[9,11] Radiographic interpretation of gestational age has not been considered sufficiently accurate for management of high-risk animals. However, no recent studies have been performed comparing digital radiography to conventional film and no study has evaluated advanced radiologic techniques, such as magnetic resonance imaging. Parameters used by the author for approximate estimation of gestation age are listed below:[9,11,12]

- The scapula, humerus and femur are first detectable 17 days (15-18) prepartum.
- The pelvis and 13 pairs of ribs are visible 11 days (9-13) prepartum.
- Teeth are visible 4 days (3-8) prepartum.

Thus, regarding prediction of whelping date, factors determined during breeding management are vital for accurate assessment and no single factor assessed during pregnancy approaches the accuracy of a known date of LH peak or rise in progesterone. However, in many instances, this information is unknown. Further, in our experience, data from breeding management or early pregnancy are insufficient in cases of high-risk pregnancy that should not be allowed to whelp naturally, as well as those cases when surgery is necessary and the aim is to perform a cesarean section at the earliest possible time. It becomes

expedient to determine fetal maturation and stress in order to affect a positive treatment outcome. Unfortunately, no single parameter is available that could determine the attainment of fetal maturity or term gestation other than the onset of labor or identification of fetal distress. Consequently, in most instances, a variety of modalities are recommended to form a "consensus opinion" of gestational age and fetal maturity prior to progressing with obstetric management.

As with early pregnancy, characteristic hormonal changes can be observed late in pregnancy, correlating with fetal maturation. Concannon and coworkers measured maternal progesterone and cortisol during late pregnancy and found that progesterone dropped from 2.6-7.8 ng/mL (average 4.5±0.6) at approximately five days prepartum to 1.9+/-0.36 ng/mL and 0.55+/-0.07 ng/mL at 24-16 and 12-8 hours prepartum, respectively.[13] In that same group of bitches, serum cortisol levels during the last 4 days of gestation ranged from 11-43 ng/mL (average 22.9 +/-1.2). In six of seven bitches, a distinct increase in cortisol occurred 8-24 hours prior to parturition reaching 42-87 ng/mL.[13] The rapid drop in progesterone is correlated with a temporary drop in maternal body temperature by one degree Fahrenheit, which was detected in approximately 85% of cases.[14] This indirect measure of "maturity" is frequently used by owners, but has insufficient sensitivity and specificity for use in a hospital setting. However, in practices that have easy access to automated endocrine assays, serial measurement of progesterone is the most definitive measure of fetal and maternal readiness for birth. In the author's practice, one to three progesterone assays are typically performed prior to timed cesarean section in order to determine fetal readiness for birth. It should be noted, however, that a single progesterone level above 2ng/mL does not have good negative predictive value (i.e., whelping can ensue on the same day even when serum progesterone concentrations are between 2 and 5 ng/mL).

Morphometric parameters, such as renal development and gastrointestinal motility, have been described as measures of fetal maturity in the past, but not gained wide traction among veterinary ultrasonographers. Gradual changes in the renal architecture may suggest fetal maturity in late gestation and gastrointestinal development and then detection of peristalsis correlate with approximately 62-64 days of gestation.[15-17] Recently, Gil and coworkers described ultrasonographic interpretation of fetal gastrointestinal characteristics based on differentiation into four phases: phase I (19-24 days prepartum) – one uniform echogenic area in the region of the bowel; phase II (14-19 days prepartum) – visualization of some bowel wall segments; phase III (8-15 days prepartum) – bowel wall segments had clearly defined intestinal wall and intraluminal content; phase IV (1-6 days prepartum) – identification of complete intestinal wall, visual distinction between the mucosal surface and intestinal wall, peristalsis in all segments of the bowel. These data provide support for the use of morphometric evaluation of fetal structures in estimating fetal age and maturity. However, the authors cautioned against using gastrointestinal appearance alone to determine fetal maturity and noted that parturition occurred naturally one to four days after this was first noted.[18]

Alterations in fetal heart rate have long been associated with impending parturition and fetal stress. Normal fetal heart rates during late pregnancy are typically 220-240 beats per minute (bpm), while fetuses that are experiencing space-constraint or metabolic stress have decreased heart rates around 180 bpm and fetuses experiencing severe distress due to hypoxia have heart rates less than 140-180 bpm.[19,20] Gil and coworkers also correlated "oscillation" (increased variability, with elevated and decreased heart rates between 180 and 240 bpm sequentially in a two to four minute period) with onset of labor.[21] Cardiotocography, or correlation of fetal heart-rate patterns with maternal contractions is a widely used, but controversial monitoring technique in human obstetrics.[22] In addition, Gil and coworkers recently described the use of Doppler ultrasound canine umbilical arteries to predict delivery time and fetal distress.[23] The results of this study suggested that the resistive index, an indirect measure of peripheral resistance, decreased significantly in the immediate prepartum period, with values measured at less than 0.7. In contrast, they found that animals taken to cesarean section had an initial decline and then significant elevation in resistive index and suggested that this modality may be useful in identifying fetal distress.

When factored together, the plethora of diagnostic potentials gives a high degree of security regarding timed cesarean section in high-risk animals and puppy vigor and survival should be excellent

after cesarean section.[24-26] The most complicated situations are those that require estimation of gestational age without breeding management and those that necessitate surgery prior to the "ideal" window based on breeding management. Nevertheless, serial evaluation and use of multiple diagnostic approaches provides adequate information for management of these animals.

References

1. Bergström A, Nødtvedt A, Lagerstedt AS, et al: Incidence and breed predilection for dystocia and risk factors for cesarean section in a Swedish population of insured dogs. Vet Surg 2006;35:786-791.
2. Concannon PW, Whaley S, Leid D, et al: Canine gestation length: vacioation related to time of mating and fertile life of sperm. Am J Vet Res 1983;44:1819-1821.
3. Yeager AE, Concannon PW: Association between preovulatory LH surge and the early ultrasonographic detection of pregnancy and fetal hearteats in beagle dogs. Theriogenology 1990;34:655-665.
4. Cohen JA, Holle DM, Meyers-Wallen VN: Accuracy of canine parturition date prediction from LH peak. Clin Therio 2009;1:570.
5. Kutzler MA, Mohammed HO, Lamb SV, et al: Accuracy of canine parturition date prediction from the initial rise in preovulatory progesterone concentration. Theriogenology 2003;60:1187-1196.
6. Luvoni GC, Beccaglia M: The prediction of parturition date in canine pregnancy. Reprod Domest Anim 2006;41:27-32.
7. Holst PA, Phemister RD: Onset of diestrus in the Beagle bitch: definition and significance. Am J Vet Res 1974;35:401-406.
8. Kim Y, Travis AJ, Meyers-Wallen VN: Parturition prediction and timing of canine pregnancy. Theriogenology 2007:68;1177-1182.
9. Lopate C: Estimation of gestational age and assessment of canine fetal marutation using radiology and ultrasonography: a review. Theriogenology 2008;70:397-402.
10. Gropetti D, Vegetti F, Bronzo V, et al: Breed-specific fetal biometry and factors affecting the prediction of whelping date in the German shepherd dog. Anim Reprod Sci 2015:152;117-122.
11. Toal RL, Walker MA, Henry GA: A comparison of real-time ultrasound, palpation and radiography in pregnancy detection and litter size determination in the bitch. Vet Radiol 1986;27:102-108.
12. Rendano VT: Radiographic evaluation of fetal development in the bitch and fetal death in the bitch and queen. In: Current veterinary therapy vol VIII. Philadelphia: WB Saunders; 1983. p. 947-952.
13. Concannon PW, Butler WR, Hansel W, et al: Parturition and lactation in the bitch: serum progesterone, cortisol and prolactin. Biol Reprod 1978;19:1113-1118.
14. Johnston SD, Root Kustritz MV, Olson PNS1: Canine parturition-eutocia and dystocia. In: Johnston SD, Root Kustritz MV, Olson PNS (eds), Canine and Feline Theriogenology. Philadelphia: WB Saunders; 2001. p. 105-128.
15. Nyland TG, Mattoon JS: Ovaries and uterus. In: Small animal diagnostic ultrasound. Philadelphia: WB Saunders; 2002. p. 231-249.
16. Yeager AE, Mohammed HO, Meyers-Wallen V, et al: Ultrasonographic appearance of the uterus, placenta, fetus, and fetal membranes throughout accurately timed pregnancy in Beagles. Am J Vet Res 1992;53:342-351.
17. Lamm CG, Makloski CL: Current advances in gestation and parturition in cats and dogs. Vet Clin North Am Small Anim Pract 2012;42:445-456.
18. Gil EMU, Gacria DAA, Froes TR: *In utero* development of the fetal intestine: Sonographic evaluation and correlation with gestational age and fetal maturity in dogs. Theriogenology 2015;84:681-686.
19. Verstegen JP, Silvia LDM, Onclin K, et al: Echocardiographic study of heart rate in dog and cat fetuses in utero. J Reprod Fertil Suppl 1993;47:174-180.
20. Zone MA, Wanke MM. Diagnosis of canine fetal death by ultrasonography J. Reprod Fertil 2001;57:215-219.
21. Gil EMU, Garcia DAA, Giannico AT, et al: Canine fetal heart rate: do accelerations or decelrations predict the parturition day in bitches? Theriogenology 2014;82:933-941.
22. Williams KP, Galerneau F: Fetal heart rate parameters predictive of neonatal outcome in the presence of a prolonged deceleration. Obstet Gynecol 2002;100(5 Pt 1):951-954.
23. Giannico AT, Gil EMU, Garcia DAA, et al: The use of Doppler evaluation of the canine umbilical artery in prediction of delivery time and fetal distress. Anim Reprod Sci 2015:154;105-112.
24. Moon PF, Erb HN, Ludders JW, et al: Periperateve risk factors for puppies delivered by cesarean section in the United States and Canada. J Am Anim Hosp Assoc 2000;36:359-368.
25. Moon-Massat PF, Erb HN: Peripoerative factors associated with puppy vigor after delivery by cesarean section. J Am Anim Hosp Assoc 2002;38:90-96.
26. Muennich A, Kuechenmeister U: Dystocia in numbers-evidence-based parameters for intervention in the dog: causes for dystocia and treatment recommendations. Reprod Domest Anim 2009;44(Suppl 2):141-147.

Analgesia for canine cesarean section recovery

S. R. Escobar, K.A. Kolster
Springfield Veterinary Center, Glen Allen, VA

Introduction

Safety studies during pregnancy and lactation have not been done or are sparsely published for a variety of drugs including analgesics for both humans and animals. This poses an issue for the use of pain management medications during pregnancy and lactation for the mother, from a safety standpoint for the fetus during gestation, and especially for the neonate during lactation.

Cesarean section and parturition itself can be a risky and painful process in animals, having adverse outcomes on health and welfare, including productivity in livestock species.[1] Postoperative analgesia presents a challenge for the small animal practitioner, especially as the canine dam and neonates are most often sent home soon after surgery and oral or topical medications are the only practical forms appropriate for the owner to administer. The most commonly considered classes of analgesics and available data on safety and efficacy will be addressed, though this is not to be considered an exhaustive review.

Opioids

Tramadol is the most commonly used oral opioid medication dispensed for analgesia in the canine. This drug presents challenges in efficacy for canine analgesia and little is known in terms of safety in suckling canine neonates. Efficacy is controversial as the pharmacokinetics and pharmacodynamics differ significantly between canine and human patients. In humans, two metabolites provide analgesia. One metabolite enhances the inhibitory neurotransmitters (serotonin and norepinephrine), and the other metabolite, O-desmethyltramadol (M1), is a weak opioid with one-onehundredth the mu receptor affinity of morphine. Dogs unfortunately produce very little of the M1 metabolite, and the half-life is very short in the dog, only 1.7 hours.[2-5] Pharmacokinetic studies have also shown that plasma levels are much lower after oral administration in dogs than humans, and that sequential dosing for several days leads to dramatic reductions of these plasma levels.[6,7] Frequent dosing is therefore necessary to maintain acceptable serum levels. However, even with frequent dosing, evidence of any significant analgesic effect using oral tramadol is not convincing.[7,8] There is evidence that parenteral administration of this drug produces a pain modifying effect, but this is not practical for at-home administration.[9-12] The safety to canine neonates suckling from treated dams is unknown, however short term use in human nursing mothers seems to be acceptable and have little effect on the neonate.[13,14] Short term use of tramadol may also be safe in the canine, but the efficacy of oral administration is questionable and in the authors' opinion not advisable, especially as a stand-alone postoperative analgesic.

Fentanyl topical preparations

There is no oral fentanyl preparation available for use in the canine and only one topical fentanyl preparation is approved in the dog (Recuvyra, Elanco, Greenfield, IN). In the past, human fentanyl patches have been used off-label in the dog. Topical preparations are not recommended for use in dams after cesarean section for several reasons, the most compelling of which is neonatal exposure. Nursing canine neonates will attempt to suckle any protruding feature on the dam, including a patch. Covering a patch is difficult and the most effective location for delivery of the patch product is on the caudal abdomen, where neonates will inevitably be exposed.[15] The approved product for dogs is a liquid topical preparation and not normally covered. The product insert warns of exposure risk to humans touching the patient, especially small children, therefore there is great concern for nursing neonates. Efficacy of topical fentanyl preparations is also a consideration. Delivery of the drug using human fentanyl patches (Duragesic, Janssen Pharmaceuticals, Beerse, Belgium) in the canine is extremely variable, affected by location, body temperature, and several other factors.[16] The recommended patch dose is 2 to 4 mcg/kg/hr, but the validity of this recommendation has never been established. Though fentanyl patches have their advocates, there are no scientific data to support their efficacy.[15] Sedation in the dam is also a risk with

these products, affecting attentiveness and care of the neonates. Topical fentanyl preparations therefore are not recommended post-cesarean section in the canine.

Gabapentin

Several studies in humans support the safety and benefit of gabapentin perioperatively for post-surgical pain, but these do not seem to correlate with efficacy in the canine.[17-23] Further studies are indicated to ensure efficacy of gabapentin as a postoperative analgesic in the dog. Safety studies and case reports in humans during breastfeeding are sparse.[24-25] Most reviews indicate the need for further studies in human medicine.[26-27] To the authors' knowledge, there are no studies or reports on milk expression of gabapentin in the canine. Somnolescence is the most common adverse effect in both canine and human patients, so attentiveness to neonates is a concern. Side effects usually spontaneously resolve after a few days of acclimation, though by this time the postoperative treatment course will be finished. The canine dose is based on the human perioperative dose of 10 mg/kg, though no studies support this recommendation.

Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are perhaps the most common analgesics used postoperatively in both human and veterinary medicine. There are many studies supporting efficacy, and several drugs in this class are approved specifically for postoperative analgesia in the dog. The availability of oral preparations and ease of administration make these drugs convenient for the owner. The primary concern for any medication used after cesarean section is safety for the nursing neonate. Human studies show low NSAID expression in breast milk.[28,29] However, NSAIDs are contraindicated in breastfeeding mothers of premature infants because the COX-2 enzymes are essential for neonatal renal development.[30] This scenario is likely a more accurate model for the canine species in which kidneys are considered immature at birth. Maturation of the canine kidney does not occur until approximately three weeks after birth, and normal function is not present until approximately six to eight weeks of age.[31] Due to the effect of NSAIDs on nephron maturation, exposure should be avoided until renal development is complete or a safe dose is determined.[32] There are limited data on milk expression of NSAIDS in the dog or on any degree of exposure to the neonate that may be safe. A literature search found milk expression studies in animals for cimicoxib, meloxicam, and carprofen, but no information on firocoxib or deracoxib.

Cimicoxib

Cimicoxib is approved in the European Union for perioperative and osteoarthritic pain in the canine.[34,35] Limited data are available on cimicoxib. One study documented non-inferiority compared to carprofen for postoperative pain; however there is a lack of overall peer reviewed published data for osteoarthritic pain.[35] Another study has shown that oral cimicoxib given at a dose of 2 mg/kg to lactating bitches had a high transfer rate into the milk, with milk to plasma ratio of 1.7 to 1.9, as the drug is very lipophilic. Twenty-eight day old neonates suckling from these dams were sampled 8.5 and 24 hours after administering a single dose of cimicoxib to the dams. All puppies had cimicoxib concentrations near or below the limit of quantification (0.01 mcg/ml). The conclusion of this study was that after administration of cimicoxib to whelping bitches, suckling puppies should be minimally exposed to the drug through the dam's milk and no serious adverse effect should occur.[36] It should be noted, however, that use in pregnant or lactating dams is labeled as a contraindication with this drug, and efficacy as a standalone analgesic in a single postoperative dose is questionable.

Meloxicam

The efficacy of meloxicam for postoperative analgesia in the canine is well documented. Reproductive studies with meloxicam have been done in the bovine, ovine, and swine species; however, a literature search found no reproductive studies in the canine. Varying effects may prevent findings in one species from being extrapolated to another species. For example, one study concluded meloxicam is safe for use in pregnant cows, but another study showed NSAID inhibition of COX-2 in term sheep caused

prolonged gestation.[37,38] Unfortunately, no studies have been done in the postpartum canine demonstrating safety in neonates exposed to milk from treated dams. Further study is indicated to determine the safety of meloxicam in the canine as a postoperative analgesic for canine caesarian section.

Carprofen

The efficacy of carprofen for postoperative analgesia in the canine is also well documented. There are multiple reproductive studies investigating carprofen use in the lactating cow. Most notably, one study showed minimal milk expression of carprofen in mastitic and normal control cows.[39] This prompted a pilot study (previously unpublished) done by the author in six Labrador retrievers with similar findings. Carprofen was administered to bitches at 2 mg/kg every 12 hours following cesarean section for four days. Validated milk assays were performed on samples collected three hours after the morning dose, when peak levels were expected, daily for five days. Milk assays were performed on three bitches 24 hours prior to caesarian section as controls. One bitch had detectable carprofen levels in five of six samples, peaking at 21 ng/ml. All other milk samples showed no detectable carprofen concentration (lower limit of assay 1 ng/ml). Matching serum samples from one bitch revealed concentrations ranging from 547 ng/ml on day two to 21,564 ng/ml on day five of administration, indicating effective absorption and serum levels. The conclusion of this pilot study was that carprofen is minimally expressed in canine milk. A larger study with paired milk and serum samples on all test subjects is indicated to confirm initial findings.

Despite the minimal likelihood of carprofen expression in canine milk, the more significant concern is determination of a safe level of carprofen exposure for nursing neonates. A study involving serum assays in neonates would prove difficult due to the low levels expressed in milk and the limits of assay sensitivity. The lower level of expected neonatal absorption may be difficult if not impossible to quantify. There are also logistical and ethical concerns with blood sampling in neonatal puppies. An alternative study (previously unpublished) evaluating offspring from dams treated with carprofen at the pilot study doses was considered and is ongoing. To date, 55 dogs have been studied from 46 litters. Blood urea nitrogen (BUN) and creatinine alone were evaluated in 15 of the 55 dogs, and complete blood counts and serum chemistries were evaluated in the remaining 40 dogs. Thirty-seven litters were represented by one dog each and nine litters by multiple dogs each. Twenty-four breeds were represented. The average age of dogs was 39.5 months (range 5 to 106 months) and average body weight was 29.0 kg (range 4.3 to 82.6 kg). Average BUN was 18.0 mg/dL (range 10 to 44 mg/dL, reference range 6 to 31 mg/dL) and average creatinine was 1.0 mg/dL (range 0.5 to 1.6 mg/dL, reference range 0.5 to 1.6 mg/dL). Two individual dogs from the same litter had BUN values outside the reference range (32 & 44 mg/dL). However, both dogs had normal creatinine (1.6 & 1.1 mg/dL, respectively) and were clinically normal. When the study population was limited to dogs over five years of age (n=11), the average BUN was 15.55 mg/dL and average creatinine was 1.0 mg/dL. In total, 394 cesarean sections have been performed at the authors' clinic from 2000 to 2015, and approximately 320 of those bitches were treated with carprofen for postoperative analgesia. There are no known cases of acquired renal abnormalities in any of those litters based on verbal follow-up with the breeders. One case of suspected renal dysplasia occurred in a puppy that was clinically abnormal at birth and was significantly smaller than its normal littermates. This puppy developed clinical signs of renal disease around nine weeks of age and was euthanized due to progressive renal failure at seven months of age. The preliminary evidence from this study is promising for the safety of neonates exposed to milk from dams treated with carprofen. Continued evaluations will be done to confirm these findings.

Conclusion

Pregnancy and lactation are pharmacologically challenging periods because administering a drug to one patient, the dam, has the potential to affect the offspring as well. While some bitches may behave stoically after cesarean section, this is unquestionably a painful procedure and providing appropriate postoperative analgesia should be the standard of care. The sparse data on drug use during lactation in the dog make decisions about drug choice difficult. The efficacy of oral opioids, such as tramadol, is

questionable, as is the safety of topical preparations like fentanyl. Efficacy and safety data are lacking for gabapentin. Nonsteroidal anti-inflammatory drugs are the most promising category of analgesics for further study. In the meantime, clinicians should consider the welfare of all affected animals, dam and offspring, when making decisions regarding postoperative analgesia.

References

1. Mainau E, Temple D, Manteca X: Experimental study on the effect of oral meloxicam administration in sows on preweaning mortality and growth and immunoglobulin G transfer to piglets. Prev Vet Med 2016;126:48-53.
2. McMillan CJ, Livingston A, Clark DR et al: Pharmacokinetics of intravenous tramadol in dogs. Can J Vet Res 2008;72:325-331.
3. Giorgi M, Saccomanni G, Lebowska-Wieruszewska B, et al: Pharmoacokinetic evaluation of tramadol and its major metabolites after single sustained tablet administration in the dog: a pilot study. Vet J 2009;180:253-255.
4. Kukanich B, Papich MG: Pharmacokinetics and antinociceptive effects of oral tramadol hydrochloride administration in Greyhounds. Am J Vet Res 2011;72:256-262.
5. Kukanich B, Papich MG: Pharmocokinetics of tramadol and the metabolite O-desmethyltramadol in dogs. J Vet Pharmacol Therap 2004;27:239-246.
6. Matthiesen T, Wohrmann T, Coogan TP, et al: The experimental toxicology of tramadol: an overview. Toxicol Lett 1998;95:63-71.
7. Malek S, Sample SJ, Schartz Z, et al: Effect of analgesic therapy on clinical outcome measures in a randomized controlled trial using client-owned dogs with hip osteoarthritis. BMC Vet Res 2012;8:185.
8. Murrell J, Flaherty D: Extending postoperative opioid analgesia in dogs 1. Oral drug administration. In Practice 2014;36:277-283.
9. Martins TL, Kahvegian MA, Noel-Morgan J, et al: Comparison of the effects of tramadol, codeine, and serum interleukin-6 in dogs undergoing maxillectomy or mandibulectomy. Am J Vet Res 2010;71:1019-1026.
10. Vettorato E. Zonca A, Isola M, et al: Pharmocokinetics and efficacy or intravenous and extradural tramadol in dogs. Vet. J 2010;183:310-315.
11. KongaraK, Chambers JP, Johnson CB: Effects of tramadol, morphine, or their combination in dogs undergoing ovariohysterectomy on peri-operative electroencephalographic responses and post-operative pain. N Z Vet J 2012;60:129-135.
12. Kongara K, Chambers JP, Johnson CB, et al: Effects of tramadol or morphine in dogs undergoing castration on intra-operative electroencephalogram responses and post-operative pain. NZ Vet J 2013;61:349-353.
13. Bloor M, Paech MJ, Kayea R: Tramadol in pregnancy and lactation. Int J of Obstet Anesth 2012;21:163-167.
14. Ilett KF, Paech J, Page Sharp M, et al: Use of a sparse sampling study design to assess transfer of tramadol and its O-desmethyl metabolite into transitional breast milk. Br J Clin Pharmacol 2008;65:661-666.
15. Murell J, Flaherty D: Extending postoperative opioid analgesia in dogs 2.Transdermal opioid administration. In Practice 2014;36:335-343.
16. Hofmeister EH, Egger CM: Transdermal fentanyl pateches in small animals. J Am Anim Hosp Assoc 2004;40:468-478.
17. HurleyRW, Cohen SP, et al: The analgesic effects of perioperative gabapentin on postoperative pain: a metaanalysis. Reg Anesth Pain Med 2006;31:237-247.
18. Ho KY,Gan TJ, Habib AS: Gabapentin and postoperative pain—a systematic review of randomized controlled trials. Pain 2006;126:91-101.
19. TippanaHamunen K, Kontinen VK, KalsoE: Do surgical patients benefit from perioperative gabapentin/pregabalin? A systematic review of efficacy and safety. Anesth Analg 2007;104:1545-1556.
20. Seib RK ,Paul E: Perioperative gabapentin for postoperative analgesia: a meta-analysis. Can J Anaesth 2006;53:461-469.
21. Clivatti J, Sakata RK, Issy AM: Review of the use of gabapentin in the control of postoperative pain. Rev Bras Anetesiol 2009;59:87-98.
22. Wagner AE, Mich PM, Uhrig SR,et al: Clinical evaluation of perioperative administration of gabapentin as an adjunct for postoperative analgesia in dogs undergoing amputation of a forelimb. J Am Vet Med Assoc 2010;236:751-756.
23. Aghighi SA, Tipold A, Piechotta M, et al: Assessment of the effects of adjunctive gabapentin on postoperative pain after intervertebral disc surgery in dogs. Vet Anaesth Analg 2012;39:636-646.
24. Kristensen JH, Ilett KF, Hackett LP, et al: Gabapentin and breastfeeding: a case report. J Hum Lact 2006;22:426-428.
25. Moore A, Costello J, Wieczorek P, et al: Gabapentin improves postcesarian delivery pain management: a randomized, placebo-controlled trial. Anesth Analg 2011;112:167-173.
26. vanderMeer DH, Wieringa A, Wegner I, et al: Lactation studies of anticonvulsants: a quality review. Br J Clin Pharmacol 2015;79:558-565.
27. Davanzo R, Dal Bo S, Bua J, et al: Antiepileptic drugs and breastfeeding. Ital J Pediatr 2013;39:50.
28. Ostrea, EM, Mantaring JB, Silvestre M: Drugs that affect the fetus and newborn infant via the placenta or breast milk. Pediatr Clin N Am 2004;51:539-579.
29. Rathmell JP, Viscomi CM, Ashburn MA: Management of nonobstetric pain during pregnancy and lactation. Anesth Analg 1997;85:1074-1087.

30. Prevot A, Mosig D, Martini S, et al: Nimesulide, a cyclooxygenase-2 preferential inhibitor, impairs renal function in the newborn rabbit. Pediatr Res 2004;55:254-260.
31. Horster M, Kember B, Valtin H: Intracortical distribution of number and volume of glomeruli during postnatal maturation in the dog. J Clin Invest 1971;50:796-800.
32. Harris RC: Cyclooxygenase-2 in the kidney. J Am Soc Nephrol 2000;11:2387-2394.
33. Grandermange E, Fournal S, Woehrle F: Efficacy and safety of cimicoxib in the control of perioperative pain in dogs. J Small Anim Pract 2013;54:304-312.
34. EMA Dimalgex:Summary to the public, EMA/532732/2010.wwwema.europa.eu/docs/en-GB/document_library/EPAR_-_ public_/veterinary/000162/wc500109401.pdf
35. Budsberg SC: Orthopedic patients. In: Grimm KA, Lamont LA, Tranquilli WJ, et al. Veterinary anesthesia and analgesia, 5th Edition. Ames: Wiley Blackwell; 2009. p. 1008.
36. Schneider M, Kuchta A, Dron F, et al: Disposition of cimicoxib in plasma and milk of whelping bitches and in their puppies. BMC Veterinary Research 2015;11:178.
37. Hirsch AC, Phillip H: Effects of meloxicam on reproduction parameters in dairy cattle. J Vet Pharmacol Ther 2009;32:566-570.
38. Rac VE, Small C, Scott CA, et al: Meloxicam effectively inhibits preterm labor uterine contractions in a chronically catheterized pregnant sheep model: Impact on fetal blood flow and fetal-maternal physiologic parameters. Am J Obstet Gynecol 2006;195:528-534.
39. Lohuis J, van Wercen T, Brand A, et al: Pharmocodynamics and pharmacokinetics of carprofen, a nonsteroidal anti-inflammatory drug, in healthy cows with Escherichia coli endotoxin-induced mastitis. J Vet Pharmacol Ther 1991;14:219-229.

Progesterone assay using variable collection tubes and variable storage techniques

William Schultz, Joe Schultz
Schultz Veterinary Clinic, Okemos, MI

Abstract

Serum progesterone testing is frequently used in veterinary practice to indirectly but accurately predict the day of ovulation in bitches for breeding. Semi-quantitative testing methods may include counter-top testing and more advanced quantitative testing. A recent study confirmed the accuracy of results between in-house laboratory and commercial laboratory quantitative testing.[1] Consistency of blood sample collection and processing is important. The purpose of this study was to determine the effect, if any, of blood collection tube type, sample handling and sample storage on the quantitative progesterone results, as determined by enzyme-linked fluorescent assay (ELFA).

Keywords: Progesterone, progesterone testing, ovulation timing, enzyme-linked fluorescent assay (ELFA).

Objectives and methods

Reliable testing of progesterone levels is useful in evaluation of reproductive events in bitches, such as the time of ovulation, the date of parturition, and so on.[2,3] Assessment of progesterone concentrations also helps in diagnosis and treatment of various reproductive disorders, such as pyometra, abnormalities of estrous cycle, ovarian remnant syndrome, diseases of pregnancy, and others.[4-6] A recent study by Fraser and colleagues[1] confirmed the accuracy of multiple types of quantitative progesterone testing methods, such as radioimmunoassay (RIA), chemiluminescence (CLIA) and enzyme-linked fluorescent assay/fluorescent enzyme immunoassay (ELFA/FEIA). The present study was designed to examine progesterone testing with the use of different variables, such as blood collection tube type, sample handling and sample storage. Within our practice, we routinely draw blood via jugular venipuncture using a 21-gauge needle and a 6cc syringe. We then place the blood in a red-top tube by removing the needle from the syringe, removing the top from the tube and gently placing the blood into the tube without the use of the vacuum. The blood is then allowed to clot for nine minutes and then centrifuged for nine minutes. After centrifugation, the serum is pipetted off the clot and transferred to another tube. The serum is then either tested immediately, or placed in the refrigerator at 35°F, pending testing. Serum progesterone of samples was measured using a mini VIDAS™ progesterone machine (BioMerieux Inc., Street, Durham, NC), which utilizes enzyme-linked fluorescent assay principals.[1] This protocol of blood collection and sample handling was followed for the present study. Testing was reviewed using one-way analysis of variance (ANOVA). Differences of $p < 0.05$ were considered significant.

Study 1

For the initial study we tested five samples and ran each sample four times in order to determine the repeatability of the test. All of the four tests were performed at the same time. All samples were collected with the methodology described above. Test results are summarized in Figure 1. Figure 2 demonstrates the results of statistical analysis of the test results.

Sample	Run 1	Run 2	Run 3	Run 4
1	7.88	7.69	7.40	8.42
2	1.75	1.77	2.03	1.70
3	4.68	4.84	4.52	4.98
4	0.46	0.42	0.45	1.00
5	22.81	21.89	22.86	23.21

Figure 1. Test repeatability results, all values are in nanograms (ng)/ml

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.79756	3	0.265853	0.003334	0.999723	3.238872
Within Groups	1275.768	16	79.7355			
Total	1276.566	19				

Figure 2. Statistics for the multiple runs of blood samples tested in Study 1.

No difference was observed in progesterone levels among the four replicates (p=0.9997). Processing of the samples using similar collection methods, sample handling and assay evaluation at the same time provides confidence in result measurement consistency.

Study 2

Good laboratory technique presupposes high consistency in the reported results.[1] Several studies have shown differences in the reported results with the use of serum separator tubes.[7-10] A more recent study by Tahir et al., conducted with silicone, lithium heparin and EDTA blood collection tubes, has demonstrated that the type of tube did not interfere with the progesterone test results.[11] Our second study involved the use of various tube types for the blood handling, i.e., serum, serum separator, lithium heparin and EDTA tubes. All samples were obtained using the described collection technique. It is not uncommon for clinics not versed in progesterone testing to use the first tube accessible at the time. It has been demonstrated, however, that improper tube choice results in unacceptable errors in progesterone measurement.[7] Figure 3 summarizes the results of sample collection performed utilizing four different types of tubes, such as serum, serum separator, lithium heparin and EDTA. Figure 4 illustrates the analysis of variance of the test results.

Sample	Serum	Serum Separator	Lithium Heparin	EDTA
1	9.11	7.69	8.27	10.71
2	3.31	2.02	2.73	2.89
3	2.32	2.56	3.16	3.16
4	4.22	3.55	4.39	5.04
5	3.04	2.71	3.35	3.17

Figure 3. Test results for various collection tubes utilized in Study #2, values are in nanograms (ng)/ml

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.15636	3	1.385453	0.193059	0.89958	3.238872
Within Groups	114.8212	16	7.176328			
Total	118.9776	19				

Figure 4. ANOVA results for Study 2 that involved blood sampling with various collection tube types

Both Figure 3 and Figure 4 demonstrate that progesterone levels for serum, serum separator, heparin and EDTA collection tubes were not statistically different (p=0.8996). Some studies report inconsistent results with variable tube type use.[7-10] In this test where the mini VIDAS™ was utilized, we found no significant changes in the progesterone levels, regardless of the blood collection tube type.

Study 3

In the next study blood samples were placed exclusively in red-top tubes. The blood was collected and processed as previously described; however the time from collection to centrifugation was varied. Each blood sample was immediately divided into two aliquots. One was allowed to clot for nine minutes, while the second fraction was allowed to clot for two hours at room temperature (72°F) before

centrifugation and progesterone measurement. The concern was that repeatability of sample testing may be affected by inconsistent laboratory technique for sample handling and storage.

The initial nine minute clotted sample was then divided into three aliquots after centrifugation. One remained at room temperature (72°F) for two days, one was refrigerated for two days at 35°F and one was frozen for two days at 7°F. A normal kitchen refrigerator was used for storage. All samples were tested for progesterone at the same time. The measured progesterone levels are demonstrated in Figure 5.

Sample	9-minute clot	2-hour clot	Counter 2 days	Fridge 2 days	Freezer 2 days
1	23.59	22.96	23.33	26.71	25.72
2	3.90	3.67	4.16	4.67	4.64
3	16.04	15.71	17.43	17.80	19.32
4	0.58	0.80	0.66	0.81	0.83
5	3.50	3.22	2.99	3.29	3.18

Figure 5. Variability in progesterone levels when different handling times and storage methods are used, values are in nanograms (ng)/ml.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.15625	1	0.15625	0.001652	0.968578	5.317655
Within Groups	756.8168	8	94.602095			
Total	756.973	9				

Figure 6. Results of the analysis of variance in progesterone levels for 9-minute clot and 2-hour clot

Figure 6 shows that progesterone levels did not differ between nine minute and two hour clot times (p=0.9686).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.237773	2	1.618886667	0.013982	0.986131	3.885294
Within Groups	1389.356	12	115.7796767			
Total	1392.594	14				

Figure 7. Statistical results for two day testing of counter, refrigerator and freezer samples
Results presented in Figure 7 demonstrate no significant difference in progesterone levels between two day counter, two day refrigerator and two day freezer samples.

Conclusion

The results of the current study suggest that the tube type, sample handling and sample storage have little to no effect on the outcome of progesterone testing. This by no means indicates that poor laboratory technique is acceptable. Consistency in sample handling and testing technique is important for accurate results. Our laboratory continues to time all samples and either test immediately after the sample collection, or store samples in the refrigerator at 35°F, pending testing.

References

1. Fraser N, Wilborn RR, Schultz WE, et al: Comparative progesterone assay. Clin Therio 2015;7:207-210.
2. Rota A, Vannozzi I, Marianelli S, et al: Laboratory and clinical evaluation of a FEIA method for canine serum progesterone assay. Reprod Domest Anim 2016;51:69-74.
3. Smith FO: Challenges in small animal parturition: timing elective and emergency cesarean sections. Theriogenology 2007;68:348-353.
4. Meyers-Wallen VN: Unusual and abnormal canine estrous cycles. Theriogenology 2007;68:1205-1210.

5. Sontas BH, Schwendenwein I, Schäfer-Somi S: Primary anestrus due to dietary hyperthyroidism in a miniature pinscher bitch. Can Vet J 2014;55:781-785.
6. Krauss Z, Krauss M: Magnetic resonance imaging and laparoscopic management of an ovarian remnant in a bitch. Tierärztl Prax 2015;43:111-114.
7. Dasgupta A, Dean R, Saldana S, et al: Absorption of therapeutic drugs by barrier gels in serum separator blood collection tubes. Volume- and time-dependent reduction in total and free drug concentrations. Am J of Clin Pathol 1994;101:456-461.
8. Ferry JD, Collins S, Sykes E: Effect of serum volume and time of exposure to gel barrier tubeson results for progesterone by Roche Diagnostics elecsys 2010. Clin Chem 1999;45:1574-1575.
9. Hilborn S, Krahn J: Effect of time of exposure of serum to gel-barrier tubes on results for progesterone and some other endocrine tests. Clin Chem 1987;33:203-204.
10. Smith RL: Effect of serum-separating gels on progesterone assays. Clin Chem 1985;31:1239.
11. Tahir MZ, Thoumire S, Raffaelli M, et al: Effect of blood handling conditions on progesterone assay results obtained by chemiluminescence in the bitch. Domest Anim Endocrinol 2013;45:141-144.

Neoplastic conditions associated with spay/neuter status in the canine

C. Scott Bailey
College of Veterinary Medicine, North Carolina State University, Raleigh, NC

Neoplasia, including malignant and non-malignant tumors, represents the single most important group of diseases in both veterinary and human medicine today. Recent studies have shown that cancer is the cause of death for 15-30% of dogs and 26% of cats. As a consequence, a large body of research focuses on the causes, diagnosis and treatment of this diverse group of conditions.

As the study of cancer in pets evolved, it was quickly recognized in the 1960's that some cancers were related to the presence or absence of a dog's reproductive tract. A series of studies demonstrated that vaginal tumors, uterine tumors, ovarian tumors and mammary tumors could all be substantially reduced or eliminated by removal of the ovaries and uterus or of the ovaries alone.[1,2] As a consequence of this work, and the wide-spread adoption of spay-neuter programs in the USA, these tumors represent a very small percentage of disease in this country. In contrast to this, mammary tumors represented the most common tumor submitted for histopathology in a Norwegian study. Mammary tumors accounted for 30% of submissions - approximately 9-13% of dogs - and 94% were malignant or pre-malignant based on modern criteria.[3] Separately, mammary tumors were among the two main reimbursement claims in female dogs (the other was pyometra) in a recent Swedish study.[4]

However, while this stark difference may be interpreted as a success for US veterinarians, recent studies in regard to other tumors have demonstrated that ovariohysterectomy has the opposite effect on a number of other important tumors, including osteosarcoma, hemangiosarcoma, cardiac tumors, mast cell tumors and lymphoma.[5-11] These data are widely available to owners and veterinarians and may be a source of confusion and frustration to both veterinarians and owners considering gonadectomy. There is an urgent need for veterinarians to develop scientifically sound recommendations that are sufficiently nuanced to educate owners and guide them in a decision-making process regarding their animal. There is further an urgent need for veterinarians to be prepared to discuss the benefits and risks of surgical gonadectomy with public interest groups and policy makers in order to serve as effective advocates for animal and human welfare in the USA.

Only mast cell tumors approach the incidence of mammary tumors, accounting for 16-21% of cutaneous tumors (approximately 2.5-3% of dogs) in several studies from the USA,[7] and 3-11% of tumors across all age groups in Norway.[3] Across all breeds, the other tumors discussed affect a very small proportion of dogs (0.2-1% each).

However, within specific breeds, the risk of developing specific tumors can be substantially higher. In Rottweilers, the incidence of osteosarcoma has been reported between 12-25% and the relative risk of diagnosis with mammary tumors or osteosarcoma was 100:1144, according to Gamlem and co-workers.[3,5-6] In contrast, the relative risk of the same tumors on the Dachshund was 194:73.[3] In addition to this source of variation, there are two other major sources of variation based on tumor type: differences in gender predilection toward tumors, and differences in response of tumors to ovariohysterectomy/neutering. In regard to the latter, mammary tumors are dramatically more common in female dogs than male dogs, and there is convincing evidence that removal of the gonads early in life results in the greatest reduction of tumor development (95%), with decreasing benefits as the animal ages to approximately a 25% reduction in tumor development after the third heat.[12-14] In contrast, delaying ovarian removal beyond 1-4 years of age attenuates the increased risk of osteosarcoma in spayed Rottweilers[5,6] A third response pattern for tumor-development is seen with several other tumors. Several studies have shown an increased risk for hemangiosarcoma and mast cell tumor in dogs spayed after seven to 12 months, compared to either dogs spayed early or intact dogs, whereas the effect of age at spay has been variable across breeds for lymphosarcoma.[10,11]

These complicated relationships between gender, gonadal status, breed and tumor risk need to be addressed through proactive conversations between owners and veterinarians. The primary factors that should be accounted for when deciding if and when to perform a spay/neuter procedure are:

- Owner's willingness to accommodate for normal reproductive physiology and behavior, and the owner's ability to prevent unwanted breedings.
- Breed of dog
- Breed predisposition to development of specific tumor types or other complications
- Relative morbidity/mortality associated with specific tumor types

Other factors that should also be taken into consideration are:

- "Family history" of lines within a given breed in regard to conditions known to be related to spay
- Purpose of the dog
- Health status of the dog prior to surgery

Unfortunately, in many cases, insufficient information is available to make clear recommendations for different breeds. Even when information is available, it is often incomplete, or the research is subject to bias based on study design and the limitations of retrospective research.[15] In addition, differing statistical terminology and methods complicate interpretation of data across studies.[5,10-11] As a consequence, it should be understood that in many cases a recommendation can only be made based on clinical experience, extrapolation from better-studied breeds and with significant input from owners. Generalizations, both by the veterinary community and by breeders/owners may not prove accurate as research in this field evolves and may cause significant harm to individual animals. They should be used only as a starting point for discussion. In the Table is a broad summary of relative risk for common tumor types and effect of neuter on the incidence.

Table. Summary of relative risk for common tumors.

	Mammary tumor risk/ risk for malignancy	Bone tumor risk	Vascular tumor risk	Lymph tumor risk	Cutaneous tumor risk
All Breeds	9-25% intact; 50-94% malignant;	0.3% intact; 98% malignant	0.3% intact; 87% malignant	0.3% intact; 100% malignant	15% intact; ~1% MCT
Effect of spay-status	25-95% reduction in tumor dev.	1.3-5 fold increase	2-9 fold increase	2-4 fold increase	2-4 fold increase (MCT)
Extrapolated risk in spayed animals	0.9-6%	0.4-5%	0.6-2.6%	0.6-1.2%	15-20%; 4% MCT

Additional information about relative risk of disease among breeds is not readily available from any source. Specific retrospective studies have now been completed on some tumors for the Rottweiler, Golden Retriever and Vizla in the USA, while Gamlem and co-authors described the relative risk of tumor development among breeds in Norway.[3-5,8,10,11] Together with this, veterinary experience and knowledge of historical disease within a line of dogs can guide a decision-making process to determine when or whether to neuter dogs. Across breeds, removal of ovarian hormones has a strong benefit of reducing the lifetime risk of mammary tumors and pyometra. These conditions affect a large proportion of un-spayed females (mammary tumors 13% by age 10, pyometra 19% by age 10) and are associated with mortality of 50% or higher.[12-14,16] Within breeds, the incidence of specific tumors, as well as other conditions affecting animal health, may outweigh this benefit. To exemplify this, two breeds in which recent research has characterized the relative tumor risks are discussed below:

In Vizlas (breed specific risk for mammary tumors was not reported), the frequency of reported cancer was 24% of animals, with frequencies of 5.9%, 2.8% and 1.8% for mast cell tumor, hemangiosarcoma and lymphoma, respectively.[11] The risk for each of these specific

tumor-types was elevated in spayed animals and the onset of cancer in general was earlier for spayed animals. Cumulatively, the risk for these tumors, which may have a higher mortality and earlier onset than mammary tumors, may outweigh the risk of mammary tumor development in intact Vizlas. Furthermore, the tumor-types studied in this breed appear to be most common after delayed ovariohysterectomy or castration. Thus, recommendations to delay sterilization beyond the first heat or first year of age may not be beneficial. To be able to make a sound recommendation, the breed-specific risk for mammary tumors should be assessed.

In Rottweilers, the risk of dying due to osteosarcoma is as much as 2x higher than the risk of dying due to mammary tumors.[4,6] Furthermore, benefits of ovarian hormones for tumor prevention have been recognized up to one to four years of age[5,6] whereas pyometra and mammary tumors commonly are seen after that age.[4] Thus, animals can be spayed prior to the most common onset of reproductive conditions and after benefits of ovarian hormones have had an effect. This approach will reduce the likelihood of an animal developing pyometra, provide some (25%) reduction in development of mammary tumors, while maximizing the preventive effect of ovarian hormones of osteosarcoma development.

In conclusion, it is increasingly recognized that the approach of "individualized medicine" is particularly important in regard to a question that was long considered settled among the veterinary community, namely whether animals benefit from surgical sterilization. As researchers expand the body of knowledge, easy answers may not be readily available and a recommendation to spay or castrate should be made after consideration of health-risks associated with specific breeds or lines of dogs, and in conversation with the owner.

References

1. MV Root Kustritz: Effects of surgical sterilization on canine and feline health and on society. Reprod Domest Anim 2012;47(Suppl 4):214-222.
2. IM Reichler: Gonadectomy in cats and dogs: a review of risks and benefits. Reprod Domest Anim 2009;449(Suppl 2):29-35.
3. Gamlem H, Nordstoga K, Glatter E: Canine neoplasia-introductory paper. APMIS Suppl 2008;125:5-18.
4. Jitpean S, Hagman R, Holst B et al: Breed variations in the incidence in mammary tumors and pyometra in swedish dogs. Reprod Domest Anim 2012;47(Suppl 6):347-350.
5. Waters DJ, Kengeri SS, Clever B, et al: Exploring the mechanisms of sex differences in longevity: ovarian influence on exceptional longevity in dogs. Aging Cell 2009;8:752-755
6. Cooley DM, Beranek BC, Schlittler DL, et al: Endogenous gonadal hormone exposure and bone sarcoma risk. Cancer Epidemiol Biomarkers Prev 2002;11:1434-1440.
7. White C, Hohenhaus A, Kelsey J, et al: Cutaneous MCTs: associations with spay/neuter status, breed, body size, and phylogenetic cluster. J Am Anim Hosp Assoc 2011;47:210-216.
8. Hart BL, Hart LA, Thigpen AP, et al: Long-term health effects of neutering dogs: comparison of Labrador Retrievers with Golden Retrievers. PLoS ONE 2014; 9: e102241. doi:10.1371/journal.pone.0102241
9. Smith A: The role of neutering in cancer development. Vet Clin North Am Small Anim Pract 2014; 44:965-975
10. Torres de la Riva G, Hart BL, Farver TB, et al: Neutering dogs: effects on joint disorders and cancers in Golden Retrievers. PLoS ONE 8(2): e55937. doi:10.1371/journal.pone.0055937
11. Zink MC, Farhoody P, Elser SE, et al: Evaluation of the risk and age of onsetof cancer and behavioral disorders in gonadectomized Vizslas. J Am Vet Med Assoc 2014;244:309-319.
12. Dorn CR, Taylor DO, Schneider R, et al: Survey of animal neoplasms in Alameda and Contra Costa Counties, California. II. Cancer morbidity in dogs and cats from Alameda County. J Natl Cancer Inst 1968;40:307-318.
13. Moe L: Population-based incidence of mammary tumors in some dogs breeds. J Reprod Fertil 2001;Suppl 57:439-443.
14. Kristiansen VM, Nødtvedt A, Breen AM, et al: Effect of ovariohysterectomy at the time of tumor removal in dogs with benign mammary tumors and hyperplastic lesions: a randomized controlled clinical trial. J Vet Intern Med 2013;27:935-942.
15. Beauvais W, Cardwell JM, Brodbelt DC: The effect of neutering on the risk of mammary tumours in dogs –a systematic review. J Small Anim Pract 2012;53:314-322.
16. Sorenmo KU, Kristiansen VM, Cofone MA et al: Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence. Vet Comp Oncol 2009;7:162-172.

Non-cancerous conditions associated with spay/neuter status in the canine

C. Scott Bailey
College of Veterinary Medicine, North Carolina State University, Raleigh, NC

Gonadecotmy (GX) represents the most common set of surgeries performed in small animal veterinary medicine in the USA. The most common surgeries in bitches are ovariohysterectomy (OHE) or ovariectomy (OX) via ventral midline approach, or via laparoscopy. The most common surgery in dogs is castration (CX) via either prescrotal or scrotal approach. These are the procedures with which students are routinely taught surgery skills and are considered extremely safe in the USA. However, in the past two decades, there has been increasing awareness of the potential ramifications of these surgeries on individual animals and much discussion both within and outside the veterinary community has focused on the potential risks associated with gonadectomy or leaving an animal intact.[1-4]

The primary societal pressure to perform GX surgeries in the USA is the continuing overpopulation of unwanted dogs and cats and the perception that routine GX will reduce this population.[5,6] Researchers found that intact animals were at greater risk for relinquishment[7] and unwanted offspring from owned and feral animals represent a major factor contributing to the population of shelter-animals.[5] Supporting the efficacy of spay-neuter programs that were established in the late 1970s and 1980's, several studies have documented declining trends in shelter intake and animals euthanized during the past two decades,[6,8] mirrored by increasing prevalence of neutered animals in private households in the USA. Recent studies suggest that 64-75% of dogs in the USA are neutered.[3,5]

In addition, GX is frequently performed to promote individual animal health. Several studies examining longevity and health in dogs found that neutered animals live longer and are less likely to suffer from serious reproductive diseases, such as pyometra and non-cancerous prostatic disease, mammary and reproductive tumors of females, roaming and associated traumatic events, disorders related to pregnancy or parturition and unwanted hormone-associated behavior.[1,9,10]

In contrast, numerous recent publications have identified specific complications associated with GX in individual animals and the effectiveness of spay/neuter programs for population control has been difficult to document, with conflicting results among studies.[1,2,6,8]

Because of the conflicting and confusing data available, there is an urgent need for veterinarians to have a nuanced approach to recommendations and to be prepared to discuss the benefits and risks of surgical GX with owners, public interest groups and policy makers. We herein make an effort to succinctly summarize the most important data regarding non-neoplastic health conditions affected by neuter-status. Neoplastic conditions will be addressed in a separate review in this journal and are not the focus of this paper.

Major non-neoplastic conditions that have been linked with neuter-status include the following: pyometra, surgical complications, ovarian remnant syndrome, behavioral problems/anxiety, prostatic disease, urinary incontinence, cystitis, obesity, hip dysplasia and cruciate ligament rupture.[4,11-21] In addition, numerous other conditions have been reported anecdotally, but are not as well supported in the literature.

Estimated incidence, effect of neuter and breed predispositions of selected conditions described in the literature have been summarized in the Table .

Keywords: Ovariohystorectomy, spay, neuter, risk,

Table. Effect of neuter and breed predispositions.

	Population most affected	Estimated incidence	Breed predisposition	Effect of neuter	Effect of early neuter
Perioperative complications	F/S	6-27%	Large dogs		unknown
Ovarian remnant syndrome	F/S	1-2%	Large Dogs		unknown
Pyometra	F/I	25% by age 10		90+% reduction	no effect
Urinary incontinence	F/S	2-20%	Boxer, Doberman, Rottweiler, Old English Sheepdog Giant Schnauzer	5+ fold increase	unknown
Urolythiasis: struvite	F/C	0.3-1%	Spaniel, Pekingese, German Shepherd Dog, Dachshund	7 fold increase	unknown
Urolythiasis: Oxalate	M/C	0.3-1%	Small, Toy Breeds	7 fold increase	unknown
Cystitis (recurrent)	F/S	14% (0.3%)	German Shepherd Dog, Dachshund, Doberman Pincher, Spaniel, Golden Retreiver	3-5 fold increase	unknown
Benign prostatic hyperplasia	M/I	50-80%	Unknown	90+% reduction	risk decreased
Joint Disorders	F/S, M/C	3-25%	Golden retriever, Boxer German Shepherd Dog, St. Bernard, Laborador retriever	1.5-5 fold increase	risk increased
Cognitive dysfunction	M/C			Increased progression	unknown
Inappropriate urination	M/I	3-8%	Beagle, Bichon Frise, Cocker Spaniel	reduction	no effect
Roaming	M/I			reduction	no effect
Aggression	M/I, M/C, F/C	5-20%	English Springer Spaniel, German Shepherd Dog, Mixed	Inter-dog aggression reduced; Aggression toward family member increased	No effect; risk increased
Anxiety-related behavior	F/C, M/C	7-10%	Vizsla, German Shepherd, Beagle, Golden Retreiver, Laborador Retreiver, Mided	2-4 fold increase	risk increased
Obesity	F/C, M/C	3-5%		3-20 fold increase	uknown

Rough calculation based on these data would suggest that GX increases the risk of an animal suffering from one or more of the disease above by approximately 20-30%, with urinary incontinence, cystitis, joint disorders and behavior being the most significant factors for longterm survival and quality

of life. This contrasts with intact animals, which are at risk for fewer diseases, but at a higher rate. Bitches are frequently affected by pyometra, while dogs are most affected by prostatic disease, with a 25% and 80% incidence, respectively.[1,2,11,22]

In addition to these health related factors, social factors must be weighed. An owner's inability to control an intact animal in order to prevent unwanted pregnancy or willingness to manage reproduction-related conditions may outweigh health concerns in many instances. On the other hand, history with orthopedic disease or chronic incontinence in a previous pet may lead other owners to delay neuter, understanding the consequences of increased security and preventive veterinary measures as the animal ages. The behavioral data present an additional conundrum. Behavioral problems represent one of the most common reasons for animal relinquishment to a shelter (40% of dogs).[3,5] The most common behavioral problems cited are aggression toward people or dogs, destructive behavior and inappropriate elimination.[3,5] Of these, inter-dog aggression has been shown to be positively affected by GX, while aggression toward people and anxiety related conditions (including submissive urination, separation anxiety and storm phobias) were increased in neutered populations and may further be exacerbated by early neuter.[14,23-26] Thus, while sterilization is an important component of population control, routine recommendation of early GX may actually increase an animal's risk of relinquishment to a shelter.[14]

In many instances, it is likely that no "ideal" choice exists and owners should be aware of the potential complications associated with either GX or leaving an animal intact. Further, as more data become available regarding the relationship between genetics, age at GX and onset of disease, the recommendation may be different for different individuals within a single household. Veterinarians are best suited to help their clients understand material read on the internet and elsewhere and are ideally situated to advise special interest groups and policy makers regarding animal health and welfare. To this end it is vital that veterinarians be able to present balanced and scientifically sound information on the health-effects of neutering, behavioral expectations of neutered animals and effective population management techniques.

References

1. Root Kustritz MV: Effects of surgical sterilization on canine and feline health and on society. Reprod Domest Anim 2012;47(Suppl 4):214-222.
2. Reichler IM: Gonadectomy in cats and dogs: a review of risks and benefits. Reprod Domst Anim 2009;44(Suppl 2):29-35.
3. Trevej R, Yang M, Lund EM: Epidemiology of surgical castration of dogs and cats in the United States. J Am Vet Med Assoc 2011;238:898-904
4. Muraro L, White RS: Complications of ovariohysterectomy procedures performed in 1880 dogs. Tierarztl Prax Ausg K Kleintiere Heimtiere 2014;42:297-302.
5. Marsh P: Replacing myth with math: using evidence-based programs to eradicate shelter overpopulation. Concord(NH): Town and Country Reprographics; 2010.
6. Scarlett J, Johnston N: Impact of a subsidized spay neuter clinic on impoundments and euthanasia in a community shelter and on service and complaint calls to animal control. J Appl Anim Welf Sci 2012;15:53-69.
7. Patronek GJ, Glickman LT, Beck AM, et al: Risk factors for relinquishment of dogs to an animal shelter. J Am Vet Med Assoc 1996; 209:572-581.
8. Morris KN, Gies DL: trends in intake and outcome data for animal shelters in a large U.S. metropolitan area, 1989 to 2010. J Appl Anim Welf Sci 2014;17:59-72.
9. Hoffman JM, Creevy KE, Promislow DEL: Reproductive capability is associated with lifespan and cause of death in companion dogs. PLoS ONE 2013;8: e61082.
10. Neilson JC, Eckstein RA, Hart BL: Effects of castration on problem behaviors in male dogs with reference to age and duration of behavior. J Am Vet Med Assoc 1997;211:180-182.
11. Hagman R, Lagerstedt AS, Hedhammer A, et al: A breed-matched casecontrol study of potential risk-factors for canine pyometra. Theriogenology 2011;75:1251-1257.
12. de Bleser B, Brodbelt DB, Gregory NG, et al: The association between acquired urinary sphincter mechanism incompetence in bitches and early spaying: a case-control study. Vet J 2011;187:42-47.
13. Ball R, Birchard SJ, May LR, et al: Ovarian remnant syndrome in dogs and cats: 21 cases (2000–2007). J Am Vet Med Assoc 2010;236:548-553.
14. Spain CV, Scarlett JM, Houpt KA: Long-term risks and benefits of early-age gonadectomy in dogs. J Am Vet Med Assoc 2004;224:380-387.

15. Thompson MF, Litster AL, Platell JL, et al: Canine bacterial urinary tract infections: new developments in old pathogens. Vet J 2011;190:22-27.
16. Seguin AM, Vaden SL, Altier C, et al: Persistent urinary tract infections and reinfections in 100 dogs(1989–1999). J Vet Intern Med 2003;17:622-631.
17. Arnoldn S, Hubler M, Reichler I: Urinary incontinence in spayed bitches: new insights into the pathophysiology and options for medical treatment. Reprod Domest Anim 2009; 44(Suppl 2):190-192.
18. Okafor CC, Pearl DL, Lefebvre SL et al: Risk factors associated with struvite urolithiasis in dogs evaluated at general care veterinary hospitals in the United States. J Am Vet Med Assoc. 2013;243:1737-1745.
19. Okafor CC, Lefebvre SL, Pearl DL, et al: Risk factors associated with calcium oxalate urolithiasis in dogs evaluated at general care veterinary hospitals in the United States. Prev Vet Med 2014;115:217-228.
20. Torres de la Riva G, Hart BL, Farver TB, et al: Neutering dogs: effects on joint disorders and cancers in golden retrievers. PLoS ONE 2013;8: e55937.
21. Witsberger TH, Villamil JA, Schultz LG, et al: Prevalence of and risk factors for hip dysplasia and cranial cruciate ligament deficiency in dogs. J Am Vet Med Assoc 2008;232:1818-1824.
22. Berry SJ, Coffey DS, Strandberg JD, et al: Effect of age, castration, and testosterone replacement on the development and restoration of canine benign prostatic hyperplasia. Prostate 1986;9:295-302.
23. Hart BJ: Effect of gonadectomy on subsequent development of age-related cognitive impairment in dogs J Am Vet Med Assoc 2001;219:51-56.
24. Bamberger M, Houpt KA: Signalment factors, comorbidity, and trends in behavior diagnoses in dogs: 1,644 cases (1991–2001). J Am Vet Med Assoc 2006;229:1591-1601.
25. O'Farrell V, Peachy E: Behavioural effects of ovariohysterectomy on bitches. J Small Anim Pract 1990;31:595-598.
26. Zink MC, Farhoody P, Elser SE, et al: Evaluation of the risk and age of onset of cancer and behavioral disorders in gonadectomized Vizslas. J Am Vet Med Assoc 2014;244:309-319

When sooner is better than later

Frances O. Smith
Smith Veterinary Hospital, Inc., Burnsville, MN

Abstract

Clinicians are commonly consulted regarding parturition in the bitch and are questioned regarding the length of gestation, labor and delivery. The client of today wants and expects a positive outcome for the health of the bitch and her puppies with minimal impact on the lifestyle of the breeder. In human obstetrics, induction of labor is commonly performed oftentimes for the preference of the mother and sometimes for the convenience of the medical team involved. This paper explores human labor induction versus parturition induction in the bitch and outlines rationale, technique and outcomes.

Keywords: Induction, parturition, bitch, aglepristone, misoprostol, cervix

A recent study on the risk of cesarean section and any maternal and /or perinatal effects of induction of labor was published in the *American Journal of Obstetrics and Gynecology*. Each of these pregnancies was a singleton in an uncomplicated full-term gestation where full-term was defined as a mother who was 39 weeks to 40 weeks gestation with intact membranes. Five randomized controlled studies including 844 women were analyzed. Primary outcome was the incidence of cesarean delivery. The incidence of cesarean delivery was similar between the groups with labor induction versus the control group (routine expectant management) 9.7% vs 7.5%, respectively. Induction of labor and the control group had similar rates of chorioretinitis but statistically less blood loss occurred in the induction group. In this study induction of labor at full term in uncomplicated singleton gestation did not results in increased risk of cesarean delivery.

An additional study involving induction of labor in twin pregnancies in Sweden yielded much different results. In this study of 462 twin pregnancies 220(48%) women had induction of labor and 242 (52%) experienced spontaneous onset of labor. Labor inductions were performed by amniotomy in 149(68%), oxytocin administration 11(5%) and cervical ripening in 60(27%). The rate of cesarean sections was 21% for induced labor and 12 % in spontaneous labor. Absolute risk of cesarean section following induction was 15% with amniotomy, 36% with oxytocin and 37% with cervical ripening. Thus induction increased the risk of cesarean section by 90% versus spontaneous labor in this group. Women with a previous cesarean section have in increased risk of uterine rupture when labor is induced. There is insufficient information available from randomized controlled trials to assess the optimal method of induction of labor in women with a previous cesarean delivery. In 2006, The American College of Obstetrics and Gynecology issued a bulletin stating that misoprostol never be used for this purpose as a significant increase in the incidence of uterine rupture could occur.

The American Congress of Obstetricians and Gynecologists have recommendations regarding the selection of cases that are appropriate for induction of labor. The rate of labor induction in the United States is 22% and has more than doubled since 1990. The rate of cesarean section in the US and Canada is 32% and has increased 50%in the US from 1996 to 2006. Prior to induction the maternal-fetal status should be assessed, the status of the cervix evaluated, and a full term pregnancy (at least 39 weeks) of gestation should be confirmed. The following conditions can be an indication for induction of labor when present: abruptio placentae, chorioamnionitis, fetal compromise, fetal death, gestational hypertension, maternal gestational diabetes of chronic kidney disease, pre eclampsia or eclampsia, premature rupture of membranes and postterm pregnancy. Logistical reasons such as distance from health care facilities or psychosocial conditions are also considered. Contrindications are prematurity, vasa previa, complete placenta previa, umbilical cord prolapse and active genital herpes.

Induction of parturition is relatively common in multiple livestock species. In swine, induction of parturition is used to ensure that there will be attendants present to assist during the farrowing time in case problems occur and to allow scheduling in farrowing facilities. Most sows will farrow within two days of

a mean gestation period of 115 days (from day 1 of estrus). Sows induced prior to 110 days of gestation may be subject to decreased piglet viability therefore accurate breeding records are essential.

Prostaglandin F2alpha (PGF) is approved in the United Sates for induction of parturition in swine within three days of expected farrowing. Synchrony of farrowing is improved if sows are given oxytocin 20-24 hours after administration of PGF. Sows are typically given 10mg dinoprost followed by 5-30 IU oxytocin 20 hours later. A high percentage of sows will farrow six hours later. In does, parturition is rarely induced except in cases of pregnancy toxemia/ketosis. Parturition can be induced by the use of prostaglandin if the doe is within two weeks of her expected kidding date. Induction of parturition in ewes can be done if the breeding date is known within an accuracy of hree days. Ewes may be induced with either dexamethasone IM or betamethasone IM after day 137 of pregnancy. Induction after day 142 results in improved fetal viability. Lambing usually occurs within 36 to 60 hours after induction. In cattle, dinoprost has been used for induction of parturition on or after 270 days of pregnancy. Indications for induction are convenience and suspected fetal oversize.

In the mare, the indications for induction of parturition are medical reasons such as rupture of the prepubic tendon, prolonged gestation, previous pelvic fracture with known compromise of the pelvic canal and convenience which can result in significant labor savings plus the opportunity for early intervention in the case of a dystocia. Parturition induction has been utilized in research projects and as a teaching tool. As with other species gestation length is a vital factor in a successful outcome for parturition induction. The mare must be at least 320 days of gestation with greater that 330 being preferred. Milk calcium levels of at least 220 ppm aid in selection of appropriate candidates for induction. Mares foaling in January, February and March have longer gestations than mares foaling in April, May or June. The most common agent used to induce parturition in the mare is oxytocin administered by either bolus or IV infusion. The dose and route vary by clinician and by the degree of cervical relaxation. Fluprostenol has been used successfully to induce parturition in mares and causes less myometrial stimulation than PGF. Fenprostalene both with and without oxytocin has been used to induce parturition in the presence of appropriate mammary secretions. Prostaglandin F2 alpha has failed to induce parturition in the mare. It can cause very strong myometrial contractions and increase the risk of fetal death due to premature placental separation. Glucocorticoids have failed to induce parturition in the mare.

The bitch depends on progesterone secreted from the corpus luteum for the maintenance of pregnancy. The ovary appears to be the only source of progesterone in the bitch. Parturition appears to be influenced by the fetus through secretions of the fetal adrenal cortex. The fetal pituitary secretes adrenocorticotropic hormone which causes glucocorticoid secretions by the fetal adrenal cortex. The fetal glucocorticoids boost the production of estrogens in the placenta through induction of aromatizing enzymes which increase the production of prostaglandin. It has been hypothesized that crowding in the uterus causes stress and subsequent cortisol release resulting in the initiation of the parturition events. Clinicians are occasionally asked about induction of parturition in the bitch. Induction of parturition in the bitch should not be attempted only for convenience. Puppies from induced parturition are at risk of fetal prematurity with accompanying respiratory distress and poor survivability. Any of the drug protocols currently published for use in pregnancy termination in the bitch can be potentially used for induction of parturition. The key factor for a successful outcome is exquisite timing which requires a very accurate history and ideally confirmed ovulation data versus simply insemination data. Historically, it has been established that glucocorticoid administration to bitches may cause premature labor hence could be used to induce parturition. In marked contrast to the species in which parturition induction is more routine, the bitch is the only one who presents with large fetal numbers. In a study published in *Theriogenology* in 2000 a group of six Greyhound bitches were treated with cloprostenol using minisosmotic pump on day 57 of pregnancy. Parturition was associated with a decrease in plasma progesterone, a reduction in body temperature and an increase in plasma concentration of 13,14-dihyro=15keto-prostaglanin F2alpha. The first puppy was born 37.7+/12.9 hours after the start of treatment (range 28-46h). Duration of whelping was 15.7+/- 2.2 hours (range 10-24 hours). The litter size was 9.2±0.8 pups(range 6 to 12 pups) and the puppy survival rate was 6.0±0.8 per litter (range 4-9

pups). While the author of that paper concluded that this protocol resulted in the birth of healthy pups, with minimal or no side effects to the bitch, neither this author nor this author's clients would have considered this protocol successful due to the time lag between induction and whelping, the length of whelping and, most importantly, puppy survival.

Is it in, is it not or is it just on top

Frances O. Smith
Smith Veterinary Hospital, Inc., Burnsville, MN

Abstract

Breeders, owners and practitioners tend to believe that medications, parasiticides applied topically have no effect on reproduction and pregnancy. In fact, many of these products can be and are absorbed systemically and have either direct or indirect effects on the reproductive system and the endocrine systems. Topical medication can have either a temporary or a more long-term effect depending upon the length of administration and the age of the animal being treated.

Keywords: Topical medications, hypothalamic pituitary axis, reproductive toxicants, steroids, endocrine disruptors

Today many practitioners and owners prefer topical medications as an alternative to oral or injectable products. As with any medication, there are risks, benefits and contraindications for these products. It is very important that label directions and recommended applications be considered when making product selection. Equally important is addressing the potential reproductive status of any animal being treated with a topical medication as some of these products can have a negative effect on future reproductive ability.

Endocrine disrupting chemicals have major risks for humans by targeting different organs and systems in the body.[1] Multiple mechanisms are involved through estrogen receptors, nuclear receptors and steroidal receptors activation. Xenobiotics are compounds with pharmacologic, endocrinologic or toxicologically active substance which are not produced within the animal and are therefore foreign to the organism. Endocrine disturbances in humans result in breast cancer, ovarian problems, thyroid disease, testicular carcinoma, Alzheimer's, schizophrenia, nerve damage and obesity. These endocrine disrupting chemicals are present in pesticides, fuels and many elements that are in routine daily use.

Topical preparations such as antibiotic preparations, antifungals (zinc undecyclenate,) antivirals, capsaicin, sunscreens, topical corticosteroids, tanning lotions and vitamin A or E all have potential for toxicity. When using any topical preparations the package labeling and packaging insert require investigation. Of particular importance are the precautions and animal safety indications.

The hypothalamic-pituitary axis (HPA) of healthy dogs can be readily suppressed with any form of glucocorticoid. The HPA axis controls development, reproduction and aging in animals. Ocular, otic and other topical products containing any glucocorticoid can be absorbed systemically and result in suppression of the HPA for weeks. The duration of action of many glucocorticoids administered either intravenously or orally is well documented however this information is scarce as it relates to topical usage. In a study published in Endocrinology, Beagle dogs were administered an otic preparation containing dexamethasone for twenty-one days.[2] The treatment resulted in a marked suppression of resting plasma concentration within the first eleven days of treatment and these levels remained reduced during the entire treatment up to nineteen days. Additionally, significant increases in serum activities of alkaline phosphatase, gamma-glutamyl transferase, alanine transaminase and aspartate transaminase were detected. Eosinophils and lymphocytes were reduced. In this study cortisol levels and hematologic parameters returned to baseline seven days after treatment cessation while liver enzymes remained elevated.

One of the important functions of the HPA axis is to regulate reproduction. The hypothalamus is located in the brain and secretes gonadotropin releasing hormone (GnRH). In females follicle stimulating hormone (FSH) and luteinizing hormone (LH) activate the ovaries to produce estrogen and inhibin. In female dogs the positive feedback loop between estrogen and LH help to prepare the follicle in the ovary and the uterus for ovulation and implantation. Once ovulation occurs the ovary begins to produce progesterone to inhibit the hypothalamus and the anterior pituitary thus stopping the estrogen-LH positive feedback loop. In males the production of these hormones are similar but the effects are different.

Follicle stimulating hormone acts on Sertoli cells of the testicle in stimulate spermatogenesis. Luteinizing hormone acts on Leydig cells to stimulate steroidogenesis. The testosterone produced works on the Sertoli cells to stimulate spermatogenesis and on the hypothalamus and anterior pituitary to inhibit GnRH, LH and FSH secretion.

Glucocorticoids affect both gonadotropin and gonadal function. Administration of corticosteroids to male dogs inhibits gonadotropin secretion as evidenced by decreased circulating testosterone concentrations and testicular atrophy. Blood levels of testosterone are two to three times lower than the levels of testosterone required for sperm production. Thus you cannot give enough systemic testosterone or any other anabolic steroid to improve sperm production. Instead any exogenous steroid including topically applied but systemically absorbed corticosteroid will interfere with the HPG axis causing a decrease in hypothalamic activity and less testosterone in the testicle itself even though libido may be enhanced.[3] Prednisone has been shown to have deleterious effects. It may impair spermatogenesis resulting in decreased sperm numbers and motility which appears to be dose related.

In female dogs, the same corticosteroids can suppress the HPA access as evidenced by interruption of estrous cycle activity. Glucocorticoid administration topically and systemically absorbed can be considered one of many xenobiotic compounds. A xenobiotic compound by definition is a pharmacologically, endocrinologically or toxicologically active substance which is not produced endogenously and is therefore foreign to an organism. The effects of these compounds can range from no effect to irreversible sterility. Other possible outcomes included premature reproductive aging and possibly increased neoplasia. In a mature female the effects are more likely to be temporary .Exposure prepuberally can disrupt reproductive function either by delaying or accelerating puberty.

When selecting topical preparations for use in our patients, reproductive status and potential reproductive demand should be considered. In patients with reproductive futures, topical preparations that contain a corticosteroid with the least potency and the shortest duration of action after withdrawal should be selected. Dexamethasone, flumethasone, betamethasone and cortisone administration to dams has been associated with increased incidence of cleft palate and other congenital malformation and may induce premature labor and abortion in dogs.

For topical parasiticides, it is important to read the package insert and to identify those the products which have been tested safe for breeding animals. The indications, safety and precautions areas of the package insert are the most informative portion of the insert. Selamectin (Revolution®, Zoetis, Florham Park, NJ) is one product that has many applications that are useful in reproducing animals. It has limited efficacy against several tick species but is very effective for control of heartworm, roundworm, hookworm and fleas. While many other products may be safe even if not labeled for use in reproducing animals, the clinician recommending these products assumes responsibility for any extra-label usage.

Many of the oral medications prescribed for dogs and cats have not been tested for their effect on pregnancy or for the effect on fetal development. Antifungal drugs are known to be teratogenic, fluoroquinolones can inhibit cartilage formation, tetracyclines and inhibit dental enamel production, chloramphenicol can suppress bone marrow, and aminoglycosides may be neurotoxic. As would be expected chemotherapeutic agents can be teratogenic or induce abortion. Cimetidine can decrease androgen production which may contribute to cryptorchidism. Exogenous hormone administration by any route may affect sexual development. Glucocorticoids may cause fetal anasarca, cleft palates and may induce abortion. Pain medications should be used with caution - opioids are the analgesics of choice during pregnancy. The US Food and Drug Administration have developed a table which categorizes drugs into five categories based on the effects of each on pregnancy in women. Whenever a drug choice is made the risk versus benefit ration should be considered.[4]

References

1. Maqbool F, Mostafalou S, Bahadar H, et al: Review of endocrine disorders associated with environmental toxicants and possible involved mechanisms. Life Sci 2016;145:265-273.
2. Abraham G, Gottschalk J, Ungemach FR: Evidence for ototopical glucocorticoid-induced decrease in hypothalamic-pituitary-adrenal axis response and liver function. Endocrinology 2005;146:3163-3173.

