AGE-RELATED EFFECTS ON MARKERS OF INFLAMMATION AND CARTILAGE METABOLISM IN RESPONSE TO AN INTRA-ARTICULAR LIPOPOLYSACCHARIDE CHALLENGE IN HORSES

A Thesis

by

MEREDITH KAHN

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Chair of Committee, Josie A. Coverdale
Committee Members, Jessica L. Lucia
Thomas H. Welsh Jr.
Tryon A. Wickersham
Head of Department, H. Russell Cross

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ABSTRACT

Eighteen Quarter Horses were utilized in a randomized complete design for a 28 d experiment to evaluate age-related effects on inflammation and cartilage turnover after induction of a single inflammatory insult using lipopolysaccharide (LPS). Horses were grouped by age, with yearlings (yearling; $n = 3$ males, $n = 3$ females), 2 to 3 yr olds (2/3; $n = 2$ males, $n = 4$ females), and mature 5 to 8 yr olds (mature; $n = 2$ males, $n = 4$ females). On d 0, all horses were housed individually and fed diets that met or exceeded NRC (2007) requirements. On d 14, horses were challenged with an intra-articular injection of LPS. Carpal joints were randomly assigned to receive 0.5 ng LPS solution obtained from *E. coli* O55:B5, or 0.8mL sterile lactated Ringer's solution as a contralateral control. Synovial fluid was collected prior to LPS injection at pre-injection h 0 (PIH 0) and 6, 12, 24, 168, and 336 h post-injection. Samples were later analyzed using commercial ELISA kits for prostaglandin E_2 (PGE_2), collagenase cleavage neoepitope (C2C), and carboxypropeptide of type II collagen (CPII). Heart rate (HR), respiratory rate (RR), and rectal temperature (RT) were monitored over the first 24 h and carpal circumference and surface temperature were recorded with additional measurements at 168 and 336 h. Data were analyzed using PROC MIXED procedure of SAS.

Values for RT, HR, and RR were within normal range. HR and RT were influenced by age ($P < 0.01$), while RR was unaffected by age ($P \leq 0.21$). Joint circumference was not influenced by age ($P = 0.84$), but circumference and surface

temperature increased ($P < 0.01$) over time across all age groups. Synovial PGE_2 concentrations tended ($P = 0.09$) to be influenced by age with yearlings having lower ($P = 0.03$) concentrations than mature horses. Synovial C2C concentrations were affected by age with yearlings and 2/3 yr olds having lower ($P < 0.01$) concentrations than mature horses. Concentrations of synovial CPII were influenced by age with yearlings and 2/3 yr old having lower ($P \leq 0.02$) concentrations than mature horses. Ratios of CPII:C2C were influenced by age with mature and 2/3 yr old horses having increased ($P < 0.01$) values compared to yearlings. These results indicate that inflammation and corresponding cartilage turnover in response to LPS administration vary with age.

DEDICATION

For Mom, Dad, and Jeremy

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CHAPTER I

INTRODUCTION

Osteoarthritis (OA) is one of the most significant causes of lameness and decreased performance that often leads to early retirement of the equine athlete (Todhunter and Lust, 1990). Surveys estimate that up to 60% of lameness is related to OA (Caron and Genovese, 2003). In young horses, adaptation of bone and soft tissue occurs during growth and training, potentially leading to the overproduction of inflammatory mediators, including interleukins and prostaglandins, and the enzymatic breakdown of the articular cartilage (Brama et al., 1998; Kidd et al., 2001; Lucia et al., 2013). A metabolic shift toward synthesis takes place in the young equine model, serving to mend damage to the cartilage framework (Lucia et al., 2013). This response is not as well understood in skeletally mature horses, although it has been speculated that the process of cartilage turnover takes place at a much slower rate (Todhunter, 1996). While mature horses may be common subjects for the development of OA, it is probable that the disease process begins during skeletal immaturity with overloading during early training and exercise. Therefore, there may be differences in inflammation and cartilage metabolism responses between horses of varying age.

Lipopolysaccharide (LPS) is an established model of inflammation that is transient, local and repeatable. Various studies have utilized this model to study biomarkers of inflammation and cartilage turnover using young or mature horses (de Grauw et al., 2009; Lucia et al., 2013). Results showed variation across studies, which

may be due to differences in age of horse under investigation. Prostaglandin E_2 (PGE_2), collagenase cleavage neoepitope (C2C) and carboxypropeptide of type II collagen (CPII) are examples of markers of inflammation and cartilage metabolism that have been shown to vary in timeline and concentration across various studies utilizing the LPS model in young or mature horses (de Grauw et al., 2009; Lucia et al., 2013). While these differences exist, it may not be accurate to compare LPS data between studies from mature horses to the young, growing model.

It is clear that OA can be life changing for the athletic horse. Since current treatments focus solely on pain management and can have negative side effects, early preventative methods are a current focus in research. A study utilizing horses of varying age, exposed to the same experimental conditions is needed to clarify the differences that have been observed across studies for inflammation and cartilage metabolism. Further exploration could help identify an age range in which addition of preventative nutritional strategies or change in exercise protocol may be the most beneficial to the working horse.

CHAPTER II

REVIEW OF THE LITERATURE

Introduction

Equine OA is a naturally occurring, progressive disease of the synovial joints that often leads to lameness, poor performance and early retirement of equine athletes (Todhunter and Lust, 1990). The osteoarthritic joint is often characterized by damage to and reduction of articular cartilage matrix components, in addition to reduced joint function (McIlwraith, 2005). For maintenance of a healthy joint, the processes of degradation and synthesis involved in cartilage metabolism are adjusted for net growth and remodeling. In the osteoarthritic state, the balance of these processes is disrupted, resulting in a shift toward tissue degeneration (Kidd et al., 2001). Prolonged exposure to inflammatory mediators and degradative enzymes with chronic inflammation perpetuates cartilage destruction. The response to prolonged inflammation and cartilage metabolism may vary with age. The osteoarthritic diseased state exists primarily in the aged horse, but the disease condition results from cartilage degradation that likely begins early in the training process.

Synovial Joint Structure and Anatomy

The synovial joint functions to enable movement and to transfer load. Its structure is designed to facilitate these two major functions. A healthy joint relies on the integrity of normal anatomy and cellular function of all components. The synovial membrane, synovial fluid, articular cartilage and bone make up these components and

are structures that contribute to the development of joint disease. Understanding normal structure and function of the healthy synovial joint is helpful for a better understanding of what happens in a diseased state.

The synovial membrane lines the joint cavity and is composed of two layers: the intima and the subintima. The intima is an incomplete layer, one to four synoviocytes thick, with no basement membrane that overlies a layer of connective tissue called the subintima (Todhunter, 1996; Frisbie, 2006). Synoviocytes are a unique feature of the membrane, which have secretory and phagocytic functions including: phagocytosis and synthesis of hyaluronan, interleukins, prostaglandins, and proteases, all of which significantly affect the state of cartilage (Goodrich and Nixon, 2006).

The intimal layer of the synovial membrane is largely responsible for composition of the synovial fluid, which is a highly viscous, lubricating fluid and the medium through which nutrients reach articular cartilage (Todhunter, 1996). Hyaluronan and lubricin glycoproteins are synthesized by the synovial membrane cells and are secreted into the synovial fluid. These molecules are thought to be important for boundary lubrication of the joint surfaces and for regulating synovial fluid composition by steric exclusion of larger molecules from the synovial cavity (Frisbie, 2006).

The articular cartilage is a specialized connective tissue, elastic and compressible in nature, which covers opposing subchondral bone. It functions to reduce the stress applied to bone and to provide the nearly frictionless movement of the joint (Frisbie, 2006). Within the synovial joint, when cartilage loading results in increased hydrostatic pressure, water is released from the extracellular matrix, contributing to lubrication of

the joint surfaces (Dijkgraaf et al., 1995). Articular cartilage is avascular and alymphatic, depending completely upon diffusion through the cartilage matrix to and from the synovial fluid for nutrition and elimination of waste products (Dijkgraaf et al., 1995). The cartilage is also anueral, and pain perception within the synovial joints is dependent on nerve endings in the synovium, capsule, muscle, and subchondral bone (Dijkgraaf et al., 1995). Chondrocytes, the cellular component of articular cartilage, are responsible for synthesis and maintenance of the extracellular matrix (Neil et al., 2005). During normal activity and exercise, joint loading encourages diffusion of chondrocyte nutrients and waste products through the matrix (Dijkgraaf et al., 1995; Todhunter, 1996). The extracellular matrix is composed primarily of water, collagens and proteoglycans, with a small percentage of glycoproteins (Fig. 1). The biochemical composition of the extracellular matrix is responsible for the biomechanical features of the tissue, including resilience and elasticity (Dijkgraaf et al., 1995).

Collagen fibrils are the main component of the extracellular matrix, constituting about 60% dry weight of hyaline cartilage and providing the fibrous, structural backbone to the matrix (Dijkgraaf et al., 1995; Poole et al., 2002). The anatomical arrangement of collagen fibrils at various depths of cartilage provides structural support (Goodrich and Nixon, 2006). The collagen network provides cartilage with flexibility and strength and serves to counteract the swelling pressure of hydrophobic proteoglycans (Dijkgraaf et al., 1995). Collagen fibrils are more concentrated at the articular surface where they are required to resist tensile strain during weight bearing (Todhunter, 1996).

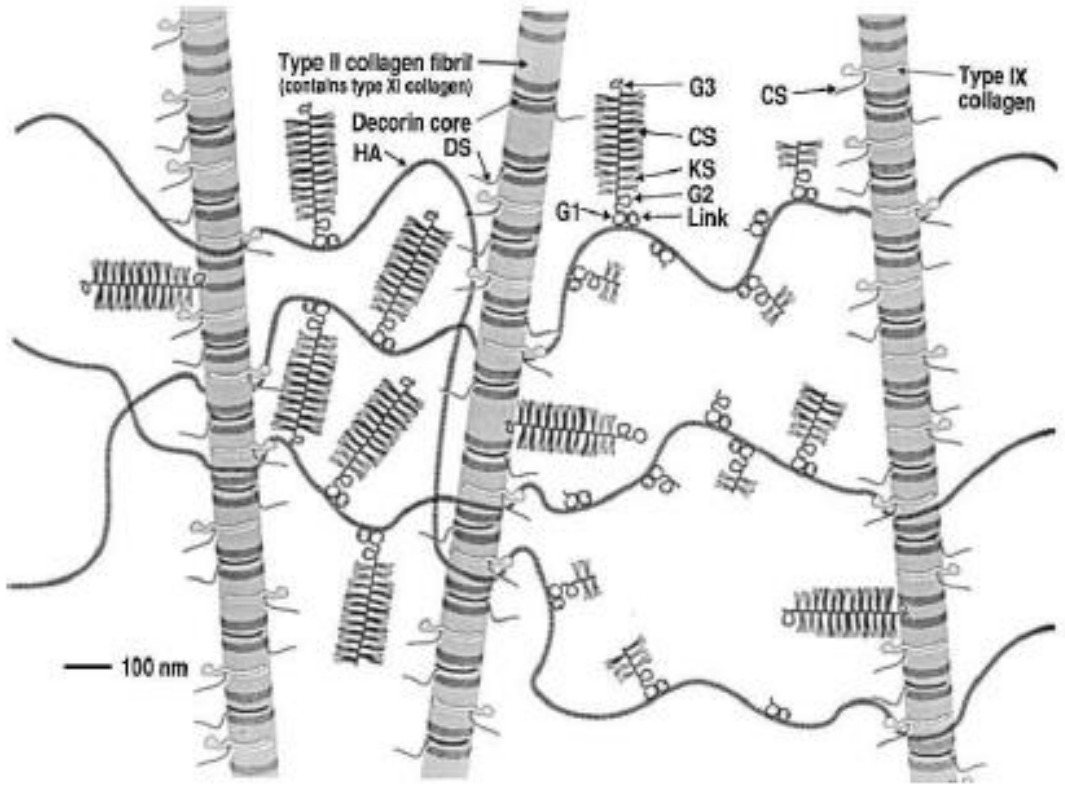

Fig. 1. Organization of the extracellular matrix components in articular cartilage. (Adapted from Frisbie, 2006).

Chondrocyte produced type II collagen is the primary collagen type found in articular cartilage, comprising about 85 to 90% of the total collagen content (Todhunter, 1996). Type II collagen is a homotrimeric right-handed helix, composed of three identical α_1(II) chains arranged in fibrils, that is thought to provide articular cartilage with its tensile strength (Todhunter, 1996; Frisbie, 2006). Each α chain contains nearly 1000 amino acid residues, and, except for the short sequences at the end of the chains, every third amino acid is glycine (Todhunter, 1996). The repeating triplet of amino

acids can be represented by Gly-X-Y, where X and Y represent other amino acids, 22% of which are proline and 4-hydroxyproline, respectively (Platt, 1996).

Fibril formation is critical for achieving the physiologic strength of the collagen framework (Ray et al., 1996). Type II collagen is synthesized and secreted from the chondrocyte as a procollagen molecule that contains nonhelical propeptide extensions at the amino and carboxy terminal ends (McIlwraith, 2005). Extracellular cleavage of these extensions yields the collagen molecule, with the cleaved extensions serving as useful markers to monitor collagen synthesis. As collagen is synthesized, the newly released type II collagen molecules associate in the matrix to form the collagen fibrils by means of intermolecular cross-linking (Platt, 1996). Cross-linking occurs once the basic fibril has been formed within the same collagen molecule and between adjacent collagen molecules, forming a 3-dimensional meshwork (McIlwraith, 2005).

