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 7, Number 3
September 2015

Clinical Theriogenology

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

Clinical Theriogenology
ISSN: 2154-3968

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The Society for Theriogenology
and
The American College of Theriogenologists

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

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

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

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Differential Diagnosis. (if relevant)
Treatment.
Outcome.
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Learning points. Three to five bullet points
References. Vancouver style
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2018
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Manuscripts not received in time to be published in the proceedings issue and manuscripts from symposia will be included in subsequent issues of *Clinical Theriogenology*.

The 2015 Bartlett Address
Musings of a comparative theriogenologist

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It truly is amazing that I find myself giving this presentation, for many reasons. To be recognized by one's peers is humbling. To know you are old enough to be considered is disconcerting. It does give one pause to go back over one's career and life to consider how you arrive at this point. I have had several significant mentors over the years as I guess we all have. I want to accept this award in the name of Dr. John Williams, a charter diplomate who had a profound influence on my journey as a theriogenologist and as a person. Dr. Williams was the best theriogenologist that I have ever had the pleasure to work with. He was known best for his work with cattle, but he had significant knowledge in horses and dogs. He was the one to encourage me to pursue my expertise in canine theriogenology that proved to be wonderful advice for me and my career. We spent many hours talking about, not just veterinary medicine, but life in general. Anyone who was ever around Dr. Williams knows what a philosopher he was.

When I was accepted into veterinary school, there were not that many women in the profession. Now, the gender ratio in our veterinary schools is heavily weighted toward women and has been for many years. I have fortunate to have been in the right place at the right time in so many different situations. Yes, I have taken advantage of these opportunities but I've had many people that have been influential along the way.

I was the only one in my class that went into an exclusively equine practice upon graduation. Travis Collins was not one that you would think would be open to hiring a woman in 1977. He was a big guy from Mississippi, an Auburn graduate, and a Lieutenant Colonel in the Army Reserves. He was a member of the pistol team and always carried a gun in his truck. He hired me without reservation, and was glad to learn from a new graduate. e wanted me to carry a gun as well, but I had a Doberman and didn't feel like I needed a gun. We practiced around Atlanta. Some of the situations I found myself, having money and drugs on the truck, could have been dangerous, but weren't. My Doberman, Brigand, was a wonderful conversation piece and a total gentleman. I practiced for two years with Travis and gained much in those years in knowledge and experience. I am a firm believer that one makes a better academic veterinarian if you have had practice experience. A taste of the real world helps a professor to train veterinary students. I had been told by equine practitioners of the day that women could not do equine work. I guess we showed them. Travis was always willing to discuss cases and was a practical teacher, himself. It was a remarkable two years.

In 1979, I found myself needing to find another job. Our practice was the typical one and a half man practice, busy in the spring-summer, slow in the fall-winter. I called John Williams, my theriogenology professor at the University of Georgia. He was well known for being the guy who knew the veterinarians who were looking for jobs and those looking for associates. He asked me if I would be interested in a residency. The next thing I knew I was on my way back to Athens, Georgia.

The theriogenology residency at the University of Georgia was primarily a bovine practice. We had many dairies and beef herds that we served as well as performing numerous bull evaluations throughout the year. We did the equine work for the university horse program during the spring. The canine work was almost nonexistent. I loved my residency. I loved the bovine work, the dairy farmers, and most of all, I loved the students. I had found my niche: Teaching.

Our residency program included doing a Master's degree simultaneously. I found that I loved being a student again. I decided on an antisperm antibody project that took advantage of all the bulls we saw for BSE's. Dr. Donald Dawe became my major professor, as he was our immunologist on the faculty. I was comparing results of our standard BSE with the presence of antisperm antibodies in the bull's serum. I learned the basics of research. I came up with the idea. I wrote the grant. I took the samples. I worked out the technique. I analyzed the results. I wrote the paper. In addition, I learned two things that were happenstance. 1. Bulls that were positive for leptospira had a higher incidence of

antisperm antibodies, and 2. My donor bull showed me that bulls do not mind being electroejaculated. He would actually come when I called, come into the chute, and stop to let me catch his head. So much for the animal welfare concerns about electroejaculation. I made an additional observation when I left Georgia to go to Virginia. When doing BSE's on bull in Georgia, the usual pass rate for bulls was around 75%. When I arrived in Virginia, I found that the pass rate was much less. I realized that I was looking at a population of bulls that had not been selected for fertility. Dr. Williams had been helping farmers select for fertile bulls for many years in the area around Athens, Geo showed me the impact a theriogenologist can have in population medicine.

When I was finishing up my residency and Master's degree, I started looking for my next job. I knew I wanted to teach which meant staying in academics. With only being board eligible, and having only a Master's degree, the jobs were not readily forthcoming. Dr. Dawe approached me with an offer of a stipend that was specifically for a veterinarian to get a PhD. Dr. Williams said if I wanted to stay in academics, I would need a PhD. Wise words. And I had finally arrived at the point that someone was paying me to go to school.

Don Dawe was a clinical immunologist and loved to work on pigs. My PhD project became a pig project. We had an innovative pig farm just south of Athens, Georgia, that was open to letting us do research on their animals. It was a shower in – shower out total confinement operation. They had aerators in their lagoon that turned the waste processing into an aerobic process cutting down considerably on the odor the farm produced. They also used the water from the lagoon for drinking water for the sows, which was shown to cut down on the incidence of E. coli infections in the piglets. I looked at the immune function in the sows during all aspects of their reproductive cycle, including pregnancy and lactation. I also examined the effect of levamisole on the immune function of these sows. Levamisole was a common anthelmintic, but had shown some immunomodulating effects. I got really good at bleeding pigs and the various immunologic laboratory techniques of the day such as lymphoblast transformation and natural killer cell assays. Right in the middle of my research, we had a TGE outbreak at the farm. The pictures we were shown of wheelbarrows full of dead piglets in pathology became a reality. There is a reason they call it Re-search. An interesting result of this disaster became apparent as we processed the data. There were significantly fewer piglets lost in sows that had been given levamisole. You never know what projects might uncover.

After completing my PhD and passing my theriogenology boards, I was once again looking for a job. With these qualifications, the job search was easier. I decided to go to Virginia Tech, who wanted someone to do canine and equine theriogenology. My kind of job! During my time at Virginia Tech, I found the wisdom of being a comparative Theriogenologist. I have been able to apply principals and techniques across species lines that have helped me many times over the years.

As I made my way as a faculty member, I followed Dr. Williams' advice and became active in veterinary associations. I started going to the local Southwest Virginia VMA meetings where I met a group of retired veterinarians who had been the movers and shakers of Veterinary Medicine in Virginia in their day. Duke Watson, Wilson Bell, Seymore Kalison to name a few. They were thrilled to have a faculty member become active in organized veterinary medicine. I became our delegate to the VVMA and eventually moved up the ranks to become President of the VVMA. I learned that the more you gave to your profession, the more you received back in satisfaction and friends throughout the state. Since many of the veterinarians around the state were Georgia graduates, we automatically had much in common. One accomplishment I still take satisfaction from is the merging of the VVMA's annual meeting with the equine and bovine practitioners organizations in the state of Virginia. To this day, our annual meeting consists of all the veterinarians in the state getting together, regardless of their chosen species.

The other veterinary association I got involved with was the Society for Theriogenology, the SFT. I don't think many know or remember we almost became the Society of Theriogenology. Fortunately Dr. Williams saved us from that fate of becoming the SOT's. I served on the board and eventually became an officer, and then President of the SFT. Yes, I think I may be what they call a glutton for punishment, but

I developed friends from all over the country and the world. I encourage anyone to become involved in rganized veterinary vedicine. What you give, you get back many times over.

Another group of individuals that have been an important part of my professional life are my residents. My first resident was Niki Parker. Then Milan Hess, Kara Kolster, and Julie Cecere. I want to thank them for their presence in my life. It is another pleasure in life to pass on the torch.

I taught at Virginia Tech for a total of 27 years. I am now retired. My theriogenology training came in handy when I married a dairy farmer, Mac Wall. Wall Brothers Dairy is a 5 generation dairy on land that has been in the Wall family since 1739. Most dairy farmers don't care much for horses, but Mac moved my horses over before he moved me over. I have been able to ride and breed a number of mares over the years. Between a dairy farmer and a theriogenologist, we think no uterus should go unused. I purchased my first Doberman in 1972. I have taken over from my old breeder, Jaima Youngblood, to breed my own line of Dobermans. I am working on my third generation of champions.

I want to thank you again for this honor. I am filled with gratitude for a professional life that has offered me so much.

I want to leave you with some words of wisdom from Dr. Williams. It is an example of the kind of a philosopher this man was. "A man is a fool to take a drink before he's forty, and a fool not to after he's forty."

Veterinary supply and demand

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The market forces that impact the economic status of the veterinary profession are complex and diverse. The recent AVMA report on veterinary markets describes the market for veterinary education, market for veterinarians and the market for veterinary services.[1] Each of these markets is impacted by the forces of supply and demand often described as the "law of supply and demand". The interaction of market forces and other factors within the veterinary profession impact the demand for veterinary services, supply of veterinarians and the level of compensation that veterinarians receive. This presentation will explore the economic relationships that are currently impacting the veterinary profession.

Introduction

In simple terms the law of supply and demand is a common sense principle that defines the generally observed relationship between demand, supply and price. The basic concept is that when the price of a good or service increases the consumer demand tends to decrease. Supply considers the relationship between the price and available supply from the perspective of the producer rather than the consumer. Increasing consumer demand for goods and services often drives the price up and producers generally respond to the increase in price by producing more goods and services. The degree to which demand or supply reacts to a change in price is described as elasticity. If a good or service is considered to be essential by the consumer the demand may not be negatively affected by increasing prices and is described as being inelastic. Factors that influence demand price elasticity are the availability of substitutes, consumer disposable income, access to capital and time. Another important economic principle is "utility". Utility is an abstract concept regarding the satisfaction or benefit that an individual gains from consuming or using a good or service. In general, a high level of utility results in an increase in the demand for goods or services and would be considered inelastic. The utility for veterinary services is often high for individuals who have strong emotional attachments to the animals in their care.

Economists assume that there is competition in the marketplace, thus prices change in response to supply and demand. Substitutes can play an important role in the market. If a product gets too expensive, consumers often substitute a less expensive product. If there is only one company producing a product that does not have a substitute, the company is said to have a monopoly on the market.

Understanding market forces

Market forces that affect supply, demand and price come in many forms. Common forces encountered in today's economy that affect the various markets in veterinary medicine include technological developments such as new products and equipment, along with the development of new communication, marketing and sales strategies. Political and governmental policies that influence regulations and tax rates affect how veterinary businesses operate. The position the profession takes on certain moral and ethical issues of our time can influence consumers' view of the veterinary profession and affect consumer spending. Weather condition can impact the profession directly and indirect due to the economic impact that these forces have on the agricultural sector. These markets forces and many others impact the various markets that define veterinary supply and demand.

Price signals are important indicators in a free market economy. Rising prices generally indicate a decrease of supply or an increase in demand. However, if prices are influenced by government policies or other forces then changes in prices may not be a reliable indicator of shortages, surpluses, or consumer preferences. The 2015 AVMA Report of Veterinary Markets points out that there are three principal markets within veterinary medicine and each is influenced by supply, demand, and price.[1] Outside forces such as governmental policies, state licensure, degree of public funding for institutions of higher education, federally backed student loan programs and many other forces can make the price signals difficult to interpret.

Another important concept to understand is the difference between need and demand. Demand is described as a consumer's desire and willingness to pay a particular price for a specific good or service. Demand often changes as the price goes up or down. Need is described as a consumer's desire for a specific good or service, yet they may not be willing to pay the current price for the good or service. If the price goes down the consumer may be willing to pay the lower price and thus in this case need is turned into demand.

The 2006 Foresight Project: Envisioning the Future of Veterinary Medical Education stated that one of the most important principles (needs) for the future of the profession was that veterinary medicine must remain relevant to the changing needs of society.[3] The report suggested that veterinary medical education could only respond to these changing needs by expanding the areas of education and that the number of graduating veterinarians should increase to address population growth and allow the profession to respond to new demands and roles.[3] While many would agree with this vision for the needs of the future, it does point out the importance of understanding the difference between need and demand.

Supply represents how much the market can offer and often refers to the amount of a certain good or service producers are willing to supply at a certain price. An oversupply exists when the supply of a certain good or service exceeds the need. If the supply of a certain good or service exceeds the demand yet fails to meet the need then excess capacity exists. The correction for oversupply is to reduce supply. Corrections for excess capacity include turning need into demand, reducing the price or reducing the supply.

The supply and demand for veterinarians, veterinary services and veterinary education are somewhat independent markets that guide the allocation of resources for veterinary medicine and provide goods and services to owners of animals, the veterinary industry, government, academia and the general public. Each of these markets has a supply and demand that influences the price providing signals to those involved in the markets about the relative supply and demand conditions.[1]

Market for veterinarians

The practice of veterinary medicine in the US is regulated by state statues that require certain standards be met to obtain a license, including the DVM degree or equivalence. Thus the supply of veterinarians is influence by the market for veterinary education. In 2014 the AVMA estimated that 100,137 veterinarians were actively practicing veterinary medicine in the United States.[1] The results of the AVMA survey of graduating seniors between 2010 and 2014 indicated that 16,267 new veterinarians (approximately 3,253 per year) were added to the veterinary workforce. Of those entering private practice, 64.5 % were employed in companion animal practice, 17.9% in mixed animal practice, 7.8% in food animal practice and 3.6% in equine practice.[1]

The demand for veterinarians is influenced by the compensation employers are willing to pay and the level of compensation is linked to the market for veterinary services. As the demand for veterinary services increases, the price for veterinary services will likely increase, resulting in increased revenue for veterinary practices. Thus, veterinary practices have the ability to increase the level of compensation they could pay veterinarians. However, if there is an oversupply or excess capacity they may be able to employ new veterinarians without increasing compensation. If the demand for veterinary services is weak then price changes for veterinary services would tend to remain flat or decrease, negatively impacting practice revenue and reducing the level of compensation the veterinary practice owners can pay new veterinarians who are entering the workforce.

Three indicators for the demand for new veterinarians are level of compensation (starting salary), level of unemployment and level of underemployment. In 2001 the nominal mean salary was approximately $45,000. Starting salaries increased at a steady rate of 7.65% annually from 2001 to 2008 at which time the starting salary was $70,000. From 2008 through 2014 the nominal mean starting salary for new graduates has remained flat at $70,000.[1] The AVMA reported the unemployment for 2014 at 3.9% and a negative underemployment that equates to room in the profession to employ an additional 951 full-time veterinarians.[4]

Market for veterinary education

Veterinary education provides the necessary training for individuals entering the veterinary workforce. The veterinary educational supply chain can be viewed by the number of seats available at accredited veterinary colleges, both domestic and international, and the price (tuition and fees) institutions are charging to provide seats. The number of seats filled at veterinary colleges determines the number of graduates produced each year. The number of graduates from US schools in 1980 was 1746 and since then the rate of increase has been approximately 2 % per year with 2977 new graduate expected in 2015.[2] There are US citizens obtaining their veterinary training from accredited international veterinary schools. The number of US citizens graduating from accredited international veterinary schools was not available until 2012. In 2012 there were 538 graduates and in 2015 the projected number of US citizens graduating by accredited international colleges is expected to be 619. The combined number of veterinary graduates in 2015, foreign and domestic is estimated to total 3596. With two new US schools admitting their first class (fall 2014) the expect number of veterinary graduates is expect to rise to 3226 (US), 644 (international) for a total of 3870 new graduates in 2018.[2] The AVMA projects that number of veterinary graduates will level out moving forward.[1]

The demand for the veterinary education market can be defined as the number of applicants who are willing to pay the current price for a seat. The price for a seat at a veterinary college varies significantly among institutions and whether or not the student pays resident tuition rates or non-resident rates. The median resident tuition for US Colleges of Veterinary Medicine, excluding the University of Pennsylvania, Tufts University and Western University of Health Sciences was reported in the 2014 AAVMC comparative data report as $21,753. The minimum was $16,546 and the maximum was $30,813. The median non-resident tuition was $45,910. The minimum was $25,809 and the maximum was $62,083.[2] Over the last decade the median resident tuition has increase at a rate of 4.3% annually and the rate of increase or non-resident tuition was 2.9%.

Data for the number of applicants that apply for a seat at a veterinary college come from the American Association of Veterinary Medical Colleges (AAVMC). The AAVMC provides an applicant service in which students wishing to apply to a veterinary college can apply using a centralized service, VMCAS. Currently 90.5% of the first year seats at US colleges of veterinary medicine are represented in VMCAS data.[2] The number of applicants per available seat as reported by AAVMC has remained steady at about 2.25 applicants per seat over the past decade.[2] However, if you consider the number of available seats at international colleges, the ratio drops.

By the mid 1980's the student debt to income was becoming an issue. In 1984 AVMA president Dr. Delano L. Proctor indicated that an in-house survey of 1984 graduates showed that 85 percent would have education related debt and the mean was $20,000. The mean starting for graduate was also about $20,000.[5] In 1999 the JAVMA published the KPMG study which stated that increased student debt was a significant issue facing many recent graduates.[6] At the time the mean starting salary for new graduates was about $42,000 and the mean debt had grown to $63,000. Since 2001, the debt of new veterinarians has been growing faster than starting salaries. The debt to income gap in 2001 was approximately $10,000 when adjust to 2014 dollars. The gap had increased to nearly $65,000 in 2014.[1]

Market for veterinary services

The general economic condition of consumers and their willingness to pay for goods and services has significant impact on market conditions for veterinary services. The amount of money that households have available for spending and saving after income taxes is referred to as disposable income.[7] The rate of growth in household disposable income is an important indicator of the money consumers have available to spend on veterinary services. Since 2000 the rate of growth in disposable income has declined as compared to historic growth (1960-2000).[1]

Currently, 66.5 percent of active veterinarians are employed in companion animal practice and would be directly impacted by the level of disposable income for the consumers in their practice area. Six point three percent of the active veterinarians are in food animal practice and would be impacted by the profit margins associated with the various livestock sectors they serve. Three point nine percent are in mixed

practice and would be impacted by the level of disposable income and profit margins in the livestock sectors. Equine practice represents 6.1 percent of active veterinarians and would be impacted by profit margins in the equine sector, along with availability of disposal income.[1]

The AVMA has been collecting aggregate economic data such as total revenue and expenses from US veterinary practices for more than two decades through the biennial economic survey. However, this survey did not contain information on the quantity and prices of specific veterinary services.[1] This information is needed to develop the supply and demand curves for specific veterinary services that can be aggregated to produce market supply and demand curves. Given the lack of data, the AVMA's Veterinary Economic Strategy Committee concluded that the currently available data would not result in a sufficiently robust analysis to provide useful information regarding the supply and demand for veterinary services.[1]

Implications

Socio-economic factors are believed to be directly correlated with lifestyle choices and are linked to patterns of drug use, disease prevalence and rates of mortality. The results from the first mental health survey of US veterinarians show that veterinarians are more likely to suffer from psychiatric disorders, experience bouts of depression and have suicidal thoughts compared with the US adult population.[8] Common predictors of suicidal behavior include hopelessness, stressful life events, substance abuse, depression and axiety.[9] It has been reported that a high percentage of veterinary students have depression levels at or above the clinical cut-off.[10] While I am not aware of any studies that have evaluate the effects of economic stress on veterinarians, it stands to reason that the widening debt to income gap could affect lifestyle choices and lead to an increase of mental health disorders.

As the future unfolds it is extremely important that as a profession we continue to gather good data and develop the necessary skills required to interpret the data and evaluate market signals. We need to develop a better understanding of how the market for veterinarians, market for veterinary education and the market for veterinary service interact and impact each other. While it is difficult to predict the future, having robust discussion regarding the factors that influence the markets of veterinary medicine and how the markets interact will be helpful to plotting the future of the profession.

Questions to consider for ongoing discussions: Can the needs of the profession, be turned into demand? How would this be accomplished? Understanding the elasticity of the many veterinary services offered and making appropriate price adjustments could improve efficiency and profitability. Should there be an adjustment in the supply of veterinarians? If so, how would this accomplished? Will the demand (ratio of applicants to seats) for veterinary education continue at same level? Will the debt to gap income continue to widen and if so at what point will applicant numbers be impacted? What are the socio-economic impacts of the growing debt to income ratio?

In closing, I would like to acknowledge the excellent work of the AVMA, Veterinary Economics Division and AAVMC for their on on-going efforts in collecting and analyzing data. Their reports are vital to understanding markets forces and how they impact the profession.

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Principles of evidence-based veterinary medicine: what is it and why does it matter?

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What is evidence-based medicine?

Evidence-based medicine (EBM) has been defined as the "conscientious, explicit, and judicious use of current best evidence in making decisions about the care of individual patients."[1] More generally, EBM is the formal application of the philosophy and methods of science to generating knowledge and making decisions in veterinary medicine. What distinguishes evidence-based veterinary medicine from other approaches is the explicit and formal integration of scientific research evidence into the clinical decision-making process. Evidence-based veterinary medicine (EBVM) is the adaption of EBM principles and techniques to the environment and circumstances of veterinary medicine.

As clinicians we need information to evaluate our patient's health problems and to provide effective preventative and therapeutic interventions. Evidence-based veterinary medicine provides tools and guidance to those who generate this information (through clinical research), those who disseminate it (through publication, continuing education, clinical practice guidelines, etc.), and those who utilize it (in clinical practice as well as public health and policy making). With better information, and more efficient information management, we are able to make better decisions, provide the best patient care possible, and more reliably achieve our intended outcomes.

Why do we need EBM?

In the absence of EBM practices, clinicians typically base their decisions on a number of sources of evidence other than formal research data. Studies of veterinary decision making have found that veterinarians rely largely on the opinions of colleagues and perceived experts.[2,3] To the extent that clinicians refer to research findings to guide their practice, they appear to utilize an informal, haphazard consultation of textbooks, journal articles, consensus statements and clinical guidelines. Above all, veterinarians, come to rely on their own clinical experience, judgment, and intuition in making diagnostic and therapeutic decisions. This collection of strategies is often referred to as opinion-based medicine.

There are a number of limitations to these approaches. Personal experience and opinion, even that of intelligent, educated, and experienced individuals, is subject to a wide range of cognitive biases and other sources of error that make it less reliable that is generally believed. Limitations in human perception, cognition, and memory and the influence of our beliefs and expectations lead us to erroneous conclusions which undermine the safety and efficacy of our interventions.[4]

There are many examples of how such error-prone assessment has supported ineffective or dangerous medical practices. Historically, interventions such as bloodletting, have been able to become ubiquitous and to persist for centuries with the best and brightest minds in medicine convinced they were effective, only to disappear rapidly when controlled scientific research revealed no benefits and significant risks.

In modern times, informal assessment not based on objective research has led to the similar widespread adoption of ineffective practices in many areas of healthcare. Based largely on the opinion of one individual, the practice of putting infants to sleep on their bellies to prevent sudden infant death syndrome (SIDS) was once nearly universal. This behavior persisted for two decades past the discovery of adequate scientific evidence to show it actually increased the risk of SIDS. Only once this external research evidence was integrated into public health recommendations and parent education did the practice, and the rate of SIDS, rapidly decrease.[5]

Surgical procedures such as internal mammary artery ligation and arthroscopic knee surgery have been widely employed until shown by controlled trials to be no more effective than sham surgery.[6,7] In the veterinary field, practices such as prescribing antibiotics for young cats with hematuria and the nearly universal use of oral glucosamine as a therapy for osteoarthritis illustrate the potential for common and persistent use of ineffective or inappropriate therapies in the absence of a rigorous evidence-based approach to evaluating our interventions.[8,9]

It has been said the three most dangerous words in medicine are "in my experience." And one definition for clinical experience is "making the same mistakes with increasing confidence over an impressive number of years." The reality is that our judgments are far less reliable than we feel them to be, and we are easily fooled by circumstances, by the complexity of the living organisms we deal with and their diseases, and by our own perceptual and cognitive biases. Evidence-based veterinary medicine offers strategies and tools to help compensate for the limitations of uncontrolled observation and judgment.

The steps of EBVM

While EBM is concerned with the production and reporting of scientific research as well as its use in guiding clinical decision making, from the point of the clinician the most important elements are the steps involved in integrating research evidence with clinical experience and the circumstances of a particular case in order to inform patient care. The basic steps of an EBVM clinical process are these:

1. Ask useful questions
2. Find relevant evidence
3. Assess the value and reliability of the evidence
4. Draw a conclusion
5. Assign a level of confidence to your conclusion

This is an iterative process that will be repeated regularly to build a body of knowledge with a known degree of uncertainty that can guide our clinical practice.

Asking useful questions

Vague or overly broad questions impede effective use of research evidence in informing clinical practices. "Does drug X work?" or "What should I do about disease Y?" are not questions that are likely to lead to the recovery of useful information from published research. There are a number of schemes for constructing questions the scientific literature can help answer. One of the easiest is the PICO scheme.

P- Patient, Problem. Define clearly the patient in terms of signalment, health status, and other factors relevant to the treatment, diagnostic test, or other intervention you are considering. Also clearly and narrowly define the problem and any relevant comorbidities. This is a routine part of good clinical practice and so does not represent "extra work" when employed as part of the EBVM process.

I- Intervention. Be specific about what you are considering doing, what test, drug, procedure, or other intervention you need information about.

C- Comparator. What might you do instead of the intervention you are considering? Nothing is done in isolation, and the value of most of our interventions can only be measured relative to the alternatives. Always remember that educating the client, rather than selling a product or procedure, should often be considered as an alternative to any intervention you are contemplating.

O- Outcome. What is the goal of doing something? What, in particular, does the client wish to accomplish. Being clear and explicit, with yourself and the client, about what you are trying to achieve (cure, extended life, improved performance, decreased discomfort, etc.) is essentially in evidence-based practice.

Find relevant evidence

Experienced clinicians typically have opinions on the value of most interventions they routinely consider. Unfortunately, we rarely know where those opinions originally came from or how consistent they are with the current best scientific evidence. And given the constraints of time and resources, practitioners will rarely have the ability to find and critically evaluate all the primary research studies relevant to a particular question. Fortunately, there are sources of evidence that can provide reliable guidance in an efficient, practical manner.

The best EBVM resource for busy clinicians is the evidence-based clinical practice guideline. These are comprehensive evaluations of the research in a general subject area that explicitly and transparently identify the relevant evidence and the quality of that evidence and make recommendations with clear disclosure of the level of confidence one should place in those recommendations based on the evidence.

Sadly, many guidelines produced in veterinary medicine are not evidence based but opinion-based (so-called GOBSAT or "good old boys sat at a table" guidelines). These are no more reliable than any other form of expert opinion. Excellent examples of truly evidence-based guidelines are those of the RECOVER Initiative for small animal CPR and the guidelines produced by the International Task Force for Canine Atopic Dermatitis.

After evidence-based guidelines, the next most useful resources are systematic reviews and critically-appraised topics (CATs). These are more focused but still explicit and transparent reviews of the available evidence on specific topics. Systematic reviews can be identified by searching the VetSRev database, a free online resource produced by the Centre for Evidence-based Veterinary Medicine (CEVM) at the University of Nottingham. Unfortunately, getting full-text copies of these reviews can be challenging for veterinarians not at universities, but there are a number of options depending on where one practices.

Critically appraised topics are also produced by CEVM and freely available on the web as BestBetsforVets. There are a number of other free CAT resources, including the Banfield Applied Research and Knowledge web site.

Finally, primary research studies are a useful source of guidance for clinicians, though they take more effort and expertise to find and critically evaluate.

Assess the value of the evidence

All research has limitations, and these must be formally assessed through the process of critical appraisal (discussed below). Only when the limitations of a study are clearly understood can we decide how much confidence to have in the results and conclusions of the study and whether it should lead to changes in our clinical practices. And even the best studies may not be applicable to our patients if the population studied differs in important ways from our patient population, or if the tools and techniques described are unavailable, impractical, or unacceptable to our clients. It is not enough merely to read published research reports. We must critically evaluate them in terms of reliability and applicability to our needs.

Draw a conclusion

Ultimately, the job of a veterinarian is to guide the client in making decisions about care for their animals. When the clinician is aware of the existing evidence and its limitations and clearly appreciates the degree of uncertainty, then he or she can best help the client to understand their options. Making evidence-informed decisions and clearly communicating with clients about the needs and choices for their animal is the core of clinical veterinary medicine, and this is what the tools and methods of EBVM exist to support.

Assign a level of confidence to your conclusions

Often, the relevant research evidence is incomplete or flawed, and sometimes there is little or no such evidence applicable to a given patient's needs. Evidence-based veterinary medicine is still useful in this situation, because it allows us to clearly, systematically identify and communicate the uncertainty inherent in our work.

Evidence-based veterinary medicine is, above all, an approach for helping clinicians reach conclusions that can guide their decisions about the diagnosis and treatment of individual patients. It is often believed that a determination in Step 3 that the evidence is weak or flawed precludes making clinical decisions and taking action, and that this limits the usefulness of EBM in the veterinary field, where research evidence is often severely limited in quantity and quality. However, this is incorrect. The purpose of assessing the reliability of the research evidence is to assign a degree of confidence to

conclusions or decisions based upon it. If the evidence is weak, it is often still be necessary to make a decision and act on it, especially if there is an urgent clinical problem. Evidence-based veterinary medicine does not prohibit or undermine such action, it simply facilitates a clear and accurate understanding of the degree of uncertainty involved. This helps the clinician and also allows fully informed consent for the client.

It is also important that we openly discuss with clients our use of evidence to inform our recommendations. Research has suggested that clients want to be told about the uncertainties involved in the treatment of their animals, and that discussing this does not reduce their confidence in their veterinarians.[10] Clients also identify truthfulness as their highest priority in communication with their veterinarian.[11] By explicitly discussing our process in identifying and evaluating relevant evidence, we enhance our clients' understanding of the role we play, and we help them to appreciate the value of our expertise, not only the products and procedures we sell.

Critical appraisal

Critical appraisal is the term used to describe the formal assessment of the quality and limitations of published research evidence. Different study designs have strengths and weaknesses that bear on how the reliability and applicability of their results. And many individual aspects of how a research study is designed, conducted, and reported influence how much weight the study results should be given in developing an answer to a specific clinical question.

The well-known hierarchy of evidence, often used as a symbol for EBVM, is simply one of many tools employed in the critical appraisal process. There are also a number of key methodological factors that need to be evaluated when deciding how much confidence to place in the conclusions of a given research study:

1. Control Group
   If a treatment is applied to a group of subjects and there is no control group receiving a placebo or alternative treatment, there is no way to be sure any changes observed in the treatment group are actually due to the treatment or are greater than would be seen if we did something else or nothing at all. Uncontrolled trials are very weak evidence.
2. Allocation
   How the subjects are assigned to the different groups in a study is important. If there is not truly randomized allocation, in which every subject has an equal chance of being assigned to any group, then there is a risk that subjects will be assigned in a biased manner and that any differences seen between the groups will be due to inherent differences between subject rather than any treatment being tested.
3. Blinding
   One of the greatest strengths of formal scientific research is that it can compensate for the cognitive biases that lead us to the wrong conclusions when making informal observations. If, however, investigators and animal caregivers are able to determine which group in a study particular subjects are in, all of these biases can operate freely, and any assessments of the subjects, especially those that are at all subjective, can be skewed by unconscious bias. Unblinded or ineffectively blinded studies almost always find what the investigators expect to find, and this is no accident. Research has shown that in veterinary clinical trials, owners perceive a response in patients on placebo treatment nearly 57% of the time, and veterinarians perceived a response in these subjects 40-45% of the time.[11]
4. Statistics
   While the details of evaluating the statistical analysis in a given paper are complex and beyond the expertise of most veterinarians, it is important to bear in mind that research has demonstrated statistical errors are extremely common in published veterinary research and that even properly applied analyses are often inappropriately used to draw conclusions.
5. Effect size
   Statistical significance is largely irrelevant to the question of whether an effect observed in a

study is real or important. The effect size, or the absolute value of the effects seen and the differences between groups, is far more important. One can often show a statistically significant difference that would be clinically undetectable and irrelevant.

6. Replication
   No single study is ever sufficient to confidently demonstrate any hypothesis to be true or false. Replication is essential to uncovering the truth in science, and any conclusions based on research that has not been replicated should be viewed as tentative at best.

The studies which have evaluated the reliability of published veterinary research is not encouraging. Significant flaws are present in the majority of published studies, and this limits the confidence that can be placed in the results or conclusions of these studies.[13-23]

Resources

Print resources

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Cockroft P, Holmes M: Handbook of evidence-based veterinary medicine. Oxford: Wiley-Blackwell; 2003.

Holmes M, Ramey DW: An introduction to evidence-based veterinary medicine. Vet Clin North Am Equine Pract 2007;23:191-200.

Schmidt PL: Evidence-based veterinary medicine: evolution, revolution, or repackaging of veterinary practice? Vet Clin North Am Small Anim Prac 2007;37:409-417.

Smith RD: Veterinary clinical epidemiology. 3rd ed. Boca Raton: CRC/Taylor and Francis; 2006.

Electronic resources

Evidence-Based Veterinary Medicine Association
http://ebvma.org (includes an extensive EBVM bibliography curated by Susan Whittaker)

Centre for Evidence-Based Veterinary Medicine
http://nottingham.ac.uk/cevm

VeSRev- A database of veterinary systematic reviews
http://webapps.nottingham.ac.uk/refbase/

BestBetsfor Vets- Critically appraised topics
http://bestbetsforvets.org/

BARK_Banfield's EBVM resources
http://www.banfield.com/veterinary-professionals/resources/research

Bottom line

Evidence-based veterinary medicine is the formal, explicit integration of controlled scientific research into clinical decision making. It can reduce error and lead to better patient outcomes. However, published clinical research is not always reliable, and clinicians must carefully assess the limitations of specific studies and the applicability of their results to individual patients.

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Just because I can, doesn't mean I should: the application of evidence-based medicine to small animal theriogenology

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"You ask of my companions. Hills, sir, and the sundown, and a dog... They are better than beings, because they know, but do not tell."

Emily Dickinson

Introduction

The stresses and demands of modern American society have triggered an increase in dog ownership; as stated by the American Veterinary Medical Association, our canine counterparts provide companionship, joy, unconditional love, a sense of safety, and often a service. This same trend has caused pet owners to become careful consumers, desiring a healthy, stable dog with traits compatible with an individual's way of life. A pedigreed dog that is carefully bred and raised is the best guarantee that a rewarding and long lasting relationship will develop, and likewise the best guarantee that fewer animals end up unwanted, in shelters or on the street.

As the importance of purebred dog ownership is increasingly appreciated, dog breeders are challenged to produce quality animals. The veterinary profession, however, had largely neglected the field of canine reproduction; despite progress in the study of reproduction in human medicine and livestock since the 1960's, little technical progress was made on behalf of dog breeders. This has changed, however, in recent years. Proper reproductive management is recognized as the foundation of a successful breeding program; genetic screening is seen to be an important factor in planned breedings; technologically advanced breeding systems are desired to give dog breeders wider options; the pathology and treatment of canine infertility is better understood. Most importantly, the veterinarian has become an active participant in the dog breeding process.

The importance of evidence

While the field has grown, and the demand has increased exponentially, training and information have remained limited. The time sensitive and multidisciplinary nature of small animal theriogenology is difficult to fit into the practical limitations of an academic system; as a result veterinary students, interns and residents have limited exposure to the type of cases that are routinely managed in a practice setting. Logistical, financial and ethical constraints severely restrict the amount and quality of research performed, and with it the evidence available to aid in clinical decision making. Nonetheless, it remains our obligation to recognize these inherent limitations, and to use the best available resources in a disciplined, science-based approach, to determine both the benefits and potential adverse effects of the procedures and treatments we recommend. By combining our collective knowledge and reasoning and maintaining the "science based" approach available to us, we can do justice to our clients and patients. By ignoring them, we risk succumbing to quackery.

Veterinary medicine offers some unique ethical challenges not faced by other health professionals. We must serve the needs of both the animals and the humans who own them, in areas as diverse as the beloved pet being given medical care as sophisticated as that provided to their human counterparts, as well as cattle in the feedlot, and laboratory animals used in biochemical research. The leaders of the profession are just beginning to learn how to address these issues.

Today we come together as veterinarians who work specifically with purebred dog breeders. Our society wants and demands healthy pets who fit a specific lifestyle and are both mentally and physically sound, but at the same time unwanted pets fill our shelters. Purpose-bred dogs are needed for military service, guide dogs and service dogs. Wealthy pet owners want to clone a beloved pet. Anti-dog and anti-dog breeding legislation is on the rise at the same time that "designer breeds" are flooding the consumer market. Veterinarians must use the advancing technology in reproduction and genetics to help

their clients make good breeding decisions to achieve their goals but not neglect the health and well-being of the individual animals or their offspring.

Evidence based medicine has been described as "the conscientious, explicit and judicious use of the best scientific evidence to inform clinical judgements with a view to improving clinical outcome at the level of the individual case".[1] In small animal reproduction, "best scientific evidence" is often absent or lacking. A recent review of the quality of current literature in theriogenology found that only 7 % of publications in canine reproduction were graded adequate to draw sound conclusions.[2] An earlier review found that only 31 % could be classified as clinical trials, with the remaining 69 % being case reports or personal opinion. Meta-analysis (the integration of data from a number of independent studies to increase the statistical relevance) could not be found in the literature of canine reproduction, and in half generally accepted and science-based conclusions could not be legitimately drawn from the collected data.[3] Nonetheless, although they are few and far between, quality research studies that are both critically designed and statistically evaluated are the best predictors of the results likely to occur in our patients. As clinicians, we are forced to make decisions regarding treatment options without significant randomized and controlled scientific studies to guide us. To deal with these limitations, it is helpful to rank the information available into categories that reflect the relative strength of the evidence, and to use this type of methodical and systematic approach to formulate clinical decisions. Several reasonable scoring systems have been described, with an example shown below:[4]

Grade I:	At least one properly designed randomized controlled clinical study performed in patients of the target species
Grade II:	Evidence from properly designed randomized controlled studies in animals of the target species with spontaneous disease in a laboratory or research colony setting
Grade III:	Appropriately controlled studies without randomization Appropriately designed case-controlled epidemiologic studies Studies using models of disease or simulations in the target species Dramatic results from uncontrolled studies Case series
Grade IV:	Studies conducted in other species Reports of expert committees Descriptive studies; Case reports Pathophysiologic justification/rationale Opinions of respected experts

Many treatment modalities and techniques have become common place in small animal reproduction based on anecdotal information, and without proof of efficacy or a critical evaluation of potential risks and benefits. While dog breeders and veterinarians clamor for "newer, better, cheaper", we must resist the temptation to embrace unproven treatments "just because we can".

Applying evidence based reproduction: choosing an insemination method

Three insemination techniques are widely used in canine artificial insemination, namely (1) vaginal, (2) intrauterine deposition via surgical insemination, and (3) intrauterine deposition via a transcervical approach, either through the use of a rigid catheter or with endoscopic guidance. The techniques involved are well described elsewhere; the specifics will not be discussed here. The choice of insemination technique should be made based on the clinical history of the animals, the quality of the semen being used, the goals of the client, the safety of the procedures, and the skills of the operator. A technique should be chosen that is likely to give the desired results using the method that is the least invasive, the least expensive, and safest. How to decide? First evaluate the evidence and grade it

according to scientific standards. Then apply the evidence to your patient. Are the outcomes of the study applicable to your circumstance? Are there differences between your patient and the animals in the study that may alter the expected response? Are there differences in the semen quality or available insemination dose from that used in the study that may alter the expected response? Will the patient's behavior, breed, or history influence the decision? How will the owner's values or financial situation affect your decision? What are the patient's likely benefits and risks from the various options? Does the patient have other health conditions that alter the potential benefits and risks of each option?

Vaginal insemination

Vaginal insemination involves deposition of semen into the cranial portion of the vagina, essentially approximating the site of semen deposition that occurs in a natural mating. This method is indicated for most instances of artificial insemination. This simple technique requires minimal equipment, experience and training, and is relatively noninvasive, with little risk to the bitch regardless of her degree of cooperation (or lack thereof). Since it is as close as possible to natural breeding, it will appeal to owners who desire a more natural approach. Studies have shown efficacy with fresh, chilled-extended and frozen semen of high quality, with conception rates of between 60 and 95 %. Relatively large numbers of healthy sperm are required, when available this should be the method of choice.

Surgical intrauterine insemination

Surgical intrauterine insemination involves deposition of semen into the cranial uterine horns, close to the site of fertilization in the oviducts, which should maximize conception. This method is indicated when using semen of low quality (either in motility, count, longevity and/or vigor), or in females in which fertility is expected to be compromised. The procedure is quick and simple, and easily performed safely by a veterinarian with basic surgical skills. It can be assumed that only bitches in good general health are voluntarily bred, and with the safe and short acting anesthetics available today, as well as modern anesthetic monitoring, the risks of general anesthesia are low. The procedure is performed using sterile technique, so the risk of bacterial introduction into the uterus is eliminated. A surgical approach allows reliable access to the uterus, so that deposition of semen into the cranial uterine horns is done with confidence, regardless of volume, and without tissue trauma to the vagina, cervix or uterus. Visualization and gentle palpation of reproductive organs can be performed to assess possible pathology in subfertile bitches. General anesthesia will minimize stress in nervous, excitable or pain sensitive bitches. However, a surgical approach for an elective procedure may be morally objectionable for some clients. In addition, some theriogenologists have limited experience and expertise in small animal anesthesia and surgery as well as limited facilities/staff to perform anesthesia and surgery safely. Studies and anecdotal reports give the highest success rates with this method.

Transcervical insemination

Transcervical insemination (TCI) involves deposition of semen into the uterine body, avoiding general anesthesia and surgery, although sedation is sometimes necessary. This method will place semen cranial to the cervix but not close to the site of conception in the oviducts. The procedure requires specialized equipment and can be difficult and time consuming to learn, as well as frustrating and time consuming to perform. The size and anatomy of some bitches create challenges that may be difficult to overcome. Since the uterine body is small and the myometrium of the uterus has significant resistance, only a small volume of semen can be used without backflow through the cervix into the vagina. This small volume may limit the insemination dose unless semen is concentrated by centrifugation, which will frequently lower sperm quality, especially in samples that are already compromised, and/or by lowering extender to semen dilution ratios which may also decrease sperm quality, as well as sperm loss by processing.[5] Since the cervix is not directly accessible to the operator, TCI has an increased risk of trauma of the reproductive tract, especially with the rigid catheter technique. In addition, since the catheter is taken from the nonsterile vagina through the cervix into the uterus, the procedure should be used with caution in bitches who are at increased risk of pyometra. Studies have shown acceptable

conception rates with fresh, chilled and frozen semen, although results have been somewhat contradictory from study to study.

What is the evidence?

A literature search showed no properly designed randomized controlled clinical studies performed in dogs comparing insemination techniques.

Three properly designed studies performed in laboratory or research animal colony settings were found. In one study, both fresh semen and frozen thawed semen were used, with both vaginal and surgical intrauterine insemination methods. The semen parameters for all groups were in range of what would be considered adequate numbers and quality. Conception rates were similar to other studies of fresh semen (100 %) and frozen semen (60 %). No significant difference was found between vaginal and surgical insemination.[6] In another study, two different semen freezing extenders were compared with both intravaginal and transcervical inseminations. Again, generally accepted adequate semen parameters for both sperm numbers and quality were used. No significant differences in pregnancy rate or number of fetuses were found between the two insemination methods.[7] The third study used a cross-over design to evaluate vaginal vs. surgical intrauterine insemination technique in bitches with decreased fertility. Surgical insemination showed a significant increase in conception over vaginal insemination when female fertility was compromised.[8] Several other experimental studies looking at conception with varying insemination methods were found in the literature, but other variables affecting fertility were not controlled. As a result, these studies could not be used to give evidence concerning the choice of insemination technique.

Numerous cohort studies, both prospective and retrospective, evaluating canine conception rates were found in the literature. Some of these compared insemination techniques, and some evaluated conception using fresh, chilled or frozen semen using one insemination method. The usefulness of these studies were all limited by the number of variables that were poorly controlled or uncontrolled, lack of randomization, and by the fact that bias was not eliminated. For example, factors such as bitch fertility, ovulation timing methods, number of inseminations performed per cycle, numbers of viable sperm per insemination dose can and do have a profound effect on conception. Even still, when evaluating the data of these studies, insemination method appeared to have little effect on conception providing adequate numbers of viable sperm were used. This held true for fresh, chilled and frozen semen inseminations.

Studies in other species (equine, porcine and human)[9-12] comparing site of deposition of compromised semen largely show an increase in conception with "deep uterine insemination" into the anterior portion of the uterine horns or close to the oviducts. Similar results may be expected in the dog, suggesting that surgical insemination might be expected to yield superior results over transcervical and intravaginal insemination when compromised sperm numbers or semen quality is used.

Safety and side effects with different insemination techniques have not been studied but are discussed as points to consider in several reports. These include the risk of anesthesia and/or surgical complications with surgical insemination, as well as the risk of bacterial introduction, trauma, pain and stress with transcervical insemination. Interestingly, in spite of voicing these concerns, several authors suggested doing transcervical inseminations for all breedings, so that the veterinarian could become competent in performing the procedure.[13,14]

Pathophysiologic knowledge may also be used as evidence and rationale to justify the choice of insemination method. Since it is known that conception occurs in the oviducts, it follows that the goal of any insemination would be to place semen in a location such that adequate numbers of sperm are present in the oviducts at a time that conception can occur. The anatomy of the bitch presents limitations that must be dealt with when approaching this challenge. In a properly performed vaginal insemination, semen is deposited in the cranial vagina, close to the site nature intended with natural mating. Sperm will then travel through the cervix, the uterine body, the uterine horns, and into the oviducts. The canine uterine horns are long, and the lumen is often tortuous, greatly increasing the length the sperm must traverse to reach the oviducts. Nonetheless, normal dogs have an extremely high efficiency of reproduction with natural mating, and it should be assumed that vaginal insemination would be successful

as well. However, when the fertility of the dogs is compromised, it would also follow that the goal of the insemination technique chosen would be to place the semen as close to the site of conception in the oviducts as is necessary for the individual case. At the same time, insemination method should not further compromise any pathology that exists. For example, transcervical insemination and to a lesser extent surgical insemination, limit insemination volume. To achieve this small volume, centrifugation of the semen may be required, which has a high chance of causing further damage and/or loss of sperm numbers in an already compromised semen sample. Bitches with decreased fertility may be more susceptible to trauma to the reproductive tract, which is difficult to control with transcervical insemination. Likewise these bitches may be less able to clear bacteria that are introduced via transcervical insemination. Another consideration might be a negative effect of mild trauma, that may occur with transcervical insemination, to cervical competence.

Recommendations

Vaginal insemination should be used for all cases involving normal fertile males and females to minimize risks but maintain high efficacy. This should hold true for chilled and frozen semen breedings, providing adequate numbers of normal sperm are available.

Surgical insemination should be used in cases involving dogs and/or bitches with compromised fertility, or when sperm numbers and/or quality is limited.

Transcervical insemination should be used as an option in normal fertile bitches when semen quality is not good enough to expect success with vaginal insemination, but surgical approach is not desired or practical.

For ethical considerations, the safest and least invasive method with the lowest risk of side effects, that is likely to achieve the desired results, should be chosen.

Conclusion

As veterinarians, we strive to achieve the best results for our clients. When making therapeutic decisions, we must consider the quality of the evidence available to support our options. Improvement in the quality of the studies performed in small animal theriogenology is necessary for our field to legitimately progress.

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Clinical methods for counting canine sperm: automated and manual techniques

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Introduction

The breeding soundness examination (BSE) in the canine primarily involves obtaining pertinent breeding history, assessment of overall health, careful examination of penis, testes and prostate, and semen analysis.[1,2] Semen analysis is arguably the most integral part of the BSE. Assessment of semen quality requires determination of ejaculate volume and pH, assessment of all cell types present in ejaculate, determination of sperm concentration, assessment of total and progressive motility, and assessment of sperm morphology.[1,3]

Clinical theriogenologists and small animal reproductive veterinarians routinely make important decisions based on semen analysis. Accurately determining quality and concentration of the sperm has become increasingly important for the small animal practitioner, as semen freezing and artificial insemination techniques have become more commonplace. Also, many companies are promoting and selling instruments to small animal veterinary practitioners that aid semen analysis. These instruments include: computer assisted sperm analysis (CASA) systems, cell counters such as the NucleoCounter®, and photometric devices such as the Spermacue® and Densimeter®. It has become evident that with the significant increase in the prevalence of more advanced artificial insemination techniques and the increasing availability of new technology used for semen analysis, there is an increased need to standardize techniques for evaluating canine semen. In fact, it has been shown in other species that external quality control and adherence to recommended, standardized procedures are extremely important for consistent, accurate semen analysis.[4,5] This is well accepted in human medicine with adherence to the World Health Organization (WHO) standards for semen analysis. The Society for Theriogenology and American College of Theriogenologists have published "Guidelines For Canine Breeding Soundness Examination" which is intended to "promote consistency" for the canine BSE.[1] In discussing these guidelines with SFT/ACT members, many find these guidelines very useful and regard them as an important standard. This paper will focus on clinical methods used for determining sperm concentration in the canine semen sample with some discussion comparing automated to manual counting methods and hopefully further the discussion on standardizing techniques for determining sperm concentration as part of the canine semen analysis.

Hemocytometer

The hemocytometer is a special microscope slide with a grid system etched into its surface and raised rails where a specifically designed cover slip sits exactly 0.1mm above the surface. The grid consists of 9 large squares, with the corner squares made up of 16 smaller squares and the remaining 5 squares made up of 25 smaller squares, each containing 16 very small squares. This pattern is replicated, as there are two identical chambers on each side of the slide. The design, when loaded and equilibrated properly, allows for a known sample volume to be evaluated. The operator counts sperm cells present on the grid system using one of a few counting methods.[6,7] The central large square is known as the "counting area" by some counting methods. The hemocytometer is considered the gold standard for sperm counting in many species, including the canine.[8-10]

Dilution of the semen sample is often necessary for proper use of the hemocytometer. One must be able to count individual sperm cells with a high degree of certainty and overcrowding of sperm cells on the hemocytometer makes proper evaluation difficult. Historically, dilution was somewhat easy and consistent for the practitioner when using the Becton Dickinson Unopette system. Semen dilution was described using acetic acid (and later ammonium oxalate) diluent (1.98ml) and the included capillary pipette (20ul) to make a 1:100 dilution. However, with the discontinuation of Unopette system, the ease and consistency of semen sample dilution changed. Practitioners were left to make their own dilutions using bulk chemicals and pipettes, or pursue alternative methods of sperm counting while searching for a commercially available alternative. A few companies now offer Unopette alternatives including

Biomedical Polymers (BMP) Leukochek™ and Animal Reproduction Systems (ARS) Thrombo-tic®. Research is ongoing comparing these, and other dilution methods, for canine semen samples for use with the hemocytometer.

Once the semen sample is appropriately diluted, several methods for counting the sperm using the hemocytometer have been described.[1,6,7] Perhaps the easiest method for counting canine semen using the hemocytometer, and the method described by the ACT/SFT guidelines, is counting all of the sperm cells present in the central square, the "counting area". The number of sperm counted in this area represents the number of sperm cells present in the sample, in millions/ml, when using a 1:100 dilution. Counting both sides of the hemocytometer using this method is important, making sure they are within 10% and taking the mean as million/ml sperm present in the sample.[1] It is advised that if each side is not within 10%, the hemocytometer should be reloaded, equilibrated and counted again.

Another method that has been described for counting sperm using the hemocytometer is to count sperm cells present in five squares of the 25 squares that make up the "counting area". The 5 squares are typically counted in either a diagonal or "star" pattern. The number of sperm cells present are multiplied by five, giving the number of sperm present in 0.1ul. Simply multiplying by 10,000 (to get number of sperm present per ml of sample) and the dilution factor, gives the number sperm cells, in millions/ml, present in the sample.[6]

The final counting method that will be considered for this paper is to count the sperm cells present in three of the large nine squares of the hemocytometer. This is typically done using three of the corner squares, not the central "counting area" square. The number of sperm cells present in each square are added together, multiplied by three and 10% of this value is added back in. The subsequent value is divided by 10 to obtain number of sperm cells present in the sample, in millions/ml, when a 1:100 dilution is used.[7]

While the hemocytometer certainly has its limitations and drawbacks, it remains the "gold standard" for determination of sperm concentration to which all other methods are compared.[8-10]

Computer-assisted sperm analysis

Computer-assisted sperm analysis (CASA) has been in use for over 35 years.[11-12] It was borne out of the need for a more objective method for assessment of sperm quality.[13-14] Computer-assisted sperm analysis assesses parameters such as: motility (overall, progressive, hyperactive), morphology, morphometry, viability and concentration. Most CASA systems work by using a camera attached to a microscope that scans several fields of a specially designed slide (typically shallow welled). The images are digitized and analyzed by a computer program.[12,15]

There are many CASA systems available for sale that are marketed for use to small animal veterinary practitioners, especially over the last 10 years. The modern systems have become increasingly user friendly and more economically feasible for use in small animal reproductive practices. These systems provide the practitioner with a quick and easy assessment of several sperm parameters. However, CASA systems have several drawbacks, and proper use is imperative for accurate results.[10,13,15]

Perhaps one of the most interesting and consistent inaccuracies with CASA is determination of sperm concentration, especially at lower concentrations. It has been written that sperm concentrations $<20 \times 10^6$ to 50×10^6/mL are most likely unreliable and should be checked using manual counting methods.[13] This illustrates the need to remain proficient in manual methods of canine sperm counting. Some of the causes that have been suggested for the inability of CASA to accurately determine sperm concentration are inappropriate slide/chamber use, Segre-Silberberg effect, mis-identification of debris as sperm and collision artifact.[10,12,14-16]

Cell counter

NucleoCounter® is a cell counter that has gained popularity over the last several years in the small animal clinical reproductive setting for counting canine sperm. It can be used with both raw and extended semen samples. The NucleoCounter® essentially works like a fluorescent microscope which captures images with a CCD camera and displays them on a computer screen. It uses an LED light instead of a

laser, like a traditional flow cytometer cell counter uses. Also, it is fully enclosed and somewhat portable. The cassettes used with the NucleoCounter®, for sperm analysis, contain propidium iodide (PI) stain and an integrated pipette that mixes the appropriate amount of stain and semen sample for analysis. The result is fluorescence and counting of nuclear material only, not debris. The NucleoCounter® been shown to be an accurate method for counting animal cells, including the counting and estimated viability of sperm cells.[17-20] The number of viable cells in the sample is determined by counting the non-viable cells and total number of cells. This is done by staining (PI) the non-lysed sample and performing a count, then comparing it to the total count (lysed sample). The (PI) will only stain nuclear material in a sperm cell whose plasma membrane has been compromised, which indicates a non-viable status, not a true live/dead status.[19]

The NucleoCounter® is an easy to use and an accurate automated device to count sperm. In fact, it has been suggested that it should become the new gold standard for determining sperm concentration.[6] Research is ongoing comparing its precision and accuracy for counting canine sperm. (personal observation)

Photometric devices

The Spermacue® and Densimeter® are photometric measuring devices that have been marketed to small animal reproductive veterinarians for several years. They work by measuring the optical density of semen samples. The light transmission data is ultimately converted to an estimate of sperm concentration and reported in million/ml. The devices are relatively inexpensive and easy to use. Due to the nature of how they estimate the sperm count (light transmission), any nonsperm cells in the sample contribute to inaccuracies in the measurment. In addition, the manufacturers caution that the estimates of sperm concentration become significantly less accurate with very dilute or very concentrated semen samples. Manufacturers of other automated sperm counting devices (CASA) also make this caution.

When using photometric devices to estimate sperm concentration, each sample should be screened using a microscope prior to use. Most practitioners place a small drop of the sample onto a warm slide and quickly scan under different magnifications. It is extremely important to perform hand counts on samples that contain excessive numbers of other cells, such as white blood cells, bacterial cells or epithelial cells. Debris, such as fat globules or cytoplasmic droplets should also be considered. Dilution, centrifugation or hand counts are also necessary for sample concentrations that do not fall within the manufacturers concentration recommendation for accuracy.

Comparative sperm analysis

Scientific articles comparing sperm counting methods have been written for many years. However, most of the comparative research has focused on species other than the canine, with few exceptions. Recently, there is increased interest in determining canine sperm concentrations, with greater accuracy, as more advanced reproductive techniques utilizing minimal numbers of sperm become commonplace in small animal reproductive practices. Besides being an integral part of the BSE, it is important to accurately determine sperm concentration when working with small or compromised semen samples and accuracy is imperative when packaging frozen canine semen samples to ensure correct insemination dose.

Historically, a dose of 150 x 10^6, progressively motile sperm cells has been described for insemination in the canine.[3] However, different insemination doses for the canine have been reported, for alternative methods of insemination, with varying success.[21-24] Ensuring that the assessed sperm concentration is accurate may lead to a better estimate of the most appropriate insemination dose needed for particular methods of insemination.

The aim of our current research is to compare several different, but commonly used dilution methods available for the small animal practitioner to use with the hemocytometer. We will also compare the previously described methods for using the hemocytometer to count canine sperm cells. Additionally, we will compare the accuracy and precision of the most common measuring devices used by small animal reproductive practitioners for determination of canine sperm concentration.

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Sperm morphological defects in dogs: causes and consequences

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Abstract

Evaluating fertility (or 'fertility potential') of a stud dog is an important part of breeding management. True indices of canine fertility are high pregnancy rates (≥ 75%) and large litters. However, both are retrospective measures and are strongly influenced by factors independent of the stud dog, including fertility of the bitch and breeding management. In clinical practice, predicting likely fertility of a male is usually required prior to breeding. Within the context of designating dogs to have 'satisfactory breeding potential,' it is generally accepted that thorough physical and reproductive examinations and conventional semen evaluation generally provide a useful alternative to actual fertility data. Although poor semen quality is a good indicator of subfertility, conversely, good semen quality is not a guarantee of acceptable fertility. Therefore, considerable effort is being invested in identifying tests and markers to determine functional sperm capacity that can more accurately predict a male's fertility. This is no easy task, given that any one test is likely to measure only one (or perhaps a few) of the many attributes that a sperm must possess for successful fertilization of an oocyte. Notwithstanding that important limitation, the objective is to describe morphological sperm defects likely to affect fertility potential of a male dog.

Introduction

Since the discovery of sperm by Van Leeuwenhoek in 1677,[1] sperm morphological assessment has been employed in semen evaluation and it is widely accepted that specific structural defects are associated with male infertility. Although new methods have been introduced for semen examination, light microscopy examination of stained semen smears are still used for routine morphological evaluation. This approach provides a general assessment of the sperm head, mid-piece and tail, whereas advanced microscopy methods yield more detailed insights regarding the inner and outer fine structures of sperm. Regardless of animal species, evaluation of sperm morphology and its clinical usefulness has always been somewhat contentious, as a subjective evaluation of sperm morphology can lack precision, repeatability and accuracy. Therefore, well-defined criteria for normal sperm morphology, and those with morphological defects, are a prerequisite for clinical evaluation of male fertility potential.

Sperm structures

Although sperm of various animal species have great variation in ultrastructure, their principal components are head, mid-piece, and tail, and their goal is to deliver an intact haploid genome to an oocyte at fertilization for a successful pregnancy. Sperm length varies among animal species, ranging from 50 μm in boars to 90 μm in bulls (Table 1).

The plasma membrane surrounding sperm has regional surface domains, consisting of specific glycoproteins and lipids. These surface domains are important for specific functions, e.g. capacitation and fertilization.[2,3] A disintegrin and metalloproteinase (ADAM) family of transmembrane peptidase protein that is essential for fertilization is localized on the sperm plasma membrane. The acrosome is a cap-like organelle covering the anterior two-thirds of the sperm head (acrosome morphology differs widely among animal species). The acrosome, which is derived from the Golgi apparatus of the spermatid, contains proteolytic enzymes such as acrosin, hyaluronidase and many other hydrolases and esterases. These enzymes promote lysis of the zona pellucida, facilitating penetration of the corona radiata of the oocyte. During an acrosome reaction, these enzymes are released when the outer acrosomal membrane coagulates with the plasma membrane.[4] Mature dog sperm (from cauda epididymis or and fresh ejaculates) have a functional membrane-bound progesterone receptor; induction of this receptor (by either progesterone or a calcium ionophore) initiates an acrosome reaction.[5] However, acrosomal damage renders a sperm incapable of binding to the zona pellucida and penetrating the corona radiate.

A mature sperm nucleus is highly condensed and very resistant to chemical and physical insults.[6] The sperm nucleus is usually dorsoventrally flattened, although the shape of the outline is very species-specific (varies from oval to falciform). Condensed nucleus and lamella-like arrangements containing protamines are apparent with transmission electron microscopy. A small region in the neck of sperm has uncondensed chromatin where transcription, translation and protein synthesis are possible; this explains the existence of sperm RNAs and their possible epigenetic and developmental functions.[7-9] Incomplete condensation of nuclear chromatin and the presence of nuclear vacuoles are two morphological abnormalities caused by protamine deficiency.[6] The concave implantation fossa serves as the attachment of the head to the mid-piece. Cytoplasm and the cytoskeleton only exist between the plasma membrane and acrosomal membrane, as well as between the acrosome and nucleus.

The neck links the sperm head and the flagellum, and contains segmented columns and a dense fibrous structure "capitulum". There are nine columns and nine outer dense fibers of the flagellum at the junction of neck and middle piece, and mitochondria with small projections between the longitudinal columns into the connecting piece. The neck serves an articular piece, whereas mitochondria supply energy.

The tail consists of mid piece, principal piece, and end piece (Figure 1). The mitochondrial sheath surrounds the axonemal complex and the nine outer dense fibers. The outer dense fibers participate in internal fertilization. The axonemal complex consists of a central pair of two single microtubules, surrounded by uniformly arranged nine double microtubules. The mitochondrial helices surround the contractile elements for high flexibility. The number of gyres and total length of mitochondria vary widely among animals. For example, there are 10-12 gyres in humans and bulls, 15-17 gyres in dogs, 90 gyres in mice and 350 gyres in rats. The principal piece is the longest segment of the tail and is enclosed by fibrous sheaths containing two longitudinal columns and circumferentially oriented connecting ribs halfway around the tail. This sheath abruptly ends in the tail, where the principal piece merges into the end piece. The fibrous sheath not only provides proteins for signaling pathways, but is also involved in regulation of sperm maturation, motility, capacitation, hyper-activation, and the acrosome reaction.[10]

Staining methods

In clinical practice, morphology of individual sperm is determined by examining an eosin-nigrosin stained semen smear under oil immersion (×1000 or ×1200 magnification). The recommended technique for the preparation of an eosin-nigrosin stained semen smear:

- Place a 4- or 5-mm drop of warm stain near the end of a warm microscopic slide.
- Place a drop of semen near the stain and mix the two on the slide using a Pasteur pipette. The size of the drop of semen varies with the density of the semen sample. For very concentrated semen use a 2-mm diameter (small) drop and for dilute semen use a 6-mm diameter (big) drop.
- To make a smear, a second slide held at 30° to 40° angle is pushed against the drop of stained semen and then pulled slowly across the slide.
- The smear should result in sperm being evenly distributed on the slide to facilitate evaluation of individual cells. If smear is too concentrated, sperm will overlap, making evaluation of individual cells difficult.

Advanced staining methods for determination of sperm structural integrity

Individual fluorochromes for specific structure, or combinations of fluorochromes for concurrently evaluating more than one sperm compartment, have been established. Various probes are utilized to determine structural and functional integrity of sperm organelles such as viability,[11] DNA fragmentation,[12] mitochondrial function,[11] and acrosome integrity (Table 2).

Morphology classification

Sperm abnormalities are often classified as primary and secondary (based on their origin) or as major and minor (effect on fertility). Regardless, it is generally recommended that morphological

abnormalities should be designated by their descriptive name. Various sperm morphological defects, including their origin and classification, are shown (Table 3).

Primary abnormalities are due to abnormal spermatogenesis, whereas secondary abnormalities are caused during transit through the epididymal duct system, during semen handling, or as a consequence of pathological conditions. A greater incidence of major defects is associated with impaired fertility probably as a result of abnormal conditions of the testis or epididymis, or from genetic defects. Minor defects are considered less important for male fertility, unless present in a large percentage.

Normal potential males should have > 70% morphologically normal sperm, with < 10% and < 20% primary and secondary abnormalities, respectively.[13,14] Regardless, some males with 50 to 70% normal sperm had higher than acceptable fertility, whereas some males with > 70 % morphologically normal sperm fail to do so. Furthermore, combining two characteristics, namely total numbers of morphologically normal and progressively motile sperm per ejaculate was more accurate for predicting fertility than a single criterion. In that regard, artificial insemination with 200 x 10^6 morphologically normal, progressively motile sperm increased pregnancy rates in bitches.

Sperm abnormalities may also be classified as compensable and uncompensable defects.[15] Males requiring more sperm to reach their optimal fertilization rate were considered to have compensable sperm deficiencies, whereas males having lowered fertility independent of sperm dosage were considered to have uncompensable sperm defects. Increasing total sperm number in an insemination dose may compensate for abnormal sperm that are not transported to the oviduct or are incapable of penetrating the zona pellucida. However, abnormal sperm not filtered by the uterus and are capable of oocyte penetration, resulting in a zona reaction that cannot be compensated for by increasing the sperm number in a breeding dose.

Morphological defects: causes and consequences

Detached heads, knobbed acrosomes, detached acrosomes, proximal and distal cytoplasmic droplets, bent midpieces, bent tails, tightly coiled tails over the midpiece and proximally coiled tails are common sperm abnormalities in dogs. Specific abnormal morphology associated with infertility in the dog include abnormalities of mid-piece attachment or ultra-structure, microcephalic sperm, and proximal retained cytoplasmic droplets.

Any insults to male reproductive organs, caused by trauma, inflammation, infection, or neoplasia, and /or indirectly by chemical, behavioral, thermal metabolic, immune-mediated or hormonal insults will decrease sperm production and increase morphological defects.

Defects of the sperm head

Sperm with intact and structurally normal plasma and acrosome membranes are generally capable of undergoing capacitation and an acrosome reaction.[16] In contrast, abnormal acrosomes are often associated with abnormal spermiogenesis, sub-fertility and infertility in several species, including stallions, bulls, boars and rams.[17-20] Acrosomal defects have several underlying causes, including prolonged sexual rest, and the fixation process for morphological evaluation. In addition, genetic causes have also been suggested.

