EFFECTS OF INTRA-ARTICULAR LIPOPOLYSACCHARIDE INJECTION ON SYSTEMIC CYTOKINE GENE EXPRESSION AND LEUKOCYTE POPULATION IN YOUNG HORSES

A Thesis

by

CARRIE LYNN MUELLER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2011

Major Subject: Animal Science

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Approved by:

Co-Chairs of Committee,	Dennis Sigler
	Josie Coverdale
Committee Members,	Noah Cohen
	Martha Vogelsang
Head of Department,	H. Russell Cross

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ABSTRACT

Effects of Intra-Articular Lipopolysaccharide Injection on Systemic Cytokine Gene Expression and Leukocyte Population in Young Horses. (December 2011)

Carrie Lynn Mueller, B.S., University of Wisconsin-River Falls

Co-Chairs of Advisory Committee: Dr. Dennis Sigler
Dr. Josie Coverdale

Nineteen yearling Quarter Horses were utilized in a randomized, complete block design to evaluate systemic cytokine gene expression and circulating leukocyte population in young horses following an intra-articular lipopolysaccharide (LPS) challenge. Horses were administered an injection of 0.25 ng (n = 7) or 0.50 ng (n = 6) of LPS or lactated Ringer′s solution (n = 6; control). Blood was collected via jugular catheter at pre-injection h 0 and at 2, 6, 12, and 24 h following aseptic injection of the left radiocarpal joint. Aseptic arthrocentesis was performed at the same times to sample synovial fluid for a companion study. Total RNA was isolated from leukocytes using a commercially available kit and real-time PCR was used to determine relative gene expression of the cytokines; interleukin (IL)-1beta (β), IL-6, IL-8, IL-10, and tumor necrosis factor-alpha (TNF-α). Determination of total leukocyte subpopulations and differentials was performed by Texas Veterinary Medical Diagnostic Laboratory.

Data were analyzed using the PROC MIX procedure of SAS. Gene expression of all cytokines analyzed was unaffected by treatment. However, changes over time were observed in some cytokines. Interleukin-1β was increased above baseline at 6, 12,

and 24 h ($P = 0.04$), IL-6 was decreased slightly at 6 and 12 h and then increased at 24 h ($P = 0.002$), and TNF-α was increased at 6 and 12 h ($P = 0.01$). Only IL-8 exceeded a 2-fold change in expression ($P = 0.01$), peaking at 12 h and indicating greater responsiveness to arthrocentesis than was observed in the other cytokines. No treatment effects on the leukocyte population were observed; however, total circulating leukocytes increased over time ($P = 0.04$), peaking at 6 h post-injection. Similarly, an increase over time was observed in monocytes ($P = 0.002$) and in platelets ($P = 0.01$) at 24 h post-injection.

The results indicate that regardless of treatment, a mild immune response was elicited, likely due to repeated arthrocentesis. Future experiments should consider the effects of arthrocentesis and potential systemic inflammatory response, even in control animals, when administering intra-articular LPS to young horses.

DEDICATION

For Cody, who held my hand through this whole crazy thing.

ACKNOWLEDGEMENTS

First and foremost, I wish to express my gratitude to my committee members, Drs. Sigler, Coverdale, Cohen, and Vogelsang, for their considerable contributions to this endeavor. Each gave me the freedom to run with this project idea, but was always there when I needed support. I am forever indebted to the members of the Equine Infectious Disease Laboratory, particularly Jessica Nerren, Courtney Brake and Angela Bordin, for their willingness to share lab equipment, teach me procedures and answer my endless supply of questions. A sincere thank you goes to Jessica Lucia for allowing me to "tag team" with her on this project, and also to the many graduate and undergraduate students who dedicated long hours to make this study a success. Thanks go to Kris Hiney and Nate Splett for their mentorship far beyond my time at UW-River Falls. Finally, to my wonderful parents and sister, Kristina: thank you for providing long-distance encouragement and for supporting all of my dreams, even when they require us to live so far apart. I am truly blessed.

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CHAPTER I

INTRODUCTION

Osteoarthritis and associated musculoskeletal injuries are commonly accepted as the most prevalent source of lameness in the equine athlete. From an epidemiological standpoint, joint disease has been deemed the primary source of inferior performance and inability to compete in the racehorse (Jeffcott et al., 1982; Wilsher et al., 2006). In one study, of 1,042 horses admitted to a large animal clinic for signs of lameness, 42% had lameness related to joint disease (Todhunter and Lust, 1990). The economic impact of this loss of performance is significant and demonstrates the need for investigation of this condition. Several experimental models have been developed such that the pathogenesis of joint disease may be studied as well as the effectiveness of potential treatment options. Previous studies have included intra-articular injection of the polyene antibiotic, filipin (McIlwraith et al., 1979), sodium monoiodoacetate (Trotter et al., 1989), and articular particles (Hurtig, 1988). While useful, these models all utilize primary cartilage destruction and fail to investigate the effects of synovial membrane inflammation alone on articular cartilage metabolism.

Intra-articular injection of *Escherichia coli*-derived lipopolysaccharide (LPS) has been used successfully as a method of inducing temporary inflammation in adult horses to mimic the progression of joint disease (Firth et al., 1987; Palmer and Bertone 1994; De Grauw et al., 2009). While this model has been useful, to our knowledge the model

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This thesis follows the style of Journal of Animal Science.

has thus far been applied solely to skeletally mature horses. Because the highest competitions in many performance disciplines such as futurities are limited to horses in their 3-year-old years, adolescent horses enter rigorous training regimens at an early age. The immature skeletal structure of a young horse in training is subject to repeated stress which induces inflammation and may ultimately lead to articular degradation. Models for experimentally-induced inflammation designed for the mature horse may not be appropriate for the growing horse due to factors including differences in body size, biomechanical forces, and immunological development. It has been suggested that the structures involved in osteoarthritis may be affected differently by trauma, degeneration, or osteochondrosis due to age (Goodrich and Nixon, 2006). Thus, determining an appropriate dosage of LPS and establishing a model for investigating joint degradation in a horse at this stage of maturity would be beneficial.

Previous studies have typically focused on biomarkers derived from synovial fluid to detect articular degradation. While this approach is beneficial in providing insight into the current condition of a particular joint, this methodology fails to consider the overall health and immune status of the animal. It is unknown whether a horse exhibiting signs of lameness may be experiencing a systemic inflammatory response. If this is the case, management decisions should take into consideration this potential overstimulation of the immune system.

Furthermore, the potential correlation between joint degradation and alterations in systemic biomarkers may provide a less invasive alternative for assessing the current status of joint health as well as providing early detection of complications prior to when

clinical signs become apparent. Gene expression of pro-inflammatory cytokines derived from synovial fluid has been successfully used as an indirect marker of joint disease (Lipsky et al., 1989). We hypothesize that analyzing the peripheral blood gene expression of a panel of cytokines known to be crucial mediators of the inflammatory response will enable determination of whether a systemic inflammatory response occurs in conjunction with a localized inflammatory insult. Therefore, the objectives of the current study were to:

1.) quantify and compare the total circulating leukocyte population and determine cellular differential; and

2.) evaluate the peripheral blood gene expression of a panel of principal cytokines involved in the inflammatory cascade in yearling horses following an intra-articular LPS injection at different dosage levels.

CHAPTER II

REVIEW OF THE LITERATURE

Anatomy of the Synovial Joint

The purpose of a synovial joint is to facilitate movement as well as disperse and transmit biochemical load. Its structure consists of two or more articulating bone surfaces, articular cartilage, the joint cavity, and the joint capsule. The capsule functions in conjunction with neighboring ligaments to maintain the proper position of the articular surfaces. This capsule consists of a superficial avascular, fibrous layer (*stratum fibrosum*) and a deeper, secreting layer (*stratum synoviale*), also referred to as the synovial membrane. The synovial membrane is a vascular connective tissue that secretes synovial fluid and contains synoviocytes, which perform phagocytosis and synthesize inflammatory mediators including prostaglandins, proteases, and interleukin (IL)-1 (Goodrich and Nixon, 2006). The joint cavity refers to the space between the adjacent bones of the joint which is surrounded by the joint capsule. This cavity is small, filled with synovial fluid, and is enclosed by the synovial membrane. Synovial fluid is a lubricating fluid containing ions and molecules similar to those found in blood plasma, with the addition of a high concentration of hyaluronan. Synovial fluid contains approximately 10% polymorphonuclear leukocytes with the remaining volume consisting of mononuclear cells (predominantly monocytes, lymphocytes, synovial lining cells; Helal and Karadi, 1968).

A layer of hyaline cartilage known as articular cartilage covers the articular surface. Its approximate composition is 75% water, 15% type II collagen, 10% proteoglycans, and 2% chondrocytes (Poole, 2001). The type II collagen fibrils form a network containing proteoglycans and hyaluronan, which provide compressive stiffness and tensile strength to the articular cartilage. The proteoglycans are actively turned over via synthesis by chondrocytes and degradation by extracellular proteinases, resulting in a delicate equilibrium. Under normal conditions, the stringent regulation of this matrix homeostasis is achieved through cytokines, growth factors, and proteinases (Tyler, 1991).

