

# *Equine Center*

January 2006



2005—2006 Research Report

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## **Research Topics**

- Genetics
- Muscle Disease
- Lameness
- Reproduction
- Medicine

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## *University of Minnesota Equine Center*

### *Improving the health, well-being, and performance of the horse*

As director of the University of Minnesota Equine Center, I invite you to read and enjoy our Research Report. These projects were supported by the University of Minnesota Equine Center, College of Veterinary Medicine, University of Minnesota, with funds provided by the Minnesota Racing Commission, Minnesota Agricultural Experimental Station and contributions from private donors.

This report contains brief, descriptive summaries of the results obtained from recently completed scientific investigations and summary reports on the initial research funded directly by the University of Minnesota Equine Center. Many of these studies served as starting points for larger projects funded by other sources such as the USDA, Morris Animal Foundation, the Grayson Jockey Research Foundation, AQHA and others. Equally important, these studies provided funding for graduate student research. The experience and knowledge gained by these talented students allows them to become the scientists leading us into a better future. We hope you will become better acquainted with both the work of our Center and some of the researchers involved in its programs.

We have, for many decades, been applying cutting-edge medical science and focusing the brightest and best of the equine medical personnel toward the betterment of the horse. The Equine Center, its researchers and clinical staff have dedicated themselves toward maximizing the health and well-being of horses everywhere. Our goal has been and will continue to be the application of medical knowledge and scientific technology toward the prevention, treatment, and cure of injuries and diseases that afflict horses.

Again, it is my pleasure to submit to you the University of Minnesota Equine Center Research Report. I sincerely hope that you find its contents both interesting and informative. We actively solicit both your comments and your support regarding our efforts on behalf of the horse. So please, do contact us regarding any of our programs, or your possible participation in them.

Cordially,

Stephanie Valberg  
DVM PhD Diplomate, ACVIM



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## *University of Minnesota Equine Center*

A renewed tradition of excellence in equine care, research, and education

### **A Program of Excellence**

The Equine Program has been identified as a “program of excellence” within the College of Veterinary Medicine in conjunction with the University of Minnesota’s initiative to become one of the top three public research universities worldwide. The sophisticated facility and program will provide innovative research opportunities, progressive undergraduate training, and advanced equine education.

### **Unparalleled Comprehensive Educational Opportunities**

This comprehensive integrated program is dedicated to educating a broad scope of future equine professionals. The Equine Center’s exceptional facilities will attract the most qualified faculty and premier students. The Center will disseminate information relevant to all aspects of equine health and performance throughout the equine community.

### **Applied Innovative Advancements in Equine Research**

Resources from many Colleges within the University of Minnesota will be integrated to generate major research support. Novel research findings will influence all aspects of equine husbandry, with the expectation of disease

prevention through genetics, nutrition, and other specialties.

### **Only The University of Minnesota can Accomplish this Essential Mission**

We are geographically located at the heart of the medical and nutrition industry and horse country itself. The University is unequalled in its knowledge base, expert resources, dedicated faculty, and reputation for integrity within the equine community.



# Genetics

## Mapping the recurrent exertional rhabdomyolysis gene in Thoroughbred horses

James R. Mickelson, Stephanie J. Valberg,  
Esther M. Gallant

### Description of the Problem:

A common episodic form of the tying-up syndrome, termed RER (recurrent exertional rhabdomyolysis), is estimated to affect approximately 5% of all Thoroughbred horses. In a series of studies we have shown that RER is an inherited muscle disorder, for which environmental, dietary, and stress factors can occasionally trigger tying-up episodes in genetically susceptible individuals. Attempts to identify the RER gene from biochemical and physiological experiments designed to test the function of genes analogous to those responsible for human myopathies have not yet and may never be successful. Our group is, however, uniquely positioned to apply advanced molecular genetic technology to identify the gene responsible for RER in horses.

### Experimental Approach:

Expand our resource herd of horses to a size of appropriate for use in mapping the RER gene. This will be accomplished through the mating of an RER stallion to three normal mares and the mating of two normal stallions to three RER affected mares.

Test all six new foals, and two other foals not already tested, with our established in vitro muscle contracture test to determine whether they are normal or RER susceptible.

Use DNA markers located on specific segments of each equine chromosome to test for co-inheritance (genetic linkage) of that DNA segment with RER trait in each horse in our herd. Linkage of a DNA marker with the RER phenotype is strong evidence that the RER gene is physically close to that marker on that chromosomal segment.

### Accomplishments/Results:

In vitro contracture test to diagnose foals. External intercostals muscle biopsies from four control foals were subjected to the caffeine and halothane in vitro contracture testing protocol. This allowed us to validate and refine our diagnostic standards of RER susceptibility. We now define RER susceptibility in vitro as the development of a contracture in muscle bundles of at least 0.5% of maximal titanic force upon their exposure to 2 mM caffeine or 1-2% halothane. Such contractures should be observed in two or more muscle bundles from each horse.

Expansion of the existing RER herd. Three six-month-old foals in our herd, TBO, TYEE and TIGR, were diagnosed using the in vitro muscle contracture test. TBO is the son of our affected mare TF and the control stallion RSTR, TYEE is the son of our affected mare AN and the control stallion RSTR, while TIGR is the daughter of our affected stallion ST and our control mare SOF. Based on our previous breedings and analysis of external pedigrees, our hypothesis is that RER is a dominantly inherited condition. As such, mating affected to unaffected horses has a 50% chance of producing an affected foal. Nevertheless, TBO, TYEE and TIGR were diagnosed as normal.

Eight pregnancies in the RER resource herd were produced in late summer/early fall of 2000 by selected matings of RER and control horses. This includes mating to the RER stallion ST to four control mares (LGCY, POLY, RSE, SOF), and the mating of four RER mares (AN, MT, TOI, and TF), to the control stallion RSTR. Eight foals were born in summer/fall of 2001 and were contracture tested in winter/spring to determine if they are RER susceptible or normal.

Acquisition of additional breeding mares. We purchased 3 additional control mares to add to our resource

### **Basis of Glycogen Storage Disease in Quarter Horse Foals**

J. Mickelson and S. Valberg

#### **Description of the Problem:**

Many inherited diseases affecting glucose and glycogen metabolism are known in humans and animal species. Glycogen Storage Disease Type IV (GSD IV) is characterized by abnormal polysaccharide accumulation in multiple tissues and by a decreased function of glycogen branching enzyme (GBE) responsible for creating glucose chain branches within the very large highly branched glycogen molecule. Neonatal mortality is a significant problem in horses. In spite of advances in intensive care of neonates, a large percentage of foals in intensive care units die, mainly for undefined reasons. We have recently identified a fatal glycogen storage disorder in Quarter horse foals that closely resembles GSD IV. Foals were treated for weakness and apparent sepsis from birth to a few weeks of age, at which time they died either of cardiac arrhythmia or were euthanized for persistent recumbency. PAS positive abnormal polysaccharide inclusions were found upon pathological evaluation of many tissues from these foals. Iodine spectra analysis of the glycogen isolated from muscle and liver of the foals showed a decrease in the number of branch points as compared to normal muscle and liver glycogen. GBE activity in multiple tissues of affected foals were less than 10% of control values, with the dams of affected foals having approximately one half of the GBE activity of normal controls. There was no immuno-detectable GBE protein in GBE Western blots of liver extracts from affected foals. This evidence strongly indicated that the affected foals were homozygous for an inherited defect in the GBE enzyme. The aim of this research is to determine the cDNA sequence of the equine GBE gene, define the chromosomal location of this gene, and identify the site of the likely GBE DNA mutation in the affected foals. Once a mutation is found, a PCR-based blood test will be developed to assess the prevalence of this mutation in Quarter Horse foals that die in intensive care units and the incidence of the carrier state in the population at large. A rapid accurate DNA-based diagnosis of equine GSD IV would prevent prolonged and costly therapy, and would provide genetic information to plan matings that avoid the birth of GBE deficient foals.

#### **Experimental Approach:**

Characterize/sequence the GBE gene in normal and GSD IV affected horses. Two different strategies are designed to sequence the equine GBE gene and identify the molecular cause of aberrant GBE function. A PCR genotyping test will be developed to clearly differentiate GSD IV-affected, normal and carrier horses, and to determine the frequency of the mutant allele in the population.

Develop a genetic test for GSD IV in horses. A blood-based PCR genotyping test would clearly differentiate between the GSD IV affected, normal and carrier horses. This familial and population study is expected to verify GSD IV as an autosomal, recessive disease and determine the frequency of the deleterious gene in the population.

Define the GSD IV gene with DNA markers. The known location of the equine GBE gene allows us to identify nearby microsatellite (MS) DNA markers to use in a genetic analysis of GSD IV. This approach will allow us to confirm the GBE as candidate gene, or deny it should our mutation search be unsuccessful.

#### **Accomplishments/Results:**

Evaluation of the pedigrees containing affected foals to date suggest a genetic defect in the equine GBE gene is responsible for this condition referred to as GSD IV. It appears that the disease is inherited in an

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autosomal recessive manner in that affected foals are produced from unaffected parents that both would be only carriers of the mutant gene. This conclusion is supported by blood GBE activity levels that are essentially 0 in affected foals and approximately 50% of control values in their dams and a number of sibs and half-sibs. Two more samples from affected foal – dam pairs have been analyzed this year and for a total now on hand of 12. One additional foal had a diagnosis of GSD IV ruled out this spring based on our measurements of significant levels of GBE in blood samples.

We have determined 95% of the DNA sequence (i.e. the cDNA) that codes for the GBE protein. PCR primers based on 500 bp segments of the known human GBE cDNA sequence were used to get started by PCR amplification of equine liver or muscle mRNA. This eventually generated the horse sequence of all the segments between the primers (approximately 2,000 bp). No sequence differences have been found between affected foals and control horses. The remainder of the cDNA sequence (which lies at both ends) has proven intransigent to our sequencing strategy. We suspect a very high GC content of this region of DNA and very large introns within the gene are to blame. We have stopped direct cDNA sequencing approaches at this time and instead are pursuing a strategy to find DNA markers within and nearby the GBE gene on its particular equine chromosome. We will then employ genetic linkage analysis, which has the power to confirm the gene and its chromosomal segment as causative of the GSD IV condition, as well as exclude this gene from consideration. Basically, we don't want to spend more time and money on completing this equine GBE sequence until it can be determined if it is indeed the correct gene or not. We have enough affected foals, mares and relatives to be successful in a simple genetic linkage analysis if the DNA marker is very close to the GBE gene.

Our attempt to identify DNA markers located within or near the GBE gene was greatly aided by determining the exact position of the GBE gene on equine chromosome 26 (ECA26). This was accomplished in part by using its chromosomal assignment on human chromosome 3 (HSA3) and obtaining equine sequences for segments of neighboring genes from the human genome map. GBE is on band 12 on the small (p) arm of human chromosome 3 (HSA3p12). Human genes nearby that we obtained equine sequence for are ROBO1, ROBO2, MITF, POU1F1, HTR1F, GLBT1, RYBP and PROS1. Collaborators at TAMU were able to place the GBE1 gene along with ROBO1, ROBO2, POU1F1, and HTR1F on ECA26. The other HSA3p genes turned out to be on equine chromosomes 16 and 19.

We have also found two new microsatellite DNA markers from within the GBE gene itself through analysis of a large segment of equine genomic DNA contained within the GBE BAC clone. Genetic linkage mapping of one microsatellite on the equine reference family enabled precise positioning of the GBE gene on ECA26 near five other known microsatellite markers on this chromosome.

We will use all these ECA26 microsatellite markers to test whether the segment of DNA containing the GBE gene has a mutation that has been passed along to all affected foals by each carrier parent. This will entail genotyping all affected foals and all relatives to see if transmission of a founder allele has occurred.

### **Mapping the polysaccharide storage myopathy gene in Quarter Horses (01-02)**

J. Mickelson, S. Valberg

#### **Description of Problem:**

Exertional rhabdomyolysis, more commonly called “tying-up”, is the medical term applied to describe the muscle cramping, stiffness and pain that can follow mild to moderate exercise. A recurrent form of tying-up, termed PSSM, occurs in Quarter Horses and related breeds. Our previous studies have demonstrated that the physiological basis for this disease is an alteration in some aspect of the regulation of the complex process of muscle glucose transport and insulin sensitivity that results in the accumulation of an abnormal sugar polymer in the muscle that damages the cells. We have also shown that PSSM is an inherited condition, implying that there is an alteration in one of the genes responsible for controlling muscle metabolism. Unfortunately, attempts to identify the PSSM gene by analogy to somewhat similar conditions in other species have thus far been unsuccessful.

We are confident that the PSSM gene can be located (mapped) in the equine genome with an approach similar to that used in humans for identifying unknown disease-causing genes such as those for colon and breast cancer. Genetic mapping technologies are now being used to identify important genes in most agricultural and companion animal species. Our aims are to define a population of clearly affected related PSSM horses, identify DNA markers from the equine genome map and the means to assay them, and then analyze for the co-inheritance of these DNA markers of known chromosomal locations with the PSSM trait.