3. Ellington J, Wilker C: Reproductive toxicology of the male companion animal. In: Peterson M, Talcott P, editors. Small animal toxicology. Philadelphia: Saunders; 2001. p. 351-370.
4. Wilker C, Ellington J: Reproductive toxicology of the female companion animal. In: Peterson M, Talcott P, editors. Small Animal Toxicology. Philadelphia: Saunders; p. 330-350.

Fetal loss in small ruminants

D.G. Pugh
Alabama Department of Agriculture and Industry; Auburn, AL

The loss of the conceptus anytime during gestation is referred to as fetal loss or wastage, or abortion. It is usually detected only during the final two months of gestation. Fetal loss may be associated with infectious conditions, or environmental or maternal factors. Sheep are not completely luteal dependent during middle and late gestation, while goats and camelids have prolonged luteal dependency. Clinical signs of embryonic or early fetal loss may include return to estral behavior, unobserved fetal loss or observation of a blood-tinged vaginal discharge. Abortion rates of 5% may be seen in sheep and goats, and may be much higher in camelids. Fetal losses appear more commonly in a sporadic pattern, but may occur in 'storms'. A complete herd/flock evaluation, epidemiologic investigation, and review of all management /husbandry procedures will be required in order to make a diagnosis, and a plan for corrective action.

Pregnant animals should be moved from areas where abortions have occurred and aborting females, placentas, aborted tissues, exudates associated with the abortion, removed to minimize exposing other pregnant females. Strict biosecurity protocols should be immediately set in place.

The veterinarian should work with farm personnel to insure that pre-planned biosecurity plan is in place to aid in diseases being spread. All 'at risk' animals should be kept in separate areas from older pregnant females.

A herd/flock health program should include the immediate complete examination of all aborting females. All clinical signs (examples: diarrhea = campylobacteriosis; encephalitis=listeriosis) should be noted. Serologic testing, should only rarely be considered a confirmatory diagnostic test for most fetal loss producing diseases. However, comparing several animals in a group, evaluation of past years' results, and/or comparing acute and convalescent titers may yield beneficial diagnostic information.

Sheep fetuses (possibly goats) will produce antibodies during early-mid gestation. During necropsy, fetal examination, or fetal loss investigations, the ability to determine fetal age is critical. The veterinarian should consult one of many texts for such information. Examination of the placenta is critical for a correct diagnosis in cases of fetal loss.

Non-infectious fetal wastage

Non-infectious congenital abnormalities (example: cleft palate, gastrointestinal atresia, heart defects, neurologic defects, etc) can contribute to sporadic abortions, weak neonates, and/or neonatal losses. Stress (malnutrition, predator attack, heat stress, re-sorting animal groups, etc) can also be a common cause pregnancy loss. Both goats and camelids are dependent on corpora lutea are more prone to stress related fetal loses than other species. Maintaining an optimal body condition score and adequate dietary intake for the ewe and doe, will help insure normal pregnancy, fetal development, and parturition. Fetal loss associated with dietary deficiencies is most commonly observed between day 90 and 120 of gestation. Mineral and vitamin deficiencies (e.g., copper, iodine, magnesium, manganese, vitamin A, and selenium, etc.) can result in fetal loss, neonatal weakness, and neonatal losses. Conditioned mineral deficiencies (e.g., copper deficiency due to excessive molybdenum, sulfur, and/or iron intake, etc.) can also depress a flock's live birth percentage.

Pharmaceuticals are also associated with fetal losses (e.g., acepromazine, estrogenic compounds, glucocorticosteriods, prostaglandins, xylazine, etc.) and their use should evaluated in all pregnant animals. Anthelmintics (e.g., phenothiazine, levamisole) given in the final months of gestation may cause abortion, whereas others (e.g., albendazole and cambendazole) given during the first trimester have been associated with fetal abnormalities. However, pharmaceuticals are often blamed for abortion when the real cause is stress related to handling the pregnant female.

Teratogenesis and/or alteration in fetal development have been associated with the intake of many plants (e.g., broomweed, locoweed, lunara lupine, poison hemlock, skunk cabbage, Sudan grass, tobacco, etc.).

Plants have also been associated with fetal loss. Nitrate accumulating plants (e.g., jimsonweed, Johnson grass, lamb's quarter, oat hay, pigweed, sorghum, sweet clover, sunflower, etc.) can result in nitrite toxicities and fetal losses. Legumes containing phytoestrogens have been implicated in increased rates of embryonic losses.

Infectious causes of fetal loss

Most infectious causes of fetal loss in small ruminants have a primary bacterial etiology. The most common microbial agents diagnosed as causes of fetal losses in small ruminants in North America are *Campylobacter fetus* subsp *fetus, Campylobacter jejuni*, *Chlamydophila abortus*, *Coxiella burnetii* and *Toxoplasma gondii*. Mycotic causes of fetal loss are rare. *Candida albicans* and *Aspergillus* spp. Both should be considered opportunistic pathogens in small ruminants. Collection of the appropriate samples are critical in the ability to make accurate diagnosis.

Chlamydiosis

Chlamydophila abortus (gram-negative, intra-cellular organism) is one of the most common causes of infectious fetal loss in goats in North America. When introduced into naive goat flocks, fetal loss rates as high as 25% to 60% may be encountered. Chlamydial organisms can cause buck epididymitis, keratoconjunctivitis, pneumonia, and polyarthritis. Most commonly the transmission of *C. abortus* is via oro-nasal contact of aborted tissues, vaginal discharges or contaminated neonates. Aborting does shed the organism in the uterine discharge, fetus, and placenta, particularly during the first three weeks after abortion. However, some birds (pigeons, sparrow) serve as reservoir hosts, while ticks and other arthropods may be vectors for disease transmission. Signs in the infected doe may include: late term abortions (100d to near term), anorexia, febrileness, bloody vaginal discharge, a fresh, autolyzed fetus, and/or weak kids, and possibly retained fetal membranes. The placenta should be examined for thickened, white, gray, yellow, or red cotyledons. Cytologic or histologic evaluation of cotyledonary impression smears (Gimenez or modified Ziehl-Neelsen stain) for elementary bodies, or necrotizing vasculitis of placental vessels will aid in the diagnosis. Aborted kids may have 'white spots' on the liver, grossly. A definitive diagnosis can be made by culturing the organism or with PCR from fresh placenta, stomach content or fetal tissue. Serological testing may also be of value, particularly if using paired serum samples of aborting does (two to three weeks apart), or by finding antibodies in blood/serum of the aborted kid. The prophylactic use of tetracycline in all pregnant does may be of value in controlling, preventing, or arresting the continued spread of abortions in a herd. Sheep vaccine may be used extra-label in goats, but the efficacy is not completely known. Quarantine of aborting does and proper disposal of aborted fetuses and tissues is critical in order to minimize the spread.

Toxoplasmosis

Toxoplasma gondii can cause abortion, fetal mummification, stillbirth, and the birth of weak lambs and kids. Domestic cats develop a transplacental infection after ingesting infected rodents or birds. Kittens that become infected *in utero*, can shed *T. gondii* oocytes in feces. Infected kittens/young/immunosuppressed cats may bury feces in hay and feed bins. Does become infected when ingesting oocyst contaminated feedstuffs. Toxoplasma can invade and multiply in the doe's placenta then infect the fetus, causing necrosis of the placenta, particularly the cotyledons, with resultant abortion, stillbirth, or the birth of weak kids. Does infected prior to breeding usually do not abort, while those infected between one and three months of gestation undergo fetal wastage or mummification. Does which are infected from three to five months of gestation abort. Most non-immunosuppressed does show no overt signs (other than the occasional increased rectal temperature) at the time of abortion. Does with concurrent immunosuppression can develop

neurologic disease. On gross examination of the placenta, the cotyledons are grey-white to yellow, with focal areas of calcification and necrosis. *T gondii* antibodies in aborted fetal fluids or presuckling blood indicates transplacental infection, and can confirm the diagnosis. The absence of *T. gondii* antibodies by one week after abortion can usually rule out Toxoplasma as the cause. High antibody titers in a doe are not diagnostic of recent infection, but the absence of antibodies can usually rule out toxoplasmosis. A positive diagnosis of toxoplasmosis requires isolation/culture of *T gondii* from the placenta or fetal tissues. All samples for culture should be transported the laboratory on ice. Toxoplasmosis is best controlled by preventing pregnant doe exposure to infective oocytes. Management protocols useful for prevention should include: (1) fetal membranes and aborted materials not used in diagnostics should be incinerated; (2) kittens and pregnant queens should be kept from pasture and feedstuffs used for feeding and care of the pregnant does; (3) spayed queens kept in barns may prevent feral pregnant queens from nesting; (4) keep feedstuffs in areas or containers that minimize cats defecating in the feedstuffs, feeders, and/or feed handling equipment. The inclusion decoquinate, monensin, or lasalocid may be useful in toxoplasmosis control, but the clinician should always be mindful to avoid the use of pharmaceuticals in an extra-label fashion. The clinician should also be mindful of the zoonotic potential for toxoplasmosis. People should only consume pasteurized goat milk.

Q (Query or Queensland) fever

Q fever is caused by *Coxiella burnetii* (an intracellular, rickettsial micro-organism), and can be a cause abortion in goats throughout much of the world. *C burnetti* can be carried and shed in the placenta, uterine fluids, colostrum, and milk of many ruminant animals. These infected animals can serve as sources for doe infection, as can grazing contaminated pastures, and vector tick bites. Pregnant does that become infected will occasionally develop placentitis, anorexia and depression, and have late term abortion, and stillbirths. Does that have aborted develop immunity. Isolation of *C. burnetii* from the placenta or aborted tissues can confirm a diagnosis of Q Fever. Identification of the organism via Ziehl-Neelsen staining of histologic cotyledonary or fetal abomasal tissue is also diagnostic. Fluorescent antibody testing of frozen placenta can also be rewarding. Antibody titers >1:20 suggest *C. burnetii* exposure, but not confirmative of the cause of fetal wastage; however a 4x increase in the aborted doe's acute and convalescent serum titers indicates a recent infection. The infected doe can carry and shed *C burnetii* indefinitely. As with other causes of abortion, all placentas and aborted tissues not used for diagnostic purposes should be incinerated. The use of some antibiotics (chlortetracycline) in susceptible does may reduce the incidence of abortions. Cats, cattle, and sheep can all serve as a source of infection, thus their monitoring and control may be of benefit in prevention. Because of the zoonotic potential Q fever, all goat milk intended for human consumption should be pasteurized, and infected does identified and culled.

Listeriosis

Listeria monocytogenes (gram positive, non-acid-fast facultative microaerophilic organism) can cause meningoencephalitis, abortion, and septicemia in goats. The causal organism can be found in feces, plant material, silage, soil, and water. Ingestion by the doe of contaminated feedstuffs in early pregnancy may result in abortion, and stillbirth or weak neonates when ingested late gestation. Both abortion and neurologic conditions may occur simultaneously in goat herds. Fetal wastage will usually occur in late gestation. The abortion may be associated with fever, inappetence, septicemia, and the expulsion of autolyzed fetal tissues. A confirmative diagnosis can be made by culturing *L monocytogenes* from the placenta, fetal tissues, or uterine discharge. The disease may be minimized or prevented by: (1) avoiding susceptible animals grazing contaminated pastures or feeding spoiled silage; (2) inclusion of chlortetracycline in feed supplements; (3) administration of long-acting oxytetracycline preparations during abortions storms; (4) use of a vaccine where available. As *Listeria* may be zoonotic, care should be taken to minimize transmission to people by exposure to humans of aborted tissues.

Leptospirosis

Goats are more resistant to *Leptospira interrogans* infections and fetal wastage than other domestic species, but are occasionally infected if exposed to infected/contaminated urine. Of the seven to eight serovars commonly encountered in most domestic animals, only *icterohaemorrhagiae, grippotyphosa*, and *pomona* have been associated with fetal wastage in goats. Infected does may present with anemia, inappetence, fever, hemoglobinuria, icterus, neuroligic disease, late term abortion, and death. Confirmative diagnosis can be made by isolation of the causal organism (which is uncommon), and by using darkfield microscopy, immunofluorescence testing, and silver stains on the placenta and fetal tissues. Paired sera from aborted does showing a 4x increase in serum titer in aborted and convalescent does is very suggestive of leptospirosis. Vaccination in endemic areas may have some value, however the clinician should avoid the extra-label use of vaccines and pharmaceuticals. Avoiding contaminated urine (rodent control, clean water, etc.) and the addition of chlortetracycline to feed supplements may all be useful in prevention. Leptospirosis is potentially a zoonotic disease, thus care should be exercised when handling suspected contaminated materials.

Mycoplasmosis

Mycoplasma organisms can cause arthritis, keratoconjunctivitis, mastitis, and vulvovaginitis and fetal wastage. Fetal wastage occurs in the final trimester of gestation, and aborting does will shed the organism in amniotic fluid, milk, and placenta. Confirmative diagnosis can be made by culture and serotyping of the organism from placenta or fetal tissues. Some antibiotics (tetracyclines and tylosin) may be of benefit to prevent the continued spread if used during abortion outbreaks.

Salmonellosis

Salmonella abortus-ovis infection in goats can produce septicemia illness, metritis, and fetal wastage. Stressful conditions (overcrowded conditions, malnutrition, parasitism, antibiotic use, etc.) may predispose or exacerbate the incidence of this condition. Birds and other wildlife, and domestic ruminants may serve as carriers and sources of contamination. In herd outbreaks up to 70% of the does may have fetal wastage, with retained fetal membranes, metritis, and signs of septicemia; all occurring in the final month of gestation. A confirmative diagnosis can be obtained by culturing Salmonella from aborted tissues. The use of antimicrobials, based on culture and sensitivity patterns, and/or autogenous vaccines may be of some benefit in both prevention and control. Salmonella organisms which are associated with abortions should be considered to be zoonotic.

Campylobacter (vibrosis)

Campylobacter fetus and/or *C jejuni* (gram negative, microaerophilic rods) are rarely documented in cases of fetal loss of goat in North America. Infection may occur when pregnant does ingest water of feedstuffs contaminated by the organism; usually after it has been shed via the gastrointestinal tract of sheep, dogs, and some birds. Fetal loss is characterized by stillbirths, weak kids, late gestational expulsion of fresh fetuses, and the doe may present with diarrhea. Findings of placental edema, with necrosis and/or swollen cotyledons, fetal subcutaneous edema, pleuritis, hepatic disease (gray targets on liver surface), and peritonitis would be indicative of Campylobacter. Isolation of the organism from placenta, fetal abomasal contents, and maternal vaginal discharge will provide a definitive diagnosis. Antibiotic therapy (penicillin, tetracycline, streptomycin) during late gestation, early kidding season, or during an outbreak, may decrease the incidence of fetal loss. A vaccine is available for sheep, and may be of use (in an extra-label fashion) for goats. On endemic premises an autogenous bacterin may be of value. *C. jejuni* is associated with mild gastroenteritis in people.

Selected references

Pugh DG: Fetal loss in goats. Clin Therio 2013;5:489-492
Pugh DG: Goat herd health reproductive management, feeding the doe, care of the kid. Clin Therio 2013;5:493-494.
Edmondson M, Roberts J, Baird AN: Theriogenology of sheep and goats. In: Pugh DG, Baird AN, editors. Sheep and goat medicine. 2nd ed. Maryland Heights(MO): Elsevier; 2012. p150-230.
Scharko P, Johnson J, Mobini S, et al: Flock/herd health. In: Pugh DG, Baird AN, editors. Sheep and goat medicine. 2nd ed. Maryland Heights(MO): Elsevier; 2012. p 539-556.
Mobini S, Heath AM, Pugh DG:Theriogenology. In: Pugh DG, editor. Sheep and goat medicine. Maryland Heights(MO): Elsevier; 2002. p. 129-186.
Mobini S, Wolfe C, Pugh DG: Flock health. In: Pugh DG, editor. Sheep and goat medicine. Maryland Heights(MO): Elsevier; 2002.

Diagnosis of endometritis in postpartum dairy cows

R.C. Lefebvre,[a] A.E. Stock,[b] N. Tison[c]

[a]Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC, Canada; [b]Service mobile de Thériogénologie-ReproVet, Chambly, QC, Canada; [c]DSAHR, Saint-Hyacinthe, QC, Canada

Abstract

The prevalence of endometritis in postpartum dairy cows is high and the condition impairs subsequent reproductive performance once the voluntary waiting period (VWP) has passed. In addition to the direct financial losses, there are also important indirect losses due to infertility and its subsequent loss of a potential profitability from genetic selection for milk production. To reduce the negative impact of endometritis on the dairy industry, an accurate diagnostic test is needed. Since there is no gold-standard to test for the condition, most diagnostic tests can only differentiate partially between animals with and without the disease. To further complicate the situation, the cause of endometritis is often multi-factorial, with several predisposing factors exhibiting different levels of impact such that the distinction between the physiological process of the involution of the uterus and a pathological process establishing disease becomes difficult.

The present article reviews the different methods used to diagnose endometritis in postpartum dairy cows. The diagnostic accuracy, the limitations and advantages of trans-rectal palpation, visual assessment of the vaginal discharge, endometrial cytology, uterine bacteriology, chemical testing, ultrasonography, and the measurement of the optical density of a uterine lavage are discussed. A better knowledge of the diagnostic methods of endometritis will allow veterinarians to be proactive in monitoring uterine health in postpartum dairy cows which is the key to restore reproductive performance in postpartum animals and to establish a strong base for an effective herd health management.

Introduction

Reproductive performance in dairy cows and farm economic efficiency are related to uterine health status at the end of the voluntary waiting period.[1-4] Uterine diseases affect about half of all dairy cows in the postpartum period,[5,6] causing infertility by disrupting uterine and ovarian function.[7] The resulting reduction in fertility leads to an estimated 50% loss of the potential profitability from genetic selection for milk production.[8] Postpartum uterine infections in dairy cows cost more than 1.4 billion, 650 million and 300 million to the European, American, and Canadian industries, respectively.[9,10] Yet, despite the significant economic impact of postpartum uterine infections, controversies still exist around the diagnosis.[11]

Postpartum uterine diseases in dairy cows include puerperal and postpartum metritis, pyometra, clinical endometritis (CE) or purulent vaginal discharge, and subclinical endometritis (SE) or cytological endometritis. Compared to puerperal metritis and pyometra, where clinical signs are more obvious, endometritis is a challenging condition for veterinarians to diagnose. Historically, endometritis has been histologically defined: a superficial inflammation limited to the endometrium and extending no deeper than the stratum spongiosum. It is characterized by disruption of the surface epithelium with infiltration of inflammatory cells, vascular congestion and stromal edema.[12,13] Diagnosis of the condition, therefore, requires endometrial tissue sampling (uterine biopsy) and histological analysis. In the last decade, a more practical definition based on clinical context and reproductive performance has been proposed.[1] This new approach allows the veterinarian to make a more straightforward, medically rational and economically based decision. The CE is defined as an infection of the endometrium with uterine exudate, varying from clear mucus with flecks of pus to purulent discharge in the vagina, 21 days or more postpartum. The SE is defined as inflammation of the endometrium based on the presence of a certain percentage of polymorphonuclear neutrophils (PMN) in the absence of purulent vaginal discharge. Because both conditions have different risk factors and that their negative effect on reproductive performance are additive, they are assumed to be two different uterine pathological entities.[14,15] Based on these definitions, it is estimated that about 20% of postpartum dairy cows have CE and 30% have SE.[9,16] To efficiently treat

CE and SE, and improve reproductive performance, the veterinarian needs accurate diagnostic methods. What are the currently available diagnostic methods, and how accurate and useful are they?

The accuracy of diagnostic tests

In veterinary medicine, decisions around animal health are based on a number of factors, including medical knowledge, professional experience, clinical intuition and clinical diagnostic testing. Diagnostic testing is a relatively objective method that reduces uncertainty about factors during diagnosis. Of primary importance is diagnostic accuracy, or the ability of a test to discriminate between the pathologic and healthy status. The discriminative ability of a test can be quantified by a variety of different measures: sensitivity (Se), specificity (Sp), negative and positive predictive values (NPV and PPV, respectively), likelihood ratio (LR), area under the ROC curve (AUC), odds ratio (OR), and kappa (K) test (Tables 1-5). Of course, there are a number of other criteria that determine the most appropriate test – criteria that are not directly linked to the medical performance of the test, but rather more to its feasibility and practicality in a given situation, such as cost, time required to perform the test, availability, and time to final results (the issue of cow-side vs laboratory tests). Moreover, measures of test performance are not absolute indicators of test quality and performance because they are sensitive to factors like population characteristics, disease prevalence and disease spectrum. For veterinarians, it is of utmost importance to know how to interpret a test, and when and under what conditions to use a test.

There is no perfect test because most diagnostic test results can make only a partial distinction between subjects with and without a disease. In bovine theriogenology, the first Koch's postulate for establishing the causative relationship between a microbe and a disease are not always applicable. The first postulate of Koch says that the microorganism must be found in abundance in all animals suffering from the disease, but should not be found in heathy organisms. The cause of uterine conditions is often multi-factorial, with several predisposing factors exhibiting different levels of impact. The concept of HOST-PATHOGEN-ENVIRONMENT is closer to the reality in the field situation.[17] This concept captures well the complex interplay of factors that result in disease at the individual and population levels. In short, a pathogen is a necessary but not sufficient cause of disease. Asymptomatic or subclinical infection carriers are known to be a common feature of many diseases as in the present case.

The result of such complexity is that there is no real gold standard for evaluating a diagnostic test. Rather, we have to evaluate a diagnosic test by examining its level of agreement with a near-gold standard or a more accepted diagnostic method. We can also assess outcomes in terms of economic, reproductive or management impacts. The kappa test measures the level of agreement between two tests beyond what would be obtained by chance alone. Note that the kappa result gives no indication of test quality per se because a good agreement may mean only that both tests are equally good or equally bad.

The overall accuracy of diagnostic tests for endometritis

In general, veterinary reproductive interventions are only beneficial if they result in pregnancy in a timely fashion. The value of reproductive examinations before the breeding period depends on the ability of veterinarians to identify cows at a higher risk of pregnancy failure and then, through a diagnostic process, identify those that could benefit from treatment for endometritis. Veterinarians need to perform diagnostic tests for a number of reasons:

- Confirm the diagnosis in symptomatic animals
- Screen for disease in asymptomatic animals (systematic postpartum examination)
- Provide prognostic information in cows with the disease
- Monitor the benefits and/or side effects of treatment (follow-up)
- Confirm disease-free status

The most important postpartum uterine disease in dairy cows is endometritis, which is prognostic for impaired reproductive performance.[14,18-22] In bovine reproduction, Se, Sp, PPV and NPV are the most important measurements for validating diagnosis methods (see above). In the case of the postpartum endometritis in dairy cows, it is very difficult to estimate the real sensitivity and specificity of diagnostic methods because there is no gold standard.[23] The diagnosis of endometritis in cows has also long been

hampered by the lack of a well-accepted definition of the condition and a simple, effective diagnostic technique. Added to these difficulties is the fact that all postpartum cows experience some degree of endometrial inflammation for a certain period of time is associated with normal uterine involution. Establishing the best time window of opportunity for testing uterine health status along the timeline of uterine involution is needed. As diagnostic tests are often interpreted using a dichotomous outcome (normal/abnormal or diseased/healthy), it is often easier to interpret results if the test itself is dichotomous, such as presence or absence of a pathogen). Interpretation is more difficult when the outcome is continuous or categorical, such as neutrophil counts or vaginal discharge categories. Variation in the cut-off point used to separate positive (disease) and negative (non-disease) results introduces a level of uncertainty (false positives/false negatives). This in turn affects estimates of prevalence. For endometritis in dairy cows, estimates of prevalence range from 0 to 68%.[24-28] This large variation is due not only to the above-mentioned differences in test cut-offs, but also to a variety of factors:

- Inconsistencies in the timing of examination during the postpartum period
- Differences in diagnostic methods
- Definition of the pathology
- True difference in prevalence between populations
- Variation between individuals
- Host-pathogen-environment interactions
- Complex interactions between etiological pathogens

Diagnostic test: transrectal palpation

In practice, transrectal palpation is always part of a complete physical examination and still the predominant method used by veterinarians to diagnose uterine diseases. However, several studies have demonstrated that this method results in a large number of false-positive diagnoses.[29] The diagnosis of endometritis by transrectal uterine palpation in the postpartum period relies on the location of the uterus (in the pelvic cavity, over the pelvic brim, or over the pelvic brim but retractable or not completely retractable), size of the uterus (enlarged or asymmetrical uterine horns), thickness of the uterine wall, presence of the uterine lumen or fluid within the lumen of the uterus,[30] and size of the cervix.[20,31] Transrectal palpation of the uterus lacks diagnostic accuracy, as a large uterus may be the result of physical damage or variation associated with breed, age or nutrition. [1,20,32] It is a subjective measure and is not reliably associated with reproductive performance.[33,34]

A cervical diameter of > 7.5 cm as diagnosed by transrectal palpation has been demonstrated to be a more reliable predictor of poor reproductive performance. However, large cervix represents less than 3% at 27-33 days in milk (DIM).[20] Cows with a large cervix have been shown to have a significantly decreased likelihood of pregnancy at first insemination and significantly increased mean days open. The greatest difference in cervical diameter between healthy cows and those with abnormal discharge is only 10 mm at 21 DIM.[31] The likelihood of returning to cyclicity decreases for each 1-cm increase in cervical diameter as measured by ultrasound between 15 and 21d postpartum.[35] Dubuc et al found a sensitivity of 40.9% and 50.8% at 35 DIM based on thresholds of > 7.5 cm and > 5.0 cm, respectively, using predicted pregnancy status at 120 DIM as the reference.[14] The overall sensitivity and specificity of rectal palpation of the cervix for all investigators, using ultrasound as the reference standard, was 37.5% and 96.2% based on thresholds of > 7.5 cm and > 5.0 cm respectively.[36] This low sensitivity may be explained in part by the considerable time lag between diagnosis and pregnancy confirmation, and by the multiple factors that influence pregnancy status.[24,37] Likewise, the repeatability of the estimate of the cervical diameter obtained through transrectal palpation is moderate, and decreases with increasing size diameter.[38] A cervical diameter of > 6.0 cm seems to confuse investigators and lead to more false-positives. By 25 DIM, both uterine horns and the cervix are expected to reach a diameter of less than 5 cm in normal cows.[39] Therefore, the lack of accuracy of the transrectal palpation of the uterine tract in identifying cows with endometritis is not surprising. A similar lack of accuracy has also been demonstrated for ovarian

examination, with significant inter-clinician variability in assessing corpus luteum function in dairy cows.[40]

Given the low inter-investigator agreement for the manual assessment of cervical and uterine horn diameter, and the lack of association between this measure and reproductive performance, the usefulness of widespread manual transrectal examination of the reproductive tract during the postpartum period in dairy cows remains controversial. The time required to perform a complete and meticulous examination is considered too long and too costly by veterinary practitioners and farmers. The main objective of characterizing the uterus and the cervix in postpartum cows is to identify those at increased risk of failure to become pregnant in a timely fashion. Transrectal palpation of the reproductive tract of dairy cows performed before 21 days postpartum may more likely overestimate the real prevalence of endometritis because of incomplete uterine involution and the difficulty in manually palpating a large uterus. However, even though the published data do not support transrectal palpation of the uterus as a useful diagnostic method for endometritis during the postpartum period, the exam is still an important part of a complete genital examination that allows the veterinarian to rapidly rule out obvious abnormalities or abnormalities that would not otherwise be noticed , such as pyometra, tumors, abscesses and lacerations.

Diagnostic test: visual examination of the vaginal discharge

There are several methods for diagnosing CE in postpartum dairy cows based on the visualization and characterization of vaginal discharge (Figure 1). They are: 1) gloved hand method,[41] 2) the Metricheck device (Metricheck, Simcro, New Zealand),[18] and 3) vaginoscopy or speculum method.[20,42] Purulent vaginal discharge resulting from bacterial infection is characterized by a mixture of neutrophils, necrotic tissue and fluid, and amount of these materials, or pus, is correlated with the growth density of pathogenic bacteria. By contrast, the growth density of bacteria categorized as opportunist contaminants of the cow reproductive tract is not associated with purulent vaginal discharge.[7] William et al proposed a 4-point scoring system for grading vaginal discharge: 0=clear mucus, 1=mucus containing flecks of pus, 2=discharge containing fewer than 50% pus, and 3=discharge containing more than 50% pus.[7] Although perceived as an inconvenient method by veterinarians, the visual assessment of vaginal discharge via vaginoscopy examination is a simple tool that can rapidly distinguish healthy from diseased cows.[1,14,22,43] In fact, vaginal examination with a speculum has been shown to be more accurate than transrectal palpation in detecting abnormal discharge.[44]

It would appear, then, that transrectal palpation is of little value in terms of monitoring uterine health in postpartum period when vaginoscopy is performed.[19,20] Yet, transrectal palpation remains an important part of a complete genital examination. Note that the stimulation of uterine contractions caused by previous transrectal palpation of the reproductive tract does not, as previously thought, increase the detection of vaginal discharge.[18,45]

The relative risk analysis does not reveal a significant effect of transrectal palpation of the uterus on the prevalence of CE as diagnosed by visual examination.[38] The prevalence of CE ranges from 10% to 20%, [20,46] however more recent studies reported prevalence > 40%.[38,45,38,47] This large variation between studies could be explained by different diagnostic criteria and methods, definitions of the disease (cutoff points), or the population and farm effect. The interobserver (k=0.47 to 0.55) and intraobserver (k=0.61-0.82) agreement for visual examination is high. The visualization of vaginal secretions by vaginoscopy does enable the detection of a fine variation in colors of purulent discharge. The repeatability of vaginoscopic examination for different investigators and different times is acceptable.[48] Furthermore, the visual assessment of the vaginal vault enables the differentiation of CE from vaginitis, cervicitis and vaginal trauma/lesions. While primary postpartum vaginitis in dairy cows is rare, the prevalence of cervicitis is estimated at 60.8%, with 29.1% of cows presenting prolapsed and hyperemic cervical mucosa.[49] This is more than the prevalence of CE. While vaginoscopy is a rapid and simple technique, it may underestimate the prevalence of endometritis because of its low sensitivity. Vaginal discharge assessed by vaginoscopy between 27 and 33 DIM had 21% sensitivity and 89% specificity when referenced to 150-day pregnancy status.[20] Barlund et al showed that vaginoscopy lacks sensitivity when compared to cytobrush endometrial cytology (Se=54%, cut-off=8%) and to 150-day pregnancy status

between 28 and 41 DIM (Se=7.1%), with moderate agreement (kappa=0.52).[24] In both studies, Sp was higher than 87% and all eligible cows were included. If the vaginoscopy is performed before 27 days postpartum, a larger number of normal cows may have mucopurulent discharge, thereby overestimating the real prevalence of CE. However, the false positive rate is probably not important because of the consistent and significant negative effect of vaginal discharge on reproductive performance,[7,14,20,24] and the clear positive effect of treatment in restoring normal fertility of cows.[22,27]

It should be noted that care is required when performing vaginal examinations to monitor uterine health in postpartum dairy cows. Performing vaginal examinations on several cows with the same instrument increases the risk of spreading transmissible diseases throughout the herd. Instruments need to be cleaned after each vaginal examination or disposable instruments used. Such examinations should always be done in a consistent and thorough manner.

Other diagnostic methods, such as Metricheck (Figure 2) or gloved hand, have been described as accurate vaginal examination techniques. With vaginoscopy as the reference diagnostic method, the Metricheck device and gloved hand have been shown to produce comparable results, although prevalence estimates tend to be higher with Metricheck.[45] This is to be expected because the device collects a considerable amount of cellular debris in the vaginal mucosa that cannot be visualized. There is a higher rate of false positives (for CE) with Metricheck, the consequence of which would be the unnecessary or inadequate treatment of cows during the postpartum period and the resulting financial losses.

The gloved hand and Metricheck methods are also unable to distinguish certain categories of vaginal discharge, such as the cloudy category of vaginal discharge without macroscopic purulent flecks, which has been associated with decreased FSCR.[22] This increases the risk of a misclassification. Cloudy mucus on vaginoscopic examination has also been associated with endometritis.[50]

It is especially important to have a good scoring system for vaginal discharge because treatment efficacy varies among discharge categories over the threshold point. It is therefore vital to have a standardized diagnostic method and to know the limitations of the diagnostic test being used. As with transrectal palpation, vaginal examination is an indirect measure of uterine inflammation. False-positive findings, such as in the case of vaginitis or cervicitis, or false-negative findings where endometritis is not diagnosed because of a closed cervix, may occur. Madoz et al showed that hysteroscopy can be used as a direct measurement of uterus health status and to test the real prevalence of false-positive and false-negative diagnosis for endometritis.[51] The Se, Sp, PPV, PNV and accuracy of the transrectal palpation and the vaginoscopy were 75%, 85%, 43%, 96% and 83%, and 100%, 85%, 50%, 100% and 86%, respectively, using hysteroscopy as the reference method.

All visual examination methods for categorizing vaginal discharge are recommended for diagnosing CE in postpartum dairy cows. They are useful routine diagnostic tools for veterinarians whose aim is to improve herd health management and maintain reproductive performance. The three visual vaginal examination methods show similar efficacy when it comes to characterizing vaginal discharge, and they have no negative effects on reproductive performance. Vaginoscopy does have the advantage of enabling the veterinary practitioner to rule out other abnormalities of the reproductive tract (Figure 3).

Diagnostic test: endometrial cytology

Subclinical endometritis is characterized by the absence of purulent vaginal discharge but inflammation of the endometrium as determined by cytology.[1] Bacterial pathogens that contaminate the uterus during the postpartum period are recognized by the innate immune system, which then attracts inflammatory cells.[52] While lymphocytes, macrophages, eosinophil leukocytes and neutrophils are all involved in eliminating uterine infections, the dominant type of professional cell, because of their ability to phagocyte bacteria, is the polymorphonuclear neutrophil (PMN, Figure#3).[53] These neutrophils are recruited from the blood stream into the uterine lumen in order to fight bacterial pathogens.[54,55] After the elimination of these pathogens, inflammation subsides and the neutrophils become limited to the fluid in the uterine lumen,[26] later to be expelled by uterine contractions (Figure 4). Thus the proportion of neutrophils relative to endometrial cells is an indicator of the inflammatory process and an important characteristic of cytological endometritis.[14,21,24,52] Gilbert and Santos showed that the proportion of

neutrophils on endometrial cytology (uterine lavage) in the first three weeks postpartum was high and likely reflecting the physiological inflammatory process of a normal uterine involution and remodeling occurring during that period and of course, the uterine infection with the large amount of bacteria.[56] All sampling in the study were from a single dairy farm representing a limitation of the study. The criterion for inflammation of the endometrium is percentage of PMN (#PMN/#PMN+#endometrial cells) in a cytological sample from the uterus.[1] As cytology of the endometrium is a direct evaluation of intrauterine cavity inflammation, and as there is no effect of estrus cycle stage on the percentage of uterine PMN in cows,[57] PMN% is the near-gold standard for diagnosing postpartum endometritis in dairy cows and the reference for evaluating surrogate tests. The presence of a large amount of neutrophils on endometrial cytology after 21 days postpartum is most likely associated with the bacterial uterine infection.[56] This is despite there being no concrete data to support its efficacy.[46] Barlund et al showed that cows with more than 8% PMN had a 1.9 greater risk of not being pregnant at 150 DIM, with a Se and Sp of 13% and 90%, respectively (n=189).[24] In a study of over a thousand cows, based on a threshold of more than 6% PMN, cytobrush cytology showed a Se, Sp, PPV, and a NPV of 42%, 80%, 85%, and 34%.[27] Cytological examination of the uterus is the most widely accepted definitive diagnosis for endometritis, although it cannot rule out other abnormalies of the caudal reproductive tract (eg. vaginitis or cervicitis, etc.).

Cytology analysis requires staining of the specimen and then examination under a microscope. The problem arises in the quantification step: there is little standardization in the number and type of cells counted, the magnification used, the number of high-power fields assessed, and the field selection on the slide. Melcher et al showed that six different slide assessments (count of 100, 300 and 500 cells at magnifications of 10x and 40x high-power fields) showed strong compliance (**r**=0.77-0.90), with the highest correlation coefficients found for counts of 100 and 300 cells.[58] Agreement between methods (k=0.30 to 0.85) and observers (k=0.79) was good. Similarly, DeGuillaume showed a strong inter- and intra-readers agreement (CI95%, 089-0.94 and CI95%, 0.80-0.97, respectively).[59] Another possible methodology for taking differential counts for neutrophils, lymphocytes, macrophages and epithelial cells is counting a maximum of 500 cells along five predetermined reading paths on the slide (Figure 5). However, counts of only neutrophils from 10 randomly selected fields at 40x magnification showed good agreement when compared to the previous method (R.C. LeFebvre, unpublished data). Different methods with different standards in different studies were designed to evaluate endometrial cytology (neutrophil counts). However, all studies reported a good reader agreement suggesting that the reading and scoring techniques used in endometrial cytology are solid.[48]

Methods to collect endometrial material to assess inflammation include uterine lavage and cytobrush, both of which are well accepted diagnostic techniques.[37,60] However, the cytobrush method (Figure 6) seems to be more reliable, rapid and consistent because it results in a better cell sample with less distortion (Figure 7).[61] With uterine lavage, up to 17% of attempts do not result in the recovery of uterine fluid in a study.[61] However, the cytobrush method is far from perfect. Like the biopsy technique, pressure on the cytobrush may influence the PMN/epithelial cell ratio. Furthermore, the small area sampled by the cytobrush may not be representative of the entire endometrium. In the mare, the technique appears to have low within-horse repeatability.[62] Sampling of the cervix, the uterine body, the right and left uterine horns showed that inflammation is not equally present through the whole reproductive tract[59]. However, SE in postpartum dairy cows diagnosed by endometrial cytobrush or uterine lavage shows similar negative effects in reproductive performance.

It is clear that more studies are needed to standardize endometrial cytology and thereby improve its accuracy. In general, the slide is prepared by rolling the cytobrush or using a centrifuge (fluid collected by uterine lavage) on a predetermined surface area of a clean glass microscope slide. The staining method of the endometrial material may also affect the analysis. Normally, the material is air-dried and the slide stained using a modified Wright-Giemsa stain (Diff-Quick™, Dabe Diagnostic, West Monroe, LA) or naphthol-AS-D-chloroacetate-esterase (Sigma, Montreal, Canada), then evaluated under the microscope.[63] Naphthol-AS-D-chloroacetate-esterase is the more effective stain for identifying and counting neutrophils.[62] However, its preparation is very labor-intensive compared to the Diff-Quick™ stain, making it impractical and not rapid enough for the field situation.

Overall, endometrial cytology with cytobrush is accepted as the best diagnostic test and the most practical in the field for postpartum endometritis. However, based on its low-to-moderate accuracy, its overall accuracy still needs to be improved.

Diagnostic test: uterine bacteriology

The uterine lumen in early postpartum dairy cows is contaminated with a variety of bacteria, but most cows will eliminate any bacterial infection by three weeks after calving. Based on the time of sampling after calving, the proportion of positive uterine bacteriology varies between 80% and 100% in the first 21 days postpartum.[7,47,56] Essentially, the uterine cavity is assumed sterile by about 28 days postpartum.[46] For cows that do not have a sterile entometrial cavity, the persisting infection will lead to endometritis.[64,65] Recognized uterine pathogens associated with uterine lesions are *Trueperalla pyogenes*, *Escherichia coli*, *Fusobacterium necrophorum* and *Prevotella melaninognica*.[7] The presence of these bacteria in the uterus causes inflammation, delays uterine involution, and causes histologic lesions in the endometrium.[66,67] Analyses of the bacteriological data of the uterine microbiome indicate that *E. coli* is the most prevalent bacteria in the first three days postpartum, with equally importance with *Streptococcus uberis* and *T. pyogenes* at nine days postpartum and where *T. pyogenes* is the most prevalent after 15 days postpartum[47]. As with *E. coli*, *Clostrium perfringens* was the most prevalent anaerobic bacteria in the first seven days postpartum.[56]

Uterine bacterial infections perturb uterine and ovarian function, and fertility.[68,69] There is a strong association between *Trueperella pyogenes* in the uterus of dairy cows during the postpartum period and CE and uterine pus.[33,70,71] Cows with a positive culture for *Trueperella pyogenes* are more at risk of clinical endometritis.[53] Although impaired fertility is more closely associated with certain pathogens, cows with intrauterine bacterial growth of any species at 21 DIM exhibit decreased fertility.[72] Uterine bacteriology has been used to investigate false-positive cases of CE as determined by vaginoscopy. At 21-28 DIM, *Trueperella pyogenes* was found in 33.5% of samples and was correlated with vaginal discharge. In total, 17.3% and 28.5% of cows with endometritis as diagnosed with vaginal discharge showed negative bacteriology and endometrial cytology, respectively. Galvao et al., reported that 41% of samples collected in cows with purulent vaginal discharge were *Trueperella pyogenes*-culture positive.[52] For sampling the uterine cavity, cytobrush and cotton swab are both acceptable techniques for generating reliable bacteriological results on uterine tract infection.[74] The Se, Sp, PPV, and NPV of the presence of *Trueperella pyogenes* were 90.7%, 13.6%, 69.6%, and 22.1% for predicting nonpregnant status at 300 DIM.[71] These accuracy measurements were similar to vaginal discharge, visual characteristic of uterine discharge and the presence of neutrophils. However, several factors can potentially affect the pregnancy status between the moment the test is performed (35±3 DIM) and 300 DIM. The uterine microbiome is very dynamic if certain bacteria are recognized as pathogens other may reduce the risk of uterine infection. For instance, Streptococcus has been reported as having a negative correlation on uterine health [56,67] although other reported a protective effect of it presence in early postpartum.[47] Several factors can explain the different results and the question is still debated.

While bacteriology would seem to be a valuable alternative diagnostic tool, the test is very expensive, not practical to perform in the field, and incapable of yielding quick results. Uterine bacteriology is more appropriate for further clinical investigation of individual infertility cases and for research.

Diagnostic test: leukocyte esterase, a new chemical test for endometritis

The cytobrush technique is simpler and more rapid than uterine lavage,[75] and more precise than ultrasonography of the uterus.[76] It is considered the reference method for the cytological diagnosis of endometritis because it generates quality samples and exhibits the highest repeatability. However, it is not a cow-side test. Although neither complex nor expensive, endometrial cytology requires special instrumentation, technical expertise and time. From the standpoint of practicality and efficiency, a diagnostic test for endometritis must be rapid and provide results at the farm. This would enable the veterinary practitioner to make a treatment decision and execute this decision at the time of the diagnosis.

An alternative method to assessing inflammatory cells in the lumen of the uterus is the leukocyte esterase (LE) test. Leukocyte esterase is released from neutrophil cells and reacts with indoxil carbonic acid ester. The esterase releases indoxil, which reacts with diazoniun salt and is oxidized, yielding a violet azo dye.[13] The intensity of color in this dye is correlated with leukocyte count. This method has been used for the rapid diagnosis of inflammation in many body fluids, including urine, pleural fluid, peritoneal fluid and cerebrospinal fluid.[77-81] It could also potentially be used as an indirect method for detecting neutrophils in the cow uterus. Santos et al., evaluated the efficacy of this method in diagnosing endometrial diagnosis in dairy cows.[82] They used a commercial urine test strip containing leukocyte esterase (Multistix 10 SG, Bayer Corporation) in uterine fluid derived through saline lavage. They reported a high correlation between endometrial cytology and leukocyte esterase activity, with 96% Se and 98% Sp. However, with a larger number of cows (n=563), Cheong et al showed that at the optimal cut-off point, the esterase test had Se, Sp, PPV, and NPV of only 77%, 52%, 38% and 85%, respectively.[26] Using a similar test, Denis-Robichaud et Dubuc showed Se, Sp, PPV, and NPV of 52%, 60%, 79% and 32%, respectively.[27] There was moderate agreement between PMN and LE (k=0.43), compared to the good agreement found by Santos et al. (**k**=0.6).[55] The LE was strongly correlated with proportion of neutrophils found in the uterus of cows with SE (Figure 8),[21] and was also associated with reproductive performance.[27]

In sum, leukocyte esterase shows promise as a cow-side test in the diagnosis of endometritis in dairy cows. However, more research is needed to further characterize and standardize the test, as well as to improve its accuracy.

Diagnostic test: uterine lavage optical density, a new test for endometritis

Several diagnostic techniques based on the visual scoring of vaginal discharge have been proposed for the diagnosis of CE. However, these techniques are indirect diagnostic methods and are subject to observer bias. A new method, uterine lavage optical density, represents a more objective, numerical measurement of vaginal discharge. The technique involves using a spectrophotometer to measure the concentration of substances in a suspension. As visible light passes through the suspension, it is scattered by particles present in the suspension. Greater scatter indicates more material in the suspension. In the case of the uterine lavage in postpartum dairy cows, optical density measures the amount of material in the uterine suspension (e.g. bacteria, exudates, cellular debris, inflammatory cells). Uterine lavage optical density (620 nm) was found to have Se, Sp, PPV, PNV and overall accuracy of 76.3%, 78.3%, 28, 96.7%, and 78.1%, respectively, when ROC and visual scoring of clinical endometritis were used as the gold standard.[83] The presence of *Trueperella pyogenes* in uterine lavage was associated with high mean uterine lavage optical density, and cows with high optical density showed a higher percentage of neutrophils.[83]

Although uterine lavage optical density is a simple and rapid test that does not require special training, it is not a cow-side test. In addition to equipment cost considerations, there are a number of other issues to be resolved. The choice of optimal wavelength changes with the type and color of the uterine suspension. Furthermore, real light absorption may not be representative of the actual number of bacterial cells because many bacteria are colorless. This technique has not been compared yet to other diagnostic techniques.

Overall perspective on diagnostic tests for endometritis

Even though endometritis in postpartum has significant consequences on reproductive performance of dairy cows and on the economic sustainability of the dairy industry, diagnosic methods for the condition are still actively investigated. Veterinarians should be able to identify cows at a higher risk to remain open and to have a diagnostic test that identifies cows that would benefit from treatment. Diagnostic tests reviewed in this article have low-to-moderate Se and PPV, and a moderate overall accuracy. As the characterization and standardization of the diagnostic tests improve, tests will become more accurate and therefore more useful to veterinarians and dairy farmers. As the number of cows per farm increases and the number of people actually working on farms decreases, less time is available to

monitor general animal health and even more so the postpartum uterine health. Therefore, a reliable, simple and rapid cow-side test is desperately needed.

Very little is known about the overall diversity and dynamics of the uterine microbiota between the calving and the end of the voluntary period. Transversal studies where sampling is performed at one point of time during postpartum period and usually more often in the second half of the VWP (between 30 to 50 DIM) have not shed sufficient light on the dynamics of the process of uterine involution. Longitudinal studies where several, subsequent samples are taken during the whole periparturient period may help researchers to understand if the postpartum uterine pathologies are associated with other body functions of the entire animal such as the general metabolic status and the immune system of high producing dairy cows. As uterine remodeling and its associated physiological inflammation are normal during uterine involution, longitudinal studies would help to draw the line between physiological and pathological processes and therefore increasing the overall accuracy of the diagnostic tests when performed at the right time. Longitudinal studies may further help to understand the carryover effect of infertility in cows with endometritis at the end of the VWP.

There is growing evidence that the female reproductive tract infection is associated with a complex immune system. It is becoming clear that several features of innate immunity play a major role in uterine defense and the normal return to fertility in dairy cows at the end of the voluntary period (Lefebvre et al., 2016 companion article). As the same mediators play a role in both immunity and female physiological reproductive functions during the postpartum uterine repair, tests on an immunological basis may become better predictive tests for endometritis. In addition, longitudinal studies will allow researchers to understand the relationship between uterine disease and infertility .

Take-home messages

1. Diagnosing endometritis in postpartum dairy cows remains challenging for veterinarians.
2. The accuracy of a diagnostic test can be quantified using a number of different measures: sensitivity, specificity, negative and positive predictive values, likelihood ratio, area under the ROC curve, and odds ratio.
3. The concept of HOST-PATHOGEN-ENVIRONMENT interactions is a useful model for describing the complex interplay of factors resulting in uterine diseases in postpartum period.
4. The most important postpartum uterine disease in dairy cows is endometritis, which is prognostic for impaired reproductive performance.
5. The large variation in reports of the prevalence of endometritis is due to a number of factors: 1) inconsistency in the timing of examination during the postpartum period, 2) difference in diagnostic methods, 3) difference in how the pathology is defined, 4) true differences in prevalence between populations, 5) variation between individuals, 6) host-pathogen-environment interactions, and 7) complexity in the interaction between etiological pathogens.
6. All visual examination methods used to characterize vaginal discharge – vaginoscopy, gloved hand and Metricheck – are recommended for the diagnosis of CE in postpartum dairy cows. They are effective routine diagnostic tools that veterinarians can use to improve herd health management and maintain reproductive performance. Risk of contamination between cows with the use of Metricheck and visual recognition of other abnormalities with the vaginoscopy (cervicitis, vaginitis, lacerations, etc.) have to be taken in account.
7. Endometrial cytology with cytobrush or uterine lavage are both accepted methods for establishing a diagnosis of SE, although the cytobrush technique results in a better sampling, but both are not cow-sided methods.
8. Even though uterine pathogens have been isolated from the uterus of cows without CE/SE, the prevalence of *Trueperella pyogenes* is higher in categories 2 and 3 of vaginal discharge and is correlated to higher numbers of PMN on endometrial cytology. When establishing a diagnosis of CE, one should expect a rate of false positives of 17-28%, depending on the threshold used.

Practical implications

In general, most currently available diagnostic tests for monitoring uterine health status do not exhibit a very high Se, PPV or overall accuracy and are not always practical for the veterinarians to perform in a routine postpartum uterine health monitoring in dairy cows. However, whatever their weakness, most of the tests described in this paper are able to predict a significant negative effect on reproductive performance. With a more accurate and practical diagnostic test, veterinarians will be more confident in monitoring uterine health in postpartum dairy cows, restoring reproductive performance in animals with endometritis, applying the appropriate treatment and therefore ensuring effective herd health management.

Issues yet to be resolved

1. The best time window during the postpartum period in which to test uterine health status.
2. The value of combining different tests for diagnosing endometritis.
3. The real sequence of events during the whole periparturient period in dairy cow which are associated with postpartum uterine infection.
4. The dynamic behavior of the whole microbiota of the reproductive tract and its relationship with each pathologic conditions affecting the high producing dairy cows.
5. With more evidence of the importance of immunity in the etiology of postpartum uterine diseases, the value of immunological tests, alone or in combination with other tests, for predicting or diagnosing endometritis will become more and more important.

Conclusion

Bovine veterinarians need a simple and reliable cow-side diagnostic test to assess herd uterine health and estimate the risk of reproductive outcomes in postpartum dairy cows. The diagnostic tests proposed in the past fifteen years all have weaknesses, and it is clear that more research is needed to standardize their use and improve their accuracy. However, the lack of consensus on what constitutes the best predictor of a negative reproductive outcome should not stop veterinarians from being proactive in monitoring uterine health, nor from taking action when it comes to the diagnosis and treatment of endometritis in postpartum dairy cows.

Acknowledgement

The authors thank Susan Lempriere for reviewing an earlier version of this manuscript.

References

1. Sheldon IM, Lewis GS, LeBlanc S, et al: Defining postpartum disease. Theriogenology 2006;65:1516-1530.
2. Bartlett PC, Kirk JH, Wilke MA, et al: Metritis complex in Michigan Holstein-Friesian cattle: Incidence, descriptive epidemiology and estimated economic impact. Prev Vet Med 1986;4:235-248.
3. Drillich M, Beetz O, Pfutzner A, et al: Evaluation of a systematic antibiotic treatment of toxic puerperal metritis in dairy cows. J Dairy Sci 2001;84:2010-2017.
4. Overton M, Fetrow J: Economics of postpartum uterine health. Dairy Cattle Reprod Council Conf; Omaha NE; 2008. p. 39-43.
5. Klucinski W, Targowski SP, Winnicka A et al: Immunological induction of endometritis-model investigations in cows. Zentralbl Veterinarmed A 1990;37:148-153.
6. Leblanc SJ: Postpartum uterine disease and dairy herd reproductive performance: a review. Vet J 2008;176:102-114.
7. Williams EJ, Fischer DP, Pfieffer DU, et al: Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the immune response in cattle. Theriogenology 2005;63:102-117.
8. Garnsworthy PC, Lock A, Mann GE, et al: Nutrition, metabolism, and fertility in dairy cows: 1. Dietary energy source and ovarian function. J Dairy Sci 2008;91:3814-3823.
9. Sheldon IM, Cronin J, Goetze L, et al: Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. Biol Reprod 2009;81:1025-1032.
10. Canadian Dairy Information Centre, https://www.cdn.ca/home.php, 2015
11. Kinsel ML: Dairy cow postpartum disease: definition and dilemma. Proc Annu Conv Am Assoc Bovine Pract; 1999. p. 3-7.
12. Kennedy PC, Miller RB: The female genital system. In: Jubb KVF, Kennedy PC, Palmer N, editors. Pathology of domestic animals. 4th ed. San Diego: Academic Press; 1993. p. 378-387.

13. Bondurant RH: Inflammation in the bovine female reproductive tract. J Anim Sci 1999;77(Suppl 2):101-110.
14. Dubuc J, Duffield TF, Leslie KE, et al: Definitions and diagnosis of endometritis in dairy cows. J Dairy Sci 2010;93:5225-5233.
15. Sens A, Heuwieser W: Presence of *Escherichia coli, Trueperella pyogenes*, a-hemolytic streptococci, and coagulase-negative staphylococci and prevalence of subclinical endometritis. J Dairy Sci 2013;10:6347-6646.
16. Dubuc J, Duffield TF, Leslie KE, et al: Risk factors for postpartum uterine diseases in dairy cows. J Dairy Sci 2010;93:5764-5771.
17. Snieszko, 1974
18. McDougall R, Macaulay R, Compton C: Association between endometritis diagnosis using a novel intravaginal device and reproductive performance in dairy cattle. Anim Reprod Sci 2007;99:9-23.
19. Runciman DJ, Anderson GA, Malmo J, et al: Use of postpartum vaginoscopic (visual vaginal) examination of dairy cows for the diagnosis of endometritis and the association of endometritis with reduced reproductive performance. Aust Vet J 2008;86:205-213.
20. Leblanc SJ, Duffield TF, Leslie KE, et al: Defining and diagnosing postpartum clinical endometritis and its impact on reproductive performance in dairy cows. J Dairy Sci 2002;85:2223-2236.
21. Couto GB, Vaillancourt DH, Lefebvre RC: Comparison of a leukocyte esterase test with endometrial cytology for diagnosis of subclinical endometritis in postpartum dairy cows. Theriogenology 2013;79:103-107.
22. Tison N, Bouchard E, DesCoteaux L, et al: Effectiveness of intrauterine treatment with cephapirin in dairy cows with purulent vaginal discharge. (submitted, 2016)
23. Gardner IA, Greiner M: Receiver-operating characteristic curves and likelihood ratios: Improvements over traditional methods for the evaluation and application of veterinary clinical pathology tests. Vet Clin Pathol 2006;35:8-17.
24. Barlund CS, Carruthers TD, Walder CL, et al: A comparison of diagnostic techniques for postpartum endometritis in dairy cattle. Theriogenology 2008;69:714-723.
25. Gautam G, Nakao T, Yusuf M, et al: Prevalence of endometrtis during the postpartum period and its impact on subsequent reproductive performance in two Japanese dairy herds. Anim Reprod Sci 2009;116:175-187.
26. Cheong SH, Nydam DV, Galvao KN, et al: Use of reagent test trips for diagnosis of endometritis in dairy cows. Theriogenology 2012;77:858-864.
27. Denis-Robichaud J et Dubuc J: Determination of optimal diagnostic criteria for purulent vaginal discharge and cytological endometritis in dairy cows. J Dairy Sci 2015;98:6848-6855.
28. Tison N, Bouchard É, DesCôteaux L, et al: Effectiveness of intrauterine treatment with cephapirin in dairy cows with purulent vaginal discharge. 2016 (submitted).
29. LeBlanc S: Field study of the diagnosis and treatment of clinical endometritis in dairy cattle. Cattle Pract 2003;11:255-261.
30. Callahan CJ, Horstman LA: Treatment of postpartum metritis in dairy cows caused by *Actinomyces pyogenes*. Bovine Pract 1993;27:162-164.
31. Oltenacu PA, Britt JH, Braun RK, et al: Relationships among type of parturition, type of discharge from genital tract, involution of cervix, and subsequent reproductive performance in Holstein cows. J Dairy Sci 1983;66:612-619.
32. Dohmen MJ, Joop K, Sturk A, et al: Relationship between intra-uterine bacterial contamination, endotoxin levels and the development of endometritis in postpartum cows with dystocia or retained placenta. Theriogenology 2000;54:1019-1032.
33. Studer E, Morrow DA: Postpartum evaluation of bovine reproductive potential: comparison of findings from genital tract examination per rectum, uterine culture, and endometrial biopsy. J Am Vet Med Assoc 1978;172:489-494.
34. Lewis GS: Uterine health and disorders. J Dairy Sci 1997;80:984-994.
35. Lopez-Helguera I, Lopez-Gatis F, Garcia-Ispierto I: The influence of genital tract status in postpartum period on the subsequent reproductive performance in high producing dairy cows. Theriogenology 2012;77:1334-1342.
36. Leutert C, Suthar V, Heuwieser W: Evaluation of transrectal examination of cervical diameter by palpation in dairy cows. J Dairy Sci 2013;96:1063-1070
37. Kasimanickam R, Duffield TF, Foster RA, et al: Endometrial cytology and ultrasonography for detection of subclinical endometritis in postpartum dairy cows. Theriogenology 2004;62:9-23.
38. Leutert.C, von Krueger X, Plöntzke J et al: Evaluation of vaginoscopy for the diagnosis of clinical endometritis in dairy cows. J Dairy Sci 2012;95:206-212.
39. Morrow DA, Roberts SJ, McEntee K, et al: Postpartum ovarian activity and uterine involution in dairy cattle. J Am Vet Med Assoc 1966;149:1596-1609.
40. Kelton DF, Leslie KE, Etherington WG, et al: Accuracy of rectal palpation and of a rapid milk progesterone enzyme-immunoassay for determining the presence of a functional corpus luteum in suboestrous dairy cows. Can Vet J 1991;32:286-291.
41. Plöntzke J, Madoz L, De La Sota R et al: Subclinical endometritis and its impact on reproductive performance in grazing dairy cattle in Argentina. Anim Reprod Sci 2010;122:52-57.
42. Sheldon IM, Noakes DE, Rycroft AN, et al: Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. Reproduction 2002;123:837-845.
43. Kaufmann TB, Westermann S, Drillich M, et al: et al: Systemic antibiotic treatment of clinical endometritis in dairy cows with ceftiofur or two doses of cloprostenol in a 14-d interval. Anim Reprod Sci 2010;121:155-162.

44. Dohmen MJ, Lohuis JAC, Huszenicza G, et al : The relationship between bacteriological and clinical finds in cows with subacute/chronic endometritis. Theriogenology 1995;43:1379-1388.
45. Pleticha S, Drillich M, Heuwieser W: Evaluation of the Metricheck device and the gloved hand for the diagnosis of clinical endometritis in dairy cows. J Dairy Sci 2009;92:5429-5435.
46. Sheldon IM, Dobson H: Postpartum uterine health in cattle. Animal Reprod Sci 2004;82-82:295-306.
47. Wagener K, Grunet T, Prunner I, et al: Dynamics of uterine infections with *Escherichia coli*, *Streptococcus uberis*, and *Trueperella pyogenes* in postpartum dairy cows and their association with clinical endometritis. Vet J 2014;202:527-532.
48. de Boer MW, Leblanc SJ, Dubuc J, et al: Systematic review of diagnostic tests for reproductive-tract infection and inflammation in dairy cows. J Dairy Sci 2014;97:3983-3999.
49. Hartmann D, Rohkohl J, Merback S, et al: Prevalence of cervicitis in dairy cows and its effect on reproduction. Theriogenology 2016;85:247-253.
50. Knutti B, Küpfer U, Busato A: Reproductive efficiency of cows with endometritis after treatment with intrauterine infusions or prostaglandin injections, or no treatment. J Vet Med A Physiol Pathol Clin Med 2000;47:609-615.
51. Madoz LV, De la Sota RL, Suzuki K, et al: Use of hysteroscopy for the diagnosis of postpartum clinical endometritis in dairy cows. Vet Rec 2010;167:142-143.
52. Galvao KN, Greco LF, Vilela JM, et al: Effect of intrauterine infusion of ceftiofur on uterine health and fertility in dairy cows. J Dairy Sci 2009;92:1532-1542.
53. Mateus L, Lopez da Costa L, Carvalho H, et al: Blood and intrauterine leukocyte profile and function in dairy cows that spontaneously recovered from postpartum endometritis. Reprod Domest Anim 2002;37:176-180.
54. Azawi OI: Postpartum uterine infection in cattle. Anim Reprod Sci 2008;105:187-208.
55. Santos NR, Roman HB, Gilbert RO: The use of leukocyte esterase reagent strips for diagnosis of subclinical endometritis in dairy cows. Theriogenology 2006;66:666-667.
56. Gilbert RO, Santos NR: Dynamics of postpartum endometrial cytology and bacteriology and their relationship to fertility ion dairy cows. Theriogenology 2016;85:1367-1374.
57. Madoz LV, Giuliodori MJ, Migliorisi AL, et al: Endometrial cytology, biopsy, and bacteriology for the diagnosis of subclinical endometritis in grazing dairy cows. J Dairy Sci 2014;97:195-201.
58. Melcher Y, Prunner I, Drillich M: Degree of variation and reproducibility of different methods for the diagnosis of subclinical endometritis. Theriogenology 2014;82:57-63.
59. DeGuillaume L: L'inflammation genital postpartum de la vache. Thèse de Doctorat, AgroParisTech, 94704 Maisons-Alfort Cedex, 2010.
60. Gilbert RO, Shin ST, Guard CL, et al: Prevalence of endometritis and its effects on reproductive performance of dairy cows. Theriogenology 2005;64:1879-1888.
61. Kasimanickam R, Dufflield TF, Foster RA, et al: A comparison of the cytobrush and uterine lavage techniques to evaluate endometrial cytology in clinically normal postpartum dairy cows. Can Vet J 2005;46:255-259.
62. Overbeck W, Jager K, Schoon HA, et al: Comparison of cytological and histological examinations in different locations of the equine uterus-an in vitro study. Theriogenology 2013;79:1262-1268.
63. Pascottini OB, Dini P, Hostens M, et al: A novel cytologic sampling technique to diagnose subclinical endometritis and comparison of staining methods for endometrial cytology samples in dairy cows. Theriogenology 2015;84:1438-1446.
64. Foldi J, Kulcsar M, Pecsi A, et al: Bacterial complications of postpartum uterine involution in cattle. Anim Reprod Sci 2006;96:265-281.
65. Sheldon IM, Williams EJ, Miller AN, et al: Uterine diseases in cattle after parturition. Vet J 2008;176:115-21.
66. Sheldon IM, Noakes DE, Rycroft AN, et al: The effect of intrauterine administration of oestradiol on postpartum uterine involution in cattle. Theriogenology 2003;59:1357-1371.
67. Bonnett BN, Martin SW, Gannon VP, et al: Endometrial biopsy in Holstein-Friesian dairy cows. III. Bacteriological analysis and correlations with histological findings. Can J Vet Res 1991;55:168-173.
68. Williams EJ, Fischer DP, Noakes DE, et al: The relationship between uterine pathogen growth and ovarian function in the postpartum dairy cow. Theriogenology 2007;68:549-559.
69. Kaneko K, Kawakami S: Influence of experimental intrauterine infusion of *Arcanobacterium pyogenes* solution in ovarian activity in cycling cows. J Vet Med Sci 2008;70:77-83.
70. Cordero OX, Wildschutte H, Kirkup B, et al: Ecological populations of bacteria act as socially cohesive units of antibiotic production and resistance. Science 2012;337:1228-1231.
71. Bicalho MLS, Lima FS, Machado VS, et al: Associations among *Trueperella pyogenes*, endometritis diagnosis, and pregnancy outcomes in dairy cows. Theriogenology 2016;85:267-274.
72. De Boer M, Buddle BM, Heuer C, et al: Associations between intrauterine bacterial infection, reproductive tract inflammation, and reproductive performance in pasture-based dairy cows. Theriogenology 2015;83:1514-1524.
73. Westermann S, Drillich M, Kaufmann TB, et al: A clinical approach to determine false positive findings of clinical endometritis by vaginoscopy by the use of uterine bacteriology and cytology in dairy cows. Theriogenology 2010;74:1248-1255.
74. Werner A, Suthar V, Plöntzke J, et al: Relationship between bacteriological findings in the second and fourth postpartum and uterine infection in dairy cows considering bacteriological results. J Dairy Sci 2012;95:7105-7114.
75. Kelly R: Inflammation mediators and cervical ripening. J Reprod Immunol 2002;57:217-224.

76. Deguillaume L, Geffré A, Desquilbet L, et al: Effect of endocervical inflammation on days to conception in dairy cows. J Dairy Sci 2012;95:1776-1783.
77. Kutter D, Figueiredo G, Klemmer I: Chemical detection of leukocytes in urine by means of a new multiple test strip. J Clin Chem Clin Bio 1987;25:91-94.
78. Levy M Tournot F, Muller C, et al: Evaluation of screening tests for urinary infection in hospital patients. Lancet 1989;2:384-385.
79. Azoulay E, Fartoukh M, Galliot R, et al: Rapid diagnosis of infectious pleural effusion by use of reagent strips. Clin Infect Dis, 2000;31:914-919.
80. Rerknimitr R, Rungsangmanoon W, Kongkam P, et al: Efficacy of leukocyte esterase dipstick test as a rapid test in diagnosis of spontaneous bacterial peritonitis. World J Gastroenterol 2006;12:7183-7187.
81. Jacobs LM, Luk SS, Burns KJ: Advanced trauma operative management course: site and instructor selection and evaluation. J Am Coll Surg 2006;203:772-779.
82. Santos NR, Lamb GC, Brown DR, et al: Postpartum endometrial cytology in beef cows. Theriogenonolgy 2009;71:739-745.
83. Machado VS, Knauer WA, Bicalho MLS, et al: A novel diagnostic technique to determine uterine health of Holstein cows at 35 days postpartum. J Dairy Sci 2012;95:1349-1357.

Table 1. Measures of diagnostic accuracy: definitions

(TP=true positive; TN=true negative; FP=false positive; FN=false negative; see Table 5)
Accuracy: Level of agreement between the test result and the true clinical state (TP+TN)/(TP+TN+FP+FN).
Sensitivity (Se): Proportion (%) among all animals with the disease that test positive (proportion of true positive subjects; TP/TP+FN). It is the probability of getting a positive test result in animals with the disease.
Specificity (Sp): Proportion (%) among all animals without the disease that test negative (proportion of true negative subjects; TN/TN+FP). It is the probability of getting a negative test result in subjects without the disease.
Positive predictive value (PPV): Probability that an animal with a positive test result actually has the disease (TP/TP+FP). PPV is largely dependent on the prevalence of the disease in the population.
Negative predictive value (NPV): Probability that an animal with a negative test result does not actually have the disease (TN/TN+FN). NPV is largely dependent on the prevalence of the disease in the population.
Likelihood ratio (LR): The likelihood that a particular test result would be expected in a subject with the disease compared to the likelihood that the test result would be expected in a subject without the disease. When both probabilities are equal to 1, the test is of no value (for classification see Table 2).
Area under the ROC curve (AUC): An overall estimate of the discriminative power of a test. A perfect diagnostic test has an AUC of 1.0, whereas an undiscriminating test has an AUC of 0.5 (for classification see Table 3).
Odds ratio (OR): An overall measure of diagnostic accuracy that is used to estimate the discriminative power of a diagnostic procedure and to compare the accuracy of two or more diagnostic tests (TP/FN)/(FN/TN). It is not affected by the prevalence of the disease in the population.
Kappa test: A measure of agreement between two test results beyond what would be expected by chance alone. The range of possible values is -1 to +1 (for classification see Table 4).

Table 2. Classifying diagnostic accuracy based on likelihood ratio

	LR+	LR-
Excellent	**10.0**	**0.1**
Very good	6.0	0.2
Fair	2.0	0.5
Useless	1.0	1

Table 3. Classifying diagnostic accuracy based on AUC

AUC	Diagnostic accuracy
0.9-1.0	Excellent
0.8-0.9	Very good
0.7-0.8	Good
0.6-0.7	Sufficient
0.5-0.6	Bad
<0.5	Useless

Table 4. Arbitrary benchmarks for kappa values (Landis and Koch, 1977)

>0.81	Almost perfect agreement
0.61-0.8	Substantial agreement
0.41-0.60	Moderate agreement
0.21-0.40	Fair agreement
0.01-0.20	Slight agreement
0.0-0.01	Poor agreement

Table 5. Criterion standard test for diagnostic accuracy

	Disease+	Disease-
Test+	TP	FP
Test-	FN	TN

Figure 1. Vaginoscopic examination on a postpartum dairy cow (R.C. LeFebvre).

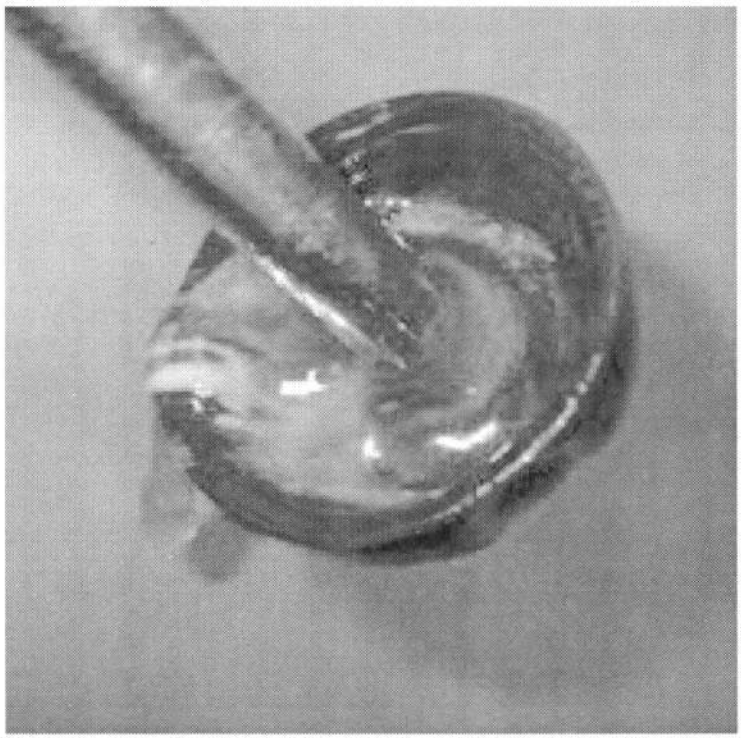


Figure 2. Postpartum genital examination using the Metricheck device (Metricure Clinic Talker, Merck 2011). Troubled vaginal mucus with less than 50% pus, cathegory #2.

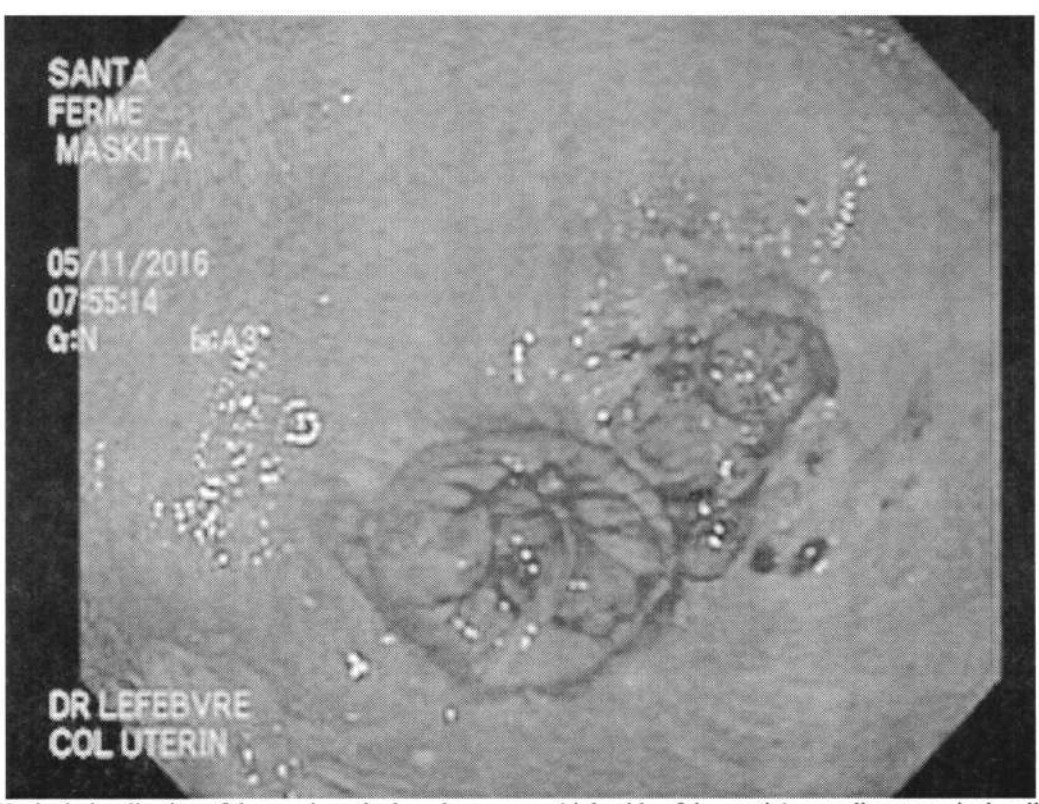


Figure 3. Vaginal visualization of the cervix and a lymphoma mass (right side of the cervix) spreading to vaginal wall and to the cervix during the vaginoscopy of the postpartum genital examination at 35 DIM.