Detached acrosomes, partially or completely lost acrosomes are frequently detected as acrosomal abnormalities. Asymmetric thickening of the acrosome cap, small acrosomes, and droplets attached to the acrosome membrane are also described. The knobbed sperm defect, a protrusion of the acrosome, has been identified in man, stallion, bull, boar, ram and dog; this defect is often associated with other morphological deviations and results in sub-fertility and infertility.[21-23] Sperm with acrosomal defects are incapable of binding and penetrating the zona pellucida and they may undergo premature capacitation and a spontaneous acrosome reaction.

In various species, nuclear and acrosomal vacuoles,[24] fluid-filled membranous cavities similar to cytoplasmic droplets,[25] membrane bound vesicles with clear fluid in the acrosomal and mid-piece region,[26] swollen acrosomes and mitochondria at the mid-piece,[27] are also observed.

Abnormal size and integrity of the nucleus and irregular chromatin condensation primarily constitute sperm head defects. Light microscopy identifies giant and dwarf heads, deformed heads and double heads, whereas electron microscopy recognizes the ultra-structure of sperm such as rolled heads and nuclear crests.[28] Giant heads are often diploid or even tri- or tetraploid. Round-head-syndrome has also been observed; this was associated with defective acrosome biogenesis and tail defects.[29] Sperm head morphological defects including, heads with small and large vacuoles, are attributed to defects in chromatin condensation or incomplete condensation.[31-33] Nuclear vacuoles have also been reported in pathologies such as inflammation of accessary sex glands, varicocele, hyperthermia, testicular tumors, and inflammatory bowel disease.[34] Incomplete condensation is a sign of immaturity and it is associated with low chromatin stability and teratozoospermia of the sperm head.[35] Sperm with abnormal chromatin are incapable of fertilizing oocytes or they result in defective development of early-stage embryos.[36] Chromatin fragmentation and defects in histone-protamine exchange[28, 37] may be attributed to an abnormal chromatin structure.

Defects of the sperm mid-piece

A lack of the mitochondrial sheath and an enlargement of the fibrous sheath constitute morphological deviations of the mid-piece. Cytoplasmic droplets are the most common defect, present at the neck region, or somewhere along the mid-piece or principal piece of the tail. In normal sperm, residual cytoplasm is released along the tail during spermiogenesis; therefore, a disturbance of maturation process causes persistent cytoplasmic droplets. Proximal cytoplasmic droplets are generally regarded as detrimental to fertility and the defect was considered as major defects of sperm. Currently, distal cytoplasmic droplets are also considered detrimental to fertility and embryonic development, due to ubiquitination.[38] Pseudo-droplets are thickening of the mid-piece.[39] Granularity of mitochondria is another abnormality of mitochondria, apparently under the electron microscope.[40] Strong folding, coiling and fracture of the distal part of the mid-piece with or without retained distal cytoplasmic droplet were regarded as a "dag" defect.[41-43] Mitochondrial sheath defects, the loss of single mitochondria and irregular axial fiber bundles are ultra-structural damages (viewed with an electron microscope). The ''corkscrew'' defect, another mid-piece defect, has also been described in bulls.[40,44]

Structural abnormalities of the sperm tail

Abnormal tubular patterns in the tail are considered detrimental and sperm having defective patterns are immotile and unable reach the site of fertilization, resulting in sub-fertility and infertility.[42,45-47] Simple coiled or broken tails and double tails are among the most common sperm defects seen on routine light microscopic examination. The "tail stump" defect was reported in bulls.[41,48,49] Hyperplasia and marked disorganization of fibrous sheath, and axonemal and microtubule doublet distortions cause the "tail stump" defect. This defect results in sterility and it is thought to result from a genetic mutation.[49-52]

Most common observed defects are deviations from the normal tubular structure 9 + 2 + 2 structure; some tails have only three or four microtubules at the distal part. The flagella sometimes lack the central pair microtubules ("9 + 0" structure; this defect causes immotility). Mutations in sperm-associated antigen 6 gene are known to cause this defect.[53] Additionally, reduced motility is caused by defects of peri-axonemal structures. Deformed or incomplete arrangements of the axial filaments, vacuolization of the axoneme, as well as an abnormal arrangement of the mitochondrial helix, have all been described in mammalian sperm. However, disorganized mitochondria, abnormal position of outer dense fiber and abnormal size of outer dense fiber are rarely present in sperm. Anomalies such as invagination or vacuolization of the outer plasma membrane can also be present. The immotile-cilia syndrome (primary ciliay dyskinesia) is caused by a lack of the dynein arms is also reported.[54,55] Dogs with fucosidosis (deficiency of the enzyme fucosidase, which metabolizes the sugar fucose) resulted in abnormal spermatogenesis and sperm maturation (retention of proximal droplets), with morphologically abnormal sperm and poor motility.[56,57]

Approaches to determining sire fertility

Male fertility can be estimated by applying the following:

1. Breeding soundness evaluation
2. Elucidation of sperm-specific organelles and their association with reproductive outcome
3. Correlation of mRNA expression of genes which are important for sperm structural and functional parameters with fertility outcome

In addition, micro RNA and associated gene regulators and genomics could also contribute to predict fertility potential. Each of these approaches has advantages and disadvantages. Even though these methods have merits over one another when applied individually, it is advisable to use combination of these tests to predict sire fertility (Fig. 2).

Application of breeding soundness evaluation

Breeding soundness evaluations (BSE) are commonly used for identifying males that have satisfactory breeding potential and those that are clearly unsatisfactory. The male should meet minimum standards (e.g. those of the Society for Theriogenology). It is important that BSE of a male should be done in a highly professional manner. Even though a BSE is the most commonly used method in the clinical field, the determination of fertility is limited to the test day.

Application of laboratory methods to determine associations of sperm organelles function and fertility

The ultimate goal of semen evaluation is to predict the fertilizing capacity of an ejaculate. Unfortunately, conventional sperm characteristics are not well correlated with the fertilizing capacity of sperm and both inter- and intra-assay variability of these characteristics are high. Furthermore, it is challenging to predict fertilizing capacity, as there is no single sperm parameter that accurately predicts fertility *in vivo*. Therefore, advanced techniques for semen evaluation are needed to increase the odds of achieving an accurate prediction. Researchers have used additional laboratory assays to accurately predict the fertilizing potential of a semen sample.[32,33,58-60] Among them are assays that evaluate sperm DNA fragmentation index (DFI), membrane integrity of sperm and other organelles. Studies[32,61] that determine the association of intactness of sperm organelles with fertility outcome, concluded that

(i) the chance of siring offspring was low for a male with higher sperm lipid peroxidation
(ii) the chance of siring offspring was low for a male with higher DFI
(iii) the chance of siring offspring was high for a male with a higher plasma membrane integrity (PMI) and
(iv) males with higher sperm lipid peroxidation were more likely to have a high DFI and low PMI

Semen cryopreservation is important for application of advanced reproductive technologies. Despite its usefulness, cryopreservation may cause deleterious changes in sperm structure and function. It is well-documented cryopreservation affects motility, morphology, viability and DNA integrity[60] to certain extent. In addition, cryopreservation induced premature capacitation of sperm reduces its epithelial cell attachment capacity at the fertilization site and impairs i fertilization capacity.[62] Thus objective morphological evaluation of cryopreserved sperm is warranted.

Sperm-oocyte interaction tests are useful for diagnosis of subtle sperm defects that cause infertility without causing severe abnormalities detected during routine semen analysis. The availability of viable oocytes still remains an important limiting factor and thus warrants clinical laboratories to apply methodology that examines sperm-oocyte interaction. Sperm-zona pellucida binding is an essential requisite during fertilization.[63] The sensitivity and specificity of sperm-zona binding results indicated the assay to be positively and significantly correlated with in vitro fertilization outcome in several species. Furthermore, there were highly significant correlations between normal sperm morphology, hyperactivated motility, sperm creatine kinase activity and the zona binding capacity of a given sperm sample. In dogs, sperm binding capacity was significantly greater in fresh versus stored oocytes. Furthermore, deep freezing of ovaries appeared to be a better method than salt storage of oocytes.[64]

Application of sperm mRNA expression

Proteins present in sperm have distinctive functions and are essential for preparing sperm for fertilization in a timely manner. Understanding the function of individual sperm protein may explain male infertility. Males with these biomarkers may possess improved fertility. We conducted several studies [65-68] to determine the association of sperm mRNA expression of genes with functions related to male fertility. It was noteworthy that these mRNAs were expressed more abundantly in high fertility compared to low fertility males (Table 4).

Conclusion

Evaluation of sperm morphology is an important component of semen evaluation. Sperm morphology provides evidence of normality or deviations in spermatogenesis and sperm maturation in the epididymis. Therefore, its results, if correctly assessed, are useful to predict male fertility. Although there are not many studies that used advanced techniques for assessment of sperm morphology in dogs, recent studies in other species using advanced techniques demonstrated a positive correlation of morphologically normal sperm with fertility. It should be noted that though total numbers of morphologically normal and progressively motile sperm per ejaculate is more important in predicting fertility, in clinical practice, advanced semen evaluation techniques may provide more information for predicting male breeding potential and for prospectively predicting infertility.

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Table 1: Length (in μM) of sperm in various animal species

Species	Length (μm)
Man	50 to 60
Stallion	60
Bull	75 to 90
Boar	50 to 60
Ram	70 to 80
Buck	60 to 70
Dog	60
Tom cat	60

Table 2. Stains used for the determination of sperm structural and functional parameters

Parameter	Stain used
Sperm viability or plasma membrane integrity	Membrane-impermeable dyes: EB, EH, PI, YoPro-1, ToPro-3, TOTO and Hoechst 3358 Acylated membrane dyes: CFDA, CAM, SYTO-1 and SYBR-14 Double staining to distinguish live, apoptic and dead sperm: SYBR-14/PI, YO-PRO-1/PI, AnnexinV-FITC/PI and AnnexinV-PE/7-ADD
Plasma membrane fluidity	Merocyanine 540/Yo-Pro-1
Acrosome	Non Fluorescent: eosin/nigrosin, Giemsa, Papanicolaou and brilliant blue. Fluorescent: Fluorescein isothiocyanate (FITC)-labeled lectins are FITC-PSA, FITC-PNA, FITC-ConA and FITC-RCA-II.
Mitochondria activity	Rhodamine 123 (R123), Mitotracker Green TM, Mito-tracker Red CMXROs, Mitotracker Deep Red 633 (M-22426), Mitotracker Orange TM, DiOD6 and JC-1 Double staining: R123/PI; DiOD6(3)/PI
Sperm DNA	Chromomycin A3, toluidine blue or aniline blue Sperm chromatin structure assay (SCSA) - Acridine orange; sperm chromatin dispersion (SCD) test - DAPI or Diff-Quik; Comet Assay – DAPI or EB; TUNEL – FITC or Texas Red; FISH- fluorescent labeled probe

Table 3. Abnormal sperm morphology (location, origin, and classification)

Location	Abnormality	Origin	Classification			Comment
			Primary (Major)	Secondary (Minor)	Compensable	
Head	Acrosome defect (knobbed, ruffled or incomplete)	Testicular	Y	-	Y/N	Dominant or sex-linked recessive; defective spermatogenesis. Normal fertility in bulls.[69]
Head	Pin or small head	Testicular	Y	-	N	Defective spermatogenesis; defective sperm DNA compaction.
Head	Detached head	Testicular/Epididymal	Y	Y	Y/N	Sex-limited recessive; Impaired spermiogenesis or maturation. Senescence following sexual rest, acute stress, testicular degeneration.[70]
Head	Round head	Testicular	Y		N	Defective spermatogenesis; with defective sperm DNA condensation and/or with no acrosome.
Head	Microcephalic and macrocephalic	Testicular	Y	Y	Y/N	Abnormal spermiogenesis; greater number affects fertility.
Head	Diadem/Crater Defect	Testicular	Y		N	Abnormal spermatogenesis; inherited?
Head	Nuclear vacuole defect	Testicular	Y		N	Abnormal spermatogenesis; number and size impacts fertility.
Head	Pyriform-shape head	Testicular	Y		Y/N	Abnormal spermiogenesis; heat stress; defective thermoregulation; greater number affects fertility.
Head/Midpiece	Abaxial tail	Testicular		Y	Y	Normal fertility.
Midpiece	Abnormal midpiece (swollen; corkscrew, asymmetry; mitochondrial sheath	Testicular	Y	Y	Y	Abnormal spermiogenesis; affects fertility with gossypol toxicity in bulls.

	defect)					
Midpiece	Proximal cytoplasmic droplet	Testicular/Epididymal	Y		Y/N	Abnormal spermiogenesis; immaturity or testicular degeneration; affects fertility with greater degree of DNA fragmentation.
Midpiece	Distal cytoplasmic droplet	Testicular/epididymal		Y	Y/N	Immaturity; Defective epididymal transit; cause infertility due to ubiquitination in boars.[38]
Tail	Distal midpiece reflex	Epididymal		Y	Y	Thermal insult; exogenous estrogen; hypothyroidism, heritable?
Tail	Bent tail	Epididymal or staining effect		Y	Y	May be real defect or caused by old stain (hypotonic effect).
Tail	Folded or coiled tail (Dag defect)	Testicular, Epididymal?	Y		Y/N	Heritable;[71] heat stress; zinc imbalance; greater ROS.
Tail	Stump tail defect	Testicular/epididymal	Y	Y	Y/N	Abnormal spermiogenesis; Heritable (chromosomal t(5;12) (p15.1; q21) translocation in human;[72] gossypol toxicity in bulls; greater numbers cause infertility.
Tail	Terminally coiled tail	Testicular/epididymal	Y	Y	N	Heat stress; gossypol toxicity in bulls.

Y-Yes; N-No;

Table 4. mRNA abundances of sperm functional and structural biomarkers and their association with fertility

Protein	Function	Association with fertility*
CRISP2	Sperm capacitation and sperm-egg fusion	Positive
PEBP1	Sperm capacitation and sperm-egg fusion	Positive
CCT8	Indicator for the presence of immature cells	Negative
AK1	Motility	Positive
IB5	Fertilization and early embryo development	Positive
Doppel	Acrosome function and fertilization	Positive
TIMP2	Acrosome function and fertilization	Positive
AQP7	Membrane water channel	Positive
Adiponectin	Fatty acid oxidation; membrane integrity	Positive

CRISP2, Cysteine-Rich Secretory Protein 2; CCT8, Chaperonin containing T complex protein 1, sub unit 8; PEBP1, Phosphatidylethanolamine binding protein 1; AK1 - Adenylate kinase 1; IB5 - Integrin beta 5; TIMP2 - Tissue inhibitors of metalloproteinases 2; AQP7 – Aquaporin 7;

*High fertile males showed increased mRNA expression compared to low fertile males;

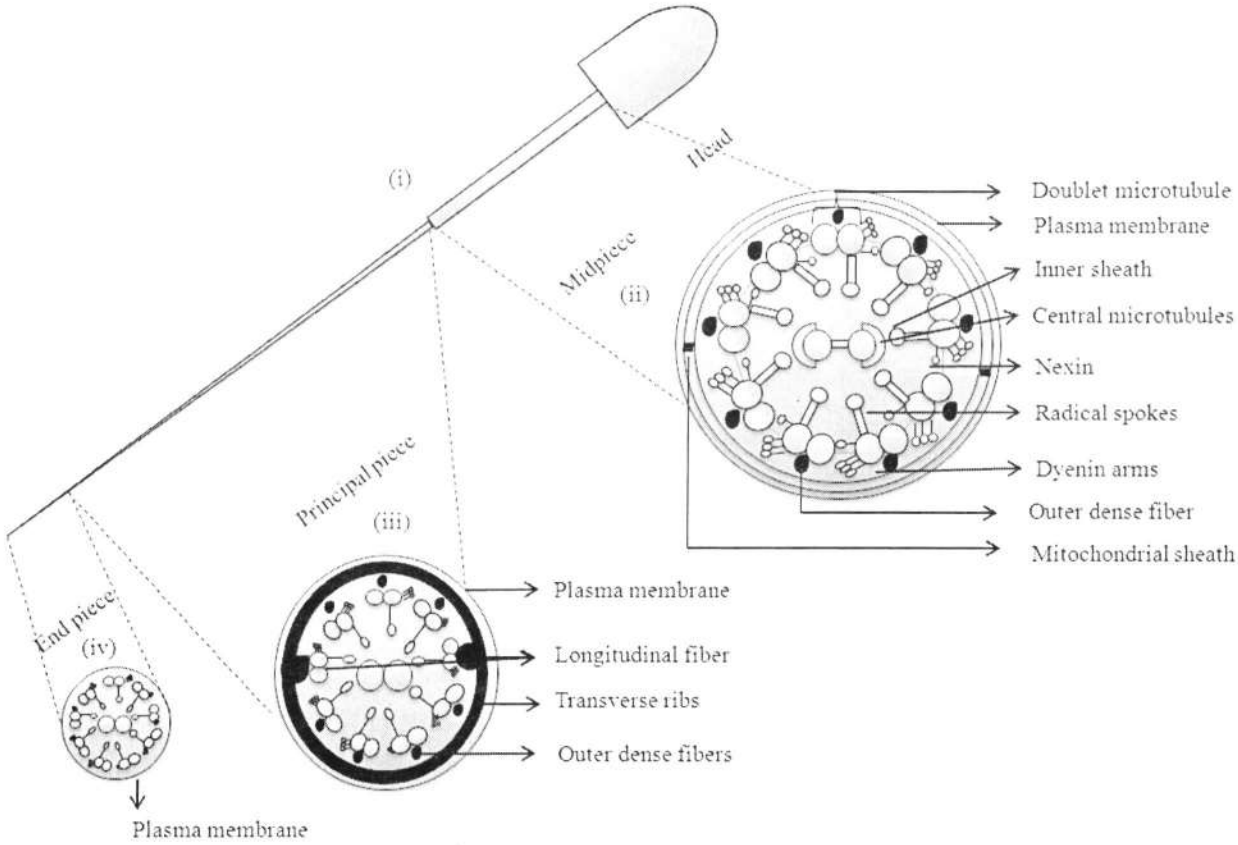

Figure. 1. Schematic representation of sperm and the ultrastructure of the flagellum: (i) Sperm (ii) Schematic cross-section of the mid-piece showing the plasma membrane and mitochondrial sheath surrounding the 9 outer dense fibers (ODFs). The ODFs has 9 outer microtubule doublets of the axoneme associated with dynein arms and radial spokes and the central pair of microtubule doublets. (iii) Schematic cross-section of the principal piece showing the plasma membrane surrounding 7 ODFs. ODFs 3 and 8 have been replaced by the two longitudinal columns of the fibrous sheath. The two columns are connected by transverse ribs. (iv) Schematic cross-section of the end piece.[73]

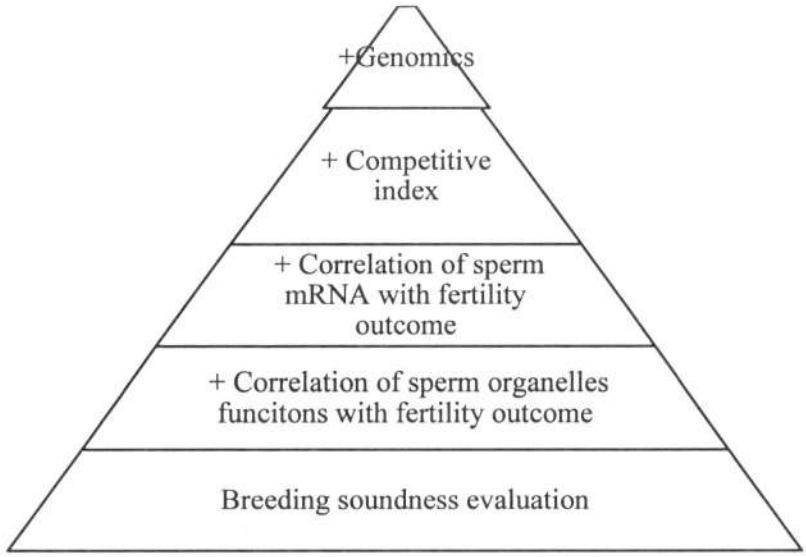

Figure. 2. Hierarchy of sire fertility evaluation methods

International shipments and Dewar maintenance

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Breeders are able to communicate more widely about their breeding programs and advance their breeds by shipping frozen semen. Due to this widespread communication, there has been an increase in both domestic and international shipments of canine frozen semen.

International shipments should be planned before the collection occurs. This should happen when the client first calls the clinic to inquire about freezing semen from their stud. The receptionist should inquire if there is a possibility for export. Additional questions from the receptionist should include:

1. Which country (ies) potentially have an interest in your stud?
2. Does the stud dog have a microchip number?
3. Is he current on his rabies vaccination?
a. Is it a one year or three year rabies vaccine?
4. When was the last time he was mated naturally?

Answering these basic questions provides a good starting point for scheduling the future collection. Gathering this information gives the reproduction technician an opportunity to obtain the most recent requirements for import before the client enters the clinic.

There are some options to obtain the most current requirements for export. The United States Department of Agriculture (USDA) provides regulatory guidelines to import and export animals and animal byproducts. This includes frozen canine semen. When we are exporting frozen canine semen to a certain country, we need to make sure we follow that country's requirements or import regulations. Key import requirements called IREGS are available on the USDA's website. However, not all countries have their qualifications posted on the USDA's IREG. One may obtain this information with the country of import's Department of Agriculture or Ministry of Agriculture.

Once the current guidelines are obtained, we will examine the key requirements:

Testing/vaccinations

1. What tests or vaccinations are required?
2. When should tests and vaccination be completed?
3. Can antibiotics be added to the buffer?
4. What type of diagnostic platform is required?
5. Does the requirement state that a specific laboratory needs to complete the tests, such as a USDA approved laboratory?
6. Reserve and freeze 2cc of serum

Physical examination

1. When should a physical examination be completed?
2. Is there a specific statement that is necessary, such as for rabies or parvovirus?
3. Do the requirements state a need for a permanent form of identification in the form of a microchip or tattoo?
4. Is this specific breed of dog able to be imported in this country?

Freezing buffers and media

1. Are there specific mentions of the method of manufacturing, for example aseptic conditions or the use of micron filters?
2. Do the buffers/media contain milk or egg substance?

Straws/vial identification

1. What is necessary for straw/vial identification?

a. Permanent identification
b. Species
c. Collection date
d. Registration name/number
e. Method of freezing

Preparation of the semen and the storage tank and vapor shipper

1. Is there a statement regarding how the semen is collected, handled, and stored?
   a. Under the supervision of an accredited veterinarian
   b. Collected and handled with equipment that is sterilized or disposable
   c. Stored with semen that meets the same requirements; "like" semen
   d. New container or disinfected/sterilized storage and vapor shipper

Expiration of the heath certificate

1. Do the requirements state expiration after signature?

USDA: signatures and sealing the tank

1. Most of the state import/export veterinary medical officers want to review the health certificate before receiving the original documentation.
2. Does a USDA veterinarian need to seal the tank?

When the health certificate is approved by the USDA, the shipment will be ready to be exported.

1. Send copies of the approved health certificate, Air Way Bill (AWB), commercial invoice to the inseminating veterinarian.
   a. The commercial invoice should state the contents, including collection and vapor shipper
   b. Four copies of the commercial invoice should be signed and placed on the outside of the container with the AWB.
   c. Copies of the approved health certificate should be located with the AWB and commercial invoice.
2. Insure the container is identified
   a. Consignee
   b. Statement that it is nonhazardous

The initial destinations of certain countries and couriers are not necessarily the airports where the customs agents or veterinarians are located. These countries will require either a direct flight or a special arrangement with the carrier.

Vapor shippers are weighed and recorded when they are shipped and when they are returned. This provides an insight into how well a shipper is maintaining its charge. Beyond recording and evaluating the vapor shipper's data, testing is completed on each unit every quarter to determine if the vapor shipper has the correct evaporation rate. A simple calculation of the evaporation rate will help determine if the vapor shipper can maintain a charge.

Date:_____ wt 1 :_____

Date:_____(24hrs from wt 1) wt 2 :_____

(first weight (wt 1) _____- second weight (wt2) _____) .5606= _____liters/day

If the evaporation rate is more than 1.9 liters/day the vapor shipper is taken out of rotation (MVE Chart Dry Vapor Shippers model numbers: SC 4/3v and SC 4/2v). Depending on the warranty from the manufacturer, the vapor shipper can be return to the manufacturer to determine if the vapor shipper can be re-vacuumed.

Tickborne and other stealth pathogen reproductive concerns

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Abstract

Tickborne and other stealth pathogens may cause illness or persist in seemingly healthy dogs and cats. Question: Might premunitive carrier status in a breeding animal have an impact on reproduction or the next generation? This review considers and compares the geographic distribution, transmission, clinical signs of illness, diagnostic tests for sick animals and for the identification of nonclinical carrier status, treatments, and prevention of anaplasmosis, babesiosis, bartonellosis, borreliosis, cytauxzoonosis, ehrlichiosis, hepatozoonosis, leishmaniasis, hemotropic mycoplasmosis, and rickettsiosis. These diseases are generally considered vector-borne but some may also be acquired vertically (mother to offspring) or horizontally via bites/fights, or by blood product transfusions.

Keywords: Carrier, cytopenia, Lyme, proteinuria, transplacental, vertical

Introduction

Most of the tickborne infectious diseases (TBDs) involve stealth pathogens, which often go undetected in long-term carriers which are nonclinical (asymptomatic) until perhaps coinfections, immunosuppression (by drugs or disease), or stress occurs. Some of the diseases cause illness more often in certain breeds, possibly due to genetic predisposition (eg, Ehrlichiosis[1,2] in German Shepherds (*E. canis)* or Lyme nephritis[3-6] in Labrador and Golden Retrievers); life-style (e.g., babesiosis[7] in Greyhounds [*B. canis]* or Pit Bull Terriers [*B. gibsoni*]); or due to vertical transmission (e.g., in babesiosis [as above][8,9] or leishmaniasis[10-15] in American Foxhounds).

Many questions may arise as reproductive concerns. Here are just the top 10:

1. What types of ticks and TBDs are we talking about, where are they endemic, and what kinds of signs might we see with illness?
2. What diagnostic tests are available for sick dogs? Should breeding dogs be screened for TBDs and which diagnostic tests should be used in those cases?
3. Can these diseases affect fertility?
4. Can these diseases be transmitted venereally or from frozen semen?
5. Should a nonclinical carrier be treated before breeding and can it be completely cleared?
6. Will the stress of pregnancy or lactation cause a carrier to become ill?
7. Can these diseases be transmitted vertically to offspring (*in utero* or during lactation)?
8. Should the gravid or nursing patient be treated?
9. How do we diagnose these diseases in the very young and should they be treated?
10. How do we best protect breeding dogs and their young offspring from these diseases?

Although we do not have all the answers to all of these questions yet, veterinarians working with breeding animals and their young offspring are wise to be on the lookout for the TBDs in their geographic area, and should also be aware of the TBDs in other areas where animals may have travelled for shows, breeding, or family vacations. They should also consider any history of bites/fights, splenectomy, blood transfusions, and illness in relatives (ancestors, littermates) or in other pets in their environs.

Q1: What types of ticks and TBDs are we talking about, where are they endemic, and what kinds of signs might we see with illness?

There are four major kinds of hard ticks, which are each 3-host ticks, including *Ixodes scapularis* (the deer tick or black-legged tick), *Dermacentor variabilis* (the American dog tick), *Rhipicephalus sanguineus* (the brown dog tick), and *Amblyomma americanum* (the Lone Star tick). Hard ticks usually quest for hosts outdoors (except *R. sanguineus*, which is found in kennels) and are found in geographic

'endemic areas' which may be extended by host migration, including bird migration. There are also soft argasid ticks (*Ornithodorus* spp) that transmit diseases. Each kind of tick may carry more than one kind of organism, for instance, *Ixodes scapularis* may carry spirochetes (*Borrelia burgdorferi* [the agent of Lyme disease], *B. miyamotoi* and *B. davisii* [these last two are among the relapsing fever group of *Borrelia* spp]), two types of rickettsia (*Anaplasma phagocytophilum* and the *Ehrlichia muris*-like agent), a protozoan (*Babesia microti*), bacteria (*Bartonella* spp), a virus (Powassan or tick-borne encephalitis virus), and possibly more! *I. scapularis* is found in the northeast, midAtlantic, and upper midwestern United States, where its hosts are usually small mammals, birds, and deer. In western states, *I. pacificus* can transmit Lyme disease, but it feeds on reptiles, which are not as good reservoirs of the agent.

Tables 1 and 2 show a variety of TBDs, with comparisons of their vectors, targetted cell types, clinical signs, diagnostic tests and whether paired titers may be needed, treatments, and whether the agent is bloodborne.

Lyme disease,[3-6] caused by *Borrelia burgdorferi*, is the most common TBD in the USA. Luckily only about 5% of Lyme-seropositive dogs show illness that is suspected due to the agent. In some areas of New England, 70-90% of healthy dogs are seropositive. Experimentally, Lyme disease induced by the tick model caused high antibody titers but no signs of illness in adult beagle dogs and puppies over six months old. In young (six-12 wk) beagle puppies experimentally infected, after a two to five month incubation, only a self-limiting illness (four days) of fever, anorexia, and arthritis in the leg closest to the tickbites occurred, with possibly a few similar recurrences in the same or different leg every two weeks. Older puppies (13-26 wks) showed milder signs (two days) and fewer recurrences. None of the experimental dogs developed proteinuria. Perhaps less than 2% of Lyme-seropositive dogs show "Lyme nephritis", a protein-losing nephropathy (PLN) due to immune-complex glomerulonephritis, for which Labrador Retrievers, Golden Retrievers, and perhaps Shetland Sheepdogs appear predisposed genetically. However, even among seropositive retrievers, proteinuria is uncommon.[16] Rash, cardiac, or neurologic signs of Lyme disease in dogs are not well-documented. Seropositive cats are even less likely to show any illness. Since Lyme Borreliosis travels by tissue migration and not generally hematogenously, seropositive animals may be used as blood donors. The diagnosis and treatment of PLN is reviewed elsewhere.[4,17-21]

Tables 1 and 2 do not include *B. lonestari* (from *Amblyomma* ticks) that causes "Southern tick-associated rash infection" (STARI) in people in southern states, mimicking the rash of Lyme disease[22] (it is unknown if this species causes illness in animals) or the tickborne relapsing fever (TBRF) group of *Borrelia* spp, which may get more attention in the future, including *B. miyamotoi* and *B. davisii* (in *I. scapularis* ticks in Lyme endemic areas) and *B. hermsii* and *B. turicatae* (transmitted by soft *Ornithodorus* ticks, which only feed for 15-90 minutes, in northwestern and southern states, respectively), associated with log cabins and sheds.[23-28] The TBRF group of *Borrelia* spp do circulate hematogenously, cause relapsing fever, myalgia/arthralgia, and neurologic signs in people and possibly illness in dogs/cats.

Anaplasmosis and ehrlichiosis[1] (rickettsial infections) are probably next most common after Lyme borreliosis. *A. phagocytophilum* may be seen alone or as a coinfection in Lyme endemic areas, with similar signs of fever, anorexia, lameness, and possibly thrombocytopenia and other cytopenias.[29,30] Coinfected dogs may be more likely to become clinically ill.[31] In the upper midwestern states, seropositivity against *A. phagocytophilum* is just as common as against *B. burgdorferi*. *A. platys*, a rickettsial parasite of platelets, is endemic in southern and Gulf states, often with *E. canis* coinfection. Ehrlichiosis is mostly seen in southcentral and eastern states. Mononuclear forms generally cause cytopenias, especially thrombocytopenia (*E. canis* and *E. chaffeensis*).[30,32] A newly recognized mononuclear form, *E. muris*-like, is found in the upper midwest and is probably transmitted by *I. scapularis* ticks.[33,34] *E. canis* may cause more severe illness (especially in German Shepherds), pancytopenia, bone marrow suppression, hypoalbuminemia, proteinuria, hyperglobulinemia (even perhaps with monoclonal gammopathy, mimicking multiple myeloma), hemorrhage, and neurologic signs. A granulocytic form, *E. ewingii*,[35] is associated with fever, polyarthropathy, and possibly cytopenias, and mimics anaplasmosis and/or Lyme disease.

Rocky Mountain spotted fever (RMSF) due to *Rickettsia rickettsii*, is more common along the eastern seaboard (transmitted by *D. variabilis*) than in the Rocky Mountains (transmitted by *D. andersoni*, the Rocky Mountain wood tick) and in the southwest by *Rhipicephalus* ticks.[22,36] Rocky Mountain spotted fever may mimic ehrlichiosis in many ways but is more likely to cause vestibular neurologic signs and unlike most of the other TBDs, RMSF is not associated with carrier status, therefore the illness is seen acutely and seasonally, occurring within one to two weeks after the tick bite.[37] In addition to doxycycline, RMSF can be treated with fluoroquinolones.

Bartonellosis, often thought of as a flea-transmitted disease, can also be transmitted by ticks, bites/fights, transfusions, and possibly vertically.[38-40] There are many species, e.g., *B. henselae* (the agent of cat scratch fever), *B. vinsonii*, *B. quintana*, *B. clarridgeiae*, *B. elizabethae*, *B. koehlerae, B. washoensis*, etc. Bartonellosis may be a coinfection with Lyme disease, Anaplasmosis, and others. In our area, 7.5% of healthy dogs and 20% of hospitalized dogs were seropositive.[41] Carriers may be nonclinical or have endocarditis, hepatitis, vasculitis, uveitis, neurologic disease, anemia, cytopenias, immune-mediated and potentially other illness (because of coinfections, it can be difficult to know if bartonellosis is the sole cause of signs).

Red blood cell TBDs such as babesiosis[7-9,42] and hemotropic mycoplasmosis[43,44] may mimic immune-mediated hemolytic anemia presentations, eg, with regenerative anemia, spherocytes, autoagglutination, Coombs' positive test results, icterus, and splenomegaly. Babesiosis may cause intravascular as well as extravascular hemolysis, with hemoglobulinemia and hemoglobinuria, as well as the bilirubinemia and bilirubinuria seen with extravascular hemolysis, whereas hemotropic mycoplasmosis (previously called hemobartonellosis) is associated with only extravascular hemolysis. Large piroplasms include *B. canis* (*B. canis canis*, *B. canis vogeli*, *B. canis rossi*, and novel as yet unnamed[45] varieties). Smaller piroplasms include *B. gibsoni*, *B. conradae*, and *B. microti*-like (also called *Theileria annae*), with possible PLN and thrombocytopenia syndromes.[46] Babesiosis is transmitted by *Rhipicephalus* all over the US, but especially seen in Greyhounds (*B. canis*) from racetracks or kennels, and in American Pit Bull and Staffordshire terriers and Tosa Inu breeds (*B. gibsoni*) possibly also transmitted by bites/fights and from mother to offspring. Mycoplasmosis may be transmitted by fleas as well as bites/fights. Mycoplasmosis due to *M. haemofelis* is more pathogenic than *M. haemominutum* in cats, and *M. haemocanis* more so than *M. hematoparvum* in dogs. Cats that are ill with mycoplasmosis should be tested for comorbidities/coinfections, e.g., FeLV/FIV. Dogs ill with mycoplasmosis may have comorbid disease, immunosuppression, splenic disease or a history of splenectomy. *Cytauxzoon felis*,[47] a feline red blood cell protozoan, is transmitted by *Amblyomma* ticks, and seen in cats mostly in the southern states, but positive reservoir bobcats have been found as far north as Pennsylvania. This hemolytic disease has been associated with vascular blockade and high mortality, however up to 15.5% of healthy domestic cats in Arkansas may be carriers, followed by a 12.9% carrier rate in Missouri and 3.4% in Oklahoma.[48]

Hepatozoonosis[49-51] is due to *H. americanum* and/or the less pathogenic (European) *H. canis* in the southeastern US and is unique in that dogs become infected by eating the tick or raw encysted meat. Chronic muscle wasting and protein-losing nephropathy may be seen but a hallmark of *H. americanum* is periosteal proliferation seen radiographically. *H. felis* may be a problem in cats travelling to other continents.

Leishmaniasis[10-15,21] is not common in the US because of the absence of the sandfly vector, however American Foxhounds, Corsicas, Spinones, and Neapolitan Mastiffs may be predisposed because of transfer of the infection vertically (mother to offspring) and possibly by bites/fights. Dogs of other breeds have been infected inadvertently via blood transfusions from carriers.[52] The disease has travelled to different areas of the US with infected dogs. The disease is associated with chronic wasting, glomerulonephritis, fever, enlarged reticuloentothelial system organs, bone marrow damage, PLN, ocular, and skin changes.

Q2: What diagnostic tests are available for sick dogs? Should breeding dogs be screened for TBDs and which diagnostic tests should be used in those cases?

Since so many of the TBDs mimic each other, e.g., by causing proteinuria, cytopenias, and/or polyarthropathy, it is important to be aware of the differential diagnoses among the TBDs as well as other differentials that can cause those signs (infectious, inflammatory, immune-mediated, neoplastic, toxic, traumatic, degenerative, genetic, etc). It is essential to keep an open mind since so many animals are nonclinical carriers (for instance, in some Lyme endemic areas, 70-90% of healthy dogs are seropositive) and the finding of a positive test result may be coincidental and not proof of the cause of illness. One study showed that 40% of dogs diagnosed with Lyme disease actually had other causes for their signs and were eventually realized to be misdiagnosed.[53] Other diseases may present during an acute stage, before seroconversion (e.g., anaplasmosis, ehrlichiosis, RMSF, leptospirosis) so paired or convalescent testing is required. Doxycycline (or minocycline) is often given when TBDs are suspected, to treat possible susceptible coinfections (spirochetes, rickettsials). But a favorable response to treatment is still not proof of cause since improvement may be coincidental over time, doxycycline (or minocycline) treats a variety of (undetected) coinfections, and the tetracycline family has antiinflammatory and antiarthritic properties.

When dogs are sick with signs of TBDs, veterinarians often wonder which tests are better, tests for antigen (cytology by blood smear, joint tap, lymph node or bone marrow aspirate as the agent requires, culture, or PCR) or tests for antibodies (IFA, ELISA, WB, etc.)? For sick animals, usually every piece of the puzzle is helpful in different ways under different circumstances, and you may need to submit multiple different types of tests on an individual.[54] Tests for antigen may be most helpful during acute stages of illness, before seroconversion and before starting treatment (to avoid a 'false' negative result). Consider PCR testing of whole blood for blood-borne agents, but realize that the small aliquot of blood sampled may be negative, especially if even one dose of treatment has been given, and that false positive PCR test results may occur due to contamination at the laboratory (controls are essential). For antibody tests, seroconversion may not occur until two to tree weeks after signs of illness present, therefore paired titers may be necessary. Consider cross-reactive antibodies, for instance, other spotted fever group rickettsial infections cross-react with serologic tests for RMSF. Some Lyme tests (OspA and OspC antibodies) may be seen in both naturally exposed and vaccinated animals. Consider that for some diseases which do not present until chronic stages, the animal should have seroconverted by the time it presents with clinical signs due to that disease (Lyme disease, leishmaniasis). If the signs of illness are chronic, testing for RMSF is likely unnecessary.

A positive test result for any TBD is a marker for tick and wildlife exposure, and coinfections with other TBDs and other infectious agents should be considered (eg, leptospirosis may mimic Lyme nephritis).[55] As another example, a dog may be found to be Lyme-seropositive but its illness may be due to Anaplasmosis or RMSF presenting during the acute phase, before seroconversion, or due to babesiosis, for which separate testing is required. See Tables 1 and 2 for a listing of some TBDs as well as leptospirosis and brucellosis, which are included for comparisons.

Since *Borrelia burgdorferi* does not circulate hematogenously, and because there are few organisms to be found in tissue samples and they are difficult to grow *in vitro* in the laboratory, antibody tests are preferred. Table 3 shows the variety of serologic tests available for antibodies against Lyme disease. Older tests (whole cell IFA, ELISA, IgM/IgG, or Western blot), while no longer as helpful for Lyme diagnosis because of cross-reactive antibodies with other spirochetal infections and vaccinal antibodies, may be interesting to study now that emerging *Borrelia* spp of the TBRF group may become a problem (TBRF antibodies do not cross-react on C6 peptide tests).

The newer SNAP-4DxPlus (IDEXX) and AccuPlex4 (Antech) are the most common screening tests done for sick and healthy animals. Websites are available that show the prevalence of positive test results (www.dogsandticks.com/diseases_in_your_area.php and www.capcvet.org/parasite-prevalence-maps/) down to the county level. The canine SNAP-3Dx, -4Dx and -4DxPlus (IDEXX) tests do not use species specific reagents and may be used on cats, horses, and other species, off label. Lyme antibodies against the C6 peptide of the VlsE antigen are specific for natural exposure antibodies. Although the height of the quantitative titer does not predict illness, the C6Quant (IDEXX) level has been shown to wane three to six months after treatment, while the other tests for antibodies have not been shown to wane with treatment (eg, ospF). Comparisons of pre- and three to six month post-treatment C6Quant are

helpful for trend, and to compare in the future if signs of illness occur, to see if there is any reason to suspect Lyme disease as cause and whether retreatment is indicated. While some tests purport to show whether the Lyme titer represents acute or chronic infection, they may merely indicate when the dog was last exposed but not when it was first exposed, and also there is no evidence that whether the infection is acute or chronic is clinically relevant. The use of IgG and IgM titers is not helpful in dogs since the incubation is two to five months before signs, and the dogs would not be presented during a time when they were IgM positive and IgG negative.

Healthy breeding dogs are often screened for brucellosis, but should they be routinely screened for TBDs? This author thinks so, certainly if they are ill with suggestive signs, but also if they are nonclinical but live or have travelled to endemic areas, or a predisposed breed, or if there have been other possible exposures (living with an affected animal, bites/fights, history of transfusions, splenectomy, etc). Using screening tests help to identify dogs at risk for proteinuria or other stealth pathogen consequences, sentinels for public health hazards, and to show if the use of tick control is adequate. The most commonly used screening tests are the IDEXX in-house kits (SNAP-3Dx, SNAP-4Dx or SNAP-4DxPlus) and the AccuPlex4 (Antech Reference Laboratories). These are qualitative tests for the presence of heartworm antigen and for antibodies against Lyme disease, anaplasmosis, and ehrlichiosis. There are differences, e.g., the *E. canis* test on the IDEXX tests may pick up cross-reacting antibodies to *E. chaffeensis* and possibly the *E. muris*-like agent, the *A. phagocytophilum* test may pick up *A. platys* antibodies, and there is also a specific test on the SNAP-4DxPlus test for antibodies to *E. ewingii*, while the AccuPlex4 may not pick up these antibodies. The AccuPlex4 claims to show antibodies 12 days earlier against *E. canis* and one week earlier against Lyme and *A. phagocytophilum* than the SNAP-4Dx tests,[56] but the SNAP-4DxPlus test was found to be more sensitive and specific for Lyme and *Anaplasma* antibodies than the AccuPlex4 in a recent study.[57]

Comprehensive tick panels including PCR and serologic tests for the more common TBDs are available, for instance at the Vector Borne Disease Diagnostic Laboratory (VBDDL, at North Carolina State University, Raleigh, NC), IDEXX Laboratories, and Antech Diagnostics. Galaxy Diagnostics (Durham, NC) does *Bartonella* BAPGM enrichment blood culture/PCR testing. The National Veterinary Laboratory, Inc (Franklin Lakes, NJ) does the *Bartonella* Western Blot antibody test (the 'FeBart' test is a species specific test but canine reagents can be used for dog samples). Less common tests and the laboratories that do them have been reviewed elsewhere.[17]

Q3: Can these diseases affect fertility?

There is no evidence that nonclinical carriers are less fertile. There is some evidence that coinfected dogs are more likely to become ill than dogs carrying only one organism,[31] possibly due to immune system modifications, which potentially could affect general health including fertility.

Q4: Can these diseases be transmitted venereally or from frozen semen?

The TBDs are generally not thought of as venereal diseases. Besides arthropod vectors, the bloodborne diseases may be transmitted by blood or blood product transfusions,[1,38,39,52,58,59] contaminated needles or surgical equipment, bites and fights, ingestion of blood,[38,39] and possibly saliva (*Bartonella* and *Mycoplasma* spp).[39,43] Since blood may be present in semen or in the vagina, it is theoretically possible that transmission during breeding could occur. Although the agent of Lyme disease may be found rarely by PCR in blood, urine, or semen, viable organisms are very rarely present.[60-65] Control dogs remained Lyme-seronegative even after being housed with seropositive dogs for more than a year.[66] The agent of visceral leishmaniasis, *L. chagasi*, has a tropism for the male genitalia and venereal transmission, as the organism is shed intermittently in the semen of infected dogs, causing infection in breeding bitches in experimental settings without the natural vector present.[67]

Q5: Should a nonclinical carrier be treated before breeding and can it be completely cleared?

Treating nonclinical, nonproteinuric dogs that are seropositive for Lyme, *Anaplasma*, or *Ehrlichia* spp is not advocated.[2,3,68,69] It may not be possible to truly clear a carrier dog with treatment anyway. For

instance, during treatment of anaplasmosis with doxycycline, the PCR test on the blood will be negative, but then positive again after treatment, or when challenged with steroids.[70,71] However, I would try to treat babesiosis in a carrier, to try to prevent illness during the stress of pregnancy, and vertical transmission to the offspring, although babesiosis is not always able to be cleared, especially in splenectomized animals.[42] Treatment may not be able to clear all animals of bartonellosis, cytauxzoonosis, mycoplasmosis, and Lyme disease.[39,44,47,72] Treatment may decrease antigenic load and produce a nonclinical premunitive carrier status, but these should not be used as blood donors (if the organism is bloodborne). In some cases of splenectomized dogs with babesiosis, if clearance with other antiprotozoal treatments fails, longterm clindamycin may be used. For hepatozoonosis, treatment is always longterm, in order to deal with organisms as they are released from muscle cysts. Similarly, treatment for leishmaniasis is longterm and may not be able to eradicate the organism.[73]

Q6: Will the stress of pregnancy or lactation cause a carrier to become ill?

It may be argued that a carrier may come out of the nonclinical premunitive state and become ill during the stress of pregnancy, therefore treatment may be warranted to try to decrease antigen load and perhaps decrease the risk for illness or transmission during the stress of breeding, pregnancy, or lactation; however, there are no supportive studies to show treatment is indicated or helpful in this regard and the animal may not be cleared.

Q7: Can these diseases be transmitted vertically to offspring (*in utero* or during lactation)?

There is evidence that babesiosis and leishmaniasis are transmitted vertically,[7-15,74] and perhaps TBRF.[75] There is evidence for vertical transmission of *H. canis* and *H. felis*, but not of *H. americanum*.[49,51] Although there is evidence for transplacental transmission of anaplasmosis in humans and cattle, there is not for dogs.[1,76] Lyme disease is probably not transmitted vertically.[66,77] Nor was Mycoplasmosis proven to be (without the presence of fleas).[38]

Q8: Should the gravid or nursing patient be treated?

If the gravid or nursing patient is clinically ill, it will need to be treated. In the past, veterinarians were worried about the use of doxycycline and the effects on the young. It appears that doxycycline is not teratogenic,[78] nor does it bind to calcium as much as tetracycline, therefore discoloration of the teeth is not as much a concern.[79]

If the positive animal is not ill but just suspected (or proven) to be a carrier, consider not treating, especially if there is no reported vertical transmission for the agent. Check for occult proteinuria, CBC, and/or biochemical changes that are associated with TBDs. The pregnancy and offspring need to be monitored carefully.

Q9: How do we diagnose these diseases in the very young and should they be treated?

Since antibodies from the mother are passively bestowed to offspring transplacentally during the last trimester and in the colostrum,[80] serologic tests alone are not diagnostic for infection in the very young. Although an experimental model utilizing intradermal injections of *B. burgdorferi* in dogs caused infection in puppies,[81] a more natural tick model[66] showed that there was no transplacental transmission of Lyme disease in pups, that they were not ill when followed for five months and their maternal antibodies waned by four weeks. The in-house IDEXX SNAP-4Dx test was used on puppies from a Lyme-seropositive dam in the field, showing positive results in the pups at seven days of age, but their C6Quant results were $\leq$10 on day 18 of life (the dam's C6Quant result was 112); these puppies were not treated, and showed no signs of illness.[82] These reports show no evidence of transplacental transmission of Lyme disease in the tick experimental model of Lyme disease nor in the field, and that passive (maternally derived) antibodies declined within the first few weeks of life.

For other TBDs, when appropriate, a search for the organism by cytology or for its DNA by PCR testing is preferable to prove infection in young puppies, to avoid confusion with material antibodies on

serologic tests. If only serology is available, passive immunity will wane over time, whereas active infection titers will stay stable or trend up over time.

If puppies are ill, they should be treated. Lyme disease can be treated with doxycycline or amoxicillin, but anaplasmosis and ehrlichiosis need to be treated with doxycycline. Although tetracycline may discolor the teeth and should not be given with milk because of calcium binding, doxycycline does not, so it can be used safely in the young.[79] Rocky Mountain spotted fever may be treated with doxycycline or fluoroquinolones, and of those, I would probably choose doxycycline in the young rather than possibly causing cartilage damage with fluoroquinolones. Doxycycline may cause esophagitis and should be given in liquid form, with care to 'wash' it into the stomach by water, milk, or food following dosing.

Q10: How do we best protect breeding dogs and their young offspring from these diseases?

While specialists still debate the pros and cons of using Lyme vaccines,[3,4,83] there is strong consensus that tick control is most important, because there are many other TBDs in Lyme endemic areas for which there are no vaccines available. Landscaping advice helps to keep pets out of brush where outdoor ticks quest, and kennels should be monitored and treated to avoid *Rhipicephalus* infestations. There are many types of products available which work very well on individuals, including amitraz and permethrin collars (put on tightly enough to have contact with skin, not just fur), topical fipronil and permethrins, and the new oral (chewable) isoxazoline products, which bind specifically to the GABA-gated chloride channel which exists in insects and ticks but not in their hosts. Products which kill the tick before or very soon after tick attachment are preferred, since many TBDs can be transmitted much faster than the two to four days of tick attachment required for the transmission of Lyme or *Babesia* spp. Permethrins (except for the Seresto collar) are toxic for cats. For dogs that swim or get bathed often, the chewable products may be ideal. See Table 4 for a comparison of tick control products.

Conscientious owners will check their pets every day for ticks and remove them with tweezers or a tick removal device, grasping the tick close to its attachment on the skin, pulling slowly but steadily. Ticks should not be covered with petroleum jelly, burned, or crushed within bare fingers (hemolymph can be infective through cracked cuticles). Tick types may be identified by checking for *Ixodes* ticks' anal groove (looks like a frown), or by images on-line. In Lyme endemic areas, if a person pulls of an engorged *Ixodes* tick, it is recommended to take one days' dose of doxycycline within 72 hours, to prevent Lyme disease.[84] No such study has been done in dogs regarding prevention of Lyme or other TBDs that are sensitive to doxycycline.

Other suggestions for prevention include planning for travel to endemic areas with tick control, screening donors before giving transfusions (or transplantation), and having a suspicion regarding TBDs when working with predisposed breeds[85,86] or animals with a history of bites/fights with those breeds.

Table 1. Some TBDs, ticks, infected cell types, diagnostic tests, treatments, etc.

	Agent	Type of Infected Cell	Major Vector	Tests Beyond Cytology	Need Paired Tests	Bite or Bloodborne	Treatment	Carrier Status
Ap	Ric	Granuloctye	Ixodes	ELISA, PCR	X	X	D	X
Ay	Ric	Platelet	Rhipi	ELISA, PCR	X	X	D	X
Bar	Bac	Endothelial cells, Epi-rbc, Macrophages	Ixodes, fleas, other ticks	PCR, WB, IFA, culture	?	X	2 of 3 D,F,Z ZR	X
Bb	Spir	Extracellular near fibroblasts	Ixodes	C6**see abbreviations	No	Rare	D (AEZ)	X
Bc	Prot	Rbc	Rhipi	PCR, IFA	X	X	Im	X
Bg	Prot	Rbc	Rhipi	PCR, IFA	X	X	Z/Q Clin	X
Bm	Prot	Rbc	Ixodes	PCR, IFA	X	X	Z/Q	X
Bru*	Bac	Lymphocyte Varied	Venereal Varied	RSAT, ELISA PCR	X	X	MD D	X
Cyt	Prot	Rbc, schizonts in macr	Ambly Derma	PCR	No	X	Z/Q Im	X
Ec	Ric	Monocytic wbc	Rhipi	ELISA, IFA, PCR	X	X	D	X
Ech	Ric	Monocytic wbc	Ambly Derma	ELISA, PCR	X	X	D	X
Ew	Ric	Granulocyte	Ambly	PCR	X	X	D	X
Hep	Prot	Myocyte, Lymphoid, Liver, Wbc	Ambly (eating tick, raw meat)	PCR Muscle biopsy	No		SPC	X
Lei*	Prot	Extracellular Macrophages	Sandfly, Vertical	IFA, PCR	No	X	PA	X
Lep*	Spir	Extracellular	Urine	MAT, PCR ELISA	X	Rare	D (A)	X
Myc	Bac	Epi-rbc	Fleas, Ticks	PCR, ELISA	(future)	X	DF	X
Rr	Ric	Endothelial cells	Derma Rhipi, Ambly	IFA, DFA	X	Rare	D F	No

*Although not TBDs, Bru, Lei, and Lep are included for comparisons

Table 1 is adapted from Goldstein RE, Brovida C, Fernandez-del Palacio MJ, et al: Consensus recommendations for treatment for dogs with serology positive glomerular disease. J Vet Intern Med 2013;27:S60–S66.[21]

See Abbreviations following Table 2 below.

Table 2. Some TBDs and the clinicopathologic changes they can cause

	Lameness	↑UPC or ↑BUN	Vasculitis or Epistaxis	Oculoneural Signs	RBC ↓	WBC ↓	Platelets ↓	Albumin ↓	Globulin ↑
Ap	X	X	X	X	X	X	X	X	
Ay		?	?	?	X	X	X	?	
Bar	X	X	X	X	X	?	?	?	?
Bb	X	X	?	Rare			X if PLN	X	
Bc		X		X	X	Varied	X	X	?
Bg		X			X	Varied	X	?	?
Bm		X?			X?		X?	X?	?
Bru	X	X		X				X	X
Cyt					X	Varied	X	X	
Ec	X	X	X	X	X	X	X	X	X
Ech	X	X	X	X	X	X	X	X	X
Ew	X	X	X	X	X	X	X	X	X
Hep	Muscle	X			X	Up		X	X
Lei	X	X	X	X	X	X	X	X	X
Lep	Muscle	Tubular	X	X	X	Varied	X	X	
Myc	?	?		X	X		X	?	?
Rr	X	X	X	X	X	X	X	X	Down

*Although not TBDs, Bru, Lei, and Lep are included for comparisons

Table 2 is adapted from Goldstein RE, Brovida C, Fernandez-del Palacio MJ, et al: Consensus recommendations for treatment for dogs with serology positive glomerular disease. J Vet Intern Med 2013;27:S60–S66.[21]

See Abbreviations following Table 2 below.

Abbreviations for tables 1-2

A – Amoxicillin
Ambly – *Amblyomma*
Ap – *Anaplasma phagocytophilum*
Ay – *Anaplasma platys*
Bac - bacterial
Bar – *Bartonella* spp
Bb – *Borrelia burgdorferi* (Lyme disease)
Bc – *Babesia canis, rossi, vogeli,* large B.
Bg – *Babesia gibsoni, conradae,* small B.
Bm – *Babesia microti*-like
Bru – *Brucella canis*
C6** – C6 peptide antigen in SNAP 3Dx, SNAP-4Dx, SNAP-4DxPLUS or Lyme Quant C6 (IDEXX); *or other Lyme antibody tests: AccuPlex4 (ospA, ospC, ospF, p39, SLP), Abaxis (VlsE, flagellin, ospC), Multiplex4 (ospA,ospC,ospF); IFA; WB; see Table 3 for comparisons
Clin – Clindamycin
Cyt – *Cytauxzoon felis*
D – Doxycycline
Derma - *Dermacentor*
E – Erythromycin
Ec – *Ehrlichia canis*
Ech – *Ehrlichia chaffeensis*
Ew – *Ehrlichia ewingii*
F – Fluoroquinolones
Hep – *Hepatazoon americanum*
IFA – indirect fluorescent antibody
Im – Imidocarb
Lei – Leishmaniasis
Lep – Leptospirosis
MAT – Microagglutination test
MD – Minocycline and dihydrostreptomycin
Myc – hemotropic *Mycoplasma* spp
PA – Pentostam (sodium stibogluconate), amphotericin B, allopurinol
PLN – protein-losing nephropathy
Proto - protozoan
Q – Atovaquone
R - Rifampin
Rhipi – *Rhipicephalus*
Ric - rickettsial
Rr – *Rickettsia rickettsii* (Rocky Mountain Spotted Fever, RMSF)
RSAT – Rapid slide agglutination
Rx – Treatment
SPC - Sulfas, pyrimethamine and clindamycin; decoquinate
Spir - spirochete
UPC – Urine protein/creatinine ratio
WB – Western blot
X - Yes
Z – Azithromycin (Zithromax)

Table 3. Lyme antibody tests available

	New	Differentiates Vaccinal vs. Natural Exposure Antibody	Qualitative	Quantitative	Bedside	Differentiates Acute vs. Chronic Infection	Heartworm *Anaplasma Ehrlichia*
Whole cell IFA or ELISA		No		X			
IgM/IgG		No		X		Possibly	
Western Blot		Possibly	X	Semi		Possibly	
SNAP-4DxPlus (IDEXX)	X	Yes, VlsE (C6)	X		X		X
C6Quant	X	Yes, VlsE (C6)		X			
VetScan (Abaxis)	X	VlsE, OspC,* Flagellin	X		X		
AccuPlex4 (Antech)	X	OspA, OspC,* OspF, p39, SLP	X			Possibly	X
Multiplex (Cornell)	X	OspA, OspC,* OspF		X		Possibly	

*OspA and OspC antibodies may be seen at times in naturally exposed and vaccinated dogs.

Antibodies to VlsE (C6) have only been seen due to natural exposure or infection, and have been shown to wane within several months after treatment.

Antibodies to OspA are usually due to vaccination, but can sometimes be seen in non-vaccinates.

Antibodies to OspC are usually due to natural exposure, but they can also be induced by the newer Lyme vaccines. OspC antibodies rise 2-3 weeks after infection and wane naturally (even without treatment) after 3-5 months, unless there is continued exposure.

Antibodies to OspF rise 6-8 weeks after natural exposure, and have not been shown to wane with treatment. See Reference 4 for a further discussion about Lyme Osp antigens.

Antibodies to flagellin may cross-react with other spirochetal/bacterial flagellins.

Table 4. Comparison of some tick control products

*	T, F	Swim	Cats	Prevents Attachment	Age, BW	Pregnancy Lactation	Frequency
Topicals							
Fipronil Frontline	T, F	Yes	Yes	No	≥8 wk	Consult vet	Monthly
Permethrins Activyl T+ Advantix II Parastar+ Vectra 3D	T, F, M	Yes	No	Yes	≥8 wk, 4# ≥7 wk, 4#	Consult vet	Monthly
Revolution	Does not kill *Ixodes*, therefore Revolution is not recommended for tick control						
Collars							
Amitraz Preventic	T only	No	No	Yes	≥12 wk	Consult vet	2-3 months
Permethrins Scalibor	T, F, M	No	No	Yes	≥12 wk	Consult vet	6 months (2-3 wk lag)
Seresto			Yes	≥ 10 wk cats	≥7 wk, 4#		8 months
Chewables							
Isoxazolines NexGard	T, F	Yes	Not yet	No but relatively fast kill	≥8 wk, 4#	Consult vet	1 month
Bravecto					≥6 months	Yes	3 months; but 2 months for *Amblyomma*

BW: body weight; F: fleas; M: mosquitos; T: ticks; wk: weeks; #: pounds

*Products, ingredients, and manufacturers:

Frontline Plus (fipronil, S-methoprene; Merial Limited, Duluth, GA 30096)

Activyl Tick Plus (indoxacarb, permethrin; Merck Animal Health, Intervet Inc, Roseland, NJ 07068)

K9 Advantix II (imidacloprid, permethrin, pyriproxyfen; Bayer Healthcare LLC, Animal Health Division, Shawnee Mission, KS 66201)

Parastar Plus for Dogs (fipronil, cyphenothrin; Novartis Animal Health US, Inc, Greensboro NC 27408)

Vectra 3D (dinotefuran, permethrin, pyriproxyfen; CEVA US, Lenexa, KS 66215)

Revolution (does not kill *Ixodes*; selamectin; Zoetis Inc, Kalamazoo, MI 49007)

Preventic collar (amitraz; Virbac Corporation, Fort Worth, TX 76137)

Scalibor Protector Band (deltamethrin; Merck Animal Health, Intervet Inc, Roseland, NJ 07068)

Seresto (flumethrin, imidoclopramid; Bayer HealthCare LLC, Animal Health Division, Shawnee Mission, KS 66201)

NexGard (afoxolaner; Frontline Vet Labs, Division of Merial Limited, Athens, GA 30601)

Bravecto (fluralaner; Merck Animal Health, Intervet Inc, Summit, NJ 07901)

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Genetic counseling for inherited kidney and urinary tract diseases

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Abstract

During their work with breeders, theriogenologists are often asked their opinion regarding which individual dogs and cats to breed and with which mates, in order to perpetuate characteristics of interest and to avoid predispositions for breed-associated health concerns. Genetic counseling can now be offered with additional support from information gleaned from studies utilizing genomic tools such as genome-wide association studies, fine sequencing of candidate genes, and specific DNA tests available for variant alleles associated with breed predispositions. For instance, for familial kidney diseases, DNA tests are available for some breeds with protein-losing nephropathy, e.g., due to hereditary nephritis with collagen IV abnormalities in the glomerular basement membrane (similar to Alport syndrome) in Samoyeds, Navasota mixbreeds, English Cocker Spaniels, and English Springer Spaniels; glomerulo-sclerosis with slit diaphragm protein abnormalities in Soft Coated Wheaten Terriers and Airedale Terriers. There are also DNA tests for polycystic kidney disease in Bull Terriers and many cat breeds, for hyperuricosuria in many dog breeds, and for some breeds predisposed to cystinuria. Theriogenologists are also in a good position to advise breeders, bank DNA from dogs and cats with well-characterized phenotypes, and work with geneticists and other veterinary specialists to help identify genotypes of breeds at risk for inherited abnormalities, as they arise in breeding programs.

Keywords: Glomerulopathy, nephropathy, polycystic kidney, hyperuricosuria, cystinuria

Introduction

When breeders and veterinarians recognize a familial predisposition for renal or urinary tract disorders, patterns of inheritance and predictive markers are sought, to try to prevent production of at-risk individuals while still maintaining genetic diversity. With genomic tools such as gene sequencing (fine mapping) of candidate genes, some DNA tests are now available for breeds (see Tables), and the molecular basis for defects is realized. When candidate genes are too numerous to study, genome-wide association studies may reveal a statistically significant interval (with fewer candidate genes for study) on one or more chromosomes which are different in affected animals compared with non-affected relatives. It is important to choose proper control animals of the same population, well past the age of onset for the phenotype, and with documentation of normalcy, possibly by blood and urine tests, imaging, and/or renal biopsy results. Mendelian and complex modes of inheritance need to be carefully explained to breeders, and the impact of breeding various genotypes to one another. If a DNA test or other predictive marker is available, and if the variant allele or test result is uncommon in the breed, then animals with the marker can be culled from the breeding program. But if the variant marker is relatively common, care must be used not to remove too many animals from the breeding pool, lest genetic diversity be lost, possibly selecting for more genetic problems in the future. Characteristics which are important to perpetuate, including health, personality, performance, conformation, and breed standards are all important concerns and need to be considered for the good of the individual as well as the breed community as a whole. Veterinarians are needed to help animals, educate owners/breeders, and help investigate genetic predispositions in potential animal models.

