DISSERTATION

EVALUATION OF ORAL N-3 POLYUNSATURATED FATTY ACID EFFECT ON THE INFLAMMATORY RESPONSE TO A NOVEL EXPERIMENTAL MODEL OF SYNOVITIS IN THE HORSE

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ABSTRACT

EVALUATION OF ORAL N-3 POLYUNSATURATED FATTY ACID EFFECT ON THE INFLAMMATORY RESPONSE TO A NOVEL EXPERIMENTAL MODEL OF SYNOVITIS IN THE HORSE

Osteoarthritis (OA) is a debilitating disease resulting in irreversible cartilage loss that can influence mobility and quality of life in multiple species. The addition of n-3 long chain polyunsaturated fatty acids (LCPUFA), specifically eicosapentaneoic acid (EPA) and docosahexanoic acid (DHA), has been reported to have beneficial effects on joint inflammation in human and mouse in-vivo and in-vitro models. Little to no information exists regarding oral n-3 supplementation and effects on inflammatory markers in the equine joint. Additionally the development of reversible *in vivo* models of synovitis is valuable for studying OA pathophysiology, and testing therapeutic modalities. A pilot study conducted by our laboratory concluded that a 90 day treatment of an oral EPA/DHA supplement resulted in significantly higher serum and synovial fluid concentrations of these specific fatty acids in healthy horses receiving the supplement compared to non-supplemented healthy horses. Therefore, a two-phase experiment was designed to 1) validate the use of an equine recombinant cytokine for inducing a humane, reversible bout of synovitis in the horse and 2) determine if oral supplementation of a n-3 LCPUFA supplement would influence the inflammatory response in an experimental model of equine synovitis.

In *Phase One* of the experiment twelve skeletally-mature mares, were utilized in a randomized block design. Synovitis was induced by an intra-articular injection of 100 ng of

recombinant equine interleukin 1 beta (reIL-1β) or 0.5 ng of lipopolysaccharide (LPS) into a middle carpal joint in 1 ml volumes. One ml of phosphate buffered saline (PBS) was injection into the contra-lateral joint to serve as a control. Lameness evaluations were conducted throughout the study trial starting at post injection hour 0 (PIH) through PIH 240. Synovial fluid samples were repeatedly taken at PIH 0, 4, 8, 24 and 48. Synovial fluid samples were analyzed for inflammatory biomarker concentrations prostaglandin E_2 (PGE_2), general matrix metalloproteinase (MMP) activity, glycosaminoglycan (GAG) and routine cytology. Synovial tissue and articular cartilage was collected at PIH 8 via arthroscopic biopsy procedures. Joint tissues were analyzed for gene expression of MMP 1 and 13; the cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β); cyclooxygenase 2 (COX-2) and the aggrecanases ADAMTS-4 (a disintegrin and metalloproteinase with a thrombospondin motif 4) and ADAMTS-5 (a disintegrin and metalloproteinase with a thrombospondin motif 5).

In the second phase of the research (*Phase Two*), twelve skeletally-mature mares, free of lameness, were randomly assigned to one of two treatment groups: a CONT (control diet) group and a N3FA (control diet + a daily EPA/DHA rich supplement) group. All horses were individually fed their assigned diet, divided into two equal feedings daily and received dietary treatment for 90 days. Serum samples and synovial fluid samples collected on days 0, 30, 60, 91 and days 0 and 91 were processed and analyzed for lipid composition. On day 91, inflammation was experimentally-induced in a single carpal joint of each horse using a reIL-1β solution and collection procedures as outlined by *Phase One* of experimental studies.

Results from the *Phase One* study indicated that a single injection of reIL-1β or LPS increased synovial white blood cell, neutrophil count, total protein, prostaglandin E_2 concentrations and general matrix metalloproteinase activity relative to control joints through

PIH 8. Injections of either reIL-1β or LPS increased mRNA expression for MMP-1 and ADAMTS-4 in synovium and for MMP-1, ADAMTS-4, and ADAMTS-5 in articular cartilage collected at PIH 8 compared to saline injected joint tissue. Synovium collected from LPS-treated joints exhibited higher expression for IL-1β and ADAMT-4. The use of reIl-1β was sufficient in presenting a robust, yet temporary bout of inflammation in the horse.

In *Phase Two*, a 90 day supplementation of approximately 36g of EPA/DHA per day to horses resulted in significantly higher serum and synovial fluid EPA and DHA compared to non-supplemented horses. Serum arachidonic acid (ARA) was significantly higher in the N3FA horses compared to CONT horses at day 30-day 90; likely due to the presence of dietary ARA in n-3 fatty acid product. Synovial fluid analysis indicated that a reIL-1β injection increased synovial white blood cell, neutrophil count, total protein, prostaglandin E_2 concentrations and general matrix metalloproteinase activity relative to control joints through PIH 8 regardless of dietary treatment. Gene expression for MMP-13, IL-1β, ADAMTS-4, and ADAMTS-5 was higher in treated cartilage samples compared to saline injections, regardless of dietary treatment. A single injection of reIL-1β resulted in increased expression for MMP-1, COX-2 and IL-1β in synovium collected from all horses with no significant difference between dietary groups. Gene expression in synovial tissue for ADAMTS-4 was significantly lower in N3FA horses compared to CONT horses.

Injections of reIL-1β into equine carpal joints resulted in a transient inflammatory response and an increase in mRNA expression of certain deleterious mediators in joint tissues that was similar in severity to the LPS injection. Given that IL-1β is a known critical mediator of traumatic arthritis and OA, this humane and temporary model may be useful in evaluating therapeutics that act against early stages of joint disease. A follow up study utilizing the reIL-1β

model to evaluate dietary n-3 LCPUFA treatment resulted in a robust, yet temporary inflammatory response characterized by significant increases in cytology parameters, biological markers of inflammation and gene expression of degradative enzymes associated with cartilage degradation. A 90 day dietary treatment was successful in significantly altering serum phospholipid and synovial fluid lipid concentrations of EPA and DHA. Dietary supplementation of n-3 LCPUFAs did not result in significant difference from control diet for clinical response (lameness), white blood cell counts, eicosanoid production, matrix metalloproteinase activity. The discovery of significantly lower expression of ADAMTS-4 in N3FA synovium supports further inquiry into the capability of n-3 LCPUFAs, in particular, EPA and DHA as potential therapies for equine OA.