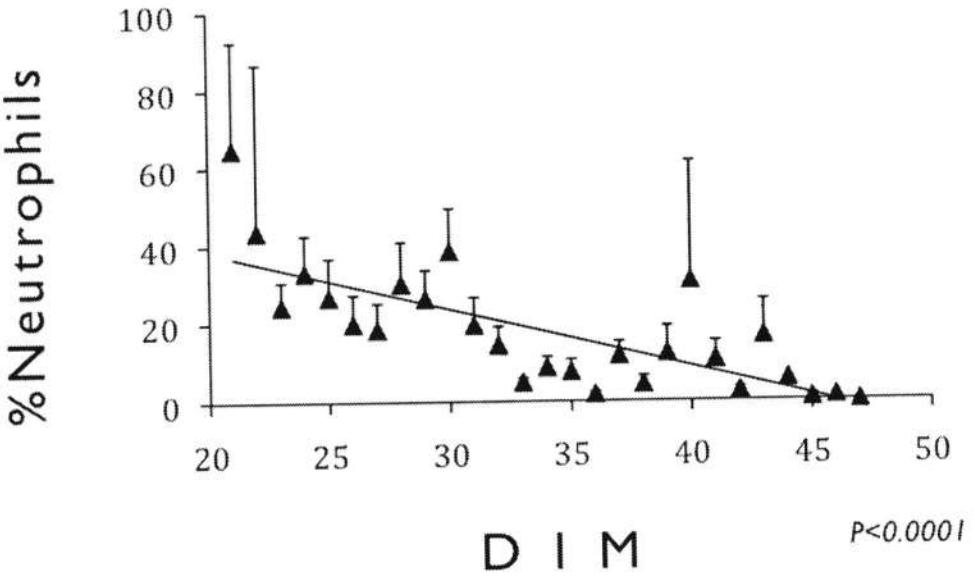


Figure 4. Correlation between endometrial cytology percentage of neutrophils and DIM. Data represent least-square means ± SEM (Couto et al., 2011)

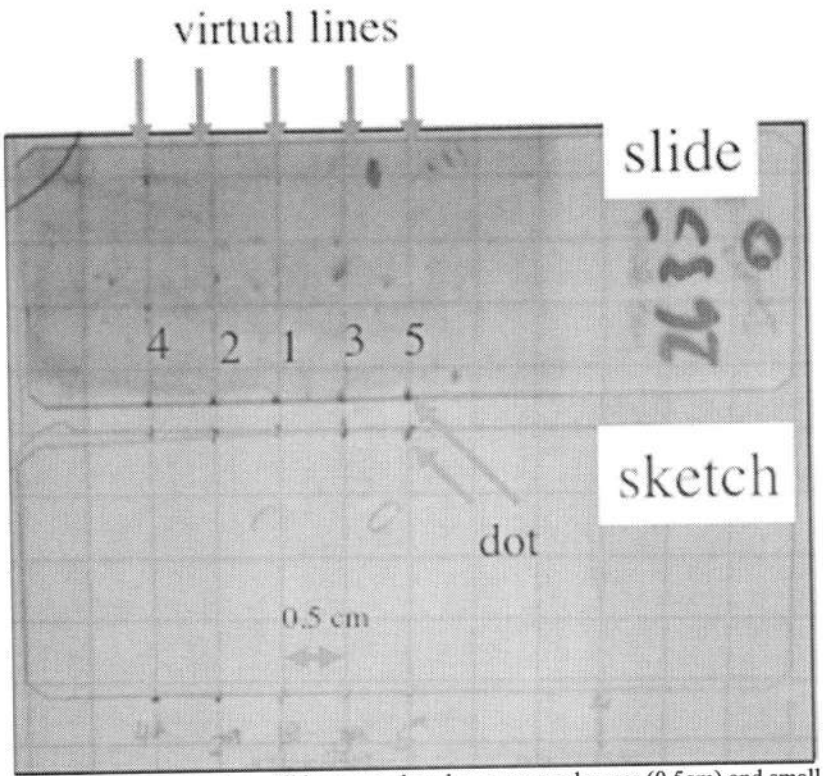


Figure 5. Methodology of RM slide evaluation. Slides were placed on a squared paper (0.5cm) and small dots were made at the bottom of the slide following the dots represented on the sketch previously made on the square paper. Following a virtual straight line bottom to top starting on 1 to 5, every 3rd field was assessed and the cells present were counted until a total of 500 cells were counted or until the top end of the 5th line was reached.

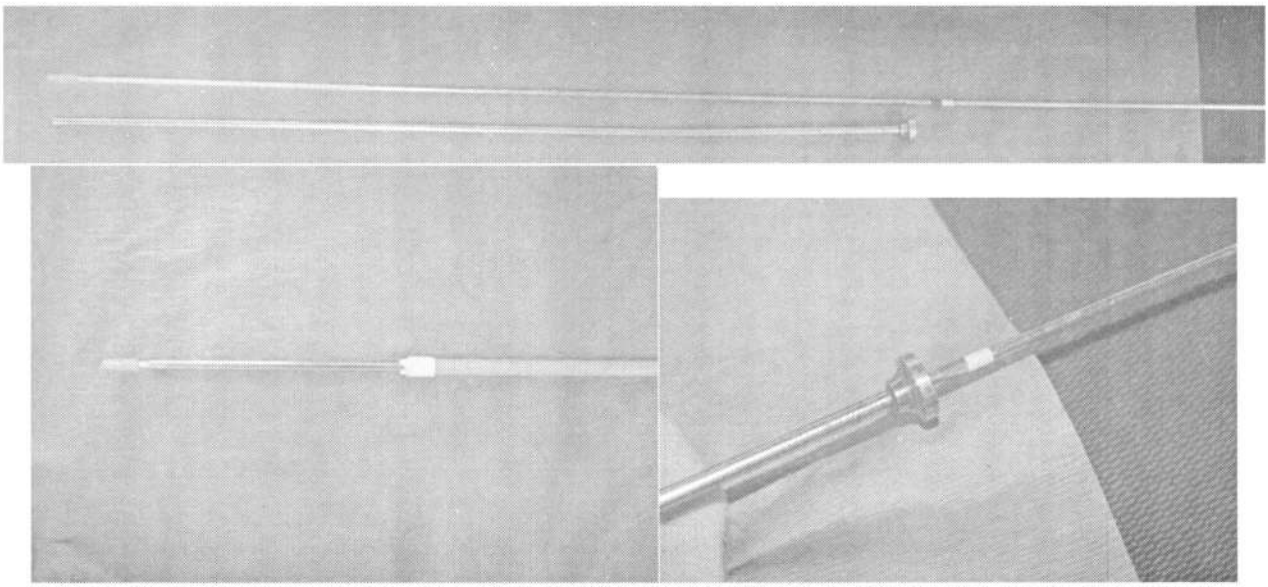


Figure 6. Uterine sample is collected using a cytobrush (VWR Canlab, Mississauga, ON, Canada). Briefly, the cytobrush is screwed onto a stainless steel rod and placed in a 65 cm-long stainless steel tube for passage through the cervix. The instrument is inserted into a guarded pipette, (Continental Plastic Corp.) for protection from vaginal contamination (R.C. LeFebvre).

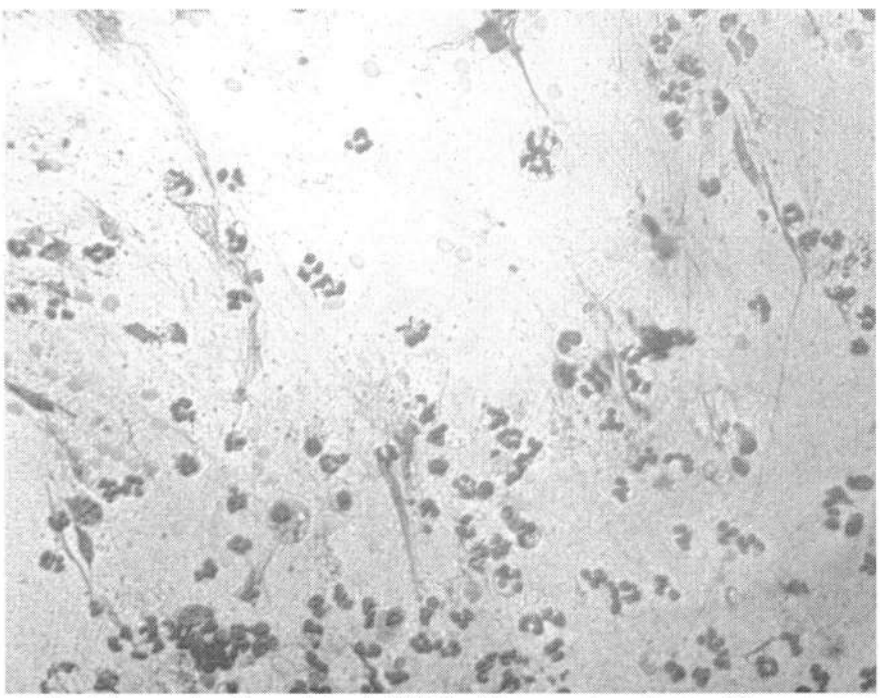


Figure 7. Endometrial cytology made with a cytobrush (400X). A large number of neutrophils are present on the slide with endometrial cells and mucus and cytoplasm

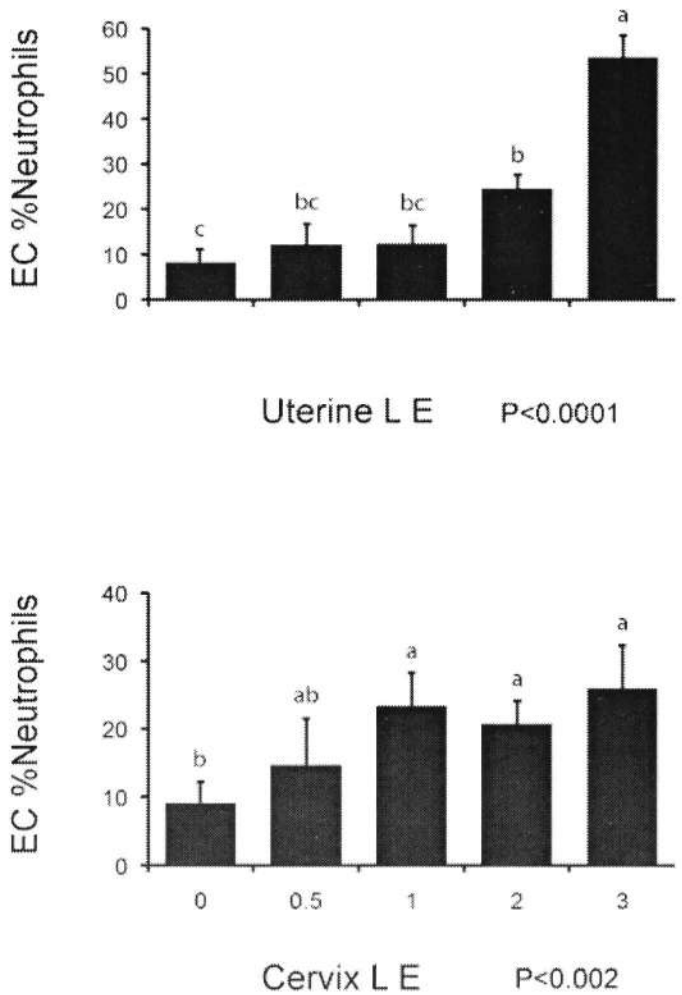


Figure 8. Relationship between endometrial cytology (EC % neutrophils) and leukocyte esterase activity scores in the uterus (n=218) and cervix (n=204). Data represent least-square means ± SEM. Bars with different superscript differ (P<0.05; Couto et al., 2011)

(Editor's note: Photographs in this manuscript are available in color in the online edition of Clinical Theriogenology.)

Uterine immune function in the dairy cow: immunomodulation/immunosuppression

R.C. Lefebvre, N. Tison
Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC, Canada

Abstract

Reproductive performance in dairy cows and farm economic efficiency are related to uterine health status at the end of the voluntary waiting period (VWP). Uterine diseases affect about half of all dairy cows in the postpartum period, causing infertility by disrupting uterine and ovarian functions. The reproductive cycle of dairy cows has several critical checkpoints where interactions with the host immune response are necessary, and where deregulation can lead to reproductive failure. As immunity of the reproductive tract appears more and more as a major player in uterine health during the periparturient period in dairy cows, a better understanding of the complex immunological processes at play in the periparturient cow is essential to design animal and herd health management strategies to prevent postpartum uterine diseases in dairy cows and to reduce the carryover negative effect on reproductive performance. The objective of the present review is to focus on the immunological processes involved in pregnancy and the periparturient period in the dairy cow that could be associated with uterine diseases, as they relate to reproductive failure after the VWP.

Introduction

In dairy cows, the incidence of disease during the periparturient period is high. During the postpartum period, the most prevalent pathologies are metabolic, mammary and uterine diseases. Although these pathologies involve different organs, they are all linked to an impairment in immune function during the periparturient period. Of these pathologies, it is postpartum uterine diseases that have the greatest impact on the health and productivity of dairy cows and, consequently, on the financial sustainability of the industry. Both reproductive performance in dairy cows and farm economic efficiency depend on the uterine health status of cows at the end of the VWP.[1-4]

The reproductive cycle of dairy cows has several critical checkpoints where interactions with the host immune response are necessary, and where deregulation can lead to reproductive failure. Several inflammatory mediators have evolved alongside reproductive physiology and signaling reproductive events rather than acting exclusively as part of the inflammatory process. Some of these inflammatory mediators are associated with fertilization (e.g., in response to allogeneic sperm), pregnancy recognition, pregnancy maintenance in the presence of an immunologically distinct fetus, parturition, and uterine involution. Of course, immune defense also plays a critical role in infectious and inflammatory processes, especially those caused by sexually transmitted pathogens. In humans, more than twenty different pathogens can be transmitted through sexual intercourse.[5] However in dairy cows, where artificial insemination has become the major mode of reproduction, sexually transmitted diseases are limited (trichomoniasis, campylobacteriosis, and leptospirosis).[6] The postpartum period is by far the most vulnerable period for infection and inflammation of the reproductive tract in dairy cows.

Reproduction and immunity are very complex processes and interlaced through common mediators. Consequently, determining etiology, establishing a diagnosis, and achieving effective treatment for a reproductive pathology are often challenging. Clearly, understanding immunological processes in reproductive physiology is critical to improving the current management for high-yielding dairy cows. This review focuses primarily on the immunological processes involved in pregnancy and the periparturient period in the dairy cow, as they relate to reproductive failure after the VWP.

Innate versus adaptive immunity: a review

The immune system is divided into two major subsystems: innate and adaptive. When protecting the body against invading pathogens, the actions of these subsystems are different but complementary.

Innate immunity, which encompasses mechanical, chemical, and cellular components, is the first responder. It is on constant standby to respond promptly at the first signs of microbial invasion.[7]

The vulva, vagina, and cervix constitute mechanical barriers that prevent ascending invaders from reaching the uterus. In short, it is the mucosa of the vagina and the uterus that prevent pathogens from invading the reproductive tract. Mucus is a family of high-molecular-weight glycoproteins that are very effective at trapping microbes[8] and inhibiting the growth of pathogens because of its acidic effect. Furthermore, epithelial cells produce antimicrobial peptides and glycoproteins that neutralize bacteria before they even reach the uterine cavity. Intact mucosal epithelial cells also play an active immunological role by sending signals to the underlying tissues immune cells, which then produces cytokines and chemokines. These substances act to destroy infected cells through necrosis, apoptosis, or phagocytosis, in addition to interacting with the adaptive immune response.

An important chemical component of the first line of immune defense is natural antimicrobial peptides (NAPs). These are produced by epithelial cells and neutrophils, and eliminate pathogens through abrogation of pH and ionic concentration gradients.[9] Natural antimicrobial peptides possess additional functions in cell proliferation, cytokine induction, chemotaxis and modulating the innate and acquired immunity.[10] The major NAPs are defensin, elafin, cathelicidin, secretory leukocyte protease inhibitor (SLPI), and lactoferrin.

As microbes evolved to become more efficient in breaking host defenses,[11] the innate immune system was forced to develop other response mechanisms. The most rapid and active immune response to bacterial infections is provided by pattern recognition receptors (PRRs) which recognize pathogens based on pathogen-associated molecular patterns (PAMPs).[12] Pattern recognition receptors can also recognize tissue and cellular damage in the host based on damage-associated molecular patterns (DAMPs) including nucleic acids released from damaged or dead cells.[13] The most studied PRR is the Toll-like receptor (TLR) family and its nine members. Toll-like receptors are expressed at the surface of several innate immune cells, including neutrophils, macrophages, dendritic cells, dermal endothelial cells, and mucosal epithelial cells.[14] This is the major sensing component of the innate immune system. Other classes of PRRs have also been identified: nucleotide oligmerization domain (NOD)-like receptors (NLRs); retinoic acid inducible gene I (RIG-I)-like receptors, or RLRs; and cytosolic DNA sensors.[15]

Toll-like receptors are the most documented form of PRR in cows. These receptors detect and identify PAMPs, gather adapters to initiate intracellular signaling pathways to recruit immune cells, secret antimicrobial factors that eradicate pathogens, and facilitate interaction (crosstalk) with the adaptive immune system.[16] Toll-like receptors are a fixed set of sensors programmed to respond the same way against all types of invaders and are highly conserved across species. Toll-like receptors signal transduction is mediated by the recruitment of different intracellular mediators like MyD88 and TRIF, which leads to the production of cytokines and chemokines, as well as prostaglandins, vasoactive mediators that manage the defense strategy and eventually cause the clinical signs associated with disease (e.g. swelling).

After TLRs have sensed and identified invading pathogens, the innate immune cellular effectors are called into play. These effectors include macrophages, neutrophils, eosinophils, basophils, and mast cells. After initiating phagocytosis, cellular effectors then release radicals, enzymes, and other molecules into the compartment in order to desactivate the contained invader and prevent the host from being exposed to dangerous elements. If the cellular effectors cannot eliminate the invader, molecular effectors like the protein complement system (e.g., C3) or mannose-binding lectin (MBL) proteins go to work in the extracellular space, although this process exposes host cells to toxic elements.

The cascade of events associated with the host response and triggered by the activation of inflammatory mediators of the innate immune system results in the cardinal signs of inflammation: fever, swelling, redness, and pain. Pus is a sign of infiltration by neutrophils and macrophages into a tissue that has been invaded by pathogens. Common inflammatory mediators in the reproductive tract include the cytokines IL-1b, IL-6, and TNF, along with chemokines such as IL-8, and the prostaglandin E2. Cytokines often produce secondary clinical effects like reduced appetite and activity.

Innate immunity is often triggered within hours of a microbial invasion; by contrast, the adaptive immunity response takes days. For example, it is not until seven days after first exposure, and two to three days after a second exposure, that the first antibody is measured in serum. In chronic situations the innate immune response remains similar in nature and efficiency over time, but in the case of the adaptive immunity, the response becomes more efficient at subsequent invasions.

The adaptive immune system functions in a very different way from the innate immune system. The primary difference lies in its ability to recognize many different unique molecular components of the invader and so block invasions in the future. The process is based on sampling and presenting pieces of the invader to adaptive immune cell responders called dendritic cells, which are differentiated monocytes that enter the invaded tissue. A molecular piece of the invader is anchored on the surface of the dendritic cells via major histocompatibility complex protein class I and class II molecules (MHC Is and MCH IIs). The dendritic cells then migrate to meet the lymphocytes in places like the lymph nodes and bone marrow. The adaptive immune system relies on two different families of lymphocytes: B cells derived from the bone marrow, and T cells derived from the thymus. All lymphocytes develop their specificity through gene rearrangement; in short, the antigen-binding domain of heavy and light chains is re-arranged, enabling it to make antibodies of different classes. B cells recognize over 1,000 different antigens, some of which become antibody factories to produce a great number of antibodies for selected antigens. The more disulfide binding between chains, the more rigid and specific the antibody molecule is. The main classes of antibodies are: IgG, which exhibit the highest concentrations; IgM, the first antibody made after the activation of B cells; IgA, which is important for mucosal surface defense; and IgE, which is associated with fighting parasites and allergies.

In T cells, the different chains (alpha, beta, gamma, and delta) are re-arranged to provide different antigen receptors (TCRs) and different specificities to lymphocytes. Gamma and delta chains are the most common chains of T cells in cows. There are two types of T cells. The first is the helper T cell, which manages adaptive immunity by making the right combination of cytokines and surface proteins to provide enough B and T cells with killing capabilities at the right time. Killer T cells can recognize the right piece of antigen using the MHC protein on their cell surface and then destroy the invader. As with the B cell, the rearrangement of TCR genes enables recognition of more than 1,000 different antigens.

Due to the complexity of immune processes, specific tissues like lymph nodes and the spleen are organized as adaptive immunity screening and production facilities. The level of adaptive immune response is based on the number of lymphocytes able to recognize and respond to antigens. Thus, the larger the number of responding B and T cells, the faster and stronger the response to the invader.

Cytokines (e.g. TNF alpha, IL-1 and IL-6) are small pleiotropic glycoprotein mediators whose biological action is locally mediated by specific receptors.[17] Chemokines (e.g., IL-8) are small chemotactic cytokines that act locally by recruiting leukocytes to the site of inflammation and activating them.[18] Basically, chemokines attract immune cells to the tissue and then cytokines differentiate and activate them. Immune cells, including monocytes, macrophages, NK cells, and DCs, are sources of immunoregulatory cytokines and chemokines in the female reproductive tract. The concentration of cytokines and chemokines in the endometrium varies during both normal physiological processes and in the presence of pathological conditions of the reproductive tract.[19] Both cytokines and chemokines have been shown to be critical for fertilization, implantation, pregnancy, and remodeling of the uterus during the menstrual cycle in women. [20]

Modulation and suppression of immunity during pregnancy and the periparturient period

Immune modulation during pregnancy

The fertility rate for dairy cows is around 90% and does not differ between low-to-moderate and high-producing animals.[21] However, the calving rate in lower producing animals is approximately 55%, compared to 35% in high-producing animals. Pregnancy losses are thought to occur primarily at the recognition or pre-implantation period.[21] In fact, pregnancy is an active and highly regulated immunologic process in which the maternal immune system adjusts to the pregnancy and ensures support of the

developing alloantigenic conceptus without threatening the mother.[22] This immune tolerance may be explained by the antigenic immaturity of the fetus because, as is the case in other mammalian species, the bovine trophoblast does not express MHC I protein in areas that are in contact with the maternal endometrium during early pregnancy.[23] This phenomenon reduces exposure of the maternal system to paternal antigens.[24] Chorionic tissue down-regulates MHC I expression throughout the pregnancy at the placental level, while it does so only during the first half of the pregnancy at the interplacentomal space.[25] Interestingly, bovine embryos produced through somatic cell nuclear transfer express MHC I earlier during pregnancy and have a much lower conception rate than *in vivo* embryos.[26] Furthermore, the genes and pathways of innate and adaptive immunity involved in the maternal immune response to the embryo during the pre-implantation period are up-regulated.[27]

Another immunological adjustment performed during pregnancy is the recruitment of macrophages. These cells play an important role in uterine remodeling before embryo implantation, immune tolerance toward fetal antigens, parturition, postpartum involution, immunomodulation of neighboring leukocytes, and uterine infection.[28] During pregnancy, there is a massive accumulation of macrophages, mainly in the superficial uterine stroma in the interplacentomal region, in particular in the stroma of the maternal-villus tree of placentomes.[29,30] A regional effect is also seen for MHC II expression on macrophages. In sheep, pregnancy is also associated with an increase in the number of macrophages in stroma.[31] Some macrophages are pro-inflammatory (M1 type) and have the capacity of presenting antigens (cell-mediated immune response). Others are anti-inflammatory cells (M2 type) are produced during wound healing and tissue remodeling; their activity is immunosuppressive[32] or promote an antibody-mediated immune response.[33] The final effect of macrophages depends on their polarization status, which is determined by the surrounding milieu. Endometrial macrophages also produce platelet-derived growth factor beta, which plays a role in tissue remodeling and proliferation.[34,35] In cattle, there is evidence that IFN-t alters peripheral and endometrial immune cell populations by increasing cells exhibiting T regulatory phenotypes (CD4+/CD25+) able to secrete IL-4, which can induce tolerance to paternal alloantigens.[29] It should be noted that since there is no increased incidence of disease during pregnancy, alteration in maternal immune regulation during pregnancy does not seem to equate with generalized immunosuppression.

Inflammatory mediators have been recognized as a necessary component for the establishment, development, and maintenance of pregnancy. However, they may also be a significant impediment to a successful periparturient period in the dairy cow because they cause a deregulation of the immune system at the end of pregnancy when there is increased demands on the cow as the fetus completes its development and the mother prepares for lactation. The prolongation of this physiological and immunological processes may transform immunomodulation during pregnancy into immunosuppression in postpartum period.

Immune suppression during the periparturient period: the prepartum-postpartum nexus

The periparturient period covers the last two months of gestation and the first two months following delivery. It is particularly the transition period, the period three weeks before and three weeks after delivery, that is associated with an increased risk of disease and, more specifically, uterine disease. This is because this period is characterized by dramatic changes in the dairy cow's metabolism and immune defense. In general, the nature of the response of uterine tissue to postpartum bacterial infection points to the important role played by the innate immune system.

There are a number of serious limiting stressors to the parturient's innate immunity that eventually lead to a derugulated inflammatory response and an increased risk of uterine disease. These include a negative energy balance (NEB) associated with final fetal growth and the initiation of milk production;[36,37] changes in the digestive tract associated with reduced dry matter intake; increased metabolic demands; changes in circulating hormones like progesterone, estrogen, cortisol, and prostaglandins associated with birth;[38] and changes in the mission of the reproductive immune system as it passes from a controlled subpressive mode during gestation to a more active mode that attempts to control an overwhelming uterine infection during the postpartum period.

While bacterial infection is present in all cows in early postpartum, about 50% of them have compromised uterine health at the end of the VWP. What determines, then, whether or not the postpartum uterus go through a normal process of involution and returns to normal status, allowing for the establishment of a new pregnancy at the end of the VWP? Researchers hypothesize that high-producing dairy cows have a compromised systemic or local immune system to start with.

Parturition is a significant stressor on immunity in the dairy cow. The stress of parturition stimulates corticotropin-releasing hormone (CRH), causing the production of adrenocorticotropic hormone (ACTH), which then activates the hypothalamic-pituitary axis and increases plasma corticosteroids.[39] During parturition, cows can exhibit a three-to-fourfold increase in baseline plasma cortisol concentrations. During metabolic disorders like hypocalcemia, a five-to sevenfold increase in the serum cortisol level can occur.[40] Similarly, dystocia increases cortisol and can suppress the immune defense of dairy cows.[41] Since glucocorticoids are elevated for only 24 hours at parturition and the immunosuppression seems to last for at least 21 days after calving,[42,43] glucocorticoids are thought to be partly responsible for periparturient immunosuppression.[44] In addition, changes in estradiol and progesterone just prior to calving may affect the immunocompetence of cows.[45]

Immediately after calving, cows are poorly positioned to fight against postpartum infection and maintain control over the process of uterine involution. Loss of integrity of the endometrial lining during the early postpartum period[46] removes an important physical barrier and neutralizes the action of mucins against bacterial invasion of the endometrial stroma.[47] Antibacterial peptides that play a role in innate immune defense are eliminated.[31] Vaginal local immunity may play an important role in uterine health. Intravaginal infusion of lactic acid bacteria decreased uterine infections and improved local and systemic immune responses in transitional dairy cows.[48] The loss of uterine epithelium also means loss of potential expression of TLRs, a major component of the innate immune defense system in postpartum infectious uterine diseases. Finally, the deep abdominal location of the uterus in early postpartum is a mechanical impediment to uterine clearance. In the mare, differences between females in their ability to clear the uterus after breeding is related to susceptibility to postbreeding endometritis.[49]

Reduced immune defense is observed not only around calving, but also during the whole transition periods. During the periparturient period, the proliferation of leukocytes (lymphocytes and neutrophils) is severely depressed, the ability of neutrophils to aggregate and phagocytose is reduced, and the cytotoxic activity of lymphocytes as well as the concentration of chemokines like IL-8 is decreased.[50] Neutrophils are recognized as being the most important cell type for protecting the mammary gland and uterus from infection.[51,52] Polymorphonuclear neutrophils (PMNs) in postpartum cows produce less reactive oxygen species (ROS) compared to PMNs in mid-lactation and prepartum cows.[53] In addition, serum levels of IgG begin to decrease 8 weeks before calving, and serum levels of IgM fur weeks before calving. IgG levels recover by four weeks postpartum but IgM levels remain low.[54] There is also a decrease in IgG1 levels during the peripartum period, most likely associated with the transport of immunoglobulins from the blood stream to the mammary gland. IgG1 is the immunoglobulin found in highest concentrations in the blood and milk of cows, and it plays an important role in antibody-mediated defense mechanisms.[55]

Whereas the dry period in cows may be considered to be a resting phase between lactations, in reality considerable fetal growth, mammary tissue remodeling and high nutrition demands occur. The transition from late gestation to early postpartum results in dramatic metabolic disturbances in the lactation cycle of the dairy cow. The sudden increase in nutrient requirements for milk production at the time when dry matter intake and nutrient supply lags behind may be associated with inflammation and dysregulated immune responses and may represent the missing link in the pathobiology of disorders during that period. Metabolic status and levels of metabolites have an impact on the immune response of dairy cows and the risk of disease. During the transitional period, the cow's intake of dry matter decreases, which further exacerbates the imbalance between energy needs and energy supply, and produces a NEB. In severe NEB cows, inflammatory immune genes are up-regulated[36] and genes involved in the acquired immune responses are down-regulated.[43] The NEB lowers glucose concentration, increases levels of ketones and fatty acids, and induces subclinical ruminal acidosis.[55,56]

Glucose is the preferred nutrient of immune cells[58] and has a stimulatory effect on the immune response. It is associated with increased proliferation and differentiation of leukocytes, and increased neutrophil chemotaxis and phagocytosis during inflammation.[59] An increase of leukocyte activation is associated with hyperglycemia.[60] Hypoglycemia is therefore associated with dysregulated immune cellular mediators. Insufficient blood glucose levels induce a decline of insulin and mobilization a triacylglycerol deposits as NEFA[61] which generates the metabolite acetyl coenzyme A (acetyl CoA) and energy via the Krebs cycle. In excess of acetyl CoA, ketones (acetoacetic acid, acetone, and B-hydroxybutyrate[BHB]) is produced. Ketone bodies negatively impact the immune response, causing a reduction in trap formation, chemotaxis and the phagocytosis of neutrophils, and reduced blastogenesis in lymphocytes.[62] Cows with subclinical ketosis as measured by BHB concentrations are at higher risk of metritis during the first two weeks postpartum.[63] Hyperketonemia has been shown to have multiple negative effects on different immune functions.[64,65] even though immune cells do not use ketones as an energy source[66] and that direct effect of ketones has not been measured in vitro.[67]

Depending on the level of saturation, non-esterified fatty acids (NEFAs) have both immunostimulatory and immunosuppressive effects on macrophage function by acting as ligands to TLRs.[56] High concentrations of NEFAs suppress DNA synthesis, IgM secretion and IFN-g production in monocytes. In general, saturated FAs decrease the phagocytic capacity of murine macrophages while unsaturated FAs increase it.[68] In dairy cows, most studies have focused on metabolic status and disease[69-70] and so the precise mode of action is unknown. Clinically, NEFA levels during the prepartum period are considered a better indicator of the risk of metritis, milk fever, and retained placenta than is plasma BHB, glucose concentration or calculated energy balance.[71] Mid-lactating cows with dietary-induced NEB show a downregulation of neutrophil expression of genes associated with antigen presentation, respiratory burst, and pro-inflammatory response.[72]

High milk producing cows demand addition of concentrates into their diet composition. High content of starch in grains increases fermentability by rumen microbes and production of short-chain fatty acids reducing ruminal pH.[73] The resulting ruminal lesions of this process allow penetration of bacteria and endotoxin with dissemination to the bloodstream.[74] The presence of LPS in the bloodstream may challenge the immune system of the animal by binding to PRR, affecting leukocyte populations, triggering the production of proinflammatory cytokines and acute phase proteins.[73] To trigger these processes, the period of ruminal acidosis most be long enough. Presence of LPS into the systemic circulation stimulates the release of proinflammatory cytokines (IL-1, IL-6 and TNFa) by mononuclear phagocytes.[75,76]

Reproductive immunity and infertility

The periparturient period in dairy cows is characterized by an activation of the innate immune system that results in nearly 50% of all parturients showing signs of a uterine disease like metritis or endometritis. The recruitment of immune cells and inflammatory mediators coordinates the host immune response, whose task is to eliminate bacterial infection, and restore normal endometrial function[77] and fertility at the end of the VWP. However, there is evidence to suggest that inflammation may persist long after the VWP and cause infertility. These long-term effects of the inflammatory process can impact endocrine signaling by the hypothalamic-pituitary-ovarian axis, uterine health, ovarian function, oocyte quality, and, ultimately, the establishment of pregnancy.

As previously mentioned, many factors produced by the oviducts and uterine endometrium are also immune mediators when there is any temporal or spatial deregulation causing an over- or under-expression of their production during infection. These immune mediators can then jeopardize embryo development either directly,[78] or indirectly via its effects on the endocrine signaling system of the hypothalamic-pituitary-ovarian axis. For example, embryos cultured in fluid extracted from an inflamed uterus show a reduction in the number of blastomeres and their allocation. This would affect the embryo-mother interaction and potentially compromise pregnancy establishment. Proinflammatory mediators like IL1, IL6, TNF, and PGE are unregulated in endometrial cells in animals with endometritis compared to healthy animals.[79, 80] The induction of these mediators is TLR-dependent, depending on the bacteria

components used.[81, 82] Finally, LPSs, acting either directly or through inflammatory mediators, change the neuroendocrine signaling pathway. Intrauterine, systemic and intramammary LPS administration reduce gonadotropin releasing hormone (GnRH) secretion, luteinizing hormone (LH) pulses, and ovulation.[83-85]

Cows exhibiting clinical disease show a longer interval to estrus, irregular ovarian cycles, prolongation of the postpartum luteal phase, delayed onset to ovarian cyclicity, and, ultimately, failure to conceive.[86] Ovarian function is perturbed both through this deregulation of the neuroendocrine axis previously described as well as directly. Although follicular fluid is free of immune cells, granulosa cells possess TLR, CD14, and MD-2 cells, which can recognize bacterial components[87] and respond to invaders by increasing Il1b, IL6, and TNFa production.[88-89] Lipopolysaccharide affects the estradiol production of granulosa cells and oocyte maturation by increasing germinal vesicle breakdown and changing spindle formation.[90] Both antral and primordial follicle activation is increased because of the loss of specific proteins like PTEN/FOXo3a. This causes a depletion in the population of primordial follicles and eventually leads to infertility.

In general, then, the same immune mediators are involved in inflammation and ovarian function. As a result, when these immune mediators are perturbed due to uterine bacterial infection, they have a negative effect on fertility performance and this effect can last after the resolution of the uterine infection per se. The potential effect of postpartum uterine disease on folliculogenesis could explain the carryover effects of uterine disease on reproduction. Potentially, oocyte can grow and ovulate even though its developmental competence is disturbed. [90]

Take-home messages

1. The preferential energy partitioning in dairy cows toward milk production as a mono-focal goal fuels immunosuppression and leads to high rates of the reproductive tract infection and inflammation. It impacts the overall health of dairy cows, in particular reproductive efficiency.
2. Several inflammatory mediators have evolved alongside reproductive physiology in dairy cows. These mediators are used for signaling reproductive events like ovulation, fertilization, pregnancy recognition, and pregnancy maintenance. Thus, inflammatory processes are both a requirement and an impediment to uterine health and successful pregnancy.
3. Innate immunity encompasses mechanical, chemical, and cellular components. It is on constant standby to respond promptly to the first signs of microbial invasion.
4. Immunosuppression results in an increased incidence and severity of infections around the time of calving and in postpartum period of dairy cows.
5. The parturient period is the most challenging metabolic stage in the lactation cycle of dairy cows because it causes a physiological imbalance and inadequate adaptation to lactation. Various metabolites and nutrients (e.g. glucose, NEFA, and BHBA) influence the cow's immune response and increase the risk of infectious diseases in the reproductive tract.
6. Understanding immunological processes in reproduction is critical to resolving reproductive problems in high-yielding dairy cows and improving the current management model.
7. Uterine bacterial infections affect immune mediators involved in both inflammation and in reproduction at the neuroendocrine, ovarian, and uterine levels. This means that the infection's negative effect on fertility performance may be carryover for a long period of time after the resolution of the uterine infection per se.

Practical implications

Starting at the drying period, all veterinary actions need to consolidate the immune strength of the dairy cow so that the parturient can meet the demand in postpartum period. Vaccination, stress control, well-being, comfort, nutrition, environment, management, genetic selection, calving care, early diagnosis of uterine infections are of equal importance toward a strong immunity, good fertility, and early establishment of a normal pregnancy.

Issues yet to be resolved

1. What imbalances tip the scale toward immunosuppression, and what are the cutoff points of these imbalances in order to negatively affect immunity and eventually, to cause infertility?
2. The importance of stress as a potent immunosuppressant in postpartum dairy cows.
3. The mechanism behind continued infertility following resolution of the uterine infection.
4. The mechanism of action by which toxins (LPS) and bacteria in the uterus and rumen during infection, affect hypothalamic-pituitary-ovarian function.
5. Characterization of cervicitis.
6. The global view of the cow immunity during the periparturient period in association with postpartum diseases.

Conclusion

There is growing evidence that the female reproductive tract is associated with a complex system of immune protection. It is becoming clear that several features (mucus, epithelial barrier, PAMPs, cytokines, chemokines by epithelial cells, and TLRs) have evolved to meet the challenges of the periparturient period. As the same mediators play a role in both immunity and female physiological reproductive function, fertility in dairy cows is intimately linked to immune system functioning. The tremendous metabolic burden that dairy cows experience during reproduction can disrupt the precarious balance achieved in the postpartum uterine defense in early postpartum, increasing the risk of disease and negatively impacting long term fertility. An understanding of the complex immunological processes at play in the periparturient cow is essential to design animal and herd health management strategies to prevent postpartum uterine diseases in dairy cows and to reduce the carryover negative effect on reproductive performance.

Acknowledgements

The authors thank Susan Lempriere and Dr. Angelika E Stock for reviewing an earlier version of this manuscript.

References

1. Sheldon IM, Dobson H: Postpartum uterine health in cattle. Anim Reprod Sci 2004;82:295-306.
2. Bartlett P, Kirk J, Wilke M, et al: Metritis complex in Michigan Hostein-Freisian cattle: incidence, descriptive epidemiology and estimated economic impact. Prev Vet Med 1986;4:235-248.
3. Drillich M, Beetz O, Pfutzner A, et al: Evaluation of a systematic antibiotic treatment of toxic puerperal metritis in dairy cows. J Dairy Sci 2001;84:2010-2017.
4. Overton M, Fetrow J: Economics of postpartum uterine health. Dairy Cattle Reprod Council Conf; Omaha NE; 2008. p. 39-43.
5. Wira CR, Patel MV, Ghosh M, et al: Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections. 2011;65:196-211.
6. Hopper RM: Bovine reproduction. Hoboken: John Wiley and Son Inc, 2015. p. 481-517.
7. Wira CR, Fahey JV, Sentman CL, et al: Innate and adaptive immunity in female genital tract: Cellular responses and interactions. Immunol Rev 2005;206:306-335.
8. Carson DD, DeSouza MM, Kardon R, et al: Mucin expression and function in the female reproductive tract. Hum Reprod Update 1998;4:459-464.
9. Wiesner J, Vilcinskas A: Antimicrobial peptides: the ancient arm of the human immune system. Virulence 2010;1:440-464.
10. Hazlett L, Wu M: Defensins in innate immunity. Cell Tissue Res 2011;343: 175-188.
11. Baxt LA, Garza-Mayers AC, Goldberg AC, et al: Bacterial subversion of host innate immune pathways. Science 2013;340:697-701.
12. Nasu K, Narahara H: Pattern recognition via the toll-like receptor system in the human female genital tract. Meidators Inflamm 2010;97:602-604.
13. Chen GY, Nunez G: Sterile inflammation: sensing and reacting to damage. Nat Rev. Immunol 2010;10:826-837.
14. Fazeli A, Bruce C, Anumba DO: Characterization of Toll-like receptors in the female reproductive tract in human. Hum Reprod 2005;20:1372-1378.
15. Kanneganti TD, Lamkanfi M, Nünez G, et al: Intracellular NOD-like receptors in host defence and disease. Immunity 2007;27:549-559.

16. Thompson MR, Kaminski JJ, Kurt-Jones EA, et al: Pattern recognition receptors and the innate immune resonse to viral infection. Viruses 2011;3:920-940.
17. Balu RB, Savitz DA, Ananth CV, et al: Bacterial vaginosis and vaginal fluid defensins during pregnancy. Am J Obstet Gynecol 2002;187:1267-1271.
18. Salamonsen LA, Hannan NJ, Dimitriadis E, et al: Cytokines and chemokines during human embryo implantation: Roles in implantation and early placentation. Semin Reprod Med 2007;25:437-444.
19. Fahey JV, Schaefer TM, Channon JY, et al: Secretion of cytokines and chemokines by polarized human epithelial cells from the female reproductive tract. Hum Reprod 2005;20:1439-1446.
20. Tabibzadeh S: Evidence of T-cell activation and potential cytokine action in human endometrium. J Clin Endocrinol Metab 1990;71:645-649.
21. Diskin MG, Murphy JJ, Screenan JM, et al: Embryo survival in dairy cows managed under partoral conditions. Anim Reprod Sci 2006;96:297-311.
22. Mor G, Cardenas I, Abrahams V, et al: Inflammation and pregnancy: the role of the immune system at the implantation site. Ann NY Acad Sci 2011;122:80-87.
23. Bainbridge DR: Evolution of mammalian pregnancy in the presence of the material immune system. Rev Reprod 2000;5:67-74.
24. Moffett-King A: Natural killer cells and pregnancy. Nat Rev Immunol 2002;2:656-663.
25. Davies CJ, Fisher PJ, Schlafer DH, et al: Temporal and regional regulation of major histocompatibility complex class I expression at the bovine uterine/placental interface. Placenta 2000;21:194-202.
26. Hill JR, Schlafer DH, Fisher PJ, et al: Abnormal expression of trophoblast major histocompatibility complex class I antigens in cloned bovine pregnancies is associated with a pronounced endometrial lymphotic response. Biol Reprod 2002;67:55-63.
27. Walker CG, Meier S, Littlejohn MD, et al: Modulation of the maternal immune system by the pre-implantation embryo. BMC Genomics 2010;11:474-487.
28. Brown MB, von Chamier M, Allam AB, et al: M1/M2 macrophages polarity in normal and complicated pregnancy. Front Immunol 2014; 5:1-9.
29. Oliveira LJ, Hansen PJ: Deviation in populations of peripheral blood mononuclear cells and endometrial macrophages in the cow during pregnancy. Reproduction 2008;136:481-490.
30. Oliveira LJ, Hansen PJ: Phenotypic characterization of macrophages in the endometrium of the pregnant cow. Am J Reprod Immunol 2009;62:418-426.
31. Tekin S, Hansen PJ: Lymphocyte-mediated lysis of sheep chorion: susceptibility of chorionic cells to third-party and maternal cytotoxic lymphocytes and presence of cells in the endometrium exhibiting cytotoxicity toward natural-killer cell targets. Theriogenology 2003;59:787-800.
32. Mantovanie A, Suzzani S, Locati M, et al: Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002;23:549-555.
33. Mantovanie A, Sica A, Allevena A, et al: the chemokine system in diverse forms of macrophage activations and polarization. Trends Immunol 2004;15:677-686.
34. Oliveira LJ, McClellan S, Hansen PJ, et al: Differentiation of the endometrial macrophage during pregnancy in the cow. PLoS One 2010; 5: e13213.
35. Barrientos S, Stojadinovic O, Golinko MS, et al: Growth factors and cytokines in wound healing. Wound Repair Regen 2008;16:585-601.
36. Wathers DC: Interactions between energy balance, the immune system and the reproductive tract with influence on dairy cow fertility. 2010;18:19-26.
37. Goff JP, Horst RL: Physiological changes at parturition ans their relationship to metabolic disorders. J Dairy Sci 1997;80:1260-1268.
38. Wood CE: Control of parturition in ruminants. J Reprod Fertil 1999;Suppl 54:115-126.
39. Mormede P, Andanson S, Auperin B, et al: Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol Beh 2007;92:317-339.
40. Horst RL, Jorgensen NA: Elevated plasma cortisol during induced and spontaneous hypocalcemia in ruminants. J Dairy Sci 1982;65:2332-2337.
41. Civelek T, Cilik A, Avci G, et al: Effect of dystocia on plasma cortisol and cholesterol levels in Holstein heifers and there newborn calves. Bull Vet Inst Pulavy 2008;52:649-654.
42. Graugnard DE, Bionaz M, Trevisi E, et al: Blood immunometabolic indices and polymorphonuclear neutrophil function in peripartum dairy cows are altered by level of dietary energy prepartum. J Dairy Sci 2012;95:1749-1758.
43. Moyes KM, Graugnard DE, Khan MJ, et al: Postpartal immunometabolic gene network expression and function in blood neutrophils are altered in response to prepartal energy intake and postpartal intrammary inflammatory Challenge. J Dairy Sci 2014;97:2165-2177.
44. Burton JL, Kehrli ME, Kapil Jr S, et al: Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethason. J Leukoc Biol 1995;57:317-325.
45. Weber PS, Madsen SA, Smith GW, et al: Pre-translational regulation of neutrophil L-selectin in glucocorticoid-challenged cattle. Vet Immunol Immunopathol 2001;83:213-240.

46. Moller K: A review of uterine involution and ovarian activity during the postparturient period in the cow. NZ Vet J 1970;18:83-90.
47. Davies D, Meade KG, Herath S, et al:Toll-like receptor and antimicrobial peptide expression in the bovine endometrium. Reprod Biol 2008;18:53-61.
48. Deng Q, Odhiambo JF, Farooq U, et al: Intravaginal lactic bacteria modulated local and systemic immune responses and lowered the incidence of uterine infections in periparturient dairy cows. Plos One/DOI:10.1371/journal.pone.0124167 2015.
49. Troedsson MH, Liu IK: Uterine clearance of non-antigenic marker (51CR) in response to a bacterial challenge in mares potentially susceptible and resistant to chronic uterine infections. J Reprod Fertil 1991;Suppl 44:283-288.
50. Kimura K, Goff JP, Schmerr MJ, et al.: Activation of immune cells in bovine mammary gland secretion by zymosan-treated bovine serum. J Dairy Sci 2008;91:1852-1864.
51. Paape M, Mehrzad J, Zhao X, et al: Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J Mammary Gland Biol Neoplasia 2002;7:109-121.
52. Schukken YH, Gunther J, Fitzpatrick J, et al: Host response patterns of intramammary infections in dairy cows. Vet immunol Immunopathol 2011;144:270-289.
53. Waldron MR, Revelo XS: Causes and effects of periparturient immunosuppression. WCDS Advance in Dairy Technology 2008;20:97-109.
54. Herr M, Bostedt H, Failing K: IgG amd IgM levels in dairy cows during the periparturient period. Theriogenology 2011;75:377-385.
55. Le Cozler Y, Guatteo R, Le drean et al: IgG1 variations in the colostrum of Holstein dairy cows. Animal 2016;10:230-237.
56. Ingvartsen KL, Moyes K: Nutrition, immunie function and health of dairy cattle. Animal 2013;7(Suppl 1):112-122.
57. Ingvartsen KL, AndersonJB: Integration of metabolism and intake regulation: a review focusing on periparturient animals. J Dairy Sci 2000;83:1573-1597.
58. Newsholme P, Newsholme, EA: Rates of utilization of glucose, glutamine, and oleate and formation of end-products by mouse peritoneal macrophages in culture. Bichem J 1989;261:211-218.
59. Pithon-Curi TC, DeMelo MP, Curi R: Glucose and glutamine utilization by rat lymphocytes, monocytes and neutrophils in culture: a comparative study. Cell Biochem Function 2004;22:321-326.
60. De Vries R, van Knegsel A, Johansson M, et al., Effect of shortening or omitting the dry period of Holstein-Friesian cows on casein composition of milk. J Dairy Sci 2015;98:8678-8678.
61. Block E, Sanchez W: Special nutritional needs of the transition cow. In: Middle South Nutrition Conference, Dallas, Tx.
62. Grinberg N, Elazar S, Rosenshine I et al: Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bacterial activity against mammary pathogenic Escherichia coli. Infect Immun 2008;76:2802-2807.
63. Duffied TF, Lissemore KD, McBride BW et al: Impact of hyperketonemia in early lactation dairy cows on health and production. J Dairy Sci 2009;92:571-589.
64. Suriyasathaporn W, Heuer C, Noordhuizen-Stassen EN, et al: Hyperketonemia and the impairment of udder defense: a review. Vet Res 2000;31:397-412.
65. Esposito G, Irons PC, Webb EC et al: Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Anim Reprod Sci 2014;144:60-71.
66. Newsholme P, Gordon S, Newsholme EA: Rates of utilization and fates of glucose, glutamine, pyruvate, fatty acids and ketone bodies by mouse macrophage. Biochemistry J 1987;242:631-636.
67. Ingvartsen KL, Moyes KM: Nutrition, immune function and health of herbivores. Adv Anim Biosci 2011;2:575-577.
68. Calder PC, Bond JA, Newsholme EA: Fatty acid inhibition of lipopolysaccharide-stimulated B lymphocyte proliferation. Biochemical Society-Transactions 1990;18:904-905.
69. Leblanc SJ: Interactions of metabolism, inflammation, and reproductive tract health in the postpartum period in dairy cattle. Reprod Domest Anim 2012;47(Suppl 5):18-30, 2012.
70. Sordillo LM, Contreras GA, Aitken SL, et al: Metabolic factors affecting the inflammatory response of periparturient dairy cows. Anim Health Res Rev 2009;10:53-63.
71. Ingvartsen KJ, Moyes KM: Factors contributing to immunosuppression in the dairy cow during the periparturient period. Jap J Vet Res 2015;63(Suppl 1):S15-S24.
72. Moyes KM, Drackley JK, Morin DE, Loor JJ: Greater expression of TLR2, TLR4, and TLR6 due to negative energy balance is associated with lower expression of HLA-DRA and HLA-A in bovine blood neutrophils after intrammary mastitis challenge with *Streptococcus uberis*. Func Integr Genomics 2010;10:53-61.
73. Redriguez-Lecompte JC, Kroeker AD, Ceballos-Marquez A, et al: Evaluation of the systematic innate response and metabolic alterations of nonlactating cows with diet-induced subacute ruminal acidosis. J Dairy Sci 2014;97:7777-7787.
74. Kleen JL, Hooijer GA, Rehage J et al: Subacute ruminal acidosis (SARA): A review. J Vet Med Physiol Pathol Clin Med 2003;50:406-414.
75. Gabay C, Kushner I: Acute–phase proteins and other systemic responses to inflammation. N Engl J Med 1999;340:448-454.
76. Dong G, Liu S, Wu Y, et al: Diet-induced bacterial immunogens in the gastrointestinal tract of dairy cows: impacts on immunity and metabolism. Acta Vet Scand 2011;53:48-54.

77. Sheldon IM, Roberts MH: Toll-like receptor 4 mediates the response of epithelial and stroma cells to lipopolysaccharide in the endometrium. PLoS ONE 2010;5:1-9.
78. Hill J, Gilbert R: Reduced quality of bovine embryos cultured in media conditioned by exposure to an inflamed endometrium. Aust Vet J 2008;86:312-316.
79. Wathes DC, Cheng Z, Chowdhury W et al: Negative energy balance alters global ene expression and immune responses in the uterus of postpartum dairy cows. Physiol Genomics 2009;39:1-13.
80. Fisher C, Drillich M, Odau S et al: Selected pro-inflammatory factor transcripts in bovine endometrial epithelial cells are regulated during the oestrus cycle and elevated in case of subclinical or clinical endometritis. Reprod Fertil Dev 2010;22:818-829.
81. Turner ML, Cronin JG, Healey D et al: Epithelial and stroma cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides in vitro via Toll-like receptors TLR2, TLR1, and TLR6. Endocrinology 2014;155:1453-1465.
82. Cronin JG, Turner ML, Goetze L, et al: Toll-like receptor4 and MYD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. Biol Reprod 2012;86:51-61.
83. Peter AT, Bosu WT, DeDecker RJ, et al: Suppression of preovulatory luteinizing hormone surges in heifers after intrauterine infusions of *Escherichia coli* endotoxin. Am J Vet Res 1989;50:368-373.
84. Suzuki C, Yoshiola K, Iwamura S, et al: Endotoxin induces delayed ovulation following endocrine aberration during the proestrous phase in Holstein heifers. Domest Anim Endocrinol 2001;20:267-278.
85. Lavon Y, Leitner G, Goshen T, et al: Exposure to endotoxin during estrus alters the timing of ovulation and hormonal concentrations in cows. Theriogenology 2008;70:956-967.
86. Ribeiro ES, Lima FS, Greco LF, et al: Prevalence of periparturient diseases and effects on fertility of seasonally calving grazing dairy cows supplemented with concentrates. J Dairy Sci 2013;96:5682-5697.
87. Bromfield JJ, Santos JE, Block J et al: Physiology and endocrinology symposium: Uterine infection: linking infection and innate immunity with infertility in the high-producing dairy cows. J Anim Sci 2015;93:2021-2033.
88. Herath S, William EJ, Lilly ST, et al: Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. Reproduction 2007;134:683-693.
89. Price JC, Sheldon IM: Granulosa cells from emerged antral follicles of the bovine ovary initiate inflammation in response to bacterial pathogen-associated molecular patterns via Toll-like receptor pathways. Biol Reprod 2013;89:119-125.
90. Bromfield JJ, Sheldon IM: Lipopolysaccharide initiates inflammation in bovine granulosa cells via the TLR4 pathway and pertubs oocyte meiotic progression in vitro. Endocrinology 2011;152:5029-5040.

20 years of modern endometritis research – a review

Robert O. Gilbert*
College of Veterinary Medicine, Cornell University, Ithaca NY

Abstract

During the last two decades it has become clear that endometritis is a common postpartum disease of dairy cows, detrimentally affecting fertility and herd life. Endometritis is a separate and distinct condition from cervicitis; they may occur separately or together. Either condition may be associated with purulent vaginal discharge but need not be. A few cases of purulent vaginal discharge exist without evidence of endometritis or cervicitis. The effect on fertility of these conditions appears to be independent and additive. Endometritis is mostly mediated by negative energy balance in the periparturient period and cervicitis by obstetrical complications. The risk of both conditions is increased in cows that suffer puerperal metritis. Cephapirin specifically formulated for intrauterine infusion is effective in improving reproductive performance in affected cows but is not available in the USA. At present no other treatment has been convincingly shown to be effective.

Keywords: Cervicitis, cow, endometritis, postpartum, purulent vaginal discharge.

Introduction

In the early 1990's there was disagreement on the importance of endometritis in bovine reproduction. While most believed intuitively that endometritis was bad for reproduction,[1,2] this was certainly not universally accepted. In fact, at least one controlled study suggested that endometritis had no effect on reproduction of dairy cows[3] and systematic studies of uterine bacteriology and histology concluded that endometritis was not a significant determinant of fertility in dairy cows.[4,5] Closer examination, however, made it clear that there was no consistent definition of endometritis. The condition was diagnosed mainly by uterine palpation in North America, where the incidence of endometritis was generally reported to be less than 20%[2,6,7] but in Europe, where vaginoscopy was a common procedure, endometritis was recognized in up to 40 % of postpartum cows.[8] Since there was no consistent definition or diagnostic consensus, it is not surprising that effects of treatment were unclear to say the least. Intrauterine infusion of an array of compounds was routine practice but none were found to be efficacious in controlled studies. In fact, it was often argued that inclusion of an untreated control group would be unethical, a stance that substantially delayed the acquisition of the necessary evidence that many then-current treatments were not only ineffective but even detrimental. It is noteworthy that the first report that intrauterine infusion as then practiced was ineffective came from Charter Diplomate Steve Roberts, at Cornell more than 60 years ago.[9] Given that intrauterine infusions were ineffective or harmful, the reports that prostaglandin treatment was as effective as intrauterine infusion[10-12] were not encouraging.

Against that background, it seemed imperative to determine whether endometritis was in fact a common disorder of postpartum cows and whether it influenced fertility. If the condition could be defined and consistently diagnosed, epidemiology and risk factors could be elucidated and potential treatments could be methodically evaluated.

Diagnosis and prevalence of endometritis

Endometritis has always had a definition – it is inflammation of the endometrium. The problem seemed to be development of a sensitive, specific and practical diagnostic method. Brenda Bonnet had

*Current address: Ross University School of Veterinary Medicine, Basseterre, St. Kitts, West Indies

used endometrial biopsy and contributed a great deal to our knowledge of endometritis but reported that the biopsy procedure itself might impair fertility[13-15]–an opinion that has been challenged recently. Endometrial cytology had long been established as a simple and reliable method of detecting endometrial inflammation in mares. We decided to apply the technique to cows, using an adaptation of the low volume flush method described by Barry Ball and others.[16] Initially we targeted cows at 40–60 days postpartum since this was shortly before the breeding period and a time at which postpartum infection and inflammation were generally believed to have resolved. We found a surprisingly high proportion of cows with evidence of inflammation, and reported that presence of cytological endometritis impaired reproduction–at first service and throughout the lactation, leaving more cows open (and therefore likely to be culled) at 300 days in milk.[17,18] These findings spurred much additional research on endometritis because the condition seemed both prevalent and detrimental, and therefore very costly to the industry, contentions soon confirmed by independent investigators.[19,20] Ram Kasimanickam and others introduced the cytobrush as an alternative method of obtaining endometrial cytology samples.[21] Using cytology as a gold standard, it was shown that ultrasonography,[19,22] presence of leukocyte esterase, protein and pH of recovered fluid[23] were useful but not excellent indicators of endometritis. Importantly, the process of obtaining endometrial samples is not itself detrimental to future reproduction.[24,25]

Purulent vaginal discharge and cervicitis

Stephen LeBlanc and others at Guelph took a different approach to diagnosis of reproductive tract disease. They studied the relationship between clinically identifiable signs in the early postpartum period (21to 35 days postpartum) and likelihood of pregnancy at 120 to 150 days in milk. They found that the presence of a purulent vaginal discharge after 21 days postpartum or a mucopurulent discharge after 28 days, or cervical diameter greater than 7.5 cm at this time significantly predicted poor reproductive performance.[26] They labeled this condition "clinical endometritis" even though there was no direct evidence of endometrial inflammation. Importantly, over 40 % of cows diagnosed with clinical endometritis would have been missed had vaginoscopy not been performed–the vaginal discharge was not always externally visible.

Because vaginoscopy is regarded as cumbersome or time consuming by many practitioners, other methods were explored for detection of exudate in the vagina. Martin Sheldon's group suggested "sweeping" the cranial vagina with a clean gloved hand and examining the recovered mucus.[27] I am not in favor of this technique because I believe it has the potential to damage the vestibulovaginal junction, which is a critical anatomic feature in defense of the cranial tubular tract. However, a valuable contribution of this publication was the proposal of a classification system for scoring the recovered mucus (Table).

Table. Scoring system for vaginal mucus from Williams et al.[27] Most investigators regard a score of 0 or 1 as "normal"; scores of 2 or 3 are associated with impaired reproduction.

Score	Description
0	Clear or translucent mucus
1	Mucus with flecks of pus
2	Mucus with up to 50 % pus
3	Mucus with more than 50 % pus
3	Serosanguinous exudate, often malodorous

A further innovation came from Scott McDougall and co-workers who introduced a device they called Metricheck® to recover vaginal mucus samples.[28] It consisted of a 50 cm stainless steel shaft at the end of which was a black rubber hemisphere. The device was inserted to the cranial vagina and when withdrawn the black "cup" collected mucus which could easily be evaluated. This device has been well tested and is now in common use.[29-33]

At first it was assumed that cows with purulent vaginal discharge simply represented a more severe form of inflammation than those with cytological evidence of endometritis in the absence of

visible discharge. However, it soon became clear that some cows with obvious purulent discharge had no evidence of cytological endometritis. This situation was clarified by Jocelyn Dubuc, working under the supervision of Stephen LeBlanc.[34,35] This group demonstrated that (cytological) endometritis and purulent vaginal discharge were separate and independent conditions, with additive detrimental effects on reproductive performance and separate risk factors. Purulent vaginal discharge was more likely to follow obstetrical complications and endometritis was associated with negative energy balance.

The source of vaginal exudate in the absence of uterine inflammation was briefly vexing but it has gradually become clearer that cervicitis is a common condition and independent of endometritis.[36,37] It is noteworthy that enlarged cervical diameter was one of the clinical findings that LeBlanc found to predict poor reproductive performance[26]

Current definitions of postpartum uterine disease

Stemming from a conversation at the 2004 ICAR meeting in Porto Seguro, Brazil, Martin Sheldon, Stephen LeBlanc, Greg Lewis and I set about developing consensus definitions of postpartum uterine disease. The manuscript we wrote was circulated for their comments to 13 international colleagues to stress the consensus nature of the publication[38] This attempt provided definitions for puerperal metritis, retained fetal membranes, pyometra, subclinical endometritis and clinical endometritis. Unfortunately it was soon out of date. Puerperal metritis was defined as an abnormally enlarged uterus and a fetid watery red-brown uterine discharge, associated with signs of systemic illness (decreased milk yield, dullness or other signs of toxemia) and fever > 39.5°C, within 21 days after parturition. In fact, this condition almost always occurs within the first 10 to 14 days and the 21 day limit was introduced to avoid a "gap" between postpartum metritis and endometritis. Later evidence has made clear that there is a great deal of physiological inflammation still active around 21 days postpartum, so any diagnosis of a clinically relevant uterine inflammatory disease at this time should be made with great caution. This paper also introduced a distinction between puerperal metritis (accompanied by fever) and the similar but afebrile condition labeled "clinical metritis." This distinction may be valid because there is evidence that reproductive performance is not impaired in cows with the afebrile form of the disease, although both puerperal metritis and clinical metritis are associated with reduced milk yield[39] This paper endorsed the concept of clinical endometritis characterized by vaginal exudate or enlarged cervix and of subclinical endometritis indicated by evidence of endometrial inflammation (usually by cytology) in the absence of visible exudate. Unfortunately, this paper perpetuated the notion that endometritis of clinical significance could be diagnosed around 21 days postpartum.

Once the independence of endometritis and purulent vaginal discharge had become clear, Dubuc et al. proposed updated definitions.[35] He proposed using the term "purulent vaginal discharge" (PVD) to replace the former "clinical endometritis" and "cytological endometritis" for the condition where inflammation was confirmed to be present in the uterus. This certainly removed some of the former confusion. These investigators stressed that purulent vaginal discharge was a distinct and separate condition from cytological endometritis and not simply a more severe form of it.

However, the source of the purulent exudate remained elusive. The logical conclusion seemed to be that cervicitis existed as a separate entity and this attracted the attention of investigators. It soon emerged that cervicitis was indeed a distinct condition that could exist separately from endometritis. However, it was not the whole answer and, indeed, it seems that cervicitis affects about 15 to 40% of dairy cows.[40] About half of cows with PVD have cervicitis and vice versa.[37] About 50 to75 % of cows with endometritis have cervicitis.[37] About 3% of cows at 35 days postpartum had PVD without any evidence of concurrent endometritis or cervicitis.[40]

Currently, puerperal metritis, clinical metritis, and pyometra seem to be adequately defined. Clearly, there is overlap between endometritis, purulent vaginal discharge and cervicitis, which may occur independently or together. This requires additional investigation and clarification.

Epidemiology

In an attempt to clarify the causes of endometritis and illuminate possible preventive strategies some attention was devoted to understanding its epidemiology. Cheong et al.[41] investigated endometritis in 38 dairy herds in Upstate New York and found the major risk factor for endometritis was clinical ketosis earlier in lactation. This was consistent with a report from Doug Hammon[42] that cows affected with endometritis at five weeks postpartum had lower voluntary dry matter intake beginning in the prepartum period and continuing after parturition, accompanied by more severe negative energy balance and impaired neutrophil function. Dubuc[34] confirmed the importance of negative energy balance for endometritis and showed that separate risk factors predisposed cows to purulent vaginal exudate, most importantly obstetrical complications. Puerperal metritis was a risk factor for endometritis and purulent vaginal discharge. At a herd level, Cheong et al. reported that risk of endometritis was reduced for cows calving on straw compared to other types of bedding.

Treatment

Establishment of clinical definitions of uterine disease laid the groundwork for systematic investigations of effective treatments to restore fertility. Prior to the 1990s an amazing array of compounds was infused into the uterus of cows despite the lack of evidence that any of these treatments was effective and some evidence that some treatments were in fact detrimental.

LeBlanc showed convincingly that cephapirin specially formulated for intrauterine administration (Metricure®, Merck Animal Health) was effective in partially restoring fertility in cows with purulent vaginal discharge.[43] Simple disappearance of clinical signs occurred as often without treatment as with it, but improved fertility required treatment. This study compared intrauterine cephapirin to systemic administration of prostaglandin F2α (PGF) and it is instructional to examine the results closely. First, cephapirin treatment improved hazard of pregnancy only when cows were diagnosed and treated after four weeks postpartum. Treatment before this time was ineffective, consistent with the concept that inflammation at three weeks postpartum is often physiological. Prostaglandin treatment was not beneficial and tended to be detrimental in the absence of a palpable corpus luteum.

Kasimanickam examined the effects of the same two treatments in the case of cytological endometritis in cows without any signs of vaginal discharge,[44] reporting that both cephapirin and cloprostenol improved reproductive performance of cows with endometritis but not of normal cows, when the cows were treated between 20 and 33 days postpartum. In contrast, Galvão et al.[45] reported results of a larger study showing that although administration of PGF increased reproductive performance, it did not do so through curing or preventing endometritis; indeed, the greatest beneficial effect of prostaglandin treatment was in cows without endometritis. Because cephapirin for intrauterine administration is not available in the USA and PGF is not specifically effective in cases of endometritis, producers and veterinarians in the USA were left without effective treatments for endometritis.

Schuenemann and colleagues have reported beneficial effects of dextrose infusion on subsequent reproductive performance of cows with purulent vaginal exudates,[46] a finding we were not able to replicate.[32] More recently success has been reported with infusion of povidone iodine into the uterus of affected cows[47] but the number of cows treated is too small to be convincing and this study requires confirmation especially since detrimental results have previously been reported with this treatment[9] and intrauterine iodine infusion has been reported to impair local neutrophil function.

This leaves US producers and veterinarians without an effective treatment against endometritis. Many have pinned their hopes on systemic prostaglandin administration. Indeed, the evidence that PGF improves reproductive performance in dairy cows is quite strong. However, its role in endometritis is not so clear. In one study PGF was administered to cows at 21, 35 and 49 days postpartum. Cows were examined at the same times for presence of cytological endometritis and their reproductive outcomes were followed. Overall, cows given PGF did have better reproduction. However, cows receiving PGF were no more likely to recover from endometritis than untreated cows, and PGF 21 days did not reduce incidence of endometritis at 35 or 49 days postpartum. Furthermore, stratification of cows by presence of

endometritis indicated that the overall beneficial effect of PGF was limited to cows without evidence of endometritis.[45]

Conclusions

Research in the last two decades has done a great deal to clarify clinical definitions of postpartum uterine disease, which in turn has permitted credible study of epidemiology, pathogenesis, consequences and treatment of these conditions. Despite intensive study, however, little information of practical significance of producers and practitioners has emerged so far.

References

1. Erb HN, Martin SW, Ison N, et al: Interrelationships between production and reproductive diseases in holstein cows. Path analysis. J Dairy Sci. 1981;64:282-289.
2. Erb HN, Martin SW, Ison N, et al: Interrelationships between production and reproductive diseases in Holstein cows. Conditional relationships between production and disease. J Dairy Sci 1981;64:272-281.
3. Miller HV, Kimsey PB, Kendrick JW, et al: Endometritis of dairy cattle: diagnosis, treatment and fertility. Bovine Pract 1980;15:13-23.
4. Griffin JF, Hartigan PJ, Nunn WR: Non-specific uterine infection and bovine fertility. I. Infection patterns and endometritis during the first seven weeks post-partum. Theriogenology 1974;1:91-106.
5. Griffin JF, Hartigan PJ, Nunn WR: Non-specific uterine infection and bovine fertility. II. Infection patterns and endometritis before and after service. Theriogenology 1974;1:107-114.
6. Curtis CR, Erb HN, Sniffen CJ, et al: Path analysis of dry period nutrition, postpartum metabolic and reproductive disorders, and mastitis in Holstein cows. J Dairy Sci 1985;68:2347-2360.
7. Bartlett PC, Kirk JH, Wilke MA, et al: Metritis complex in Michigan Holstein-Friesian cattle: incidence, descriptive epidemiology and estimated economic impact. Prev Vet Med 1986;4:235-248.
8. de Kruif A, Gunnink JW, de Bois CH: Diagnosis and treatment of postpartum endometritis in cattle. Tijdschr Diergeneeskd 1982;107:717-725.
9. Roberts SJ: An evaluation of uterine infusions for the treatment of infertility in cattle. Cornell Vet 1956;46:21-38.
10. Murray RD, Allison JD, Gard RP: Bovine endometritis: comparative efficacy of alfaprostol and intrauterine therapies, and other factors influencing clinical success. Vet Rec 1990;127:86-90.
11. Bretzlaff K: Rationale for treatment of endometritis in the dairy cow. Vet Clin North Am Food Anim Pract 1987;3:593-607.
12. Vaillancourt D: Physiopathologie et therapeutique de l'uterus en periode puerperale chez la vache laitiere: revue. Can Vet J 1987;28:330-337.
13. Bonnett BN, Martin SW, Gannon VP, et al: Endometrial biopsy in Holstein-Friesian dairy cows. III. Bacteriological analysis and correlations with histological findings. Can J Vet Res 1991;55:168-173.
14. Bonnett BN, Miller RB, Martin SW, et al: Endometrial biopsy in Holstein-Friesian dairy cows. II. Correlations between histological criteria. Can J Vet Res 1991;55:162-167.
15. Bonnett BN, Martin SW, Meek AH: Associations of clinical findings, bacteriological and histological results of endometrial biopsy with reproductive performance of postpartum dairy cows. Prev Vet Med 1993;15:205-220.
16. Ball BA, Shin SJ, Patten VH, et al: Use of a low-volume uterine flush for microbiologic and cytologic examination of the mare's endometrium. Theriogenology 1988;29:1269-1283.
17. Gilbert RO, Shin ST, Guard CL, et al: Incidence of endometritis and effects on reproductive performance of dairy cows. Theriogenology 1998;49:251. [abstract]
18. Gilbert RO, Shin ST, Guard CL, et al: Prevalence of endometritis and its effects on reproductive performance of dairy cows. Theriogenology 2005;64:1879-1888.
19. Kasimanickam R, Duffield TF, Foster RA, et al: Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows. Theriogenology 2004;62:9-23.
20. Hammon DS, Holyoak GR, Jenson J, et al: Effects of endometritis at the beginning of the breeding period on reproductive performance in dairy cows. Am Assoc Bovine Pract 2001;34:142-143.
21. Kasimanickam R, Duffield TF, Foster RA, et al: A comparison of the cytobrush and uterine lavage techniques to evaluate endometrial cytology in clinically normal postpartum dairy cows. Can Vet J 2005;46:255-259.
22. Barlund CS, Carruthers TD, Waldner CL, et al: A comparison of diagnostic techniques for postpartum endometritis in dairy cattle. Theriogenology 2008;69:714-723.
23. Cheong SH, Nydam DV, Galvao KN, et al: Use of reagent test strips for diagnosis of endometritis in dairy cows. Theriogenology 2012;77:858-864.
24. Cheong SH, Nydam DV, Galvao KN, et al: Effects of diagnostic low-volume uterine lavage shortly before first service on reproductive performance, culling and milk production. Theriogenology 2012;77:1217-1222.
25. Thome HE, Arruda RP, Oliveira BM, et al: Uterine lavage is efficient to recover endometrial cytology sample and does not interfere with fertility rate after artificial insemination in cows. Theriogenology 2016;1585:1549-1554.