Table 1*: Inherited urinary tract abnormalities in dogs and cats

Breed	Phenotype	Site	Mode of Inheritance	Genotype (Test Available)**
Airedale Terrier	Podocytopathy/GS (as SCWT)	G	Complex	NPHS1 c.3067G>A and KIRREL2 c.1877C>G[l,PH]
Akita	Possibly amyloidosis	K		
Alaskan Malamute	JRD	K		
American Foxhound	ICGN (Leishmaniasis)	G		
American Staffordshire Terrier	Cystinuria Hyperuricosuria	T T	AR	SLC2A9 c.616G>T (many labs)
Australian Cattle Dog & Stumpy Tail Cattle Dog	Cystinuria, Type II-A	T	AD (IP)	SLC3A1 c.1095_1100del[n,o,p]
Australian Labradoodle	Cystinuria, Type I-A	T	AR	SLC3A1 c.350delG[n,p,r]
Australian Shepherd	Cobalamin malabsorption and mild proteinuria Cystinuria Hyperuricosuria	GI/K T T	AR AR	AMN c.3G>A[p] SLC2A9 c.616G>T (many labs)
Basenji	GN (with SIIPD) Fanconi syndrome Cystinuria	G T T	AR	FAN1 321bp deletion,[c,o] urine metabolic screening[p]
Bassett Hound	Cystinuria	T		
Beagle	Renal agenesis Amyloidosis Glomerulopathy (HN?) Cobalamin malabsorption and mild proteinuria TCC	K K G GI/K L	AR	CBN c.786delC[j,l]
Bernese Mountain Dog	MPGN	G	AR, possible sex-linked modifier	

Black Russian Terrier	Hyperuricosuria	T	AR	SLC2A9 c.616G>T (many labs)
Border Collie	Cobalamin malabsorption and mild proteinuria	GI/ K	AR	CBN c.8392delC[l,r]
Border Terrier	JRD/Fanconi syndrome Ectopic ureter	K,T L		
Boston Terrier	Urethral prolapse Hypospadias	L L		
Boxer	JRD/Reflux nephropathy	K		
Briard	Ectopic ureter	L		
Brittany Spaniel	MPGN Complement deficiency	G	AR	C3 c.2136delC[o]
Bullmastiff	Glomerulopathy/FSGS	G	AR	
Bull Terrier, English Bull Terrier	GBM defect PCKD	G K	AD AD	PKD1 c.9772G>A[d,o]
Cairn	PCKD (infantile)	K	AR	
Cavalier King Charles Spaniel	Renal agenesis Xanthinuria	K T		
Chihuahua	Cystinuria	T		
Chow Chow	JRD, cystic glomeruli	K		
Coton de Tulear	Hyperoxaluria (infantile)	T	AR	AGXT c.996G>A[o,r]
Dachshund	Cystinuria	T		
Dalmation	GBM defect Hyperuricosuria Hypospadias	G T L	AD AR	SLC2A9 c.616G>T (many labs); Test LUA (low uric acid) dogs; http://luadalmatians-world.com/wordpress/about-lua/
Doberman	Renal agenesis JRD/Glomerulopathy/HN? GN (sulfonamides) Urinary incontinence/ intrapelvic bladder?	K K,G G L		
Dutch Kooiker	JRD	K		

English Bulldog	Renal/ureteral duplication Cystinuria Hyperuricosuria Ectopic ureter, urethrorectal fistula, urethral prolapse, urethral duplication	K T T L	 AR	 SLC3A1 c.574A>G, c.2091A>G also SLC7A9 c.649G>A (linked)[k,n,s] SLC2A9 c.616G>T (many labs)
English Cocker Spaniel	GBM defect	G	AR	COL4A4 c.115A>T[d,f,g,h,j,m,n,u]
English Foxhound	Amyloidosis	K		
English Springer Spaniel	GBM defect	G	AR	COL4A4 c.2712C>T[n,o]
Entlebucher Mountain Dog (Swiss dog)	Ectopic ureter	L	Complex	
Finnish Harrier	JRD (Davidson)	K		
Fox Terrier	Ectopic ureter	L		
French Bulldog	Cystinuria, as English Bulldog Hyperuricosuria	T T	 AR	SLC3A1 c.574A>G, c.2091A>G also SLC7A9 c.649G>A (linked)[k,n,s] SLC2A9 c.616G>T (many labs)
French Mastiff (Bordeaux)	Juvenile glomerulopathy	G	AR	
German Shepherd	ICGN (*Ehrlichia canis*) Cystadenocarcinomas (with nodular dermatofibrosis, uterine leiomyomas) Hyperuricosuria	G K T	 AD (homozygous lethal in embryo) AR	 FLCN c.764A>G[k,o,r,t] SLC2A9 c.616G>T (many labs)
German Spitz	Hyperuricosuria	T	AR	SLC2A9 c.616G>T (many labs)
Giant Schnauzer	Cobalamin malabsorption and mild proteinuria Hyperuricosuria	GI/K T	AR AR	AMN c.1113_1145del[p,r] SLC2A9 c.616G>T (many labs)
Golden	ICGN (Lyme nephritis)	G		

Retriever	JRD Ectopic ureter	K L		
Gordon Setter	JRD/Reflux nephropathy	K		
Greyhound	Vasculopathy (skin, renal)	G		
Griffon	Ectopic ureter	L		
Irish Terrier	Cystinuria, Type III	T	Sex-limited	
Jack and Parson Russell Terrier	Hyperuricosuria	T	AR	SLC2A9 c.616G>T (many labs)
Keeshond	JRD	K		
Labradoodle	Cystinuria, Type I-A	T	AR	SLC3A1 c.350delG[n,p,r]
Labrador Retriever	ICGN (Lyme nephritis) Liver disease/Fanconi Cystinuria, Type I-A Ectopic ureter	G T T L	 AR	 SLC3A1 c.350delG[n,p,r]
Landseer	Cystinuria, Type I-A	T	AR	SLC3A1 c.586C>T (16 labs)
Large Munsterlander	Hyperuricosuria	T	AR	SLC2A9 c.616G>T (many labs)
Lhasa apso	JRD	K		See text
Mastiff	Cystinuria, Type III	T	Sex-limited	Linked test[p]
Miniature Pinscher	Cystinuria, Type II-B	T	AD (homozygous lethal)	SLC7A9 c.964G>A[n,p]
Miniature Poodle Miniature and Toy Poodle	Urethrorectal fistula, urethroperineal fistula, urethral duplication Ectopic ureter	L L		
Miniature Schnauzer	GS or possibly HN JRD Persistent Muellerian Duct Syndrome	G K L	 AR (sex-limited)	 AMHR2 c.238C>T, ie, anti-Muellerian hormone receptor 2, aka MISRII, Muellerian Inhibiting Substance Type II receptor[e,m,o]
Native Amer. Indian Dog	2-8 dihydroxyadenine urolithiasis	L	AR	APRT c.260G>A (no matching laboratories)
Navasota, TX mixbreed	GBM defect	G	X-linked dominant	COL4A5 c.689_699delTAATCCAGGA[o]
Newfound-land	Juvenile collagenofibrotic	G	AR	

	glomerulopathy Cystinuria, Type I-A Ectopic ureter	 T L	AR	SLC3A1 c.586C>T (16 labs)
Norwegian Elkhound	Periglomerular fibrosis Glucosuria	G T		
Pekingese	Renal agenesis	K		
Pembroke Welsh Corgi	Glomerulopathy Renal telangiectasia Cystinuria	G K T		
Rottweiler	Glomerulopathy (HN?)	G		
Samoyed	GBM defect (females are mosaics)	G	X-linked recessive	COL4A5 c.3079G>T[j,o,r]
Scottish Deerhound	Cystinuria, Type III	T	sex-limited	
Scottish Terrier	Cystinuria Glucosuria TCC	T T L	AR	
Shar pei (Chinese)	Amyloidosis	G		HAS2 g.23,746,089_23,762,189dup16.1kb (no matching laboratories)
Shetland Sheepdog	ICGN (Lyme nephritis) Renal agenesis TCC	G K L		
Shih Tzu	JRD	K	AD (IP)	See text
Siberian Husky	Ectopic ureter	L		
Skye terrier	Ectopic ureter	L		
Soft Coated Wheaten Terrier	Podocytopathy/GS ± ICGN, see text JRD	G K	Complex	NPHS1 c.3067G>A, KIRREL2 c.1877C>G[l,PH]
South African Boerboel	Hyperuricosuria	T	AR	SLC2A9 c.616G>T (many labs)
Standard Poodle	JRD	K		
Weimeraner	Hyperuricosuria	T	AR	SLC2A9 c.616G>T (many labs)
West Highland White Terrier	PCKD (infantile) Ectopic ureter TCC	K L L	AR	

Wire Hair Fox Terrier	TCC	L		
CATS				
Abyssinian	Amyloidosis (T > G) Proliferative glomerulopathy	K G	AD (IP) AR	
British Long-hair; British Shorthair	PCKD	K	AD	PKD1 c.10063C>A (many labs)
Burmilla	PCKD	K	AD	PKD1 c.10063C>A (many labs)
Domestic Shorthair	Hyperoxaluria (infantile) Cystinuria	T T	AR	GRHPR Intron 4 acceptor site G>A (no matching laboratories) SLC3A1 c.1342C>T (no matching laboratories)
Exotic Shorthair	PCKD	K	AD	PKD1 c.10063C>A (many labs)
Himalayan	PCKD	K	AD	PKD1 c.10063C>A (many labs)
Maine Coon	PCKD (different)	K		See text
Persian	PCKD	K	AD	PKD1 c.10063C>A (many labs)
Ragdoll	PCKD	K	AD	PKD1 c.10063C>A (many labs)
Scottish Fold	PCKD	K	AD	PKD1 c.10063C>A (many labs)
Selkirk Rex	PCKD	K	AD	PKD1 c.10063C>A (many labs)
Siamese	Amyloidosis (T>G)	K		

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A: autosomal; D: dominant; G: glomerular; GBM: glomerular basement membrane; GI: gastrointestinal; GS: glomerulosclerosis; ICGN: immune-complex glomerulonephritis; IP: incomplete penetrance; JRD: juvenile renal disease; K: kidney; L: lower urinary tract; MP: membranoproliferative; PCKD: polycystic kidney disease; R: recessive; SIIPD: small intestinal immunoproliferative disease; T: tubular; TCC: transitional cell carcinoma

Table 2: Laboratories (See http://www.wsava.org/HereditaryDefects.htm)
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Ultrasound of the reproductive system: female dog

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Preparation and scanning procedure

Ultrasound of the ovaries and the uterus can be very useful in dogs presenting for pregnancy identification, assessment of normal fetal development and viability, vaginal discharge, clinical signs compatible with hormonal imbalances suggesting ovarian dysfunction, and abdominal mass lesions in intact queens and bitches. While the ovaries and uterus should be readily identified, the normal oviducts are usually too small to be seen, and the vulva and vagina are positioned within the pelvic canal, which precluded visualization. Mammary glands are infrequently examined.

The ultrasound examination is typically performed with the animal in dorsal recumbency. A 5-MHz transducer is usually sufficient to visualize an enlarged fluid-filled uterus, fetal structures, or abdominal mass lesions; however, a 7.5- or 10-MHz transducer provides better detail in the examination of smaller structures.

Normal sonographic anatomy

Ovaries

The main landmark for finding both the left and right ovary is the kidney on the respective side. The ovaries are oval structures located caudal, and often lateral, to the caudal poles of the kidneys. Depending on the phase of the cycle, they measure approximately 1-2□cm long in dogs. During anestrus and early proestrus, the ovary is small (approximately 1 cm in length), oval and uniform in echogenicity. During proestrus, the ovary enlarges and becomes more oval. Follicles begin to appear as anechoic oval fluid cavities up to 1 cm in diameter ranging in number from 0-10 per ovary. On the day of ovulation, the follicle number decreases to 0-2 per ovary and the remaining follicles reduce in size. The contour of the ovary may be irregular and a scant amount of fluid may be seen surrounding it. During estrus, the maximum ovarian size is reached equaling a 300-400% increase over size during anestrus. The contour of the ovary remains irregular and fluid filled corpora lutea may be seen. Corpora lutea are 5-9 mm diameter and tend to have thicker walls than preovulatory follicles. During diestrus, the ovarian contour remains irregular but the size reduces. The corpora lutea gradually decrease in size while increasing in echogenicity. Around 10-14 days after ovulation, the corpora lutea appear solid and remain as such for the duration of diestrus. Although ultrasonographic changes during the ovarian cycle have been well studied in dogs, the exact time of ovulation cannot be predicted with ultrasound.[1]

Non-gravid uterus

The normal nongravid uterus is inconspicuous and is often difficult to identify particularly in young dogs. It is best seen dorsal to the urinary bladder, where it appears as a tubular structure between the urinary bladder (ventral) and the descending colon (dorsal). Its size and appearance depend on the size of the animal, previous pregnancies, and stage of the estrus cycle. After identification of the cervix or the uterine body, the uterus is traced cranially to the level of the bifurcation and the uterine horns. An alternative approach is the identification of the uterine horns close to the ovaries; however, their small diameter at this location makes this approach more challenging. Even if the uterine body and the cervix are seen in a nongravid animal, the uterine horns may not be visible because of their small size and surrounding intestinal segments. The lack of identifiable wall layers helps in differentiating uterine horns from intestinal loops.

In spayed dogs, the uterine stump is usually inconspicuous and may be visible as a blind-ended tubular structure between urinary bladder and colon.

Uncomplicated pregnancy

Ultrasonography is a reliable method for diagnosing pregnancy in dogs. Inconsistency exists in the literature regarding the time of the earliest definitive diagnosis, partially because it is difficult to determine the time of conception in dogs.

The most commonly used definition of gestational age is the number of days after luteinizing hormone (LH) peak in dogs. According to these definitions, the length of normal pregnancy is 65 ± 1 day for dogs. A practical problem is that information on hormone assays is often unavailable to animal owners and ultrasonographers. If the time of breeding is known, pregnancy can usually be ruled out 30-33 days after the last breeding based on a negative ultrasonographic examination.

Ultrasonography is useful in monitoring normal embryonic and fetal development.[2] The first reliable ultrasonographic indicator of pregnancy is the detection of gestational chambers, which appear as small, thin-walled anechoic structures within the uterus. Embryos can be discerned at days 23-25. The fetus develops rapidly after day 30, enabling the identification of internal organs. Formulas have been developed and published to determine gestational age and predict time of parturition based on measurements of fetal dimensions.[3] Using these parameters, time of parturition can be predicted with an accuracy of 1–3 days.[4] Ultrasonographic determination of litter size is not reliable.[4]

Postpartum

Ultrasonographic changes during normal involution of the postpartum uterus have been described. Uterine wall thickness and volume of intraluminal fluid decreases, and the uterus becomes less conspicuous over time. Uterine involution usually takes 3-4 weeks in dogs.[5]

Sonographic findings with common reproductive disorders

Because of the high rate of ovariohysterectomy, ovarian diseases are uncommon in dogs in the United States. In many cases, a presumptive diagnosis of an ovarian abnormality is made based on clinical findings, and ultrasound is used to confirm the suspicion rather than serving as the primary means of diagnosis.

Benign lesions such as ovarian cysts appear as anechoic, well-circumscribed, and thin-walled structures with acoustic enhancement. Hormonally inactive cysts arising from the ovarian bursa and hormone-producing follicular and luteinizing cysts cannot be differentiated with ultrasound. Large follicles and corpora lutea may be confused with ovarian cysts and can only be ultrasonographically differentiated by serial examinations. Follicles should not persist longer than 30 days and corpora lutea for no more than 60 days. As a result, the presence of fluid-filled structures associated with the ovary has to be interpreted in light of the clinical presentation when serial examinations are not being performed.

Ovarian tumors appear as nodules or masses of variable size and echogenicity and may have a cystic or mineral component. Possible tumor types include epithelial tumors, sex-cord stromal tumors, and germ-cell tumors and cannot be differentiated ultrasonographically, although teratomas and teratocarcinomas have the tendency to become very large and contain shadowing material (bone or mineral). Concurrent findings may include ascites, pyometra, and cystic endometrial hyperplasia.

Ultrasound can be used to search for ovarian tissue left behind during ovariohysterectomy. Dogs usually present with clinical signs of estrus or stump pyometra. In most circumstances, residual ovarian tissue is positioned in the normal anatomic location for an ovary. However, the entire area from the caudal poles of the kidneys to the level of the bladder should be searched for residual ovarian tissue.[6]

The most common abnormalities of pregnancy in dogs are resorption (embryonic death before 25 days) and abortion (fetal death after 35 days). Embryonic resorption manifests as loss of the normal anechoic gestational chamber, accumulation of echogenic material within the chamber lumen, loss of embryonic heartbeat, embryonic disintegration, and ultimately collapse of the gestational chamber with thickening of the uterine wall.

Signs of fetal death include absence of heartbeat and fetal movement, abnormal fetal posture, reduced volume and increased echogenicity of fluid in the gestational sack, accumulation of gas within fetus or uterus, and fetal disintegration.[7] Failure of implantation of the conceptus, small size or

underdevelopment of the conceptus for true gestational age, and abnormal location of the conceptus within the uterus can usually not be diagnosed. Ultrasonography is of particular value in assessing fetal viability and distress. Normal fetal heart rate has been reported to be twice that of maternal heart rate and is a reliable indicator of fetal viability. Bradycardia is the normal response of a fetus to hypoxia and should be assessed in cases of dystocia.

Although a large number of congenital defects can occur in dogs and cats, these defects are very rarely diagnosed in utero. Examples of fetal abnormalities that can be detected by means of ultrasound include hydrocephalus, fetal pleural effusion, and hydrops fetalis or anasarca.[8] Uterine torsion is a potentially life-threatening condition that is characterized by infarction of the affected uterine segment, with subsequent wall thickening, increased echogenicity of the uterine wall and fetal fluids, and fetal death.

Fluid within the uterus is easily visualized by means of ultrasound. Echogenicity of the luminal contents is variable. Although hydrometra and mucometra are usually characterized by anechoic luminal fluid, and pyometra tends to have echogenic luminal contents, ultrasonographic differentiation of these entities is often not possible. Concurrent uterine wall thickening, endometrial cysts, and polyps are common. Uterine stump pyometra manifests as a fluid-filled, blind-ended pouch between the urinary bladder and descending colon.

Cystic endometrial hyperplasia causes thickening of the endometrium, with cystic lesions embedded in the uterine wall because of proliferation of endometrial glands.[9] The hyperplasia is commonly associated with fluid accumulation within the uterine lumen and may precede the development of mucometra or pyometra or be associated with endometritis.

Tumors of the uterus or uterine stump include polyps, leiomyomas, leiomyosarcomas, or adenocarcinomas. They appear as nodules or masses of variable shape, size, and echogenicity and may be associated with fluid accumulation within the uterine lumen. Vaginal masses can be visualized when they become large enough to extend from the pelvic canal into the abdomen. Uterine stump granulomas manifest as mass lesions of variable echogenicity between the bladder and colon. Hematoma formation with or without abscessation can occur at the uterine stump following ovariohysterectomy and appear mass-like when ligatures loosen. Differentiation of neoplastic from non-neoplastic uterine or vaginal mass lesions and ultrasonographic distinction among different tumor types is not possible.

Percutaneous procedures

Depending on size and location, fine-needle aspiration or biopsy of mass lesions associated with the ovary or the uterus can be performed under ultrasound guidance following the same principles and precautions as in other organ systems. Because of the risk of leakage into the peritoneal cavity, uterine fluid is usually not aspirated. Amniocentesis is not a routine procedure in the assessment of pregnant dogs or cats.

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Ultrasound of the reproductive system: male dog

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Preparation and scanning procedure

Ultrasonographic examination of male reproductive organs is commonly performed for andrologic evaluation of breeding dogs, localization of retained testicles, difficulties or abnormalities in urination or defecation, abdominal, scrotal, and penile pain or discomfort, caudal abdominal mass lesions, perineal hernia, clinical signs compatible with hormonal imbalances (hyperestrogenism), scrotal or penile trauma, and palpable scrotal abnormalities.

The dog is usually positioned in dorsal recumbency. A 5.0-MHz curvilinear transducer is appropriate in larger dogs but a 7.5- or 10-MHz transducer provides better detail and is recommended for most examinations. The prostate can be readily seen from a transabdominal approach and is located by following the bladder caudally to its terminus. Examination is performed in transverse and sagittal planes. In neutered dogs with an empty or intrapelvic bladder, ultrasonographic assessment of the prostate may identify only a small hypoechoic widening of the urethra.

The testicles should be examined with a high-frequency transducer (at least 7.5□MHz). A linear transducer with broad contact area and good resolution in the near field is preferable. Clipping of the scrotum is usually unnecessary.

The penis is occasionally examined to identify urethral abnormalities or to assess integrity of the os penis. Dependent on the examiner's preference, a linear or curvilinear high-frequency transducer (7.5□MHz or higher) may be used. The examination is started at the level of the os penis and is continued proximally to the level of the ischium. It is important to recognize that the intrapelvic portion of the urethera cannot be visualized with ultrasound.

Normal sonographic anatomy

Prostate

The location, size, and echogenicity of the prostate varies with age, previous disease, and neutering status.[1] In intact dogs, the prostate is of medium echogenicity and homogeneous, with a fine to medium coarse echotexture and smooth margins.[2] In the sagittal imaging plane, the prostate is rounded to ovoid. In transverse, the two prostatic lobes can be seen distinctly and should appear symmetrical. The vertical raphe and prostatic urethra with surrounding urethralis muscle are generally visible as a hypoechoic area between both lobes. The urethral structures may be associated with edge shadowing on transverse images, which should not be misinterpreted as a lesion. Normal aging changes the appearance of the prostate resulting in increased size and mottled echogenicity. Prostatic cysts are a common incidental finding in older dogs.[2] Prostatic size in intact dogs is significantly correlated with age and body weight.[3] In neutered dogs, the prostate is small, inconspicuous, hypoechoic, and homogeneous. The two lobes cannot be distinguished.

Testicles

Normal testicles are of medium echogenicity and have a fine, homogeneous echotexture.[4] In the sagittal imaging plane, a central hyperechoic line is visible that represents the mediastinum testis. In transverse, the mediastinum testis appears as a centrally located hyperechoic dot.

The head and tail of the epididymis are located at the cranial and caudal poles of the testicle, respectively, and the body is found dorsal to the testicle. In comparison with testicular parenchyma, the epidiymis is hypoechoic and has coarse echotexture. The spermatic cord can be followed from the head of the epididymis to the inguinal ring and is characterized by the large tortuous anechoic venous structures of the pampiniform plexus.

Penis

At the level of the distal penis, the smooth, hyperechoic interface of the os penis is surrounded by penile soft tissues (glans penis) and the prepuce. The urethra is located within a V-shaped ventral groove in the os penis and is usually not visible unless distended. Proximal to the osseous part, penile soft tissues (corpus cavernosum, corpus spongiosum, and muscles of the penis) are of medium echogenicity and inconspicuous.

Sonographic findings with common reproductive disorders

Benign prostatic hyperplasia (BPH) is a common condition in older dogs and is often an incidental finding. The prostate is enlarged, of normal to increased echogenicity, and of homogeneous or inhomogeneous echotexture with multiple variably sized cysts frequently being present. Enlargement is typically symmetrical and surrounding structures are unaffected.

Benign prostatic hyperplasia is often present in conjunction with acute or chronic prostatic infection.[5] The prostate may be of normal size or enlarged. Echogenicity and echotexture of the prostatic parenchyma are variable, ranging from normal to heterogeneous. Although changes in echogenicity and echotexture tend to be more striking than those seen with BPH, ultrasonographic differentiation of these conditions is often not possible, and prostatitis in many cases complicates preexisting BPH. Assessment of the surrounding structures is essential as prostatitis frequently causes regional peritonitis, which manifests as hyperechoic, hazy peri-prostatic fat and pocketing of fluid. Prostatic abscesses arise in some dogs with prostatitis and appear as fluid filled cavities within the parenchyma of the gland. Abscessess usually have a thick wall around the abscess cavity, contain echogenic fluid, occasionally contain gas shadows in the case of infection with gas-producing bacteria, and may develop septation. Fungal prostatitis is rare but when present causes variable ultrasonographic changes and may mimic prostatic neoplasia.

Paraprostatic cysts are fluid-filled remnants of the Müllerian duct system that occur predominantly in older large-breed dogs.[6] Unlike intraprostatic cysts, paraprostatic cysts are located in the vicinity of the prostate, but reside predominantly in the abdominal cavity. They may communicate with intraprostatic cavitations and can usually be traced to their origin at the prostate. The cyst wall is of variable thickness. Paraprostatic cysts contain anechoic to echogenic fluid, can become very large, may contain internal septa, and may be peripherally mineralized. Paraprostatic cysts must be differentiated from the urinary bladder.

Ultrasonographic findings with prostatic neoplasia are variable and include asymmetric prostatomegaly, mottled and heterogenous parenchyma, large cystic cavities and regions of mineralization. Extension of tumors in to the surrounding tissue or urethra may occur. Both intact and neutered dogs may be affected. Assessment of regional structures such as sublumbar lymph nodes and caudal lumbar vertebral bodies is suggested as local metasteses are common with prostatic tumors.

Ultrasound is very useful for locating cryptorchid testicles which can be found anywhere between the caudal pole of the kidneys and the inguinal area. If the mediastinum testis is not developed, identification of an undescended testicle may be difficult. However, in most cases, the cryptorchid testicle appears as a smaller version of a scrotal testicle and can be readily identified. It is important to remember that abdominally and inguinally located testicles are predisposed to neoplastic transformation and can reach considerable size in this instance.

Benign testicular tumors are common and frequently incidental findings (Leydig and interstitial cell tumors). Seminomas and Sertoli cell tumors are more aggressive and can affect cryptorchid and descended testicles. These tumors have the potential for hormone production and metastases. Ultrasonographic features of testicular tumors range from circumscribed small nodules to large complex masses with disruption of normal testicular anatomy. Different tumor types cannot be distinguished

ultrasonographically.[7] Concurrent prostatic changes such as BPH or squamous metaplasia are common, especially in hormone-producing tumors. In case of metastatic neoplasia, enlarged medial iliac lymph nodes may be seen.

Orchitis and epididymitis may occur subsequent to hematogenous spread of infectious organisms, may result from urinary tract or prostatic inflammation, or may be caused by scrotal trauma. Inflammatory scrotal disorders exhibit variable ultrasonographic characteristics, ranging from diffuse echogenicity changes of the testicle and/or epididymis to complex masses and anechoic areas subsequent to abscess formation.[7,8] Fluid may accumulate within the scrotum or the scrotum may thicken. Testicular and epididymal size often increase during acute inflammation and decrease in chronic cases.

Testicular torsion most commonly affects retained neoplastic testicles. In this instance, the ultrasonographic examination shows an abdominal mass of variable size and echogenicity, with decreased or absent blood flow on color Doppler examination.[9] Intra-abdominal and intrascrotal torsion of non-neoplastic testicles and vascular compromise of other etiology (infarction or space-occupying lesions within the inguinal ring) are rare. Depending on the degree and duration of vascular occlusion, the affected testicle may appear hyperechoic or hypoechoic, increased, normal or decreased in size, with initially normal architecture. Concurrent abdominal or scrotal effusion is common, especially in acute cases.

Scrotal trauma may result in hematoma, hematocele, contusion, intratesticular hematoma, and testicular rupture. Scrotal hematomas with accumulation of blood within scrotal soft tissues manifest as space-occupying lesions of variable echogenicity that displace the testicle and epididymis. With hematocele formation, there is intrascrotal fluid accumulation of variable echogenicity. Testicular contusions and hematomas appear as diffuse echogenic changes to the testicular parenchyma or mass lesions of variable echogenicity. Differentiation from testicular lesions of inflammatory or neoplastic etiology is mainly based on medical history rather than ultrasonographic characteristics. Inhomogeneous echotexture of the testicular parenchyma with loss of contour definition indicates testicular rupture.

Hydrocele manifests as anechoic to echogenic fluid adjacent to the testicles and within the scrotal sac. This may be an incidental finding if fluid quantity is small, but is more commonly found secondary to scrotal disorders or when abdominal fluid descends through the inguinal ring.

With inguinal or scrotal hernia, abnormal contents (e.g., bowel loops or mesenteric fat) may be found within the inguinal ring or scrotum. Concurrent findings include hydrocele, testicular congestion or infarction.

Common abnormalities of the penis that warrant ultrasonographic examination include urethral calculi, fracture or neoplasia of the os penis, or urethral lesions such as tumor or stricture. Lesions of the os penis cause discontinuity of its hyperchoic, smooth contour.

Percutaneous procedures

Percutaneous fine-needle aspiration or biopsy of the prostate is easily performed with ultrasound guidance. The same principles and precautions used in other interventional procedures apply. In cases of suspected prostatic neoplasia, sampling by means of traumatic catheterization or prostatic massage should be considered due to potential risk of implantation of tumor cells along the needle tract after percutaneous aspiration.[10]

Percutaneous drainage of prostatic abscesses and in situ injection of antibiotics is a valid alternative method to surgical intervention, especially in immunocompromised patients.[11] Percutaneous drainage of paraprostatic cysts can be performed to temporarily relieve patient discomfort. However, recurrent filling usually warrants surgery at a later stage.

Fine-needle aspiration or biopsy of intra-abdominal testicular tumors is commonly performed, following the same principles and precautions as in biopsies of other abdominal organs. Fine-needle

aspiration of intrascrotal testicles is infrequently performed in veterinary medicine. However, the procedure has a high accuracy in the diagnosis of testicular neoplasms, with a low risk of adverse effects.

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Comparative progesterone assay

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Abstract

Analysis of systemic progesterone concentrations is routinely used for ovulation timing in bitches. Ability to accurately determine when ovulation has occurred can improve pregnancy rates, litter size, and reduce the need for multiple inseminations during an estrous cycle; it is of utmost importance when utilizing cooled shipped or frozen semen to correctly identify the appropriate time for insemination. It has anecdotally been recommended that progesterone analysis be performed at the same laboratory for a given estrous cycle to reduce confounding factors related to differences between sample handling and different analysis techniques. However, it is sometimes necessary to send samples to different laboratories during a given estrous cycle which can lead to difficulties in interpretation and determination of the fertile window.

There are many options available for quantitative progesterone assay available to the practitioner today. Radioimmunoassay (RIA) is considered the gold standard for endocrinology testing, but is only available at reference laboratories and may have delayed turnaround times as a result. Point-of-care assay is becoming increasingly common in private practice settings, which utilizes methods such as chemiluminescence (CLIA), enzyme-linked fluorescent assay (ELFA), or fluorescent enzyme immunoassay (FEIA). It is important to determine the level of agreement between different point-of-care analyzers as well as to determine whether point-of-care analyzers are consistent with gold-standard testing techniques such as RIA. These differences could impact the accuracy of ovulation timing in bitches with a subsequent negative impact on pregnancy rate and litter size.

Keywords: Progesterone, radioimmunoassay, chemiluminescence, enzyme-linked fluorescent assay, fluorescent enzyme immunoassay, ovulation timing

Introduction

A variety of diagnostic assessments are utilized for ovulation timing in bitches, including vaginal cytology, vaginoscopy, behavior, serum luteinizing hormone (LH) measurements, and serum progesterone measurements. Great variability has been noted regarding estrus behavior and ovulation, so progesterone assay has been utilized to further define and identify the fertile window.[1] The most common cause of failure to conceive is poor ovulation timing and breeding management, which makes accurate progesterone assay of vital importance to bitch owners and veterinarians alike.[2-4] The bitch is unique among the domestic species in that preluteinization of follicles results in a preovulatory rise in progesterone above baseline, allowing both estimation of the LH surge and ovulation indirectly. Conveniently, progesterone is not a species-specific molecule which allows human progesterone assays to be utilized effectively in a variety of species.[5] Several immunoassay methods have been evaluated in a variety of species, including dissociation enhanced lanthanide fluorescence immunoassay (DELFIA), multianylate immunoassay, luminescence immunoassay, and enzyme-linked immunosorbent assay (ELISA).[5-9] However, there is very little research examining the repeatability of these methods or directly comparing the agreement of these methods against each other.

With the increased use of artificial insemination, determination of ovulation and the optimal time of breeding have become even more important. Likewise, some veterinarians are utilizing point-of-care machines, with quantitative progesterone results available the same day. Semi-quantitative ELISA test kits are available for patient-side use such as Target Canine Ovulation Test (BioMetallics; Princeton, NJ) and Ovucheck® Premate 10 (Zoetis; Florham Park, NJ). These tests are not considered highly accurate, even when correlating results with other parameters.[11] Some veterinarians consider these semi-quantitative test kits to be most useful in confirming progesterone values that are very high (>10 ng/ml) or

very low (<2 ng/ml), but they are not accurate enough for determination of quantitative values for the purposes of breeding management, and thus these types of tests are excluded from this discussion. Rather, the purpose of this review is to compare different types of quantitative progesterone assays that are commonly used for ovulation timing in the bitch (RIA, CLIA, and ELFA/FEIA).

Radioimmunoassay has long been considered the gold standard for assessment of hormones and endocrine compounds as it is considered to be both highly accurate and repeatable. However, RIA is only available at select reference laboratories due to equipment cost, maintenance and handling of radioactive materials; this often results in 24 hours or longer prior to reporting of results. Recent changes in availability of reagents utilized for RIA has led to a decrease in the number of reference laboratories offering progesterone assay via this method. At the time of publication, the only reference laboratory in the United States utilizing RIA that was identified by the authors was Colorado State University Reproductive Endocrinology Laboratory. The reference laboratories that the authors previously utilized for RIA have since transitioned to CLIA as a result of reagent unavailability.

Chemiluminescence (CLIA) has become an increasingly popular method of progesterone analysis due to rapid turnaround time and decreased costs compared to RIA. Some practitioners report anecdotally that this assay type is less reliable than other methods, but the literature reports that chemiluminescence is equivalent to, or even more accurate than RIA.[10] For perspective, one commonly used CLIA machine is the Immulite® system (Siemens Medical Solutions USA, Inc, Malvern, PA) now used by at least two universities that have recently switched from RIA to CLIA.. Even more recently, ELFA/FEIA machines have become available and affordable for individual practitioners to maintain within the hospital setting to allow rapid, cost-effective, quantitative progesterone assays that have been specifically validated for use in dogs.[12] Some common models for ELFA/FEIA testing include mini VIDAS® (Biomerieux, France) and TOSOH™ (Tosoh USA, Inc, Grove City, OH). To date, no research has directly compared multiple methods to each other, nor compared the effects of ovulation timing when multiple laboratories must be used for analysis. When new diagnostic methods are validated, they are typically assessed by testing known concentrations of progesterone created from stock solutions rather than comparing to another assay type. Determining agreement between the most commonly utilized methods (RIA, CLIA, and ELFA/FEIA) could confirm commonly accepted anecdotal paradigms regarding the necessity of ideally utilizing a single laboratory for progesterone analysis, and may provide insight into means to adapt data when multiple laboratories or analysis techniques are used.

There are several machines available for in-clinic progesterone assay, including the mini VIDAS® and TOSOH™ models. These utilize enzyme-linked fluorescent assay and fluorescent enzyme immunoassay, respectively. Dependent on the particular machine, a conversion chart may be utilized to convert raw data. Initial set up costs and individual test costs will vary with the particular brand and model purchased, but are estimated to range between $8-15 per test (clinic cost). Cost of testing at reference laboratories (RIA or CLIA) can vary considerably, ranging from $20 per test to considerably more. Practice owners will have to balance the cost of purchase/rental and maintenance of equipment relative to the number of blood samples assayed on a regular basis.

Sample handling

Good laboratory technique suggests that consistent handling of blood samples should result in greater consistency in reported results. Several studies have reported alterations in reported results when serum separator tubes are used.[13-16] therefore plain glass (red top) tubes are recommended. Progesterone appears to remain relatively stable in canine blood, with no significant differences noted from freeze-thaw cycles or prolonged storage up to 14 days at room temperature.[17] Significant differences were also not noted between silicone, lithium heparin, and EDTA blood tubes.[17]

Determining agreement between samples

Comparison of different laboratory techniques can be achieved by measuring accuracy, precision, repeatability, and/or the level of agreement between sample values. *Accuracy* is defined as the degree of conformity of a measure to a standard or "true" value. *Precision* describes the closeness of two or more

measurements to each other, which is its repeatability. Therefore, it is possible for any type of measurement to be very precise (repeatable), yet inaccurate. Another question that must be answered is how to best determine the level of agreement between samples. Even if samples between different laboratories had poor agreement, if the bias between analytical technique is consistent this can be accounted for, and values adjusted accordingly. Bland-Altman methods[18] of statistical analysis can be utilized to account for both of these issues. Other statistical tests, such as analyzing the coefficient of variance, may give a false sense of security as the values between different laboratories are likely to be closely associated. The agreement between samples can be summarized by calculating the bias as a function of progesterone concentration; if the bias is consistent, this would allow adjusted values to be calculated between those two methods. However, differences in values (and potentially standard deviation in those differences) will likely vary depending on the range that the actual progesterone measurement falls within. As the progesterone level rises, the limits of agreement for clinical acceptability increase as well, which is another factor to consider when determining if two methods are interchangeable.

While a proper comparison of these testing methods can seem confusing and academic in nature, such comparisons are necessary in order for veterinarians to make sound clinical decisions regarding ovulation timing and breeding management. At the time of publication, a collaborative study involving all authors is underway to determine these comparisons using different methodologies and different laboratories. Formal results are not yet available but will be presented at the August meeting in San Antonio, and will also be available for reference in a separate publication at that time.

Acknowledgement

The authors thank the Theriogenology Foundation for generous financial support for this project.

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A review and update of research on pregnancy associated glycoproteins (PAGs) in cattle

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Key points

- PAGs are a large gene family found in all ruminants.
- The function that PAGs play during gestation remains a mystery. In this review speculation about putative roles for the PAGs will be highlighted.
- The PAGs display a wide range of expression patterns and localization. These proteins may be playing different roles depending on the exact location or time of pregnancy that they arise.
- Understanding the roles that PAGs play in ruminant pregnancy may lead to a better understanding of pregnancy success and failure.

Introduction

The placenta is a multifaceted organ that has a critical role in maintaining and protecting the developing fetus by transferring nutrients and metabolic wastes, acting as a regulator of the maternal immune system, and serving as a major endocrine organ.[1] In the ruminant placenta there is a unique cell type (binucleate cells) that constitutes 15 - 20% of the fetal placental trophoblasts. These cells become visible around d 19-20 of gestation in cattle and have been shown to secret a plethora of hormones and proteins, including placental lactogen (PL) and pregnancy associated glycoproteins (PAGs). Although much of the focus on PAGs has been directed toward PAGs expressed in bincucleate cells, there are PAGs that have been shown to be expressed by mononucleated cells as well. This section will focus on the characterization and functions of PAGs in cattle and attempt to link data presented in the review of "Application of PAGs to manage reproductive efficiency in cattle."

Characterization of pregnancy associated glycoproteins (PAGs)

PAGs were first reported by Butler[2] after isolation of two proteins from fetal membrane extracts, referred to at the time as pregnancy specific proteins A and B (PSPA/PSPB). In the same study it was determined that PSPA was alpha-fetoprotein and that PSPB was specific to the placenta. Sasser[3] developed a PSPB specific radioimmunoassay which they used to measure PSPB in the maternal circulation. In subsequent reports, they demonstrated that measurement of PSPB in females could be used to successfully detect pregnancy in dairy cattle,[4] sheep,[5] and goats.[6] Around the same time, Zoli[7] reported the purification of another pregnancy specific protein that they called 'bovine pregnancy associated glycoprotein' (PAG). Bovine PAG and PSPB had very similar amino acid sequences at the amino-terminus[8] suggesting that these two proteins were similar, if not identical.

Initial immunolocalization studies determined that PAG was synthesized by the trophoblast binucleate cells and stored in large secretory granules prior to delivery into the maternal circulation.[9] The exact function of PAG remained unclear; however, Xie[10] reported that 60% of the nucleotide sequence of PAG was shared with pepsinogens. Furthermore, it was shown that mutations in and around the active site rendered PAG inactive as a proteinase.[10,11]

As time progressed, new members of the PAG family were discovered in cattle and many species within the Ruminantia suborder. Consequently, the original PAG was renamed PAG1 and PAGs discovered afterwards were numbered sequentially. It is now known that PAGs comprise a large diverse gene family belonging to the aspartic proteinase superfamily.[12-14] In cattle alone, there are >20 distinct PAG cDNAs represented in Genbank.[15]

Hughes[16] reported that PAGs can be divided into two distinct groups: 1) *ancient* PAGs which are estimated to have originated about 83 million years ago (around the time the Artiodactyla order is thought to have arisen), and 2) *modern* PAGs which are estimated to have originated approximately 54 million years ago. These two distinct groups of PAGs have been studied extensively and characterized based on

their mRNA expression. Ancient PAG mRNAs are usually expressed throughout gestation in both mononucleate and binucleate trophoblast cells of the cotyledons; whereas, modern PAGs are synthesized primarily in binucleate cells of the trophoblast and their expression seems to change during gestation.[13]

Ancient PAGs

The ancient lineage of PAGs seems to have arisen from duplication of a single pepsinogen F-like gene around 85 million years ago.[16] Kumar[17] also reported the divergence of the even-toed ungulates (Artiodactyla) and odd toed ungulates (Perissodactyla) around this same time period suggesting that these two events may be closely related. In cattle, the ancient PAGs are comprised of a relatively small group of about six genes.[13] These six bovine (b) PAGs are expressed in cotyledons from early placentation to term, in both uninucleate and binucleate trophoblast cells. Following secretion, some of the ancient PAGs accumulate at the microvillar junction between the maternal and fetal interface.[18] Their function is still unknown, however, based on their localization. Wooding[18] suggested that ancient PAGs may be important for the following: 1) adhesion of the uterus and trophoblast cells to maintain appropriate transport, 2) proteolytic processing, 3) activation of growth factors or bioactive molecules, or 4) protection of trophoblast cells from the maternal immune system. There is also speculation that the ancient PAGs and modern PAGs may work together throughout gestation. Collectively, the ancient PAGs were thought to be peptidases, although there had been no solid evidence until Telugu[19] reported that bPAG2, which is the most abundant transcript reported in the PAG family possessed proteolytic activity. A similar PAG, bPAG12, has also been shown to be proteolytically active. The preceding authors concluded that ancient PAGs exhibiting proteolytic activity may function as sheddases to activate latent biomolecules, which could be important for placental development and growth.

Modern PAGs

The burst in gene duplication that led to the lineage of modern PAGs has been linked to the emergence of the synepitheliochorial placenta of the ruminant ungulates.[16,18] The modern family of ruminant PAGs includes a larger number of genes than their ancient counterparts.[13] Wooding[(18)] suggested that the modern PAG family expansion could potentially have evolved to deliver a variety of fetal products and hormones to the mother by bypassing the uterine epithelial barrier. These PAGs are restricted to ruminant species and are expressed primarily in trophoblast binucleate cells from which they are released into the maternal system, with some accumulating in the stromal layer within the maternal caruncles.[18] The authors concluded that the preceding localization pattern could potentially place PAGs in a position to engage in immunological protection, such as blocking lymphocyte or polymorphonuclear leukocyte migration and activation. To date there have been no clear functions related to modern PAGs; however, PAGs have been shown to inhibit different immune cells, in vitro, and may camouflage fetal/placental antigens from the immune system.[20] Alternatively, PAGs have been suggested to have a luteotrophic action based on a report that addition of PSPB/PAG1 to endometrial cells increased the production of the luteal-promoting prostaglandin, prostaglandin E_2 (PGE_2);[21,22)] however, the evidence for a luteotrophic or antiluteolytic action of PAGs is not compelling at this time.

Possible potential function(s) of the PAG

To date the function of PAGs is not clear. However, their expression patterns by the placenta of cattle and related species as well as the proteolytic activity of some PAGs could provide some insight into their roles during pregnancy. For instance; many PAGs such as bPAG-2 and porcine PAG-2 (belong to the ancient PAG group) are found to accumulate at the placental feto-maternal interface. Bovine PAG-2 and procine PAG-2 are known to have a proteolytic activity,[19,23)] which is suggestive of possible roles involving protein turnover or remodeling at the trophoblast-uterine epithelial interface[18] or they could be acting to proteolytically activate bioactive molecules and latent growth factors located at the interface.[24,25)]

There are some PAGs that lack the ability to act as proteinases; these may have another role, such as peptide binding at the uterine- fetal interface. Interestingly, PAGs are able to interact with peptide

ligands via their substrate- binding cleft.[26] Conceivably, binding to other proteins through the substrate-binding cleft could position PAGs to interact with other proteins at the maternal-fetal interface or with transmembrane receptors (e.g. integrins). Furthermore, the carbohydrates displayed on the surface of PAGs could bind to a lectin (carbohydrate-binding protein) at the maternal-fetal interface to sequester them to that location. Pregnancy associated glycoproteins with enzymatic activity typically exhibit proteolysis of substrates at comparatively low pH.[19,23)] The positioning of proteinases at the interface could facilitate release of the cotyledon from the caruncular crypts around parturition when the pH of the interface microenvironment falls and proteolytic activity of these PAGs would be expected to increase.[18]

Pregnancy associated glycoproteins may have an effect on the maternal immunological system in cattle. For example, bPAG-1/PSPB treatment of bovine bone marrow has been shown to cause a drop in bovine hematopoietic cells proliferation.[20] In other experiments, bPAG-1/PSPB treatment of bovine endometrial (BEND) cells induced release of granulocyte chemotactic protein 2 (GCP2).[27] Pregnancy associated glycoproteins have also been shown to associate with the peripheral blood lymphocytes and with endometrial serpin-like proteins in vitro.[28,29]

In cattle and sheep PAG1/PSPB could have an effect on luteolytic activity. For instance, bovine luteal cells progesterone and prostaglandin E2 (PGE_2) production increased in response to PSPB treatment.[30-32] This observation of an increase in bovine luteal cells progesterone production might be due to a luteotrophic effect of PGE_2 but it was not observed consistently.[30-32] It seems to be that PAGs can play multiple roles during pregnancy in regard to placental development and function.[33]

In addition, PAGs may be playing a totally different role than any of the data above suggest. Recent data generated out of Dr. Jon Green's lab at the University of Missouri is potentially pointing to a role of PAGs and uterine remodeling around the time of early placental attachment. These preliminary data found that the addition of purified bovine PAGs to endometrial explants, collected on day 18 from pregnant and nonpregnant heifers increased the expression of members of the matrix metalloproteinase gene family which have a role in tissue remodeling. Furthermore, we have demonstrated that cows likely to undergo late embryonic mortality (between day 28-60 of gestation) have decreased circulating concentrations of PAGs on day 28 compared to cows that will maintain a pregnancy.[34] These results support the idea that cattle pregnant on day 28 of gestation, with decreased circulating concentrations of PAGs, may be undergoing pregnancy loss based on the failure of placental and endometrial crosstalk along with a failure of tissue remodeling that is critical for early placentome formation.

Summary

The bovine PAG family is a large group of related proteins that are encoded by more than 20 genes. The function that PAGs play during gestation have not been elucidated, but preliminary data point to having a role in manipulation of the maternal immunological system, regulation of luteolytic activity, and(or) tissue remodeling. As our understanding of mammalian genomes increases and the advancement of biological tools increase it is likely that the exact function that PAGs play during pregnancy will be discovered.

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Application of pregnancy associated glycoproteins (PAGs) to improve reproductive efficiency in cattle

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Key points

- Pregnancy diagnosis from a blood sample enables the detection of nonpregnant cows earlier than transrectal palpation after insemination
- Pregnancy diagnosis using PAGs is an efficient method that is based on detecting the presence of a pregnancy-specific protein
- PAG testing is commercially available in both blood and milk
- PAG testing may also provide a useful tool for detection of pregnancies that have a high probability of undergoing late embryonic morality

Introduction

Increasing profitability of a beef or dairy herd is dependent upon increasing reproductive efficiency. In the U.S., the annual cost of reproductive failure to the beef and dairy industries is estimated to be $600 million and 1.4 billion, respectively, which is most likely a gross underestimate of the actual cost. The exact causes of reproductive failure include management issues, cow infertility, bull infertility, heat stress, and embryonic mortality. In order to increase reproductive efficiency there has been a rapid development and utilization of reproductive technologies (e.g. fixed–time artificial insemination [FTAI], estrus synchronization [ES], real-time ultrasonography, and chemical based pregnancy testing [PAGs]) that can improve both the reproductive management and genetic merit of a cattle herd. In fact, Seidel[1] stated: "ES and AI are among the most powerful and applicable technologies for genetic improvement of a beef herd." However, the primary challenge to adopting these technologies has been the commitment of time and labor associated with their implementation. Traditional methods of ES and AI require monitoring estrous behavior at least twice daily during the synchronization process. This traditional requirement of estrous detection is not only tedious and time consuming, but it also eliminates the insemination of animals that are either anestrus or not detected in estrus. Consequently, ES protocols that specifically synchronize the timing of ovulation in relation to the time of semen deposition have eliminated the need for estrous detection while achieving pregnancy rates similar to estrous detection-based ES protocols. Therefore, the adoption of FTAI by producers has increased. Based on the rate of FTAI adoption, it has been clear that these technologies are practically useful in increasing reproductive efficiency in cattle.[2] The bottom line is that these protocols work effectively at synchronizing ovulation for appointment breeding with acceptable pregnancy rates. The question is: "How can we as producers further increase pregnancy rates to a single insemination or increase reproductive efficiency?" The answer to that question is not so easily resolved and it has prompted further investigations of how to increase dominant follicle maturity, increase oocyte competence, improve the uterine environment and promote placental health, to name a few. Our group has chosen to explore the use of PAGs (chemical based pregnancy testing) to increase reproductive efficiency in cattle.

Maternal circulating of PAGs as tools for reproductive management in cattle

PAGs and pregnancy establishment

Members of the modern PAG family are detectable in the maternal circulation by multiple tests (e.g. RIA and ELISA) starting soon after the time of binucleate cell formation (day 19-20 of gestation)[3] until a few weeks after parturition.[4,5] Circulating concentrations of bovine PAGs can be influenced by a number of factors including breed, weight, parity status of the dam, fetal sex, fetal number, and fetal birth

weight, along with pregnancy stage and status.[6,7] However, the role that PAGs play during gestation remains undefined.

A majority of the work on PAGs has focused on the development of a reliable tool for diagnosing pregnancy in multiple ruminant species including cattle, sheep, goats, buffalo, bison, moose, and elk.[8] Pregnancy associated glycoproteins are unique compared to other biochemical methods of pregnancy detection in cattle because these proteins are pregnancy specific. Pregnancy associated glycoprotein 1 (PAG1; also known as pregnancy specific protein B; PSPB) has been the primary PAG of most interest in relation to early pregnancy diagnosis because of the ability to detect PAG1 in the maternal circulation throughout gestation.[4,9] However, Green[10] highlighted two disadvantages in using PAG1 for pregnancy detection: 1) pregnancy diagnoses in the first month of pregnancy could be compromised due to the low and variable circulating concentrations of PAG1, and 2) the long half-life of these proteins (~8 days) in the maternal circulation after partition or fetal loss. Due to these concerns, there has been interest in detecting other PAGs for pregnancy detection. Green[5] reported the establishment of an ELISA based test for early pregnancy PAGs with a relatively short half-life (4.3 day). It has also been shown that PAG concentrations first significantly increase in circulating around day 24 of gestation followed by a transient rise out to partition in cattle (Figure 1A)[5,11] which is similar to that of other small ruminants (Figure 1B). In the preceding study, PAGs were detected in all cattle by d 28 of gestation, PAG concentrations peaked around the time of parturition. After parturition PAGs were undetectable by eight weeks postpartum in 38 of the 40 cows, thus concluding that choosing different PAGs helps overcome the persistence of PAG immunoreactivity far into the postpartum period. In similar studies, after induced embryonic morality, the half- life of circulating concentrations of PAGs was determined to be 35.8 ±21.9 h (mean±SD; Figure 2).[11] These differences in PAGs half-life are presumably a result of distinct forms of the PAG family present earlier in gestation compared to term or a result of different clearance mechanisms between early and late pregnancy.

There are currently three commercial PAG testing platforms available for use, 1) BioPRYN (BioTracking, LLC, Moscow, ID), 2) DG29 (Conception Animal Reproduction Technologies, Beaumount, QC), and IDEXX Bovine Pregnancy Test (IDEXX Laboratories, Inc. Westbrook, ME). Current PAG assays have been documented to accurately diagnosis pregnancy in cattle with an average accuracy ranging from 93 to 96% in both blood and milk.[5,11-17]

PAGs as a predictor of late embryonic mortality and as a biochemical marker for placental function

In cattle, the incidence of late embryonic/early fetal loss around the time of embryo uterine attachment is approximately 4 to 10%.[11,17-21] The mechanisms associated with reproductive loss around the time of placentation are unknown, but may be associated with inadequate placental development or function. Along with the ability to use PAG assays as tools for pregnancy-detection, PAGs may also serve as a marker for monitoring embryonic/fetal viability along with placental function. For example, beef cows that successfully carried a pregnancy past day 72 of gestation had higher circulating concentrations of PAGs on day 28 compared to cows that exhibited late embryonic/fetal mortality between day 28 to 72 (using a sandwich ELISA).[11,20] In the preceding studies, all cows had an embryo with a heartbeat on day 28 of gestation; however, cows that experienced late embryonic/fetal mortality after day 28 and before day 72 had decreased circulating concentrations of PAGs on day 28 (Figure 3). Similar data have been reported in dairy cows[17,22,23] and sheep[24] in which circulating concentrations of PAGs were higher or lower in animals that maintained or lost a pregnancy, respectively. However, Ricci[14] reported that PAGs were not predictive of late embryonic mortality in dairy cattle. The preceding discrepancy in the efficacy of utilizing circulating concentration of PAGs on day 28 to 30 to predict late embryonic mortality in cattle may be explained by the specific PAG assay that was employed. The studies above that reported an association between circulating PAG concentrations and embryonic mortality utilized an antisera directed against placentomes collected during early gestation and developed by Jon Green[11] that has been shown to accurately predict late embryonic mortality in beef and dairy cattle. Thompson[17]also reported circulating concentrations of PAGs on day 30 were lower in dairy cows that experienced late embryonic mortality. In that study, circulating concentrations of PAGs were measured

with a sandwich ELISA validated by Green[5] and the antisera in that ELISA was also raised against PAGs secreted during early-mid gestation. In addition, there seems to be no correlation between embryonic size (crown rump length), embryonic width or embryonic volume at day 35 or 56 of gestation in beef cattle suggesting that these lower concentrations of PAGs in the maternal circulation are not purely reflective of a smaller embryo.

In cattle the use of somatic cell nuclear transfer (SCNT) usually results in high levels of fetal loss that occur throughout gestation. These losses are thought to be the result of abnormal placental development with lower placentome numbers.[25,26] Hashizume[27] reported that placentomes from SCNT pregnancies were decreased in number and that expression of PL and PAG genes was reduced compared to control pregnancies. There was a significant difference at d 35 in PAG secretion between control and SCNT pregnancies[28] along with reports of significant increases in PAG concentrations in recipient SCNT cows at d 35[25] and d 50[29] of gestation that aborted during the first trimester. This increase in PAG concentrations was speculated to be from one of the following: 1) placental hypertrophy with a greater percentage of binucleate cells, 2) an increase in the synthetic activity of binucleate cells, or 3) a higher degree of PAG glycosylation which leads to an increase in their half-life in the maternal circulation.[30] However, Constant[30] reported that the increase in maternal concentrations of PAGs in abnormal SCNT pregnancies was not the result of any of the above and concluded that it was most likely due to an augmentation of PAG half-life, but speculated that it was not mediated through a higher degree of glycosylation.

Summary

Overall, using a biochemical marker such as PAG in ruminant ungulates may provide a powerful technique for a producer for identifying pregnant animals along with selecting cows that are most likely to experience embryonic/fetal loss thus increasing reproductive efficiency.

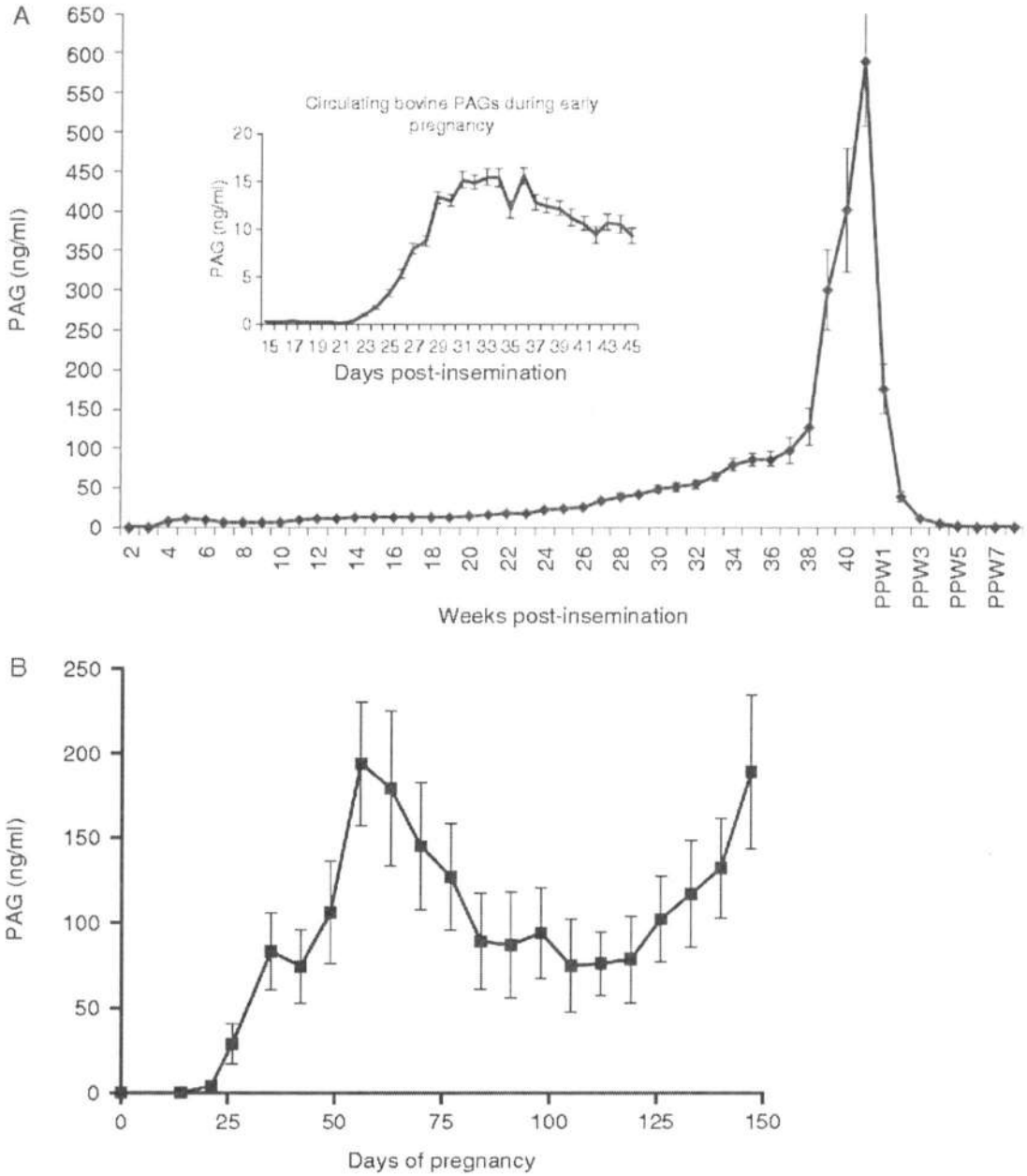

Figure 1. Circulating concentrations of PAGs during gestation in cattle (A) and sheep (B). Adopted and modified from Green/Wallace.[5,31]

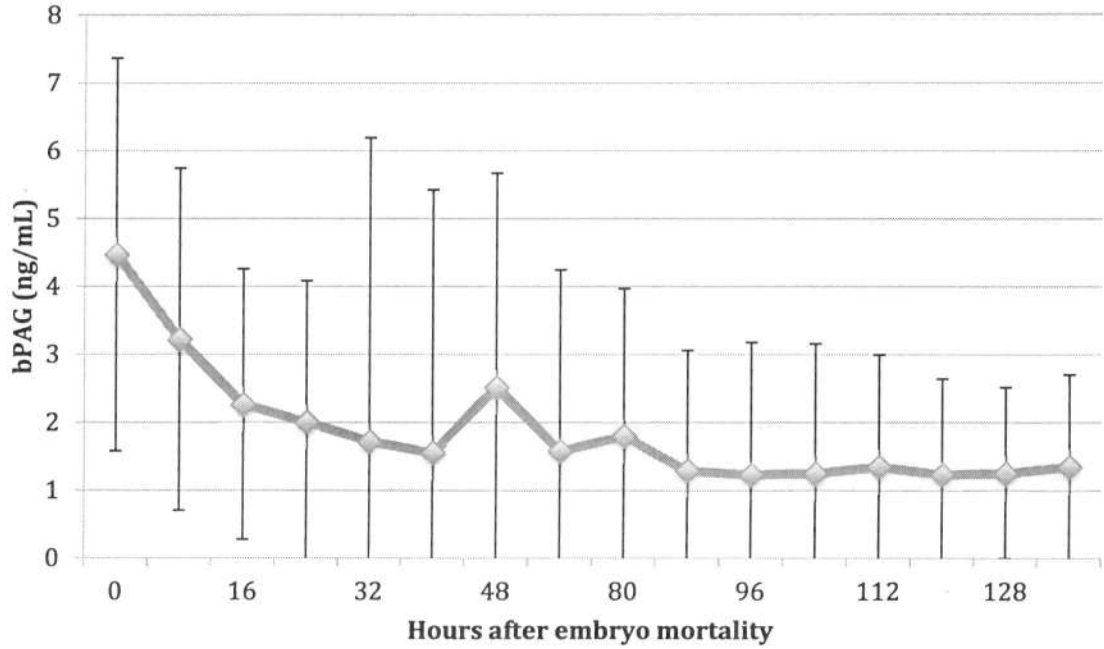

Figure 2. Half- life of circulating concentrations of PAGs after induced embryonic mortality (0h). Modified from Pohler.[11]

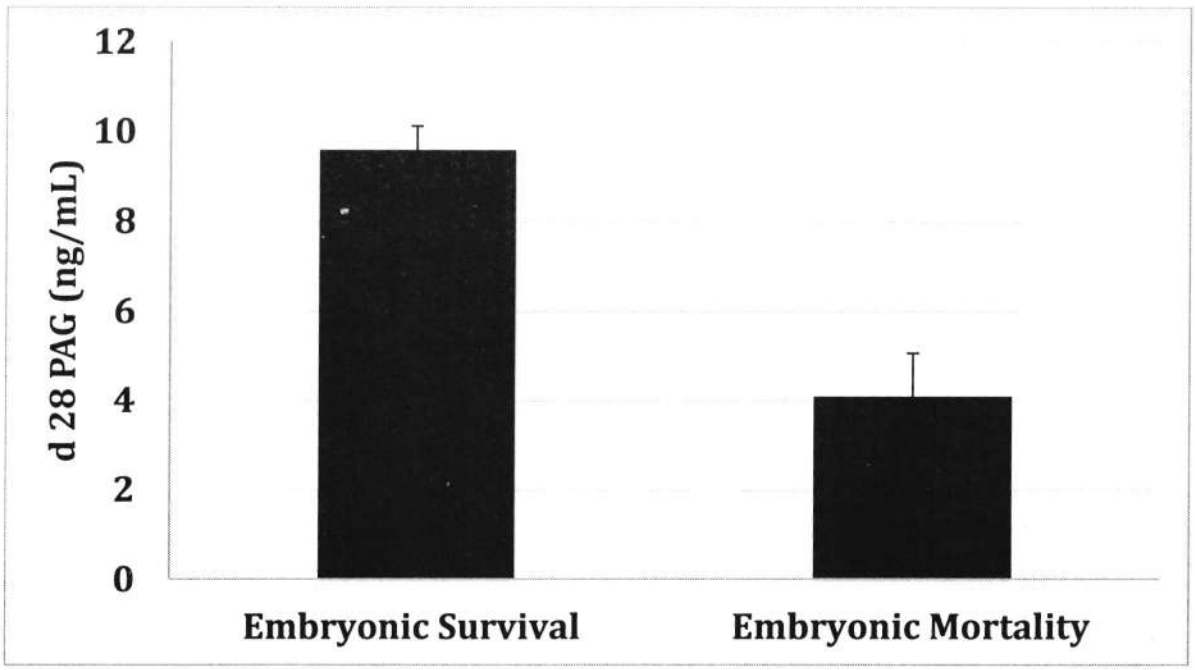

Figure 3. Serum concentrations of PAGs in samples collected on day 28 of gestation from pregnancy cows with a viable embryo based on fetal heartbeat. Cows were then divided into whether they maintained pregnancy until day 72 of gestation (Embryonic survival) or embryonic mortality (between day 29-72). Modified from Pohler.[11]

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Applying ultrasonographic evaluation of antral follicle count to improve reproductive management in heifers

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Abstract

Ultrasonography is a powerful technology that can be used to improve reproductive management in heifers. By counting the number of antral follicles observed on an ultrasound screen the practitioner can gather additional information when reproductive tract scoring, because the number of antral follicles is predictive of the status of the ovarian reserve. The number of antral follicles is also predictive of response to exogenous gonadotropins and can be used as a screening tool to remove poor responders before time and money is squandered trying to recover oocytes or embryos.

Introduction

Palpation of the ovaries and reproductive tract of the cow has been used by veterinarians for decades to evaluate reproductive status. Advantages of this technique are that the reproductive tract is easily accessible, examinations can provide information on ovulatory status and size of the ovaries and the uterus, results are instantaneous allowing management decisions to be made at time of examination, and labor is used efficiently because a relatively large number of heifers can be evaluated in a relatively short period of time. These advantages led to development of the Reproductive Tract Score, a formalized method of evaluating reproductive tract development and ovulatory status in heifers[1] and cows.[2] Especially in the beef industry where access to the heifers can be limited under some management practices, this has provided a method for evaluating replacement heifers that did not require observation for behavioral estrus over several months to determine pubertal status. Nevertheless, adoption by producers has been slow because of need for highly-trained individuals with palpation skills, costs associated with procuring services of such trained individuals, need for suitable working facilities, and need to gather heifers and bring them into the working facilities. From a veterinarian's perspective, strain on the practitioner's arm and shoulder deters enthusiasm for providing such a service. In cases where an extension arm can be used, such as pregnancy diagnosis, ultrasonography can eliminate this strain on the shoulder. Furthermore, ultrasonography can provide additional information not acquired by traditional palpation, such as antral follicle count and blood flow, leading to better decision making about females to retain for breeding purposes.

Ultrasound and its application to reproductive management in bovine females

Ultrasound machines are composed of a primary electronic processing unit and a piezoelectric transducer that emits and detects high frequency sound waves. Based on density of tissue being scanned, the sound waves emitted by the transducer either reflect back to the transducer or continue to penetrate the tissue. Returning sound waves are detected by the transducer and converted to a real-time image displayed on the screen of the processing unit. Fluid filled structures such as the antrum of a follicle or a gravid uterus do not reflect sound waves and appear on a standard screen as a black area.

Transducers may comprise linear or sector (convex) crystal arrays. Linear transducers have crystals arrayed in a single plane and emit waves at a ninety degree angle from the transducer. Sector or convex transducers have a crescent-shaped array of crystals and emit waves across a broader area. In the past, transducers had a fixed frequency and there was a trade-off because a greater frequency gave a better image quality but decreased depth of tissue penetration. In newer machines, frequency for a transducer can be changed and this allows greater flexibility in the information that can be obtained from a single examination.

Compared with traditional palpation, ultrasonography increases the information that can be collected as part of an examination process. Ultrasonography can be useful in detecting multiple

ovulations, size of corpora lutea, number and gender of fetuses, heart beat of fetuses, abnormalities of the genital tract, and other indicators of fertility.[3,4] For example, analysis of gray scale patterns of uterine images may provide some indication of fertility.[5,6] Researchers began using ultrasonography to measure diameters of antral follicles as a method to understand follicular development and ovulation.[7,8] From this research, they discovered that diameter of the ovulatory follicle is related to estradiol production and overall fertility;[9,10] however, follicle diameter measurements must be taken at consistent times during the follicular phase to generate measurements that can be compared across cows and be useful for making decisions. Counting the number of antral follicles that are visible on the ultrasound screen can be used as an indicator of the number of microscopic follicles within an ovary.[11-13] This measurement is less dependent on stage of the estrous cycle because small (< 5 mm) antral follicles comprise the majority of the count and do not fluctuate during follicle wave progression.