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Introduction

Equine Osteoarthritis

Osteoarthritis (OA) is a debilitating condition affecting one or more joints and characterized by degenerative changes in articular cartilage and is associated with varying degrees of synovial inflammation and joint tissue fibrosis (McIlwraith 2005a). Cartilage loss contributes to a decrease in motility and function of the joint, chronic pain, and inflammation, which ultimately compromises the quality of life for the animal or individual. Osteoarthritis affects multiple species, including humans, canines and equines. Approximately 21% of adult humans (Helmick et al., 2008) and 20% of mature dogs (Johnston, 1997) are affected, while OA accounts for 60% of equine lameness cases in the United States (Caron and Genovese, 2003). While often related to the natural process of aging, the development of OA is more commonly caused by direct trauma or repetitive mechanical loading reflective of athletic training and is also associated with synovitis or capsulitis (McIlwraith, 2005b) which can be chronic in nature. Joint inflammation causes the release of soluble mediators that are associated with disease development (Sellam and Berenbaum, 2010), ultimately lead to the thickening of joint tissues and irreversible thinning of articular cartilage (McIlwraith et al., 1979). Due to the aneural nature of articular cartilage, clinical signs of OA may become apparent only after permanent damage to the cartilage has occurred; therefore, identifying indicators of early stages of the disease may be helpful in minimizing the development of OA.

Economic Impact

The diagnosis and treatment of OA as well as the loss of animal use results in a significant negative economic impact for the horse industry (Frisbie, 2006). Estimated direct costs for diagnosis and treatment of OA can be substantial, an annual average of $3,000 per horse, with indirect costs being much higher (Oke and McIlwraith, 2010). Due to the prevalence and significance of OA, there is a considerable market for treatment methods and products. In addition to surgical treatments, conventional medicinal therapies include administration of corticosteroids, hyaluronan (HA), polysulfated glycosaminoglycan (PSGAG) and non-steroidal anti-inflammatory drugs (NSAIDS), all of which has been shown to provide symptomatic relief. With the advancement in research and technology, newer specialized treatments have been developed ranging from biological-based therapies such as mesenchymal stem cells (MSCs) and cytokine-inhibitor treatments to the use of physical therapy including massage and aqua treadmills. The use of oral nutraceuticals has also received attention for their potential of being symptom and/or disease-modifying. Common ingredients found in oral joint nutraceuticals include chondroitin sulfate (CS), glucosamine sulfate (GS), methylsufonlymethane (MSM), avocado and soybean unsaponifiable (ASU) extracts and n-3 polyunsaturated fatty acids (PUFAs). Nutraceuticals formulated for dogs, cats and horses are a $1.2 billion industry in the United States, with 34% of total sales being joint supplements (Oke and McIlwraith, 2010). However, there is little *in vivo* evidence to support the use of nutraceuticals in the horse and available products may not contain adequate ingredients to support their claims of being beneficial (Oke and McIlwraith, 2010). Nutraceutical products are not regulated by the Federal Drug Administration (FDA); therefore, biological relevance is unclear as product testing is not required to market such goods. With scientific evaluations of joint nutraceuticals lacking,

(Pearson and Lindinger, 2009) an established *in vivo* research model for assessing nutraceuticals would greatly benefit veterinarians, owners and horses by providing evidence for their worth as an OA therapy.

Anatomical structures involved in Osteoarthritis

A joint is an area within the body where two or more bones converge. There are three types of joints: fibrous, cartilaginous and synovial. The synovial (or diarthroidial) joint is the most prevalent in the horse and functions to facilitate movement and load transfer of adjacent bones. This type of joint is also the most affected by lameness and OA. The anatomical structure of a synovial joint (Figure 1.1) consists of the joint capsule, intra-articular ligaments, subchondral bone and, in some joints, cartilaginous menisci. The joint capsule contains a fibrous layer and a synovial membrane layer (synovium) that connects to the adjacent bone ends, with articular cartilage covering the articulating surface of the bones. Intra-articular ligaments attach to each bone and help stabilize the joint by limiting the degree of rotation (Todhunter, 1996). The subchondral bone is an integral part of the underlying bone and also provides structure to the articular cartilage. When considering the pathogenesis of osteoarthritis, the synovial joint capsule is of primary interest as this is the site for development and progression of the disease.

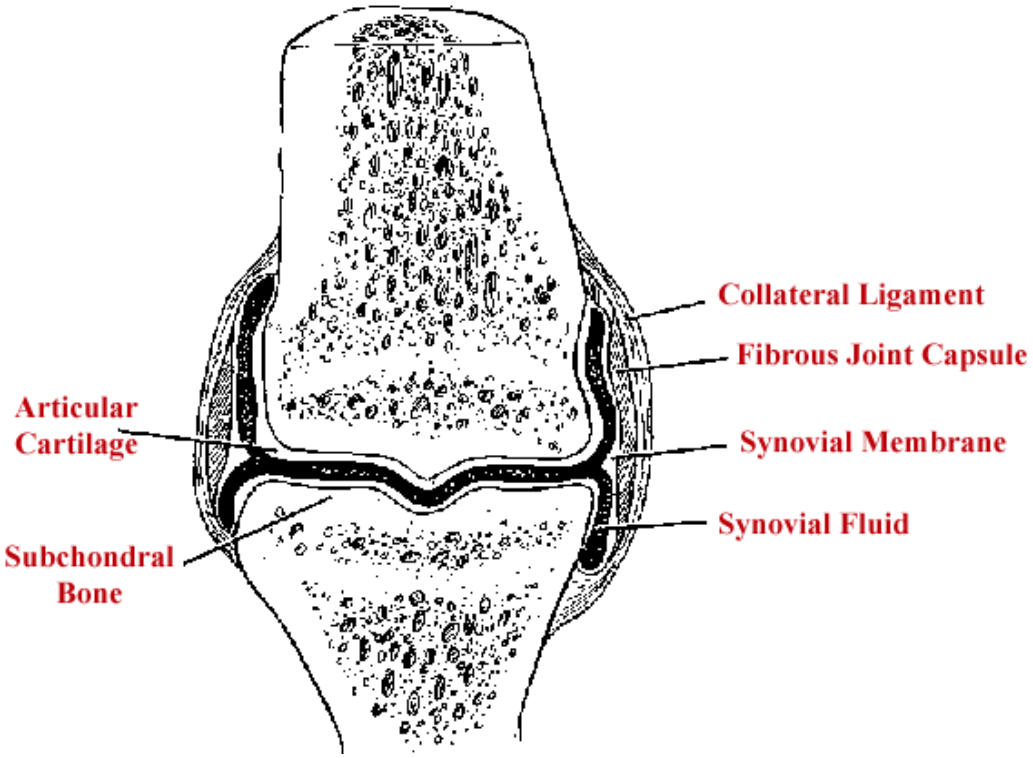

Figure 1.1 Diagram of synovial joint. Reproduced from McIlwraith CW. Diseases of Joints, Tendons, Ligaments and related structures. In: Adam's Lameness in Horses. 1987. 4th Ed. Pg. 339. Lea and Febiger. Philadelphia.