Pathology of Osteoarthritis

Osteoarthritis is a degenerative, chronic disease which occurs when there is an imbalance of extracellular matrix destruction and repair and is characterized by erosion of type II collagen and cartilage-specific proteoglycans (Todhunter et al., 1996). Numerous causes of osteoarthritis have been implicated; however, the most prevalent cause in young horses is trauma and the concomitant synovitis (McIlwraith and Trotter, 1996). Repetitive overuse and deficiencies in conformation are contributing factors due to excessive biomechanical forces overwhelming the structural integrity of the legs. The joints of the young horse, favoring tissue turnover and growth, have higher synthesis rates of total protein and stromelysin in articular cartilage as well as greater metabolic activity overall when compared with adult horses (Morris and Treadwell, 1994).

Typically, the inflammatory process is initiated in the synovial membrane, cartilage, or subchondral bone. While the interactions between tissues are complex and

not fully understood, some traumatic event triggers the release of inflammatory mediators, principally cytokines IL-1 and TNF-α, from the primary tissue of insult. These cytokines inhibit proteoglycan synthesis and cause the release of metalloproteinases and prostaglandins from synoviocytes and chondrocytes (McIlwraith and Trotter, 1996). In addition, IL-1 stimulates chondrocytes to secrete stromelysin, which cleaves collagenase to its active form (Towle et al., 1987). Cysteine proteases, also released from the synovial membrane by IL-1 or TNF-α signaling, digest articular cartilage matrix, releasing degradative particles into the synovial fluid (Huet et al., 1993). Chondrocytes, synoviocytes, bone cells, and leukocytes release a host of pro-inflammatory cytokines, which upregulate the activity of various metalloproteinases and signal for additional release of inflammatory mediators. Collagenase is the principal proteinase involved, disturbing the triple helix structure and effectively disrupting the integrity of collagen tissue.

This imbalance of metabolism results in a loss of proteoglycans and collagen from the articular matrix as well as the subsequent biomechanical failure and pain associated with osteoarthritis (Poole, 1990). As degradation ensues, proteoglycan particles from the cartilage are released into the synovial fluid. This exacerbates the progression of the disease as products of proteoglycan catabolism are antigenic and stimulate synovial inflammation (Glant et al., 1993).

It is well recognized that with current technology, identifying early stages of cartilage degradation and resulting osteoarthritis is difficult and often impossible prior to the appearance of radiographic evidence. Much research has been devoted to the

development of biomarkers which can be used to monitor degradative changes in the articular structures of the joint. Cytokines, particularly IL-1 and TNF-α, are one such area under investigation for potential correlation between gene expression and inflammatory status of an individual.

Cytokines

Cytokine, also known as peptide regulatory factor, is an expansive term which encompasses the small, cell-signaling protein molecules secreted by various cells of the body, particularly those of the immune system. Cytokines influence the function of a target cell by altering gene expression in a positive or negative manner. The cytokine family includes the interleukins, lymphokines, and signaling molecules including tumor necrosis factor and the interferons. These polypeptides are structurally diverse but possess many shared properties (O'Shea and Murray, 2008).

Cytokines are produced in response to inflammatory or antigenic stimuli and can exhibit local or systemic effects via autocrine, paracrine, or endocrine actions. These proteins provide a crucial link between innate and adaptive immunity by mediating the immune responses of both systems. Resting lymphocytes and other cell types of the immune system do not produce cytokines routinely (Lipsky et al., 1989). Rather, synthesis of cytokines is generally induced by new gene transcription resulting from transient transcriptional activation. The instability of encoding messenger RNA results in cytokine synthesis being transient as well. Further control over cytokine production exists in RNA and proteolytic processing. Upon synthesis, cytokines are secreted in a rapid surge (Abbas et al., 2010).

Circulating cytokines bind to specific transmembrane receptors which are regulated by external signals, such as B or T lymphocyte stimulation. Other cytokines can also up- or down-regulate the expression of receptors. Cytokines bound to the extracellular portion of the receptor initiate intracellular signaling pathways by altering gene expression in target cells. Typical effects induced by cytokines include lymphocyte differentiation and activation of effector cells. The secretion of cytokines triggers a cascade of events which has diverse effects on cells involved in immunity and inflammation. Cellular responses are tightly regulated through negative feedback mechanisms and inhibitors, and signals are fine-tuned through the synergistic and antagonistic properties of cytokines (Abbas et al., 2010).

The biomedical field has expressed an increasing interest in cytokine therapy as more is discovered about the mechanisms of action and effects of individual cytokines. Certain cytokines are commonly used as therapeutic agents as well as targets for antagonists in many inflammatory and immune diseases (Abbas et al., 2010). A difficulty with this approach, however, is the pleiotropic and redundant tendencies of cytokines; treatment with one cytokine may have unintended additional effects, while an antagonist against one cytokine may not be effective due to compensation by other cytokines. The inflammatory cascade initiated by cytokine release is crucial to the pathological events that ensue, and thus understanding these events is fundamental to effectively disrupting the cascade and preventing damaging biochemical changes.

Tumor Necrosis Factor-alpha

Tumor necrosis factor-alpha (TNF-α) is considered to be the primary mediator of the acute inflammatory response and is responsible for systemic complications derived from serious infections. TNF-α is primarily secreted by activated mononuclear phagocytes; however, additional sources include antigen-stimulated T cells, natural killer cells, and mast cells. The strongest stimulus for macrophage secretion of TNF-α is the interaction of Toll-like receptors with LPS and other microbial products (Abbas et al., 2010). Natural killer cells and T lymphocytes produce interferon-gamma, which amplifies TNF-α production by LPS-stimulated macrophages. Activation and recruitment of neutrophils and monocytes to sites of infection are the primary roles of TNF-α. This is accomplished through a variety of actions. Tumor necrosis factor-α stimulates chemokine production by macrophages, which generates leukocyte chemotaxis and recruitment. Additionally, TNF-α initiates IL-1 secretion, induces apoptosis, and stimulates endothelial cells to produce adhesion molecules for leukocyte accumulation. When acute trauma is severe, TNF-α is produced in substantial quantities and causes systemic pathologic anomalies (Abbas et al., 2010). Séguin and Bernier reported that TNF-α disrupts the synthesis of articular cartilage matrix and glycoproteins during inflammation by down-regulating link protein and type II collagen formation at the transcriptional level (2003).

Interleukin-1

Many of the cytokines which are synthesized by leukocytes and exhibit their effects on leukocytes are classified as interleukins. Interleukin-1 exists in two isoforms (alpha and beta) and is released from a multitude of cells including macrophages, neutrophils, epithelial cells, and endothelial cells. However, the largest amounts of IL-1 seem to be released from mononuclear phagocytic cells at sites of inflammation (Lipsky et al., 1989). It is typical for these cells to store large amounts of IL-1 in the cytoplasm, ready for release upon stimulation. While sharing less than 30% amino acid homology, IL-1α and IL-1β appear to share the same surface receptor and have similar biologic activities (Auron et al., 1984). Equine IL-1 produced from peripheral blood monocytes stimulated with LPS has been identified as the equine form of IL-1β (May et al., 1990). Both equine IL-1α and IL-1β inhibit proteoglycan synthesis of articular cartilage explants (Takafuji et al., 2002). The IL-1β precursor is inactive and must be cleaved enzymatically, which is likely the rate-limiting step in IL-1 secretion (Dinarello, 1989). This cytokine works in conjunction with TNF-α to mediate the host inflammatory response. It also stimulates the secretion of other inflammatory cytokines including IL-2 and IL-6 and induces cytokine receptors to be expressed on T cells and hemopoietic stem cells (Dinarello, 1989).

Extensive research implicates TNF-α and IL-1 as the key mediators in the progression of joint disease. Numerous *in vitro* studies support the notion that these cytokines work synergistically with almost identical effects, stimulating proteinase synthesis by chondrocytes and production of neutral metalloproteinases (Dinarello,

1986; Ratcliffe et al., 1986; Loyau and Pujol, 1990). Injection of IL-1 into the knee joints of mice stimulates *in vivo* proteoglycan degradation and inhibits resynthesis (van Beuningen et al., 1991).

Interleukin-6

Another cytokine integral to both innate and adaptive immunity is IL-6. The presence of microbes, IL-1, and TNF-α stimulate synthesis of IL-6 by cells including mononuclear phagocytes, endothelial cells, and fibroblasts. Interleukin-6 contributes to the acute-phase response by inducing synthesis of hepatic acute-phase proteins and stimulating neutrophil production from bone marrow progenitors. Stimulating B lymphocytes and pro-inflammatory cytokine production, serving as a growth factor for myeloma cells, and inhibiting regulatory T cells are additional attributes of IL-6 (Abbas et al., 2010). High synovial fluid concentrations of IL-6 have been reported in an equine severe joint disease model (Bertone et al., 2001) as well as in humans with progressive rheumatoid arthritis (Kutukculer et al., 1998).

Interleukin-8

Interleukin-8 is classified as a chemotactic cytokine, or chemokine. Its primary purpose is to induce chemotaxis in neutrophil granulocytes. Interleukin-8 functions as a mediator of the innate immune system and is secreted by cells with toll-like receptors. Production of IL-8 has been reported *in vitro* in LPS-stimulated macrophages, endothelial cells, and fibroblasts (Larsen et al., 1989). A synovial fluid source of this cytokine has not been demonstrated (Lipsky et al., 1989), thus its potential effects in the peripheral blood merit investigation.