Identifying a DNA marker that is inherited with the PSSM trait in essence maps the PSSM gene to a known segment of an equine chromosome. This process therefore narrows the search for the PSSM gene from one of the approximately 40,000 genes present in the equine genome to one of the several hundred that lie on that particular chromosome segment defined by the marker. Characterization of this PSSM-linked DNA marker will then form a logical basis for tests designed to identify the precise genetic alteration, tests to predict PSSM susceptibility, and the development of specific therapies and treatments to deal with this major disorder affecting health and performance of Quarter Horses.

#### **Accomplishments / Results:**

The equine genome map is no longer the primary limiting factor in the success of simple single gene disease mapping projects in horses. Rather, it is the access to pedigrees and samples from affected horses, as well as the accuracy of the diagnosis, that limits progress in defining the disease-causing genes. We now have over 150 muscle tissue samples from horses that are moderately to severely affected with PSSM that have been diagnosed in Dr. Valberg’s equine neuromuscular disease diagnostic laboratory. These samples are stored in an ultra-low temperature freezer for future use. 120 of the samples are from Quarter Horses and 24 samples are from Paints or Quarter Horse crosses. We have isolated DNA from blood samples of 18 of the most severely affected Quarter Horses and plan to isolate DNA from the frozen muscle biopsies of an additional 24 – 36 severely affected horses.

What is desperately needed for genetic linkage analysis to succeed is the ability to track and obtain samples from their relatives; sibs, half-sibs, parents and beyond. At this stage of the project we are performing an association analysis between the allelic composition of markers from affected horses versus a control population. The hypothesis is that the closer a DNA marker is to the PSSM gene on its chromosome, the more likely the alleles of that marker are to being the same in all severely affected



horses. This is because genetic recombination between loci on a chromosome at meiosis is a function of the distance between the loci. The closer the marker to the PSSM gene the more likely it will be to still have the founder's allele. Thus, all affected horses should share at least one and possibly both alleles of markers very near the PSSM gene, while in random control horses there will not be this skewed distribution of alleles of markers close to the normal allele of the PSSM gene.

The microsatellite markers selected for this first phase of the work are the first generation genome scanning panel developed by the International Equine Genome

Mapping Workshop and distributed to us through the auspices of the USDA and the Equine Workshop. This set contains 101 markers selected from the 250 known at the time. All but one of the 31 equine chromosomes had at least one marker, with the largest chromosome, chromosome 1 having the most markers (8), but with several of the smallest chromosomes having only one marker. These markers were judged to be the most informative for such analyses since they had the most alleles demonstrated across all breeds of horses.

Genotypes to determine allelic profiles were determined by incorporating radioactive nucleotide into the PCR reaction with the microsatellite marker-specific PCR primers, separating the different-sized DNA fragments representing the different alleles by electrophoresis, and visualizing them with autoradiography on X-ray film. We have now analyzed the allelic profile of 12 PSSM horses across the 101 microsatellite marker screening panel. The DNA isolated from all the horses was of high quality as judged by the reproducibility of the PCR amplifications. All but one of the markers did not work well.

The number of alleles of the markers in the PSSM population ranged from 1 to 9, with an average number of alleles of 4.7. Thus, these are highly polymorphic markers and demonstrate excellent utility in this work. The 100 markers that could be genotyped were divided into three groups: those which appeared to have only one allele in the PSSM population (2), those that have an allele representing more than 50% of the total (28), and those that had many alleles with no allele greater than 50% of the total (70).

Sixteen of the microsatellite markers that showed the lowest number of alleles in the PSSM population were also analyzed in a 25-horse control population. Here, 12 of these 16 markers were demonstrated to have approximately the same number of alleles as in the PSSM population, and are unlikely to remain as candidate markers.

One of the markers appeared to have only one allele in the PSSM population, but when analyzed on the control population had genotyping problems that confused the result. Three of the markers had preliminary results suggesting a low prevalence of the PSSM population allele in the control population, makes them candidates for further genotyping work in a larger sample population.

At this time we have sampled only a fraction of the DNA markers and many very large segments of chromosomes have not yet been analyzed with a marker. If a suggestive marker identified in this manner remains a candidate following analysis of more PSSM and control horses, it must still be confirmed by a genetic linkage analysis in a pedigree.

#### **Class I MHC allele frequencies in the Appaloosa and Thoroughbred breeds**

M. Rutherford, S. Valberg, H. Kaese

### **Description of the Problem:**

The goal of this work is to lay the foundation for population studies that will investigate a potential genetic link between specific alleles of immune response genes and immune-mediated uveitis in Appaloosas (also known as moon blindness). The gene in question is a class I gene of the major histocompatibility complex that encodes proteins used to present antigens to other immune cells. This locus was targeted initially because there appears to be a link with a specific form of this MHC class I gene in an autoimmune human uveitis. To determine if there is an association in horses, we proposed three specific aims.

### **Study Objectives:**

Prior to being able to genotype normal and affected horses, we developed the molecular tools to be used to perform genotyping experiments. This has involved isolating genomic DNA from horses and designing primers for polymerase chain amplification of the specific MHC class I gene in question. This has involved several trials in order to standardize DNA extraction procedures, quantify the DNA yields, and set the correct temperature profiles to accurately and reproducibly amplify the gene target.

It is possible that the two copies of the gene can be different in sequence, meaning each protein produced might have a slightly different function. This is especially true for MHC molecules where many alleles usually exist and most animals will carry two different versions of the gene. Once we have designed and tested the necessary PCR primers, we will use these reagents to genotype horses in order to determine normal allele frequencies in various breeds. This process involves cloning and sequencing of the gene region. These numbers are necessary in order to determine if animals affected by moon blindness have a significantly higher frequency of one or more MHC class I alleles.

Once we have standardized the necessary tools and determined what normal distributions for various alleles may be in different horse breeds, we will collect blood from horses affected by moon blindness. Genomic DNA will be prepared, and the animals will be genotyped as above. Once we have collected enough unrelated animals (at least 30), we can determine if the presence of a given allele for this gene is associated with a higher incidence of immune-mediated uveitis. If so, it may be possible to select animals that do not carry this detrimental allele.

### **Accomplishments/Results:**

The conclusions drawn from our work to date are that more sequence information for all Class I loci and pseudogenes is absolutely necessary in order to design locus-specific primers. We also note that great similarity exists between Class I loci in horses, even with intronic regions surrounding exon 2, the putative antigen binding site. The goal of this work is to lay the foundation for population studies that will investigate a potential genetic link between specific alleles of immune response genes and immune-mediated uveitis in Appaloosas. The gene in question is a Class I gene of the major histocompatibility complex that encodes proteins used to present antigens to other immune cells. This locus was targeted initially because there appears to be a link with a specific form of this MHC class I gene in an autoimmune human uveitis. Importantly, a recent publication using blood stereotyping suggests a link with Class I genes and uveitis in horses.

### **Class I MHC allele association with recurrent uvetitis**

M. Rutherford, H. Kaese

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### **Description of the Problem:**

The goal of this work is to lay the foundation for population studies that will investigate a potential genetic link between specific alleles of immune response genes and immune-mediated uveitis in Appaloosas (also known as moon blindness). The gene in question is a class I gene of the major histocompatibility complex that encodes proteins used to present antigens to other immune cells. This locus was targeted initially because there appears to be a link with a specific form of this MHC class I gene in an autoimmune human uveitis. Importantly, a recent publication using blood typing suggests a link with the class I genes and uveitis in horses. Thus, we expect to achieve these goals.

### **Study Objectives:**

Prior to being able to genotype normal and affected horses, we are developing the molecular tools to be used to perform genotyping experiments. This has involved isolating genomic DNA from horses and designing primers for polymerase chain amplification of the specific MHC class I gene in question. This has involved several trials in order to standardize DNA extraction procedures, quantify the DNA yields, and set the correct temperature profiles to accurately and reproducibly amplify the gene target. This aim has been accomplished and over 100 DNA samples have been collected from Standardbred and Appaloosas.

It is possible that the two copies of the gene can be different in sequence, meaning each protein produced might have a slightly different function. This is especially true for MHC molecules where many alleles usually exist and most animals will carry two different versions of the gene. We have designed and tested the necessary PCR primers, and we attempted to use these reagents to genotype horses in order to determine normal allele frequencies in various breeds. Cloning of the amplified gene products revealed that the class I genes of the equine MHC are extremely similar in sequence, and thus the primers were not specific to a single gene. We are now attempting to isolate all class I genes from horses in order to obtain exact sequence information that will allow better genotyping primer design. We have obtained bacterial artificial chromosomes for this purpose.



Once we have standardized the necessary tools and determined what normal distributions for various alleles may be in different horse breeds, we will continue to collect blood from horses affected by moon blindness. Genomic DNA will be prepared, and the animals will be genotyped as above. Once we have collected enough unrelated animals (at least 30), we can determine if the presence of a given allele for this gene is associated with a higher incidence of immune-mediated uveitis. If so, it may be possible to select animals that do not carry this detrimental allele. In the meantime, we have obtained PCR primers for amplification of MS markers within the equine MHC. We are currently using automated genotyping to provide genotypes at 5 MS loci across the entire equine MHC (~2.5 kb). Although this will not identify which alleles are associated with uveitis, the genotypes can be used to confirm its linkage with a given MHC locus.

## Muscle Disease

### **Calcium imaging for diagnosis of recurrent exertional rhabdomyolysis in Thoroughbred horses**

E. Gallant, S. Valberg

#### **Description of Problem:**

Recurrent exertional rhabdomyolysis (RER) affects approximately 5% of Thoroughbred horses. Our recent research suggests that RER in most Thoroughbreds is due to an inherited abnormality of intracellular calcium ( $\text{Ca}^{2+}$ ) regulation, which intermittently results in painful muscle contractures. The long term goal of our research is to define the specific calcium regulatory protein that is abnormal in order to develop specific treatments/prevention of RER and better diagnostic tests for this form of tying-up.

#### **Study Objectives:**

To determine the effect of those activators and inhibitors that alter muscle contraction in specific aim 1, on intracellular calcium levels in muscle cell cultures from RER and normal horses using a calcium sensitive indicator.

To determine the myoplasmic  $\text{Ca}^{2+}$  concentration at rest and in response to activators and inhibitors that alter muscle contraction in specific aim 1. Fluorescent (Fura2)  $\text{Ca}^{2+}$  imaging will be used to determine the intracellular  $\text{Ca}^{2+}$  concentrations in myotubes of normal and RER horses in response to varying concentrations of activators (including caffeine) and inhibitors of the  $\text{Ca}^{2+}$  ion channels/exchangers. Fura2 imaging with myotubes is an accurate means of determining the specific effects of the pharmacologic agents on myoplasmic  $\text{Ca}^{2+}$  concentrations. These experiments will allow us to correlate the effects of these agents on muscle contraction with the actual intracellular  $\text{Ca}^{2+}$  concentrations.

#### **Experimental Approach:**

Four Thoroughbred horses with a history of recurrent exertional rhabdomyolysis were donated to the University of Minnesota. Intact intercostal muscle bundles from all of those horses were found to have a lower threshold for contracture in response to caffeine and halothane compared to control horses. Three offspring of 2 of those mares were contracture tested and found to have a lower contracture threshold than control horses. Thus the RER group was comprised of 7 horses. All horses were between 2 and 14 yrs of age when studied. Ten control horses were selected from the University of Minnesota teaching herd. Specific Aim 2) Intracellular Calcium Concentrations: Fluorescent imaging of intracellular  $\text{Ca}^{2+}$  levels was used to determine whether resting intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) is abnormally elevated in RER myotubes, and whether either resting  $[\text{Ca}^{2+}]_i$  or sarcoplasmic reticulum  $\text{Ca}^{2+}$  release is affected abnormally by agents acting on sarcoplasmic reticulum  $\text{Ca}^{2+}$  regulatory systems. The lower threshold for caffeine-induced contracture in RER muscles could be due either to elevated resting  $[\text{Ca}^{2+}]_i$ , to a change in sarcoplasmic reticulum  $\text{Ca}^{2+}$  release with no change in resting  $[\text{Ca}^{2+}]_i$  or a delay in removal of calcium from the myoplasm after stimulation of release. A number of recent studies have used fluorescent indicators to determine  $[\text{Ca}^{2+}]_i$  in myotubes. We determined whether caffeine causes abnormally high basal or transient myoplasmic  $[\text{Ca}^{2+}]_i$  in RER as compared to normal myotubes using the calcium sensitive fluorescent indicator

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**Results:**

We found that there were differences in the calcium levels in RER as compared to normal muscle cells consistent with our earlier observations of abnormal drug sensitivity. When the RER cells were exposed to a drug that increases contraction more in RER than in normal muscles (e.g., caffeine), the calcium concentration was greater in the RER muscles. When a drug that has similar effects on RER and normal muscle force was tested (e.g., 4-chloro-m-cresol), the calcium concentrations were similar. In the absence of any added drugs, the calcium concentrations within the RER and normal cells were similar indicating normal calcium in the resting cell. This information is important step in understanding the defect in RER muscles and should be helpful as we seek to identify the defective gene. However, the differences that we observed were small and not likely to be useful as the basis for a diagnostic test.