Synovium

The synovial membrane or synovium is a connective tissue that lines the joint capsule, extending to but not covering the articular cartilage. The membrane consists of two layers; a thick subsynovial stroma that functions as a lymphatic filter (Blewis et al., 2007) removing transported molecules, and a thinner synovial intima that contains a diverse population of cells (Revell, 1989; Caron, 2011). Cells within the synovium, known as synoviocytes, have secretory and phagocytic functions, and are instrumental in maintaining homeostasis within the joint capsule (Todhunter, 1996). Synoviocytes congregate along the outer boundary of the synovial intima and can be classified into three cell types: type A cells with macrophage-like properties (Revell, 1989); type B cells that are fibroblast-derived (Wilkinson et al., 1992); and type C cells

that are intermediate between types A and B (Caron, 2011).Type B cells synthesize proteoglycan 4 and surface-active phospholipids and hyaluronan (HA) that are released into the synovial fluid to provide low-friction and low-wear properties for lubricating the articulating joint (Barland et al., 1962; Wilkinson et al., 1992; Schwarz and Hills, 1998). Hyaluronan, a non-sulfated, glycosaminoglycan is synthesized in the cell's plasma membrane and yields a very large molecular weight (2-6 million Daltons) that contributes to its unique biological properties (Ogston and Stainer, 1953). The osmotic activity and disportional relationship to its molecular weight allows HA to effectively maintain water homeostasis and regulate plasma protein distribution (Fraser et al., 1997) adding to its primary function of lubricating the joint.

Synoviocytes synthesize and release anabolic and catabolic mediators such as growth factors and cytokines that act to maintain normal joint homeostasis (Palmer and Bertone, 1994a). Intercellular gaps exists among the discontinuous layer of synovial cells, containing collagen and a variety of macromolecules including HA, chondroitin sulfate (CS) and biglycan (Steel, 2008). The synovium functions as a semi-permeable barrier to surrounding blood vessels and selectively allows plasma components to enter from the bloodstream, thus determining the composition of the synovial fluid (Palmer and Bertone, 1994a). Serving as a macromolecular sieve, the synovium influences transport of proteins and molecules via a physical barrier of overlapping synoviocytes and a chemical barrier of hyaluronan within the intercellular matrix of the membrane (Barland et al., 1962). Molecules transferred through the synovium via the synovial fluid include glucose, urea and electrolytes as a nutrient source for the articular cartilage and the removal of transported molecules and byproducts of cartilage degradation (Barland et al., 1962; Sellam and Berenbaum, 2010).

Synovial fluid

Located within the joint cavity, synovial fluid normally functions as a biological lubricant and a nutrient transporter to the joint tissues. This fluid is secreted by the synovium as a result of interstitial fluid being filtered from the bloodstream through the membrane. Glucose and electrolytes found in limited concentrations in the fluid (Steel, 2008) are primary sources of nutrients for the articular cartilage (Hamerman et al., 1963) while other molecules such as proteoglycan 4, HA and surface-active phospholipids contribute to lubricating functions of the fluid (Blewis et al., 2007). Hyaluronan makes up the largest molecular portion of synovial fluid and its presence and degree of polymerization contribute to the fluid's viscosity properties (Todhunter, 1996). Under inflammatory conditions, fluid viscosity may decrease due to changes in the fluid's HA composition (Hamerman et al., 1963) likely due to joint effusion of inflammatory products and decreased concentration of HA (Hardy et al., 1998) that can compromise the normal functions of the synovial fluid.

Articular cartilage

The articular cartilage is a specialized connective tissue, avascular, aneural and alymphatic when mature, that functions to dissipate loading in the joint while providing a near friction-less articulating surface (Edwards, 1967). Cartilage has three distinct, unmineralized zones (Figure 1.2): a superficial (zone I), middle (zone II) and deep (zone III), that are separated by a tidemark from a calcified zone (zone IV) (as reviewed in Caron, 2011).

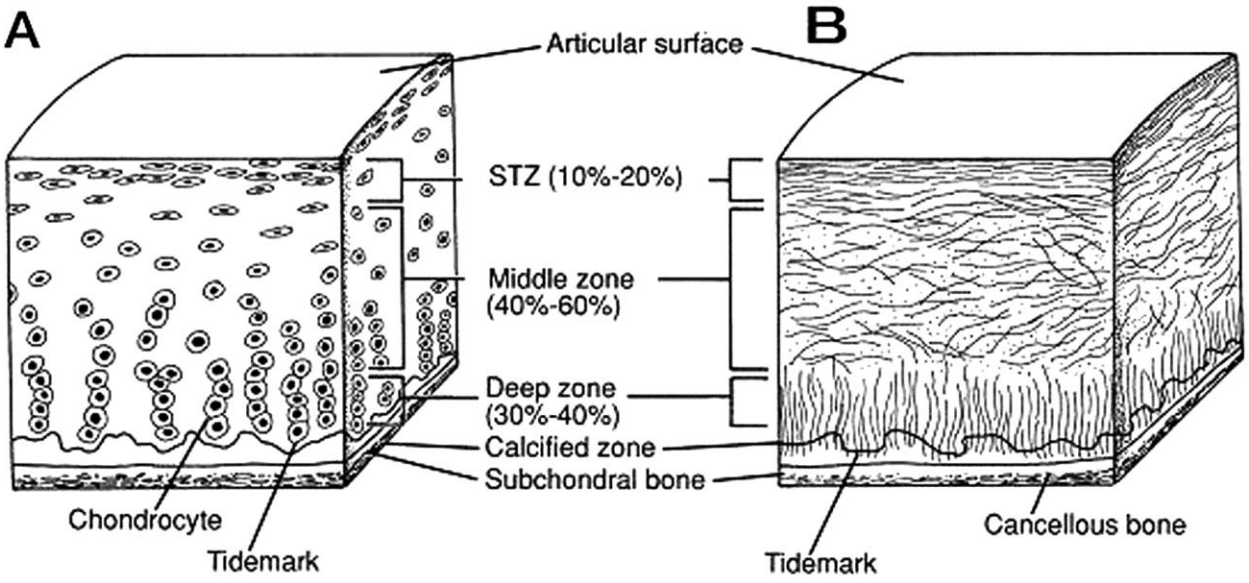

Figure 1.2 Articular cartilage morphology. In: J Am Acad Orthop Surg 1994; 2(4):192-201

Within the superficial zone, cells are in the highest density and lie flat and parallel to the joint surface, with cells becoming fewer in density, rounder and more perpendicular through the middle and deep zones (Schenk et al., 1986). The cartilage surface covers the end of long bones, attaching to the underlying subchondral bone at the zone of calcified cartilage tissue.