Color Doppler ultrasonography makes use of the Doppler effect to determine direction and rate of movement relative to the position of the transducer. It is most commonly used to measure blood flow with the colors (blue and red) simply indicating orientation of the flow to the transducer and having nothing to do with arterial or venous blood flow. Some researchers feel that it has application for early pregnancy diagnosis in cows by focusing on the function of the corpus luteum (CL), because during luteal regression blood flow to the CL decreases. Therefore, by examining blood flow at days 18 to 19 after breeding the technician may be able to identify corpora lutea that are regressing in the absence of pregnancy.[14,15] It is still difficult to time this correctly because bovine estrous cycles with three waves of follicular development will average 24 days in length.[16,17] Color Doppler ultrasonography can also be used to measure blood flow to a gravid uterus and understand the role of blood flow in fetal development.[4] The diagnostic uses of this for reproductive management of cows have yet to be established.

Relative distance from the surface of the transducer to the surface of the tissue being examined along with the frequency of the sound waves being emitted affect the size of structures measured. For pregnancy diagnoses in cattle, curved handles that enclose the probe and connecting cable can be inserted per rectum to decrease strain on the arm and shoulder that are characteristic of palpation. In contrast, the transducer must be held and manipulated by hand to insure consistent measurements of ovaries, diameters of follicles or CL, and cross-sections of uterus. There may be future solutions for this requirement, but the current technology can provide very informative data that helps veterinarians and producers make profitable decisions.

Ultrasound machines have become more compact and mobile with greater computing capabilities. As computing capabilities and image analysis software improve, applications of the machines to improve reproductive management will continue to increase. Ability to store digital video clips of reproductive tract scans of individual animals will create permanent records that are managed, stored, and queried more easily than with the video tapes used in the past. Imaging software will attain a level of sophistication to instantaneously provide pixel densities of the ovaries as a proxy for antral follicle count and blood flow measurements to better evaluate CL and reproductive tract function. This should improve diagnostic capabilities and perhaps decrease the amount of time a diagnostician has to spend with their arm inserted into a rectum. Wireless technologies and adoption of electronic identification will eliminate need for a practitioner to record animal identifications by hand, thereby decreasing the error rate and the time required to complete an examination of an animal.

Antral follicle count as an indicator of fertility

Compared with traditional palpation, ultrasonography allows a practitioner to visualize and count the number of antral follicles present on an ovary. By the early 1990's, human medicine had moved in this direction as a method to assess reproductive status of women with fertility issues. Women that were approaching menopause or that suffered from primary ovarian insufficiency had lower numbers of antral follicles detectable by ultrasonography.[18] Because basic mechanisms controlling numbers of follicles in the ovaries are similar in all mammalian females, we began to investigate the role of antral follicles counts in bovine reproduction, and it has spread as a tool to a number of mammalian species as a way of

addressing reproductive management in endangered species. Antral follicle counts provide practitioners with a non-invasive estimate of the number of follicles in an ovary, because histological studies have demonstrated that there is a positive relationship between the number of microscopic follicles and the number of antral follicles in a bovine ovary.[11-13,19]

Antral follicle counts are associated with a number of important reproductive traits. Heifers that give birth early in their first calving season have greater numbers of antral follicles at pre-breeding ultrasonographic examination than those that give birth later in the first calving season.[20] Similarly, dairy cows with high numbers (≥ 25) of antral follicles required fewer inseminations per conception than those with low numbers (≤ 15) of antral follicles.[21] Because beef heifers that give birth early in their first calving season have greater reproductive longevity,[22] it is tempting to speculate that differences in germ cells numbers may contribute to this increased reproductive longevity. However, there is little information on the mechanisms influencing the rate of depletion of the follicles. If a heifer with high numbers of follicles has a high rate of depletion and a heifer with a low number of follicles has a low rate of depletion, they could still both reach reproductive senescence at the same age. Further research is needed to understand the mechanisms controlling depletion of the ovarian reserve.

Dairy heifers with high numbers of antral follicles have greater serum progesterone concentrations during the luteal phase and greater endometrial area during the first 6 days of the estrous cycle than those with low numbers of antral follicles.[23] Serum follicle stimulating hormone (FSH) concentrations are decreased and follicular fluid estradiol concentrations are increased in cows with high numbers of antral follicles compared to cows with low numbers of antral follicles.[24] Furthermore, ovaries from cows with high numbers of antral follicles are larger, but this is not just due to the increased volume of the antral follicles, because these ovaries have more microscopic follicles on a per gram of tissue basis.[13,19] Thus, there are clearly differences in hormonal milieu and reproductive tract function that contribute to improved conception in cows with high numbers of antral follicles.

Whether there is a difference in oocyte competence between cows with high and low numbers of antral follicles is unclear. The majority of studies have reported increased numbers of blastocysts produced in vitro, but no difference in percentage of blastocysts produced in vitro between cows with high numbers of follicles and cows with low numbers of follicles.[24,25] However, there is one study that reported an increase in percentage of blastocysts produced in vitro from oocytes collected from cows with high numbers of follicles compared to those with low numbers of antral follicles.[26]

Response to exogenous gonadotropins

The use of exogenous gonadotropins either for multiple ovulation embryo transfer or ultrasound-guided oocyte pick up (OPU) is a way to rapidly increase the number of progeny of a genetically superior heifer while decreasing the generation interval. The decision to use a heifer in such a program should not be based solely on genetic merit, but should also be based on reproductive capacity. We demonstrated that the number of microscopic and antral follicles in one ovary was predictive of the response to exogenous FSH in the other ovary.[12] Cows with low numbers of primordial follicles had fewer antral follicles and poor ovulatory response to exogenous gonadotropins while cows with greater numbers of primordial follicles had more antral follicles and a strong ovulatory response to exogenous gonadotropins.

These results led others to investigate the use of ultrasonography to determine antral follicle numbers as a method to predict response to exogenous gonadotropins in cows.[27] One hundred and forty one cows were submitted for ultrasonography and based on total number of follicles ≥ 2 mm that were observed at follicle wave emergence, cows in the top 10% and cows in the bottom 10% were chosen. The cows in the high group had more than twice the number of follicles >5 mm after treatment with FSH for three days than the cows in the low group. The authors concluded that screening of cows prior to the start of treatment with exogenous gonadotropins was a useful way to remove poor responders.

Similar results were reported for OPU,[28] demonstrating that cows with high numbers of follicles had greater numbers of cumulus-oocye-complexes recovered after treatment with exogenous gonadotropin. A follow-up study proposed the need for individual protocols based on follicle numbers, but giving greater doses of FSH in the low follicle number cows did not improve response.[29] This is most

likely because others have demonstrated that cows with low numbers of follicles already have greater circulating concentrations of FSH due to less negative feedback.[24]

Conclusions

Ultrasonography is a powerful tool that can contribute greatly to reproductive management in heifers and cows. The ability to visualize physiological functions that would not be palpable such as heart beat or blood flow and to record these measurements for future diagnostic reference provide advantages beyond traditional palpation. It provides a method for early and accurate pregnancy detection and diagnosis of ovarian cysts. One advantage that it has over traditional palpation for evaluating reproductive capacity is the ability to visualize and count the number of antral follicles. As a predictor of the number of microscopic follicles in the ovaries, antral follicle counts can help to identify replacement females that may not produce enough calves to pay for their development costs or identify poor responders to treatments with exogenous gonadotropins. Continued research on antral follicle counts and the ovarian reserve will identify the mechanisms that contribute to decreased fertility and provide better decision making tools for reproductive management.

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Mechanisms influencing establishment of the ovarian reserve in heifers

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Abstract

Ultrasonography serves as a diagnostic tool for understanding what is occurring at the microscopic level in the bovine ovary. Situations exist, however, in which genetic or nutritional influences can alter the population of microscopic follicles without altering the number of antral follicles observed on the ultrasonically, because correlations between preantral and antral follicle numbers are not 100%. Investigation of the mechanisms controlling establishment of the ovarian reserve and regulation of preantral follicle growth may improve both reproductive management of developing heifers and diagnostic capabilities.

Introduction

Using transrectal ultrasonography to determine the number of antral follicles in the bovine ovary is a powerful tool that can predict changes in the ovarian reserve (e.g., population of microscopic follicles in the ovary) and improve reproductive management. A greater understanding of what is occurring at the microscopic level is necessary to continue to improve development and application of ultrasonography. Genomic tools have enabled identification of genes involved in establishment of ovarian reserves and regulation of preantral follicle activation and growth. Research in this field provides evidence that variation in both gene sequence and gene function are contributing to differences in antral follicle count among cows, and that nutritional programming at specific developmental stages can change the function of a number of ovarian genes, presumably through epigenetic modifications to the genome.

Establishment of the ovarian reserve and follicle development in heifers

In bovine females, primordial germ cells migrate to the germinal ridge by d 30 of gestation.[1] Sexual differentiation of the ovaries occurs by d 40 of gestation, and germ cells proliferate until mid-gestation, reaching a peak of approximately 2.7 million oogonia. During the second half of gestation this number declines. By d 60 to 90 of gestation oogonia begin to form the functional units of the ovarian reserve, primordial follicles, composed of an oocyte surrounded by a single layer of flattened pre-granulosa cells. Primordial follicles are dormant until they enter the growing pool of follicles through an undefined process known as activation. Because of the decline in germ cell number during the second half of gestation, a heifer is born with approximately 100,000 primordial follicles in her ovaries,[2] and as with all mammalian females, it is the slow depletion of those primordial follicles over time that eventually leads to reproductive senescence.[3]

During activation, the flattened pre-granulosa cells become cuboidal granulosa cells and the oocyte enlarges, forming a primary follicle.[4] Activation of primordial follicles is most likely controlled by a combination of stimulatory and inhibitory factors,[4] the most well-described being anti-Müllerian hormone.[5,6] Anti-Müllerian hormone inhibits primordial follicle activation in cows and other mammalian species. Our research indicated that supra-physiological doses of estradiol given to cows over a 60-d period could stimulate primordial follicle activation.[7] This follicular stimulation provides indirect evidence that during normal follicle development, estradiol from the dominant follicle may inhibit ovarian anti-Müllerian hormone production, thereby ameliorating the inhibitory effects of anti-Müllerian hormone and providing a window of opportunity for some primordial follicles to enter the growing pool. However, this potential mechanism does not explain the mechanisms by which primordial follicles of the ovarian reserve are activate during any one of those windows of opportunity.

The cuboidal granulosa cells surrounding the oocyte proliferate forming a secondary follicle consisting of an oocyte surrounded by multiple layers of granulosa cell. Proliferation of granulosa cells is

controlled by a number of local growth factors, many belonging to the transforming growth factor-β super-family.[8] After three layers of granulosa cells are established, theca cells begin to differentiate. Once secondary follicles have attained six layers of granulosa cells, a fluid filled antrum begins to form. Transition from secondary to antral follicle is believed to be controlled mainly by insulin-like growth factor-1 and follicle stimulating hormone, because model systems that remove those two hormones stall progression of follicles at the secondary follicle stage.[9] Once the antral follicle attains 3 mm in diameter, it is distinctly visible via standard real-time ultrasonography.

The number of follicles is repeatable among cows and heifers over time.[10] Although the absolute number of antral follicles differs between heifers and lactating cows, their rank from high to low has a positive relationship[of about 80%. Furthermore, the number of follicles in one ovary is predictive of the number of follicles in the other ovary and the other ovary's response to exogenous gonadotropins.[11]

Genetic selection for numbers of follicles in heifers

Rapid response to genetic selection requires a selection criterion with high predictive value based on repeated measurements obtained at a young age. Conversely, response to genetic selection for reproductive traits in cattle has been limited by their low estimates of heritability, long generation intervals for progeny testing, and sex linkage. In a long-term genetic selection experiment at the U.S. Meat Animal Research Center to increase the frequency of multiple ovulations, ovulation rate was measured by ovarian palpation per rectum in 12- to 18-month-old pubertal heifers for six to ten consecutive estrous cycles.[12-14] Ovulation data were combined with ancestral records and utilized in a repeated-records animal model to estimate an animal's predicted breeding value for ovulation rate.[15] Repeated measurements of ovulation estimated heritability for ovulation rate based on a single observation to be 0.11 in this population; this heritability increased to 0.35 for mean ovulation rate for observations during six consecutive estrous cycles and to 0.43 for the mean ovulation rate for observations during ten consecutive estrous cycles.[16] In addition to an increased frequency of multiple ovulations, ovaries of the selected females contained more preantral and antral follicles compared with control females. The increased frequency indicates that selection for improved ovarian function modified the regulatory system of ovarian folliculogenesis, enabling activation and development of more preantral follicles, maintenance of larger pools of small antral follicles, recruitment of more follicles within the cohort of developing follicles, selection of two or more ovulatory follicles within a follicular wave, and/or a reduced rate of atresia of preantral and antral follicles.[17,18]

The ovarian reserve in *Bos indicus* females

Bos indicus cattle have greater numbers of antral follicles and greater ovarian stroma volume than *Bos taurus* cattle.[19] There is no evidence of increased fertility in *Bos indicus* cattle compared to *Bos taurus* cattle leading investigators to question relationships between follicle count and fertility. *Bos indicus* cows have larger ovaries than *Bos taurus* cows; however, a number of histological studies have reported no difference in the number of primordial, primary, or secondary follicles between age-matched *Bos indicus* and *Bos taurus* cows.[20,21] Taken together, those data indicate that *Bos indicus* cows have fewer primordial follicles per gram of ovarian tissue and appear similar in that regard to *Bos taurus* cows with small antral follicle numbers. Fewer antral follicles further indicates that there is enhanced secondary to antral follicle transition in *Bos* indicus cows, which is hypothesized to be a product of increased serum insulin-like growth factor-1 concentrations in *Bos indicus* cows.[22]

Genome wide association studies for antral follicle count in heifers

Genetic selection was able to increase numbers of follicles in heifers and that *Bos indicus* heifers have greater numbers of antral follicles detectable by ultrasonography; therefore, it was logical to hypothesize that it would be possible to identify chromosomal regions associated with antral follicle numbers. Our hypothesis was that this would aid us in identifying genes involved in establishing the number of primordial follicles, controlling primordial to primary follicle transition, stimulating granulosa

cell proliferation in secondary follicles, controlling secondary to antral follicle transition, and selection of ovulatory follicles.

We used the BovineSNP50 BeadChip to perform a genome wide association study, and identified a number of nominally significant regions of the bovine genome that associated with antral follicle number in yearling beef heifers.[23,24] From our results, the genomic heritability of the trait was 0.44, and subsequently a genetic heritability of 0.25 to 0.31 was reported in dairy heifers and cows.[25] Pathway analysis of genes with a start codon within 50,000 base pairs of a nominally significant polymorphism determined that the top upstream regulator of antral follicle count in heifers was transforming growth factor-β signaling, a result that was in strong agreement with the reported roles of anti-Müllerian hormone, growth differentiation factors, and bone morphogenic proteins in follicle development.

Nutritional programming of the bovine ovary

Variation in gene sequence does not control all of the variation observed in follicle number. Studies have provided evidence that alterations to the diet may regulate the epigenome, thereby changing gene function. This is referred to as nutritional programming. Nutritional programming of the bovine ovary seems to occur at multiple stages during development.

Maternal nutrition during pregnancy can influence follicle numbers and reproductive capacity in daughters. Daughters of dams that were fed 60% of maintenance during the first and second trimester of pregnancy had fewer antral follicles at slaughter at a year of age compared with daughters of dams fed 120% of maintenance.[26] The first half of gestation when oogonia are proliferating seems to be a particularly sensitive time for influencing follicle numbers in daughters. A high protein diet during the first two trimesters resulted in daughters with fewer primordial and antral follicles as yearlings. Thus, it may be inferred that conditions influencing forage quantity or forage quality during the first two trimesters may impact reproductive capacity of the daughters.[27] Additional research is necessary to understand the long-term impacts of such nutritional or environmental conditions on daughter performance.

When nutrient intake was limited to 75% of maintenance during the second and third trimester, no change was detected in daughter antral follicle numbers.[28] Combined with the previous results, lack of a change in antral follicle number indicates that the truly sensitive window of maternal nutritional status is during the first trimester. In this study, cows that were provided a diet containing 125% of maintenance during the third trimester produced daughters that conceived earlier in their first breeding season. Those daughters did not have any change in antral follicle counts before their first breeding season when compared with daughters from the cows fed the control diet (100% of maintenance). Protein supplementation with 0.45 kg/d of a 42% crude protein cake in the third trimester had the same influence on first service conception in daughters.[29] Supplementation with dried distiller's grains with solubles during the third trimester did not change antral follicle numbers in daughters but did improve conception to timed artificial insemination.[30] Taken together, these results indicate that maternal nutrition during the third trimester can improve daughter reproductive performance without altering daughter antral follicle number. It is not clear, however, what impact this nutritional programming might have on daughter preantral follicle numbers.

The peri-pubertal period is another window of development when the ovarian reserve can be influenced by nutrient intake. Caloric restriction between eight and 11 months of age as part of a single phase stair-step development regimen increased the number of primordial follicles, but did not change the number of antral follicles.[31] Similarly, nutrient restriction from weaning through breeding did not change antral follicle numbers in beef heifers.[32] This indicates that nutrient restriction in the peri-pubertal period may have a sparing effect on the ovarian reserve without changing the rate of primordial follicle activation or preantral follicle growth. The mechanism responsible could be a slowing of activation of primordial follicles or a stimulation of formation of primordial follicles. Although primordial follicle formation is reported to cease before birth in heifers, our preliminary data indicates an increase in the mRNA abundance of SLIT/ROBO members, specifically SLIT2, SLIT3, and roundabout, axon guidance receptor, homolog 4 (ROBO4) in the ovarian cortex of the Stair-Step heifers. Those genes are up-

regulated in the ovine fetal ovary during the time of primordial follicle formation.[33] Thus, calorie restriction may be stimulating primordial follicle formation, but further research is warranted. On-going research at the U.S. Meat Animal Research Center also is examining the reproductive longevity of heifers developed using the stair-step diet to determine how such changes in ovarian function impact production efficiency.

Conclusions

Research into mechanisms controlling establishment of the ovarian reserve and preantral follicle growth has demonstrated that gene products are involved in regulating function of the ovarian reserve. Although it is important to understand how variation in DNA sequence contributes to phenotypic differences, understanding how nutritional programming of those genes alters their function in replacement heifers may have the greatest impact on improving reproductive efficiency and the quality of replacement heifers.

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Bovine temperament impacts immunity, metabolism, and reproduction: a review

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Abstract

Temperament, or excitability, is a behavioral trait that has been shown to impact physiology and performance. Temperament in cattle alters the function of the hypothalamic-pituitary-adrenal axis, thereby influencing circulating concentrations of catecholamines and glucocorticoids. The physiological changes associated with temperament have also been reported to alter immune responsiveness and associated biomarkers. While temperament has been demonstrated to influence metabolism, which can impact growth and carcass performance, the implications of this relationship remains unclear at the present time. With regard to reproductive efficiency and progeny performance, several studies have reported that temperament not only negatively influences the reproductive system efficiency, but also the growth and performance of offspring. Overall, the literature supports the idea that cattle temperament impacts major physiological mechanisms that ultimately impact reproduction, growth, performance and profitability of beef cattle. Therefore, this review is aimed at elucidating the interactions between temperament and immunity, metabolism, performance, and reproduction in cattle.

Keywords: Temperament, reproduction, immunity, metabolism

Introduction

Cattle temperament is known to modify physiological responses with regards to immunity, metabolism, performance and reproduction that ultimately impact production parameters such as milk production, carcass yield and carcass quality. Additionally, temperamental cattle pose a risk to livestock handlers, facilities, and equipment. Temperament may be defined as the fear response or the degree of reactivity to humans or to novel environments. Cattle classified as temperamental have been reported to exhibit reduced weight gain[1] and decreased hot carcass weights.[2] Other production parameters including carcass performance are also hindered in temperamental cattle such as decreases in tenderness[3] and milk yield[4] and increases in carcass bruising.[5] Differences in cattle temperament may become more pronounced during stressful events, and these are detectable by visual observation, as well as physiologically. During illness or an immunological insult, more temperamental cattle do not display as dramatic signs of illness as calmer cattle.[6] Curley et al[7] reported differences in stress hormone concentrations, specifically cortisol, between calm and temperamental cattle. With respect to production parameters and animal health, it is essential that producers minimize the negative physiological impacts of stress to maintain and enhance growth performance and animal health.

Separation of cattle into temperament categories may be accomplished in a variety of ways. However, two of the most common ways are exit velocity (i.e., flight speed) and pen score (Figures 1 and 2). Exit velocity is an objective measurement of temperament that measures the time it takes the animal to traverse a set distance upon release from a squeeze chute.[1] In this manner, an animal with a slower exit velocity is considered more calm than an animal with a faster exit velocity (temperamental). Pen score is a subjective measurement of temperament and is measured by separating small groups of cattle (n = 3 to 5) in a pen and ranking them on a scale of 1 (calm) to 5 (very temperamental) based on their response to a human observer.[8] The pen score and exit velocity of each animal can be averaged to determine a temperament score, comprising elements of both the subjective and objective measures. Schmidt et al.[9] reported that temperament is moderately heritable between generations of cattle. While temperament is not only heritable, epigenetic effects can also play a role in temperament such as the age of the dam, the age of the calf at weaning, environmental events, the herd in which they reside, breed, sex, and other factors such as human acclimation.[10-12]

The effects of cattle temperament can be further elucidated when specific physiological systems in the animal are evaluated. Temperament has been reported to impact immune function in cattle.[13] Cattle temperament has also been reported to influence optimal metabolism and nutrient partitioning.[14] Additionally, temperament may influence cattle reproductive physiology and subsequently their offspring.[15] Therefore, temperament can play a pivotal role in the health and performance of cattle, and this review is aimed at elucidating some of the interactions of temperament associated with immunity, metabolism and performance, and reproduction.

Immunity

The effects of temperament on immunity in cattle have not been fully elucidated. Historically, the prevailing opinion has been that temperament negatively impacts the immune system due to increased basal concentrations of stress hormones exhibited by temperamental cattle. However, more recent research both supports and refutes this idea. In response to stress and activation of the hypothalamic-pituitary-adrenal (HPA) axis, temperamental mice have been reported to over-express phenylethanolamine-n-methyl transferase which coverts norepinephrine to epinephrine, thereby leading to more aggression.[16] Similarly, more temperamental cattle have been found to have greater basal concentrations of circulating cortisol in comparison to calmer cattle.[17-19] Consequently in mice, Cavigelli et al.[20] reported that aggressive mice exhibit increased corticosterone concentrations and decreased tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine, thus suggesting that temperament negatively impacts immune function. Curley et al.[18] also reported that there was a positive correlation between temperament (measured via exit velocity) and cortisol concentrations. Furthermore, Fell et al.[17] reported that temperamental cattle entering the feedlot had significantly greater concentrations of circulating cortisol. After transportation, temperamental Brahman bulls exhibited increased cortisol in comparison to their calmer counterparts; however, TNF-α was unaffected by temperament.[13] Generally, temperamental cattle have differential responses to stressors when compared to calmer cattle, and this is evident by increased concentrations of cortisol and catecholamines and further by the decreased stress hormone responses when challenged with corticotropin releasing hormone or adrenocorticotropic hormone.[7,21]

A major factor in determining whether or not to treat sick cattle is their behavioral sickness response, which is a basic visual observation of the general health of the animal. Animals respond to illnesses in different ways and their response is generally correlated with physiological changes within the animal, such as body temperature.[22] In a study by Burdick et al., temperamental animals displayed diminished sickness behavior compared to their calmer cohorts.[6] Though unpublished, this phenomenon has been observed in other studies in which cattle experienced an immunological insult. Thus, since temperamental cattle do not display as dramatic visual signs of illness or sickness behaviors, they may be passed up for treatment when they are ill. Untreated temperamental cattle may represent morbidity and mortality in a herd or pen and may function to further infect the herd.

Temperament may also impact immune function in response to vaccination. Oliphint reported that temperamental calves had a diminished immunological response to Clostridial vaccination.[23] Specifically, Oliphint reported that temperamental Brahman bull calves had decreased lymphocyte proliferation and vaccine-specific IgG concentrations in comparison to calm bull calves. Bauer et al. similarly reported that temperament was negatively correlated with circulating concentrations of IgG and the ability of lymphocytes to produce IgM and proliferate.[24] Therefore, it has been speculated that temperament may negatively influence adaptive immunity. While the inherent differences in temperamental cattle may be partially beneficial with regard to regulation of the innate immune system, these apparent beneficial effects do not necessarily carry over to the adaptive immune response after vaccination.

Temperament, according to the literature, seems to have an overall negative impact on acute stress; however, little is known about the role temperament plays in chronic stress. As mentioned previously, more temperamental cattle have elevated basal circulating stress hormone concentrations. If one attempts to elucidate how temperament, specifically elevated concentrations of cortisol, impacts chronic stress, one may note that induction of chronic stress in mice has resulted in glucocorticoid

resistance[25] which may enhance the ability of an animal to clear bacteria from superficial wounds and heal faster. However, the relationship between hormonal changes associated with temperament, and the subsequent impact on overall cattle immunity and health have not been fully elucidated.[26] Thus, cattle temperament may differentially affect aspects the immune response of cattle, and further investigations are needed in order to reveal and understand the mechanisms of immune modulation within both the innate and adaptive immune systems in concert with temperament.

Metabolism and performance

Temperament has been demonstrated to have effects on metabolism and overall production. Previous work[27] reported that temperament was related to energy requirements; temperamental cattle required more energy to sustain maintenance and growth because of their inefficiency to metabolize and utilize nutrients. Other studies report that temperamental cattle have decreased average daily gains (ADG)[28,29] and calmer cattle exhibited greater body condition scores than more temperamental cattle.[30] One might assume that the activity and/or arousal level of calm versus temperamental cattle could impact their overall energy requirements. This supports the inefficiency data associated with temperamental cattle. An additional aspect associated with efficiency is found in reports that temperamental cows have been observed to produce less milk compared to less temperamental cows.[4,31]

In addition to overall performance, temperament may also play a role in terms of meat quality. Temperament has been reported to decrease overall carcass fat[32] and hot carcass weight,[19] which can decrease carcass value. Furthermore, more temperamental cattle have been observed to produce meat that is less tender in comparison to their less excitable cohorts.[19,33] Voisenet et al.[3] reported that temperamental cattle exhibited darker lean color with greater pH (i.e. dark cutter) which can be detrimental to fresh meat palatability. Off-flavor development (i.e. non-beef like flavor profiles) has also been correlated with temperament when evaluated by taste panelists.[19]

The mechanisms by which temperament influences performance have not been fully elucidated; however, glucocorticoids may be primary factors that impact metabolic changes between these animals.[14] Cortisol, the primary glucocorticoid hormone released by cattle in response to stress during activation of the HPA axis, can influence glycogen release and the production and allocation of glucose within the body. As stated previously, more temperamental cattle have been reported to have greater concentrations of stress hormones such as catecholamines and cortisol.[7,18,19,21] Bradbury[14] reported that temperamental cattle had increased circulating concentrations of insulin and glucose compared to their less excitable counterparts. During a glucose challenge, Bradbury[14] observed a tendency for increased cortisol and insulin concentrations in temperamental cattle. The results of a glucose challenge test suggest that temperamental cattle do not utilize glucose as efficiently as calm cattle, which may thereby alter performance and growth. Bradbury also observed insulin resistance in temperamental heifers which may hinder performance.[14] Temperamental cattle have been reported to maintain greater concentrations of non-esterified fatty acids (NEFAs) in comparison to calmer cohorts, and these differences may impact glucose regulation and utilization.[34,35] In the same studies, Burdick Sanchez et al.[34,35] reported decreased blood urea nitrogen (BUN) and insulin sensitivity in more temperamental steers. These data suggest that temperament may impact nutrient utilization and repartitioning of energy, specifically in relation to what types of tissues are catabolized for energy and maintenance. The endogenous hormonal and physiological differences exhibited by temperamental cattle may contribute to metabolic differences between calm and temperamental cattle. Overall, the effects of temperament on metabolism and performance may be detrimental to feedlot performance, carcass merit, and meat quality, thus decreasing profitability of temperamental cattle in comparison to calmer cattle.

Reproduction

Cattle temperament can impact reproductive performance and may ultimately influence offspring performance either epigenetically or through heritable traits. Reproductive performance and efficiency is a critical component needed to profitably sustain a viable cow-calf operation in the U.S.[15] A study by Cooke et al.[15] reported a negative correlation between temperament and artificial insemination conception

rates of beef cows. Additional studies have also reported decreased conception rates in more nervous cattle.[36-38]

Temperament may impact reproduction via multiple mechanisms. Specifically, temperament may inhibit feed intake and optimal metabolic efficiency.[39-40] Additionally, cortisol and other stress hormones may alter reproductive physiology by altering the mechanisms involved in ovulation and conception through physiological pathways.[41] Cortisol, and its association with stressors, is known to influence reproduction beginning at the level of the hypothalamus.[42] Specifically, elevated cortisol concentrations may inhibit the release of gonadotrophins from the anterior pituitary gland.[43,44] Typically, short-term or acute stress has little impact on reproduction while chronic stress may interfere with various reproductive processes,[42] which may explain why temperamental cattle with chronically elevated basal cortisol have decreased reproductive efficiency. Echternkamp reported that calmer cattle have reduced basal cortisol concentrations and increased concentrations of luteinizing hormone which enhances the onset of puberty and ovulation.[45] In concert with findings from Echternkamp, Cooke et al. reported that calmer heifers reached puberty earlier in life when compared to more temperamental heifers.[15] However, it is important to note that Dobson and Smith[46] reported that stress, regardless of animal temperament, inhibited fertility and oocyte production.

Not only does temperament play a role in conception, but temperament may also impact the offspring of temperamental cows. Temperamental cattle may have offspring with decreased body weights,[1] while Phocas et al.[47] suggested that a calm temperament in dams was correlated with enhanced calf performance. Additionally, milk yield may be increased in calmer dams, which may partially explain one of the mechanisms that contribute to calf performance.[47] While temperament has been reported to be detrimental to reproductive performance, acclimation to human handling may reduce the hormonal stress response in heifers and mitigate some of the negative effects of temperament on reproduction.[45,48,49] It is noteworthy to mention that while some of the effects on reproduction may be slightly mitigated through acclimation, it is important to remember that this acclimation is only applicable to a specific situation/environment, and any alteration in that environment may reduce the positive effects of acclimation.

Conclusion

Temperament has broad effects on cattle physiology ranging from immunity to metabolism to reproduction. All of the factors associated with temperament ultimately impact growth performance, carcass and meat quality, reproductive efficiency, and health, all of which ultimately impact profitability and the sustainability of cattle-based operations from conception to consumption. While most of the aspects associated with cattle temperament imply negative implications with regard to overall production, the stress and metabolic alterations associated with temperament may play a pivotal role in priming the innate immune system. Priming the innate immune system may promote pathogen clearance, which decreases the duration of infection and may have positive implications on performance, health, and overall hardiness. Future research efforts must focus on elucidating the mechanisms associated with these physiological differences between calm and temperamental cattle. Furthermore, research should focus on management strategies to alleviate the negative impacts of temperament on reproduction and growth.

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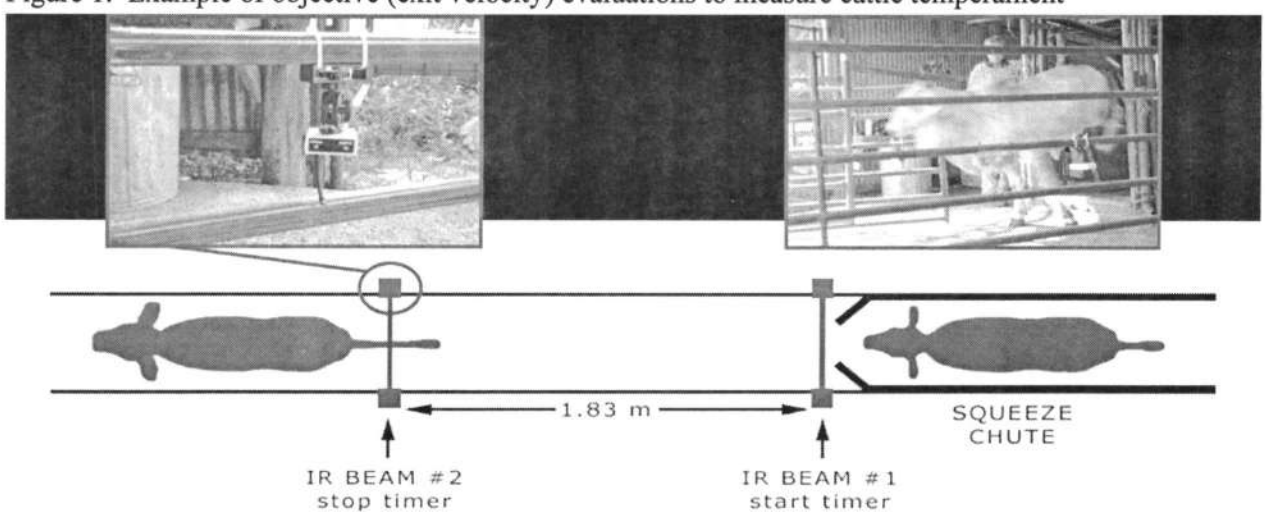
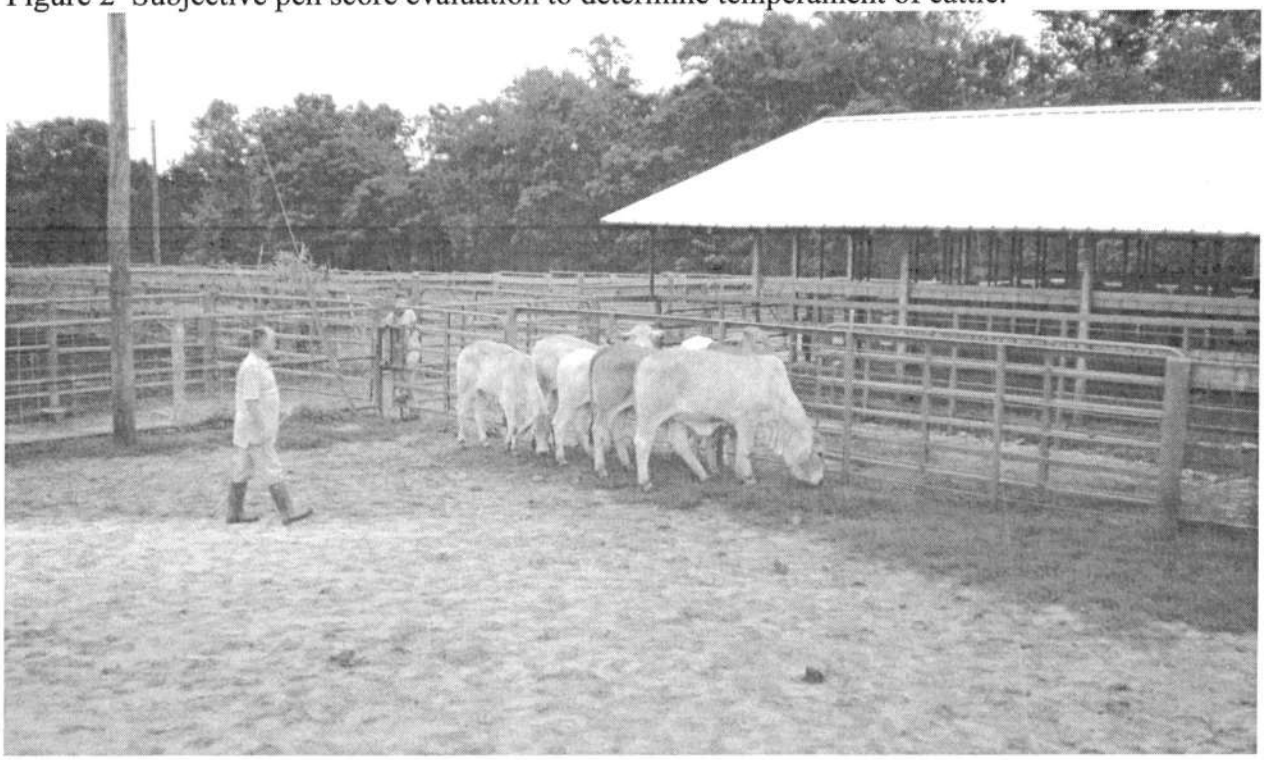
Figure 1. Example of objective (exit velocity) evaluations to measure cattle temperament*



* The exit velocity method of classifying temperament measures the time it takes for cattle to travel a set distance after being released from a working chute or handling equipment.

Figure 2 Subjective pen score evaluation to determine temperament of cattle.†



† The pen scoring method assesses the response of cattle to a human observer.

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Diagnostic techniques for assessing bull infertility

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Abstract

Investigating causes for bull infertility requires thorough understanding of the anatomy and physiology of erection, coitus and ejaculation. This presentation will review techniques not commonly used during a routine breeding soundness examination for exploring reproductive failure in bulls. Additionally limited therapeutic options will be discussed for management of chronic seminal vesiculitis as well as use of the pudendal nerve block to assist with extension of the penis of the bull.

Keywords: bull, breeding, erection, infertility, cavernosography

Introduction

Erection in the bull occurs when blood flow increases in the deep artery of the penis and into the crus penis and subsequently into the corpus cavernosum penis (CCP) following olfactory or visual sexual stimulation. The CCP in the bull is a closed system in that erectile blood flows into the penis from the crus and leaves this same area during detumescence following erection. The stimulation that causes this reflex dilation of the deep artery of the penis also causes relaxation of the retractor penis muscles which hold the penis in the preputial cavity. As the retractor penis muscles relax, the sigmoid flexure relaxes and the mildly engorged penis protrudes from the sheath. With continued sexual stimulation the ischiocavernosus muscles (ICM) begin rhythmic contraction which raises blood pressure from the normal resting state of 15 mmHg within the CCP. Peak pressure within the CCP may be greater than 14,000 mm Hg. This rapid increase in blood pressure within the CCP causes complete penile extension and erection. Following ejaculation the ICM relax, detumescence occurs as blood pressure within the CCP decreases and the penis is withdrawn back into the preputial cavity.[1-6]

Erection may be induced in the bull with an ejaculator although the optimal method for evaluating erection is with observed test mating. Normal function of the penile nerves is essential for coitus and is most accurately assessed by observed test mating or by semen collection by artificial vagina.[7]

Test mating

Bulls with erectile dysfunction do not achieve sufficient erection pressure to complete coitus.[6-8] Bulls with nerve dysfunction mount the cow but there are no penile searching motions near the vulva and the bull fails to make intromission.[9,10] Usually the penis is placed along the cow's hip or below the vulva in the escutcheon area above the cow's udder.

Semen collection with an artificial vagina

Semen collection with a properly prepared artificial vagina (AV) can confirm that a bull has the sufficient erection and sensation to ejaculate. The temperature within the AV should be 45 to 50°C and sufficiently filled to provide mild pressure on the erect penis. Most reasonably docile beef bulls can safely be collected with an AV as they mount a female in estrus.[9] Failure to ejaculate into the AV could result from in sufficient pressure within the AV or in appropriate liner temperature for that particular bull. More likely failure to ejaculate would be due to a painful condition of the back or hind limbs or to lack of nerve sensitivity of the dorsal nerves of the penis.

Contrast cavernosography for evaluation of the erection failure or penile deviation

Contrast radiography of the corpus cavernosum penis may confirm vascular defects in the penis.[10-12] The procedure is most easily accomplished with the bull restrained on a table in lateral recumbency. Manually extend the penis and place a towel clamp under the dorsal apical ligament

approximately 6-10 cm from the distal end of the penis to aid in manipulation of the penis. Percutaneously place a double strand of heavy suture (0.6 mm) between the retractor penis muscles and the penis to retract the penis away from the abdominal wall in order to enhance visualization of the sigmoid flexure of the penis. The radiographic series will consist of two or three film exposures taken as quickly as practicable progressing from the free portion to the distal bend of the sigmoid flexure.

On the dorsum of the penis near the towel clamp insert a 16-gauge x 3.8 cm needle at a 45^0 angle proximally through the tunica albuginea and into the CCP. After attaching a sterile extension set to the needle for ease of injection and to position the hands away from the radiographic field inject 10 ml sterile saline which should flow into the CCP with ease. Place a radiographic cassette under the penis then rapidly inject 15 ml of water-soluble radiographic contrast medium (Renograffin 76, Squibb Diagnostic, New Brunswick, NJ) and expose the film. Slowly inject an additional 15 to 30 ml of medium as the radiographic series is performed. Remove the cassette and quickly place another cassette more proximal under the penis. By using 43 cm-long cassettes the entire penis up to the sigmoid flexure may be radiographed with two or three exposures. Ideally all radiographic exposures should be completed within 60 seconds.

The normal bovine penis has no vascular communications from the CCP to peripenile vasculature.[10-12] Presence of contrast medium outside the CCP is evidence of a vascular shunt as a potential cause for erection failure. Alternatively, failure of complete filling of the vascular spaces within the CCP may indicate fibrosis or failure of proper development of the internal architecture of the penis. These conditions usually result in partial erection failure or deviation of the erect penis.

Erection failure due to corpus cavernosal shunts

Congenital vascular shunts

Occasionally young bulls fail to achieve intromission due to congenital corpus cavernosal vascular shunts. These bulls usually are normal on physical examination but fail to achieve adequate intracorporeal pressure for erection. When observed during erection, either by test mating or with electroejaculation the free portion of the penis becomes noticeably bluish during attempted erection. The bluish discoloration is due to blood from a relatively porous tunica albuginea of the penis exiting the corpus cavernosum penis and being removed by subcutaneous capillaries and veins. These shunts may be confirmed by cavernosography. Typically the shunts are multiple and not considered repairable.

Acquired vascular shunts

The most common cause of acquired corpus cavernosal shunts is penile hematoma due to rupture of the tunica albuginea of the penis on the dorsum of the distal bend of the sigmoid flexure.[8,11] Shunts in this area of the penis may be surgically repaired thereby restoring a bull's ability to achieve erection.

Electro diagnostics for evaluation of penile nerves

Determination of sensory nerve conduction velocity is a well-established modality for evaluating peripheral neuropathy. Injury of the dorsal nerves of the penis may occur during or after rupture of the tunica albuginea of the penis at the distal bend of the sigmoid flexure or due to trauma elsewhere along the course of these nerves along the penis.[15]

The procedure is most easily accomplished with the bull restrained in lateral recumbency. Sedation or anesthesia is not required and most bulls tolerate the procedure very well. Manually extend the penis and place a gauze loop around the glans penis to hold the penis in extension for approximately 15 minutes while the procedure is conducted. Place two spring-type ring electrodes 2.0 cm apart around the middle third of the glans penis. The proximal electrode is the cathode and the distal electrode the anode. Place a 1cm disk electrode as a ground 2.o cm proximal to the stimulating electrodes. Electrode conductivity gel is applied under each electrode.

Recording electrodes are paired needle electrodes placed 1 cm apart at three sites along the penis. Insert the needle electrodes through the skin to the dorsum of the tunica albuginea ensuring that the tip is

near the dorsal nerves of the penis. The distal pair of needles is inserted one half the distance between the urethral orifice and the attachment of the prepuce to the free portion of the penis, the middle site is one half the distance between the attachment of the prepuce to the insertion of the retractor penis muscles at the distal bend of the sigmoid flexure, and the proximal site is just proximal to the distal bend of the sigmoid flexure.

Mean conduction velocity is 55.1 $\pm$ 5.1 m/s for normal bulls.[15] This procedure can confirm loss of innervation of the dorsal penile nerves and also may localize the lesion on the nerves. If the denervation involves the glans or distal few centimeters of the penis the bull will be incapable of intromission.[7] However, if innervation is intact to distal end of the prepuce and proximal few centimeters of the penis the bull should be able to ejaculate into an artificial vagina for semen collection for cryopreservation.[9]

Pudendal nerve block to assist penile extension

The internal pudendal nerve is made up of fibers originating from the ventral branches of the third and fourth sacral and the pelvic splanchnic nerves. Achieve caudal epidural anesthesia then introduce the hand into the rectum to the depth of the wrist and direct the fingers laterally and ventrally to locate the lesser sacrosciatic notch and foramen by rectal palpation. Locate the internal pudendal artery by its pulsations at the cranial angle of the notch and palpate the pudendal nerve approximately 1 cm caudodorsal to the artery. Insert an 18-gauge, 10 cm spinal needle through the skin in the ischiorectal fossa beside the tail and direct the needle forward and slightly ventrally to a depth of 5 to 7 cm. Palpate the tip of the needle through the rectal wall and direct the needle in the direction of the nerve in the foramen. Inject approximately 10 ml of 2% lidocaine hydrochloride along the nerve then withdraw the needle 2 to 3 cm and inject an additional 10 to 15 cm at the cranial border of the foramen to desensitize the muscle branches of the rectal nerve. Repeat the procedure on the opposite side of the pelvis.[16,17]

The advantage of the technique is that the penis and prepuce are easily extended by this procedure and the animal can remain standing. A disadvantage of the technique is that a bull will not be able to retract the penis and prepuce for approximately 30 minutes following the procedure.[17]

Infrared thermography

Infrared thermography provides a non-invasive measure and map of skin surface temperatures.[18] Skin surface temperatures vary according to blood flow regulation to the skin surface and are affected by both internal and external factors. The cutaneous circulation is under sympathetic vasomotor control and peripheral nerve injuries and nerve compression can result in skin surface vascular changes that can be detected by infrared imaging. Inflammation and nerve irritation may result in vasoconstriction, causing cooler thermograms in the afflicted areas. Transection of a nerve and/or nerve damage to the extent that there is a loss of nerve conduction results in a loss in sympathetic tone causing vasodilatation indicated by an increase in the thermogram temperature.

This technology is useful for localizing neuromuscular or vascular pathology and may be particularly helpful when examining a bull for pain in the back or hind limb area. This technology is also useful for graphically depicting issues with scrotal or testicular thermoregulation which may lead to impaired spermatogenesis.[18,19]

Treatment of chronic seminal vesiculitis

The paired seminal vesicles of bulls are 2 to 4 cm wide and 10 to 15 cm long and are located on the pelvic floor lateral to the ampullae and dorsal to the neck of the bladder. The glands are lobulated and secrete a clear fluid, containing nutrients and buffers, which is discharged immediately before and during ejaculation through ducts that open into the urethra adjacent to the colliculus seminalis.[20]

Inflammation or infection of the vesicular glands is fairly common in young bulls housed together on and high energy diets. These peripubertal bulls may spontaneously recover from this condition or respond well to antimicrobial treatment. However, aged or chronically infected bulls rarely recover from seminal vesiculitis Based on clinical and abattoir evaluation of reproductive tracts of bulls the prevalence

of infection of the seminal vesicles is reported to range from less than 1 percent to greater than 9 percent. Peripubertal bulls may spontaneously recover from septic seminal vesiculitis, but aged or chronically infected bulls rarely recover.[21-23]

Bulls with septic seminal vesiculitis are often classified as unsatisfactory potential breeders as their semen may be grossly contaminated with exudate and blood, but often, red blood cells and white blood cells can be detected only microscopically.[2] Abnormal concentrations of polymorphonuclear cells (PMNs), poor sperm motility, low fructose concentrations, and an elevated seminal pH are characteristics of semen of bulls affected with vesiculitis. Semen of bulls with septic seminal vesiculitis freezes poorly, and antibiotics used in extenders often do not significantly diminish the large number of bacteria in the ejaculate. Although chronic, unresponsive, septic seminal vesiculitis does not occur commonly in breeding bulls, the economic impact of this disease is considerable. The greatest economic loss associated with septic seminal vesiculitis occurs in bulls whose genetic value qualifies them for inclusion in an artificial insemination program.

The prognosis for bulls with chronic septic seminal vesiculitis is guarded at best. Prolonged antimicrobial therapy is often unsuccessful and complete surgical removal of affected glands is technically difficult.

The author has successfully treated bulls with chronic seminal vesiculitis by chemical ablation of the glands with 4% formaldehyde.[24] Restrain the bull in a chute and achieve caudal epidural anesthesia and introduce the hand into the rectum and identify the vesicular gland. Approximately 4 to 6 cm ventrolateral to the anus on the side adjacent to the infected vesicular gland introduce an 18 gauge x 30 cm stainless steel needle through the skin parallel to the rectum to a depth of 10 to 12 cm. With the aid of the hand in the rectum advance the needle and guide the tip into the vesicular gland. Inject 10 to 15 ml of sterile saline into the gland and palpate for gland enlargement to verify needle placement. Inject 4% formaldehyde into the gland until swells and its surface is quite firm. Withdraw the needle and repeat the procedure on the opposite vesicular gland if indicated. Immediately administer flunixin meglumine as bulls so treated frequently display signs of abdominal pain following the procedure. Allow sexual rest for 45 to 60 days before examining the ejaculate for evidence of seminal vesiculitis.

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Lameness in breeding bulls

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Abstract

Lameness or structural unsoundness often prevents a bull from being classified as a Satisfactory Potential Breeder. This presentation will review the anatomy and etiology of common causes of conformation flaws and lameness in breeding bulls. Lameness accounts for tremendous production loss in the cattle industry and has been identified as a particular concern in animal welfare. Cattle are relatively stoic animals and often do not show lameness until significant pathology is present. This discussion will explore anatomic and management relationships with common orthopedic conditions of the back, hip, stifle and feet in cattle. Additionally, diagnostic and therapeutic options will be reviewed.

Keywords: Bull, lameness, hoof, claw, stifle, fibroma

Introduction

The Computer Generated Breeding Soundness Evaluation (CG-BSE or eBSE) form was introduced at the Society for Theriogenology Annual Conference.[1] The ability to capture photographs of the bull onto the form is a valuable asset in documenting conformation, body condition and structural soundness of that animal.

In 2014, Dr. Robert Carson reported on trends of bull breeding soundness examinations utilizing records over a twenty year period.[2] For comparison, three time periods were evaluated: 1993-1995, 2006-2008, and 2009-2013. Data from bulls examined between 2009 and 2013 were put into the eBSE. Evaluation of those records between 1993 and 2013 revealed 5.4% to 10.4% of bulls presented to the Auburn University Large Animal Teaching Hospital were classified as unsatisfactory or deferred because of physical conditions. A large portion of these bulls were either lame or had conformational flaws that prevented them being classified as Satisfactory Potential Breeders. This paper will review the most common causes of lameness or conformational flaws in bulls.

Anatomy and conformation

Lameness in cattle is frequently associated with conformation flaws that create abnormal forces on the back, limbs and feet.[3-7] Additional causes of lameness are due to trauma or degenerative changes associated with heavy weight and aging. Conformation describes the dimensions and shape of an animal, and deviation from ideal conformation may lead to unsoundness as animals grow due to abnormal stressors on bones, tendons, ligaments and joints.[4] Posture refers to the manner in which an animal stands, and gait refers to the manner in which an animal moves.

When cattle are viewed from the rear, they should stand with the hind feet approximately as far apart as their hips. It is unusual to see cattle that are base-wide, that is, their feet are farther apart than the width of their hips; however, cattle that are base narrow with their feet closer together than the width of their hips are quite common, especially among beef breeds.[6]

Screw claw

Cattle that are base-narrow in their rear limbs frequently develop screw claw as the animal grows. Due to the conformation and abnormal forces on the hind feet, the tendency is for the hind feet to tend toward supination and the hoof wall of lateral claw (abaxial) hoof wall grows under the hoof displacing the sole dorsomedially.[5,7] Cattle may develop bruises on the sole of that lateral claw which frequently lead to sole ulcers or subsolar abscesses. Likewise, due to abnormal lines of stress on the hoof during weight bearing, vertical cracks frequently develop in the axial hoof wall. Additionally, degenerative arthritis frequently develops prematurely in the coffin, pastern and fetlock joints of affected animals.[7] This condition is common in continental breeds of beef cattle and their crosses and, in the opinion of the author, is becoming more prevalent in other beef breeds as greater selection is being applied to enhance

musculature of the beef carcass. Although the classic twisted or cork screw claw frequently does not develop until the animal is two or more years of age, the conformation leading to this condition is evident at weaning. Animals with this conformation are usually heavily muscled and base narrow when viewed from the rear.

When viewed from the side, there should be obvious but not excessive angulation of the joints of the rear limb. There should be approximately 130° between the femur and tibia and approximately 145° of angulation between the tibia and the metatarsus with the tuber calcis directly below the caudal aspect of the tuber Ischia . Animals with excessively straight rear legs (post legs) are more prone to develop joint, tendon and hoof diseases.[7] In the author's experience, large breed beef cattle with this conformation are more likely to develop cartilage disease in the stifle than more moderate conformation cattle.

Front limb conformation is also correlated with soundness. When viewed from the front the distance between the feet should be slightly less than the width of the shoulders, and the hooves should point straight ahead. Cattle that have toes that point out (laterally) are more likely to develop abnormal hoof growth similar to screw claw in the rear hooves. The adjacent toes on one hoof should be equal length, and the distance from the coronary band to the sole at the heel should also be equal.[7]

Laminitis

Chronic or sub-clinical laminitis is quite common in beef cattle due to the common practice of high concentrate feeding to achieve rapid growth and high yearling weights. Clinical or sub-clinical laminitis in cattle may cause an array of hoof problems and is frequently the predisposing cause of lameness.[8-10] Cattle develop overgrown "slipper" hooves and frequently suffer vertical or horizontal fissures in the hoof wall due to loss of flexibility of the hoof. Subsolar hemorrhage, bruising and ulceration are frequent sequelae to chronic laminitis. Additionally, the affected hooves often grow excessively long which changes the angles of the coffin, pastern and fetlock joints thereby leading to abnormal stresses on these joints and their supporting soft tissue structures. These hoof growth changes lead to white line disease and separation of the hoof wall from the lamina of the hoof. Premature degenerative joint disease is a common occurrence in severely affected cattle.

Interdigital fibroma

Interdigital fibromas, also called interdigital hyperplasia or corns, are proliferative growths of the skin of the interdigital space caused by dermatitis or chronic irritation.[8,10] The condition is more common in bulls than females and more common in heavy versus lighter weight animals. *Bos indicus* crossbred cattle appear to have a higher incidence of interdigital fibroma than *Bos taurus* cattle. Cattle that have a very wide interdigital space and cattle that are extremely narrow in the interdigital space appear more at risk for development of this condition than cattle with normal interdigital conformation. Rarely does the problem develop in cattle less than two years of age and most animals presented for treatment are four to seven years of age. It is troubling that in recent years significantly more two year-old Angus bulls have been presented to our teaching hospital for removal of these growths.

Treatment for interdigital fibroma involves surgical excision of the hyperplastic tissue utilizing local or regional anesthesia.[10] After thoroughly cleansing the area for aseptic surgery, grasp the apex of the interdigital mass with towel forceps. Begin on the dorsal surface and make a longitudinal skin incision along each side of the mass being careful to preserve the axial coronary band. Continue the dissection caudally until the entire hyperplastic tissue is removed. Remove any protruding interdigital fat with blunt dissection and apply topical antibacterial powder on sterile non-adherent surgical gauze over the incision. Wire the toes together to hold the bandage and to prevent separation of the claws. Remove the bandage in approximately five days and continue to confine the animal to a dry area for another two weeks. Systemic antibiotics are rarely indicated unless the interdigital fat pad is infected prior to surgery. Some surgeons routinely remove the interdigital fat pad; although, this technique slightly prolongs postoperative healing.

Stifle injuries

Stifle injuries are common in cattle, and one or more structures may be involved.[11] Rupture of the collateral ligament produces the least degree of lameness, and cattle with this condition are only slightly lame. Cattle with stifle injuries are generally reluctant to kick and are easier to examine than an animal without injury in this joint. The injury is most easily diagnosed by watching them walk away from you to observe instability during the weight bearing portion of the stride. Medial-to-lateral instability will cause the stifle to deviate either medially or laterally toward the affected side when the animal is bearing full weight. While standing behind the restrained animal, place fingers of one hand on the medial aspect of the stifle joint while abducting the lower limb. If the medial collateral ligament is torn there will be excessive joint space while the leg is abducted. Place the fingers of one hand on the lateral aspect of the stifle and adduct the lower limb to examine for excessive motion if the lateral collateral ligament is torn. The torn collateral ligament may be visualized by a skilled ultrasonographer.[12]

Meniscal injuries cause the next most severe degree of lameness in cattle. The most common meniscal injury is similar to other species in that the posterior horn of the medial meniscus is injured more commonly than the lateral meniscus. With acute injury, there may be evidence of joint effusion. Lameness will be evident during weight bearing and because the animal does not advance the limb normally while walking. The injury appears to occur more commonly in heavy muscled beef bulls than in other cattle. There may be an audible or palpable "click" during the weight-bearing portion of the stride. This damaged meniscus may be visualized by a skilled ultrasonographer.

The third common and most severe stifle injury is rupture of the anterior crucial ligament (ACL). This injury causes marked lameness and usually obvious joint effusion. The animal is very reluctant to bear weight on the affected limb, and the mass of the animal usually precludes palpation of the classical anterior drawer sign as may be detected in dogs. However, many beef cattle with this injury will tolerate flexion of the affected limb whereby the veterinarian may be able to detect excessive motion in the stifle joint and perhaps grating of bony surfaces due to loss of articular cartilage.

These stifle injuries are discussed together as they all appreciably shorten the productive life of cattle. Additionally, animals with an initial collateral ligament tear may quickly develop degenerative joint disease due to joint instability and abnormal wear of joint surfaces. Cattle with an initial meniscal tear likewise have the added risk of suffering cruciate ligament tears due to the atrophy of leg muscle that frequently and rapidly accompanies this injury and more severe loss of stability of the stifle joint. Cattle with cruciate ligament tears suffer severe joint instability, rapid muscle atrophy and frequently quickly develop meniscal tearing and loss of articular cartilage.

Therapy for any of the above conditions consists of confining the animal to a stall or small paddock that is level and free of mud for six to eight weeks. Bulls with anterior cruciate ruptures should not be used for breeding for a minimum of six months. Animals with this injury usually do not return to soundness and have permanent muscle atrophy on the injured limb. Analgesics are not recommended during the acute phase of the injury as animals so treated may use the limb excessively and sustain additional trauma to the joint. However, anti-inflammatory agents, joint lavage, polysulfated glycosaminoglycans and other therapies utilized in management of joint injuries in the equine athlete may prove beneficial in conjunction with a few months convalescence to assist a bull through a breeding season.

Shoulder injuries in cattle

Fortunately shoulder injuries are relatively uncommon in breeding cattle. Fractures or bruises of the shoulder are occasionally encountered in lightweight cattle while being worked in a chute which is usually due to excessively wild or excited cattle and/or inadequate footing, maintenance, or design of the working chute.

Fractures of the scapula or humerus occasionally result from bulls fighting. These injuries are readily diagnosed by the degree of lameness and swelling accompanying the injury. The spine of the scapula, humerus and shoulder joint are difficult to palpate in heavily muscled beef cattle, especially beef

bulls. Additionally, quality diagnostic radiographs are difficult to obtain due to the size and conformation of these animals.

Fortunately, fractures of the scapula or humerus often heal with stall rest in beef cattle. Contracture and swelling of the heavy muscles of these animals serve to reasonably splint the injured bones. These animals should be confined to a stall for a minimum of eight weeks followed by at least four months confinement in a flat paddock area. We do not recommend analgesic therapy as freedom from pain may induce the animal to excessively use the injured limb creating additional traumatic injury and potentially further displacing bone fragments.

Spinal injuries or disease

Diseases or injury of the spine are common among large bulls. Discospondylosis or spondylosis is commonly caused by repetitive trauma and increases with age and activity. The resulting, severe proliferative bony and fibrotic arthritis of the intervertebral joints may cause nerve root entrapment, and potentially spinal compression and pain. This condition may be confined to only one intervertebral joint but more often affects several in varying stages of progression. The hind limbs and spine are mainly affected. The proliferative bone growth may form arthritic bridges between vertebrae. These bony bridges may fracture, producing an acute crisis episode of pain or weakness. Probably less likely, fibrocartilagenous emboli may enter the blood stream resulting in stroke-like symptoms of the spinal cord.

Another spinal cord condition which may affect breeding soundness is spastic syndrome which is a latent recessive condition generally developing at two to seven years of age.[13] This syndrome is characterized by spastic contractions of the muscles of the hind limbs and back. These contractions are often mild and are most evident when the animal first rises after lying down. The syndrome usually persists for the lifetime of the animal and contractions are often exacerbated by arthritis or other painful conditions. In some bulls, the contractions tend to progress to more frequent and more severe episodes in the standing animal. Spastic syndrome condition is probably inherited as a single recessive trait with incomplete penetrance.

Conventional radiography or nuclear scintigraphy or bone scan may be useful for identifying or localizing lesions in the vertebral column, hips or limbs of bulls. Bone scan has been used for various applications in horses for many years and currently many private practices and most veterinary schools have gamma cameras. These cameras are used to image an injected radionuclide in the animal. Skeletal scintigraphy is quite sensitive and is well suited for detecting acute abnormalities as radionuclide uptake often precedes radiographic detection. Scintigraphy can also be useful in locating potential areas of abnormal osseous turnover in cattle with chronic or vague lameness.[14]

The authors have used radiography, ultrasonography and nuclear scintigraphy to localize and characterize sources of lameness in cattle. Several of these bulls returned to soundness and had semen collected for cryopreservation following intra-articular corticosteroid injection or steroid epidural therapy delivered through a spinal catheter. Similar to the equine patient, bulls may benefit from systemic or intra-articular glycosaminoglycan therapy and other anti-inflammatory modalities.

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Trichomoniasis in cattle

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Abstract

Bovine trichomoniasis is a sexually transmitted disease caused by the extracellular protozoa *Tritrichomonas foetus*, an obligate parasite of the reproductive tract of cattle. Infected bulls are often asymptomatic carriers of *T. foetus*. However, these infected bulls are capable of transmitting the organism to a cow during coitus. Infections in cows cause endometritis, cervicitis, vaginitis which may result in early embryonic death, abortion, pyometra, fetal maceration, or infertility. The major economic losses associated with *T. foetus* are due to: 1) reduced calf crop due to early embryonic loss or abortion, 2) reduced weaning weight due to delayed conception, and 3) culling and replacement of infected cattle . Due to the inability to use efficacious drugs, such as the nitromidazoles, for control and prevention of *T. foetus* infections in food animals, most control efforts have targeted identification and elimination of positive bulls, systemic immunization of cows and bulls, and management strategies to prevent introduction of the organism into the herd. This paper will review trichomoniasis in cattle and discuss pathogenesis of disease, transmission, consequences of infection, immunity, diagnostic techniques, and control and prevention strategies.

Keywords: *Tritrichomonas foetus*, trichomoniasis, bovine, cow, bull, prevention, control

Introduction

Bovine trichomoniasis is a sexually transmitted disease caused by the extracellular protozoa *Tritrichomonas foetus*, an obligate parasite of the reproductive tract of the cow and the folds on the mucosal surfaces of the bull's penis and prepuce. Infected bulls are often asymptomatic carriers of *T. foetus*. However, these infected bulls are capable of transmitting the organism to a cow during coitus.[1] Infections in cows cause endometritis, cervicitis, vaginitis which may result in early embryonic death, abortion, pyometra, fetal maceration, or infertility.[1] The major economic losses associated with *T. foetus* are due to: 1) reduced calf crop due to early embryonic loss or abortion, 2) reduced weaning weight due to delayed conception, and 3) culling and replacement of infected cattle. Due to the inability to use efficacious drugs, such as the nitromidazoles, for control and prevention of *T. foetus* infections in food animals, most control efforts have targeted identification and elimination of positive bulls, systemic immunization of cows and bulls, and management strategies to prevent introduction of the organism into the herd.

Pathogenesis in the female

Life cycle

The life cycle of *T. foetus* is thought involve two forms 1) a tear-shaped trophozoite form and 2) a pseudocyst form. The trophozoite is 10-25μm long and possesses three posterior flagella, one anterior flagellum and an undulating membrane. Trophozoite multiply asexually through binary fission.[2] Pseudocysts usually appear as a result of unfavorable conditions; although, a small percentage of pseudocysts exist under normal conditions.[3] Pseudocysts occur when *T. foetus* trophozoites round up and internalize their flagella in response to assorted stimuli.[3-5] The pseudocyst form lacks a protective cyst wall and does not represent a true cyst form.[4]

Trophozoites of *T. foetus* are transmitted between cows and bulls during coitus and remain in the genito-urinary tract where they multiply by longitudinal binary fission. Under stressful conditions trophozoites will internalize their flagella and replication of the nuclei and other cellular structures will occur, resulting in a multinucleated pseudocyst form. When conditions become desirable once more, mononucleate trophozoites will bud from the pseudocyst. In bulls, infections are usually chronic and asymptomatic and often persist for the life of the animal. Infected cows will initially experience vaginitis

which may or may not resolve spontaneously. In some cases, endometritis can occur resulting in complete sterility. Tritrichomonas infections may also result in fetal loss during pregnancy.[6]

Studies have revealed that pseudocyst formation and reversal can be rapidly and simply effected by certain cooling and warming patterns.[4] However, the induction of pseudocysts by chemicals, dependent of exposure time and concentration, can lead to an irreversible process that leads to the death of the cells.[7] Historically, there has been some uncertainty about whether pseudocysts represent a normal or infective form rather than a degenerative form. More recent research indicates that *T. foetus* is easily stimulated into the pseudocyst form and that these immotile pseudocysts are able to proceed with the process of adhesion to the vaginal epithelial cells.[5] In addition, it has been demonstrated that the pseudocysts are more cytotoxic when in contact with host cells when compared to trophozoites.[8]

Transmission

Cows become infected with *T. foetus* primarily through coital exposure with an infected bull. Subsequently, a mild vaginitis occurs that may go undetected. The organism gains entry into the uterine lumen via the cervix during estrus. Colonization of the entire reproductive tract with *T. foetus* occurs within one to two weeks.[9] Although, contaminated semen or contaminated insemination equipment may also be minor sources of infection.[1] Penetration of the vagina is seemingly necessary because swabbing the vulvar area with high numbers of organisms does not result in vaginal or uterine infection.[10] Infected cows conceive but infection causes endometritis, cervicitis, or vaginitis which results in death of the conceptus within the first half of gestation, abortion, pyometra, fetal maceration, or infertility.[1] These infected cows usually remain infertile for a period of two to six months. In heifers, the duration of infection is reported to be as short as 95 days[11] or as long as 22 months.[12] *Tritrichomonas foetus* has been detected in the reproductive tract for 13 to 28 weeks after experimental infection in heifers.[13]

Consequences of infection

T. foetus organisms arrive in the female reproductive tract concurrently with spermatozoa. However in most cases, fertilization occurs in spite of the presence of the pathogen. In vitro studies have demonstrated that fertilization and early embryonic development to the hatching stage (8-10 days) are not significantly affected by simultaneous culture with *T. foetus*.[14] Conceptus deaths most commonly occur between 50-70 days of gestation. Therefore, the majority of pregnancy loss is during the fetal period (>42 days of gestation). Although unusual, occasional abortions can occur of fetuses greater than four months of gestation.