Conversely, during the remodeling process, when type II collagen fibrils are cleaved by collagenases, normally hidden epitopes are uncovered and can be quantified to study cartilage metabolism (Todhunter, 1996). The helical portion of the type II collagen molecule can be cleaved across all three chains or in nonhelical sites (Platt, 1996; Billinghurst, 1997). During intrahelical cleavage, collagenases cleave the fibrillar type II collagen at a single site (Gly_{775}-Leu/Ile_{776}) within each α chain of the triple helical collagen molecule, about three quarters of the distance from the amino-terminal end of each chain (Billinghurst, 1997). This yields both three-quarter and one-quarter length collagen fragments (Billinghurst, 1997). These cleaved collagen fragments spontaneously denature and become susceptible to continued degradation by

collagenases, including MMP-1, -8, and -13, proteinases, such as MMP-3, and the gelatinases MMP-2 and -9 (Billinghurst, 1997).

Proteoglycans are complex macromolecules that make up the remaining 40% of the matrix. They consist of one or more glycosaminoglycan (GAG) chains covalently attached to a protein core (Todhunter, 1996). Negatively charged GAG side-chains repel each other and draw water into the extracellular matrix, providing compressive stiffness (Frisbie, 2006; Goodrich and Nixon, 2006). Aggrecan, the most abundant proteoglycan found in cartilage, associates with collagens and other proteoglycans, including hyaluronan. Link protein simultaneously binds hyaluronan and the aggrecan monomer, decreasing the dissociation constant. Hyaluronan functions to perform a space-filling or lubrication role within the synovial joint (Todhunter, 1996). As many as 100 aggrecan monomers associate with hyaluronan in the form of large aggregates, providing compressive stiffness, resiliency and durability (McDonald et al., 1994). The concentration of proteoglycan increases with increasing depth from the articular surface, most likely to resist compression (Todhunter, 1996).

The articular cartilage extracellular matrix is a dynamic system, constantly exposed to catabolic and anabolic factors (Dijkgraaf et al., 1995). In healthy cartilage, at each stage of development, relative rates of matrix synthesis and degradation are adjusted in order to achieve net growth or remodeling. Chondrocytes, while only constituting a small percentage of the cartilage, maintain and turn over the entire matrix (Todhunter, 1996). Dynamic load and cytokines, such as interleukins (IL) and tumor necrosis factor α (TNFα) have been shown to accelerate matrix turnover (Todhunter,

1996). These factors influence production and activation of degradative enzymes that can break down the matrix. This process is offset by the synthesis of enzyme inhibitors and growth factors, including insulin-like growth factor 1 (IGF-1) and transforming growth factor β (TGF β), that have been shown to increase the production of collagens and proteoglycans while reducing their degradation (Platt, 1996). Normal degradation of the matrix is under tight regulation. Matrix metalloproteinases 1 and 3 (MMP 1 and MMP 3), collagenase and stromelysin, respectively, degrade collagen and proteoglycan components of the matrix and are inhibited by tissue inhibitors of metalloproteinase 1 and 2 (TIMP 1 and TIMP 2; Todhunter, 1996). In normal articular cartilage, there is a slight excess of TIMPs over MMPs (Dean et al., 1989). Pathologic processes, such as degenerative joint disease (DJD), that involve gradual deterioration of the extracellular matrix of articular cartilage may result from a shift in the tight regulatory control of chondrocyte metabolism.

By increasing the rate of degradation and decreasing synthesis, cytokines induce the depletion of proteoglycan in normal cartilage (Todhunter, 1996). Interleukin-1 (α and β; IL-1α and β) has been studied with regard to its effect on chondrocyte and synovial lining cell metabolism. Activity of IL-1 is present in normal equine synovial fluid, and induction of equine articular cartilage degradation occurred when recombinant human IL-1 was added to explant culture (McDonald et al., 1994; May et al., 1994a). *In vitro* studies have confirmed IL-1 as a potent stimulator of MMP secretion by chondrocytes in normal equine cartilage (McDonald et al., 1994). The effects of IL-1 on articular cartilage metabolism can be repressed by IGF-1 and transforming growth

factor-β (TGF-β). Hyaluronan has also been shown to inhibit IL-1 mediated proteoglycan degradation in bovine articular cartilage explants (Morris et al., 1992). Antagonistic effects of IL-1 on synovial lining cells may occur through the action of inhibitors residing in equine synovial fluid (May et al., 1994a).

The synovial joint relies on the health of each of its components to function properly, with particular emphasis placed on cartilage maintenance. In order to remodel and maintain articular cartilage, a certain amount of joint loading is required, which can be accomplished by normal activity and exercise. However, overloading of cartilage can lead to overproduction and prolonged exposure to deleterious enzymes and inflammatory mediators. Chronic exposure to inflammation and overloading of the joint, which can result from overtraining, can shift normal homeostatic processes of cartilage metabolism. This response to loading and overloading for the joint varies with age of horse.

Effects of Age on Articular Cartilage Metabolism

There are few reports on the effects of ageing and maturation on healthy and diseased equine articular cartilage. Adaptation of bone and soft tissue occurs during growth and training in the young equine model (Brama et al., 1998). During this adaptation, horses experience repetitive trauma and stress to synovial joints, which can result in an overproduction of inflammatory mediators, eventually leading to breakdown of the articular cartilage (Kidd et al., 2001). Early modifications can influence mature cartilage, known to have little capacity for repair and remodeling (Todhunter, 1996). Maroudas et al. (1992) stated that due to slow turnover of collagen in mature articular

cartilage, the collagen framework is laid down prior to maturity and undergoes little change thereafter. This statement was supported by a later study, which demonstrated that enzymatic modifications of the collagen network are not affected by age after maturity in both human and equine articular cartilage (Brama et al., 1999). Little and Ghosh (1997) hypothesized that the regional chondrocyte phenotype of mature cartilage results from factors such as weight-bearing and articulation, imposed on the joint postpartum. If heterogeneous characteristics of the collagen network are induced by biomechanical loading, early changes may be decisive for the final makeup of the tissue and, consequently for future strength and resilience to injury.

During periods of rapid growth, in the initial stages of life, composition of the extracellular matrix changes dramatically. Brama et al. (1998) found MMP activity in the synovial fluid of joints of juvenile horses, 5 to 11 mo in age, to be almost twice that of adult joints, aged 4-30, indicative of a greater matrix turnover in juvenile horses. With the completion of this maturation phase, the cartilage remodeling continues, but at a slower rate, as the need for greater tissue turnover for net growth decreases (Todhunter, 1996; Brama et al., 2000). Brama et al. (2000) investigated functional adaptations of equine articular cartilage, and discovered that the foal is born with a biochemically homogeneous joint with regard to proteoglycan and collagen content, as well as, the posttranslational modifications of the collagen network, such as cross-linking. It was also determined that the content of water, DNA and GAGs declines significantly during maturation, while the content of collagen and hydroxylysine increase (Brama et al., 2000). The water content of articular cartilage has been shown to

decrease with age in numerous species, including horses (Kempson, 1980; Todhunter, 1996). Decreases in DNA content have also been shown in other species, verifying cellular density of articular cartilage declines with age up to the time of skeletal maturity (Stockwell, 1971; Igarashi and Hayashi, 1980).

With increasing age, composition of the extracellular matrix changes and this is accompanied by reduced sensitivity of chondrocytes to cytokines, demonstrated by McDonald et al. (1994) using recombinant human IL-1 (Todhunter, 1996). Results showed increased GAG synthesis in cartilage explants from young horses, aged 11 mo to 1 yr, in response to recombinant human IL-1 exposure. It was also shown that for a given increase in IL-1 dose, the change in GAG synthesis was greater for cartilage from young horses than mature horses. Fubini et al. (1993) concluded similar results *in vivo*, demonstrating an age-effect response on equine articular cartilage injected with the corticosteroid methylprednisolone acetate. Younger ponies, aged less than 10 yr, showed more severe depletion of proteoglycan than older ponies, aged greater than 10 yr, signifying decreased chondrocyte responsiveness as horses age. Therefore, depending on the age under investigation, healing and restoration of articular cartilage will vary.

According to the available research, it appears that the composition and responsiveness of equine articular cartilage changes with age. The processes of degradation and synthesis involved in cartilage metabolism differ depending on the age of horse, with younger, growing horses experiencing a metabolic shift toward synthesis to achieve net cartilage synthesis. While this continues in the mature horse, it does so at

a much slower rate. Although normal articular cartilage metabolism seems to vary among immature and mature horses, differences may also exist when the natural balance of degradation and synthesis is disrupted in a diseased state.

Joint Disease in Horses

The term OA is a broad description of a group of disorders characterized by varying degrees of articular cartilage deterioration, often accompanied by changes in the bone and soft tissue (Todhunter, 1996). Factors associated with the pathogenesis of OA include age, genetic predisposition, and excessive mechanical loading of the joint (Briston et al., 2009). Clinical manifestation mostly commonly involves a slowly progressive lameness with probable joint pain, decreased range of motion and variable joint effusion and inflammation (Kidd, et al. 2001). Inflammation that gives rise to secondary synovitis within the synovial lining and joint capsule usually manifests later from irritation due to the release of articular cartilage degradation products (Briston et al., 2009). Within a diseased joint there is a disturbance in the normal balance between degradation and repair of articular cartilage as evidenced by net cartilage degeneration (Kidd et al., 2001). The pathogenesis of OA is complex and the disease can develop in a number of different ways. Still, the result is consistent, often producing clinical symptoms of pain and lameness in the horse.

Inflammation as a result of repeated trauma, also termed use trauma, is common in the joints of athletic horses (Palmer and Bertone, 1994b; Kidd et al., 2001). A common clinical characteristic in the carpal and fetlock joints of young, actively training racehorses, for example, is primary synovitis in the absence of articular cartilage

damage, defined as inflammation of the synovial membrane (McIlwraith, 2002). Primary synovitis occurs as a result of severe injury or inadequately treated injuries. With sufficient rehabilitation and rest, horses can recover. Yet, in reality, the rest period is often shortened or neglected completely and horses often return to work too soon since it can be difficult to objectively monitor recovery. The result is prolonged production and exposure to inflammatory mediators, such as IL-1 and prostaglandin E_2 (PGE_2), and the subsequent release of enzymes, such as MMPs, with potential to damage the articular cartilage matrix. Chronic joint inflammation may eventually result in an imbalance of normal articular cartilage metabolism, with an increase in matrix destruction, as evident by alteration in the biomechanical properties of the articular cartilage and resultant limitations in the athletic capabilities of the horse (Palmer and Bertone, 1994b). Matrix degradation is often accompanied by an increase in the synthesis of type II collagen and aggrecan, however these newly synthesized molecules are often damaged, compromising any effective attempts at repair (Poole et al., 2002). Ultimately, the combination of insufficient rest and sustained work will lead to reduced performance and lameness.

Mechanical factors of joint loading play a role in the pathogenesis of OA via use trauma. In the healthy joint, a certain level of joint loading is required for lubrication and production of the matrix components. Through repeated loading and hyperextension of the limbs, the equine athlete is constantly testing the ability of the joint to withstand mechanical demand. Under normal conditions, stimulation of extracellular matrix synthesis, namely proteoglycan, is dependent on the magnitude and duration of applied

load. Strenuous exercise decreases GAG concentrations and can promote remodeling of the subchondral bone, decreasing the natural compressive stiffness of the articular cartilage (Palmer and Bertone, 1994b). Therefore, while moderate and cyclic compression is beneficial, heavy and/or continuous loads can be harmful to the joints (Palmer and Bertone, 1996).

Release of cytokines and inflammatory mediators is up regulated in OA compared to the healthy joint. Catabolic cytokines IL-1 and TNFα increase beyond normal levels, promoting production of MMPs and PGE_2 and inhibiting synthesis of key matrix components such as aggrecan and type II collagen (Frisbie, 2006). Bertone et al. (2001) found significant differences in PGE_2 concentrations between normal and diseased joints from 89 horses aged 3 to 10 yr and subsequently described PGE_2 above a concentration of 22.5 pg/mL as a good-excellent marker for any joint disease. IL-1 has also been shown to amplify the catabolic cascade by inhibiting production of naturally occurring anti-arthritic molecules, including interleukin-1 receptor antagonist (IL-Ra) and TIMPs (Frisbie, 2006). Anabolic cytokines such as IGF and TGF promote chondrocytes to synthesize proteoglycans and type II collagen (Frisbie, 2006). Such molecules play a role in osteoarthritis by promoting reparative efforts in diseased joints (Frisbie, 2006).