A hydrated tissue with approximately 70% of the wet weight being water, the dry matter composition of mature articular cartilage in the horse is approximately 60% collagen, 30% proteoglycans and a10% composition of glycoproteins, chrondocytes, lipids and minerals (McDevitt, 1973). The collagen network in articular cartilage is almost entirely Type II collagen, which is the most abundant extra-cellular matrix (ECM) molecule (Figure 1.3) and serves to provide tensile strength to the cartilage (Vachon et al., 1990). Type II collagen is comprised of three identical alpha chains that form a triple helical structure when wound together during collagen biosynthesis. Newly formed collagen molecules are arranged within the ECM through intermolecular cross-linking with proteoglycans and hyaluronan, creating a dense,

fibrillar network (Platt, 1998). Collagen fibers are positioned in a similar manner as chondrocytes within the cartilage zones (Figure 1.2); being more abundant and parallel in orientation closer to the joint surface to provide resistance to tensile force during loading (as reviewed by Palmer and Bertone, 1994). The primary proteoglycan, in the form of aggrecan, connects multiple proteoglycans and attaches to an 'anchor' proteoglycan; hyaluronan. The aggrecan molecule contains the glycosaminoglycan (GAG) chains chondroitin sulfate and keratin sulfate, which due to their negatively charged sulfate groups create high osmotic swelling pressure (van den Boom, et al., 2005; Palmer and Bertone, 1994). Three globular regions exist within the aggrecan molecule; two structurally related N-terminal domains known as G1 and G2; and a third, G3, located at the C-terminal end of the proteoglycan structure. An interglobular domain (IGD) separates G1 and G2 and is the primary site of cleavage during aggrecan turnover due to its proteinase-sensitive sequences (Hardingham et al., 1994). Keratan sulfate and chrondrotin sulfate chains are linked to the aggrecan protein core in the extended region between the G2 and G3 domains. The entire aggrecan molecule binds non-covalently to the HA molecule through the G1 domain and is stabilized by parallel 'linking' with a link protein. Each HA molecule has the capability of binding up to 100 aggrecan core proteins (Hardingham and Fosang, 1992) and provides the anchor to Type II collagen fibers within the ECM (Figure 1.3). The high molecular weight of the aggrecan molecule also helps to limit diffusion out of the ECM. This unique balance between collagen tension and the osmotic properties of the proteoglycan structures provides the biomechanical function to the articular cartilage (Hardingham et al., 1994; Platt, 1998). Alteration to any of these components leads to a change in the cartilage's ability to resist loading.

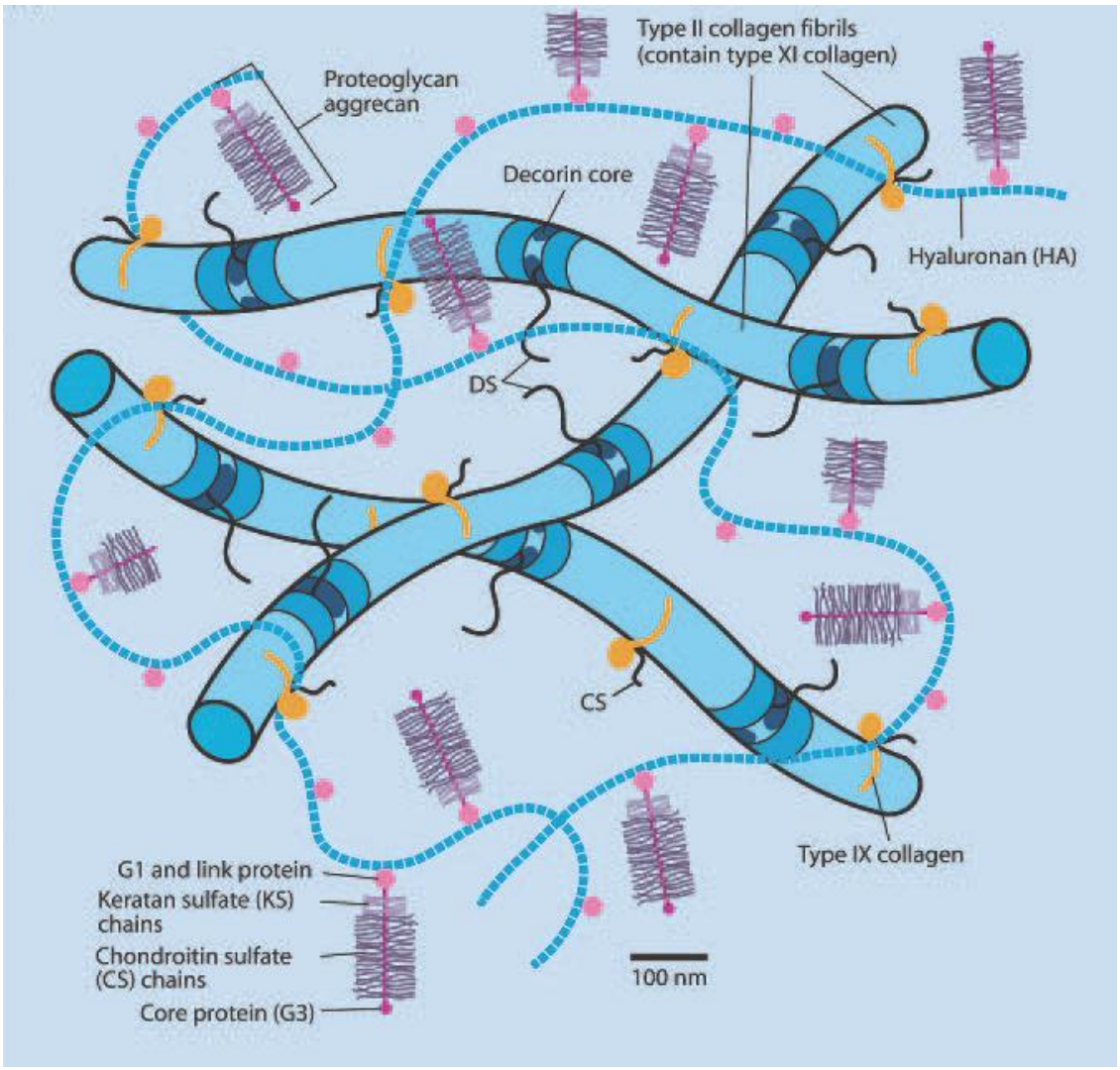

Figure 1.3 Extra-cellular matrix of cartilage. In: McIlwraith CW (2005) Frank Milne Lecture: From arthroscopy to gene therapy-30 years of looking in joints, in *Proceedings. Am Assoc Equine Pract* 51:65-113

Articular cartilage is unique in that it is an avascular, non-innervated tissue that contains only one type of cell: the chondrocyte. Chondrocytes are embedded within the extra-cellular matrix, possessing the ability to synthesize and degrade matrix components such as collagens, proteoglycans, glycoproteins and hyaluronic acid (Archer and Francis-West, 2003). Remodeling of the ECM is essential for joint homeostasis; chondrocytes contribute by synthesizing and

releasing catabolic enzymes such as matrix metalloproteinases (MMP), serine proteinases and aggrecanases. During normal joint homeostasis, there is a balance in turnover of the extra-cellular molecules; however, during prolonged or excessive production of MMPs or aggrecanases, degradation supersedes synthesis resulting in breakdown of the extra-cellular matrix (Dean, 1991) and loss of the functional integrity of the cartilage.