*Our recent research suggests that RER in most Thoroughbreds is due to an inherited abnormality of intracellular calcium (Ca<sup>2+</sup>) regulation, which intermittently results in painful muscle contractures.*



### **Corticosteroids as adjunct treatment for polysaccharide storage**

S. Valberg , A. Firshman

#### **Description of the Problem:**

Polysaccharide Storage Myopathy (PSSM) is a devastating form of hereditary exertional rhabdomyolysis. It commonly presents as “tying-up” in adults but is also responsible for severe rhabdomyolysis and death in young Quarter Horses. PSSM can be classified as a glycogenosis based on the accumulation of muscle glycogen, glucose-6-phosphate and abnormal polysaccharide inclusions in skeletal muscle. Horses with PSSM have functional glyco(genol)ytic enzymes, but exhibit increased synthesis of glycogen and abnormal polysaccharide. Our research suggests this increased glycogen synthesis is due to enhanced insulin sensitivity, based on IV and oral glucose tolerance testing, hyperinsulinemic euglycemic clamping and insulin tolerance testing. Insulin stimulates the uptake of glucose from the blood into skeletal muscle via translocation of an intracellular pool of glucose transporter proteins called GLUT4 into the cell membrane. An alteration in the insulin signaling cascade could explain the enhancement of the cellular glucose uptake and glycogen synthesis seen in PSSM horses. To date, we have found that PSSM muscle has 1.5 X more insulin receptors than control horses, similar total GLUT4 content and large cytoplasmic deposits of GLUT4 in those fibers that contained abnormal polysaccharide. These findings indicate that there may be upregulated insulin signaling and/or an abnormality in the transport of GLUT4 into and out of the sarcolemma in horses with PSSM. Long-term management of horses with PSSM can be successful by providing a low starch/fat supplemented diet to minimize glucose uptake into muscle cells, combined with a gradual increase and maintenance of daily exercise. Currently, however, there is no therapy that could be used in the short term to treat acute life threatening rhabdomyolysis or to facilitate the return to exercise for the first few weeks after diet changes without inducing rhabdomyolysis. Dexamethasone is often used to manage immune-mediated disorders in horses. A secondary effect of dexamethasone is the induction of insulin resistance by directly inhibiting the translocation of GLUT4 to the plasma membrane in response to insulin. The specific aims of this research are 1) to determine if dexamethasone treatment significantly decreases insulin sensitivity in PSSM horses compared to placebo and 2) to determine if dexamethasone treatment decreases 4-hour post exercise serum CK activity compared to placebo. The results will determine whether dexamethasone is a useful adjunct to the treatment of PSSM through induction of insulin resistance.

#### **Experimental Approach:**

Four adult Quarter Horses (A, B, C, D) with PSSM have been donated to the University of Minnesota. These horses have a history of rhabdomyolysis (based on clinical signs and elevation of muscle enzymes after exercise) and biopsies of the gluteus muscle show abnormal polysaccharide accumulation. Throughout the trial, horses were fed grass hay and a combination of sweet feed and rice bran. We have previously shown that this diet provides moderate elevations in CK activity without painful muscle stiffness.

#### **Accomplishments/Results:**

Cortisol: At 48 hours post treatment with dexamethasone serum cortisol was lower ( $0.38 \pm 0.08$ mg/dl) than placebo ( $4.15 \pm 0.3$ mg/dl) ( $P= 0.0009$ ). At the end of the washout period serum cortisol level was found to be  $3.25 \pm 0.57$ mg/dl indicating sufficient time had been allowed for discontinuation of dexamethasone action.

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Serum CK activity: No significant effect of treatment on 4 hour post exercise CK activity was observed ( $P = 0.959$ ). In addition no effect was found of the day of treatment on serum CK activity. Daily 4-hour post exercise CK activity for all horses combined ranged from 216 to 24060 U/L when treated with dexamethasone and from 222 U/L to 44100 U/L with placebo treatment. Mean serum daily 4-hour post exercise CK activity ( $3,920 \pm 1,110$ U/L) on placebo was not significantly different from dexamethasone treatment ( $3,900 \pm 743$  U/L).

**Benefits to the Equine Industry:**

This study showed that dexamethasone treatment significantly decreased (by an average of 2.5 times) the rate of glucose infusion necessary to maintain euglycemia during the clamp compared to placebo, despite having higher insulin levels, indicating that horses with PSSM develop a marked reduction in insulin sensitivity with dexamethasone treatment. Interestingly, rates of glucose infused in PSSM horses treated with dexamethasone during hyperinsulinemic euglycemic clamping compared closely with rates of control horses without PSSM reported by Annandale et al, 2004 indicating that dexamethasone had altered insulin sensitivity in horses with PSSM to that of normal horses as measured by a clamp.

Regardless of the reduction in insulin sensitivity, no beneficial effect of dexamethasone treatment was found on post-exercise CK activity or muscle glycogen content after three weeks of treatment compared to placebo. Thus, the results of this study indicate that although dexamethasone treatment can significantly reduce insulin sensitivity in PSSM horses over a 3-week period it has no impact on reducing exercise induced rhabdomyolysis or decreasing muscle glycogen concentrations in PSSM horses. Thus, for the duration of this study, enhanced insulin stimulated glucose uptake did not appear to be the primary factor responsible for increased glycogen synthesis and exertional rhabdomyolysis in PSSM horses.

**The glycemic and hormonal response to diets varying in starch availability and fat content in unfit and thoroughbred horses with recurrent exertional rhabdomyolysis (01-02)**

Stephanie Valberg



### Description of Problem:

Recurrent exertional rhabdomyolysis (RER), the most common muscle disease in Thoroughbred racehorses, is characterized by intermittent episodes of muscle pain and high post-exercise serum creatine kinase (CK) activity. RER appears to be an inherited abnormality in the regulation of muscle contraction that is similar but not identical to malignant hyperthermia. Factors that trigger episodes of rhabdomyolysis in susceptible horses include halothane anesthesia, a nervous temperament, excitement, and diet composition. A focus of our research has been finding the best diet to minimize rhabdomyolysis in susceptible horses. In nutritional trials using our herd of RER horses, we found that a high caloric intake where >40% of energy is supplied by starch produces high post-exercise serum CK activity (mean >2700 U/L). The form of starch also appears to influence post-exercise serum CK activity with a 3.6 fold higher CK in horses consuming a processed pellet of ground corn and oats compared to sweet feed. Dramatically decreasing the starch content and increasing the fat content of the diet results in an 8 fold lower post-exercise serum CK activity. Interestingly, the decrease in muscle damage with a high fat diet was not associated with any measurable changes in muscle metabolism. We hypothesize that one beneficial effect of replacing soluble starches with fat or more complex starch in the diet of RER horses is a calming behavior due to lower blood glucose insulin and cortisol concentrations. Persistent hyperglycemia and hormonal alterations caused by processed starch diets may increase excitability of RER horses and increase the likelihood of triggering rhabdomyolysis. Furthermore, we hypothesize that state of fitness will affect the character of the glucose, insulin and cortisol responses to isocaloric diets varying in fat and starch availability.

### Experimental Approach:

**Diets:** Three isocaloric diets were tested. Diet 1: High starch - 0.5% bodyweight of standard sweet feed mixture, Diet 2: High processed starch - 0.5% bodyweight in pelleted concentrate where the starch content is composed of ground corn, oats and wheat middlings, and Diet 3: High fat - 0.5% bodyweight in a pelleted concentrate high in fat (Re-Leve).

**Treadmill training:** Four horses were trained for 6 weeks on the treadmill for 30 min a day performing repeated intervals of walk, trot and canter. Horses were then fed one of three isocaloric rations in randomized order for 5 days. Horses were exercised for the first 3 days of this accommodation period. The glycemic index was performed after a 12 hour fast and then horses were switched to the next ration for 5 days. The procedure was repeated until all 4 horses had been tested on the three diets.

**Detraining:** The horses were rested for 3 months (n=2) and 6 months (n=2) and then the diet trial was repeated.

**Glycemic index:** Jugular venous catheters were placed the day prior to the experiment. Following a 12 h fast horses were fed ½ of their total daily meal of concentrate. Blood samples were drawn before feeding and at 15, 30, 60, 120, 180, 240, 300, 360 and 420 min after feeding. Blood glucose was measured immediately using a Precision QID glucometer. Plasma samples were centrifuged immediately and frozen at -80 C until insulin and cortisol could be measured by RIA.



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**Accomplishments / Results:**

The Re-Leve diet resulted in lower glucose and insulin after feeding compared to the sweet feed diets regardless of fitness. The glycemic or insulinemic responses to the sweet feed compared to the pelleted sweet feed diet were similar. The insulin, insulin:glucose (I:G) ratios and [cortisol] were much higher in fit vs unfit horses after consuming sweet feed diets. The magnitude of the difference in insulin responses between fit vs unfit horses was less for the Re-Leve diet.

*A high fat, low starch diet in RER horses results in significantly lower insulin and blood glucose responses to a meal and significantly less muscle damage during treadmill exercise compared to a low fat high starch diet.*



### **Starch Intake and excitability in recurrent exertional rhabdomyolysis**

S. Valberg and P. Mertens

#### **Description of the Problem:**

Recurrent exertional rhabdomyolysis (RER) is a common, heritable disorder of intracellular calcium regulation in Thoroughbred in which muscle damage may be triggered by excitability as well as by high calorie, high starch diets. Diet is known to affect reactive behavior in many species by modulating uptake of dietary neurotransmitter precursors as well as lipids that become incorporated into neuronal membranes in the brain. We seek to find a means to feed RER horses the high calorie diet they require for peak performance without producing muscle damage. We hypothesize that a high calorie-high starch diet, compared to a high calorie-fat enriched diet will produce high post-exercise serum CK activity in RER horses and will produce greater behavioral reactivity. Furthermore, we hypothesize that the detrimental effect of high starch on skeletal muscle is due to the hormonal or metabolic effects of increased reactivity to the environment and not due to an effect on muscle glycogen or lactate concentrations in RER horses. A 2x2 latin square design using a 29 MCal high grain diet and a 29 MCal high fat/low grain diet will be fed for 3 weeks per block with horses performing 30 min of exercise 5 days/week.

#### **Study Objectives:**

To determine 4 h post-exercise serum CK activity in fit RER horses consuming, a 29 MCal sweet feed diet and a 29 MCal high fat diet. To determine the reactivity of RER horses to their normal environment while consuming a 29 MCal high starch diet and a 29 MCal high fat diet. To determine the glycemic and insulin response to the 2 diets and their effect on daily blood glucose, insulin, amino acids, free fatty acid and cortisol concentrations.

#### **Experimental Approach:**

4 RER mares that had positive intercostal muscle biopsy contracture tests in response to caffeine and clinical episodes of RER were used in this study.

Diets: Throughout the trial all horses were fed 1.2% of their body weight in grass hay that was obtained from one University of Minnesota pasture. The high grain diet provided 29 MCal of energy (40% DE starch, 5% DE as fat) per day/500 kg horse from hay and additional sweet feed (approximately 10 lbs/day). The sweet feed was manufactured specifically for the trial by Hallway feeds and contained 45% ground corn, 32% wheat middlings, 45% oats, 10% molasses. The high fat diet provided 29 MCal of energy (7% DE starch and 20 % DE as fat) per day/500 kg horse from hay and a textured feed called ReLeve made by Hallway Feeds (approximately 10 lbs/day). ReLeve is 13% fat by weight and composed of beet pulp, soy hulls, rice bran, corn oil and vitamins and minerals.

Experimental Design: Horses were trained on the treadmill for 3 weeks before the study. A replicated 2 x2 latin square design using 4 horses and 2 diets high grain and high fat, with a one week wash out period between diets and a 3 week block per diet was used. During the wash out period horses were gradually switched from the proceeding diet to the next diet in order to avoid colic.

Exercise regime: Horses will be exercised at the same time of day each day for 30 min on a flat treadmill. The exercise will consist of 2 minutes of walk, followed by repeating 2 minute intervals of walk (1.9 m/s), trot (4 m/s) and canter (8 m/s). During the wash out period horses will be exercised for 15 min every other day. Blood samples will be obtained 4 h after exercise to determine serum CK activity, and blood glucose concentrations. Cortisol was measured twice each week 4 h post-exercise.

Glycemic and insulinemic response: In the final week on each diet, the blood glucose and insulin response were measured before, after consumption of a meal (time to total consumption measured), and at 15, 30, 60, 120, 180, 240, 300 and 360 min following finishing the meal. Horses were fasted for 12 h prior to the glycemic tests.

Reactivity Testing: Once a week a reactivity test was performed on each horse at the same time of day. A galloping boot was placed on the horse and a pedometer attached to record total spontaneous activity over a 4 h period. A heart rate monitor was also worn during this period to record mean heart rate over 4 h and the mean of 4 highest heart rates recorded during this time. A PCV was obtained from a blood sample of the facial sinus during the test. A reactivity index was obtained from a scoring system. Reactivity tests were video taped and scored for the magnitude of response to specific startle stimuli performed once per hour. The test consisted of 1) the reaction to obtaining a blood sample from facial sinus during hour 1. 2) the reaction to a startle test during hour two when a ball was thrown into the stall. 3) the reaction to opening an umbrella during hour 3. The magnitude of startle response was determined by 2 blinded observers from video tapes and the response was graded from 0 (no response) to 4 (maximum response). An average response to all 3 stimuli was determined for each horse. The heart rate monitor recorded mean, minimum and maximum heart rates for each hour and the pedometer attached to a galloping boot measured spontaneous activity.

### Accomplishments/Results:

Glycemic and insulinemic response: Figure 1.

