EVALUATION OF CONJUGATED LINOLEIC ACID SUPPLEMENTATION ON MARKERS OF JOINT INFLAMMATION AND CARTILAGE METABOLISM IN YOUNG HORSES CHALLENGED WITH LIPOPOLYSACCHARIDE

A Thesis

by

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ABSTRACT

Seventeen yearling Quarter Horses were used in a randomized complete block design for a 56 d trial to determine ability of dietary conjugated linoleic acid (CLA) to reach circulating levels in plasma and mitigate joint inflammation and cartilage turnover. Horses were blocked by age, sex and BW and randomly assigned to treatments consisting of a commercial concentrate offered at 1% BW (as-fed) supplemented with either 1% soybean oil (CON; n = 6), 0.5% soybean oil and 0.5% CLA (LOW; n = 5; Lutalin®, BASF Corp.), or 1% CLA (HIGH; n = 6; 55% purity) top-dressed daily. Horses were fed individually every 12 h and offered 1% BW daily (as-fed) coastal bermudagrass (*Cynodon dactylon*) hay.

This study was separated into two phases: phase 1 determined incorporation of CLA to circulating levels in plasma; phase 2 evaluated the potential of CLA to mitigate induced intra-articular inflammation and cartilage metabolism stimulated by lipopolysaccharide (LPS). Phase 1 comprised the first 41 d. Phase 2 began on d 42 and extended to d 56. Weekly, beginning at d 0, physical growth measurements were recorded and blood samples were collected and analyzed for fatty acid concentrations.

On d 42, an LPS challenge was conducted. Carpal joints within each horse were randomly assigned to receive intra-articular injections of 0.5 ng LPS derived from *Escherichia coli* 055:B5 or sterile lactated Ringer's solution as a contralateral control. Synovial fluid samples were obtained via arthrocentesis at pre-injection h 0

and 6, 12, 24, 168 and 336 h post-injection, and subsequently analyzed by ELISA for prostaglandin E_2 (PGE_2), carboxypeptide of type II collagen (CPII), and collagenase cleavage neopeptide (C2C). Vitals were monitored at 0, 6, 12 and 24 h; and carpal circumference and surface temperatures were also recorded. All data were analyzed using PROC MIXED procedure of SAS.

All physical measurements increased ($P < 0.01$) over time with no influence of CLA supplementation. Isomers of CLA were detectable in plasma by d 14, and HIGH horses had greatest concentrations ($P < 0.04$). Horses fed CON had undetectable levels of CLA. Arachidonic acid levels were lower ($P < 0.06$) in HIGH horses compared to both LOW and CON. Vitals were not different across dietary treatments ($P > 0.13$) and remained within normal.

Synovial PGE_2 was not affected by dietary treatment ($P = 0.15$). Synovial C2C responded to dietary treatment ($P = 0.05$) with HIGH horses having lower C2C than LOW. There was an effect ($P < 0.01$) of time with C2C values peaking between h 12 and 24, and decreasing by h 336. Levels of CPII tended to be influenced by dietary treatment ($P = 0.10$) with LOW horses having greater CPII compared to CON, and HIGH horses were intermediate with no difference from CON or LOW. Regardless of diet, CPII increased to h 24 ($P < 0.01$) and decreased to h 336. In conclusion, dietary CLA reached circulating levels in plasma prior to the LPS challenge. Dietary CLA supplementation did not influence PGE_2; however, horses receiving CLA had lesser C2C and greater CPII, indicating less degradation and greater synthesis of cartilage in response to an acute inflammatory condition.

DEDICATION

For Coach T.

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CHAPTER I

INTRODUCTION

Osteoarthritis (OA) is the leading cause of lameness in the horse and is characterized by the degradation of articular cartilage (Rossdale et al., 1985). Often, detrimental changes to articular cartilage occur because of recurring inflammation caused by trauma or overloading to the synovial joint (McIlwraith et al., 2012). When an inflammatory response is initiated, macrophages are recruited to the site of damage and release cytokines including Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α; McIlwraith, 1996). These cytokines stimulate cells of the synovium and articular cartilage, synoviocytes and chondrocytes, respectively, to produce and release matrix metalloproteinases (MMPs) and eicosanoids, including Prostaglandin E_2 (PGE_2; McIlwraith et al., 2012).

These products of inflammation can be released into the synovial fluid within the joint cavity. When an animal moves, articular cartilage acts as a sponge and absorbs cytokines, eicosanoids and MMPs (Caola, 2003). When articular cartilage is exposed to these inflammatory products, the response is the breakdown of articular cartilage and degradation of the extracellular matrix. When the fibrous portion of articular cartilage, type II collagen, is broken down, hidden epitopes are exposed and can be used as biomarkers to measure the metabolism of articular cartilage (Poole, 1992). One such epitope is collagenase cleavage neoepitope (C2C), which has been successfully used as a measurement of cartilage degradation in the horse (Lucia et al., 2013; de Grauw et al.,

2009). The use of such biomarkers is beneficial to diagnosis since current radiographic imaging cannot detect early stages of OA. As such, current treatments are used to mask the pain of already present OA, and include non-steroidal anti-inflammatory drugs (NSAIDS), intra-articular corticosteroids, and IV or IM polysulfated glycosaminoglycans (PSGAGs).

With the discovery of reliable biomarkers to measure inflammation and cartilage metabolism such as PGE_2, C2C and carboxypeptide of type II collagen (CPII), current research has focused on options to mitigate inflammation through dietary supplementation. One method that has been extensively researched is through the use of polyunsaturated fatty acids. These are most commonly supplemented as ω-3 fatty acids in the form of fish oil or flaxseed oil. They have been found to successfully reduce cytokine production and alter the ω-3:ω-6 of membrane phospholipids (Gallai et al., 1993; McCann et al., 2000). However, many studies have noted increases in arachidonic acid (20:4) levels following supplementation through fish oil or flaxseed oil, which increases PGE_2 production. Therefore, it is desirable to find a way to supplement fatty acids in a manner that mitigates inflammation without increasing 20:4 levels.

Conjugated linoleic acid (CLA) is an 18-carbon fatty acid with 2 double bonds in a conjugated system. It originally generated interest for its anti-carcinogenic properties and has since been studied extensively in pigs. It has been found to enter circulating levels in plasma and incorporate into muscle tissue following dietary supplementation in pigs (Kramer et al., 1998). Recently, it has been discovered that CLA may have anti-inflammatory properties, and following *in vitro* treatment to macrophages, was found to

reduce cytokine production following an inflammatory insult (Yang and Cook, 2003). When cytokines are produced during an inflammatory response, they signal neighboring cells to produce other inflammatory products including eicosanoids. In a study supplementing CLA to pigs, Changhua et al. (2005) found a reduction in eicosanoid production.

Currently, only one study has been performed in which horses were supplemented CLA. The supplemented CLA reached measureable circulating levels in equine plasma after 14 d, and reached maximum concentrations 28 d following the onset of supplementation (Headley et al., 2011). With this understanding, further investigation into the effects of CLA supplementation in the horse could provide essential information into its anti-inflammatory effects and ability to mediate cartilage degradation.

CHAPTER II

REVIEW OF THE LITERATURE

Introduction

Lameness in horses is described as an abnormality of gait, such that a horse cannot be used for its intended purpose (McIlwraith et al., 2012). In Thoroughbred racehorses, lameness is the primary factor in loss of use and delayed race starts (Rossdale et al., 1985). Osteoarthritis, a common joint disease in humans, is the leading cause of lameness in the equine industry, and is believed to contribute to approximately 60% of lameness in the horse (McIlwraith et al., 2012). The pathophysiology of OA in humans and horses is very similar, making horses an ideal model for research, which may be beneficial to both the equine industry and human medicine.

The definition of OA is the degeneration of articular cartilage, but it also affects the underlying bone and soft tissues (Sandell and Aigner, 2001). When a healthy joint undergoes repeated trauma or overloading, excessive inflammatory responses can lead to changes in articular cartilage metabolism. Articular cartilage damage can be classified as primary or secondary, with primary cartilage damage being caused by abnormal loading to healthy cartilage (McIlwraith et al., 2012). Secondary cartilage damage occurs because of damage to another area of the synovial joint. For example, damage to the subchondral bone can lead to articular cartilage damage as a secondary response (McIlwraith et al., 2012).

Currently, diagnostic techniques for equine OA are limited to imaging, such as radiography, ultrasound, or MRI. These techniques are often not used until there are

clinical signs of OA, including lameness, heat, swelling, pain on flexion and decreased range of motion (Jansson, 1996). It has become widely accepted that radiographic imaging techniques often do not pick up early degeneration of articular cartilage. It is desirable to find a diagnosing technique that can detect the early metabolic changes within a joint capsule. The investigation of synovial fluid biomarkers as indicators of bone, cartilage and soft tissue health is a growing area of research and can provide information about joint health prior to visual radiographic changes.

Once clinical signs of OA appear, there are multiple therapeutic methods available; however, there is very little research to support preventative measures. Preventative treatments to reduce inflammation prior to changes in articular cartilage need to be investigated due to the small success of treatments of existing OA. Existing therapies to treat OA do not reverse the damage to articular cartilage, but manage symptoms and could limit further inflammation from continuing the deleterious effects on articular cartilage (Jansson, 1996). Following the onset of clinical symptoms, the most common treatments include non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular corticosteroids, and PSGAGs (Trotter and McIlwraith, 1996). A more recent area of research is evaluating gene therapy as a possible treatment for OA to alleviate pain and preserve articular cartilage (Frisbie et al., 2002).

The therapies described above are utilized once clinical symptoms have initiated. Therefore, it is desirable to find preventative measures to delay the onset of osteoarthritic changes in the joints of humans and horses. Glucosamine, an amino sugar, has been researched as a possible dietary supplement to mitigate changes in

markers of inflammation and cartilage metabolism in young horses (Lucia, 2013). Others have studied dietary supplementation of polyunsaturated fatty acids (PUFAs), such as ω-3s, as anti-inflammatories in horses and humans (Gallai et al., 1993; Lucia et al., 2009). Recently, however, researchers have begun to test the effects of CLA on inflammation through in vitro and in vivo studies (Yang and Cook, 2003; Changhua et al., 2005).

Conjugated linoleic acid describes a group of fatty acid isomers containing 18 carbons with two double bonds in a conjugated orientation. There are 28 CLA isomers, the most common of which are cis-9, trans-11 and trans-10, cis-12 (c9, t11; t10, c12; Bhattacharya et al., 2006). Both isomers are naturally occurring in beef and dairy products (Hur and Park, 2007). While CLA has been studied in mice, swine and human subjects to evaluate anti-inflammatory, anti-carcinogenic, anti-adipogenic, anti-arthrogenic and anti-diabetogenic properties, only one study has evaluated effects of CLA supplementation in horses (Bhattacharya et al., 2006; Headley et al., 2011). The anti-inflammatory properties of CLA are of greatest interest as a preventative strategy for OA in performance horses.

Synovial Joint Anatomy and Physiology

Synovial joints, also known as diarthrodial joints, in all species function to facilitate motion and transfer load (Todhunter, 1996). They are composed of subchondral bone, articular cartilage and a joint capsule, which are supported by collateral ligaments (van Weeren and de Grauw, 2010; Todhunter, 1996). The joint capsule is made up of two major components: the fibrous portion and inner synovium.

The fibrous portion of the joint capsule is made up of collagen fibers to provide structural support for the joint capsule. The synovium, sometimes referred to as the synovial membrane, is located just inside the fibrous portion of the joint capsule and is highly vascularized and innervated (Todhunter, 1996). During movement, the synovium is able to simultaneously contract and distend as the horse extends and flexes to ensure adequate lubrication across the joint to protect articular cartilage and subchondral bone (Caola, 2003).

Synoviocytes are cells that make up the synovium, and are responsible for the exchange of compounds from blood to synovial fluid via an ultrafiltrate system (van Weeren and de Grauw, 2010). The production of hyaluronan, a non-sulfated glucosaminoglycan, is a significant role of synoviocytes. Following production, hyaluronan is secreted into synovial fluid and provides its viscous nature for lubrication of articular cartilage (Todhunter, 1996). Further, these cells play a significant role in the inflammatory response to trauma and the onset of OA.

Synovial fluid is located within the joint capsule, beneath the synovium, and functions to lubricate the joint system and provide essential nourishment to articular cartilage and intra-articular ligaments (Todhunter, 1996). Articular cartilage lies on the articulating surface of subchondral bone to provide protection to the boney processes and allow for smooth movement during motion. Made up of approximately 70% water, articular cartilage is translucent in appearance, and is composed of chondrocytes and an extracellular matrix (Neil et al., 2005). During loading, articular cartilage self-compresses, releasing its water contents into the synovial fluid cavity. When the load is

removed, cartilage acts as a sponge and rehydrates (Caola, 2003). During rehydration through unloading, articular cartilage is able to uptake essential nutrients from the synovial fluid, thereby maintaining its integrity (Caola, 2003). For this reason, movement is essential to sustain joint health; however, during excessive loading, this is also where articular cartilage uptakes degradative enzymes produced by synoviocytes.

The extracellular matrix of articular cartilage is made up of collagen, proteoglycans and glycoproteins, which make up approximately 50, 35 and 10% of articular cartilage on a dry weight basis, respectively (Todhunter, 1996). Type II collagen constitutes the fibrous portion of the extracellular matrix and is responsible for tensile strength of articular cartilage (Neil et al, 2005). The other major solid component of articular cartilage is proteoglycan. Proteoglycans are glycosylated proteins, whose function is dependent upon the glycosaminoglycans present (Yanagishita, 1993). In general, they function to provide hydration and swelling pressure to articular cartilage, which allows the joint to withstand compression forces (Yanagishita, 1993). Aggrecan is the largest, most abundant and most extensively studied proteoglycan within articular cartilage. It is produced by chondrocytes and is made up of covalently attaching glycosaminoglycans chondroitin sulfate and keratin sulfate to the proteoglycan protein core (Neil et al., 2005; Todhunter, 1996). A comprehensive picture of synovial joint and extracellular matrix anatomy is provided in Fig. 1.

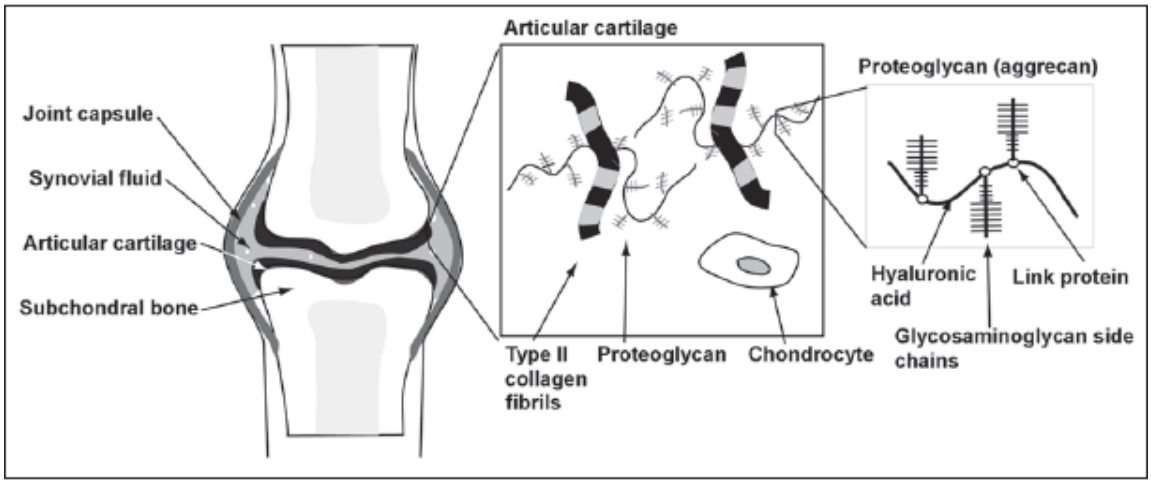


Figure 1: Synovial joint anatomy with articular cartilage composition (adapted from Neil et al., 2005).