Pathophysiology of Equine Osteoarthritis

Overview

Two overall pathways can cause osteoarthritis in the horse; the result of normal stresses (aging or athletic stress) on abnormal cartilage (due to aging or genetic defects) or abnormal stress (fracture or ligament failure; microfractures) on normal cartilage, causing biochemical responses that lead to breakdown of the articular cartilage (McIlwraith, 2005). While OA has disease of the articular cartilage as the critical component, the synovial membrane and subchondral bone are tissues of importance as their response to abuse or injury contributes to disease development. Synovitis and joint effusion commonly precede the disease of cartilage and may contribute to the development of OA as levels of inflammatory biomarkers can be correlated with progression of cartilage change in OA (Maiotti et al., 2000; Marini et al., 2003). Early events of equine OA (such as trauma) reflect an articular inflammatory response; synoviocytes and articular chondrocytes synthesize the cytokines interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α), prompting the release of other cytokines (IL-6 and IL-8) and additional inflammatory mediators such as eicosanoids (notably, prostaglandin E_2), matrix metalloproteinases (MMPs) and aggrecanases (Figure 1.4). These mediators additionally heighten pain receptors and contribute to joint effusion resulting in clinical signs such as lameness, decreased motion and heat around the affected joint. Continued imbalance of normal

homeostatic processes within the joint can lead to synovial tissue thickening, fibrillation and erosion of the articular surfaces of cartilage and may be accompanied by sclerosis of the subchondral bone (McIlwraith, 1996a); articular changes that are common in advanced OA.

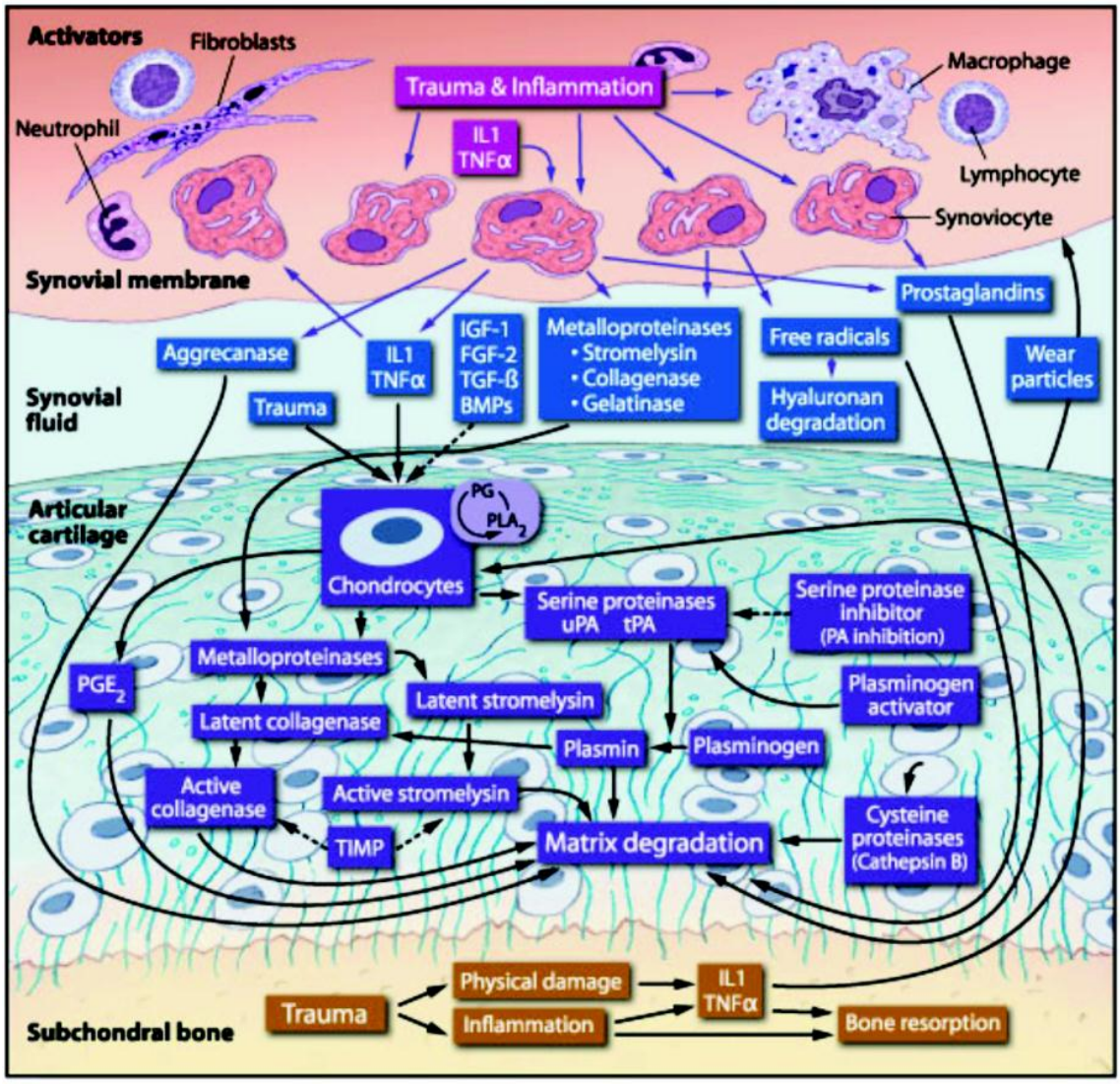

Figure 1.4 Factors involved in enzymatic degradation of articular cartilage In: McIlwraith, CW. 1996; in Joint disease of the horse; Ch. 3 pg. 50. W.B. Saunders, Philadelphia

The role of synovitis (joint inflammation)

Proliferation of synoviocytes, hyperemia and lymphocytic infiltration of the synovium are active processes involved in synovitis (Hamerman et al., 1963) and results in clinical signs such as lameness, pain, palpable joint swelling and heat around the joint. Latent forms of plasma-derived mediators such as kinin, histamine, fibrinogen, plasminogen and trypsin enter the joint (Palmer and Bertone, 1994) increasing the protein concentration of the synovial fluid. Within the joint, the presence of these non-specific mediators stimulate monocytes, synoviocytes and chondrocytes to synthesize pro-inflammatory molecules, such as IL-1β, TNF-α, IL-6, PGE_2 and LTB_4, (Bondeson et al., 2006) as well as anti-inflammatory meditators, such as IL-4 and IL-1Ra, to maintain homeostasis (Sellam and Berenbaum, 2010). The response is further characterized by chondrocytes producing additional MMPs and aggrecanase, a proteinase that cleaves the large proteoglycan aggrecan of the cartilage ECM (Fernandes et al., 2002). The release of type II collagen fragments such as proteoglycans, glycosaminoglycans (GAGs) and HA into the synovial fluid can further stimulate the inflammatory response by the synovium (Boniface et al., 1988; Sellam and Berenbaum, 2010) enhancing the catabolic effects and ultimately resulting in irreversible cartilage loss. Additionally, there are measureable changes in the composition of synovial fluid (Table 1.1) with degree of change indicating level of inflammation. Trauma, inflammation or bacterial infection can cause changes to synovial fluid composition resulting in a reduction in the lubricating properties of the fluid and subsequently negatively affecting the articular cartilage, leading to degradation (Bertone et al., 1998b; Elsaid et al., 2005).