Serum CK responses: Horses on the high starch diet had a significantly greater serum CK response to exercise than horses fed the low starch, high fat diet.

Reactivity Testing: Reactivity test scores showed large inter-individual variation. In general, the individual reactivity scores reflected the subjective assessment of the horse's temperaments with high scores were found in the most nervous horses. There was however, no measurable effect of diet on the reactivity score identified. In addition, no measurable effect of diet on heart rate, steps taken during the reactivity test (fidgeting), blood glucose or PCV during the reactivity test was identified.

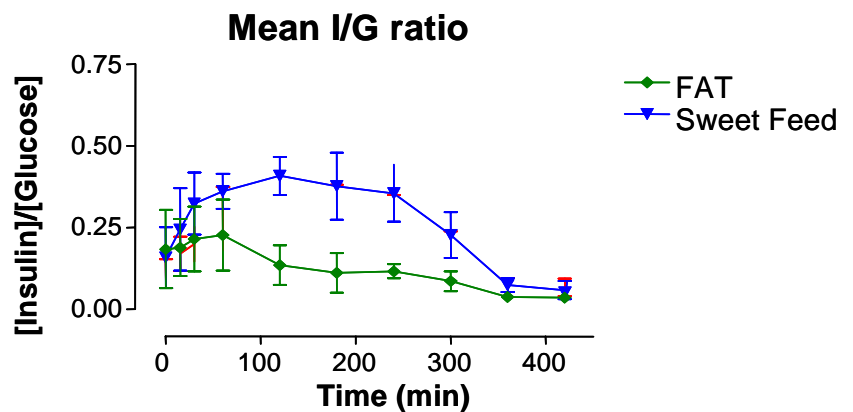


Figure 1. The mean  $\pm$  se for the insulin/glucose ratio in fit RER horses after consuming a high starch (Sweet feed), and low starch, high fat (FAT) diet. Note that horses on a high starch diet

### Benefits to the Equine Industry:

A high fat, low starch diet in RER horses results in significantly lower insulin and blood glucose responses to a meal and significantly less muscle damage during treadmill exercise compared to a low fat high starch diet.

The reactivity test used in this study was not able to detect an effect of diet on the reactivity of horses to stimuli in their environment. More sensitive testing, such as computer analysis of behavior over longer time periods, may be necessary to accurately assess behavior.

## Lameness

### **Detailed ultrasonographic mapping of the normal and diseased equine pelvis**

A. Sage, T. Turner, J. Tomlinson, and D. Feeney

#### **Study Objectives:**

To devise a technique to map the normal equine pelvis using ultrasonography, validated by computed tomography, magnetic resonance imaging and frozen cadaver slices in sagittal and cross sectional planes. To provide a reference range of measurements for each structure imaged and so apply this knowledge to the imaging of abnormalities in the equine pelvis. The technique and measurements determined for normal horses will be applied to clinical cases of fractures, hematomas, subluxations and ligament damage. Measurements of normal horses can be used to recognize displacements of bone surfaces relative to each other and ligament enlargement or disruption due to damage or inflammation.

#### **Accomplishments / Results:**

Phase 1 has been completed; the frozen sections also verified ultrasonographic measurements ( $r^2 0.99$ ,  $p < 0.05$ ). So far 36 clinical cases with lameness of the upper hind limb have been examined. Ultrasonography has been pivotal in reaching a diagnosis. Of the 36 cases examined, 16 were diagnosed with dorsal sacroiliac ligament desmitis. Of these 16 cases, 12 had a chronic history of lameness in one hindlimb and 5 of these had asymmetry of the tuber sacrale. One horse had a history of refusing to jump. One horse had previously raced and was back sore all over, the injury was suspected to be old and only contributing to the soreness. The chronic cases had a decrease in ligament size on the affected side when compared with the normal ranges (mean  $\pm$  SE normal,  $1.09 \pm 0.07 \text{cm}^2$ ; mean  $\pm$  SE affected,  $0.66 \pm 0.04 \text{cm}^2$ ;  $p < 0.001$ ). Long term follow up (2 – 11 months) was achieved in 3 chronic cases, little change in the sonographic appearance occurred. Three of the 16 cases had an acute history of lameness, enlargement of the dorsal sacroiliac ligament was seen on the affected side. Hypoechoic areas were noted in the affected ligament in two cases. The remaining case was associated with a chip fracture of the tuber sacrale. Reexamination of 1 case 4 months later revealed the hypoechoic area was no longer present, at 9 months the ligament had reduced to the normal size of the left. The ligament size in these acute cases fell outside the normal range but the numbers were too small for statistical analysis. Four cases were diagnosed with sacroiliac subluxation, one case showed no other abnormalities, 1 was associated with a sacral fracture. The remaining cases also had dorsal sacroiliac ligament desmitis.

A total of 15 fractures were diagnosed. The ultrasonographic appearance was characterized by discontinuity of the linear echo produced by the bone surface. In cases of comminution, multiple small echogenic foci were seen. Six horses were diagnosed with fractures of the pelvic body. Three of the cases were managed with stall rest; each had improved at a 4-month recheck the fractures were confirmed with radiographs or scintigraphy in all but one case and were complete but minimally displaced. The remaining 3 were euthanized due to extensive involvement of bone. Findings were confirmed at necropsy. Two sacral fractures were diagnosed; one associated with sacroiliac subluxation and was comminuted. This was evident as disruption of the normal architecture; the sacral spine could not be visualized on midline due to the dorsomedial luxation of the tuber sacrale. The horse was euthanized, and the findings confirmed at necropsy. The remaining sacral fracture was chronic and appeared healed and

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was associated with chronic dorsal sacroiliac desmitis. Six cases were diagnosed with fractures of the tuber coxae. 2 were acute and associated with a hematoma, 1 had a sequestrum removed surgically. The remaining 3 had a more chronic history of lameness. Each case improved with rest. One horse had a fractured capital physis, the horse was euthanized. Four horses had disease of the coxofemoral joint. Two had septic arthritis that was confirmed with arthrocentesis. The remaining 2 had arthritis, the femoral head and acetabulum appeared irregular on sonography. Clinical findings were confirmed with intraarticular anesthesia and in one case radiographs.

### **Preliminary Isolation of Multipotent Bone Marrow-Derived Stem Cells from the Adult Horse**

T. O'Brien, M. Troedsson, and C. Clarkson

The primary goal of this project was to test whether the procedures used to develop multipotent stem cell lines from bone marrow of adult humans and mice would also work for the horse. These multipotent stem cells are able, with appropriate growth conditions, to form virtually any tissue type including brain cells, liver cells, and connective tissue cells such as chondrocytes, which form cartilage. To accomplish this goal we obtained samples of bone marrow from two adult horses immediately after they had been euthanized (for reasons unrelated to this project). Red blood cells were separated from the samples and eliminated and the remaining bone marrow cells were placed in culture dishes with cell culture medium specifically designed from growing stem cells from marrow. Most adult cell types when placed in cell culture, including the conditions that we were using, will grow only for a short while, usually no more than 1 or 2 weeks, and generally will only undergo a few cell divisions or may not divide at all. In contrast, the human and mouse stem cells will typically persist in cell culture and will expand up to and often beyond 60 population doublings. We were able to grow the cell lines from these horses up to nearly 60 population doublings which is good preliminary evidence that we did indeed establish stem cell lines. Another feature of the stem cells that goes along with their ability to grow in cell culture for long periods of time is their ability to maintain their chromosomes in a state similar to those of young animals. We were also able to show that our horse cell lines maintained their chromosomal telomere lengths, which is an indicator of "youthful" chromosomes.

To prove that the cell lines are actually multipotent (able to form many tissue types), it is necessary to take some of the cells and direct them to form specific tissues in culture dishes. Because injuries to joint cartilage are such an important health concern in horses, one of the tissues we were most interested in being able to develop in the horse is cartilage. We were indeed able to accomplish this using our horse stem cells. The horse cells under the cartilage-inducing conditions assumed the typical shape and orientation for chondrocytes and produced the extracellular matrix typical of cartilage. In addition we were able to demonstrate both type II and type X collagen in this tissue, which confirms that the cells were making proteins characteristic of cartilage. We are currently developing additional methods to confirm and identify the presence of additional characteristic proteins and appropriate gene expression in the cartilage tissue. This will help to further confirm the identity and proper function of the cartilage cells. The other goal of this project that we have yet to accomplish is to differentiate the equine stem cells into neuroectodermal tissues. However, before we can accomplish this we need to develop methods that allow us to identify equine nerve cells and nerve-supporting cells. Therefore, we are currently developing new tests to locate important "marker" proteins in cells, Western blots to help confirm the identity of proteins, and RT-PCR methods to assess gene expression all aimed at allowing us to identify stem cells that have differentiated into neurons and glial cells.

### **Effects of extracorporeal shock wave therapy on local tissue metabolism (03-04)**

T. Turner

#### **Description of the Problem:**

There is a general assumption that shock waves increase bone remodelling and blood supply. However, there is little documentation that demonstrates this effect on equine bone. The objective of this study was to determine the effects of shock wave therapy, using focus and radial hand-pieces (Swiss Dolor Clast Vet System), on the soft tissues and bone of the metacarpal/metatarsal region in horses as evaluated by thermography, nuclear scintigraphy, and radiology.

Six sound horses with normal metacarpi and metatarsi were used. One metacarpus and metatarsus per horse was random assigned for treatment and the contralateral limb served as the control. The Swiss Dolor Clast® Vet System was used with the radial or focus devices to treat the dorso-middle third of each cannon bone every 2 weeks for a total of 4 treatment cycles. Thermography was performed daily for 11 days beginning 24 h after each treatment cycle. Twelve days post-treatment, bone phase scintigraphy was performed. Scintigraphic images of the metacarpi and metatarsi were obtained 90 min after a dose of 150 mCi 99mTc-HDP IV. Two days after the final scintigraphy, radiographs were repeated. Statistical analysis of the thermographic and scintigraphic data consisted of repeated measures ANOVA with treatment vs. control, and hand-piece applied (focus vs. radial) as factors. Significance was set at  $p < 0.05$ .

There were no significant differences between treated and control limbs based on evaluation by scintigraphy or thermography. There was no significant difference when the scintigraphic and thermographic values or radiographic appearance were compared between focus and radial hand pieces devices. We concluded that no measurable effect on bone remodeling and thermal pattern occurs when shock waves therapy are apply to the equine bone minimally cover by soft tissue as are the metacarpus/metatarsus bones.

ESWT is increasingly used by veterinarians to treat several musculoskeletal conditions in horses, including suspensory ligament desmitis and osteoarthritis of the low motion joints of the tarsus (bone spavin). ESWT is also used to treat dorsal metacarpal disease or bucked shins that occurs commonly during training of Thoroughbred racehorses. Despite the vast amount of information supporting the efficacy of ESWT in clinical trials, there is not a complete understanding of the mechanism of action of this new therapy on equine bone and its surrounding tissues. Drawn from results seen in human medicine and laboratory animal, there is a general belief, that ESWT increases equine bone remodeling. However, to our knowledge, there is no scientific evidence to substantiate a substantial increase in bone remodeling in horses undergoing shockwave therapy.

Thermography and scintigraphy have been shown to be excellent measures of local tissue temperature (associated with metabolic rate), and bone remodeling, respectively. Thermography is a noninvasive, non-contacting technique that measures infrared emissions. The level of infrared emissions correlates directly with skin temperature. Thus, by measuring infrared emission from the skin, a pictorial representation of skin temperature may be produced. As skin derives its heat from local metabolism and circulation, variation in tissue perfusion and blood flow in superficial vessels will correspond to changes in skin temperature. Thus, changes in skin temperature that are produced by the application of ESWT can be objectively measured.

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Bone scintigraphy is a highly sensitive measure of remodeling in bone tissue. It can detect as little as 10-13 g of radiopharmaceutical in bone, whereas, changes measured in grams must occur before a sign of remodeling can be detected using radiography.

### **Study Objectives:**

The objective of this study was to determine the metabolic effects of ESWT on the soft tissues and bone of the metacarpal/metatarsal region in horses using both radial and focus hand-pieces. To determine the thermal changes caused by ESWT. To determine the thermal changes associated with multiple treatments with ESWT. To determine the scintigraphic changes caused by ESWT. To determine the scintigraphic changes associated with multiple ESWT treatments on the metacarpal/metatarsal bone. To determine the radiographic changes associated with multiple ESWT treatments on the metacarpal/metatarsal bone. To determine any difference between the focus and radial hand-piece devices (Swiss Dolor Clast Vet) as a way to deliver shock wave therapy.

### **Experimental Approach:**

Six adult mares, mean age 6 (range 3 to 8 year), mean weight 480 Kg (range 450 to 550) with radiographically, thermography and scintigraphy normal metacarpus & metatarsus (MC/MT) area were used for this investigation. These horses were resident members of the University's teaching horse herd. The criteria for choosing horses of this age were that the animals are no longer growing; therefore, the likelihood of a physiological increase in bone metabolic rate was diminished. Horses were evaluated before each session for any signs of pain upon palpation of the metacarpal/metatarsal area, and for evidence of lameness at the trot in a straight line.