While the extracellular matrix provides structure for articular cartilage, chondrocytes play a major role in the homogeneity of articular cartilage. Chondrocytes are the cellular component of articular cartilage and amount to approximately 1 to 12% of total dry matter (Todhunter, 1996). They obtain nutrients from synovial fluid, which include glucose, oxygen and amino acids that diffuse into the synovial fluid from the vasculature in the synovium via the ultrafiltrate system described previously. As a general rule, any molecule smaller than hemoglobin can diffuse into the extracellular matrix and to chondrocytes (Todhunter, 1996). Some of the glucose taken up by the chondrocytes is converted to glucosaminoglycans (GAGs), and later covalently bound to a protein core for proteoglycan production to maintain the integrity of the extracellular matrix (Fig. 2 Neil et al., 2005). Furthermore, chondrocytes produce type II collagen; however, the complex processes and multiple cellular compartments involved in its

production cause its turnover rate to be longer than that of proteoglycans. For example, it is estimated that the average type II collagen turnover times in dogs and humans is 120 and 350 years, respectively (Todhunter, 1996). Due to this slow turnover it is important to maintain the integrity of the collagen portion of the extracellular matrix to maintain joint health and stability.

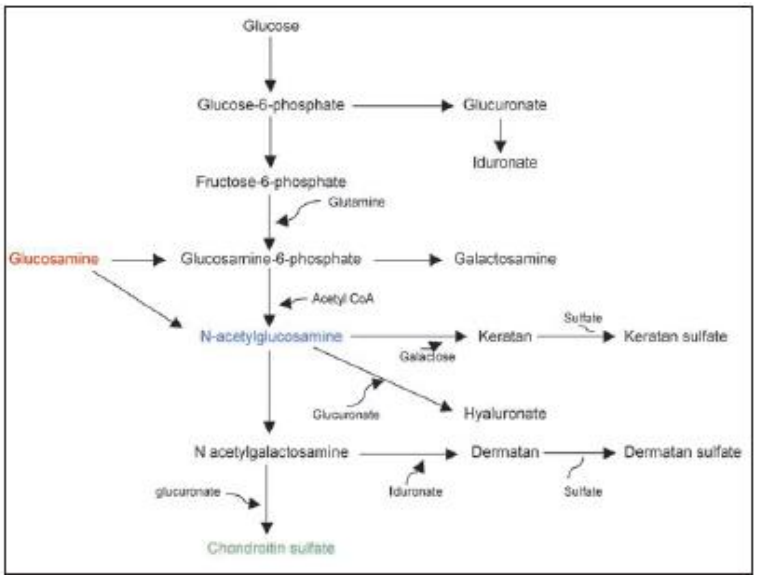


Figure 2: Production of glucosaminoglycans keratin sulfate, dermatan sulfate and chondroitin sulfate from glucose. (Adapted from Neil et al., 2005).

Finally, subchondral bone is the innermost component of the synovial joint system and provides the structural support for the animal. The subchondral bone can be subdivided into the subchondral bone plate and trabecular bone, which separates

calcified cartilage from marrow-containing spaces of bone (Kawcak et al., 2001). The trabecular bone contains blood vessels that channel into the calcified cartilage layer and provide nourishment to deeper portions of articular cartilage that cannot receive nutrients from the synovial fluid (Duncan et al., 1987). Subchondral bone is extensively adaptable and undergoes modeling and remodeling as a response to loading. Modeling is described as solely bone deposition, while remodeling indicates the removal and replacement of bone tissue. Osteocytes regulate the modeling and remodeling of subchondral bone and can sense local changes in loading intensities and duration (Duncan and Hruska, 1994). As with articular cartilage, regular exercise is advantageous for the maintenance and strengthening of subchondral bone. Bone formation tends to slow when loading duration or intensity decreases. For example, when Thoroughbred foals were moved from pasture to stable housing, there was a reduction in bone formation due to decreased physical activity (Maenpaa et al., 1988). When assessing subchondral bone, it is normal for different areas to have varying densities and thickness due to variations in mechanical processes across the joint (Kawcak et al., 2001).

In growing animals, modeling of bone should exceed bone resorption for normal skeletal development. The modeling of bone is due to the presence of peptide growth factors such as: TGF-β, IGFs, platelet-derived growth factor and fibroblast growth factor (Todhunter, 1996). These growth factors affect cellular proliferation, which gives rise to increased calcification. However, during excess loading or trauma, all cells within the

synovial joint are able to produce cytokines and eicosanoids, which prompt tissue degradation and lead to joint disease.

Pathophysiology of Inflammation and Osteoarthritis

A degenerative joint disease, OA, is characterized by degradation of articular cartilage, and can encompass changes to subchondral bone as well. Simply put, OA is caused by abnormal loading on normal cartilage or normal loading on abnormal cartilage (McIlwraith et al., 2012). Recurring or severe acute synovitis has also been identified as a precursor to equine OA, and can account for the inaugural changes in articular cartilage. Each cell type within the synovial joint has the capacity to initiate an inflammatory response. Regular exercise and movement is encouraged to maintain the health and homogeneity of the synovial joint; however, too much can shift joint metabolism from anabolic to catabolic, leading to a net degeneration of joint tissue.

Inflammation

The inflammatory response occurs following excess loading or trauma to the joint. The purpose of an inflammatory response is to protect against foreign agents and remove damaged tissues. With the occurrence of trauma, cells are damaged and the inflammatory response is initiated to destroy and remove the damaged cells to protect remaining healthy cells from secondary damage. Inflammation within the synovial joint can occur individually in the synovial membrane, articular cartilage or subchondral bone, or in combination; however, the responses vary between tissues due to vasculature (McIlwraith, 1996).

When inflammation occurs in the synovial membrane, it is referred to as synovitis. Synoviocytes play a major role in initiating the inflammatory response; however, following trauma, neutrophils, macrophages and lymphocytes are recruited to the site to assist in degradation and removal of damaged cells (Jansson, 1996; McIlwraith et al., 2012). The macrophages and neutrophils produce the primary inflammatory cytokines including IL-1 and TNFα. Equine IL-1 was purified from monocytes following the use of lipopolysaccharide (LPS) to induce an inflammatory response confirming their origin from leukocytes recruited to a site of inflammation (May et al., 1990). Cytokines TNFα and IL-1 perform similar functions within the joint. Both inhibit the production of proteoglycans in articular cartilage and initiate a response by synoviocytes and chondrocytes (Arner and Pratta, 1989). In an in vitro study performed using bovine and human articular cartilage slices, it was found that TNFα both degrades and inhibits the synthesis of proteoglycans (Kowanko et al., 1990).

Chondrocytes and synoviocytes respond to trauma directly, but also respond to cytokines IL-1 and TNFα as a secondary up-regulation. In response to injury and presence of cytokines, synoviocytes release aggrecanase, MMPs, free radicals and prostaglandins (McIlwraith et al., 2012). Each product is released into the synovial fluid and can be absorbed by the articular cartilage where chondrocytes become exposed to IL-1 and TNFα, and exhibit their own inflammatory response (Jansson, 1996). This evidence suggests that the inflammatory response extends into the synovial fluid and articular cartilage whether the injury affects those areas or not.

The MMPs released by synoviocytes and chondrocytes include stromelysin, collagenase and gelatinase enzymes (McIlwraith et al., 2012). They are characterized as such because of their requirement for Zn^{2+} in their active sites (McIlwraith, 1996). These enzymes collectively contribute to degradation of the extracellular matrix of articular cartilage. In healthy articular cartilage, there is a balance between MMPs and tissue inhibitor of metalloproteinases to maintain the extracellular matrix; however, following trauma, this balance is disturbed, leading to degradation of type II collagen and proteoglycan turnover (Palmer and Bertone, 1994). The MMP stromelysin is believed to cleave proteoglycans such that the glycosaminoglycan-bearing region is released (Fosang et al., 1991). Stromelysin is also capable of denaturing collagen, as well as activating the zymogen procollagenase to collagenase for further fiber degradation (Fig. 3; Murphy et al., 1987).

When a type II collagen fibril is exposed to MMPs such as collagenase, hidden epitopes within the collagen fiber become exposed (Trumble et al., 2009). The exposed epitopes can then be used as biomarkers to indicate metabolism of type II collagen at the time of collection. Recent research has focused on the use of these hidden epitopes to diagnose early changes in articular cartilage indicative of OA before radiographic changes become evident. The most reliable source of measurement of such epitopes is through synovial fluid samples, which provide the most direct assessment of type II collagen metabolism. One such biomarker is C2C, which has been used frequently in equine research as a biomarker of collagen metabolism.

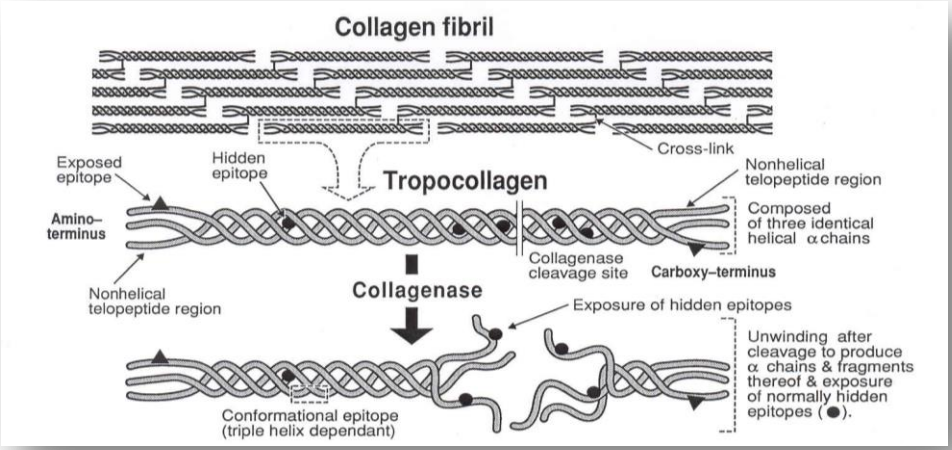


Figure 3: Collagenase degradation of type II collagen (adapted from Poole, 1992).

In addition to the MMPs, eicosanoids, including prostaglandins, are released from synoviocytes and chondrocytes. The most significant prostaglandin in the inflammatory response is PGE_2. It is synthesized from 20:4 via the cyclooxygenase-2 (COX-2) pathway (Fig. 4; Vane et al., 1998). The COX enzyme is located on macrophages released during an inflammatory response (Iwakiri et al., 2002). While 20:4 is present in all cell membranes, repetitive trauma increases 20:4 concentrations within the membranes of chondrocytes (Chrisman et al., 1981). The functions of PGE_2 include vasodilation, enhancement of pain perception, proteoglycan depletion and bone demineralization (McIlwraith, 1996). The enhancement of pain perception is a suggested correlation with substance P, which is a neurotransmitter present in the joint capsule (Kirker-Head et al., 2000).

Prolonged or repeated exposure to inflammatory cytokines and eicosanoids can damage articular cartilage beyond the capacity of regeneration. When this occurs, OA is the unfortunate development. Deterioration in the integrity of articular cartilage affects the health and strength of subchondral bone. If inflammation can be abated, the onset of OA can be delayed. Therefore, it is desirable to seek ways in which to limit the release of inflammatory cytokines and eicosanoids as an approach to mitigating cartilage degradation.

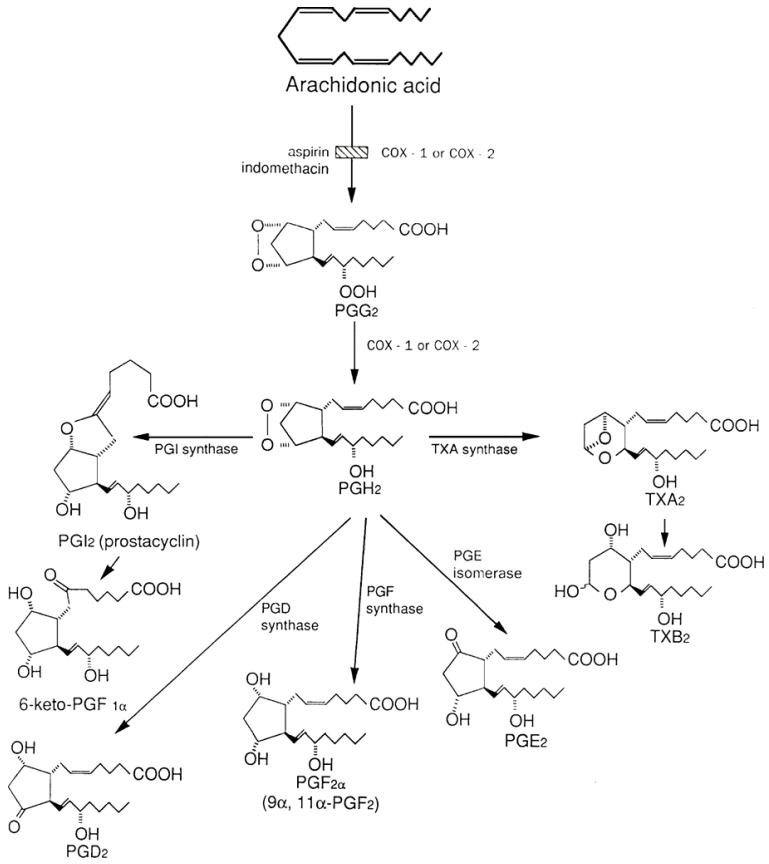


Figure 4: Synthesis of PGE_2 from 20:4 (adapted from Vane et al., 1998)

Effects on Subchondral Bone

Damage to subchondral bone can occur after the integrity of articular cartilage is compromised, or changes in subchondral bone can lead to changes in articular cartilage leading to OA. For this reason, researchers often induce OA by creating an osteochondral fragment in the subchondral bone of a synovial joint (McIlwraith et al., 2012). Through this process, pathogenesis of OA can be monitored at various stages without creating significant pain.

Like articular cartilage, bone is constantly turning over. In growing animals, bone formation should exceed bone resorption, and in adults, formation and resorption should be balanced. However, when this balance is obscured, net bone loss or excess calcification occurs, which can damage articular cartilage. The two primary cell types associated with bone formation and resorption are osteoblasts and osteoclasts, respectively (Harada and Rodan, 2003). When mechanical loads are increased, bone formation is stimulated and bone resorption is suppressed. During bone formation, osteoblasts synthesize bone alkaline phosphatase, which likely contributes to the calcification of bone matrix (Trumble et al., 2007).

When articular cartilage is damaged, the response of subchondral bone is usually microdamange (Kawcak et al., 2001). The microdamage can lead to an inflammatory response being initiated by cells within subchondral bone. It has been suggested that the eicosanoid, PGE_2 that is also associated with synovitis, can cause bone demineralization (Raisz, 1995; Schlueter and Orth, 2004). It is likely that inhibiting bone resorption will also inhibit prostaglandin release during OA (Kawaguchi et al., 1994).