The release of cytokines and inflammatory mediators initiates action of deleterious enzymes that are also active in healthy joints. The MMPs collectively possess the ability to degrade all major components of articular cartilage and, therefore, play a large role in both health and disease of the articular cartilage. A key deleterious

effect of acute inflammation resides with the ability of IL-1 to encourage production and activation of matrix destructive MMPs. In a diseased joint, the normal homeostasis of anabolic chondrocyte synthetic activity and enzymatic breakdown of the matrix is disrupted. Research suggests MMPs 1 and 3 are particularly important in the pathogenesis of OA (Todhunter, 1996). Brama et al. (1998) found elevated MMP activity in the synovial fluid of OA adult metacarpophalangeal joints compared to normal joints, indicative of a shift toward net degradation of tissue.

Structural changes with OA include alterations in the articular cartilage. Mild OA manifests as fibrillation of the cartilage surface with some loss of chondrocytes, accompanied by initial increases in the water content (Kidd et al., 2001). As the collagen framework of the matrix is further disrupted, water is increasingly attracted to the negatively charged GAGs, causing cartilage to swell. Eventually resistance to force is reduced and deep clefts develop with the progression of OA. This process can be characterized microscopically by a loss of proteoglycan. With continued damage to the cartilage, small pieces of cartilage and subchondral bone are released into the joint space, further encouraging the inflammatory cycle (Kidd et al., 2001).

The joint capsule becomes increasingly vascular and starts to thicken in response to inflammation in OA. A cyclic inflammatory cycle begins with the release of inflammatory mediators and cytokines, such as IL-1 and TNFα from synoviocytes and chondrocytes. With the release of MMPs and cytokines the inflammatory cycle is perpetuated, causing hypertrophy of the synovial lining of the joint (Kidd et al., 2001). While the synovial membrane itself has no known role in the biomechanics of the joint,

injury to this area may have negative consequences for the joint. Damage to synoviocytes potentially causes release of degradative enzymes and cytokines, which ultimately contribute to changes in the articular cartilage (Evans, 1992).

Pain associated with OA is a common reason for the development of joint-related problems in the horse. While the articular cartilage itself is aneural, surrounding tissues are not and have been defined as sources of pain in cases of OA (Frisbie, 2006). Sensory nerves in these tissues respond to chemical mediators and mechanical stimuli. With joint inflammation, local mediators are released that act on these nerves to stimulate activation directly or lower the threshold to other stimuli (Schaible et al., 2002; Hinz and Brune, 2004). Substance P, an 11-amino acid neuropeptide, is released into the synovial fluid from articular type C nerve fibers, and has been suggested as a mediator responsible for equine articular pain (Caron, 1996; de Grauw et al., 2006). Multiple studies have demonstrated increased levels of synovial fluid substance P in arthritic versus normal equine joints (Caron et al., 1992; Kirker-Head et al., 2000). de Grauw et al. (2006) found higher levels of substance P in clinically painful joints that responded positively to intra-articular anaesthesia (IAA), suggesting a direct role in articular nociception in horses.

Prostaglandins, specifically PGE_2, are also thought to play a role in pain associate with equine OA because of the pain relief from treatment with non-steroidal anti-inflammatory (NSAID) drugs (May et al., 1994b). Elevated levels of PGE_2 have been found in previous experiments with horses diagnosed with various types of joint disease when compared to sound, non-arthritic horses (May et al., 1994b; Bertone et al.,

2001). Researchers have concluded that PGE_2 is a good indicator of joint disease and correlates with pain and severity of lameness. The pathological changes and clinical manifestations associated with equine OA vary and are often undetectable before the disease is well established. Early detection is key for prevention and current research is focusing on methods for continued improvement in this area by studying OA in the horse using various models to induce experimental osteoarthritic conditions.

Experimental Joint Disease

A model that produces joint disease is necessary for solving important questions related to equine joint pathophysiology and repair. Previous studies have utilized intra-articular injection with cartilaginous particles or monoiodoacetate to stimulate cartilage destruction (Hurtig, 1988; Gustafson et al., 1992). These models failed to provide information on the effects of synovial inflammation on articular cartilage in the absence of primary cartilage damage. Other studies have applied more extreme techniques such as euthanasia or permanent mechanical alteration to the joint to mimic acute joint disease (Frisbie et al., 2008). Utilization of small doses of intra-articular *E. coli* derived LPS, an integral structural component of the outer leaflet of the outer membrane of all gram-negative bacteria, has been established as a model that mimics acute, local synovitis in horses and provides a method for evaluation of cartilage metabolism and inflammatory markers without permanent alteration to the joint (Raetz et al., 1991; Palmer and Bertone, 1994a). Horses are thought to be 10 times more susceptible to LPS when compared to other mammals, suggesting that equine chondrocytes may also be more responsive to LPS (MacDonald et al., 1994). This high level of sensitivity may

predispose horses to rapid cartilage breakdown when synovial joints are exposed to LPS. This is an important consideration for the utilization of this model to produce severe, but transient joint inflammation.

The LPS endotoxin acts by reacting with receptors present on the membrane of target cells, resulting in release of various inflammatory mediators including cytokines and eicosanoids (Morris, 1991). When interacting with the cell surface of macrophages, LPS activates membrane-bound phospholipase A_2, liberating arachidonic acid and increasing its metabolism via the cyclooxygenase and lipoxygenase enzyme systems (Fig. 2). This ultimately produces physiologically active eicosanoids including PGE_2, a cyclooxygenase end product of arachidonic acid (Morris, 1991). Previous investigators have used varying doses of *E. coli* derived LPS in the horse to produce a temporary, local inflammatory response similar to clinical inflammatory conditions. Firth et al. (1987) utilized high doses of LPS, ranging from 25 to 45 μg, in the middle carpal joint of ponies to produce a severe, sterile arthritis as a model to study infectious arthritis. All ponies were reported as lame within 2 h post-injection and all showed systemic signs of endotoxemia, including fever and depression, all of which subsided by 36 h post-injection (Firth et al., 1987). A decreased dosage of 3 μg/joint was used more recently to study the response of the antebrachiocarpal joint to a severe inflammation with respect to eicosanoid and cytokine production (Hawkins et al., 1993). Injection induced signs of acute inflammatory arthritis, including increases in PGE_2 concentration in principal

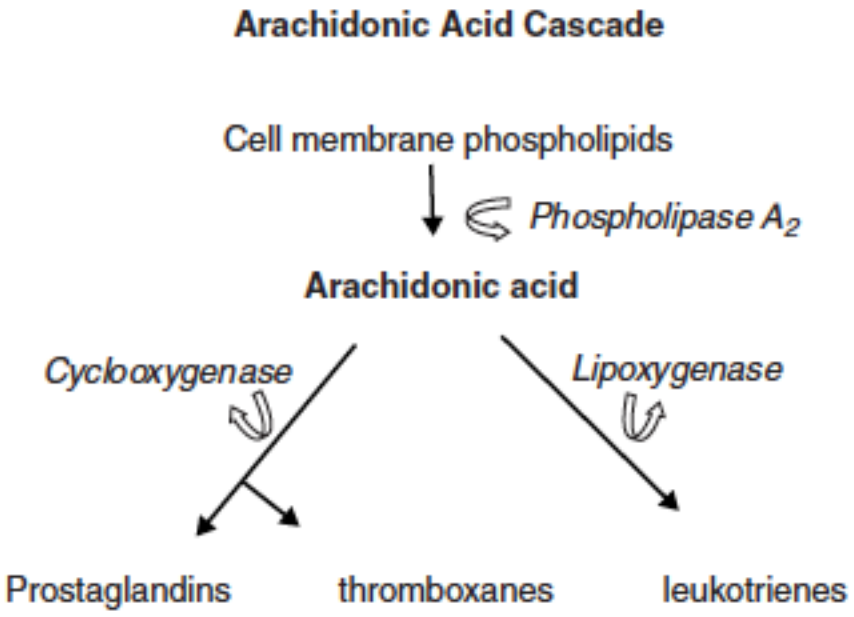

Fig. 2. The arachidonic acid cascade (Adapted from Caron and Genovese, 1993).

versus control joints, yet only mild, transient discomfort and minimal systemic effect. Palmer and Bertone (1994a), utilizing mature horses, found LPS doses of 0.125 ng to 5,000 ng per joint adequate to induce synovitis clinically similar to acute synovitis in the horse. Synovial fluid total nucleated cell count and total protein were linearly related to increasing dosages of intra-articular LPS ranging from 0.125 to 0.5 ng. Intra-articular LPS injections of 0.5 ng resulted in mild to moderate joint effusion without any signs of systemic illness, while doses greater than 0.5 ng caused fever, depression and non-weight-bearing lameness (Palmer and Bertone, 1994a). The 0.125 ng dose produced mild to moderate effusion and warmth of the injected joint, mild resistance to palpation and grade 2 lameness, yet in order to maintain synovitis, repeated injections were

performed at 48, 96 and 144 h. Other studies since have used 0.25 ng and 0.5 ng of LPS to prevent the need for multiple injections.

More recent studies have confirmed use of LPS in small doses for the induction of temporary, local joint inflammation. Meulyzer et al. (2009) observed no signs of systemic illness in mature horses (aged 14.4 ± 2.1 yr) injected with 0.25 and 0.5 ng intra-articular LPS into both radiocarpal joints. Resultant pain and lameness was resolved by 48 h post-injection. Also using mature horses (aged 5 to 8 yr), de Grauw et al. (2009) found the clinical response to LPS and changes in routine synovial fluid parameters to closely parallel previous results reported by Meulyzer et al. (2009) and Palmer and Bertone (1994a). An overt inflammatory response, prompted by 0.5 ng intra-articular LPS assigned to a random carpal joint, was transient and did not produce systemic signs of endotoxemia, confirming effects as localized and limited to the injected joint. Following a similar protocol to induce inflammation, Lucia et al. (2013) demonstrated differences in the relative time course and concentrations of cartilage and inflammatory mediators in the carpal joints of weanling horses (aged 184 to 327 d) injected with 0.25 ng and 0.5 ng intra-articular LPS. A contralateral control, injected with 0.8 mL sterile lactated Ringer's solution (LRS), was utilized to establish the effects of intra-articular LPS compared to the effects of repeated arthrocentesis alone. Synovial PGE_2 levels increased in both the LPS and LRS-injected joints, yet levels were higher in response to 0.25 and 0.5 ng intra-articular LPS, demonstrating the importance of a sham-injected control joint in these types of studies (Lucia et al., 2013).

LPS in ng quantities is an established model that can be used to study the inflammatory response in equine synovial joints, without permanent alteration to the joint. Previous studies have utilized this model to investigate levels of PGE_2, however other processes, including the process of cartilage metabolism in response to a single inflammatory insult using LPS, are also the subject of much of the current research. Measurement and analysis of synovial fluid biomarkers can provide valuable information for investigating diagnostic differences between diseased and healthy joints, to distinguish the degree of degradation in articular cartilage and to assess potential variation in these factors between horses of different ages.

Synovial Fluid Biomarkers

The term biomarker is a general term used to describe direct or indirect indicators of abnormal tissue turnover (McIlwraith, 2005). These markers are usually molecular products formed during normal metabolic processes. However, with diseases like OA, when the natural balance of anabolism and catabolism is altered, concentrations of biomarkers may increase or decrease. With disruption to the articular cartilage, biomarkers are released into the synovial fluid. These markers can indicate repair, synthesis, and degradation of articular cartilage, increased bone turnover or inflammation, and can be used to help quantify these processes (Kidd et al., 2001). When comparing concentrations of biomarkers utilized across several previous studies, it seems there is an age related response to induced inflammation.

Several biomarkers have been implicated for measuring inflammation and cartilage metabolism. A number of cytokines and eicosanoids can be used as indirect

biomarkers that are not derived primarily from the tissues that make up the joint, but have the potential to influence the metabolism of these tissues or the integrity of the matrix (McIlwraith, 2005). Bertone et al. (2001) concluded that IL-6 is sensitive and specific, while TNFα and IL-1β cannot adequately detect the presence of joint disease in general. van den Boom et al. (2005) observed increases in synovial fluid TNFα 2 h post exercise in response to exercise and repeated arthrocentesis. However, Jouglin et al. (2000) found no correlation between degree of joint damage and TNFα levels. Conclusions have also been made about eicosanoids, including PGE_2, which has been indicated a good to excellent marker of joint disease (Bertone et al., 2001). Gibson et al. (1996) reported greater PGE_2 concentrations in diseased middle carpal or antebrachiocarpal joints compared to control joints. No differences were found in mean concentrations of other eicosanoids: prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), and leukotriene B_4 (LTB_4) (Gibson et al., 1996). Bertone et al. (2001) found that concentrations of thromboxane B_2 (TXB_2) were actually less in severe, acute, and chronic joints than normal joints, and therefore only concentrations below 31.5 pg/mL were reported as a very good predictor of joint disease. Eicosanoids are released into synovial fluid with most any joint disease, with some variation among eicosanoids, and are therefore a more sensitive indicator of joint disease in general (Bertone et al., 2001).

Synovial PGE_2 is an eicosanoid and mediator of local inflammation that has been identified as an excellent marker of OA in naturally occurring arthritis (Bertone et al., 2001). Eicosanoids are released into the synovial fluid with most any joint disease, and

are a sensitive indicator of joint disease in general. While PGE_2 is elevated with any joint disease, there is little information in the literature to establish normal values (Bertone et al., 2001). Synovial PGE_2 concentrations increase in response to intra-articular LPS in both young and skeletally mature horses (de Grauw et al., 2009; Lucia et al., 2013). Lucia et al. (2013) reported peak PGE_2 values at 12 h post LPS injection in young horses while de Grauw et al. (2009) saw increases at 8 h post-injection in mature horses. Both studies observed sharp, short-lived increases in synovial PGE_2 concentrations, yet the variations between studies may be due to differences in the age of horse under investigation.