26. LeBlanc SJ, Duffield TF, Leslie KE, et al: Defining and diagnosing postpartum clinical endometritis and its impact on reproductive performance in dairy cows. J Dairy Sci 2002;85:2223-2236.
27. Williams EJ, Fischer DP, Pfeiffer DU, et al: Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the immune response in cattle. Theriogenology 2005;63:102-117.
28. McDougall S, Macaulay R, Compton C: Association between endometritis diagnosis using a novel intravaginal device and reproductive performance in dairy cattle. Anim Reprod Sci 2007;99:9-23.
29. Runciman DJ, Anderson GA, Malmo J: Comparison of two methods of detecting purulent vaginal discharge in postpartum dairy cows and effect of intrauterine cephapirin on reproductive performance. Aust Vet J 2009;87:369-378.
30. Pleticha S, Drillich M, Heuwieser W: Evaluation of the Metricheck device and the gloved hand for the diagnosis of clinical endometritis in dairy cows. J Dairy Sci. 2009;92:5429-5435.
31. Senosy W, Uchiza M, Tameoka N, et al: Evaluation of reproductive tract infection during early post-partum period and its relationship with subsequent reproductive performance in high milk producing dairy cows. Reprod Domest Anim 2012;47:203-207.
32. Machado VS, Oikonomou G, Ganda EK, et al: The effect of intrauterine infusion of dextrose on clinical endometritis cure rate and reproductive performance of dairy cows. J Dairy Sci 2015;98:3849-3858.
33. Bicalho ML, Lima FS, Machado VS, et al: Associations among *Trueperella pyogenes*, endometritis diagnosis, and pregnancy outcomes in dairy cows. Theriogenology 2016;85:267-274.
34. Dubuc J, Duffield TF, Leslie KE, et al: Risk factors for postpartum uterine diseases in dairy cows. J Dairy Sci. 2010;93:5764-5771.
35. Dubuc J, Duffield TF, Leslie KE, et al: Definitions and diagnosis of postpartum endometritis in dairy cows. J Dairy Sci. 2010;93:5225-5233.
36. Hartmann D, Rohkohl J, Merbach S, et al: Prevalence of cervicitis in dairy cows and its effect on reproduction. Theriogenology 2016;85:247-253.
37. Deguillaume L, Geffre A, Desquilbet L, et al: Effect of endocervical inflammation on days to conception in dairy cows. J Dairy Sci 2012;95:1776-1783.
38. Sheldon IM, Lewis GS, LeBlanc S, et al: Defining postpartum uterine disease in cattle. Theriogenology 2006;65:1516-1530.
39. Giuliodori MJ, Magnasco RP, Becu-Villalobos D, et al: Metritis in dairy cows: risk factors and reproductive performance. J Dairy Sci 2013;96:3621-3631.
40. LeBlanc SJ: Reproductive tract inflammatory disease in postpartum dairy cows. Animal 2014;8(Suppl 1):54-63.
41. Cheong SH, Nydam DV, Galvão KN, et al: Cow-level and herd-level risk factors for subclinical endometritis in lactating Holstein cows. J Dairy Sci 2011;94:762-770.
42. Hammon DS, Evjen IM, Dhiman TR, et al: Negative energy balance during the periparturient period is associated with uterine health disorders and fever in Holstein cows. J Dairy Sci 2004;87(Suppl 1):279.
43. LeBlanc SJ, Duffield TF, Leslie KE, et al: The effect of treatment of clinical endometritis on reproductive performance in dairy cows. J Dairy Sci 2002;85:2237-2249.
44. Kasimanickam R, Duffield TF, Foster RA, et al: The effect of a single administration of cephapirin or cloprostenol on the reproductive performance of dairy cows with subclinical endometritis. Theriogenology 2005;63:818-830.
45. Galvão KN, Frajblat M, Brittin SB, et al: Effect of prostaglandin F-2 alpha on subclinical endometritis and fertility in dairy cows. J Dairy Sci 2009;92:4906-4913.
46. Brick TA, Schuenemann GM, Bas S, et al: Effect of intrauterine dextrose or antibiotic therapy on reproductive performance of lactating dairy cows diagnosed with clinical endometritis. J Dairy Sci 2012;95:1894-1905.
47. Mido S, Murata N, Rawy MS, et al. Effects of intrauterine infusion of povidone-iodine on endometrial cytology and bacteriology in dairy cows with clinical endometritis. J Vet Med Sci. 2015.

New frontiers in molecular endometritis research

Robert O. Gilbert*
College of Veterinary Medicine, Cornell University, Ithaca, NY

Abstract

Postpartum uterine disease is common in dairy cows. The earliest pathogen seems to be *E. coli*, but it soon disappears, giving way to *Trueperella pyogenes* and the gram negative anaerobes, especially *Fusobacterium necrophorum*, *Prevotella melaninogenica* and *Bacteroides* spp. Specific strains of the organisms, expressing specific virulence factors appear to be responsible for mediating uterine disease. Susceptibility to postpartum uterine disease is increased by negative energy balance and deficiency of other nutrients. These deficiencies blunt the postpartum inflammatory response resulting in more sluggish uterine recruitment of leukocytes in the early postpartum period and a more prolonged course of infection and inflammation, with profoundly detrimental consequences for reproduction. Uterine inflammation impairs reproduction locally by influencing sperm function and longevity as well as zygote and embryo development, but also more systemically by reducing pituitary secretion of gonadotrophins and altering follicular function to reduce steroidogenesis and oocyte competence. Some of the pathways accounting for these effects are reviewed here.

Keywords: Cow, embryo, infertility, inflammation, uterus

Introduction

Postpartum uterine disease is common in dairy cows, with as many as 50% of animals per lactation being affected by at least one of metritis, purulent vaginal discharge, endometritis or cervicitis.[1] Since these conditions contribute to infertility and increase the risk of culling they are extremely costly to producers. Furthermore, our current ability to prevent or treat uterine disease is limited. These diseases constitute a welfare issue since they cause discomfort to cows.[2] Collectively, the prevalence of postpartum uterine disease, its effect on fertility and welfare and its contribution to antibiotic use in food production make the disease complex important to producers and society as a whole and it is an active field of investigation.

This presentation will focus on a few themes:

1. Which pathogens are primarily responsible for endometritis?
2. Which metabolic and immune factors mediate susceptibility to and pathogenesis of endometritis?
3. How does endometritis mediate infertility?

Which pathogens cause endometritis?

Most cows have postpartum bacterial infection or contamination of the uterus. The mixed bacterial population characteristic of the early postpartum period was originally judged to be insignificant but persistence of *Trueperella pyogenes* (formerly *Actinobacillus pyogenes*, formerly *Corynebacterium pyogenes*) after 21 days postpartum was shown to be detrimental to subsequent reproduction.[3] The synergistic relationship between *T. pyogenes* and the gram negative anaerobes *Fusobacterium necrophorum*, *Prevotella melaninogenica* and *Bacteroides* spp. in the context of pathological uterine infection was also recognized early. Interestingly, the infection rate increases after the first postpartum day, reaching a maximum at seven to 11 days after parturition.[4,5]

Classical, culture-based, bacteriological methodology has confirmed the importance of the traditional pathogens in bovine uterine disease, but has also revealed an important role for *Escherichia coli*. Although *E. coli* are seldom isolated from diseased uteri, early invasion of the uterus by *E. coli*

* Current address: Ross University School of Veterinary Medicine, Basseterre, St. Kitts, West Indies

seems to be an important step in paving the way for the traditional pathogens. On the basis of measuring lipopolysaccharide (LPS) concentration in lochia Dohmen et al. concluded that the early presence of *E. coli* predisposed cows to infection with *T. pyogenes* and gram negative anaerobes, particularly *P. melaninogenica.*[6] We found *E. coli* to be the most common isolate from the bovine uterus in the early postpartum period and that its presence predisposed to later infection with *T. pyogenes* and *F. necrophorum.*[5,7,8] The mechanism whereby *E. coli* paves the way for later infection is not known but this organism has been reported to impair neutrophil function.[9,10] Specific strains of *E. coli* are associated with bovine uterine disease.[11] Uteropathogenic *E. coli* belonged to various phylogenetic groups, serotypes and multilocus sequence types (MLST), but showed some characteristics in common: they were more likely to adhere to cultured uterine epithelial cells, and were more likely to invade these cells (becoming intracellular), and they caused more severe disease when infused into the uteri of mice.[11]

Cows are more likely to be infected with uterine *E. coli* in the early postpartum period if they gave birth to twins or stillborn calves, had retained fetal membranes, or were in poor body condition,[12] all factors known to increase the risk of metritis. Strains of *E. coli* that were pathogenic in the uterus were more likely to contain the virulence factors *fimH*, *astA*, *ibeA*, *cdt*, *hlyA* and *kpsMII*. Several of these virulence factors were found to increase the risk of purulent vaginal exudate later in the postpartum period and to be associated with impaired reproduction.

Metagenomic methods allow identification and quantification of bacteria independent of culture. This means that relative abundance of bacteria, some of which may be difficult to culture in vitro, can be determined accurately. Given the importance of anaerobic organisms in pathogenesis of uterine disease, this is an important attribute. Initial studies showed significantly different bacterial microbiomes in cows with healthy or unhealthy postpartum course.[13]. Cows that went on to develop metritis had a more diverse microbiome and, in particular, had far greater representation of the phyla Fusobacteria, Bacteroidetes (including *Flavobacteria* spp., *Porphyomonas* spp, and *Prevotella* spp.), and uncultured bacteria.

Machado, et al. obtained uterine samples at 35 days postpartum and identified bacteria by individual amplification of 16S rRNA followed by pyrosequencing. *Bacteroides* spp., *Ureaplasma* spp., *Fusobacterium* spp., *Peptostreptococcus* spp., *Sneathia* spp., *Prevotella* spp. and *Trueperella* spp. prevalence was significantly ($P < 0.05$) higher in samples derived from cows that had concurrent endometritis. *Bacteroides* spp., *Ureaplasma* spp., *Fusobacterium* spp., and *Trueperella* spp. prevalence was significantly ($P < 0.05$) higher in samples derived from cows that were not pregnant by 200 DIM.[14] Subsequent studies have yielded similar, but not identical results, suggesting some farm-specific or regional variation in pathogen importance. For example, Jeon, et al.[15] reported that *Bacteroides* spp. was much more important than *F. necrophorum* in the mediation of metritis. Interestingly, Knudsen, et al.,[16] while reporting presence of generally similar bacterial phyla, showed a difference in the microbiota of the endometrium and the uterine lumen, a finding that should receive more attention.

Virulence factors for major bacteria involved in pathogenesis of postpartum uterine disease have been investigated. By using major virulence factors as markers for specific species of bacteria, increasing diversity of pathogen load from calving to ten DIM was again confirmed. The *E. coli* virulence factor *fimH*, when present in the early postpartum period increased the odds of developing metritis by almost 5-fold while the *lktA* virulence factor (*F.necrophorum*) increased odds by 2.6.[17] Virulence factors associated with risk of development of purulent vaginal discharge diagnosed at 35 DIM included *E. coli fimH* at 1-3 DIM and *T. pyogenes fimA* at 8-10 or 34-36 DIM.[17] Cows in which the *E. coli* virulence factor fimH was detected at 1-3 DIM were significantly less likely to become pregnant, regardless of other findings in the postpartum period.[17]

The close association of specific virulence factors with development of postpartum uterine disease prompted us to attempt a vaccine trial. We tested several permutations of bacteria and virulence factors (recombinant virulence factors alone, inactivated bacteria alone, or a combination of bacteria and virulence factors) and two routes of administration (subcutaneous or intravaginal). All three vaccines, when administered subcutaneously but not intravaginally, reduced incidence of metritis and postpartum fever and improved subsequent reproduction.[18] Follow up testing is under way. Additional research should be devoted to sequential changes in uterine microbiome in the healthy or complicated postpartum

period, with a view to understanding mechanisms of synergism and antagonism between bacteria and potentially also viruses.

Metabolic and immune mechanisms mediating endometritis

Epidemiologic and experimental studies consistently demonstrate that negative energy balance in the periparturient period is a major risk factor for endometritis.[19-22] The mechanisms linking energy deficit and endometritis are emerging. Cows that develop metritis or endometritis have diminished intracellular reserves of glycogen in circulating neutrophils in the period preceding development of disease. This may directly reflect lack of available energy substrates and, since neutrophils depend on intracellular energy sources for migration, phagocytosis and killing, it directly impairs immune function.[23] We have recently shown that cows with the healthiest postpartum course recruit neutrophils to the uterus in greater numbers in the immediate postpartum period. These cows have fewer intrauterine bacteria and resolve postpartum infection and inflammation more promptly than cows with a more sluggish initial recruitment of leukocytes to the uterus, in which infection and inflammation linger, eventually being manifest as cytological endometritis.[5] This robust recruitment of neutrophils to the postpartum uterus is reflected in increased endometrial expression of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα or interleukin (IL) 1β in the immediate postpartum period.[24] The effect may be generalized as circulating monocytes of cows destined to develop metritis similarly express less mRNA for proinflammatory cytokines when challenged in vitro with *E. coli*.[25] Consistent with these results from our group, others have also shown that endometrial gene expression is pro-inflammatory in the early postpartum period but changes to a predominantly tissue-remodeling environment by about 21 days postpartum in cows with a healthy postpartum course compared to diseased cows in which the proinflammatory environment persists.[26] Other workers have also linked this persistent state of inflammation with severe negative energy balance[27] and have confirmed that cows with more severe negative energy balance recruit uterine neutrophils less effectively in the first week postpartum.[28] Several investigators have investigated the pathways linking negative energy balance, metabolic status and immune function, with particular reference to the uterus.[29] Uterine inflammatory responses are diminished by lack of glucose availability. Restricting glucose resulted in dose responsive reduction in IL-1β, IL-6 and IL-8 in response to LPS challenge. This effect appears to be mediated by AMP-activated protein kinase (which senses the AMP:ATP ratio as a measure of energy availability). mTOR, a more general sensor of nutrient deficit did not affect response to LPS in this experiment. Importantly, this paper also showed that uterine response to LPS is energetically expensive.[30] Interestingly, many differences have emerged between first lactation cows and multiparous animals,[23,31,32] and these differences should be accounted for in future studies.

Fundamental to the function of the innate immune system is recognition of potential pathogens by so-called pattern recognition receptors,[33] the Toll-like receptors TLR), NOD-like receptors (NLR), retinoic acid-inducible gene I (RIG-1)-like receptors and C-type lectin receptors (CLR).[34] Expression of most of the pattern recognition receptors has been found in uterine tissue. Interestingly, purified populations of uterine epithelial or stromal cells also express many of these molecules, meaning that these cells themselves, and not just professional immune cells within the uterus, can respond directly to pathogens. Stimulation of the pattern recognition receptors in the bovine endometrium results in marked increases in MAPH p38 and ERK 1/2and nuclear translocation of NFκB, and increased expression of IL-1β, IL-6 and IL-8.[35] Accumulation of IL-6 in peripheral circulation stimulated production of acute phase proteins in the liver. Cows express α1-acid glycoprotein, haptoglobin and ceruloplasmin after parturition,[36] and their increase is further stimulated in cattle experiencing severe uterine infection.[21,37]

Another metabolic indicator of increased susceptibility to postpartum uterine disease is cholesterol. Diminished circulating cholesterol has long been associated with uterine disease and poorer fertility[38,39] and cholesterol, in turn, is reduced in cows with more severe negative energy balance.[40] It has recently been shown that manipulation of the mevalonate pathway, specifically inhibition of squalene synthase, dampens inflammatory response to LPS in cultured bovine endometrium,[41] providing a novel

potential therapeutic or preventive approach. Dietary manipulation of periparturient lipid metabolism may also hold potential for management of postpartum disease.[42]

How does endometritis mediate infertility?

It may seem intuitive that endometritis should be associated with infertility since loss of function is a hallmark of inflammation in general. However, much work has been devoted to understanding mechanisms of endometritis-related infertility with some interesting results. Detrimental effects may be mediated by direct effect of pathogens themselves, or indirectly by the effects of inflammatory mediators such as cytokines, eicosanoids, nitric oxide and oxidative stress on sperm, ovarian, uterine and embryonic and endocrine function.[43]

Uterine infection and inflammation have several effects at distant sites. Uterine infection is associated with slower postpartum follicular growth, diminished follicular estradiol synthesis, increased risk of anovulation.[8,44-46] Delayed postpartum ovulation is associated with impaired reproductive performance,[47,48] and this effect is independent of and additive to, the effect of endometritis.[49] The effects of uterine infection on ovarian function are mediated in part by an effect at the hypophysis. Uterine infection of administration of exogenous LPS reduce luteinizing hormone (LH) pulsatility.[50-52] However, LPS from the uterus has a direct effect on follicular function.[53] Follicular cells express pattern recognition molecules, and their stimulation can perturb follicular steroid synthesis. Lipopolysaccharide has been found in follicular fluid and its concentration was proportional to the degree of endometrial inflammation.[53]

It also seems likely that endometritis may directly affect oocyte quality, an effect that persists after resolution of the active inflammation. Evidence for this lies in the observation that fertilization failure, rather than (or in addition to) embryonic death, is frequently observed in cows that previously had endometritis.[54,55] A similar observation has been made in embryo donors with mastitis; fertilization rate improves once mastitis is resolved (R.J. Mapletoft, personal communication, 2011). We have also observed an increased number of unfertilized oocytes and fewer transferrable embryos from donor cows with cytologic evidence of endometritis (R.O. Gilbert and M. Frajblat, unpublished observations). Exposure of bovine oocytes to LPS during in vitro maturation reduced the proportion developing to blastocysts but exposure to LPS after fertilization had no effect.[56] In consideration of the effect of endometritis on follicular function it is important to recall the nature of the utero-ovarian circulation and the ease with which inflammatory products could be transferred from uterine vein to ovarian artery, using the mechanism described for ipsilateral transfer of prostaglandin.[57-60] The basement membrane of the follicle is porous to diffusion of molecules of up to 850 kD.[61] In addition to LPS, inflammatory mediators TNFα and IL-1 inhibit follicular steroidogenesis.[62,63]

Local inflammation in the uterus could affect sperm survival and function. Sperm motility and zona binding are impaired in an inflammatory environment[63,64] and sperm phagocytosis is increased[65] (in women and horses). Bovine sperm exposed to oxidative stress suffer impaired motility, oocytes exposed to such sperm are less likely to undergo cleavage, and those that do cleave are less likely to result in blastocysts.[66]

In vitro produced bovine embryos co-cultured with neutrophils, even for as long as six days, suffer only very minor developmental impediment[67] while those exposed even briefly to cell-free uterine secretion of cows with experimentally induced aseptic endometritis have fewer trophoectoderm cells, possibly resulting in defective pregnancy recognition.[68] Several inflammatory mediators impair embryo development. Exposure to nitric oxide completely inhibits development to blastocyst stage in vitro[56] and development may be improved in the presence of a nitric oxide scavenger.[69] TNFα increases blastocyst apoptosis.[70] Loss of established pregnancy (after 28 days) is common in dairy cows[71] and is increased in cows previously diagnosed with uterine disease.[72]

Conclusion

Postpartum uterine disease in cattle is common and appears to be mediated by specific pathogens expressing specific virulence factors. Susceptibility to such infection is greatest in cows suffering deficits

in energy and other nutrients. The effect of these deficiencies seems to be to blunt the immediate postpartum recruitment of leukocytes to the uterus, with a consequently prolonged course of interaction with pathogens and longer residual inflammation. Endometritis exerts detrimental effects on inflammation at the pituitary and ovarian levels as well as in the uterus itself with consequences on fertility that persist after the resolution of the initial inflammation and may be seen as fertilization failure, early or late embryonic death. Progress in understanding of mechanisms involved is likely to enhance our ability to prevent endometritis and mitigate its effects.

References

1. LeBlanc SJ: Reproductive tract inflammatory disease in postpartum dairy cows. Animal 2014;8(Suppl 1):54-63.
2. Stojkov J, von Keyserlingk MA, Marchant-Forde JN, et al: Assessment of visceral pain associated with metritis in dairy cows. J Dairy Sci 2015;98:5352-5361.
3. Griffin JF, Hartigan PJ, Nunn WR: Non-specific uterine infection and bovine fertility. I. Infection patterns and endometritis during the first seven weeks post-partum. Theriogenology 1974;1:91-106.
4. Radoslavov V: [Quantitative changes in the uterine microflora during the puerperal period and conception in cows]. Vet Med Nauki 1975;12:49-54.
5. Gilbert RO, Santos NR: Dynamics of postpartum endometrial cytology and bacteriology and their relationship to fertility in dairy cows. Theriogenology 2016;85.
6. Dohmen MJ, Joop K, Sturk A, et al: Relationship between intra-uterine bacterial contamination, endotoxin levels and the development of endometritis in postpartum cows with dystocia or retained placenta. Theriogenology 2000;54:1019-1032.
7. Gilbert RO, Santos NR, Galvão KN, et al: The relationship between postpartum uterine bacterial infection (BI) and subclinical endometritis (SE). J Dairy Sci 2007;90:469-470.
8. Santos NR, Galvão KN, Brittin SB, et al: The significance of uterine E. coli infection in the early postpartum period of diary cows. Reprod Domest Anim 2008;43:63.
9. Zerbe H, Ossadnik C, Leibold W, et al: Influence of *Escherichia coli* and *Arcanobacterium pyogenes* isolated from bovine puerperal uteri on phenotypic and functional properties of neutrophils. Vet Microbiol 2001;79:351-365.
10. Zerbe H, Ossadnik C, Leibold W, et al: Lochial secretions of *Escherichia coli*- or *Arcanobacterium pyogenes*-infected bovine uteri modulate the phenotype and the functional capacity of neutrophilic granulocytes. Theriogenology 2002;57:1161-1177.
11. Sheldon IM, Rycroft AN, Dogan B, et al: Specific strains of Escherichia coli are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. PLoS One. 2010;5(2):e9192.
12. Bicalho RC, Machado VS, Bicalho ML, et al: Molecular and epidemiological characterization of bovine intrauterine *Escherichia coli*. J Dairy Sci 2010;93:5818-5830.
13. Santos TM, Gilbert RO, Bicalho RC: Metagenomic analysis of the uterine bacterial microbiota in healthy and metritic postpartum dairy cows. J Dairy Sci 2011;94:291-302.
14. Machado VS, Oikonomou G, Bicalho ML, et al: Investigation of postpartum dairy cows' uterine microbial diversity using metagenomic pyrosequencing of the 16S rRNA gene. Vet Microbiol 2012;159:460-469.
15. Jeon SJ, Vieira-Neto A, Gobikrushanth M, et al: Uterine microbiota progression from calving until establishment of metritis in dairy cows. Appl Environ Microbiol 2015;81:6324-6332.
16. Knudsen LR, Karstrup CC, Pedersen HG, et al: An investigation of the microbiota in uterine flush samples and endometrial biopsies from dairy cows during the first 7 weeks postpartum. Theriogenology 2016;doi: 10.1016/j.theriogenology.2016.02.016.
17. Bicalho ML, Machado VS, Oikonomou G, et al: Association between virulence factors of *Escherichia coli*, *Fusobacterium necrophorum*, and *Arcanobacterium pyogenes* and uterine diseases of dairy cows. Vet Microbiol 2012;157:125-131.
18. Machado VS, Bicalho ML, Meira Junior EB, et al: Subcutaneous immunization with inactivated bacterial components and purified protein of *Escherichia coli*, *Fusobacterium necrophorum* and *Trueperella pyogenes* prevents puerperal metritis in Holstein dairy cows. PLoS One 2014;9(3):e91734.
19. Hammon DS, Evjen IM, Dhiman TR, et al: Negative energy balance during the periparturient period is associated with uterine health disorders and fever in Holstein cows. J Dairy Sci 2004;87(Suppl.1):279.
20. Cheong SH, Nydam DV, Galvão KN, et al: Cow-level and herd-level risk factors for subclinical endometritis in lactating Holstein cows. J Dairy Sci 2011;94:762-770.
21. Dubuc J, Duffield TF, Leslie KE, et al: Risk factors for postpartum uterine diseases in dairy cows. J Dairy Sci 2010;93:5764-5771.
22. Yasui T, McCann K, Gilbert RO, et al: Associations of cytological endometritis with energy metabolism and inflammation during the periparturient period and early lactation in dairy cows. J Dairy Sci 2014;97:2763-2770.
23. Galvão KN, Brittin SB, Caixeta L, et al: Association between metritis and glycogen concentration in neutrophils of lactating Holstein cows. Reprod Domest Anim 2008;43:41.

24. Galvão KN, Santos NR, Galvao JS, et al: Association between endometritis and endometrial cytokine expression in postpartum Holstein cows. Theriogenology 2011;76:290-299.
25. Galvão KN, Felippe MJ, Brittin SB, et al: Evaluation of cytokine expression by blood monocytes of lactating Holstein cows with or without postpartum uterine disease. Theriogenology 2012;77:356-372.
26. Foley C, Chapwanya A, Callanan JJ, et al: Integrated analysis of the local and systemic changes preceding the development of post-partum cytological endometritis. BMC Genomics 2015;16:811.
27. Swangchan-Uthai T, Chen Q, Kirton SE, et al: Influence of energy balance on the antimicrobial peptides S100A8 and S100A9 in the endometrium of the post-partum dairy cow. Reproduction 2013;145:527-539.
28. Yasui T, McArt JA, Ryan CM, et al: Effects of chromium propionate supplementation during the periparturient period and early lactation on metabolism, performance, and cytological endometritis in dairy cows. J Dairy Sci 2014;97:6400-6410.
29. Esposito G, Irons PC, Webb EC, et al: Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Anim Reprod Sci 2014;144:60-71.
30. Turner ML, Cronin JG, Noleto PG, et al: Glucose availability and AMP-activated protein kinase link energy metabolism and innate immunity in the bovine endometrium. PLoS One 2016;11(3):e0151416.
31. Cheong SH, Nydam DV, Galvão KN, et al: Cow-level and herd-level risk factors for subclinical endometritis in lactating Holstein cows. J Dairy Sci 2011;94:762-770.
32. Cheong SH, Nydam DV, Galvao KN, et al: Effects of diagnostic low-volume uterine lavage shortly before first service on reproductive performance, culling and milk production. Theriogenology 2012;77:1217-1222.
33. Beutler B: Inferences, questions and possibilities in Toll-like receptor signalling. Nature 2004;430:257-263.
34. Takeuchi O, Akira S: Pattern recognition receptors and inflammation. Cell 2010;140:805-820.
35. Turner ML, Healey GD, Sheldon IM: Immunity and inflammation in the uterus. Reprod Domest Anim 2012;47(Suppl 4):402-409.
36. Sheldon IM, Noakes DE, Rycroft A, et al: Acute phase protein responses to uterine bacterial contamination in cattle after calving. Vet Rec 2001;148:172-175.
37. Galvão KN, Flaminio MJ, Brittin SB, et al: Association between uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows. J Dairy Sci 2010;93:2926-2937.
38. Sommer H, Marx D: [Fertility of cattle and its relationship to metabolism. Preliminary report on blood serum values (GOT, LDH, bilirubin, cholesterol, glucose) and disposition endometritis]. Berl Munch Tierarztl Wochenschr 1969;82:201-204.
39. Sepulveda-Varas P, Weary DM, Noro M, et al: Transition diseases in grazing dairy cows are related to serum cholesterol and other analytes. PLoS One 2015;10(3):e0122317.
40. Newman A, Mann S, Nydam DV, et al: Impact of dietary plane of energy during the dry period on lipoprotein parameters in the transition period in dairy cattle. J Anim Physiol Anim Nutr (Berl). 2016;100:118-126.
41. Healey GD, Collier C, Griffin S, et al: Mevalonate biosynthesis intermediates are key regulators of innate immunity in bovine endometritis. J Immunol 2016;196:823-831.
42. Raphael W, Sordillo LM: Dietary polyunsaturated fatty acids and inflammation: the role of phospholipid biosynthesis. Int J Mol Sci 2013;14:21167-88.
43. Gilbert RO: The effects of endometritis on the establishment of pregnancy in cattle. Reprod Fertil Dev 2011;24:252-257.
44. Sheldon IM, Noakes DE, Rycroft AN, et al: Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. Reproduction 2002;123:837-845.
45. Williams EJ, Fischer DP, Noakes DE, et al: The relationship between uterine pathogen growth density and ovarian function in the postpartum dairy cow. Theriogenology 2007;68:549-559.
46. Opsomer G, Grohn YT, Hertl J, et al: Risk factors for post partum ovarian dysfunction in high producing dairy cows in Belgium: a field study. Theriogenology 2000;53:841-857.
47. Gilbert RO, Frajblat M, Butler WR, et al: The importance of early postpartum ovulation for reproduction in dairy cows. Proc 24th World Buiatrics Conf; 2006.
48. Galvão K, Frajblat M, Butler W, et al: Effect of early postpartum ovulation on fertility in dairy cows. Reprod Domest Anim 2010;45:e207-e11.
49. Vieira-Neto A, Gilbert RO, Butler WR, et al: Individual and combined effects of anovulation and cytological endometritis on the reproductive performance of dairy cows. J Dairy Sci 2014;97:5415-5425.
50. Peter AT, Bosu WT, DeDecker RJ: Suppression of preovulatory luteinizing hormone surges in heifers after intrauterine infusions of *Escherichia coli* endotoxin. Am J Vet Res 1989;50:368-373.
51. Battaglia DF, Krasa HB, Padmanabhan V, et al: Endocrine alterations that underlie endotoxin-induced disruption of the follicular phase in ewes. Biol Reprod 2000;62:45-53.
52. Karsch FJ, Battaglia DF, Breen KM, et al: Mechanisms for ovarian cycle disruption by immune/inflammatory stress. Stress 2002;5:101-112.
53. Herath S, Williams EJ, Lilly ST, et al: Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. Reproduction 2007;134:683-693.
54. Cerri RL, Rutigliano HM, Lima FS, et al: Effect of source of supplemental selenium on uterine health and embryo quality in high-producing dairy cows. Theriogenology 2009;71:1127-1137.

55. Bender RW, Hackbart KS, Dresch AR, et al: Effects of acute feed restriction combined with targeted use of increasing luteinizing hormone content of follicle-stimulating hormone preparations on ovarian superstimulation, fertilization, and embryo quality in lactating dairy cows. J Dairy Sci 2014;97:764-778.
56. Soto P, Natzke RP, Hansen PJ: Identification of possible mediators of embryonic mortality caused by mastitis: actions of lipopolysaccharide, prostaglandin F2alpha, and the nitric oxide generator, sodium nitroprusside dihydrate, on oocyte maturation and embryonic development in cattle. Am J Reprod Immunol 2003;50:263-272.
57. Mapletoft RJ, Del Campo MR, Ginther OJ: Unilateral luteotropic effect of uterine venous effluent of a gravid uterine horn in sheep. Proc Soc Exp Biol Med 1975;150:129-133.
58. Mapletoft RJ, Ginther OJ: Adequacy of main uterine vein and the ovarian artery in the local venoarterial pathway for uterine-induced luteolysis in ewes. Am J Vet Res 1975;36:957-963.
59. Mapletoft RJ, Del Campo MR, Ginther OJ: Local venoarterial pathway for uterine-induced luteolysis in cows. Proc Soc Exp Biol Med 1976;153:289-294.
60. Mapletoft RJ, Lapin DR, Ginther OJ: The ovarian artery as the final component of the local luteotropic pathway between a gravid uterine horn and ovary in ewes. Biol Reprod 1976;15:414-421.
61. Rodgers RJ, Lavranos TC, van Wezel IL, et al: Development of the ovarian follicular epithelium. Mol Cell Endocrinol 1999;151:171-179.
62. Williams EJ, Herath S, England GC, et al: Effect of *Escherichia coli* infection of the bovine uterus from the whole animal to the cell. Animal 2008;2:1153-1157.
63. Halis G, Arici A: Endometriosis and inflammation in infertility. Ann NY Acad Sci 2004;1034:300-315.
64. Alghamdi A, Troedsson MH, Laschkewitsch T, et al: Uterine secretion from mares with post-breeding endometritis alters sperm motion characteristics in vitro. Theriogenology 2001;55:1019-1028.
65. Troedsson MH, Loset K, Alghamdi AM, et al: Interaction between equine semen and the endometrium: the inflammatory response to semen. Anim Reprod Sci 2001;68:273-278.
66. Hendricks KE, Hansen PJ: Consequences for the bovine embryo of being derived from a spermatozoon subjected to oxidative stress. Aust Vet J 2010;88:307-310.
67. Gilbert RO, Yang X, Jiang S, et al., editors: Effects of aseptic inflammation on development of bovine embryos in vivo and in vitro. Proc Annu Meet Soc Therio; 1995.
68. Hill J, Gilbert R: Reduced quality of bovine embryos cultured in media conditioned by exposure to an inflamed endometrium. Aust Vet J 2008;86:312-316.
69. Lim JM, Hansel W: Improved development of in vitro-derived bovine embryos by use of a nitric oxide scavenger in a cumulus-granulosa cell coculture system. Mol Reprod Dev 1998;50:45-53.
70. Soto P, Natzke RP, Hansen PJ: Actions of tumor necrosis factor-alpha on oocyte maturation and embryonic development in cattle. Am J Reprod Immunol 2003;50:380-388.
71. Diskin MG, Waters SM, Parr MH, et al: Pregnancy losses in cattle: potential for improvement. Reprod Fertil Dev 2015;28:83-93.
72. Ribeiro ES, Gomes G, Greco LF, et al: Carryover effect of postpartum inflammatory diseases on developmental biology and fertility in lactating dairy cows. J Dairy Sci 2016;99:2201-220.

Current understanding of bacterial biofilms and latent infections

Ryan A Ferris
Equine Reproduction Laboratory, Colorado State University, Fort Collins, CO

Introduction

Most encounters between bacteria and the equine endometrium lead to an acute period of subclinical infection and occasionally clinical signs. Following an acute infection in the majority of mares the invading bacteria will be eliminated and the infection resolved. However, in a minority of cases, small numbers of bacteria survive and cause persistent infections that can be difficult to eliminate. The development of acute and chronic cases of endometritis is the result of deficiencies in the mare's ability to eliminate an infection and the causative bacteria's unique pathogenic properties.

The mare's uterine defense mechanisms to bacterial infection are well understood and consist of physical, immunological, and mechanical barriers. Bacteria utilize numerous methods to survive degradation by the host immune system and antibiotic therapy. One survival tool utilized by bacteria is the production of a biofilm. Biofilms allow bacteria to be unrecognized by the host immune system, prevent exposure to antibiotics, and allow for exchange of genetic material leading to antibiotic resistance.

The purpose of this review is to describe how alterations to host defenses in combination with the pathogenicity of bacteria result in chronic cases of bacterial endometritis.

Pathophysiology

Host defense mechanisms

The mare has three main defense mechanisms to prevent bacterial infections in the uterus, physical barriers of the reproductive tract, the innate immune system, and mechanical uterine clearance. The physical barriers include the vulva, vagino-vestibular sphincter, and cervix. These barriers prevent feces, air and environmental pathogens from reaching the uterus. A reduction in the pathogenicity and quantity of bacteria occurs from the vulva to the cervix. Any disturbance in conformation of the reproductive tract will increase the likelihood of bacteria entering the uterus. Consequently, this results in a decrease in pregnancy rates. Once bacteria have reached the uterus the mare's innate immune system is activated.

The presence of bacteria within the uterine lumen results in a rapid influx of neutrophils, immunoglobulins, and serum proteins. This binding of complement and opsonins to bacteria greatly increase the ability and rate at which neutrophils phagocytize bacteria. Neutrophils from susceptible mares have reduced in vitro ability to phagocytize bacteria as compared to resistant mares. The inflammation associated the innate immune system results in fluid production into the uterine lumen.

The final defense mechanism against bacterial endometritis is mechanical uterine clearance of bacteria and inflammatory products. Several studies have shown that mares susceptible to uterine infections have decreased clearance of uterine fluid as compared to resistant mares. After intrauterine inoculation with bacteria susceptible and resistant mares have similar uterine myometrial contractions for 6-8 hours following inoculation, but contractions are depressed in susceptible mares after eight hours. Failure to clear bacteria and inflammatory products from the uterus, results in continued activation of the innate immune system and results in a further increase in inflammatory cells, immunoglobulins, and serum proteins reaching the uterus that continue to activate the innate immune system.

A single alteration to any of the defense mechanisms of a mare may allow for colonization of the uterus with a bacterial pathogen leading to a chronic infection.

Bacterial lifestyle

Bacterial are capable of living in two different lifestyles planktonic or biofilm states. Planktonic bacteria are single bacterial cells free flowing in suspension. Bacteria in this lifestyle are utilizing available nutrients for procreation. These individual cells are relatively susceptible to recognition and

degradation by the host immune system, susceptible to changes in environment (desiccation, lack of nutrients, etc), and sensitivity to antibiotics. However, the planktonic cell paradigm does not accurately reflect the growth of bacteria in nature associated with a biofilm.

In the last several decades the biofilm state has been considered to be the more prevalent lifestyle with ~99% of the overall world bacterial biomass living in a biofilm. In natural environments these biofilms are invariably a multispecies microbial community harboring bacteria that stay and leave with purpose, share their genetic material at high rates and fill distinct niches within the biofilm.

The first step in biofilm formation is migration and adherence to a surface. This is typically performed through the use of flagella and type IV pili in *E. coli, P. aeruginosa,* and *K. pneumonia. Strep. equi* subsp. *zooepidemicus* bacteria are non-motile and rely on movement from environmental or host factors. Individual bacteria will migrate (if capable) until other bacteria (same species or other) are encountered and micro-colonies start to form. At this point planktonic and biofilm lifestyles start to diverge, genes associated with flagella are down regulated and genes associated with polysaccharide production increase. This exopolysaccaride matrix forms the scaffold for the biofilm community.

As the community of bacteria grows in size the environment within the biofilm because heterogeneous with higher concentrations of oxygen and a more neutral pH on the outside of the biofilm as compared to the core which is relatively low in available oxygen with a slightly acidic pH. Bacteria are not organized randomly distributed within a biofilm but rather organized to best meet the needs of individual and the group.

Intercellular communication or quorum sensing is carried out through the production of bacterial products that are able to diffuse away from one cells and enter another cell. Signaling between cells is critical in the development of a viable biofilm and in reacting to outside environmental stress.

One of the biggest advantages of biofilm living is the ability to acquire transmissible, genetic elements at accelerated rates. Conjugation occurs naturally among bacteria but appears to be accelerated when bacteria are in a biofilm lifestyle. This allows for the rapid horizontal transfer of genetic material making a biofilm a perfect milieu for emergence of new pathogens by acquisition of antibiotic resistance, virulence factors and environmental survival capabilities.

Clinically biofilms can cause significant difficulty for clinician to eliminate these chronic infections once established. Bacteria within a biofilm are protected from the host immune system as white blood cells have reduced ability for movement and function, and the thick layer of exopolysaccaride (EPS) prevents antibodies from reaching bacteria deep within the biofilm. Biofilms protect bacteria from antibiotics by providing a diffusion barrier that decreases the amount of antibiotics that reach the protected bacterial colonies and creates a microenvironment that slows down the metabolism and therefore the replication rate of bacteria, which also makes them more resistant to antimicrobial agents. Ultimately, biofilms are associated with development and maintenance of subpopulations of 'persister cells'.

As antimicrobial agents come in contact with the biofilm, the agents must traverse through a layer of thick EPS, DNA, RNA, lipids and proteins in order to reach bacteria buried deep within this protective barrier. Bacteria in the outer region may be killed, but a decrease in the level of antibiotics reaching the inner layer bacteria contributes to the formation of a nidus for chronic infection.

The thick layer of EPS found in biofilms not only prevents antibiotics from penetrating, but limits the diffusion of oxygen and nutrients. Oxygen and nutrient deprivation consequently results in a decrease in metabolic rate as compared to planktonic or free individual bacteria. This reduction in metabolic rate provides additional antimicrobial resistance as antibiotics typically only act upon rapidly multiplying bacteria.

A popular theory currently is that growth of bacteria in biofilms produces 'persister cells'. These cells are unique in that they do not appear to grow and are highly multi-drug resistant to a wide variety antimicrobials. Further work is warranted to understand the role of 'persister cells' in chronic infections and biofilms.

The innate factors of antimicrobial resistance in bacterial biofilms have led to significant challenges in human medicine. It is estimated that 65% of nosocomial infections are associated with

biofilms, and that treatments for biofilm based infections cost >$1 billion annually. In equine medicine, we have just started investigating the role of biofilms in chronic infections.

It has been proposed that biofilms play an important role in chronic infections in the horse including chronic uterine infections resistant to antimicrobials may be due to biofilm production. Acute and chronic non-healing wounds on the distal equine limb contained a significantly greater incidence of biofilm producing bacteria as compared to a skin sample near the wound.

Evaluation of bacteria isolated from the equine uterus suggests that the majority of isolates of *Streptococcus equi* subsp. *zooepidemicus, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumonia* are capable of producing a biofilm *in vitro*. However, to date *in vivo* biofilm production and identification has not occurred in the endometrium of the mare. Unfortunately, no clinical diagnostic tests are available for the detection of a biofilm related infection. In human medicine a biofilm is suspected if appropriate antibiotic therapy is administered and the infection is unable to be eliminated.

Persister cells and infections

Persister cells represent about 1% of all bacteria in a free-floating state, an are characterized by being tolerant to antibiotics with no change in genetic expression. It is often thought that these bacteria are potentially dormant and metabolically inactive. This phenomenon was originally described in the 1940's in that cultures of *Staphylococcus aureus* exposed to lethal doses of penicillin resulted in <1% of the original CFUs surviving penicillin exposure. While this work was conducted before genetic sequencing was available the authors did not feel the acquired antibiotic resistance was due to a mutation in the bacteria as subsequent culturing and exposure to antibiotics resulted in continued susceptibility of these previous tolerant colonies.

In the mare it has been clearly identified that some mares can have a population of dormant *Streptococcus equi* subsp *zooepidemicus* deep in the uterine glands. This population of bacteria would not be identified on routine culture (not actively dividing bacteria) or cause significant inflammation or infection. However, if these bacteria were to leave this dormant stage the resulting bacterial growth will result in inflammation and infection leading to pregnancy loss.

Conclusion

Development of chronic infections is dependent upon a decrease in host susceptibility and the pathogenicity of causative bacteria. The idea of persistent bacteria and bacteria living within a biofilm are becoming solidified in human medicine and recent research is suggesting that these bacterial states play a significant role in chronic infections in equine reproduction.

Selected references

Causey RC: Making sense of equine uterine infections: the many faces of physical clearance. Vet J 2006;172:405-421.
Costerton JW, Stewart PS, Greenberg EP: Bacterial biofilms: a common cause of persistent infections. Science. 1999;284:1318-1322.
Gray GW, Wilkinson SG: The effect of ethylenediaminetetra-acetic acid on the cell walls of some gram-negative bacteria. J Gen Microbiol. 1965;39:385-399.
Kirkland KD, Fales WH, Blanchard TL, et al: The in vitro effects of EDTA-tris, EDTA-tris-lysozyme, and antimicrobial agents on equine genital isolants of Pseudomonas aeruginosa. Theriogenology 1983;20:287-295.
LeBlanc MM: Advances in the diagnosis and treatment of chronic infectious and post-mating-induced endometritis in the mare. Reprod Domest Anim 2010;45:21-27.
LeBlanc MM, Asbury AC, Lyle SK: Uterine clearance mechanisms during the early postovulatory period in mares. Am J Vet Res 1989;50:864-867.
Licking E: Getting a grip on bacterial slime. Bloomberg; Sept 12, 1999.
Lindsay D, Holy von A: Bacterial biofilms within the clinical setting: what healthcare professionals should know. J Hosp Inf 2006;64:313-325.
Stewart PS, Costerton JW: Antibiotic resistance of bacteria in biofilms. Lancet 2001;358:135-138.
Watnick P, Kolter R: Biofilm, city of microbes. J Bacteriol 2000;182:2675-2679.
Webb JS, Givskov M, Kjelleberg S: Bacterial biofilms: prokaryotic adventures in multicellularity. Curr Opin Microbiol. 2003;6:578-585.
Ferris RA, McCue PM, Borlee GI, et al: In vitro efficacy of nonantibiotic treatments on biofilm disruption of gram-negative

pathogens and an in vivo model of infectious endometritis utilizing isolates from the equine uterus. J Clin Microbiol 2016;54:631-639.
Petersen MR, Skive B, Christoffersen M, et al: Activation of persistent Streptococcus equi subspecies zooepidemicus in mares with subclinical endometritis. Vet Microbiol 2015;179:119-125.
Petersen MR, Nielsen JM, Lehn-Jensen H, et al: Streptococcus equi subspecies zooepidemicus resides deep in the chronically infected endometrium of mares. Clin Therio 2009;1:161-165.

Treatments for latent infections, bacterial biofilms, and fungal endometritis

Ryan A Ferris
Equine Reproduction Laboratory, Colorado State University,,Fort Collins, CO

Treatment for latent bacteria

The goal for treating mares with latent or dormant bacteria is to get the bacteria to move from the dormant state into a metabolically active state in which identification and treatment can be performed. Recent work by Petersen et al (2015) has shown that dormant *Streptococcus zooepidemicus* can be activated by infusing a proprietary medium (bActivate) into the uterus. After the infusion (24 hours) 64% (15/25) mares were culture positive for *Streptococcus zooepidemicus* as compared to 8% (1/12) mares infused with phosphate buffered saline (PBS). The proprietary medium is capable is able to get bacteria to convert from the dormant state to a metabolically active state and treatment can be initiated.

Interestingly, it should be noted that breeding also may result in bacteria being activated from a dormant state. Recent work by Christoffersen et al (2015) showed that 55% (16 of 29) of mares with a negative culture prior to breeding but retained fluid after breeding were positive for growth of *Streptococcus zooepidemicus*. The authors conclude that it was more likely to be dormant *Streptococcus zooepidemicus* that was reactivated as compared to introduction at the time of breeding. The development of post-mating fluid in barren mares could be due to both inflammation from breeding but also reactivation of dormant bacteria.

Treatment options for biofilms

Bacteria residing in a biofilm can be up 1000 times more resistant to treatment with antibiotics as compared to free-living (planktonic) bacteria. The simple administration of antibiotics has been unable to eliminate chronic infections suspected of involving a biofilm in both human and veterinary medicine. The goal in treating a biofilm associated infection is to remove the biofilm material and kill the bacteria residing within the biofilm.

We have tested various products in the last year for efficacy to disrupt a biofilm or kill the bacteria within a preformed biofilm. This is an *in vitro* assay looking at the effects of these agents specifically on bacteria residing in a biofilm and the effects observed. The overall results are presented in Table 1.

N-acetylcysteine

Treatment with 3.3% N-acetylcysteine (NAC) significantly ($p<0.05$) reduced the number of CFUs in all *P. aeruginosa* isolates evaluated, and no significant reduction in biofilm biomass was observed (Table 1). The *E. coli* and *S. zooepidemicus* isolates had a significant reduction in CFUs and biofilm biomass as compared to the untreated control (Table 1). The MIC for NAC was found to be 3.3% dilution to 1.6% did not disrupt preformed biofilms or kill the bacteria within the biofilm. Treatment with NAC did not reduce biofilm biomass or reduced the number of CFUs for *K. pneumoniae* (Table 1).

Hydrogen peroxide

Challenge with 1% hydrogen peroxide significantly reduced the number of CFUs for nine of the ten *K. pneumoniae* isolates, but did not reduce the biofilm biomass (Table 1). *E. coli* isolates were significantly reduced in biofilm biomass and had a reduction in CFUs following challenge (Table 1). *P. aeruginosa* isolates had reduced biofilm biomass in five isolates but no reduction in CFUs following treatment with 1% hydrogen peroxide (Table 1). *S. zooepidemicus* isolates had significantly reduced biofilm biomass and CFUs as compared to untreated controls. Dilution to 0.5% was found to be just as effective as a 1% concentration.

Chelating agents

No significant difference in biofilm biomass or CFUs was observed following a 6h challenge with either tris-EDTA (50 mM and 3.5 mM, respectively) or THAM-EDTA (20 mM and 8 mM, respectively)

for isolates of *P. aeruginosa*, or *K. pneumoniae* (Table 1). *E. coli* had a significant reduction in biofilm biomass following challenge with Tris-EDTA or THAM-EDTA (Table 1). *S. zooepidemicus* isolates had significantly reduced biofilm biomass and CFUs as compared to untreated controls. Dilution from recommended concentrations failed to disrupt a preformed biofilm or kill the bacteria within the biofilm.

Hypochlorous acid

No significant reduction in biofilm biomass or CFUs was observed following a six hour challenge with either Vetricyn® (Vetricyn, Riverside Ca) or Omniphase® (Integrated Healing Technologies, Franklin TN) for *P. aeruginosa, E. coli, or K. pneumonia.* Isolates of *S. zooepidemicus* had a significant reduction in biofilm biomass and CFUs following challenge with Vetricyn® or Omniphase® (Table 1).

Antimicrobial peptide mimic

A significant reduction in biofilm biomass was observed for isolates of *E. coli and K. pneumoniae* but no change in CFUs was detected following challenge. In *P. aeruginosa* 50% of isolates had reduced biofilm biomass, but none of these isolates had decreased CFUs. Ceragyn® (Ceragyn, Spanish Fork, UT) effectively reduced the biofilm biomass and reduced the number of CFUs for isolates of *S. zooepidemicus (*Table 1). For *K. pneumoniae* and *E. coli* isolates we were able to dilute this product to 1.5%, and 0.5%, respectively and still maintain equal disruption and killing to the recommended concentration.

Ozone

No significant changes in biofilm biomass or CFUs was observed following a six hour challenge with ozone for *E. coli, K. pneumonia, P. aeruginosa,* and *S. zooepidemicus.*

Treatment of fungal infections

Therapy for fungal endometritis involves treating the active infection via methods such as uterine lavage with dilute acetic acid or dilute povidone-iodine, plus systemic and/or intrauterine infusion of anti-fungal agents, in addition to correction of predisposing factors that could result in treatment failure. Administration of more than one anti-fungal agent may be indicated in refractory or recurrent clinical cases. Uterine lavage is indicated to remove retained fluid, reduce organism load, kill fungal organisms, and remove biofilm. It may also be beneficial to apply topical antifungal medication to the vagina and clitoris as these areas may act as a reservoir or nidus for reinfection.

Ideally, selection of an antifungal agent would be based on results of susceptibility tests for each case of fungal endometritis. Unfortunately, antifungal susceptibility tests are not performed in many diagnostic laboratories and several weeks are often required from sample submission to when results are obtained. Clinicians often rely on empirical choices for initial antifungal therapy drugs based on published susceptibility patterns while awaiting organism identification and susceptibility testing (Table 1).

Table 1. Susceptibility patterns of fungi to antifungal drugs commonly used to treat fungal endometritis in mares (from Coutinho da Silva and Alvarenga, 2011).

Antifungal Agent	Susceptibility pattern (% of isolates)		
	Susceptible	Intermediate	Resistant
Polyenes			
Amphotericin B	96	0	4
Natamycin	100	0	0
Nystatin	100	0	0
Azoles			
Clotrimazole	80	13	7
Ketoconazole	81	15	4
Miconazole	43	41	16
Itraconazole	62	38	0
Fluconazole	44	14	42
Flurocytosin	83	0	17

Several anti-fungal agents are available for intra-uterine therapy (Table 2). Fluconazole and itraconazole are reported to be absorbed following oral administration, with fluconazole being the most cost effective therapy in the horse. Oral administration of an antifungal agent can provide long term anti-mycotic activity and may be an important component of a multifaceted treatment program for fungal endometritis. Lufenuron has also been reported to be an effective treatment against fungal endometritis by inhibiting chitin synthesis in the cell wall. Lufenuron may not be effective in all cases as not all fungal organisms have chitin in their cell walls.

Tables 2, 3, and 4 list systemic medications and uterine therapies used in the treatment of fungal endometritis.

Table 2. Intrauterine medications used in the treatment of fungal endometritis.

Medication	Dosage, Route, Frequency
Amphoteracin B (50 mg/vial)	100 to 200 mg reconstituted in 50 to 100 mls sterile saline
Clotrimazole	500-700 mg in 50 to 100 mls sterile saline
Fluconazole (200 mg/tablet)	100 to 250 mg in 50 to 100 mls sterile water; to reconstitute, add 5 mls DMSO to 1 gram (5 tablets) of fluconazole to dissolve; divide into 4 aliquots of 250 mg each; qs to 50 to 100 mls with sterile water
Lufenuron (Program®) (270 mg/packet)	540 mg in uterus suspended in 60 mls sterile saline, 270 mg applied to vaginal vault and clitoral area
Miconazole (1,200 mg insert)	1,200 mg insert deposited into uterus
Nystatin (100,000 IU/gram; 30 gram vial)	5 grams suspended in 50 to 100 mls sterile water; or 0.5 to 2.5 million units

Table 3. Systemic medications that may be used in the treatment of fungal endometritis.

Medication	Dosage, Route, Frequency
Fluconazole (200 mg/tablet)	14 mg/kg, PO, loading dose, followed by 5 mg/kg q 24h
Itraconazole (3 grams/packet)	3 - 5 mg/kg PO q 24h for 2 to 3 weeks or longer

Table 4. Uterine lavage therapies that may be used in the treatment of fungal endometritis.

Medication	Dosage, Route, Frequency
N-Acetylcysteine solution (20 %) (200 mg/ml)	30 mls (6 grams) diluted into 150 mls sterile saline infused into uterus
Dimethyl sulfoxide (DMSO) (99%)	50 ml DMSO per liter saline; may repeat as needed; follow with lavage with 1 liter saline or LRS
Hydrogen Peroxide (3 %)	60 to 120 mls infused into uterus; follow the next day with lavage using sterile saline or lactated Ringer's solution (LRS)
Lactated Ringer's Solution (LRS)	1 to 4+ liters; repeat lavage until effluent fluid is clear
Povidone-Iodine (Betadine® Solution) (1 %)	10 -15 mls added to 1 liter sterile saline
Saline (0.9 %)	1 to 4+ liters; repeat lavage until effluent fluid is clear
Tris-EDTA (Tricide®)	250 to 500 mls infused into uterus; followed by uterine lavage with lactated Ringer's solution (LRS)
Acetic Acid (Distilled White Vinegar) (2 %)	20 – 100 mls added to 1 liter sterile saline

Mares are often treated empirically while awaiting results of antifungal susceptibility tests. A combination of two antifungal agents may be warranted in the event that the fungal organism is resistant to one of the agents. A potential protocol for treatment of a mare with fungal endometritis is presented below:

1. Uterine lavage during early estrus with sterile saline (plus acetic acid or other agent) and administer oxytocin (20 units, IM or IV) to promote evacuation of uterine fluid. May be repeated daily as needed.
2. Administer systemic fluconazole therapy (14 mg/kg loading dose PO, once; followed by 5 mg/kg maintenance dose PO q 24h for 2 to 3 weeks)
3. Administer intrauterine antifungal therapy using either nystatin (500,000 IU in 50 mls saline, IU q 24h x 5 days) or one miconazole pod (1,200 mg IU; once) as indicated by organism and antimicrobial sensitivity tests
4. Lavage uterus (± oxytocin) at conclusion of intrauterine therapy
5. Short-cycle mare with prostaglandin
6. Re-culture when back in estrus
7. Anticipate treatment for secondary bacterial infection (esp. *S. equi* subsp. *zooepidemicus*)

It is common for a moderate to heavy growth of a bacterial organism such as *Streptococcus equi* subsp. *zooepidemicus* to be detected in mares following treatment of fungal endometritis. Consequently, it is often necessary to treat for bacterial endometritis along with or after treatment for fungal endometritis.

Conclusion

Treatment of infectious endometritis due to latent bacteria, biofilm, or fungal organisms can be difficult. However, an understanding of the pathophysiology of the organisms in their current states can help when selecting treatment protocols.

Selected references

Christoffersen M, Söderlind M, Rudefalk SR, et al: Risk factors associated with uterine fluid after breeding caused by Streptococcus zooepidemicus. Theriogenology 2015;84:1283-1290.

Coutinho da Silva MM and Alvarenga MA: Fungal endometritis. In: McKinnon AO, Squires EL, Vaala WE, et al, editors. Equine reproduction. Ames: Wiley-Blackwell; 2011. p. 2643-2651.

Ferris RA, McCue PM, Borlee GI, et al: In vitro efficacy of nonantibiotic treatments on biofilm disruption of gram-negative pathogens and an in vivo model of infectious endometritis utilizing isolates from the equine uterus. J Clin Microbiol 2016;54:631-6639.
Petersen MR, Skive B, Christoffersen M, et al: Activation of persistent Streptococcus equi subspecies zooepidemicus in mares with subclinical endometritis. Vet Microbiol 2015;179:119-125.

Table 5. List of agents effective at either degrading a preformed biofilm or killing the bacteria within a preformed biofilm. If no agent was 100% effective against a particular bacterial species the most effective agents were listed.

	Degradation Of Biofilm Biomass (% isolates susceptible)	Killing Of Bacteria Within A Biofilm
E coli	Tris-EDTA/Tricide-100% Acetylcysteine-100% H_2O_2-100% DMSO-100% Ceragyn®- 100%	Acetylcysteine H_2O_2
K pneumonia	Ceragyn®- 90%	H_2O_2
P aeruginosa	Tris-EDTA/Tricide- 38% H_2O_2- 50% Ceragyn®- 50%	Acetylcysteine
S equi* subsp *zooepidemicus	Tris-EDTA/Tricide H_2O_2 DMSO Hypochlorous Acid Ceragyn®	Tris-EDTA/Tricide H_2O_2 DMSO Hypochlorous Acid Ceragyn®

New diagnostic biomarkers to evaluate late term pregnancy in mares

Barry A. Ball
Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY

Introduction

In populations of Thoroughbred mares in central Kentucky and in Great Britain, pregnancy loss rates in mares from approximately Day 40 of gestation to term vary between 6.3 to 12.9%.[1-3] As such, these losses represent a substantial economic loss to breeders, and considerable effort in clinical approach and in research have been directed toward the identification of risk factors, diagnosis and treatment of the many and varied causes of these losses. In particular, a majority of abortions and stillbirths in the mare are associated with placental insufficiency or placentitis, and biomarkers for placental function have been of considerable interest.[4,5] This review will examine current understanding of biomarkers for evaluation of equine pregnancy during the late fetal period (last trimester of pregnancy).

Steroid hormones

The equine fetoplacental unit produces pregnanes, androgens and estrogens during pregnancy which can be detected in maternal circulation. The application of techniques such as mass spectrometry has provided a better understanding of the specific steroids present in circulation compared to immunoassay techniques which may lack specificity in identifying individual steroids because of antibody cross-reactivity to related molecules. In a recent study, we characterized changes in maternal concentrations of steroids in pregnant mares throughout gestation.[6]. Although mass spectrometry has been used previously to examine changes in steroid concentrations during pregnancy, this is among the first study to examine these changes across the whole of gestation in the mare.

Estrogens

Production of estrogens by the equine conceptus has been identified as early as Days 8 to 10[7,8] although changes in circulating concentrations of estrogen in maternal blood are not detectable until about Day 40 of pregnancy when secretion of estradiol from the corpus luteum results in an elevated serum estradiol in pregnant mares.[9] Production of estrogens by the fetoplacental unit beginning at around Day 80 result in an elevated estrogen concentration in maternal blood which peaks at around Day 210 of gestation[10] and parallels the hypertrophy of the equine fetal gonads. Estrone and its sulfoconjugate are the predominant estrogen formed by the equine fetoplacental unit, and synthesis of estrogens requires steroidogenic activity by both the fetal gonads and placenta. The equine fetal gonads synthesize dehydroepiandrosterone (DHEA) while placental conversion of DHEA to androstenedione and subsequently estrone and estradiol complete the synthesis of estrogens by the placenta.[11,12] In addition to the classic phenolic estrogens, the pregnant mare also has high concentrations of ring B unsaturated estrogens (equilin and equilenin) which are produced via secretion of an androgen (7-dehydro-DHEA) by the fetal gonads with subsequent aromatization by the placenta to the ring B unsaturated estrogens.[13] Unfortunately, little is known about the biological significance of these estrogens nor their role as possible biomarkers for pregnancy well-being in the mare.

A number of studies have attempted to assess fetal well-being based upon determination of various estrogens in the blood of pregnant mares. Measurement of estrone sulfate concentrations have been used for pregnancy detection in mares[14-17] with elevations in peripheral estrone sulfate concentrations as early as Day 37 of gestation. Because the initial elevation in maternal estrogens is likely associated with luteal production estrogens under the influence of increasing equine chorionic gonadotropin (eCG),[9] fetoplacental estrogen synthesis does not appear to be increased until approximately Day 80. Induction of fetal death by administration of prostaglandin F2□ or surgical removal of the fetus in pregnant mares at Days 44-89 resulted in a decline in maternal estrone sulfate concentrations that was coincident with fetal death. Determination of estrogens has been suggested as a method to assess fetal well-being during later gestation in the mare. Experimental induction of placentitis in mares at nine

months of gestation by intracervical inoculation of *Streptococcus equi* subsp *zooepidemicus* resulted in decline in maternal estradiol-17□ that was detectable within four days after inoculation (three to six days prior to abortion) compared to gestationally age matched control mares.[18]. In contrast, estrone sulfate concentrations were decreased only at the day of abortion in mares with experimentally induced placentitis. The differences in the timing of changes between free estradiol-17□ and estrone sulfate are likely due to the relatively longer half-life of the conjugated estrone in blood compared to estradiol-17□. In a large field study conducted on Thoroughbred mares in Japan, serum estradiol concentrations after Day 201 of gestation were lower in mares that aborted or produced dead foals compared to mares that delivered live foals.[19] Similarly, clinical observations reported by Douglas,[20] found lower "total estrogen" concentrations between Days 150-280 in pregnant mares that aborted with placentitis than in mares delivering live foals. Together, these studies indicate that determinations of serum estradiol concentrations in late gestation may be useful for assessment of fetoplacental well-being in equine pregnancy. A few caveats apply in making this statement. Conjugated estrone sulfate concentrations appear to change more slowly and may not be useful for assessment of fetal well-being in the mare. Interpretation of estradiol concentrations in the late pregnant mare requires normal reference values for the gestational age being evaluated. These reference values will be particular for the assay being used since these immunoassays may vary in their cross-reactivity and specificity for estrogens in equine sera. Interpretation of estradiol concentrations in late pregnancy (> 300 days) may be more difficult because estradiol concentrations are falling in normal mares during this period.

Androgens

As noted above, fetal gonadal production of androgens increases along with gonadal hypertrophy with a peak at approximately 175 days GA. In particular, concentrations of dehydroepiandrosterone (DHEA), and its sulfoconjugate (DHEA-S), increase in maternal circulation peaking at approximately 175 days of pregnancy.[18] We hypothesized that concentrations of DHEA-S appearing in maternal blood might be increased in mares in which placental dysfunction secondary to placentitis occurred. To test this hypothesis, we examined changes in maternal DHEA-S concentrations in mares with experimentally induced placentitis and control mares. Unfortunately, no significant differences were found in mares with or without placentitis indicating that maternal androgens do not appear to be as useful biomarker for placental dysfunction in mares.

Pregnanes

The mare has a complex steroidogenic scheme for the production of pregnanes during pregnancy which involves both luteal as well as fetoplacental contributions to progestational support of pregnancy in the mare.[8] Initially, the primary corpus luteum formed at ovulation is responsible for progestogen production; however, progesterone concentrations are low to undetectable during the second half of gestation in mares.[21] The secretion of eCG initiates the formation of accessory corpora lutea which result in an increase in maternal progesterone concentrations beginning around Day 40. Although progesterone is the major progestogen secreted during this time, concentrations of 5□-dihydroprogesterone (5□-DHP) are also elevated after ovulation, and DHP is a bioactive progestogen which is capable of supporting pregnancy in the mare in the absence of progesterone.[22] As the endometrial cups and accessory CLs regress, there is a shift from luteal to fetoplacental production of progestogens around Day 110 of gestation.[6] Placental production of pregnanes is characterized by increasing 5□-DHP for the remainder of pregnancy, and it is likely that 5□-DHP is the major progestagenic steroid during this portion of gestation. In addition, a number of other pregnanes are also produced by the equine placenta, some in very high concentrations.[6,23] The biologic activity, if any, of these remaining pregnanes in equine pregnancy are unknown although some are present in very high concentrations during late pregnancy.[6,23]

A number of investigators have examined the use of pregnanes to assess fetoplacental well-being in the mare.[19,20,24-26] Because all of the studies except one, relied upon immunoassays for detection of pregnanes, it is difficult to make direct comparisons across these studies. Immunoassays vary widely in their cross-reactivity to different pregnanes which may be present in the mare in late gestation.[27]

Therefore the reported values obtained may vary widely from laboratory to laboratory. In one study where determination of pregnane concentrations was based upon GC-MS, mares with clinical placentitis had elevated peripheral concentrations of several metabolites including pregnenolone (P5) and/or progesterone as well as metabolites of 5□-DHP: P5ββ, ββ-diol, βα-diol, 20α-5P compared to normal pregnant mares.[24] When measured by immunoassay, total pregnanes were elevated in all seven mares affected with placentitis during late gestation.[24] It appears that metabolism of progesterone to 5□-DHP is increased in mares with placentitis and that several other metabolites of 5□-DHP are increased in maternal circulation. These increases appear more likely to occur with chronic placental disease, and it has been hypothesized that chronic fetal stress leads to an increased P5 production by the fetal adrenal glands which subsequently drives an increased pregnane production by the placenta.[24,28] Conversely, more acute disease was associated with a reduced fetal P5 production and a decrease in pregnane concentrations in the mare.[24,28] Based upon immunoassay data, several studies have demonstrated an increase in pregnane concentrations in mares with placentitis or other abnormalities in pregnancy during late gestation. In a large field study conducted on Thoroughbred mares in Japan, serum progesterone concentrations after Day 201 of gestation were higher in mares that aborted or produced dead foals compared to mares that delivered live foals.[19] Douglas also reported that mares with placentitis had higher serum progestogen concentrations between Days 150-280 than did mares with normal pregnancies.[20] In summary, it appears that placentitis, particularly as it becomes more chronic, is often associated with elevations in serum progestogen concentrations in the mare. More acute placentitis may be associated with a decline in serum pregnane concentrations although this may occur very shortly before abortion. Future studies using more specific techniques such as mass spectrometry may shed more light on which pregnanes are more related to placental pathology. Clinical application of progestogen determinations in pregnant mares during late gestation currently is based upon evaluation of serial (three or more) samples taken at 48-72 hour intervals looking for a greater than 50% increase or decrease in progestogen concentrations compared to the initial sample.[28] Again, these changes are more difficult to interpret after 300 days GA because progestogen concentrations are normally increasing beyond this time in normal pregnancy. Interestingly, mares exposed to endophyte-infected tall fescue (fescue-toxicosis) fail to demonstrate a rise in serum progestogen concentrations during the last month of gestation.[29]

Relaxin

Relaxin in the mare is a polypeptide hormone produced specifically by the placental trophoblast with increases in circulating concentrations noted from around Day 80 of gestation to term.[30] Relaxin concentrations increase during labor,[30,31] and relaxin has been evaluated as a possible biomarker for placental function in mares.[32] Although the function of relaxin during pregnancy and labor in mares is not well studied, there is some information suggesting that relaxin secretion is altered in abnormal pregnancy in the mare. In a series of clinical cases, relaxin concentrations were reduced in mares with abnormalities such as hydrops, placentitis, and premature placental separation compared to normal pregnant mares.[32] However, relaxin concentrations were also highly variable between mares with breed differences in relaxin concentrations noted in one study.[33] At present, equine-specific immunoassays for relaxin are not available clinically although this biomarker warrants further research to assess its utility in evaluating fetal well-being and placental function in the mare.

Alpha-fetoprotein

Alpha-fetoprotein (AFP) is a major protein present in fetal circulation as well as in allantoic and amniotic fluids. An early description of an immunoassay for equine fetal protein described an increase in measured concentrations of equine fetal protein (EQFP) in serum of mares with clinical placentitis, embryonic loss and twin pregnancy.[34] Unfortunately, characterization of EQFP was not provided, and it is unclear if this protein was equine AFP. Further studies using this ELISA for EQFP were not reported in the literature and so no additional information regarding this assay is available. Alpha-fetoprotein is a major protein constituent of equine allantoic and amniotic fluids,[35] and AFP was identified in high concentrations in equine fetal serum as well as in equine fetal fluids based upon a heterologous assay for

human AFP (Immulite® 1000 platform; Siemens Healthcare Diagnostics Tarrytown, NY). Alpha-fetoprotein was detected based upon the heterologous ELISA in plasma from pregnant mares, but not in plasma from geldings or nonpregnant mares.[35] In mares with experimentally induced ascending placentitis, plasma AFP concentrations were increased by 7 days after inoculation compared to control mares. We hypothesize that increased concentrations of AFP in maternal plasma of mares with placentitis may be related to altered vascular permeability, but this mechanism requires confirmation. In women, elevated AFP concentrations in maternal circulation have been associated with increased risk for preterm birth;[36] however, meta-analysis of 24 studies incorporating more than 200,000 pregnancies concluded that elevated concentrations of AFP in maternal circulation alone were not a predictive marker for preterm birth unless AFP was combined with other markers including hCG and estriol.[37] In mares, AFP may have utility to predict abnormal pregnancy outcome; however, larger studies are required and incorporation of other markers such as estradiol or progestogens may be required to realize this potential.

Acute phase proteins

Acute phase proteins (APP) are synthesized primarily by the liver as part of the acute response of the innate immune system to stimuli such as trauma, infection, neoplasia or inflammation.[38] In horses, acute phase proteins include serum amyloid A (SAA), haptoglobin, and fibrinogen. Fibrinogen is probably the most commonly used APP in horses; however, a number of recent studies have examined SAA and haptoglobin as indicators of acute inflammation in the horse. In particular, two studies reported to date have examined changes in APP in mares with experimentally induced placentitis. In the first study, maternal concentrations of SAA increased (>7mg/L) within two to seven days after experimentally induced placentitis,[39] and peak concentrations of SAA varied from 274 to 4386 mg/L. In a group of nine mares that were experimentally inoculated with *Streptococcus equi* subsp *zooepidemicus*, treatment (trimethoprim-sulfa, altrenogest, pentoxifylline) was initiated at onset of clinical signs of placentitis.[39] In these treated mares, SAA did not increase in six of nine mares, and all of these six mares delivered a live foal. In the remaining three mares, SAA was persistently elevated in one mare which aborted whereas SAA was transiently elevated in the remaining two mares which delivered live foals. These data indicated that changes in SAA in the mare are rapid after experimentally induced placentitis with *Streptococcus* and that changes in SAA concentrations may be predictive of outcome after initiation of treatment.[39] In a second study, changes in the APP, SAA, haptoglobin and fibrinogen were examined in mares with experimentally induced placentitis secondary to inoculation of *Streptococcus equi* subsp *zooepidemicus*.[40] In this study, SAA concentrations increased significantly within two days after inoculation whereas haptoglobin concentrations increased significantly at Day 3 after inoculation.[40] Interestingly, neither fibrinogen nor total white blood cell counts differed between control and inoculated mares in this study.

Although APP appear to be a very sensitive biomarkers for models of experimental placentitis, it is important to note that they are also very nonspecific. Other inflammatory stimuli can elicit a pronounced rise in SAA, and we have seen marked rises in SAA in mares subsequent to routine immunization, for example. In clinical cases of placentitis, SAA appears to be a much less consistent biomarker for placental inflammation. In a large prospective field study in which mares (n = 700) were sampled weekly during late gestation, SAA was not consistently elevated in mares with placentitis diagnosed by histopathologic evaluation of the placenta (unpublished data). It is unclear whether this is due to differences in the causative organism, duration or chronicity of the disease or possibly some other factor.

microRNAs

MicroRNAs (miRNAs) are small (18–24 nucleotides) non-coding RNAs that function to regulate translation and degradation of specific messenger RNAs (mRNA).[41] MicroRNAs make up approximately 1% of the genome of multiple species, are highly conserved across species and are believed to regulate at least of 30% of genes.[42] Approximately 10 x more stable than messenger RNAs, miRNAs have an average half-life of approximately five days and are abundant in circulation where they are found bound

to carrier proteins or within small membrane bound vesicles termed exosomes.[43-45] MicroRNAs appear to have a wide range of biological functions and have attracted considerable attention as potential diagnostic markers as well as therapeutic targets for a wide range of diseases.[46-48] MicroRNA represents a source of cell-free nucleic acid derived from the placenta which is readily available in maternal circulation.[49]

Although much work remains to decipher the roles of placental-derived microRNAs, it appears likely that these small, regulatory RNAs function in fetal-maternal communication in areas such as immunoprotection of the fetal allograft.[50,51] Other proposed roles include implantation and placentation,[52] angiogenesis, proliferation and decidualization, apposition/adhesion of the embryo to the endometrium and embryonic migration and invasion (reviewed in[53]). There are a number of pregnancy-associated miRNAs in women,[41,49,50,54-60] and it appears that fetal miRNAs in the maternal circulation increase exponentially during the first trimester.[44] In the human, many of the miRNAs found in maternal circulation are derived from a single cluster located on chromosome 19 which appear to play a role in protecting the placenta against viral infections.[61] Additionally, miRNA levels change significantly with abnormal pregnancies such as ectopic pregnancy,[62] idiopathic, recurrent pregnancy loss,[63] low fetal birth weight,[64] high fetal birth weight,[65] pre-term labor[55,66] and pre-eclampsia[66-69] in women. Our laboratory is currently investigating the use of miRNA in the horse as potential biomarkers for placental function.

Conclusions

Determination of reliable biomarkers for fetoplacental well-being in the mare has many potential applications but also present a number of challenges. Steroids, particularly progestogens and estradiol appear to be good biomarkers for diseases such as placentitis; however, normal values need to be established across the gestational ages since these steroids have variable serum concentrations across different stages of pregnancy. Likewise, normal values need to be established for the particular immunoassay being used for measurement of steroids, particularly progestogens, due to varying cross reactivities of different assays for progestogens. Alpha-fetoprotein may also be a possible biomarker for placentitis in the mare since elevated concentrations of AFP were observed in mares with experimental placentitis. Although acute-phase proteins such as SAA demonstrate a rapid and large increase after experimentally induced placentitis, experience to date suggests that SAA is not consistently elevated in clinical cases. It is likely that ultimately a multiparameter biomarker panel will be required to assess fetoplacental well-being in mares. At present, such a panel might constitute estradiol, progestogens and alpha fetoprotein.