Most producers do not recognize a problem in the early breeding season as conception occurs normally. The conceptus in most infected cows typically survives long enough to release sufficient interferon tau to prevent the prostaglandin $F_{2\alpha}$-mediated lysis of the corpus luteum. Fetal death in infected cows occurs between seven to ten weeks of gestation. Death of the conceptus during the early stages of pregnancy results in a prolonged interestrous interval.[9,15] Due to abortions and subsequent immunity, the distribution of pregnancies is unusually skewed with a higher proportion of pregnancies conceived towards the end of the breeding season. Although in many progressively managed herds with a limited breeding season, the bulls may no longer be available by the time the cow aborts and clears the infection. Therefore, *T. foetus* infection in a herd may go unnoticed until the time of pregnancy diagnosis when a high percentage of females are diagnosed not pregnant. Pyometra, along with abortions, may be the first physical signs of *T. foetus* infection in a herd, but are thought to occur in less than 5% of infected cows.[16] Pyometra results as the corpus luteum of pregnancy is maintained with a large purulent response which may cause damage to the uterine endometrium.[17]

Most infected cows will clear the organism and develop short-lived immunity of six months to one year. However, carrier cows do occur and are capable of spreading the protozoa. In the case of carrier cows, a very small percentage of cows (<1%) in infected herds have been shown to remain infected throughout pregnancy and into the following breeding season. Thus, the carrier cow has the potential to be quite devastating to control efforts and emphasizes that control programs must focus on the entire herd, not just the bull.[9]

Pathologic changes have been reported in several late-term, *T. foetus* aborted fetuses.[18] The placentas had focal or diffuse invasion of the chorionic stroma by *T. foetus* as seen on hematoxylin and eosin (HE) stained sections of placentas. There was also evidence of a moderate inflammatory cell infiltrate comprised mostly of mononuclear cells. Six of eleven fetuses that were examined had bronchopneumonia with identifiable trichomonads in the airways. Another examination of late term abortions associated with *T. foetus* described a necrotizing enteritis and pyogranulamatous bronchopneumonia with tissue invasion by trichomonads. The exact mechanism that leads to the death of the conceptus is not fully understood. Although, cytotoxic and hemolytic effects by *T. foetus* on mammalian cells have been described.[19]

The preputial cavity of the bull provides an ideal environment for *T. foetus* as the organism localizes in the preputial smegma of the epithelium of the bull's penis and prepuce. The organism does not penetrate the epithelium and does not cause any observable gross pathology or affect semen quality or libido. Histological changes are subtle at first with an increase in the number of neutrophils in the nonkeratinized, stratified squamous epithelium of the glans penis and preputial epithelium followed by an infiltration of lymphocytes and plasma cells penetrating into the intraepithelial area which coalesce in the subepithelium to form lymphoid nodules.[20]

The duration of infection with *T. foetus* for bulls is not clearly understood. There are two theories regarding this debate: 1) transient infection and 2) chronic carrier state. Bulls with the chronic carrier infection of *T.foetus* rarely clear the infection regardless of time. The pathophysiology of infection regarding the carrier state in mature bulls in not fully understood. *T. foetus* infections in bulls less than three to four years of age are more likely to have a transient infection. Younger bulls may not efficiently transmit the organism to a noninfected cow unless the sexual contact occurs within minutes to days of breeding an infected cow. Thus, transmission of *t. foetus* by a young bull is thought to be more passive, mechanical transmission as compared to transmission in older, chronically infected bulls. Nonetheless, any bull exposed to a *T. foetus* infected cow as a result of natural breeding is capable of becoming chronically infected, regardless of age.

Immunity

In the female, *T. foetus* induces inflammation of the mucosa of the vagina, the cervix, the endometrium and the oviductal mucosa. In the first one to two weeks after infection, neutrophils and eosinophils predominate; however, this is followed by a moderate to severe mononuclear infiltration of lymphocytes and plasma cells. Subepithelial and periglandular lymphoid nodules resembling lymphoid follicles begin to develop at almost six weeks post infection. In addition, there is also an apparent degranulation of mast cells between six to nine weeks after infection.[20]

T. foetus specific IgA and IgG_1 antibodies are detectable in uterine and vaginal secretions by the fifth to sixth week after infection. The IgA antibodies do not kill the organisms but may be responsible for immobilization and agglutination of parasites as well as preventing adhesion of the organisms to the mucosal surfaces. The IgG_1 antibodies are presumed to facilitate complement mediated lysis of the parasites as well as opsonization and enhanced phagocytic killing by neutrophils or macrophages. Immunity following natural infection and clearance of *T. foetus* is short-lived with females becoming susceptible within a year, in time for the following breeding season. Because *T. foetus* is an extracellular pathogen, the immune response from the host is predominately humoral and the result of the short-lived immunity. The uterine mucosal inflammation that is seen with infection may allow systemically derived IgG and complement to gain access to the lumen of the uterus and, thus, clear the organism. A relative lack of IgG from the vagina or possibly blocking of IgG effects by vaginal IgA binding of organisms may help explain the carrier state that can be seen in infected herds.[20]

Although specific immunoglobulins have been detected in small amounts in preputial smegma by some researchers, there seems to be no effective acquired immunity to *T. foetus* in the mature bull.

Diagnosis

The comparison of diagnostic assays for detection of *T. foetus* infections has primarily focused on the bull. Collection of *T. foetus* samples from bulls involves recovering the organism from the preputial cavity of the bull. Several techniques have been described for collection of diagnostic specimens in the bull and include a dry pipette technique, a wet pipette technique a douche technique and a swab technique. While the douche method is preferred in Europe, the dry pipette technique is most commonly used in the United States. Regardless of which technique is used, it is generally recommended that bulls be given two weeks of sexual rest prior to sample collection in order to allow accumulation of the organism on the bull's penis and prepuce and a greater chance of recovery.

Isolation of *T. foetus* from the female is reported to be less sensitive when compared with techniques used for bulls.[21,22] In one study, the InPouch™ TF system (BioMed Diagnostics, Inc; White City, OR) was more effective than Diamond's medium (88% versus 68% in detecting heifers that had been experimentally infected with *T. foetus*.[23] The accuracy of prevalence in the cow most likely depends on the timing of sampling relative to exposure. The immune response in females begins to eliminate the infection within eight to ten weeks after exposure in unvaccinated females.[13] Therefore, cultures from females are best performed before the infection is possibly eliminated by the immune response.[23]

Sample handling is also crucial for accurate detection of *T. foetus*. When evaluating temperature and media type it has been found that when laboratory of field isolates were cultured in Diamond's medium or InPouch™ TF, all cultures were positive for *T. foetus* when maintained for up to four days at either 22° or 37°C. However, samples maintained at 4°C or less resulted in inconsistent sensitivity.[24] It is important to remember that time, temperature, type of isolate, and type of medium all have an effect on the sensitivity of *T. foetus* culture.

Microscopic evaluation of cultured organisms is not sufficient to differentiate *T. foetus* from nonpathogenic intestinal or coprophilic trichomonads (*Pentatrichomonas hominis*, *Simplicimonas moskowitzi*, *Tetratrichomonas* spp., etc).[25] Therefore, several conventional and real-time polymerase chain reaction (PCR) assays have been developed for the definitive diagnosis of trichomoniasis, and this methodology has demonstrated some advantages over culture.[25] However, accurate PCR results are directly related to the quality of the sample, which can be affected by transport condition parameters such as temperature and time of transport to the laboratory. There have been a number of issues that have limited the sensitivity of various conventional PCR assays for the detection of *T. foetus*. These problems include DNA degradation, accumulation of inhibitory compounds, sample contamination, and unexpected amplification products.[26] One study demonstrated a decrease in sensitivity of PCR testing with samples that were stored for five days or more. However, PCR was in 100% agreement with culture as long as the PCR was performed within 24 hours of the sample being submitted.[26]

A more recent study evaluated the effect of different simulated transport conditions on samples containing *T. foetus* for the diagnosis of trichomoniasis using culture and quantitative PCR (qPCR).[25] This study demonstrated that transport temperatures of 4-20°C for one to three days before culture reduced or temporarily inhibited parasite replication but maintained viability. Samples tested by either culture or qPCR would have been expected to give positive results. However, diagnosis of trichomonads by both methods was negatively affected when specimens were maintained at transport temperatures of 42°C for 24 hours or more. This study emphasizes the importance of ensuring that clinical samples arrive to the diagnostic laboratory within 24-48 hours and of avoiding temperature transport conditions above 37°C in order to achieve an accurate diagnosis of *T. foetus*. The effects of high incubation temperatures on culture and real-time PCR for *T.* foetus have also been evaluated following inoculation into the InPouch™ TF system.[27] This study showed that *T. foetus* was detectable at microscopically in inoculated pouches incubated at 37°C regardless of exposure time (1, 3, 6 and 24 hours), whereas those samples incubated at 46.1 °C detected *T. foetus* only after one and three hours of incubation. *T. foetus* was detected in samples incubated at 54.4°C after only one hour. Testing using real-time PCR for all inoculated medium samples (37°C, 46.1°C, and 54.4°C at 1, 3, 6 and 24 hours) produced positive results for all inoculated medium samples. This study suggests that samples collected for culture alone should be protected from high temperatures.

Prevention and control

One complicating factor with bovine trichomoniasis in the United States is the lack of effective treatments with Food and Drug Administration approval. Historically, the most successful treatment for bulls with trichomoniasis was systemic treatment with nitromidazole derivatives.[28] Currently, the use of nitromidazole derivatives is illegal in food-producing animals in the U.S., and no effective alternative treatments are available. The lack of effective, approved therapies for bovine trichomoniasis emphasizes the need for appropriate preventive and control measures. Prevention of trichomoniasis includes the following recommendations: 1) avoid movement of animals (co-grazing, leasing of bulls, good fences); 2) utilize artificial insemination, if possible; 3) use a defined breeding season and cull all non-pregnant females after the breeding season; 4) purchase virgin bulls and heifers as replacements; 5) test all bulls for *T. foetus* prior to introduction into the herd and maintain a young population of bulls; and 6) breed purchased cows and heifers in a separate herd.[9]

Once *T. foetus* has been confirmed in a herd, there are additional measures that should be considered in order to "clean up" the herd. These measures include 1) testing and culling all infected bulls and purchasing *T. foetus* negative bulls; 2) intense management of bulls so that smaller breeding units are used and bulls are bred to the same cattle until trichomoniasis is under control; 3) create high and low risk herds; and 4) vaccinate all herd females with an approved *T. foetus* vaccine.[9] Vaccination is an important aspect of any control program as it has been shown to reduce pregnancy wastage associated with *T. foetus* infection in cattle herds. Currently, TrichGuard® (Boehringer Ingelheim Vetmedica, Inc.) is the only commercially available vaccine licensed by the USDA for the control of trichomoniasis in the United States. TrichGuard® is a proprietary vaccine that is a Freund adjuvant killed *T.* foetus-derived vaccine that requires two doses subcutaneous injections administered two to four weeks apart with the last injection to be given four weeks prior to the breeding season.[9] One study compared pregnancy and calving rates between beef heifers vaccinated with TrichGuard® and control heifers after heifers were exposed to *T. foetus* infected bulls and intravaginally inoculated with a large number (10 million) of *T. foetus* organisms.[29] At calving twice as many vaccinated heifers calved when compared to control heifers (61% versus 31%). Thus, the vaccine appeared to offer at least some protection against *T. foetus*.

Conclusion

Trichomoniasis can be an economically devastating infection in cattle herd with losses due to reduced calf crop due to early embryonic loss or abortion, reduced weaning weight due to delayed conception, and culling and replacement of infected cattle. Carrier females and concerns with diagnostic sampling and testing have made the control of trichomoniasis in cattle even more complex. Control and prevention of *T. foetus* infections in cattle must focus on identification and elimination of positive cows and bulls, systemic immunization of cows and bulls, and management strategies to prevent introduction of the organism into the herd.

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Semen evaluation and overview of common sperm abnormalities

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Abstract

A veterinary breeding soundness examination as directed by Society for Theriogenology (SFT) standards provides information that is vital for the cowman and in turn the beef cattle industry. An incomplete exam, specifically the omission of a microscopic evaluation of sperm morphology compromises the results and thus its value. This is also the case with a poorly performed morphology exam whether due to sample handling, equipment (microscope) quality or competency of the veterinarian.

From the standpoint of specific sperm abnormalities, when identified in significant numbers, there is utility in assigning prognosis if possible. Likewise, a suspected etiology can also differentiate bulls that might be held for re-testing versus immediate culling. Thus the category of abnormality, suspected etiology, and age of the bull represent information useful in providing both a prognosis and the scheduling of a date for re-test.

Keywords: Bull, breeding soundness, morphology, sperm abnormality

Introduction

The economic justification for a veterinary breeding soundness examination (VBSE) is based on the identification and the subsequent culling of individuals that will potentially have a negative effect on cowherd productivity. Basically, this effect can be expressed both by decreases in pregnancy percentage and sale weight, with secondary resulting effects being increased culling of open cows and those that appear to be less productive, and also the insidious propagation of low fertility offspring. While percent pregnant has an obvious and easily observed impact on profitability, decreased sale weights due to calves being conceived late in the breeding season might be less readily identified by a producer, as is the decrease in fertility of retained females . Specifically, low pregnancy rates and/or a wide distribution of calvings are the hallmarks of a subfertile bull and as the completely sterile bull is rare, it is the identification of this individual (subfertile bull), which in the general population can represent 20-40% of all bulls,[1] that is the focus of our efforts. For a variety of reasons, evaluation of sperm morphology is the single best tool for identifying these bulls. Morphology and indeed every aspect of the VBSE must be performed conscientiously, consistently and competently in order for the results to be valid. The purpose of the following narrative to provide guidance toward a consistent approach with regard to semen evaluation.

Sample collection, handling and slide preparation

A detailed review of semen collection is beyond both the scope and mission of this manuscript, but needless to say a suitable sample is most efficiently obtained via electroejaculation, following rectal palpation in which the urethralis muscle is stimulated and the rectum fatigued. Additionally, a good quality, uncontaminated (absence of dirt/hair/debris) sample is more easily obtained when the penis is extended and gross observation indicates a color change of the ejaculatory fluid from clear to milky and preferably creamy white. As sperm motility must be assessed, careful handling from collection to microscopic evaluation is crucial and can be accomplished by utilizing a disposable, Styrofoam™ coffee cup as a receptacle followed by examination within 3-5 minutes with a pre-warmed slide. The process described works fairly well as long as the environmental temperature is greater than 40° F and duration of time to evaluation is short. When ambient temperatures are below 40° F utilization of a warmed collection vial is recommended.

The evaluation of semen begins with a gross examination, following this visual assessment, motility is evaluated microscopically, and following preparation of a suitably stained semen smear morphology is performed. Because of both the importance of the morphology examination and the need,

when evaluating large numbers of bulls, to proceed quickly, the microscopic evaluation of morphology can be postponed until a later time.

Evaluation of sperm morphology is facilitated by the use of a good semen smear. It is crucial to start with a clean, warm slide. Some "new" slides may have detergent coatings that interfere with staining. India ink is an acceptable stain but does not actually stain the sperm cells and is considered a "background" stain. Eosin-nigrosin (E-N) is a vital stain and is the currently recommended (SFT) stain. It is easy to use and has consistent staining properties. The eosin portion will penetrate dead sperm cells, staining them pink (red is dead) while leaving live cells unstained (white) against a dark background provided by the nigrosin component. A smear stained with Diff Quik™ is also adequate for visualizing sperm morphology and provides the advantage of allowing easy visualization of white blood cells. Another useful staining procedure is the Feulgen staining method. This technique begins with preparing a smear and then allowing it to dry for an hour. Next place the slide in 5 N HCl for 30 minutes. Wash the slide by running water into the corner of a staining dish containing the slide for two minutes. Then place the slide in Schiff's reagent for 30 minutes. Wash again as before and air dry. The Feulgen technique is superior for identifying the nuclear vacuole (crater) defect and because the process removes fat globules, it is an excellent staining technique for smears from extended semen.

My approach utilizing the SFT E-N stain is to first place a small droplet of stain on the slide, then add a drop of semen and mix. Placing the semen drop first followed by the stain can result in contamination of the stain solution if the tip of the bottle or dropper inadvertently touches the semen. Once the stain and semen is mixed a second slide is used to push (spread) the semen across the slide in the same manner a blood smear is prepared. Because the feathered edge is the best area for evaluation, an alternative method is to create several thickness gradients by stopping and starting as the mixture is spread. It is also often a good idea to make a second slide at the same time, as it is faster to make two, than to come back later and make another slide on the occasion that the first slide was not of diagnostic quality.

Equipment

The type and quality of microscope utilized is likely to be one of the causes of the inconsistency in results cited as a problem with VBSE's and indeed there seems to exist among theriogenologists a strong preference for the use of phase contrast microscopy.[2] A study compared the evaluation of sperm morphology with a wet mount (fixed with isotonic formol saline) sample utilizing differential interference phase contrast (DIC) or an E-N stained dry-mount bright field (BF) microscopy provides insight on this issue.[3]. In this study the sample slides were examined at 1000X. The DIC was superior in identifying morphologic abnormalities of the sperm head such as the presence of vacuoles but the percentage normal was the same with both types of microscopy. Since the percent normal did not differ, clinical results should not be affected. So based on these findings, for the routine VBSE the use of BF microscopy and the E-N stain is adequate. Another study comparing the use of the E-N stain with BF microscopy, Feulgen-stained with BF microscopy, or phase contrast microscopy revealed that the Feulgen identified more head defects and the phase contrast revealed more distal cytoplasmic droplets.[4] Again, the authors felt that the differences were not enough to be clinically important.

With respect to microscope quality if maintenance and cleaning are assumed and the oil immersion objective utilized to evaluate a properly prepared sample a simple guideline follows: if you can readily identify the diadem defect in a spermogram, the microscope you are using is of adequate quality. If this defect is never observed, you should likely upgrade your microscope.

Gross evaluation of semen

After collection the semen should first be observed grossly. A rough estimation of the concentration can be made based on the opacity or lack of and the color of the semen. Very concentrated samples look like heavy cream while very dilute samples have the appearance of watered down skim milk. Yellow tinted semen can result from urine contamination and this can be substantiated by smell or the use of a blood urea nitrogen test strip. Additionally, semen contaminated with urine will not be motile

or at least will display rapidly declining motility when examined microscopically. Conversely a gold appearance is also associated with very highly concentrated semen and the presence of riboflavin.[5] This is a common finding in many Jersey and some Angus bulls. Red or brown colored semen indicates the presence of blood or blood pigments and the source should be determined.

Evaluation of sperm motility

A small "standing" drop is placed on a pre-warmed slide and evaluated under low power microscopy (40X-200X) for gross motility. Thick, dark, rapidly oscillating swirls are indicative of excellent motility (defined as a high velocity or high speed motility), a high percentage of sperm that are progressively motile, and a sample of high concentration. That sample would typically be classified as Very Good. A sample that displays slower moving swirls is classified as Good. A Fair sample displays no swirls, but significant individual sperm movement. A Poor sample has no or very little movement/oscillation. Because the concentration of a sample impacts the gross motility designation, individual motility should be assessed if there is any question about the validity of a motility rating based on gross motility. Individual motility can be assessed utilizing 200X-400X microscopy and depending on the concentration either a cover slip over either the previously examined droplet or a diluted droplet (dilute w/ warmed sodium citrate solution). Individual motility greater than 70% is Very Good, 50-69% is Good, 30-49% is Fair, and if less than 30% the sample is categorized as Poor.[6]

Based on our current standards (SFT), bulls must have a minimum of Fair sperm motility based on either individual or gross assessment.[7] While this may seem to be low, it is a minimum threshold and while there is a positive correlation between motility and fertility with the use of artificial insemination, this is not reported to be the case with natural service bull fertility.[8]

Evaluation of sperm morphology (the spermogram)

There is no aspect of the VBSE in which there are greater concerns with respect to the delivery of accurate and repeatable results than the portion dealing with sperm morphology. Specifically, inconsistency among veterinarians due to differences in their ability to evaluate a spermogram[9,10] and this does not take into account veterinarians or others that perform VBSEs or the euphemistic "semen check", fertility examination, etc. but do not include the evaluation of sperm morphology. This underscores the need for adequate training for veterinarians who wish to provide this service and the protection of the public (cattlemen) from those that pass off a substandard service as a VBSE.

Current SFT- VBSE standards set a morphology threshold of 70% normal with no distinction in regard to classification of abnormalities.[7] The 70% normal metric can be justified by a study[11] in which cows and heifers exposed to bulls with either 70% or 80% morphologically normal sperm had similar pregnancy rates and these pregnancy rates were statistically higher than cows and heifers exposed to untested bulls. An additional argument can be made due to discrepancies among the various classification systems of sperm abnormalities- Primary vs Secondary, Major vs Minor, Compensable vs Uncompensable; and the realization that as more becomes known about a specific abnormality it's classification status may change.

For comparison sake, the standards set forth by the Western Canadian Association of Bovine Practitioners sets a minimum morphology standard as no more than 30% total sperm defects **OR** 20% nuclear (head) defects.[12] This approach provides, at least to some degree, a safeguard with respect to the accounting of those defects believed to be more significant in their impact on fertility, specifically by the fact that their occurrence in an ejaculate is predictive of the presence of defective cohort sperm that appear morphologically normal.[13-15] Therefore, it appears to me that for those of us utilizing the SFT standards, we might at the very least more closely scrutinize those morphology smears that display greater than 20% head abnormalities and take that into consideration when evaluating the marginal bull.

Generally speaking, the important thing to remember when approaching the evaluation of a spermogram is that the presence of a specific sperm abnormality in significant numbers represents the clinical manifestation of a problem occurring during either spermatogenesis or sperm transport. This "problem" could be the pathological response to a transient insult as is seen with stress or exposure to

environmental temperature extremes of short duration. It can also represent permanent or at least semi-permanent pathology such as found with testicular degeneration. Insult to the seminiferous epithelium resulting in abnormal spermatogenesis and in turn the presence of abnormal sperm also results in the production of sperm that while morphologically normal in appearance undoubtedly have a compromised ability to fertilize an ovum. Thus an ejaculate with 30% abnormal sperm present does not mean that we can say with certainty that there are 70% completely normal sperm, but instead is representative of a threshold by which we can reasonably assure the fertility of that bull to a level that meets the standard described in the introduction.

Commonly encountered sperm abnormalities

Detached head

The normal detached head, which is found in small numbers in virtually all ejaculates, is often present in high numbers in bulls following sexual rest ("rusty load" scenario), in peripubertal bulls, and from bulls that have experienced a recent stress with or without a high fever. It is also found in young bulls with testicular hypoplasia, but these should have already been excluded from further testing based on an inadequate scrotal circumference measurement. It is categorized as both a secondary and minor abnormality and is considered to be compensable due to the obvious lack of motility . Detached heads that are abnormal are categorized based on that abnormality.

If this defect is found in threshold levels, the bull can often be re-collected immediately and the number will be dramatically decreased. When placed in the deferred category, bulls with a history of recent stress etc. can be re-tested as early as two weeks, because it is an abnormality of epididymal origin and epididymal transit is around 11 days. Bulls believed to be peripubertal may benefit from a longer wait time before re-testing.

A very rarely encountered version of the detached head that has a genetic basis and in which affected bulls are sterile is the presence of separated and motile tails. This is a different, distinct abnormality in which 80-100% of sperm in an ejaculate will be affected.[16]

Distal midpiece reflex

The distal midpiece reflex (DMR) is the most common abnormality of the sperm tail.[17]. It is considered to be epididymal in origin and therefore a secondary defect. It is also categorized as a minor defect and compensable. This defect is compensable due to the lack of forward, progressive motility. Evidence for its origin is based on its rapid appearance in the ejaculate of bulls within a few days of a thermal insult. This defect appears as a sharp hairpin bend at the distal midpiece[18] with a cytoplasmic droplet within the bend. If a droplet is not observed to be present, it is likely that the "bend" is due to contact with a hypotonic solution, presumably the stain that was used. This defect can often be identified during the evaluation of motility as the affected sperm will appear to be swimming backwards.

The etiology is believed to be due to a negative effect on epididymal function due to depressed testosterone levels which can in turn be caused by stress, thermal stress (either high or low), exogenous estrogen, or induced hypothyroidism; although normal, fertile bulls can have up to 25% of this defect in an ejaculate[17] due presumably to its compensable nature. However, I have observed that when this defect is present at a level of 20-25% in the ejaculate of a bull that meets standards (>70% normal) when tested at a time of moderate weather and absence of stress, the same bull when re-evaluated during times of environmental temperature extremes will have an increased percentage of this defect in his ejaculate. I now closely scrutinize these bulls and discuss this issue with the owner with respect to the time of year that the bull will be placed into service. Additionally, that this defect could very well have a genetic etiology or at least predisposition in some of the beef breeds is something that should be considered.[19] It has definitely been shown to be heritable in Jersey bulls, some of which would have up to 100% DMR defective sperm in an ejaculate.[20]

Cytoplasmic droplets

The distally located cytoplasmic droplet is thought to be epididymal in origin and its significance or rather status as an abnormality is debatable. As there is no correlation between a high incidence of this sperm type and infertility and also due to the fact that these sperm will often shed their droplets during even short periods of incubation,[21] these should be re-categorized as a variation of normal.

Proximally located cytoplasmic droplets are a result of abnormal spermiogenesis and are categorized as uncompensable.[17,20] However, the placement of the proximal droplet defect in the uncompensable category might be problematic as a study that used ejaculates of either high or low numbers of sperm with that defect for in vitro fertilization revealed that while fertilization was decreased as the percentage of proximal droplets in an ejaculate was increased (this meets the definition of uncompensable), of the ova that were fertilized, cleavage rates were similar.[22] Indeed the presence of abnormal sperm (proximal droplets) did not impact the fertilizing ability of the normal cohort sperm insinuating that increasing the number of normal sperm could "compensate" for the presence of the defect. This reiterates the problem of becoming overly concerned with the categorization of certain sperm abnormalities instead of focusing on the likely etiology and in turn prognosis. Specifically, in the case of this defect, as previously stated the cause is undoubtedly due to abnormal spermiogenesis with the potentially underlying etiology either immaturity or conversely testicular degeneration. So in the case of the young peripubertal bull we can place that individual in a deferred status with the reasonable assurance that with age (maturity) his spermiogram will improve and in the case of the older bull we can safely assume testicular degeneration and therefore less chance for a return to fertility.

Abnormal midpiece

I will include in this category the "pseudodroplet" defect and the various mitochondrial sheath defects as well as "Dag like" defects. Additionally, the midpiece may appear swollen, "corkscrew", bent, or asymmetric. These defects are all designated as compensable because of the obvious impact on motility and all are classified as a primary defects in the SFT system. Since the development of this sperm region occurs almost completely during spermiogenesis the specific origin for most of these defects is undoubtedly testicular. It has been shown that some forms of this group of defects can be caused by increased levels of gossypol,[23] a compound found in the cotton plant and specifically cottonseed, in the diet of bulls. Bulls fed diets high in gossypol appear to be especially sensitive to this compound during puberty.[24] The etiology of defects caused by gossypol appears to result from damage to sperm structure during spermiogenesis with further damage occurring during epididymal transit.[23] From a practical standpoint, simply limiting the intake of whole cottonseed to less than five pounds per day for bulls of an age presumed to coincide with the attainment of puberty should be sufficient to avoid this problem. Also, this seems to be more common in Brahman bulls and indeed in the Chenoweth report[24] the bulls described were Brahman. This or at least a similar defect can be created in rats fed gossypol and also rats deprived of selenium.[17]

The specific abnormality referred to as a pseudodroplet is actually not common and may have a genetic component.[17] It is best described as local thickening at and slight thickening of the midpiece.

Pyriform head

This is the most common defect of the sperm head[17] and is usually found in low numbers even in the ejaculates of fertile bulls.[18] Because there are bulls of normal fertility that have narrowed sperm and there appear to be variations in the range of "taperedness", it can be hard to distinguish at what point a designation is made between normal and pyriform.[17] For example, in the human, sperm formerly categorized as pyriform or pear-shaped sperm are no longer considered as abnormal.[25] However, there was not a clearly defined distinction with regard to degree of taperedness. In veterinary literature this is a defect and categorized as both a primary and major defect. The evidence for whether or not this abnormality is compensable is equivocal. In general, sperm with misshaped heads do not transverse the reproductive tract, but sperm with this defect apparently do,[26] although that in fact, appears to be dependent on the level of deformity.[27] The level of deformity also apparently impacts fertilization rates as

trials evaluating this defect reveal decreased levels of zona penetration, fertilization, and cleavage rates.[27,28] For example the previously cited work revealed that semen containing this defect at high percentages (85% pyriform heads) had zona penetration at about half the rate of control (90% normal) semen. Considering that semen containing a high percentage of pyriform head defects still resulted in some, albeit much lower, fertilization, these authors came to the conclusion that this could be due to the presence of a small number of normal sperm as well as a percentage of less affected pyriform sperm that may be capable of successful fertilization suggesting that this abnormality could be partially compensable.[28]

With respect to etiology this defect is seen following environmental heat stress, validated by scrotal insulation studies,[13] and also from bulls with testicular hypoplasia.[17] In addition to environmental causes of heat stress, the scrotal insulation effects of fat deposition around the scrotum that results from heavy feeding during gain tests has the same deleterious effect. Bulls examined after recently coming off a gain test or going through adverse environmental extremes that have this abnormality in numbers that contribute to not meeting the metric for percent normal sperm should be deferred. In the case of bulls with testicular hypoplasia, they typically do not meet VBSE standards for scrotal circumference anyway and older, mature bulls that do not have a history of a transient insult that would provide a reason for a disruption in spermatogenesis carry a poor prognosis.[17] But remember, slight degrees of taperedness may be normal. Also those young, over-fitted bulls that are deferred, might need more than 60 days to recover and meet standards.

Terminally coiled tail (coiled principal piece)

The terminally coiled tail defect also termed a coiled principal piece has been described to not be as commonly found,[17] but we seem to encounter bulls with significant numbers of this defect especially following environmental temperature stress. It will be seen with other heat stress related defects and this has been documented by a scrotal insulation trial.[13] It is also a defect that is increased proportionally following gossypol toxicity [29]. Due to poor motility it is compensable.

Less commonly encountered sperm abnormalities that are important due to their significance

Knobbed acrosome

The knobbed acrosome defect can be identified as an apical swelling that may protrude from or fold over the head[30] but appears most often as a flattening or indention of the apex.[17] This defect was identified as having a genetic etiology, specifically being an autosomal sex-linked recessive trait in the Friesian breed.[16,30] A genetic etiology should be considered when this defect is prominent in the ejaculate over time, but when identified with several other defects an environmental (temperature related)[13,14,17] or other transient cause is likely. Thus when this defect is encountered with other head defects there is a better prognosis for recovery[31] and it would be prudent to defer and recheck the bull in 60-90 days. It is considered to be both a major and primary defect; and the best current evidence is that it is uncompensable.[14] The uncompensable nature of this defect is not straightforward as it actually appears to be compensable based on the fact that sperm with this defect don't transverse the reproductive tract of cows efficiently[26] and those that do are unable to penetrate the zona pellucid.[14] However, this defect is the perfect example of a defect the presence of which denotes the occurrence of normal appearing, but defective cohorts.[13-15] These defective, but morphologically normal cells although able to penetrate ova, had lower rates of fertilization and reduced cleavage by zygotes.[14,15] Therefore from a practical perspective we should scrutinize more closely those bulls whose ejaculate displays this defect predominately in large numbers (>20%) as we know it can have a genetic basis and that it expresses infertility at levels higher than its occurrence within an ejaculate.

Nuclear vacuole

The nuclear vacuole defect is also termed as a crater defect and includes the diadem defect, which is a string or line of vacuoles around the acrosome-nuclear cap junction.[32] While small numbers (<15%)

of this defect in an ejaculate can be compatible with fertility, larger numbers suggest a disturbance in spermatogenesis and in fact most instances in which this defect is present at 10% or greater it is accompanied by other defects that reduce semen quality.[33]

The etiology of this defect is undoubtedly environmental stress as the appearance of this sperm abnormality follows within days of the administration of dexamethasone or the application of scrotal insulation[34] and the possibility of a genetic etiology has been ruled out.[35] The prognosis for recovery is good if the inciting cause is eliminated.[34,36]

Dag

The Dag defect named for the Jersey bull from which it was identified has a genetic etiology. Because up to 100% of the sperm in an ejaculate can be affected[21] it has proven to be largely self-limiting. "Dag like" defects seem to be an etiologically distinct abnormality and were grouped with the midpiece defects.

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Protecting your investment: nutrition for the broodmare

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Introduction

The American College of Veterinary Nutrition (ACVN)'s circle of nutrition includes three components of a nutritional assessment: patient assessment, diet/ration assessment, and feeding management assessment. For this talk I will address the three components for the broodmare. The nutritional goals for the mare are to maximize health (i.e. fertility, fetal development, milk production) and prevent disease/abnormalities (i.e. reduced fertility, altered fetal development).

Assess the mare

The two main components of patient nutrition assessment are body weight and body condition score (BCS).[1] Body condition is important for determining whether the current body weight is appropriate or not and thus will influence what and how you feed. Ideal BCS for adult horses ranges from 4-6/9, but a BCS of 6/9-7/9 is generally considered ideal for the reproducing mare. Increasing body condition at conception has favorable results on mare fertility.[2] Having a BCS of <5/9 may negatively affect the brain-hypothalamic-pituitary axis and has been associated with a prolonged anovulatory period, more cycles to conceive, and lower conception rates in mares.[1,3] An obese body condition (>7/9) does not appear to reduce reproductive efficiency; however, there are other obesity comorbidities (i.e. laminitis, abnormal thermoregulation) that can compromise the mare's health. Furthermore, there is compelling evidence from the human and rodent world that gestational obesity can lead to long-term problems in offspring.

Ideally, the mare would be in good condition *before* breeding and improvements in the mare's BCS should ideally be completed prior to breeding. Declining BCS and weight loss increases the transition period and time to first ovulation in mares.[2] The negative affect of weight loss on fertility appears to be greater in overweight mares and may be due to the effect of adipose tissue-derived fatty acids on hormones.[2] The duration of time needed to achieve ideal BCS depends on how much change is needed. In general, aim to change BCS at a rate of 0.5-1 BCS point on a 9-point scale each month.

In addition to body weight and BCS, the mare's physiologic status, future plans for the mare, and presence of clinical abnormality should also be considered. The physiologic stages of the mare include maintenance (maiden/barren, early gestation), late gestation, early lactation, and late lactation.

Maintenance and early gestation

Mares in early gestation may be generally managed as a barren/maiden mare because fetal growth is slow and thus nutrient requirements are similar to maintenance, with the exception of higher vitamin E requirements. Expected dry matter intake during maintenance is 2% BW, but may be higher for exercising mares. Energy requirements may be higher during mid-gestation (5-8 months); monitoring of BCS throughout gestation will help determine when the mare's energy requirements are no longer similar to maintenance. Protein quantity and quality positively influences reproductive efficiency and thus protein requirements appear to be higher for the reproducing mare as compared to a mare at maintenance.[3]

Late gestation

Late gestation represents the last trimester (months 9-11) when the majority of fetal gain occurs. Expected weight gain during gestation is 12-16% of pre-gestational body weight.[4] Compared to maintenance, the mare in late gestation has higher requirements for digestible energy, protein, calcium, phosphorus, iodine, copper, iron, and vitamin E. Energy excess in late gestation will promote mare, but not fetal, weight gain and has no effect on foaling.[5] Conversely, underfeeding energy may prolong gestation potentially due to the effects of negative energy balance on prostaglandin concentrations and

glucose homeostasis.[6,7] Energy source, regardless of quantity, in the mare's ration may influence foal's insulin sensitivity. Thoroughbred foals of late gestation mares fed a concentrate with 39% starch, on a dry matter basis, had lower insulin sensitivity compared to Thoroughbred foals from mares fed a concentrate with 4% starch, on a dry matter basis.[8] The high starch concentrate also had lower fat and fiber; the effect of these cannot be teased out. Maternal diet positively influences fetal trace mineral stores (i.e. hepatic copper, selenium), which are essential for early pre-weaning growth because milk is generally a poor source of trace minerals like copper. Copper is a key nutrient in the prevention of nutrition-related developmental orthopedic disease in foals. Mares should be fed 0.25 mg copper for each kg of body weight (125mg for a 500kg mare) during the last trimester.[3] Folate is a key nutrient during gestation and is important for neural tube development; however, B vitamin requirements for the mare appear to be met by gastrointestinal microbes alone.[3]

Early lactation

Early lactation represents the first three months of lactation when milk production is about 3-4% of body weight per day. Nutrient requirements for lactation are influenced by the nutrient profile of the dam's milk (accounting for individual variation), increased nutrient assimilation (due to higher DM intake/higher plane of nutrition), increased waste production (i.e. elimination of metabolic waste products), and increased physical activity.[3] The mare in early lactation, as compared to maintenance, has higher requirements for digestible energy, protein, calcium, phosphorus, iodine, copper, iron, and vitamin E. Furthermore, compared to late gestation, the mare in early lactation has higher requirements for zinc and selenium. Energy restriction of the mare during lactation has negative effects on mare body condition and milk production and subsequent foal growth.[9] However, my observation is that foal growth, and presumably milk production, is effected to a less degree than mare body condition. Relative to colostrum, there is no evidence that supplementation of a *complete* ration improves colostral immunoglobulin concentration or foal immunoglobulin status.

Late lactation

Late lactation represents the fourth month of lactation through weaning when milk production begins to decline; by the fifth month of lactation, milk production is about 2% of body weight per day.[3] Compared to early lactation, the mare in late lactation has lower requirements for digestible energy, protein, calcium, and phosphorus.

Assess the mare's ration & feeding management

Water is always the most important ingredient in a mare's ration. Water requirements are higher for the reproducing mare, especially during lactation. Enhancing specific nutrients does not appear to be of benefit unless deficiencies are present. Therefore, the focus for the mare's ration should be to feed a ration that meets the nutrient requirements of the mare to maximize the mare's health reproductive function.

Expected daily dry matter intake during maintenance, late gestation, and lactation is 2% BW, 2% BW, and 2.5% BW, respectively.[3] This is important to consider with respect to expectations of voluntary consumption and maintenance of satiety. The ability of forage to meet the mare's energy and protein requirements largely depends on the mare's BCS and forage quality. Feeding volume of each feed should be tailored to mare BCS, energy and nutrient density of selected feeds, nutrient requirements, and other feeds in the mare's ration. Considerations should be given to forage quality and seasonal changes in pasture forage.

Forage

Forage is the next most important and makes up 50-100% of a mare's diet/ration. Forage includes both grasses and legumes. Forage comes fresh, dried, and processed forms, which all serve a unique purpose. There are *general* differences between grasses and legumes with respect to nutritional quality. Legumes are *generally* more energy dense, higher in protein, and higher in calcium. However, it

is important to consider the stage of plant maturity at which the pasture forage is consumed on pasture or cut to make hay. An immature grass could have a higher energy density and higher protein content than a more mature legume.

I am not going to provide an in-depth review of forage types as commonly fed forages vary by region in the US. However, Tall Fescue (*Festuca arundinacea*) is a dominant forage across the country and is an important forage to consider when feeding broodmares, particularly in late gestation. Endophyte-infected Tall Fescue is associated with placentitis and agalactia in mares. Ergot alkaloid produced by an endophytic fungus concentrates in the grass seed head. Taking the mare off a Tall Fescue pasture/hay 30 days prior to foaling will prevent the negative effects of the ergot alkaloid. Alternatives to this include grazing/feeding a Tall Fescue mixed pasture/hay (i.e. legume mix or mixed grass) and mowing to remove the seed heads.

Regardless of a mare's life-stage, forage is a good source of energy and several essential nutrients (i.e. protein, calcium, phosphorus, and vitamin E) provided the forage quality compliments the mare's needs. Forage is an important source of fiber carbohydrates. The focus of a mare's ration must be on what forage is available and the quality of that forage. The rest of the ration should be built around the forage. How do you know if the forage you are feeding is appropriate and appropriately fed? Check the mare's body condition and evaluate the forage quality.

Stage of plant maturity is the single most influential factor on forage quality. The most accurate way to determine forage quality is to submit a representative sample for chemical nutrient analysis (see guidelines below). Forage quality analysis should include analyses of neutral detergent fiber (NDF), acid detergent fiber (ADF), and crude protein (CP). Neutral detergent fiber represents cellulose, lignin, and hemicellulose; this fraction is used as a positive indicator of forage bulk. The higher the NDF the more bulky the hay and the less the mare is willing to consume. Acid detergent fiber (ADF) represents cellulose and lignin; this fraction is used as a negative indicator of digestibility (total tract digestibility, including microbial fermentation of fiber). As forage matures, CP generally decreases while NDF and ADF increase. I generally like to see NDF less than 70%, on a dry matter basis, and ADF less than 40%, on a dry matter basis, for maintenance. Therefore, for reproduction, I advise feeding forages with NDF and ADF well below 70% and 40%, respectively, on a dry matter basis. Energy density is another variable to consider when evaluating forage quality. The energy density of grass hays vary, in *general*, from 0.8-1.0 Mcal per pound on an as fed basis (AF); therefore, if fed at equal weight (i.e. 2% BW per day in total dry matter [dry matter = 100% of a feed - % moisture]) a 1100lb barren mare fed 24 pounds of hay, as fed, per day could consume anywhere from 17.6 to 22.0 Mcal (17,600 – 22,000 kcal) per day from forage alone. Furthermore, voluntary dry matter intake of various forages by adult mares ranges from 1.4-5.4 % BW per day with consumption typically greatest for legume-based forages and lowest for high NDF forages.[3] Therefore, open mares and early gestation mares could quite easily over consume calories relative to requirements when fed a moderate to good quality forage free choice. Conversely, a forage of poor quality ($\geq$ 70% NDF, on a dry matter basis) could be offered free choice, but the mare may become satiated far before she meets her energy requirements. If a mare is to consume all forage in the form of fresh pasture, ensure that the pasture contains enough forage (grass +/- legume), meaning that leaf height is no less than 3-4 inches and there is at least a ½ acre to graze per horse. When pasture forage is not adequate (especially in the winter months), the hay fed should be of adequate quality for that mare's physiologic status.

If a forage is used exclusively to meet the mare's energy and protein requirements, then the forage should contain adequate protein.[3] If you chose to feed a forage that does not meet your protein goal, you will need to add a more concentrated source of protein to the diet (i.e. alfalfa, balancer pellet, life-stage feed). Legumes are *generally* higher in protein than grasses; however, this is influenced by forage maturity. An immature grass could have a higher protein content than a more mature alfalfa.

Concentrates

Concentrates include grains and commercial feeds (i.e. life-stage feeds like a Mare & Foal feed); they are a concentrated source of energy and often protein too. Mares in early gestation do not

necessarily need concentrates in their ration as they may be able to consume enough energy and protein from their forage. Concentrates are more energy dense than forage and thus can help to meet enhanced energy requirements. In general, concentrates are needed to compliment energy and nutrients lacking in the mare's forage, particularly when forage alone does not meet the mare's energy or protein requirements. Due to the higher energy and protein requirements of the mare, concentrates are often needed. In general, up to 50% of a horse's diet may include concentrates (i.e. grains and commercial feeds) if the forage does not meet the horse's energy and protein requirements alone. There are reports of successful reproductive programs where the mares never receive concentrates.[10,11] The pasture forage in the Collas *et al*[10] study was high; however, there was neither mention of the vitamin/mineral content of the forage nor the use of vitamin/mineral supplementation. Furthermore, Lepeule *et al*[11] reported that the incidence of orthopedic lesions was lower in foals when their mares were provided concentrate in late gestation and vitamin/mineral supplementation of the mare's (gestation and lactation) diet was high. In my experience, concentrates are needed to meet the mare's requirements during late gestation and lactation.

Concentrates provide vitamins and minerals, in addition to calories in protein. A g*eneral* rule of thumb is that a horse would need to consume at least 0.5% of body weight per day (i.e. 5 pounds of concentrate per day for a 1,000lb horse) in a fortified concentrate in order to not need further trace mineral supplementation to meet maintenance requirements. However, keep in mind, this is a *general* rule of thumb and will not apply across all concentrates since different feeds have different concentrations of vitamins and minerals.

The forage: concentrate ratio of a mare's ration, particularly with respect to fiber and starch influences the macronutrient composition of mare's milk. High starch rations (low forage: concentrate) result in higher milk lactose and milk yields while high fiber rations (high forage: concentrate) result in higher milk fat and lower milk yields. Due to the higher energy density of fat, the total daily energy consumed by the foal may not be effected. Dietary fat quantity and fatty acid profile positively and directly influences mare's milk fat content and fatty acid profile. Unlike in ruminants, dietary fatty acids are not conjugated and thus the polyunsaturated fatty acid content of mare's milk is higher as compared to ruminants.

Feeding of straight grains should be viewed with caution because grains are high in phosphorus and not balanced in total mineral content. The feeding of straight grains are not fed as commonly as they were in the past due to the development of numerous commercial concentrate options. In *general*, if feeding a horse (maintenance) 0.7% of body weight per day in grain and 1.3% body weight per day in grass forage, on a dry matter basis, (or a grass forage:grain ratio of < 2:1), the calcium:phosphorus ratio in the total ration will likely be < 1:1. Note, the calcium and phosphorus content of the total ration is influenced by the mineral content of the forage, which is determined by the forage type (i.e. legume vs. grass) and soil that the forage was harvested from. Furthermore, the grain will not contain the needed trace minerals and vitamin E.

Ideally, concentrate should be fed individually, divided into at least two meals per day. Feeding large volumes of concentrates, especially low fiber and high starch concentrates, increases horse's risk for colic. General rule of thumb is to feed no more than five pounds of concentrate per meal. However, when feeding concentrates, particularly high starch concentrates, dividing the total daily amount into more frequent small meals should help to reduce the risk of colic. If mares are feed in groups, mares should be grouped based on reproductive stage. For large operations or when possible, mares should be sub-grouped by body condition.

Generally, the amount of concentrate fed should decrease in late lactation, provided that the mare is at a BCS of ~6/9 and maintains ideal BCS throughout the remainder of gestation.
In general, concentrate would be discontinued one to two weeks prior to weaning as concentrate intake will slow the decline in milk production during late gestation. However, mare BCS and rebreeding status should be considered.

Supplements

Supplements is a broad classification, but for the purpose of this talk I am focusing on the use of vitamin-mineral supplements to balance the mare's ration. Trace mineral requirements must be supplied through a mineral supplement. Forage alone will not meet a horse's trace mineral requirements. Furthermore, with a low commercial concentrate intake, trace mineral requirements and vitamin E may not be met. Aside from commercial concentrates, trace minerals may be provided through a low sodium trace mineral supplement or balancer pellet. Trace mineral salt blocks (i.e. 95-99% NaCl) are a fine source of sodium and chloride, but a horse would have to overconsume sodium chloride to meet the trace mineral requirements. Also, not all trace mineral blocks contain selenium, which is deficient in some regions of the US (I am from a Se-deficient region). Low sodium trace mineral sources are often still a good source of sodium chloride. In summer months you may want to provide an additional sodium chloride source (white or trace mineral salt) if not year-long. Balancer pellets are handy as it is easier to ensure adequate intake of individually fed balancer pellet as compared to communal mineral supplement. I cannot emphasize enough the importance of focusing on maintaining ideal BCS and feeding a complete and balanced ration. Ensure the ration is meeting the mare's needs; this is more important for reaching for fancy supplements.

Make any changes to a mare's ration (or any horse's ration) gradually. Generally, allow a two week transition period; modest changes may be made safely within a one week period.

Submitting a forage sample for nutrient analysis

To take a sample, sample for forage, follow instructions on the "Taking a Hay Sample" form http://www.equi-analytical.com/TakingASample/TakingAHaySample.pdf. Note, that each batch of forage (i.e. first cutting orchard grass or second cutting orchard grass, or alfalfa, etc.) must be sampled and nutritionally analyzed separately. Dried forage (hay) is most commonly sampled, but fresh forage (i.e. grass pasture) can be sampled and analyzed as well. For hay (grass or alfalfa hay), 10 representative bales (or 10%) from each batch should be sampled with a hay corer. You can either borrow a hay corer from your local extension office or ask to have an extension agent come to your farm to sample the hay. To contact your local extension agent, search online your state with "ext". For example, to get a list of Virginia extension offices, you would search "Virginia ext", select the "Virginia Cooperative Extension" site, then select "Local Offices" to search offices by county. If you would like for us to contact your local extension agent, we are happy to do so.

To submit the sample for analysis, complete the "Sample Information" form http://www.equi-analytical.com/Services/ForageInfoEqui.pdf. I prefer the "TRAINER" analysis. Include pertinent information on the "Sample Information" sheet to properly identify the sample. Include whether it is hay or pasture, which cutting, and what type of forage as specific as possible (i.e. timothy versus cool-season grass). Send samples to Equi-analytical Laboratories, 730 Warren Road, Ithaca, New York 14850, 1-877-819-4110; service@equi-analytical.com; http://www.equi-analytical.com/default.htm. There are other forage laboratries, but Equi-analytical is the one I am most familiar with.

Questions

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Also, contact our Clinical Nutrition Consultation Service
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http://www.vetmed.vt.edu/vth/nutrition.asp

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Protecting your investment: nutrition for the foal

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Introduction

The American College of Veterinary Nutrition (ACVN)'s circle of nutrition includes three components of a nutritional assessment: patient assessment, diet/ration assessment, and feeding management assessment. For this talk I will address the three components for the foal. The nutritional goals for the foal are to maximize health (i.e. promote moderate and even growth) and prevent disease (i.e. developmental orthopedic disease). Nutrition isn't always to blame for developmental orthopedic disease; however, ensuring adequate nutrient intake may be an easier factor to manage than other developmental orthopedic disease factors like genetics or abnormal physical stress.

Patient assessment

The two main components of patient nutrition assessment are body weight and body condition score (BCS).[1] Body condition is important for determining whether the current body weight is appropriate or not and thus will influence what and how you feed. Although the 9-point BCS chart is not designed for foals, it is still helpful in identifying foals that are under or over conditioned. Ideal BCS for adult horses ranges from 4-6/9. A BCS of 4/9-5/9 is likely ideal for the foal. Promoting a lean body condition may promote longevity and reduce the risk of developmental orthopedic disease.[2]

Foals achieve nearly 50% of adult body weight by the time of weaning. Therefore, malnutrition before weaning is likely to result in abnormalities more rapidly than after weaning. Tracking serial body weight over time is critical for determining if rate of growth is appropriate. While body weight increases with age, average daily gain reduces with age.[3] Thoroughbred foal average daily gain (ADG) declines from 1.25 kg/day at 1 month of age to 0.5kg/day at one year of age.[4] Mare's nutritional status and season also influences growth rate. Growth rates slow if the dam's body condition is declining and growth rates speed up during spring and summer months.[5,6] Foals appear to obtain maximal wither height growth rate (cm/day) by two months of age, at which time height growth rate dramatically declines.[5] Promoting rapid growth will not influence adult stature; size is genetically determined and only with chronic malnutrition is mature stature negatively affected. Foals display catch-up growth after periods of malnutrition; however, catch-up growth is successful when malnutrition spans over a relatively short period of time (i.e. one season). Skeletal growth, represented by gains in wither height, is complete around two years of age; therefore, growth after two years of age is soft tissue development.

The patient's physiologic status, including signalment, life-stage (suckling, weanling, yearling), and if a clinical abnormality is present, should also be considered.

Nutrient requirements of foals

Relative requirements for energy and nutrients are higher for growth as compared to adult maintenance. Furthermore, absolute daily requirements for protein, calcium, and phosphorus are higher for a suckling or weanling foal than for an adult at the same anticipated body weight despite being at a lower body weight currently. While all nutrients are important during growth, those that have received the most press with regards to ties to developmental orthopedic disease include energy (excess, deficiency, source), protein (deficiency), calcium (relative deficiency), phosphorus (relative excess), copper (deficiency) and zinc (deficiency). Interestingly, even though the first four months of life are the most critical with respect to growth rate and consequences of malnutrition, nutrient requirements for this stage of growth aren't well described.[4] Therefore, requirements of the suckling foal are largely extrapolated from data on mare's milk composition.

Energy requirements for horses is expressed as digestible energy (DE), which is simply the difference in the energy in the food and in the feces. Foal DE requirements are defined as such, (Mcal/day) as $(((56.5x^{-0.145})xBW)/1000)+((1.99+1.21x-0.02x^2)xADG)$, where x = age in months, BW =

body weight in kg, ADG = average daily gain in kg/day.[4] Digestible energy requirements are negatively associated with environmental temperature, particularly with low temperatures. Foals have a lower critical temperature than adult horses.[4] Cymbaluk[7] reported that digestible energy should be increased by 1.3% daily for each degree Celsius below the lower critical temperature. Rapid growth, influenced by both nutrition (energy excess) and genetics, has been associated with orthopedic disease in foals.[8] The impact of excess energy on developmental orthopedic disease in foals is likely exacerbated in the face of protein and mineral deficiency.

Protein is expensive and is more costly to metabolize than carbohydrates and fats. Protein quality is important because essential amino acids must be supplied by the diet. Lysine and threonine are the first two limiting amino acids in growth. Supplementing a low protein (9% crude protein DM) diet with lysine and threonine lead to similar growth rates as for foals fed a high protein (14% crude protein DM) diet.[9] While it is important to meet the foal's protein requirements, excess protein has no benefit and results in excessive nitrogenous waste excretion.

Incidence of orthopedic lesions in foals was lower when the mares were provided concentrate in late gestation, vitamin/mineral supplementation of the mare's (gestation and lactation) and foal's diet was high, and copper zinc content of the mare's gestation diet was high.[8] Not only should mineral requirements be met, but done so in balance. Calcium and phosphorus are certainly important for developing bone. A relative phosphorus excess, paired with a relatively low calcium intake, can lead to developmental bone problems. Feeding a diet with an inverse calcium: phosphorus ratio leads to nutritional secondary hyperparathyroidism and osteopenia and predisposes the foal to osteochondrosis. Phosphorus can also interfere with trace mineral availability. Target a calcium: phosphorus ratio of 1:1 – 3:1, avoiding < 1:1, during growth. Copper is a cofactor for lysyl oxidase, an enzyme essential for healthy cartilage development. Milk is a poor source of copper; therefore, fetal hepatic copper storage, which is influence by the mare's diet, is critical. Multiple studies have reported an association between low copper intake and developmental orthopedic disease in foals like physitis and osteochondrosis. Zinc deficiency has also been associated with developmental orthopedic disease; however, excess zinc could interfere with copper availability and thus should be avoided. Therefore, target a zinc: copper ratio of 3:1-5:1.

Assess the foal's ration & feeding management

Suckling foal

Foals typically consume 15-25% body weight in milk per day at one week of age. In general, the dam's milk will meet the nutrient requirements of the foal until about two months of age, when milk production begins to decline. However, foals typically begin consuming solid feeds as early as the first week of age.

A growth concentrate should be introduced to the foal at two months of age and fed such that it is only available to the foal(s) and not the mare(s) (i.e. creep feeding). Mare's milk is low in trace minerals and foal tissue stores (i.e. hepatic stores) are a source of minerals during the early suckling period. These stores need to be repleted through the foal's diet. Creep feeding also prepares the foal for solid food and may reduce the stress of weaning. The macronutrient profile of the creep feed may also reduce stress of weaning. Higher fat and fiber concentrate was associated with less distress in foals undergoing weaning as compared to foals fed a high starch and sugar concentrate.[10] General recommendations are to feed the growth concentrate at a rate of 0.5-0.75 kg for every 100kg of the foal's current body weight.[11]

If the foal is an orphan then the diet and management before four months of age will be different. Milk differs among species; the author recommends mare's milk replacers and follow label recommendations. If the milk replacer is reconstituted to a greater dilution, which is not recommended but observed, the energy density of the reconstituted milk replacer is lower and thus more needs to be fed to meet the foal's requirement. Start feeding milk replacer at 10% of body weight per day, on an as fed basis, and gradually increase to 20-25% body weight per day by ten days of age. Daily milk/milk replacer should be divided into multiple small meals; every two to four hours during the first two weeks, reducing

to four times per day at two to four weeks of age, then to three times per day after one month of age. Teaching the foal to drink from a bucket will make the caregiver's life easier. Introducing milk replacer pellets in the first week of age and transitioning the orphan to milk replacer pellets early will further simplify management. Once the foal's milk replacer pellet consumption is about 1-2% of current body weight, on a dry matter basis, introduce a suckling foal growth concentrate (labeled for suckling foals, not just weanlings, i.e. mare and foal feed) into the diet and gradually transition the foal off of the milk replacer pellets. Discontinue liquid milk replacer once the foal's solid feed consumption is about 2-3% of current body weight, on a dry matter basis.

Weanling

In general, expected total daily dry matter intake during growth is 2.5% BW.[4] Feeding volume of each feed should be tailored to foal BCS, energy and nutrient density of selected feeds, nutrient requirements, and other feeds in the foal's ration. Considerations should be given to forage quality and seasonal changes in pasture forage. Forage is the basis of every ration, more so for adults (by volume). A forage only diet will typically not meet the energy and protein demands of the foal. Furthermore, mature (lower quality) forages will need to be complimented with larger volumes of concentrate to ensure the foals energy and protein requirements are met. Therefore, feeding concentrates during growth is typically warranted due to the high nutrient demands during growth. General recommendations are to feed a growth concentrate at a rate of 1.7-2 kg for every 100kg of the foal's current body weight.[11] Of course, this should be titrated by BCS. Ideally, concentrate should be fed individually. However, for large operations, group feeding is more practical.

Energy source, regardless of quantity, may influence growth. Gray et al.[12] reported that Quarter Horse weanlings fed a concentrate with 52% non-structural carbohydrates (NSC) on a dry matter (DM) basis had higher postprandial glycemic and insulinemic responses and altered growth hormone secretion compared to Quarter Horse weanlings fed a 11% NSC DM. The authors proposed one mechanism to be attributed to insulin's positive effect on hypothalamic somatostatin release, which has a negative effect on growth hormone secretion. However, the significance of this on growth has yet to be determined.

Yearling

Due to slower growth rates in yearlings, forage may meet the energy and protein requirements for yearlings provide the quality is adequate. A yearling ration could be as simple as forage complimented with a single vitamin/mineral supplement. Fresh forages are generally higher in vitamin content. Preserved forage is lower in fat soluble vitamins (i.e. vitamin E) than fresh forages; therefore vitamin supplementation is important when horses are fed preserved forages, especially if preserved forage is fed year around. The addition of salt may be needed if the vitamin/mineral supplement doesn't meet sodium chloride requirements.

Questions

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Epigenetics and fetal programming: what do we know?

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Introduction

A multitude of factors, including undernutrition, overnutrition, uterine capacity, as well as heat and other environmental stressors, can strongly influence the uterine environment during gestation. A rapidly growing body of evidence is uncovering a correlation between a suboptimal uterine environment during gestation and the incidence of metabolic diseases in the adult offspring . A variety of animal models, including sheep, cattle, rats, mice, and non-human primates, have been developed for investigation of this link between *in utero* environment and development of disease in response to a variety of environmental stressors. Maternal malnutrition and uterine capacity are perhaps the two more common factors influencing *in utero* development in horses. However, there is still a lack of experimental evidence regarding the developmental origins of disease in the horse. Given the observed incidence of equine metabolic disorders in veterinary practices, it is probable that similar mechanisms of fetal programming exist in the horse. The aim of this review is to provide a broad understanding of how fetal and neonatal environmental factors affect fetal programming, which may be applicable across of variety of species, including the horse.

Keywords: Epigenetics, fetal programming, pregnancy, nutrition, equine

Fetal programming

During gestation, the fetus is said to be in a state of developmental plasticity, a phenomenon by which a single genotype (genetic sequence) can give rise to many different phenotypes (physiological states) based on the *in utero* environmental conditions.[1] Fetal programming can be defined as the process by which the fetus adapts to its uterine environment. This adaptive developmental process is thought to be evolutionary and intended to be advantageous for the fetus to thrive in suboptimal environments. The "thrifty phenotype hypothesis" proposes that a poor uterine environment induces permanent changes so that offspring can "thrive or more accurately survive" in a suboptimal postnatal environment.[2] Practical examples of this in horses and other livestock species are "easy-keepers" which demonstrate a greater ability to convert nutrient intake into fat stores. This is likely a response to metabolic programming *in utero* rather than an inherited trait. When matched appropriately, predictive adaptive responses allow a species to survive in a compromised environment.[3] Conversely, the "mismatch concept" suggests that the degree of disparity between the developmental environment and the environment of adulthood influences the risk of disease.[4,5]

Maternal undernutrition

It is not surprising that the prenatal growth trajectory is both directly and indirectly impacted by maternal nutrition during gestation.[6-9] Various weather conditions make undernutrition during gestation a common occurrence in livestock production. Furthermore, twinning in species that are typically monotocous, such as the horse, requires the division of nutrients between two fetuses and causes reduced uterine space for adequate fetal development.

Birth weight is the principal indicator of prior nutrient availability *in utero* and predictor of postnatal viability utilized in livestock. However, it is important to note that not all offspring of nutrient restricted mothers are born at a low birth weight or as intrauterine growth restricted (IUGR). In cattle exposed to nutrient restriction during early to mid gestation, IUGR was induced in only a subset of offspring.[10] A similar model in sheep induced IUGR-like classification at mid gestation but at birth these lambs were of an average birth weight.[11-13]

In horses, both experimentally induced maternal undernutrition, as well as undernourishment induced by maternal illness, had no influence on birth weight.[14,15] However, the degree of undernourishment and amount of weight lost in these mares are make comparisons of these results with

those observed in other animal models difficult. Nevertheless, the strong correlation between low birth weight and metabolic diseases emphasize the importance of this simple measure on properly managing potential health risks.

Various livestock models of maternal nutrient restriction have shown a disruption in the pattern of fetal organ development that ultimately results in impaired organ function during postnatal life. Sheep models have shown that maternal undernutrition results in impaired cardiovascular and renal function, as well as increased whole-body obesity.[16-18] In sheep and pigs, IUGR is associated with a decreased number of skeletal muscle fibers, an increase in connective tissue content, and increased adiposity.[19-22] Reduced musculature and increased adiposity may substantially reduce growth performance and nutrient utilization. In horses, it has been hypothesized that a suboptimal uterine environment leads to reduced skeletal muscle mass, impaired bone and cartilage formation, and weakened pulmonary and vascular systems, all of which can negatively impact athletic performance.[23]

To further complicate things, the effects of fetal programming have been shown to persist in multiple generations. For example, maternal protein restriction in rats results in vascular dysfunction and hypertension in the F1 generations that was also maintained in the F2 generation.[24,25] Such heritable developmental adaptations do not alter the DNA sequence and therefore are not genetic alterations. Instead, they are "epigenetic" alterations or alterations in how the DNA is utilized. Many epigenetic alterations are lost or "reset" when sperm and oocytes are produced. However, some epigenetic changes may be passed on to the subsequent generations.

Maternal overnutrition

Like with undernutrition, data has shown that maternal overnutrition perturbs fetal growth in various species.[26-28] Furthermore, overnutrition and obesity during gestation are strongly associated with an increased risk of metabolic disorders during the postnatal life.[29,30] Of the livestock species, obesity is most often encountered in horses. This largely stems from a shift towards the utilization of the horse for recreation and companionship. Indeed, it has been shown that obesity in horses is associated with increased occurrence of disorders such as equine metabolic syndrome, insulin resistance, diabetes mellitus, laminitis and equine motor neuron disease.[31-35,49,50]

A link between maternal obesity and metabolic perturbations in the offspring has been shown in a number of species. Feeding rats a high-fat diet during pregnancy or lactation can induce cardiovascular dysfunction and high systolic blood pressure.[36] Interestingly, the response to a maternal high-fat diet differed based on sex of the offspring. With potential application to the horse, impairment in glucose homeostasis of the offspring, with elevated plasma insulin levels at 1 year of age, were also seen in response to a maternal high-fat diet.[37] Maternal diets high in fat have also been shown to induce similar disorders in mice and have resulted in increased levels of circulating markers of systemic inflammation.[38,39,40]

In sheep, maternal overnutrition results in increased fetal weight at mid-gestation, with numerous organ and tissue weights also showing a proportional increase.[41] However, at parturition, the birth weights of lambs from overnourished and obese dams did not differ compared to normal fed ewes.[42,43] Subsequent sheep studies have shown that maternal obesity impairs skeletal muscle and liver metabolic function through downregulation of genes associated with muscle and liver development in the fetus.[44,45] Alterations in these genes also resulted in increased fetal adiposity.[41,46] Consequently, even though an alteration in birth weight is not seen, body composition with less muscle and increased adiposity has the ability to substantially impact postnatal growth and performance.

Feeding a high starch diet in non-pregnant horses results in reduced insulin sensitivity and increased occurrence of obesity and laminitis.[47] Feeding these same high starch diets to pregnant mares during late gestation reduced insulin sensitivity in foals from postnatal day 5 to 160, as well.[47] In addition, maternal overnutrition late in gestation has been shown to decrease IgG content in the colostrum, but no change was detected in the colostrum fat or protein content.[48] Diets for other livestock species are often higher in fat compared to equine diets. This illustrates the need for equine studies

utilizing high caloric diets with high levels of starch to induce obesity during gestation and the consequences of this diet composition on offspring development.