Use of Inflammatory and Cartilage Biomarkers

Currently, diagnosis of OA is performed through the use of radiography to evaluate thinning of articular cartilage. Since a major precursor to OA is inflammation, the diagnosis of acute or recurring inflammation is desirable for the prevention or delay of OA onset. A popular area of research has been investigating biomarkers and their correlation with the inflammatory response and degradation of articular cartilage. Early research in biomarkers began with urinary analysis, as it is a less invasive means by which to collect a sample. More recently, however, blood has become a popular means to analyze inflammation, but synovial fluid biomarkers will give the most direct assessment of the status of the synovial joint.

Prior to biomarker analysis, hormones were analyzed as indicators of joint health; however, they can be unreliable. Early urinary analysis investigated bone resorption through markers released into circulation and excreted through urine. Two such markers include pyridinoline and deoxypyridinoline, which provide information on ongoing bone resorption in disorders such as osteoporosis, hyperparathyroidism and metastatic bone disease (Seibel et al., 1992). Urinary markers were soon after replaced with blood and synovial fluid markers. The majority of the biomarkers analyzed through blood and synovial fluid are the same; however, there is no way of knowing that the biomarkers present in blood in fact originated in the joint of interest.

Markers of Inflammation

Markers of inflammation are the same as the inflammatory response markers described previously; however, cartilage and bone biomarkers are representative of

products released during degradation. Many studies have researched the presence of synovial fluid cytokines and eicosanoids present with inflammation (Bertone et al., 2001; De Grauw et al., 2006; Frisbie et al., 2008). Products such as TNFα, IL-1 and PGE_2 are the most extensively studied of the inflammatory biomarkers. Inflammatory markers PGE_2, IL-1 and TNFα were analyzed in a study performed using horses with naturally occurring joint disease (Bertone et al., 2001). PGE_2 concentrations had the greatest correlation to joint disease, and was determined to be a significant indicator of any joint disease at varying levels of severity with values greater than 63.5 pg/mL (Bertone et al., 2001). However, in this study, IL-1 concentrations varied considerably with individual horses making it a poor indicator of joint disease severity (Bertone et al., 2001). When OA was experimentally induced via an osteochondral fragment, PGE_2 was found to peak approximately 7 d following surgical OA induction (Frisbie et al., 2008). However, other studies have used LPS to induce a local inflammatory response in the joint. When this method was used, PGE_2 concentrations peaked between 8 and 12 h following LPS injection (de Grauw et al., 2009; Lucia et al., 2013). It is important to note, however, that when synovial fluid samples are repeatedly collected via arthrocentesis, the invasive procedure further stimulates inflammation and could lead to confounding effects when evaluating inflammation (van den Boom et al., 2005).

Markers of Articular Cartilage

When evaluating biomarkers as an indication of articular cartilage health, inflammatory markers do not provide any indication of the current cartilage metabolism. Therefore, several other markers have been used to provide indications of cartilage

remodeling or degradation. The biomarkers produced by articular cartilage enter the synovial fluid and concentrations can be analyzed without taking articular cartilage samples, providing a less invasive technique to study articular cartilage metabolism. It has been suggested that changes in GAG concentration throughout the extracellular matrix is an indication of matrix breakdown; therefore, the GAG content of synovial fluid can be assessed for articular cartilage metabolism (McIlwraith et al., 2012). The GAG content of articular cartilage increased following induction of OA by osteochondral fragmentation, indicating that it could be a possible indicator of OA (Frisbie et al., 2008). However, in a study performed in horses with naturally occurring lameness, no correlation was seen between GAG content and articular cartilage degradation; therefore, GAG content may not be the best indicator of articular cartilage degradation (Fuller et al., 2001).

Matrix MMPs have also been considered as possible markers to indicate proteoglycan degradation of the extracellular matrix; however, in a study of young horses with evidence of osteochondrosis, matrix MMP concentrations were unreliable as a possible marker (de Grauw et al., 2011). One marker that has proven reliable to evaluate proteoglycan stability is chondroitin sulfate 846 (CS846). Presence of CS846 indicates regeneration of proteoglycan content in articular cartilage. In all studies analyzed, CS846 proved to be a reliable marker of articular cartilage regeneration (Frisbie et al., 2008; de Grauw et al., 2011; de Grauw et al., 2009). In a study using osteochondral fragmentation to initiate osteochondrosis, CS846 concentrations in synovial fluid increased after 7 d and peaked 42 d after fragmentation (Frisbie et al.,

2008). In naturally occurring osteochondrosis, CS846 was greater in horses with osteochondrosis than horses with normal joints (de Grauw et al., 2011). Thirdly, in a study using intra-articular LPS injection to induce an inflammatory response, CS846 peaked approximately 24 h post-injection and returned to normal values 168 h post-injection (de Grauw et al., 2009). The combined analysis of these studies indicate that CS846 is a reliable marker of proteoglycan regeneration in naturally occurring osteochondrosis, osteochondral fragmentation and LPS models, but provides no information on the metabolism of type II collagen, which is the structural component of articular cartilage..

There are two contrasting markers of type II collagen turnover that have been studied extensively and have correlations with extracellular matrix metabolism. Carboxypeptide of type II collagen is an indicator of collagen regeneration. Billinghurst et al. (2003) evaluated foals separated into three treatment groups: stalled, exercised and stalled, and pasture housed. Serum CPII was greatest in foals housed in pasture (522.4 ± 35.8 ng/mL), and lowest in animals undergoing forced exercise (387.8 ± 26.2 ng/mL; Billinghurst et al., 2003). In a second study, CPII was evaluated following an inflammatory response initiated via intra-articular LPS injection and concentrations in synovial fluid peaked 24 h post-injection (Lucia et al., 2013). When OA was induced by creating an osteochondral fragment, CPII concentrations in synovial fluid increased approximately 21 d following surgery (Frisbie et al., 2008). These studies indicate that following loading or inflammation, type II collagen aims to repair and regenerate itself;

however, as stated previously, the turnover rate of type II collagen is extremely slow (Billinghurst et al., 2003; Frisbie et al., 2008; Lucia et al., 2013).

The second marker of type II collagen turnover, C2C, is a marker of type II collagen degradation. It is found in synovial fluid when a collagen fiber becomes exposed to collagenase. When the collagen fiber begins to denature, the C2C epitope is exposed and released into synovial fluid where it can then be measured. Several studies have used both C2C and CPII as contrasting markers of cartilage metabolism. One such study, following intra-articular LPS injection, measured concentrations of synovial fluid C2C, and observed peak values at 24 h post-injection, similar to CPII, with values of 344.97 ± 23.34 ng/mL (Lucia et al., 2013). Lucia et al. (2013) also indicated that C2C concentrations increase with increasing levels of inflammation through the use of three levels of LPS, further confirming the efficacy of C2C as a measurement of cartilage metabolism. Billinghurst et al. (2003) found elevated values in foals undergoing extensive training (1933.4 ± 598.6 ng/mL) and lower values in horses housed on pasture (906.5 ± 242.6 ng/mL) indicating increased loading to joints can promote cartilage degradation. These studies also found a correlation between the two type II collagen biomarkers. When type II collagen degradation increases via C2C measurements, regeneration also increases as measured through CPII.

All described biomarkers have been used to measure magnitudes of inflammation and cartilage metabolism with PGE_2 being a reliable marker of joint inflammation, and C2C and CPII contributing to type II collagen. More recent research has used these markers to determine efficacy of treatments and mitigation techniques. By reducing

concentrations of inflammatory markers, including PGE_2, cartilage degradation can be tempered to delay the onset of OA. Among these mitigation techniques, diet is the most popular due to ease of administration.

Polyunsaturated Fatty Acid Supplementation

Dietary supplementation of PUFAs, including ω-3 and ω-6, regulate inflammatory mediators including proinflammatory cytokines, anti-inflammatory cytokines, eicosanoids and nitric oxide (Bhattacharya et al., 2006). Linoleic acid, an ω-6 fatty acid, and α-linolenic acid (ALA), an ω-3 fatty acid, are essential fatty acids required in the diet of horses. Monogastrics are unable to synthesize linoleic and ALA because of the lack of necessary desaturases to place the double bonds appropriately. Once the two essential fatty acids are supplemented, available desaturases and elongases can act to produce other fatty acids including 20:4, eicosopentanoic acid (EPA), and docosohexanoic acid (DHA). In equine nutrition research, both EPA and DHA are commonly supplemented ω-3 fatty acids, and are believed to have anti-inflammatory effects, particularly EPA. The most common means of supplementation of EPA and DHA is fish oil; however, flaxseed oil is commonly used to supplement ALA, which can be converted to EPA and DHA (Hansen et al., 2002).

Dietary supplementation requires an adaptation period to the new product, which allows time for the added fatty acids to achieve circulating levels in plasma and incorporate into tissue (cellular) lipid membranes. Following flaxseed oil supplementation, plasma concentrations of ALA were greater compared to horses receiving a control diet. Because ALA is the precursor to EPA and DHA, both of these

isomers were also higher in plasma after 28 d compared to non-supplemented horses (Hansen et al., 2002). Fish oil, on the other hand, is a direct source of both EPA and DHA, and after supplementation, both fatty acids reached peak circulating levels in equine plasma by the first collection period at 6 weeks following the onset of supplementation (Hall et al., 2004). Protected fatty acids are meant to remain intact through the digestive process. After feeding protected fatty acids to horses, EPA and DHA were maximized in plasma after 17 d (King et al., 2008). These studies lend confidence that supplemented fatty acids can reach circulating levels in plasma by 28 d following the onset of supplementation.

It has been suggested that supplementation of products such as fish oil with EPA and DHA decreases cytokine (IL-1 and TNFα) production by macrophages during an inflammatory response (Calder, 1996). Whether humans consumed fish oil for 6 or 24 weeks, there was a notable decrease in IL-1 and TNFα concentrations (Gallai et al., 1993; Endres et al, 1993). Since IL-1 and TNFα stimulate synoviocytes and chondrocytes to produce eicosanoids and MMPs, the reduction in cytokine production is likely to reduce the overall inflammatory response. By reducing exposure of articular cartilage to products of inflammation, the integrity of articular cartilage can likely be sustained without damage for an extended period.

As well as reducing cytokine production, ω-3 supplementation can also shift the ω-3:ω-6 in cell membranes (McCann et al., 2000). The ω-6 fatty acids are precursors to 20:4, which produces the prostaglandins associated with inflammation. By lowering the ω-6 concentration of membrane phospholipids, precursors to PGE_2 production are

restricted, which can further reduce the inflammatory response following trauma. For example, chicks fed a diet higher in ω-3 than ω-6 fatty acids had lower concentrations of 20:4 in bone (Watkins et al., 1997). It is important to note that, although fish oil is the most common means of EPA and DHA supplementation and has been found to reduce cytokine and eicosanoid production, it also contains a significant level of 20:4, which is the ω-6 precursor to PGE_2. For example, corn oil supplemented to horses contained undetectable levels of 20:4 and was used as the control diet; however, the fish oil supplemented contained 6.7% 20:4 (Hall et al., 2004). The data suggests that fatty acid supplementation can mitigate the inflammatory response to possibly delay the onset of OA; however, a fat supplement low in 20:4 may be necessary to maximize anti-inflammatory properties.

Conjugated Linoleic Acid Supplementation

CLA was originally extracted in 1985 from ground beef and found to have anti-carcinogenic effects. It has since been heavily researched in areas of bone remodeling, attenuation of inflammation, and effects on body composition in multiple species (Pariza and Hargraves; Bhattacharya et al., 2006). Most commonly, CLA has been supplemented as a top-dressed oil. Supplemental forms of CLA contain predominantly c9,t11 and t10,c12 isomers, which are the primary isomers of focus in research as they are believed to be attributed to the majority of health benefits (Hur and Park, 2007; Bhattacharya et al., 2006). Because these isomers are generally supplemented in combination, they do have independent, additive and antagonistic effects (Park and Pariza, 2007).

CLA and Body Composition

The t10,c12 isomer is most closely associated with changes in body composition and reduction in body fat (Park et al., 1999). This isomer is able to reduce body fat by inhibiting stearoyl-CoA desaturase, also known as the delta-9 desaturase, which is responsible for adding a double bond at the 9-carbon of a fatty acid chain (Hur and Park, 2007). The t10,c12 isomer is also capable of reducing hepatic release of apolipoprotein B, which is an integral part in the transport of triacylglycerols to tissues for storage in the body (Storkson et al., 2005). Currently, there is only one study to determine the effects of CLA on body fat in the horse. When fed a mixed isomer CLA supplement containing 28.5% c9,t11 and 28.1% t10,c12 over a 6-wk period, horses showed no changes in body fat composition (Headley et al., 2011).

CLA and Inflammation

More recently, research has shifted to investigate the anti-inflammatory effects of CLA. The majority of research studies utilizing CLA have fed mixed isomers rather than single isomers. Therefore, it is difficult to determine the effect of each isomer on inflammation and bone remodeling, however, it has been suggested that the c9,t11 isomer is likely the primary contributor. As described previously, macrophages and other white blood cells are sent to the location of injury and initiate an inflammatory response via the release of cytokines. When CLA is supplemented and circulating in blood plasma, it has an affect on macrophage function as it reaches the site of trauma (Yang and Cook, 2003).

Following in vitro LPS injection into macrophages treated with CLA, TNFα concentrations were lower in macrophages receiving CLA than control cells (Yang and Cook, 2003). A similar study aimed to determine the COX-2 activity with CLA supplementation in which macrophages were activated using LPS (Iwakiri et al., 2002). Those cells treated with CLA had 78% lower PGE_2 production than cells maintained in a lipid-free medium (Iwakiri et al., 2002). It has been hypothesized that depression in PGE_2 production may be due to the suppression of the transcription of COX-2 (Iwakiri et al., 2002). An alternative anti-inflammatory mechanism of CLA is perhaps a reduction in the activity of phospholipase A2. This enzyme functions to cleave fatty acids off of the second position of the glycerol backbone. By limiting the enzyme's activity, the availability of 20:4 for the COX-2 pathway is reduced; therefore, lower concentrations of PGE_2 would be expected following an inflammatory response (Eder et al., 2003). Eder et al. (2003) confirmed CLA's ability to lower the activity of phospholipase A2 through the use of human aortic endothelial cells.

Changhua et al. (2005) performed an in vivo study using pigs challenged with LPS at 14 and 21 d following the onset of dietary supplementation of CLA at a level equal to 2% of the diet. On d 21, concentrations of IL-1 were 61.2 and 94.8 ng/L for CLA and control pigs, respectively (Changhua et al., 2005). Similarly, PGE_2 concentrations on d 21 were 703.4 and 998.7 pg/mL in pigs receiving CLA vs. control, respectively (Changhua et al., 2005). The previous in vitro studies validated CLA's use as an anti-inflammatory; however, the data presented by Changhua et al. (2005) confirms that CLA can be reach circulating levels through dietary supplementation and

function to reduce eicosanoid production. The flow chart illustrated in Fig. 5 summarizes these anti-inflammatory properties of CLA and how they act to delay the onset of OA.