Acknowledgement

Information on miRNAs was kindly provided by Dr. Shavahn Loux.

References

1. Allen WR, Brown L, Wright M, et al: Reproductive efficiency of Flatrace and National Hunt Thoroughbred mares and stallions in England. Equine Vet J 2007;39:438-45.
2. Bosh KA, Powell D, Shelton B, et al: Reproductive performance measures among Thoroughbred mares in central Kentucky, during the 2004 mating season. Equine Vet J 2009;41:883-888.
3. Morris LHA, Allen WR: Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket. Equine Vet J 2002;34:51-60.
4. Giles RC, Donahue JM, Hong CB, et al: Causes of abortion, stillbirth, and perinatal death in horses: 3, 527 cases (1986-1991). J Am Vet Med Assoc 1993;203:1170-1175.
5. Ryan PL, Greene JM, Christiansen DL, et al: Emerging diagnostic approaches for evaluation of fetal and pregnancy well-being in the mare. Clin Therio 2010;2:149-161.
6. Legacki EL, Scholtz EL, Ball BA, et al: The dynamic steroid landscape of equine pregnancy mapped by mass spectrometry. Reproduction 2016;151:421-430.
7. Zavy MT, Mayer R, Vernon MW, et al: An investigation of the uterine luminal environment of nonpregnant and pregnant pony mares. J Reprod Fertil 1979;Suppl27:403-411.
8. Ousey J, McKinnon A, Squires E, Vaala W, Varner D. Endocrinology of pregnancy. Equine reproduction, Volume 2. 2011(Ed. 2):2222-33.
9. Daels PF, Shideler S, Lasley BL, et al: Source of oestrogen in early pregnancy in the mare. J Reprod Fertil 1990;90:55-61.

10. Raeside J, Liptrap R: Patterns of urinary oestrogen excretion in individual pregnant mares. J Reprod Fertil 1975;Suppl 23:649-675.
11. Raeside J, Liptrap R, McDonell W, et al: A precursor role for DHA in a feto-placental unit for oestrogen formation in the mare. J Reprod Fertil 1978;Suppl27:493-47.
12. Pashen RL, Allen WR: The role of the fetal gonads and placenta in sterod production, maintenance of pregnancy and parturition in the mare. J Reprod Fertil 1979;Suppl27:499-509.
13. Bhavnani BR. The saga of the ring B unsaturated equine estrogens. Endocr Rev 1988;9:396-416.
14. Stabenfeldt GH, Daels PF, Munro CJ, et al: An oestrogen conjugate enzyme immunoassay for monitoring pregnancy in the mare: limitations of the assay between days 40 and 70 of gestation. J Reprod Fertil1991;Suppl44:37-44.
15. Daels PF, Ammon DC, Stabenfeldt GH, et al: Urinary and plasma estrogen conjugates, estradiol and estrone concentrations in nonpregnant and early pregnant mares. Theriogenology 1991;35:1001-1017.
16. Jeffcott LB, Hyland JH, MacLean AA, et al: Changes in maternal hormone concentrations associated with induction of fetal death at day 45 of gestation in mares. J Reprod Fertil 1987;Suppl35:461-467.
17. Kasman LH, Hughes JP, Stabenfeldt GH, et al: Estrone sulfate concentrations as an indicator of fetal demise in horses. Am J Vet Res 1988;49:184-187.
18. Canisso IF, Ball BA, Esteller-Vico A, et al: Changes in maternal androgens and oestrogens in mares with experimentally induced ascending placentitis. Equine Vet J 2016;Jan 5. doi: 10.1111/evj. 12556.
19. Shikichi M, Iwata K, Ito K, et al: Diagnosis of abnormal pregnancy by serum progestins and estrogens in late pregnant mares. J Equine Vet Sci 2014;34:231-233.
20. Douglas RH: Endocrine diagnostics in the broodmare: what you need to know about progestins and estrogens. Proc Soc Theriogenol. 2004.
21. Short RV: Progesterone in blood. J Endocrinol 1959;19:207-210.
22. Scholtz EL, Krishnan S, Ball BA, et al: Pregnancy without progesterone in horses defines a second endogenous biopotent progesterone receptor agonist, 5+¦-dihydroprogesterone. Proc Nat Acad Sci 2014;111:3365-3370.
23. Ousey JC, Forhead AJ, Rossdale PD, et al: Ontogeny of uteroplacental progestagen production in pregnant mares during the second half of gestation. Biol Reprod 2003;69:540-548.
24. Ousey JC, Houghton E, Grainger L, et al: Progestagen profiles during the last trimester of gestation in Thoroughbred mares with normal or compromised pregnancies. Theriogenology 2004;63:1844-1856.
25. Morris S, Kelleman AA, Stawicki RJ, et al: Transrectal ultrasonography and plasma progestin profiles identifies feto-placental compromise in mares with experimentally induced placentitis. Theriogenology 2007;67:681-691.
26. Rossdale P, Ousey J, Cottrill C, et al: Effects of placental pathology on maternal plasma progestagen and mammary secretion calcium concentrations and on neonatal adrenocortical function in the horse. J Reprod Fertil 1990;Suppl44:579-590.
27. Wynn M, Legacki E, Conley A, et al: Determination of peripheral progestin concentrations in the late pregnant mare based upon immunoassays and liquid chromatography-tandem mass spectrometry. J Equine Vet Sci 2015;35:441-442.
28. Morris S, Kelleman AA, Stawicki RJ, et al: Transrectal ultrasonography and plasma progestin profiles identifies feto-placental compromise in mares with experimentally induced placentitis. Theriogenology 2007;67:681-691.
29. Evans TJ: Endocrine alterations associated with ergopeptine alkaloid exposure during equine pregnancy. Vet Clin North Am Equine Pract 2002;18:371-378.
30. Stewart DR, Stabenfeldt GH, Hughes JP, et al: Determination of the source of equine relaxin. Biol Reprod 1982;27:17-24.
31. Stewart DR, Kindahl H, Stabenfeldt GH, et al: Concentrations of 15-keto-13, 14-dihydroprostaglandin F2a in the mare during spontaneous and oxytocin induced foaling. Equine Vet J 1984;16:270-274.
32. Ryan PL, Christiansen DL, Hopper RM, et al: Evaluation of systemic relaxin blood profiles in horses as a means of assessing placental function in high risk pregnancies and responsiveness to therapeutic strategies. Ann N Y Acad Sci 2009;1160:169-178.
33. Stewart DR, Addiego LA, Pascoe DR, et al: Breed differences in circulating equine relaxin. Biol Reprod 1992;46:648.
34. Sorensen K, Neely DP, Read W, et al: Measurement and clinical significance of equine fetal protein in pregnant mares serum. J Equine Vet Sci. 1990;10:417-422.
35. Canisso IF, Ball BA, Scoggin KE, et al: Alpha-fetoprotein is present in the fetal fluids and is increased in plasma of mares with experimentally induced ascending placentitis. Anim Reprod Sci 2015;154:48-55.
36. Jelliffe-Pawlowski LL, Baer RJ, Currier RJ: Second trimester serum predictors of preterm birth in a population-based sample of low-risk pregnancies. Prenat Diagn 2010;30:727-733.
37. Yuan W, Chen L, López Bernal A: Is elevated maternal serum alpha-fetoprotein in the second trimester of pregnancy associated with increased preterm birth risk?: A systematic review and meta-analysis. Eur J Obstet Gynecol Reprod Biol 2009;145:57-64.
38. Cray C, Zaias J, Altman NH: Acute phase response in animals: a review. Comp Med 2009;59:517-526.
39. Coutinho da Silva MA, Canisso IF, Macpherson ML, et al: Serum amyloid A concentration in healthy periparturient mares and mares with ascending placentitis. Equine Vet J 2013;45:619-624..
40. Canisso IF, Ball BA, Cray C, et al: Serum amyloid A and haptoglobin concentrations are increased in plasma of mares with ascending placentitis in the absence of changes in peripheral leukocyte counts or fibrinogen concentration. Am J Reprod Immunol 2014;72:376-385.

41. Ladomery MR, Maddocks DG, Wilson ID: MicroRNAs: their discovery, biogenesis, function and potential use as biomarkers in non-invasive prenatal diagnostics. Int J Mol Epidemiol Genet 2011;2:253-260.
42. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-297.
43. Gantier MP, McCoy CE, Rusinova I, et al. Analysis of microRNA turnover in mammalian cells following Dicer1 ablation. Nucleic Acids Res 2011;39:5692-5703.
44. Sarker S, Scholz-Romero K, Perez A, et al: Placenta-derived exosomes continuously increase in maternal circulation over the first trimester of pregnancy. J Transl Med 2014;12:204.
45. Salido-Guadarrama I, Romero-Cordoba S, Peralta-Zaragoza O, et al: MicroRNAs transported by exosomes in body fluids as mediators of intercellular communication in cancer. Onco Targets Ther 2014;7:1327-1338.
46. Berindan-Neagoe I, Monroig PD, Pasculli B, et al: MicroRNAome genome: a treasure for cancer diagnosis and therapy. CA Cancer J Clin 2014;64:311-336.
47. Khazaie Y, Nasr Esfahani MH: MicroRNA and male infertility: a potential for diagnosis. Int J Fertil Steril 2014;8:113-118.
48. Santamaria X, Taylor H: MicroRNA and gynecological reproductive diseases. Fertil Steril 2014;101:1545-1551.
49. Chim SS, Shing TK, Hung EC, et al: Detection and characterization of placental microRNAs in maternal plasma. Clin Chem 2008;54:482-490.
50. Chiu RW, Lo YM: Pregnancy-associated microRNAs in maternal plasma: a channel for fetal-maternal communication? Clin Chem 2010;56:1656-1657.
51. Bidarimath M, Khalaj K, Wessels JM, et al: MicroRNAs, immune cells and pregnancy. Cell Mol Immunol 2014;11:538-547.
52. Su L, Liu R, Cheng W, et al: Expression patterns of microRNAs in porcine endometrium and their potential roles in embryo implantation and placentation. PloS one. 2014;9(2):e87867.
53. Dior UP, Kogan L, Chill HH, et al: Emerging roles of microRNA in the embryo-endometrium cross talk. Semin Reprod Med 2014;32:402-409.
54. Hung EC, Chiu RW, Lo YM: Detection of circulating fetal nucleic acids: a review of methods and applications. J Clin Pathol 2009;62:308-313.
55. Kotlabova K, Doucha J, Hromadnikova I: Placental-specific microRNA in maternal circulation--identification of appropriate pregnancy-associated microRNAs with diagnostic potential. J Reprod Immunol 2011;89:185-191.
56. Luo SS, Ishibashi O, Ishikawa G, et al: Human villous trophoblasts express and secrete placenta-specific microRNAs into maternal circulation via exosomes. Biol Reprod 2009;81:717-729.
57. Miura K, Miura S, Yamasaki K, et al. Identification of pregnancy-associated microRNAs in maternal plasma. Clin Chem 2010;56:1767-1771.
58. Miura S, Nozawa M, Nei M: Evolutionary changes of the target sites of two microRNAs encoded in the Hox gene cluster of Drosophila and other insect species. Genome Biol Evol 2011;3:129-139.
59. Montenegro D, Romero R, Kim SS, et al. Expression patterns of microRNAs in the chorioamniotic membranes: a role for microRNAs in human pregnancy and parturition. J Pathol 2009;217:113-121.
60. Morales Prieto DM, Markert UR: MicroRNAs in pregnancy. J Reprod Immunol 2011;88:106-111.
61. Mouillet JF, Ouyang Y, Bayer A, et al: The role of trophoblastic microRNAs in placental viral infection. Int J Dev Biol 2014;58:281-289.
62. Dominguez F, Moreno-Moya JM, Lozoya T, et al: Embryonic miRNA profiles of normal and ectopic pregnancies. PloS one 2014;9(7):e102185.
63. Jung YW, Jeon YJ, Rah H, et al: Genetic variants in microRNA machinery genes are associate with idiopathic recurrent pregnancy loss risk. PloS one 2014;9(4):e95803.
64. Wang D, Na Q, Song WW, et al: Altered expression of miR-518b and miR-519a in the placenta is associated with low fetal birth weight. Am J Perinatol 2014;31:729-734.
65. Jiang H, Wu W, Zhang M, et al: Aberrant upregulation of miR-21 in placental tissues of macrosomia. J Perinatol 2014;34:658-663.
66. Mayor-Lynn K, Toloubeydokhti T, Cruz AC, et al: Expression profile of microRNAs and mRNAs in human placentas from pregnancies complicated by preeclampsia and preterm labor. Reprod Sci 2011;18:46-56.
67. Choi SY, Yun J, Lee OJ, et al: MicroRNA expression profiles in placenta with severe preeclampsia using a PNA-based microarray. Placenta. 2013;34:799-804.
68. Ura B, Feriotto G, Monasta L, et al: Potential role of circulating microRNAs as early markers of preeclampsia. Taiwan J Obstet Gynecol. 2014;53:232-234.
69. Xu P, Zhao Y, Liu M, et al: Variations of microRNAs in human placentas and plasma from preeclamptic pregnancy. Hypertension 2014;63:1276-84.

Applications of anti-Müllerian hormone (AMH) in equine reproduction

Barry A. Ball
Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY

Introduction

Anti-Müllerian hormone (AMH) is best known for its role in inducing regression of the paramesonephric (Müllerian) ducts in the male fetus during differentiation of the tubular reproductive system.[1] Anti-Müllerian hormone is secreted by Sertoli cells in the developing testis and induces regression of the Müllerian ducts by acting through its receptor, AMHRII. After birth, the sexually dimorphic expression of AMH is lost, and AMH is also expressed in the granulosa cells of the ovarian follicle. Anti-Müllerian hormone is a disulfide-linked, homodimeric protein that is a member of the transforming growth factor-beta superfamily of hormones.

In the male after birth, there is a continued strong expression of AMH by the Sertoli cells which is thought to act at the level of the testis to reduce the expression of 17□-hydroxylase/17,20-lyase cytochrome P450 (P450c17), a key steroidogenic enzyme in the synthesis of androgens by Leydig cells. At puberty in the male, AMH secretion appears to shift from an endocrine pattern (with secretion into the blood) to an exocrine pattern with secretion into the seminiferous tubule.[2] Consequently, circulating concentrations of AMH in blood are reduced at puberty with a concomitant appearance of AMH in seminal fluid. The role of AMH in the postpubertal male remains unclear although decreased circulating concentrations of AMH have been associated with fertility of males in some species.[3] More studies are required to determine whether AMH is a potential marker for Sertoli cell number or function in the stallion.

In the female, AMH is expressed exclusively by the granulosa cells of growing preantral and small antral follicles up to a diameter corresponding to follicular size at the time of follicle selection. In the mare, AMH is produced principally by follicles up to approximately 20 mm in diameter. Beyond this size, AMH secretion declines sharply and is markedly reduced in preovulatory compared to smaller growing follicles. Anti-Müllerian hormone in the female appears to regulate the number of primordial follicles which enter the growing or dynamic pool of follicles and thereby regulates the number of antral follicles preset on the ovary which are available for recruitment by gonadotropins in subsequent follicular waves.

AMH in the stallion

As noted above, circulating concentrations of AMH are high in the prepubertal stallion and decrease after puberty although concentrations remain detectable in the mature stallion.[4] Concentrations of AMH vary with season with a peak AMH concentration occurring in late spring/summer and a nadir in AMH concentrations occurring in the fall and winter (Figure 1). This seasonal pattern of AMH secretion corresponds to circannual changes in gonadotropins and steroids in the stallion; however, the mechanisms regulating AMH secretion in the stallion remain uncertain. Downregulation of the hypothalamic-pituitary-gonadal axis with a GnRH-antagonist did not appear to alter AMH secretion in stallions (Davolli, unpublished) so the mechanism regulating circannual AMH secretion remains undefined.

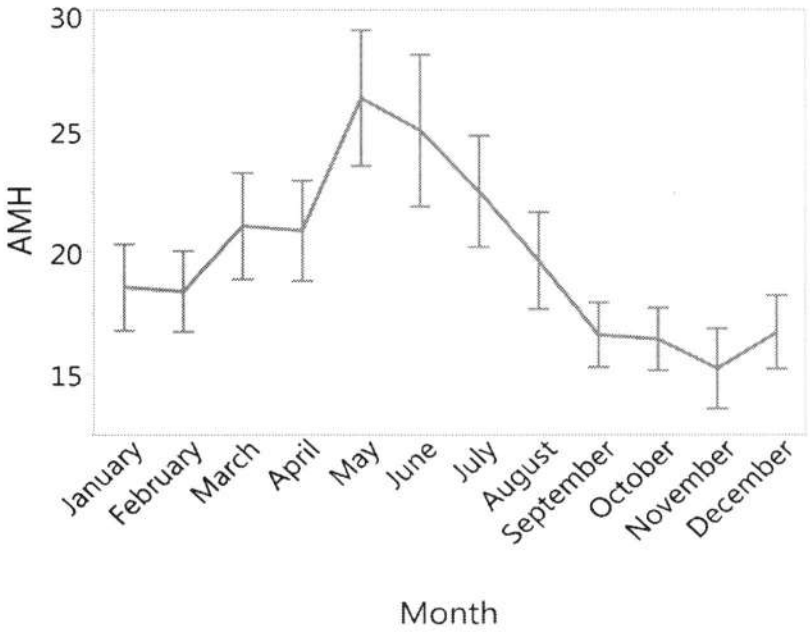


Figure 1. Seasonal changes in serum concentrations of AMH in stallions (n = 9). Data are mean ± SEM.

At puberty, formation of the blood-testis barrier appears to alter the secretion of AMH from an endocrine to an exocrine secretion with AMH concentrations declining in blood and AMH appearing in the seminal fluids.[5] In cryptorchid testes, formation of the blood-testis barrier is perturbed, and it appears that AMH secretion into the circulation continues with continued high expression of AMH by Sertoli cells of the cryptorchid testis. We hypothesized that this differences in cryptorchid testes would be associated with elevations in AMH concentrations in blood of stallions with a retained testis. Because AMH is produced exclusively by the Sertoli cells, peripheral AMH concentrations in geldings are low (< 0.1 ng/mL), and the continued secretion of AMH by the Sertoli cells in cryptorchid testes makes AMH a good marker for the detection of retained testes in suspected cryptorchids.[4] To this end, we examined AMH concentrations in known geldings (n = 41), intact stallions (n = 15) and cryptorchid stallions with one retained testis (n = 41).[4] Anti-Müllerian hormone concentrations were significantly higher in cryptorchids vs geldings (36.3 ± 6.4 ng/mL vs. 0.07± 0.01 ng/mL, respectively), and AMH concentrations were higher in cryptorchid stallions versus intact stallions with two descended testes. Because baseline concentrations of AMH in geldings are very low and cryptorchid stallions appear to have an elevated AMH, AMH determinations for diagnosis of cryptorchidism require only a single sample.[6] In a series of three stallions with unilaterally retained testes, baseline determinations for serum testosterone concentrations yielded equivocal results whereas a single determination of AMH concentrations was diagnostic in all three cases.[7] Age of the stallion remains as one caveat in the use of serum AMH determinations for diagnosis of retained testis in stallions. Although AMH was present in cryptorchid testes from an older (9-yr-old) stallion, the sample set initially evaluated from cryptorchid testes did not include very many older males. It is possible that in the older male, severe degeneration of Sertoli cells in the cryptorchid testis might lead to false negative results when using AMH as a biomarker. Further studies are required to determine the incidence of possible false negative results for older cryptorchid stallions.

In addition to cryptorchid testes, AMH expression has also been characterized in equine testis tumors as well as intersex gonads.[8] Sertoli cell tumors appeared to express AMH (based upon immunohistochemistry) whereas seminomas and teratomas did not show evidence of AMH expression based upon IHC. Data are lacking on changes in serum AMH concentrations in stallions with Sertoli cell tumors; however, a recent report in dogs identified a markedly elevated serum AMH concentration in animals with Sertoli cell tumors. Although uncommon in stallions, serum AMH concentrations might be a useful marker for Sertoli cell tumors as well.

AMH for diagnosis of Granulosa-cell tumors

As noted earlier, AMH appears to be secreted exclusively by the granulosa cell of ovarian follicles in females. Elevated concentrations of AMH have been reported in granulosa-cell tumors (GCT)

in women,[9] and we hypothesized that AMH would be a useful biomarker for GCTs in the mare.[10] Previously, endocrine diagnosis of equine GCTs was based upon combined assays for inhibin, testosterone and progesterone. Although this GCT panel was a useful aid for endocrine diagnosis of GCTs in the mare, there were several problems which limited its utility. In pregnant mares, both testosterone and inhibin may be elevated during late gestation, thereby confounding the interpretation of these analytes in pregnant mares. Furthermore, the diagnostic accuracy of these analytes was somewhat limited with approximately 87% of mares with GCTs reporting elevated inhibin, and 50% reporting elevated testosterone in mares with GCTs.

Based upon immunohistochemistry and quantitative PCR, AMH is strongly expressed in the granulosa-like cells in equine GCTs.[11] In a retrospective analysis of samples (n = 403) previously submitted to the Clinical Endocrinology Laboratory at UC Davis, we evaluated the utility of serum AMH concentrations for diagnosis of equine GCTs. These samples were stratified based upon serum inhibin concentrations (normal < 0.7 ng/mL) as well as serum testosterone concentrations (normal < 45 pg/mL) and were assayed for serum AMH concentrations using a human ELISA for AMH.[12] For this study, the diagnostic threshold for serum AMH concentrations was set at 4 ng/mL based upon previous studies in normal cycling and pregnant mares. Samples were further stratified based upon serum progesterone concentrations (≥1 ng/mL or < 1 ng/mL) to assess the relationship between serum AMH, inhibin and testosterone concentrations in mares with suspect GCTs. In mares with serum progesterone concentrations ≥1 ng/mL, serum inhibin or serum inhibin and testosterone yielded a significantly larger proportion of abnormally elevated values (inhibin > 0.7 ng/mL; testosterone > 45 pg/mL) than did serum AMH (> 4ng/mL). Although definitive case outcome (confirmed GCT) was not available for all of these mares, this finding suggests that in the presence of elevated progesterone (presumably either cycling or pregnant) inhibin or the combination of inhibin / testosterone were more likely to yield a false positive result than was AMH for the diagnosis of GCT in mares. Conversely, when serum progesterone concentrations were < 1 ng/mL (suggesting anestrus or estrus), serum AMH concentrations were elevated in a significantly greater proportion of mares than were inhibin or inhibin/testosterone. These data suggest that AMH was more likely to identify a GCT in mares with low serum progesterone concentrations. To examine the sensitivity of AMH for diagnosis of GCTs, a subset of samples in which the presence of a GCT had been definitively diagnosed (based upon histopathology; n = 44) were examined. In these samples 98% (43/44 mares) with a GCT had elevated AMH compared to 80% with elevated inhibin, 45.5% with elevated testosterone or 84.1% with elevations in inhibin or testosterone. Therefore, the presence of elevated AMH concentrations in this group of 44 mares had a significantly higher sensitivity for diagnosis of GCT in mares compared to inhibin, testosterone or the combination of inhibin and testosterone.

In seven mares with confirmed GCTs, multiple serum samples were submitted for determination of a GCT panel (Table). In four of seven mares, inhibin or testosterone concentrations were not diagnostic at the initial examination whereas serum AMH concentrations were > 4ng/mL. These observations suggest that AMH concentrations may rise earlier in serum than either inhibin or testosterone concentrations. Part of the difference between AMH and inhibin relative to early detection of GCTs may be related to the longer half-life of AMH (1.5-2 days)[13] compared to approximately one hour for inhibin.[10] The long half-life for AMH should be considered when resampling mares after ovariectomy to confirm a decline in serum AMH concentrations after removal of a GCT. Because of the earlier ability of serum AMH concentrations to diagnosis of GCTs in the mare, elevations of serum AMH concentrations have been identified in both pregnant mares as well as mares that were apparently still undergoing normal estrous cycles based upon elevations of serum progesterone > 1 ng/mL.[13]

Table: Serum anti-Müllerian hormone (AMH), inhibin and testosterone concentrations for mares with multiple sample submissions along with clinical diagnosis.

Case number	Sampling interval (days)*	AMH (ng/mL)	Inhibin (ng/mL)	Progesterone (ng/mL)	Testosterone (pg/mL)	Diagnosis
	350	**10.6**	0.19	14.7	21.6	
2		**64.4**	**2.55**	0.2	**51.2**	at surgery
	14	1.518	0.71	5.9	22.9	
		1.05	0.71	5.5	23.4	
	162					
4		1.684	0.79	13.9	20.3	at surgery
	266	**13.1**	0.74	0.2	**58.6**	
5		**531.2**	**0.90**	1.1	**161.5**	at surgery
	16	---	**0.92**	0.1	7.6	
6		**45.1**	**1.08**	0.3	16.7	by histopathology
	399	**145.6**	**2.33**	0.1	**105.9**	at surgery
7		0.424	0.32	19.2	13.7	
		11.2	0.55	0.1	13.3	
	119	**6.8**	0.22	0.1	11.7	
	210	---	0.41	0.2	27.9	
9	350	**14.1**	0.35	0.2	35.7	by histopathology
	41	**41.2**	0.62	0.5	**74.4**	
10		**40.5**	0.81	0.1	27.4	at surgery

Adapted from: Ball et al, Equine Vet J 2013;45:199-203. **Values in bold exceed the diagnostic threshold.**

Because the original study described for AMH determinations in mares with GCTs was conducted as a retrospective study and because the status of mares with normal endocrine parameters was unknown (i.e. we were unable to confirm that they were indeed reproductively normal mares, only that they had normal endocrine parameters), it was not possible to accurately estimate specificity of AMH for detection of GCTs in the mare. Subsequent clinical experience in the Clinical Endocrinology Laboratory at UC Davis (Dr. Al Conley, personal communication) suggests that in the absence of clinical signs of a GCT (ovarian enlargement, ultrasonographic appearance) that AMH concentrations between 4 to 10 ng/mL are considered a grey zone for endocrine diagnosis of GCTs in the mare. Anti-Müllerian hormone concentrations > 10 ng/mL are almost certainly associated with endocrine evidence of a developing or existing GCT.

AMH as a marker for follicular reserve, follicular function and fertility in the mare

In mammals, the number of primordial follicles present on the ovaries are thought to be fixed at or near the time of birth for most species studied. The number of oocytes present on the ovaries at birth appear hugely variable between individuals, and this variation is thought to contribute to the variation in reproductive longevity of females. In single ovulating species, the number of oocytes which actually undergo ovulation is relatively small, and most oocytes are lost through the process of atresia during repeated follicular waves throughout the reproductive lifespan of that individual. In women, follicular populations are eventually depleted at menopause. Domestic animals do not undergo menopause per se; however, in species such as the horse which may be maintained in the breeding herd until a relatively late age, there is a process of reproductive senescence which appears to be related to longer interovulatory intervals, increased peripheral FSH concentrations, longer follicular phases, reduced fertility and eventually cessation of estrous cycles.[14,15]

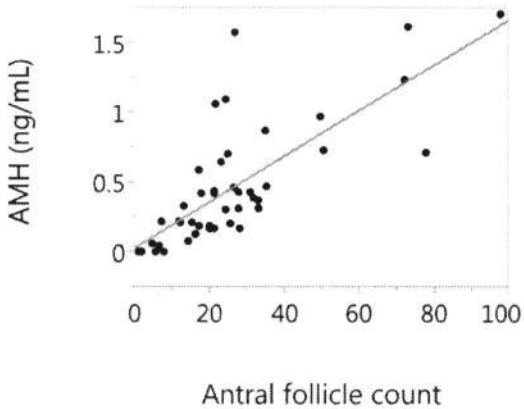


Figure 2. Relationship between total antral follicle count and serum AMH concentrations in a group of 40 mares.

Over the past several years, there has been a considerable interest in the use of AMH as a marker for ovarian reserve in several species including women and cattle. Concentrations of AMH are closely related to the total number of antral follicles present on the ovaries (antral follicle count - AFC). Antral follicle counts in turn are closely related to the number of primordial follicles remaining on the ovaries in species studied to date (women, cattle, mice).[16-18] Therefore, peripheral AMH concentrations appear to be a good marker for follicular reserve, and determinations of serum AMH has become widely used in women to predict their response to ovarian stimulation protocols as well as fertility outcomes with assisted reproduction techniques. [19,20] Likewise in cattle, a single determination of AMH appears adequate to measure follicular reserve, and AMH determinations are becoming more widely used to assess potential response to superovulation.[21-23]

In mares, serum concentrations of AMH do not appear to vary significantly during the estrous cycle but there is considerable inter-individual variation in AMH concentrations.[10] To examine the relationship between serum AMH concentrations, AFC and age in mares, we examined young (3–8 years; n = 10), middle-aged (9–18 years; n = 16) and old mares (19–27 years; n = 19) across three estrous cycles.[24] Total AFC was determined by mapping the ovary with transrectal ultrasonography, and serum AMH concentrations were measured across three estrous cycles. Overall, serum AMH and total AFC were highly related ($\rho = 0.77$, $P<0.0001$). Serum AMH and total AFC were lower in older mares, and AMH and AFC were strongly related ($\rho = 0.86$, $P<0.0001$) in older mares. Interestingly, there was only a moderate correlation between AMH and AFC in middle-aged mares ($\rho = 0.60$, $P<0.01$), and there was no significant correlation between AMH and AFC in the young mares. Older mares had longer interovulatory intervals that middle-aged or young mares which were characterized by a longer follicular phase. Within the old-mare group, mares with low AFC were more likely to have prolonged interovulatory intervals than were aged mares with high AFC suggesting that mares may undergo reproductive aging or senescence at different rates. Both AFC and AMH were highly repeatable within mares both within and across estrous cycles with correlations of >0.8 for serum AMH concentrations and >0.9 for AFC within mares across or within estrous cycles. Therefore, in mares reproductive age may be better related to follicular reserve than calendar age.

In an additional study, we examined molecular and endocrine differences in growing and preovulatory follicles in mares in relation to the AFC and serum AMH concentrations.[25] Mares with low AFC (and concordantly low serum AMH concentrations) had alterations in expression of a number of

genes in the granulosa cells of growing (15-20 mm) follicles when compared to mares with a high AFC (and high serum AMH concentrations). In particular, gene expression of AMH, the AMH receptor (AMHRII), estrogen receptors □ and □, and the FSH receptor were lower in mares with low AFC. Although data are not yet available for the mare, in cattle, it appears that females with low AFC have chronically elevated serum follicle stimulating hormone (FSH) concentrations which are thought to result in FSH-resistance in growing follicles with changes similar to those described in the mare.[23,26] This perturbation of normal follicular development in cattle with low AFC has been associated with an increased incidence of aneuploid oocytes, reduced serum progesterone concentrations and a reduced fertility in these females.[23,26,27]

In a retrospective field study, we examined the relationship between fertility outcome (pregnant vs nonpregnant at Days 13-18) serum AMH concentrations in a group of 419 Thoroughbred mares. Mares in the lowest AMH quartile had a greater chance of being open at the initial pregnancy check compared to mares in the middle or upper quartile of serum AMH concentrations. These data should be confirmed by a prospective field study to examine the relationship between serum AMH concentrations and fertility in mares.

Conclusions

Anti-Müllerian hormone is a versatile endocrine marker that is first produced by Sertoli cells of the fetal testis and acts to induce regression of the paramesonephric ducts. After birth, AMH is secreted by granulosa cells; the female homolog of Sertoli cells. In males, AMH appears to be a testis-specific marker and as such, can be used to detect the presence of undescended testes in suspect cryptorchid stallions. In females, AMH is produced by granulosa-cell tumors and appears to be a sensitive marker for the presence of these tumors in the mare. Furthermore, AMH in the mare is associated with antral follicle counts which are in turn related to follicular reserve. Because follicular reserve undergoes depletion at different ages in the mare (likely owing to differences in the number of primordial follicles present on the ovary at birth and the subsequent depletion of these reserves), it appears that AMH and AFC may be a better predictor of reproductive longevity in mares than their calendar age.

References

1. Josso N, Picard JY: Anti-Mullerian hormone. Physiol Rev 1986;66:1038-1090.
2. Rey R, Lukas-Croisier C, Lasala C, et al: AMH/MIS: what we know already about the gene, the protein and its regulation. Mol Cell Endocrinol 2003;211:21-31.
3. Goulis DG, Iliadou PK, Tsametis C, et al: Serum anti-Mullerian hormone levels differentiate control from subfertile men but not men with different causes of subfertility. Gynecol Endocrinol 2008;24:158-160.
4. Claes A, Ball BA, Almeida J, et al: Serum anti-Mullerian hormone concentrations in stallions: developmental changes, seasonal variation, and differences between intact stallions, cryptorchid stallions, and geldings. Theriogenology 2013;79:1229-1235.
5. Almeida J, Conley AJ, Ball BA: Expression of anti-Mullerian hormone, CDKN1B, connexin 43, androgen receptor and steroidogenic enzymes in the equine cryptorchid testis. Equine Vet J 2013;45:538-545.
6. Murase H, Saito S, Amaya T, et al: Anti-Müllerian hormone as an indicator of hemi-castrated unilateral cryptorchid horses. J Equine Sci 2015;26:15.
7. Claes A, Ball BA, Corbin CJ, et al: Anti-Mullerian hormone as a diagnostic marker for equine cryptorchidism in three cases with equivocal testosterone concentrations. J Equine Vet Sci 2014;34:442-445.
8. Ball BA, Conley AJ, Grundy SA, et al: Expression of anti-Müllerian hormone (AMH) in the equine testis. Theriogenology 2008;69:624-631.
9. Rey RA, Lhomme C, Marcillac I, et al: Antimullerian hormone as a serum marker of granulosa cell tumors of the ovary: Comparative study with serum [alpha]-inhibin and estradiol. Am J Obstet Gynecol 1996;174:958-965.
10. Almeida J, Ball BA, Conley AJ, et al: Biological and clinical significance of anti-Mullerian hormone determination in blood serum of the mare. Theriogenology 2011;76:1393-1403.
11. Ball BA, Conley AJ, Grundy SA, et al: Expression of anti-Müllerian hormone (AMH) in equine granulosa-cell tumors and in normal equine ovaries. Theriogenology 2008;70:968-977.
12. Ball BA, Almeida J, Conley AJ: Determination of serum anti-Müllerian hormone concentrations for the diagnosis of granulosa-cell tumors in mares. Equine Vet J 2013;45:199-203.
13. Ball BA, Conley AJ, Almeida J, et al: A retrospective analysis of 2,253 cases submitted for endocrine diagnosis of possible granulosa cell tumors in mares. J Equine Vet Sci 2014;34:307-313.

14. Carnevale EM, Bergfelt DR, Ginther OJ: Aging effects on follicular activity and concentrations of FSH,LH, and progesterone in mares. Anim Reprod Sci 1993;31:287-299.
15. Carnevale EM, Bergfelt DR, Ginther OJ: Follicular activity and concentrations of FSH and LH associated with senescence in mares. Anim Reprod Sci. 1994;35:231-246.
16. Kevenaar ME, Meerasahib MF, Kramer P, et al: Serum anti-Mullerian hormone levels reflect the size of the primordial follicle pool in mice. Endocrinology 2006;147:3228-3234.
17. Ireland JJ, Smith GW, Scheetz D, et al: Does size matter in females? An overview of the impact of the high variation in the ovarian reserve on ovarian function and fertility, utility of anti-M++llerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle. Reprod Fertil Dev 2010;23:1-14.
18. Hansen KR, Hodnett GM, Knowlton N, et al: Correlation of ovarian reserve tests with histologically determined primordial follicle number. Fertil Steril 2011;95:170-175.
19. La MA, Sighinolfi G, Radi D, et al: Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). Hum Reprod Update 2010;16:113-130.
20. Nardo LG, Gelbaya TA, Wilkinson H, et al: Circulating basal anti-Müllerian hormone levels as predictor of ovarian response in women undergoing ovarian stimulation for in vitro fertilization. Fertil Steril 2009;92:1586-1593.
21. Vernunft A, Schwerhoff M, Viergutz T, et al: Anti-Muellerian hormone levels in plasma of Holstein-Friesian heifers as a predictive parameter for ovum pick-up and embryo production outcomes. J Reprod Dev 2015;61:74.
22. Ribeiro E, Bisinotto R, Lima F, et al: Plasma anti-Müllerian hormone in adult dairy cows and associations with fertility. J Dairy Sci 2014;97:6888-6900.
23. Mossa F, Walsh SW, Butler ST, et al: Low numbers of ovarian follicles >/=3mm in diameter are associated with low fertility in dairy cows. J Dairy Sci 2012;95:2355-2361.
24. Claes A, Ball BA, Scoggin KE, et al: The interrelationship between antral follicle count, anti-Mullerian hormone concentration, and age in mares. J Equine Vet Sci 2014;34:110.
25. Claes A, Ball B, Troedsson M, et al: Molecular changes in the equine follicle in relation to variations in antral follicle count and anti-Müllerian hormone concentrations. Equine Vet J 2015. Sep 25. doi: 10.111/evj. 12514.
26. Jimenez-Krassel F, Folger JK, Ireland JL, et al: Evidence that high variation in ovarian reserves of healthy young adults has a negative impact on the corpus luteum and endometrium during estrous cycles in cattle. Biol Reprod 2009;80:1272-1281.
27. Luciano AM, Franciosi F, Lodde V, et al: Oocytes isolated from dairy cows with reduced ovarian reserve have a high frequency of aneuploidy and alterations in the localization of progesterone receptor membrane component 1 and aurora kinase B. Biol Reprod 2013;88:58.

Equine oocyte recovery and ICSI in clinical practice: what can I expect?

Katrin Hinrichs
College of Veterinary Medicine and Biomedical Sciences, Texas A&M University,
College Station, TX

Abstract

Embryo production by ICSI is becoming a common clinical procedure in the horse, but little information is available on the expected outcomes of this procedure. Our clinical ICSI program at Texas A&M University sees about 450 cases per year (case = all the oocytes recovered from the ovaries of a mare on one aspiration session), both of oocytes recovered from immature follicles and those recovered from the dominant stimulated follicle. Over half of these cases are of oocytes shipped to the laboratory by referring veterinarians. The average number of oocytes recovered per aspiration of immature follicles at Texas A&M University is seven; after in vitro maturation this yields 4.5 mature oocytes per aspiration. The blastocyst rate achieved is 23% per injected mature oocyte, or about one blastocyst per aspiration session. Approximately half of these blastocysts produce ongoing pregnancies after transfer, for about one foal produced for every two aspiration sessions performed. These averages reflect only the findings of our laboratory; other laboratories may have different results.

Keywords: Embryo, equine, in vitro embryo production, intracytoplasmic sperm injection

Introduction

The first report on commercial equine use of intracytoplasmic sperm injection (ICSI) for foal production was presented by Galli and coworkers in 2007.[1] They reported a 58% oocyte recovery rate on transvaginal ultrasound-guided aspiration (TVA) of immature follicles, providing an average of 10 immature oocytes per aspiration, of which 6.6 matured after in vitro culture. The blastocyst rate was 12% per injected oocyte, providing 0.85 blastocysts per aspiration, with a 55% ongoing pregnancy/foaling rate per blastocyst transferred. We presented a brief report on the findings from our clinical ICSI service for in vitro embryo production after aspiration of immature follicles for February through May of 2013.[2] During this period, we performed 103 aspiration sessions and produced 119 blastocysts, a 20% blastocyst production per injected oocyte.[2] Fresh transfer of 101 embryos yielded 82 pregnancies (82%). The final number of foals produced from these 82 pregnancies was 60, for a foaling rate per embryo transferred of 59%. Galli and coworkers summarized more recent results from their laboratory in a review paper in 2014,[3] in which they reported a 66 to 70% oocyte recovery rate from immature follicles, providing nine to 12 oocytes per aspiration, depending upon mare breed. The blastocyst rate was 2% to 8% per injected oocyte, again depending upon breed, providing 0.3 to 0.8 blastocysts per aspiration. To the best of our knowledge, these are the only reports available on the expected efficiency of ICSI as a clinical method for foal production.

Currently, there is increasing interest in recovery of oocytes by referring practitioners for shipment to ICSI laboratories, and increasing awareness of ICSI on the part of horse breeders. Our laboratory at Texas A&M University started research on in vitro embryo production via ICSI in 2000, and has been offering ICSI clinically since 2009. We saw < 60 cases per year from 2009 to 2012. In 2013, the owner of a popular American Quarter Horse sire which had aged to the point of subfertility announced that the stallion was available for breeding only via ICSI with frozen semen. This had a notable effect on our ICSI caseload; it went from 63 cases in 2012 to 176 cases in 2013. In 2014, two more owners of popular aged stallions followed suit; our program increased in participation to over 450 cases. We have limited the caseload to this level subsequently. We define "case" as all the oocytes recovered from a given mare at one aspiration; for the purpose of this summary, if both immature and mature oocytes were recovered at the same time, i.e., subordinate follicles were aspirated at the time of aspiration of a dominant stimulated follicle (DSF), then this is counted as two cases, an immature oocyte case and a DSF case.

Here we discuss, based on our laboratory findings over the last three years, the basic parameters that can be expected by the referring practitioner in offering embryo production via ICSI to their client. These parameters are applicable only to our laboratory, as other laboratories may have different results.

Oocyte recovery

Recovery from the dominant stimulated follicle

Aspiration of the oocyte from the dominant stimulated follicle (DSF) immediately before ovulation is fairly easy to learn, so is a good place to start for the practitioner wanting to apply oocyte aspiration in their practice. Due to the large size of the follicle and the fact that it is being aspirated after a gonadotropin stimulus (thus the oocyte-cumulus complex is expanding and loosening from the follicle wall), the procedure is relatively simple and the recovery rate is high. Oocyte recovery from the DSF was first described by Vogelsang et al., in 1983; they used a needle placed through the flank.[4] Aspiration of the DSF via transvaginal ultrasound-guided aspiration (TVA) was first described by Brück et al., in 1992.[5] Subsequently, aspiration of the maturing oocyte from the DSF has been performed, both via the flank puncture and via TVA, for many purposes including study of oocyte maturation,[6,7] study of follicle-oocyte interactions,[8] recovery of oocytes for oocyte transfer,[9,10] and recovery of oocytes for ICSI.[11]

Detailed methods for oocyte recovery from the DSF, by both flank approach and TVA, have been presented previously.[12] Whether done by flank approach or by TVA, the oocyte recovery rate from DSF should be 70% or better. Because the oocyte is maturing (is in the process of meiosis) at the time of recovery, care should be taken to keep the oocyte at body temperature (37 to 38.2°C) during handling and transport to the ICSI laboratory. Detailed methods for handling and packaging of DSF oocytes for shipment have been presented previously.[12]

Briefly, to recover oocytes from the DSF, mares are monitored for follicle development during estrus, and an ovulatory stimulus (human chorionic gonadotropin [hCG] or a gonadotropin releasing hormone [GnRH] analog such as deslorelin) is administered when the operator thinks that the follicle will respond to it. The stimulus must be given before the mare's endogenous luteinizing hormone (LH) signal has been received by the follicle, so that the stage of oocyte development at the time of aspiration can be timed definitively from the time of administration of the stimulus. Typically the DSF is aspirated 24 to 35 h after the ovulatory stimulus was administered. The oocyte will be cultured in vitro until the anticipated time that the donor would have ovulated, i.e. until about 40 h after administration of the ovulatory stimulus, and then fertilized.

However, because of the need for a) monitoring of estrus and follicle activity in the mare; b) timing of gonadotropin stimulation in relation to follicle maturity; c) timing of aspiration at a set point after administration of gonadotropin stimulation; d) special handling of the maturing oocyte; e) inability to schedule these procedures beforehand, due to variations in follicle growth, and f) the fact that aspiration of the DSF recovers at most only one oocyte, our laboratory rarely performs aspiration of the DSF. Aspiration of immature oocytes from non-preovulatory (immature) follicles allows exact scheduling of aspiration times, increases the number of oocytes available and thus the blastocysts produced per aspiration session, and simplifies oocyte handling.

Recovery from immature follicles

Aspiration of oocytes from immature follicles in the mare, via TVA, was first described by Cook et al. in 1992.[13] Use of TVA is essentially required when immature follicles are aspirated; aspiration through the flank, which is done based on transrectal palpation, can be used to attempt to aspirate larger immature follicles (>20 mm) but visualization via ultrasound is necessary to locate and puncture smaller follicles. Typically, on TVA of immature follicles, all follicles ≥ 5 mm in diameter are aspirated.

Detailed methods for performing TVA of immature follicles has been presented previously.[12] Because TVA involves placing a needle through the vaginal wall into the ovary numerous times per aspiration session, we evaluated the effect of this procedure on mare health in mares in our research herd over a three year period.[14] Over almost 400 aspiration procedures and > 3,000 follicle punctures,

performed on all immature follicles on the ovary once every 14 days throughout the breeding season and without administration of antibiotics, only one complication, an ovarian abscess, was noted. Evaluation of ovaries on laparoscopy and after removal via ovariectomy showed minimal gross or histological changes. It should be noted, however, that ovarian abscess,[14,15] rectal tear, peritonitis and even death due to hemorrage[16] remain potential complications of performing TVA.

The most important facet of TVA of immature follicles that the practitioner should take note of is that there is a very long learning curve, during which the procedure may be both frustrating and unproductive. At first, simply holding the ovary and probe in such a way as to puncture a follicle can be difficult, even for an experienced equine reproduction practitioner. We find that practitioners may attempt to perform TVA in their practice several times, and after finding it not possible to do effectively, abandon it. However, in our laboratory, we have found that essentially every veterinarian, given enough experience, can learn this technique. Since the closure of all equine slaughterhouses in the US in 2007, the source of oocytes for our research has been TVA in our herd of research mares. Thus we perform TVA of immature follicles, typically on three to five mares per day, one or two days per week, to obtain oocytes for our studies. All veterinarians in the laboratory participate in TVA, including those employed by the laboratory, graduate students, and visiting scholars. Given this schedule, and that for laboratory members there is no option not to perform TVA, we have found that after a variable period (typically 10 to 20 TVA attempts) all operators learn to perform the technique effectively.

In performing TVA of immature follicles, each follicle punctured should be recorded so that the operator can evaluate the rate of oocyte recovery per aspirated follicle. The operator should attempt to aspirate all follicles $\geq$ 5 mm diameter. This should lead to an average about 12-14 follicles aspirated per mare for Quarter-type mares; other breeds may have a higher average follicle number. Young mares may have more than this number of follicles, and older mares fewer. The operator should strive for an average recovery rate over a series of mares of 50% or greater. The recovery rate depends greatly on the technique of both the operator manipulating the ovary and the operator manipulating the needle. For each follicle we puncture, we aspirate the fluid, then rotate the needle to knock granulosa cells (and hopefully the cumulus-oocyte complex) free of the follicle wall, while moving the ovary so that the needle contacts different aspects of the follicle. We then fill the follicle with flush medium to suspend these cells, aspirate the fluid out, and repeat, for a total of six flushes. We also record the size of each follicle punctured. The recovery rate from smaller follicles is higher than that from larger follicles,[17] probably because in the larger follicles the surface area is greater, decreasing the likelihood of knocking the cumulus-oocyte complex free with the needle.

The recovered immature oocytes are sent to the ICSI laboratory, where they will be subjected to in vitro maturation. Oocytes that mature (progress to metaphase II) will undergo ICSI. Detailed methods for handling, packaging and shipping immature oocytes have been presented previously.[12] The most important thing for the practitioner to note is that oocytes are exquisitely sensitive to toxins; these can include volatile organic compounds in the air, fly spray, lingering antiseptic from washing the perineum, or deodorant or perfume from handling dishes or medium in a non-sterile manner. All media and supplies used for oocyte collection and shipment should be embryo-quality (tested to be non-toxic to embryos). Before sending client oocytes to ICSI laboratories for fertilization and embryo development, it is strongly recommended that the referring veterinarian send "practice" oocytes, recovered from non-valuable mares such as embryo recipient mares, to determine if the oocyte developmental competence has been maintained during handling, as evidenced by successful blastocyst production after ICSI with a control stallion.

Blastocyst production by ICSI

Immature oocytes

When immature oocytes are recovered, they are matured in vitro in the ICSI laboratory. About 65% of oocytes, on average, will mature to metaphase II and go on to ICSI. In about 9% of cases with

which we deal, no oocytes mature to undergo ICSI; this can be due to a low number of oocytes recovered, or to a low maturation rate for that lot of oocytes.

The results from ICSI with oocytes recovered from aspiration of immature follicles are variable. Overall, 20-25% of mature oocytes that undergo ICSI will produce blastocysts. However, the variability in blastocyst production is reflected in our finding that no blastocysts are produced in about 40% of cases. The proportion of cases resulting in one, two, three to five, and six or more blastocysts per case are approximately 30%, 15%, 13%, and 2%, respectively. The most blastocysts we have produced from one aspirate was 10 (from 15 recovered oocytes). Thus, the AVERAGE blastocyst production is 1.1 blastocysts per case, but the most likely outcome for any given mare is to produce no blastocyst. Currently there is little information about why this variation exists, for example it is not known whether aspiration of follicles at a certain time of the cycle, or a certain time in relationship to follicle wave growth, provides oocytes with greater developmental competence. We have found no difference in blastocyst development between oocytes recovered at the same time as is a DSF (thus, the immature follicles are at the decline of their follicular wave, and oocytes are recovered from them after hCG or deslorelin administration) and those aspirated during the luteal phase.

Oocytes from the dominant stimulated follicle

Since only one oocyte is available for recovery from the DSF, blastocyst production is limited to "yes" or "no." The DSF oocyte has a higher intrinsic developmental competence, because it is the oocyte naturally selected to ovulate, and has undergone the majority of its meiotic progression in vivo. This is in contrast to immature oocytes, which may be from growing or atretic follicles at any stage of their development.

Theoretically, the DSF oocyte should give us the same blastocyst rate in vitro as we see with it after oocyte transfer, that is, surgical transfer of the mature oocyte to the oviduct of an inseminated recipient mare. This is not the case, however: Pregnancy rates after OT when research mares and stallions are used are typically $\geq$ 75%,[9, 10] whereas the blastocyst rate after ICSI and in vitro culture of DSF oocytes in our laboratory, even for research animals, is only ~40%.[18]

In our clinical program, the blastocyst rate for DSF oocytes is higher than that for in vitro-matured oocytes (38% vs. 23%); however since only one DSF oocyte may be recovered per aspiration, and is recovered only about 80% of the time, the blastocyst rate per aspiration is much lower than is the blastocyst rate after recovery of immature oocytes (estimated 0.8 x 0.38 blastocysts/oocyte = 0.3 blastocysts per DSF aspiration, vs. 1.1 blastocysts per aspiration of all immature follicles).

Mare and stallion effects

Blastocyst production varies by both mare and stallion used. Colleoni et al. reported no difference in blastocyst rate after ICSI for eight mares with reproductive disorders compared to 22 normal mares.[1] Unfortunately, we do not have good histories to determine which mares are presented for subfertility and which are presented because of stallion semen availability, especially in oocytes shipped to us by practitioners.

While both practitioners and ICSI operators are quick to point to mare age as a possible reason for low efficiency, the only significant finding we have had in relation to advancing mare age is a decrease in the number of follicles present on the ovaries at the time of aspiration.[2] However, this can significantly affect results, because when few follicles are present, few oocytes are recovered, few mature oocytes are available and thus few blastocysts are produced.

We sometimes find low cleavage and blastocyst rates for sperm from a given stallion.[19] This may not be related to the field fertility of that stallion or of that frozen semen; low cleavage rates could be due to either inadequate sperm quality, or, theoretically, to sperm having robust membranes that resist dissolution after ICSI. We have found that the method used for sperm preparation can significantly affect embryo development after ICSI in stallions with low embryo production.[19]

In stallions that have "aged out" and for whom only frozen semen is available, we recommend using semen that was frozen as early as possible in the stallion's reproductive life. In humans, it has been

shown that paternal age can affect both the ability of sperm to produce embryos in vitro, and the health of the offspring produced, both in vitro and by natural conception.[20]

Results from shipped oocytes

Currently, over half of the cases in our clinical program originate from oocytes shipped to us from referring veterinarians. These include both oocytes recovered from the DSF and oocytes recovered from immature follicles. Recovery and shipment of oocytes from referring veterinarians has produced results equal to those for in-house aspiration. The maturation rate of shipped and in house-recovered immature oocytes is similar at 60-65%, and the blastocyst rate achieved in our program with shipped immature oocytes is similar to that for immature oocytes recovered in-house (20 to 25% per injected mature oocyte). Results with recovery and shipment of oocytes from DSF by referring veterinarians (36 to 48% blastocysts per injected oocyte) have also been equivalent to what we obtain with in-house DSF aspirations (41%).

Pregnancy and pregnancy loss

In our program, all blastocysts produced are shipped for transfer, or are vitrified for later transfer, as we do not have a recipient mare herd. We recommend that in vitro-produced blastocysts be treated as for Day-6 in vivo embryos; that is, transferred to recipients that have ovulated 4 to 6 days previously. This is true no matter what day of culture the in vitro-produced blastocyst is seen to develop.

In our program, we find that in vitro-produced blastocysts have a good initial pregnancy rate (over 70%) but that about 25% of these pregnancies are lost, most before a heartbeat is recognized. This loss rate appears to be intrinsic to the in vitro system as the rates are almost exactly the same between oocytes collected from immature follicles and those collected from DSF, which should have optimal viability. A major current focus of our laboratory is to modify the in vitro system to minimize this pregnancy loss.

Conclusion

Overall, given a blastocyst rate of about 1 blastocyst per immature follicle aspiration procedure and an ongoing pregnancy/foaling rate of about 50% per transferred blastocyst, the clinical ICSI program at Texas A&M produces on average one foal for every two immature-follicle aspiration procedures. This is approximately the same efficiency as the per-cycle pregnancy rate for embryo transfer in normally-fertile mares; however, the potential for injury to the donor mare, the labor involved, and the cost are much greater for aspiration and ICSI than for direct ET. For this reason, ICSI should not be considered as a method to produce more foals from a normal mare in a given year. Intracytoplasmic sperm injection is an effective method to produce foals from mares that cannot produce embryos for transfer, or from stallions with limited semen stores.

The averages presented here are applicable to our laboratory only; other laboratories may have very different findings. Equine oocyte maturation, ICSI and embryo culture are complex systems that take both equipment and expertise to address effectively. Laboratories seeking to develop equine ICSI programs may find it difficult to establish a effective system, even when utilizing embryologists experienced with in vitro embryo production in other species.[21] When using a laboratory for clinical ICSI, the referring veterinarian should first gain information on the number of cases the laboratory has done, the blastocyst rate per injected oocyte, and the ongoing pregnancy rate per transferred embryo, to determine if the laboratory is efficient enough to justify the expense to the client.

References

1. Colleoni S, Barbacini S, Necci D, et al. Application of ovum pick-up, intracytoplasmic sperm injection and embryo culture in equine practice. Proc Am Assoc Equine Pract 2007. p. 554-559.
2. Hinrichs K, Choi YH, Love CC, et al.: Use of intracytoplasmic sperm injection and in vitro culture to the blastocyst stage in a commercial equine assisted reproduction program. J Equine Vet Sci 2014;34:176.
3. Galli C, Duchi R, Colleoni S, et al: Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice. Theriogenology 2014;81:138-151.

4. Vogelsang MM, Keider JL, Potter GD: Recovery of pre-ovulatory equine oocytes by follicular aspiration. Proc 8th Equine Nutrition and Physiology Symposium; 1983. p. 285-288.
5. Brück I, Raun K, Synnestvedt B, et al: Follicle aspiration in the mare using a transvaginal ultrasound-guided technique (short communication). Equine Vet J 1992;24:58-59.
6. Bézard J, Magistrini M, Duchamp G, et al: Chronology of equine fertilisation and embryonic development in vivo and in vitro. Equine Vet J 1989;Suppl8:105-110.
7. King WA, Bézard J, Bousquet D, et al: The meiotic stage of preovulatory oocytes in mares. Genome 1987;29:679-682.
8. Hinrichs K, Rand WM, Palmer E: Effect of aspiration of the preovulatory follicle on luteinization, corpus luteum function, and peripheral plasma gonadotropin concentrations in the mare. Biol Reprod 1991;44:292-298.
9. Carnevale EM, Ginther OJ: Defective oocytes as a cause of subfertility in old mares. Biol Reprod 1995;Monograph 1:209-214.
10. Hinrichs K, Matthews GL, Freeman DA, et al: Oocyte transfer in mares. J Am Vet Med Assoc 1998;212:982-986.
11. Altermatt JL, Suh TK, Stokes JE, et al: Effects of age and equine follicle-stimulating hormone (eFSH) on collection and viability of equine oocytes assessed by morphology and developmental competency after intracytoplasmic sperm injection (ICSI). Reprod Fertil Dev 2009;21:615-623.
12. Hinrichs K, editor: Application of assisted reproductive technologies (ART) to clinical practice. Proc Am Assoc Equine Pract; 2010.
13. Cook NL, Squires EL, Ray BS, et al: Transvaginal ultrasonically guided follicular aspiration of equine oocytes: preliminary results. J Equine Vet Sci 1992;12:204-207.
14. Velez IC, Arnold C, Jacobson CC, et al: Effects of repeated transvaginal aspiration of immature follicles on mare health and ovarian status. Equine Vet J 2012;Suppl43:78-83.
15. Bøgh IB, Brink P, Jensen HE, et al: Ovarian function and morphology in the mare after multiple follicular punctures. Equine Vet J 2003;35:575-579.
16. Vanderwall DK, Woods GL: Severe internal hemorrhage resulting from transvaginal ultrasound-guided follicle aspiration in a mare. J Equine Vet Sci 2002;22:84-86.
17. Cook NL, Squires EL, Ray BS, et al: Transvaginal ultrasound-guided follicular aspiration of equine oocytes. Equine Vet J 1993;Suppl15:71-74.
18. Jacobson CC, Choi YH, Hayden SS, et al: Recovery of mare oocytes on a fixed biweekly schedule, and resulting blastocyst formation after intracytoplasmic sperm injection. Theriogenology 2010;73:1116-1126.
19. Choi YH, Velez IC, Macias-Garcia B, et al: Effect of clinically-related factors on in vitro blastocyst development after equine ICSI. Theriogenology 2016;85:1289-1296.
20. Sharma R, Agarwal A, Rohra VK, et al.: Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. Reprod Biol Endocrinol 2015;13:1-20.
21. Lewis N, Hinrichs K, Schnaufer M, et al: Effect of oocyte source and transport time on rates of equine oocyte maturaiton and cleavage after fertilization by ICSI, with a note on the validation of equine embryo morphological classification Clin Therio 2016;8:25-39.

Update on equine embryo biopsy and vitrification

Katrin Hinrichs
College of Veterinary Medicine and Biomedical Sciences, Texas A&M University,
College Station, TX

Abstract

Methods to successfully biopsy and vitrify equine embryos have been under development for many years. Both techniques have been generally successful in small embryos (morulae and early blastocysts <300 μm diameter). However, both biopsy and vitrification were initially unsuccessful in large embryos (expanded blastocysts > 300 μm). Successful biopsy of expanded equine blastocysts via micropipette, with subsequent normal pregnancy rates, was first reported in 2010. Since then, several other laboratories have reported similar normal pregnancy rates after biopsy via micromanipulation. Attention must be paid to obtain efficient genetic diagnosis from embryo biopsy samples, due to the small number of cells obtained. Direct PCR may be performed if only one gene is being evaluated, such as for embryo sexing, but the methods so far reported have variable efficiency and accuracy. Preliminary findings suggest that it may be possible to perform some genetic testing using only the blastocoele fluid. Whole genome amplification is effective for subsequent multiplex PCR of a variety of genes, and can be highly accurate (> 97%), but results depend upon the method used for amplification. Methods for effective vitrification of small embryos were established in 2005 and are currently commercially available. Successful vitrification of expanded equine blastocysts was reported in 2011; this was dependent upon micromanipulation to puncture the embryo and remove the majority of blastocoele fluid. This method of blastocoele collapse appears to be robust as a method to prepare blastocysts for cryopreservation, as it has been reported to be successful by other laboratories using different cyroprotectant protocols and both open and closed vitrification loading devices. A method for blastocoele collapse that avoids the need for micromanipulation equipment is needed, to allow blastocyst cryopreservation to become widespread in practice.

Keywords: Equine, embryo, preimplantation genetic testing, vitrification

Introduction

The last 15 years have seen a great increase in interest in assisted reproduction techniques (ART) in horses. Efficient in vitro production (IVP) of equine blastocysts via intracytoplasmic sperm injection (ICSI) and in vitro embryo culture was reported in 2005.[1] Galli and coworkers presented their findings on use of ICSI/IVP for clinical embryo production in 2007.[2] Since that time, several laboratories have begun using micromanipulation methods to not only perform ICSI but also to attempt other embryo procedures, leading to an expanding base of knowledge regarding handling and manipulation of the equine embryo. This was an important step in development of methods for both biopsy and cryopreservation of expanded equine blastocysts.

The early development of methods for equine embryo biopsy and cryopreservation were performed using in vivo-recovered embryos, as IVP of equine embryos was not yet established. Progress in commercial application of both methods was hindered by two aspects of equine embryo physiology: the rapid expansion of the blastocyst after entry of the embryo into the uterus, and the presence of the embryonic capsule. Early studies showed that if small equine embryos (< 300 μm diameter) were recovered, they could be biopsied or vitrified fairly efficiently, using methods developed for cattle embryos,[3-6] however, use of these methods on large equine embryos (expanded blastocysts > 300 μm) was problematic.[3,6,7]

It is possible to effectively recover small embryos from the uterus. Late morulae and small early blastocysts may be obtained by flushing the uterus on Day 6 after ovulation, and timing of embryo flush after administration of an ovulation stimulus yielded a good embryo recovery rate at Day 6.5.[6] Mechanistically, however, searching for this small embryo takes more time, and in practice, equine embryo recovery on Day 6 has been associated with lower embryo recovery rates than on Days 7 or 8.[8,9]

While the decreased recovery rate on Day 6 has led some to state that the equine embryo is not in the uterus at Day 6,[8] a study in which the entire tract was removed and both the oviducts and the uterus were flushed[10] determined that embryos started entering the uterus at 5 d 10 h after ovulation and that after 5 d 22 h, all but one of the subsequent 14 embryos were in the uterus. It is possible that flush on Day 6 tended to yield a lower embryo recovery rate, especially when embryo collection was done by sedimentation,[8] because of differences in buoyancy between morulae and blastocysts[11] or the possibility of missing small embryos when searching. Nevertheless, the problems with embryo recovery on Day 6 provided stimulus for establishing methods for biopsy and vitrification of Day 7 and 8 embryos, that is, larger, expanded blastocysts, so that these methods could be used with embryos recovered under standard clinical embryo recovery conditions. Beginning in 2010, methods were developed for successful biopsy for preimplantation genetic testing and, in 2011, cryopreservation, of equine expanded blastocysts. Further research has expanded on the efficiency and clinical utility of these methods.

Embryo biopsy

Embryo biopsy in other species

Work with equine embryo biopsy for preimplantation genetic testing has benefitted from the fact that this technique is already being used extensively in humans and in cattle. One of the applications of human ART (in vitro embryo production) is to allow preimplantation genetic testing of embryos for couples that carry mutations for a given disease.[12] In addition, human IVP embryos--and probably human in vivo-produced embryos as well--have a high rate of aneuploidy, so some human ART programs screen all embryos produced for chromosomal abnormalities (deletion or duplication of a chromosome or segment of a chromosome).[13] These programs are continually striving to develop better methods for determining embryo status, to select the best embryo for transfer. In the horse, similar genetic screening would seem to have limited application. There are no data suggesting a high rate of aneuploidy in equine in-vivo produced embryos. In vitro-produced embryos may have a higher pregnancy loss rate than do in vivo-produced embryos[14] and genetic screening could potentially show that some of this loss is related to aneuploidy; however, there is no information available yet in this area. The main difference in interest in genetic screening in the horse is that, in contrast to the situation in humans in which many embryos of variable viability are available, but only one healthy pregnancy is desired, in the horse all embryos produced will be transferred into separate recipient mares, and most owners are eager to obtain as many foals as possible.

Embryo biopsy for genetic testing is also performed in livestock species. This was initially done in cattle for determination of embryo sex, and commercial kits are available for this purpose. Now, with the advent of commercial microarrays for evaluation of production-related nucleotide sequences (single-nucleotide polymorphisms, or SNPs), bovine embryos can be assessed for genetic merit before being transferred. These "SNP Chips" may test for 50,000 or more SNPs that relate to important production traits such as ease of calving, rate of growth, carcass fat, milk yield and reproductive efficiency. Several SNP microarrays are available for use in the horse, but it may take time before enough information is available to make effective use of this technology for selection of equine embryos.

Embryo biopsy in the horse

Preimplantation genetic testing (PGT) in the horse has several applications. Embryo sex may be important to owners in some cases, such as in Polo Ponies, in which females are desired as they are considered to be better performers. In many breeds, PGT would be useful to determine the presence of disease-related alleles in the embryo before transfer, especially for diseases that have been propagated to a high prevalence in a breed due to selection for related traits, such as hereditary equine regional dermal asthenia and hyperkalemic periodic paralysis in the American Quarter Horse. Owners may also want to know the coat color or, in some cases, parentage of embryos, or, in the future, to perform microarray analysis for SNPs that they wish to select for or against.

Biopsy of small embryos, as typically recovered on Day 6 after ovulation in mares, was first reported in 1997.[15] Embryos were biopsied by cutting a section of the zona-enclosed embryo with a microblade, and a pregnancy rate of 3/14 was obtained. Subsequently, several laboratories reported successful biopsy of small embryos using the microblade technique, or by aspiration of cells under micromanipulation.[16,17] Transfer of the embryos after biopsy resulted in pregnancy rates of 21 to 75%. The cells recovered on biopsy were successfully analyzed for genetic sex in these studies. However, biopsy of larger, expanded blastocysts was associated with a lower pregnancy rate (29%).[17]

Biopsy of expanded equine blastocysts necessitates penetration of the embryonic capsule. There is some evidence that small embryos can repair damage to the capsule, as found after bisection of early embryos: these embryos lost their zonae pellucidae and nascent embryonic capsule when bisected; however after they were transferred to the uterus and then flushed out, they were found to have formed a complete capsule.[18] However, in larger, expanded blastocysts the capsule apparently cannot repair extensive damage, and several studies showed that destruction of the capsule in expanded blastocysts yields non-viable embryos.[11,18-20]

In 2010, our laboratory at Texas A&M reported a successful method for biopsy of large expanded equine blastocysts.[21] We performed aspiration of trophoblast cells using a micropipette with the Piezo drill, as we already utilized the Piezo technique for many procedures associated with somatic cell nuclear transfer and intracytoplasmic sperm injection in the horse. We first found that aspiration of cells from small embryos could be performed using this method, with normal pregnancy rates after transfer. We then applied this technique to expanded blastocysts (300 – 611μm diameter) and found that the capsule could be breached with the micropipette, allowing recovery of sufficient embryonic cells for analysis. The cells are recovered from the periphery of the embryo (the trophoblast), being careful to avoid the inner network of cells (in early blastocysts or IVP embryos), or the inner cell mass in expanded blastocysts. Expanded blastocysts collapsed after being biopsied, due to loss of fluid from the blastocoele, but re-formed the blastocyst within a few hours in culture, and yielded a normal pregnancy rate after transfer (10/12). We concluded either that the embryos could repair the small hole in the capsule that was produced from the biopsy procedure (the diameter of the pipette being 8 to 15 um) or that the embryo was viable after transfer even with this small hole in the capsule. This procedure was equally effective on embryos shipped overnight to the laboratory before transfer as it has with fresh embryos.[21]

Jarazo et al.[22] reported successful biopsy of equine expanded blastocysts using a standard sharp micropipette, with resulting pregnancy rates of 53 to 75%. No significant difference in pregnancy rates was observed related to embryo diameter. Herrera et al.[23] evaluated the micropipette biopsy technique on a large scale, using almost 300 embryos in a commercial equine embryo transfer program. There was no difference in pregnancy rates between biopsied and non-biopsied embryos (61% for each). Within biopsied embryos, there was no difference in pregnancy rates related to the time of holding between biopsy and transfer (59 to 63%). These authors also reported no effect of blastocyst diameter before biopsy on pregnancy rate (62% pregnancy both for embryos 300 to 1000 μm, and for embryos >1000 μm). Guignot et al.[24] reported a reduced pregnancy rate with small embryos biopsied using a microblade (1/11) vs. that obtained with embryos recovered at Day 7, of varied sizes, biopsied using a micropipette (4/8).

Recently, Herrera et al.[25] reported that aspiration of blastocoele fluid only may provide sufficient DNA for genetic analysis. The DNA that was recovered from the fluid was apoptotic, however, it was successfully analyzed to determine embryo sex in 15 of 18 embryos. This technique (aspiration of only the blastocoele fluid) presents a distinct advantage over cellular biopsy for commercial application, as a smaller pipette may be used, and no manipulation of the embryonic tissue itself is required. The DNA obtained, while degenerating, appeared to be adequate for the simple interrogation of sex. It must be determined if the DNA obtained from blastocoele fluid can be used for accurate analysis of multiple genetic loci.

In their study of PGT for genetic sex using DNA from blastocoele fluid, Herrera et al.[25] reported equivalent efficiency between fluid from IVP vs. in vivo-recovered embryos. In a recent study, we found

equal high efficiency (97%) of genetic analysis of biopsy samples recovered from IVP embryos or from in vivo-recovered embryos.[26] These studies establish that preimplantation genetic testing is applicable to embryos produced by ART.

Genetic analysis

The cells, or fluid, collected from the embryos may be analyzed for the genes of interest by PCR. Here the problem of very small amounts of DNA obtained by biopsy must be overcome. While theoretically one cell can provide the entire genome for analysis, inefficiencies in performing PCR make it advisable to amplify the DNA obtained before attempting to analyze it, especially if more than one gene is to be interrogated. It should be noted that when very small amounts of DNA are used, even whole-genome amplification (WGA) can be associated with problems such as amplification failure, and contamination with exogenous DNA becomes a major problem. One of the most important factors to consider in accuracy of genetic analysis is allele dropout, that is, detecting only one allele in an individual that is actually heterozygous. In this case, the interrogation gives a signal from only one allele, and it is not possible to tell without additional testing that the analysis is not accurate, and that the other allele was present but was missed. This is why methods for genetic analysis must be tested on heterozygous loci to determine their true accuracy.

Successful evaluation for equine embryo sex has been obtained with using direct PCR of DNA from biopsied cells with efficiencies from 40 to 100%.[15,16,23,27] The higher efficiencies tended to be reported in studies with lower pregnancy rates, perhaps due to more embryo damage associated with the recovery of more cells. Jarazo et al.[27] reported that success of genetic analysis of biopsies obtained by micropipette was influenced by embryo size; significantly higher rates of sex determination were obtained in embryos 350-550 μm than in embryos >550 μm (80% vs. 42.1%), probably due to the tighter connections among cells in the large embryos leading to greater difficulty in obtaining an adequate biopsy sample. Evaluation of DNA recovered from blastocoele fluid was performed using direct PCR, and sex was determined in 15 of 18 samples; the remaining three samples gave no signal (all samples with no signal were from embryos that were later established to be female).[25]

For determination of multiple genes from biopsy samples, use of WGA followed by multiplex PCR can be extremely accurate. In collaboration with the Veterinary Genetics Laboratory at the University of California at Davis, we evaluated methods to investigate a large array of loci in single biopsies, including loci for sex, coat color (representing commonly heterozygous protein-coding genes), disease-related mutations, and identification microsatellites.[26] We found a significant difference in accuracy between two methods for whole genome amplification: the Qiagen Repli-g Midi kit was more accurate than was the Illustra Genomiphi V2 kit (98.2% vs 25.8% of alleles reported correctly, respectively, after whole genome amplification and multiplex PCR).

We went on to use the Repli-g WGA method to compare biopsy sample efficiency between IVP and in vivo-recovered embryos; these samples yielded equivalent results (> 99% accuracy of interrogated loci; with equal accuracy in detection of heterozygous loci).[26] Interestingly, we found that after WGA, the biopsy obtained with the micropipette gave the same fidelity as did analysis of an entire demi-embryo containing hundreds to thousands of cells. An important finding was that three of 81 biopsy samples (4%) were not useable (returned < 50% accuracy for interrogated loci); we hypothesized that either not enough cells were obtained at the time of biopsy, or that cells were lost during handling before WGA was performed. These non-performing biopsy samples could be identified by a lack of detected heterozygosity at microsatellite identification markers (allele dropout), as these markers are selected to have a high degree of polymorphism and are commonly heterozygous. We recommend that microsatellite markers be interrogated when performing genetic evaluation analysis commercially, to validate that the biopsy sample was sufficient for accurate analysis–this is important in order to have a high degree of confidence that a homozygous read at a locus of interest is accurate (that allele drop-out has not occurred).

Guignot et al.[24] also used the Repli-g kit for WGA before query for multiple genetic loci in biopsy samples from Welsh Pony embryos. As discussed above for direct PCR studies, they found a

higher accuracy, but lower pregnancy rate, when samples were obtained by microblade dissection, probably due to the greater number of cells obtained. For samples obtained by micropipette, they reported 82% success in determination of sex, and on evaluation of multiple other genes (degree of heterozygosity not evaluated), a 93.8 % signal rate, with an error rate of 6.6%.