Briston et al. (2009) reported age-related differences in PGE_2 synthesis by equine cartilage explants from 3 age groups: A ($\leq$ 10 yr), B (11-20 yr), and C ($\geq$ 20 yr). Explants from group A were the least sensitive to LPS, while explants from group C were the most responsive. Increases in PGE_2 concentrations were observed for aged horses (group C) in response to LPS concentrations of 10 μg/mL by 18 h and by 24 h and all subsequent times for LPS concentrations of 1.0 μg/mL. Explants from young horses (group A) were resistant at all concentrations (0.1 to 100 μg/mL) and at all times between 12 and 48 h. Group B horses demonstrated a strong initial response to 10 μg/mL LPS at both 12 and 18 h, however PGE_2 concentrations did not increase after this time. Results from this study demonstrate increased PGE_2 concentrations with age when exposed to LPS, and the continued release of inflammatory mediators like PGE_2. Synovial concentrations of PGE_2 and length of exposure may contribute to the activation of degradative pathways via chondrocytes and play a role in the progression of OA.

Ultimately this could lead to degenerative changes in articular cartilage in response to mechanical load or the enhanced degradative sensitivity in older horses (Briston et al., 2009).

Cartilage metabolism can be measured using biomarkers for the degradation and synthesis of the two major articular cartilage components: type II collagen and proteoglycan. Antibodies have been developed to identify type II collagen fragments that have been cleaved and/or denatured, uncovering neoepitopes that were formerly inaccessible (Fig. 3; Hollander et al., 1994). Elevated levels of degraded type II collagen have been demonstrated in synovial fluid and serum from horses, dogs, and rabbits using various experimental models for OA (Billinghurst et al., 1997). The COL2-3/4C_{short}, also known as C1,2C, immunoassay detects collagenase-cleaved collagen fragments, but is not specific, detecting the degradation of both types I and II collagens (McIlwraith, 2005). Keratan sulphate (KS) is a specific GAG molecule found in proteoglycan molecules of aggrecan that has been evaluated as an indicator of cartilage turnover. However, in one study, synovial fluid KS concentrations were found to be less valuable indicators of joint disease despite findings that total sulphated GAG levels were found to be useful (Palmer et al., 1995). Additionally, Frisbie et al. (1999) concluded KS to be a marker with little potential value in the horse. Chondroitin sulphate (CS) is the major GAG of aggrecan and has proven to be a useful marker of aggrecan synthesis (McIlwraith, 2005). Frisbie et al. (1999) demonstrated higher synovial fluid CS-846 concentrations in joints with osteochondral fragmentation than in control joints, indicating higher re-synthesis of aggrecan in OA compared to normal joints. The CS-

846 epitope is found in fetal and OA cartilage, but is almost completely absent in healthy, mature cartilage, indicating that it may not be a reliable biomarker to measure the effects of age on cartilage metabolism (McIlwraith, 2005).

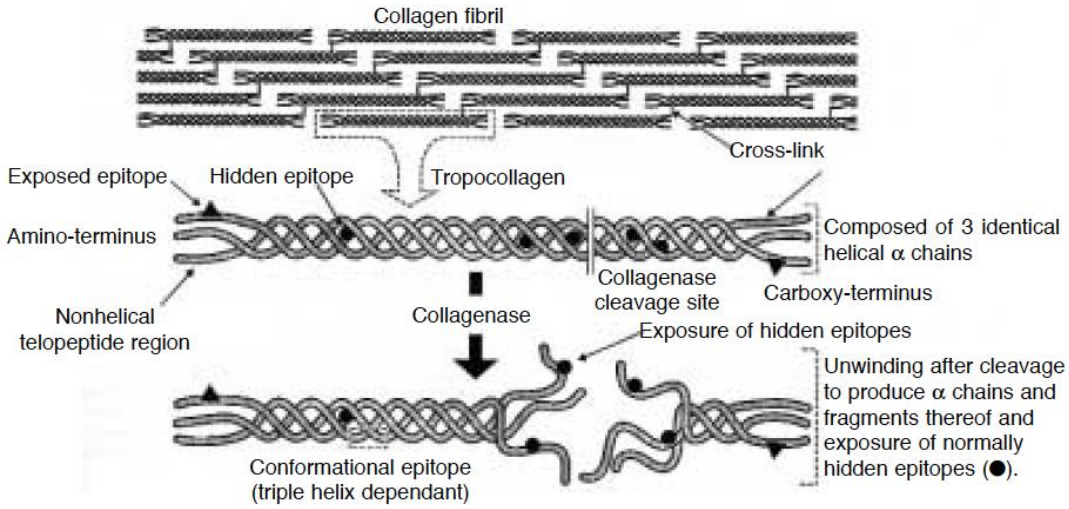

Fig. 3. Illustration of the principle behind detecting a marker of collagen degradation. Specific epitopes are direct markers, but most indirect markers are also detected with help of antibodies. (Adapted from McIlwraith, 2005).

Direct biomarkers of cartilage turnover include breakdown products of type II collagen and proteoglycan fragments, which are liberated in increased concentrations into the synovial fluid (Ray et al., 1996). Initiated by IL-1, this catabolic process in articular cartilage leads to the stimulated release of MMPs that have been implicated as useful biomarkers to monitor early OA (Brama et al., 1998; McIlwraith, 2005). In

humans, total MMP activity was shown to be 136% increased in OA joints compared to control joints. van den Boom et al. (2005a) found increased concentrations of equine synovial MMP-1 in response to inflammation as a result of repeated arthrocentesis. In another study, synovial MMP activity was higher in in adult (mean age 13.4, range 4-30) osteoarthritic metacarpophalangeal joints than in normal joints (Brama et al., 1998). Results from the same study also demonstrated an age-related effect on MMP activity with decreased activity in normal adult metacarpophalangeal joints compared to normal juvenile (aged 5 to 11 mo) joints (Brama et al., 1998). Since MMPs are essential for matrix turnover and because they play a key role in OA, they have the potential to act as useful tools to assess the status of articular cartilage.

In healthy cartilage, rates of matrix synthesis and degradation vary depending on maturity and are adjusted to achieve net growth or remodeling (Todhunter, 1996). The major biomarkers of collagen turnover in cartilage are epitopes of type II collagen (Garvican et al., 2010). With OA, normal joint metabolism homeostasis is disrupted. Degradation exceeds repair and the result is a net loss of articular cartilage. With continued exposure to inflammation, the reparative efforts of articular cartilage fail and collagen is cleaved by collagenases. This degradative process forms tropocollagen, which is composed of three identical α helixes each containing an amino and a carboxy terminus. Further degradation of tropocollagen by collagenases produces α chains and peptide fragments that expose epitopes that are normally hidden (Todhunter, 1996). These small peptide fragments are taken up by the chondrocytes, enter into circulation, and are further degraded by lysosomal enzymes or diffuse into the synovial fluid.

Peptide fragments can be easily measured in synovial fluid or serum and can potentially help identify early or subtle tissue damage.

Type II collagen is the major component of the extracellular matrix and in OA, degradation of type II collagen increases (Poole et al., 2002). Analysis of these breakdown product fragments can provide valuable information with regard to cartilage turnover. The C2C antibody recognizes collagenase-cleaved type II collagen fragments Garvican et al., 2010). The concentration of C2C increases in anterior cruciate transection in dogs (Matyas et al., 2004). Chu et al. (2002) found increased synovial fluid C2C concentrations at 4, 8 and 16 weeks after induction of experimental arthritis using monopolar radiofrequency energy applied arthroscopically to the cranial cruciate ligament compared to normal dogs. These findings indicate that the C2C antibody may serve as a useful biomarker to determine the condition of a joint, and particularly articular cartilage

There are limited studies investigating effect of age on levels of synovial fluid C2C in horses. However, two different studies found increased levels of catabolic C2C in weanling and skeletally mature horses subjected to a single inflammatory insult using 0.5 ng LPS and a similar arthrocentesis protocol (de Grauw et al., 2009; Lucia et al., 2013). In young horses, C2C concentrations decreased from 0 to 6 h, but peaking at 24 h post LPS injection (Lucia et al., 2013). A similar rise in C2C was seen in mature horses, with peak values also occurring at 24 h post-injection, however, values were 2 times greater (800 ng/mL; de Grauw et al., 2009) in mature horses than those (400 ng/mL; Lucia et al., 2013) observed in young horses. While these results suggest that

levels of C2C are influenced by LPS injection, there also may be a varied response with age of horse.

During fibril formation, carboxypropeptide of type II collagen (CPII) is proteolytically cleaved from the procollagen strand and, thus represents a marker of type II collagen synthesis (Fig. 4). The concentration of this peptide has been directly related to the rate of collagen synthesis, which increases in arthritic joints (de Grauw et al., 2006). A metabolic shift towards cartilage synthesis that serves to mend damage to the collagen framework has been observed in the young equine model. Lucia et al. (2013) discovered increasing concentrations of CPII with increasing dosage of intra-articular LPS. Anabolic CPII was linearly influenced by treatment with the exception of 12 and 168 h post-injection, when the 0.25 ng dosage of LPS yielded greater concentrations than 0.5 ng. At 6 and 24 h, horses receiving the greatest LPS dosage had greater CPII concentrations than horses that received intermediate and control doses. Since the half-life within synovial fluid is only about 16 h, the cleaved propeptide is a good indicator of recent collagen synthesis (Garvican et al., 2010). Type II procollagen is expressed at much lower levels in adult horses, which may have relevance to changes that occur naturally in the cartilage of older horses (McIlwraith, 2002).

The CPII molecule increases in response to 0.5 ng LPS in the joints of young and skeletally mature horses. Frisbie et al. (1999) found increased CPII concentrations in young (2 to 4 yr) exercising horses in response to osteochondral fragmentation. Lucia et al. (2013) observed peak values of about 3500 ng/mL at 24 h post LPS injection in young horses. These values are about 2 times lower than those observed in mature

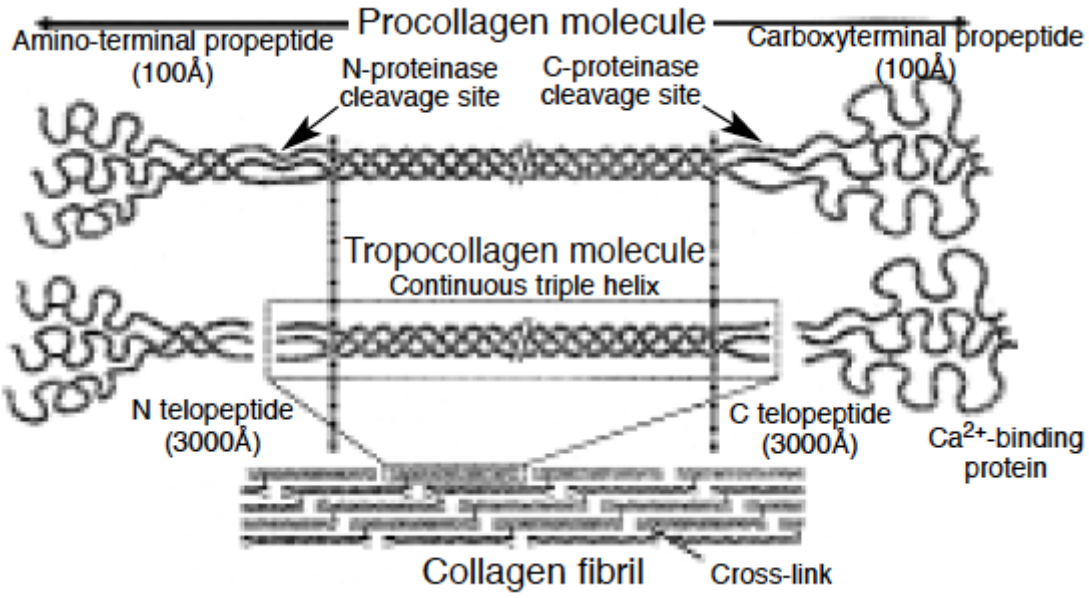

Fig. 4. Type II procollagen synthesis and fibril formation, showing removal of propeptides as the triple helix is formed. The released propeptides form the basis of the carboxypropeptide of type II collagen (CPII) marker (Adapted from McIlwraith, 2005).

horses by de Grauw et al. (2009) who observed an increase in CPII concentrations, peaking at 6000 ng/mL at 24 h and remaining elevated above baseline at 168 h post LPS injection. Variation between studies indicates that there may be an effect of age and growth, however further studies are needed to clarify effects on the time course and concentration of anabolic CPII in horses across various age groups within the same study.

Conclusion

Osteoarthritis is a major concern for athletic horses of all ages. While some loading is required for the maintenance of a healthy synovial joint, overloading can lead to degenerative changes in the articular cartilage. Early diagnosis and prevention are

key and the subject of much of the current research. In order to conduct research regarding OA in young horses, a model of inflammation must be used. Previously LPS has been used as a model for the study of transient, local inflammation. Biomarkers, which show promise for early detection of equine OA and serve as a sensitive indicator for experimental measurements, can be detected in synovial fluid from horses exposed to a single inflammatory insult using ng quantities of LPS to quantify inflammation and cartilage metabolism.