Uterine capacity

It is well documented that maternal size has a profound influence on fetal growth *in utero*, primarily through placental size and function.[49] A number of studies in the mare have illustrated that the uterine environment dictates fetal growth rates, irrespective of the embryo's genetic makeup. The original study illustrating the dramatic impact of uterine size was conducted by Walton and Hammond utilizing Shire horse and Shetland pony crosses.[50] Shire mares were artificially inseminated with Shetland pony semen and Shetland pony mares being artificially inseminated with semen from Shire stallions. The Shire mare and Shetland stallion foal was much taller and heavier at birth than the reciprocal cross foal, with this growth and size differential continuing at maturity.[49,50]

Various laboratories have employed the use of embryo transfer to investigate the impact of uterine capacity on fetal growth and development in the horse.[49,51-57] In these studies, reciprocal embryo transfer between large framed horses and small framed ponies, along with within-breed embryo transfers were compared. Foals carried by large framed mares were considerably heavier and taller than their genetically similar siblings carried by pony recipient mares.[49,51-57] In addition, birth weights of foals from between-breed transfers were intermediate to those of either within-breed transfer.[58] Furthermore, these size disparities remained to adulthood.[49,51-57] In one study, increased uterine capacity for the pony in Thoroughbred recipient (P-T) transfers also resulted in altered cardiovascular function and response to stress.[57] These P-T foals further displayed elevated basal insulin levels.[56] Conversely, Thoroughbred foals gestated in pony recipient mares exhibited an increased production of catecholamines following acute stress.[56,57] Another study, utilizing pony, Saddlebred, and draft control and reciprocal crosses not only found that maternal size regulated fetal growth but also found altered concentrations of thyroid hormone and impaired glucose homeostasis.[35] These data show that maternal size and the *in utero* environment plays a potentially greater role in fetal growth determination than genotype and that the effects of either a large or small uterine environment have significant postnatal ramifications for growth and health.

Amino acid nutrition and epigenetics

Amino acids serve many fundamental functions beyond the basic building blocks for protein synthesis including serving as precursors for nitric oxide, purine and pyrimidine nucleotides, neurotransmitters, and other essential molecules. Amino acids, particularly methionine, serve as methyl group donors and have a critical role in regulating and maintaining the epigenome.[59] Studies in species such as the pig and sheep have begun to illustrate the importance of individual amino acids in supporting optimal fetal growth and development. As example, dietary restriction of methionine, essential B vitamins, and folate to ewes during the peri-conceptual period induced alterations in DNA methylation and resulted in the development of insulin resistance and elevated blood pressure of the lambs. Interestingly, the male offspring appeared to be more susceptible to this dietary restriction.[60] In contrast, supplementation of pregnant sows with arginine increased litter size at parturition by two piglets.[61] In sheep, administration of arginine during late gestation increased fetal peri-renal brown adipose tissue development (MC Satterfield, unpublished observation). An increased amount of brown adipose tissue will likely enhance neonatal survival by increasing the lamb's ability to combat cold temperatures.

Equine nutritional management is the most varied among the domestic livestock species. In consequence, an encompassing standard of nutritional requirements or recommendations has yet to be composed. Foundational studies emphasizing functions and importance of amino acids and other micronutrient requirements, have not been established.[62] Yet, there is an overwhelming availability of equine dietary supplements. Therefore, it is necessary to utilize caution in supplementing pregnant mares as administering an inappropriate supplement, dose, or combination of these could have permanent consequences on the developing fetus, persisting throughout postnatal life.

Behavioral programming

During the early neonatal and postnatal life offspring are still susceptible to alterations in their epigenome. More specifically, genes associated with behavioral traits are largely influenced by maternal behavior and glucocorticoid exposure. Thus, it is not surprising that behavioral programming in the horse may be profoundly impacted due to typical managerial practices allowing for increased interaction with humans. The benefits or consequences of this can profoundly impact a horse's willingness to train or compete.

The process of "imprinting" foals during early neonatal life has shown that conditioning foals through tactile interaction at birth and postnatal day one reduced foals' resistance to touching of their legs and hind feet at three months of age.[63] Interestingly, it is known that orphan foals reared without the presence of a mare are difficult to train. Assisting with a foal's first suckling interferes with the development of the maternal/fetal bond and can cause the foal to avoid human approach and physical contact at a few weeks of age.[64] Moreover, forced handling of foals during early postnatal life did not improve human/foal interaction later in life but exposure to a motionless human did improve this interaction.[64]

Studies have investigated the effects of early neonatal exposure to glucocorticoids in foals. Induction of parturition through the use of oxytocin approximately 24 to 48 hours prior to parturition altered pancreatic function.[65] Cortisol levels were also increased in foals born by induced delivery. Induced delivery may alter pancreatic sensitivity to glucose and/or cause tissue insulin resistance, along with increased cortisol secretion.[65] In another study, spontaneously delivered foals receiving long-acting ACTH endogenously during the neonatal period resulted in increased plasma cortisol concentrations at three but not 13 weeks age.[66] This treatment also lead to impaired pancreatic function.[67] It is possible that ACTH-induced affects may occur much later in life but this warrants further investigation.

Limited evidence suggests that the early neonatal life is critical for physiological and behavioral adaptations, which may be altered by environmental and biological stressors. Not surprisingly, this small amount of data fails to elucidate the long-term consequences of adaptations occurring during this time period, but justifies the need for controlled studies to evaluate the extent to which the physiology of the foal can be programmed by environmental stressors in the early postnatal period. Future studies in this area may provide valuable insight for development of new managerial and training practices that can be utilized to maximize genetic and physiological potential of foals.

Conclusions

The prenatal uterine milieu, as well as the early neonatal environment, may profoundly impact the health, metabolism, physiology, and behavior of livestock throughout life. Importantly, a scarcity of information regarding these mechanisms, adaptations, and responses exists. More specifically, there is a paucity of data elucidating the impact of nutritional supplementation during gestation and the importance that individual amino acids or other nutrients serve in the equine diet. Furthermore, both the mechanisms and consequences of human and foal interaction during the neonatal period remain unknown. Collectively, these early findings support the need for continued and more rigorous investigation into these areas.

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Developmental orthopedic disorders in foals

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Abstract

Developmental orthopedic disease (DOD) is a broad topic in the horse. There are several categories of disease processes that fall under the realm of DOD including osteochondrosis (OC), osteochondrosis dessicans (OCD), subchondral cystic lesions, angular limb deformities, flexural deformities, physitis, cuboidal bone abnormalities, and juvenile osteoarthritis. Historically these issues have had a major financial impact on the equine racing and performance horse industries and veterinarians alike. The etiopathology of these processes is multifactorial.[1] There are many physiologic and phenotypic considerations with respect to disease manifestation such as body size, conformation, and growth rate. Exercise and body condition are variables that effect biomechanical stress on developing bone and cartilage. Environmental factors such as confinement versus pasture housing and availability of nutrients from pasture that may be deficient or low in necessary minerals can play a role in development of clinical abnormalities. Genetic and epigenetic relationships also are linked to expression of pathology.[1,2]

Keywords: Equine, osteochondrosis, angular limb deformity, nutrition

Defining the problem

Osteochondrosis is a disease that results from the disruption of normal endochondral ossification of bone at the physes and epiphyses. This is an active process in juvenile animals that allows lengthening and thickening of bone.[3-5] Osteochondrosis dessicans occurs within the epiphysis involving the articular cartilage. A core of cartilage is retained within the subchondral bone; this site undergoes necrosis and results in a defect in the articular cartilage that becomes a lesion in high motion, weight bearing surfaces within joints. Subchondral bone cysts occur if the defective endochondral ossification occurs at a deeper level within the bone than the articular surface. These lesions occur in high weight bearing areas and can also be secondary to trauma.[4]

There are several known sites of predilection for the appearance of OC defects.[3,4] Radiographic screening is the most common means of diagnosis.[4,5] Radiographing both limbs is recommended as OCD lesions are frequently bilateral.[3-5] Chronologically with respect to the timing of endochondral ossification OCD lesions should be present by seven to eight months of age. Spontaneous resolution of lesions may occur, but generally not after eight to ten months of age.[3,4] The exception to this is OC of the sagittal ridge in the fetlock which may resolve later with conservative medical management.[4]

The tarsus is one of the most commonly effected joints with lesions visible as young as three months of age. The three consistently named locations of problems within the joint are the distal intermediate ridge of the trochlea (DIRT), lateral trochlear ridge of the talus, and the medial malleolus of the tibia. The OC lesions at these sites usually appear as round, smoothly marginated fragments with an underlying subchondral bone defect. Medial malleolus fragments are typically very small. Lateral trochlear ridge lesions may have more than one fragment and are fairly large involving a significant surface area of the ridge. Radiographically, it should be noted that "teardrop" fragments involving the medial trochlear ridge are considered incidental findings. This is characterized by focal dorsal flattening and small distal smoothly marginated fragments.[3,4] Distal intermediate ridge of the trochlea lesion fragments and those of the lateral trochlear ridge if displaced may be found distally, cranial to the talus or central tarsal bone.[4]

Stifle OC may be visualized as early as five months of age. It should be cautioned that around four to five months of age the femoral trochlear ridges appear highly irregular and poorly defined on radiographs. The most common site of OCD is the proximal to middle third of the lateral trochlear ridge of the femur. Subchondral bone cysts occur more commonly on the medial femoral condyle.[3,4]

Fetlock OCD of the sagittal ridge of MT3 or MC3 may affect all four limbs. A flexed lateromedial position gives the best diagnostic radiographic view as it removes superimposition.[6] Often a

subchondral bone defect and flattening of the sagittal ridge are the only radiographic changes. Axial, proximal plantar or the palmar eminence of P1 lesions occur and are shown to have a hereditary component in Standardbreds. These may be seen prior to initiating training, but trauma has been proposed as an etiology as well.[4]

Shoulder lesions are less common but tend to carry a poor prognosis for soundness. Anatomically the humeral head is the site of pathology with or without secondary involvement of the glenoid cavity articular surface.[3,4]

Subchondral bone cysts appear as radiolucent round to oval lesions that are well circumscribed and may have a thin sclerotic edge. The stifle is the most common location but they are also found in the distal third of the metacarpal and metatarsal condyles, proximal or distal P1 and P2, and proximal P3. Computerized tomography and magnetic resonance imaging are very helpful to determine if there is communication to and involvement of the articular surface.[4]

Normal endochondral ossification

Normal endochondral ossification occurs in the axial and appendicular skeleton. Chondrogenesis is the result of accumulation and condensation of mesenchymal cells into cartilage, which then differentiate into chondrocytes. Next proliferation, hypertrophy, and ossification transform the chondrocytes creating bone at the diaphyseal and epiphyseal sites of ossification.[7] This provides a constantly changing template for longitudinal bone growth that begins at the center of the avascular cartilage and radiates outward until the skeleton is ossified, leaving only articular surfaces.[8] As endochondral ossification occurs, the bone and extracellular matrix is remodeled; this is controlled by factors including angiogenic factors such as vascular endothelial growth factor (VEGF) along the growth plate.[7] If this process is disturbed in a young, dynamically growing individual, irregular ossification of the bone may occur, manifesting itself as osteochondrosis in the horse.[9]

Pathophysiology of OC and OCD

Damage to the micro-vasculature of the arterial supply of the articular epiphyseal growth cartilage has an important role in the early pathogenesis of OC. In fetuses there is a defined change in collagen structure from the proliferative to hypertrophic zones within the physis. The critical time period when this tissue is vulnerable is during the transition in the arterial source of vessels in the cartilage canals of the growth cartilage from perichondrial to vessels crossing the ossification center.[1,10-12] This transition coincides with the time the lesions are known to occur in the hock and stifle joints. Biomechanical influences explain the mechanism of initiation of this type of pathology and the existence of predilection sites.[1,12] This is supported by the inability to find OC lesions in fetuses.[1,12,13]

Treatment of OC and OCD

The question of treatment is circumstantial; many smaller lesions that are not accompanied by clinical signs such as lameness and joint effusion may not require treatment. Studies have shown that the long term effects on joint health and performance are minimal with the exception of the lesions that involve a large area of articular surface or involve fragmentation of the cartilage.[1,3] Medical management options for OCD include rest, control of nutrient intake, administration of chondroitin sulfate and hyaluronic acid with the intention of providing matrix for cartilage repair. Selection criteria for medical management is that the lesion is <2 cm long and <5mm deep. The standard of care for OC with fragments, osteochondrosis dessicans, is typically arthroscopic surgery to remove the fragments. In the case of large OCD flaps the use of polydioxanone pins (PDS) to reattach fragments that maintained perimeter continuity with the attached cartilage and did not have excessive mineralization or fissures has been successful.[3,13,14]

Treatment of subchondral bone cysts, particularly in the femoropatellar joint involves debridement via arthroscopy plus or minus additional medical therapy.[3] Subchondral bone lesions have been treated using intralesional injections of corticosteroid. Cancellous bone grafting has been performed but a six month follow up study revealed similar outcome for grafting versus surgical debridement

alone.[3,15] Mosaic arthroplasty using osteochondral grafting has been successful for return to performance but has limitations for larger lesions with respect to rejection of the graft and it is a technically difficult procedure to perform.[3,16] More recently developed techniques include the use of allogenic chondrocytes combined with human IGF-1 as a graft following surgical debridement. This was particularly effective for older horses with evidence of osteoarthritis.[3,17] Mesenchymal stem cells in fibrin have also been used intralesionally after debridement.[3]

Role of nutrition

Nutrition is a key component in normal bone and cartilage development. Often the problem lies with excess available nutrition and energy, particularly associated with carbohydrate availability and metabolism. Research has shown the necessity for adequate levels of trace minerals such as zinc and copper. Our understanding of the role of copper has been refined to learn that the adequate levels of copper in the foal's liver at birth is related to the repair of cartilage lesions but not pathogenesis. Mare's milk is very low in copper therefore ensuring that the mare receives adequate dietary copper during gestation is a part of nutritional prenatal programming.[18] Lysyl oxidase is the copper-dependent enzyme necessary for normal maturation of connective tissue. In older foals it has been shown that low copper diets at 7-15 ppm result in increased incidence of osteochondrosis lesions.[19] The NRC suggests a diet containing 125mg per day during late gestation.[2] Zinc has an antagonistic effect on copper and it has been proposed that diets with excessive zinc could cause a secondary copper deficiency.[20] Adequate protein intake for mares increases by about 20% in late gestation and from maintenance requirements during early lactation.[2] The calcium to phosphorous ratio of the diet of the mare and foals is important. The ratio should be no less than 1:1 or there is risk of impairment of calcium absorption. It may result in skeletal abnormalities and in rare cases nutritional secondary hypoparathyroidism. Although quite high, it has been documented that the ratio in the diet of the growing horse may be as high as 6:1 if the phosphorous intake is adequate. The Ca:P ratio found in mare's milk between 16-24 weeks lactation is between 1.8 and 2.5:1.[2] One of the key difficulties producers face in balancing the diet appropriately is the variation in forage quality and content. Hay analysis is helpful if the source of the forage is consistent. Additionally, it is difficult to gauge the overall amount of energy and caloric intake for horses raised on pasture. All of the variables of nutrients in the diet, body condition, and the biomechanical stressors of excess weight could potentially have an epigenetic effect on the expression of genes associated with osteochondrosis.

Genetics of OC

There have been a number of studies to determine the factors that control OC in the horse, including predilection sites, predisposed breeds, heritability, and genetics. Low T3 and T4 levels that have been altered by insulin in a growing horse may be responsible for a lack of capillary penetration into the bone matrix that in turn gives rise to perceptible OC when compounded with the effects of mechanical exercise and environmental factors.[21]

Osteochondrosis can occur in a number of joints including the coffin joint, pastern, shoulder, hock, and stifle, with the fetlock being the most affected.[22] Heritability in these joints varies across breeds, with the hock joint having the greatest heritability (typically presenting lesions from birth) at 0.3-0.4 in most populations[23] and the stifle joint having the least heritability, as lesions were typically formed during the early stages of growth. In addition to this, both the metacarpophalangeal and metatarsophalangeal joints display medium levels of heritability.[9] Although there has been no relation between birth weight and the appearance of OC in the growing horse, Warmblood foals presenting as OC positive appear to have a higher rate of weight gain in the third and fifth month of growth than foals that do not have any lesions present and are also taller at both the withers and the croup (withers 1.56 ± 0.004 m and croup height 1.60 ± 0.007 m in OC positive foals; withers 1.49 ± 0.004 m and croup 1.52 ± 0.03 m in OC negative foals) at the age of 11 months than OC negative foals.[24]

There do not appear to be consistent quantitative trait loci (QTL) shared across different breeds of horses, however next-generation sequencing of equine DNA and RNA samples may allow for a more

thorough understanding of the differences in the genetic regulation of OC.[23] In a genome-wide association study (GWAS) on OCD conducted on Norwegian Standardbred horses, of 162 horses chosen for genotyping from 22 sires, it was determined that *Equus caballus* chromosomes (ECA) 5, 10, 27, and 28 were the ECA showing a moderate level of association with tibotarsal OCD. In this case, ECA10 posessed two single-nucleotides (SN), BIEC2-132748 and BIEC2-132753, which showed the most significant hits.[25] Osteochondrosis dessicans in Thoroughbreds, however, is associated with ECA3, a suggestion of complex genetic inheritance of OCD lesions.[26]

A number of QTL for palmar/plantar osteochondral fragments (POF) have been identified as well in Norwegian Standardbred trotters, with medial POF identifiable on ECA1, 2, 7, 9, and 31 and lateral POF identifiable on ECA7, 11, 27, and X. Medial POF occurs most frequently in these horses. Of 176 yearlings studied, 82 were POF-negative controls, 82 presented medial POF, 33 were diagnosed with lateral POF, and 21 had both.[25] In another genome-wide OC study on 201 Dutch Warmblood horses, four significant SNP wer discovered on chromosomes 3 (BIEC2-808543) and 10 (BIEC2-121323, BIEC2-121320, and BIEC20121337), which suggests a potentially novel susceptibility locus[27] that is different from the loci associations in Norwegian Standardbreds[25] and further indicates that there are both intricate genetic relationships to heritability as well as breed differences and environmental factors.

Warmblood horses tend to have a very high occurrence of OC in the coffin joint, whereas Standardbreds have the highest occurrence of lesions in the pastern joint.[22] In the shoulders and hocks, they are seen in Quarter Horses frequently; Standardbreds also have a high occurrence of hock fragments, but they rarely appear in their shoulders.[22] Dutch Warmblood horses also historically have a high incidence of lesions in the hock joint, with the distal end of the talus lateral trochlear ridge, the distal tibia medial malleolus, and the distal intermediate ridge of the tibia at the cranial apex all representing various predilection sites.[25] Ponies, conversely, are typically not affected by OC to the extent that horses are.[28]

Angular limb deformities

There are a number of different types of presentations of angular limb deformities (ALD) ranging from slight deformities with the ability to potentially resolve over time to those requiring less passive forms of intervention. There are two main forms of ALD; if the limb deviates laterally from the site of the deformity it is referred to as being valgus, whereas a limb that deviates medially is referred to as varus.[29] Common conformational areas in which these deformities occur include the fetlock as well as both the carpal region and tarsal region in the foal. Mild fetlock valgus (5°), carpal valgus less than 15 degrees, and slight tarsal valgus in foals have been found to typically correct on their own given time and do not need intervention; conversely, fetlock varus, carpal varus deformity, and tarsal varus often require intervention or corrective surgery.[30] It is important to diagnose these angular limb deformities early in life. If there is a physical deformity as well as an articular deformity present, intervention is necessary, but once ossification reaches the cartilage periphery, the deformity is considered uncorrectable; this typically occurs at around four months.[29] These deformities are often bilateral, although they can present as unilateral, and in some studies show a linkage to both genetic and physical factors including both breed and size.[31]

Treatment of angular limb deformity

Much of the correction of angular limb deformities relies on foot balance adjustment made on a regular basis, every two to four weeks by a skilled farrier. Foals grow hoof at a rate of 15mm per month which is much greater than adults at 9 mm per month.[32] Many of the mild valgal conformation imperfections will correct as the foal grows and the chest broadens.[32,33] With valgal conditions there is more weight bearing on the medial side of the hoof capsule which pushes the hoof capsule laterally and the medial heel bulb proximally. Foal feet not only grow distally but also expand. The expansion occurs proximally which gives the foot a tapered shape. This places the weight more on the dorsal aspect of the foot. Mild rasping of the heel increases the weight bearing surface area and moves it to the rear of the foot. Trimming to include a round or square toe will promote better break over easing stress on the toe. Aggressive trimming can lead to distortion of the hoof capsule. The use of glue on shoes and extensions

to extend the weight bearing surface in the direction of axial alignment in moderate to severe cases helps to prevent deformity of the foot. Confinement is recommended for severe cases to reduce the risk of damage to the growth plates.[32,33]

Surgical procedures are reserved for moderate to severe cases. Common techniques are the placement of single transphyseal screws, and the screws and wires technique. Correction of the deformity occurs by creating pressure across the growth plate which reduces chondrocyte proliferation and hypertrophy leading to decreased longitudinal bone growth. A second procedure is required after the desired correction has been achieved to remove the hardware to avoid overcorrection.[34] Periosteal transection with or without periosteal elevation on the convex side of the deformity is another corrective method. The timing of physeal closure must be considered in the treatment approach. Fetlock deformities must be addressed sooner, typically between four to six weeks of age as MC3 and MT3 physes functionally close at 12 weeks of age. Carpal conformation should also be evaluated concurrently with fetlock issues as offset carpi tend to impair effective correction of fetlock varus.[32] Radial physeal closure occurs much later thus carpal deviations may be surgically addressed at several months of age. However, the highest rate of growth at the distal physis is from birth to ten weeks of age. Carpal valgus of up to 4 degrees are considered normal in the horse.[33]

Conclusions

Developmental orthopedic disease will continue to persist as a complex issue in the horse. Progress has been made to manage clinical manifestations with a better understanding of dietary needs as well as continually improving medical and surgical techniques. Genetic associations with specific locations of osteochondrosis lesions and breed correlations may give breeders another selection tool when considering pairings. Ultimately, the goal is to produce a horse that is a functional athlete. Promoting client understanding of which lesions are relevant to function versus those that should be considered a cosmetic defect should be a focus of veterinarians as it is in the best interest of the horse. Many imperfect physical specimens have accomplished great athletic feats.

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Feeding and supplementing the stallion for maximum fertility

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Introduction

Nutrition plays a major role in maintaining the health and condition of the stallion. Ideally, stallions should be maintained in moderate body condition (BCS; condition score of five or six) before, during and after the breeding season. If the stallion tends to lose weight during the breeding season, having a BCS of six or seven prior to the start of the breeding season will help ensure that the stallion does not become too thin when energy demands are high during the season. With regards to nutritional requirements, breeding can be considered "work" and as such, the stallion should be on a similar feeding program to that of working or performance horses. The nutrient requirements in dietary dry matter for breeding stallions are 1.15 - 1.3 Mcal/lb, 10-11% protein, 0.3% calcium, and 0.25% phosphorus.[1] When fed in sufficient quantities (1.5 - 2% of body weight), good quality forage can meet the breeding stallion's minimum protein, calcium, and phosphorus requirements, but will fall short of the energy requirements. Therefore, the additional energy will need to be supplied in the form of grains and/or oils. So, for most stallions, breeding does not require an increase in any nutrient other than digestible energy and the requisite increase in crude protein. However, for stallions with marginal or low fertility, dietary modifications may be necessary to optimize their semen quality.

Vitamins and antioxidants

Vitamins C and E are well known for their antioxidant properties and are those that have been the most extensively examined for their effects on semen quality. In a number of species, dietary supplementation with vitamin C, vitamin E or a combination of these increased, total sperm output, sperm concentration and sperm motility while decreasing dead and abnormal sperm.[2-4] In humans, vitamin C supplementation was associated with higher sperm numbers and concentrations in ejaculates, whereas vitamin E appeared to exert its effects by improving sperm motility.[3,5] In semen from infertile men, supplementation with vitamin C and vitamin E also resulted in a significant reduction in sperm DNA fragmentation.[6] German and Russian investigators reported improved semen quality by supplementing stallions with vitamins A, D and E.[7,8] While the intake of high levels of antioxidant vitamins was associated with better semen quality, moderate intake did not appear to be effective. Results such as these are not universal, and there have been numerous studies that have failed to demonstrate beneficial effects on semen quality, even by giving large doses of vitamins.

Another antioxidant, showing promise for improving semen quality is levocarnitine (L-carnitine). Along with its antioxidant properties, L-carnitine is essential for mitochondrial energy metabolism. Both L-carnitine and L-acetyl-carnitine are found in high concentrations in the epididymis and both are accumulated by sperm and in men with asthenozoospemia, combined treatment with L-carnitine and L-acetyl-carnitine was effective in increasing sperm motility.[9] The most significant improvements were seen in men with the lowest numbers of motile sperm prior to treatment. Feeding L-carnitine to boars resulted in higher semen volumes and sperm concentrations thereby increasing the total number of available sperm in ejaculates for artificial insemination.[10]

Combining antioxidant vitamins and micronutrients may also have some benefit. When the standard diet of stallions with normal fertility was supplemented with 1500 mg of alpha-tocopherol acetate, 360 mg of zinc, and 2.5 mg of organic selenium on a daily basis for 60 days, a significant improvement in average path velocity, straightness, viability, progressive motility, and sperm morphology was reported.[11]

Micronutrients

The supplementation of organic selenium has been shown to improve the progressive motility of boar sperm and increased their resistance to thermal challenges. Organic selenium supplementation also improved the short term storage ability of preserved semen and increasing the fertility rate in gilts.[12]

Bulls supplemented with organic trace minerals (Zn, Cu, Co, and Mn) had a greater percentage of motile, progressively motile sperm, and rapidly motile sperm than those supplemented with inorganic trace minerals.[13] After 60 days of supplementation with both zinc and selenium, the semen quality of caprine bucks was enhanced in terms of a significant increase in sperm numbers, progressive motility, percentage of live spermatozoa, acrosomal integrity and a decrease in abnormal spermatozoa.[14] A recent study in humans showed that treatment of asthenospermic patients with zinc supplementation leads to, among other things, restored nitric oxide synthase (NOS) activity to normal values and improvement of semen parameters.[15] However in another study, zinc sulphate and folic acid supplementation did not improve semen quality in infertile men with severely compromised sperm parameters due to oligoasthenoteratozoospermia.[16]

Polyamines

Polyamines are products of metabolism of the amino acid arginine and are found in seminal plasma in relatively high concentrations.[17] L-arginine is a substrate for the NOS producing nitric oxide (NO), a reactive molecule that participates in a variety of male reproductive functions. At low levels, NO is necessary for spermatogenesis, spermiogenesis, sperm motility, sperm capacitation, the acrosome reaction, and sperm/oocyte fusion.[18-20] However, high levels of NO can result in adverse effects on sperm motility, morphology and DNA stability.[21,22] L-arginine has been shown to have a protective effect on spermatozoa against the sperm plasma membrane lipid peroxidation as well as to enhance sperm metabolism and maintain sperm motility.[23,24] In frozen stallion semen, NO production was positively correlated with sperm motility and velocity after thawing.[25] Oral L-arginine supplementation for 62 days improved both sperm motility and morphology (from 40% to 69%) in semen from sub-fertile dogs with oligoasthenoteratozoospermia, and also increased sperm motility in fertile dogs with normal semen characteristics.[26] Dietary supplementation with 1% L-arginine-HCl for 30 days increased both sperm numbers and sperm motility in boar semen.[17] Interestingly, the human fertility supplement, "Sperm-Aid" is a tablet containing 500 mg of L-arginine. Note however, that as with NO, there is evidence that too much L-arginine can adversely affect sperm motility and fertility.[27,28]

Anecdotal information exists from equine practitioners using a compounded herbal supplement for stallions called "SpermAid". The active ingredients in this product are spermine and spermidine, which are found in radish leaves, radish root, cucumber fruit, and oats. Spermine and spermidine are polyamines that are produced by the prostate and found in the semen of most mammals. In rams, ejaculates with sperm motility greater than 85% had almost double the spermine and total sperm polyamine content than ejaculates with lower motility.[29] Lower levels of spermidine are found in the seminal plasma of men with idiopathic asthenozoospermia as well as those with asthenozoospermia associated with diabetes.[30] For stallions, feeding of the supplement is typically initiated three weeks prior to the breeding season. While significant improvements in sperm motility have not been reported with the use of this product, a number of slow breeding stallions have apparently shown dramatic improvements in libido.

Fatty acids

Semen from virtually all species examined contains relatively large amounts of lipid, in the form of polyunsaturated fatty acids (PUFAs) which plays a major role in motion characteristics, sensitivity to cold shock and fertilizing capacity of sperm.[31] In particular, docosahexaenoic acid (DHA; an omega-3 fatty acid) and docosapentaenoic acid (DPA; an omega-6 fatty acid) are the major polyunsaturated fatty acids (PUFAs) in semen.[32] The level of DHA in seminal plasma as well as the ratio of omega-3 to omega-6 fatty acids in sperm of men with poor sperm motility, was found to be significantly lower than in men with normal semen quality.[33] Increasing the ratio of DHA to DPA in semen has been shown to increase fertilizing capacity and semen quality; whereas higher levels of DPA relative to DHA results in reduced fertility.[34,35]

Since animals are unable to synthesize PUFAs, they must acquire them from precursor PUFAs in their diet and the transfer of PUFAs from the diet to semen occurs in a number of species, including the

horse.[36] Vegetable oils, such as corn and soybean oil, found in most equine diets, contain high levels of linoleic acid, the parent compound of DPA, while the precursors for omega-3 fatty acids, such as DHA, are very low. A diet of this nature would favor the formation of DPA over DHA since the conversion of precursors to DPA and DHA uses the same competitive enzymatic pathway. Omega-3 fatty acids cannot be converted to omega-6 fatty acids or vice-versa. Since high DPA to DHA ratios in semen are associated with reduced sperm quality and fertility, typical equine diets could have a negative impact on quality of stallion semen and its tolerance to cooling and freezing.[34,36]

Simply supplementing the stallion's diet with precursors to omega-3 fatty acids such as cod liver oil or flaxseed oil can increase the overall level of omega-3 fatty acids in semen, but this may not result in the desired effects of improved semen quality. However, supplementing the diet with omega-3 precursors along with pre-formed DHA and antioxidants has been shown to increase semen quality in boars and stallions.[34,36]

Researchers at Texas A&M fed a supplement, which resulted in a three-fold increase in semen DHA levels and a doubling of the ratio of DHA to DPA in the semen. Beneficial effects including increases in total motility, progressive motility and rapid motility were most apparent after 48 hours of cooling and storage.[36] The sperm concentration of stallions fed the supplement was almost double that of those fed the control diet. Total motility, progressive motility, and percentage of sperm exhibiting rapid motility were also significantly higher in frozen-thawed semen of stallions being fed the supplement. Subsequently, similar studies carried out at other institutions resulted in improved total numbers, morphology and percentages of live sperm.[37,38] In all of these studies, the improvements were most noticeable for stallions that initially had poorest semen quality.

In spite of the improvements in semen quality observed in the Texas A&M study, the level of DPA in semen remained higher than DHA. The stallions' rations were typical equine formulations containing corn and soybean oils. It was hypothesized that even more dramatic improvements in semen quality may have been observed if the fat content of the stallion diets were modified to favor more DHA precursors and incorporated with the DHA supplement.[36] Flaxseed (meal and oil), which is very rich in the omega-3 fatty acid alpha-linolenic acid (ALA), may be such an alternative energy source. However, since most of the ALA is converted to another omega-3 fatty acid, eicosapentaenoic acid (EPA), the conversion of ALA to DHA in horses does not appear to be very efficient.[39] Even so, because of the competitive enzymatic pathway, reducing the amount of omega-6 precursors in the diet by substituting them with omega-3 precursors and supplementing with preformed DHA, should be beneficial for some stallions with poor semen quality. Recently, Austrian workers reported that dietary supplementation of stallions with linseed oil (flaxseed oil) plus antioxidants mitigated the decline in motility and membrane integrity of cooled-stored stallion semen during winter, a similar effect was not observed for frozen-thawed semen.[40] As early as the 1950's, it was reported that adding fish meal to the diet improved sperm motility and survival in stallion semen.[41] Supplementing the diet of miniature Caspian stallions with a combination of fish oil and thyme (*Thymus vulgaris*), which is rich in antioxidative substances, improved total and progressive motility, plasma membrane integrity and functionality of cooled stored sperm.[42] While marine sources of DHA and its precursors (fish oil or algae) may be superior to flaxseed for increasing DHA levels in stallions, but palatability and supplement refusal can be problematic.

Conclusions

It is clear that dietary alterations can have an effect on semen quality and in some cases, fertility. Controlled studies in stallions are few, but those investigating fatty acids, in particular omega-3 fatty acids such as DHA, have shown real potential. Supplementing the diet of highly fertile stallions or those that produce sperm that survive cooling and freezing well does not appear warranted. However, stallions of marginal fertility and those whose sperm have poor tolerance to cooling and freezing would be horses that might benefit most from being fed dietary supplements.

Altering the diet of marginally fertile stallions by optimizing levels of DHA and its precursors, as well as adding antioxidants and micronutrients may improve their semen quality sufficiently enough to make them commercially viable for cooling or freezing. Further studies involving optimal levels of

individual supplements and combinations of supplements which could act synergistically to improve stallion semen quality are needed. Since maintaining a healthy balance of all nutrients is imperative to overall health, one should exert caution and an equine nutritionist should be consulted before making any dramatic changes to the horse's diet.

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Granulosa-theca cell tumor in a dairy doe: endocrinology and surgical treatment

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A 7 year old multiparous Alpine doe was presented for evaluation of infertility. For the previous two seasons, she had only shown heat after progesterone treatment and continued to show estrus at weekly intervals despite gonadotropin releasing hormone administration at breeding. Three months prior to presentation she had been bred using laparoscopic artificial insemination but did not become pregnant. She was bred twice more by natural cover but did not become pregnant. On examination, the doe was systemically healthy. Complete blood count and serum biochemistry panels were unremarkable. On reproductive examination the uterus contained traces of fluid, the left ovary was small an inactive, and the right ovary was large, multiloculated, and with appearance suggestive of granulosa-theca cell tumor (GTCT) or cystic ovarian disease. Vaginoscopy demonstrated a normal cervix and vagina with mucus present. Endocrinology panels showed high inhibin and anti-Mullerian hormone (AMH), supporting diagnosis of GTCT (Table). The right ovary was surgically removed using a ventral midline celiotomy under general anesthesia, and measured 7.5 x 6 x 4 cm. Histopathology confirmed the diagnosis of GTCT. Endocrinology performed 96 hours post-operatively demonstrated significant decreases in AMH and inhibin. Peri-operative treatments included broad-spectrum antibiotics and anti-inflammatories. The doe remained healthy post-operatively but did not become pregnant the following breeding season. Re-examination demonstrated cystic ovarian disease of the remaining ovary. This case suggests that endocrinology including AMH and inhibin can be used to confirm the diagnosis of GTCT in goats. In fact, AMH recently has been used to diagnose GTCT in cattle.[1] Unfortunately, although the remaining ovary resumed follicular activity, cystic ovarian disease developed and infertility persisted.

Table. Serum endocrinology findings of a doe with granulosa theca cell tumor of the right ovary and shortened interestrus periods.

	Pre-operatively	96 hours post-operatively
Inhibin	10.01 ng/mL	1.1 ng/mL
Anti-Mullerian Hormone	7.7 ng/mL	0.82 ng/mL
Testosterone	16.2 ng/mL	n/a
Progesterone	0.6 ng/mL	n/a

Keywords: Anti-Mullerian hormone, inhibin, ovariectomy, neoplasia

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Progesterone levels at the expected time of luteolysis in diestrus, pregnant, carbetocin and oxytocin treated mares

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Oxytocin is an inexpensive, safe and reversible means of estrus suppression when administered on days 7 to14 of the estrous cycle, however a related compound, carbetocin, was reported to shorten the luteal phase.[1] To better understand the ovarian response to these related hormones a study was designed with the objective of comparing progesterone (P4) profiles in diestrus (Diest), pregnant (Preg), carbetocin (Carb) and oxytcocin (Oxy) treated mares at the expected time of luteolysis. We hypothesized that Carb administration would result in premature luteal regression. Light horse mares were examined to determine if they had a normal interovulatory interval and were then examined daily in estrus until the day (D) of ovulation (D0), and then every other day during an estrous cycle using transrectal palpation and ultrasonography. Jugular blood was drawn on D12, D14 and D15, centrifuged and serum stored until assayed (Siemens Coat a Count Progesterone RIA, Los Angeles, CA). Mares were randomly assigned to treatment and studied over two estrous cycles with a rest cycle in between treatment cycles. Groups were: Diest (n=5), Preg (n=6), (bred using artificial insemination with >200 normal and motile sperm from one fertile stallion every other day in estrus), Oxy (n=6) (Oxytocin, Bimeda–MTC, Cambridge ON) 60 IU BID D7 to 14) and Carb (n=10) (T.R.C, North York, ON, Canada, 1.9 mg SID D7 to 14). Luteal tissue was sampled on either D12 and D15, or D10 and D12 as part of another study. Proprietary software (Statistix version 10, Tallahassee, FL) using $p<0.05$ was used to evaluate the normality of the P4 data using a Shapiro – Wilk test, and Kruskal Wallis was used to evaluate the effect of treatment and day on P4. Post hoc analysis was performed using Dunn's all pair wise test. There was a significant effect of treatment ($p=0.0000$), but not time on P4 levels. The P4 levels ng/ml [median (quartiles)] by group were: diestrus [5.1 (1.0, 14.0)], Oxy [10.4 (5.5, 16.1)], Carb [0.13 (0.03, 0.90)], and Preg [10.2 (7.2, 14.4)]. The lowest P4 levels were in the Carb treated mares. An examination of the data showed that P4 levels in Carb treated mares were low at day 12. We concluded that Carb administration shortens the luteal phase by inducing premature luteolysis. The underlying basis for this effect of Carb requires further investigation.

Keywords: Carbetocin, mare, luteal, progesterone, diestrus

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In vitro maturation of goat oocytes recovered during the non-breeding season

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The present study utilized a 2 x 2 factorial design examining the effects of gonadotropin stimulation (follicle stimulating hormone [FSH] vs. equine chorionic gonadotropin [eCG]) and medium supplementation (with or without alpha-tocopherol) on in vitro maturation rates of goat oocytes recovered during the non-breeding season. We hypothesized that follicular stimulation would not differ between gonadotropin treatments but oocytes incubated in medium supplemented with alpha-tocopherol would show increased rates of maturation compared to oocytes incubated in medium without it. Healthy does were randomly distributed into two groups: FSH (n = 8) and eCG (n = 5). A controlled intravaginal drug release (CIDR-G) device was inserted for six days starting at day 0. Animals received 10 mg dinoprost and a single dose of 160 mg FSH IM or 500 IU eCG IM 24 hours before CIDR-G removal (day 5). Follicular aspirations were performed by laparoscopic ovum pick-up 36 hours after gonadotropin treatments. For follicular punctures, an 18-gauge 3.5 inch long with a short bevel needle attached to a vacuum system with pressure not exceeding 60 mmHg was used. Oocytes were recovered into 50-mL centrifuge tubes with medium composed of PBS supplemented with 10 IU/mL of heparin and kept at 36°C. All recovered oocytes were placed into maturation medium (M199 with Earle's salts) and incubated for 24 h. After incubation, oocytes were examined for the presence of the first polar body. Proportions of oocytes reaching maturation between and within experimental groups (FSH vs. eCG; with or without alpha-tocopherol) were compared using Chi-square and Fischer's exact test whenever indicated, with significance set at $P \leq 0.05$ and with a trend for significance set at $0.05<P\leq 0.1$. The overall oocyte maturation rate was 40.63% (26/64). There was no effect of medium supplementation with (33.3%; 11/33) or without (48.4%; 15/31) alpha-tocopherol. Similarly, there was no effect of gonadotropin stimulation on oocyte maturation, FSH (43.2%; 19/44) vs. eCG (35%; 7/20). We conclude that addition of alpha-tocopherol to in vitro maturation medium did not improve oocyte maturation in does during the non-breeding season.

Keywords: Alpha-tocopherol, goat, gonadotropin, oocyte, laparoscopy

XX/XY chimerism in an infertile female alpaca born as a singleton cria

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Repeat breeding represents 74% of complaints in female South American Camelids seen at the Theriogenology Service at Washington State University, and of those, ovarian hypoplasia and chromosomal abnormalities represent the majority of diagnoses in nulliparous females.[1] A three year old, 61.4 kg, Huacaya alpaca was presented for infertility. She had been bred multiple times over one year to two fertile males, with no resultant pregnancies and had been examined by several veterinarians with no diagnosis. Transrectal ultrasonography demonstrated a small uterus with small, inactive ovaries. Serial ultrasonography was performed over nine days, with no follicular development observed. The cervix was easily catheterized and uterine cultures were negative for pathogenic organisms. Laparoscopic examination confirmed the diagnosis of ovarian hypoplasia, with each ovary 3 mm in diameter with no follicular development. Cytogenetic evaluation of peripheral lymphocytes demonstrated a karyotype of 74,XX/74,XY. Chromosomal abnormalities are a common cause of ovarian hypoplasia in camelids. However, in this case, presence of the Y chromosome cell line suggests the possibility that this female, although born singleton, may have been co-twin to a male which perished in utero. A case of freemartinism in a llama has been previously reported, born co-twin to a male.[2] This case exemplifies how diagnosis of the cause of infertility can often only be confirmed using advanced techniques, and that birth of a singleton cria should not rule out chimerism or freemartinism. Furthermore, owners of female camelids which are repeatedly bred without conceiving should seek veterinary examination, as females with ovarian hypoplasia will not reject the male, and repeated breeding becomes a welfare issue.

Keywords: Cytogenetics, karyotype, laparoscopy, freemartinism, camelid

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Sperm-bound antisperm antibodies affect the oviductal binding index of bovine spermatozoa

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The effect of sperm-bound antisperm antibodies (ASAs) on bull fertility is poorly understood. It is thought that ASAs affect sperm-oviduct interactions and impair the ability to form a sperm reservoir. The objective of the present study was to assess the effect of sperm-bound IgG and IgA on the ability of bovine spermatozoa to bind to oviductal epithelial cells *in vitro*. The *in vitro* binding index (BI) was hypothesized to decrease in the presence of sperm-bound IgG and IgA. Three ejaculates were cryopreserved from each of four ASA-negative satisfactory breeder yearling bulls. Bulls were then immunized three times 21-d apart with autologous spermatozoa. Three ASA-positive ejaculates were cryopreserved from each bull after immunization. Presence of ASAs was evaluated with flow cytometry before cryopreservation.[1] Frozen/thawed washed spermatozoa were incubated in SP-TALP with bovine oviductal explants for 30 min at 37 °C in 5% CO_2.[2] The fluorescent stain JC-1 was then added to improve visualization of spermatozoa. The number of spermatozoa bound to each explant was counted with phase contrast and fluorescence microscopy at 40 X. At least 10 explants were evaluated per ejaculate and digital images were captured. The area of the explants was measured on the photos with image analysis software (ImageJ, National Institute of Health). The binding index (BI) was calculated as the number of spermatozoa bound per 0.1 mm^2 of explant. The BI was compared between the ASA-negative and ASA-positive group with a Wilcoxon Rank Test, and the correlation between BI and the percentage of IgG- and IgA-bound spermatozoa was analyzed. The BI was lower in ASA-positive (114.9; 0 to 201.8 sperm/0.1 mm^2) than ASA-negative samples (218.9; 24.7 to 276.8 sperm/0.1 mm^2) (median; interquartile range; P=0.0002). There was a low but significant negative correlation between BI and the percentage of IgG-bound spermatozoa (P= 0.024; R^2 = -0.119) but not IgA-bound spermatozoa (P=0.091). The percentage of IgG-bound spermatozoa was higher after (55.4; 45.1 to 77.4 %) than before immunization (0.04; 0 to 0.6 %) (P<0.0001). Similarly, the percentage of IgA-bound spermatozoa increased after immunization (11.7; 7.3 to 24.3 %) compared with pre-immunization samples (0.9; 0.5 to 2.5 %) (P=0.002). Sperm morphology and motility did not differ significantly between pre-and post-immunization ejaculates. In conclusion, presence of sperm-bound antisperm antibodies significantly reduced the ability of bovine spermatozoa to bind to oviductal explants *in vitro*. This effect seemed to be mediated primarily by IgG given the negative correlation between BI and the percentage of IgG-bound spermatozoa but not IgA-bound spermatozoa. Sperm-bound IgG may therefore affect bovine fertility by reducing the ability of spermatozoa to form an oviductal sperm reservoir.

Keywords: Bovine, bull, antisperm antibodies, oviductal binding, sperm reservoir

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A comparison of different extenders for cryopreservation of semen in white-tailed deer

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Deer farming is an economically important and continuously growing industry in the United States, with its sustainability relying on the use of artificial insemination with frozen semen to effectively disseminate valuable genetics. While anecdotally, egg yolk-based semen extenders have been used with some success in white-tailed deer, there are currently no data comparing its use to soybean-based (AM, AndroMed®) extenders. The objective of the current study was to compare the use of AM extender to different egg yolk-based extenders (OR, Ovine Red®; TR, Triladyl®; BI4, Biladyl® 4%; BI6, Biladyl® 6%; BI8, Biladyl® 8%). Our hypothesis was that semen extended in egg yolk-based extenders would exhibit a greater decline in sperm motility than semen extended in AM extender. White-tailed deer (n = 6) were anesthetized with tiletamine-zolazepam (0.4 mg/lb each) and xylazine (1 mg/lb) intramuscularly via dart gun. Semen was collected using electroejaculation, and the ejaculate from each buck was divided evenly amongst six extenders: AM, OR, TR, BI4, BI6, BI8. Each sample was diluted to a concentration of 120 million sperm/mL, cooled to 5°C, and then incubated for 2 to 4 h. 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 m before submerging them into the liquid nitrogen for final freezing and storage. Each semen straw was thawed in a 37°C water bath for 30 s for post-thaw analysis. Overall and progressive sperm motilities were assessed in each extended sample using computer-automated semen analysis before and after freezing, and percent motility decline was calculated for each parameter. Data were analyzed using a General Linear Models procedure for all analyses of variance in SPSS with a Tukey HSD test for post-hoc analysis. Percent decline in overall motility for AM (49 ± 7.9%) tended to be less than for BI4 (71 ± 3.6%; $P = 0.09$); however no differences in overall motility percent decline existed between the extenders OR (50 ± 3.8%), TR (51 ± 9.2%), BI8 (62 ± 3.7%), and BI6 (69 ± 3.2%; $P \geq 0.14$). Percent decline in progressive motility for AM (51 ± 8.3%) was less than for BI4 (90 ± 1.8%), BI6 (85 ± 2.6%), BI8 (82 ± 3.6%), and TR (69 ± 9.9%; $P \leq 0.01$). Percent decline in progressive motility for OR (67 ± 2.4%) also differed from that of BI4 ($P = 0.02$), but did not differ from AM ($P = 0.16$) or any of the other extenders ($P \geq 0.11$). The use of soybean-based AM semen extender resulted in less of a decline in progressive sperm motility following cryopreservation compared to all egg yolk-based extenders used, except OR, and can be considered a better option for semen cryopreservation in white-tailed deer.

Keywords: Cryopreservation, extender, semen motility, white-tailed deer

Parturition augmentation in mares–efficacy and safety

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Foaling complications require prompt intervention to maximize foal viability. The majority of spontaneous foaling occurs late at night when personnel and support services are not readily available, making prompt identification of obstetrical problems difficult. Augmentation of parturition is the advancement of progression from stage I labor to stage II and is safely used in human obstetrics. We hypothesized that parturition augmentation can be used to safely and efficaciously advance foaling to ensure presence of personnel and support services. Our objectives were: 1) to determine the efficacy of augmentation; 2) determine the expected range of time between augmentation and foaling; and 3) to determine if there were side effects to mare or foal from the augmentation.

Mares were assigned to augmentation group (AUG) or control (CTL) and outcomes of foaling recorded. This is an ongoing study and this abstract reports the preliminary findings from 2013 and 2014. Seven mares were augmented (AUG group) with 3 IU of oxytocin i.v. when milk secretions were at pH < 6.5, and calcium > 250 ppm, with other signs of impending parturition (elongated vulva, relaxed pelvic ligaments, waxed teats). Eleven control mares (CTL group) were allowed to foal naturally. These mares foaled in the same barn during the same time frame. Differences between groups were evaluated using 2-way ANOVA in JMP Pro version 11.

Efficacy of augmentation resulted in 1 of the 7 augmented mares requiring a second dose of oxytocin. Average time from oxytocin injection to foaling was 40 minutes (range 20-70 minutes). There were no differences ($P > 0.10$) in the time between rupture of the chorioallantois and foaling (12 min CTL, 13 min AUG); foal weight (94 lbs CTL and 97 lbs AUG); interval between birth and standing (57 min CTL, 49 min AUG); and placental weight (11 lbs CTL and 13 lbs AUG) between the AUG and CTL mares. There was however, a delay in the time to suckle (81 min CTL and 124 min AUG; $P = 0.03$) and passing fetal membranes (49 min CTL and 116 min AUG; $P = 0.04$) in the AUG group compared with CTL group. One mare in the CTL and one mare in the AUG group (the mare that required the second oxytocin injection) had premature separation of the choriolallantois. In both cases, the chorioallantois was ruptured immediately and the foals were viable but required supportive treatment. In addition, one foal each in the CTL and AUG group had malposture (flexion of the elbow) that was corrected and the foals were viable. Preliminary results are promising for this augmentation protocol to advance parturition to ensure adequate personnel and support services to be available if needed. The augmentation protocol is efficacious and the only mare requiring a second dose of oxytocin had other underlying conditions. Response to augmentation was rapid with only one mare taking longer than one hour to foal after oxytocin injection. As a teaching tool, this protocol has allowed a greater number of students to observe live foalings and the timing from the rupture of the chorioallantois to completion of foaling was similar between AUG and CTL mares. Delay in the time from foaling until the first suckle is a concern. Three foals in the AUG and only one foal in the CTL group were tube fed colostrum at two hours after birth. There was also a significant delay in the time from parturition until passing of the fetal membranes; however, all of the mares passed the fetal membranes within the normal three hour window after foaling. In conclusion, the augmentation protocol at this preliminary stage of the experiment has been shown to be efficacious, with rapid response, but with minor side effects.

Keywords: Mare, parturition, augmentation, oxytocin

8 ISO prostaglandin $F_{2\alpha}$ is produced during in vitro incubation of stallion spermatozoa and correlates with sperm death

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Although the generation of reactive oxygen species (ROS) is required for numerous normal sperm physiologic functions, oxidative stress occurs when endogenous antioxidants and oxygen scavengers are overwhelmed by ROS, resulting in cellular injury and cell death. Moreover current sperm technologies in the functionality of the male gamete, involves different forms of oxidative stress. Reactive oxygen species have been largely considered a detrimental factor, but recent research challenges this assumption.[1] We hypothesize that apparently paradoxical issues around ROS arise due a simplified view of the role of ROS. We propose that detrimental effects of ROS are related to the toxic adducts resulting from oxidation of the lipids of the membranes, and to the exhaustion of the intrinsic antioxidant defenses of the sperm. To test this hypothesis we investigated the presence of 8-ISO-PGF2α, a toxic adduct of the peroxidation of the lipids of the sperm membranes, and the intrinsic levels of intracellular reduced gluthation. Semen was obtained from seven Pure Spanish horses individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Spain. The ejaculate samples were diluted 1:1 in INRA-96, centrifuged (600 g x 10 min), and re-suspended in Biggers-Whitten-Whittingham medium supplemented with 1% PVA to obtain a concentration of $50x10^6$ spermatozoa/mL and incubated at 37°C up to six hours. After one hour and six hours of incubation levels of 8-ISO-PGF2α, and reduced glutathion (GSH) were investigated. The spermatozoa (1×10^6/mL) then were washed with saline-HEPES medium and fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 15 minutes. After fixation, the cells were washed twice with PBS and incubated in the same buffer with 2 µL/ml of a solution containing 0.1 mg/mL of anti 8 iso prostaglandin F2α primary antibody and incubated for 30 minutes in the dark at room temperature. Then samples were washed in PBS and the secondary antibody (2 µL/ml) added (Antirrabit Alexa Fluo 402) and further incubated 30 minutes in the dark at room temperature. Samples were then washed again in PBS and run in the flow cytometer. Thiol tracker violet reagent was used to monitor free intracellular GSH. Thiol tracker stock solution was prepared in DMSO (20 mM) and sperm cells ($1\text{-}5x10^6$ sperm in 1 ml) were stainded with 1 µL of Thiol tracker and 1 µl of Live death far red stain and incubated in the dark for 30 minutes before reading in the flow cytometer. The presence of significant amounts of 8 Iso PGF2α in stallion spermatozoa increased from 1% at 60 minutes of incubation to 12 % after 6 hours of incubation ($p<0.01$). Interestingly significant negative correlations were found between 8 iso PGF2α levels and live sperm ($r=-0.406$ $p<0.01$). The intracellular GSH content was reduced after the incubation period from 24% at time 1 to 14% after six hours of incubation ($p<0.01$). Overall these results may indicate that adducts produced as result of lipid peroxidation may be responsible for the lose of stallion sperm quality during conservation and that intrinsic antioxidant defenses exhaust during in vitro incubation contributing to sperm death

Keywords: Stallion, sperm, ROS, 8 iso prostaglandin F2α,Thiols, flow cytometry

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Lipid content and cryopreservation of Jersey cattle embryos

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Cryopreservation of in vivo derived Jersey bovine embryos have resulted in 10% lower pregnancy rate compared to other dairy breeds. Poor embryo survival after cryopreservation has been partially attributed to the high lipid content of Jersey cattle embryos. We hypothesized that the lipid content of in vivo and in vitro produced (IVP) Jersey embryos is higher than respective Holstein embryos. The objectives of this experiment were (1) to analyze lipid content of in vivo and IVP Jersey cattle embryos and (2) evaluate Jersey IVP embryo survival rates after three cryopreservation procedures. For experiment one, IVP embryos (n=60) were produced by standard procedures, briefly oocytes were aspirated from 2 to 8 mm follicles from slaughterhouse ovaries then matured for 24h in SMM medium (BoviPro, MOFA Global, Verona, WI). Matured oocytes were fertilized using semen from two different bulls for each breed, and embryos were cultured in BBH7 medium (BoviPro, MOFA Global, Verona, WI) at 38.5°C in 5% O_2, 5% CO_2, and 90% N_2. In vivo produced embryos (n=27) were collected by standard procedures 7 days after artificial insemination. The lipid content of embryos was quantified by staining Day 7 blastocysts with 1 µg/mL Nile red dye (580-596nm), after which a digital photograph of the equatorial region of the embryo was taken at 40x, and fluorescence intensity (FI) was measured with Image Pro software. Comparisons within and between breeds were evaluated by T-Test. For experiment two, Grade 1 Jersey IVP blastocysts (n=356) were divided into six treatments using a 2x3 factorial design comparing intact (IB) vs collapsed blastocoele (CB) and three cryopreservation methods: slow freezing (SF) vs vitrification using open pulled straws (OPS) or cryotop (CT). Slow freezing embryos were equilibrated in 0.7 M glycerol and 0.1 M galactose in holding media for 5 min, held for 10 min at -6°C, seeded after 5 min, decreased to -32 °C at 0.5 °C /min and, held at -32°C for 5 min, and plunged into liquid nitrogen. Vitrified embryos were equilibrated in 1.5 M ethylene glycol (EG) for 5 min, exposed to 7 M EG + 0.6 M galactose for 30 s while loaded into OPS or placed onto CT, then immediately plunged into liquid nitrogen. SF embryos were thawed in air for 10 s and placed in a water bath at 37°C for 45 s. Vitrified embryos were warmed directly into holding medium at 37°C supplemented with 1.0 M , 0.5 M and 0.25 M galactose for 3 minutes each. Subsequently, embryos were cultured in BBH7 and re-expansion rates were assessed at 24 and 48h after warming and data were evaluated by GLIMX. For experiment 1, Jersey and Holstein IVP embryos had higher lipid content than Holstein in vivo produced embryos (56, 55 vs 43 ± 4 FI; $p<0.05$), but were not different than Jersey in vivo-derived embryos (49 ± 4 FI; $p>0.1$). For experiment 2, re-expansion rates were higher for CT, than OPS, and SF (85 vs. 66 vs. 72% ± 0.4, respectively; $p<0.05$). Main effect means for re-expansion were higher for CB than IB (79 vs 68% ± 0.3; $p<0.05$). In conclusion, IVP embryos have higher lipid accumulation over Holstein in vivo embryos. The CT method and collapsing the blastocoele prior to cryopreservation resulted in higher blastocyst survival rate. Further studies including transfer of embryos to recipients are necessary to corroborate these results.

Keywords: Lipid, cryopreservation, Jersey, embryo, Nile red

Diagnosis and effects of urine contamination on stallion semen cooling

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Contamination of semen with urine (urospermia) is known to affect raw semen quality in stallions. While semen extension has been shown to mitigate the effects of urospermia on raw semen, the effects of urine on the motility of cooled stallion semen remain unknown. Practitioners have no proven means to confirm urine contamination in extended cooled-shipped semen. We hypothesized that urine contamination affects semen motility after cooling and extension, and that semen levels of creatinine, urea and pH can be used to detect urospermia. The objectives of this study were: (i) to assess the effects of variable amounts of urine contamination on total and progressive motility of extended fresh and cooled semen and (ii) to test whether pH, creatinine and urea can detect urine contamination in extended-cooled stallion semen. Eleven reproductively healthy light breed stallions with no known history of urospermia were enrolled in the study. Free catch urine samples were obtained from three of the enrolled stallions, pooled and frozen at -20°C until further use in the study. Each stallion was collected using a phantom and a Missouri artificial vagina at two to three day intervals, and a total of thirty-seven ejaculates were obtained. Each ejaculate was assessed for initial motility using Computer-Assisted Sperm Analysis (CASA, Spermvision Minitube of America, Verona, WI), sperm concentration using an Equine Densimeter (Animal Reproduction Systems, Chino, CA), and semen pH using a LAQUA Twin pH meter (Horiba Instruments, Irvine, CA). The ejaculates were then divided into five 5 ml aliquots, and either 0, 0.25, 1, 1.5, or 5 mls of pooled stallion urine was added. Each semen sample was then reassessed for semen pH, creatinine and urea concentrations and extended with INRA 96 (IMV Technologies, Maple Grove, MN) to a final concentration of 25 million sperm/ml. Total and progressive motility were re-assessed, and the samples were then packaged in Whirl-Paks (Nasco, Fort Atkinson, WI) and stored in commercial semen containers (Equitainer I, Hamilton Research Inc, Ipswich, MA) for 24 hours. At 24 hours, the semen containers were opened and semen motility and pH were assessed for all samples. Two ml aliquots of all samples were frozen for later analysis using an automated analyzer (Beckman Coulter, Pasadena, CA). Statistical analyses were performed using mixed models and when significant, post hoc comparisons were made with LSD (JMP 11, SAS Institute, Cary, NC). There were no stallion effects ($p>0.05$). As expected for raw semen samples, urea, creatinine and pH increased with urine contamination ($p<0.0001$). Motility decreased in all samples pre- and post-cooling, with pronounced reduction in groups contaminated with 1 to 5 ml of urine ($p<0.05$). Creatinine and urea measurements in cooled extended semen enable identification of urine contamination and were different than the control group ($p<0.05$). There were no differences in pH among the groups after cooling for 24 hours ($p>0.05$). In conclusion, urospermia not only affected raw semen motility, but small amounts of urine contamination affected motility after 24 hours of cooling. Extension of semen with INRA 96 did not prevent detection of urine contamination using creatinine and urea measurements. However, pH can only be used to detect urine contamination in raw semen and not for cooled-extended semen. This is the first study to demonstrate the use of creatinine and urea to detect urospermia in cooled extended stallion semen.

Keywords: Urospermia, stallion, semen cooling, creatinine, urea

Circulating microRNAs and associated gene regulation in puerperal metritis in dairy cows

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Dairy cows that calved recently are prone to uterine and metabolic disorders due to suppressed immune function and negative energy balance. Metritis is one of the most common uterine diseases of dairy cows diagnosed during puerperal period (first 10 days in milk). It is defined as presence of fetid reddish-brown watery vaginal discharge, systemic signs of illness with fever (rectal temperature of 103°F or greater). Cows that suffer from metritis associated with poor reproductive performance, including irregular estrous cycles, lower conception rates and greater intervals from calving to pregnancy. Although uterine infection occurs most commonly after calving complicated by dystocia, retained fetal membranes, twins or stillbirth, cows with poor immune function are most likely to develop metritis. MicroRNAs (miRNAs) are small non-coding molecules that are partially complementary to one or more messenger RNA (mRNA). Their main function is to down-regulate gene expression in a variety of manners, including translational repression, mRNA cleavage and deadenylation. The objective was to compare circulating miRNAs and their integrated genes in cows suffering from metritis and normal cows. Dairy cows diagnosed with (n=4) or without (n=4) metritis from a single farm were included in this study. Blood samples were collected via coccygeal venipuncture at the time of diagnosis. In individual serum samples, we investigated 84 prioritized cow-specific miRNAs using RT-PCR method. Total RNA, including miRNAs, was isolated from frozen-thawed serum, complementary DNA was synthesized and mature miRNA expression profiling was performed using real time PCR. MiRNA-specific forward primer and universal reverse primer were used to amplify mature miRNAs. *Caenorhabditis elegans* miRNA, cel-miR-39-3p was used as endogenous control to normalize target miRNA expression. Data were analyzed using the ΔΔCT method of relative quantification using the computational software at http://pcrdataanalysis.sabiosciences.com/mirna. Circulating miRNAs (n=34) were identified in differential abundance in cows with metritis compared to normal cows. Of those 34 miRNA, 18 were observed in abundance and 16 were scarce among cows with metritis compared to normal cows. Specifically several miRNA families were scarce, including bta-let-7f (-31.3), bta-miR-10a (-20.1), bta-miR-127 (-4.2) and bta-miR-148b-3p (-61.8); and several families were found to be in abundance, including bta-let-7a-5p (25.6), bta-miR-101 (88.2), bta-miR-142-3p (77.5), bta-miR-150 (16.4), bta-miR-16b (27.18), bta-miR-181a (4.2), bta-miR-191 (21.9), bta-miR-192 (8.2), bta-miR-21-5p (3.1), bta-miR-24-3p (2.8), bta-miR-25 (3.1), bta-miR-26b (169.3), bta-miR-30d (2.5) and bta-miR-30e-5p (4.0) in cows with metritis compared to normal cows ($P<0.01$). A considerable number of miRNAs were predicted to inhibit the expression of genes associated to proinflammatory and immune-related responses, angiogenesis, cell-cycle progression, and adhesion molecules. In most of the cases, the levels of these miRNAs were abundant in cows with metritis compared to those without metritis. In conclusion, the presence of distinct miRNA profiles between cows with metritis and normal cows indicates that miRNA may have a role in the pathophysiology of metritis. It is possible that these miRNA could be targeted for treatment using inhibitors and/or mimics.

Keywords: Dairy cows, postpartum, metritis; miRNA, genes

Ovarian function in pony mares undergoing porcine zona pellucida immunocontraception

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An advantage of the porcine zona pellucida (pZP) vaccine over other immunocontraceptives is the preservation of reproductive cyclicity and associated behaviors. Few studies have investigated ovarian function following pZP vaccination in the mare despite reported ovarian dysfunction in other species. The objectives of this study were to investigate ovarian function and estrous cyclicity in pony mares following treatment with the conventional pZP vaccine. Fourteen mares were randomized into two groups of seven. Group I received 100 μg of pZP with Freund's complete modified adjuvant (FCMA; V1), followed by 100 μg of pZP with Freund's incomplete adjuvant (FIA; V2). Group II (controls) received saline with FCMA (V1) and saline with FIA (V2). Treatments were administered into the gluteal muscles. Data were collected by an investigator blinded to treatment group over a period of 24 weeks during the physiological breeding season, with V1 and V2 administered during weeks four and nine, respectively. Mares underwent examination by trans-rectal palpation and ultrasound of the internal reproductive tract on days 7 and 14 of each estrous cycle, with daily monitoring from day 14 until ovulation (day 0). Artificial insemination was performed using fresh semen for up to two consecutive estrous cycles, commencing five weeks after V2. Serum samples were collected weekly for the analysis of ovarian steroid (progesterone and estradiol) levels. Data were compared using Mann-Whitney U tests using commercially available software (IBM SPSS Statistics Version 22, International Business Machines Corp., Armonk, NY). Statistical significance was set as $P < 0.05$. All Group II mares showed normal estrous cyclicity throughout the study. Six Group I mares showed intermittent to sustained evidence of ovarian suppression > five weeks after V2, characterized by small, inactive ovaries and baseline progesterone and estradiol levels. Ovarian volumes, follicle counts and maximal follicle diameters in Group I were significantly lower than Group II > five weeks after V2 (Table). Per-cycle pregnancy proportions in Groups I and II were 0% and 78%, respectively. This study demonstrated suppression of ovarian function in six of seven (86%) pony mares during pZP immunocontraception.

Table. Median (range) ovarian volumes (cm^3), follicle counts, and maximal follicle diameters (mm) recorded during one week > five weeks after V2*.

	Group I: pZP	**Group II: Controls**	P value
Left ovary volume	6.3 (6.3 - 131.8)	78.5 (23.5 - 131.8)	0.017
Right ovary volume	6.3 (2.1 - 78.5)	41.8 (18.8 - 131.8)	0.017
Left ovary follicle count	0 (0 - 2)	4 (1 - 10)	0.004
Right ovary follicle count	0 (0 - 1)	3 (2 - 8)	0.001
Maximal follicle diameter	0.0 (0.0 - 47.2)	33.6 (22.0 - 47.0)	0.026

* If more than one data point for a mare existed, the data point recording the greatest follicle diameter was included.