Prior to beginning ESWT, baseline radiographs and scintigraphy were obtained of each limb. One metacarpus and metatarsus per horse was randomly assigned for treatment and the contralateral limb served as the control. The area selected to have treatment was the middle third dorsal-medial aspect of the metacarpus/metatarsus bone. Treatment protocol was standardized within each individual, such that the treated leg was always the same for each horse during the entire project. The type of device to be use was randomly assigned between forelimbs and hindlimbs. ESWT treatment was performed once every 2 weeks for a total of 4 treatment cycles. Following each ESWT, the horses were stabled for 12 days with 4 hours of daily turn-out. Thermography was performed daily for 11 days beginning 24 h after each treatment cycle. Twelve days after each treatment bone phase scintigraphy was performed. Radiographs were repeated at the completion of the 4th treatment.

The Swiss Dolor Cast® Vet System (EMS Corp. USA, Dallas TX) was used to generate and apply the shock waves. It consisted of a control unit, a medical air compressor and one of two hand-pieces (Radial and Focus\*). The compressor created pneumatic energy that was used to accelerate a projectile inside the hand piece. When the projectile struck the applicator, a shock wave was created that is distributed from the tip of the applicator to the desired area. The radial hand-piece had a convex tip to distributed the waves eccentrically (radially), whereas the focus hand-piece had a concave tip that was suggested to deliver the waves concentrically.

Prior to each ESWT the treated and control areas were clipped and prepared with coupling gel to obtain maximum skin contact and minimize the loss of shock wave energy at the applicator tip/skin interface before each treatment cycle. The patient was sedated with detomidine (0.2 mg/kg) intravenously and placed in a stocks. Each session consisted of 2,000 impulses per treatment at a pressure of 2.5 bars and a frequency of 8 Hz. The coupling gel was removed after treatment.

Radiography: Radiographs of each leg were taken with the MiniXray HF80+, standardized at 70 Kv

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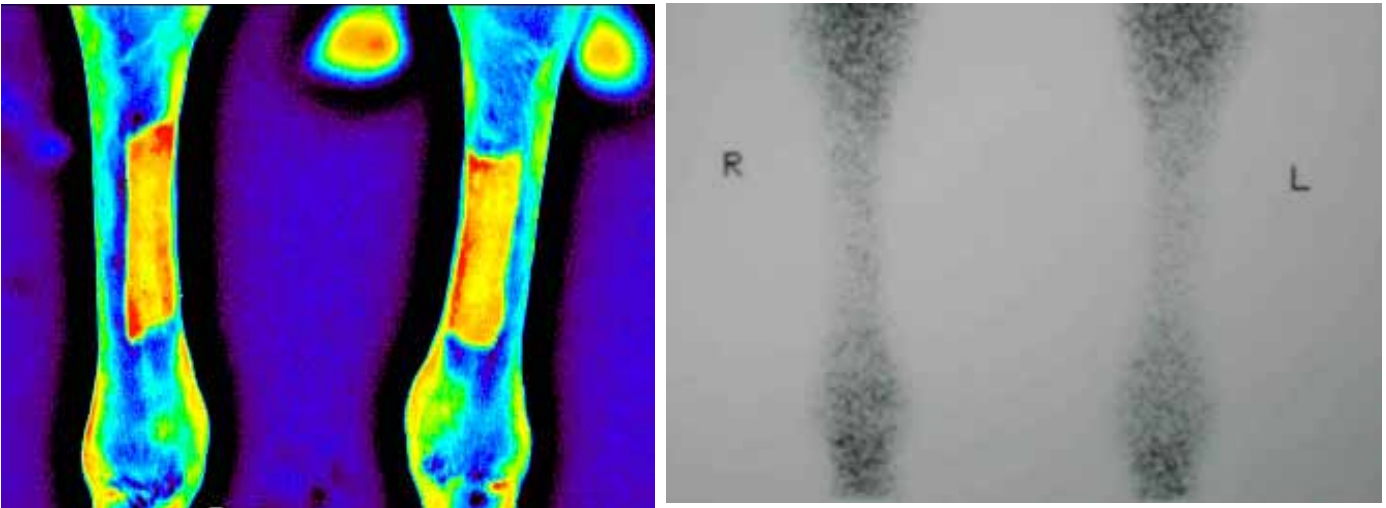
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and .1 sec for each view. Two radiographic projections of each leg (Latero-medial and 45° Dorsolatero-palmaro/plantaro medial view) were obtained before and after the ESWT treatment protocol. A board certified radiologist reviewed the films blinded to whether they were baseline or post-treatment films.

**Thermography:** Thermographic images were obtained using a camera Emerge Vison DTIS 500.

**Scintigraphy:** Bone phase scintigraphic images were taken utilizing 150 millicuries of technetium 99m MDP, administered intravenously 90 minutes before the scan.

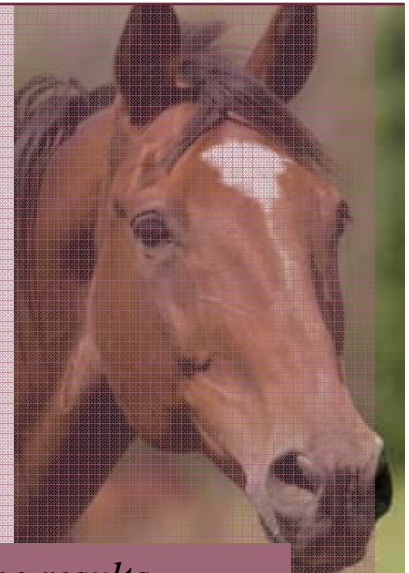
Repeated measures ANOVA was used to determine if there were differences in thermographic and scintigraphic data between treatment and control limbs, as well as between device hand pieces (focus vs. radial). Effect of time and subject were also evaluated. Significance was set at  $p < 0.05$ . Radiographs were visually assessed for density changes caused by the treatment. **Scintigraphy Results.** The average count



density in the treated mid-diaphysis was  $13.73 \text{ counts} \pm 0.48$  compared to  $13.44 \text{ counts} \pm 0.09$  for the control leg ( $p=0.67$ ). The mean ratio between the mid-diaphysis counts and the proximal diaphysis counts for treated limbs was  $0.76 \pm 0.14$ , compared to  $0.74 \pm 0.14$  ( $p=0.43$ ). When the ratio of the mid-diaphysis and proximal diaphysis were evaluated over a period of time, a significant difference ( $p=0.046$ ) was perceived between the baseline and treatment having a mean of 0.72, 0.81, 0.79, 0.77, 0.67, respectively. However, there was not a significant difference between the control and treated leg over a period of time ( $p=0.41$ ). **Radiography:** No radiographic differences were subjectively perceived between baseline and post-treatment radiographs.

**Benefits to the Equine Industry:** Although this study evaluates the effect of ESWT on bone metabolism and the soft tissue blood supply using scintigraphy and thermography and not microscopic evaluation of the bone or soft tissue, the results from this study suggest that extracorporeal shock wave therapy does not have any appreciable effect on bone remodeling or modeling as evaluated by sequential scintigraphy and radiology in adult non-trained horses. Also, no significant increase in temperature, correlated with an increase in blood supply necessary to modify the healing rate was evident. Therefore the successful outcomes described in patients with dorsal metacarpal disease treated with radial extracorporeal shock wave therapy may not be due to an increase in bone remodeling or an increased blood supply that speeds the healing process. However, further studies are necessary to demonstrate if there are not any variations with younger patients in training.

These results suggest that the presence of subperiosteal and endosteal hemorrhage seen acutely after treatment or the small significant increase in microcracks that has been described are not enough to stimulate the remodeling rate of the bone treated with ESWT. Possibly, the differences in our research stem from applying the ESWT on larger areas (10 x5 cm vs. 2 cm area) and utilizing the amount of energy indicated for clinical cases, trying to mimic as much as possible the field conditions that has been described. The results of the study suggests that extracorporeal shock wave therapy does not have any appreciable effect on thermal variation in the short (1 treatment) or long term (a full series of 4 treatments); using focus or radial hand-piece devices; as evaluated by sequential thermography, in adult non-trained horses. The results of the study suggests that extracorporeal shock wave therapy does not have any appreciable effect on bone remodeling in the short (1 treatment) or long term (a full series of 4 treatment); as evaluated by sequential scintigraphy, in adult non-trained horses.



*The results suggest that extracorporeal shock wave therapy does not have any appreciable effect on bone remodeling or modeling as evaluated by sequential scintigraphy and radiology in adult non-trained horses*

Pre-treat



Post-treat



### **Concentration of Nitric Oxide in the uterine secretion from mares susceptible and resistant to chronic post-breeding Endometritis (00-01)**

M. Troedsson, A. Alghamdi

#### **Description of Problem:**

Breeding of mares result in a physiological inflammation that helps to clear excess and defective spermatozoa and other contaminants from the uterus. This is an important mechanism in preparing the uterus for the arrival of the embryo and for maintaining the pregnancy. Normal mares are capable of achieving this goal and usually clear the uterus within 24 – 48 hours after insemination and these mares are classified as resistant. Unfortunately, 10-15% of all brood mares fail to clear the uterus and the inflammation progress to a persistent inflammation with lower fertility as a result. These mares are classified as susceptible to chronic or persistent uterine inflammation. Failure to clear the uteri before embryo arrival results in an incompatible environment for the embryo, which will be destroyed by inflammatory products in the uterus. We have previously shown that the main mechanism involved in clearing fluid and inflammatory products from the uterus is uterine contractions. Uterine contractility is similar for both resistant and susceptible mares in the absence of an inflammation. However, between 7-19 hours after breeding, resistant mares sustain their increased uterine contractions while susceptible mares showed decreased contraction and reached muscle activity well below baseline at 12 hours after breeding. The cause of the impaired uterine muscle activity in the absence of inflammation, and also respond with strong contractions in response to drugs such as oxytocin, a substance or a factor that causes muscle relaxation may be released in increased levels, or accumulated in the uterus in susceptible mares. Prostaglandin F<sub>2α</sub> (PG F<sub>2α</sub>) and nitric oxide (NO) are both inflammatory mediators with contrasting effects on uterine contractions. While PGF<sub>2α</sub> stimulates smooth muscle contractions, NO causes smooth muscle relaxation. Since uterine contractions in resistant and susceptible mares are similar during the first 6 hours after breeding, the decreased activity in susceptible mares between 7-19 hours may be due to evacuate inflammatory products as it is formed due to a horizontal position of the uterus. However, susceptible mares with their pendulous uteri, fail to clear inflammatory products resulting in NO build up, which causes uterine relaxation.

#### **Study Objectives:**

To measure and compare the concentration of nitric oxide in uterine secretion collected at 13 hours after insemination from resistant and susceptible mares.

#### **Experimental Approach:**

Mares were assigned to either a susceptible or a resistant group based on their reproductive history, clinical examination, and a breeding challenge. Mares were inseminated when in heat, and uterine secretion was collected at 13 hours after insemination. All samples were stored frozen at -20C until assayed. Nitric oxide concentration in uterine secretion was measured by the use of a commercially available assay kit (BIOXYTECH, OXIS International Inc. Portland, OR). The total amount of nitric oxide was calculated based on the total volume of uterine secretion at the time of sampling.

Prior to the experiment, the NO assay needed to be validated for (1) NO in uterine secretion, and (2) to determine whether freezing of samples affected NO concentration.

#### **Accomplishments / Results:**

NO was detectable in uterine secretion from all mares. There was no effect of freezing the samples on

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NO concentrations. This agrees with previous reports that nitrate and nitrite are stable in frozen biological fluids.

NO concentrations in susceptible and resistant mares: NO concentrations were significantly higher in uterine secretion from susceptible mares compared to resistant mares at 13 hours after insemination. This supports our hypothesis that increased NO concentrations may be responsible for impaired uterine contractility and failure to clear the uterus from fluid and inflammatory products in susceptible mares.

**Benefits to the Equine Industry:**

This is the first time that NO concentrations have been measured in uterine secretions. Results from this study are encouraging, and we will continue to explore increased NO levels as a key factor in the mechanism of impaired muscle contractions during inflammation in susceptible mares. If NO is responsible for the reduced uterine contractions in susceptible mares, we expect to be able to develop additional therapeutic strategies to prevent nitric oxide production and/or accumulation, which should improve the fertility of susceptible mares.

**Artificial insemination (AI) with fresh, cooled and frozen/thawed semen: Does lubrication of the artificial vagina affect semen quality?** M. Trodeson

Artificial insemination (AI) allows for an efficient and safe use of stallions. Current techniques to freeze semen indefinitely, or to cool semen for short term storage, eliminate the need to transport mares and foals great distances for breeding, and allow breeders to make decisions based on genetic suitability rather than geographic convenience. Pregnancy rates following AI with fresh semen are comparable with natural breeding, but mares bred with frozen/thawed semen have significantly lower pregnancy rates. Variations in sperm motility and viability have been observed between stallions, but also between collections from the same stallion. The reason for these variations is not fully understood. In addition to biological variations between stallions, inconsistent temperature of the collection equipment, or other external factors could affect the quality of the ejaculate. We have found that the osmotic pressure of semen collected by an artificial vagina (AV) generally is higher than what is reported in the literature to be normal for equine semen. We also observed great variations between stallions and between collections from the same stallion. Further investigations revealed that changes in the osmotic pressure may have been caused by contamination of the semen with a water soluble lubricating gel in the AV. Much attention has been directed towards the use of a non-spermicidal, non-irritable, and easily removable lubricant, but the effect of hypertonic water soluble lubricants on the sperm cells has not been investigated before. Since the temperature in the AV is 45-50 C and the pressure of the AV is relatively high, there is a good chance for contamination of the semen by a water soluble lubricant during collection.