Interleukin-10

Interleukin-10 is an important cytokine in the anti-inflammatory cascade and is produced chiefly by macrophages as well as regulatory T lymphocytes. The primary role of IL-10 is to inhibit the pro-inflammatory effects of activated macrophages, mainly by down-regulating reactions which produce inflammatory products such as IL-12, interferon-gamma, and class II major histocompatibility complex (MHC) surface molecules. Interleukin-10 impairs the activation of T lymphocytes and abolishes cell-mediated immune reactions, limiting and ultimately terminating inflammatory reactions. The TNF-α-induced release of prostaglandin E_2, a crucial mediator of inflammation, is also impeded by IL-10 (Alaaeddine et al., 1999). Consequently, IL-10 is considered to be a critical signal for the regulation of both innate and cell-mediated inflammatory responses.

Synovial Fluid Cytokines as Biomarkers for Joint Disease

Previous work has largely relied upon biomarkers within the synovial fluid to assess joint condition. Cytokines are crucial mediators of the inflammatory response, and pro-inflammatory cytokines have been used widely as indirect markers of joint disease (Lipsky et al., 1989). In a study evaluating the usefulness of several synovial fluid cytokines to detect, diagnose, and characterize joint disease, it was found that IL-6 was an excellent marker, being both sensitive and specific; presence of synovial fluid IL-6 indicated joint disease and was highly correlated to synovial fluid leukocyte counts (Bertone et al., 2001). The same study, which used ELISAs on synovial fluid samples from 119 joints, identified TNF-α and IL-1β as good predictors of joint disease (Bertone

et al., 2001). All three of these cytokines have been determined to be fundamentally involved in stimulating an acute inflammatory response (Molloy et al., 1993) as well as synovial metabolism, joint inflammation, and stimulating articular cartilage degradation (Shinmei et al., 1989). Studies have reported increased expression of IL-1β and TNF-α in the synovial fluid of osteoarthritic horses (Alwan et al., 1991; Hawkins et al., 1993), indicating their usefulness as secondary biomarkers of joint degradation.

The anti-inflammatory cytokines detected at increased levels in synovial fluid of osteoarthritic joints are IL-4, IL-10, and IL-13 (Sutton et al, 2009). An *in vitro* study investigating LPS-stimulated human monocytes reported the production of high levels of IL-10, which strongly inhibited the expression of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and granulocyte colony-stimulating factor (de Waal Malefyt et al., 1991). This inhibition has been reported to be similar in both the synovial fluid and peripheral blood mononuclear cells (Hart et al., 1995).

Systemic Cytokine Response to Intravenous LPS Infusion

To our knowledge, cytokine expression in equine plasma has not been measured in response to an intra-articular LPS challenge. However, LPS has been alternatively introduced in several studies investigating equine response to endotoxin. MacKay and colleagues (1991) identified TNF as a critical early mediator in the cellular response to LPS based on its effects on cytotoxicity in an *in vitro* study. Inflammatory cytokine expression has also been evaluated in peripheral blood of horses following an intravenous infusion of LPS. Up-regulation of IL-1α, IL-1β, IL-6, IL-8, and TNF-α was quantified by real-time PCR, with expression peaking at 60 min (IL-1α, IL-1β, IL-6, and

TNF-α) and 90 min (IL-6) post-infusion (Nieto et al., 2009). A similar increase in serum IL-1β, IL-6, and TNF-α was reported in intra-arterially LPS-challenged rats (Givalois et al., 1994). Lipopolysaccharide appears to reliably induce a detectable alteration in gene expression of several cytokines involved in the inflammatory cascade.

Intra-Articular Injection of LPS to Induce Acute Inflammation

LPS Structure

Previous studies in numerous species have utilized intra-articular LPS injection to induce temporary inflammation. As the name indicates, LPS is a molecule consisting of a polysaccharide covalently-linked to a lipid moiety. An *O*-specific chain and core oligosaccharide are anchored in the outer bacterial membrane and attached to lipid A, which is a phosphorylated glucosamine disaccharide linked to several fatty acids. The Lipid A portion is responsible for the endotoxic effects of LPS (Silipo et al., 2002; Figure 1).

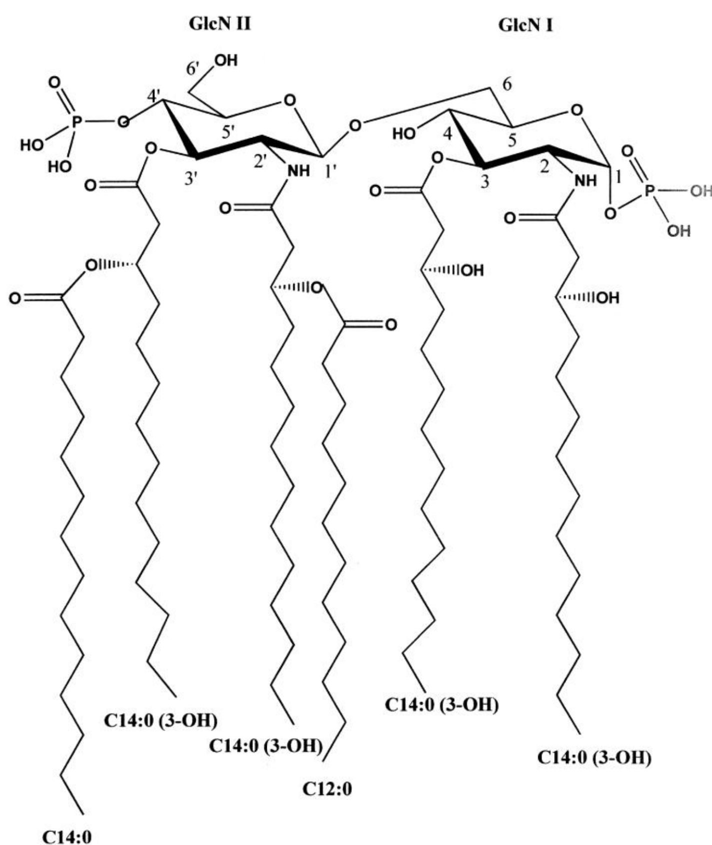

Figure 1. Lipid A structure of *Escherichia coli* (Adapted from Silipo et al., 2002). GlcN = glucosamine.

Lipopolysaccharide is a major structural component of the cell wall of gram-negative bacteria and acts as a strong endotoxin. Its ability to elicit *in vivo* cartilage degradation has been previously demonstrated in horses (De Grauw et al., 2009) and rabbits (Sáez-Llorens et al., 1991). *In vitro* studies have produced similar effects in cartilage of equine (Todhunter et al., 1996), bovine (Morales et al., 1984), and canine (Todhunter et al., 1995) species. Lipopolysaccharide is recognized by Toll-like receptor 4, which activates macrophages to release cytokines (principally TNF, IL-1, and IL-6) and initiate an inflammatory cascade (Warren et al., 2010). The primary cytokine

released in response to LPS is TNF-α, which then stimulates the release of a number of products from macrophages and initiates the cascade of events which may ultimately lead to septic shock or death (Mathison et al., 1988).

Previous Studies Utilizing Intra-Articular LPS

Hawkins et al. (1993) reported that injection of 3 μg of LPS into the antebrachiocarpal joint of horses resulted in signs of acute synovitis (increased surface temperature of LPS-injected carpi, increased synovial fluid leukocyte count, increased prostaglandin E_2) within 2 h of injection. These findings are in agreement with Jacobsen et al. (2006), who reported fever, tachycardia, tachypnea, acute lameness, and joint swelling within 4-8 h following 1 μl and 3 μl LPS injection. An even larger dosage of 0.1 μg of LPS/kg has elicited acute fever, depression, increased pulse and respiratory rates, and lameness in ponies (Firth et al., 1987). Palmer and Bertone (1994) sought to compare dosage levels of 0.125 ng, 0.17 ng, 0.25 ng, 0.5 ng, 25 ng, and 5,000 ng of LPS per joint in adult horses. The authors reported signs of endotoxemia (i.e., fever, depression, lack of appetite, severe lameness) at doses larger than 0.5 ng per joint (Palmer and Bertone 1994). At 12 h post-LPS injection, the magnitude of systemic signs presented by these horses required that they be treated with phenylbutazone and excluded from the remainder of the study.

The dosage of 0.5 ng LPS successfully altered markers of collagen II turnover (collagenase cleavage neopeptide and carboxypeptide) and inflammatory mediators (prostaglandin E_2, substance P, and bradykinin) when injected into the midcarpal joint of adult horses (De Grauw et al., 2009). These data suggest that investigation of varying

dosage levels is warranted, specifically for the target population of adolescent equines, which has thus far been largely underrepresented in studies of equine joint health. Ideally, the lowest effective dosage that elicits detectable joint inflammation should be employed. This is to minimize discomfort to the experimental subject as well as to more accurately mimic the pathophysiology of equine joint disease. While injection of large doses of LPS elicits rapid, significant changes, these models represent a severe arthritis or acute joint sepsis. A model which induces a less severe synovitis would more accurately mimic the progression of joint disease in a typical clinical case and thus may prove more beneficial for making inferences about the development of osteoarthritis and developing preventative strategies to combat early progression of the disease.