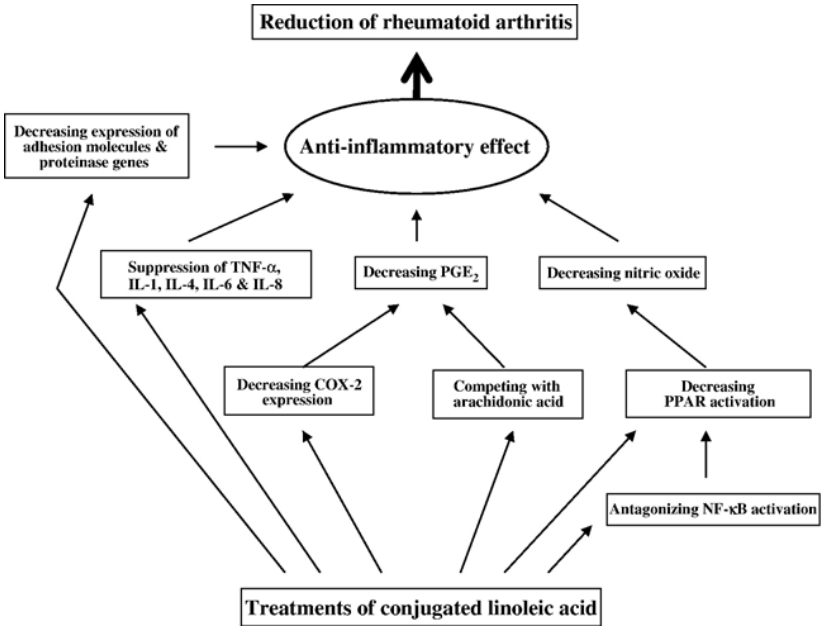


Figure 5: Mechanism of CLA effects on inflammation to prevent osteoarthritis (adapted from Hur and Park, 2007).

CLA and Bone Metabolism

With these data regarding the effects of CLA on inflammation, questions have arisen about its effects on bone metabolism. At this time, data to support CLA's effects on bone metabolism are variable and few conclusions can be made; however, it is still

hypothesized that CLA supplementation can have a positive effect on bone. Osteoblasts, cells within bone responsible for resorption, produce PGE_2 that initiates bone loss (Hur and Park, 2007). As described previously, fatty acid supplementation can reduce 20:4 levels in cell membranes, but CLA-specific supplementation may also reduce the activity of COX-2 to reduce PGE_2 production. This provides the basis for the theory of CLA's effects on bone metabolism; however, the current data are unreliable. For example, Kelly et al. (2003) suggested that CLA supplementation increased Ca absorption from the small intestine; however, they saw no effect on biomarkers of bone formation or resorption. Similar results were observed in a study performed in adult men where markers of bone formation and resorption were analyzed and showed no difference between CLA and placebo treatments (Doyle et al., 2005). In ovariectomized rats, however, CLA supplementation at 5 and 10 g/kg diet significantly lowered PGE_2 production, providing some evidence that CLA may, in fact, reduce bone resorption (Kelly and Cashman, 2004).

The extensive variability of data on the effects of CLA supplementation on bone health and metabolism make it difficult to formulate significant conclusions. Measurements of articular cartilage through the use of biomarkers can provide valuable understanding of the effects of CLA. As of yet, no studies have been performed to determine the effects of dietary CLA on markers of articular cartilage metabolism and could be a reliable means to better determine the effects of CLA on joint health.

Conclusion

In summary, OA is the leading cause of lameness in the equine industry (McIlwraith et al., 2012). Its characterization as loss of articular cartilage makes it desirable to search for a solution to stop or reverse the degradation process. When an animal undergoes overloading or trauma to a synovial joint, an inflammatory response is initiated. The goal of the inflammatory response is to remove damaged cells; however, healthy cells and matrix tissues can become damaged in the process. When inflammation occurs, white blood cells move to the location of trauma and release pro-inflammatory cytokines such as IL-1 and TNFα. The trauma and presence of the cytokines signal local cells such as synoviocytes or chondrocytes to release degradative enzymes, eicosanoids and free radicals. Each of these products plays a role in the degradation of the extracellular matrix of articular cartilage.

The extracellular matrix is made up of type II collagen and proteoglycans, which provide its elastic properties. Proteoglycans can undergo degradation and regeneration more rapidly than type II collagen; therefore, the loss of type II collagen is paramount in the occurrence of OA. At this point, diagnosis of OA cannot be performed until significant changes in the integrity of articular cartilage can be seen through radiographic imaging. The use of inflammatory biomarkers could lead to earlier diagnosis, and the ability to provide treatment before excess damage exists.

Mitigation of inflammation is likely the best way to delay the onset of OA. The supplementation of PUFAs such as ALA, EPA and DHA has been successful in reducing the inflammatory response by reducing cytokine and eicosanoid production.

Fish oil is the most common means of supplementation of ω-3 fatty acids; however, it also has a significant level of 20:4 within the oil. Since 20:4 is a direct precursor to prostaglandin production, it is desirable to find a way to maximize anti-inflammatory properties without also supplementing a pro-inflammatory. The use of CLA-rich oil, which is often plant-derived and contains very little 20:4, could provide a better method by which to mitigate inflammation through dietary supplementation.

Therefore, the objective of this study was to determine the incorporation of supplemental CLA into circulating levels of plasma and to evaluate the effects of dietary CLA supplementation on joint inflammation following an LPS challenge. This overall objective was tested in two phases with phase one used to determine the incorporation of supplemental CLA to circulating levels in plasma. Phase two was then be used to evaluate the potential of dietary CLA to mitigate induced intra-articular inflammation and cartilage metabolism. The long-term goal of this study is to determine the influence of diet on joint inflammation in young horses and its effect on joint health as an adult.

CHAPTER III

MATERIALS AND METHODS

All procedures and handling of horses was approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP # 2011-170)

Horses and Dietary Treatments

Seventeen Quarter Horse yearlings (n = 8 males, n = 9 females) with average age of 470 ± 26 d and BW of 354.0 ± 16.0 kg were used in a randomized complete block design for a 56-d trail separated into two phases. Phase 1 was used to determine incorporation of dietary CLA supplementation into plasma. Phase 2 immediately followed phase 1, on d 42 of the study. During phase 2, an intra-articular LPS challenge was conducted to induce a local, acute inflammatory response in the radiocarpal joint. Prior to the start of the experiment, horses were blocked by age, sex and BW and randomly assigned to one of three treatment groups. Throughout the experiment, horses were housed in 3.0 m x 3.0 m box stalls from 1830 h to 0930 h daily. Between 0930 h and 1830 h, horses were allowed free exercise in dry-lots, with the exception of d 42 when the LPS challenge was conducted.

All diets were formulated to be isocaloric and isonitrogenous. Horses were fed individually at 1% BW (as-fed) commercial concentrate feed (14% CP textured feed, Producers Cooperative Association, Bryan, TX) and 1% BW (as fed) coastal bermudagrass (cynodon dactylon) hay daily separated into two feedings at 0630 and 1830 h. All dietary treatments were top-dressed on the concentrate feed and fed as a

percent of total diet. Horses received either a control diet containing 1% soybean oil (CON; n = 6), 0.5% soybean oil + 0.5% CLA (LOW; n = 5), or 1% CLA (HIGH; n = 6). The CLA supplement contained 55% CLA with a mixture of cis-9, trans-11; trans-10, cis-12; and trans-9, trans-11 isomers (Lutalin®, BASF Corp., Florham Park, NJ). Days 0 to 7 were used as a dietary adaptation period where horses were slowly adapted to concentrate from their previous diet to treatments by increasing treatment diet and decreasing the previous diet by 25% every 2 d until horses received only the treatment diet on d 7. Oil supplementation began on d 1. Starting on d 7, no significant refusals of diet or treatments were observed. Samples of concentrate, hay, soybean oil and CLA were all analyzed by a commercial laboratory for nutrient content (Table 1) and fatty acid concentrations (Table 2).

Table 1. Nutrient analysis of the commercial concentrate and coastal bermudagrass hay (*cynodon dactylon*).

	Concentrate[a]	Coastal Bermudagrass Hay
Dry Matter, %	92.35	92.45
Crude Protein, % DM	18.82	11.26
NDF, % DM	16.83	61.05
ADF, % DM	9.47	33.87
DE, Mcal/kg DM	3.42	2.51
EE, % DM	6.84	3.53
Ca, % DM	1.14	0.6
P, % DM	0.73	0.22
K, % DM	1.35	1.12
Mg, % DM	0.26	0.34

[a] 14% CP textured feed (Producer's Cooperative, Bryan, TX)

Table 2. Concentration of CLA isomers and arachidonic acid in feedstuffs and supplements.

Fatty Acid	Concentrate[a]	Coastal Bermudagrass Hay	Soybean Oil	CLA[b]
cis-9, trans-11 CLA, %	ND[c]	ND	ND	26.18
trans-10, cis-12 CLA, %	ND	ND	ND	26.12
Arachidonic acid, %	0.25	0.52	0.32	0.57

[a] 14% CP textured feed (Producer's Cooperative, Bryan, TX)
[b] Lutalin® (BASF Corp., Florham Park, NJ)
[c] ND, not detectable (minimum detectable level 0.003%)

Sample Collection

Phase 1

Weekly, beginning at d 0, physical growth measurements were recorded including BW, wither and hip heights, body length, heart girth circumference, rump fat thickness and body condition score (BCS). The BCS was determined using the 1 to 9 scale outlined by Henneke et al. (1983). Rump fat thickness was measured via ultrasound images on the left hip at a point 5 cm dorsal of halfway between the first coccygeal vertebrae and the ischium (Westervelt et al., 1976) using an ultrasound instrument (Aloak SSD-500V, Aloka Inc., Tokyo, Japan). Approximately 10 mL whole blood were collected weekly via jugular venipuncture into 10-mL evacuated tubes containing 0.1 mL 15% buffering solution and 15 mg K_3 EDTA (BD Vacutainer, Franklin Lakes, NJ). Samples were immediately placed on ice and centrifuged at 2,000 x *g* for 20 min at 4°C. Plasma was collected into pour-off tubes and stored at -20°C for subsequent analysis.

Phase 2

On d 41, prior to LPS challenge, radiocarpal joints were clipped to allow for ease of aseptic preparation before arthrocentesis. On d 42, an LPS challenge was conducted. For each horse, radial carpal joints were randomly assigned as either treatment (LPS) or contralateral control (LRS). At pre-injection h 0 (PIH 0), carpal joints were aseptically prepared and horses were sedated with 1.5 mL Sedivet® (romifidine hydrochloride 1% injection, Boehringer Ingelheim, Fremont, CA). Approximately 4 mL synovial fluid was collected via arthrocentesis by veterinarians from the Texas A&M University Large Animal Clinic (College Station, TX). Immediately following PIH 0 sample collection, 0.5 ng *Escherichia coli* O55:B5 derived lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) was injected to the randomly assigned LPS joint. The 0.5 ng LPS was reconstituted in 0.8 mL sterile lactated Ringer's solution (LRS). The LRS joint received 0.8 mL sterile LRS only. Injections were given 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 a depth of approximately 12.7 mm to avoid unnecessary contact with articular cartilage (McIlwraith and Trotter, 1996).

Following LPS injection, synovial fluid samples were collected via arthrocentesis at 6, 12, 24, 168 and 336 h. Synovial fluid samples were transferred to non-additive 10 mL vacutainers (serum blood collection tubes, BD Vacutainer, Franklin Lakes, NJ) and immediately placed on ice. Synovial fluid was then divided into small aliquots in 1.5 mL snaplock microtubes and stored at -20°C for later analysis. Prior to collection times PIH 0, and 6, 12 and 24 h post-injection, rectal temperature (RT; °C), heart rate (HR;

beats per min), and respiration rate (RR; breaths per min) were recorded. Prior to each collection time, carpal circumference was measured at the level of the accessory carpal bone using a soft tape measure, and surface temperature of each carpal joint was determined at the same location and from a distance of 1.2 m using an infrared camera (FLIR E60 Series, Flir Systems Inc., Wilsonville, OR).

Sample Analysis

Nutrient Analysis

Hay and grain samples were dried for 48 h at 38°C (Lindberg/Blue M, Asheville, NC), then ground through a 1 mm mesh screen (Thomas Model 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ) prior to analysis. Fatty acids from hay, grain, soybean oil and CLA supplements was extracted and methylated using a modified Folch method and analyzed using a gas chromatograph (Folch et al., 1957; model CP-3800 fixed with a CP-8200 autosampler, Varian Inc., Walnut Creek, CA)). Nutrient content was analyzed by a commercial laboratory (SDK Laboratories, Hutchinson, KS).

Plasma Analysis

Total lipids were extracted by a modification of the method of Folch et al. (1957). One mL of plasma was extracted in chloroform:methanol (2:1, v/v) and fatty acid methyl esters (FAME) were prepared as described by Morrison and Smith (1964), modified to include an additional saponification step (Archibeque et al., 2005). The FAME were analyzed using a Varian gas chromatograph (model CP-3800 fixed with a CP-8200 autosampler, Varian Inc., Walnut Creek, CA). Separation of FAME was accomplished on a fused silica capillary column CP-Sil88 [100 m × 0.25 mm (i.d.)]

(Chrompack Inc., Middleburg, The Netherlands), with hydrogen as the carrier gas (flow rate = 35 mL/min). After 32 min at 180°C, oven temperature was increased at 20°C/min to 225°C and held for 13.75 min. Total run time was 48 min. Injector and detector temperatures were at 270°C and 300°C, respectively. Individual fatty acids were identified using genuine external standards (Nu-Chek Prep, Inc., Elysian, MN).

Synovial Fluid Analysis

Synovial fluid collected during the LPS challenge was analyzed for PGE_2, CPII, and C2C using commercial ELISA kits previously validated for use in horses (Bertone et al., 2001; de Grauw et al., 2006). The PGE_2 assay was a 96-well microplate with an anti-mouse monoclonal antibody (R&D Systems, Inc., Minneapolis, MN). Synovial fluid samples were diluted at 2:1, 1:2, 1:3, or 1:4 depending on time post-injection using calibrator diluent provided in the kit. Following dilution, all procedures followed the protocol outlined by the kit. The PGE_2 ELISA had intra-assay precision between 5.0 and 9.0% and inter-assay precision between 9.0 and 12.9%.

Also a 96-well microplate, the CPII commercial ELISA kit used a rabbit polyclonal antibody specific for CPII peptide (IBEX Pharmaceuticals Inc., Montreal, Quebec, Canada). Synovial fluid samples were diluted using buffer III provided by the kit at 1:4, 1:5, 1:6 or 1:7 depending on time post-injection. The protocol outlined by the kit was followed for all procedures. The CPII plates ran with inter-assay precision within plates between 0.5% and 9.5% and intra-assay precision between plates between 0% and 8.8%.

Finally, C2C commercial kits using a mouse monoclonal antibody ran with inter-

assay precision between 0.6 and 9.5% and intra-assay precision between 1.0 and 6.0% (IBEX Pharmaceuticals Inc., Montreal, Quebec, Canada). All samples were diluted on a 1:3 dilution using buffer III provided in the kit. Final concentrations of all markers were read using a microplate reader with optical density set at 450 nm (BioRad 680 Microplate Reader, BioRad Laboratories, Hercules,CA).

Statistical Analysis

All data were analyzed using the PROC MIXED method of SAS (SAS Inst. Inc., Cary, NC). The model contained effects for treatment, time, knee, treatment by time, and treatment by time by knee interactions. The 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 treatments at individual time points. Significance was established for all variables at $P \leq 0.05$ and $P \leq 0.10$ was defined as a trend toward significance.

CHAPTER IV

RESULTS

Phase 1

Physical Measurements

Wither height, hip height, BW, BCS, length, heart girth circumference and rump fat thickness were not affected ($P > 0.40$) by dietary treatment, but all physical measurements, with the exception of BCS, increased over time ($P < 0.01$; Table 3).

Plasma Fatty Acid Concentrations

A treatment by time interaction was observed for both isomers of CLA ($P < 0.01$). The c9,t11 isomer was detectable in LOW and HIGH horses by d 14 of supplementation with horses fed HIGH having greater ($P = 0.04$) concentrations. Similar results were observed for the t10,c12 CLA isomer with d 14 values tending to be greater ($P = 0.08$) in HIGH horses than LOW. Concentrations of both CLA isomers in LOW horses peaked and plateaued d 14 after dietary treatments began, but concentrations continued to increase to d 42 for horses fed HIGH. Plasma concentrations of both CLA isomers then declined ($P < 0.01$) in HIGH horses between d 42 and 56 to levels similar to those observed at d 28.