Table 1.1 Composition of equine synovial fluid (Adapted from Steel 2008; Bertone et al., 2001)

Characteristics	Normal	Abnormal
Color	Pale Yellow	Amber-red
Clarity	Clear	Cloudy
Viscosity	Viscous	Watery
WBC (X 10 cells/μl)	<1.0	>15.0
Neutrophils	<10%	>90%
Total protein (g/dl)	<2.5	>4.0

Inflammatory mediators involved in synovitis

Cytokines (IL-1β and TNF-α)

Classified as intercellular signaling molecules, cytokines are soluble glycoproteins, released by a variety of cell types and responding non-enzymatically in picomolar to nanomolar concentrations to regulate tissue cell functions (Nathan and Sporn, 1991). Tumor necrosis factor alpha (TNF-α) and Interleukin-1 (IL-1) are both produced in OA associated synovitis and have identical functions; however, IL-1 is more potent and each cytokine's action is independent of each other; contrary to human rheumatoid arthritis (RA) where TNF-α is the dominant cytokine and regulates the production of IL-1 (Bondeson et al., 2006). Interleukin-1 and interleukin-1 derived mediators have been identified in numerous animal models of OA, human osteoarthritic tissues (as reviewed by Malemud et al., 2010) equine OA models (Hawkins et al., 1993; Theoret et al., 1998; Frisbie et al., 2008) and naturally-occurring equine OA (Trumble et al., 2001; Von Rechenberg et al., 2001; Ley et al., 2007) establishing IL-1 as the most recognized cytokine in human (Scott et al., 1994) and equine OA (McIlwraith, 2005). Given the identical functions of TNF-α and IL-1 and IL-1's central role in the development of OA, further reference in this review of cytokine activity will focus on IL-1.

The large precursor form of IL-1 is enzymatically cleaved into two low weight isoforms, IL-1α and IL-1β and both have equal affinities for binding to cell surface receptors in order to be activated (Oppenheim et al., 1996). Two forms of interleukin-1 in the horse have been identified as equine IL-1α and IL-1β (May et al., 1990). Both have been cloned and evaluated for stimulatory effect in an *in vitro* model, with equine IL-1β being more potent than equine IL-1α (Takafufi et al., 2002, May et al., 1992a). Previous research has focused heavily on the β isoform of IL-1; further reference to IL-1 will be restricted to IL-1β.

In a response to insult or injury, various stimuli including immune complexes and neuropeptides promote IL-1β production within the tissue (Scott et al., 1994). Activation of IL-1 occurs through its binding to a specific cell-surface receptor, prompting macrophages, synoviocytes and articular chondrocytes to synthesize and release neutral metalloproteinases, collagenases and eicosanoids; causing proliferation of synovial cells and the inhibition of chondrocyte production of collagen and matrix macromolecules and aggrecan assembly (Oppenheim et al., 1986; Scott et al., 1994; Palmer and Bertone, 1996). Additionally, IL-1β upregulates the production of other pro-inflammatory cytokines, notably IL-6 and IL-8 (Daheshia and Yao, 2008), both of which may induce a concurrent wave of proteinase and eicosanoid products. Elevated protein concentrations of IL-6 have been reported in the synovial fluid of horses from experimental (Hawkins et al., 1993) and natural (Ley et al., 2007; Ley et al., 2009) OA studies and it is regarded as an excellent predictor of joint disease in the horse (Bertone et al., 2001). Further review of experimental synovitis models in the horse will be presented later in this chapter.

Natural inhibitors of IL-1β include IL-1 receptor antagonist (IL-1Ra), a protein that attaches to the IL-1 receptor site, inhibiting IL-1 binding and affecting IL-1's signaling and

regulatory abilities. The endogenous production of IL-1Ra in numerous experimental models of disease and experimental neutralization of anti-IL1Ra antibodies has demonstrated the importance of IL-1Ra anti-inflammatory effects in arthritis and other diseases (Arend et al., 1998). The importance of IL-1β's natural role in cartilage degradation is evident by the reduction of OA associated articular cartilage lesions in dogs (Pelletier et al., 1997), pain alleviation, reduced macroscopic and microscopic cartilage lesion scores along with elevated cartilage IL-1Ra expression in horses (Frisbie et al., 2002) and pain respite in a clinical trial of human OA (Chevalier et al., 2005) when all were treated with IL-1Ra protein therapies.

Eicosanoids (Prostaglandin E_2)

Eicosanoids are a family of potent lipid mediators derived from the cellular membrane bound 20-carbon unsaturated fatty acids. While other long chain polyunsaturated fatty acids produce eicosanoids, arachidonic acid (ARA) is greatest in abundance in the cell phospholipid bilayer; therefore, its byproducts are synthesized in the largest quantities. During joint inflammation, IL-1β activates cystolic phospholipase A_2 ($cPLA_2$), a lipolytic enzyme, to catalyze the hydrolysis of ARA from the cell's phospholipid layer (Leistad et al., 2011) releasing ARA to be freely oxidized by cycloxygenase and lipoxygenase enzymes; yielding inflammatory prostaglandins, thromboxanes and leukotrienes (Funk, 2001).

Prostaglandin E_2 (PGE_2) is one of the main prostaglandins involved in the inflammatory response is the primary PG in the joint and is produced by several classifications of mammalian cells, like macrophages, mast cells and fibroblasts, and is an important arbitrator of several biological processes, specifically immune responses. As part of its inflammatory functions, PGE_2 stimulates vasodilation (causing swelling and redness), increased permeability (joint effusion) and a hyperalgesic response; an increase in sensitivity, particularly to pain (Hardy et

al., 1998, Kirker-Head et al., 2000; Funk 2001). Synovial PGE_2 levels have been correlated to level of lameness in horses (May et al., 1994) and dogs (Trumble et al., 2004). Clinical and functional improvement have been reported in cases where steroidal (i.e. corticosteroids) and non-steroidal anti-inflammatory drugs (NSAIDS) were used to treat clinical signs associated with OA. The primary function of these drugs are to inhibit ARA conversion's to PGE_2 resulting in symptomatic relief.

In addition to immune responses, PGE_2 inhibits chondrocyte synthesis and stimulates latent MMP production (Bertone and Palmer, 1996) and bone resorption (Watkins et al., 2001). Levels are elevated in horses suffering from acute synovitis (Hawkins et al., 1993, May et al., 1994; Bertone et al., 2001; de Grauw et al., 2009) or osteoarthritis (Tamanini et al., 1980; Gibson et al., 1994; Kirker-Head et al., 2000; Frisbie et al., 2008).