In addition, it is of note that studies of this nature often treat each experimental animal with intra-articular LPS, utilizing the contralateral joint as a saline-injected control (Hawkins et al., 1993; De Grauw et al., 2009). This may be of importance, as an earlier study has reported signs of synovitis, enhanced cytokine immunoreactivity, and cartilage degradation in the saline-injected, contralateral joint of horses injected with LPS (Todhunter et al., 1996). These findings are in agreement with studies which have observed loss of proteoglycans in the saline-injected joint contralateral to either the hyaluronate-polylysine-injected joint in rabbits (Smith et al., 1994) or the IL-1-injected joint in rats (Chandrasekhar et al., 1992). Saline solution injections did not appear to directly cause proteoglycan loss, as control animals treated with saline alone experienced no alterations in proteoglycan content (Smith et al., 1994). Rather, these results suggest a potential systemic effect induced by the inflammatory agent. It is possible that

cytokine-producing leukocytes may have been recruited and released into circulation, ultimately reaching the contralateral joint and altering proteoglycan metabolism at this site as well as the primary site of insult. Alterations in serum concentrations of a direct indicator of the predominant protein in cartilage (N-propeptide of type IIA procollagen) have been reported in human osteoarthritic patients, indicating that systemic effects are generated by the disease (Garnero et al., 2002). Due to the potential for unintended systemic consequences, an investigation into the effects of a singular intra-articular challenge utilizing appropriate controls is clearly warranted.

Conclusion

In summary, osteoarthritis is a prevalent disease which contributes profound losses to the performance horse industry. The mechanisms of the disease are poorly understood, and a reliable early detection method has not been developed. Current models for the disease utilize the adult horse; however, a young horse model may be more appropriate due to industry standards placing high physical demands on skeletally immature horses. While numerous studies have utilized primary cartilage destruction to investigate the pathophysiology of osteoarthritis, synovial inflammation has been largely ignored. However, research suggests that inflammatory cytokines are crucial bio-signaling molecules which have a large role in the development of osteoarthritis by stimulating chondrocytes and synoviocytes to secrete cartilage-degrading proteinases. The use of these proteins as biomarkers may provide a more practical, less invasive means of monitoring joint health and identifying individuals at risk for development of osteoarthritis. Reported systemic alterations in response to both osteoarthritis and a

single inflammatory insult merit the use of a separate group of control animals, a measure not typically practiced in prior studies. Therefore based on previous literature, a study utilizing a young horse model to investigate the potential systemic effects of a single, intra-articular challenge with separate control animals may provide valuable insight on the metabolic response to synovial inflammation in the young horse.

CHAPTER III

MATERIALS AND METHODS

All procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP #2010-221).

Animals and Treatments

Nineteen yearling Quarter Horses from the Texas A&M University Horse Center herd were utilized in a randomized, complete-block design. Horses were of similar breeding and aged 184 to 327 d with initial BW ranging from 220 to 384 kg. Horses were blocked by age, sex, and BW and randomly assigned within block to one of three treatments. Treatments were an injection containing 0.25 ng (n = 7) or 0.5 ng (n = 6) of LPS or lactated Ringer's solution (n = 6; control). The LPS was obtained from *E. coli* 055:B5 and diluted in sterile lactated Ringer's solution to the desired concentration.

Horses were housed in groups of four or five by BW in 25 m x 25 m dry lots for a back-grounding period of 21 d prior to the start of the study. Throughout the experiment horses were group fed by pen and received approximately 1% BW (as fed) of a commercially prepared pelleted concentrate (Producers 14% Horse Pellet, Producer's Cooperative Association, Bryan, TX; Table A.1) and 1.5% BW (as fed) of Coastal Bermudagrass hay (*Cynodon dactylon*; Table A.1), divided into two daily feedings. Biweekly BW measurements were determined using a digital platform scale (CAS Corp. Seoul, Rep. of Korea) and diets were adjusted accordingly. Horses were provided free access to water at all times. Throughout the back grounding period horses

were individually handled a minimum of two times per week to mimic procedures used throughout the LPS challenge, with activities including being haltered, standing tied, leading quietly at the walk and trot, walking across the scale, and picking up both front feet.

LPS Challenge (Time 0)

On the day of the LPS challenge, horses were individually confined in 3 m x 3 m stalls and had a 14-gauge catheter inserted aseptically in the left jugular vein following subcutaneous administration of 1.5 ml sterile lidocaine hydrochloride 2% (RXV Products, Westlake, TX). Catheters were maintained every 2 h throughout the sampling period with 6 ml of heparinized saline solution. Horses were unobtrusively monitored for at least 2 h following catheter placement prior to sample collection. Subsequently, time 0 was established, and heart rate (HR) and respiratory rate (RR) were recorded as well as rectal temperature via thermometer. Each horse was then removed from its stall and trotted in-hand across the concrete barn-aisle for determination of a lameness score, according to the American Association of Equine Practitioners' (AAEP) lameness grading system (range 0-5, with 0 assigned to horses with no detectable lameness and 5 for non-weight bearing lameness; AAEP 1999). The same evaluators scored all 19 horses for consistency. Circumference of the left carpal joint, which had been clipped 24 h prior, was measured in centimeters using a flexible measuring tape at the level of the accessory carpal bone by a single observer. The carpus was prepared immediately prior to arthrocentesis by scrubbing the surface, alternating diluted Betadine scrub and 70% isopropyl alcohol. A licensed veterinarian from the Texas A&M University Large

Animal Clinic (College Station, TX) performed arthrocentesis of the left radiocarpal joint, extracting synovial fluid samples with a 20-gauge needle and 6-ml syringe. Synovial fluid samples were processed for analysis of markers of joint inflammation and cartilage markers in a related study (Lucia et al., 2011). Immediately following synovial fluid aspiration, 0.8 ml was injected into the same joint containing LPS according to assigned treatments.

Blood Sampling

Subsequently, blood was collected via the jugular catheter into ethylenediaminetetraacetic acid (EDTA)-containing evacuated tubes (Kendall Co., Mansfield, MA). Air-dried, unstained blood-smear slides were prepared from each sample and accompanied chilled blood collection tubes which were promptly transported to the Texas Veterinary Medical Diagnostic Laboratory (College Station, TX) for analysis. Total leukocyte population was determined using a hematology analyzer (Abbott CELL-DYN 3700, Global Medical Instrumentation, Inc., Ramsey, MN) and differentials were determined manually. An additional 8 to 10 ml peripheral blood sample collected into tubes containing EDTA was passed through a LeukoLOCK™ filter[1] to capture the total leukocyte population for later gene expression analysis. Filters were flushed with 3 ml of phosphate-buffered saline (PBS) to remove residual red blood cells and 3 ml of RNA*later*™ solution[1] to stabilize RNA. Stabilized filters were stored at room temperature for 24 h, at which point trapped leukocytes were lysed with 2.5 ml of LeukoLOCK™ lysis/binding solution[1] and the lysate was collected in 15-ml conical

[1] Ambion Inc, Austin, TX

tubes and stored at -80°C until further analysis.

Post-Injection Hours 2, 6, 12, 24

Horses were maintained in stalls under constant supervision for the 24 h duration of the sample collection period. Determination of vital signs, lameness scores, joint circumference, and synovial fluid collection occurred at 2, 6, 12, and 24 h post-injection of LPS.

Laboratory Analysis

RNA Isolation

RNA-containing lysate was thawed at 23°C and RNA was extracted from the total leukocyte population using a commercial system (LeukoLOCK™ Total RNA Isolation System, Ambion Inc, Austin, TX) following the manufacturer's protocol. Briefly, nuclease-free water was added to each tube along with proteinase K and incubated for 5 min at 23°C with intermittent inversion to degrade cellular proteins. Isopropyl alcohol (100%) and 50 µl of RNA binding beads were added to each tube, mixed, and incubated at 23°C with intermittent mixing for 5 min to allow RNA to bind to the beads. Beads were pelleted using a centrifuge at 2,000 x g for 3 min (5810R, Eppendorf North America, Westbury, NY). Supernatant was aspirated and discarded. Beads were then washed twice with a wash solution and transferred to a 1.5-ml microcentrifuge tube. Samples were spun in a centrifuge (5415C, Eppendorf North America, Westbury, NY) at 16,000 x g for 30 s to pellet the beads and supernatant was discarded upon aspiration. A second wash solution was added, followed by brief centrifugation and aspiration as described. An optional DNase treatment included with

the kit was then performed. A master mix of DNase and a buffer solution was added to each tube, which was then agitated gently for 10 min at 23°C to release nucleic acids from RNA binding beads and degrade genomic DNA. Lysis/binding solution and 100% isopropyl alcohol were added and tubes were incubated at room temperature for 3 min. Two further cycles of centrifugation, aspiration, and washing steps were repeated. Following the final aspiration, tubes were left open at room temperature for 3 min to allow residual liquid to evaporate. Approximately 150 µl of an elution solution was added to the tubes which were then mixed vigorously for 30 s. A final centrifugation at 16,000 x g for 2 min pelleted the beads, and the RNA-containing supernatant was aspirated and transferred to a microcentrifuge tube and stored at -20°C.

Treatment for DNA Contamination

Nucleic acid concentration and purity were assessed using a micro-volume spectrophotometer (NanoDrop 1000, Thermo Scientific, Wilmington, DE). Samples were treated to remove residual contaminating DNA using a commercial kit (TURBO DNA-free™ Kit, Ambion Inc, Austin, TX) using the manufacturer's protocol. Briefly, 15 µl of provided 10X DNase Buffer and 1 µl DNase were added to each microcentrifuge tube containing RNA and gently mixed. Following 20 min incubation at 37°C, 16.6 µl of resuspended DNase Inactivation Reagent was added. The tubes were mixed thoroughly and incubated 5 min at 23°C. Finally, tubes were centrifuged at 10,000 x g for 1.5 min. Purified RNA was transferred to a fresh tube and nucleic acid concentration and purity were reassessed by spectrophotometry. Samples were then stored at -20°C until further analysis.

cDNA Synthesis

DNase/RNase-free distilled H_20 was used to dilute samples such that each aliquot contained 500 ng of RNA and had a final volume of 10 µl. Synthesis of cDNA was performed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Corporation, Grand Island, NY) following the manufacturer's instructions. Briefly, 2 µl of cDNA first-strand mix consisting of equal parts oligo $(dT)_{20}$ and dNTPs were added to each tube and mixed gently. Tubes were incubated on a thermal cycler at 65°C for 5 min, then at 4°C for at least 1 min. A cDNA master mix was prepared and 10 µl were added to each reaction, followed by incubation on the thermal cycler at 50°C for 50 min, then at 85°C for 5 min. Subsequently samples were diluted with 28 µl nuclease-free water so that the final concentration of cDNA was 10 ng/µl and aliquots were stored at -80°C.