In conclusion, equine embryo biopsy by aspiration of trophoblast cells under micromanipulation is an efficient method for obtaining biopsies for preimplantation genetic analyses in the horse. The micropipette biopsy procedure appears to have little effect on embryo viability, with normal pregnancy rates after transfer being reported by several laboratories. It is also possible that aspiration of blastocoele fluid could be used to provide DNA for simple genetic analyses, but more information on accuracy of this method is needed. For biopsy samples, the accuracy of the genetic analysis appears to be dependent on the number of cells recovered, the method used for amplification (direct PCR vs. WGA then PCR), and the method used for WGA. If practitioners have the capacity to obtain biopsies or blastocoele fluid, but not the facilities to analyze them, shipping biopsy samples to a reputable laboratory that is set up to perform WGA and multiplex PCR, and that accepts biopsy samples commercially, is recommended.

Equine embryo vitrification

Numerous studies have shown that cryopreservation of small equine embryos can be performed by either slow freezing (using methods similar to those for bovine embryos) or by vitrification, and result in good pregnancy rates (45-67%) after warming and transfer.[3-6] Similarly, slow freezing of IVP equine blastocysts, that are also typically < 300 μm diameter, results in pregnancy rates, and ongoing pregnancy rates, similar to those for fresh IVP embryos.[28] Eldridge-Panuska et al.[6] reported a method for vitrification of small equine embryos using a cryoprotectant system of glycerol plus ethylene glycol, in 0.25 mL straws, that resulted in 45 to 67% pregnancy after transfer. A modification of this method was made available commercially as an equine embryo vitrification kit.

In contrast, pregnancy rates after direct cryopreservation and transfer of large, expanded equine blastocysts (>300 μm in diameter) have been low (0 to 38%).[3,6,7,29-31] This is thought to be related to the large amount of fluid in the blastocoele of expanded equine embryos, or perhaps to some action of the embryonic capsule interfering with penetration of cryoprotectants.

We became interested in embryo cryopreservation during our study on embryo biopsy, as we needed to develop a method for holding embryos while waiting for the results of genetic analysis of the biopsy sample. We were intrigued by the fact that the blastocyst collapsed after biopsy, but recovered quickly in culture and provided normal pregnancy rates after transfer. We thus investigated whether collapse of the blastocyst via micromanipulation would enable successful vitrification of expanded blastocysts.

We initially investigated the standard embryo vitrification method of Eldridge-Panuska et al.,[6] which utilizes 0.25-mL straws, for vitrification of equine embryos after biopsy; however, 0 of 5 embryos we vitrified by this method established pregnancy after warming and transfer.[32] We went on to investigate use of a smaller-diameter pipette (a microloader tip) that had been reported to be successful for vitrification of ferret embryos.[33] We also investigated two different cryoprotectant protocols, one using dimethylsulfoxide (DMSO), ethylene glycol and a sugar (DM method) and one using only ethylene glycol and a sugar (EG/s method). We found that expanded blastocysts > 300 to 700 μ diameter could be successfully vitrified after blastocoele collapse. While initial pregnancy rates with the two cryoprotectant protocols were similar (46 to 50%), pregnancies produced using embryos vitrified via EG/s were significantly more likely to be normal (yield a conceptus with an embryonic heartbeat) than were pregnancies produced using embryos vitrified via DM (6/6, 100% vs. 2/8, 25%). On investigating use of the EG/s-micropipette method further, we found that the degree of blastocoele collapse, i.e., the amount of fluid loss from the blastocoele, was related to the success of vitrification. Blastocysts recorded as losing 10%, 20 to 30%, and > 70% of the blastocoele fluid after puncture gave pregnancy rates of 0/3, 2/5 and 4/5, respectively. In the final study of this project, we used the EG/s-microloader vitrification method and collapsed blastocysts entirely by aspirating fluid with a micropipette positioned at the periphery of the

embryo. These expanded blastocysts, initially 407-565 μm in diameter, produced a pregnancy rate (with heartbeat) of 71% after warming and transfer.[32]

The microloader tip is awkward to handle, and has a training curve before operators can achieve successful warming, thus for commercial use they have the drawback of requiring warming at our laboratory and shipment of the warmed embryo. For commercial use, it would be better to have a system that can be warmed mare-side at the embryo transfer center. We have investigated the use of the EG/s cryoprotectant protocol using open vitrification systems, both commercial (Cryolock; Biotech Inc., Cumming, GA) and a hand-made modification of the microloader tip to create a shelf on which the embryo can be placed, which we designated the "Sujo." These methods provided excellent embryo viability after warming when assessed in vitro (18 of 18 vitrified/thawed embryos on Sujo grew in culture[34]) but we have not transferred an adequate number of embryos to assess viability in vivo.

Interestingly, only a few studies on vitrification of equine expanded blastocysts after blastocoele collapse have been reported subsequently. Guignot et al.[24] vitrified a small number of embryos after biopsy and blastocoele collapse. They used a cryoprotectant protocol similar to EG/s, above, and loaded the embryos into open-pulled straws for vitrification. After warming, the embryos were cultured in vitro for three hours before transfer. These authors reported pregnancy rates at 30 days of 1/2 and 2/5, for small and large blastocysts, respectively.

Diaz et al.[35] reported on the use of a commercial co-axial microinjection system, the Embryo Cradle (Genesearch, Bozeman, MT; initially called the Dracula Pipette) for collapse of equine Day-8 embryos before vitrification. "Co-axial" refers to the fact that the small micropipette used to puncture the embryo is extruded within a larger holding pipette. Use of this system on Day-8 blastocysts appeared to be associated with a high incidence of loss of the embryonic capsule (25 to 31% of manipulated embryos), which presumably would render them non-viable. For vitrification, these authors used the cryoprotectant protocol of Eldridge-Panuska et al.,[6] with an open vitrification device (Cryolock). They investigated whether injection of cryoprotectant into the blastocoele cavity before aspiration of blastocoele fluid would increase viability after vitrification; they found no difference in embryo growth in vitro after warming, but reported that embryos undergoing cryoprotectant injection had a higher incidence of capsule loss (70%, vs. 31% for collapse only). Subsequently, they applied the blastocoele collapse only / vitrification method to Day-8 expanded blastocysts (448-1168 μm diameter) and reported a pregnancy rate, with heartbeat, of 5/6 (83%) after warming and transfer.

These studies show that blastocoele collapse is a repeatable method to achieve successful vitrification of expanded equine blastocysts. Good pregnancy rates were achieved using two different cyroprotectant protocols, and with both closed (microloader tips and open-pulled straw) and open systems. The widespread application of blastocoele collapse for equine embryo vitrification in commercial ET programs would be facilitated by the development of a simple, commercially-available method to puncture the blastocyst and aspirate fluid from it.

Acknowledgments

Work in the author's laboratory was supported by the Link Equine Research Endowment Fund, Texas A&M University, the Clinical Equine ICSI Program, Texas A&M University, and the American Quarter Horse Foundation.

References

1. Hinrichs K, Choi YH, Love LB, et al: Chromatin configuration within the germinal vesicle of horse oocytes: changes post mortem and relationship to meiotic and developmental competence. Biol Reprod 2005;72:1142-50.
2. Colleoni S, Barbacini S, Necci D, et al: Application of ovum pick-up, intracytoplasmic sperm injection and embryo culture in equine practice. Proc Am Assoc Equine Pract 2007. p. 554-559.
3. Slade NP, Takeda T, Squires EL, et al: A new procedure for the cryopreservation of equine embryos. Theriogenology 1985;24:45-58.
4. Maclellan LJ, Carnevale EM, Coutinho da Silva MA, et al: Cryopreservation of small and large equine embryos pre-treated with cytochalasin-B and/or trypsin. Theriogenology 2002;58:717-720. [abstract]
5. Hochi S, Maruyama K, Oguri N: Direct transfer of equine blastocysts frozen-thawed in the presence of ethylene glycol and sucrose. Theriogenology 1996;46:1217-1224.

6. Eldridge-Panuska WD, Caracciolo di Brienza V, Seidel Jr GE, et al: Establishment of pregnancies after serial dilution or direct transfer by vitrified equine embryos. Theriogenology 2005;63:1308-1319.
7. Squires EL, Seidel GE, McKinnon AO: Transfer of cryopreserved equine embryos to progestin-treated ovariectomised mares. Equine Vet J 1989;Suppl 8:89-91.
8. Battut I, Colchen S, Fieni F, et al: Success rates when attempting to nonsurgically collect equine embryos at 144, 156 or 168 hours after ovulation. Equine Vet J 1998;Suppl 25:60-62.
9. Jacob JCF, Haag KT, Santos GO, et al: Effect of embryo age and recipient asynchrony on pregnancy rates in a commercial equine embryo transfer program. Theriogenology 2012;77:1159-1166.
10. Freeman DA, Weber JA, Geary RT, et al: Time of embryo transport through the mare oviduct. Theriogenology 1991;36:823-830.
11. Müller Z, Cikryt P: A simple method for bisecting horse embryos. Equine Vet J 1989;Suppl 8:123-125.
12. Spits C, Sermon K: PGD for monogenic disorders: aspects of molecular biology. Prenat Diagn 2009;29:50-56.
13. Gardner DK, Meseguer M, Rubio C, et al: Diagnosis of human preimplantation embryo viability. Hum Reprod Update 2015;21:727-747.
14. Hinrichs K, Choi YH, Love CC, et al: Use of intracytoplasmic sperm injection and in vitro culture to the blastocyst stage in a commercial equine assisted reproduction program. J Equine Vet Sci 2014;34:176.
15. Huhtinen M, Peippo J, Bredbacka P: Successful transfer of biopsied equine embryos. Theriogenology 1997;48:361-367.
16. Troedsson MHT, Paprocki AM, Koppang RW, et al: Transfer success of biopsied and vitrified equine embryos. Anim Reprod Sci 2010;121S:295-296. [abstract]
17. Seidel Jr GE, Cullingford EL, Stokes JE, et al: Pregnancy rates following transfer of biopsied and/or vitrified equine embryos: evaluation of two biopsy techniques. Anim Reprod Sci 2010;121S:297-298. [abstract]
18. McKinnon AO, Carnevale EM, Squires EL, et al: Bisection of equine embryos. Equine Vet J 1989;Suppl 8:129-133.
19. Skidmore J, Boyle MS, Cran D, et al: Micromanipulation of equine embryos to produce monozygotic twins. Equine Vet J 1989;Suppl 8:126-128.
20. Stout TA, Meadows S, Allen WR: Stage-specific formation of the equine blastocyst capsule is instrumental to hatching and to embryonic survival in vivo. Anim Reprod Sci 2005;87:269-281.
21. Choi YH, Gustafson-Seabury A, Velez IC, et al: Viability of equine embryos after puncture of the capsule and biopsy for preimplantation genetic diagnosis. Reproduction 2010;140:893-902.
22. Jarazo J, Gambini A, Muredas L, et al: Horse embryo biopsy: effect on pregnancy rates and successsful sex determination depending on size of the embryo. Reprod Fertil Dev 2012;25:224.
23. Herrera C, Morikawa MI, Bello MB, et al: Setting up equine embryo gender determination by preimplantation genetic diagnosis in a commercial embryo transfer program. Theriogenology 2014;81:758-763.
24. Guignot F, Reigner F, Perreau C, et al: Preimplantation genetic diagnosis in Welsh pony embryos after biopsy and cryopreservation. J Anim Sci 2015;93:5222-5231.
25. Herrera C, Morikawa MI, Castex CB, et al: Blastocele fluid from in vitro- and in vivo-produced equine embryos contains nuclear DNA. Theriogenology 2015;83:415-420.
26. Choi YH, Penedo MC, Daftari P, et al: Accuracy of preimplantation genetic diagnosis in equine in vivo-recovered and in vitro-produced blastocysts. Reprod Fertil Dev 2015;doi: 10.1071/RD14419.
27. Gambini A, Jarazo J, Olivera R, et al: Equine cloning: in vitro and in vivo development of aggregated embryos. Biol Reprod 2012;15:1-9.
28. Galli C, Colleoni S, Duchi R, et al: Developmental competence of equine oocytes and embryos obtained by in vitro procedures ranging from in vitro maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. Anim Reprod Sci 2007;98:39-55.
29. Young CA, Squires EL, Seidel Jr GE, et al: Cryopreservation procedures for Day 7-8 equine embryos. Equine Vet J 1997;Suppl 25:98-102.
30. Bass LD, Denniston DJ, Maclellan LJ, et al: Methanol as a cryoprotectant for equine embryos. Theriogenology 2004;62:1153-1159.
31. Barfield JP, McCue PM, Squires EL, et al: Effect of dehydration prior to cryopreservation of large equine embryos. Cryobiology 2009;59:36-41.
32. Choi YH, Velez IC, Riera FL, et al: Successful cryopreservation of expanded equine blastocysts. Theriogenology 2011;76:143-152.
33. Sun X, Li Z, Yi Y, et al: Efficient term development of vitrified ferret embryos using a novel pipette chamber technique. Biol Reprod 2008;79:832-840.
34. Choi YH, Velez IC, Macias-Garcia B, et al: Application of an open device to vitrify equine in vitro-produced embryos. Reprod Fertil Dev 2013;25:178.
35. Diaz F, Bondiolli K, Paccamonti D, et al: Cryopreservation of Day 8 equine embryos after blastocyst micromanipulation and vitrification. Theriogenology 2016;85:894-903.

Characterization of serum, uterine and vaginal IgG response against *Tritrichomonas foetus* surface antigen after administration of a commercial killed whole *T. foetus* vaccine in beef cows

R.A. Palomares,[a] L.B. Corbeil,[b] D.J. Hurley,[a] E. Rollin,[a] T. Collop,[c] A. Williard,[a] J. Felton,[a] J. Parrish[a]
[a]Department of Population Health, College of Veterinary Medicine, The University of Georgia, Athens GA; [b]Department of Pathology, University of California, San Diego, CA; [c]Veterinary Service, Rutledge, MO

Bovine trichomoniasis is a sexually transmitted disease caused by *Tritrichomonas foetus*, which affects cattle reproductive performance and profitability of beef farms. Infections with *T. foetus* result in vaginitis, cervicitis, and endometritis which are associated with embryonic death and abortion. Since *T. foetus* is an extracellular microorganism, immunoglobulins in vaginal and uterine mucosa are believed to be essential to protection. The objective of this study was to determine the level and duration of IgG antibodies induced against a *T. fetus* purified surface antigen (TF1.17) in serum, vaginal and uterine secretions after systemic immunization of beef cows with a commercial vaccine containing killed whole *T. foetus*. We hypothesize that administration of a commercial vaccine containing *T. foetus* provides a significant induction of specific IgG to a *T. foetus* surface antigen in serum, vaginal secretions and uterine flush samples of beef cows, and that the levels of IgG remain elevated over baseline for several weeks. Twenty beef cows (> 30 days postpartum) were randomly assigned to vaccine or control groups as follows: vaccine (n=10): cows received 2 mL of a commercial vaccine containing killed whole *T. foetus* (TrichGuard®, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) subcutaneously and a 2-mL booster 2 weeks later. Control (n=10): cows received 2 mL of sterile saline on the same schedule. Vaginal secretions and blood samples were collected on days 0, 8, 15, 22, 29, 36, 43, 50, 60, 75, 89, 110, 146, and 182 relative to day of vaccination. Uterine flush fluid was collected on days 0, 15, 29, and 43 after the day of primary vaccination. Serum, vaginal secretions and uterine flush samples were assayed for IgG antibodies to the immunoaffinity purified-lipophosphoglycan (LPG)/protein antigen TF1.17 of *T. foetus* using ELISA as described previously. IgG antibody levels were compared between groups for each day using a two independent sample t-test, and over time using a repeated measure analysis of Statistical Analysis System (SAS®). Serum *T. foetus*-specific IgG levels were significantly increased (between days 8 and 182) following vaccination with *T. foetus* compared to the values on day 0 ($P < 0.001$) and the control group during the same days ($P < 0.001$). Serum IgG levels remained elevated for at least 26 weeks (day 182). A significant rise in TF1.17-specific IgG levels was observed in vaginal and uterine fluids from day 15 after vaccination compared to the baseline levels ($P < 0.001$). These levels remained elevated in vaginal and uterine secretions through days 75 and 43 after primary vaccination, respectively. Antibody levels in serum, vaginal and uterine secretions remained low in the control group throughout the study. In conclusion, vaccination of beef cows with a commercial vaccine containing *T. foetus* induced significant increase in the levels of IgG to the *T. foetus* surface antigen in serum, vaginal secretions and uterine fluid, which remained elevated through days 43, 75 and 182 in uterine fluids, vaginal secretions and serum, respectively. The induced TF1.17-specific IgG response is likely to be important in the prevention of trichomoniasis in beef cattle.

Keywords: *Tritrichomonas foetus*, TF 1.17 specific IgG, vaccine, beef cows

The effects of EC-Oxyrase® and coconut water on equine sperm cryopreservation

K.T. London,[a] B.W. Christensen,[a] C.J. Scott,[a] C.D. Renaudin,[a] G.A. Dujovne,[a] K. Klooster,[b] P.H. Kass,[a] S.A. Meyers[b]

[a]Department of Population Health and Reproduction, [b]Department of Anatomy, Physiology & Cell Biology, University of California, Davis, CA

The use of milk and egg yolk in semen extenders raises the possibility of xenobiotic contamination and increases variability between batches. Alternative sources of lipoproteins could replace animal byproducts. Oxidative damage is thought to be a major cause of damage occurring during cryopreservation. EC-Oxyrase® (Oxyrase, Inc, Mansfield, OH) is an *E. coli* origin membrane-derived enzyme system that removes oxygen from liquids. We hypothesized that: 1) post-thaw semen parameters and pregnancy rates would not be different in coconut water treated samples compared with egg yolk treated samples and, 2) the use of EC-Oxyrase would improve post-thaw sperm motility and membrane integrity and decrease lipid peroxidation.

Experiment 1: Three ejaculates each from five stallions were frozen using EquiPlus semen extender (MOFA Global, Verona, WI) in one of four treatments: 2% egg yolk (EY), 2% coconut water (CW), 2% egg yolk with 8.69% EC-Oxyrase (EYO), or 2% coconut water with 8.69% EC-Oxyrase (CWO). Fresh semen was added to a basic extender (EquiPlus part A) lacking CW or EY and progressive motility (PM) was measured using a computer assisted sperm analysis system (SpermVision, MOFA Global, Verona, WI). A portion of the ejaculate was evaluated with flow cytometry using propidium iodide, annexin, and BODIPY probes to assess apoptosis, membrane integrity, and lipid peroxidation, respectively. Samples were frozen according to EquiPlus instructions at a concentration of $200x10^6$ cells per mL using a controlled rate freezer (Planer, Middlesex, UK) and then thawed for 30s in a 37°C water bath. A post-hoc comparison between all treatments was done using a Bonferroni adjustment for multiple comparisons. CW showed better post-thaw PM than EY (dropping 51.4% and 56.3%, respectively, $P<0.05$). There were no differences in apoptosis observed among groups. Membrane damage increased in EY, EYO, and CWO when compared to CW ($P<0.05$). No differences were seen in membrane oxidation between EC-Oxyrase and control samples.

Experiment 2: One ejaculate was divided into two aliquots and frozen using the same method as in Experiment 1 in either coconut water (CW) or egg yolk (EY) extender. Mares ($n = 12$) were randomly assigned to either CW or EY, inseminated at 24 and 42 h after deslorelin injection (when in estrus with a follicle greater than 35mm) with thawed semen (50×10^6 PMS/dose), and examined for pregnancy 14 d after ovulation. The pregnancies were terminated immediately after diagnosis and the trial was repeated with the alternate treatment in each mare. Each mare thus served as her own internal control in this crossover experimental design. Statistical analysis of the crossover data used Prescott and McNemar tests and employed exact inference using Crossover-1 statistical software (Cytel Software Corporation, Cambridge, MA). More CW mares became pregnant (11/12) than the EY mares (6/12) ($P = 0.0373$). In both experiments, CW performed better than EY in preserving semen parameters and fertility ($P<0.05$). EC-Oxyrase did not seem to positively affect semen parameters ($P>0.05$).

Keywords: Coconut water, egg yolk, semen extender, stallion, oxidative stress, EC-Oxyrase

Do differences in maternal immunoglobulin G influence passive transfer and subsequent cria growth in alpacas?

K. Gleason,[a] L.Edens,[b] A. Pena[b]
[a]Dancing Horse Farm, Pemberton, NJ; [b]Little Creek Farm, North Salem, NY

Undetected failure of passive transfer (FPT) of immunoglobulin is a major determinant of mortality in newborn alpaca cria, and early detection with proactive management can reduce mortality rates in cases of suspect FPT. The goal of this prospective observational study was to evaluate maternal serum IgG levels as a predictor for subsequent cria FPT (IgG <400 mg/dl) and/or poor cria early development. Prepartum blood samples were collected from dams within 60 days of anticipated birth, and from suckling cria within 36 hours after birth, and analyzed for serum IgG concentration by use of a commercial spectrophotomic assay. Any cria with a birth IgG < 400 mg/dl was offered oral maternal colostrum as routine practice. Birth weights, and weights at two weeks and two months were recorded for all cria to establish growth patterns. Dams were divided into three groups based on natural breaks in prepartum IgG levels of 1000 to 1499 mg/dl (n = 25), 1500 to 2000 mg/dl (n = 22), and >2000 mg/dl (n = 13). Analysis of variance within maternal groups revealed significant differences in cria birth IgG levels, whereby the highest levels were observed when dam IgG measured 1000-1499 mg/dl, followed by 1500-2000 mg/dl and > 2000 mg/dl (640, 554 and 545 mg/dl, respectively). Although there were cria with birth IgG <400 mg/dl in every dam group, there were 20% more in the latter group. Cria that were handfed maternal colostrum tended to have lower measured IgG ($p<.05$). Despite differences in birth IgG levels, cria birth weights, weights at two weeks and at two months did not differ amongst maternal groups. Interestingly, while the maternal group with the highest IgG had more births in the spring (7 vs 5 and 2), their cria subsequently had the lowest IgG than the other groups, raising concerns for a seasonal effect on immunoglobulin passive transfer. Results of this study suggest that maternal IgG could be an early, gestational indicator of potential FPT births in North American alpacas. Early identification of possible problems can negate the need for costly and invasive treatments such as plasma transfer, which often requires hospitalization. More research is needed to follow cria development to weaning, and to investigate possible seasonal influences on passive transfer.

Keywords: Immunoglobulin G, passive transfer, alpaca, cria

Prevalence of *Brucella melitensis* seropositive goats in rural Uganda

C. Card, L. McDonald
Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Brucella melitensis (B. melitensis) is a zoonotic disease present in sub-Saharan Africa. However, few studies have evaluated the prevalence of the disease in goats. Infection with *B. melitensis* is associated with human morbidity, and in goats: abortion, weak premature neonates and infertility. Among poor rural women farmers who typically own goats, it is a major cause of financial loss. We hypothesized that the disease was endemic. A key event in 2012 was a government program offering non-*Brucella* tested goats at low prices to farmers, which spread the disease throughout the region. The specific objectives of the study were to characterize and determine changes in the seroprevalence of Brucellosis in goats owned by women in predominately female-headed households in rural communities in Isingiro district, Uganda. The majority of the goats were raised using communal grazing areas and partial pen confinement with supplemental feeding of plantain peelings, napier grass (*Pennisetum purpureum*), fodder, and mineral. A few goats were raised in 100% zero grazing confinement. All goats in the study were identified using permanent ear tags. Management interventions against Brucellosis began in 2009, which included educating owners, training paravets, testing goats, vaccinating against *B. melitensis,* and encouraging culling of seropositive goats. Jugular blood was drawn from every third goat (n = 213 samples) from eight communities in 2009 during a Clostridial disease vaccination campaign prior to vaccination for *B. melitensis.* In 2012-2015 serum samples were obtained from goats in eight to 12 communities with n=479, 603, 681 and 332 goats sampled and tested, respectively. Serum was evaluated using a serum agglutination test, which was performed according to the manufacturer's directions. Pooled control positive and negative serum was included on each plate. In 2012-2015 vaccination commenced from eight to 12 communities with n=479, 603, 353 and 107 seronegative goats vaccinated, respectively. Seronegative goats were vaccinated once with Rev-1 in 2012 or with intra-ocular *B. melitensis* in 2014-2015. Fisher's exact test was used for data analysis with significance set at $p<0.05$. Interviews with women and group meetings and biannual meetings of paravets to discuss disease management were used to determine the effectiveness of the vaccination. In 2009, the majority of the goats were local Mubende cross type (76%) with a small percentage of dairy and dairy cross (19%) and Boer, Boer-cross goats (5%). In 2009 the overall seroprevalence was 19.7%, which was significantly greater in dairy (29%) and Boer (50%) goats when compared with local goats (15%; $p<0.001$). Seroprevalence from 2012-2015 in non-vaccinates was 34%, 40%, 30% and 12%, respectively and decreased significantly from 2014 to 2015 ($p<0.001$). Women farmers and paravets reported very few abortions among vaccinated goats. In conclusion, *B. melitensis* infection is endemic in goats in Isingiro district, Uganda. Higher rates of seropositive dairy and Boer goats in 2009 were likely related to their herds of origin. A single lifetime vaccination under these conditions appeared to prevent abortion, and may have decreased the seroprevalence of *B. melitensis.*

Acknowledgements: Special thanks to Foundation for AIDS Orphaned Children, the women and paravets of Isingiro and the eight veterinarians, 11 Global Vets students and 21 veterinary student volunteers with Veterinarians Without Borders Canada.

Keywords: *Brucella melitensis*, goat, seropositive, vaccine

Gene expression and changes in biological function in placenta and uterus of ewes with subclinical pregnancy toxemia

S. Bynum, R. Kasimanickam
Department of Veterinary Clinical Sciences, Washington State University, Pullman, WA

The ovine placenta produces angiogenic factors throughout gestation, and tissue- and cell-specific patterns of gene expression have been reported in normal and abnormal pregnancies. The objective of this study was to elucidate differences in utero-placental gene expressions between ewes with subclinical pregnancy toxemia (SCPT) and healthy ewes, as well as to identify associated biological functions and pathways involved in pregnancy toxemia. The hypothesis was that ewes with SCPT will have altered utero-placental gene expressions associated with angiogenesis and hypoxia. Samples were collected and some methods were performed during a previously reported clinical trial.[1] Eighteen pregnant ewes (3.1±0.11 years of age; impregnated by different sires [n=2] by natural service), with similar breeding dates were selected. On Day 136 (±1 day) after breeding all ewes had body condition score (BCS; 1 to 5; 1, emaciated; 5, obese) assessed and blood samples were collected for plasma glucose and β-hydroxybutyrate (BHBA) analyses. The ewes were euthanized and tissue samples were collected from the gravid uterus and placentomes. Tissue samples were placed in RNAlater (Qiagen Inc., Valencia, CA) in 5 mL cryogenic vials (Nalgene®, Sigma-Aldrich, St. Louis, MO), snap frozen immediately and stored at -70°C until analyses. Based on BCS (2.0 ± 0.02), glucose (2.4 ± 0.33) and BHBA (0.97 ± 0.06) concentrations, ewes (n=10) were grouped as healthy (n=5) and subclinical SCPT (n=5) ewes.[2] The mRNA expressions were determined by RT-PCR method (House-keeping gene, 18Sr and β-actin). The RT-PCR data $2^{\Delta\Delta Ct}$ values for mRNA expressions of genes were analyzed by ANOVA to ascertain statistical significances. Prediction of miRNA partners and target genes for the predicted miRNA were identified using miRDB (http://mirdb.org/miRDB/). Top ranked target genes were used to identify associated biological functions and pathways in response to pregnancy toxemia using PANTHER. The angiogenesis genes VEGF, PlGF, sFlt1, KDR, AdipoQ, AdipoR2, PPARG, LEP, IGF1, IGF2, IL1b and TNFa mRNA expressions were lower in abundances, and hypoxia genes eNOS, HIF1a, and HIF 2a mRNA expressions were greater in abundance in cotyledon, caruncle and uterus of SCPT ewes compared to healthy ewes ($P<0.05$). The predicted miRNA and associated target genes contributed to several biological processes, including apoptosis, biological adhesion, biological regulation, cellular component organization or biogenesis, cellular process, developmental process, immune system process, localization, metabolic process, multicellular organismal process, reproduction, and response to stimulus. The target genes were involved in several pathways including angiogenesis, cytoskeletal regulation, hypoxia response via HIF activation, interleukin signaling, ubiquitin proteasome and VEGF signaling pathway. In conclusion, the findings of the study indicate that expressions of the genes associated with blood vessel remodeling were lower in abundance, and that the genes associated with hypoxic conditions were greater in abundance in the utero-placental compartment of SCPT ewes. It is obvious that the factors that influence placental vascular remodeling set the course for hemodynamic changes and hence have a major impact on the rate of transplacental nutrient exchange, fetal growth and health of the dam. To our knowledge, this is the first study to elucidate utero-placental gene expressions in ewes with subclinical pregnancy toxemia.

Keywords: Sheep, pregnancy toxemia, uterus, placenta, gene expression

References

1. Kasimanickam RK, Kasimanickam VR, Rodriguez JS, et al: Tocopherol induced angiogenesis in placental vascular network in late pregnant ewes. Reprod Biol Endocrinol 2010;8:86.
2. Lacetera N, Bernabucci U, Ronchi B, et al: Effects of subclinical pregnancy toxemia on immune responses in sheep. Am J Vet Res 2001;62:1020-1024.

Evaluation of oral folic acid supplementation on the incidence of cleft palates in Labrador retrievers, Golden retrievers, and Labrador/Golden cross puppies in the Guide Dogs for the Blind breeding colony

K. Gonzales,[a] T. Famula,[b] H. Power,[a] J. Bullis[a]

[a]Guide Dogs for the Blind, San Rafael, CA; [b]Department of Animal Science, University of California, Davis, CA

In canine fetuses, cleft palates result when there is a disruption in the sequential steps of palate development during embryogenesis. In dogs craniofacial defects can appear in any breed, but the brachycephalic breeds and dogs with wide skulls seem to be most predisposed. The incidence rate of cleft palates in puppies whelped at Guide Dogs for the Blind between 2004-2015 ranges from 1.2% to 2.3% annually. A small number of studies in Boston Terriers, Pugs, Chihuahuas, and French Bulldogs have shown a reduction in puppies with cleft palates when their dams were supplemented with folic acid during breeding and pregnancy.[1-3] We hypothesized that supplementing Labrador, Golden retriever, and Labrador/Golden cross dams with oral folic acid during breeding and pregnancy would reduce the incidence of cleft palates in their puppies.

Breeding bitches (n=111) were given 5mg oral folic acid daily at the start of proestrus through day 40 of gestation. Puppies (n=739) were born in the trial period of folic acid supplementation. The incidence of cleft palates in the folic acid supplemented puppies was compared to the control puppies (n=5,334). Because the control group was larger than the study group, puppies were randomly drawn from the control population in numbers, gender and breed to equal those in the study population (5 random control samples were compared), allowing identical matching of each puppy of a given breed and sex by an untreated puppy of the same breed and sex. The goal for this analysis was to assess the impact of feeding folic acid on the prevalence of this disease. Recognizing the binary nature of the phenotype, combined with the extensive pedigree in the colony, we fit a mixed logistic model under a Bayesian framework. The mean of the posterior density for the odds ratio of disease across the two feeding groups, with dogs not fed folic acid in the numerator, was 0.66 with a 95% HPD interval of [0.33, 1.05], indicating that folic acid feeding had no significant impact on reducing the presence of cleft palate. Subsequently, a more refined analysis, using a subset of 53 females (producing 687 progeny) who were fed or not fed folic acid over a two-year period revealed a mean odds ratio across the feeding regimes of 1.06 and a 95% HPD interval of [0.53, 1.88], again indicating no significant impact of folic acid supplementation on disease. Further study will include investigation into the genetic influence of cleft palates.

Keywords: cleft palate, folic acid

References

1. Elwood JM, Colquhoun TA: Observations on the prevention of cleft palate in dogs by folic acid and potential relevance to humans. NZ Vet J 1997;45:254-256.
2. Domoslawska A, Jurczak A, Janowski T: Oral folic acid supplementation decreases palate and/or lip cleft occurrence in Pug and Chihuahua puppies and elevates folic acid blood levels in pregnant bitches. Pol J Vet Sci 2013;16:33-37.
3. Guilloteau A, Servet E, Biourge V, et al: Folic acid and cleft palate in brachycephalic dogs. Waltham Focus 2006;16:30-33.

16S rDNA-based profiling of canine reproductive tract microbiota reveals a complex microbial ecosystem

C. Lyman,[a] K. Meinkoth,[a] U. DeSilva,[b] E. Coffman,[a] G.R. Holyoak[a]
[a]Center for Veterinary Health Sciences and [b]Department of Animal Science, Oklahoma State University, Stillwater, OK

While it is established that the cranial vagina of mammals harbors a thriving microbial ecosystem, the uterus was long considered to be a sterile environment in order to sustain a successful pregnancy. This paradigm was recently challenged by the observation that human placentae harbors a diverse microbiome. There is very little information available on canine reproductive tract microbiota and the few published studies were conducted using culture-based techniques that fails to detect >90% of resident microflora.[1] As a proof-of-concept study in an effort to identify the normal flora of healthy canine vagina and uterus, we performed a comprehensive analysis of their resident microbiota. Twenty five young, healthy bitches presented for elective ovariohysterectomy (OVH) were anesthetically induced and samples from the cranial vagina were taken for cytology. Serum samples for progesterone analysis were taken to confirm the stage of estrous. During OVH the uterus and ovaries were removed using sterile technique. Endometrial biopsy samples and vaginal swabs were collected for microbial analysis. Animals were assigned to pre-pubertal, anestrus, pro-estrus, estrus or diestrus stages (n=5 per group). Vaginal swab and endometrial samples were collected under strict aseptic conditions to prevent contamination and a vaginal swab and an endometrial biopsy sample from each animal was used for DNA extraction. Integrity of extracted DNA was verified spectrophotometrically as well as by gel analysis. A 300bp fragment from 16S ribosomal v3/v4 region was PCR amplified. Each DNA sample was barcoded to identify the individual animal and the tissue (vagina/uterus) of origin and over 4 million paired-end reads were generated from the samples. After removing ambiguous sequences, barcodes and misalignments, sequences were further analyzed using MOTHUR and taxonomic assignments were made using the SILVA database. The results showed that the canine reproductive tract consists of a rich and diverse microbial environment with over 300 different operational taxonomic units (OTUs) of organisms identified. A comprehensive analysis of the microbiomes across different stages of estrus as well as a core microbiome of the canine reproductive tract will be provided.

Keywords: Endometrial microbiome, canine, estrous cycle

Reference

1. Olson PNS, Mather EC: Canine vaginal and uterine bacterial flora. J Am Vet Med Assoc 1978;172:708-711.

Time-dependent changes in pregnancy-associated glycoproteins and serum progesterone in a commercial crossbred sheep during pregnancy and the postpartum period

J. Roberts,[a] K.J. May,[b] A. Veiga-Lopez[c]

[a]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, MI; [b]Antel Biosystems, Lansing, MI; [c]Department of Animal Science, College of Agriculture and Natural Resources, Michigan State University, East Lansing, MI

In ruminants, the trophoblastic placental layer secretes pregnancy-associated glycoproteins (PAG), a family of aspartic proteinases, also referred to as pregnancy specific protein B (PSPB). Pregnancy-associated glycoproteins are detectable in maternal circulation after placental attachment. Since PAGs are secreted specifically by placental binucleate cells, they may be used to assess feto-placental unit viability. However, variability of PAG profiles amongst sheep breeds[1] warrants establishment of standard breed profiles prior to relying on a particular PAG assay for pregnancy diagnosis. This study aimed to assess serum PAGs, their correlation to progesterone (P4), and persistence in newborns and postpartum ewes in a crossbred sheep. Two groups of Polypay X Dorsett sheep, housed at Michigan State University Sheep Research Farm were used to study early to mid-pregnancy (Study 1) and late pregnancy to postpartum (Study 2) hormonal and PAG dynamics. In Study 1, maternal serum samples were collected from seven females every two weeks from gestational day (GD) 30 to GD120 and assayed using two ELISAs for PAG-1 (IDEXX; Westbrook ME) and PSPB (BioPRYN, BioTracking, Moscow ID). Progesterone was assessed at GD45, GD75, and GD105. In Study 2, maternal serum samples were collected from 12 females weekly starting at GD120 until 11 weeks postpartum. Serum samples from newborns were collected at birth and every three days until day 12 after birth. All samples were assayed for PAG1. The following statistical analyses were conducted: one-way repeated measures analysis of variance, Pearson and Spearman's correlations, linear regression, and independent T-test. Significance was defined as $P < 0.05$. Circulating PAG1 levels steadily increased from GD30 until GD120 while PSPB exhibited a bimodal pattern of secretion. A strong positive correlation was observed between P4 and PAG1 ($r^2 = 0.779$, $P < 0.0001$), but not between PSPB and P4. Maternal PAG1 concentrations declined until 10 weeks after parturition ($P < 0.05$). Pregnancy-associated glycoprotein1 concentrations were lower before and after parturition in singleton compared to twin pregnancies ($P < 0.05$). Pregnancy-associated glycoprotein1 levels continuously declined in singleton and twin newborns ($P < 0.05$) and cleared from newborns by 12 days after birth. Therefore, PAG1 can be used to distinguish singleton from multiple pregnancies in sheep. Our findings demonstrate for the first time how different PAG assays provide unique gestational profiles. Also, because the placenta is the main source of P4 in sheep beyond GD50, the strong correlation between PAG1 and P4 through mid-gestation demonstrates that PAG1, but not PSPB can be effectively used as a marker of placental endocrine function.

Keywords: Pregnancy-associated glycoproteins, gestation, postpartum, sheep

Reference

1. Ranilla MJ, Sulon J, Carro MD, et al: Plasmatic profiles of pregnancy-associated glycoprotein and progesterone levels during gestation in Churra and Merino sheep. Theriogenology 1994;42:537-545.

Nitric oxide's dose-dependent inhibition of uterine contractility: a potential mechanism underlying persistent breeding-induced endometritis in the mare

F.A. Khan,[a] T.S. Chenier,[a] C.L. Murrant,[b] R.A. Foster,[c] J. Hewson,[d] E.L. Scholtz[a]
[a]Department of Population Medicine, [b]Department of Human Health and Nutritional Sciences, [c]Department of Pathobiology, [d]Department of Clinical Studies, University of Guelph, Guelph, ON, Canada

Persistent breeding-induced endometritis (PBIE) is a major cause of equine infertility. Mares susceptible to PBIE have increased uterine nitric oxide (NO) concentrations and decreased uterine contractility. Nitric oxide may have a role in the development of PBIE in mares through an inhibitory effect on uterine contractility. The objectives of this study were to test the effect of NO on uterine contractility in-vitro and to evaluate whether this effect varied between the longitudinal and circular muscle layers of the uterus. It was hypothesized that NO would have a dose-dependent inhibitory effect on spontaneous uterine contractility irrespective of the muscle layer. Reproductive tracts were collected postmortem from eight non-pregnant mares (age 4 to 19 years; body weight 405 to 530 kg). Transrectal examination of the reproductive tract was performed before euthanasia to evaluate stage of the estrous cycle and presence of any apparent abnormality. After euthanasia, one uterine tissue sample was collected for histological evaluation and four full-thickness uterine tissue strips (10–12 mm × 2 mm), two parallel to each muscle layer, were excised for in-vitro contractility evaluation. Strips were suspended in tissue chambers containing Krebs–Henseleit solution, with continuous aeration (95% O_2–5% CO_2; pH 7.4) at 37°C. After equilibration, spontaneous contractility was recorded (pre-treatment) and strips excised in each direction were randomly allocated to each of two groups: 1) SNAP (S-nitroso-N-acetylpenicillamine, an NO donor); or 2) NAP (N-acetyl-D-penicillamine, vehicle and time-matched control). These were treated at 15 min intervals with increasing concentrations (10^{-7} M to 10^{-3} M) of SNAP and NAP, respectively. Contractility data were recorded throughout the experiment. Data were log transformed and analyzed for main effects and appropriate interactions using a linear mixed-effects model (PROC MIXED, SAS) with repeated measures. Significance was set at $P<0.05$ with a Bonferroni correction for multiple comparisons. An interaction effect of group-by-concentration was observed ($P<0.0001$). The mean contractility after treatment with 10^{-4} M and 10^{-3} M SNAP were significantly lower than the pre-treatment contractility and the mean contractility after treatment with lower SNAP concentrations. In contrast, contractility did not change significantly in the NAP treated controls. The main effect of muscle layer and its interactions with group, concentration, and stage of estrous cycle were not significant. Other secondary findings included significant main effects of stage of the estrous cycle (increased contractility in estrus compared to diestrus), uterine histology grade (decreased contractility in grade IIB compared to grade I) and age (decreased contractility in mares aged > 8 years compared to mares aged ≤ 8 years). In conclusion, results of this study indicate that NO has a dose-dependent inhibitory effect on spontaneous uterine contractility irrespective of the muscle layer in the mare. The presence of increased NO concentrations in the uteri of mares susceptible to PBIE coupled with our findings that NO decreases uterine contractility constitutes a potential mechanism underlying development of PBIE in the mare.

Keywords: Equine, nitric oxide, uterine contractility, breeding-induced endometritis

The significance of eosinophils in equine uterine cytology: a retrospective study

V.C.L. Gomes,[a] M.R. Schnobrich,[b] K. Clemons,[b] M.S. Ferrer[a]
[a]Department of Large Animal Medicine, University of Georgia, Athens GA; [b]Rood and Riddle Equine Hospital, Lexington, KY

Eosinophils are occasionally seen in equine uterine cytology. Their presence is usually attributed to pneumometra, urometra, or fungal endometritis. However, the significance of these cells is still poorly understood. It was hypothesized that presence of eosinophils could be associated with a reduction in pregnancy rate. Therefore, a retrospective study was conducted to determine the prevalence of eosinophils on uterine cytologies, their association with endometritis, and their implication on reproductive performance. Records of uterine cytologies performed at Rood and Riddle Equine Hospital during 2013 and 2014 were analyzed (n=6,783), and mares with complete data sets were included in the analysis (n=231). Uterine cytology was considered positive for eosinophils if at least one eosinophil was seen in 10 high power fields. The reproductive history, cytological interpretation, uterine culture results, and pregnancy rate at 14 days after ovulation were evaluated. Thoroughbred mares presenting eosinophils on cytology and complete data sets were included in the analysis (n=79). The mares were divided into five groups according to findings on culture and cytology: 1) growth, inflammatory cytology (>2 neutrophils/field) and eosinophils; 2) growth, inflammatory cytology and no eosinophils; 3) no growth, non-inflammatory cytology and eosinophils; 4) growth, non-inflammatory cytology and no eosinophils; and 5) no growth, non-inflammatory cytology and no eosinophils. Frequency distribution of the data was analyzed using Chi-Square tests. Eosinophils were present in 1.69% of all cytologies (115/6783). Of the 111 mares that presented eosinophils, eight presented either urometra, repaired or unrepaired cervical defects (7.2%). Eosinophils were seen in cytologies of 78.4% (40/51) barren mares, 20.7% (36/174) foaling mares and 25% (1/4) maiden mares ($P<0.0001$). Eosinophils were present in 85.7% (30/35) of the mares with severe inflammatory cytology (>5 neutrophils/field), 53% (17/32) of the mares with moderate inflammation (2-5 neutrophils/field) and 19.5% (32/164) of the mares with non-inflammatory cytology ($P<0.0001$). The growth of a mixed population of organisms was seen more frequently in mares that presented eosinophils (12.8%) than in mares without eosinophils (1.3%). The culture results for mares presenting eosinophils included growth of a mixed population of organisms (12.8%), gram positive bacteria (19.2%), gram negative bacteria (9%), fungi (2.6%) and no growth (56.4%) ($P<0.001$). Only two mares grew fungi on culture, both of which had eosinophils on cytology. Pregnancy rates differed with mare classification, and were 42.2% (19/45) in group 1, 57.9% (11/19) in group 2, 65.7% (23/35) in group 3, 75% (18/24) in group 4, and 70.4% (76/108) in group 5 ($P<0.02$). Although there was a low prevalence of eosinophils in equine uterine cytologies, their presence was associated with reduced pregnancy rates. As expected, the pregnancy rate was reduced in mares that presented neutrophilic endometritis, but it was even lower in mares with concurrent neutrophilic and eosinophilic endometritis. Therefore, the presence of both cell types seems to indicate a more severe stimulus or inflammatory response, and a worse prognosis for fertility.

Keywords: Eosinophils, cytology, equine endometritis, fertility

A rodent model for non-surgical sterilization using an antibody-guided lipid nanoparticle cytotoxin delivery system

K. Meadows,[a] Q. Xu,[b] S. Ayres[a]

[a]Department of Biomedical Sciences, Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA; [b]Department of Biomedical Engineering, Tufts University, Medford, MA

According to the ASPCA, it is estimated that there are over 70 million stray dogs and cats in the United States. The development of a non-surgical method of sterilization has the potential to decrease pet overpopulation by reducing the mortality, cost and inconvenience associated with surgical sterilization. The goal of the current study was to develop a rodent model of non-surgical sterilization using an antibody-guided nanoparticle cytotoxin delivery system. We hypothesized that this could be accomplished by targeting the anti-Mullerian hormone receptor II (AMHRII), which is expressed primarily in the gonads. PEGylated lipids were used to form stable nanocomplexes tagged with a commercially available AMHRII antibody (Sigma Aldrich, St. Louis, MO) and loaded with the cytotoxin, saporin. Male and female Sprague-Dawley rats were injected intravenously (IV) with either saline, 2 nmol, 5 nmol, or 10 nmol of the antibody-nanoparticle-saporin complex. Additionally, two animals were injected with 10 nmol of complex directly into the testes or ovaries. Twenty four hours after injection, gonads were collected and flash frozen. Gonads were cryostat-sectioned and 20 μm sections were thaw-mounted directly onto charged microscope slides. A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit was used to detect DNA fragmentation as a result of apoptotic signaling cascades (Roche Applied Science, Indianapolis, IN). Fluorescent images were obtained with a Zeiss Axiovert 200M fluorescent microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) and quantification of tissue apoptosis was performed using ImageJ software (NIH, Bethesda, MD). Data were analyzed with GraphPad Prism (La Jolla, CA) using two-way analysis of variance (ANOVA) to compare the number of TUNEL labeled apoptotic cells with sex and dose as the condition factors. Post hoc analyses were conducted using Tukey's test and significance was set to $p<0.05$. There was a main effect of sex ($F_{[1,84]} = 446.2$, $p<0.01$), with males displaying more apoptotic cells than females. In addition, there was a main effect of dose ($F_{[3,84]} = 7.669$, $p<0.01$) and an interaction between sex and dose ($F_{[3,84]} = 6.545$, $p<0.01$). Male animals injected IV with 2 nmol of complex showed less TUNEL labeling than all other males. Female animals directly injected into the ovaries showed more TUNEL labeling than all other females. Saline injected animals showed negligible TUNEL signal. These data indicate that an AMHRII antibody-guided nanoparticle is capable of delivering cytotoxin and inducing apoptosis, particularly in the male rodent gonad. Future studies are underway to determine the specific cell types affected, the extent of sterilization, and the distribution of AMHRII binding using an in vivo imaging system (IVIS®).

Keywords: Sterilization, non-surgical, anti-Mullerian hormone, nanoparticles

Fertility following control of follicular wave emergence with GnRH and timing of insemination in 14-day CIDR protocol in Angus cross beef heifers

S. White,[a] R. Kasimanickam,[a] J.B. Hall[b]
[a]Department of Veterinary Clinical Sciences, Washington State University, Pullman, WA; [b]Nancy M. Cummings REEC, University of Idaho, Carmen, ID

Timing of insemination is critical for the success of artificial insemination (AI) programs. Success following early insemination in relation to time of estrus appeared to be limited by sperm life, leading to fertilization failure. Conversely, late timing of insemination yields reduced success due to declining embryo quality. This study compared AI pregnancy rate (PR) in Angus cross beef heifers between the 14-day CIDR-GnRH-PGF2α-GnRH and the 14-day CIDR-PGF2α-GnRH synchronization protocol with two insemination times, either 56 or 72 h after PGF2α administration. We hypothesized that administration of GnRH would provide better synchrony and improve AI-PR irrespective of timing of insemination in a 14-d CIDR protocol in beef heifers. On Day 0, heifers (n=1070) from seven locations in WA, ID and OR received a body condition score (BCS; 1 to 9; 1-emaciated, 9-obese), reproductive tract score (RTS; 1 to 5; 1-immature, acyclic; 5-mature, cyclic), and temperament score based on chute exit and gait (ES; 0-calm, walk; 1-excited, jump/trot/run). Heifers also received a controlled internal drug release insert (CIDR, 1.38 g of progesterone, inserted vaginally) for 14 days. Within the herd, heifers were randomly assigned to either no-GnRH (n=514) or GnRH (n=556) groups. On Day 23, heifers in the GnRH group received 100 μg GnRH (gonadorelin HCl, 2 mL, IM) and heifers in the no-GnRH group did not. On Day 30, all heifers were treated with 25 mg of PGF2α (dinoprost, 5 mL, IM). Heifers in both GnRH and no-GnRH groups were randomly assigned to either AI-56 or AI-72 groups, and artificially inseminated at either 56 or 72 h after PGF2α administration, respectively, and were concurrently given 100μg of GnRH. Heifers were examined for pregnancy status 50 to 70 days after AI by ultrasonography (Sonosite-S8, Universal Diagnostic Solutions, Oceanside, CA) of the uterus and its contents to differentiate whether heifers became pregnant after AI or natural service. The criteria considered were size of the amniotic vesicle, fetus and placentomes. The AI-PR was calculated as the number of heifers pregnant to AI, divided by the total number of heifers inseminated. The data were analyzed using a mixed model (Proc Glimmix of SAS, SAS version 9.4 Cary, NC) to determine the differences in the AI-PR. The variables included in the model were GnRH treatment, AI time, RTS, BCS, ES and GnRH treatment and AI time interactions. Artificial insemination sire and AI technicians, animal handlers and location nested within states were considered as random effects. Accounting for RTS ($P<0.05$), BCS ($P<0.1$), and temperament ($P<0.01$), AI-PR differed between GnRH (yes, 63.7% vs. no, 57.6%; $P=0.04$) and time of insemination (AI-56, 57.7% vs. AI-72, 64.0%; ($P=0.02$) groups. The GnRH treatment by AI time interaction influenced AI pregnancy (GnRH56, 63.2%; GnRH72, 64.1%; No-GnRH56, 51.9%; No-GnRH72, 63.8%; $P<0.05$). In conclusion, heifers synchronized with 14-d CIDR protocol that were inseminated at 72 h after PGF resulted in greater AI-PR irrespective of whether they received GnRH on Day 23 or not. Heifers required GnRH on Day 23 if they were to be inseminated at 56 h after PGF in order to achieve greater AI-PR.

Keywords: Beef heifers, synchronization, 14-d CIDR, GnRH, PGF2a, AI timing, AI pregnancy

Evaluation of post-thaw semen parameters for different extenders in white-tailed deer

J.L. Stewart, I.F. Canisso, E. Po, R.E. Ellerbrock, F.S. Lima, C.F. Shipley
Department of Veterinary Clinical Medicine, University of Illinois, Urbana, IL

White-tailed deer farming in the United States relies heavily on the use of frozen semen for dissemination of valuable genetics, yet there are limited data available on the most suitable extenders for deer sperm cryopreservation. Previous studies suggest that the use of soybean-based extenders is superior to most egg-yolk based extenders. However, to date, the use of a synthetic liposome-derived extender in white-tailed deer has not been critically evaluated. The objective of the current study was to compare semen parameters of white-tailed deer semen cryopreserved using three different extenders: (1) soybean-based (AM, Andromed®); (2) liposome-based (OC, OptiXcell®); and (3) egg yolk-based (OR, Ovine Red®). Our hypothesis was that white-tailed deer semen cryopreserved with AM or OC extenders would present superior post-thaw sperm motility, increased viability and acrosome integrity, and reduced DNA fragmentation when compared to OR extender. White-tailed deer (n = 8, mean age of 1.6 ± 0.1 yr, range 1-2 yr) were anesthetized with tiletamine-zolazepam (0.4 mg/lb) and xylazine (1 mg/lb) intramuscularly. Semen was collected by electroejaculation, and the ejaculate from each buck was divided equally amongst the three extenders. Each aliquot was extended to a final concentration of 120 million sperm/mL, cooled to 5°C, and then incubated at 5°C for 3 to 4 hr, according to manufacturers' recommendations. Semen was loaded into 0.5 mL straws and frozen manually by placing straws on a rack in liquid nitrogen vapor at a distance of 4 cm horizontally above the liquid nitrogen level for 10 min before submerging into the liquid nitrogen for final freezing and storage. Each semen straw was thawed in a 37°C water bath for 30s before post-thaw analysis. Percent of total sperm motility (TM) and progressive sperm motility (PM) were assessed for each sample using computer-automated semen analysis. Additionally, samples were stained with fluorescent probes for evaluation of sperm viability (SYBR-14/PI), acrosomal integrity (FITC-PNA/PI), and chromatin stability (acridine orange) using flow cytometry. Data were analyzed using a General Linear Models procedure for all analyses of variance in R. Data are expressed as mean ± SEM. Significance was set at $p < 0.05$. Total and progressive sperm motilities for AM (TM: 46 ± 8.8% and PM: 33 ± 7.7%), OC (TM: 56 ± 6.7% and PM: 40 ± 6.9%), and OR (TM: 51 ± 5.7% and PM: 28 ± 5.1) were not different ($p \geq 0.49$). There were no differences in sperm viability ($p \geq 0.31$), with the post-thaw population containing 78 ± 6.6%, 87 ± 3.5%, and 73 ± 8.0% viable sperm when cryopreserved with AM, OC, and OR extenders, respectively. There were no differences in acrosome integrity ($p \geq 0.18$) with 90 ± 7.4% (AM), 80 ± 12% (OC), and 77 ± 3.6% (OR) of viable sperm having an intact acrosome. The DNA fragmentation index also did not differ ($p = 0.68$) and was 7.2 ± 2.5% (AM), 7.3 ± 2.4% (OC), and 9.8 ± 2.0% (OR), which is consistent with normal fertility in other mammalian species. These results suggest that non-egg yolk based extenders are equally effective as a traditional egg-yolk based extender for cryopreservation of white-tailed deer semen. Although we did not directly assess fertility in this study, the semen parameters herein evaluated appear to indicate that all three extenders are suitable for use in clinical practice.

Keywords: Cryopreservation, flow cytometry, sperm quality, white-tailed deer.

Risk factors for canine dystocia and stillbirths

A.J. Cornelius, S.H. Cheong
College of Veterinary Medicine, Cornell University, Ithaca, NY

Studies that describe risk factors for canine dystocia and stillbirths typically do not have appropriate non-dystocia controls. This study aimed to 1) evaluate risk factors for canine dystocia; 2) assess risk factors for stillbirth in puppies, by examining all whelping and puppies born in a breeding population during a defined period. We hypothesize that dystocia risk is affected by maternal factors, and stillbirth risk is influenced by whelping and puppy parameters. A retrospective observational epidemiologic study was performed. Data were collected from all whelpings in a guide dog breeding facility from 2003 to 2015. The breeding colony consisted mostly of Labrador Retrievers with some German Shepherds and Golden Retrievers. Variables tested were: dam signalment (age, breed, parity, and dam ID), whelping information (duration, litter size, abnormalities, contraction quality, difficulty of whelping), interventions (calcium management, oxytocin management, cesarean section performed), and information on the puppy outcomes (survived or stillborn, weight). Statistical analysis was done using mixed-effect logistic regression models PROC GLIMMIX of SAS version 9.4. Repeated measures analysis was used for dystocia risk with parity and dam while litter was used as the random effect for stillbirth risk. The final model was built using a backward-stepwise method. A total of 696 litters from 265 bitches was analyzed. Overall dystocia rate was 24.9%. Parity and maternal age ranged from one to eight and dystocia decreases as age-of-dam and parity number increases. First-parity litters had odds ratios (OR) of 1.6, 1.7, and 5.4 for dystocia compared with second, third, and fifth-parity-litters, respectively. Maternal age was also associated with dystocia risk. Bitches under two-years-of-age had a dystocia rate of 19% and increased to 31% in two-year-olds. Then dystocia rates decreased to 26%, 21%, 11% and 6%, respectively for three, four, five, and six-year-olds before increasing again to 50% in bitches > six years. Age-at-first-parity also affected dystocia risk where bitches having her first litter after turning two-years-old had 2.4 times higher likelihood of dystocia compared with bitches having her first litter between one and two years-of-age. There was a significant effect of the bitch on dystocia risk, suggesting individual predisposition. A total of 5,455 puppies were born with a 4.7% stillbirth rate. Factors that affected stillbirths were: large (OR = 2.3) and small (OR = 9.2) puppies in the top and bottom 2.5% of birth weights, puppies that were assisted in delivery (OR = 3.9), animals delivered by cesarean section (OR = 2.1), unusual birth position (OR = 4.8), and oxytocin use (OR = 1.5). There was a tendency for litter size effect on dystocia, but calcium use and age of dam were not associated. Dystocia risk generally decreases with increasing maternal age and parity. There were only six litters born from bitches >six years-of-age but 50% required intervention. Stillbirth risk increased as expected in large and small puppies, abnormal position and obstetrical interventions such as cesarean section and oxytocin use. Surprisingly litter size, maternal age, and calcium use did not increase stillbirth rates. We report the significant risk factors and for canine dystocia and stillbirths from data of all whelping and births from a breeding colony.

Keywords: Canine, dystocia, stillbirth, risk-factors,

Evaluation of lipid content, mitochondrial polarity, and cryotolerance of Holstein *in vitro*-produced embryos following culture with vitamin K_2, forskolin, and conditioned medium

M.A. Roberts,[a] D.J. Gumber,[a] M. Barceló-Fimbres,[b] J.L. Altermatt,[a] L.F. Campos-Chillón[a]
[a]Department of Animal Sciences, Cal Poly, San Luis Obispo, CA; [b]AniCell Biotech LLC, Chandler, AZ

Accumulations of intracellular lipids and reactive oxygen species in Holstein *in vitro*-produced (IVP) embryos decrease their developmental competence and subsequent cryotolerance. We hypothesized that during culture combining metabolic regulators and conditioned media (PYC) would reduce oxidative stress, promote lipolysis, and improve post-thaw survival of IVP embryos. Study objectives were to analyze individual and combined effects of vitamin K_2 (VK_2), forskolin (FSK), and PYC on lipid content, mitochondrial polarity, and percent of apoptotic cells after thawing. Embryos were produced in vitro by standard procedures. Briefly, oocytes (n=1745) were aspirated from 2 to 8 mm follicles of abattoir ovaries, matured for 23 h, fertilized using semen from two different bulls, and cultured in a continuous chemically defined medium at 38.5°C in 5% O_2, 5% CO_2, and 90% N_2. All chemicals were obtained from Sigma-Aldrich, St. Louis, MO. Presumptive zygotes were randomly assigned to treatment groups during culture: 0 and 0.5 mM VK_2 at day 3; 0 and 10 µM FSK at day 5; 0 and 5% PYC at day 1, and all combinations of these in four replicates. Blastocysts (stage 7) were stained with 1 µg/mL Nile red and 300 nM MitoTracker Red CMX-rosamine to quantify lipid content and mitochondrial polarity, respectively, or cryopreserved by standard slow freezing methods intended for direct transfer (1.5 M ethylene glycol and 0.5 M sucrose in holding medium). Frozen embryos were thawed and re-expansion rates assessed at 36 h. Surviving embryos were stained with DAPI and underwent TUNEL assay to determine percentage of apoptotic cells. Ten images per embryo were acquired by confocal microscopy using a 5µm step size at 40X magnification. Image PRO software was used to measure fluorescence intensity of Nile red and MitoTracker red, as well as number of cells after TUNEL assay using a cell counter plug-in. Data (Table) were analyzed by ANOVA and means compared by LSD using SAS. Results indicate that each additive had no effect on at least one qualitative parameter; however, an interaction may exist between additives which improves individual effects. In conclusion, a combination of VK_2, FSK, and PYC improves cryotolerance of Holstein IVP embryos by increasing developmental competence.

Table.

	Control	PYC	VK_2	FSK	PYC + VK_2	PYC + FSK	VK_2 + FSK	PYC + VK_2 + FSK
Blastocyst (%)	22.5^{b}	29.4^{ab}	30.5^{ab}	23.5^{b}	33.1^{a}	27.4^{ab}	28.0^{ab}	33.3^{a}
Nile red (AFU)	197^{ab}	206^{a}	131^{c}	129^{c}	169^{abc}	157^{bc}	141^{c}	143^{c}
MitoTracker red (AFU)	5584^{b}	7267^{a}	3940^{cd}	4832^{bc}	5189^{bc}	4113^{cd}	2807^{d}	4034^{cd}
Apoptotic Cells (%)	31.2^{ab}	28.1^{ab}	34.5^{a}	32.8^{ab}	17.5^{ab}	24.2^{ab}	16.5^{b}	20.2^{ab}
Re-expansion Rate (%)	31.0^{b}	39.3^{ab}	51.9^{ab}	38.9^{ab}	81.3^{ab}	54.5^{ab}	77.7^{ab}	91.4^{a}

[a,b,c] Values within rows with different superscripts differ ($P<0.05$).

Keywords: Vitamin K_2, forskolin, lipid, mitochondrial polarity, cryopreservation

Role of biofilm in infectious endometritis in the horse

M.L. Hennet,[a] R.A. Ferris,[a] G.I. Borlee,[b] P.M. McCue,[a] B.R. Borlee[b]
[a]Department of Clinical Sciences and [b]Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO

Treatment of chronic bacterial endometritis with antimicrobials is often unsuccessful. Bacteria have developed multiple mechanisms to tolerate antimicrobial treatment, which include the production of biofilm. Therefore, this project aimed to investigate the localization of bacteria within a biofilm, determine the best diagnostic techniques to detect a biofilm, and evaluate host immune reaction to bacteria in a biofilm using a model of equine bacterial infection. We hypothesized that a bacterial biofilm would be detected in focal areas on the surface of the endometrium, Bouin's solution would be the ideal fixative for detection of biofilm matrix, and the host immune response would be similar in areas with and without the presence of a biofilm. Six mares were inoculated with 10^6 CFUs of three equine *Pseudomonas aeruginosa* isolates genetically modified to constitutively express luminescence genes. The infection was allowed to develop for five days, at which point the mares were euthanized and the reproductive tracts were removed. The endometrial surface was gently rinsed to remove non-adherent cells and imaged for luminescence to localize the adherent labeled bacteria. Samples from areas of endometrium with and without adherent bacteria were collected for cytology, histopathology, carbohydrate analysis, and gene expression of inflammatory cytokines. Categorical data were compared by a contingency table utilizing Fishers Exact test, and continuous data were compared using least square means. A significant difference was considered if $p<0.05$. Adherent bacteria were present in focal areas between the endometrial folds at the base of and extending into both uterine horns (6/6 mares). The biofilm exopolysaccharide Pel and cyclic di-GMP, a small molecule that regulates biofilm formation, were detected in endometrial samples with adherent bacteria and had a greater incidence (5/6 mares and 6/6 mares respectively) as compared to endometrial samples without bacteria (0/2 and 0/2 mares, respectively) ($p<0.05$). A greater ($p<0.05$) incidence of adherent material was present on histology for samples fixed in Bouin's solution (18/18) as compared to buffered formalin fixed tissue (0/18). There were no differences ($p>0.05$) in the number of inflammatory cells in the endometrium between Bouin's and formalin fixation, the uterine body and horn, or endometrium with and without adherent bacteria. No differences ($p>0.05$) in gene expression were detected for ten host inflammatory genes in the endometrium from areas with and without adherent bacteria. There were increased ($p<0.05$) neutrophils on cytology between areas without adherent material (>5 WBC/HPF) and areas with adherent material (0-2 WBC/HPF).

In conclusion, a biofilm was identified in all six *in vivo* experimental cases of equine endometritis, and the resulting inflammatory response was consistent throughout the uterus. It is recommended that uterine biopsy samples be fixed in Bouin's solution in order to detect the presence of a biofilm. Future studies will focus on therapeutic options for elimination of bacterial biofilms in the equine uterus.

Keywords: Equine, bacterial endometritis, *Pseudomonas aeruginosa*, biofilm

Performance of a rapid slide agglutination test for *Brucella canis* with canine sera containing antibodies to *Leptospira spp.*

M. Krecic,[a] J. Brooks,[a] J. Lizer[b]
[a]Zoetis, Florham Park, NJ; [b]Zoetis, Kalamazoo, MI

Brucella canis is a gram negative bacterium that is a cause of canine infertility and abortion. Veterinarians screen for antibodies to *B. canis* with a variety of serological tests including the point-of-care rapid slide agglutination test (RSAT), D-Tec® CB (Zoetis, Florham Park, NJ). False positive results are possible because of cross-reactivity to antibodies produced to other gram negative bacteria, such as *Leptospira* spp. Cross-reactivity between *Brucella* spp. and *Leptospira* spp. antibodies produced through infection or vaccination with the serological tests for brucellosis has been reported for cattle;[1,2] yet, to our knowledge has not been confirmed for dogs. Therefore, we evaluated D-Tec® CB with the sera from dogs experimentally-infected with *Leptospira kirschneri* serovar Grippotyphosa and from dogs suspected for natural infection with *Leptospira* spp. We evaluated sera collected at day zero and between days six and 13 from eight dogs experimentally-infected with Grippotyphosa and sera collected from 20 client-owned field dogs with acute clinical signs compatible with leptospirosis. Microscopic agglutination testing (MAT) was used to confirm experimental and natural infections. All eight experimentally-infected dogs seroconverted (MAT $\geq$ 800) between days 3 and 10. To diagnose leptospirosis for the field dogs, a MAT cut off of $\geq$ 800 was used when the dog was known to have no previous leptospirosis vaccination or the last vaccination was > one year prior. When the vaccination history was unknown, positive confirmation was defined by three possible MAT outcomes: 1) at least one serovar $\geq$ 800 and at least one other serovar $\geq$ 400; 2) a single serovar $\geq$ 3200; or 3) a non-vaccine serovar $\geq$ 800. The sera from 10 of the 20 field dogs fulfilled these criteria. We tested a total of 36 samples with D-Tec® CB. The sera collected pre- and post-experimental Grippotyphosa infection (n = 16) yielded negative test results for canine brucellosis. The sera from the 10 MAT-negative field dogs also yielded negative test results for brucellosis. Of the sera from the 10 MAT-positive field dogs, only one sample yielded an initial weak positive test result but tested negative with the addition of 2-mercaptoethanol. Although antibodies produced to *Leptospira* spp. may cross-react with *Brucella* antigen with the D-Tec® CB,[3-5] cross-reactivity may not be as common as purported.

Keywords: Brucellosis; leptospirosis; canine

References

1. de Faria Naves JHF, Rezende LM, Ramos GC, et al: Interference in diagnostic tests for brucellosis in cattle recently vaccinated against leptospirosis. J Vet Diag Invest 2012;24:283-287.
2. Moshkelani S, Javaheri-Koupaei M, Rabiee S, et al: Detection of *Brucella* spp. and *Leptospira* spp. by multiplex polymerase chain reaction (PCR) from aborted bovine, ovine and caprine fetuses in Iran. African J Microbiol Res 2011;5:4627-4630.
3. de Oliveira MZD, Vale V, Keid L, et al: Validation of an ELISA method for the serological diagnosis of canine brucellosis due to *Brucella canis*. Res Vet Sci 2011;90:425-431.
4. Carmichael LE, Zoha SJ, and Flores-Castro R: Problems in the serodiagnosis of canine brucellosis: dog responses to cell wall and internal antigens of *Brucella canis*. Develop Biol Standard 1984;56: 371-383.
5. Hollett RB: Canine brucellosis: outbreaks and compliance. Theriogenology 2006;66:575-587.

Effect of methanolic extract of *Spondias mombin* on estrous cycle, conception rate and gestation in rabbits

A.T. Ayisat, A. Deborah, S. Frederick
Department of Veterinary Public Health and Reproduction, Federal University of Agriculture, Abeokuta, PMB 2240, Nigeria

Several hormonal contraceptive pills have been developed with none being free from side effects. *Spondias mombin* (SM) plant is reported with possible contraceptive effect, however, the reproductive effects of SM appears to be at variance . This study evaluated the effect of SM on estrous cycles, conception and pregnancy in rabbits. Sexually matured Chinchilla rabbit does (25; Mean weight: 1.94 ± 1.06kg) were used for the study. In phase one, fifteen rabbits were synchronized with follicle stimulating hormone (FSH; 2 mg/kg i.m.) at 12 hour intervals for three days. Three days after, they were randomly assigned into three groups of five rabbits each. Group 1 (control group) received 1ml of saline solution orally for thirty days. Group 2 received 800 mg/kg methanolic extract of SM orally for thirty days, while Group 3 received a single treatment of melengesterol acetate (50mg/kg i.m.). Blood was obtained from the jugular vein at intervals of ten days for thirty days for determination of plasma concentration of luteinizing hormone (LH), FSH, estrogen and progesterone. Thereafter, the does were mated and subjected to laparotomy one week after maing to determine the number of embryonal sacs. In phase two, ten does were mated and confirmed pregnant seven days after mating using transabdominal ultrasonography. They were treated with 800 mg/kg methanolic extract of SM orally at ten days after mating and twenty days after mating. The does were observed daily until they either gave birth or aborted. Data were presented as mean ± SD and compared between groups using either student's t test or repeated measures ANOVA, with significance set at P=0.05. Luteinizing hormone was significantly lower in Group 3 than control and Group 2 by day 20 after treatment. However, FSH was significantly lower in Group 3 compared with control and Group 2. Concentrations of plasma progesterone significantly decreased in both Group 3 and 2 up to day 20 after treatments, while concentrations of plasma estrogen were significantly greater in control and Group 3 than Group 2. There were no embryonal sacs in Group 3 treated rabbits at day 7 after mating, while the number of embryonal sacs in Group 1(5.6 ± 1.3) and Group 2(5.6 ± 1.4) were not significantly different. Following the oral administration of SM by day 10 after mating all does had vaginal bleeding 24 hours following treatments; one had a complete abortion and the remaining four does kidded with a mean litter size of 3.25 kits. Does treated with methanolic extract of SM at twenty days after mating did not have vaginal bleeding and kidded with an average litter size of 6.4 kits. It was concluded that methanolic extract of SM at 800 mg/kg did not adversely affect reproductive cyclicity and pregnancy but caused abortion during early pregnancy in Chinchilla rabbits.

Keywords: Contraceptive, hormonal, chinchilla rabbit, ultrasound, *Spondias mombin*

Mammary hyperplasia in a Main Coon queen

D.M. Best,[a] C. Card,[b] M. Diel de Amorim[a]
[a]Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada; [b]Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK Canada

Mammary fibroepithelial hyperplasia or fibroadenomatous hyperplasia (FAH) in cats is a benign generalized enlargement of one or more mammary glands reported in young queens during early pregnancy or pseudopregnancy, or in cats that have received an exogenous source of progesterone. A two-year old Main Coon queen that had been imported from Russia was presented to the Atlantic Veterinary College (AVC) due to a severe mammary gland enlargement. The queen had been exposed to a tomcat, and breeding. A typical after-mating reaction was reported to have occurred on several occasions. Mating occurred a month prior to presentation. On presentation, the queen was active, bright, alert and responsive, and physiological parameters were within normal range. The owner reported that the queen had a normal appetite and was still very playful. The chief complaint was the presence of severe mammary gland enlargement. Both right and left mammary glands, which included the most cranial thoracic and the most caudal abdominal/inguinal were swollen. However, both caudal mammary glands were the most affected and showed excessive enlargement with areas of purplish coloration, and areas suggestive of mild self-inflicted trauma. A presumptive diagnosis of FAH was made based on the reproductive history and examination findings. Transabdominal ultrasound revealed the queen to be non-pregnant. Palpation of the mammary gland did not elicit signs of pain and discomfort. The owner opted to not measure the queen's blood concentration of progesterone, and agreed to have the queen started on therapy with aglepristone, a progesterone-receptor antagonist. The queen was treated with two injections of aglepristone 24 hours apart at 15 mg/kg subcutaneously, and seven days later two additional treatments were administered seven days apart at a dose of 10 mg/kg subcutaneously. No injection site reaction was noted. Before every treatment, the queen was revaluated for mentation, physiological parameters and reassessment of the mammary glands was performed using serial photographs to evaluate and document the degree of improvement reflected by the decrease of mammary gland size. Pain management, anti-inflammatory, and progesterone measurement were considered in this case, however due to financial constraints, clinical presentation and continuous improvement, progesterone receptor antagonist treatment alone was chosen. Three days after the second injection the cat had a remarkable improvement and continued to have the mammary gland decrease in size every week. Complete resolution of the mammary hyperplasia occurred by three weeks after the initial treatment. A lower dose of aglepristone once weekly was sufficient to resolve the FAH problem within three weeks in this case. Further studies are warranted on the understanding the full pathogenesis of this disease.

Keywords: Mammary hyperplasia, fibroadenomatous hyperplasia, queen, aglepristone

Treatment of clitoral hypertrophy by urethral transposition in an intersex dog

E. Wu, C. Hall, S. Sathe
Department of Veterinary Clinical Science, College of Veterinary Medicine, Iowa State University, Ames IA

The normal canine chromosomal make-up is 78, XX for females and 78, XY for males. Chromosomal disturbances can lead to various syndromes and associated complications such as clitoral hypertrophy in females and ambiguous clinical syndromes in males. A seven-month-old spayed female pit bull mix presented with a history of a reddish colored mass protruding from the ventral vulvar commissure. She had experienced vulvar irritation and polyuria for the past several weeks likely due to the vulvar mass. The female was also suffering from occasional urinary incontinence. Further investigation found the bitch was adopted two months prior from a local shelter and it was noted during a routine spay procedure that testicular tissue instead of ovaries was present along with an otherwise complete internal female genital tract.