Table 3. Growth measurements of yearling horses fed increasing levels of CLA from d 0 to 56[1].

		Treatment[2]				*P*-values[3]		
Measurement	Day	CON (n = 6)	LOW (n = 5)	HIGH (n = 6)	SEM	Trt	Time	Trt * Time
BW, kg	0	353	353	354.8	10.4	0.97	< 0.01	0.93
	7	358	361	360.4				
	14	357	362	362.3				
	21	361	364	365.6				
	28	364	370	367.9				
	35	367	374	371.0				
	42	370	376	370.6				
	49	371	374	371.0				
	56	373	375	373.2				
Wither height, cm	0	137	139.3	138.1	1.9	0.81	< 0.01	0.42
	7	139.2	140.0	139.0				
	14	135.6	140.1	140.3				
	21	139.9	140.3	141.3				
	28	140.2	140.6	141.4				
	35	140.9	141.2	141.6				
	42	140.3	141.0	141.4				
	49	140.7	141.0	141.7				
	56	140.7	141.4	141.9				
Hip height, cm	0	143.5	144.7	143.8	1.5	0.93	< 0.01	0.89
	7	143.9	145.4	144.7				
	14	144.8	145.3	144.7				
	21	145.4	146.2	145.5				
	28	145.4	146.2	146.2				
	35	145.7	146.7	146.4				

Table 3. Continued

		Treatment[2]				*P*-values[3]		
Measurement	Day	CON (n = 6)	LOW (n = 5)	HIGH (n = 6)	SEM	Trt	Time	Trt * Time
	42	145.7	146.4	146.5				
	49	146.3	146.4	146.5				
	56	147.9	147.7	147.6				
Length, cm	0	147.2	146.2	145.2	1.7	0.97	< 0.01	0.73
	7	147.3	147.6	147.3				
	14	149.0	148.2	149.0				
	21	151.3	150.4	150.5				
	28	151.5	150.6	150.7				
	35	151.8	151.2	152.5				
	42	152.3	152.4	153.8				
	49	153.3	153.3	153.6				
	56	153.3	153.7	153.6				
Heart girth, cm	0	159.8	157.5	160.5	1.9	0.87	< 0.01	0.71
	7	160.3	160.0	161.7				
	14	161.3	160.4	162.5				
	21	162.2	162.2	163.3				
	28	162.7	163.0	163.5				
	35	163.0	163.0	163.8				
	42	163.0	163.6	163.8				
	49	163.9	162.8	164.0				
	56	164.0	163.6	164.0				
BCS	0	5.5	5.8	5.4	0.2	0.81	< 0.01	0.72
	7	5.4	5.7	5.4				

Table 3. Continued

Measurement	Day	Treatment[2] CON (n = 6)	LOW (n = 5)	HIGH (n = 6)	SEM	*P*-values[3] Trt	Time	Trt * Time
	14	5.4	5.7	5.6				
	21	5.4	5.5	5.4				
	28	5.6	5.7	5.5				
	35	5.7	5.7	5.5				
	42	5.5	5.4	5.5				
	49	5.3	5.7	5.6				
	56	5.8	5.9	5.8				
Rump fat thickness, cm	0	0.28	0.29	0.28	0.01	0.78	< 0.01	0.79
	7	0.29	0.30	0.29				
	14	0.29	0.30	0.30				
	21	0.29	0.31	0.31				
	28	0.31	0.32	0.33				
	35	0.32	0.32	0.33				
	42	0.32	0.32	0.33				
	49	0.33	0.34	0.33				
	56	0.33	0.34	0.34				

[1]Least square means
[2]Dietary treatments consisted of 1% diet soybean oil (CON), 0.5% diet soybean oil with 0.5% diet CLA (LOW), and 1% diet CLA (HIGH).
[3]Trt, treatment; Trt*time, treatment by time interaction

Both CLA isomers (c9,t11; t10,c12) were affected by dietary treatment ($P <$ 0.01) with horses in the HIGH group having the greatest plasma concentrations, followed by LOW, with lowest concentrations observed in CON. Horses fed CON had undetectable levels (minimum detectable level 0.0003%) of CLA throughout the 56 d trial, and both isomers increased over time in supplemented horses (HIGH and LOW; P < 0.01). Figures 6 and 7 illustrate concentrations of c9,t11 and t10,c12 CLA isomers expressed as a percentage of total fatty acid content. Concentrations of 20:4, represented in Fig. 8, were also influenced by CLA supplementation with HIGH horses having lower (P < 0.01) concentrations than CON and tending to be lower (P = 0.06) than LOW.

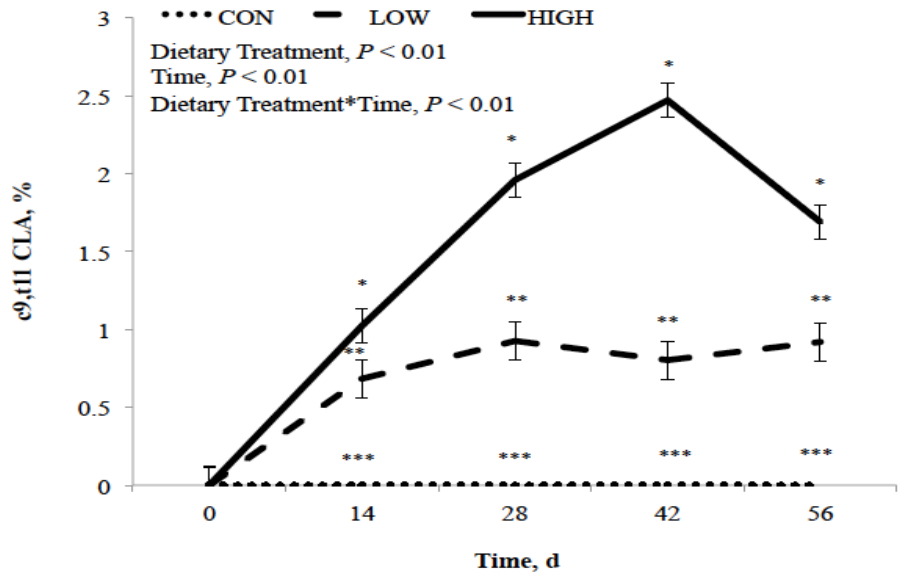


Figure 6. Changes in circulating levels of cis-9, trans-11 isomer of CLA in plasma (c9,t11; % total plasma fatty acid content) over time (d; least squares means ± SEM). Treatments were 1% diet soybean oil (CON; n = 6), 0.5% diet soybean oil with 0.5% diet CLA (LOW; n = 5), and 1% diet CLA (HIGH; n = 6). $^{*, **, ***}$ Superscripts denote a difference among treatments at each time (P < 0.05).

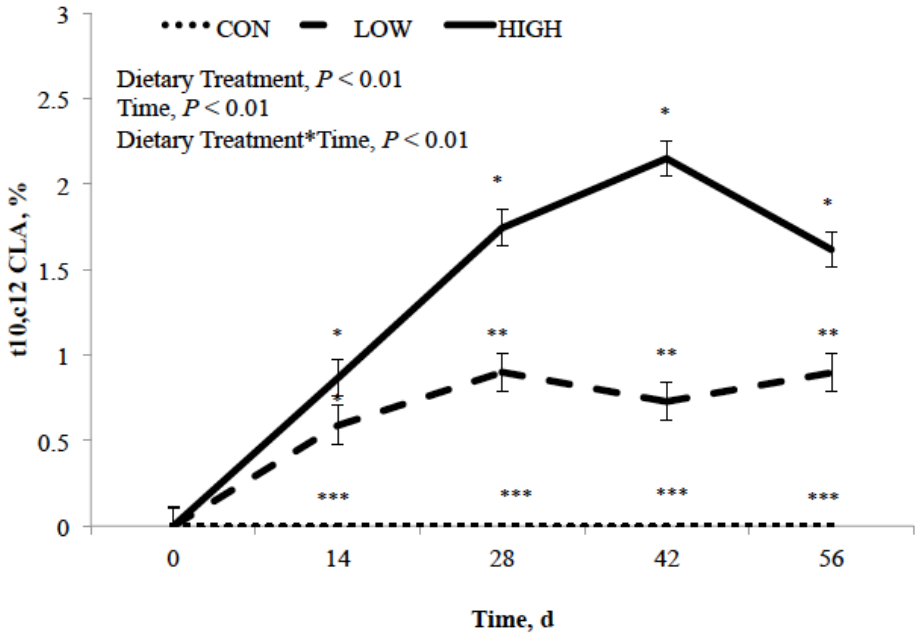


Figure 7. Changes in circulating levels of trans-10, cis-12 isomer of CLA in plasma (t10,c12; % total plasma fatty acid content) over time (d; least squares means ± SEM). Treatments were 1% diet soybean oil (CON; n = 6), 0.5% diet soybean oil with 0.5% diet CLA (LOW; n = 5), and 1% diet CLA (HIGH; n = 6). *, **, *** Superscripts denote a difference among treatments at each time ($P < 0.05$)

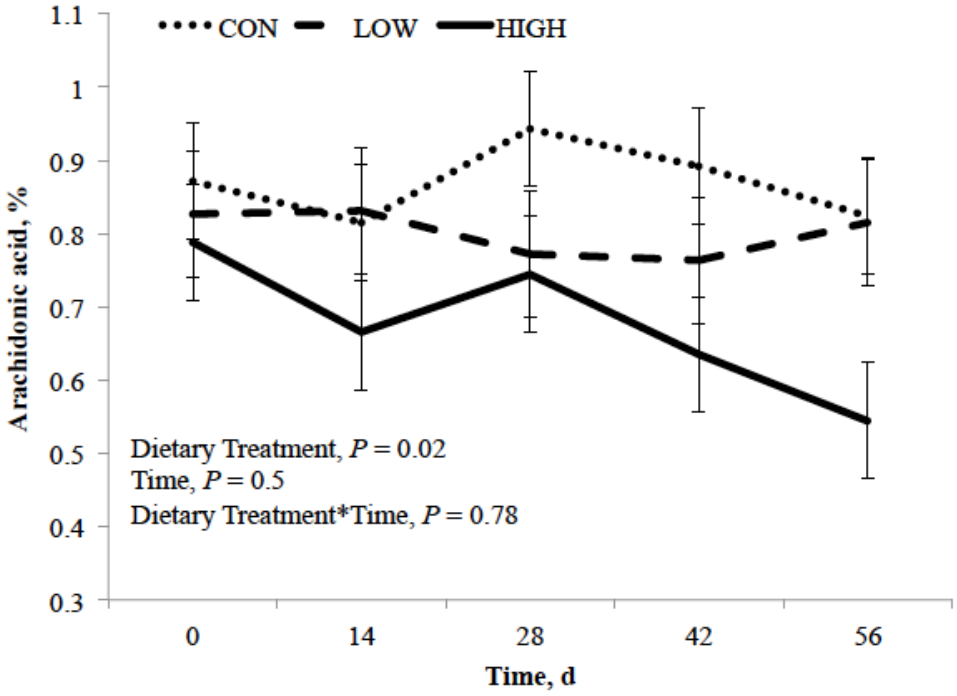


Figure 8. Changes in circulating arachidonic acid levels in plasma (% total plasma fatty acid content) over time (d; least squares means ± SEM). Treatments were 1% diet soybean oil (CON; n = 6), 0.5% diet soybean oil with 0.5% diet CLA (LOW; n = 5), and 1% diet CLA (HIGH; n = 6).

Additionally, plasma concentrations of 18:0 and 20:2 were influenced by dietary treatment ($P = 0.04$ and $P = 0.02$, respectively). Stearic acid (18:0) concentrations were higher ($P = 0.01$) in LOW horses than CON, and increased ($P < 0.01$) in all horses over time, regardless of dietary treatment. Plasma concentrations of 20:2 were lower ($P < 0.01$) in HIGH horses compared to CON, and also tended to increase ($P = 0.08$) over time for all horses, regardless of dietary treatment. Plasma concentrations of other fatty acids changed over time, regardless of dietary treatment, and included: 16:0, 18:1, 18:2, 18:3, and 22:6 ($P < 0.01$). An overall increase over time ($P \leq 0.01$) was observed for plasma concentrations of 16:0, 18:1, and 18:2 for all horses while concentrations of 18:3 and 22:6 decreased ($P < 0.01$) over time with 18:3 showing a sharp decrease from d 0 to 14. Plasma fatty acid concentrations are illustrated in Table 4.

Table 4. Plasma fatty acid concentrations of yearling horses fed increasing levels of CLA from d 0 to 56.[1]

		Treatment[2]				P-values[3]		
Fatty Acid	Day	CON (n = 6)	LOW (n = 5)	HIGH (n = 6)	SEM	Trt	Time	Trt*Time
14:0. %	0	0.27	0.27	0.20	0.08	0.71	0.76	0.07
	14	0.14	0.22	0.18				
	28	0.24	0.24	0.10				
	42	0.13	0.30	0.19				
	56	0.19	0.11	0.43				
16:0, %	0	10.57	9.66	7.63	1.25	0.37	0.01	0.02
	14	8.15	8.45	7.30				
	28	10.47	10.75	8.68				
	42	8.30	13.31	9.86				
	56	9.59	9.99	12.95				
16:1, %	0	0.29	0.27	0.44	0.21	0.09	0.43	0.54
	14	0.60	0.38	0.64				
	28	0.96	0.37	0.32				
	42	0.60	0.18	0.62				
	56	0.38	0.09	0.19				
18:0. %	0	13.24	12.86	12.87	1.39	0.04	< 0.01	0.29
	14	12.70	13.81	12.02				
	28	13.47	15.44	15.20				
	42	12.43	18.96	15.11				
	56	14.39	19.17	17.86				
18:1, %	0	8.39	7.22	7.65	1.07	0.33	0.01	0.16
	14	6.22	6.25	5.43				
	28	7.23	7.59	7.52				
	42	6.42	9.17	8.38				

Table 4. Continued

		Treatment[2]				P-values[3]		
Fatty Acid	Day	CON (n = 6)	LOW (n = 5)	HIGH (n = 6)	SEM	Trt	Time	Trt*Time
	56	7.32	8.25	11.52				
18:1 trans-11, %	0	0.57	0.51	0.52	0.06	< 0.01	0.52	0.75
	14	0.54	0.50	0.39				
	28	0.67	0.53	0.43				
	42	0.59	0.54	0.40				
	56	0.59	0.54	0.49				
18:2, %	0	40.36	37.87	37.82	3.40	0.48	< 0.01	0.08
	14	40.16	41.15	35.67				
	28	44.30	40.87	47.22				
	42	42.21	50.74	47.81				
	56	47.52	54.34	41.37				
18:3, %	0	1.89	1.34	1.70	0.13	0.15	< 0.01	0.28
	14	0.86	0.74	0.77				
	28	1.00	0.71	0.81				
	42	0.92	0.87	0.89				
	56	0.93	0.97	0.77				
cis-9,trans-11 CLA, %	0	ND[4]	ND	ND	0.13	< 0.01	< 0.01	< 0.01
	14	ND	0.68	1.02				
	28	ND	0.92	1.96				
	42	ND	0.80	2.47				
	56	ND	0.92	1.69				
trans-10,cis-12 CLA, %	0	ND	ND	ND	0.11	< 0.01	< 0.01	< 0.01
	14	ND	0.59	0.86				
	28	ND	0.90	1.75				