Previous studies using LPS and a number of other models have used immature or mature horses to investigate the processes of OA. Adaptation of bone and soft tissue takes place in the young equine model during growth and early training. In comparison to the immature model, the response of bone and soft tissue in mature horses is questionable. Comparing data across studies may not be accurate because horses may have been exposed to different variables. A study using horses exposed to the same experimental protocol and conditions, investigating inflammation and cartilage metabolism to study the effects of age on these processes is needed. Therefore, the objective of this study is to evaluate the influence of age on inflammation and cartilage metabolism in horses in response to an acute inflammatory insult using LPS. The long-term goal of this work will be to properly evaluate response of the equine joint, specifically inflammation and cartilage metabolism, to an acute inflammatory insult using LPS.

CHAPTER III

MATERIALS AND METHODS

All care, handling, and sampling of horses were approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP #2011-170).

Horses and Treatments

Eighteen Quarter Horses (n = 7 males, n = 11 females) were used in a randomized complete design for a 28 d experiment. All horses were selected for age from the Texas A&M University herd and had no history of lameness or use of anti-inflammatory drugs for one year prior to the experiment. Horses were assigned to treatment groups according to age, with yearlings (yearling; n = 3 males; n = 3 females), 2 to 3 yr olds (2/3; n = 2 males; n = 4 females), or skeletally mature 5 to 8 yr olds (mature; n = 2 males; n = 4 females). Starting on d 0, horses were housed in individual 3.0 × 3.0 m stalls and fed diets that met or exceeded NRC (2007) requirements for the age group. Horses were fed a commercially available 14% CP sweet feed (Producer's Cooperative Association, Bryan, TX) and coastal Bermuda grass hay (*Cynodon dactylon*) twice daily at 0600 and 1730 (Table 1). They were allowed *ad libitum* access to water and provided free exercise in dry lots from 0930 to 1700.

On d 14, horses were challenged with an intra-articular injection of LPS derived from *Escherichia coli* O55:B5 (Sigma-Aldrich, St. Louis, MO). Intra-articular treatments of LPS were assigned to a randomly selected radial carpal joint with the remaining carpal joint serving as the contralateral control (CON). The LPS was

administered at 0.5 ng as previously described by Lucia et al. (2013) using weanling horses. Purified LPS was reconstituted in sterile lactated Ringer's solution (LRS) to yield a stock solution of 1 mg/mL. Serial dilutions were performed from the stock concentration to yield individual doses of 0.8 ml as described by de Grauw et al. (2009). CON joints were injected with 0.8 ml of LRS.

Table 1. Nutrient analysis of diet components

	Component	
	Concentrate[1]	Coastal Bermudagrass Hay (*Cynodon dactylon)*
Moisture, %	7.65	7.55
Dry Matter, %	92.35	92.45
Crude Protein,[2] %	18.82	11.26
ADF,[2] %	9.47	33.87
NDF,[2] %	16.83	61.05
DE,[2] Mcal/kg	3.42	2.51
EE,[2] %	6.84	3.53
Ca,[2] %	1.14	0.6
P,[2] %	0.73	0.22
K,[2] %	1.35	1.12
Mg,[2] %	0.26	0.34

[1]14% CP sweet feed concentration (Producer's Cooperative Association, Bryan, TX)
[2]Dry matter basis

Sample Collection

Prior to arthrocentesis, at pre-injection h 0 (PIH 0), both carpal joints were clipped and aseptically prepared. Horses were sedated using 1.5 mL Sedivet® (romifidine hydrochloride 1% Injection, Boehringer Ingelheim, Fremont, CA). Additional lip and neck twitch restraint was used when necessary. Arthrocentesis was performed by veterinarians from the Texas A&M University Large Animal Clinic.

Treatments (0.8 mL) were administered aseptically at a location medial to the extensor carpi radialis tendon in the palpable depression between the radial carpal bone and the third carpal bone to the depth of approximately 12.7 nm to avoid unnecessary contact with the articular cartilage (McIlwraith and Trotter, 1996).

Synovial fluid samples (0.5 to 5.8 mL) were collected via arthrocentesis at PIH 0 and 6, 12, 24, 168 and 336 h post-injection and transferred into sterile, non-additive tubes (BD Vacutainer® Blood Serum Collection Tubes, Franklin Lakes, NJ). Following collection, samples were immediately placed on ice and further divided into small aliquots (1.25 mL) that were stored at -20°C for later analysis. Heart rate (HR; beats/min), respiratory rate (RR; breaths/min), and rectal temperature (RT; °C) were recorded prior to arthrocentesis at PIH 0, and 6, 12, an 24 h post-injection. Prior to all sample collections, at PIH 0, and 6, 12, 24, 168, and 336 h post-injection, carpal circumference (cm) was measured at the level of the accessory carpal bone using a soft tape measure and surface temperature (°C) of each carpal joint was determined using an infrared camera (FLIR E60, FLIR Systems, Boston, MA). Wall temperature was consistently measured to monitor ambient temperature and to provide consistent temperature data. Horses were continuously monitored for signs of anaphylaxis throughout the first 24 hours post LPS injection.

Synovial Fluid Analysis

Synovial fluid samples were analyzed to determine concentrations of PGE_2 using commercial ELISA kits (R&D Systems, Minneapolis, MN) previously validated for use in horses (Bertone et al., 2001; de Grauw et al., 2006). Synovial fluid samples required a

1:1.5, 1:2, 1:3, 1:4 or 1:5 dilution, depending on time post-injection using the calibrator diluent provided in the kit. The mean minimum detectable dose was 30.9 pg/mL. The interassay coefficient of variation (cv) averaged 10.6% and the mean intraassay cv was 6.7%.

A commercially available ELISA kit (IBEX Pharmaceuticals Inc., Quebec, Montreal, Canada), previously validated for use in horses (Billinghurst et al., 2001; Frisbie et al., 2008), was used to measure concentrations of synovial C2C. Standards were diluted following kit recommendations, and samples were diluted at a 1:4 or a 1:5 dilution, depending on time post-injection. Samples and standards were diluted using an assay buffer solution provided by the manufacturer. The minimum detectable dose for C2C was 10 ng/mL. The interassay cv averaged 2.9% and the average intraassay cv was 4.8%.

A commercially available ELISA kit (IBEX Pharmaceuticals Inc., Quebec, Montreal, Canada) with a similar protocol to the C2C ELISA kit was used to measure concentrations of synovial CPII. Samples required a 1:4, 1:5, 1:6 or 1:7 dilution, depending on time post-injection and standards were diluted following kit recommendations using an assay buffer solution provided with the kit. The minimum detectable dose for CPII was 50 ng/mL. The interassay cv averaged 3.0% and the mean intraassay cv was 4.8%.

Statistical Analysis

Data were analyzed using PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The model contained effects for age, time, knee, age by time interaction, and

age by time by knee interaction. This model uses both RANDOM and REPEATED statements. This covariance structure specifies a random effect of differences between animals and creates a correlation structure within animals that decreases with increasing amount of time between measurements (Littell et al., 1998). A paired t-test was used to compare age groups and individual time points. Significance differences were declared as $P \leq 0.05$, and $P \leq 0.10$ was considered a trend toward significance.

CHAPTER IV

RESULTS

Physical Variables

All values for HR, RR, and RT were within normal range for each age group (Gore et al., 2008). There was an age by time interaction observed for RT, with values for yearlings being higher ($P \leq 0.03$) than 2/3 yr olds and mature horses at 0, 12 and 24 h post-injection (Table 2). Both HR and RT were affected by age, regardless of time, with yearlings having greater ($P \leq 0.02$) values (44.5 ± 0.7 beats/min; 38.1 ± 0.1°C) compared to 2/3 yr olds and mature horses (37.0 ± 0.7 beats/min; 37.8 ± 0.1 °C and 33.2 ± 0.7 beats/min; 37.7 ± 0.1 °C, respectively). In addition, 2/3 yr olds had faster ($P < 0.01$) HR values than mature horses. Values for HR and RT were influenced by time ($P < 0.01$) for all horses, with peak values at 12 h post-injection (Table 2). Peak HR and RT values correlated with measures of inflammation in all horses, as indicated by peak synovial PGE_2 concentrations at 12 h post-injection. Values for RR were unaffected by age of horse, with no difference ($P = 0.21$) between age groups; however, RR tended to be affected by time ($P = 0.07$), also peaking at 12 h post-injection for all horses (Table 2).

Table 2. Clinical assessment of equine vital signs following intra-articular lipopolysaccharide (LPS) injection.

	Age[1]				*P*-Value		
Measure	Yearling	2/3	Mature	SEM	Age[1]	Time	Age[1] × Time
HR[4], beats/min	44.5	37.0	33.2	0.7	< 0.01	< 0.01	0.50
Hour							
0[2]	45.3	38.0	36.7	1.6			
6[3]	41.3	34.7	28.7	1.6			
12[3]	46.7	36.3	35.3	1.6			
24[3]	44.7	39.0	32.0	1.6			
RR[5], breaths/min	22.2	19.7	20.0	1.0	0.21	0.07	0.15
Hour							
0[2]	21.0	22.3	21.3	1.7			
6[3]	18.7	19.0	19.7	1.7			
12[3]	25.0	19.7	20.0	1.7			
24[3]	24.0	17.7	18.7	1.7			
RT[6], °C	38.1	37.8	37.7	0.1	0.01	< 0.01	0.01
Hour							
0[2]	38.3	37.9	37.6	0.1			
6[3]	37.7	37.6	37.5	0.1			
12[3]	38.3	38.0	37.9	0.1			
24[3]	38.2	37.9	37.8	0.1			

[1]Age group: yearlings (yearling; $n = 6$), 2 to 3 yr olds (2/3; $n = 6$), and skeletally mature 5 to 8 yr olds (Mature; $n = 6$).

[2]Pre-injection hour: prior to intra-articular LPS injection.

[3]Post-injection hour: after intra-articular LPS injection.

[4]Heart rate.

[5]Respiratory rate.

[6]Rectal temperature.

There were no differences ($P = 0.58$) in joint circumference (cm) between LPS and CON knees, therefore data was combined into a single graph (Fig. 5). Joint circumference was not influenced by age of horse ($P = 0.84$), however circumference increased ($P < 0.01$) in both LPS and CON joints for all horses over time, with peak values at 168 h post-injection (Fig. 5). Joint surface temperature (°C) was not different ($P = 0.24$) in LPS and CON knees; therefore the data was combined and is shown in Fig. 6. There was an age by time interaction for carpal joint surface temperature that was evident at PIH 0, and 6, 12, 168, and 336 h post-injection, when yearlings had higher ($P < 0.01$) temperature values than mature horses (Fig. 6). At PIH 0 and 6, 12, 24, 168 and 336 h post-injection, yearlings had greater ($P \leq 0.05$) or tended to have greater ($P \leq 0.09$) carpal surface temperatures than 2/3 yr olds (Fig. 6). Carpal joint surface temperature was influenced by age, with yearlings having elevated ($P < 0.01$) values (34.7 ± 0.1 °C) compared to 2/3 yr old and mature horses at all time points (33.8 ± 0.1 °C and 33.6 ± 0.1 °C, respectively; Fig. 6). There was also an effect of time ($P < 0.01$) on surface temperature in LPS and CON joints, with values peaking at 6 h post-injection for all horses (Fig. 6).

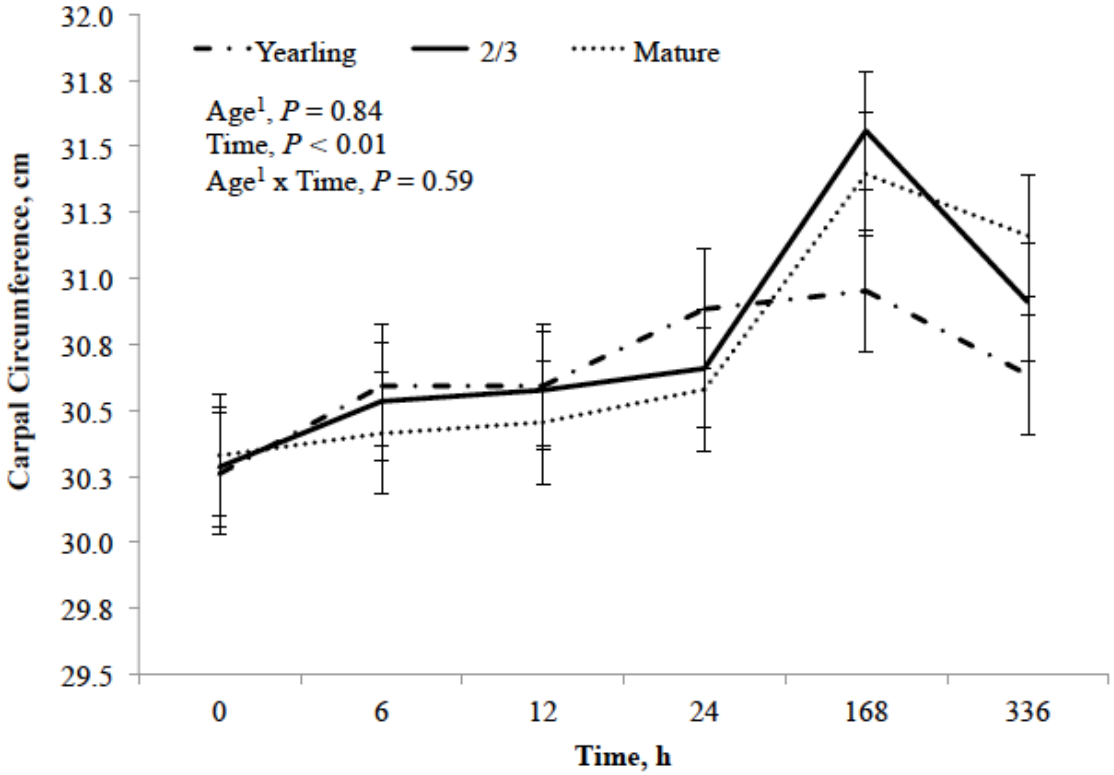

Fig. 5. Carpal circumference (cm; LS mean ± SEM) in horses after intra-articular injection with 0.5 ng lipopolysaccharide (LPS: derived from *Escherichia coli* O55:B5) or 0.8 mL sterile lactated Ringer's solution (CON) at 0, 6, 12, 24, 168 and 336 h post-injection. [1]Age group: yearlings (yearling; $n = 6$), 2 to 3 yr olds (2/3; $n = 6$), and skeletally mature 5 to 8 yr olds (Mature; $n = 6$).