Upon physical examination, a reddish pink mass was noted protruding from the ventral commissure of an infantile vulvar opening. On palpation, the mass was non-painful and a firm structure, suspected as an os clitoris, extended deep into the vaginal canal. The urethra was closely attached to the mass and the urethral opening was identified near the tip of the structure. Surgical removal of the mass and urethral transposition were elected. After an episiotomy incision, urethral catheterization allowed identification of the underlying clitoral mass that was dissected using electrocautery. Urethral transposition to the underlying vaginal mucosa was performed and its patency confirmed with catheterization. The dissected tissue was approximately six centimeters in length and bony tissue was identified on gross examination. A blood sample was collected in sodium heparin and the plasma was sent to the University of California Davis Veterinary Genetics Laboratory for karyotyping. Results of the karyotyping showed a SRY negative, typical female profile (78, XX). The bitch recovered uneventfully from surgery and did not experience any further vulvar irritation or polyuria-like episodes. For continued urinary incontinence, treatment with oral estriol (Incurin™, Merck Animal Health, Summit, NJ) at 2 mg per day was initiated shortly after surgery. Unfortunately, the long-term efficacy of surgical treatment is unknown. Sexual differentiation in females occurs earlier than males with the regression of the Wolffian (mesonephric) ducts and maintenance of the Mullerian (paramesonephric) ducts which eventually form the uterine tubes, uterus and cranial vagina. In males, the Sertoli cells release anti-Mullerian hormone (AMH) causing the regression of the Mullerian duct while the Wolffian ducts continue to differentiate into the epididymis and vas deferens. XX sex reversal is a condition associated with a normal female phenotype but in the presence of testicular tissue. Females are usually phenotypically true and discovery of the condition is through infertility or a secondary finding during a routine spay procedure. In veterinary medicine, current treatments vary from hormonal therapy to surgical intervention.

Keywords: Dog, XX sex reversal, intersexuality, clitoral hypertrophy

Canine thyroid gland expresses luteinizing hormone receptors

K. Zwida, M. Kutzler
Department of Animal and Rangeland Sciences, Oregon State University, Corvallis, OR

Hypothyroidism is a complex disease with a net effect of inadequate thyroid hormone receptor activation.[1] The incidence of hypothyroidism is 10-15% in postmenopausal women[2] and 30% in gonadectomized dogs.[3] Circulating luteinizing hormone (LH) concentrations are significantly and persistently elevated in both of these populations. Luteinizing hormone receptors are expressed in normal human thyroid glands.[4] We hypothesized that LH receptors were also present in normal canine thyroid glands. The aim of this study was to determine if LH receptors were expressed and to quantify the level of cellular expression. Thyroid and bladder tissue were removed from two dogs (two month old Labrador Retriever female and six month old Labrador Retriever mix spayed female) postmortem, formalin-fixed, paraffin-embedded, and sectioned (6 μm) onto charged slides. Testicular tissue from a separate dog obtained following castration was treated in the same manner. All slides were deparaffinized, rehydrated, subjected to heat-induced epitope retrieval (#S1700, Dako, Carpinteria, CA). Endogenous peroxidase activity was inactivated with 3% H_2O_2 and nonspecific binding was blocked with 1% horse serum. Goat polyclonal anti-human LHR antibody (SC-26341, Santa Cruz Biotechnology, Dallas, TX,) was applied at a 1:50 dilution. Negative controls from each tissue were treated in the same way except in absence of primary antibody. Slides were then reacted with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA) and incubated with preformed avidin-biotin-peroxidase complex (#PK6105, ABC kit, Vector Laboratories) followed by Nova Red Peroxidase substrate (#SK4800, Vector Laboratories). Slides were counter-stained with hematoxylin, dehydrated, and mounted. The percentage of cells positive for LHR was determined at 400X magnification. Canine thyrocytes expressed LH receptors in 5% and 19% of the cells counted. Luteinizing hormone receptor expression was also evident in bladder tissue from the same animals as well as in positive control testis. There was no positive staining evident in any of the negative control tissue sections. This is the first report of LH receptor expression in the canine thyroid. Additional studies are needed to determine if the unregulated hypersecretion of LH after gonadectomy is responsible for the incidence of hypothyroidism observed in these dogs.

Keywords: Dog, gonadectomy, hypothyroidism, immunohistochemistry

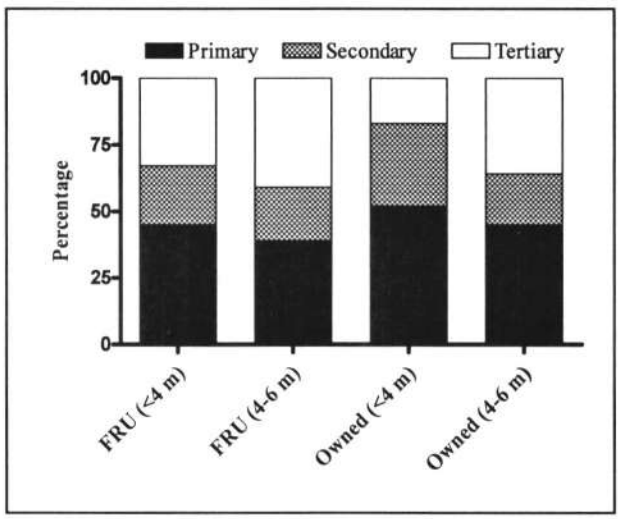
References

1. Mooney CT: Canine hypothyroidism: a review of aetiology and diagnosis. N Z Vet J 2011;59:105-114.
2. Giri A, Edwards TL, LeGrys VA, et al: Subclinical hypothyroidism and risk for incident ischemic stroke among postmenopausal women. Thyroid 2014;24:1210-1217.
3. Milne KL, Hayes HM Jr: Epidemiologic features of canine hypothyroidism. Cornell Vet 1981;71:3-14.
4. Liu J, Chen G, et al:. Serum levels of sex hormones and expression of their receptors in thyroid tissue in female patients with various types of thyroid neoplasms. Pathol Res Pract 2014;210:830-835.

Early onset of reproductive capacity in free-roaming unowned queens

Ellie Bohrer, Michelle Kutzler
Department of Animal and Rangeland Sciences, Oregon State University, Corvallis, OR

Our laboratory is interested in determining if an underlying biological cause exists for the exuberant reproductive success observed in free-roaming unowned (FRU) cats. The hypothesis of the current study was that FRU queens had more tertiary follicles at a younger age compared to owned queens. The study objective was to evaluate histological ovarian follicle classifications from FRU and owned cats at <4 months of age and 4-6 months of age. Queens were presented for ovariohysterectomy at a local humane society in August and September 2015. Queens were grouped by age (<4 months (owned n=5, FRU n=10) and 4-6 months (owned n=2, FRU n=7)). Age and life history data from cat colony managers were combined with dental eruption patterns to accurately estimate the age for FRU queens. A routine ovariohysterectomy was performed under general anesthesia. The total ovarian uterine weights from FRU queens were also recorded.



Both ovaries were hemi-sectioned, formalin-fixed, paraffin-embedded, cut into sections (6 μm), and stained with hematoxylin and eosin. Slides were analyzed using bright field microscopy at 200X by a single observer (EB), blinded to the individual's age group and living status. Follicles were counted and classified as primary, secondary, or tertiary. Mean±SD percentages for tertiary follicles were compared between FRU and owned cats <4 months of age using a Student's t test. Total ovarian uterine weights were analyzed via linear regression. Significance was defined as $p<0.05$.FRU cats <4 months old had more tertiary follicles compared with owned cats <4 months of age (33% and 17%, respectively; $p<0.05$; see Figure). As evidence of ovarian follicle endocrine function, total ovarian uterine weights were significantly greater in 4-6 month old FRU queens compared to <4 months (1.18±0.31 g vs 0.93±0.28 g, respectively). Unlike the owned cats in the current study, female FRU cats appear to be developing reproductive capacity at earlier ages (<4 months). This observation was supported by work in research colony cats.[1] The implication of this indicates that sterilization programs need to include kittens in their efforts.

Keywords: Feral cats, folliculogenesis, ovary, uterus

Reference

1. Faya M, Carranza A, Miotti R, et al: Fecal estradiol-17b and testosterone in prepubertal domestic cats. Theriogenology 2013;80:584-586.

Hormonal, biochemical and hematological changes during gestation in rabbit does synchronized with prostaglandin F_2 alpha

A.T. Ayisat, N. Mercy, S. Fredrick
Department of Veterinary Public Health and Reproduction, Federal University of Agriculture, Abeokuta, PMB 2240, Nigeria

Assessment of the physiological parameters such as hormonal, biochemistry and hematology of animals at different stages of gestation is helpful to monitor the health and nutritional status of animals. This study therefore evaluated the changes in hormonal levels, biochemical and hematological parameters during gestation in domestic rabbit (*Oryctolagus cuniculus*) does following estrus synchronization with prostaglandin F2alpha ($PGF_2\alpha$). Eight nulliparous, sexually matured intact New Zealand rabbit does with mean weight of 1.9±0.1kg were used for the study. They were distributed into eight hutches, and were synchronized with 0.7 mg/kg BW i.m. injection of $PGF_2\alpha$ prior to mating. After 48 hours, the eight does were naturally mated with four bucks each (within two hours each doe was allowed to mate with four bucks to maximize chances of pregnancy). Does were examined for pregnancy using ultrasonography seven days after mating. Blood was sampled once every week from the jugular vein before mating (BM), 7 days after mating (7DPM), 14 days after mating (14DPM), 21 days after mating (21DPM), 28 days after mating (28DPM) and 3 days after parturition (3DPP), respectively. Blood plasma progesterone, FSH, estrogen and prolactin were assayed using enzyme linked immunosorbent assay. Hematological and biochemical parameters determined were packed cell volume (PCV), hemoglobin (Hb) concentration, red blood cell (RBC) count, white blood cell (WBC) count, cholesterol, triglycerides, high density lipoproteins (HDL) and low density lipoproteins (LDL). Data obtained for hormone and serum biochemistry were subjected to descriptive statistics, while other data were subjected to analysis of variance using general linear model procedure of statistical software. Results revealed that mean values for progesterone, FSH, estrogen and prolactin during gestation significantly varied at different periods of the experiment ($p<0.05$). Progesterone secretion during gestation peaked at 14DPM (32.1 ± 0.27 ng/ml). Estrogen secretion was at BM (857.2 ± 3.22 ng/ml), at 14DPM (857.5 ± 3.80 ng/ml) and increased to 866.6 ± 2.17 ng/ml at 28DPM but subsequently declined to 850.7 ± 6.04 ng/ml at 3DPP. Prolactin increased from 92.3 ± 0.13 ng/ml at BM to 92.8 ± 0.06 ng/ml at 7DPM, but decreased to 91.8 ± 0.36 ng/ml at 14DPM then increased to 92.5 ± 0.20 ng/ml at 3DPP. Cholesterol, triglyceride and LDL were not significantly ($p>0.05$) influenced by the period of sampling. The PCV, RBC, Hb, and WBC significantly varied from BM to 3DPP ($p<0.05$). The RBC, PVC, Hb and WBC counts decreased gradually from BM to 28DPM and subsequently increased until 3DPP. The study concluded that there were changes in hormonal parameters, PCV, RBC, Hb, WBC, and LDL while cholesterol, triglyceride, HDL and the WBC differential counts showed no changes during the study period.

Keywords: Gestation, New Zealand white rabbit, progesterone, parturition

Expression of retinoic acid synthesizing enzymes, ALDH1A1, ALDH1A2 and ALDH1A3 in canine testis during post-natal development

Vanmathy Kasimanickam
Department of Veterinary Clinical Sciences and Center for Reproductive Biology, Washington State University, Pullman, WA

Spermatogenesis is a highly regulated process of cell division and differentiation. Sperm production is organized temporally and spatially and is carefully controlled by several signaling molecular processes. Among these signaling events, retinoic acid (RA) signaling is considered as indispensable since the RA promotes spermatogonial differentiation, adhesion of germ cells to Sertoli cells, and release of mature spermatids into the lumen of seminiferous tubules. Evidence suggests that RA induces expression of the critical pre-meiosis gene, Stra8, in germ cells of pubertal testis and adult testis. To appropriately activate the retinoid signaling pathway during spermatogenesis, optimal testicular RA level is necessary. The suitable testicular RA level is maintained via RA synthesis and degradation. The metabolism of RA is tightly regulated by a group of RA synthesizing- and degrading-enzymes. The retinaldehyde dehydrogenase enzymes (ALDH1A1, ALDH1A2 and ALDH1A3) are responsible for the oxidation of RA precursors to produce RA. The goal of the study was to investigate the expression pattern of the retinoic acid synthesizing enzymes ALDH1A1 (XM_533525), ALDH1A2 (XM_535494) and ALDH1A3 (XM_003638965) in canine testis during post-natal development at both mRNA and protein levels. Real-time polymerase chain reaction was performed to determine the relative quantity of ALDH1A1, ALDH1A2 and ALDH1A3 at mRNA level in young, peripubertal adult dog testes, and Western blotting was used to specifically detect their proteins' presence in testes. Canine β-actin was used as a reference gene for normalization of CT (cycle threshold) values. The $2^{-\Delta\Delta CT}$ model was used for relative comparison, and the p-value was derived using ΔΔCT estimation to determine the statistical significance ($p \leq 0.05$). Expression of ALDH1A2 transcript was significantly greater in young, peripubertal and adult testes on comparison of three enzymes within age groups. Within enzyme groups, ALDH1A2 expression highly varied among young, peripubertal and adult testes, and the peripubertal testis had the greatest expression of ALDH1A2. The proteins' presence for all three enzymes was found in young, peripubertal and adult testes, but the protein level was not quantified in the study. Although genes encoding all three RA synthesizing enzymes were expressed in dog testes at various developmental ages, the study concludes that ALDH1A2 might be a critical enzyme in pertinent to spermatogenesis and testicular function, based on the highest level of expression identified at the peripubertal stage in which re-entry of meiosis happens, and the significant level seen at adult stage during which continuous sperm production occurs.

Keywords: Retinoic acid signaling, post-natal testicular development, dog; real-time PCR, immunoblotting

Fusion of boar sperm with nanoliposomes prepared from synthetic phospholipids

Vanmathy Kasimanickam*
Department of Animal and Poultry Science, University of Guelph, Guelph, ON, Canada

Liposomes are artificial membrane vesicles that can be used to test and model the functions and interactions of various biological membranes, as a carrier system to deliver biologically active substances into the cells, or to incorporate lipids into the plasma membrane of target cells to modify membrane structure-function relationships. Sperm plasma membrane undergoes lipid modification during maturation in epididymis, and during capacitation in the female reproductive tract to facilitate fertilization. Natural variation in the amounts and composition of lipids in the sperm plasma membrane may contribute to the species-specific sperm sensitivities to handling and storage conditions. Boar sperm are notoriously susceptible to membrane damage, and are resistant to compositional alteration by artificial liposomes. The current study used flow cytometry to demonstrate highly efficient and stable incorporation of nanoliposomes prepared from a complex mixture of various phospholipids (phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylserine, phosphatidylinositol) into boar sperm. Small unilamellar liposomes ranging from 20nm-100nm size were prepared using bath sonication. A fluorescent dye, 5 octadecanoylamino fluorescein at 2 mole % final concentration was incorporated in the liposomes to monitor the fusion. Five ejaculates (replicates) from three mature Yorkshire boars were used. Sperm at concentrations of 1×10^7 or 4×10^7 sperm/mL were mixed with fluorescently-labelled liposomes (final concentration 0.3241μmole/mL) and analyzed at 1, 10, 30 or 60 min using an Epics flow cytometer. Unlabelled sperm, fluorescently-labelled liposomes and fluorescently-labelled fused events (sperm & liposome) were identified and quantified. Cytograms and dot plots assessed the percentage of sperm fused to liposomes and the percentage of liposomes fused to sperm, using the number of events in the appropriate gated area. The GLM procedure analyzed the square root and arcsin-transformed percentage data. Variances were found to be homogenous for the treatment means. The transformed data were analyzed by factorial design and compared by using orthogonal contrasts. The two sperm concentration were compared by ANOVA, analyzing each time period separately because their interaction was significant. Time, sperm concentration and their interaction significantly ($p \leq 0.001$) affected the percentage of sperm fused to liposomes. Tukey's test showed that the percentage of sperm fused to liposomes at 1 min was significantly lower than that at other time periods for 4×10^7 sperm/mL concentration, but did not change over time for 1×10^7 sperm/mL concentration. Overall, the percentage of liposomes used by both concentrations of sperm did not change significantly over time. In conclusion, over 90% of sperm rapidly took up fluorescently labelled liposomes and retained the lipids for at least 60 minutes, in a significant time- and concentration-dependent manner. This unique fusion efficacy could be used to alter sperm plasma membrane composition and hence membrane-based functional responses.

Keywords: Nanoliposomes, boar sperm, flow cytometry, fusion efficiency

*Current address: College of Veterinary Medicine, Washington State University, Pullman, WA

Do sperm and seminal plasma microRNAs have similar patterns of expression in boar semen?

Vanmathy Kasimanickam
Department of Veterinary Clinical Sciences and Center for Reproductive Biology, Washington State University, Pullman, WA

Although sperm and seminal plasma differ in their origin, physical and chemical properties of seminal plasma influence sperm function. Seminal plasma is a fluid medium containing substances from the testes, epididymides and accessory glands. Composition of seminal plasma varies among animal species and in boars, vesicular glands, prostate and bulbourethral glands are major contributors to the volume and contents. While the origin of some components of seminal plasma are known, the source of recently discovered seminal plasma microRNAs is unknown, in part because of the difficulty of recovering and characterizing RNA from porcine sperm and seminal plasma. To test the hypothesis that seminal plasma miRNAs interact with sperm, the current study first validated protocols for recovering RNAs from porcine seminal plasma and sperm. We then characterized the expression pattern of 84 prioritized microRNAs employing a real time PCR strategy. For RNA isolation protocol validation, nine sperm samples and nine seminal plasma samples from nine superior Landrace boars were used. Three sperm samples and three seminal plasma samples from three Landrace boars were utilized for miRNA profiling. Total RNA containing small RNAs was isolated from each boar sperm using RNeasy plus Universal Mini Kit, following the manufacturer's instructions with some modifications. Small RNAs were purified from individual boar seminal plasma using miRNeasy serum/plasma kit. Mature miRNAs from sperm and seminal plasma were reverse transcribed into cDNA using miScript II RT kit. Real-time PCR was performed using miScript miRNA PCR arrays in combination with the miScript SYBR Green PCR Kit. C_T values of sperm miRNAs were normalized using RNU6-6P, and the C_T values of seminal plasma miRNAs were normalized using cel-miR-39-3p. Normalized C_T values of 84 sperm and seminal plasma miRNAs were viewed plotting the normalized C_T values on the y-axis and miRNAs on the x-axis. Within microRNAs, normalized C_T values were compared using Student t-test. The study identified a relationship between sperm and seminal plasma microRNAs, based on the normalized threshold cycle of amplifying cDNA in sperm and seminal plasma from the same semen of Landrace boars. Therefore, it was concluded that seminal plasma miRNAs may originate from sperm or these miRNAs may shuttle between sperm and seminal plasma in order to facilitate cell-to-cell communication.

Keywords: Boar, sperm, seminal plasma, microRNA

Morphological characteristics of ovarian follicular dysplasia (OFD) observed by ultrasound in four Florida beef herds

J. Roberts,[a] E. Steel,[b] J. Gard,[c] J. Yelvington,[d] J. Yelich,[e] N. Siegel,[f] K. Norton,[e] J. Hinds,[g] W. Freel,[h] C. Armstrong,[i] S. Lee,[j] I. Hardee,[b] O. Rae[k]

[a]Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn AL; [b] Steele Equine Veterinary Services and Performance Horse Center, Zolfo Springs FL; [c] Department of Veterinary Clinical Services, College of Veterinary Medicine, Auburn University, Auburn AL; [d] Ridge Large Animal Services, Lake Placid, FL; [e]Animal Science Department, University of Florida, Gainesville FL; [f]Animal Science Department, Auburn University, Auburn AL; [g] Citrus Animal Clinic, Lake Placid, FL; [h]Freel Veterinary Services, Ocala FL; [i]Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA; [j]Adena Springs Ranch, Ocala FL; [k]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL

A slaughterhouse study commissioned by Florida Cattleman's Association in 2007, identified ovarian follicular dysplasia (OFD) as a primary cause of infertility in Florida beef cows. Ovaries with OFD have progressive bilateral development of solid clustered follicles containing multiple Call-Exner bodies that originate in the rete ovarii and the hilar region, and progress into the cortex to eventually form bilateral Sertoli-type granulosa theca cell tumors (GTCT). The goal of this study was to access the usefulness of ultrasound by bovine practitioners for on-farm diagnosis of ovarian follicular dysplasia. Ultrasound images of right and left ovaries from 390 cull cows and heifers representing four Florida ranches were made with 5 MHz linear probes (Aloka, Ibex) and 10-12 females per ranch were followed to slaughter the following day for collection of reproductive tracts. Fixed ovaries were measured, sectioned para-sagittal through the hilus, photographed, and arranged in histology cassettes for complete examination of the cut surface. Large ovarian structures including corpus luteum, Graafian follicles, atretic follicles, dysplastic follicles, rete ovarii, dysplastic follicles and tumors were counted and measured for each ovary. Ovaries with OFD were graded I to IV. Grade I OFD contained small individual dysplastic follicles with diameter less than 200 μm mostly limited to the rete ovarii and medulla. Grade II OFD possessed dysplastic follicles greater than 200 μm diameter that were present in the medulla and cortex. Grade III OFD had extensive multi-sized dysplastic follicles scattered throughout the entire cortex of the ovary and Grade IV OFD had Sertoli-type GTCT. Grade II-IV often had dystrophic mineralization of dysplastic follicles. Gross morphology of fixed sagittal sections and ultrasound images were blindly compared against OFD grade in 40 individual ovaries. Ovarian follicular dysplasia was identified at slaughter in 29/41 cows and in 1/5 of heifers. The distribution of OFD for 30 affected females was Gr I 16/30, Gr II 9/30, Gr III 4/30 and Gr IV 1/30. Characteristics that could be detected by routine ultrasound included increased size and length, increased hyperechogenicity and decreased number of fluid filled follicles. Hyperechogenic shadows were evident in higher grade OFD. The study demonstrated that Grade III and IV OFD can be observed by routine ultrasound but Grade I and II may require higher resolution ultrasound probes, imaging analysis software or Doppler ultrasound.

Keywords: Follicular dysplasia, bovine, ultrasound, Call-Exner bodies, Sertoli-type granulosa theca cell tumor

Sperm and seminal plasma proteomics of bulls with differing fertility

R. Kasimanickam,[a] V. Kasimanickam,[a] A. Arangasamy[b]
[a]Department of Veterinary Clinical Sciences, Washington State University, Pullman, WA;[b]National Institute of Animal Nutrition and Physiology, Bangalore, KA, India

Sperm are highly specialized compartmentalized cells, with unique compositional, morphological and functional properties. The plasma membrane of sperm undergoes dynamic protein remodeling and surface modifications that are critical for the events essential to fertilization including maturation, capacitation and sperm-egg communication. Seminal plasma proteins protect sperm against oxidative stress, and immune responses in the female reproductive tract. The objective of the study was to determine sperm and seminal plasma proteomes of bulls with differing fertility and to associate its biological process. The hypothesis was that bulls with differing fertility will have differences in proteome expression and to associate these proteomes with their biological processes. Bulls with high (n=4) and low (n=4) fertility (daughter pregnancy rates) were selected. Following isolation and purification of protein from sperm and seminal plasma for individual bulls, protein concentration was determined and 2-D gel electrophoresis was performed including gel staining. Protein identification and gene ontology were performed. Differences in the intensity of spots/proteins between high and low fertile bulls were calculated using PDQuest and ImageJ, and the data were analyzed using SAS (version 9.4) using ANOVA. Spots in the sperm and seminal plasma maps were identified as binder of sperm proteins (BSP)-1, -3 and -5, and spermadhesin-1; multiple isoforms of CLU, ALB, and TIMP were also identified. Others included HSPA1A, GPX 3, CATHL1, OPN-K, NPC2, APOA-1, CATHL3, CATHL7, ENO1, TPP1, AZGP1, SERPINA5, B2M, PSMB4, ACTB, CTSL, CTSS, NUCB1, S100A9, HBA1, CDH1, ANG1, FGA, EFNA1, PARK7, SerpinA3, SRN4, PTGDS, PAFAH, PGK1A2M, ANXA1, FGB, CFB, and PIGR. The protein intensity differed between high and low fertile bulls (Fig.1; $P<0.05$).

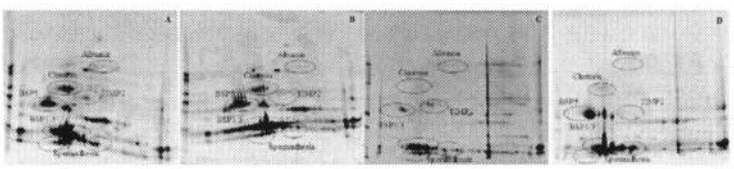


Figure1: 2-D maps of sperm and seminal plasma proteins of Holstein bulls. Images A and B, and C and D are from seminal plasma and semen of high and low fertile bulls, respectively. Circle represents the main clusters of proteins.

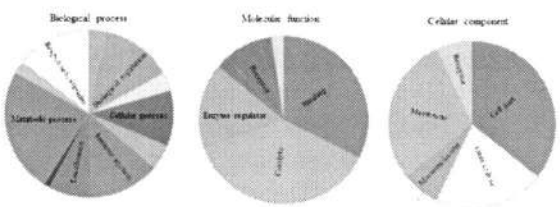


Figure 2: Pie charts show biological process, molecular function and cellular component for the proteins differentially seen between high and low fertile groups.

The two key biological processes of the proteins differentially seen in high and low fertile bulls are metabolic process and biological regulation (Fig.2). The most prominent molecular functions for these proteins that differed are binding, catalytic and receptor activities (Fig.2). The main cellular components for these proteins that differed are cellular, extracellular, and plasma membrane (Fig.2). Since proteins level differs in high and low fertile bulls, the efficiency of associated functions that are necessary for the sperm function may also differ between high and low fertile semen. In conclusion, varying protein levels of sperm and seminal plasma of high and low fertile bulls likely related to the differences in fertility.

Keywords: Bulls, seminal plasma, sperm, proteome, fertility

(Editor's note: Photographs in this manuscript are available in color in the online edition of Clinical Therogenology.)

The effect of early postpartum intervention on the reproductive performance of anovulatory anestrus New Zealand dairy cows

J. Hyatt, G. Jarratt, F. Hollinshead, D. Hanlon
Matamata Veterinary Services, Matamata, New Zealand

Due to the seasonal nature of New Zealand's dairy industry all cows within a herd commence breeding on the same calendar date regardless of their individual calving date (planned start of mating). Cows which have not resumed normal ovulatory activity postpartum by this date are a significant cause of reproductive wastage. The objective of the current study was to determine the reproductive performance of anovulatory anestrus (AA) cows treated with intravaginal progesterone (P4), equine chorionic gonadotropin (eCG) and gonadotropin releasing hormone (GnRH) at 21-35d postpartum. We hypothesized that the early intervention and treatment of AA cows would improve their reproductive performance. A prospective cohort study was performed involving 6642 cows in 18 herds. All cows without visible signs of estrus 21-35d postpartum were examined. Each cow was body condition scored (BCS), and a per-rectum ultrasound examination of the reproductive tract performed to measure combined uterine horn diameter, ovarian diameter and ovarian follicle diameters. Cows without a corpus luteum (CL) were classified as AA (n=232) and treated as follows: d0 - intravaginal P4 device inserted (Cue Mate 1.56 g w/w P4); d6 - device removed and eCG (400 IU) administered IM; d 8 - GnRH (100 ug) administered IM; fixed-time AI (FTAI) performed 16-20 h later. Each cow was examined for pregnancy and fetal ageing performed by per-rectum ultrasonography at 42 and 100 d after FTAI. Fetal ageing enabled accurate determination of pregnancy rate (PR) to FTAI and at 21d after FTAI (21 day in-calf rate). Multivariable logistic regression was used to determine the effects of cow age, parity, days calved, uterine horn diameter, ovarian diameter, ovarian follicle diameter and BCS on each of the following outcomes: PR to FTAI, 21 day in-calf rate, and the in-calf rate at the end of the season. All analyses were performed using the software package R Version 3.1.3 (www.r-project.org). The only variable remaining in all multivariable models (PR to FTAI, 21 day in-calf rate and the in-calf rate at the end of the season) was days calved. Cows that were 21-24d postpartum when compared to those 25-35d postpartum had improved PR to FTAI (46% vs. 23% respectively; $P < 0.01$), 21 day in- calf rate (61% vs. 35% respectively; $P < 0.01$) and end of season in-calf rate (83% vs. 69% respectively; $P < 0.01$). Additionally, cows with a uterine horn diameter of < 4.5 cm at the time of P4 device insertion had a higher PR to FTAI compared to cows with a uterine horn diameter of > 4.5 cm (31% vs. 21% respectively; $P = 0.03$). The results of this study suggest that early intervention and treatment of AA in cows 21-28d postpartum, and thus earlier than the current "industry standard" of waiting until at least 28d postpartum, can result in positive reproductive outcomes. In addition, this study highlighted the use of ultrasound examination to aid in determining the predicted benefits of AA treatment in individual cows. In particular, uterine horn diameter is likely to be an indicator of uterine involution and a positive response to treatment.

Keywords: Bovine, anestrus, intravaginal progesterone

Corynebacterium pseudotuberculosis as a cause of bilateral orchitis in a Boer buck

J.L. Stewart, R. McCann, I.F. Canisso
Department of Veterinary Clinical Medicine, University of Illinois Urbana-Champaign, Urbana, IL

Orchitis is an important cause of subfertility/infertility in small ruminants (SR). Several organisms have been implicated as a cause of non-venereal orchitis in bucks, such as *Arcanobacterium pyogenes*. *Corynebacterium pseudotuberculosis* is a highly contagious bacteria associated with caseous lymphadenitis and is characterized by chronic, focal/multifocal abscessation in various body systems of SR. Despite its widespread occurrence, there have been few reports documenting *C. pseudotuberculosis* as a cause of orchitis in bucks. Herein, we describe a non-venereal orchitis caused by *C. pseudotuberculosis* in an eight year-old buck concomitantly suffering with chronic respiratory disease. Upon presentation, physical examination revealed a BCS of 1.5/5 with generalized muscle atrophy, bilateral mucoid nasal discharge, and harsh lung sounds in the left chest. The left testis was firm on palpation and asymmetrically larger than the right testis which was palpably normally. The epididymides were palpably normal with no obvious adhesions. A CBC, blood chemistry, and fibrinogen were performed, which revealed a severe neutrophilia (24.3×10^3 /μL; RR: $1.2\text{-}7.2\times10^3$/μL), hyperfibrinogenemia (504 mg/dL; RR: 100-400 mg/dL), and hyperglobulinemia (6.0 g/dL; RR: 2.7-4.1g/dL), consistent with active infection. Semen was collected by electroejaculation using routine procedures with a handheld ram ejaculator. Gross semen assessment revealed a mildly cloudy and yellow-colored fluid, and microscopic evaluation showed obvious oligozoospermia and asthenozoospermia (subjective total motility of 10% and negligible progressive motility). Sperm morphology was 57% normal with 8% simple bent tails, 6% proximal droplets, 14% head detachment, 3% mid-piece defects, and 12% strongly folded tails. Thoracic ultrasound showed a small amount of mildly hyperechoic pleural free fluid, found to be modified transudate on cytology with no visible bacteria. Testicular ultrasound exposed a 15 cm, hypoechoic nodule with a hyperechoic rim near the craniodorsal region of the left testicle. Hyperechoic foci were dispersed throughout both the left and right testicles. Differential diagnoses included sperm granuloma, abscess, or neoplasia. A fine needle aspirate (FNA) was performed on both testicles for cytology and aerobic culture. Cytology revealed suppurative inflammation with marked necrosis within both testicles and short, rod-shaped bacteria in the left testicle. *Staphylococcus xylosus* was cultured from the left testicle, and *Corynebacterium pseudotuberculosis* was cultured from the right testicle. A semen culture was also obtained and isolated *Bacillus* sp. and *S. xylosus*. The presence of *S. xylosus* on the semen culture and left testicular FNA is most likely due to contamination, as this organism has been previously isolated from both scrotal skin and prepuce and appears not to be associated with reproductive diseases in SR. The presence of lesions in both testicles, as indicated by ultrasound, with subsequent isolation of *C. pseudotuberculosis* provided a grave prognosis for recovery of breeding ability. An interesting aspect about this case is the fact that *C. pseudotuberculosis* was only cultured using FNA from one testicle, suggesting that diagnosis of testicular orchitis due to this bacteria may not be successfully confirmed by semen culture alone and that multiple FNA sites may be required for detection. As abscesses in caseous lymphadenitis tend to be highly constricted to a specific area, we suggest that the bacteria might not be secreted in the semen unless the abscess is ruptured or that connecting ducts (efferents) were completely destroyed by the abscess.

Keywords: Caprine, *Corynebacterium pseudotuberculosis*, infertility, oligozoospermia, orchitis

In vitro efficacy of novel anti-protozoal compounds against *Tritrichomonas foetus*

J.H. Koziol,[a] D.F. Wolfe,[a] M.A. Edmondson,[a] S.H. Duran,[a] T. Passler,[a] J.A. Gard,[a] H.S. Maxwell,[a] K. Joiner[b]

[a]Department of Clinical Sciences and [b]Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL

There are no legal treatments for cattle infected with *Tritrichomonas foetus*. This obligate parasite of the reproductive tract in the bovine species represents a serious economic threat to the cattle industry in the United States. The hypothesis of this study was that oxfendazole combined with a pluronic lecithin organogel (PLO) to form a topical formulation may be an effective treatment for bulls infected with *T. foetus*. The specific aim of this experiment was to conduct *in vitro* testing of the antiprotozoal effects of the oxfendazole formulation and its components on *T. foetus* organisms. Oxfendazole powder was reconstituted to a solution (OXF) with 70% ethanol (EtOH) and mixed with Velvachol (VC), an emollient, prior to addition of PLO. Viable *T. foetus* trophozoites (5×10^4 mL^{-1}) were added to tissue culture wells containing 4 mL of Diamond's medium (DM). At time 0, each of the following treatments were added to tissue culture wells in duplicate: 1) positive control 4 mL DM; 2) negative control 4 mL EtOH; 3) 150 mg OXF (1 mL) + 2 mL VC + 1 mL PLO + 4 mL DM; 4) 150 mg OXF (1 mL) + 3 mL VC + 4 mL DM; 5) 150 mg OXF (1 mL) + 3 mL EtOH + 4 mL DM; 6) 150 mg OXF (1 mL) + 2 mL EtOH + 1 mL PLO + 4 mL DM; 7) 1 mL EtOH + 2 mL VC + 1 mL PLO + 4 mL DM; 8) 1 mL EtOH + 3 mL VC + 4 mL DM; 9) 3 mL EtOH + 1 mL PLO + 4 mL DM; 10) 4 mL EtOH + 4 mL DM; 11) 4 mL VC + 4 mL DM; 12) 150 mg oxfendazole dissolved in 4 mL 99% DMSO + 4 mL DM; 13) 4 mL 99% DMSO + 4 mL DM (a known control). Cultures were incubated at 37°C and aliquots taken every 8 hours over 24 hours to assess the number of viable organisms. A 20 µL sample was collected from each well. Surviving organisms were counted utilizing disposable Neubauer hemocytometers and the presence of pseudocysts noted. At 24 hours after treatment, contents were removed from the tissue culture wells, placed in centrifuge tube, and centrifuged at 4000g for 10 minutes. The supernatant was removed and placed in a labeled vial for evaluation of drug concentration. The pellet was re-suspended in DM and placed in a tissue culture well containing drug free DM and re-incubated at 37°C for 24 hours. This process was repeated every 24 hours for a total of 5 passages (120 hours) to evaluate for re-emergence of *T. foetus*. Each sample was examined microscopically during this period for the presence of *T. foetus* organisms by counting the live organisms as described above. Non-motile trophozoites were characterized by the presence of the pear shaped bodies with externalized flagella but lack of motion. Pseudocysts were characterized by rounding of the cell with internalized flagella. Multiple formulations rapidly induced the state of non-motile trophozoites and pseudocysts. The antiprotozoal and topical components inhibited the *in vitro* growth of *T. foetus* to varying extents. However, formulations 2-13 lead to the complete kill of *T. foetus* by 24 hours. Re-emergence to the trophozoite state was not observed in samples from cultures 2-13. In conclusion, this study demonstrated that oxfendazole combined with PLO gel can inhibit the growth of the bovine strain CDTf3 of *Tritrichomonas foetus* in *in vitro* cultures with efficacy similar to 70% ethanol which is commonly used to destroy the organism in the laboratory.

Keywords: *Tritrichomonas foetus*, bull, topical treatment, oxfendazole

Bovine reproductive palpation training: what methods make a difference and do our skills really transfer?

H. French,[a] J. Williamson,[b] G. E. Gilbert,[a] J. Dascanio[b]
[a]Ross University School of Veterinary Medicine, St. Kitts, West Indies; [b]College of Veterinary Medicine, Lincoln Memorial University, Harrogate, TN

Gaining experience and dexterity for trans-rectal cattle palpation requires time and training. Studies conducted with simulation models allow students to perform palpation without any risks and obtain feedback, but many believe that live cattle palpation is essential for training. There is limited research on the proper method for training live animal trans-rectal palpation skills. To examine this topic, we conducted two investigations. The first study compared student improvement in palpation skills when assigned to the same cows weekly versus choosing a cow at random over an eight week period. The hypothesis for the study was: assigned students will be more accurate at palpation since they are examining the same cows each week; will learn what structures are present on the ovaries and what sizes the reproductive tract measures; and will be able to follow the cyclicity of the cow over time. Cervical diameter, uterine tone, diameter of left and right uterine horns, pregnancy status, and ovarian structures were recorded over time. Responses were compared to laboratory instructor's responses and z-tests for proportions were used to test the differences in percent correct at each time point for each palpation exercise. Although significant differences were seen at different time points, no overall difference was seen between training methods. Veterinary educators are challenged to teach large and small animal palpation skills. Often students' interests are in the former or latter, but not usually for both. The second experiment conducted assessed student's small animal trans-abdominal palpation skills before and after large animal trans-rectal palpation training. The hypothesis for this study was: students' small animal palpation skills will improve after large animal palpation training. Students palpated four feline models prior to participating in eight weeks of bovine palpation labs with supplemental palpating exercises. A palpation questionnaire and scoring rubric were used for student responses on the presence, measurement, texture and tone of four organs (abdominal mass, right/left kidney, and bladder) within the four feline models. No improvement was seen in the trans-abdominal palpation skills of students after eight weeks of laboratories. Although statistically significant improvement was not seen in either study, both experiments led to changes in how skills are taught and further improvement in creating an integrated curriculum. The palpation study demonstrated cow assignment did not improve skills for live animal palpation, but gave students more direct instructions and key structures to identify. This allowed instructors to have more productive learning time with students. While the lack of transferability of palpation skills between large and small animals may seem surprising, this is normal for early learners. Novices learn clinical skills by breaking them into components and rigidly following a sequence of events. It is not until a learner reaches proficiency that he/she is able to make generalizations and act intuitively in new situations that require the same skills. The trans-abdominal study justified the continued allocation of time in the curriculum for teaching both bovine trans-rectal palpation and small animal trans-abdominal palpation thoroughly, without the expectation that a novice is able to transfer skills between these two methods of palpation.

Keywords: Veterinary education, palpation, model, clinical skills, simulation

Results of hysteroscopic examinations performed as part of breeding soundness evaluations on mares presented for infertility–a retrospective study

E.A. Bradecamp,[a] M.R. Schnobrich,[a] P.C. Sheerin,[b] C.E. Howard[a]
[a]Rood and Riddle Equine Hospital, Lexington, KY; [b]Veterinary Associates, New Freedom, PA

Hysteroscopy is a valuable diagnostic tool when trying to determine the cause of infertility in the mare. While traditional reproductive diagnostics (transrectal palpation and ultrasonography of the reproductive tract, endometrial culture, cytology and biopsy) identify the majority of pathologies, hysteroscopy is useful as an advanced means of assessing the uterine environment. Hysteroscopy is included as part of a breeding soundness exam for most mares presented for infertility at Rood and Riddle Equine Hospital. A review of all hysteroscopic examinations performed on mares presented for infertility between 2008 and 2015 was conducted to determine the incidence of pathology identified with this diagnostic tool. Hysteroscopic evaluations were performed during diestrus with the mare restrained and sedated in stocks. A one meter flexible endoscope was used to perform this procedure with air used to insufflate the uterus for visualization. A total of 121 hysteroscopic examinations were performed. Fifteen of these examinations were performed on mares presented for hydrointubation of the oviducts. Two of these examinations revealed abnormal findings that explained the mares' infertility. A piece of foreign debris was identified in one mare's uterus and a marble was found in the other. Of the 108 examinations performed solely as part of the breeding soundness examination pathology was identified in 39% (45/108) of the cases. Abnormalities identified included discrete, discolored endometrial plaques 14.8% (16/108) associated with fungal or bacterial growth, intraluminal foreign debris 1.9% (2/108), excessive and overly viscous mucus 6.5% (7/108), retained endometrial cups 5.5% (6/108), excessive fibrosis or abnormal anatomy due to cesarean section scars 2.7% (3/108), adhesions/scarring 3.7% (4/108), diffuse fungal endometritis cases 1.9% (2/108), and cases classified as other 4.6% (5/108). These cases included a cervical diverticulum, an endometrial diverticulum, lymphosarcoma, endometrial discoloration, and punctate lesions of unknown etiology. In several cases the traditional reproductive diagnostics (endometrial culture/cytology/biopsy and transrectal palpation and ultrasound) failed to reveal any pathology, and only following hysteroscopic identification of the pathology could an appropriate treatment plan for the mare be initiated. The high percentage of abnormalities identified with this diagnostic modality highlights the importance of this procedure as part of an infertility evaluation.

Keywords: Hysteroscopy, uterus, endometritis, infertility

Endocrinologic and somatic changes during the peripubertal period in Standardbred colts

R. Ellerbrock,[a] I.F. Canisso,[a] F. Lima,[a] C. Shipley,[a] K. Kline[b]
[a]Department of Veterinary Clinical Medicine, [b]Department of Animal Sciences, University of Illinois Urbana-Champaign, Urbana, IL

The onset of puberty and its association with somatic growth and testicular development is well-characterized in many species of livestock, and neonatal and peripubertal somatic development has been shown to influence the sperm production in future bull sires. In contrast there are no studies assessing the association between sexual and somatic development in peripubertal colts. We hypothesized that onset of puberty would correspond with an increase in total body fat and exponential testicular growth and that it is associated with an increase in gonadotropins and sex steroid hormones. The objectives of this study were: (i) to determine plasma follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol 17β concentrations during the peripubertal period in colts and (ii) to characterize changes in body fat deposition and testicular development during the peripubertal period. Twenty-five healthy Standardbred colts were enrolled in the study when they reached six months of age. All colts were managed and fed in similar conditions and comparable to industry standards. Blood samples were collected via jugular venipuncture between 8 and 10 am every four weeks for twelve months, and plasma samples were stored at -80°C until analysis. The colts were weighed monthly (WE) using a weight tape. Testicular volume (TV) was estimated using measurements acquired by B-mode ultrasound according to the formula (length x width x height x 0.5233), and percent body fat (BF) was estimated using the following equation BF = 3.83 + 5.58x; x = rump fat (cm). Rump fat was measured over the hindquarters approximately 5 cm lateral to midline at the center of the pelvis. Serum testosterone was analyzed by radioimmune assay (RIA) from six to eighteen months of age, and the onset of puberty was determined to be the month when testosterone was at least two standard deviations above the previous mean. Plasma FSH and LH were analyzed for the seven month peripubertal period by RIA. Data were analyzed using RStudio v 0.99.489 (RStudio Team, Boston, MA). Continuous variables not normally distributed were log-transformed and analyzed with mixed models. Pearson's coefficient of correlations (r) were performed between variables. Data are expressed as mean ± SEM and significance was set at $p < 0.05$. At the onset of puberty, colts were 13.0 ± 0.3 months of age, weighed 759 ± 17 lbs, with a BF of 6.2 ± 0.2%, and a TV of 53.5 ± 8.6 cm^3; plasma testosterone was 3.01 ± 0.32 ng/ml, estradiol 17 β was 42.1 ± 5.2 pg/ml, LH was 0.52 ± 0.06 ng/ml, and FSH was 10.97 ± 0.89 ng/ml. Age was significantly correlated with weight (r=0.67), testosterone (r=0.57), and TV (r=0.68). Testosterone was significantly correlated with weight (r=0.4) and with testicular volume (r=0.5). Follicle stimulating hormone was significantly different from the peripubertal period at the onset of puberty than pre and post-pubertal months, but was not correlated with age. There was no significant change in LH during the peripubertal period. In conclusion, spring born Standardbred colts undergo puberty at 13 months of age, and the onset of puberty coincides with exponential testicular growth. The onset of puberty does not coincide with an increase in cutaneous body fat deposition. Knowledge of normal testicular development in the colt lays the groundwork for future studies into long term testicular function and the relationship between testicular development and future total sperm output and fertility.

In vivo embryo production during induced aluteal cycles in the mare

C.A. Leisinger,[a] G.M. Davolli,[a] B.A. Foster,[b] S. Whisnant,[c] D.L. Paccamonti,[a] C.R.F. Pinto[a]
[a]Department of Veterinary Clinical Sciences, School of Veterinary Medicine, and [b] School of Animal Sciences, Louisiana State University, Baton Rouge, LA; [c]Department of Animal Science, North Carolina State University, Raleigh, NC

A proof of concept experiment was designed to investigate the feasibility of generating equine embryos during aluteal cycles induced by antiluteogenic treatments administered shortly after ovulation. Four cyclic mares with a median age of 9.5 years (range 9 to 16) were utilized. Mares were monitored until a preovulatory follicle ≥ 35 mm was detected on the ovary in the presence of uterine edema as determined by ultrasonography. A fertile stallion was used for artificial insemination with each breeding dose comprised of ≥ 1 x 10^9 total motile sperm. Mares were artificially inseminated and human chorionic gonadotropin (hCG; 2000 IU, IV; Chorulon, Merck Animal Health, Kenilworth, NJ) administered. Blood was collected every other day until ovulation was detected by twice daily ultrasonography. Once ovulation was detected, dinoprost (10 mg, IM; Lutalyse, Zoetis, Florham Park, NJ) was administered once daily for 5 days. Daily blood samples were collected from ovulation until the day of embryo collection 8 days after ovulation. Following embryo collection, mares were monitored until they returned to estrus, and then artificially inseminated as described above. After ovulation was detected, mares were again subjected to the 5 day antiluteogenic treatment. Then on day 6 after ovulation long acting biorelease altrenogest (225 mg, IM; BET Pharm, Lexington, KY) was administered to evaluate the ability to establish pregnancy after progesterone deprivation during early embryogenesis. Data are reported as mean ± S.E.M. The mean interovulatory interval between subsequent antiluteogenic cycles was 13.5 ± 0.87 d. The mean daily progesterone concentration from ovulation to embryo collection was 0.40 ± 0.15 ng/mL. After the first ovulation, two of four mares produced embryos. Two mares became pregnant with heartbeats detected 22 days after the second ovulation. The mean daily progesterone concentration from ovulation to 22 days after ovulation was 0.64 ± 0.36 ng/mL. Only one mare failed to produce an embryo and become pregnant during both cycles. This study demonstrated that embryos could be collected from mares when antiluteogenic treatment was initiated immediately after ovulation resulting in an aluteal cycle. Furthermore, viable pregnancies were established after progesterone deprivation during early embryogenesis.

Keywords: Antiluteogenesis, embryo collection, aluteal, pregnancy, progesterone

Pathogenicity of *Escherichia coli* isolated from the equine uterus

J.K. Morrissey,[a] R.A. Ferris,[a] B.R. Borlee,[b] P.M. McCue[a]
[a]Department of Clinical Sciences; [b]Department Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO

Escherichia coli is one of the most common bacteria isolated from the equine uterus, representing 30-50% of all cases of infectious endometritis. *E. coli* isolates are recognized to have different virulence characteristics based on phylogenetic analysis. The goal of this study was to characterize *E. coli* isolates from the equine reproductive tract based on phylogenetic group, antibiotic susceptibility and *in vitro* biofilm production. The hypothesis was that the *E. coli* isolates could be classified into different phylogenetic groups that would correlate with their antibiotic resistance and biofilm propensity. *E. coli* isolates (n=43) were obtained from equine clinical cases in Lexington, Kentucky. Bacterial identification was first confirmed using MALDI-TOF. Antibiotic resistance was then determined using Kirby-Bauer disc diffusion for the following antibiotics: gentamicin, enrofloxacin, ceftiofur, amikacin, ampicillin, trimethoprim sulfamethoxazole, ticarcillin with clavulanic acid, and penicillin. *In vitro* biofilm production was evaluated by crystal violet staining and phylogenetic groups classified using multiplex PCR. Data were compared using Fisher's exact test for antibiotic resistance and an unpaired t-test for biofilm formation. Results are reported as the mean ± SEM. Phylogenetic analysis showed that a majority of samples ($p<0.05$) could be classified into either B1 (n=22) or B2 (n=16) groups, with the remaining isolates belonging to group A (n=2) or were undetermined (n=3). There was a greater ($p<0.05$) percentage of isolates resistant to ticarcillin with clavulanic acid and ampicillin for group B2 (9 of 16 and 14 of 16, respectively) as compared to the B1 group (5 of 22 and 8 of 22, respectively). Biofilm formation was greater ($p<0.05$) in the B1 group (0.52 ± 0.09 OD_{600}) compared to the non-biofilm-forming B2 group (0.16 ± 0.01 OD_{600}). In summary, a majority of *E. coli* isolated from the equine reproductive tract were divided into two distinct groups (B1 and B2). Clinical B2 *E. coli* did not form a biofilm *in vitro*, but had greater antibiotic resistance as compared to the B1 group. Future studies will focus on differences in *in vivo* pathogenicity between B1 and B2 groups of *E. coli*.

Keywords: Equine, endometritis, *E. coli*, biofilm, antibiotic resistance

Immunolocalization of UCH-L1 in the cryptorchid stallion

J.B. Andrews,[a] J.L. Rose,[b] C.S. Perumamthadathil,[a] C.J. Scott,[a] S. Norman[a]
[a]School of Animal and Veterinary Sciences; [b]School of Biomedical Sciences; Charles Sturt University, Wagga Wagga, NSW, Australia

The etiology of idiopathic testicular degeneration (ITD) is unknown, with it being unclear whether the problem lies with the Sertoli cells, Leydig cells, or germ cells. The equine cryptorchid offers a model for the investigation of complete spermatogenic arrest, being histologically analogous to that seen in advanced ITD. De-ubiquitinating enzymes (DUBs), including ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), may regulate the balance between spermatogonial quiescence, self-renewal and differentiation. Ubiquitin carboxy-terminal hydrolase is required for first round spermatogenesis in neonatal mice as well as sperm maturation in the adult. Testes in mice deficient in UCH-L1, have a decrease in germ cell apoptosis, an increase in premeiotic cells, a decrease in sperm numbers, and an increase in defective spermatozoa. We hypothesize that if germ cell dysfunction resulted in spermatogenic arrest in the cryptorchid then the population characteristics of the UCH-L1 positive cell would be at variance to that in the normal descended testis, assuming that UCH-L1 acts at the control point between self-renewal, differentiation, or apoptosis in spermatogenesis. Ubiquitin carboxy-terminal hydrolase was localized in the retained and descended testis of a 3 year old unilateral cryptorchid colt using fluorescence immunohistochemistry. In both the retained and descended testis, UCH-L1 was expressed in the A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia. Positively labelled spermatogonia were also shown to be restricted to the seminiferous tubules basal compartment in both conditions. One hundred tubules were analyzed for their UCH-L1 positive spermatogonia, with tubules for analysis chosen on the basis of their roundness (diameter $_{max} \approx$ diameter $_{min}$). The mean number of spermatogonia per tubule was 12.4±0.5 (retained, SEM) and 11.1±0.4 (descended), P=0.07. It was concluded that there was not a significant difference in the UCH-L1 population per tubule nor in their location. The consistency in the population numbers and location of UCH-L1 positive spermatogonia suggest that spermatogonial function is unimpaired in the cryptorchid and hence the cause of spermatogenic arrest does not lie with the germ cells. This adds to the body of evidence suggesting cryptorchid testis, and possibly ITD dysfunction, is associated with perturbations of the Sertoli or Leydig cells. From this preliminary study we suggest that UCH-L1 may be a suitable biomarker for identifying functional spermatogonia in the normal testis, the cryptorchid testis and possibly testes with ITD. Although the role of DUBs at this spermatogenic control point remains to be fully elucidated, this study suggests that UCH-L1 could be used to identify and harvest functional type A spermatogonia from the abnormal testis for use in stem-cell technologies.

Keywords: UCH-L1, ubiquitin spermatogonia, cryptorchid, stallion

Alpha-fetoprotein as a marker for equine neonatal disease

M. Prell,[a] I. F. Canisso,[a] M. Schnobrich,[b] T. Riddle,[b] R. E. Ellerbrock,[a] P. Wilkins[a]
[a]Department of Veterinary Clinical Medicine, University of Illinois Urbana-Champaign, Urbana, IL;
[b]Rood & Riddle Equine Hospital, Lexington, KY

Most equine neonatal losses have been associated with intrauterine infections such as placentitis. Alpha-fetoprotein (AFP), a protein produced by the fetal liver, was recently shown to be increased in the plasma of mares with ascending placentitis. While AFP is present in high amounts in both allantoic and amniotic fluids, and in fetal plasma, AFP profiles in healthy and sick newborn foals are not well defined. Theriogenologists/equine practitioners working in broodmare farms frequently face the challenge to determine whether a foal is ill and requires tertiary care. We hypothesize that AFP is present in high concentrations in the fetus and it will also be increased in plasma of foals suffering neonatal disease. Therefore, it may be a useful diagnostic and prognostic marker for neonatal disease. The objectives of this study were to (i) describe AFP concentrations in healthy newborn foals during the first week of life, (ii) compare AFP concentrations in healthy foals to septic foals. In study I, sixteen clinically healthy newborn Standardbred foals had blood samples collected daily for seven days, at 24 hour intervals. Plasma was harvested and stored at -80°C until further analyses. Concentrations of AFP were determined using a heterologous commercial chemiluminescence assay previously validated for use in the horse. In study II, fifty newborn Thoroughbred and Standardbred having normal deliveries had complete blood count (CBC), fibrinogen (Fb) concentration, determination of IgG concentration, and blood collected for determination of AFP concentration by 12 to 24 hours after delivery. All foals were thoroughly examined by well-experienced equine veterinarians during routine normal mare/new foal check. Based on physical examination, CBC, and Fb results, foals were grouped as healthy (white blood cell <12,000, Fb <400mg/dl) or clinically septic (white blood cell count >12,000 and Fb >400). Statistical analyses were performed with a commercial software (JMP-Pro12, SAS Institute, Cary, NC). Data on both studies were analyzed by mixed models, and expressed as means ± SEM, with foal accounted as random variable. Significance was set as $p<0.05$. There was an effect of time for AFP during the first week of life ($p=0.003$). There was a significant reduction in AFP concentration from day 1 (1118 ± 118 ng/ml) of birth to day 7 (538 ± 58 ng/ml). Although it was outside the scope of this study, it remains to be determined when AFP concentrations are negligible in plasma of newborn foals. For study II, nine foals met the criteria of clinically septic and 41 were clinically healthy. Concentrations of AFP were different between clinically healthy foals (1148 ± 147 ng/ml) and clinically septic foals for AFP concentrations (1430 ± 205 ng/ml) ($p=0.03$). Our findings suggest that AFP may serve as a useful maker that can be used to assess neonatal health under field conditions. Its uniqueness for being present in high concentration both in the fetus and newborn foal may open a new field of investigation in understanding neonatal losses in horses.

Keywords: Foal, sepsis, diagnostic marker

Hysteroscopic hydrotubation of the oviducts as a treatment for idiopathic infertility in the mare–a retrospective study

E.A. Bradecamp, M.R. Schnobrich
Rood and Riddle Equine Hospital, Lexington, KY

In a small subset of mares the cause of infertility cannot be determined by routine diagnostic tests (trans-rectal ultrasound, vaginal speculum examination, digital examination of the cervix, uterine culture and cytology, hysteroscopic examination, uterine biopsy). When the results of all diagnostics performed are negative and no pathology of the reproductive tract can be identified, it is hypothesized that bilateral obstruction of the oviducts may be the cause of the infertility. Several studies have demonstrated the presence of proteinaceous plugs within the oviducts of 42% to 87% of mares and there is the possibility that these plugs may obstruct passage of the oocyte and or embryo.[1] In recent years, two procedures (laproscopic application of PGE2 gel to the oviducts and hydrotubation of the oviducts) have been described to treat the oviducts of mares suspected of oviductal blockage as a cause of their infertility.[2,3] This is a retrospective study to determine pregnancy rates in mares after hysteroscopic hydrotubation of the oviducts was performed as a treatment for idiopathic infertility. During the 2014 and 2015 breeding seasons fourteen mares presented to the LeBlanc Reproduction Center at Rood and Riddle Equine Hospital for hydrotubation of the oviducts as a treatment for idiopathic infertility where no other cause could be determined. All of the mares had been bred three or more cycles (range 3-7) to a fertile stallion without establishing a pregnancy or obtaining an embryo. Hydrotubation of the oviducts is performed during diestrus. A one meter endoscope is advanced through the cervix and the uterus is insufflated with air. A 200 cm polyethylene tube (1.7 mm outer diameter), with a 22 gauge 4.45 cm injection catheter attached to one end and a human angiography guide wire passed through, is passed through the endoscope and advanced into the orifice of the oviductal papilla. The oviduct is then flushed with 10 mls of saline. The procedure is repeated on the other oviduct. Mares are then bred the following cycle. After hydrotubation of the oviducts 78.6% (11/14) of the mares were pregnant or an embryo was obtained within two cycles. Hysteroscopic hydrotubation of the oviducts has proven to be a valuable treatment modality for re-establishing fertility in mares that have been unsuccessfully bred to fertile stallions for multiple cycles where no reproductive pathology can be identified.

Keywords: Oviduct, oviductal plugs, hysteroscopic hydrotubation, infertility

References

1. Arnold C, Love C: Laparoscopic evaluation of oviductal patency in the standing mare. Theriogenology 2013;79:905-910.
2. Inoue Y. Hysteroscopic hydrotubation of the equine oviduct. Equine Vet J 2013;45:761-765.
3. Allen W, Wilsher S, Morris L, et al: Laparoscopic application of PGE2 to re-establish oviductal patency and fertility in infertile mares: a preliminary study. Equine Vet J 2006;38:454-459.

Update on foal production using oocytes collected from ovaries shipped postmortem in a clinical program

Y.H. Choi, K. Rader, S.G. Spacek, H.S. Canesin, K. Hinrichs
College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX

Oocytes recovered postmortem are the last resource to obtain foals from a mare that suffers untimely death. We previously reported the clinical production of 10 foals via intracytoplasmic sperm injection (ICSI) of oocytes collected postmortem from ovaries of 16 mares (2006-2009).[1] We have also briefly presented clinical postmortem ICSI results in relation to ovary shipping temperature and duration (20 mares, 2010-2012).[2] In that study, there was a tendency for higher blastocyst formation but a lower pregnancy rate for oocytes from ovaries transported at 10-19°C than for those shipped at higher temperatures. Here we evaluate cases received in 2012-2014 to determine if that tendency was repeated. We categorized ovaries from 47 mares, 2-26 years of age, by ovary temperature at arrival: A) < 10°C (3-13 h transportation time, 2 mares); B) 10-19°C (6-9.5 h, 5 mares); C) 20-29°C (0.5-9 h, 22 mares); and D) 30-38°C (< 5 h, 18 mares). Packaging recommendation was that ovaries shipped < 2 h be packed with ballast near body temperature, and those shipped > 2 h be packed at room temperature or cooler (~13–22°C). Oocytes were collected by scraping of follicles, and matured oocytes were subjected to ICSI and cultured in vitro for blastocyst formation. Overall, 621 oocytes were collected from 938 follicles (13 oocytes per mare; 66% oocyte recovery rate per follicle). Of these oocytes, 63 were degenerating (10%) and 560 were cultured for maturation. The overall oocyte maturation rate was 49% (273/558); the rate of blastocyst development per injected oocyte was 20% (54/270). Out of 26 blastocysts transferred fresh, 10 foals were produced (38%). Eight blastocysts were vitrified, then warmed and transferred, for 5 foals (63%). Some vitrified blastocysts have not yet been transferred. When examined by category, oocyte maturation rates were 28, 50, 50, and 49% for Groups A, B, C, and D, respectively. Blastocyst rates per injected oocyte were 0 (0/5), 31 (11/35), 18 (24/132), and 19% (19/98), respectively; Group B tended to have a higher blastocyst rate than did Group C (P=0.07, Fisher's exact test). The foaling rates for transferred embryos (fresh + vitrified-warmed) in Groups B, C and D were 71% (5/7), 47% (9/19) and 13% (1/8) (B vs D, P<0.05). These data show, surprisingly, that transport of ovaries for 6-18 h at 10 to 19°C resulted in higher foal production efficiency than did transport for < 5 h at 30 to 38°C.

Keywords: Equine, oocytes, intracytoplasmic sperm injection, embryo culture

Acknowledgements: This work was supported by the Clinical Equine ICSI Program, Texas A&M University and the Link Equine Research Endowment Fund, Texas A&M University.

References

1. Hinrichs K, Choi YH, Norris JD, et al: Evaluation of foal production following intracytoplasmic sperm injection and blastocyst culture of oocytes from ovaries collected immediately before euthanasia or after death of mares under field conditions. J Am Vet Med Assoc 2012;241:1070-1074.
2. Choi YH, Velez IC, Macías-García B, et al: Factors affecting equine embryo development after post-mortem ICSI in a clinical program. J Equine Vet Sci 2012;32:409-410 (abstract).

Fecal contamination of the vagina and vestibule caused infertility of a Toggenburg doe due to a third-degree perineal laceration

A.P. Drexler, T.M. Prado, N.T. Jenkins
Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, TN

Trauma during parturition may inflict various degrees of lesions in the cervix, vagina, and/or vestibule in domestic females. A third-degree perineal laceration has been shown to occur when a foal's or calf's foot or nose catches the annular folds of the hymen at the vaginovestibular junction in mares and cows, but we were unable to find similar reports in the doe. This case report describes a doe unable to become pregnant due to fecal contamination of the vestibule and vagina caused by a third-degree perineal laceration. An eight-year-old Toggenburg doe was presented to the University of Tennessee's Veterinary Medical Center in January 2016 because of failure to conceive. The doe had a normal parturition in the spring of 2015, delivering four kids without any assistance. During the fall of 2015 she was artificially inseminated twice via transcervical approach and naturally mated twice, but reported to be open on her last pregnancy check. A thorough breeding soundness examination performed at our clinic revealed a third-degree perineal laceration. Vaginal speculum evaluation confirmed fecal contamination of the vestibule and vagina with a normal cervix. The doe was confirmed to be not pregnant by transrectal ultrasound. Failure to become pregnant due to fecal contamination of the vagina was suspected. A third-degree perineal laceration repair was recommended. The perineal laceration was repaired in a manner similar to that described for mares and cows using a six-bite suture pattern. However, the submucosa was dissected further cranially, horizontal to the tear, allowing placement of an extra suture pattern that inverted the vaginal submucosa and mucosa into the vaginal lumen and the rectal submucosa into the rectal lumen relieving tension. This also decreases the likelihood of a fistula forming at the cranial aspect of the repair. Both stages of the rectovestibular reconstruction and the anoperineal reconstruction were performed during the same operation. Once the surgical site was healed, 10 days after surgery, a synchronization protocol for fixed time artificial insemination with a controlled internal drug release (CIDR) was established. Before inserting the CIDR, the surgical site was determined to be intact and healing. The doe was bred 52 hours after removal the CIDR by laparoscopic artificial insemination. A 30 day pregnancy examination is pending.

Keywords: Doe, perineal, laceration, vagina, contamination

Reproductive findings in two trisomy X (65,XXX) Thoroughbred mares

M. R. Schnobrich,[a] E. A. Bradecamp,[a] T.L. Lear[b]
[a]Rood and Riddle Equine Hospital; [b]Maxwell H. Gluck Equine Research Center, Lexington, KY

This abstract describes trisomy X (65,XXX) in two four-year old Thoroughbred mares. While human individuals with trisomy X appear to be fertile, this is not the case in horses. Horses with X trisomy exhibit normal external genitalia but gonadal dysgenesis. The first mare was presented for breeding soundness evaluation following two years of infertility. The mare was bred unsuccessfully multiple times the first year. The second year the mare was bred when two follicles >30 mm developed on the left ovary. An ovulation inducing agent was administered and ovulation was detected within 48 hrs. The mare was administered a post-breeding lavage, and long-acting progesterone five days after ovulation but no pregnancy was detected 14 days later. Referred for a breeding soundness evaluation in mid-April, the mare's external genitalia appeared normal. Trans-rectal ultrasound revealed small, hypoplastic ovaries (< 2 cm of ovarian stroma). The left ovary had a 20 mm follicle, the right ovary had a 35 mm follicle, and the uterus had moderate endometrial edema with no intra-luminal fluid. On vaginoscopy the cervix appeared small, pale pink and elevated off of the vaginal floor. Digital examination of the cervix revealed a shortened canal (3 cm long). Hysteroscopic examination revealed no abnormal findings. Small volume uterine lavage was performed and no bacterial growth was recovered on aerobic culture and there was no evidence of inflammation following cytologic evaluation. A uterine biopsy (category IIA) revealed mild lymphocytic infiltration. Based on the presence of small dysplastic ovaries, a small cervix and the history of infertility, a karyotype was performed. The karyotype revealed that the mare had three copies of the X chromosome (65,XXX) and no further breeding was performed. The second mare was presented for routine evaluation following one unsuccessful breeding that season. Reportedly the mare developed a 30mm follicle and ovulated following breeding but no pregnancy was detected. The mare was evaluated in December and was normal on physical examination. The mare's external genitalia appeared normal and transrectal ultrasonography revealed small ovaries (1.5 cm x 2 cm) with no palpable ovulation fossae and no follicular development. The uterus was flaccid and small. Evaluation of the cervix revealed a shortened (2.5 cm long) cervical canal with no tone. Due to suspicion of a chromosomal abnormality a karyotype was performed and revealed the mare's karyotype to be 65,XXX. These cases describe the general phenotypic and karyotype findings of two confirmed 65,XXX mares and may be helpful in identifying future cases. The incidence of 65,XXX karyotype in the mare is rare. X chromosome monosomy (63,X) is the most frequently reported sex chromosome abnormality reported in horses followed by 64,XY,SRY negative sex-reversal. Both 63,X and 64,XY,SRY negative cases present with normal external genitalia but an infantile internal reproductive tract and no follicular development. In these two 65,XXX mares there was follicular development, highlighting a phenotype to be aware of when suspecting chromosomal abnormalities in horses.

Keywords: Trisomy X, karyotype, reproductive dysfunction, reproductive pathology, infertility

Diprosopia, cerebral, cerebellar and pituitary aplasia in a Charolais-cross fetus

E.P. Cambier,[a] M. Diel de Amorim,[a] Y. Huang,[b] C. Card[c]

[a]Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PI, Canada; [b]Prairie Diagnostic Services, Saskatoon, SK, Canada; [c]Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Diprosopia refers to a form of incomplete monozygotic twinning where there is a single body with a head that has a variety of craniofacial duplication abnormalities. A three year-old Charolais-cross heifer was presented with history of being off-feed and dull. Two weeks before presentation the heifer had a prolapsed vagina which was replaced using a Buhner stitch, four days before presentation at our clinic, the vaginal prolapse relapsed. The pregnancy status was unknown and the heifer had been exposed to a bull for the previous 13 months before presentation. Upon presentation in our clinic, fetal membranes were protruding from the vulva and a vaginal discharge of one day's duration was present. Physical examination revealed tachycardia (HR 112), tachypneia (RR 66), hyperthermia (39.8°C), and 7% dehydration. The heifer did not have any characteristic pre-calving udder development. Obstetric examination showed a partially dilated cervix with an autolyzed calf in a posterior longitudinal presentation, with bilateral hip flexure posture and dorso-sacral position. A paramedian ventral abdominal celiotomy was used to deliver a 60 kg bull calf with a crown rump length of 100 cm. Gross findings included macrosomia, hursuitism, and fully erupted molar and premolar teeth. The fetus was diprosopic with hypertelorism and complete medial cheilopalatoschisis. The non-fused halves of the maxilla each had a nostril and were separated by a large skin fold. At necropsy the fetus was noted to have cerebral, cerebellar and pituitary aplasia. Cranial to the atlanto-occipital joint there were bone structures in the cranium resembling vertebrae. These vertebrae were directed ventrally, which created a partial separation between two spaces; a caudal space that contained spinal cord like material, but that lacked a clear distinction between grey and white matter, and a cranial space that contained soft tissue that encapsulated a developed eye with lens, uvea, and vitreous. The appearance of the fetus including the macrosomia, hirsuitism, and fully erupted teeth was compatible with prolonged gestation. The unusual features of this case included musculoskeletal defects such as severe cheilopalatoschisis, in combination with triophthalmia, and aplasia of the central nervous tissue and pituitary. The pituitary aplasia was the underlying cause of the failure to initiate parturition, resulted in a prolonged gestation and fetal macrosomia.

Keywords: Diprosopia, pituitary, cheilopalatoschisis, cerebral, aplasia

Evaluation of a sex cord-gonadal stromal tumor arising during pregnancy in a mare

A.A. Kelleman,[a] A.J. Conley[b]
[a]College of Veterinary Medicine, University of Florida, Gainesville, FL; [b]School of Veterinary Medicine, University of California, Davis, CA

Beginning in the 2013 breeding season, a 13-year-old Thoroughbred mare was followed reproductively until sale of the mare in 2016. In 2013, one 4.6 x 4 cm follicle was present within the left ovary. The mare was bred. One day later, a presumptive left ovarian hemorrhagic anovulatory follicle (HAF) of 6.8 x 5.2cm diameter was present on ultrasonography. Fifteen days later, there was no evidence of pregnancy, the HAF was 11cm and the mare was administered prostaglandin F2alpha. Streptococcal endometritis was managed pre- and post-mating with daily intra-uterine infusion of 2g of buffered gentamicin, postmating saline uterine lavage, and ceftiofur crystalline free acid 6.6mg/kg, IM. The mare double ovulated from the right ovary spontaneously, while the HAF remained on the left. At 15 days, an embryonic vesicle was present, the left ovarian 7cm HAF was fibrin filled, and the right ovary contained corpora lutea. At days 18, 24, 30, 71, and 105, normal pregnancy was observed. At 71days, the left ovary first appeared multi-cystic ("honeycomb") suggesting granulosa theca cell tumor (GTCT) and was similar at 105 days. Subsequently, the ovary was beyond reach transrectally. The mare was assessed throughout pregnancy at 71, 105, 201, 269 and 309 days, at 1, 3, 107, and 154 days after foaling, and on subsequent pregnancy on day 276, utilizing anti-Müllerian hormone (AMH), inhibin, testosterone, and progesterone. Anti-Müllerian hormone concentrations were elevated at >3.8ng/ml in 4/5 specimens obtained during pregnancy, and 2/4 non-gravid. Inhibin was elevated at >0.7ng/ml in only 2/9 specimens, at 71 and 105 d gestation. Progesterone was normally present in all pregnancy specimens. Testosterone was normally elevated during pregnancy, but was abnormally high at 71 and 105 d gestation, at 720 and 364 pg/ml, respectively, and elevated at >45pg/ml in 2/4 samples while non-gravid. Postpartum, the left ovary appeared multi-cystic, but normal estrus and spontaneous ovulation from the right ovary was documented. The mare was not re-bred. The left ovary was excised in October, 2014. Histopathological diagnosis was sex cord-gonadal stromal tumor, consistent with a GTCT. Immuno-histochemistry showed strong specific staining for AMH on the granulosa derived components of the ovary. The following season (2015), the mare was rebred, double ovulated, managed for recurrent streptococcal endometritis, achieved pregnancy with twin vesicles, one of which was manually reduced, and a normal single pregnancy was followed. At 276 days of gestation, endocrinological parameters were normal. The mare was sold. In the diagnosis of GTCT, AMH provided the most consistently useful result during pregnancy and non-pregnancy in this mare.