Matrix Metalloproteinases

Tissue turnover, the act of degradation and re-synthesis, is a normal process that occurs throughout the body. Degradation of tissue can be orchestrated by matrix metalloproteinases (MMPs), whose activity is regulated, to an extent, by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs). In-vitro cartilage degradation was reported when increased activity of MMPs occurred in concert with low levels of TIMPs (Cawston et al., 1991). A unique balance between MMPs and TIMPs is necessary for tissue maintenance and homeostasis. Imbalances have been detected in human OA cartilage (Dean et al., 1989) and may contribute to the progression of the disease.

Matrix metalloproteinases are zinc-dependent extracellular enzymes that function to cleave extracellular matrix components and degrade cell surface and pericellular proteins (Clutterbuck et al., 2010). Biomolecules, such as IL-1β, promote the synthesis and release of

MMPs in a latent (inactive) form due to zinc being restricted from binding within the MMPs. Once the MMPs leave the cell, the presence of other enzymes, such as plasmin, allows zinc to bind to active site, causing conformational change in the MMP, activating the enzyme. (Woessner, 1991). Additionally, MMPs have been shown to regulate IL-1β, the very cytokine that stimulates MMP production, indicating a negative feedback loop may be involved to keep MMP activity in balance (Clutterbuck et al., 2010). Activation of MMPs is tightly regulated due to their destructive nature. The primary regulatory units are tissue inhibitors of matrix metalloproteinases (TIMP), which function to prevent binding of zinc, which down-regulates the activation of MMPs. Synovial fluid collected from healthy human knees was reported to have higher concentrations of TIMPs compared to latent-MMPs; however, synovial fluid from injured knees had increased concentrations of MMPs that exceeded increases in TIMPS (Tchetverikov et al., 2005).

Matrix metalloproteinases are classified according to their target substrate, with numerous MMPs having the capability of degrading one or more components of tissues' extracelluar matrix (Woessner, 1991) and have a known cleavage site on the aggrecan molecule that differs from other proteinases (as reviewed by Caterson et al., 2000). Classifications of MMPs and their primary target are as follows: collagenases (MMP-1, MMP-8 and MMP-13), cleavage of interstitial collagen helix; stromelysins (MMP-3, MMP-10), degradation of proteoglycans; and gelatinases (MMP-2, MMP-9), cleavage of unwound collagen within the extra-cellular matrix (van den Boom, et al., 2005). Matrix metalloproteinases identified to be most prevalent in joint tissue maintenance are MMP-1, MMP-13, MMP-3, MMP-2 and MMP-9 (Trumble et al., 2001) with MMP-13 preferential for type II collagen (Knauper et al., 1996). Elevated concentrations of synovial MMP-2 and 9 and articular cartilage expression of MMP-13

have been reported in horses with OA (Trumble et al.,2001) and synovial fluid MMP concentrations increase significantly under experimental synovitis in the horse (de Grauw et al., 2009). Due to their ability to degrade various constituents of articular cartilage, MMPs are a common therapeutic target for minimizing cartilage degradation and managing OA in the horse.

Aggrecanases (ADAMTS)

A disintegrin-like and metalloproteinase with thrombospondin type I motifs (ADAMTS) are a class of extracellular matrix-anchored enzymes that have been identified as major contributors to cartilage degradation (Kuno et al., 2000). These enzymes are similar to Zn-dependent MMP proteases in that they have the ability to target and degrade the extra-cellular matrix within tissues; yet their action may be independent of MMPs, particularly during late OA (as reviewed by Caterson et al., 2000) and they are not considered to be part of the MMP family (Hughes et al., 1998; Cawston et al., 1999). The ADAMTS are specialized in that their extra domain: thrombospondin (TS) motifs are specific for aggrecan glycoaminoglycan chains (De Rienzo et al., 2009). Several aggrecanases have been identified, yet ones specific to cartilage degradation and most heavily researched are aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5). While ADAMTS-4 and -5 can cleave multiple locations within the aggrecan molecule, the proteolysis of Glu^{373}-Ala^{374} bond within the interglobular domain portion of the molecule is the predominant site of cleavage generating fragments (Flannery et al., 1992) that can be detected within the synovial fluid (Sandy et al., 1992). While both ADAMTS 4 and 5 have been implicated in cartilage degradation, in-vitro research indicates ADAMTS 5 may be the more efficient of the two (Gendron et al., 2007) and cleavage activity of both can be driven by IL-1 and TNF (Tortorella et al., 2001). Increasing the production of naturally occurring aggrecanase inhibitors, such as TIMPS, has been shown to regulate aggrecanse activity in-vitro

(Bondeson et al., 2008). Blocking of their activity in murine models has resulted in decreased aggrecan levels in synovial fluid and decreased cartilage erosion (Little et al., 2007). Additionally, dietary n-3 polyunsaturated fatty acids (PUFAs) have recently been implicated in mediating gene expression of aggrecanases in *in-vitro* studies using bovine chondrocytes (Zanial et al., 2009; Wann et al., 2010) indicating the potential of these fatty acids to have disease-modifying properties.

Conventional anti-inflammatory therapies for equine OA

Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are customary therapies that provide analgesia and pain relief to inflammatory conditions. The primary function of NSAIDS are to decrease production of prostaglandin E_2 and thromboxanes causing a reduction in vascular permeability, platelet aggregation and vasodilation; common physiological effects that occur during inflammation (Simon and Strand, 2007). The primary mechanism for altering prostaglandin synthesis is interrupting the cyclooxygenase pathway within the arachidonic-cascade. Two isoforms of cyclooxygenase, COX-1 and COX-2, are responsible for yielding eicosanoids (prostaglandins and thromboxanes) from ARA (Levin et l., 2002) ; COX-1 derived eicosanoids are produced for general physiological responses such as smooth muscle contraction while COX-2 derived eicosanoids, are specific to the inflammatory process (as reviewed by Smith, 2007).

Traditional NSAIDS target both COXs; however, long term application can cause detrimental effects, specifically to the gastrointestinal tract due to the prolonged inhibition of COX-1. Phenylbutazone (PBZ) is the primary NSAID given to horses for symptomatic relief of

musculosketetal-related pain. Inexpensive and easy to administer (oral dosages are available); PBZ is a practical medicinal for horses owners. However, long term application or inappropriate doses in the horse can inhibit necessary functions of prostaglandins in the gastrointestinal tract resulting in reduced blood flow to intestinal villi causing necrosis of intestinal microvilli that can lead to ulceration (Reed et al., 2006) and leaking of plasma proteins (McConnico et al., 2008). The development of selective COX-2 inhibitors has gained popularity given the specific inhibition of COX-2 without influencing COX-1 functions. The use of carprofen, a preferential COX-2 inhibitor, in horses has shown promise as an NSAID treatment, providing suppression of adverse PBZ side effects (McIlwraith, 2011).