Primer and Probe Sequences

Previously designed equine-specific sequences for B2M, IL-6, and IL-8 were used (Nerren, 2008) as well as for IL-10 (J. Nerren, unpublished data). Specific primer-probe premixes were designed for equine TNF-α and IL-1β using the Assays-by-Design software program (Applied Biosystems, Foster City, CA). All sequences are listed in Table 1.

Real-Time PCR

Quantitative real-time polymerase chain reaction (PCR) was carried out in 10-µl reactions, each containing 1 µl template cDNA and 5 µl TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA). The remaining volume consisted of either 0.5 µl primer-probe premix and 3.5 µl nuclease-free water (Invitrogen Corporation, Grand Island, NY; TNF-α, IL-1β), or 0.45 µl of each primer, 0.5 µl probe, and 2.6 µl water (B2M, IL-6, IL-8, IL-10). A GeneAmp 7500 Sequence Detection System was used for all cDNA amplification and data analysis (Applied Biosystems, Foster City, CA). The thermal profile consisted of an initial hold at 50ºC for 2 min, followed by denaturation at 95°C for 10 min, then 40 cycles of 95°C for 15 s, 60°C for 60 s.

Beta-2 Microglobulin (B2M) served as the endogenous control and was used to normalize all resulting cycle threshold (Ct) values. Target genes were calibrated to baseline expression (Time 0) and relative expression levels were determined using the $2^{-\Delta\Delta C_T}$ method of quantification (Livak and Schmittgen, 2001).

Table 1. Oligonucleotide primer and probe sequences for equine cytokine targets and endogenous control.

Gene	Primer/probe	Sequence (5'-3')
B2M*	Forward	CGGGCTACTCTCCCTGACT
	Reverse	GGGTGACGTGAGTAAACCTGAAC
	Probe	CCGTCCCGCGTGTTC
IL-1β	Forward	GAATGACCTGTTCTTTGAGGAGGAT
	Reverse	GAGCTGAGGTCCAGGTCTTG
	Probe	AAGCTGCCCTTCATCTGT
IL-6*	Forward	GAAAAAGACGGATGCTTCCAATCTG
	Reverse	TCCGAAAGACCAGTGGTGATTTT
	Probe	CAGGTCTCCTGATTGAAC
IL-8*	Forward	GCCACACTGCGAAAACTCA
	Reverse	GCACAATAATCTGCACCCACTTTG
	Probe	ACGAGCTTTACAATGATTTC
IL-10**	Forward	GACATCAAGGAGCACGTGAACTC
	Reverse	CAGGGCAGAAATCGATGACA
	Probe	AGCCTCACTCGGAGGGTCTTCAGCTT
TNF-α	Forward	TTACCGAATGCCTTCCAGTCAAT
	Reverse	GGGCTACAGGCTTGTCACT
	Probe	CCAGACACTCAGATCAT

* (Nerren, 2008)
** J. Nerren, unpublished data

Statistical Analysis

All data were analyzed as a randomized complete block design using the PROC MIXED procedure of SAS (SAS v 9.1; SAS Inst. Inc., Cary, NC). Treatment, time, and their interaction were included in the model. Main effects were considered significant when $P \leq 0.05$ and a trend toward significance when $P \leq 0.10$.

CHAPTER IV

RESULTS AND DISCUSSION

Physical Parameters

No effects of treatment were observed on heart rate, respiratory rate, or rectal temperature ($P = 0.77$, $P = 0.16$, and $P = 0.60$ respectively; Table 2). However, all three decreased over time ($P < 0.01$). This change can be attributed to the young horses becoming acclimated to the repeated handling procedures, resulting in a diminished stress response. This finding agrees with that of Palmer and Bertone (1994), who observed no changes in peripheral temperature, pulse rate, or respiratory rate in horses that received intra-articular injection of 0.125 ng, 0.17 ng, 0.25 ng, or 0.5 ng LPS.

At 0 h post-injection, all horses were determined to be clinically sound (lameness score 0). Lameness scores increased across all groups over the 24 h following LPS challenge, with peak scores ranging from 1 to 2, regardless of treatment ($P < 0.01$; Table 3). This observation demonstrates the necessity for studies utilizing repeated arthrocentesis to include a group of control animals so that effects of treatment are not confounded by changes associated with repeated arthrocentesis alone.

Additionally, the lameness scores of horses receiving any dosage of LPS injection were increased at 12 and 24 h post-injection compared to the control group ($P \leq 0.05$). These data suggest that the effect of the endotoxin was sufficient to induce discomfort beyond that induced by arthrocentesis alone. While lameness was still evident at the conclusion of the 24 h study, we predict that this would have been resolved within 48 h, as was the case in the Palmer and Bertone study (1994), which reported lameness scores ranging from 1 to 3 in the 36 h following LPS injection of up to 0.5 ng but all lameness was resolved by 48 h post-injection.

Treatments did not affect joint circumference ($P = 0.96$); however, circumference increased 0.8 to 1.5 cm above baseline in all groups regardless of treatment ($P < 0.05$; Table 4). Once again this observation can be attributed to the process of repeated arthrocentesis, and the merit of having a control group in similar studies is clearly warranted. We expect that this mild joint effusion would have been resolved within 48 h post-injection, as was observed in the Palmer and Bertone study (1994) which elicited a similar increase in joint circumference of 1 to 1.5 cm at the LPS dosages up to 0.5 ng.

Table 2. Least square means for heart rate (HR), respiration rate (RR), and rectal temperature (RT) by treatment across yearling Quarter Horses for a 24 h period in response to intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

	Treatment			
	0 ng LPS	0.25 ng LPS	0.5 ng LPS	SE
HR (beats/min)				
0	48.5^{a,b,c}	46.5	48.5^{a}	1.61
2	49.5^{a}	45.5	48.5^{a}	1.61
6	45.5^{b,e}	45.5	46.5^{a}	1.61
12	45.0^{c,e}	44.5	42.5^{b}	1.61
24	42.0^{d,e}	43.0	41.5^{b}	1.61
RR (breaths/min)				
0	19.0^{a}	16.0^{a,b}	17.5^{a}	1.41
2	19.5^{a}	16.0^{a}	16.5^{a}	1.41
6	14.5^{b}	12.5^{b,c}	13.0^{b}	1.41
12	12.5^{b}	11.0^{c}	13.0^{b}	1.41
24	12.5^{b}	10.0^{c}	11.0^{b}	1.41
RT (°C)				
0	38.1^{a}	37.9^{a,b}	38.1^{a,b}	0.14
2	38.1^{a}	38.0^{a,e}	38.1^{a,b}	0.14
6	37.9^{b}	37.9^{b}	37.9^{a}	0.14
12	38.2^{a}	38.1^{c,e}	38.1^{b}	0.14
24	37.8^{b}	37.7^{d}	37.7^{c}	0.14

$^{a-e}$Within a column for a given parameter, means that do not have a common superscript differ ($P \leq 0.05$).

Table 3. Lameness scores (grading system 0-5, AAEP 1999) in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

Treatment	Hour					
	0	2	6	12	24	SEM
Control	0.0^{a}	0.8^{b}	1.0^{b}	1.0^{b}	0.8^{b}	0.22
0.25 ng LPS	0.0^{a}	1.7^{b}	1.5^{b}	1.7$^{\dagger b}$	2.0$^{\dagger b}$	0.22
0.50 ng LPS	0.0^{a}	1.2^{b}	1.3^{b}	1.7$^{\dagger b}$	1.7$^{\dagger b}$	0.22

†Indicates significant difference within a column ($P \leq 0.05$).
$^{a-c}$Within a row, means that do not have a common superscript differ ($P \leq 0.05$).

Table 4. Carpal circumference (cm) in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

Treatment	Hour					
	0	2	6	12	24	SEM
Control	28.3[a,b]	28.8[a]	29.4[b]	29.7[b]	29.8[b]	0.66
0.25 ng LPS	28.3[a]	28.5[a]	28.8[a]	29.4[b]	29.7[b]	0.66
0.50 ng LPS	28.5[a]	29.0[b]	29.0[b]	29.3[b]	29.3[b]	0.66

[a-b]Within a row, means that do not have a common superscript differ ($P \leq 0.05$).

Circulating Leukocyte Population

Similar studies have focused on biomarkers of inflammation located in the synovial fluid rather than peripheral blood. Synovial fluid leukocyte data have been reported but to our knowledge, there are relatively few published accounts of leukocyte dynamics in the peripheral blood over the course of an intra-articular LPS challenge. The current study aimed to quantify and compare the total circulating leukocyte population and determine cellular differential in yearling horses following an intra-articular LPS injection at different dosage levels. No effects of LPS treatment were observed on the total leukocyte count or differential.