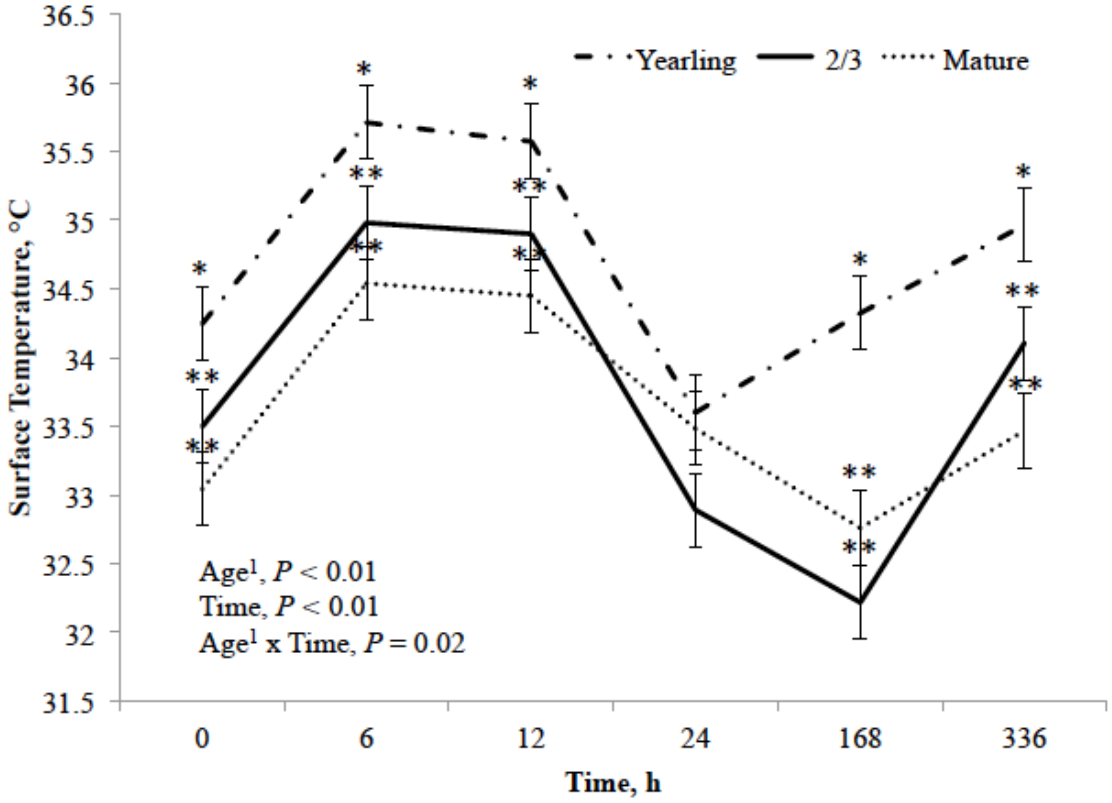

Fig. 6. Carpal joint surface temperature (°C; LS mean ± SEM) in horses after intra-articular injection with 0.5 ng lipopolysaccharide (LPS: derived from Escherichia coli O55:B5) or 0.8 mL sterile lactated Ringer's solution (CON) at 0, 6, 12, 24, 168 and 336 h post-injection. [1]Age group: yearlings (yearling; $n = 6$), 2 to 3 yr olds (2/3; $n = 6$), and skeletally mature 5 to 8 yr olds (Mature; $n = 6$). *,**,***Different superscripts indicate differences ($P \leq 0.05$) in carpal joint surface temperature among different age groups at specific time points post-injection.

Synovial Joint Inflammation

PGE_2

Synovial PGE_2 concentrations in LPS and CON joints from PIH 0, over 336 h are shown in Fig. 7. Synovial PGE_2 tended ($P = 0.09$) to be influenced by age, regardless of time post-injection, with yearlings having decreased ($P = 0.03$) concentrations (528.8 ± 108.8 pg/mL) compared to mature horses (870.4 ± 101.3 pg/mL). Synovial PGE_2 was influenced by time post-injection for all horses, increasing ($P < 0.01$) to peak values at 12 h post-injection and returning to baseline by 336 h post- injection (Fig. 7). There was a tendency for PGE_2 values to be affected by knee for all horses across time, with LPS injected joints tending to have higher ($P = 0.06$) concentrations (827.9 ± 84.5 pg/mL) than CON joints (590.9 ± 87.2 pg/mL). Additionally, LPS joints had increased ($P < 0.01$) concentrations of synovial PGE_2 at 6 and 12 h post-injection compared to CON joints.

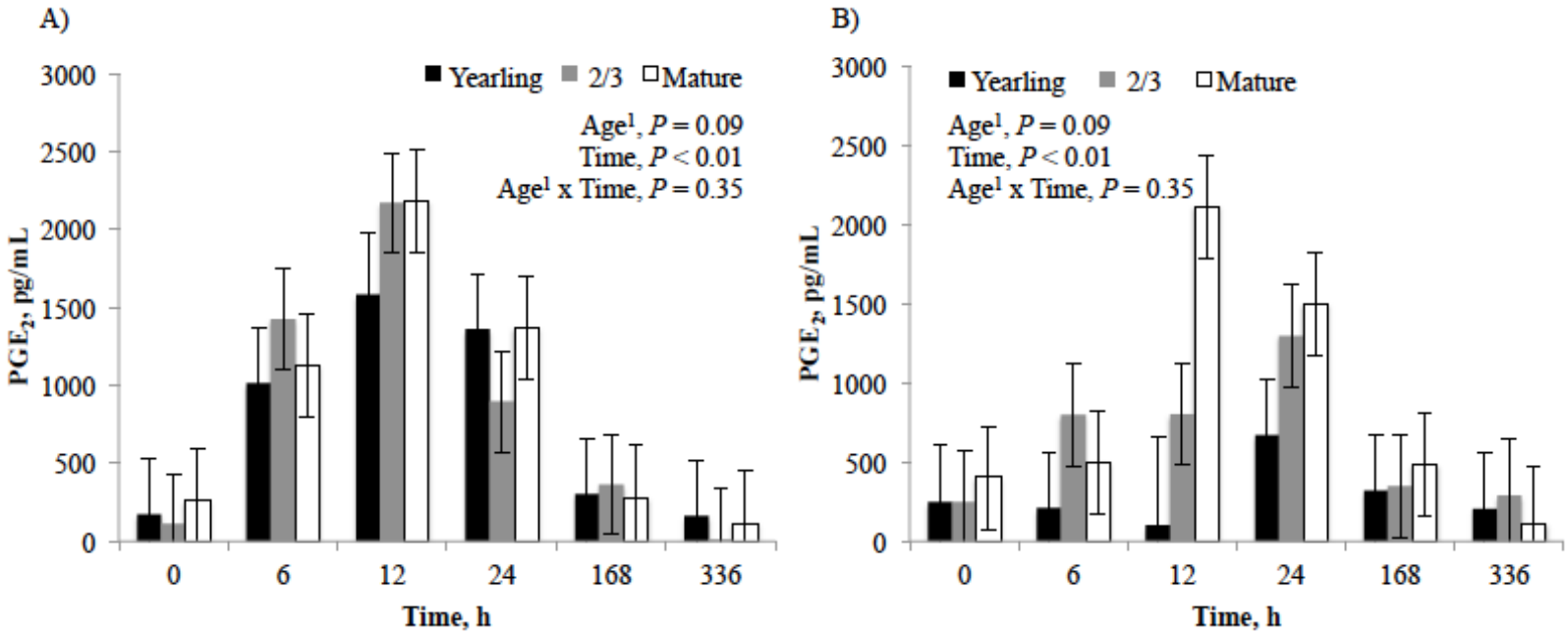

Fig. 7. Mean synovial fluid concentrations (pg/mL) of PGE_2 in horses in the LPS knee (Panel A; 0.5 ng of intra-articular lipopolysaccharide derived from *Escherichia coli* O55:B5) and in the CON knee (Panel B; 0.8 mL of intra-articular sterile lactated Ringer's solution) at 0, 6, 12, 24, 168 and 336 h post-injection. [1]Age group: yearlings (yearling; $n = 6$), 2 to 3 yr olds (2/3; $n = 6$), and 5 to 8 yr olds (Mature; $n = 6$).

Biomarkers of Cartilage Metabolism

Catabolic C2C

Synovial concentrations of catabolic C2C over 336 h in LPS and CON injected joints are displayed in Fig. 8. At 6 and 168 h post-injection, yearling horses had decreased ($P < 0.01$) C2C concentrations compared to 2/3 yr olds and lower ($P \leq 0.03$) values compared to mature horses at 6, 24, 168, and 336 h (Fig. 8). At 12 h post-injection 2/3 yr olds had lower ($P = 0.03$) concentrations of C2C than mature horses and tended to have decreased ($P = 0.07$) concentrations over mature horses at 168 h. Yearlings had increased ($P < 0.01$) levels of C2C over 2/3 yr olds and tended to have increased ($P = 0.09$) levels over mature horses at 12 h (Fig. 8). Yearlings and 2/3 yr olds had lower ($P < 0.01$) concentrations (200.1 ± 11.0 ng/mL and 219.7 ± 11.0 ng/mL) than mature horses (275.1 ± 11.0 ng/mL) at all time points post-injection. For all age groups, the LPS injected joint had higher ($P = 0.02$) concentrations (246.8 ± 9.0 ng/mL) compared to CON joints (216.5 ± 9.0 ng/mL). There was an effect of time for all horses, with concentrations increasing ($P < 0.01$) to peak values at 24 h (342.6 ± 13.1 ng/mL), and returning to baseline by 336 h (Fig. 8).

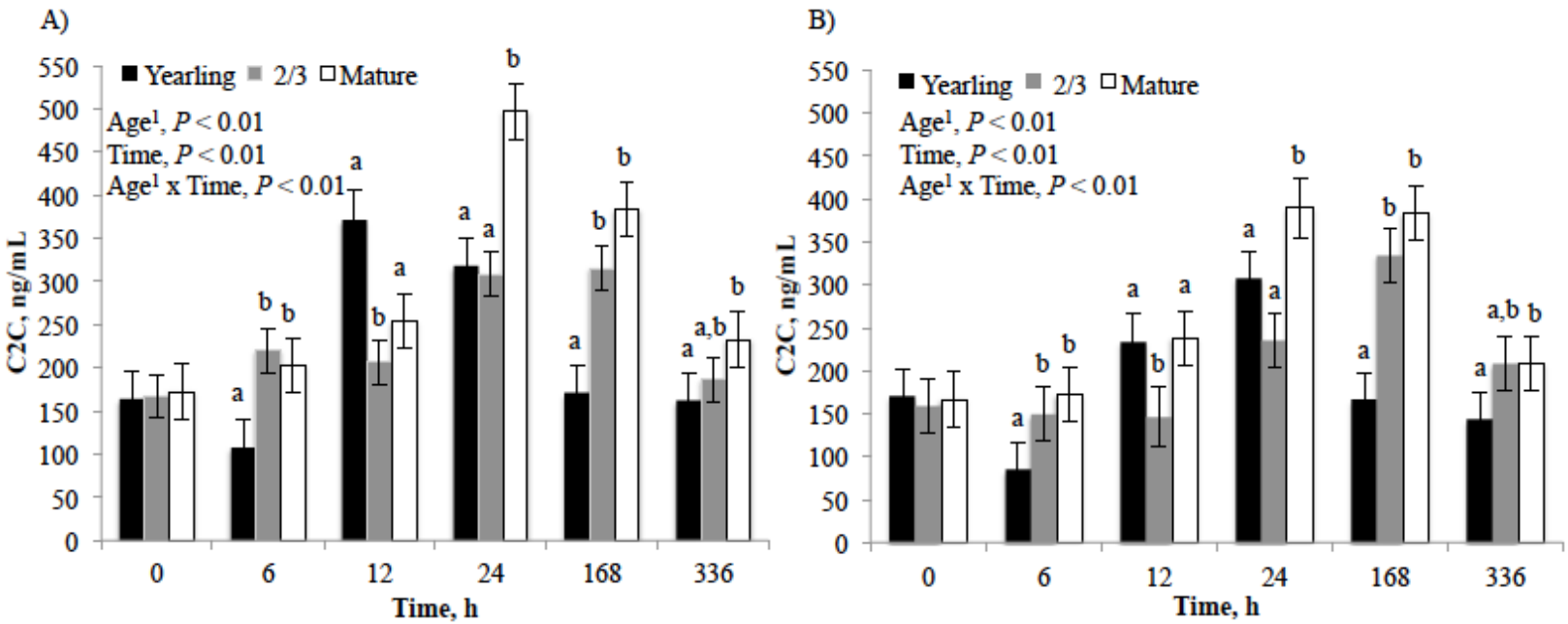

Fig. 8. Mean synovial fluid concentrations (ng/mL) of catabolic collagenase cleavage neoepitope of type II collagen (C2C) in horses in the LPS knee (Panel A; 0.5 ng of intra-articular lipopolysaccharide derived from *Escherichia coli* O55:B5) and in the CON knee (Panel B; 0.8 mL of intra-articular sterile lactated Ringer's solution) at 0, 6, 12, 24, 168 and 336 h post-injection. [1]Age group: yearlings (yearling; $n = 6$), 2 to 3 yr olds (2/3; $n = 6$), and 5 to 8 yr olds (Mature; $n = 6$). [a,b]Different superscripts indicate differences ($P \leq 0.05$) in synovial C2C concentration among different age groups at specific time points after injection.