Table 4. Continued

		Treatment[2]				P-values[3]		
Fatty Acid	Day	CON (n = 6)	LOW (n = 5)	HIGH (n = 6)	SEM	Trt	Time	Trt*Time
	42	ND	0.73	2.15				
	56	ND	0.90	1.62				
20:1, %	0	0.28	0.22	0.22	0.04	0.47	< 0.01	0.16
	14	0.13	0.09	0.17				
	28	0.20	0.12	0.25				
	42	0.28	0.20	0.25				
	56	0.28	0.26	0.21				
20:2, %	0	0.26	0.21	0.16	0.05	0.02	0.08	0.73
	14	0.18	0.18	0.16				
	28	0.27	0.18	0.18				
	42	0.32	0.21	0.14				
	56	0.33	0.31	0.20				
20:4, %	0	0.87	0.83	0.79	0.09	0.02	0.5	0.78
	14	0.81	0.83	0.67				
	28	0.94	0.77	0.74				
	42	0.89	0.76	0.64				
	56	0.82	0.82	0.54				
20:5, %	0	ND	0.05	0.01	0.07	0.278	0.22	0.44
	14	ND	ND	ND				
	28	ND	0.29	0.03				
	42	ND	ND	ND				
	56	ND	ND	ND				

Table 4. Continued

		Treatment[2]				P-values[3]		
Fatty Acid	Day	CON (n = 6)	LOW (n = 5)	HIGH (n = 6)	SEM	Trt	Time	Trt*Time
22:6, %	0	1.42	2.12	1.92	0.55	0.59	< 0.01	0.79
	14	2.16	1.65	2.53				
	28	1.11	0.89	1.10				
	42	1.17	ND	0.50				
	56	0.74	0.03	0.40				

[1] Least square means
[2] Dietary treatments consisted of 1% diet soybean oil (CON), 0.5% diet soybean oil with 0.5% diet CLA (LOW), and 1% diet CLA (HIGH)
[3] Trt, treatment; Trt*time, treatment by time interaction
[4] ND, not detectable (minimum detectable level is 0.0003%)

Phase 2

Heart Rate, Respiration Rate and Rectal Temperature

During the LPS challenge, dietary treatment did not affect HR, RR and RT ($P \geq 0.13$), but all measurements were influenced by time post LPS injection (Fig. 9). Both HR and RR decreased ($P < 0.01$) from PIH 0 to 6 h post-injection. Maximum values for all three vital signs were recorded at 12 h post-injection, however returned to baseline levels by h 24.

A)

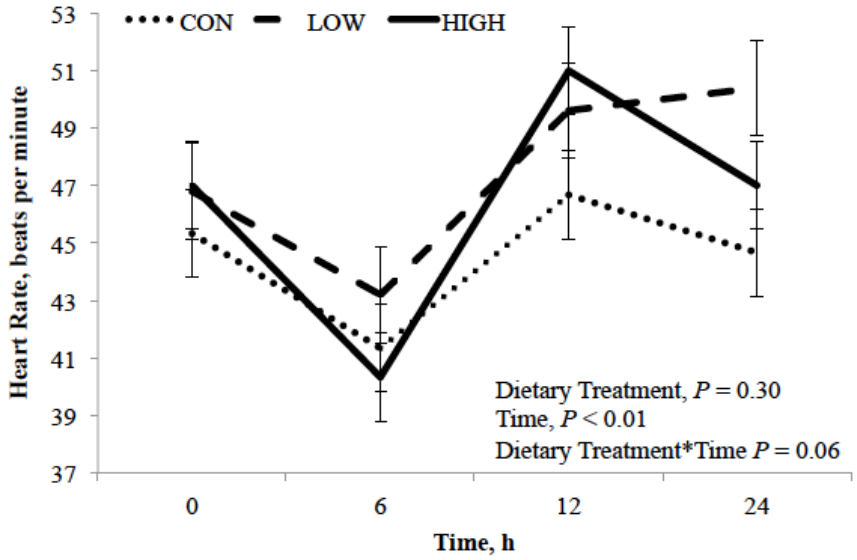
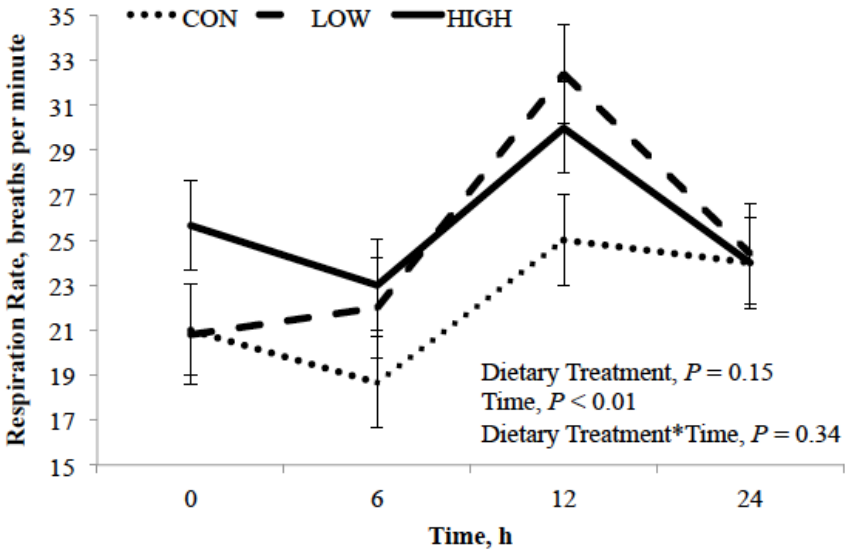


Figure 9. Clinical assessment of heart rate (panel A), respiration rate (panel B), and rectal temperature (panel C) after intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection. Treatments were 1% diet soybean oil (CON; n = 6), 0.5% diet soybean oil with 0.5% diet CLA (LOW; n = 5), and 1% diet CLA (HIGH; n = 6). *, ** superscripts denote differences between treatments at each time ($P < 0.05$).

B)



C)

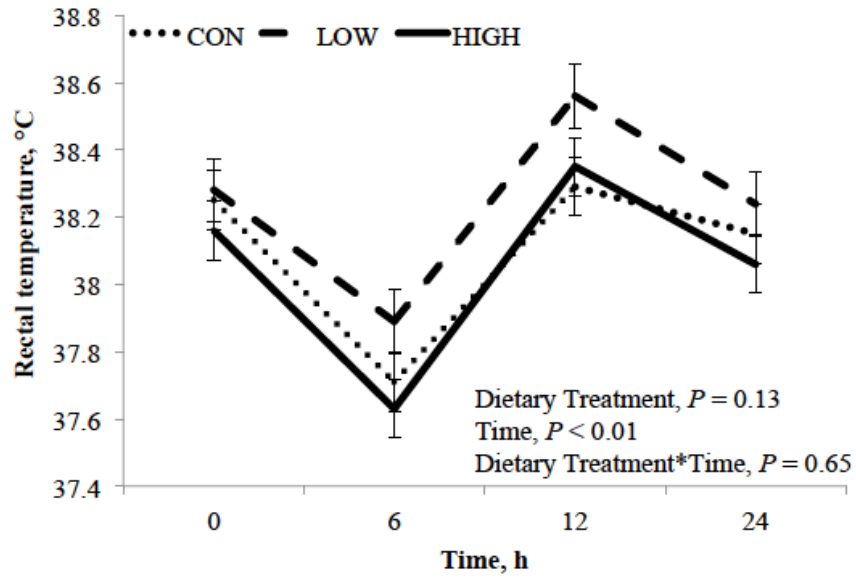


Figure 9. Continued

Carpal Circumference and Surface Temperature

A treatment by time interaction ($P < 0.01$) for carpal circumference was observed and characterized by delayed peak values in CON horses to h 168 when compared to peak values at h 24 by both the HIGH and LOW treatment groups. Carpal circumference was not affected by dietary treatment or knee ($P = 0.74$), and because carpal circumference was not influenced by knee ($P < 0.74$), Fig. 10 represents combined mean carpal circumference for both the LRS and LPS joint. There was an effect of time ($P < 0.01$) with peak circumference values noted at 24 h post-injection, followed by a decrease at h 168.

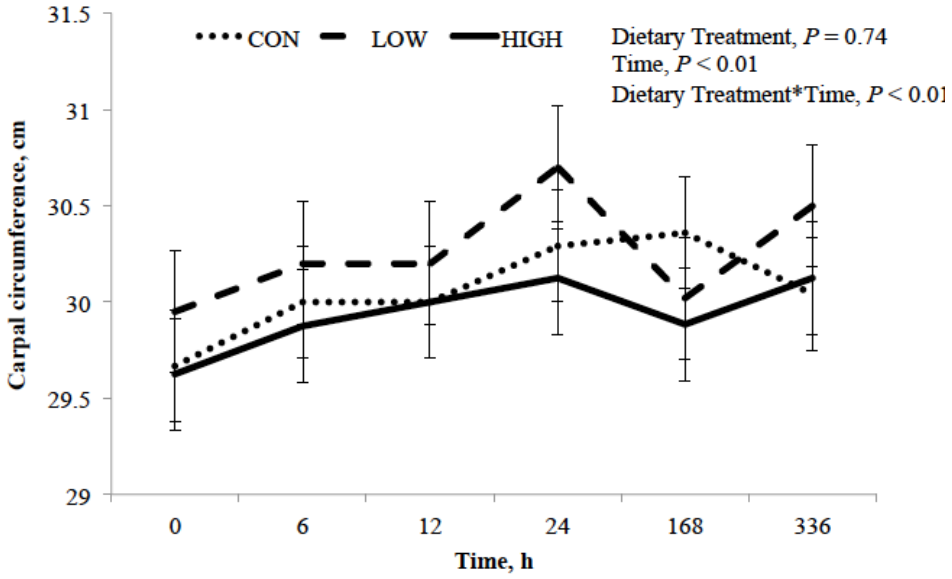


Figure 10. Carpal joint circumference (cm; least squares mean ± SEM) after intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at pre-injection h 0 and 6, 12, 24, 168, and 336 h post-injection. Treatments were 1% diet soybean oil (CON; n = 6), 0.5% diet soybean oil with 0.5% diet CLA (LOW; n = 5), and 1% diet CLA (HIGH; n = 6).

There was a tendency toward a dietary treatment by time interaction for carpal surface temperature ($P = 0.10$), which was evident at pre-injection h 0 and post-injection h 24. Surface temperatures recorded at h 0 were higher ($P < 0.01$) for LOW horses versus HIGH, and tended to be higher ($P = 0.06$) than CON horses. At 24 h post-injection, the CON group's carpal surface temperatures were lower ($P = 0.03$) than both HIGH and LOW horses.

Carpal surface temperatures tended to be influenced by dietary treatment ($P = 0.10$) with horses fed the LOW diet having higher surface temperatures than CON ($P = 0.04$). Since no effect of knee was noted ($P < 0.27$), Fig. 11 represents mean carpal surface temperatures for both carpal joints. Regardless of dietary treatment, carpal surface temperatures were influenced by time ($P < 0.01$) with peak values noted at 6 h post-injection with an increase from baseline levels at h 0 ($P < 0.01$). Values remained elevated at 12 h post-injection and fell from h 12 to 24 ($P < 0.01$) to levels tending to be below baseline ($P = 0.06$).

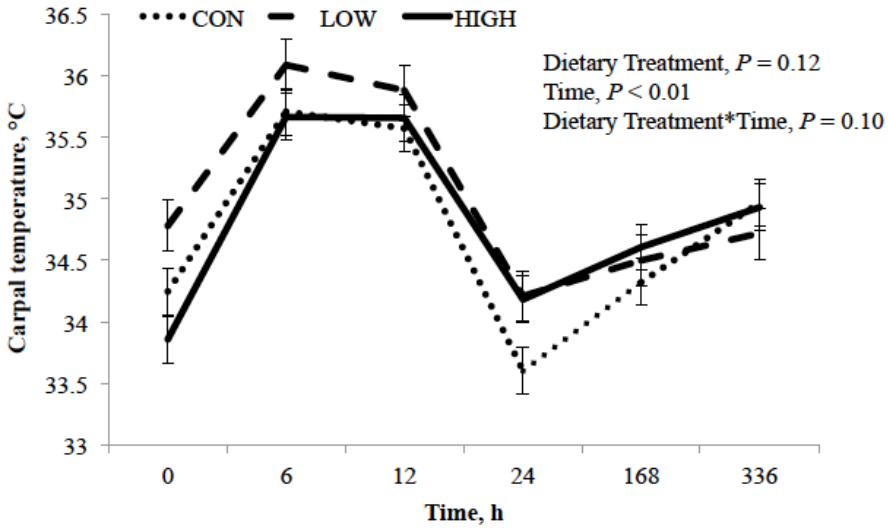


Figure 11. Carpal joint surface temperature (°C; least squares mean ± SEM) at pre-injection h 0 and 6, 12, 24, 168, and 336 h post LPS injection. Treatments were 1% diet soybean oil (CON; n = 6), 0.5% diet soybean oil with 0.5% diet CLA (LOW; n = 5), and 1% diet CLA (HIGH; n = 6).

Synovial Fluid Biomarkers

Synovial PGE_2 concentrations exhibited a time by knee interaction ($P < 0.01$), with the LPS knee showing greater changes in concentration over time compared to the LRS knee (Fig. 12). Peak PGE_2 concentrations were observed at 6 and 12 h post-injection with a return to baseline by 168 h post-injection in the LPS knee. In the LRS knee, concentrations peaked 24 h post-injection ($P = 0.02$) and returned to baseline by h 168. There was a significant knee effect with the LPS knee having greater ($P = 0.01$) PGE_2 concentrations than the LRS knee. Synovial PGE_2 concentrations were not influenced by dietary treatment ($P \geq 0.15$); however were affected by time ($P < 0.01$) with concentrations increasing to peak values at 6 h post-injection and decreasing to baseline by 336 h for all horses.