Similarly, no changes in systemic leukocyte totals or differentials were observed when ponies received an intra-articular LPS injection of 0.02 μg/kg of bodyweight; however, peripheral blood was analyzed only at 0 and 8 h post-injection (Todhunter et al., 1996). Hawkins et al. (1993) analyzed equine peripheral blood leukocyte counts at nine time intervals concluding at 144 h following intra-articular injection of 3 μg LPS and also reported no changes due to treatment. These findings as well as those of the current study are in agreement with Palmer and Bertone (1994), who reported no

changes in total peripheral leukocyte count in any horse, regardless of intra-articular LPS dosage ranging from 0.125 ng to 5,000 ng. Palmer and Bertone (1994) observed an alteration in peripheral blood leukocyte differential only at the 5,000 ng dose; however this is exponentially larger than the dosage levels (0.25 ng and 0.5 ng LPS) utilized in the current study. It is likely that our LPS dosages were not large enough to induce a detectable deviation in systemic leukocyte dynamics.

However, in the current study, total circulating leukocytes increased over time, regardless of treatment ($P = 0.04$; Table 5), with the highest values at 6 and 24 h post-injection. Similarly, an increase over time was observed in subpopulations of monocytes ($P = 0.002$; Table 6) and in platelets ($P = 0.01$; Table 7) by 24 h post-injection. These observations further reinforce the conclusion that repeated sterile arthrocentesis alone is sufficient to induce physiological changes, including hematological parameters. Nevertheless, the magnitude of these changes is minor and mean effects are well within the accepted reference ranges. A similar study found that 24 h was the most sensitive time to detect changes induced by repeated arthrocentesis in the equine midcarpal joint (White et al., 1989). Thus, future studies might benefit from including sampling time points beyond 24 h to further characterize these changes and identify the duration of the response until return to baseline values.

Table 5. Blood leukocyte count (cells × 1,000/µl) in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

Treatment	Hour					
	0	2	6	12	24	SEM
Control	9.8	9.6	9.8	9.6	10.4	0.95
0.25 ng LPS	10.3[a,b]	10.2[a]	11.5[c]	10.9[a,b]	10.9[a,c]	0.95
0.50 ng LPS	10.5	11.1	11.1	11.2	10.7	0.95

[a-c]Within a row, means that do not have a common superscript differ ($P \leq 0.05$).

Table 6. Blood monocyte count (%) in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

Treatment	Hour					
	0	2	6	12	24	SEM
Control	1.2[a,b]	1.0[a]	0.6[a,b]	1.4[a,b]	2.8[b]	0.81
0.25 ng LPS	1.4[a]	1.4[a]	1.4[a]	1.1[a]	3.4[b]	0.81
0.50 ng LPS	1.7[a,b]	1.2[a]	1.2[a,b]	0.8[a]	3.0[b]	0.81

[a-b]Within a row, means that do not have a common superscript differ ($P \leq 0.05$).

Table 7. Blood platelet count (cells × 1,000/µl) in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

Treatment	Hour					
	0	2	6	12	24	SEM
Control	217[a,b,c]	258[a,b]	202[c]	242[a,b,c,d]	275[d]	27
0.25 ng LPS	231[a]	234[a,b]	225[a,b]	253[a,b]	280[b]	27
0.50 ng LPS	242[a,b]	245[a,b]	219[a,b]	210[a]	265[b]	27

[a-d]Within a row, means that do not have a common superscript differ ($P \leq 0.05$).

Cytokine Gene Expression

Relative gene expression of IL-1β, IL-6, IL-8, IL-10, and TNF-α in the peripheral blood following an intra-articular LPS challenge was quantified using real-time PCR. Data were analyzed using the equation,

target amount = $2^{-\Delta\Delta C_T}$,

where $\Delta\Delta C_t = (C_{T,target} - C_{t,B2M})$Time x – $(C_{t,target} - C_{t,B2M})$Time 0.

Time x is post-injection hour 2, 6, 12, or 24 and Time 0 represents the 1X expression of the target gene normalized to B2M.

Interleukin-1β

No treatment effects were observed on IL-1β ($P = 0.90$); however, an effect of time was observed ($P = 0.04$; Table 8).

Table 8. Relative systemic gene expression[a] of IL-1β in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

Treatment	Hour					
	0	2	6	12	24	SEM
Control	1.00[b]	0.99[b]	1.46[b,c]	1.93[c]	1.44[b,c]	0.24
0.25 ng LPS	1.00[b]	1.55[c,d]	1.30[b,d]	1.39[b,d]	1.54[b,d]	0.22
0.50 ng LPS	1.00	1.32	1.45	1.41	1.25	0.24

[a]Data were normalized to the house keeping gene B2M and calibrated to baseline expression (time 0).
[b-d]Within a row, means that do not have a common superscript differ ($P \leq 0.05$).

Interleukin-6

Treatment did not affect IL-6 expression ($P = 0.18$); however, an effect of time was observed ($P = 0.002$; Table 9).

Table 9. Relative systemic gene expression[a] of IL-6 in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

Treatment	Hour					
	0	2	6	12	24	SEM
Control	1.00	0.83	0.76	0.75	0.79	0.15
0.25 ng LPS	1.00[b]	1.27[c]	0.99[b]	0.91[b]	1.36[c]	0.13
0.50 ng LPS	1.00[d,e,f]	1.19[b,d]	0.78[c,f]	0.87[c,e]	1.20[b,d]	0.15

[a]Data were normalized to the house keeping gene B2M and calibrated to baseline expression (time 0).
[b-f]Within a row, means that do not have a common superscript differ ($P \leq 0.05$).

Interleukin-8

No treatment effects were observed on IL-8 ($P = 0.24$), but time did affect expression ($P = 0.01$; Table 10). Interleukin-8 was the only cytokine to surpass a 2-fold change in expression, which is conventionally considered the threshold for meaningful alterations in gene expression. This finding may reflect the recruitment of polymorphonuclear (PMN) leukocytes, which secrete large quantities of IL-8. As a chemokine, IL-8 stimulates and regulates migration of leukocytes from the blood to the tissues. Its production is enhanced by IL-1 and TNF-α (Lipsky et al., 1989), thus it is possible that slight increases in IL-1 and TNF-α expression acted synergistically to amplify IL-8 gene expression in the peripheral blood in response to repeated arthrocentesis. Future investigators may consider separateing PMN cells and peripheral blood mononuclear cells to explore cytokine expression from distinct subpopulations rather than the total circulating leukocyte population, as reported here.

While statistically insignificant, IL-8 appears to be the most responsive to repeated arthrocentesis of the cytokines analyzed here and may be considered for future

studies which utilize peripheral blood cytokines as markers of inflammatory response. Increases in synovial fluid cytokine expression or immunoreactivity in response to small amounts of LPS injection have been reported (Hawkins et al., 1993; Todhunter et al., 1996; Bertone et al., 2001). This suggests that the response to intra-articular endotoxin injection is more readily detected locally within the synovial fluid.

Table 10. Relative systemic gene expression[a] of IL-8 in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

Treatment	Hour					
	0	2	6	12	24	SEM
Control	1.00[b]	1.90[b]	3.63[b]	8.87[c]	4.62[b]	1.66
0.25 ng LPS	1.00	1.52	0.97	2.43	1.53	1.53
0.50 ng LPS	1.00	1.16	1.19	1.63	1.26	1.66

[a]Data were normalized to the house keeping gene B2M and calibrated to baseline expression (time 0).
[b-c]Within a row, means that do not have a common superscript differ ($P \leq 0.05$).

Interleukin-10

Treatment did not affect IL-10 expression (P = 0.48). However, trends toward both a time effect and a treatment x time interaction were observed (P = 0.08 and P = 0.07, respectively; Table 11).

Table 11. Relative systemic gene expression[a] of IL-10 in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

Treatment	Hour					
	0	2	6	12	24	SEM
Control	1.00	0.96	1.02	0.85	1.06	1.10
0.25 ng LPS	1.00^{b}	1.36^{c}	1.04^{b}	0.96^{b}	0.93^{b}	0.09
0.50 ng LPS	1.00	1.15	1.08	1.10	1.15	0.10

[a]Data were normalized to the house keeping gene B2M and calibrated to baseline expression (time 0).
[b-c]Within a row, means that do not have a common superscript differ ($P \leq 0.05$).

Tumor Necrosis Factor-α

No treatment effects were observed on TNF-α ($P = 0.71$); however, time influenced expression ($P = 0.01$; Table 12).

Table 12. Relative systemic gene expression[a] of TNF-α in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

Treatment	Hour					
	0	2	6	12	24	SEM
Control	1.00	1.08	1.31	1.14	1.19	0.17
0.25 ng LPS	1.00^{b}	$1.36^{b,d}$	$1.35^{b,d}$	$1.55^{c,d}$	1.04^{b}	0.16
0.50 ng LPS	1.00^{b}	$1.17^{b,d}$	$1.27^{b,d}$	$1.52^{c,d}$	1.08^{b}	0.17

[a]Data were normalized to the house keeping gene B2M and calibrated to baseline expression (time 0).
[b-d]Within a row, means that do not have a common superscript differ ($P \leq 0.05$).

Intravenous infusion of LPS has been previously shown to up-regulate gene expression of inflammatory cytokines including IL-1α, IL-1β, IL-6, IL-8, and TNF-α in horses, while expression levels remained at baseline for saline-treated control horses (Nieto et al., 2009). These findings confirm that LPS is a potent agonist for inducing

inflammatory cytokine release. It is possible that the low response to LPS in the current study may be attributed to the intra-articular route of administration. The associated inflammatory response might have been limited to local recruitment of leukocytes within the synovial fluid, which would account for the failure to detect a pronounced systemic response.