Anabolic CPII

Synovial concentrations of anabolic CPII in LPS and CON joints from PIH 0, over 336 h are shown in Fig. 9. There was an age by time interaction, most notable at 0, 24, 168 and 336 h. At PIH 0 and 336 h yearlings had lower ($P \leq 0.03$) CPII concentrations than 2/3 and mature horses (Fig. 9). Anabolic CPII levels in yearlings and 2/3 yr olds were lower ($P < 0.01$) than mature horses at 24 h post-injection, while at 168 h yearling CPII tended to be lower ($P = 0.06$) than 2/3 yr olds and was decreased ($P < 0.01$) compared to mature horses (Fig. 9). Anabolic CPII was influenced by age at all time points post-injection, with yearlings tending to have lower ($P = 0.06$) concentrations than 2/3 yr olds (2252.4 ± 196.4 ng/mL) and lower ($P \leq 0.02$) concentrations (1560.3 ± 253.9 ng/mL) compared to mature horses (2891.2 ± 216.1 ng/mL). Anabolic CPII was affected by time, in both LPS and CON joints for all horses, with concentrations increasing ($P \leq 0.02$) to 168 h, and returning to baseline by 336 h post-injection (Fig. 9).

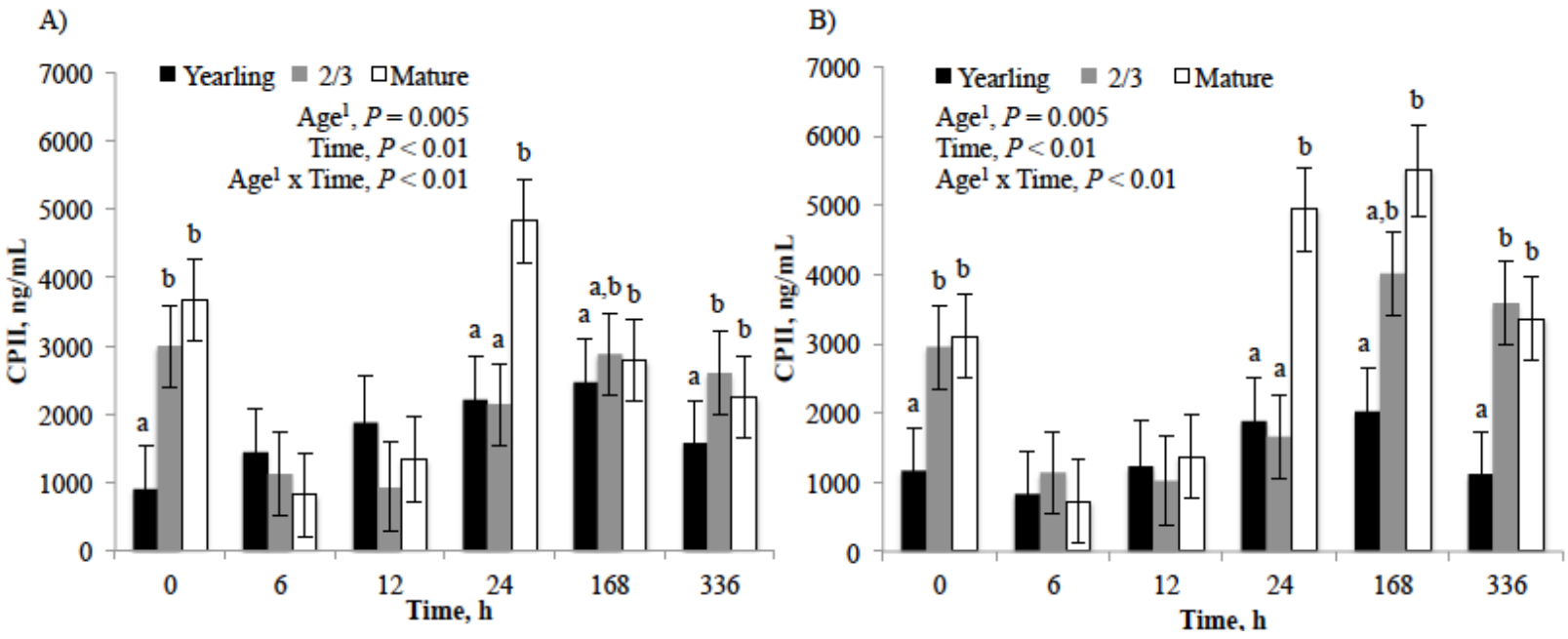

Fig. 9. Mean synovial fluid concentrations (ng/mL) of anabolic carboxypropeptide of type II collagen (CPII) in horses in the LPS knee (Panel A; 0.5 ng of intra-articular lipopolysaccharide derived from *Escherichia coli* O55:B5) and in the CON knee (Panel B; 0.8 mL of intra-articular sterile lactated Ringer's solution) at 0, 6, 12, 24, 168 and 336 h post-injection. [1]Age group: yearlings (yearling; $n = 6$), 2 to 3 yr olds (2/3; $n = 6$), and 5 to 8 yr olds (Mature; $n = 6$). [a,b]Different superscripts indicate differences ($P \leq 0.05$) in synovial CPII concentration among different age groups at specific time points after injection.

CPII:C2C

Ratios of synovial fluid CPII to C2C concentrations in LPS and CON joints from PIH 0, over 336 h are shown in Fig. 10. An age by time interaction for both LPS and CON knees was observed and was most notable at 6 h post-injection when yearlings showed higher ($P = 0.04$) ratio values than mature horses and at PIH 0 and 336 h post-injection when yearling horses had lower ($P < 0.01$) ratio values than both 2/3 and mature horses (Fig. 10). CPII:C2C was influenced by age, regardless of time post-injection, with mature and 2/3 yr old horses having increased ($P < 0.01$) values compared to yearlings. There was also an effect of time, with peak values for all horses increasing ($P < 0.01$) up to 336 h post-injection.

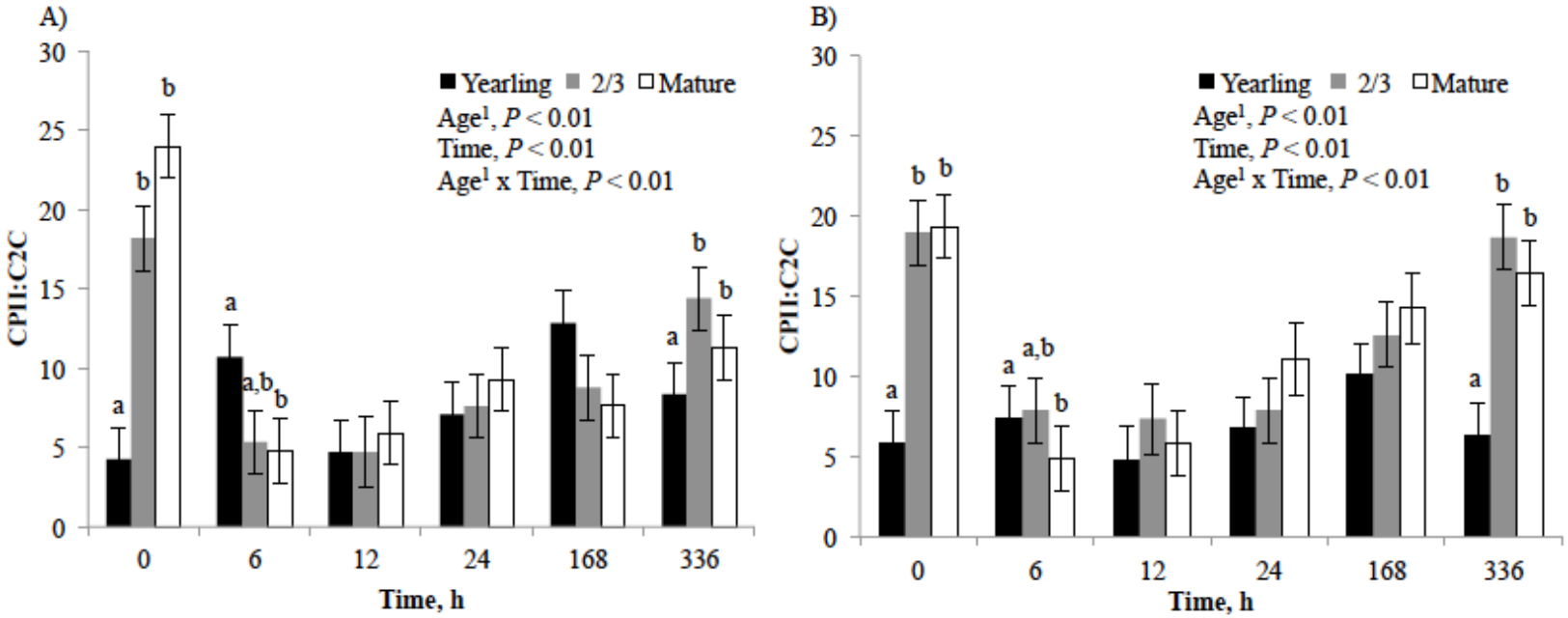

Fig. 10. Ratio of the mean synovial fluid concentration of carboxypropeptide of type II collagen (CPII) to the mean synovial fluid concentration of collagenase cleavage neoepitope of type II collagen (C2C) in horses in the LPS knee (Panel A; 0.5 ng of intra-articular lipopolysaccharide derived from *Escherichia coli* O55:B5) and in the CON knee (Panel B; 0.8 mL of intra-articular sterile lactated Ringer's solution) at 0, 6, 12, 24, 168 and 336 h post-injection. [1]Age group: yearlings (yearling; n = 6), 2 to 3 yr olds (2/3; n = 6), and 5 to 8 yr olds (Mature; n = 6). [a,b]Different superscripts indicate differences ($P \leq 0.05$) in synovial CPII:C2C among different age groups at specific time points after injection.

CHAPTER V

DISCUSSION

In the current study, measurements for HR, RR, and RT were all within normal range for each age group. Values for HR and RT were greater in younger horses compared older horses, however normal vitals are expected to be elevated in young horses compared to mature (Gore et al., 2008). Additionally, HR, RR, and RT values peaked at 12 h post LPS injection for all horses, which corresponds to peak inflammation, as indicated by peak synovial PGE_2 concentrations. All vitals returned to baseline values by 24 h post- injection, which is most likely an indication of horses becoming more comfortable with handling procedures. Clinical responses of HR, RR, and RT showed no signs of systemic illness and were similar to those previously reported in studies utilizing young or mature horses exposed to a single inflammatory insult using nanogram quantities of LPS (de Grauw et al., 2009; Lucia et al., 2013).

Joint effusion as a result of LPS-induced inflammation and/or repeated arthrocentesis, can lead to increased joint circumference. Joint circumference did not differ between age groups, however circumference values did increase above baseline by 168 h in all horses in response to LPS injection and repeated arthrocentesis. This differs from a previous study that showed peak circumference values at 24 h post-injection (Lucia et al., 2013). However, Lucia et al. (2013) only recorded circumference values up to 24 h post-injection, and did not report values at168 h. In the current study, surface temperature of carpal joints was greater in yearlings than the 2/3 yr olds and mature horses. Values peaked 6 h post-injection, prior to peak synovial PGE_2 concentrations

and decreased back to baseline by 24 h in all horses as inflammation subsided. No previous studies examining the effects of an LPS challenge on carpal joint surface temperature have been found, therefore no comparisons can be made at this time.