Keywords: *Equus caballus*, mare, immunocontraception, porcine zona pellucida, ovarian function

Long-term effects of clinical applications of pyrethrin and cyfluthrin, a synthetic pyrethroid, on bull reproductive parameters

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Effective fly control is crucial for cattle health and well-being; however, pyrethrin and pyrethroid insecticides can impair semen quality and inhibit testosterone production in mammals. Previous experiments demonstrated pour-on, ear tag, and spray applications of pyrethrin and pyrethroid insecticides had no effects on bull semen quality in the short-term (zero to nine weeks). However, spray applications of these insecticides decreased serum testosterone concentrations at nine weeks, suggesting potential detrimental effects on reproductive parameters if used long-term. The objectives of the current study were to determine the effects on bull reproductive parameters of pyrethrin and beta-cyfluthrin spray applications, used at labeled dosages over 18 weeks, in combination with cyfluthrin pour-on and ear tags. Our hypothesis was that addition of spray applications would negatively impact reproductive parameters in bulls after nine weeks. Angus, Simmental, and Angus x Simmental bulls (n = 27) were randomly assigned to one of three treatment groups: (1) no exposure to pyrethrins/pyrethroids (CONT; n = 10), (2) fly tags and pour-on (TRT1; n = 9), or (3) fly tags, pour-on, premise spray, and fog spray (TRT2; n = 8). The TRT1 and TRT2 bulls were treated with Cylence® pour-on (active ingredient cyfluthrin; 1%) at the labeled dose every three weeks and had two Cylence Ultra® fly tags (active ingredients beta-cyfluthrin; 8% and piperonyl butoxide; 20%) inserted, one in each ear, at week 0. Bulls receiving TRT2 also had Tempo® premise spray (active ingredients beta-cyfluthrin, cyano and methyl 3; 11.8%) applied to their barn once weekly and a fogging spray (pyrethrins; 0.5% and piperonyl butoxide; 4%) applied to the bull once daily at labeled dosages. Body weight (BW), body condition score (BCS) and scrotal circumference (SC) were assessed on weeks 0, 9, and 18. Semen was collected every three weeks via electroejaculation and assessed, using computer-assisted semen analysis, for overall and progressive sperm motility, and morphology. Whole blood, as a source of serum, was collected from the tail vein at approximately the same time every three weeks, and serum testosterone concentrations were measured by RIA. Data were analyzed using the MIXED procedures in SAS with repeated measures for sperm motility, sperm morphology, serum testosterone, and BW. There was a treatment x week interaction ($P < 0.01$) for sperm with primary defects; bulls in CONT group had a greater ($P = 0.01$) percentage of sperm with primary defects than bulls treated with insecticides at week 18. Overall and progressive sperm motility, normal sperm morphology, and serum testosterone concentrations changed ($P < 0.01$) over time in all bulls; however, treatment group did not affect ($P \geq 0.25$) any of these parameters. There were also no treatment effects ($P \geq 0.14$) on bull BW, BCS, and SC. The use of pyrethrin and cyfluthrin based insecticides, regardless of application, did not alter reproductive parameters in beef bulls when administered over 18 weeks.

Key words: Cattle, pyrethroid, reproduction, semen quality, testosterone

Seminal plasma microRNAs: potential biomarkers for bull fertility

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MicroRNAs (miRNAs) are small, endogenous non-coding RNAs, approximately 22 nucleotides in length responsible for regulating gene expression at the transcriptional and post-transcriptional level. MiRNAs are highly conserved, with close to 90% sequence similarity between humans and animals and play a critical role in many cellular processes, such as spermatogenesis and sperm physiology, including fertilization, oocyte activation and embryo development. Several studies suggest miRNA repression occurs during spermatogenesis; therefore, there is a proposed association between aberrant miRNA levels and male infertility. Stable seminal plasma-specific miRNAs present may serve as a diagnostic biomarker and potential predictor for male infertility in both humans and animals. A comprehensive spectrum of seminal plasma miRNAs and their regulation on spermatogenesis-associated genes have not been well elucidated. The objective of this study was to profile and differentiate seminal plasma miRNAs in high and low fertile Holstein bulls. It was hypothesized that bull seminal plasma miRNA levels vary between high and low fertility groups. Sire conception rate (SCR; fertility index) was used for Holstein bull selection, where SCR estimates were based on at least 500 services. To accomplish the described objectives, semen samples from low (SCR -4; n=3) and high (SCR +7; n=3) fertile Holstein bulls were collected using an artificial vagina. Seminal plasma was separated using centrifugation steps, aliquoted, and stored at -80°C until miRNA profiling. Total RNA, including miRNA, was isolated from frozen-thawed seminal plasma, complementary DNA was synthesized, and mature miRNA expression profiling was performed using real time PCR for each individual sample. MiRNA-specific forward primers and universal reverse primers were used to amplify mature miRNAs. Data were analyzed using the$\Delta\Delta$CT method of relative quantification using the computational software at http://pcrdataanalysis.sabiosciences.com/mirna. The software calculated the standard deviation for CT and delta CT values when more than one animal per treatment group was used. *Caenorhabditis elegans* miRNA (cel-miR-39-3p) was used as an endogenous control to normalize target miRNAs' expression. Interestingly, it was found that eighty four prioritized bovine-specific miRNAs were present in seminal plasma. Thirty two miRNAs were differentially seen at a 5-fold level in the seminal plasma of low and high fertile bulls. Twenty miRNAs, including bta-miR-214 (23.68 fold), bta-miR-199a-5p (20.11 fold), bta-miR20b (17.78 fold) and bta-miR-21-3p (13.48 fold) were highly abundant at a significant level ($n = 3$, $p < 0.05$) in the seminal plasma of high fertile bulls. Twelve miRNAs, including bta-miR-16 (-32.60 times), bta-miR-29c (-14.58 times), bta-miR-200a (-11.03 times) and bta-miR-101 (-10.99 times) were significantly lower in abundance ($n = 3$, $p< 0.05$) in seminal plasma of high fertile bulls compared to the levels in low fertile seminal plasma. The coefficient of variation values between bull samples were found to be similar. The amplified measurements of miRNAs in seminal plasma provide a novel, non-invasive approach to categorizing bulls in terms of fertility and can be used for diagnosing male infertility and reproductive pathology in both animals and humans.

Keywords: MicroRNA, seminal plasma, bull fertility, biomarkers

Fertility following two doses of PGF2 concurrently or at 6-hour interval on the day of CIDR removal in 5-day CO-Synch progesterone-based synchronization protocols in beef heifers

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Timed artificial insemination protocols in beef cattle are designed to result in highly synchronized estrus while simultaneously achieving acceptable pregnancy rates and a concise calving season. Protocols attaining such goals reduce time and labor associated with estrus detection, and make advanced reproductive technologies implementable for beef producers. We hypothesized two doses of prostaglandin F2α (PGF) administered at a six hour interval would attain the highest pregnancy rate as the corpus luteum requires additional PGF to achieve complete luteolysis at day 5 of development in 5-day CO-Synch progesterone-based synchronization protocols. The objective of the study was to determine the effect of three different PGF dosage schemes on artificial insemination (AI) pregnancy rates in beef cattle. Angus heifers (n=875; 14 to 16 months of age) at six locations in Washington, Idaho and Oregon were included in this study. Among a subset of heifers (n=493), 63.3% were cyclic. All heifers were assigned a body condition score (BCS) and received a controlled internal drug release (Eazi-Breed CIDR Cattle Insert®, Zoetis Animal Health, New York, NY) and 100 μg IM of gonadotropin releasing hormone (GnRH; Factrel®, Zoetis Animal Health) on Day 0. CIDRs were removed on Day 5, and heifers within locations were randomly allocated to and received one of three protocols: 1PGF (n=291) received 25 mg IM of dinoprost (Lutalyse®, Zoetis Animal Health); 2CO-PGF (n=291) received 50 mg IM of dinoprost at CIDR removal, and 2PGF (n=293) received 25 mg IM of dinoprost at CIDR removal and an additional 25 mg IM of dinoprost 6 hours later. Each heifer was given GnRH (100 μg, IM) and concurrently artificially inseminated 56 h after CIDR removal. Heifers were examined for pregnancy status between 50 and 70 d after AI. The AI pregnancy rate was calculated by number of heifers pregnant divided by number of heifers inseminated. A mixed model procedure (PROC GLIMMIX of SAS) was used to evaluate the effect of treatments (1PGF, 2CO-PGF and 2PGF) on AI pregnancy rates. This model included treatments, BCS categories (≤ 5 and > 5), and appropriate interactions. Location (state), handling facilities, handlers, inseminators and AI sires were included as a random effect in the model. Additionally, univariate analysis (PROC GLM, SAS) was used to determine location effect. The *P* value was set at ≤ 0.1 for inclusion and > 0.1 for exclusion until only significant main and interaction effects were retained in the model. The 2PGF group yielded a greater AI pregnancy rate of 63.6±3.2% (185/291), compared to the 2CO-PGF group at 51.9±2.6% (151/291) and 1PGF group at 54.9±2.5% (161/293) ($P<0.001$). An AI pregnancy rate of 50±2.3% (104/208) was observed for heifers with BCS ≤ 5, versus 58.9±3.0% (393/667) for heifers with BCS >5 ($P<0.05$). Location did not influence the AI pregnancy rate ($P>0.1$). In conclusion, heifers synchronized for fixed time AI with 5-d CO-Synch progesterone based protocols require two administrations of PGF at 6 h interval for optimal AI pregnancy rates.

Keywords: Beef heifers, CIDR, PGF, estrous synchronization, insemination, pregnancy

Histologic and morphometric evaluation of testes of feral tom kittens and cats

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Even with numerous successful trap-neuter-release programs, feral cat populations continue to grow. Our laboratory is interested in determining if an underlying biological cause exists for the exuberant reproductive success in this once domestic subspecies. An earlier age for developing reproductive capacity (onset of spermatogenesis in males) may be one factor. For domestic toms, puberty is reported to occur around 8 months of age.[1] Previous work by our laboratory has shown that normal morphologic sperm are present in vas deferens secretions of feral toms before 6 months of age.[2] Therefore, our hypothesis was that in feral toms the onset of spermatogenesis occurred before 6 months of age. The study objective was to histologically evaluate testes from weanling (2 months of age) through adulthood (24 months of age) to determine when the onset of spermatogenesis occurs in feral toms.

Feral toms were presented for castration at a local humane society during August-October 2014. Age was determined by records provided from feral cat colony managers and confirmed with dental eruption patterns. The age groups were: 2-2.5 months (weanling; n=6), 3-4 months (juvenile; n=6), 5-6 months (pubertal; n=6), and 12-24 months (adult; n=6). General anesthesia was induced and a routine open castration was performed. Both testicles from each cat were hemi-sectioned, formalin-fixed, paraffin-embedded, cut into sections (6 μm), and stained with hematoxylin and eosin. The slides were evaluated by a single observer (EB) blinded to age group using bright field microscopy (200X). Evidence of spermatogenesis was determined on the basis of presence of spermatozoa in the seminiferous tubule lumen. In addition, perpendicular diameters measured from 5 tubules for each testis were averaged and mean ± SD was determined for each age group. Tubular diameter was compared using a Student's t test where $p < 0.05$ was defined as significant. The presence of spermatozoa in the lumen was compared using a χ^2- test.

Evidence of spermatogenesis in weanling, juvenile, pubertal, and adult toms was 0%, 17%, 67%, and 100%, respectively ($p < 0.05$ between successive age groups). The seminiferous tubular diameter was significantly larger in each successive age group (weanlings 88.10 ± 10.88 μm; juveniles 109.8 ± 8.89 μm; pubertal 142.2 ± 16.89 μm; adult 237.90 ± 52.45 μm).

Juvenile feral toms in the current study had significantly wider tubular diameter than previously reported for domestic toms under 5 months of age (86 μm),[3] which supports our hypothesis that spermatogenesis is occurring at an earlier age in ferals. However, the time of year the previous measurements in domestics was not reported. If these measurements were made outside of the breeding season, this may explain why the tubular diameters were smaller. Future studies are planned to determine if folliculogenesis occurs earlier in queens as well.

Keywords: Castration, feline, puberty, seminiferous tubule, spermatogenesis

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Intrauterine marbles for estrus suppression in mares – two marbles are not always better than one

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Estrous behavior in performance mares is undesirable (aggression, hyperexcitability, musculoskeletal pain).[1] Methods of estrus suppression include progestin supplementation, administration of oxytocin during diestrus, and intrauterine marbles. Marbles can prolong the luteal phase and decrease behavioral estrus in some mares. The efficacy of intrauterine marbles is debatable[2-4] and severe complications with their use have been reported.[5] The following describes two separate instances of previously unreported complications associated with intrauterine marbles.

Case 1: A 13-year-old Quarter horse mare presented for chronic intermittent colic of 80 days duration. Eight years prior to presentation for colic, two marbles were individually placed into the uterus at three-week intervals to prevent unwanted estrous behavior. Diagnostic tests (complete blood count, serum chemistry, palpation per rectum, transabdominal and transrectal ultrasonography, and gastroscopy), failed to determine the cause of colic. Abdominal radiographs revealed two 4-cm, round, radiopaque structures in the caudal abdomen consistent with enteroliths, uroliths, or uterine marbles. Following induction of estrus with prostaglandin F2alpha and cervical dilation with prostaglandin E1, both marbles were removed and the mare was discharged to the owner's care. Follow-up 12 months later confirmed no further colic, suggesting that intrauterine marbles were likely the cause of the abdominal discomfort.

Case 2: A 4-year-old Thoroughbred filly had marbles placed in her uterus during her racing career. Before breeding, the attending veterinarian removed one marble. She was mated in Kentucky, pronounced pregnant, and returned to a breeding farm in Louisiana. Three months later she aborted and the uterine contents were submitted for evaluation. Examination of the abortus revealed a second marble that was encased in extra-fetal membranes attached by a pedunculated stalk to the amnion. The stalk was wrapped around the amniotic umbilical cord, resulting in fetal death and abortion.

These cases illustrate that intrauterine marbles are not innocuous and careful examination for multiple marbles is necessary.

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Sperm immotility as a cause of infertility in a bull

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A three year old, 800 kg Charolais bull was presented to the Theriogenology Service for a breeding soundness evaluation (BSE) after he failed to produce any pregnancies in a herd of 40 cows and a herd of 30 heifers. The owner claimed that he had purchased the animal with a satisfactory breeding soundness certificate. The bull had never been ill and had been seen to breed many females. During a routine BSE[1], > 85% of the bull's spermatozoa lacked progressive motility. Many spermatozoa were alive and displayed some degree of slow non-progressive motility. Eosin-nigrosin staining confirmed that over 90% of his spermatozoa were alive immediately after collection and that 62% had abnormal midpieces (mitochondrial helix disruptions and abaxially attached flagella).

As the bull was obviously infertile, the client elected to have him slaughtered. The bull's testes could not be procured, but semen and blood samples were saved for further study. Electron microscopic evaluation revealed spermatozoa with deranged flagellar microtubules and disrupted mitochondrial helix. Further tests identified high incidence of DNA fragmentation detected by TUNEL assay,[2] and subcellular defects detected by lectin (PNA and LCA) and ubiquitin labeling.[3] Genotyping results are pending, but it is hypothesized that there may be one or more mutations associated with the abnormal maturation of this bull's spermatozoa.

Sperm immotility has not previously been reported as a congenital defect in bulls. Should this prove to be a genetic defect and a specific mutation can be identified as its cause, other bulls in this pedigree and the breed can be tested. It stands to reason that the defect described here was congenital. Therefore, this case illustrates the grave consequences of an improperly executed BSE prior to the sale of any young bull.

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Domperidone treatment for agalactia in a queen

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A 2-year old female Himalayan cat with a history of neonatal loss in her previous three litters presented to Auburn after queening three kittens. Physical examination revealed poor mammary development and agalactia. The kittens were supplemented with milk replacer, but all died within two days. Two kittens were submitted for necropsy, and the cause of death was undetermined. Other causes for fading kitten syndrome were ruled out, including blood type incompatibility and obvious infection.

The queen was presented six months later for suspected pregnancy. Pregnancy was confirmed and gestational age estimated with consecutive ultrasound examinations. Domperidone therapy was initiated six days before queening and continued one week following. The day of queening, the kittens were administered serum from a healthy adult donor cat of known blood type as a colostrum replacement. Therapy was successful; three of five kittens thrived and agalactia was resolved.

Primary agalactia occurs with defects of the pituitary-ovarian-mammary gland axis.[1] Secondary agalactia occurs as failure of ejection, occurring secondary to stress, premature parturition, mastitis, or metritis[1]. Iatrogenic agalactia may follow progesterone supplementation[2]. Oxytocin and metoclopramide are often used for treatment at time of delivery in affected animals[3].

There is little literature on diagnosis and treatment of agalactia in queens. Domperidone is a dopamine inhibitor used for treatment of fescue toxicosis in pregnant mares and agalactia in bitches[4]. However, the use of domperidone in queens for agalactia is not well described.

Primary agalactia in queens is rare, but this case shows potential for improving outcome in affected queens. The timing of initiating domperidone is essential. If too early, milk production may increase too soon before queening, thus lack of active nursing would cause milk to be resorbed. If treatment is too late, the drug would not have adequate time to be effective before kittens attempt to nurse.

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Use of behavioral and pharmacological manipulations followed by castration and gamete rescue in securing offspring from a challenging stallion

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A 4-year-old Paint stallion was presented for semen evaluation and phantom training. As a foal, the stallion had sustained a penile laceration and during erection a 10 cm long band of scar tissue was noted along the dorsal aspect of the penis. During initial semen collection attempts using an artificial vagina the stallion showed good arousal and response but did not ejaculate. Differential diagnoses included pain during erection due to scar tissue and decreased sensation to the distal penis.

Subsequent semen collection attempts included administration of imipramine (2mg/kg PO), xylazine (300mg IV), and gonadorelin (100mg SQ), the use of manual and heat stimulation of the penis, and providing a variety of ovariectomized mount and natural estrous stimulus/mount mares.[1-3] At best only pre-ejaculate fluid was collected. Castration and gamete rescue was elected, resulting in 16,663 million total sperm (total motility = 79%; progressive motility = 69%). Post-thaw total and progressive motility were 71% and 60%, respectively, producing 103 straws with 50 million progressively motile sperm per straw.[4]

Using 150 million progressively motile sperm with the addition of 15% seminal plasma from a fertile stallion, a 9-year-old Paint maiden mare was bred using deep uterine insemination before and after each of two ovulations.[5] The mare was not presented until Day 17 for pregnancy diagnosis, when twin embryonic vesicles were found fixed to the base of contralateral horns. The smaller of the two vesicles was manually reduced. On Day 27, one embryo with a visible heartbeat was documented.

There are many options for semen collection from challenging stallions, including behavioral and pharmacological manipulations. When other modalities are not productive, castration and gamete rescue provide a final option for genetic preservation. In this case, gamete rescue in conjunction with deep uterine insemination techniques resulted in a successful pregnancy, where one would not have been possible otherwise.

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Endometrial cyst ablation in a 23-year old Dutch Warmblood mare

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A 23-year old Dutch Warmblood mare presented for breeding management with cooled transported semen. Her uterus contained 15-20 endometrial cysts, several measuring 3-4 cm in diameter with multiple 1-2 cm cysts scattered throughout the endometrium. Multiple breedings were attempted but failed. Endometrial cyst ablation was recommended before attempting to breed again. A uterine biopsy was evaluated prior to cyst ablation. A "cyst map" was made to mark size and location of all cysts.

A hysteroscope was inserted through the cervix into the uterus. Each cyst was identified and ablated with a diode laser. Following ablation, the uterus was lavaged once daily for three days. On the third day, one gram of ceftiofur was infused to re-establish a healthy uterine environment. The mare returned for breeding management and insemination with cooled transported semen 30 days later. She was confirmed pregnant at 14 days. The mare carried the foal to term and delivered without complication.

Endometrial cysts form from dilations of endometrial lymphatics or glandular tissue.[1,2] Presence of endometrial cysts can interfere with early pregnancy identification, embryonic movement, embryonic fixation, and delivery of placental nutrition. Diagnosis is made by transrectal ultrasonographic examination. Mares with five or more cysts greater than 10 mm have increased embryonic loss[3]. Treatment of endometrial cysts is necessary when the mare fails to conceive or suffers early embryonic loss for undiagnosed reasons. Treatment options include rupture using a biopsy instrument or ablation via diathermy or laser. Ablation with a laser has been shown to decrease endometrial scarring and adhesion formation.[1]

If overall uterine health is adequate, treatment leads to improved reproductive ability; however, recurrence is common. Breeding should be attempted soon after treatment to minimize regrowth of cysts. This case represents how appropriate identification and treatment of a common equine infertility condition can result in a positive outcome.

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Equine pregnancy via intracytoplasmic sperm injection following remote transvaginal follicular aspiration

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An 11-year-old Shire/Thoroughbred cross mare presented to University of Calfornia Davis (UCD) for breeding management with 20-year-old frozen semen (total and progressive motility of 36% and 26%, respectively). Three unsuccessful attempts were made to breed the mare using deep horn insemination and a two-dose protocol (250×10^6 progressively motile sperm/dose). Intracytoplasmic sperm injection (ICSI) was then considered; semen was sent to Texas A&M University (TAMU) where blastocysts were produced using oocytes from resident mares, confirming the fertility of the sperm. Transvaginal follicular aspiration (TVA) was then performed on the donor mare at UCD. Six oocytes were aspirated and shipped to TAMU, where the oocytes were incubated for 30 hours, at which time four oocytes matured to metaphase II. Intracytoplasmic sperm injection was performed on the four oocytes by holding each oocyte in place with a micromanipulator and injecting an immobilized spermatozoon into the oocyte cytoplasm through a hole formed by a Piezo drill. The resultant zygotes were then returned to the incubator. Seven days following ICSI, one 6-day, blastocyst had developed, was shipped chilled to UCD, and transferred into a recipient mare that had ovulated five days previously. Ten days following ICSI, another blastocyst had developed and was vitrified. Six days following transfer of the first embryo to the recipient mare, an 8 mm vesicle was detected, which continued to grow with a heartbeat detected at 27 days and determined to be a colt fetus at 60 days.

Traditionally, ICSI requires the mare's presence at the facility where ICSI is to be performed. In this case, off-site TVA and subsequent transport and *in vitro* maturation of the oocytes allowed the mare to remain off site for the entirety of the procedure. This aspect of the oocyte collection process signifies an improvement in the availability of ICSI and potentiates its widespread utilization.

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Evaluation of a novel swim-up protocol and protein source on canine sperm progressive motility, concentration and acrosome reaction

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Introduction

Swim-up has been considered a preferred method for separating non-motile sperm in an ejaculate.[1] However, the long incubation time and equipment necessary for swim-up make this process clinically impractical.[2] Our laboratory hypothesized that these problems could be avoided for canine applications with a shorter incubation time at room temperature (RT). The objective was to compare the standard swim-up method (STAN) to this novel method (NOVEL). In addition, we hypothesized that incubation with heat-treated canine serum (HTCS) would improve swim-up results compared to bovine serum albumin (BSA) or fetal calf serum (FCS).

Methods

Ejaculates were collected manually from eight dogs and divided equally into 6 aliquots. Ham's F-10 (Cellgro, Manassas, VA; 1 mL) supplemented with 10% BSA, FCS or HTCS was overlaid on each semen aliquot. For the STAN, the samples were incubated at 45° angle for 1 hour at 37°C in 5% CO_2. For the NOVEL, the samples were incubated upright at RT for 5 min. After incubation, 0.5 mL was removed from the top of the sample and progressive motility (PM), sperm concentration (SC), and acrosomal reaction (AR; Spermac, FertiPro N.V., Beernem, Belgium) were evaluated. NOVEL was compared to STAN via a paired Student's t test (Microsoft® Office Excel 2007, Redmond, WA) and BSA, FCS, and HTCS were compared via a one-way ANOVA (GraphPad Prism®, La Jolla, CA). Significance was defined as $p<0.05$.

Results

NOVEL-HTCS and NOVEL-FCS yielded higher SC compared to the STAN-HTCS and STAN-FCS, respectively ($p<0.05$), with the same trend observed between NOVEL-BSA and STAN-BSA ($p=0.05$). Irrespective of protein source, NOVEL always yielded a higher PM and lower AR compared to STAN ($p<0.05$). NOVEL-FCS and NOVEL-BSA had a higher PM compared to NOVEL-HTCS, and NOVEL-FCS had a higher PM than NOVEL-BSA ($p<0.05$). NOVEL-FCS had the lowest AR compared to NOVEL-BSA and NOVEL-HTCS ($p<0.05$).

Discussion

This novel swim-up method can be easily applied in a clinical setting. Based upon these results, Ham's F-10 with 10% FCS is superior to either BSA or HTCS when separating the highest concentrations of progressively motile sperm that are not acrosome-reacted.

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Keywords: Bovine serum albumin, fetal calf serum, heat-treated canine serum, Spermac

Concentration dependent effect of prostatic fluid on seminal parameters of cooled canine semen

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The aim of this project was to determine the optimal dilution ratio of prostatic fluid (PF) to sperm rich fraction (SRF) for use with cooled, shipped canine semen, by evaluation of *in vitro* seminal parameters. Our hypothesis was that increasing PF concentration during cooled storage would decrease motility parameters while increasing membrane stability.

The SRF and PF were collected consecutively from four fertile stud dogs (produced a litter in the last six months) on three occasions. The ejaculates were pooled, extended (1:1, v/v) in a tris-based egg yolk extender (EYT, Uppsala Equex I), then divided into four aliquots and centrifuged (700 x g, 8 min). The pellets were re-suspended with EYT modified with 50%, 25%, 10% or 0% PF (Treatment PF50, PF25, PF10 and PF0 = control) and adjusted to a final concentration of 200 x 10^6 cells/mL. Sperm motility (CASA), sperm membrane integrity (HOST) and sperm membrane stability (YO-Pro-1/EthD-1, flow cytometry) were evaluated at 0, 24 and 48 h following storage at 4°C. The data were analyzed using repeated measures ANOVA, mixed linear model (SAS 9.4). Effects of time, treatment and interaction of time x treatment were evaluated with significance level being < 0.05.

A significant effect of time was seen on the variables total motility and progressive motility with no significant differences between treatments. No significant differences of treatment or time on plasma membrane integrity were detected with HOST. There was an effect of treatment on plasma membrane stability: the percentage of cells with a stable plasma membrane (YO-Pro-1 - / EthD-1 -) with PF0 was higher than with PF10 ($p < 0.05$) or PF25 ($p < 0.01$) but not PF50 ($p = 0.052$). Percentage of early unstable cells (YO-Pro-1 + / EthD-1 -) was higher with PF25 than PF0 ($p < 0.03$) or PF50 ($p < 0.01$), but not PF10 ($p = 0.18$). Percentage of late unstable cells (YO-Pro-1 + / EthD-1 +) with PF50 was higher than with PF0 ($p < 0.01$), PF10 ($p < 0.01$) or PF25 ($p < 0.01$). Percentage of necrotic cells (YO-Pro-1 - / EthD-1 +) was higher with PF50 than PF0 ($p < 0.03$) or PF10 ($p < 0.03$), but not PF25 ($p = 0.51$). All other unreported pair-wise comparisons between treatments were not significantly different.

Our data suggest that motility parameters decline over time during storage at 4°C over 48h without significant influence of PF concentration. The presence of a high concentration of PF was shown to have a detrimental effect on plasma membrane stability.

Keywords: Canine semen, prostatic fluid, concentration, cooled storage, seminal parameters

Canine vaginal lactic acid producing bacteria exhibit characteristics which may antagonize common urogenital pathogens

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Lactic acid-producing bacteria (LAB) positively affect vaginal health of women and other species. Relevant mechanisms include superior cellular adhesion to competitively displace other organisms and antimicrobial activity. In our laboratory, we aimed to characterize the ability of LAB obtained from vaginal swabs of healthy estrus bitches to exhibit those same characteristics. We hypothesized that LAB from canine vaginal samples would bind to vaginal epithelial cells and that they would inhibit growth of common uropathogens.

Washed vaginal epithelial cells (CVEC) obtained from estrus or anestrus bitches were incubated for eight hours with either a saline control or one of twelve LAB obtained from canine vaginal swabs. Cells were then collected by filtration (10μm filter) and bacteria adhered to 26 cells were counted by a blinded operator. Isolates demonstrating superior adhesive characteristics were tested using an agar spot assay to quantify their antimicrobial activity against five common urogenital pathogens. Selected isolates were plated onto MRS agar and incubated anaerobically. After 24 h, an agar overlay inoculated with a standard concentration of challenge organism (*Enterococcus faecalis/faecium; Klebsiella pneumoniae, Proteus vulgaris, Echerichia coli)* was performed in triplicate. After overnight incubation, the zone of inhibition was measured. Those isolates were further subjected to lyophilization and samples were plated weekly for eight weeks to test their stability at room temperature.

Bacterial adhesion to anestrual and estrual CVEC was significantly higher for two LAB (*Weisella sp.* and *Enterococous canintestini,* strain 1) than control cells ($p=0.01$ and $p=0.00001$ respectively). Of these, *Weisella* demonstrated the highest degree of inhibition against *E. coli* and *P. vulgaris,* while the *E. canintestini* demonstrated the highest degree of inhibition against *E. faecalis, E. faecium* and Klebsiella. Lyophilized organisms exhibited minimal declines in colony forming units over eight weeks.

These studies demonstrate potential protective characteristics of two LAB obtained from canine vaginal samples and demonstrate stability of organisms after lyophilization. However, additional work is needed to demonstrate the safety of these organisms to the canine urogenital tract, including the absence of a proinflammatory action and the absence of genes encoding antibiotic resistance.

Keywords: Canine, lactic acid producing bacteria, vaginal microbiome, urogenital disease.

Effect of male age on semen parameters in a purebred dog breeding program with established fertility parameters

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Sperm fertility parameters in male dogs have largely been derived from research performed in other species. Age-related changes are expected to occur as dogs mature, affecting the overall fertility of the animal. Our objectives with this study were to 1) establish baseline parameters of fresh semen for a healthy population of actively breeding stud dogs with known fertility, and 2) compare semen quality between different age groups of studs. We hypothesized that older dogs have decreased fertility and semen quality in their ejaculates, as compared to younger dogs. A breeding group of Labrador retrievers (n=39; age range 1-10 years) were subdivided into three groups: young (Y, 1-3 years; n=21), middle-aged (M, 4-6 years; n=13), and senior (S, >7 years; n=5) for comparison. Lifetime conception rate and average number of puppies per litter were documented for each dog. Two semen collections from each dog were acquired using manual collection and fractionation. An aliquot of each sperm-rich fraction was extended 1:1 (semen:extender; AndroPRO ChillGuard, MOFA Global, Verona, WI) and transported at 24°C to the laboratory. All samples were evaluated within six hours of collection. Each ejaculate was evaluated for total motility, progressive motility, and velocity of the average path (VAP) using computer assisted sperm analysis (SpermVision, MOFA Global, Verona, WI). Morphology was evaluated using eosin-nigrosin stain. An additional aliquot, extended 1:3, was slowly chilled to 4°C and warmed 48 h later to 24°C for analysis with flow cytometry using dihydroethidium (DHE) and BODIPY fluorescence probes to evaluate cellular oxidation and membrane lipid peroxidation, respectively. All data were analyzed using mixed effects models. Post-hoc multiple comparisons were conducted. Significance was set at $p<0.05$. No differences were noted between the two ejaculates from each dog. Significant differences in sperm velocity existed among all age groups, with velocity decreasing as age increased (Y>M>S). Percent morphologically normal sperm was significantly lower in older dogs (S<Y&M). No differences were noted in total motility, progressive motility, superoxide anion production, membrane lipid peroxidation, conception rate, or average litter size between any age groups. In this population of dogs, fertility does not appear to decrease with age, though sperm velocity and normal morphology decrease with age. To our knowledge, this study is the first to evaluate semen and fertility parameters as they relate to canine age. The use of advanced laboratory tests to evaluate sperm parameters beyond the classic motility, morphology, and concentration may open the door to more specific and sensitive fertility tests in canine reproduction.

Acknowledgement

The American Kennel Club generously made funds available for this research in canine fertility.

Keywords: Canine sperm, male fertility, reproductive senescence

Gene expression of retinoic acid receptors in post-natal canine testis

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Canine spermatogenesis is a complex and tightly controlled cell differentiation process, producing mature spermatozoa from spermatogonia stem cells. The role of dietary vitamin A and retinoid acid signaling for normal testicular development and spermatogenesis has been recognized for many years. Retinoic acid (RA), which is the active metabolite of vitamin A, binds to and activates nuclear RA receptors (RARA, RARB, and RARG) for the desired downstream functions in target tissues. Although there is little published research concerning testis-specific RARs, a few previous gene ablation studies in mice suggested that RA signals through RARA either in the germ cells or in the supporting cells are necessary for normal testicular function and thus for spermatogenesis. Hence, the objective of this study was to elucidate the gene expression of three major isomers of RARs (RARA, RARB and RARG) in young, peripubertal and adult canine testis and to identify their protein localization in adult testis.

The gene expression of RARA, RARB and RARG was analyzed in young (N = 8), peripubertal (n = 6) and adult (n = 8) testes of mixed-breed, medium-sized dogs using real time polymerase chain amplification technique. A non-specific SYBR chemistry approach was employed to detect target DNA sequences using specific set of primers for RARA, RARB and RARG. Relative quantification of RARB and RARG gene expression to RARA gene expression in three aged groups was calculated following normalization with the endogenous control, canine beta actin. Related fold changes were analyzed by ANOVA using $2\Delta\Delta CT$ values to ascertain statistical significance of any differences in gene expression. Protein localization in adult testes was visualized using two-step immunohistochemistry. These receptors were labeled with primary antibodies on frozen adult testes sections. The ligand-primary antibody complex was then tagged with FITC-conjugated secondary antibodies. Images were captured using a white light laser confocal microscope.

RARA gene expression was highly abundant in young, peripubertal and adult testis compared to the mRNA expressions of RARB and RARG ($p < 0.05$). On immunohistochemistry images, RARA protein localization was more intense when compared to RARB and RARG protein localization. Together, gene expression data and protein localization images from this study suggest that RARA plays a critical role in RA signal transduction for the normal function of spermatogenesis in the canine testis.

Keywords: RAR, RA signaling, spermatogenesis, testis, dog

Does cell enrichment influence the gene expression of Sertoli cells, Leydig cells and spermatogonia cells specific markers in canine testis?

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Sertoli cells, Leydig cells and spermatogonia actively proliferate and differentiate after birth in all mammalian species and the enrichment of these cells are considered stable in adulthood. These cells' enrichment were well studied in mice. At day 35, complete spermatogenesis is observed in mice. Spermatogonia increase in number until the day 21 and Sertoli and Leydig cells amplify in numbers during first three weeks. These cell numbers are essentially constant from day 35 in mice. Cell enrichment occurs in the canine testis until puberty. Several genes are associated to testicular function and spermatogenesis. Our hypothesis was that spermatogenesis-associated genes expressions are not enhanced with the mere increase of these cells numbers. To accomplish the objective, we investigated cell specific markers such as FSHR and AMH (Sertoli cell specific), LHR and INSL3 (Leydig cell specific) and THY1 and CDH1 (spermatogonia specific) in immature and mature canine testis.

Testes of four biological replicates of immature and mature groups were processed to elucidate cell specific markers. Complementary DNA was synthesized from 1 μg of total RNA. Real-time PCR was performed using specific primers. Threshold cycles (C_T) were used to analyze mRNA expressions. Fold comparisons were made between immature and mature testes. The normalized threshold cycles data were analyzed by ANOVA, using $2^{\Delta\Delta CT}$ to ascertain statistical significance of any differences in mRNA expressions.

Gene expressions of these cells' specific molecular markers were down-regulated ($P<0.05$) in adult canine testis in our investigation. Albeit, there is obvious enrichment of these cells from the immature dog testis to the mature dog testis, the cells' specific markers were not enriched from immature testes to mature testes in this study. The results supports that the gene expressions do not directly correlate with the mere increase of the cell numbers during post-natal development, but changes in gene expressions warrant functional significance.

Key words: Spermatogonia, Sertoli cells, Leydig cells, testis, dog

Computer-assisted semen analysis (CASA) to determine sperm concentration: a comparison of slide preparation methods using the Hamilton-Thorne and Minitube systems

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Abstract

This study was conducted to test the hypothesis that different slide preparations will provide accurate sperm concentration values when evaluated using CASA systems. The sperm concentration of samples prepared using six different slide preparation methods were evaluated using two CASA machines (Hamilton-Thorne TOX IVOS II; Hamilton Thorne, Inc., Beverly, MA) and Minitube SpermVision Therio Version (MOFA, Verona, WI). The slide preparation methods included a DRM-600 CELL-VU® Sperm Counting Chamber (Millenium Sciences Inc., New York, NY), using a 22 x 22 mm #2 cover slip (Method 1); - a plain glass microscope slide using a 22 x 22 mm #2 cover slip with 7 uL of semen (Method 2); - a plain glass slide using an 18 x 18 mm #1 cover slip with 7 uL of semen (Method 3); - a plain glass slide using a #2 cover slip with a "hanging drop" from a Pasteur pipette (Fisher Scientific, Waltham, MA; Method 4); - a Leja counting chamber (IMV International Corp., Maple Grove, MN) (Method 5); and a Minitube standard counting chamber (Method 6). Each method was run with samples of known sperm concentrations (230 million sperm/mL, 115 million sperm/mL and 57 million sperm/mL). These six methods were evaluated for precision when compared to one another and accuracy when compared to the known sample . The concentration of the known sample was determined by hemacytometer count (Method 7), and verified by a nucleo-counter and densimeter (control). For sperm at 230 million cells/mL, the average sperm concentrations and standard deviations determined for each method were 111 ± 21, 141 ± 53, 161 ± 46, 316 ± 101, 276 ± 20, 289 ± 31, and 230 ± 11, respectively. The repeatability determined for each method was 19, 37, 28, 32, 7, 11, and 5%, respectively. Only method 5 (Leja slide) and the control (hemacytometer) methods provided results that were within the desired 10% repeatability. In addition, the sperm concentrations determined using the CASA methods were different from the control ($P<0.05$), and no slide preparation accurately determined sperm concentration ($P <0.05$). Precision among methods was not different ($P >0.05$) except for Method 4 (hanging drop), which was less precise than the other methods. Furthermore, the accuracy of the sperm concentrations was lower at the lowest sperm concentration. The results from the two CASA machines were not different from each other ($P >0.05$). Therefore, regardless of sperm preparation method, CASA does not accurately determine sperm concentration in canine semen. However, repeatability and precision of CASA-derived concentrations are maximized using fixed-chambered slides.

Keywords: Canine sperm concentration, computer-aided semen analysis, CASA slide preparation

Failure of anti-Müllerian hormone to diagnose cryptorchidism in a pot-bellied pig

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Cryptorchidism is common in domestic swine with a prevalence of 12%.[1] Most production pigs are castrated at a very young age and issues may arise if a cryptorchid animal is not identified. Little research has been done on endocrine diagnosis of cryptorchidism in swine. The objective of this case report is to determine if anti-Müllerian hormone (AMH) could be used as a diagnostic marker for cryptorchidism in a companion pot-bellied pig.

A one-year-old castrated pot-bellied pig was presented to the Veterinary Teaching Hospital at WSU with a history of increased sexual behavior including mounting, aggressiveness and the typical boar odor. The owner reported that a diagnosis of right unilateral cryptorchidism was made at birth. The left descended testicle was removed at three weeks of age by the breeder. The cryptorchid testis was reportedly surgically removed at five weeks of age at a veterinary clinic. On presentation all physical examination parameters were normal. The prepuce and penis were normal and no testicles were visible or palpable externally. An abdominally located testicle was visualized on transabdominal ultrasonography. The owner declined surgical removal of the abdominal testis. To study the endocrine profile in this case, blood samples were collected and sent to an endocrinology laboratory for testosterone, inhibin and AMH assays. Serum samples from a known castrated pot-bellied pig were submitted as a negative control. The hormone panel revealed high testosterone (1277.3 pg/mL) and high inhibin (1.6 ng/mL) consistent with the presence of testicular tissue. Anti-Müllerian hormone was low and equal in both the cryptorchid and control pig (0.01 ng/mL).

Contrary to other species, AMH was not helpful in the diagnosis of cryptorchidism in this pot-bellied pig. Possible reasons for this finding are low endogenous AMH in postpubertal boars or species incompatibility for the equine AMH kit that was used. Unpublished data from the laboratory used in this case show that AMH levels are elevated in prepubertal cryptorchid pigs.[2] Further studies are needed to determine the normal AMH levels at the different stages of sexual maturity in swine.

Keywords: Anti-Müllerian Hormone; endocrinology; sexual behavior; pot-bellied pig

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MicroRNA expression profiling in porcine spermatozoa of different breeds

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MicroRNAs (MiRNAs) are non-coding RNAs that regulate gene expression at post-transcriptional level and fine tune the expression of about 30% of all mammalian protein coding genes. Genetic diversity among pig breeds has been well documented. However, genetic variability at the miRNA expression level, which could imply modifications of genes at post-transcriptional level, has not yet been well explored although it may correlate to great phenotypic differences and/or pathological disorders. Semen traits such as semen volume, sperm concentration, sperm vitality and sperm motility have been found to be significantly different among breeds, which may be the result of differences in miRNA. The goal of this study was to determine the breed differences in sperm miRNAs transcriptome in Landrace, Yorkshire and Duroc breeds.

Sperm from each of three Yorkshire (n = 3), Landrace (n = 3) and Duroc boars (n = 3) were utilized in this study. Total RNA that contains small RNAs such as miRNA was isolated from sperm. Mature miRNA was reverse transcribed into cDNA. Real-time PCR profiling of sperm mature miRNAs using miScript miRNA PCR arrays in combination with the miScript SYBR Green PCR Kit which contains the miScript Universal reverse primer and QuantiTect SYBR Green PCR Master Mix was performed. Human miRNome miScript miRNA PCR array was used in this study since all mature miRNAs are conserved between human and pig. This array interestingly profiled the expression of 252 most abundantly expressed and best characterized miRNA sequences in boar sperm (hsa-miR-142-5p to hsa-miR-758-3p). Data analysis was performed using the 2^ΔΔCT method of relative quantification. Relative quantification was performed between Duroc and Landrace, and Duroc and Yorkshire.

Out of 252 miRNAs, 11 miRNAs (hsa-miR-196a-5p, hsa-miR-196b-5p, hsa-miR-372-3p, hsa-miR-504-5p, hsa-miR-558, hsa-miR-563, hsa-miR-579-3p, hsa-miR-624-5p, hsa-miR-626, hsa-miR-639 and hsa-miR-648) were down-regulated in Landrace and Yorkshire when compared to Duroc at 3-fold cut-off. These breed-specific sperm miRNAs may be integrated to different phenotypes and may be a tool to study the genetic variability underlying complex phenotypic traits.

Keywords: MicroRNA, sperm, boar, Duroc, Landrace, Yorkshire

In vitro maturation of *Cuniculus paca* oocytes recovered by laparoscopic ovum pick-up (lapOPU)

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The aim of this study was to establish optimal hormonal protocols for recovery of oocytes in *Cuniculus paca* (spotted paca) for in vitro maturation. We hypothesized that gonadotropin treatments would induce greater ovarian stimulation, oocyte recovery and in vitro oocyte maturation than untreated animals. Eight healthy adult females were subjected to each of four treatments to stimulate ovarian follicular growth. All females were subjected to a short estrus synchronization protocol, adapted from studies in small ruminants using a single dose of 45 mg of injectable progesterone. Ovarian stimulation was carried out as follows: in Group TFE (FSHp and eCG), animals were treated with a single dose of 80 mg of follicle stimulating hormone (FSHp) and 200 IU of equine chorionic gonadotropin (eCG) intramuscularly on day 6 after application of progesterone; in Group TF (FSHp), they were treated with a single dose of 80 mg of FSHp intramuscularly on day 6 after application of progesterone; in Group TE (eCG), they were treated with 200 IU of eCG intramuscularly on day 6 after application of progesterone; and in Group TC (saline solution), 1 ml of saline solution was administered to control does. All females received a single intramuscular injection of 0.075 mg d-cloprostenol on day 6. The laparoscopic ovum pick-ups (lapOPUs) were performed between 22 to 26 hours after gonadotropin treatments. For follicular punctures, an 18-gauge 3.5 inch long needle attached to a vacuum system with pressure not exceeding 65 mmHg was used. Oocytes were recovered into 50-mL centrifuge tubes with medium composed of PBS supplemented with 10 IU/mL of heparin and kept at 36°C. All recovered oocytes were placed into maturation medium and incubated for 24 h according to procedures previously described (Mohammadi-Roushandeh et al., 2006). After incubation, oocytes were fixed in a solution of acetic acid:ethanol (1:3) and stained with 1% (wt/vol) lacmoid for assessment of nuclear maturation according to Wang et al., 1988. Data are expressed as mean ±SD and were analyzed using ANOVA with $p \leq 0.05$. There were no differences among the mean number of observed follicles, aspirated follicles and oocytes recovered per treatment, TFE = 17.63 ± 14.51; 12.0 ± 9.8; 5.5 ± 5.5; TF = 10.75 ± 9.59; 10.0 ± 8.5; 4.6 ± 5.2, TE = 12.75 ± 12.37; 11.0 ± 11.0; 2.1 ± 3.5, and TC = 14.88 ± 15.53; 13.0 ± 13.0; 5.3 ± 5.5; respectively. Oocyte maturation rates did not differ among groups: TFE = 40.0%; TF = 57.1%; TE = 46.1%; and TC = 20.0%; except, TF oocytes had greater maturation rates than TC oocytes (57.1% vs. 20.0%, respectively; $p \leq 0.05$). Despite the feasibility of the procedure, further studies are needed to develop and refine hormonal protocols for oocyte recovery and in vitro maturation in this species.

Keywords: Gonadotropin, laparoscopy, oocyte, ovum pick-up, spotted paca.

Acknowlegement

Financial support: FAPESP–2012/00724-6 and CNPq–481073/2012-4

Cesarean section in camels (*Camelus dromedarius*): complications and post-surgical fertility

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Little information is available on cesarean section in camels. The objectives of this retrospective study were to evaluate the indications for cesarean section, survival rate of dams and calves, and postoperative complications and fertility.

Seventy-six cases were included. Surgery was performed with the female restrained in sternal recumbency using a left flank approach. Sedation was obtained with xylazine (0.25 mg/kg, IV) alone or in combination with butorphanol (0.05 mg/kg IV). Local analgesia was provided with an inverted L block using lidocaine. Caudal epidural analgesia with lidocaine was performed on only six animals. Females were multiparous and either bred naturally (n=11) or had received an embryo at eight days post-ovulation (n=65). The mean ± SEM gestational age at presentation was 374.1 ± 1.4 days (range 352-398) for embryo transfer recipients and 376.1 ± 6.2 days (range= 278-393) for naturally mated females. The time from observation of the second stage of labor to surgery was less than six hours for embryo transfer recipients and between eight and 48 hours in naturally mated females. Failure of cervical dilation was the most common indication for cesarean section (81.6%; n=62). Other causes included uterine torsion (0.5%; n=3), fetal lateral head and neck deviation (0.5%; n=3) and one case each of bilateral carpal flexure, fetotomy complication, fetal emphysema, bilateral hip flexure and vaginal evisceration. All females with failure of cervical dilation were part of a study on embryo recipients where pregnancy was maintained by daily progesterone injection until two weeks prior to the due date.

Overall dam survival rate was 92.1% (n= 70). Cause of death included peritonitis (n=3), septic metritis (n=2) and one euthanasia due to vaginal evisceration. The most common non-lethal postsurgical complications were surgical site infection or dehiscence (mostly due to myiasis; 20%) and retained placenta (15.7%). Calf viability at delivery, after one week of life and at weaning was 81.6%, 75% and 73.7%, respectively. Complete placental separation was present in all cases where the calf was delivered dead or died within 15 minutes after surgery. Causes of death in the first week included sepsis (n=2), encephalopathy (n=2) and trauma (n=1). Forty-eight females were used as recipients in an embryo transfer program one year after surgery. Of these, 70.8% (34/48) became pregnant following one to three embryo transfers.

This study shows that survival of the dam and calf are excellent for field cesarean section in camels if intervention is early. Complications due to incision site dehiscence were reduced with administration of local and systemic ivermectin treatment. The high rate of failure of cervical dilation in this case series may have been due to maintenance of pregnancy with exogenous progesterone.

Keywords: Embryo transfer, dystocia, surgery, neonate

Pregnancy length, cria birth weight, placental weight, and IgG concentration in Suri alpacas

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The objective of this study was to evaluate the associations between parity, season of breeding, pregnancy length, cria birth weight, placental weight, and IgG concentrations (mg/dl) in Suri alpacas. Breeding and neonatal care records from a large Suri farm were evaluated. Records of 34 maiden and 75 multiparous (2-10 pregnancies) females were included in the study. Cria and placental weights were measured following parturition. The IgG concentrations were measured between 24 and 36 hours following parturition.

Mean (± SD) pregnancy length was 345.9 ± 10.5 days (range 314-372), cria birth weight was 7.95 ± 1.18 kg (range 3.76-11.25), and mean placental weight was 0.97 ± 0.52 kg (range 0.45-4.13). A significant positive correlation was found between cria birth weight and placental weight ($p=0.02$). The mean IgG concentration was 1424.5 ± 842.7 mg/dl (range 149-3845). A significant positive correlation was found between cria birth weight and IgG concentrations ($p=0.03$), with heavier crias having a higher IgG concentration.

There was no significant difference between maiden and multiparous females for pregnancy length, cria birth weight, or placental weight. There was no significant effect of sex of the cria on weight at birth, placental weight, or IgG concentrations. Pregnancy length was significantly longer for male (348.06 ± 10.34 days) than for female crias (342.96 ± 10.05 days) ($p=0.01$). The mean IgG concentrations in crias born to maiden and multiparous females were 1190 ±723.7 mg/dl and 1524.6 ± 874.02 mg/dl, respectively ($p=0.06$). The effect of dam parity on passive transfer of immunity may have been masked by other factors. IgG concentrations were classified into three categories of passive transfer: adequate (IgG > 1000 mg/dl), marginal (IgG 500-1000 mg/dl), and failure (IgG < 500 mg/dl). Records for 108 crias revealed 70 crias (64.8%) were classified as adequate passive transfer, 18 crias (16.7%) were classified as marginal passive transfer, and 20 crias (18.5%) were classified as failure of passive transfer. A significant effect of month of breeding on pregnancy rate ($p<0.05$) was observed with females bred during November-January period having longer gestations (351.92 ± 10.64 days) than those bred in May-July (344.49 ± 9.0 days). Females breed in November-January had a significantly heavier placenta (1.52 ± 1.17 kg) compared to the other groups ($p=0.01$). No significant effect of month of breeding was observed with respect to cria weight or IgG concentration.

Results of this study confirmed the effect of season on pregnancy length. Longer pregnancies do not result in increased cria birth weights. There does not appear to be an effect of pregnancy length on the incidence of failure of passive transfer. Further research is needed to determine mechanisms regulating pregnancy length, cria birth weight, and placental weight.

Keywords: Alpaca, IgG, failure of passive transfer, neonate, seasonality

Evaluation of novel sampling technique for bovine trichomoniasis (*Tritrichomonas foetus*)

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Bovine trichomoniasis is of great concern to producers and veterinarians in the beef industry. A standard breeding soundness examination (BSE) does not usually include evaluation for venereal diseases, such as *Tritrichomonas foetus (T. foetus)*, and typically goes undiagnosed and is commonly suspected during pregnancy diagnosis which reveals poor conception rates, early embryonic deaths, and extended calving seasons. Trichomoniasis can be adequately diagnosed via preputial/penile scraping; however research has exposed detection rate limitations including: sample collection methods and techniques, sample numbers, media selection and sample processing, and diagnostic laboratory modalities. The aim of the study was to identify a potential technique that increased sensitivity and could easily be used at a BSE or during reproductive investigations. Commercial and purebred bulls (n=76, age = 1 to 5 years, 8 different locations), of unknown infection, were sampled for detection of *T. foetus* using two different collection methods: 1) traditional preputial/penile scraping (TPS) and 2) preputial/penile swabbing (PPS). All (n=76) bulls were subjected to both procedures on the same day or within one week. Traditional preputial/penile scraping samples were taken by vigorously scraping preputial/penile mucosa using a rigid insemination pipette while applying negative pressure with a syringe. Preputial/penile swabbing samples were obtained by briskly swabbing the preputial/penile mucosa with gauze (4x4) during full extension of the penis either by manual stimulation or by use of electrostimulation. All samples were processed using InPouch™ TF (Biomed, White City, OR) media and submitted under similar conditions for polymerase chain reaction (PCR) (Vetmax™ Gold Trich Detection Kit, Life Technologies, Grand Island, NY) testing at Iowa State University Veterinary Diagnostic Laboratory (Ames, IA). Samples were analyzed based on laboratory specifications for positive and negative cycle cutoff points. Positive PCR results were observed in 25/76 (33%) bulls using TPS technique, however 28/76 (37%) were positive using PPS technique. Comparatively, 25/28 (89%) bulls were confirmed positive on TPS technique versus PPS. Fundamentally, PPS technique was 11% more sensitive compared to TPS. Data were analyzed using McNemar's Exact Test and significance was defined as $P \leq 0.05$. There was not a significant difference between the two methods, even though there was a numerical difference. This newer technique alone reduces false negative rates (numerically), which can increase proper diagnosis using one-sample testing. These data indicate that the new PPS technique is a viable alternative to the TPS method which allows practitioners to choose between the collection methods. Further studies with a larger sample size are required to prove the significant difference between the two methods.

Keywords: Bovine, trichomoniasis, venereal, polymerase chain reaction (PCR)

Comparison of estrus, breeding and pregnancy rates in goats during the non-breeding season using a short P4 protocol with and without GnRH or hCG

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The purpose of this project was to compare induction of estrus and pregnancy rates in goats bred during the non-breeding season using a short progesterone priming protocol with either gonadotropin releasing hormone (GnRH) or human chorionic gonadotropin (hCG) to induce ovulation. Previous work on a small number of animals suggested that the use of hCG (stimulates ovary directly) resulted in good estrus and increased willingness to be bred as well as a slightly better pregnancy rate compared to using GnRH (stimulates anterior pituitary). Our hypothesis was that during the non-breeding season using hCG vs. GnRH would increase the percentage of animals coming into estrus and breeding rate, as well as increasing pregnancy rates. A larger study was performed to test this hypothesis.

Thirty mixed breed Alpine and Saanen dairy goats were assigned to Group 1 (GnRH, n=10), Group 2 (hCG n=10), or controls (n=10). In all three groups, progesterone was administered over three days via a vaginally placed controlled internal drug releaser (Eazi-Breed CIDR Sheep & Goat; Pharmacia and Upjohn, New York, NY), follicle stimulating hormone (Follitropin-V; Bioniche, Belleville, Ontario, Canada) was administered once daily on the second and third days of progesterone priming (16 mg IM, SID) and prostaglandin F2α (Lutalyse; Pfizer, New York, NY) was given (5 mg IM) on the final day of CIDR removal. Two days after CIDR removal, Group1 received 50 mcg IM of GnRH (Cystorellin; Merial, Duluth, GA), Group 2 received 500 IU IM of hCG (Chorulon, Intervet, Millsboro, DE) and control animals received no additional treatment. All does were naturally bred by one of two bucks wearing marking harnesses to indicate if and when breeding had taken place. Ultrasound was used to evaluate for pregnancies starting at day 35 after breeding.

In summary, there were no statistical differences found among the groups for any of the parameters evaluated. Additionally, there were no significant differences seen between bucks for either breeding or pregnancy rates.

Group	% In Estrus/bred	% Pregnant US (35 days)	% Delivering	Number of kids	% Rate of preg to term
GnRH (n=9)*	66 (6/9)	50 (3/6)	33 (1/3)	4	11(1/9)
hCG (n=10)	90 (9/10)	33 (3/10)	33(1/3)	2	10 (1/10)
Control (n=9)*	55 (5/9)	60 (3/5)	40(2/5)	4	22(2/9)

*1 animal removed due to illness

In conclusion, neither hCG nor GnRH administration improved breeding or pregnancy rates compared to controls in this protocol.

Keywords: Non-breeding season, estrous synchronization, pregnancy, goats

Prevalence of *Tritrichomonas foetus* in Tennessee beef bulls

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Tritrichomonas foetus is a venereally transmitted protozoan of cattle in which diseased bulls tend to be asymptomatic carriers, while infections in cows and heifers may result in embryonic and fetal loss, vaginitis, or pyometra. It has been estimated the disease has the potential to cost the U.S. beef industry over $650 million annually. While the reported individual bull prevalence rates of trichomoniasis in the U.S. were 0 to 7.8 %, the prevalence in Tennessee beef bulls and the direct cost to the Tennessee beef industry were unknown. The objective of this study was to estimate the prevalence of *T. foetus* infections in Tennessee beef bulls through prospective and retrospective surveys. According to the National Agricultural Statistics Service, there were approximately 950,000 beef cows in the state of Tennessee in 2012, and assuming a bull:cow ratio of 1:20,there were approximately 47,500 beef herd bulls in the state. The target sample size was calculated using the Epi Info Version 7.0 software from the Centers for Disease Control and Prevention (Atlanta, GA). Given an estimated prevalence of 3% (and not less than 1.5%) a confidence interval of 95% and a population of 47,500 herd bulls, 492 bulls were needed to estimate the prevalence of *T. foetus* in Tennessee beef bulls. To date, the prospective survey has included 380 Tennessee beef bulls sampled between March 2014 and January 2015. Preputial smegma was collected from the 380 bulls with a bull rasper (Tricamper™) and cultured in an InPouch™ *T. foetus* culture pouch (BioMed Diagnostics). All bulls were aged via dentition, individual identification numbers, as well as the breed of the bull recorded at the time of collection. The samples were evaluated microscopically every other day for seven days for any growth resembling *T. foetus.* An aliquot of the culture medium from each sample was used for DNA extraction and subsequent real-time PCR using the VetMAX-Gold Trich Detection *T. foetus* DNA detection Kit (Life Technologies, licensed by the USDA). Of the 380 bulls cultured in the prospective survey, two (0.53 %) cultures were considered suspect on microscopic evaluation. However, all real-time PCR-based assays were negative for *T. foetus*, suggesting that the samples were most likely contaminated and contained fecal trichomonads. The retrospective analysis included 1,118 *T. foetus* tests (culture and/or real-time PCR) performed at the Tennessee Department of Agriculture Kord Animal Health Diagnostic Laboratory in Nashville, TN and the University of Tennessee College of Veterinary Medicine Biomedical and Diagnostic Sciences in Knoxville, TN between November 2013 and January 2015. These dates were chosen according to the time of diagnosis of two confirmed cases of bovine Trichomoniasis in Tennessee. Of the 1,118 samples from Tennessee bulls included in the retrospective analysis, *T. foetus* was observed and subsequently confirmed by real-time PCR in samples from two (0.18 %) of the bulls. When considering the results of the prospective and retrospective surveys, the estimated prevalence of bovine Trichomoniasis may be lower for Tennessee compared to other surveyed areas.

Keywords: Bovine trichomoniasis, beef bulls, Tennessee

Investigation of *in vitro* conditions required for biofilm formation in *Escherichia coli* isolated from mare reproductive tract

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Biofilm formation has been suggested to be important for chronic bacterial endometritis of mares. The crystal violet (CV) assay is used commonly in human medicine to evaluate the biofilm forming potential of bacterial isolates. The CV assay evaluates the early steps in biofilm formation, i.e. bacterial attachment and extracellular polymatrix (EPS) production. The CV dye binds to both cells and EPS, and it is a measure of total biofilm biomass formed. However the CV assay is not standardized across biomedical disciplines, as published methodologies vary considerably among laboratories. The aim of this project was to evaluate the effects of two incubation conditions (time and orbiting) on the biofilm forming potential of *Escherichia coli* (*E.coli*) isolates from the mare reproductive tract. We hypothesized that different methods of incubation affect biofilm development. The biofilm forming potential of 101 *E.coli* (49 clitoral fossa and 51 uterine isolates) was assessed under differing incubation conditions using the CV assay: orbiting for 24 h (O-24), non-orbiting for 24 h (NO-24) and orbiting for 48 h (O-48) at 37°C. Aside from the specific conditions being tested, all isolates were tested with the same CV assay protocol. Isolates with an optical density (OD) of greater than 0.4 were considered strongly adherent (a strong biofilm forming isolate). Additionally, 25 strong biofilm forming *E. coli* isolates were evaluated at 4 h intervals under orbiting incubation at 37°C for biofilm formation using the CV assay. The mean number of isolates and OD of the different incubation conditions and times were evaluated using t-test. Significance was set at $P<0.05$. The number of strong biofilm forming isolates for O-24, NO-24 and O-48 h, were 30, 28 and 27, respectively (n=101), with no significance between treatments. Similarly, there was no significant difference between the mean ± SEM OD of strong biofilm forming isolates at the end of each incubation period (1.54 ± 0.12, 1.51 ± 0.14, 1.28±0.11 for O-24, NO-24 and O-48 h, respectively). The mean ± SEM OD at 4, 8, 12, 16, 20 and 24 h were 1.09 ± 0.11, 1.57 ± 0.12, 1.77 ± 0.13, 1.83 ± 0.13, 1.89 ± 0.13 and 1.76 ± 10.13, respectively. Although the OD for the 4 h incubation time was significantly lower than any group, all incubation periods resulted in OD > 0.4 (strong biofilm formation). Our results show some *E. coli* isolates collected from the endometrium and clitoral fossa demonstrate strong biofilm forming potential when tested *in vitro*, and formation occurs rapidly and repeatedly under variable incubation conditions.

Keywords: Equine endometritis, bacterial biofilm, crystal violet assay

Pregnancy outcomes in Thoroughbred mares administered different doses of cloprostenol

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The dose of prostaglandin $F_{2\alpha}$ (PGF), age of the corpus luteum, and follicle size have effects on the interval between PGF administration and ovulation, and the type of luteolytic response in mares.[1] The objectives of this study were to determine: (i) if mares with large diestrus follicles (≥30 mm) at the time of PGF administration had a difference in pregnancy rate at 13-16 days, 25-28 days, and 40-45 days post-ovulation when compared to mares bred on a natural estrus, (ii) if there was a difference in pregnancy rate at the same time points between mares administered PGF with small diestrus follicles (<30 mm), and mares bred on a natural estrus, and (ii) associations between follicle size and days to ovulation following PGF administration Retrospectively, 522 estrous cycles from one practitioner's records during the 2013 and 2014 breeding season were evaluated. Thoroughbred mares were divided into three groups: 1) mares that were administered 125µg of cloprostenol (Estrumate®, Schering-Plough Animal Health Corp., Summit, NJ) with ≥ 30mm diestrus follicles and were bred (n=23), 2) mares that received 250µg of cloprostenol with < 30mm diestrus follicles and were bred (n=87), and 3) mares bred on a natural estrus (n=412). All mares were administered an ovulation inducing agent (SucroMate™, Bioniche Animal Health, Louisville, KY) 24 hours prior to breeding, when the dominant follicle was ≥35mm. Data were compared using one-way ANOVA for continuous response variables and Chi-square test for two nominal variables. The pregnancy rate was significantly lower in mares that received 125µg of cloprostenol with large diestrus follicles when compared to mares bred on natural heat (13-16 days: 50% vs 71%, 25-28 days: 41% vs 68%, and 40-45 days: 41% vs 65%). There was no significant difference in pregnancy rate at the same time points for mares with <30mm diestrus follicles administered 250µg cloprostenol compared to mares bred on a natural heat. There was a significant difference in the mean interval from cloprostenol administration to ovulation for mares receiving 125 µg of cloprostenol with >30 mm follicles (4.1 days) when compared to mares given 250µg with <30mm follicles (8 days). The mean interval from cloprostenol administration to ovulation was inversely proportional to follicle size when looking at mares administered 125µg of cloprostenol (5.9 days for 30-39 mm and 4.7 days for 40-49 mm) and mares administered 250µg of cloprostenol (7.7 days when follicle size was 20-29 mm). Administration of cloprostenol to mares with a large diestrus follicle appears to be associated with a lower pregnancy rate when compared to mares bred on a natural estrus.

Keywords: Prostaglandin $F2_{\alpha}$, cloprostenol, pregnancy rate, diestrus follicle

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Ultrsonographic imaging of cervical defects in two mares with chronic infertility

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The following cases demonstrate the use of trans-rectal ultrasound to image and diagnose cervical defects. In case one, a 16 year old Thoroughbred mare was presented for a breeding soundness examination due to a history of chronic infertility. The mare had foaled the previous year and did not become pregnant after being bred for three estrous cycles that year and two additional cycles in the subsequent year. The mare had been managed appropriately and treated aggressively during those cycles. Upon presentation the mare was 16 days post-ovulation and was in estrus (40mm follicle left ovary, moderate endometrial edema, a trace of intra-luminal fluid and had a relaxed cervix). No progestagen supplementation had been administered, and trans-rectal ultrasound revealed three small (2-5 mm) hypechoic defects in the ventral musculature of the cervix. Digital palpation of the cervix confirmed three depression-like defects in the ventral floor of the cervix that corresponded with the areas visualized via trans-rectal ultrasound. Surgical repair to correct the defects was performed and the defects were not visible via trans-rectal ultrasound after repair. The mare was bred and became pregnant the second cycle she was bred after surgery. In case two, a 13 year old Oldenburg mare was presented for a breeding soundness examination due to a history of chronic infertility. The mare had a history of a difficult foaling four years prior and had failed to become pregnant after being bred to a fertile stallion two consecutive years. Trans-rectal palpation and ultrasound of the reproductive tract revealed the mare was in early estrus with a 30 mm follicle, mild endometrial edema, a trace of intra-luminal uterine fluid and a slightly relaxed cervix. No progestagens had been administered to the mare prior to evaluation. Transrectal ultrasound of the cervix revealed a hypoechoic circular region 3 cm in diameter in the ventral musculature of the cervix approximately 6 cm from the external cervical os. Digital palpation of the cervix revealed a defect in the ventral cervical musculature that extended from 3-9 o'clock and was approximately 3 cm in the cranio-caudal direction. The cranial cervical canal extended from this defect another 4 cm cranially. Insufflation of the cervical canal during an endoscopic examination revealed a large opaque mucoid mass within the defect of the ventral cervical musculature. Microscopic examination of a sample aspirated from this mass yielded neutrophils and hyphae, consistent with a fungal infection. Consultation with a surgeon determined that due to the defect's large size and being located relatively far cranially in the cervical canal surgical repair would be difficult and might not yield complete resolution of the defect. This information combined with the poor prognosis associated with fungal endometritis cases led the owner to decide against pursuing treatment. These cases describe cervical defects that were identified initially via trans-rectal ultrasound examination. The defects identified during digital palpation corresponded with those visualized via trans-rectal ultrasound. Thorough ultrasound examination of the cervix may aid in the identification of cervical defects that have been undetected previously and should be used in conjunction with digital cervical examination. While not all defects are identifiable via trans-rectal ultrasound, visualization of the defect provided valuable information regarding the size, extent and contents of the defect. Surgical correction was beneficial in one of the cases, but not possible in the second one.