Synovial Fluid Biomarkers

A concurrent study analyzed markers of inflammation and articular cartilage metabolism in synovial fluid extracted at 0, 2, 6, 12, and 24 h (Lucia et al., 2011). Lucia et al. (2011) detected no changes in collagenase cleavage neopeptide, a marker of catabolic type II cartilage metabolism; however, both prostaglandin E_2 (a critical inflammatory mediator) and carboxypeptide (an established indicator of anabolic type II cartilage turnover) were linearly influenced by treatment, with concentrations increasing with LPS dosage ($P \leq 0.01$). This observation indicates that a difference of only 0.25 ng LPS was successful in inducing dosage-dependent effects in a young horse model. Further investigation with more than 2 dosage levels would be beneficial in identifying the most appropriate level of intra-articular LPS injection for the young horse. In addition, all three biomarkers were influenced by time ($P \leq 0.01$; Lucia et al., 2011), further suggesting a role of repeated arthrocentesis in modifying physiological parameters.

CHAPTER V

SUMMARY

Previous work has established LPS as a useful model for inducing temporary inflammation in the mature equine for the purpose of investigating the progression of joint disease. However, little research has been devoted to developing an appropriate model for use in the young horse. With many athletic competitions limited to horses during their 3-year-old years, the industry is requiring horses to undergo rigorous training regimens at a young age, prior to skeletal maturity. Thus, investigation into a model of acute inflammation for this stage of musculoskeletal development is clearly warranted. In addition, the potential for a systemic effect of the inflammatory agent has been given little attention in previous studies and merits exploration. The existence of such an effect would produce confounding results and may affect conclusions drawn from similar studies which use the common approach of employing a saline-injected contralateral joint as the control.

The results of the current study indicate that a singular intra-articular injection of 0.25 ng or 0.50 ng LPS did not alter the total circulating leukocyte population or differential in yearling horses. However, an effect of time was observed in total leukocytes as well as in cellular subpopulations of monocytes and platelets. This response is likely due to the repeated arthrocentesis procedure. Similarly, intra-articular injection of 0.25 ng or 0.50 ng LPS did not affect the expression of five cytokines which are crucial to the inflammatory cascade. Once again an effect of time was observed,

indicating that the repeated arthrocentesis alone was sufficient to induce mild changes in expression levels which were similar across all groups. Increased concentrations of IL-8 suggest possible recruitment of PMN leukocytes in response to arthrocentesis. Future studies may benefit from investigating the use of alternative biomarkers of inflammation in the peripheral blood and synovial fluid which may prove more sensitive to physiological changes.

From these results, we conclude that performing repeated arthrocentesis on a particular joint likely stimulates a mild inflammatory response, inducing effects which can be manifested systemically. Including a negative control might be beneficial to establish physiological normal values for the parameters measured. Future studies of a similar nature should utilize a separate group of control animals to eliminate any confounding effects of treatment and arthrocentesis.

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APPENDIX A

DIET

Table A.1 Nutrient Composition (DM) of Diet Fed to Yearling Quarter Horses.

Item	Pelleted concentrate[1]	Hay[2]
DM, %	87.76	91.34
CP, %	17.60	8.90
ADF, %	11.39	33.45
NDF, %	23.79	59.71
Ca, %	0.83	0.37
P, %	0.68	0.14
K, %	1.37	1.10
Mg, %	0.33	0.19
Na, %	0.21	0.09
S, %	0.38	0.24
Al, ppm	170.00	457.00
Co, ppm	1.53	1.02
Cu, ppm	53.20	9.28
Fe, ppm	261.00	704.00
Mn, ppm	128.00	33.70
Mo, ppm	1.44	2.03
Zn, ppm	110.00	29.20
DE, Mcal/lb	1.52	1.15

[1] Commercially prepared pelleted concentrate (Producers 14% Horse Pellet, Producer's Cooperative Association, Bryan, TX).
[2] Coastal Bermudagrass (*Cynodon dactylon*).

APPENDIX B

PHYSICAL PARAMETERS

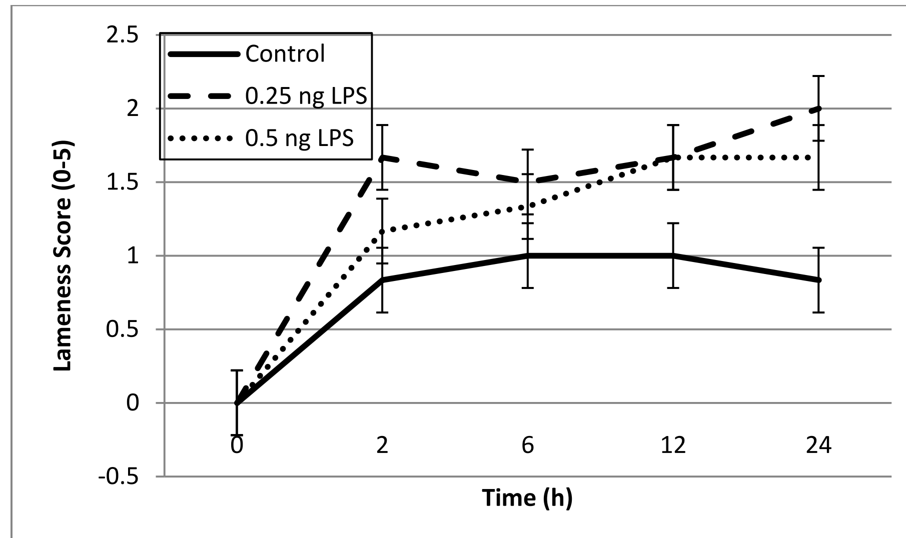

Figure B.1 Lameness scores (grading system 0-5, AAEP 1999) in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

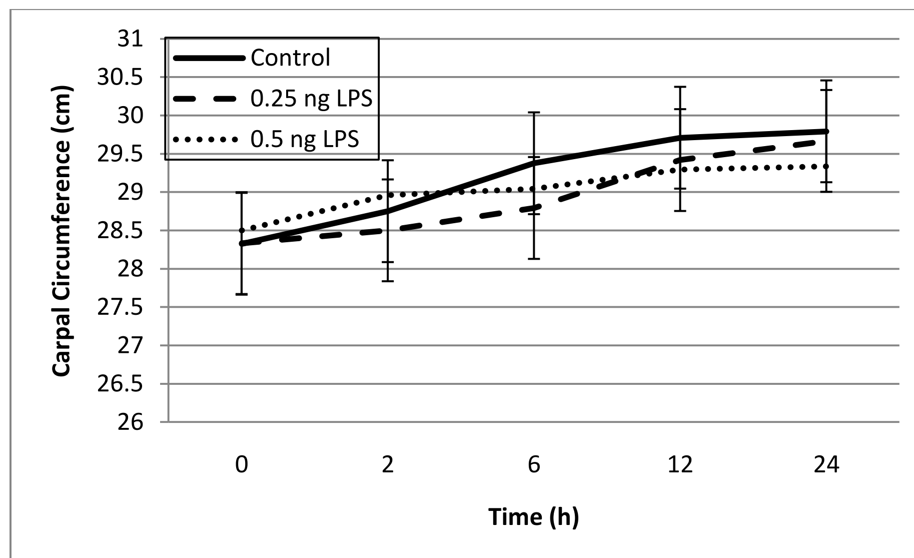

Figure B.2 Carpal circumference (cm) in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

APPENDIX C

LEUKOCYTES

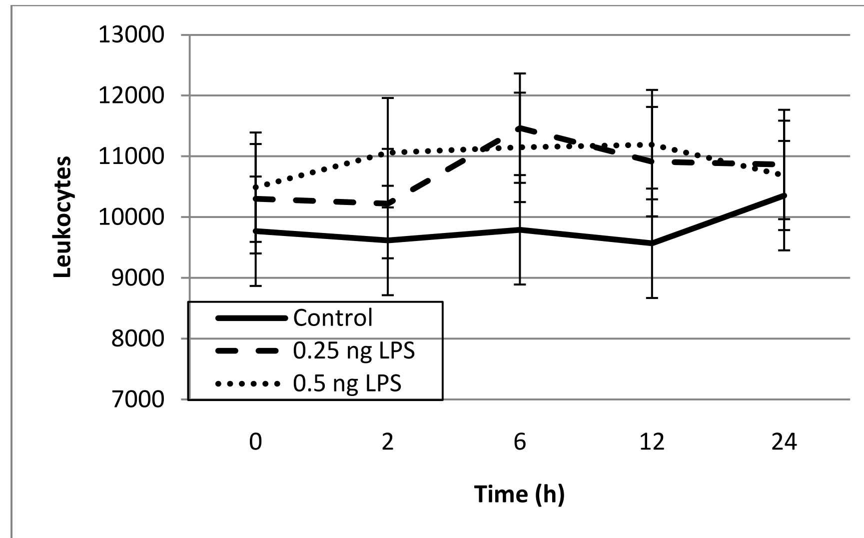

Figure C.1 Blood leukocyte count (cells × 1,000/µl) in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