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In a series of UMEC-funded experiments carried out by researchers at the University of Minnesota, College of Veterinary Medicine and the Medical School, we found that sperm motility was significantly lower in semen with higher than normal osmotic pressure. We also found that high osmotic pressure in an ejaculate resulted in poor quality of frozen/thawed semen. The increased osmotic pressure in the ejaculates could be traced to the use of water soluble lubricants in the AV, particularly when the lube was used excessively in combination with high pressure in the AV. Further experiments showed that contamination of semen with more than 10% of a water soluble lubricant results in an increase in osmotic pressure by more than 50%. This had a detrimental effect on the quality of fresh, as well as cooled stored, and frozen/thawed semen. It was concluded that the damage of the sperm cells occurred at the time of exposure to the lubricant, which would be at the time of collection under practical conditions.

The portion of lubricating gel in a collected ejaculate is dependent on the pressure of the AV, the amount of lubricant that is used, and the volume of the ejaculate. We have observed osmotic pressure between 450-800 mOsm (normal is 320-349 mOsm) when a water soluble gel is used for semen collection. These observations suggest that a 10-25% contamination of semen may occur under normal clinical conditions. Therefore, we recommend that the use of a water soluble lubricant should be avoided, or be used with precaution when semen is collected by the use of an AV.

### **Binding between equine spermatozoa and polymorph nuclear neutrophils; P-selectin role and the impact on reproductive efficiency. (01 – 02)**

M. Troedsson, D. Foster, and G. Al-Ghamdi

#### **Description of Problem:**

Due to the long estrus period in the mare and the relatively short life span of gametes, insemination needs to be performed close to ovulation, especially for preserved semen. The difficulties in predicting the time of ovulation make the timing for optimal breeding difficult. Therefore, mares are commonly inseminated more than



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once per estrus cycle. With cooled and frozen semen, the second insemination is usually performed within 24 hours after the first insemination. Since all inseminations result in a physiological inflammation of the uterus for up to 36 hours in normal mares, the second insemination will occur at times when the uterus is laden with inflammatory cells (mainly polymorphonuclear neutrophils; PMNs). Our in vitro studies have showed that sperm cells incubated in uterine secretions collected 6 – 24 hours after insemination result in intensive sperm-PMN binding that reduces sperm motility. In addition, good spermatozoa removed from seminal fluid and inseminated in an inflamed uterus, resulted in a low pregnancy rate. Therefore, sperm-PMN binding may be responsible, at least in part, for the lower fertility of preserved semen compared to natural breeding. The mechanism of sperm-PMN binding is not clear and its elucidation may lead to better management and our techniques for equine breeding, especially with preserved semen. The goal of this study was to investigate whether P-selectin, one of the most common adhesion molecules involved is leukocyte mediated binding, is responsible for equine sperm-PMN binding.

### **Experimental Approach:**

**Recombinant P-selectin production:** Because purification of equine P-selectin is costly and because commercial P-selectins are not specific to equines, production of equine-specific P-selectin was necessary. Total mRNA was isolated from endothelial tissue of the mare, and consensus PCR primers derived from other species was used to isolate a cDNA segment of equine P-selectin that was associated with its binding properties. Sequencing and comparison of the putative equine P-selectin to other species confirmed the identity of the cDNA. The cDNA fragment was then engineered into an expression construct, and introduced into bacterial cells for P-selectin production and purification. The recombinant protein was confirmed as the target protein but the use of antibodies against a 6x-His tag incorporated into the recombinant protein. Further confirmation the recombinant protein was equine P-selectin was the fact that it reacted with anti-mouse P-selectin antibodies.

**Anti-equine P-selectin antibody production:** The recombinant equine P-selectin was used to immunize New Zealand rabbits. Rabbit serum was first collected before immunization for a negative control, and all rabbits were then immunized with 0.7 mg of recombinant protein. A month later, rabbits were boosted and immune serum was collected at 3 intervals of 20 days. Immune serum from each rabbit was tested separately for binding to the recombinant protein produced to confirm the specificity and to determine dilution ratios. The highest dilution that resulted in a positive reaction with recombinant protein was 1:5000. One rabbit failed to respond to the immunization and another responded only after the second booster. Immune serum was frozen at -80° C to be used in a sperm-PMN binding assay to determine whether these antibodies would prevent sperm-PMNs binding.

**Sperm and PMN preparation and binding assay:** Blood was collected from a healthy mare and PMNs were isolated and responded to 14 million per mL. Semen was collected from a normal stallion and sperm cells were suspended in semen extender at a concentration of 50 million per mL and divided to 5 aliquots to be treated as follows: sperm preparation was incubated with semen extender alone; sperm preparation was incubated with pre-immune serum; sperm preparation were incubated with immune serum from the three responding rabbits, respectively. Incubation was at 37C for 30 minutes and the sperm preparations were then extended 1:1 with PMN preparations and incubated for another 30 minutes. Binding was measured as the percentage of PMNs that bound to at least 1 sperm cell. All treatments resulted in a similar percentage of PMN bound to sperm cells.

**Detection of P-selectin on equine spermatozoa:** Because sperm-PMN binding was not prevented by our antibodies, antibody specific to equine P-selectin was tested on platelets and spermatozoa. In addition,



since it has been speculated that acrosome-reacted sperm cells express P-selectin, we attempted to induce acrosome reaction by: intrauterine insemination and recovery; freezing and thawing; and calcium ionophore. Once these treatments to induce the acrosome reaction were completed, a fresh sperm preparation was added as an acrosome-intact sample. All sperm samples as well as platelet sample were treated with the recombinant P-selectin antibodies. Only on platelets did the antibodies reacted with a protein of the expected size (~140 kDa). On sperm cells treated to induce acrosome reaction, the antibodies reacted with a protein of about 32 kDa. Fresh spermatozoa did not react with the antibodies.

**Accomplishments / Results:**

The results strongly suggest that P-selectin is not responsible for equine sperm binding to PMN. The fact that the portion of P-selectin responsible for binding is made of two common domains (epidermal growth factor and lectin), may be responsible for the cross-reaction of our antibody against a smaller protein on the acrosome reacted sperm cells. However, other investigators have demonstrated that P-selectin is present on acrosome reacted boar

sperm cells. Although our study was not designed to determine if equine sperm cells contain P-selectin, the finding that anti P-selectin antibody reacts with a protein on acrosome reacted spermatozoa may suggest the presence of either P-selectin or some other protein(s) that contain epidermal growth factor and/or selectin.

Our results from other studies that seminal plasma prevents sperm-PMN binding may lead to the identification of the protein(s) responsible for this binding. Understanding of the mechanism(s) of sperm-PMN binding may result in better management and/or technique to improve equine fertility especially with preserved spermatozoa. In conclusion, we believe that equine sperm-PMNs binding is not mediated by P-selectin.

**Characterization of Crisp-1 and SP22 in stallion sperm: correlation of specific sperm function marker molecules to fertility status in stallions (01 – 02)**

K. Roberts and M. Troedsson

**Description of the Problem:**

Reproductive success in large animals such as horses is difficult to predict and the causes of fertility problems in these species are equally as difficult to diagnose. A tremendous amount of time and money is invested in breeding of horses and reproductive problems serve to increase this cost. Since the majority of equine breed registers in the US have approved the use of artificial insemination (AI), we need to be able to predict fertility of stallions used for natural breeding and for various forms of semen intended for AI. Currently, stallions are evaluated for fertility using a breeding soundness examination (BSE) protocol that was established almost 20 years ago. While this protocol has been clinically useful to estimate a stallion's potential as a breeder, there are numerous examples showing that the test is imprecise at best. The BSE

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protocol is based on the number of spermatozoa in an ejaculate, their motility, and their morphological characteristics. This assay was established to evaluate fertility in stallions used for natural breeding. Since the majority of breed registers in the US register foals conceived by artificial insemination (AI), an accurate prediction of fertility of semen using various forms of this breeding technique is also needed. Pregnancy rates following AI with frozen/thawed semen are generally lower compared to AI with fresh semen from the same stallion. The cause of the lower fertility with frozen/thawed semen is not fully understood, but is undoubtedly the result of damage to the sperm that occurs during cooling and thawing. There is currently no reliable method to assess the fertility of frozen and thawed sperm. Progressive motility is most often used clinically for frozen/thawed equine sperm, but there is only a low correlation between post-thaw motility and fertility. AI with cooled stored semen is also a common procedure, since it allows shipping of semen to different parts of the country with less damage to the sperm than that caused by cryopreservation. Fertility of the cooled semen from most stallions is acceptable within 24 – 48 hours after collection. However, we have observed that in some cases, the semen quality is below the acceptable level after only 24 hours of cooled storage. Thus, it is important to be able to predict low fertility of cooled stored semen.

The availability of assays for fertility that do not depend on breeding outcomes and that can be used in advance of breeding or assisted reproductive procedures would greatly benefit the equine breeding industry. With the goal of ultimately establishing an assay to determine the fertility potential of equine sperm, we have undertaken to correlate the amount and localization of sperm proteins to the fertility of sperm. We hypothesize that fertility of stallion semen is associated with specific sperm function marker molecules.

### **Experimental Approach:**

For our initial studies we chose to investigate the sperm protein SP22. SP22 is a protein of 22 kilodaltons molecular weight found on the surface of sperm from many species of animal. The function of this protein is unknown, but in rats the amount of SP22 on sperm correlates to the fertility of the animal. This appears to be true for bovine sperm as well. To date, no studies of SP22 relative to fertility have been carried out in the equine species. To determine if SP22 might serve as a marker protein for equine sperm function, we set out to characterize the localization of this protein on sperm. The localization of SP22 on horse sperm that have been fixed using a protein cross-linking reagent and stained using an immunofluorescent anti-SP22 antibody staining technique. The predominant staining pattern on normal horse sperm prepared in this fashion is overlying the acrosome (upper half of the head) and in the equatorial band region the sperm head with light staining on the upper part of the sperm tail. This staining is characteristic of normal, undamaged, fertile sperm and almost 70% of the sperm stain in this pattern. A minority of the sperm in such a sample stains just over the acrosome (13%), just over the equatorial region (13%), or on the neck (5%). These sperm likely represent damaged or malformed sperm in the sample.

Since the fertility of cryopreserved sperm decreased relative to freshly prepared sperm, we choose to evaluate the staining of SP22 these sperm to determine if the decrease in sperm function due to freezing and thawing processes is reflected by changes in the staining pattern for SP22. The sperm were frozen and thawed according to clinically accepted protocols. After thawing the



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sperm were fixed and immuno-stained for SP22. The most common staining for SP22 observed in frozen/thawed sperm prepared in this manner, which was over the equatorial region (47%). Only 15% of the sperm in this sample stained over both the acrosome and the equatorial region. The pattern most common in freshly prepared sperm. A substantial portion (18%) showed a completely different staining pattern with staining scattered over the head. Although the decreased fertility of the frozen/thawed sperm used in this experiment has not been tested, it is likely that this change in staining reflects a loss of fertility in these sperm.

### **Accomplishments / Results:**

Very little is currently known about fertility diagnosis in the stallion. Once decreased fertility is detected, the exact cause of the problem is often left unknown. Consequently, there are few scientifically based therapeutic recommendations to offer once a stallion has been determined to have a fertility problem. Advances in our understanding of basic semen biology in stallions is necessary to overcome this deficit in our knowledge. It is our goal to establish molecular markers of sperm function and fertility in the equine species that will advance our knowledge of semen biology and advance our ability to detect and treat infertility in stallions. We hope that the results from this study will contribute to the development of improved fertility evaluations of individual stallions, and semen used for different forms of assisted reproductive techniques.



## **Assessment of endotoxin-induced alterations in jejunal activity in ponies (00-01)**

Erin Malone

### **Description of the Problem:**

Endotoxemia remains one of the leading causes of death in horses due in part to its intimate involvement in colic and diarrhea. Endotoxins are present in the wall of certain bacteria and enter the bloodstream when the gut is damaged. Endotoxemia is believed to be one of the main factors associated with the development of “reflux” (the back-up of fluid in the stomach) due to ineffective gut motility or ileus. Ileus is most likely to occur following small intestinal (SI) surgery and with a syndrome of small intestinal inflammation known as proximal enteritis. Persistent ileus decreases the prognosis for these types of colic. Endotoxin has been detected in the blood of horses that required SI surgery and in horses with proximal enteritis. Experimentally, endotoxin has been shown to alter the function of the equine stomach, SI and cecum. The aim of this study was to evaluate changes that occur in the mechanisms regulating SI motility following endotoxemia in the pony.

Movement of food through the SI is controlled by a balance of excitatory and inhibitory neurotransmitters. These agents are released from nerves within the gut and act on other nerves or on the gut muscle to regulate intestinal activity. Four neurotransmitters have been found to function within the equine SI. Norepinephrine (adrenaline, NE) primarily inhibits motility. Acetylcholine (Ach) primarily stimulates motility. Nitric oxide (NO) and substance P (SP) can either inhibit or stimulate motility depending on the situation. Normal motility requires a delicate balance between these neurotransmitters. Either excessive inhibition or excessive stimulation of bowel may occur if this balance is upset. The stress of colic may alter NE levels and both NE and NO levels are known to be elevated in endotoxemia.