Keywords: Cervix, ultrasonography, infertility

Comparison of chemical and surgical vasectomy on testicular activity in free-roaming stallions

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Following surgical vasectomy in rats, epididymal distension and testicular interstitial changes occur. These effects are due to pressure-mediated damage to the seminiferous epithelium. Overpopulation of free-roaming horses in the United States causes significant adverse impacts to the ecological integrity of the environment. These impacts have reduced the health and function of native habitats necessary to support native wildlife (e.g. pronghorn, greater sage grouse). The objective of this study was to determine if both surgical and chemical vasectomy induce morphologic changes within the equine testis. The hypothesis was that both vasectomy methods increase seminiferous tubular diameter and impair spermatogenesis. Left and right testicular tissue sections were collected from stallions castrated following surgical vasectomy an average of 4.84 years previously (SURG; n=19) or chemical vasectomy an average of 2.25 years previously (CHEM; n=16). Unvasectomized stallions were used as controls (CONT; n=10). Tissues were formalin-fixed and embedded in paraffin. Cut sections (4 um) were stained with hematoxylin and eosin. Randomly selected fields (two to three fields per testis) were examined using bright field light microscopy (200X) and images were digitally captured. A single observer (RLL) blinded to the treatments performed all histomorphometry measurements. Briefly, two perpendicular diameter measurements across five seminiferous tubules with accompanying tubular basement membrane thicknesses were recorded for each testis. In addition, a single observer (KMP) blinded to the treatments scored spermatogenesis according to Yoshida et al.[1] Treatment group results were reported as mean ± SD in the table below.

	CONT	SURG	CHEM
Seminiferous tubule diameter (μm)	201.54 ± 28.81	192.25 ± 15.37	194.69 ± 18.81
Basement membrane thickness (μm)	3.18 ± 0.53	3.12 ± 0.53	2.83 ± 0.16
Spermatogenesis score	12.0 ± 0.0	11.8 ± 0.3	12.0 ± 0.0

It is unknown if either surgical or chemical vasectomy caused acute pressure damage within the testes as they were not evaluated until several years after vasectomy. Based upon the present findings, it appears that there is no evidence of permanent damage to the seminiferous epithelium.

Keywords: Equine, seminiferous epithelium, seminiferous tubule, spermatogenesis, testis

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A novel approach to removing retained fetal membranes in the mare

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A ten year old primparous Arabian X Australian Riding Pony was presented with retained fetal membranes. Physical examination revealed that all vital parameters were within normal limits and a portion of desiccated amnion and the umbilical cord were protruding from the vulva. It was reported that foaling was unattended and that the mare had not been examined between 4 pm and 6 am. The mare was found in a paddock with a foal by her side. The mare and foal were observed on the farm for approximately six to eight hours prior to veterinary examination. The aim of this case report is to describe a novel technique for removal of retained fetal membranes in a mare. The technique was first reported by Dr. Mark Meijer to the remaining authors in March 2014 at the Proveto Equine Conference, the Netherlands. The mare was restrained in a stock, the tail was tied to the side and the perineum was cleaned. The umbilical cord was transected and a single 2cm longitudinal incision was made in an umbilical vessel. A 9mm diameter nasogastric tube (foal tube) was introduced into the incision and fed into the umbilical vessel toward the root of the umbilical attachment on the placenta. The nasogastric tube was attached to a water (garden) hose using a modified garden spray nozzle. Water was continuously infused into the umbilical vessel under low pressure for approximately five minutes. As the umbilical vessel distended, the umbilical cord and nasogastric tube were held tightly by hand to prevent retrograde leakage of water from the umbilical vessels. It appears that the infusion of water induces edema and swelling of the tissue causing separation of the chorioallantois from the endometrium. While water was continuously infused into the vessel, gentle traction was applied to the umbilical cord which facilitated removal of the entire placenta in less than ten minutes. The mare did not demonstrate any signs of discomfort during the procedure. The reproductive tract of the mare was examined using transrectal palpation and ultrasonography for two days after removal of the fetal membranes and no abnormalities were noted. Large volume uterine lavage was performed on the mare for two days. The mare was administered flunixin meglumine (1.1mg/kg, PO, Q24hr) and trimethoprim sulfamethoxazole (30mg/kg, PO, Q12hr) for three and five days after foaling, respectively. The mare was discharged four days after foaling. The mare was served naturally 68 days after removal of the fetal membranes, and she became pregnant. In summary, this novel technique can be easily used in a field setting for successful removal of uncomplicated, retained fetal membranes.

Keywords: Equine, placenta, post-partum

Progesterone levels and interovulatory intervals of mares treated with intrauterine fractionated coconut oil

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Intrauterine plant oil infusion, including one milliliter (ml) of fractionated coconut oil, was reported to be a safe, cheap and reversible option to prolong the luteal phase in mares when administered on day (D)10 of the estrous cycle. The objective of this study was to understand and compare the utero-ovarian response to one ml and 0.5 ml of coconut oil administered into the uterus using an insemination pipette and an embryo transfer gun, respectively, on D10 of diestrus. We hypothesized that intrauterine administration on D10 of both volumes of coconut oil with either infusion device would result in prolonged luteal function. Light horse mares (n=12) were examined using transrectal palpation and ultrasonography to determine if they had a normal interovulatory interval, and then were examined daily in estrus until the D of ovulation (D0), and every other D during one estrous cycle. Mares were randomly assigned to treatment and studied over one to two estrous cycles with a resting ("washout") cycle after each treatment cycle. Jugular blood was drawn on D11,13,15 and 17, centrifuged and serum stored until further assaying for progesterone (P4; Siemens Coat-a-Count Progesterone RIA, Los Angeles, CA). Groups were: diestrus control (n=5), coconut oil 1.0 ml (Miglyol 810, Sasol Oil, Witten, Germany) infused in the uterus with an artificial insemination pipette on D10 (n=5) and coconut oil 0.5 ml infused in the uterus with an embryo transfer gun on D10 (n=5). All statistical analyses were performed using Stata, version 13.1, College Station, TX at $p<0.05$. Normality was evaluated using the Shapiro-Wilk tests and non-normal data analyzed using non-parametric tests. Days to luteolysis (DTL) defined as P4 <2.0 ng/ml, was examined using ANOVA, and a post hoc Bonferroni test. There was a significant difference ($p=0.0083$) in DTL (mean $\pm$ SD) between the control (15.8 $\pm$ 1.09), coconut oil infused 1.0 ml (12.2 $\pm$ 0.45) and coconut oil infused 0.5 ml (15.2 $\pm$ 2.48), mare groups, with the control group greater than the 1.0 ml group ($p=0.011$), and the 0.5 ml group greater than the 1.0 ml group ($p=0.034$). The effect of treatment and D on P4 levels were analyzed using the Kruskal-Wallis, and Dunn's tests. There was a significant effect of treatment ($p=0.0098$) on P4 levels with control group P4 levels higher than the 1.0 ml group ($p=0.0012$), and the 0.5 ml group higher than the 1.0 ml group ($p=0.0495$). There was a significant effect ($p<0.0001$) of D on P4. The overall median P4 levels in ng/ml [median (quartiles)] for the four D were: control [8.0 (1.5, 13.8)], coconut oil 1.0 ml [0.7 (0.005, 6.1)], and coconut oil 0.5 ml [3.9 (0.35, 12.3)]. Post hoc tests showed significant differences ($p<0.03$) between all D except D11 vs D13, and D15 vs D17. We concluded that intrauterine coconut oil administration lowered progesterone levels during diestrus and did not prolonged the luteal phase of the mares.

Keywords: Coconut oil, mare, interovulatory, progesterone.

The effect of repeated $PGF_{2\alpha}$-induced antiluteogenesis in the interovulatory interval of mares

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Serial administration of prostaglandin F2alpha ($PGF_{2\alpha}$) in the early post-ovulatory period has been recently shown to prevent the formation of the corpus luteum.[1] In the present study, we proposed to assess the effects of PGF2α-induced antiluteogenesis on follicular dynamics and luteal function of cycling mares treated during two consecutive cycles. We hypothesized that: 1) serial administration of multiple doses of $PGF_{2\alpha}$ to mares during early diestrus would prevent luteal function (antiluteogenesis) and induce return to estrus and normal ovulation; 2) the interovulatory interval (IOI) between ovulations following antiluteogenic treatments would be shorter than the IOI of control cycles. Palpations per rectum and transrectal ultrasonography were used to examine four cycling mixed light breed mares (9-16 years old) during the months of June, July and August in the Northern hemisphere housed at Louisiana State University. On cycle 1, within 12 hours from detection of ovulation, mares were treated with 10 mg $PGF_{2\alpha}$ (dinoprost) twice daily on days 0, 1, and 2 and then once daily on days 3 and 4; this treatment was repeated when mares ovulated again in the post-treatment cycle following the initial antiluteogenic protocol (cycle 2). Plasma samples were obtained daily for progesterone RIA analyses. Interovulatory intervals (days) and concentrations of plasma progesterone (ng/mL) were analyzed by ANOVA repeated measures with significance set at $P \leq 0.05$. Administration of exogenous $PGF_{2\alpha}$ successfully prevented formation of the corpus luteum in all mares as mean concentrations of plasma progesterone remained below 1.0 ng/mL during all IOI. Mean (±SD) days for IOI following for both antiluteogenesis treatment periods were significantly reduced (11.5±2.6 and 13.2±2.6) when compared with the IOI for control cycles (21±1.4; $P < 0.05$). Ovulations in mares in the post-treatment cycles (cycle 3) following two periods of antiluteogenic treatments were followed by normal luteal development and function. Antiluteogenesis can be reliably applied with serial $PGF_{2\alpha}$ administrations that result in the induction of apparently normal ovulatory cycles. Repeated antiluteogenic protocol may provide a novel approach to manipulate the equine estrous cycle.

Keywords: antiluteogenesis, dinoprost, horses, ovulation, $PGF_{2\alpha}$, luteal function

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Fetoplacental steroids and eCG concentrations in a pregnant mare receiving intrauterine cloprostenol sodium

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Recently, a new therapeutic procedure involving intrauterine (IU) administration of cloprostenol (CLP) was described to terminate unwanted pregnancies in surrogate mares.[1] Despite widespread use of this technique in South America, there are limited data on fetoplacental steroids and eCG concentrations following IU administration of CLP. The objectives were to determine clinical reproductive parameters, progesterone, estrogens and equine chorionic gonadotropin (eCG) concentrations following IU administration of CLP in a controlled abortion case. A seven year-old maiden Quarter Horse mare was presented for pregnancy termination at 60 days after ovulation. Transrectal palpation and ultrasonography examination revealed a single live fetus, normal fetal fluids and ovaries with multiple corpora lutea consistent with 60 day pregnancy. The mare's perineal region was cleaned using povidone iodine. Cloprostenol sodium (500 mcg) extended in 10 mL of physiological saline was administered IU via transcervical route with an insemination pipette. The mare was observed for signs of discomfort for one hour following the treatment. Transrectal ultrasonography was performed at 12, 24, 48, 72, 96 h and 7 and 15 days after the treatment. Blood was collected simultaneously for hormone analyses. Estrone sulfate, estradiol 17 β, progesterone and eCG were measured by immunoassays. The mare showed no signs of discomfort following treatment. Transrectal ultrasound showed decreased blood flow to the corpora lutea by 24h. Following treatment, fetal movement was observed up to 48h and by 72h, the fetus was not active but still had a heartbeat. Since the mare had not aborted by 72h, second dose of IU cloprostenol (500 mcg in 10 mL of saline) was administered. During the treatment the mare's cervix was observed relaxed and softened. The mare passed the fetus and the fetal membranes 94 and 22h after first and second treatment, respectively. Uterine lavage was performed with 3L of lactated Ringer's solution 4h after abortion, and oxytocin (20 units/IM/BID) was administered for three days. Seven days after abortion retained endometrial cups and a trace amount of anechoic intrauterine fluid accumulation were noted. By 15d after abortion, no IU fluid, a trace amount of endometrial edema, and multiple small follicles were observed. At that time, no endometrial cups were visualized, although serum eCG concentrations remained elevated (ranging from 6,930 to 8,000 mIU/ml). Serum progesterone declined to baseline concentration by 96h (9.11, 3.02 1.48 and 0.18 ng/ml at 0, 12, 72 and 96h, respectively). Estrone sulfate and estradiol were 28.92 and 74.29 ng/ml initially at 0 h, increased to 50.15 and 98.25 ng/ml at 48 h and declined to 3.71 and 36.66 ng/ml, at 96h. In conclusion, this new approach effectively induced abortion in this mare with minimal side effects, however, the mare aborted 94h after initial prostaglandin administration (~22h after the second dose), which is remarkably longer than the average (i.e. 24h) reported by Aguilar et al.[1] and longer than reported by the standard protocol using prostaglandin (60-72h) intramuscularly. Clearly, the endometrial cups were still actively secreting eCG at 15 days after abortion and it is uncertain how long endometrial cups will persist in this mare.

Keywords: Abortion, prostaglandin, fetoplacental unit, steroids

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Using color flow Doppler ultrasonography to estimate progesterone concentrations at embryo transfer and during early pregnancy in recipient mares

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Color-flow Doppler sonography (CF) has been described as a means of rapidly assessing corpus luteum (CL) function in cycling mares because luteal blood flow correlates with circulating progesterone (P_4) concentrations. The hypothesis was that CL size and blood flow would provide an indication of luteal function as represented by circulating levels of P_4, in mares at and following embryo transfer (ET). Day 8 equine embryos (n=48) were transferred into available recipient mares (ovulated 1 day before to 3 days after donor) as part of a commercial program. B-mode and CF sonography were performed using a MyLab™Five (Esaote, Maastricht, The Netherlands) immediately prior to ET (ET+0), and blood was collected from the jugular vein to measure plasma [P_4] (Coat-A-Count TKPG; Siemens Healthcare Diagnostic BV, Los Angeles, CA). For every detectable CL, fixed settings of 10 cm depth in B-mode (frequency 7.5MHz, maximum gain) and color mode (frequency 5.0MHz, 70% gain, pulse repetition frequency 1.0kHz) were used to examine size and vascularity. Three cross-sectional images at the position of maximal size and area of blood vessels of the respective CL were captured and stored. Measurements were repeated at day four after ET for all mares, and days 11, 18 and 25 in pregnant mares. The cross sectional area (corrected for presence of lacunae) and area of color pixels within the cross-section were analyzed using ImageJ software (National Health Institutes, Bathesda, MD), and statistical analysis was performed using SAS® (Version 9.4, SAS Inst., Cary, NC). None of CL area, area of color pixels or [P_4] at the time of ET were predictive of pregnancy outcome when analyzed with binary logistic regression. The total area of color pixels in the CL correlated significantly ($r = 0.35$ to 0.45), if only moderately, with [P_4] at all-time points except day 18 after ET (Spearman's rank-order correlation). A significant correlation between CL area and [P_4] was evident until day 11 ($r = 0.37$ to 0.60). Corpus luteum vascularity (area of blood vessels) decreased significantly after day 18, whereas CL area had already decreased from day four (Wilcoxon signed rank test). These findings confirm that area of color pixels in the CL cross-section is a reasonable index of circulating [P_4] at the time of ET and during early pregnancy and can be used to indicate luteal insufficiency.

Keywords: Equine, mare, color flow Doppler, embryo transfer, progesterone

Infectious endometritis is associated with endometrial expression of transforming growth factor-β and integrin α5β1

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Persistent endometritis is a leading reproductive health concern in mares. Despite increasing knowledge and improved treatment strategies, the multifactorial pathogenesis of the disease complex still remains a puzzle. In this study it was hypothesized that the endometrial gene expression of transforming growth factor –β1 (TGF-β1) and alpha-5 beta-1 integrin (integrin α5β1) was correlated to uterine growth of pathogens in brood mares.

Endometrial biopsies were obtained from brood mares at a Danish AI-center during the 2014 breeding season. Mares with clinical signs of endometritis and/or a history of previous unsuccessful breeding were selected for the study. Two biopsies were obtained from each mare. One biopsy was used for bacterial culture and cytology and one biopsy was used for RNA extraction. Relative gene-expression analyses were performed by quantitative reverse transcriptase PCR (qRT-PCR) using validated primers and SYBR green detection. Infectious endometritis was diagnosed in 49% of the mares (29/59) with *S. equi* subspecies *zooepidemicus* isolated most frequently (45%). Expression of TGF-β1 and integrin α5β1 was significantly increased in mares with infectious endometritis compared to mares with no growth of uterine pathogens and/or endometrial inflammation (positive cytology). Mares with growth of *S. zooepidemicus* had an increased endometrial expression of TGF-β1 compared to mares with no growth or growth of other pathogens although it was not significant ($p=0.06$).

The results indicate that TGF-β1 and integrin α5β1 play a role in the pathogenesis of persistent infectious endometrits. A similar observation has been demonstrated in tonsillary epithelium.[1] This study showed that bacterial invasion was critically dependent on a TGF-β1 promoted presence of α5β integrins. Further studies are however needed to investigate this hypothesis in relation to persistent infectious endometritis in the mare.

Keywords: Infectious endometritis, transforming growth factor-β1, integrin α5β1, *S. zooepidemicus*

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Breeding soundness of weaned bull calves treated with bolus injections of trace minerals

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Young bulls that fail to pass a breeding soundness examination (BSE) represent a financial loss to cattle breeders. We hypothesized that two bolus injections of trace minerals would increase the proportion of bulls assigned satisfactory BSE scores. Weaned, 7-mo-old bull calves (n = 491; initial BW = 314 ± 45 kg) of 3 breeds (Charolais, Angus, and Charolais × Angus) and originating from 12 ranches in the Great Plains and intermountain west were blocked by breed type and ranch of origin and assigned randomly at the beginning of the study (D0) to 1 of 2 treatment groups: 1) supplemental s.c. trace mineral injection containing 15 mg/mL Cu, 10 mg/mL Mn, 5 mg/mL Se, and 60 mg/mL Zn (TM) or 2) s.c. injection of physiological saline (SA). Injections (SA or TM) were administered at weaning (D0; 1 mL/45 kg BW) and again 90 D after weaning (1 mL/68 kg BW). Bulls were fed a growing diet *ad libitum* at a common location for 225 d. The diet consisted of ground hay, corn silage, corn grain, SBM, macro-minerals, and trace-minerals and was formulated to promote a 1.5 kg ADG at a DMI of 2.6% BW (DM basis). Initial weights and blood samples via caudal vessel puncture were collected on D0. Semen samples were collected on D90 and D150 by electroejaculation. Scrotal circumference was measured and semen samples were evaluated by a single technician. Breeding soundness classifications as approved by the Society for Theriogenology were assigned by a licensed veterinarian. Initial blood serum samples were analyzed for Cu, Mn, Se, and Zn concentrations. Bulls with initial serum Se < 70 μg/kg were more likely ($P = 0.03$) to fail the BSE on D90 than contemporaries with serum Se > 70 μg/kg (48 vs 52% respectively). Scrotal circumference did not differ ($P \geq 0.27$) between treatments and averaged 34 ± 2.9 cm on D90 and 37 ± 2.7 cm on D150. Proportions of TM- and SA-treated bulls receiving satisfactory BSE scores were not different ($P \geq 0.54$) on D90 (49 vs 49 %, respectively) or D150 (89 vs 86 % respectively). Conversely, motility scores were greater ($P = 0.06$) for TM-treated bulls on D150 than for SA-treated bulls (88 vs 86 %, respectively). During the development period, ADG differed ($P < 0.01$) between ranch of origin and breed but not ($P = 0.96$) between treatment groups. In summary, bulls with relatively-low initial serum Se were more likely to fail BSE. Cumulative BSE scores of young bulls given bolus injections of TM were not different from those given bolus injections of SA; however, sperm motility on D150 was greater for bulls treated with TM than bulls treated with SA.

Keywords: Breeding soundness, bulls, selenium, sperm motility

The effect of progesterone intravaginal devices (CIDRS), P.G. 600 and ram effect on hastening the onset of cyclicity of transitional Targhee ewes

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Sheep are seasonally polyestrous breeders, cycling in late summer and early autumn. Breeding out of season or as early as possible in the season has many management and economical benefits. Efforts to hasten the transition period of ewes into cyclicity involve the use of vasectomized rams to induce the "ram effect"(RE) and progesterone devices.

The objective of the study was to compare the use of a recently-approved intravaginal progesterone device (CIDR; EAZI-BREED™ CIDR® Sheep Insert, Zoetis, Florham Park, NJ) with the ram effect (vasectomized rams), a combination of CIDR and ram effect and the addition of P.G.600® (eCG+hCG; Merck Animal Health/Intervet, Madison, NJ) to determine their contribution to induce the onset of cyclicity and alter reproductive efficiency. We hypothsized a positive effect in the treatment groups as compared with controls, and no additive effect when treatments were combined.

Taregee ewes (n=159) at the University of California Hopland research facility were randomly divided into 6 groups: G1:(n=21) RE+CIDR+PG600, G2:(n=23) CIDR+PG600, G3:(n=21) RE+CIDR, G4:(n=22) CIDR, G5:(n=32) RE, G6:(n=40) control. All the ewes where exposed to intact rams at the end of the treatments and lambing data were collected. Earliest expected lambing date to actual lambing was analyzed using survival analysis and Cox regression and analyzed for distribution of lambing with SPSS 22 software (Table).

Available data suggest that the use of CIDR (G1, G2, G3 and G4) with or without RE or P.G.600® induce a faster onset to cyclicity in transition ewes than when not used (G5 and G6). Ewes exposed to a vasectomized ram only tended to have shorter time to lambing than controls ($p= 0.07$) but in groups exposed to CIDR, the RE was of no additional benefit. In multivariate Cox regression analysis, only exposure to CIDR was a significant covariate for time to event ($p<0.001$).

Table.

Variable	Hazard	Sig.
CIDR	3.3	<0.001
PG600	0.7	0.283
RAM	1.1	0.651

The results of this study show that the use of a CIDR is superior in achieving earlier lambing dates to other methods or a combination of methods. Use of P.G.600® does not seem to influence time to lambing.

Tissue oxytocinase activity in diestrus mares

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Oxytocin is important in the regulation of key physiologic processes in mares such as: uterine clearance, luteal maintenance, milk ejection, passage of the fetal membranes, and maternal foal bonding, however while it is known that oxytocin has a short half-life, there is a lack of understanding of how oxytocin is metabolized. In most species the metabolism of oxytocin occurs through serum and tissue oxytocinase (OTase) often referred to as insulin regulated aminopeptidase (IRAP) or leucyl-cystinyl aminopeptidase (LNPEP). We hypothesized that OTase in mares would be similar to other species and would be found widely distributed in tissues and serum. The objective of this study was to characterize OTase activity in serum and in various tissues in diestrous mares. Jugular blood samples were obtained from five mares following the ultrasound detection of ovulation (day 0), every other day from day 3 to 15 of diestrus. Serum was separated and stored frozen at -20°C until analysis. Tissue samples (liver, kidney, myometrium, endometrium, corpus luteum, and follicle wall) from six other healthy mares, that were euthanized for another study, were collected at necropsy and stored frozen at -80°C. Examination of the ovaries in these six mares showed active luteal tissue. Tissue samples were thawed, rinsed in ice-cold 0.02 mmol/L PBS pH 7.2, and minced. Tissue samples (10 mg) were homogenized in PBS using 1.4 mm ceramic beads (Omni International, Bead Kit, Kennesaw, GA) and then centrifuged at 1500 x g for 15 minutes. The resulting supernatant was stored at -80°C and assayed at a later time. A commercial ELISA (LNPEP for horses, MyBioSource, San Diego, CA) which was validated in our laboratory, with a detection range of 6.25 – 200 U/L and an intra and interassay coefficient of variation of <15%, was used for the analysis. The diestrus serum OTase levels were variable with daily mean levels ranging from 1.3-6.4 U/L, with an overall mean diestrus serum OTase (mean ± SD) level of 5.49 ± 0.9 U/L. The mean tissue OTase activity U/L ± SD (range) per 10 mg of tissue ranked from highest to lowest activity was: myometrium 48.4 ± 3.1 (44.3-51.6), endometrium 45.6 ± 1.3 (42.6-50.8), liver 41.7 ± 3.1 (35.9-44.9), corpus luteum 24.1 ± 3.0 (20.1-33.6), and follicle 22.8 ± 2.3 (18.7-25.9). This is the first description of the presence of OTase in equine tissues. In mares both serum and tissues have detectable levels of OTase activity that likely play an important role in the metabolism of oxytocin. Further investigation is required to determine if serum and tissue OTase activity varies during the estrous cycle and pregnancy.

Keywords: Oxytocinase, mare, tissue, diestrus, serum

Evaluation of dexamethasone on fetal maturation and delivery in mares when administered on days 305 to 307 of gestation

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In many species corticosteroids are administered to the dam to induce precocious fetal maturation when the pregnancy is at risk; however in the mare this has met with mixed results. Previously we showed that 24 mg betamethasone administered to pregnant mares on d305 to 307 of pregnancy tended to hasten delivery[1] and more recently Ousey, et al[2] demonstrated mares receiving 100 mg dexamethasone (dex) on d315 to 317 had significantly decreased gestation length. Thus, we hypothesized mares receiving 100 mgs dex on d305 to 307 of gestation would significantly advance parturition and our objective was to determine if this treatment safely induced precocious fetal maturation and delivery at this stage of gestation.

Ten light breed mares were stratified by age and breeding date into two groups: 100 mg dex IM (DEX, n=5) or 50 mL saline IM (CON, n=5) on d305 to 307 of gestation. Jugular blood samples were obtained daily from d304 to 310 and then every other day until parturition to assess serum progesterone (P4) and cortisol concentration. All foals were APGAR scored and weighed at birth and blood samples were obtained at birth, 24 and 48 h to evaluate serum P4 and cortisol. Additionally, at 24 hours IgG concentration was measured and a complete blood count was performed.

Mixed model analysis with repeated measures was used to analyze the treatment and time effect on mare and foal P4 concentration and foal weights. T-tests were used to determine significance of other variables. DEX mares had a shorter gestation length (328+/-14 vs. 341+/-10); but not significantly different. DEX mares had significantly higher P4 on days 307, 308, and 309 and significantly lower P4 between both 0 to 48 and 48 to 96 h prior to foaling compared to CON mares. DEX mares delivered lighter foals at birth (P=0.007) and had lower IgG concentrations (P=0.01). Foals of the DEX mares did not have significantly different indices of maturity (neutrophil:lymphocyte ratio, white blood cell count and APGAR score) compared to CON foals. Although dex treatment on d305 to 307 did not significantly shorten gestation length this could be related to the small sample size of this study. In addition, decreased mammary development of DEX mares may indicate parturition was hastened with treatment and further studies are on-going.

Keywords: Horse, fetus, maturation, glucocorticoids, parturition

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Comparison of Monday-Friday 4-day versus 5-day Co-Synch + controlled internal drug release (CIDR) + timed artificial insemination (TAI) protocols in beef heifers

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Previous studies demonstrated that the gonadotropin releasing hormone (GnRH) dose at the day of controlled internal drug release (CIDR) insertion and the second injection of prostaglandin F2alpha (PGF) 8-12 h after CIDR removal are not essential to optimize pregnancy after timed artificial insemination (P/TAI) in heifers treated with a 5-day Co-Synch + CIDR protocol.[1] A major factor limiting the success of TAI programs is failure to apply treatments at the prescribed day and time. A Monday-Friday 4-day Co-Synch + CIDR protocol would simplify reproductive management of heifers, as treatment administration is facilitated (Monday: CIDR insertion; Friday: CIDR withdrawal + $PGF_2\alpha$; next Monday: GnRH +TAI). In a recent study conducted in dairy heifers, the application of a 4-day Co-Synch + CIDR protocol resulted in optimal P/TAI (55.0%), similar to that observed with the 5-day Co-Synch + CIDR protocol (63.3%).[2] The objective of this study was to compare the P/TAI in beef heifers treated with 4-day or 5-day Co-Synch + CIDR protocols. We hypothesize that the use of a Monday-Friday 4 day Co-Synch + CIDR protocol will result in similar P/TAI to a 5 day Co-Synch + CIDR protocol. A total of 222 Angus heifers, 12-14 months old, were randomly assigned at each of two locations to one of two treatment groups. The heifers received an intravaginal CIDR insert (Eazi-Breed CIDR®, Zoetis Animal Health, Florham Park, NJ) containing 1.38 g of progesterone for 4 days (Monday-Friday 4-day Co-Synch + CIDR, n=113) or 5 days (5-day Co-Synch + CIDR, n=109). On the day of CIDR removal 25 mg of PGF (Lutalyse®, Zoetis Animal Health) was injected IM, and 72 h after CIDR removal heifers received 100 μg of GnRH (Factrel®, Zoetis Animal Health) IM and TAI. The heifers were artificially inseminated by an experienced technician using commercial frozen-thawed semen from a single sire. Pregnancy diagnosis was performed using ultrasonography per rectum at ≥ 36 days after TAI. Data were analyzed using proc logistic and Chi-square test of SAS®. Pregnancy rate did not statistically differ in heifers in the 4-day Co-Synch + CIDR group (46.0%; 52/113) compared with those in the 5-day Co-Synch + CIDR group (52.2%; 57/109) In conclusion, no difference in pregnancy rate was observed between the 4-day and 5-day Co-Synch + CIDR protocols, confirming the hypothesis. Both protocols resulted in acceptable P/TAI. As seen previously in dairy heifers, the Monday-Friday 4-day Co-Synch + CIDR protocol may also be a promising hormonal treatment for TAI in beef heifers, allowing for simple reproductive management, while preserving acceptable fertility.

Keywords: 4-day Co-Synch, CIDR, beef heifers, pregnancy, timed artificial insemination.

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Uterine and corpus luteal vascular dynamics on day 34 of pregnancy do not differ between dairy cattle which abort or carry pregnancy to term

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The objective of this experiment was to determine if uterine or ovarian vascular dynamics could be used to identify cows at risk for pregnancy loss. Our hypothesis was cows which aborted would have decreased corpus luteal (CL) perfusion and/or an increased resistance index (RI; indicative of decreased uterine blood flow) on D 34 of pregnancy. This experiment was performed in two replicates from November 2011 to April 2012 (n=69) and November 2012 to April 1013 (n=53). Cows were bred via timed artificial insemination (AI) using OvSynch-56 and examined for pregnancy on D 32 after AI. On D 34, cows confirmed pregnant were examined via transrectal Doppler ultrasonography (Micromaxx; Sonosite, Bothell, WA) and blood samples collected via coccygeal vein to measure progesterone concentrations using radioimmunoassay. Diameter of the CL and crown-rump length of the fetus were measured. Color power Doppler ultrasonography was utilized to determine vascular perfusion to the CL and RI was measured for the uterine arteries as previously described.[1] Records were later examined to determine which cows aborted and at what day of pregnancy. Abortion rates between replicates were compared using the SAS FREQ (version 9.4; SAS Institute Inc., Cary, NC) procedure invoking a Chi square statistic. Resistance index, CL diameter, CL perfusion, crown-rump length, and progesterone concentrations were analyzed using the SAS LOGISTIC procedure. Data are presented as mean ± SEM. Abortion rate was 11.6% (8 of 69 cows) for the first replicate (2011 to 2012) was not different than the second replicate (2012 to 2013;9.4%; 5 of 53 cows; $P = 0.77$) with a combined abortion rate of 10.7%. Of the cows which aborted, 6 cows aborted by D 46, 1 cow by D 56, 2 cows by D 67, 1 cow by D 74, 1 cow by D 136 and 1 cow by 144 d. There were no differences ($P > 0.05$) in mean RI to the gravid uterine horn (aborted = 0.90 ± 0.05, term = 0.89 ± 0.02), mean RI to the non-gravid uterine horn (aborted = 1.04 ± 0.05, term = 1.01 ± 0.02), mean CL diameter (aborted = 24.5 ± 1.2 mm, term = 25.5 ± 0.5 mm), or mean CL perfusion (aborted = 11.0 ± 2.3 %, term = 15.2 ± 1.1 %) in cows which aborted compared to cows which went to term. Mean crown-rump length was less ($P \leq 0.05$) in aborted cows (13.2 ± 0.5 mm) compared to term cows (14.2 ± 0.2 cm). Mean serum progesterone concentrations were less ($P \leq 0.05$) in aborted cows (7.5 ± 0.5 ng/dL) than term cows (9.1 ± 0.3 ng/dL). This study demonstrates that monitoring uterine and ovarian vascular dynamics on D 34 do not offer a method to identify cows at risk for pregnancy loss.

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Removal of an intrauterine mineralized caruncle from a Holstein cow by colpotomy and hysterotomy

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During routine prebreeding transrectal examination at 50 days postpartum a high producing, second lactation cow was found to have a firm, movable structure in the base of the left uterine horn. Further examination by ultrasonography revealed a hyperechoic mass not associated with the endometrium. This cow had delivered a live heifer calf with no obstetrical or postpartum reproductive complications. She was considered valuable based on her mature equivalent (ME) 305 (35620 lbs). It was decided to remove the mass by colpotomy and hysterotomy. Surgery was performed 57 days postpartum in a chute. An epidural injection consist of 2% lidocaine (120mg) with xylazine (24mg; 100 mg/mL) was administered. The cow's tail was restrained to the side and her perineal and perivulvar area was prepared for surgery. The cranial vagina was anesthetized using 2% lidocaine on sterile gauze held in contact with the mucosa for a few minutes. A Kimberling-Rupp spay instrument was used to make a small incision in the left vaginal fornix, at 10 o'clock position. The surgeon's hand was used to enlarge the incision. The uterus was retracted into the vagina and held by an assistant. A 5 cm incision was made along the greater curvature of the left horn about 4 cm from the tip of the uterine horn. The mass was removed and the uterus was closed using 2-0 polydioxanon in a Utrecht pattern. The serosa of the uterus was lavaged with sterile water and replaced in the peritoneum. The cow was given 750 mg of flunixin meglumine IV (1.1mg/kg) and 750 mg ceftiofur hydrochloride IM (1.1 mg/kg) for three days. The mass was a yellowish-brown, hard substance measuring 4.5 by 5 by 1.5 cm. It was submitted for histologic identification. Despite considerable necrosis and mineralization, there was sufficient cellular detail to confirm histologically that the removed tissue was indeed a caruncle. The cow was examined by transrectal palpation one and two weeks after surgery in which minor adhesions were palpated on the ventral aspect of the serosal uterine wall. The cow was seen in estrus two weeks later and re-examined with mild thickening of the left uterine wall but no obstructive adhesions palpable, and was artificially inseminated at that time. Mineralized caruncles are thought to occur postpartum when the blood supply to the caruncle is lost and the whole caruncle is shed intact rather than in smaller pieces. Detachment of the entire superficial layer of the caruncle is a normal phenomenon, but occasionally the detached cap is too large and inspissated and does not get passed through the cervix or broken down into normal lochia. These caruncles may further inspissate and one or two may be palpated several weeks later as firm masses. It should be noted that this surgical technique has been described for removal of mummified fetuses but not for removal of mineralized caruncles and could be used to remove pathological uterine contents.

Keywords: Mineralized caruncle, colpotomy, hysterotomy

Clinical and metabolic evaluation in hyperlactatemics foals from mares with placentitis

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Lactate is an important marker of sepsis and neonatal hypoxemia and clinical evaluation of this and other metabolites may be relevant in the prognosis of foals from mares with placentitis. We hypothesized that hyperlactatemic foals from mares with placentitis will have altered biochemistry profiles of hepatic and renal functions. The aim of this study was to determine changes in biochemistry profiles in response to hyperlactatemia in newborn foals from mares with placentitis. We included twenty-four foals with high blood lactate concentrations (≥3mmol/L) from mares with placentitis. Placentitis was confirmed by histologic evaluation. All folas were born at term (≥ 320 days). From the assessment of neuromuscular reflexes and behavioral signs, the foals were devided into two groups: 1) mature foals (n=14), with normal neuromuscular reflexes and behavioral signs and, 2) dysmature foals (n=10), with abnormal signs and behavior consistent with immaturity, including the following: low birth weight, laxity of the flexor tendons, domed forehead, fine, silky haircoat with soft ears and lips. Blood samples were collected from all foals at birth (within 5 minutes) and at 24 hrs for the estimation of lactate (mmol/L), glucose (mg/dL), creatinine (mg/dL), albumin (g/dL), total bilirubin (mg/dL), alkaline phosphatase (ALP; U/L), creatine kinase (CK; U/L) and gamma glutamyl transferase (GGT; U/L). Normality of the data was assessed by Shapiro-Wilk test. One-way ANOVA was used to evaluate the difference in the biochemistry profile between groups. Significance was set at $p<0.05$. The results of biochemical evaluation (mean+SD) in mature and dysmature foals were, respectively: at 5 minutes after birth: lactate (4.3±0.4; 4.7±0.5), glucose (114±13; 114±17), creatinine (2.6±0.4; 4.3±0.5), albumin (3.3±0.1; 2.6±0.1), total bilirubin (6.1±0.8; 8.8±0.9), ALP (943±77; 975±91), CK (185±28; 167±33) and GGT (13±3; 31±4). At 24 hrs: lactate (3.9±0.3; 3.9±0.4), glucose (170±9; 160±11), creatinine (1.8±0.1; 1.9±0.2), albumin (3.1±0.2; 2.4±0.4), total bilirubin (8.0±1.1; 8.3±1.5), ALP (850±102; 957±121), CK (379±45; 212±49) and GGT (27±4; 24±5). In dysmature foals higher concentrations of creatinine and GGT at birth ($p<0.05$) and lower albumin concentrations, at birth and at 24h ($p<0.05$) were observed. Furhter a trend for an increase in bilirubin concentrations at birth was noted in dysmature foals ($p = 0.07$). Hypercreatinemia in the first hours of life could be a result of placental injury and fetal stress. The high concentration of GGT and low albumin in dysmature foals at birth, may be indicative of hepatic disorder. Low albumin concentrations may also be related to protein catabolism in these foals. In conclusion, assessments of creatinine, GGT and albumin are important to identify hight-risk newborn foals from mares with placentitis, in addition to blood lactate. More studies are required to relate the use of these metabolites in the prognosis of foals.

Keywords: Lactate, GGT, creatinine, albumin, immaturity

The detection of Tritrichomonas foetus in bovine semen with centrifugation and PCR

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Bovine trichomoniasis caused by *Tritrichomonas foetus*, is a true venereal disease of cattle that is spread only through coitus. Diagnostic techniques have recently improved with the emergence of a more sensitive and specific polymerase chain reaction (PCR) techniques. This is an improvement from traditional culture methods, but better diagnostic and collection methods are still needed given the serious consequences of inaccurate diagnosis. The objective of the study was to determine if a *T. foetus* infection could be detected using PCR in pre-seminal fluid and a seminal sample collected from known positive bulls.

Mature beef bulls (n=20) of various breeds from several south Florida ranches that were previously diagnosed to be positive for *T. foetus* by routine culture and real-time PCR on preputial smegma at an external state diagnostic laboratory were used for this study. These bulls underwent routine electroejaculation, and a dry preputial scraping sample was collected from each bull using a 52.5 cm infusion pipette with a flex adaptor and a 20 ml syringe. The samples collected from urethral emissions were fractionated into a pre-seminal sample and seminal sample based on gross appearance. The bulls all achieved an erection and the penis extended completely outside of the sheath. The preputial sample was immediately suspended in 2 ml of trypticase-yeast extract-maltose medium (TYM) with agar (Diamond's medium). The samples were transported to the laboratory in a commercial incubator at 37°C. Samples from each bull were centrifuged at 4000g for nine minutes at room temperature and the resulting pellet was used for DNA isolation prior to processing for conventional PCR. Analytical sensitivity and specificity of preputial scrapes were calculated based on previous confirmation of *T. foetus* positive status using an online calculator (http://vassarstats.net/clin1.html). Overall, 13 of 20 bulls were positive by traditional preputial scraping resulting in a test sensitivity of 0.65 (95% CI: 0.41-0.84). Four of these 13 positive bulls were also positive on the pre-seminal sample. One bull tested positive on the pre-seminal sample, but was negative on preputial scraping. The test sensitivity and specificity for detection of *T. foetus* by conventional PCR in pre-seminal fluid was 0.30 (95% CI: 0.1-0.61) and 0.86 (95% CI: 0.42-0.99), respectively. None of the semen samples were found to be positive for *T. foetus*. The combined sensitivity for the preputial scrape and pre-seminal fluid in this study was [1 - (1- 0.308) x (1- 0.65) = .758], suggesting that the diagnosis of *T. foetus* in infected bulls can be improved by additionally testing pre-seminal fluid during routine collection of a preputial scrape.

Keywords: Bulls, *Tritrichomonas foetus*, PCR, centrifugation, pre-seminal, seminal

Pharmacokinetics after intrauterine infusion of ciprofloxacin in the mare

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Infectious endometritis has serious impacts on the reproductive management of broodmares. Enrofloxacin causes irritation to the endometrium following intrauterine infusion. The aim of the present study was to examine concentrations of ciprofloxacin (Hospira, Inc. Lake Forest, IL) in the uterine lumen, endometrium and plasma over a 24-hour period following intrauterine infusion. Mares (n=12) were evaluated by ultrasound and infused with 600 mg ciprofloxacin when in estrus. Intraluminal contents and blood were collected at 0, 2, 4, 8, 12 and 24 hours after infusion. Three mares had uterine biopsies performed at 2 and 24 hours after infusion. Ciprofloxacin was measured using high performance liquid chromatography-tandem mass spectrometry. Peak average intraluminal and endometrial concentration of ciprofloxacin post infusion was 6,178.82 ± 2,774.47 μg/mL and 7,433.33 ± 4,027.51 μg/g, respectively (mean ± SD). Peak average plasma ciprofloxacin concentrations was 0.158 ± 0.128 μg/mL. Endometrial cytology samples were collected at the end of the study. Seven out of twelve mares had a normal cytology; five mares had scattered neutrophils. C_{max} in intrauterine luminal and endometrial tissue for ciprofloxacin reached a target of at least 10x the minimum inhibitory concentration for common bacterial species associated with equine endometritis. Absorption of ciprofloxacin into the endometrium may be beneficial in treating deep-seated infections. No significant adverse reactions were noted after uterine infusion of ciprofloxacin, based on ultrasonography and uterine cytology. Ciprofloxacin may be a good option for treatment of infectious endometritis due to susceptible organisms in the mare.

Keywords: Mare, ciprofloxacin, infectious endometritis

Embryo stage, quality and number of ovulations in recipient mare affect pregnancy rates in embryo transfer recipient mares

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The aim of the study was to investigate whether stage and quality of embryos, and number of ovulations in recipient mare influenced pregnancy outcome in an embryo transfer program. Embryos (n = 181) from 76 donor mares aged 3-25 years were recovered 8 to 9 days after ovulation. The embryos were classified according to the International Embryo Transfer Society (IETS) manual (Stages: 4 - morula, 5 - early blastocyst, 6 - blastocyst and 7 - expanded blastocyst; Quality: excellent or good and fair). Warmblood recipient mares aged 4 to15 years were scanned every second day for ovulations and embryos were transferred on days 2 to 7 after ovulation. The recipient mares were scanned for pregnancy on day 18 after ovulation. The overall pregnancy rate on day 18 was 74% (134/181). Pregnancy rates in recipients that had embryos transferred at day 2, 3, 4, 5, 6 and 7 days after ovulation were 100% (n=1), 62% (n=8), 73% (n=44), 74% (n=57), 77% (n=61) and 70% (n=10), respectively (P>0.05). Good quality embryos at stages 5, 6 or 7 resulted in higher pregnancy rates (79%; 110/139) than fair quality embryos at stages 4, 5, 6, or 7 (57%; 24/42) (P = 0.008). Recipient mares with two or more ovulations became pregnant at a higher rate (86%; 37/43) than recipients with a single ovulation (70%; 97/138) (P = 0.047). In conclusion, embryos graded as excellent or good resulted in higher pregnancy rates than embryos graded as fair, and recipient mares with multiple ovulations became pregnant at a higher rate than mares with one ovulation.

Keywords: Embryo transfer, mare, double ovulation

Non-infectious abnormal placenta and its association with obstetric and neonatal parameters in mares

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The placenta is responsible for metabolic and gaseous exchange between mares and fetus during gestation. Any placental damage can affect fetal development and/or foal survival. The aim of the study was to describe the histopathological features of non-infectious placentas from Thoroughbred mares. Immediately after foaling, the placentas (n=148) were weighed and samples from nine placental points (cervical star, uterine body, gravid horn, non-gravid horn, bifurcation, amnion, and tree points from umbilical cord) were collected in 10% formalin, processed, and stained using hematoxylin and eosin and periodic-acid-Schiff (PAS) reagents for evaluation under optical microscopy. In addition, the following data were recorded: mare's age, number of parturition, gestational age, third stage labor (placental elimination time), umbilical-cord length, placental weight and foal weight. Following histological evaluation the placentas were grouped in to three categories: normal (n=99) - no changes were observed; vacuolated (n=30) - no pathologic findings but with intense cytoplasmic vacuolization of the epithelial areolar cells. The chorionic epithelium consisted of large cells containing vesiculated nuclei and granular cytoplasm characterized by translucent material containing eosinophilic granules, compatible with histotrophic secretory (uterine milk) glands, suggestive of the presence of mucopolysaccharides; hypoplastic (n=19) - exhibited microcotyledonary hypoplasia or atrophy, characterized by the presence of short villi some of which had a narrow base, or lack of villi and with necrotic microcotyledons at the chorionic surface. The results for the normal, vacuolated and hypoplasic placentas were: age of mare (9.4±0.3; 13±0.8; 9.5±1), number of parturition (4.1±0.2; 6.4±0.7; 4±0.8), umbilical cord length (48±1.1, 48±1.9, 42±1.8) and placental weight (6.9±0.1; 6.8±0.2; 6.2±0.2). The other obstetric parameters, gestational age, placental elimination time and foal weight, did not differ among groups. The mares with vacuolated placenta were older and with more numbers of parturitions ($p<0.05$). Older mares are recognized as high risk, due to endometrial fibrosis. However, the presence of vacuolated cells indicate higher production of histotrophic secretion and suggest that these mature mares had a more integrated utero-placental unit. The mares with hypoplasic placentas had a lower umbilical-cord length and placental weight, when compared with normal placentas ($p<0.05$). Hypoplasia/atrophy of microcotyledons are a major cause of non-infective abortions or premature delivery. These lead to intra-uterine growth restriction and thus resulting in lower chance of neonatal foal survival. However, this was not observed in this study. It should be noted that even in non-infectious conditions placental histologic findings are associated with placental and mare parameters. In conclusion, older mares with more numbers of parturitions showed placental abnormalities and hypoplasia of microcotyledons which leads to low placental weight.

Keywords: Placenta, mares, non-infectious, histology, obstetric dates

Evaluation of salivary progesterone profiles as an indicator of reproductive status in equines

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Diagnostic assays of reproductive hormones are usually performed by veterinarians on mares to confirm the findings of trans-rectal palpation and/or reproductive ultrasound examination. Of the reproductive hormones, progesterone (P4) is one of the most commonly measured hormones in the field of equine reproduction. Serum P4 concentration in mares is usually evaluated using an extracted radioimmunoassay (RIA), which is generally considered as the gold standard in reference laboratories. However, the use of RIA for serum P4 measurement usually involves a lengthy process to extract the steroids from carrier proteins. Up to 95% of the steroid hormone binds to carrier proteins. Estimation of salivary steroid hormones presents an attractive alternative, since the salivary steroids are not protein bound and appear to be in biologically active form. The objective of this study was to compare serum and salivary progesterone values during the estrous cycle (n=13) and early pregnancy in mares and to evaluate whether saliva could serve as an alternate biological fluid to monitor the luteal phase in mares. Serum and saliva samples were collected on selected days of the estrous cycle days (1, 3, 5, 8, 14, 17 and 20) and during early pregnancy (days 1, 3, 5, 8, 14, 17, 25, 35, 45, and 65) from mares and were validated using a liquid phase RIA. Serum samples were extracted and processed using charcoal dextran adsorption before subjecting to the RIA. Salivary samples did not require solvent extraction and were subject to RIA without this step. The inter-assay coefficient of variation (CV) for low and high controls was 6.91% ± 0.81 (Mean ± S.E.) and 5.06 % ± 0.57 (Mean ± S.E.) for paired serum and saliva samples run in the same assay, while the intra-assay CV averaged 13.19% for saliva and 11.71% for serum. Mean saliva: serum ratio was elevated in cycling (35%) and pregnant (25%) mare until day 3 of the cycle and then dropped to and remained between 8 to 12% for the remaining duration in cycling (until day 17) and pregnant mares (up to day 65) ($p > 0.05$). Statistical analysis was performed using Friedman's one way ANOVA. Serum P4 for cycling and pregnant mares differed by day of sampling ($p < 0.0001$). In sharp contrast there was no significant difference in salivary progesterone levels by day of sampling for cycling mares ($p > 0.05$). In pregnant mares it was different for only on days 1 and 3 after ovulation. A significant difference was seen with the saliva to serum ratio in cycling mares while this difference was only observed on day 1 after ovulation in the pregnant mare group. Pearson's correlation analysis showed that the correlation between salivary and serum progesterone levels was not significant except on day 5 (after ovulation) for the cycling mare group ($p >0.05$), and days 5 and 8 ($p < 0.05$) for pregnant mares. Luteal phase saliva P4 levels were observed to be consistently above 0.5 ng/ml in both groups and could be used as a cutoff range to differentiate between the follicular and luteal phase. The study shows that salivary P4 concentration was not correlated with serum values. Nevertheless it can be utilized to monitor luteal phase of the estrous cycle and early pregnancy.

Keywords: Mare, estrous cycle, pregnancy, progesterone, saliva, serum

Effect of epidermal growth factor supplementation during different culture periods on *in vitro* maturation of canine oocytes

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Despite many attempts to improve *in vitro* maturation (IVM) of canine oocytes using different culture conditions, the efficiency of canine IVM remains very low compared to IVM of other domestic animals. The molecules involved in maturation and culture time to induce meiotic resumption remain unclear . Thus, this study was aimed to evaluate the influence of epidermal growth factor (EGF) in IVM media on IVM of canine oocytes. The study was approved by the Institution's Animal Care and Experimentation Ethics Committee (protocol number 176/2011). The ovaries were collected from 40 bitches in anestrus or diestrus after ovariohysterectomy, transported in phosphate buffer saline (PBS) at 4°C to the laboratory and sliced to recover the cumulus oocyte complexes (COCs). Only grade I COCs were used in this study. The COCs were cultured in HEPES-buffered TCM 199 medium supplemented with 50 μg/mL gentamicin, 26 mM sodium bicarbonate, 1.5 mM sodium pyruvate, 0.6 mM cysteine, 0.03 UI/mL hCG, 0.5 μg/mL FSH and 20 mg/mL E_2. Further, EGF (10 ng/mL, Sigma Chemical Co., St. Louis, MO) was added to treatment group culture medium and no EGF was added to control group culture medium. The COCs from both treatment groups were cultured for 24 h, 48 h or 72 h at 38.5°C and 5% CO_2. After IVM, the oocytes were denuded, fixed and stained with Hoescht 33342 to evaluate nuclear status. Statistical analysis was carried out by ANOVA and Fisher's test at 5% significance level. In this study, the time of exposure of canine oocytes to EGF affected *in vitro* maturation. There is no effect of EGF at 24 h on nuclear progression of germinal vesicle (GV) to germinal vesicle breakdown (GVBD) stage (EGF: 30.5 and 44.2%; No-EGF: 27.5 and 63.5%, respectively, $p>0.05$). However, oocytes cultured for 24h in supplemented medium showed increased rate of nuclear progression to metaphase I stage (M-I) (13.7 *vs* 6%, $p<0.05$). We found increased percentage ($p<0.05$) of oocytes in M-I and metaphase II (M-II) stage (31% and 2.5%, respectively) in oocyte cultured for 48h in supplemented medium compared to free-EGF medium (16% and 0% respectively). Similarly, at 72 h maturation, there is increased percentage ($p<0.01$) of oocytes in M-I (36%) and M-II (12%) cultured in EGF than in free-EGF medium (16% and 3%, respectively). It is noteworthy that the acquisition of meiotic competence is a two-step process; the oocyte first achieves the ability to undergo germinal vesicle breakdown and gradually becomes more capable of completing meiotic maturation to metaphase II. In this study we observed that the highest percentage ($p<0.05$) of oocytes at metaphase II was observed after 72 h of culture. In conclusion, the time of exposure of canine oocytes to EGF affected *in vitro* maturation and that the advancement of meiosis was directly associated to the time of maturation. According to these results, we suggest that EGF supplementation in culture medium plays essential role in resumption of meiosis in the transition to M-I and M-II stages.

Keywords: Bitch, oocytes, IVM, EGF, bitch

Acknowledgment

The authors acknowledge FAPESP (2013/21667-3) for the financial support.

Evaluation of p34^{cdc2} protein kinase activity during *in vitro* maturation of canine oocytes

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The efficiency of *in vitro* maturation (IVM) of canine oocytes is still very low compared to other mammalian species after being cultured for 48 h to 72 h *in vitro*. Control of the cell cycle is regulated by a cascade of coordinated events, which influences expression or repression of protein activity related to resumption of meiosis. Studies indicate that the activity of proteins involved in this process appears to be time-dependent and thus could help to establish optimal culture time. This work was aimed to evaluate the p34^{cdc2} protein kinase activity during IVM of canine oocytes cultured for 24 h, 48 h and 72 h. The study was approved by the Institution's Animal Care and Experimentation Ethics Committee (protocol number 176/2011). Ovaries were obtained from 40 bitches submitted to ovariohysterectomy (OH). After OH, the ovaries were immersed in sterile 0.9% NaCl solution and immediately transported at 4°C. In the laboratory, the ovaries were sliced repeatedly to recover cumulus-oocyte complexes (COCs). Only grade 1 COCs were selected and cultured in HEPES-buffered TCM 199 medium supplemented with 50 μg/mL gentamicin, 26 mM sodium bicarbonate, 1.5 mM sodium pyruvate, 0.6 mM cysteine, 0.03 UI/mL hCG, 0.5 μg/mL FSH, 20 mg/mL E_2 and 10 ng/mL EGF (Sigma Chemical Co., St. Louis, MO) for 24 h, 48 h and 72 h at 38.5°C and 5% CO_2 in air in a humidified atmosphere. After IVM, oocytes were collected, denuded, fixed and stained with Hoescht 33342 to evaluate nuclear status. In addition, oocytes were washed in PBS, and placed in tubes containing lysis buffer (20 mM TRIS, 150 mM NaCl, 1.0 EGTA, 1.0 mM EDTA, 2.5 mM sodium phosphate, 1.0 mM β-glicerophosphate, 1.0 mM Na_3VO_4, 1.0 mg/mL leupeptin). The samples were frozen in liquid nitrogen and sonicated (five times during 25 seconds, 10% amplitude and 1 minute interval). Cell extracts were stored at -80°C until use. The Cdc2 assay was performed using the MESACUP® cdc2 kinase assay kit (MBL, Nagoya, Japan). Statistical analysis was carried out by ANOVA and Fisher's test at 5% significance level. The results indicated that the p34^{cdc2} protein activity was time-dependent, reaching a peak at 48 h of IVM ($p<0.01$), and at 72 h, the activity decreased. The proportion of germinal vesicle (30.5%) and germinal vesicle breakdown (44.2%) in oocytes cultured for 24 h were similar to those cultured for 48 h and were significantly higher ($p<0,05$) than those in oocytes cultured for 72 h (9.4% and 23% respectively). The percentage of oocytes in metaphase I (37%) and metaphase II (3%) was higher in oocytes cultured for 48 h than in those cultured for 24 h (13.7% and 1%, respectively) ($p<0.05$). The proportion of metaphase I oocytes cultured for 72 h were similar to those cultured for 48h (3%) and were significantly higher ($p<0.01$) than those in oocytes cultured for 24 h (1%). In conclusion, p34^{cdc2} protein kinase plays an important role in meiosis progression, especially in germinal vesicle breakdown to metaphase I and metaphase II transition.

Keywords: Protein kinase, oocytes, p34^{cdc2}, maturation, bitch

We acknowledge FAPESP (2013/21667-3) for the financial support.

Ovariohysterectomy following uterine rupture in a ewe

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Small ruminant periparturient emergencies account for the majority of flock losses.[1] Despite the complicated nature of these cases, some breeders attempt to manage them without veterinary assistance, compromising the animal's welfare and prognosis. This report describes uterine torsion in a ewe followed by an unsuspected uterine rupture and surgical treatment by ovariohysterectomy.

A two–year-old parous Icelandic ewe was presented for failure of parturition at the expected due date. The ewe was progressing normally one week prior to the due date and showed mammary gland enlargement, then became lethargic and weak. The owner managed her at home for pregnancy toxemia. The ewe recovered clinically but her mammary regressed. On presentation five weeks following mammary development, physical examination and thorough reproductive evaluation were performed. Vaginal speculum examination revealed a closed cervix. Ultrasonographic evaluation showed an irregular outline of the uterus and fetus(es) that appeared macerated. A cesarean section was elected using a ventral midline approach. Following the skin incision, the linea alba was visibly congested and thickened. Incising the linea immediately exposed the fetuses, suggesting a uterine rupture. The fetuses were removed. The uterus was adhered to the body wall sealing the uterine contents from the viscera. Careful dissection of the adhesions freed the uterus from the abdominal wall. Exploration revealed a 180° uterine torsion and advanced uterine necrosis. Ovariohysterectomy was performed. Post-operatively the ewe received antibiotics and was discharged. The ewe suffered no surgical complications and returned to fiber production.

The clinical presentation for pregnancy toxemia, failure of cervical dilation, and uterine torsion can be similar. Uterine torsion and rupture is rarely reported but should be considered a differential diagnosis in overdue, recumbent ewes. Breeders should consider an overdue ewe with mammary development and regression without parturition an emergency. Owners should be educated on the welfare issues associated with peripartum emergencies.

Keywords: Management, welfare, dystocia, reproduction, emergencies

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Clitoridectomy following vulvar laceration in a pregnant mare

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Treatment of vulvar trauma is common in equine practice; however, cases are likely underreported, particularly in pregnant mares. Vulvar or vaginal surgeries during pregnancy can predispose to ascendant infection or pregnancy loss. A 15 year old multiparous Arabian broodmare was presented at 93 days of gestation following a kick to the vulva. Hemorrhage and swelling of the vulva and clitoris were treated locally by placement of sutures and clitoral cerclage. Systemic treatments included anti-inflammatories and antibiotics. The mare displayed increasing pain and digital pulses over the next two days as the clitoris developed a large hematoma. Due to inability to control her pain, surgical clitoridectomy using electrocautery was performed under epidural anesthesia and systemic sedation. Post-operatively the mare immediately became comfortable. Transrectal evaluation of the fetoplacental unit demonstrated contraction of the uterus and position of the fetus at the cervix. Due to risk of ascendant infection and potential effects of systemic inflammation on pregnancy, the mare was treated with progesterone, antibiotics, and anti-inflammatories. Subsequently the uterus was found to be relaxed with normal uteroplacental thickness. The mare healed well postoperatively and foaled without complication or assistance at 325 days of gestation.

This case is an example of successful surgical treatment of a vulvar injury which was nonresponsive to medical management. Vulvovaginal surgeries in pregnant mares increase risk for ascendant infection and abortion. In this case, the mare foaled normally, supporting that surgical treatment of this case to manage pain was the best treatment for welfare of the mare and foal.

Keywords: Broodmare, surgery, clitoris, ultrasonography

Diagnosis and treatment a of granulosa cell tumor in a 10 year old mare

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Abstract

A 10 year old American Quarter Horse mare was presented to her veterinarian with a history of stallion-like behavior and aggression. A diagnosis of granulosa cell tumor was made based on history, and the mare was referred to Auburn University for definitive diagnosis and ovariectomy. On trans-rectal palpation and ultrasound, the left ovary was enlarged and firm, measuring 7 cm, with no palpable ovulation fossa and a multicystic appearance. The right ovary was small and inactive, measuring 1.5 cm in diameter. A diagnosis of granulosa cell tumor of the left ovary was made based on history and characteristic ultrasonographic findings. Hormone analysis was pending at the time of admission.

Ovariectomy of the left ovary was performed using laparoscopy through a left paralumbar flank approach. Three 1.5 cm incisions were made in the left paralumbar fossa, the first caudal to the eighteenth rib, and the next two cranial to the tuber coxae, 6.5 cm apart on a vertical axis. After incision, cannulas were placed to aid instrumentation. The ovary was identified and a LigaSure™ electrocautery instrument was used to obtain hemostasis of and to transect the mesovarium. The two incisions cranial to the tuber coxae were connected and the ovary was removed. Following removal, histopathology confirmed the diagnosis of granulosa cell tumor.

In mares, granulosa cell tumors are the most common neoplasm involving the ovary.[1] Tentative diagnosis of a granulosa cell tumor is commonly made based on behavioral history and trans-rectal palpation and ultrasonography findings.[2] Diagnosis is usually confirmed with elevated blood testosterone (67% of cases) and inhibin (87% of cases).[1] Recently, studies have indicated elevated anti-Mullerian hormone (AMH) may diagnose the presence of granulosa cell tumor with 98% accuracy.[3] This case describes characteristics of a typical granulosa cell tumor presentation, with diagnostic and treatment options.

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Priapism in a Thoroughbred gelding associated with metastatic *S. equi* infection

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A 12-year-old Thoroughbred gelding was presented to the North Carolina State University College of Veterinary Medicine for acute priapism and weight loss. A physical examination was unremarkable except for poor body condition (BCS 2/9) and complete protrusion of the erect penis. The penis was reduced into the prepuce following irrigation of the corpus cavernosum with heparinized saline, injection of 10 mg of phenylephrine into the corpus cavernosum, and manual massage using nitrofurazone and DMSO ointment. A purse-string suture and penile sling were placed to assist in penile retention and support. The erection recurred approximately eight hours after the procedure, and treatment was repeated. The purse-string was removed after 48 hours due to superficial trauma. The penis remained moderately erect throughout hospitalization and was treated conservatively with a sling to prevent edema, application of tetracycline wound ointment, and hydrotherapy. Diagnostic procedures to pursue the weight loss included thoracic and abdominal ultrasound, complete blood count and chemistry, urinalysis, urine culture, rectal biopsy, and *Streptococcus equi* ELISA. Urinalysis revealed 2+ hematuria and pyuria, likely due to preputial inflammation. The serum ELISA assay for *S. equi* returned strongly positive (1:25,600), consistent with metastatic strangles abscesses. Treatment with trimethoprim sulfamethoxazole (TMS; 30 mg/kg PO BID) and rifampin (5 mg/kg PO BID) was instituted for eight weeks based on evidence that rifampin may prevent resistance and improve efficacy of the TMS against *Streptococcus* spp. sequestered in abscesses.[1] When reexamined seventeen weeks after discharge, the gelding's BCS was 5/9 and his priapism had improved, although the penis still protruded from the prepuce two inches at most times. Thirty weeks after discharge the animal had returned to a normal weight and the priapism was completely resolved. Priapism is rare is horses, especially geldings, and there are no reports of priapism secondary to metastatic strangles.

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Return to cylicity after diagnosis of granulosa cell tumor in 16 month old Simmental heifer

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Abstract

A 16 month old Simmental heifer was presented with history of udder development and masculine behavior. A left ovarian mass was confirmed via transrectal palpation and ultrasonography revealed a 10cm by 7cm cavitated mass typical in appearance to a granulosa-theca cell tumor (GTCT) and an abnormally small right ovary. Granulosa-theca cell tumors are rare; one study of 1489 slaughter cattle showed GTCT incidence rate of 0.7% .[1] If present in bovine species it is more commonly reported in lactating dairy cattle and rarely in beef heifers.[2]

Granulosa-theca cell tumors occur most commonly in equids which have a higher rate of return to fertility than bovids.[3] Normal presentation of this tumor in cattle is similar to that seen in this heifer with the addition of nymphomania. Diagnosis, as in this case, is generally made through clinical signs, transrectal palpation, ultrasonography of the reproductive tract and confirmed with post-operative histopathology to determine if tumor was of granulosa or theca cell origin. Additionally clinical diagnosis may be confirmed through inhibin and testosterone concentration in the blood. Ovariectomy is considered the treatment of choice for affected females. The affected ovary may be removed by either a colpotomy or a flank approach. In this case, a standard left flank laparotomy was chosen due to the size of tumor. Post-operative medications included antibiotics and anti-inflammatory therapy. There should be suspicion of these tumors if abnormal estrus, nymphomania or virilism is reported. Diagnosis is confirmed with hormone analysis and histopathology. This case allows a rare opportunity to study the pathology and treatment of these tumors and differentiation of granulosa verses theca cell tumors in beef cattle. Histopathology confirmed that this tumor was exclusively of granulosa cell origin. This heifer resumed to normal estrous cycles but was not fertile which further supports the theory that equids have a better prognosis for future fertility after tumor removal.

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Stallion-like behavior in male castrated Thoroughbred with non-secretory inguinal mass

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A six-year-old castrated male Thoroughbred was presented to the North Carolina State Theriogenology Service for stallion-like behavior and a serum estrone sulfate concentration of 17.50 ng/ml (reference: gelding <5 ng/ml; stallion/cryptorchid >10 ng/ml), consistent with cryptorchidism. Transrectal ultrasonography detected no structures resembling a retained testicle. A human chorionic gonadotropin (hCG) stimulation test was performed and blood collected before and two hours after administration of 6000 IU of hCG intravenously. Serum was submitted to University of Caifornia- Davis Clinical Endocrinology Laboratory for measurement of anti-Mullerian hormone (AMH) and testosterone concentrations. The concentration of AMH was 0.1 ng/ml (reference: cryptorchid >0.15 ng/ml) and testosterone concentrations pre-hCG and post-hCG stimulation were 7.7 ng/ml and 17.7 ng/mL, respectively (reference: gelding <15 ng/ml; inconclusive 50-100 ng/ml; cryptorchid 100-500 ng/ml). These results were not diagnostic for cryptorchidism, however, due to persistent stallion-like behavior, the owner elected to pursue exploratory laparoscopy. During the standard flank laparotomy, a 4x4 cm tissue structure was observed hanging in the left flank and retrieved from the left inguinal ring region. Histopathology examination showed arterial and venous profiles with large nerve bundles arranged in a loose connective tissue and adipose matrix, normal vaginal tunic constituents not normally seen in castrated animals. After a post-surgical rest-period, the gelding was returned to work and displayed none of the previous behavioral issues.

Although hormone testing and histopathology did not conclusively support cryptorchidism, the improved behavior postoperatively indicates the mass in the inguinal region may have induced the stallion-like behavior.

Retained testicular tissue is a common cause of behavioral problems and increases risk of neoplasia for affected animals. This case demonstrates the role of both serologic and laparoscopic diagnoses for animals with persistent behavioral issues.

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