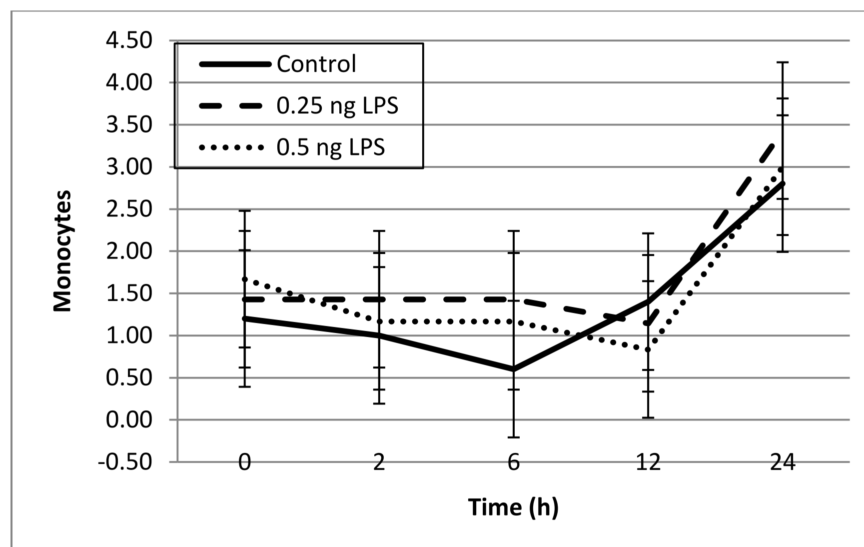

Figure C.2 Blood monocyte count (%) in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

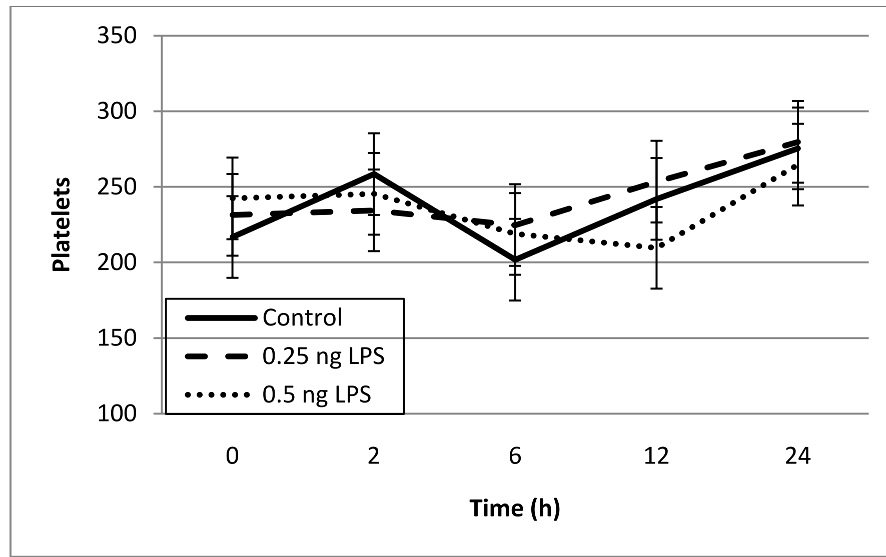

Figure C.3 Blood platelet count (cells × 1,000/μl) in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

APPENDIX D

GENE EXPRESSION

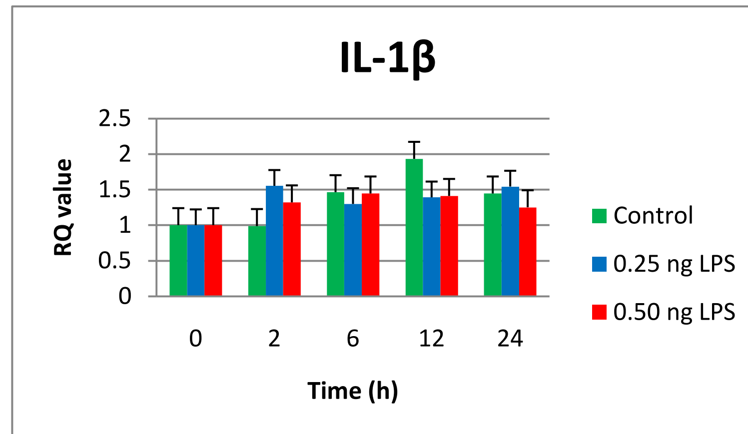

Figure D.1 Relative systemic gene expression of IL-1β in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

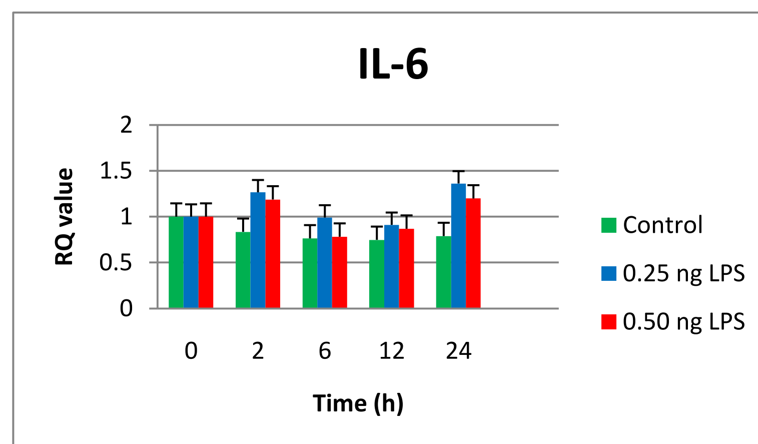

Figure D.2 Relative systemic gene expression of IL-6 in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

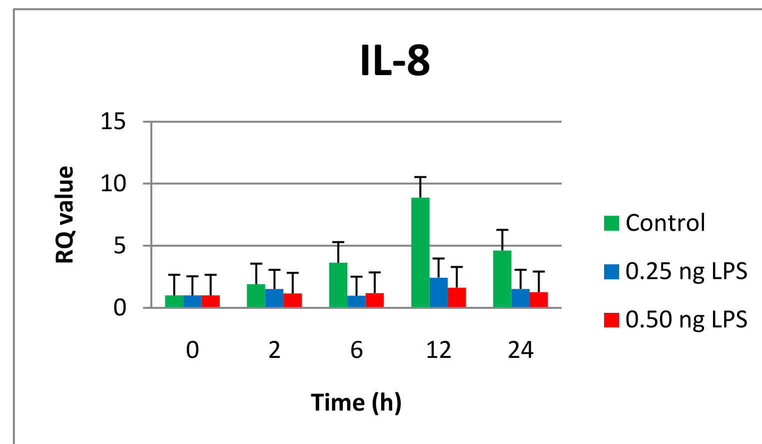

Figure D.3 Relative systemic gene expression of IL-8 in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

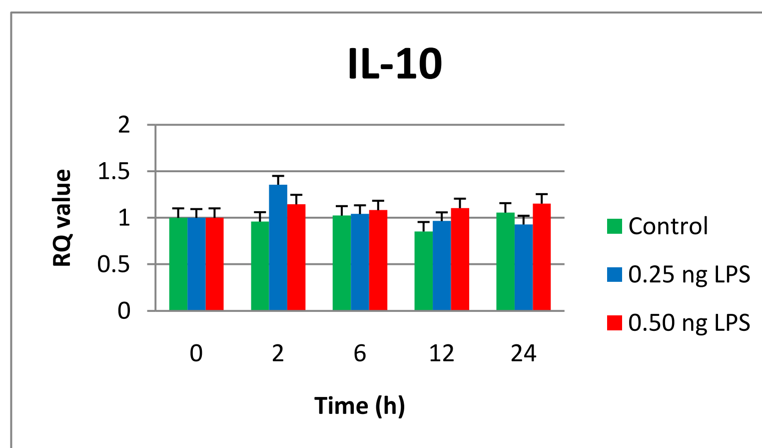

Figure D.4 Relative systemic gene expression of IL-10 in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

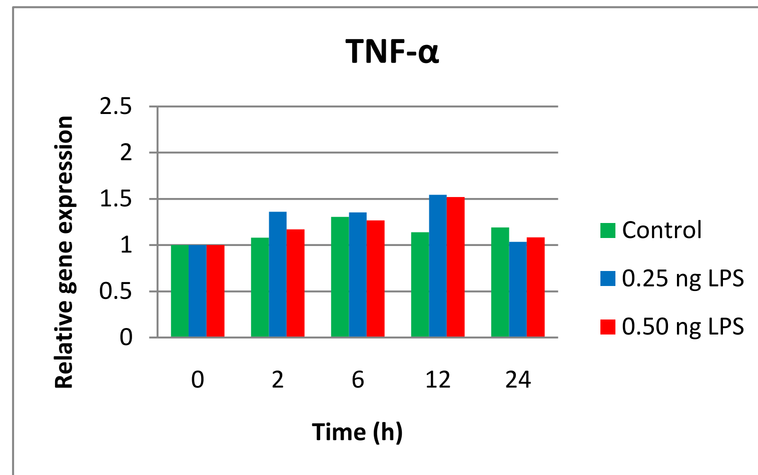

Figure D.5 Relative systemic gene expression of TNF-α in yearling Quarter Horses over the 24 h period following intra-articular lipopolysaccharide (LPS) injection of 0.25 ng, 0.5 ng, or 0 ng (control).

VITA

Name:	Carrie Lynn Mueller
Address:	Department of Animal Science c/o Dr. Sigler 2471 TAMU College Station, TX 77843-2471
Email Address:	CMueller@medicine.tamhsc.edu

Education: B.S., Animal Science, University of Wisconsin at River Falls, 2009
M.S., Animal Science, Texas A&M University, 2011