Synovial PGE_2 is a mediator of inflammation that has been described as an excellent indicator of equine OA (Bertone et al., 2001). Increases in synovial PGE_2 have been demonstrated using various experimental models including exercise, osteochondral fragmentation, and LPS (Frisbie et al., 2008; de Grauw et al., 2009; Lucia et al., 2013). With experimental induction of inflammation using LPS, eicosanoid concentrations, including PGE_2, vary depending on time post-injection. In the current study, an inflammatory response was observed across age groups as indicated by increases in synovial PGE_2 concentrations. Synovial PGE_2 tended to be greater in mature horses compared to yearling horses. These results indicate an increased inflammatory response in older horses in response to LPS injection. This is consistent with a previous *in vitro* study that demonstrated age-related differences in PGE_2 production by equine cartilage explants (Briston et al., 2009). Explants from aged horses were the most sensitive to LPS, producing significantly greater concentrations of PGE_2, suggesting that PGE_2 concentrations increase with age (Briston et al., 2009). Over time, with chronic exposure to inflammatory mediators like PGE_2, degradative changes to the articular cartilage could result from mechanical load or an increased sensitivity to degradation in older horses.

Values for PGE_2 peaked for all horses at 12 h post-injection, returning to baseline by 336 h, indicating a transient inflammation in response to intra-articular LPS

injection. These results are consistent with a previous study that utilized 0.5 ng intra-articular LPS to induce inflammation in weanling horses (Lucia et al., 2013). Another study by de Grauw et al. (2009) also utilized 0.5 ng LPS solution and a similar arthrocentesis protocol in mature horses and observed sharp, short lived increases in synovial PGE_2 at 8 h post- injection. These peak concentrations are numerically lower than peak values observed at 12 h by Lucia et al. (2013) in weanling horses. However, it was postulated by Lucia et al. (2013) that the delayed increase in PGE_2 concentrations in younger horses could be a reflection of the more frequent number of samples obtained via arthrocentesis. Results from the current study support this hypothesis since all age groups demonstrated peak PGE_2 concentrations at 12 h post-injection. Had measurements been taken at 12 h post-injection by de Grauw et al. (2009), elevated concentrations of synovial PGE_2 may have been noted at that time period.

Concentrations of PGE_2 tended to be higher in LPS joints compared to CON joints for all horses, across all time periods. This was most evident at 6 and 12 h post-injection. This demonstrates the importance of using contralateral controls for direct comparison of the effects of LPS injection versus arthrocentesis alone on synovial fluid biomarker concentrations. van den Boom et al. (2005b) saw increases in synovial PGE_2 when arthrocentesis was performed at 12 h intervals, with concentrations remaining elevated through 60 h. Frisbie et al. (2008) utilized repeated arthrocentesis in another study, but noted no significant effects on synovial PGE_2, most likely due to arthrocentesis being 1 wk apart. Data from this study and a previous study by Lucia et al. (2013) indicate that there is an inflammatory response from repeated arthrocentesis

alone. However, this response appears to be decreased compared to LPS injection and the accompanying arthrocentesis protocol.

The C2C antibody recognizes fragments of collagenase-cleaved type II collagen. A previous study observed increases in type II collagen degradation in response to exercise and joints subjected to osteochondral fragmentation (Frisbie et al., 2008). Increased concentrations of catabolic C2C have been observed using LPS-induced inflammation in both young and skeletally mature horses (de Grauw et al., 2009; Lucia et al., 2013). Variation in C2C concentrations exists across studies, with peak concentrations at 24 h observed by de Grauw et al. (2009) in mature horses being two times greater than values seen by Lucia et al. (2013) in weanlings also at 24 h. In the current study, peak synovial C2C values occurred at 24 h post-injection for all age groups. At this time, mature horses had concentrations of synovial C2C (443.17 ± 23.46 ng/mL) that were approximately one and a half times higher than yearling and 2/3 yr old horses (312.83 ± 22.40 ng/mL and 271.76 ± 22.41 ng/mL, respectively). The results from the current study, in conjunction with those from previous studies, show increased C2C concentrations with age, indicating an increased degradative response in mature horses exposed to intra-articular LPS compared to younger horses.

Synovial C2C concentrations were also elevated in LPS joints compared to CON joints in the current study. An increased inflammatory response in LPS joints, as indicated by greater levels of synovial PGE_2, can lead to cartilage breakdown. Degradation of the articular cartilage, quantified using concentrations of synovial C2C, is most likely increased in joints exposed to LPS injection rather than repeated

arthrocentesis alone, due to an increased inflammatory response (Garvican et al., 2010). Concentrations of synovial C2C have been shown to increase with joint inflammation in rabbits and with osteochondral injury in Thoroughbred racehorses (Kojima et al., 2001; Trumble et al., 2009). Therefore, it is probable that LPS injection, which is an established model of experimental inflammation, has an effect on synovial C2C concentrations, through its ability to induce the release of inflammatory mediators such as PGE_2, leading to the enzymatic breakdown of type II collagen (de Grauw et al., 2009; Lucia et al., 2013).

Associated with the increased cleavage of collagen by collagenases is a rise in the synthesis of matrix molecules, including type II collagen, which is proteolytically cleaved from the procollagen strand during fibril formation. Frisbie et al. (2008) demonstrated increased collagen synthesis with exercise and osteochondral fragmentation as evidenced by increased synovial fluid CPII under these conditions. Values for CPII have also been found to increase in response to intra-articular LPS injection in both growing and mature horses, with variation in concentration between studies (de Grauw et al., 2009; Lucia et al., 2013). Results from the current study are consistent with the previous data that reported synovial CPII values in mature horses to be two times greater than young horses. In the current study peak CPII values (4,886.74 ± 435.74 ng/mL) at 24 h in mature horses are approximately two times greater than those observed at 24 h for yearling and 2/3 yr old horses (2,046.67 ± 455.35 ng/mL and 1901.20 ± 426.08 ng/mL, respectively), indicating increased synthesis of the type II collagen component of articular cartilage in mature horses.

A metabolic shift toward re-synthesis serves to mend damage to the cartilage framework after exposure to inflammatory mediators and deleterious enzymes. While articular cartilage repair involves replacement of the damaged matrix, the tissue does not necessarily return to its original structure or function (Frisbie, 2006). Newly synthesized matrix molecules are often damaged, compromising any effective attempts at cartilage matrix repair (Poole et al., 2002). Matrix turnover in mature horses is slow and in young individuals metabolism is sustained at a higher level to allow for growth and remodeling (Brama et al., 1998). In the current study ratios of CPII to C2C showed a shift toward re-synthesis in both the young and the mature equine model, which, for young horses, correlates with results by Lucia et al. (2013). In the current study, mature horses showed increased ability for articular cartilage repair over young horses, possibly because the amount of degradation in these horses was higher overall. This elevated ability towards re-synthesis in mature horses may also be accompanied by increased damage to matrix molecules shown to be important for the integrity of the joint and its ability to function. The results in the current study were surprising, however values for mature horses did peak at 336 h, later than younger horses at 168 h, possibly indicating the slower turnover rate in mature horses compared to young horses. Therefore, the current study suggests that mature horses may have an increased ability to re-synthesize cartilage in response to inflammation and enzymatic breakdown when compared to younger horses, however the process takes longer and may be accompanied by increased damage to newly synthesized matrix molecules. Further studies are needed to clarify this relationship between horses of varying age.

CHAPTER VI

SUMMARY

Although OA is believed to exist primarily in the aged horse, the disease is thought to result from cartilage damage that may develop when training occurs early in life. Results from this study indicate an increasing inflammatory response with age in horses. This increased inflammation is accompanied by greater amounts of cartilage degradation and repair and, likely, greater amounts of damage in the newly synthesized cartilage. Additionally, with age, the rate of cartilage re-synthesis decreases. After a single inflammatory insult, this decreased rate does not appear to limit the ability of the mature horse to repair damaged cartilage. However, it is possible that without an adequate rest period, the older equine athlete returning to work may not have time to sufficiently complete the cartilage repair process. Therefore, further studies are needed, to confirm the effects of age on inflammation and cartilage metabolism in exercising horses exposed to chronic joint inflammation.

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APPENDIX

ELISA KIT PROTOCOLS

PGE_2 (R&D Systems, Minneapolis, MN)

Synovial fluid samples were analyzed to determine concentrations of PGE_2 using commercial ELISA kits (R&D Systems, Minneapolis, MN) previously validated for use in horses (Bertone et al., 2001; de Grauw et al., 2006). Synovial fluid samples required a 1:1.5, 1:2, 1:3, 1:4 or 1:5 dilution, depending on time post-injection using the calibrator diluent provided in the kit. The mean minimum detectable dose was 30.9 pg/mL. The interassay coefficient of variation (cv) averaged 10.6% and the mean intraassay cv was 6.7%.

First, 200 µL calibrator diluent was added to the non-specific binding (NSB) wells and 150 µL to the zero standard (B_0) wells. Approximately 150 µL of standard or sample were then added to all remaining wells. The NSB, B_0, samples and standards were all run in duplicate. Next, 50 µL of primary mouse monoclonal antibody solution was added to all wells excluding the NSB wells. The covered ELISA plate was then incubated at room temperature for 1 h on a horizontal orbital microplate shaker (VWR Microplate Shaker 120 V, Henry Troemner, LLC, Thorofare, NJ) at 525 rpm. Approximately 50 µL of PGE_2 conjugated to horseradish peroxidase was added to each well. The covered plate was then incubated for 2 h at room temperature on the shaker at 525 rpm. The plate was washed (Labsystems Wellwash 4, Thermo Fisher Sci, Waltham, MA) 4 times and blotted after the final wash. Substrate solution (200 µL) was added to

each well and the plate was incubated for another 30 min at room temperature, protected from light. Stop solution (100 µL) was added to each well, changing the color of the solution from blue to yellow. The optical density (OD) was determined within 30 min of the final incubation using a microplate reader (BioRad 680 Microplate Reader, BioRad Laboratories, Hercules, CA) set to 450 nm. The OD is inversely proportional to the amount of PGE_2 that is present in the samples.

Catabolic C2C (IBEX Pharmaceuticals Inc., Quebec, Montreal, Canada)

A commercially available ELISA kit (IBEX Pharmaceuticals Inc., Quebec, Montreal, Canada), previously validated for use in horses (Billinghurst et al., 2001; Frisbie et al., 2008), was used to measure concentrations of synovial C2C. Standards were diluted following kit recommendations, and samples were diluted at a 1:4 or a 1:5 dilution, depending on time post-injection. Samples and standards were diluted using an assay buffer solution provided by the manufacturer. The minimum detectable dose for C2C was 10 ng/mL. The interassay cv averaged 2.9% and the average intraassay cv was 4.8%.

Sample (50 µL) was then added to a polypropylene mixing plate followed by the addition of 50 µL of C2C antibody diluted in assay buffer. The mixing plate was pre-incubated on the plate shaker for 30 min at room temperature (20 to 25°C) at 650 rpm. All C2C incubations were performed at room temperature at 650 rpm. Approximately 80µL of the antigen-antibody mixture was then transferred to its corresponding well on the C2C ELISA plate. The ELISA plate was then incubated for 1 h, allowing the

antibody to bind. The plate was washed 3 times and blotted dry after the final wash. Goat anti-rabbit horseradish peroxidase (GAR-HRP; 100 µL) diluted in assay buffer was then added to the plate and incubated for another 30 min. The ELISA plate was then washed 6 times and blotted dry after the final wash. 100 µL tetra-methylbenzidine (TMB) buffer warmed to room temperature was added to each well. The plate was incubated again for 30 min and color development was monitored. The reaction was then stopped by the addition of 100 µL stop solution/well, which changed the product color from blue to yellow. Samples were quantified within 10 min of the completion of the final incubation using a plate reader set at an OD of 450 nm. The OD is inversely proportional to the amount of C2C that is present in the samples.

Anabolic CPII (IBEX Pharmaceuticals Inc., Quebec, Montreal, Canada)

A commercially available ELISA kit (IBEX Pharmaceuticals Inc., Quebec, Montreal, Canada) with a similar protocol to the C2C ELISA kit was used to measure concentrations of synovial CPII. Samples required a 1:4, 1:5, 1:6 or 1:7 dilution, depending on time post-injection and standards were diluted following kit recommendations using an assay buffer solution provided with the kit. The minimum detectable dose for CPII was 50 ng/mL. The interassay cv averaged 3.0% and the mean intraassay cv was 4.8%.

CPII samples and antibody were added to the polypropylene mixing plate following the same protocol as the C2C ELISA kit. The mixing plate was then pre-incubated for 60 min at 20 to 25°C at 650 rpm. All CPII incubations were performed at

room temperature at 650 rpm. The CPII antigen-antibody mixture was transferred to the CPII ELISA plate using the same methods as the C2C kit. The CPII ELISA plate was covered and allowed to incubate for 2 h. The ELISA plate was then washed 6 times and blotted dry following the final wash. GAR-HRP was added to each well following the same procedure as the C2C kit. The covered plate was then incubated for 30 min. The plate was then washed another 6 times and blotted dry. TMB was added to each well and a final 30 min incubation was performed. Color development was monitored and stop solution was added. Samples were quantified using the same plate reader at an OD of 450 nm. The OD is inversely proportional to the amount of CPII that is present in the samples.