A) B)

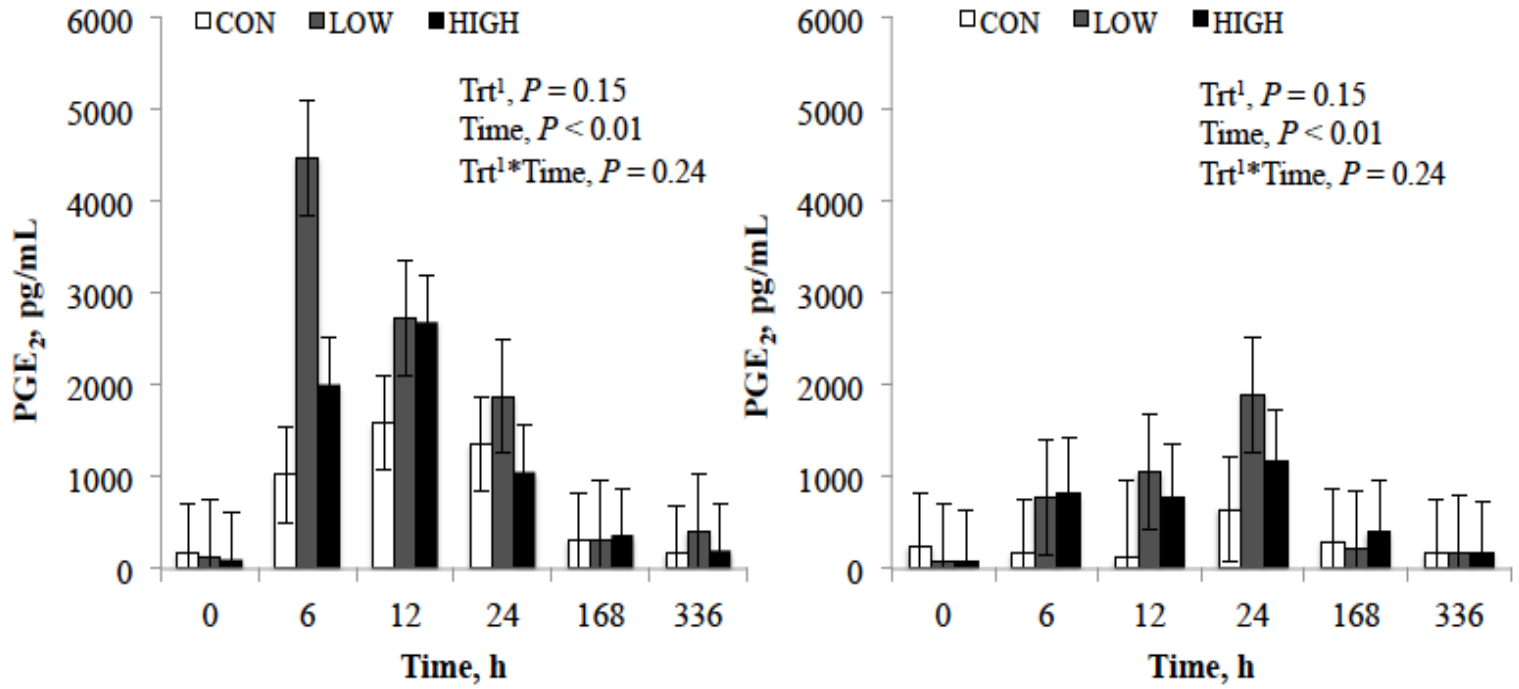


Figure 12. Mean synovial fluid concentrations (pg/mL) of prostaglandin E_2 (PGE_2) over time (h) after intra-articular injection of 0.5 ng lipopolysaccharide (Panel A; LPS; derived from *Escherichia coli* 055:B5) or 0.8 mL sterile lactated Ringer's solution (Panel B; LRS). Treatments were 1% diet soybean oil (CON; n = 6), 0.5% diet soybean oil with 0.5% diet CLA (LOW; n = 5), and 1% diet CLA (HIGH; n = 6). [1]Trt, dietary treatment.

There was a treatment by time interaction ($P = 0.03$) for synovial fluid C2C concentrations, where, at PIH 0, HIGH horses had lower ($P = 0.02$) values than LOW, and CON horses tended to have lower concentrations ($P = 0.10$) than LOW horses (Fig. 13). Also, at 24 h post-injection, HIGH fed horses had lower concentrations than both the LOW and CON groups ($P = 0.02$ and $P < 0.01$, respectively). Synovial C2C concentrations were influenced by dietary treatment with horses in the HIGH group having lower ($P = 0.01$) concentrations than LOW. There was also an effect of time ($P < 0.01$) with peak values seen at 12 and 24 h post-injection and returning to baseline by h 336. Concentrations of C2C tended to be influenced by knee ($P = 0.09$) with the LPS injected joint having greater concentrations than the LRS joint over the 24 h challenge.

Horses receiving the LOW dose CLA treatment tended to have higher ($P = 0.10$) synovial CPII concentrations than the other treatment groups. Synovial concentrations of CPII also tended to be higher ($P = 0.07$) in samples from the LPS knee compared to LRS. There was an effect of time ($P < 0.01$) with concentrations increasing ($P = 0.03$) from PIH 0 to h 12 followed by a sharp increase ($P < 0.01$) to peak values from 12 to 24 h post-injection (Fig. 14). Concentrations of CPII decreased ($P < 0.01$) from h 168 to 336, but did not reach baseline values (Fig. 14).

A) B)

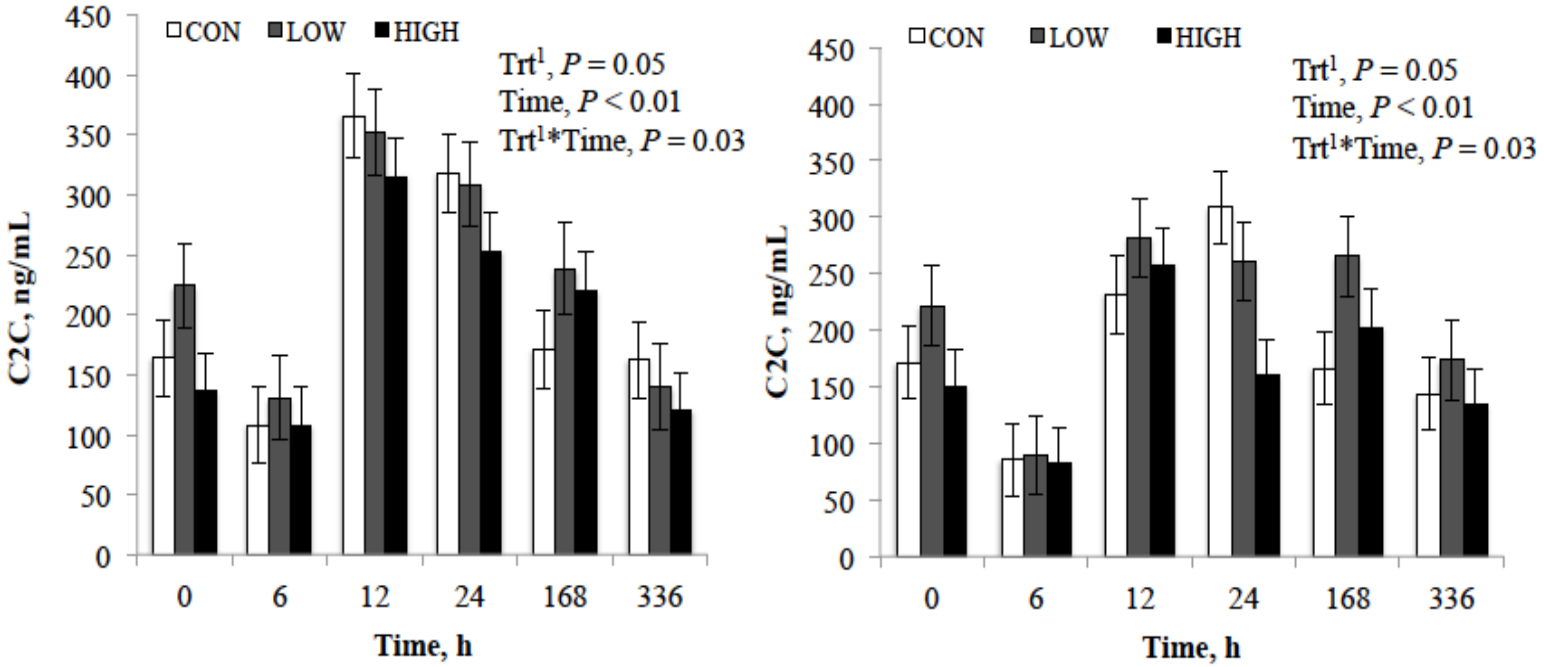
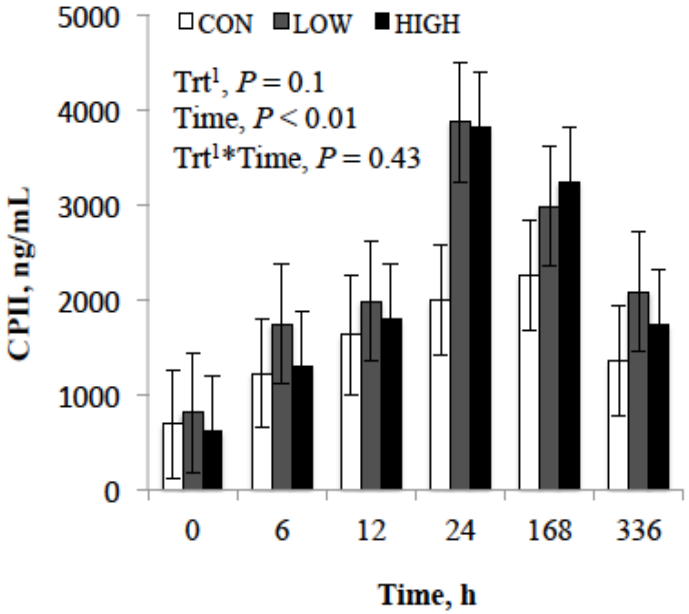


Figure 13. Mean synovial fluid concentrations (ng/mL) of collagen cleaving neopeptide (C2C) over time (h) after intra-articular injection of 0.5 ng lipopolysaccharide (Panel A; LPS; derived from *Escherichia coli* 055:B5) or 0.8 mL sterile lactated Ringer's solution (Panel B; LRS). Treatments were 1% diet soybean oil (CON; n = 6), 0.5% diet soybean oil with 0.5% diet CLA (LOW; n = 5), and 1% diet CLA (HIGH; n = 6). [1]Trt, dietary treatment.

A)



B)

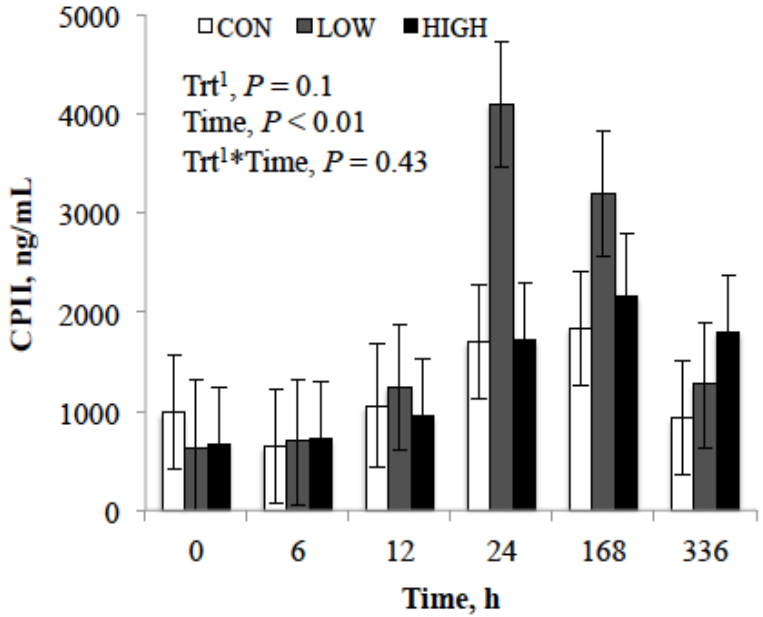


Figure 14. Mean synovial fluid concentrations (ng/mL) of carboxypeptide of type II collagen (CPII) after intra-articular injection of 0.5 ng lipopolysaccharide (Panel A; LPS; derived from *Escherichia coli* O55:B5) or 0.8 mL sterile lactated Ringer's solution (Panel B; LRS). Treatments were 1% diet soybean oil (CON; n = 6), 0.5% diet soybean oil with 0.5% diet CLA (LOW; n = 5), and 1% diet CLA (HIGH; n = 6). [1]Trt, dietary treatment.

CHAPTER V

DISCUSSION

During the 56 d trial, BW, hip and wither heights, length, and heart girth circumference increased over time, which was expected for these yearling horses. The magnitude of increase in the first 21 d followed by a plateau is likely indicative of compensatory growth. It is important to note that all horses were fed at or above requirements outlined by the NRC (2007). Horses in the current study received approximately 22 Mcal/d DE compared to the 18 Mcal/d DE requirement for a 500 kg mature horse indicated by the NRC (2007). As discussed by Hornick et al. (2000) through a compilation of studies, compensatory growth in livestock typically occurs within the first 30 d after an increase in the animal's plane of nutrition, and data in the current study agrees with this assessment. The lack of a treatment effect across all physical parameters through the duration of the study supports isocaloric formulation of diets, and indicates that soybean oil and CLA supplements contribute similar energetics in the horse.

In addition to the other physical measurements, there was also an increase in rump fat thickness over time, regardless of dietary treatment. Previous research in pigs and mice indicates that CLA supplementation reduces adiposity; however, results with human CLA supplementation vary. Weanling mice supplemented with 0.5% diet CLA had body fat values that were less than half the value of corn oil supplemented controls (Park et al., 1997). In a similar study performed with grower-finisher pigs fed varying

levels of CLA, the impact of fat depth varied with body location with some areas having decreased fat thickness with increased CLA supplementation, and other locations not being affected (Thiel-Cooper et al., 2001). Therefore, the singular measurement of rump fat in the current study may not give the best indication of body adiposity. Only one study evaluated the effect of dietary CLA on rump fat thickness in mature horses and produced similar results as the current study (Headley et al., 2011). Additionally, it is possible that stage of growth and plane of nutrition has an effect on adipose versus lean deposition in the horse.

Plasma CLA concentrations were not detectable in the CON at any time point measured, which was expected since the CON diet contained no detectable levels of CLA. Changes in plasma CLA concentrations in the current study for horses in the LOW group followed the same trend found by Headley et al. (2011) following dietary CLA supplementation to mature mares in which measureable levels were detected at d 14 with peak concentrations at d 28. Interestingly though, horses in the HIGH group did not follow the same trend, and had steady increases in plasma CLA concentrations to d 42, followed by a notable decrease in circulating levels at d 56. The decrease in CLA concentrations from d 42 to 56 may be explained by the inflammatory response caused by LPS injection affecting circulating fatty acid concentrations for the final 14 d of the study. During peak inflammation, fatty acids are recruited from the phospholipid bilayer for the production of pro-inflammatory prostaglandins. The re-filling of the phospholipid bilayer could potentially explain the reduction in circulating CLA levels in plasma. The additional time effects observed in fatty acids other than CLA and 20:4

reveal the change in diet at the onset of the study. The greatest magnitude of change in fatty acid concentrations occurred between d 14 and 42, and since it takes approximately 28 d to fully adapt to a new source of dietary fat, this was to be expected (Julen et al., 1995).

Eicosadienoic acid (20:2), like 18:0, was affected by dietary treatment with lower concentrations in horses consuming HIGH compared to CON. The CLA supplement contained greater concentrations of 20:2 than the soybean oil supplement; therefore, it is interesting that circulating concentrations were lower in horses that received the CLA supplement. Muscle and adipose tissue of pigs showed similar results following 1% diet CLA supplementation where pigs supplemented with CLA had lower 20:2 concentrations than those that received no CLA (Bee et al., 2008). Lo Fiego et al. (2005) observed a reduction in 20:2 in similar tissues following supplementation of CLA at levels as low as 0.25% of the diet. In further analysis of 20:2, Huang et al. (2011) found macrophages exposed to increased levels of 20:2 resulted in increased 20:4 concentration and PGE_2 production following treatment with LPS. Therefore, the reduction of 20:2 in horses supplemented with CLA could support the hypothesis that CLA supplementation may reduce prostaglandin production during an inflammatory response.

Plasma levels of 20:4 were lower in horses supplemented with the HIGH dose of CLA when compared to both the LOW and CON. This corresponds to findings by Headley et al. (2011) in which lower concentrations of 20:4 were measured in horses supplemented at the same level as the current study at 0.01% BW CLA than corn oil

supplemented controls. In addition, 20:4 levels in breast tissue of broilers was lower in those that received the CLA supplement than both flaxseed and fish oil treatment groups (Shin et al., 2011). In regards to LOW horses having similar 20:4 levels as CON, it may be that 0.5% diet CLA may not be sufficient to reduce circulating 20:4 levels in the horse. During an inflammatory response, 20:4 present in cell membranes is converted to inflammatory prostaglandins, including PGE_2, through the COX-2 pathway. A successful reduction in circulating 20:4 levels through 1% diet CLA supplementation reduces the availability of a direct precursor to inflammation.