Keywords: Horse, anti-Müllerian hormone, ovarian neoplasia, pregnancy

Outcomes on four mares undergoing perineal body reconstruction

L. Rothrock, R. Ellerbrock, I. F. Canisso
Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois Urbana-Champaign, Urbana, IL

Perineal body laceration is a common foaling-induced lesion in broodmares. The condition is underdiagnosed as the lesion may go unnoticed, even by experienced personnel. A diagnosis of perineal body tear can be attained by inspecting the perineal body through exposure of the vestibule mucosa by everting the dorsal aspect of the labia and by external digital palpation of the perineal body. Once the mare has been scrubbed, one finger can be introduced into the anus and one in the dorsal commissure, pressuring both fingers towards the roof of the vestibule and floor of the anus. In the normal mare, a musculofibrous node of tissue between the anus and urogenital tract is felt. However, mares with a torn perineal body will have little to no tissue in between the two fingers. These mares present a mucosa with a rough aspect and lines that that resemble striae. Perineal body reconstruction, also known as Gadd's procedure and deep Caslick's surgery, is the treatment of choice for a torn perineal body. This technique is sometimes applied to young maiden mares presenting pneumovagina or pneumouterus uncontrolled by standard Caslick's stitches. The present case report describes the outcome of four broodmares (1 Andalusian, 2 Quarter horses, 1 Standardbred) needing perineal body reconstruction during the summer of 2015. Two mares had a history of dystocia and two were reported to have normal deliveries. A diagnosis of perineal body defect was made as described above. In two mares, perineal body reconstruction was carried out within the first week after foaling; for the other two, the condition was diagnosed in the subsequent breeding season when the mares were presented for breeding management. Each mare was placed in stocks, sedated with detomidine (0.01 mg/kg IV), and the perineal area was thoroughly scrubbed with povidone iodine. In three mares a bilateral pudendal nerve block was performed using an 18gx 6in spinal needle to infiltrate 20 mL of lidocaine (2%) into the paravaginal space, guided by a gloved-hand introduced through the vestibule. The vulva was infiltrated with an in-line lidocaine block. One or two stay sutures were placed on each side of the vulvar labia depending on the vulvar length. After suitable exposure of the affected area, a triangular portion of the dorsal caudal vestibular mucosa was removed on each side. The ventral border of the incision was closed with absorbable suture (polyglactin 910, 2/0) in a simple continuous pattern to create a floor for the perineal body, whereas the top part was closed with continuous interrupted sutures until the dead space was completely reduced. Externally, the mucocutaneous junction vulva was closed with a Ford interlocking pattern using non-absorbable suture as any standard modified Caslick's surgery. There were no surgical complications noted. Profound bleeding and improper healing of the surgical site are reported to be the most common side effects. Three mares were re-bred and then had the perineal body reconstruction performed the day of ovulation. Three mares were confirmed pregnant by 15 d after ovulation, and one mare was not re-bred. Of the three bred mares, one was euthanized three weeks after surgery due to colic and two mares remained pregnant. Our rationale for this case series was to increase awareness of the condition and surgical correction for this important cause of subfertility in broodmares. These cases also highlight that perineal body reconstruction can be performed without apparently complications.

Keywords: Perineal body repair, horse, mare, foaling lesions

Mucometra of the uterus and vagina in a La Mancha doe caused by vestibular and vaginal stricture secondary to dystocia

N.T. Jenkins, T.M. Prado, A.P. Drexler
Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, TN

Persistent hymen, cervical laceration, fibrosis, and congenital strictures are commonly reported causes of mucometra in the doe and other species; mucometra caused by stenosis of the vestibule and the caudal vagina, due to dystocia, has been poorly documented in goats. This case report describes the treatment of a doe with mucometra caused by fibrosis and stenosis of the vestibule and caudal vagina. A 1.5-year-old La Mancha doe was presented to the University of Tennessee's Veterinary Medical Center in January 2016 to determine the cause of her infertility. The doe had a dystocia in the spring of 2015 that caused severe vaginal and vestibular damage. A thorough breeding soundness examination revealed fibrous tissue producing a stricture approximately 3 cm cranial to the vulva, as well as anechoic fluid in the uterus and vagina seen on transrectal ultrasound. Hydrometra or mucometra of the uterus and vagina as a consequence of vestibular/vaginal fibrosis/stenosis was diagnosed, and surgical correction of the obstruction was deemed necessary for both general and reproductive health. The patient was anesthetized and a midline episiotomy was performed and extended 6 cm cranially to allow visualization of the stricture. Transrectal ultrasound was used to guide placement of a 10 cm teat cannula through approximately 3 cm of fibrotic tissue into the fluid filled vagina. Metzenbaum scissors were used to dissect the tissue cranially past the stricture. Enlargement of the opening was accomplished with a 1 cm diameter trephine, followed by progressively larger trocars. After a copious amount of clear, mucus was evacuated from the uterus and vagina, a one inch rectal prolapse ring was placed at the level of the obstruction and secured with non-absorbable suture to maintain the opening while healing. A urethral extension was performed using a technique described for cows and the episiotomy was repaired as a second degree perineal laceration. The rectal prolapse ring was removed six days after surgery; a synchronization protocol for a fixed time insemination with a controlled intravaginal drug release (CIDR) was started 18 days after surgery. The doe was bred 52 hours after removal of the CIDR by laparoscopic artificial insemination. The patient was diagnosed as pregnant by a blood test for pregnancy-specific protein B (bioPRYN) 47 days after artificial insemination.

Keywords- Doe, dystocia, fibrosis, vagina, mucometra

Examination of the equine cervix by high magnification video-endoscopy

R.C. Causey, E.B. Christian, M.A. Hawkes, A.T. Diver, M. Stokes
School of Food and Agriculture, University of Maine, Orono, ME

The equine cervix is thought to be essential for reproductive health in the mare. Based on published anatomical descriptions of its folded architecture and heavily ciliated epithelium, it was hypothesized that the cervix has a functional mucociliary apparatus. The objectives of this study were to: (i) develop a method for high magnification (200 X) video endoscopy of the equine cervix; and (ii) demonstrate the behavior of carbon particles in aqueous suspension applied to the cervical epithelium. Three reproductively sound Standardbred mares were used for the study. To reduce movement, mares were sedated with 5 mg detomidine IV and examined in stocks during estrus. A high magnification endoscope (Fuginon Inc, Wayne, NJ; #EG-590ZW) was introduced manually *per vaginam* to the level of the external cervical os on the first examination in the first mare. In all subsequent examinations the endoscope was immobilized by introducing it through a 14 mm x 52 cm endotracheal tube (Jorgensen, Loveland, CO) anchored to the internal cervical os by an inflatable cuff, the close fit serving to stabilize the endoscope. A 1 cm x 3 cm window (with edges smoothed) was cut in the endotracheal tube, with the long axis oriented longitudinally. Cervical folds flowed through the cut window into the lumen of the tube, allowing visualization by the endoscope. A suspension of 100 mg of carbon powder in 3 ml of sterile water was deposited, via aspiration catheter, onto the visualized cervix. Behavior of visible carbon particles was then video recorded for up to 20 minutes. Following initial survey of the recordings, more detailed observations were performed in which the following events were searched for: bulk flow of carbon suspension resulting from mare movement; unidirectional movement of carbon, not due to bulk flow, relative to a fixed reference point on the cervical epithelium; bidirectional movement in which carbon particles in close proximity were seen moving in different directions or at different speeds; tumbling of carbon particles. Due to high magnification, breathing movements, which could not be eliminated, were sufficient to cause frequent loss of focus. However, for periods of a few seconds, observations were made in each mare, including movement of red blood cells in capillaries, possible lymphatic flow, and bulk movement of the suspension, all correlated with breathing or body movement. In one mare, consistent with mucociliary clearance, unidirectional and bidirectional flow were observed, along cervical folds, at high speed, with possible tumbling. However, body movement may facilitate propulsion, by the cervix, of fluid, carbon, blood and lymph.

Keywords: Equine, mucociliary, endoscopy, cervix, carbon

Creatinine measurements of accumulated intra-uterine fluid can be used to objectively diagnose urometra in mares

M. Schnobrich,[a] I. Canisso[b]

[a]Rood and Riddle Equine Hospital, Lexington, KY; [b]Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois Urbana-Champaign, Urbana, IL

The detection of urine in the vaginal vault or uterus in a mare more than two months after foaling is abnormal and can have a significant negative impact on fertility. Urine is alkaline (pH ≥8), has a high osmolality and crystals, and its presence in the uterine lumen and cranial vagina can negatively affect fertility by causing endometrial inflammation and by interfering directly with sperm viability. Identification of urometra can be somewhat challenging, particularly in mares presenting with the condition intermittently or when the urine flows into the uterus but is undetectable in the vagina. Nonetheless, up to now there have been no reported objective methods to differentiate intra-fluid accumulation without or with urine contamination (i.e. urometra). As creatinine is present in high concentrations in urine and does not diffuse across cell membranes, creatinine concentration in uterine fluid should be negligible in normal mares, but elevated if urine contamination is occurring. To test this hypothesis, we measured creatinine concentrations in the intrauterine fluid accumulation of four mares presented for evaluation and suspected of urine accumulation in the uterus. All mares were examined by vaginoscopy, transrectal palpation, and ultrasonography. Samples for creatinine testing were obtained by placing a sterile plastic, soft-tipped deep-horn insemination pipette into the uterus and aspirating fluid with a syringe. Harvested samples were immediately brought to the laboratory and concentrations of creatinine were determined using an automated analyzer (AU-480 Analyzer, Beckman Coulter, Inc). Interestingly, all four mares in the present report were found to have high creatinine concentrations in the uterine fluid (Table).

Table: Signalments and creatinine concentrations (mg/dl) in intra-luminal uterine fluid

Mare age (yrs)	Breed	Urovagina on vaginoscopy	Suggested urometra on ultrasound	Creatinine (mg/dl)before surgery	After surgery (mg/dl)
8	Thoroughbred	Yes	Yes	42.5	0.2
12	Arabian	No	Yes	47.0	NE
17	Thoroughbred	Yes	Yes	9.3	NE
17	Thoroughbred	No	Yes	4.1	0.4

N.E.: not evaluated.

In two of the subjects a urethral extension was performed and the mares were evaluated 21 days after the procedure. Evaluation of creatinine concentrations in the uterine fluid after surgical correction revealed creatinine concentrations (10 to 200 fold reduction) (Table). These four clinical cases described here highlight a previously undocumented approach to assist the diagnosis of urometra in mares. In addition, in two of the four mares, determination of creatinine concentrations in the intra-uterine fluid accumulations were a useful tool in assessing whether urethral extension was effectively performed. We hope that following the presentation of this case series other clinicians will apply the approach herein described, and with more studies this may become a new diagnostic tool for identifying urometra in mares.

Keywords: Urometra, urovagina, creatine, urine

Vascular shunts of the corpus cavernosum penis unidentified by cavernosography in a Brangus bull

J. Ball, R. Hopper, K. Walters, E. H. King
Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS

A two-year-old Brangus bull was presented to the Mississippi State College of Veterinary Medicine with a history of being unable to extend his penis and failure to service females. During a breeding soundness examination (BSE), the bull required manual assistance to fully extend the penis and a characteristic blushing of the penis was observed. Contrast cavernosography was conducted but did not illustrate the presence of a shunt. New methylene blue dye was then injected into the corpus cavernosum penis (CCP) and following stimulation via electroejaculation multiple vascular shunts were identified as areas of blue coloration appeared in the subcutaneous tissue in the free portion of the penis.

The failure of a breeding bull to attain an erection and achieve intromission is a cause of infertility that is not easily or consistently identified with a BSE. Identification of these impotent bulls allows for timely culling or potential surgical correction. Shunts can be the result of either a congenital weakness in the structural integrity of the tunica albuginea, or acquired via trauma or as a complication following a penile hematoma.[1,2] The presence of single or multiple shunts prevents the "closed system" of the penis from attaining the blood pressure necessary for an erection.[1,2,4] Diagnosis of vascular shunts is typically confirmed by cavernosography in which a contrast medium is injected into the CCP and serial radiographs of the penis are captured.[1-3] Congenital shunts are usually multiple and not considered repairable; acquired shunts can be repaired surgically with a wedge resection of the defect in the tunica albuginea.[4]

Despite the diagnosis of multiple shunts (congenital), surgical repair of the largest shunt was attempted and the bull was placed on a 60-day period of sexual rest. At this time, the bull has not returned for a subsequent examination.

References

1. Maxwell, H: Inability to breed due to injury or abnormality of the external genitalia of bulls. In: Hopper RM, editor. Bovine reproduction. Ames: Wiley 2015. p. 113-127.
2. Wolfe D: Restorative surgery of the penis. In: Hopper RM, editor. Bovine reproduction. Ames: Wiley; 2015. p. 155-171.
3. Gilbert R, Van den Berg S: Communication between the corpus cavernosum penis and the corpus spongiosum penis in a bull diagnosed by modified contrast cavernosography: a case report. Theriogenology 1990;33:577-581.
4. Van Camp S: Common causes of infertility in the bull. Vet Clin North Am Food Anim Pract 1997:13; 203-231.

Unilateral ovariohysterectomy in a 3-year old German Shepherd Dog

C. Garrett, A.K. Johnson, R.R. Wilborn
College of Veterinary Medicine, Auburn University, Auburn, AL

A 3-year old intact female German Shepherd Dog was presented for suspected abortion. She was bred naturally without progesterone timing and whelping was not expected for two weeks. Physical examination abnormalities included hemorrhagic vaginal discharge, delayed capillary refill time, and pale mucous membranes. Blood work findings included anemia and stress leukogram. Ultrasonographically, the uterus was distended with fluid, and a singleton fetus without a heartbeat was identified.

Surgical management was chosen due to the hematologic status of the bitch and non-viability of the fetus. During cesarean section, haematometra and necrosis of the right uterine horn were noted. A single fetus was located within the necrotic horn. The left horn was in good condition macroscopically. The fetus was removed, followed by unilateral ovariohysterectomy to salvage potential fertility. Recovery was uneventful. The bitch was bred naturally on her second heat postoperatively. She was confirmed pregnant and delivered two puppies without veterinary assistance.

Traditionally, the treatment for compromised uterine tissue has been complete ovariohysterectomy.[1,2] Successful unilateral ovariohysterectomy followed by conception and normal parturition have been reported in the bitch, queen, mare, and doe.[3] Indications for unilateral ovariohysterectomy include fetal retention, uterine torsion, uterine rupture, and haematometra associated with cystic endometrial hyperplasia.[4] Potential concerns for future pregnancies include uterine rupture or cornuectomy site perforation, uterine torsion, luteal insufficiency, and dystocia or reduced neonatal viability.[3,4,5] Unilateral ovariohysterectomy does appear to affect future estrous cycles though timing from surgery to first estrous is variable.[5]

Prognosis for fertility is good in unilateral ovariohysterectomy cases if recovery is uneventful and the bitch cycles normally. Clients should expect reduced litter size.[5] Ovulation timing, artificial insemination, progesterone monitoring, and cesarean section should be recommended for future breedings due to potential complications. This case represents an alternative to complete ovariohysterectomy allowing for preservation of fertility in reproductively valuable females.

References

1. Gunzel-Apel AR, Fehr M, Seefeldt A, et al: Prolonged foetal retention in a bitch resulting in trichogranulomatous panmetritis and re-establishment of fertility after unilateral ovariohysterectomy. Reprod Domest Anim 2008;43:117-120.
2. Kumru IH, Seyrek-Intas K, Seyrek-Intas D, et al: Clinical case: unilateral en bloc ovariocornuectomy as a treatment for uterine torsion in a bitch. Rev Med Vet 2011;162:76-78.
3. Jurka P, Kacprazak KJ, Degorska B: Pregnancy in a unilaterally ovariohysterectomised queen. J Feline Med Surg 2015;17:364-366.
4. Seyrek-Intas K, Kumru IH, Seyrek-Intas D, et al: Utero-ovarian torsion in a bitch and subsequent fertility after unilateral ovariohysterectomy. Tierarztl Prax 2011;39:268-270.
5. Seyrek-Intas K, Axel W, Yavuz N, et al: Unilateral hysterectomy (cornuectomy) in the bitch and its effect on subsequent fertility. Theriogenology 2004;61:1713-1717.

Does maternal adrenocorticotrophic hormone (ACTH) administration hasten the onset of parturition in the dog?

J.M. Lection, T.M. Beachler, A.E. Dunlap, C.S. Bailey, S.K. Lyle
Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC

A rescued pregnant mixed-breed dog of unknown age and gestation length with chronic lacerations and hind limb and pelvic fractures was referred to the North Carolina State University Veterinary Hospital. Initial workup included a computerized tomography scan to evaluate the fractures and radiographs to age the fetuses (estimated 54 days). On transabdominal ultrasonographic examination, fetal heart rates ranged from 200 to 250 bpm, but no intestinal peristalsis was imaged, indicating a lack of fetal maturity. Two treatment plans were considered: immediate cesarean section despite fetal immaturity, or delaying surgery and monitoring for fetal maturity using daily ultrasound examinations and measurement of progesterone (P4) concentrations. The second option was elected by ther rescue group. Serum P4 concentrations were 4.9 and 7.4 ng/ml, on days 2 and 3 of hospitalization, respectively. On day 3, due to concern for hypoadrenocorticism, the primary care clinician performed an ACTH stimulation test (cortisol pre-treatment: 4.6 µg/dl, post-treatment 12.5 µg/dl, suggesting normal adrenal function). On day four, mammary development and mild bradycardia of multiple fetuses were detected, and the P4 concentration was 3.7 ng/ml. With concern that endogenous cortisol release initiated parturition, cesarean section was elected. Five of six puppies were successfully resuscitated and the dam recovered without major complications.

This case raises two questions: can dogs be ready for parturition with a P4 concentration approaching 4 ng/mL and can ACTH stimulation initiate parturition in dogs? A marked decrease in P4 concentration prior to parturition in dogs is typical,[1] but was not observed in this case. Intra-fetal ACTH infusion in lambs and kids results in preterm birth,[2,3] while maternal ACTH infusion induces labor in goats[4] but not in sheep.[5]. The difference of effect is possibly related to each species' main P4 source. This case highlights the paucity of literature regarding initiation of premature labor in dogs by maternal ACTH administration.

References

1. Concannon PW, Butler WR, Hansel W, et al: Parturition and lactation in the bitch: serum progesterone, cortisol and prolactin. Biol Reprod 1978 19: 1113-118
2. Challis JRG, Kendall JZ, Robinson JS, et al: The regulation of corticosteroids during late pregnancy and their role in parturition. Biol Reprod 1977 16: 57-69
3. Currie WB, Thorburn GD: Parturition in goats: studies on the interactions between the foetus, placenta, prostaglandin F and progesterone before parturition, at term, or at parturition induced prematurely by corticotrophin infusion of the foetus. J Endocrinol 1977 73: 263-278.
4. Jimenez Escobar C, Basrur PK, Gartley C, et al: A comparison of the adrenal cortical response to ACTH stimulation in Angora and non-Angora goats. Vet Res Commun 1997 22:119-129.
5. Liggins GC, Fairclough RJ, Grieves SA, et al: The mechanism of initiation of parturition in the ewe. Recent Prog Horm Res1973 29: 111-159.

Poor reproductive performance on a beef operation – a population based approach

R. Shutter, R. Kasimanickam, V. Kasimanickam
Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA

Beef operation productivity is determined by annual calf crop (pounds of beef produced per number of cows exposed). Pregnancy achieved earlier in the breeding season and overall breeding season pregnancy rates (BSPR) are two critical factors affecting annual calf crop. Commercial beef producers should aim for a 95% cow calving rate during a nine-week period, with a calving distribution of 65-70% calved in the first three weeks, followed by 20% in the second three weeks, and 10% in the third three weeks.

A pasture based cow-calf operation in northern Washington was investigated for poor BSPR and calving spread, with an average BSPR of 80.2% and a cumulative calving distribution of 51, 70 and 81% in the 30, 45 and 60 day window, respectively. The operation raised 350 females with an 85 day breeding season. Management included comprehensive vaccinations and loose mineral supplementation programs. Investigation revealed a 1:20 bull to cow ratio, with all bulls trichomoniasis negative and considered satisfactory for breeding potential. Body condition scores (BCS) revealed 68, 23 and 9% of females moderate to good, thin, and obese, respectively. Plasma and serum from 20 individuals revealed normal abortion panel titers, but were found to be deficient in trace minerals, including copper, selenium, and zinc.

An enriched mineral supplementation program for all females, and energy supplementation to thin females was implemented. Injectable minerals were given. Additional loose mineral supplements were placed and positioned closer to water resources. In the subsequent season, essential blood trace mineral concentrations were found to be within normal range. Average BSPR improved by 13.8% and 30, 45 and 60 day calving distribution was 64, 19 and 11%, respectively.

Minerals are important for bodily functions and processes. Nutritional management should address mineral requirements throughout the year, particularly for pasture based operations in mineral deficient locations.

Suggested references

1. Ahola JK, Baker DS, Burns PD, et al: Effect of copper, zinc, and manganese supplementation and source on reproduction, mineral status, and performance in grazing beef cattle over a two-year period. J Anim Sci 2004;82:2375-2383.
2. Hemingway RG: The influences of dietary intakes and supplementation with selenium and vitamin E on reproduction diseases and reproductive efficiency in cattle and sheep. Vet Res Commun. 2003;27:159-174.
3. Waldner C, Campbell J, Jim GK, et al: Comparison of 3 methods of selenium assessment in cattle. Can Vet J 1998;39:225-231.
4. Naylor JM, Kasari TR, Blakley BR, et al: Diagnosis of copper deficiency and effects of supplementation in beef cows. Can J Vet Res 1989;53:343-348.

Cystic ovarian disease with inappropriate lactation in a doe

K.K DiMiceli, C.L. Armstrong
Department of Veterinary Clinical Science, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA

Cystic ovarian disease is a cause of reproductive failure in ruminants. The incidence has been reported as high as 12% in goats and cattle.[1] In most cases, follicular cysts can be treated with gonadotropin releasing hormone (GnRH)-induced luteinization followed by luteolysis with prostaglandin in 10 days. Medical therapy with GnRH is reported to have an 80% success rate.[2] It can be difficult to discern the exact etiology of inappropriate lactation in goats.[3]

A seven-year-old intact female pygmy goat was presented displaying persistent signs of estrus and aggressive behavior for the past year. This doe was never exposed to a male. On the physical examination it was noted that the udder was well developed, produced a milk-like substance, but was not palpably abnormally warm. Transrectal ultrasonography revealed round anechoic structures >10mm on both ovaries. A progesterone assay was performed and a concentration of 0.63 ng/ml supported the diagnosis of cystic ovarian disease. Human chorionic gonadotropin (1500 IU) was administered to induce luteinization of the cystic follicles.

A month after treatment there was no significant improvement in the doe's condition. The udder engorgement and milk production persisted. Male-like behavior included urine marking, snorting, and spitting at her owners. Transrectal ultrasonography revealed that the cystic structures on both ovaries persisted. A serum sample was submitted to measure anti-Mullerian hormone, inhibin, progesterone, and testosterone levels. The results helped rule out a possible granulosa cell tumor. Ovariectomy was recommended due to failure of the medical treatment. During the procedure multiple cystic structures were discovered on both ovaries. The ovaries were submitted for histopathology and the diagnosis of cystic ovarian disease was confirmed. Following surgery, the doe's udder regressed in size and was no longer productive. The aggressive, male-like behavior subsided.

This case revealed that ovariectomy should be considered in refractory cases of persistent estrus in pet goats.

Suggested references

1. Medan MS, Watanabe G, Sasaki K, et al: Transrectal ultrasonic diagnosis of ovarian follicular cysts in goats and treatment with GnRH. Domest Anim Endocrinol 2004;27:115-124.
2. Miller CC, Williamson LH, Miller-Liebl DM, et al: Lactation associated with acidophilic pituitary adenoma, pheochromocytoma, and cystic endometrial hyperplasia in two goats. J Am Vet Med Ass 1997;210:378-381.

Successful medical management of pregnancy toxemia in a two-year old Katahdin ewe

K. McCombs, H. Maxwell, J. Stockler, A.K. Johnson
College of Veterinary Medicine, Auburn University, Auburn AL

A two-year old pregnant ewe was presented 128 days following breeding with ataxia, lethargy, and anorexia. Physical examination findings were unremarkable. The ewe was hypoglycemic and blood beta-hydroxybutyrate was elevated. A transabdominal ultrasound detected at least one fetal heartbeat, but more fetuses were likely present. Pregnancy toxemia was suspected.

Initial treatment included intravenous glucose, oral propylene glycol, and Vitamin B complex. Blood ketone and glucose values had improved by the next morning. The rate of dextrose administration was decreased. On day two, flocculent intrauterine fluid was noted on ultrasound and a fetal heartbeat could not be detected. Induction of parturition was elected.

Dinoprost tromethamine (25 mg) and dexamethasone (20 mg) were administered intramuscularly. Blood ketones remained elevated and glucose was poorly regulated following initiation of the induction protocol. Vaginal discharge was noted 24 hours later and two live male lambs were born 41 hours following induction. The ewe and lambs were bright, alert, and responsive following delivery. The dam and lambs were discharged the same day with instructions to monitor for evidence of worsening condition.

Pregnancy toxemia is a metabolic disease affecting small ruminants during late gestation. Characteristics include some degree of anorexia, depression, neurologic deficits, progressing to recumbency and death.[1] The disease is associated with a negative energy balance in late gestation. Females under-conditioned, over-conditioned, or carrying multiple fetuses are at increased risk.[2] Increased blood beta-hydroxybutyrate concentration is diagnostic.[3]

Medical treatment with propylene glycol and intravenous dextrose may be sufficient following early diagnosis.[4] Induction of parturition or cesarean section may be necessary to preserve the life of the dam. This case is significant to the study of theriogenology because it illustrates the value of medical management in cases of pregnancy toxemia in small ruminants. Medically managing these cases can not only result in rewarding outcomes, but also avoids adverse consequences associated with cesarean section.

References

1. Pugh DG, Baird DK: Sheep and goat medicine 2nd edition. Maryland: Elsevier Saunders; 2012. p. 200-201.
2. Ogilvie TH: Large Animal Internal Medicine 1st edition. Baltimore: Williams & Wilkins; 1998. p. 219.
3. Pichler M, Damberger A, Schwendenwein I, et al: Thresholds of whole-blood β-hydroxybutyrate and glucose concentrations measured with an electronic hand-held device to identify ovine hyperketonemia. J Dairy Sci 2014;97:1388-1399.
4. Cal-Pereyra L, Gonzalez-Montana JR, Benech A: Evaluation of three therapeutic alternatives for the early treatment of ovine pregnancy toxaemia. Irish Vet J 2015; 68:1-7.

Bacteriological quality of frozen-thawed stallion epididymal sperm

M. Ciccarelli,[a] D. Diaz-Campos,[b,c] A.J. Campbell,[a] A. Tibary[a]
[a]Comparative Theriogenology, Department of Veterinary Clinical Sciences and [b]Department of Veterinary Microbiology and Pathology, [c]Washington Animal Disease Diagnostic Laboratory, Washington State University, College of Veterinary Medicine, Pullman, WA

Stallion epididymal sperm collection and freezing is a valuable tool to preserve genetics from superior stallions after castration or euthanasia. Retrograde flushing of the cauda epididymis is the most common and efficient technique to collect sperm. This process may expose samples to contamination. In the present study we evaluated the bacteriology of samples collected using this technique. We hypothesized that retrograde flushing of the cauda epididymis with a commercial extender will result in minimal contamination making this frozen-thawed spermatozoa safe for use in mares. The objective of this study was to determine the risk for contamination of stallion epididymal spermatozoa collection and to identify the bacteria most likely isolated. Epididymal sperm was harvested from 13 stallions following closed castration. The samples were cryopreserved using a standard technique for ejaculated semen involving dilution in INRA 96® (antibiotics listed: penicillin [27 mg/L], gentamycin [76 mg/L], amphotericin), centrifugation, and re-suspension of the pellet in a freezing extender (EZ Freezin LE® that contains ticarcillin disodium). All samples had a final concentration of $400x10^6$ spz/mL and frozen in 0.5 mL straws. One straw per stallion was thawed and the contents cultured for aerobic and anaerobic bacteria. Conventional culture media was inoculated, incubated at 37°C and examined for seven days. Bacterial identification was achieved by the use of Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) technology. One sample (7.7%) showed high number of mixed aerobic bacteria (*Bacillus* spp, coagulase negative *Staphylococcus* spp, alpha and beta *Streptococcus* spp) suggesting a high-level environmental contamination. Anaerobic cultures revealed moderate contamination in 6 of 13 stallions (46%) with non-pathogenic bacteria. The most common isolates were *Lactobacillus* spp, *Peptostreptococcus* spp and *Propionobacterium* spp. Bacterial contamination occurs frequently in ejaculates of stallions collected by artificial vagina. Antimicrobials present in the extender help to reduce this contamination. In this study, the use of antibiotic-containing extender for flushing and freezing seemed to be sufficient to control the contamination with pathogenic aerobic bacteria. The presence of anaerobic bacteria, though non-pathogenic, in 46% of the samples is interesting and warrants further investigation. In conclusion, harvesting epididymal spermatozoa with antibiotic-containing extenders seems to be a safe technique as far as risk for contamination of the uterus upon use for artificial insemination.

Keywords: Cryopreservation, artificial insemination, equine.

Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) and asymmetrical spermatogonial division in the stallion

J. B. Andrews,[a] J. L. Rose,[b] C. S. Perumamthadathil,[b] C. J. Scott,[b] S. T. Norman[a]
[a]School of Animal and Veterinary Sciences; [b]School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia

Undifferentiated spermatogonia have been identified as having stem-cell-like activity. They either undergo indefinite self-replication or differentiate into spermatozoa. It has been postulated that spermatogonia can divide symmetrically and asymmetrically. UCH-LI (PGP 9.5) is expressed in undifferentiated spermatogonia of other species. Luo, et al[1] demonstrated that high expression levels of UCH-LI were associated with self-renewal and low expression levels were associated with differentiation. Furthermore they provided evidence for the asymmetrical division of spermatogonia in the boar.[1] The purpose of this study was to immunolocalize UCH-L1 in the stallion testis and to determine if UCH-L1 levels were differentially expressed in spermatogonia of the stallion. Testes were collected from spermatogenically active (evaluated histologically) stallions (2.5 to 5 years of age, n=4) presented for routine castration at the university clinic. Testicular tissues were fixed in Bouin's solution for 24 hrs, embedded in paraffin and processed for immunohistochemistry. Localization of UCH-L1 to spermatogonia was observed following initial immunohistochemistry in all tissue samples. Immunofluorescent localization of UCH-L1 was then used to examine differences in expression levels (fluorescent intensity). For each stallion, approximately 20 seminiferous tubules from a single section were digitally captured and converted to a 32-bit gray-scale image before being analyzed using ImageJ software (http://imagej.nih.gov/). UCH-L1 expression in spermatogonia was analyzed by determining their mean gray-scale intensity and their modal gray value (most frequently occurring gray value). Gray value plot profiles and threshold intensity masking were employed to demonstrate different expression levels in representative spermatogonia. These cells were characterized as either having high or low expression of UCH-L1based on the observed intensity of individual spermatogonium within the same seminiferous tubule. When analysed using a two-tailed t-test, the respective results for low versus high UCH-L1 expressions were: spermatogonium per seminiferous tubule 7.5±2.5 (SD) and 12±3.1 (P=0.02); mean gray-scale intensity of 45±2.1 versus 61.2±3.3 gray scale units (P = 0.002); and modal gray intensity of 45.5±1.7 versus 62.3±6.1 gray scale units (P=0.001). Furthermore, paired or chains of spermatogonia were observed in which adjacent spermatogonium displayed either low, high or mixed levels of expression.

These results demonstrate two populations of spermatogonia with differential UCH-L1 expression levels thus supporting the asymmetrical division model. As it has been proposed that high levels of UCH-L1 expression is associated with proliferation and lower levels with differentiation, we hypothesize that asymmetrical division resulted in the differential UCH-L1 expression levels observed in adjacent spermatogonia. In conclusion, UCH-L1 was identified in spermatogonia of the stallion, however the role of UCH-L1 in spermatogonial differentiation remains to be elucidated.

Keywords: UCH-L1, spermatogenesis, stallion, ubiquitin, spermatogonia

Reference

1. Luo J, Megee S, Dobrinski I: Asymmetric distribution of UCH-L1 in spermatogonia is associated with maintenance and differentiation of spermatogonial stem cells. J Cell Physiol 2009;220:460-468.

Can exogenous insulin improve sperm motility in cooled-stored stallion semen?

S.M. Adcock,[a] R. Ellerbrock,[a] L. Rothrock,[a] V. Muller,[b] I.F. Canisso[a]
[a]Department of Veterinary Clinical Medicine, University of Illinois, Urbana, IL; [b]Universidade Federal de Pelotas, Pelotas, Rio Grande do Sul, Brazil

Unlike many of the domesticated large animal species, stallions are selected based on athletic and phenotypic characteristics rather than fertility. Stallion semen is often extended, cooled, and shipped to the location of the mare prior to breeding, and sperm motility inevitably decreases as semen ages despite the addition of semen extender. There is active interest in identifying additives to extend the life and improve motility of sperm, to increase the likelihood of successfully impregnating mares. Recent studies in human males showed that insulin may play a role in sperm metabolism and motility. We hypothesized that addition of insulin to stallion semen would have a positive effect on sperm viability and motility, compared to an insulin-negative control. To test this idea, semen was collected from eight light breed stallions using the Missouri artificial vagina for a total of 24 separate ejaculates, three from each stallion. Semen extender (EquiPro Cool Gard, MOFA Global, Verona, WI) was used to dilute each ejaculate to a final volume of 50 ml and a concentration of 25 million sperm/ml. The extended ejaculate was divided into three separate 15 ml aliquots (control, 0.25 and 1.0 IU insulin/ml). Aliquots were further divided into three smaller 5-ml aliquots and stored in a passive cooling device (Equitainer, Hamilton Research Inc., Ipswich, MA), to simulate real-life shipping conditions, for subsequent examination of motility. A computer-aided sperm analysis machine (SpermVision II, MOFA Global, Verona, WI) was used for the testing. Total and progressive motilities were analyzed at 0, 24, 48, and 72 hours after collection, and then statistical analyses performed. The motility parameters were log-transformed and analyzed by mixed models. The data were expressed as means +/- SEM, with significance set at $p<0.05$. There were significant effects of time on the total and progressive motilities ($p<0.05$), but no time-group interaction ($p>0.05$). There was no significant difference in motility for each of the three treatment groups at the time points recorded, with total and progressive motilities having the same average whether insulin was in the sample or not. Sperm viability was not directly measured. Therefore, in the present study, the addition of insulin to the semen extender does not improve sperm progressive motility during cooling over a 72 hour time period. Differences in endogenous insulin levels between equine and human semen could possibly account for the different results in similar experiments in the two species.

Keywords: Stallion, semen, motility, insulin

Serum oxytocinase activity in control, pregnant, oxytocin, and carbetocin treated mares at the expected time of luteolysis

M. Diel de Amorim,[a] K. Nielsen,[b] C. Klein,[c] C. Card[b]

[a]Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada; [b]Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada; [c]Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, Canada

Oxytocin in horses is involved in uterine clearance, luteal maintenance, parturition, passage of fetal membranes, maternal-foal bonding, and milk let-down. Oxytocinase/insulin regulated aminopeptidase (IRAP) or leucyl-cystinyl aminopeptidase (LNPEP) is an enzyme that is involved in the regulation of hormones such as oxytocin and vasopressin, and little is known how oxytocin is metabolized. The objective of this study was to characterize oxytocinase (OTase) activity in serum in control, pregnant, oxytocin, and carbetocin treated mares at the expected time of luteolysis. Light horse mares were examined daily in estrus until the day (D) of ovulation (D0), and then on D12, D13, D14, D15 with transrectal palpation and ultrasonography. Jugular blood samples were obtained from D12 to D15 after ovulation. Mares were randomly assigned to treatment, with a rest cycle in between treatment cycles. Groups were: control (n=8), pregnant (Preg) (n=6), (bred using artificial insemination with >200 million normal and motile sperm from one fertile stallion every other day in estrus), oxytocin (Oxy) (n=6) (Oxytocin, Bimeda, MTC, Cambridge, ON, Canada) 60 IU BID IM D7 to 14) and carb (n=4) (T.R.C., North York, ON, Canada,;1.19 mg SID IM D7 to 14). Serum was separated and stored frozen until analysis. A commercial ELISA (LNPEP for horses, MyBioSource, San Diego, CA) with a detection range of 6.25–200 U/L and an intra and interassay coefficient of variation of <15%, which was validated in our laboratory, was used for the analysis. Proprietary software (STATA/SE version 13.1, College Station, TX) using $p<0.05$ was used to evaluate the normality of the OTase data using a Shapiro–Wilk test, and Kruskal Wallis was used to evaluate the effect of treatment and day on OTase. Post hoc analysis was performed using Dunn's all pair wise test. There was a significant effect of treatment ($p=0.0001$), but not day on OTase levels. The OTase levels ng/ml [median (quartiles)] by group were: control [4.9 (2.7, 14.1)], Preg [8.8 (6.0, 19.6)], Oxy [3.0 (0, 8.9)], and Carb [0 (0, 0)]. Oxytocin and carbetocin treated mares had the lowest OTase serum levels, and carbetocin administration lowered OTase serum to below detection limit. The regulation of OTase activity in serum requires further investigation, however it can be speculated that the changes in OTase activity are due to treatment.

Keywords: Oxytocinase, pregnant, carbetocin, oxytocin, serum

Effects of time of insemination relative to time of ovulation on embryonic sex ratio in mares

A.P. Drexler, T.M. Prado, L.L. Amelse, B.K. Whitlock
Department of Veterinary Clinical Sciences College of Veterinary Medicine, University of Tennessee, Knoxville, TN

Oocyte fertilization can occur in most mammalian species by sperm recently deposited in the female reproductive tract or several days after. It has been reported that the timing of artificial insemination (AI) in cattle, sheep, deer, and mice relative to the time of ovulation influences the sex ratio of embryos. The objective of this study was to determine the effects of timing of insemination relative to ovulation on the embryonic sex ratio in mares. One stallion and seventeen mares were used for preliminary study. The stallion was collected using an artificial vagina and the semen was evaluated for concentration, motility, and morphology. Semen was extended at one to one ratio using fresh semen extender (Animal Reproduction Systems, Chino, CA) and a minimum of five hundred million normal motile sperm cells were used to AI each mare within two hours of collection. Mares were examined by trans-rectal ultrasonography to determine follicular diameters and were divided into three groups. To increase the range of ovulation times, the first group was induced with 3000 IU of human chorionic gonadotrophin intravenously while the other two groups were not. Group 1: mares that had a 35 mm diameter follicle, were induced then AI 35 hours after injection. Group 2: mares with follicles that were 35 mm were AI. Group 3: mares that had any follicle greater than or equal to 35 mm were AI. All mares were examined by ultrasound every 24 hours until ovulation was confirmed. Mares were flushed between 8 and 10 days after ovulation and embryonic sex was determined by amplification of the ZFY and ZFX loci by PCR. Once an embryo was recovered from a mare, that mare was moved to an alternate treatment group. The sexed embryos were assigned to one of two categories: 1) those from ovulations occurring less than or equal to 48 hours after AI, and 2) those from ovulations occurring greater than 48 hours after AI. Of the 16 embryos in category one, nine were male (57%) and seven were female (43%). Of the 10 embryos in category two, three were male (30%) and seven were females (70%). The results of this preliminary study suggest that timing of insemination relative to ovulation may influence embryonic sex ratio in the mare. The study will need to be expanded to a minimum of 74 embryos before a statistically significant difference with a 0.8 power can be demonstrated in support of a 30 to 70 ratio, male to female due to ovulations occurring greater than 48 hours after AI.

Keywords: Semen, ovulation, embryo, sex, mares

Effects of lidocaine on fresh equine sperm membrane permeability, motility, and morphology parameters 0 to 48 hours after collection

J.K. Boye,[a] G.A. Dujovne,[b] S.A. Katzman,[c] P.H. Kass,[b] B.W. Christensen[b]
[a]William R. Pritchard Veterinary Medical Teaching Hospital; [b]Department of Population Health and Reproduction; [c]Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, CA

Lidocaine is commonly used for castration in stallions. Local anesthesia facilitates maintenance of a lighter plane of anesthesia and less resistance to mobilization of the testes during surgery. When horses are castrated with subsequent epididymal flush, surgeons are generally advised not to use lidocaine in order to prevent any negative affects local anesthetics may have on spermatozoa and subsequent fertility. However, to date there are no reports evaluating potential detrimental effects lidocaine may have on equine spermatozoa. The aim of this study was to determine effects of different concentrations of lidocaine on sperm morphology and motility after mixing fresh equine spermatozoa with lidocaine. We hypothesized that exposure to different concentrations of lidocaine would decrease total motility (TM), progressive motility (PM), normal morphology (M) and membrane permeability (MP) of the spermatozoa. Semen was collected at two time points from four fertile stallions; samples with TM, PM, and M greater that 80%, 70%, and 70%, respectively, were included in the study. The semen was diluted with INRA96 (IMV technologies, L'Aogle, France) semen extender to a final concentration of 30 x 10^6 sperm/ml, and was mixed with 2% lidocaine to final concentrations of 1 μg/ml, 10 μg/ml, 100 μg/ml, 1,000 μg/ml and 10,000 μg/ml. A sample without lidocaine served as the control. Motility was assessed with a computer assisted sperm analysis system (Spermvision® MOFA Global, Verona, WI). Sperm concentration and MP were measured with a NucleoCounter® SP-100™ (Chemometec, Allerod, Denmark). Morphology was assessed under 1000x phase contrast microscope using a wet mount preparation of semen fixed in buffered formalin. Total motility and PM were compared to the control sample at 10 min, 2 h, 4 h, 6 h, 24 h, and 48 h. Normal morhology and MP were assessed before mixing the spermatozoa with lidocaine and at 48 h. Statistical analysis was performed using mixed effects analysis of variance, with horse as the random effect, and ejaculation (first or second), concentration of lidocaine, and time as categorical fixed effects (Stata/IC 13.1, StataCorp LP, College Station, TX). Models with interactions were compared to main effects models using likelihood ratio tests. There was no significant difference for TM ($p=0.46$ and $p=0.65$) and PM ($p=0.16$ and $p=0.78$) between the control and the lower concentrations of lidocaine 1 μg/ml and 10 ug/ml, respectively, when controlling for time. There were significant decreases in TM and PM at higher concentrations of 100 μg/ml ($p=0.034$ and $p<0.001$, respectively), 1,000 μg/ml ($p < 0.001$), and 10,000 μg/ml ($p < 0.001$) compared to the control (no lidocaine). Addition of interactions between concentration and time did not significantly improve model fit. Normal morphology did not change negatively over time at any concentration. Membrane permeability decreased significantly at 10,000 μg/ml ($p < 0.001$). In conclusion, low concentrations of lidocaine (1 μg/ml to 10 μg/ml) did not significantly affect the parameters TM, PM, or MP when it was mixed with freshly collected semen and stored for 48 hours. Lidocaine concentrations of 100 μg/ml to 10,000 μg/ml decreased TM and PM. MP was negatively affected only at concentration 10,000 μg/ml.

Keywords: Lidocaine toxicity, sperm, stallion, lidocaine

Hydroallantois and prepubic tendon rupture in a Standardbred mare

Madonna Jayne Wilda Morrison,[a] Bradley Back,[a] J. Trenton McClure,[a] Paul Hanna,[a] Claire Card,[b] Mariana Diel de Amorim[a]

[a]Atlantic Veterinary College, University of Prince Edward Island, Charlottown, PE, Canada; [b]Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Hydroallantois in mares is a rare and life-threatening condition,[1,2] which may lead to abdominal wall disease.[2,3] A fourteen-year-old Standardbred mare was presented with a chief complaint of anorexia, colic, excessive ventral edema and an overly enlarged abdomen at nine months of gestation. The mare was tachycardic (112 bpm), 7% dehydrated, and had labored breathing. A serum biochemistry panel showed an elevated creatinine kinase (CK; 1927 U/L), and aspartate aminotransferase (AST; 292 U/L), and elevated serum amyloid A (SAA; 295 μg/ml). A CBC showed a mild leukocytosis with 1+ toxic change. Transrectal palpation and ultrasound findings were: an enlarged uterus domed with no palpable fetus, with an excessive amount (> 15cm) of anechoic allantoic fluid. Transabdominal ultrasound examination revealed that the uterus extended to the xyphoid area. The mammary secretion was bloody. Two jugular catheters were placed and intravenous fluid therapy started with lactated Ringer's solution (LRS). A 24F trocar catheter was inserted transcervically into the allantoic compartment. Allantoic fluid drainage (>170 liters) was performed over 4.5 hours concurrent with aggressive intravenous LRS fluid therapy. The cervix dilated over four hours and the foal was delivered with minor repositioning by traction and was immediately euthanized with intracardiac pentobarbital. After the delivery an umbilical vein infusion using 40 liters of water was performed to assist in placental passage, and the placenta was delivered six hours later. After delivery of the fetus the mare was diagnosed with a complete prepubic tendon tear. There were no gross abnormalities noted on postmortem examination of placenta and fetus, and no signs associated with placentitis were noted on histological evaluation. This the first report of the SAA levels in a mare with a hydropic pregnancy and impending prepubic tendon rupture, and the use of the umbilical vein water infusion technique in a hydroallantoic case.

References

1. Blanchard TL, Varner DL, Buonanno AM, et al. Hydrallantois in two mares. Equine Vet Sci 1987;7:222-225.
2. Waelchli RO: Hydrops. In: McKinnon AO, Squires EL, Vaala, WE, et al, editors. Equine reproduction. 2nd ed. West Sussex (UK): Blackwell Publishing Ltd. p 2368-2371.
3. Card C, Dedden I, Ripley E, Nairn LD. Features of ventrolateral abdominal wall muscles in non-pregnant mares and pregnant mares with and without abdominal wall disease. J Equine Vet Sci 2014:229.

Use of progesterone levels to determine due date in a 1.5-year old English bulldog bitch

B. Shumack, A.K. Johnson, R.R. Wilborn
College of Veterinary Medicine,Auburn University, Auburn, AL

A 1.5 year old English bulldog was presented for a pregnancy examination and whelping management. She was bred without hormonal testing 40-41 days previously using vaginal artificial insemination with fresh semen at another location. Pregnancy was confirmed by transabdominal ultrasonography and a cesarean section was recommended due to the high incidence of dystocia with this breed. Breeding dates, fetal abdominal measurements, and biparietal parameter were used to estimate her parturition date.[1]

Progesterone concentrations were evaluated daily, starting five days prior to delivery and were as follows: 5.3, 6.0, 5.9, 5.3, and 1.7 ng/ml. Transabdominal ultrasound was also performed daily to assess the maturity of the puppies. Once the progesterone concentration decreased below 2 ng/ml and transabdominal ultrasound confirmed fetal maturity (presence of intestinal lumen and kidney definition), an elective cesarean section was performed. The surgery was successful with five puppies delivered.

Accurate due dates can be determined at the time of breeding by documenting a rise in progesterone concentration. A luteinizing hormone (LH) surge will occur once progesterone concentrations reach 2-3 ng/ml with ovulation occurring when progesterone concentrations are 4-10 ng/ml. A parturition date can be expected 65 +/- 1 days after the LH surge.[2,3] Progesterone concentrations are also used to determine a parturition date when no previous breeding management was performed. Parturition typically occurs within 24 hours once progesterone concentrations below 2 ng/ml.[3]

Breeding days can be used to estimate a due date, but a large window of variability occurs with this method. Gestational aging using ultrasonography can narrow the window of accuracy, but may not accurately predict the exact delivery date. This case represents the importance of progesterone concentrations and cesarean section intervention for breeds with high dystocia incidence. It documents case management for determining the timing of an elective cesarean section when hormonal breeding management was not performed.

References

1. Lenard ZM, Hopper BJ, Lester NV, et al: Accuracy of prediction of canine litter size and gestational age with ultrasound. Aust Vet J 2007;85:222–225.
2. Luvoni GC, Beccaglia, M: The prediction of parturition date in canine pregnancy. Reprod Domest Anim 2006;41:27–32.
3. Kim Y, Travis AJ, Meyers-Wallen VN: Parturition prediction and timing of canine pregnancy. Theriogenology 2007; 68:1177-1182.

Short gestation length in Cavalier King Charles spaniels

K.S. Baltutis, K. Settle, T. Beachler, S. K. Lyle, F. Panis, C.S. Bailey
Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC

The Cavalier King Charles spaniel (CKCS) is a toy breed that ranks as the 19th most popular dog breed by the American Kennel Club. There has been anecdotal evidence from breeders of CKCS that their bitches consistently whelp early. In this case series, the gestation length of 17 CKCS bitches was evaluated based on timing of the luteinizing hormone (LH) surge. As a control, the gestation length of 17 bitches of other, non-CKCS breeds was evaluated in the same manner. The bitches selected for the control group included a Lucas terrier, Tibetan terrier, French bulldog, English springer spaniel, border collie, Airedale terrier, Nova Scotia duck tolling retriever, Labrador retriever, Polish lowland sheepdog, German shorthaired pointer, Briard, black and tan coonhound, German shepherd dog, Gordon setter, Doberman pinscher, Rottweiler, and a great Dane. The date of the LH surge was estimated as the date at which progesterone was measured between 2.0 and 3.0 ng/mL. Gestation length was compared between groups using a Wilcoxan Rank Sum test. Gestation length for the CKCS bitches was calculated to be 62.8 ± 2.0 days (range = 60 to 66 days) whereas the gestation length for the control group was calculated to be 64.5 ± 1.4 days (range = 62 to 68 days). The difference between the two groups is statistically significant ($p < 0.05$). On average, the gestation length measured for the CKCS bitches was 3.2% shorter than the reported canine gestation length of 65 days from the LH surge. This difference has clinical implications for pregnancy management of this breed, including recommendations for scheduling a timed cesarean section and approaches to managing late-term complications.

Estrus staging via vaginal cytology and behavior in Aye-Ayes (*Daubentonia madagascariensis*)

E.W. Hyde, C.S. Bailey, R.L. Schopler, C.V. Williams, J. McKinney, M. Dye
College of Veterinary Medicine, North Carolina State University, Raleigh, NC

Aye-ayes (*Daubentonia madagascariensis)* are listed as endangered by the International Union for Conservation of Nature. There are only 52 captive aye-ayes in the world. Duke Lemur Center is home to the largest population in North America, with fourteen individuals. The very small genetic pool for propagation of captive populations, combined with slow maturation and relatively low fecundity of the aye-ayes makes population management difficult. Consequently, it is vital to develop an understanding of their physiology. The current case describes the correlation of vaginal cytology and physical changes of the external genitalia from a single adult aye-aye that has been trained to allow collection of regular vaginal swabs.

Between February 6, 2015 and April 23 2015, twelve vaginal swabs were obtained successfully with intervals of two to twenty-four days between samples. Seven additional swabs resulted in non-diagnostic, acellular slides. During this time, physical changes of the external genitalia were also observed and graded subjectively on a 0-4 score, in accordance with established policy of the center. Cellular "cornification" gradually increased between February 6th and March 4th, with 99% superficial cells noted between March 4th and March 6th followed by reversal to 0% superficial cells over seven days. On April 22nd, greater than 90% superficial cells were noted again. These findings are consistent with changes typically seen in canids during proestrus, estrus, and diestrus and corresponded with development of vulvar edema with a subjective grade of 1-2 in this animal during periods of "cornification", compared to a grade of 0 during periods when predominately parabasal cells were noted.

This case is the first to characterize the entire cycle of an aye-aye using both physical and cytological observations. Systematic hormonal assays, combined with cytologic, physical, and behavioral observations are urgently needed to define the normal cycle of aye-ayes.

Bilateral testicular Sertoli cell tumors in an American Paint Horse

Kayla Nielsen,[a] Mariana Diel de Amorim,[b] Moira Kerr,[c] Travis Smyth,[a] Claire Card[a]
[a]Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Candda; [b]Atlantic Veterinary College, University of Prince Edward Island, Charolettetown, PI, Canada; [c]Prairie Diagnostic Services, Saskatoon, SK, Canada

Testicular neoplasms are infrequently reported in stallions, and Sertoli cell tumours are extremely rare. Between the ages of 6 and 16 years old a healthy American Paint Horse (APH) breeding stallion was collected for breeding purposes. The number of progressively motile and morphologically normal sperm (PMMN), in billions by year by collection was: in 2005 0.0945 PMMN, 0.823 PMMN, in 2008 0.188 PMMN, in 2009 0.128 PMMN and 0.066 PMMN, respectively. In 2015 the stallion was sold, and in November a breeding soundness examination (BSE) was performed. Findings included an elevated respiratory rate (30 rpm), and a body condition score of 8.5/9. He displayed normal libido. In two semen collections spaced one hour apart he ejaculated 0.078 PMMN, and 0.061PMMN billion sperm. His testes were small and soft bilaterally and measured LxWxH cm and volume (cm^3) as: left 9x5x5.7, (256.5); and right 8.2x5x5.6, (229.6), respectively. His calculated daily sperm output was 4.85 billion sperm. An ultrasound evaluation of his testes showed bilateral hypoechoic areas with the right testis having an oval lesion measuring 20.1 x 17.7 cm, and left having the entire central region abnormal surrounded by testicular parenchyma. The stallion was classified as unsatisfactory due to oligospermia, spermiostasis (36% and 28% detached heads) and bilateral testicular neoplasia. In January 2016 the stallion was castrated and the testes submitted for histopathology, which showed bilateral sertoli cell tumors. This is the first report of bilateral Sertoli cell tumors in a stallion along with reported semen parameters. From the history it is not possible to ascertain the onset of the testicular neoplasia but the tumor tissue grossly replaced a large portion of the testicular parenchyma and may be responsible for the stallion's low sperm production.

Suggested references

Pratt SM, Stacy BA, Whitcomb MB, et al: Malignant Sertoli cell tumor in the retained testis of a unilaterally cryptorchid horse. J Am Vet Med Assoc 2003;222:486-490.
Rahaley RJ, Gordon BJ, Leipold HW, et al: Sertoli cell tumour in a horse. Equine Vet J 1983;15:68-70.

Estradiol-17β and alpha-fetoprotein as diagnostic markers for ascending placentitis in a Quarter Horse broodmare

L. Rothrock, R.E. Ellerbrock, I. F. Canisso
Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois Urbana-Champaign, Urbana, IL

Placentitis is the leading cause of late-term pregnancy wastage in mares.[1] Diagnosis of placentitis is based on clinical signs (vulvar discharge and premature lactation) and transrectal ultrasonography.[1,2] While transrectal ultrasonography is a valuable diagnostic tool, early stages of ascending placentitis can be missed.[1] Recently, estradiol-17β and alpha-fetoprotein (products of the fetoplacental unit) were shown to be reduced and elevated, respectively, in plasma of mares with experimentally induced placentitis.[3,4] It remains to be determined whether these molecules are useful markers for spontaneous placentitis. A 14 year-old mare at approximately 300d gestation was presented to our clinic for premature lactation. Physical examination revealed scant greyish vulvar discharge and pronounced udder development. Transrectal ultrasonography demonstrated a combined thickness of uterus and placenta slightly above normal (13 mm) and a small area of placental separation at the cervical star. Vaginoscopy revealed an open cervix with scant purulent discharge. A clinical diagnosis of ascending placentitis was made. The mare was started on a 5d-course of gentamicin (6.6 mg/kg IV, SID), procaine penicillin G (22,000 units/IM BID), flunixin meglumine (1.1 mg/kg PO, SID), and altrenogest (0.088mg/kg PO, SID). Plasma samples were collected daily and preserved at -80°C for analyses of estradiol-17β and alpha-fetoprotein with commercial immunoassays[3,4]. Following initial 5d-treatment, the mare was continued on altrenogest and trimethoprim-sulfamethoxazole (30mg/kg PO, BID) until uneventful delivery of a live premature filly 17d after admission. Treatment for sepsis was initiated but discontinued due to financial restrictions. Estradiol-17β varied from 40 to 110 mg/mL, and alpha-fetoprotein was highly variable but remarkably elevated (2-20ng/mL); both were consistent with experimentally induced placentitis. This case illustrates the usefulness of estradiol-17β and alpha-fetoprotein in a mare with spontaneous placentitis. Ultrasonographic changes were subtle enough that practitioners with limited experience scanning late-term pregnant mares could have missed the lesions present, thus both estradiol-17β and alpha-fetoprotein aided in the diagnosis.

References

1. Canisso IF, Ball BA, Erol E, et al: Comprehensive review on equine placentitis. Proc Annu Meet Am Assoc Equine Pract 2015;64; 350-370.
2. Troedsson MH, Zent WW: Clinical ultrasonographic evaluation of the equine placenta as a method to successfully identify and treat mares with placentitis. K. Betteridge, editor. Workshop on the Equine Placenta, University of Kentucky, Agricultural Station, Lexington, KY; 2004. p.66-67.
3. Canisso IF, Ball BA, Esteller-Vico A, et al: Changes in maternal androgens and oestrogens in mares with experimentally induced ascending placentitis. Equine Vet J 2016; doi: 10.1111/evj.12556.
4. Canisso IF, Ball BA, Scoggin KE, et al: Alpha-fetoprotein is present in the fetal fluids and is increased in plasma of mares with experimentally induced ascending placentitis. Anim Reprod Sci 2015;154:48-55.

Pyometra in a Standardbred mare

David Best,[a] Bradley Back,[a] Bronwyn Crane,[a] Claire Card,[b] Mariana Diel de Amorim[a]
[a]Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PI, Canada; [b]Western College of Veterinary Medicine, Univsrity of Saskatchewan, Saskatoon, SK. Canada

Pyometra in mares requires intensive treatment, as it leads to endometrial degeneration.[1] An asymptomatic 19 year-old Standardbred mare was presented with a chief complaint of an abnormally enlarged uterus, discovered by palpation. Her reproductive history included untreated chronic uterine fluid accumulation, and breeding by a stallion ten months earlier. The differential diagnosis included pyometra, endometritis and pregnancy. Examination showed an inward sloping vulva and sunken anus. A transrectal palpation and ultrasonography revealed the mare was non-pregnant, had a toned cervix, an enlarged uterus containing > 10 cm grade IV fluid, a corpus luteum on the right ovary, and no significant structures on the left ovary. There was no vaginal discharge, and no cervical abnormalities. A self-retaining catheter was passed into the uterus and 15L of purulent exudate was drained, which grew >2+ *Streptococcus zooepidemicus,* sensitive to ampicillin and sulfadiazine/trimethoprim (TMS). The uterus was lavaged with saline, and 200mls of 3.3% of N-acetylcysteine (NAC) was infused into the uterus and left overnight. The mare was then sedated for hysteroscopy, which revealed a hyperemic endometrium, and purulent exudate tightly adhered to the uterine wall. A second uterine infusion of NAC, removed by uterine saline lavage 12 hours later, was performed, and followed by infusion of 500mg of ampicillin in 60mls of sterile water. The mare was placed on oral TMS at 30mg/kg, twice daily for five days. Reevaluation a week after the last treatment, showed no fluid accumulation, and Caslick's surgery was performed. Three months later, the mare was found to be in early transition with 2cm of grade 0 intrauterine fluid. This case was unusual in that the exudate adhered to the endometrium. The use of NAC was important to assist with the removal of the adherent exudate, and the breakdown of a pathologic biofilm.

Reference

1. Lu KG: Pyometra. In: McKinnon AO, Squires EL, Vaala, WE, et al, editors. Equine reproduction. 2nd ed. West Sussex (UK): Blackwell Publishing Ltd. p 2652-2654.

Intentional superfecundation through surgical insemination with cooled, shipped semen in the bitch

Rebecca Cooper, Dietrich Volkmann
Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO

A review of the scientific literature failed to produce any reports on the deliberate creation of a multi-sire litter of pups. Previous studies illustrate superfecundation occurring naturally in a colony of feral cats[1] as well as in unplanned matings of dogs.[2] Historically, breeding a bitch to two sires in succession or mixing the semen prior to artificial insemination usually produces a litter sired by just one of the males. The goal for this case was to intentionally produce a dual-sired litter with approximately equal proportions of each sire.

The bitch was inseminated with two sires via surgical insemination using cooled, shipped semen 5 days after the luteinizing hormone (LH) surge. Ultrasonography confirmed at least five ovulation sites per ovary at the time of insemination. Semen of Dog A (1.2 mL; 240 million; 65% progressively motile) and Dog B (1.2 mL; 300 million; 80% progressively motile) was deposited in the left and right horns, respectively. Prior to semen deposition and for five minutes after, each uterine horn was occluded at the base using digital pressure so the semen of each donor remained trapped in the horn into which it was deposited.

The bitch became pregnant and her five live pups (2 and 3 from the left and right horns, respectively) were delivered by elective cesarean section 64 days after the LH surge. DNA testing determined one pup was from Dog A and four were from Dog B. The pup sired by Dog A was located in the tip of the left uterine horn where Dog A's semen was deposited. The pups located in the right uterine horn and at the base of the left uterine horn, nearest the bifurcation, were sired by Dog B.

Explanations for the observed outcome and suggestions for an improved fertilization rate and puppy distribution will be presented.

References

1. Natoli E, Schmid M, Say L, et al: Male reproductive success in a social group of urban feral cats (*Felis catus* L.). Ethology 2007;133:283-289.
2. Senger PL:Pathways to pregnancy and parturition, 3rd ed. Redmund: Current Conceptions, 2012.

Death following acute respiratory distress in a two-day old embryo transfer kid after dystocia and cesarean section

Z. Deutsch, M. Ciccarelli, A.J. Campbell
Comparative Theriogenology, Department of Veterinary Clinical Sciences, Washington State University College of Veterinary Medicine, Pullman, WA

Neonatal losses in embryo transfer programs are devastating for producers. This report describes dystocia management with cesarean section in an embryo transfer recipient doe followed by sudden death in the kid.

A two-year old Nubian doe recipient presented for dystocia following prolonged second stage labor without progression. Cesarean section was elected. The doe and kid recovered uneventfully and were discharged. Two days following surgery, the kid was readmitted due to lethargy, anorexia, and exercise intolerance. Physical examination revealed pale mucous membranes, tachycardia, hyperpnea with abdominal effort, and occasional open-mouth breathing. Complete blood count and serum biochemistry were performed and demonstrated decreased hemoglobin, elevated GGT, elevated CK, elevated BUN, hypoproteinemia (due to hypoalbuminemia), hyperphosphatemia, and hypomagnesemia. Pneumonia was suspected initially and abdominal and thoracic ultrasonography were performed. Thoracic ultrasonography revealed several comet-tail artifacts, areas of increased echogenicity bilaterally along the pleural surface, and suspected cardiomegaly. Antibiotics and oxygen therapy were initiated, however clinical signs continued to worsen resulting in death within 12 hours of presentation.

Gross necropsy revealed a patent foramen ovale with right auricular and ventricular dilation, right ventricular hypertrophy, moderate ascites and severe, diffuse, pulmonary edema. Histopathology revealed pulmonary edema and hepatic congestion. A congenital patent foramen ovale, a condition sparsely reported in the literature, was diagnosed as the cause of death in this kid.

This case illustrates the importance of thorough evaluation of neonates with sudden onset of weakness or lethargy for congenital abnormalities. Many neonatal deaths can go undiagnosed, and the clinical presentation for acute right-sided heart failure and primary lung disease can be similar. Ultrasonography of the thoracic in neonates is an important clinical skill for theriogenologists. In this case, ultrasonography and postmortem evaluation were essential for determining the definitive diagnosis.

Keywords: Congenital defects, dystocia, cesarean section, ultrasonography

References

1. Bormann CL, Ongeri E, Krisher RL: The effect of vitamins during maturation of caprine oocytes on subsequent developmental potential in vitro.Theriogenology 2003;59:1373-1380.
2. Parry BW, Wrigley RH, Reuter RE: Ventricular septal defects in three familially-related female Saanen goats. Aust Vet J 1982;59:72-76.
3. Schrama HJ, Nie CJV, Venneman W: Congenital hypoplasia of the pulmonary trunk without a ventricular septal defect in a goat lamb. Vet Q 1982;4:186-189.

Hydrops allantois in a mare with twin pregnancy

Lillian P. Baker, Elvia R. Bridges, Andrew Lovelady
Large Animal Teaching Hospital, College of Veterinary Medicine, Tuskegee University, Tuskegee, AL

This case is important to theriogenology because it will educate veterinarians on recognizing the clinical presentation of this condition, treating it, and subsequently reestablishing the reproductive health of the patient.

A 15-year old Spotted Racking Mare was presented for severe ventral edema and reluctance to move. The owner suspected that she was exposed to the neighbor's stallion. Physical examination identified hard and warm bilateral edema extending from the ventral thorax to the inguinal area.

Rectal palpation revealed uterine atony and no palpable fetus. Transabdominal ultrasonnography identified hyperechoic fluid in the uterus and severe ventral edema in the abdominal wall musculature. A vaginal examination determined that the cervix was two centimeters dilated. The patient was diagnosed with hydrops of the fetal membranes.

Treatment therapy was initiated by manually dilating the cervix. Five liters of balanced electrolytes in water; 500 mL of calcium borogluconate, magnesium borogluconate, and calcium hypophosphate solution, and 120 mEq of potassium chloride was administered intravenously (IV). Five hundred mg of flunixin meglumine and one gram of prednisilone sodium succinate were also administered IV to decrease the risk of hypovolemic shock. Ninty-four liters of fluid was slowly drained from the uterus using a sterile tube following fluid therapy.

Parturition was induced by administering twenty units of oxytocin IV. Once the amniotic sac became visible, it was manually ruptured. The first foal was delivered in a posterior position. A second foal was then detected. An additional 20 units of oxytocin was administered IV after a brief rest period. Fifteen minutes later, the second foal was manually removed. The umbilical cord was extremely twisted. Each foal was euthanized after birth. The presence of twins and the twisted umbilical cord could have each contributed to the accumulation of fluid that lead to the presentation of hydrops. Balanced electrolytes in water, flunixin meglumine and ceftiofur sodium were administered for postpartum management. The mare had an uneventful recovery.

Keywords: Hydrops, ventral edema, twins, parturition, pregnancy

Use of midazolam to enhance copulatory behavior in a maiden Standardbred stallion during semen collection

H. Lynaugh, R.E. Ellerbrock, I.F. Canisso
Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois Urbana-Champaign, Urbana, IL

Midazolam, an imidazobenzodiazepine, is used as a pre-anesthetic, with good anxiolytic properties.[1] Low doses of midazolam (20-25mg/horse) have been incorporated into the management of low libido and fractious stallions. It is unknown how the drug benefits stallions, however, its use is becoming more common when stallions presented for mating/semen collection refuse to mount the phantom or a receptive estrus mare. Reasons for this refusal can be multifactorial, and may have challenging etiologies. Here we describe the use of midazolam to enhance mating behavior in a six-year-old stallion presented for semen collection. Since this horse was raised as an orphan and had received very little handling, the horse received reconditioning behavior outside the breeding shed during his stay. Upon arrival, he was given six 15min-training sessions on a phantom in the presence of an estrous mare. During the first session the stallion exteriorized the penis, but never tried to mount the mare or phantom. In the afternoon, he allowed washing of his penis, but did not mount the mare or phantom despite strong encouragement. The next day the stallion was teased with the mare wearing breeding hobbles, and despite a full erection, the stallion did not mount. The stallion was given 25 mg of midazolam intravenously, and five minutes later re-introduced to the mare, his penis was washed, and a successful ejaculate ($8.4x10^9$ sperm) was obtained using Missouri model artificial vagina. A second ejaculate ($4x10^9$ sperm) was obtained one-hour later; this time the stallion rapidly achieved an erection and mounted the mare on one jump. Both ejaculates were extended and combined to breed the mare. Administration of midazolam enhanced copulatory behavior on this fractious stallion. It is unclear whether the horse would have mounted without the use of midazolam, but based on his demeanor, he probably would have required several more training sessions to achieve a semen collection.

Reference

1. Hubbell JA, Kelly EM, Aarnes TK, et al. Pharmacokinetics of midazolam after intravenous administration to horses. Equine Vet J 2013;45:721-725.

A rare case of ovarian leiomyoma in a lion (*Panthera leo*)

Adeline R. Noger, C. Scott Bailey, Luke B. Borst
College of Veterinary Medicine, North Carolina State University, Raleigh, NC

A twenty year old intact female lion (*Panthera leo*), owned a by a wildlife facility in North Carolina, was presented to its primary care veterinarian for progressive anorexia, weight loss, and lethargy of three months duration. Following sedation and chemical immobilization, physical examination and palpation revealed a large mass in the caudoventral abdomen. Given the animal's age and recent decline, euthanasia was elected and a necropsy was performed. The left ovary was enlarged with a multinodular firm surface measuring 11x9x3.2 cm. On cut surface the ovary showed both solid and cystic portions. Histopathology revealed a well-circumscribed, non-encapsulated mass completely replacing normal parenchyma and blending with the adjacent smooth muscle suggesting ovarian leiomyoma. Female reproductive tract leiomyomas are well documented in the literature, typically arising from uterine tissue. Ovarian leiomyomas have been reported in several veterinary species,[1-4] but they remain rare, and to the authors' knowledge this is one of two reports of this neoplasm in a lion.[3] While reports of tumors in large felid species are limited in the literature, the most commonly reported reproductive tract tumors are uterine leiomyomas. Due to the benign nature of leiomyomas they typically do not cause substantial morbidity or mortality, however when arising from the reproductive tract they can present complications in captive conservation breeding efforts.

References

1. Baumwart CA, Prado TM, Anderson MP, et al: Successful treatment of a Vietnamese potbellied pig with ovariin leiomyoma. J Am Vet Med Assoc 2010;236:558-561.
2. Hermes R, Hildebrandt TB, Walter C, et al: The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (Ceratptherium simum simum, C.s. cottoni) Theriogenology 2006;65:1492-1515.
3. Junginger J, Hansmann F, Herder V, et al: Pathology in captive wild felids at German zoological gardens. PLoS One, *10*(6). doi:10.1371/journal.pone.0130573
4. Shawa N, Boonsriroj H, Kimitsuki K, et al: The co-existence of a myxoid liposarcoma and leiomyoma in the same ovarian mass of a dog. J Vet Med Sci 2016; 78:467-471.
5. Siegal-Willott JL, Henrikson T, Carpenter JW, et al: Chronic obstipation in a leopard (*Panthera pardus*) caused by intrapelvic uterine leiomyoma compression of the distal colon. J Zoo Wildl Med 2005;36:534-537.

An unusual case of fetal maceration in a cow

Devin Gardner, Dietrich Volkmann
Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO

The presentation of this case to the theriogenology service was initiated by discovery of a 2.75 inch mandible in the cervix of a cow, with additional fetal bones in the uterus. The nine-year-old Guernsey cow was due to calve two weeks prior to presentation. No calf observed at that time, but fetal membranes were passed. The size of the recovered mandible indicated a half-term fetus. It was hypothesized that only one fetus died earlier in gestation, followed by birth of a twin at full term, evidenced by the passage of fetal membranes.

The cow was previously treated for a uterine infection based on persistent, odiferous vaginal discharge. Transrectal ultrasonography revealed fluid and fetal bones within the uterus. The fetal bones were localized in the left uterine horn. The cervix was flaccid and open. The owners elected a hysterotomy by left flank incision. The fetal bones were removed manually through incisions in both uterine horns, and the uterus was thoroughly lavaged. The endometrium of the left horn was noticeably deteriorated. The cow was treated with antibiotics, anti-inflammatory drugs and recovered well. Months later, the cow was diagnosed with intraluminal and serosal uterine adhesions. Oocyte recovery, in vitro fertilization, and embryo transfer will be attempted, but to date no oocytes have been recovered.

The fascination in this case is the concurrent discovery of a mandible consistent with a rather small fetus, and expelled fetal membranes, which indicates the completion of parturition. It is speculated that this cow conceived twins nine months prior to passing the fetal membranes. One fetus died and was mummified while the other was carried to term. The mummy was retained inside the uterus after calving, and an ascending infection from the open cervix resulted in maceration. Pathophysiology and alternate mechanisms for these events will be presented.

Selected reference

1. Mehta JS, Kumar P, Purohit GN: Surgical management of a macerated bovine fetus. Ruminant Science 2013;2:107-108.

Intermittent hemospermia and pregnancy rate in a Quarter Horse stallion

Erin Patricia Cambier,[a] Mariana Diel de Amorim,[a] Claire Card[b]
[a]Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PI, Canada; [b]Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Hermospermia has been reported to be related to urethral rents, penile/urethral injury or infection, neoplasia, and granulomatous lesions, and experimentally has been reported to effect fertility. A 10-year old Quarter Horse stallion was referred for a lameness and fertility evaluation. Prior to referral his stifle had been injected with intra-articular steroids. Significant findings included left stifle osteoarthritis and a damaged meniscus. The stallion was maintained on stall rest and intermittent oral phenylbutazone. The stallion was administered (n=12) a combination of 400mg of imipramine IV and one hour later 150 mg xylazine IV for ex copula semen collection, and was collected using a breeding phantom and artificial vagina (AV) (n=20). Semen was collected in 2/12 ex copula attempts, and 19/20 attempts with an AV. The ex copula collected semen contained no red blood cells, but six of the stallion's ejaculates collected with an AV contained a large amount of blood. Following hemospermic semen collections with an AV blood was seen in the urethral process. The stallion was sedated, urethroscopy was performed which revealed that the blood was associated with a fibrotic enlarged urethral process. The stallion was treated with trimethoprim sulfa orally at 30 mg/kg for one week. The semen was extended using INRA96 in a 1:1 ratio. Fifteen mares were bred with peri-ovulatory insemination(s) in 19 cycles. The first cycle pregnancy rate (PR) was 11/15 (73.3%), per cycle PR 12/19 (63%) and seasonal PR was 12/15 (80%). Six of twenty-one ejaculates were hemospermic; PR with and without hemospermia were similar.

Selected references

1. Pearson LK, Campbell AJ, Tibary A. How to diagnose and treat hemospermia: a review and case series. Proc Annu Meet Am Assoc Equine Pract 2013;59:40-50.
2. McKinnon AO, Voss J, Trotter GW, et al: Hemospermia of the stallion. Equine Pract 1988;10:17-23.

Society for Theriogenology
"Veterinarians Dedicated to Animal Reproduction"
761 Tiger Oak Drive
Pike Road, AL 36064-3063
334-395-4666 (voice)
334-270-3399 (fax)
http://www.therio.org