### **Study Objectives:**

To evaluate changes that occurs in the mechanisms regulating SI motility following endotoxemia in the pony.

### **Experimental Approach:**

The effects of endotoxin were evaluated in two ways. Eight ponies undergoing nonsurvival abdominal surgery as part of the student surgery laboratory were used. After the ponies were anesthetized, a section of SI was taken as a control. The ponies were then given a low dose of endotoxin intravenously and a second section of SI was taken one hour later. The level of the neurotransmitters was examined using microscopic studies and special stains for all eight ponies. Additionally, for six ponies, muscle strips from the same SI sections were compared to determine if there is a functional difference in the way the gut responds in general as well as to NE and NO. Further studies were performed using different drug combinations to further determine the mechanism of action of these compounds on the SI.

### **Accomplishments / Results:**

The muscle strips responded fairly normally in terms of regular activity with the exception of decreased maximal contraction levels. However, when neurotransmitters were released using electrical stimulation, the muscles exposed to endotoxin reacted differently than did control samples. The alterations were most pronounced in the longitudinal muscle layer. Furthermore, the endotoxin-exposed muscles responded differently to blockage of NO release and to added NO release. However, no differences were detected in the staining results for control or endotoxin-exposed samples.

### **Benefits to the Equine Industry:**

The results confirm that endotoxin can affect intestinal activity and are consistent with previous studies in the live horse. Therefore, we have also helped confirm the validity of muscle bath studies such as these. Furthermore, the results are different from those obtained when samples of muscle were used that had been exposed to intestine that had lost its blood supply. In that study, the muscle seemed to have decreased its intrinsic ability to respond whereas in this study the muscle can function but is altered by differing neurotransmitter release. This would suggest differing approaches should be used for the treatment of the two conditions, despite the fact that the clinical picture is similar. This is consistent with results of treatment of refluxing horses with intravenous lidocaine: horses refluxing due to postoperative ileus (usually due to removal of necrotic intestine) respond better than do horses with proximal enteritis (a primarily endotoxin-mediated disease).

### **Alterations in Jejunal Neurotransmitter Activity Following Adjacent Intestinal Ischemia in the Horse** **Erin Malone**



Despite advances in medical and surgical treatment of colics, the survival rates for horses undergoing small intestinal (SI) surgery remains significantly less than that for horses undergoing large intestinal surgery, due in part to the development of postoperative ileus (POI), a motility disorder preventing movement of food down the intestines. In a recent study, persistent POI was a significant complication in 16% of horses undergoing surgery to remove a portion of the SI. 13% of the horses with POI died. This meant 40% of the deaths which occurred shortly after surgery were attributable to POI.

Since POI is frequently associated with surgery to resect damaged bowel, the objective of this research was to evaluate the effects of damaged bowel on adjacent bowel, in particular the sections of bowel that would be “reattached” once the damaged bowel was removed. If alterations in the more normal bowel were detected, they were to be analyzed to determine what types of changes were occurring in order to better design treatment protocols for these horses.

Ten ponies being euthanized following a teaching surgery laboratory were used. While the ponies were under anesthesia, a section of small intestine was damaged by cutting off its blood supply for 60-90 minutes. A biopsy was taken of the bowel prior to the damage and then sections of bowel proximal and distal to the damaged area removed at the end of the time period. Sections were examined in muscle baths for their activity, response to neuron stimulation, and response to norepinephrine (adrenalin). Because stress is often believed to cause or worsen colic in horses, the type of response to norepinephrine in normal gut was also evaluated. Sections of intestine were also taken for microscopic studies to examine if changes occurred in the neurons which control gut motility.

Even after this short period of damage, changes were detected in the gut motility. The portions of small intestine proximal to the area of damage could not contract as well as normal gut and did not contract as

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frequently. The portions of intestinal distally contracted even less frequently and had problems relaxing. These discrepancies could lead to severe problems in coordinating the movement of food down the intestinal tract past the area of reattached bowel.

The presence of norepinephrine did affect the type of response to neuronal stimulation in normal gut and had a different effect in intestine that had been adjacent to damaged bowel. Two of the neurotransmitters (agents released from neurons) examined appeared to be involved in the response to norepinephrine and could potentially be altered in cases of POI.

The microscopic studies did not indicate any changes in the amount of the neurotransmitters or provide any evidence for changes in function of the neurons. Therefore, the changes in muscle activity most likely reflect either the muscle's inability to contract and/or changes in the muscle's response to the neurotransmitters.

In summary, it appears that both the normal regulatory mechanisms of the bowel and the response to local neurons are altered in bowel that is adjacent to damaged bowel for at least 1 hour. Either or both may be responsible for some of the motility problems observed postoperatively in horses and may provide potential routes of therapy. In this study most changes occurred in the longitudinal muscle layer. Most motility modifiers have been evaluated for their effects on the circular muscle; the effects of many of the available drugs on longitudinal muscle function are unknown and need to be explored further. Finally, increased stress does have the potential to affect gut function in postoperative colics. More attention may need to be paid to minimizing the release of adrenalin in these cases, perhaps by minimizing pain and metabolic demands.

### **Evaluation of gastrointestinal activity patterns in healthy horses using B mode and Doppler ultrasonography** Erin Malone and Eli Hendrickson

Ultrasound is frequently used in horses with colic as a rapid and noninvasive test to determine if the intestines are in the normal position. This helps determine if surgery is required and is particularly useful for examining portions of the intestinal tract that cannot be examined in any other way. Ultrasound may also be useful in differentiating abnormal from normal intestinal activity in horses with colic; however, no studies have been performed to



determine normal activity. It is also difficult to determine if the activity observed via B mode ultrasound (routinely performed) is truly functional (eg indicates that ingesta is moving toward the anus) or is just mixing activity (moving the ingesta back and forth). In people, Doppler ultrasonography is a new technique that uses the same type of ultrasound used to assess blood flow to assess ingesta flow. This technique has not been used in horses to assess intestinal motility but the capability exists. Our goal was to increase the usefulness of ultrasound in horses with colic by collecting data on the intestinal activity of normal horses and to determine if Doppler ultrasonography would be a useful tool in the horse. However, horses with colic are often off feed, sedated, and may have a tube placed into the stomach. These factors have the potential of affecting intestinal activity. Therefore, a healthy unsedated horse on full feed may not serve as a good model for a colicky horse and our model was expanded to include healthy horses under conditions mimicking a colic examination.

For this study we used 13 horses. Transabdominal ultrasound was performed in all horses to evaluate

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activity of the stomach, small intestine, cecum and large intestine. Changes in visibility and contraction rates were analyzed. Doppler ultrasound was used in an attempt to assess small intestinal peristaltic (true movement) activity and allow comparison to B mode activity. Each horse was examined on multiple occasions under colic-type conditions: fed, fasted, fed and sedated, fasted and sedated, and fasted with an indwelling stomach tube.

We found that healthy horses placed under conditions mimicking a colic examination do have changes in their intestinal activity and that Doppler ultrasonography should be further investigated as a potential useful monitor of forward ingesta movement.

In fasted horses the small intestine was more visible and contraction rates of the small intestine, cecum and colon were decreased. In addition, the stomach was located very low in the abdomen. In the past, we have assumed small intestine should never be visible except potentially near the inguinal area. This study found it to be a common finding when horses were off feed. We suspect the small intestine may be more evident due to decreases in the size of other viscera. Similarly, the stomach was likely empty and collapsed, making it difficult to visualize in its normal position. Lack of food in the other organs is also the most likely cause of the decreased activity.

In sedated horses that were also fasted, our results correlate strongly with previous studies that show xylazine decreases intestinal activity. However, this finding was not observed in fed horses. Reviewing the literature, we found most studies evaluated the effects of xylazine only in fasted horses! The few studies that looked at fed horses found minimal changes in activity.

Leaving a stomach tube in place for at least 30 minutes had little effect on intestinal activity but did make the stomach “reappear” at its normal location despite the fact that these horses were also fasted. We suspect there was local irritation of the stomach by the tube, leading to gas or fluid build-up.

When we examined the small intestinal activity using both B mode and Doppler ultrasonography, the results were similar. At this stage we cannot determine which is the better test for assessing true motility but Doppler shows good potential. At the present time, most machines are not capable of performing Doppler ultrasound simultaneously with B mode. As this technology improves, the usefulness of Doppler can be better assessed and it should be easier to perform.

In summary, we found many alterations in healthy horses that have been previously considered abnormal and were able to determine ranges of expected intestinal contraction rates that should be useful for evaluating colics. It will be important to compare findings in colics to the appropriate controls depending upon the feeding and sedation status of the patient.

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## **Role of activated neutrophils in the mortality associated with equine colic (01-02)**

D. Weiss, H. Kaese, O. Evanson

**Study Objective:** Determine if activated neutrophils are present in the blood of horses with colic. Determine if activated neutrophils predict the severity or mortality associated with colic.

### **Experimental Approach:**

Thirty horses with colic and 30 healthy control horses were evaluated. Each horse was evaluated without knowledge of the type or outcome of the colic. To detect activated neutrophils, we analyzed surface CD11/CD18 expression, neutrophils size and granularity, neutrophils degranulation, and neutrophils filterability. Of these test neutrophils filterability and neutrophils size and granularity have provided results that were distinctly different from those of control horses but the magnitude of the difference was not great. Tests for neutrophils degranulation did not result in significant differences between colic horses and control horses.

### **Accomplishments / Results:**

After comparing colic horses to control horses we divided colic horses into subgroups that included gas/impaction colic, strangulating obstruction, and inflammatory bowel disease. Horses with impaction/gas colic had no evidence of activated neutrophils. Horses with inflammatory bowel disease consistently had evidence of circulating activated neutrophils including decreased leukocyte deformability, increased CD11/CD18 expression, increased neutrophils size, and decreased neutrophils granularity. Horses with strangulating colic had variable results. Of 14 horses with strangulating colic, 7 had marked changes in filtration pressures, 5 had increased CD11/CD18 expression, and 6 had changes in neutrophils size. Other horses had slight or no changes. Among the horses with strangulating colic, changes in neutrophils deformability and neutrophils size and granularity correlated with adverse outcome. The correlation of activated neutrophils with mortality in strangulating colic indicates that activated neutrophils are a negative prognostic indicator. Further studies are needed to determine if activated neutrophils contribute directly to the adverse outcome in strangulating colic.

*Among the horses with strangulating colic, changes in neutrophils deformability and neutrophils size and granularity correlated with adverse outcome.*

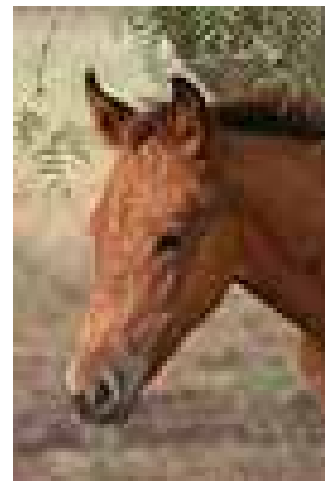


### Optimization of Methods for Diagnosis of Proliferative Enteritis in Horses (01-02)

T. Ames, G. Al-Ghamdi, C. Gebhart, J. Wilson, D. Hayden

#### Description of Problem:

*Lawsonia intracellularis* has been associated with an intestinal disease in horses named Proliferative enteropathy (PE). PE has been reported in various animal species including the pig, dog, blue fox, ferret, guinea pig, hamster, ostrich and rabbit. Young animals are mostly affected. The disease has recently been reported in foals occurring mostly at the time of weaning. Clinical signs may include, depression, elevated body temperature, decreased appetite, dehydration, and tissue swelling of the ventral abdomen. Diarrhea is commonly seen in sick foals, which can be watery and projectile. PE affected horses may have significant loss of body weight or fail to maintain normal body condition. Signs of inflammation such as elevated fibrinogen in the serum and increased white blood cell count may be detected. The most common finding is marked reduction in the blood proteins.



Clinical signs of PE mimic other intestinal diseases of horses, therefore careful examination is necessary to implement the appropriate treatment. *Salmonella* needs to be differentiated based on serial fecal culture. Potomac horse fever caused by *Ehrlichia risticii* is excluded based on serologic testing. Bacterial infection with *Clostridium* spp is ruled out based on culture and detection of toxins in the feces. Finally, infection of the gastrointestinal tract with the bacteria *Rhodococcus equi* may be excluded via examination with electron microscope. Parasitic infection including round worms and tapeworms as well as protozoa such as cryptosporidia are usually differentiated based on fecal flotation. Finally, non-infectious causes of diarrhea such as antibiotic associated diarrhea, toxicosis, and nutritional/management changes, may be ruled out based on thorough evaluation of the history of individual cases.