Clinical responses of HR, RR, and RT recorded prior to arthrocentesis in the first 24 h of the LPS challenge were unaffected by dietary treatment; however, the increased handling and stress of repeated arthrocentesis may explain the changes in vitals over the 24 h period. Values recorded at PIH 0 and post-injection h 24 were not different indicating that all vitals returned to baseline by 24 h post LPS injection. Residual effects from sedation prior to arthrocentesis may explain the decreased values of each vital sign at 6 h post-injection. Peak values at 12 h post-injection correspond to the increased inflammatory response as indicated by peak PGE_2 concentrations, causing a slight, but noticeable increase in vital signs. It is important to note, however, that vitals were never outside the normal range confirming that no animal had an adverse, systemic reaction to the LPS injection.

Carpal circumference and surface temperature values were also measured during the LPS challenge on both the LPS and LRS knees, using the LRS knee as a contralateral control. The increase over time in carpal circumference without an effect of knee

indicates increased joint effusion from the initiation of an inflammatory response in both carpal joints. The increased effusion reflected by increased carpal circumference in the LRS joint is likely due to repeated arthrocentesis and has been noted in other studies (van den Boom et al., 2005). Both carpal joints had greatest values at 24 h post-injection, which is consistent with findings in a similar study performed by Lucia et al. (2013) using LPS in weanling horses.

Increased surface temperatures during an inflammatory response are caused by hyperaemia at the site of inflammation due to increased metabolic waste accumulation (Bliss, 1998). In the current study, carpal surface temperatures peaked at 6 and 12 h post-injection, which were expected due to the inflammatory response caused by LPS injection. The peak temperature value coincides with peak PGE_2 concentrations measured in the synovial fluid. Surface temperatures in both knees returned to baseline levels by 24 h post-injection, which indicate a return to normal blood flow and reduction in inflammation. This is one of the first uses of infrared images during an intra-articular LPS challenge, and values appear to correspond closely with inflammatory markers in synovial fluid.

Analyses of synovial fluid biomarkers indicate time effects for PGE_2, CPII, and C2C, validating the LPS model and the ability to stimulate an acute and transient inflammatory response. In addition, the LPS carpal joint had consistently greater concentrations of each biomarker compared to the LRS joint, further confirming the effectiveness of LPS to induce an acute inflammatory response. Peak concentrations for PGE_2, CPII and C2C relate to values previously recorded in studies performed by Lucia

et al. (2013) and de Grauw et al. (2009) measuring similar parameters in carpal joints of weanling and mature horses following intra-articular LPS injection. Furthermore, both PGE_2 and C2C returned to baseline prior to 336 h post-injection proving that inflammation caused by intra-articular LPS injection is transient.

Prostaglandins released during an inflammatory response function to regulate inflammation. Specifically, PGE_2 has been determined as an excellent marker of equine joint disease, and has been used as a marker of inflammation in numerous studies in the horse (Bertone et al., 2001). In regards to the effect of CLA on synovial PGE_2 concentrations, no treatment effect or treatment by time interaction was observed. The limited number of horses in each treatment group may explain this lack of a treatment effect. Following CLA supplementation to pigs using larger sample sizes, Changhua et al. (2005) found lower PGE_2 concentrations in those supplemented with CLA (916.3 ± 53.5 pg/mL) than the control diet (1211.0 ± 53.5 pg/mL) following systemic LPS injection. The reduction in circulating 20:4 and 20:2 concentrations in the current study would suggest a potential to reduce the inflammatory response, as 20:4 is a direct precursor to prostaglandin production during inflammation. Previous studies have confirmed incorporation of CLA into both lean and adipose tissues, indicating the possibility for CLA to successfully incorporate into synovial tissue where it could potentially decrease 20:4 levels and suppress the COX-2 pathway (Kramer et al., 1998; Thiel-Cooper et al., 2001). Examination of the effects of dietary CLA supplementation on 20:4 levels in synovial fluid would provide a more direct indication of PGE_2

precursor availability during inflammation. At this time, further research is needed to determine the effects of CLA on the inflammatory eicosanoid PGE_2.

Although no dietary treatment effect was noted in synovial PGE_2 concentrations, dietary CLA supplementation did have an effect on biomarkers of articular cartilage metabolism. Following an inflammatory insult, the fibrous type II collagen begins to unwind and reveal hidden epitopes, including C2C, which can be used as a marker for articular cartilage degradation. This epitope has been previously measured following exercise, LPS injection, and osteochondral fragmentation in horses (Frisbie et al., 2008; Lucia et al., 2013). In the current study, there was an initial decrease in synovial C2C concentrations from PIH 0 to 6 h post-injection, which is likely due to a shift in cartilage metabolism at the onset of an inflammatory response. The same decrease from PIH 0 to 6 h post-injection was observed in a similar study performed by Lucia et al. (2013) following LPS injection in weanling horses. Peak C2C values were observed at 24 h post-injection, which is consistent with peak times recorded by Lucia et al. (2013) and de Grauw et al. (2009). Peak concentrations of C2C in the current study are similar to those recorded by Lucia et al. (2013) using weanling horses.

A reduction in type II collagen degrading biomarker, C2C, was observed in horses fed HIGH CLA when compared to the LOW dose. This demonstrates less cartilage degradation when horses received 1% diet CLA supplementation prior to LPS administration. At PIH 0, HIGH horses had lower C2C concentrations than LOW, which can be attributed to the previous 42 d of CLA supplementation. Furthermore, at 24 h post-injection when peak values were recorded, HIGH fed horses had lower

concentrations of C2C compared to both LOW and CON horses. This indicates CLA supplemented at 1% diet is effective in reducing cartilage degradation both prior to and during an inflammatory insult.

When type II collagen degrades, there is an attempt to repair the fibers. During this repair, CPII is released from the type II collagen precursor, procollagen, and can be used as a marker of type II collagen regeneration. This biomarker is often used as a measurement to counter the degradative marker C2C. Concentrations of CPII peaked at 24 h post-injection, which agrees with both Lucia et al. (2013) and de Grauw et al. (2009). Peak values recorded in the current study more closely resemble those seen by Lucia et al. (2013) in weanling horses, which are lower than those recorded by de Grauw et al. (2009) using mature horses. It is expected that yearling horses would have values similar to weanlings as opposed to mature horses (5 to 8 y). Unlike PGE_2 and C2C, concentrations of CPII in the current study did not return to baseline levels by 336 h post-injection, which was not anticipated since CPII concentrations in weanlings following LPS injection returned to baseline by 336 h post-injection (Lucia et al., 2013). This delay indicates recurring cartilage regeneration at 2 wk following LPS injection. An additional sample could have provided baseline measurements; however, concentrations of CPII were on the decline by 336 h post-injection.

In regards to the effect of CLA on cartilage metabolism, there was a tendency toward a treatment effect for CPII, indicating that horses fed the LOW dose CLA had greater cartilage regeneration than CON fed horses. No previous studies have yet been performed to determine the effects of CLA on the metabolism of type II collagen;

however, a similar study was performed to determine the effect of oral glucosamine supplementation on inflammation in yearling horses using the LPS model (Lucia, 2013). Lucia (2013) found that oral glucosamine reduced synovial PGE_2 and C2C, but increases CPII concentrations in carpal joints challenged with LPS. Although this gives little insight into the effectiveness of dietary CLA, it does support the potential of CLA to reduce inflammation and cartilage degradation while increasing cartilage repair through dietary supplementation in the horse.

CHAPTER VI

SUMMARY

Based on these results, horses that received CLA supplementation had less cartilage degradation and tended to have greater cartilage regeneration when inflammation was induced via LPS, but there was no effect on PGE_2 concentrations. The results of fatty acid analysis, in particular reduction of 20:4, and cartilage metabolism, however, support the hypothesis that CLA may be capable of reducing inflammation. There are multiple biomarkers for inflammation, and PGE_2 was chosen due to its relationship to fatty acids, and its reliability as an indicator of joint disease (Bertone et al., 2001). The lack of a response by PGE_2 to CLA treatment may be explained by individual response to inflammation, as well as the small sample sizes in the current study. Therefore, in future studies, it may be beneficial to have larger numbers of animals to account for the individual variability if PGE_2 is the marker of choice.

Both 20:4 and 20:2 were measured at lower circulating concentrations in horses that received the HIGH dosage of CLA at 0.01% BW. The successful reduction in circulating 20:4 levels may reduce precursor availability to inflammatory prostaglandins, including PGE_2. Huang et al. (2011) confirmed the pro-inflammatory effects of 20:2 in mice macrophages, and its reduction in circulating levels could provide further evidence into CLA's capability to mitigate inflammation. Additionally, further research is needed to determine the effects of CLA on adiposity in the young horse. Because horses in the current study showed evidence of compensatory growth, this may have been a poor

model to determine effects on fat deposition. Further study of dietary CLA inclusion in the horse may require a longer period of supplementation, evaluation of body fat deposits other than just rump fat, or mature horses in a more obese condition. In conclusion, dietary CLA supplementation may be a method to mitigate inflammation and cartilage metabolism in young horses. Dietary CLA was well accepted by horses, beneficially altered plasma fatty acid concentrations, and decreased cartilage degradation when acute inflammation was induced. Therefore, during an inflammatory response, CLA has the potential of delaying or minimizing cartilage turnover, and may have application for OA models and exercising horses.

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APPENDIX A

EXTRACTION AND METHYLATION OF FATTY ACIDS

Extraction

Feed samples were dried and ground, and 5 g of both hay and grain were added to a 50 mL tube with 10 mL 2:1 chloroform:methanol Folch solution to extract the lipid. Each feed sample was homogenized using a Polytron homogenizer for 1 min, and allowed to sit on a shaker for 6 hours. Feed samples were then filtered through a Whatman filter apparatus using 2.4 cm GF/C filters into a second 50 mL tube. Approximately 8mL of 74% KCl was added to every sample, vortexed and then centrifuged for 30 min at ½ max speed. Plasma samples were extracted similarly, with 1 mL homogenized in 10 mL Folch solution for 1 min. Each plasma sample received 5 mL 74% KCl, and also centrifuged for 30 min at ½ max speed. Lastly, the bottom chloroform phase containing the extracted lipid was removed from all samples and transferred to 20 mL test tubes.

Methylation

All feed and plasma samples were methylated using the same protocol. After the chloroform phase was dried using N-evap at 40°C, the lipid was saponified and methylated by adding 1 mL of 0.5 N KOH in MeOH and heated in a 70°C water bath for 10 min to separate the fatty acids. Next, 1mL of 14% BF3 in MeOH was added to each sample, flushed with N_2 and placed in a 70°C water bath for 30 min. Finally, the fatty acids were constituted in hexane by adding 2 mL hexane to each sample with 2mL

saturated NaCl and vortexed. The top phase was transferred to a 20 mL tube containing approximately 1 g of $NaSO_4$ to remove any remaining H_2O from the sample. This step was repeated twice and all hexane was transferred to a glass scintillation vial, dried, reconstituted with 500 μL hexane and placed in a GC vial for analysis using the gas chromatograph.

APPENDIX B

ELISA PROTOCOL

PGE_2

All synovial fluid samples were diluted with calibrator diluent provided by the commercial PGE_2 ELISA kit (R&D Systems, Inc., Minneapolis, MN) prior to plating depending on collection time post-injection. Synovial fluid samples taken at preinjection h 0 and 6, 24, 168 and 336 h postinjection had dilutions of 2:1, 1:2, 1:3, 1:2, and 2:1, with diluent provided by the manufacturer, respectively. At 12 h postinjection, 1:3 and 1:4 dilutions were used for CON and LPS joints, respectively due to the greater variability in PGE_2 concentrations at this time point.

The diluted synovial fluid was pipetted at 150 µL into each well with 50 µL of mouse monoclonal antibody to PGE_2, and allowed to incubate for 1 h at room temperature on an orbital microplate shaker (VWR Microplate Shaker 120 V, Henry Troemner, LLC, Thorofare, NJ) at 525 rpm. Following the incubation period, 50 µL of a horseradish peroxidase-labeled PGE_2 was added to each well and allowed to incubate for 2 h on the orbital microplate shaker at 550 rpm. The 96 well microplate was washed using a wash buffer, and 200 µL of a color reagent was added and allowed to sit for 30 min. Each well received 100 µL of a stop solution and a microplate reader with optical density set at 450 nm was used to read each plate (BioRad 680 Microplate Reader, BioRad Laboratories, Hercules,CA).

C2C

The C2C ELISA is a 96 well plate coated with C2C peptide conjugate within a stabilizing matrix (IBEX Pharmaceuticals Inc., Montreal, Quebec, Canada). A test plate was used to determine optimum dilutions for time points and treatment joints. All synovial fluid samples were run on a 1:3 dilution. Diluted samples were plated at 50 µL in each well on the polypropylene mixing plate with 50 µL of a C2C antibody and allowed to pre-incubate for on an orbital microplate shaker (VWR Microplate Shaker 120 V, Henry Troemner, LLC, Thorofare, NJ) for 30 min at 650 rpm. Following pre-incubation, 80 µL of the sample mixture was transferred to the ELISA microplate and incubated on the orbital microplate shaker for 1 h at 650 rpm. The ELISA microplate was washed 3x using the provided wash buffer and 100 µL of a goat anti-mouse horseradish peroxidase was added to each well. Following another 30 min incubation on the orbital microplate shaker at 650 rpm, the ELISA microplate was washed 6x using the provided wash buffer and 100 µL of the color reagent was added to each well. The ELISA microplate was incubated a final time on the orbital microplate shaker at 650 rpm for 30 min. Finally, 100 µL of a stop solution was added and a microplate reader was used to determine concentration with an optical density at 450 nm.

CPII

The CPII ELISA is a 96-well microplate coated with bovine CPII antigen (IBEX Pharmaceuticals Inc., Montreal, Quebec, Canada). Dilutions did have to be modified based on time postinjection for the concentrations in synovial fluid. Synovial fluid from both the LRS and LPS joints at pre-injection h 0 were diluted with Buffer III with a 1:4

dilution. Some samples were more concentrated and required a 1:5 dilution. LPS and LRS samples taken at 6 and 336 h postinjection were run on a 1:4 dilution. Samples from 12 h postinjection were diluted 1:4 and 1:5 for CON and LPS joints, respectively. At 24 h postinjection, CPII concentrations were expected to be the highest; therefore, dilutions of 1:6 and 1:7 were used for CON and LPS joint samples, respectively. Lastly, postinjection h 168 CON samples were diluted at 1:4, and LPS samples were diluted at 1:5.

After diluting the synovial fluid samples in Buffer III and plating each sample on a polypropylene mixing plate, 50 μL of a rabbit polyclonal antiserum specific for CPII is added and allowed to pre-incubate for 1 h on an orbital microplate shaker (VWR Microplate Shaker 120 V, Henry Troemner, LLC, Thorofare, NJ) at 650 rpm prior to transferring the mixture to the ELISA microplate. Once on the ELISA microplate, the sample was allowed to incubate for 2 h on the orbital microplate shaker at 650 rpm. The microplate was then washed 6x using a wash buffer provided with the kit. Each well received 100 μL of a goat anti-rabbit horseradish peroxidase conjugate and placed on the orbital microplate shaker at 650 rpm for 30 min. The microplate was washed 6x again using the provided wash buffer and 100 μL of the color reagent was added. After an additional 30 min at 650 rpm on the orbital microplate shaker, a stop solution was added and a microplate reader with wavelength set at 450 nm was used to read the concentrations.