Diagnosis of PE in horses has been very challenging for veterinary diagnosticians. The causative agent, *L. intracellularis*, lives only inside the cells lining the inside of the small intestine. Routine culturing of this bacteria is not possible on regular culture plated because the bacteria will only grow in culture containing mammalian cells. Clinical signs may be indicative but they are not specific. Laboratory testing of blood cells and serum electrolytes while of value for patient monitoring, is not conclusive for diagnosis. Most of the equine cases reported in the literature were confirmed during necropsy examination after death. In swine, detection of antibody titers against *L. intracellularis* has been evaluated using an immunofluorescence assay. The test is promising and may indicate the status of PE in swineherds. In addition detection of *L. intracellularis* DNA in feces using polymerase chain reaction (PCR) that specifically targets *Lawsonia* genomic DNA have been optimized and the sensitivity of the test has been improved. The data from swineherds would indicate that serologic testing and PCR might be the most appropriate testing to detect PE in live animals.. Such testing has not been developed to us in horses to evaluate their diagnostic potential.

Treatment of PE in horses has to be specific for this organism in order to be effective in eliminating the bacteria. The combination of Erythromycin and rifampin is the treatment of choice at this point. Additional antibiotic treatment options may include chloramphenicol and oxytetracycline. Supportive treatment may be necessary including intravenous fluid for dehydration, plasma transfusion for decreased plasma protein and anti-inflammatory drugs to minimize the signs of inflammation.

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### **Study Objectives:**

To optimize and evaluate ante mortem testing to detect PE in live horses.

**Accomplishments / Results:** During the last five years, researchers have reported diagnosis of PE in horses in various scientific journals. All these cases were in North America (Kentucky, Indiana, Iowa, and Canada). In the majority of the reports; PE was confirmed during postmortem examination. NO ante mortem testing successfully identified the causative agent in these cases. These reports point out the importance of developing and evaluating definitive, accurate, and reliable testing to detect PE in live horses. The overall goal of this study was to optimize and evaluate ante mortem testing to detect PE in live horses. To achieve this goal to main approaches were chosen. First, detection of antibody using serologic testing was performed. Second, a PCR method to detect *L. intracellularis* DNA in fecal samples was optimized. In addition, a retrospective study was initiated to investigate the potential existence of PE among horses admitted to the Veterinary Diagnostic Laboratory.

Serologic testing has been optimized to detect antibody in horse serum that develops specifically against *L. intracellularis*. During the year of 1999 and 2000, 93 serum samples have been tested. Results have shown 52-PE positive horses, which represent approximately 56% of the total examined samples. Antibody titers ranged between just positive to very high. These samples were obtained from Minnesota, Kentucky, Texas, Georgia, Florida, California, Indiana, Alabama, and Canada. This distribution indicates that the disease is widely distributed around the nation. Most of these samples were collected from animals showing signs of depression, diarrhea, weight loss, and low plasma protein.

PCR has been used widely to detect animals shedding pathogens. PCR can be a sensitive and specific tool to diagnose *L. intracellularis* shedding in PE affected horses. The application of this method has required a number of modifications to be successful in horses. During the years of 1999 and 2000, 23 fecal samples obtained from clinical cases were tested. Four-PE positive horses were detected that correspond to approximately 17% of the total examined cases. The low prevalence of PCR positive cases among the sample population might be affected by several factors such as handling and shipping of samples, the amount of shedding of the organism in PE affected horses and the presence of any unknown substances in horse feces that might reduce the sensitivity of the test.

In a retrospective study, an immunohistochemistry test was implemented to determine if PE positive cases were among horses admitted to the Veterinary Diagnostic Laboratory, University of Minnesota. Such testing is based on the use of a specific antibody that reacts only with Lawsonia antigen in tested materials. Three groups of horses were tested. The first group included young horses less than 12 months of age that died as a result of an intestinal disorder. The second group included horses older than 12 months of age that died as a result of an intestinal related disorder. The third group includes horses less than 12 months of age that died with non-intestinal related diseases. Two horses tested PE positive among the first group using this approach. These horses were 6 and 8 months old. No PE positive cases were detected among the other two groups. These findings reinforce the belief that PE is mostly a disease of young animals.

### **Benefits to the Equine Industry:**

Looking at these data, it can be concluded that PE is a serious problem among horse population. Signs of the disease are not specific and may be confused with other intestinal diseases. Usually non-specific treatment is not successful therefore accurate diagnosis of the disease is extremely important to successfully treat affected horses. The diagnostic tests listed above are currently being evaluated under experimental conditions to determine the correlation between diagnostic testing and the clinical

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progression of the disease in horses. The role of management of factors and other possible risk factors that might influence the existence and transmission of the disease are being evaluated in farms with known occurrence of the disease.

### **Epidemiology of Proliferative Enteropathy in Horses (00-01)**

T. Ames, G. Al-Ghamdi, C. Gebhart, J. Wilson, D. Hayden

Proliferative enteropathy (PE) is an enteric disease that affects horses and several other animal species. The disease is caused by *Lawsonia intracellularis*. In horses, individual cases and outbreaks of the disease among horses have been reported. Diagnosis of the disease has been limited to detection of pathologic lesions in intestinal tissue or detection of the organism in tissue after post mortem. Recently, serologic tests and a PCR test have been optimized by researchers at the University of Minnesota. The use of these tests to monitor horses during an outbreak of PE will provide information on the epidemiology of PE in horses. In addition, since PE is a disease that involves several animal species, the use of DNA fingerprinting of the bacteria will determine the relationship between bacterial isolates obtained from multiple animal sources. Such approach may aid in determining the possibility of cross species transmission of the disease.



**Study Objectives:** To study PE during a farm outbreak, morbidity of the diseases during a PE episode, mortality as a result of the disease

### **Accomplishments / Results:**

One goal of this work was to study PE during a farm outbreak. Morbidity of the diseases during a PE episode, and mortality as a result of the disease was estimated. Risk factors related to management practice were evaluated. To address these questions, six horse farms were selected for this study via cooperation with local equine veterinarians. Four farms had a history of PE during the past year that was confirmed via clinical examination and using serology. These farms were assigned as affected in our study. Two farms that had no history of PE during the past two years were chosen as control. Farms were sampled once every three months. Sampling procedure included fecal and serum samples. A survey was submitted to the local veterinarian to obtain detailed information about management practice and health aspects of affected horses on each farm. In the meantime, three additional farms that had foals that experienced an outbreak of PE were included in this study. TO date, we have completed the last sampling period for the farms. We have also obtained the surveys from the veterinarians observing the farms. We are in the process of analyzing the data.

The molecular epidemiology study of the *L. intracellularis* isolates obtained from various animal species has been completed. Six isolates, one obtained from a horse, one from a hamster, and four from pigs were

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tested. The DNA fingerprinting utilizing repetitive element based polymerase chain reaction (rep-PCR) showed that these isolates can be differentiated into three subtypes. These subtypes were not species specific. Using computer software, Molecular Analyst, to analyze the rep-PCR results the horse isolate was found similar to two swine isolates. Two swine isolates were found similar and the hamster isolate was found genetically distinct from all isolates. These findings may aid in determining the role of other animal species in the epidemiology of PE horses if the organism culturing methodology can be modified.

### **Evaluation of Cellular Immune Responses Involved in Resistance to *Rhodococcus equi* Infection (99-00)**

D. Weiss, M. Blauvelt

#### **Description of Problem:**

*Rhodococcus equi* causes a severe, often fatal, pneumonia in foals 1 to 6 month of age. Affected foals usually have a prolonged course of chronic pneumonia, fever, and swollen joints and 40 to 80% of infected foals die. *R. equi* organisms live in soil are present in the gut of most horses. Some farms, which the soil is highly contaminated with organisms have repeated outbreaks of foal pneumonia. However, most farms experience no outbreaks or only sporadic outbreaks of the disease. Recent epidemiological studies indicate that prevalence of infection is directly related to the number of *R. equi* organisms in the soil.



#### **Study Objectives:**

To determine why foals are uniquely susceptible to *R. equi* infection. Evaluate how macrophages respond when *R. equi* organisms are ingested.

#### **Experimental Approach:**

Unlike foals, adult horses are resistant to infection of *R. equi*. The goal of our research is to determine why foals are uniquely susceptible to *R. equi* infection. We expect that this knowledge will lead to development of effective vaccines to prevent this disease. To accomplish our goal, we are comparing the cellular immune responses of foals and horse to *R. equi* infection. In our initial studies, we evaluated the capacity of foal and horse neutrophils and macrophages to kill *R. equi* organisms. When neutrophils were incubated with *R. equi* organisms, the organisms were readily ingested and killed. However, there was no difference in the capacity of foal and horse neutrophils to kill the organisms. When macrophages, from both foals and horses, were incubated with *R. equi* organisms, the organisms were readily ingested but were not killed.

In the second part of this study, we are evaluating how macrophages respond when *R. equi* organisms are ingested. To date, we have shown that horse macrophages produce two antibacterial products, nitric oxide and superoxide, when *R. equi* organisms are ingested. Initial studies indicate that foals produce similar concentrations of nitric oxide and superoxide. We have also initiated a study of the expression of inflammatory cytokines by foal and horse macrophages. Horse macrophage that phagocytize *R. equi*

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express high amounts of tumor necrosis factor, interleukin-8, interleukin-10, and interleukin-12. Foals macrophages have not been tested. Therefore, to date, we have not identified characteristics of foal neutrophils and macrophages that would make them uniquely susceptible to *R. equi* infection.

### **Molecular Characterization of *Streptococcus equi* isolates collected from Horses Experiencing Post-Vaccinal Complications (00-01)**

T. Ames, G. Al-Ghamdi, C. Gebhart

**Description of Problem:** Vaccination against strangles using the intranasal vaccine (Pinnacle™ I.N.) has been associated with several complications. Localized abscesses in the neck of vaccinated horses as well as additional signs of strangles have been reported. *Streptococcus equi* has been isolated from sick horses experiencing these postvaccinal problems. The goal of this study is to utilize DNA fingerprinting to determine whether the organisms isolated from sick horses are the same organism (strain) as that used in the vaccine.

#### **Study Objectives:**

To utilize DNA fingerprinting to determine whether the organisms isolated from sick horses are the same organism (strain) as that used in the vaccine.

#### **Experimental Approach:**


*S. equi* isolates have been obtained from horses developing signs of complications following vaccination with the Pinnacle™ I.N. vaccine. These samples have been collected from horses residing farms in Minnesota, Colorado, Kentucky, Kansas, and Ohio. The bacteria have been grown under the appropriate laboratory conditions and the genomic DNA has been extracted using a DNA extraction kit. DNA fingerprinting has been completed utilizing repetitive element based polymerase chain reaction (rep-PCR) and amplified fragment length polymorphism (AFLP). Currently, the preliminary results have indicated that most of the *S. equi* isolates obtained from sick horses were genetically identical to the vaccine strain. Additional analysis of these data with specialized computer software, Molecular Analyst, has been completed for the AFLP results and in progress for the rep-PCR results. The use of Molecular Analyst to analyze the AFLP data showed more the 96% of similarity between the vaccine strain and all the *S. equi* samples. This finding indicates that the vaccine strain is commonly caused of the post Vaccinal complications in these horses.

# Mammalian Genome

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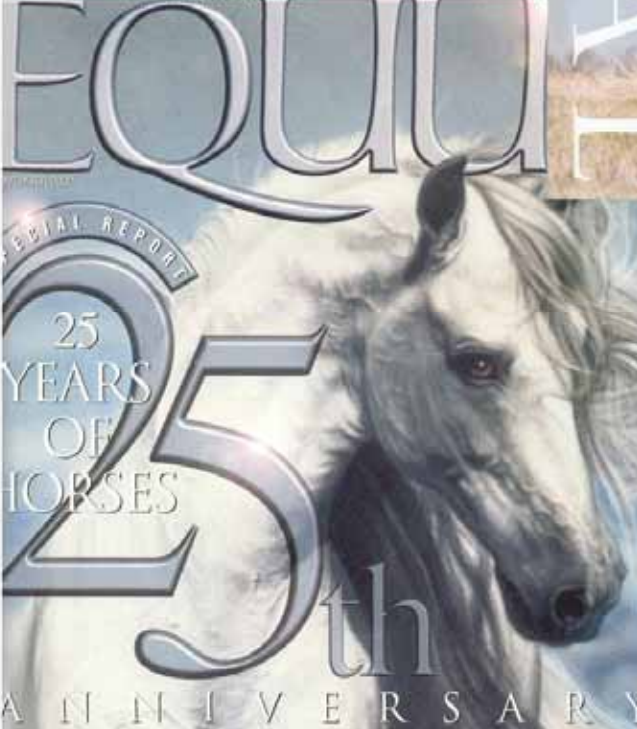
# EQUUS

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# PSSM

POLYSACCHARIDE STORAGE MYOPATHY



learn the most by realizing how wrong you really were," says Valberg, adding that the recommendations traditionally made for tying up included stall rest, which can be very harmful to horses with PSSM.

The study of PSSM also illustrates the importance and efficacy of funding for specific diseases, says Valberg. "My research was funded in large part by the American Quarter Horse Association," she says. "So it's no surprise that most of the information we have on it involves that breed. I suspect other breeds experience this, but until there are specific funding efforts by those associations, there just aren't the resources to pursue it."

- ◆ **The future:** Genetic research could one day yield a DNA-based test for PSSM, but that may be a long way off yet, says Valberg, because the gene or genes involved have not yet been identified.
- ◆ **Further reading:** "Tying Up," EQUUS 257; "Muscle-Saving Rations," EQUUS 299; "Malfunctioning Muscles," EQUUS 225.