Intra-articular corticosteroids

Another method for treating OA is the use of intra-articular (IA) corticosteroid therapy. Corticosteroids are potent anti-inflammatory compounds that are effective in reducing and improving OA-related clinical signs, such as pain and effusion (Richardson and Dodge, 2003). Customary corticosteroids used for joint disease therapy include betamethasone acetate, methy-prednisolone acetate and triamcinolone acetonide. The primary function of these and other corticosteriods are to inhibit cellular and humoral inflammatory processes, through the inhibition of phoshoplipase A_2 activation, negating the synthesis of PGE_2 (Masferrer et al., 1992). Eliminating the availability of free ARA minimizes all eicosanoid production, making these drugs very potent analgesics. Corticosteroids cause decreases in capillary dilation, margination and migration of inflammatory cells, and inhibition of the synthesis and release of cytokine, prostaglandin and nitric oxide-derived meditators involved in the development of osteoarthritic symptoms and tissue damage (Caron, 2005). The use of triamcinolone acetonide (TA) has demonstrated strong analgesic effects (reduced lameness) and anti-inflammatory effects (reduced

synovial total protein concentrations, synovial membrane edema) in an equine experimental model of synovitis (Kay et al., 2008) and improved lameness scores, decreased inflammatory response in synovium and improved cartilage morphological parameters compared to controls in an experimental model of equine OA (Frisbie et al., 1997)

A review paper by Caron (2005) reported inconsistency in IA corticosteroid studies; effects of high doses and or repeated use of IA corticosteroids ranged from the development of cartilage lesions to no evidence of cartilage-sparing effects when compared to placebo treatments. An *in-vivo* model of experimental equine osteoarthritis evaluated intra-articular corticosteroid use of methylprednisolone acetate (MPA) reporting a tendency for lower synovial PGE_2 concentrations, reduced lameness scores and lower synovium vascularity scores, yet also significantly higher modified Mankin scores (indicator of negative articular cartilage histopathological change) and inhibition of development of repair tissue in surgically-created cartilage defects (Frisbie et al., 1998).

Nutraceutical therapies for equine OA

The use of oral nutraceuticals has received attention for their potential of being symptom and/or disease-modifying. Nutraceuticals are defined as "a nondrug substance that is produced in a purified or extracted form and administered orally to provide agents required for normal body structure and function with the intent of improving the health and well-being of animals" (Shotwell, 1999). Traditional oral joint nutraceuticals are glucosamine-based, containing glucosamine (GS) and/or chondroitin sulfate (CS) while newer products are derived from avocado and soybean unsaponifiable (ASU) extracts and n-3 polyunsaturated fatty acids (PUFAs). Oral joint supplements are theorized to be readily absorbed across the gastro-intestinal tract, transported through the hepatic system and into the joint capsule for uptake and utilization

by the tissues for protective or modifying effects against OA. Absorption and tissue uptake of oral GC have been demonstrated in various species including dogs (Setnikar et al., 1986) and humans (Stenkiar et al., 1993); however, information is inconclusive in the horse. Reports of analgesic effects (Hanson et al., 1997) and improved articular function (Forsyth et al., 2006) have been published; however, a majority of *in vivo* studies of oral GC based supplementation in the horse have numerous deficiencies that may question the efficacy of these products. Poor trial design, small sample size, lack of long term application or follow up and potential bias with commercial sponsors exists in human (Goggs et al., 2005) and equine (Pearson and Lindinger, 2009) studies. With the lack of scientific evidence and the low quality of reported *in vivo* studies, there is a need for improved application of scientific techniques to better understand the biological relevance these products have to joint health.

Dietary n-3 long chain polyunsaturated fatty acids (LCPUFA)

Dietary fats are required to support absorption of fat-soluble vitamins and provide the essential fatty acids, linoleic acid (LA) and alpha linolenic acid (ALA) (NRC, 2007). The long chain fatty acid family of n-6 and n-3 are important components of the diet and are necessary in daily physiological functions as well as for fetal development and neonatal growth. The parent fatty acids are linoleic acid (LA) for the n-6 family and alpha-linolenic acid (ALA) for the n-3 family. These 'parent' fatty acids are considered essential in mammalian diets; the lack of proper enzymes prevents their endogenous synthesis. The derivatives of these 'parents' are likely of even greater importance; LA can be elongated and converted to arachidonic acid (ARA), where the primary role of this 20 carbon polyunsaturated fatty acid is to produce 20 carbon signaling molecules known as eicosanoids. Eicosanoids have short half-lives and are localized in their production; influencing events within and around the cells that produce them. Arachidonic acid

and other eicosanoid- producing fatty acids must be present in tissue in order for these signaling molecules to be effective. Eicosanoids are instrumental in regulating a variety of cellular functions during both physiological (normal) and pathophysiological (inflammatory) events. The best well-known classes of eicosanoids are prostaglandins (PG), thromboxanes (TX), leukotrienes (LT) and lipoxins (LX), with PGs and TXs being synthesized via the cyclooxygenase pathway and LTs and LXs being converted from ARA by lipoxgenases. While all classes are vital physiological components, prostaglandins are important as they are utilized by all major organ systems ranging from reproductive to gastrointestinal and to neurological function. Of these active eicosanoids, prostaglandin E_2 is the primary PG, synthesized exclusively from ARA, playing an important role in the inflammatory response. In terms of joint health and the development of OA; PGE_2 has been implicated as therapeutic target as it is elevated in early stages of the disease and contributes to down-stream production of degradative cartilage enzymes.

Long chain derivatives of alpha-linolenic acid (ALA); specifically eicosapentanoic acid (EPA), docosapentanoic acid (DPA) and docosahexanoic acid (DHA), have equally important roles as ARA in cellular function and physiologic homeostasis. While all ALA derivatives have the ability to produce eicosanoids, EPA is the most widely recognized n-3 long chain fatty acid for producing anti-inflammatory PGs, TX, LTs and LXs. However, more research is indicating that DPA and DHA play vital roles in mediating the inflammatory response in conjunction with EPA. One of the premier sources of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is marine fish oil, also known as menhaden oil. Eicosapentaenoic acid, though found in the largest quantities in marine fish oil, originates from alpha linolenic acid (ALA) which is in high concentrations in plants. Plant oils such as linseed, soybean and flaxseed oils are all robust

sources of ALA, therefore, when consumed; the animal may be able to synthesis EPA, DPA and DHA from these plant oils. Alpha-linolenic acid is converted to EPA, DPA and DHA via a desaturation and elongation pathway (Figure 1.5). The initial step, the addition of a double bond to ALA by the Δ6-desaturased enzyme, is the rate-limiting step in the pathway and may contribute to the reported low conversion efficiency of ALA to the longer chain PUFAs. Both LA and ALA share a need for Δ6-desaturase and while the enzyme has a higher affinity for ALA, higher dietary intake of LA may be the limiting factor in ALA conversion (Tu et al., 2010). A review of human studies reported that biological conversion of dietary ALA to EPA ranged from 8-10% with conversion of ALA to DHA being as low at 4%. (Williams and Burdge, 2006). Due to the evidence of a poor conversion rate, it is recommended to supply EPA and DHA directly in the diet.

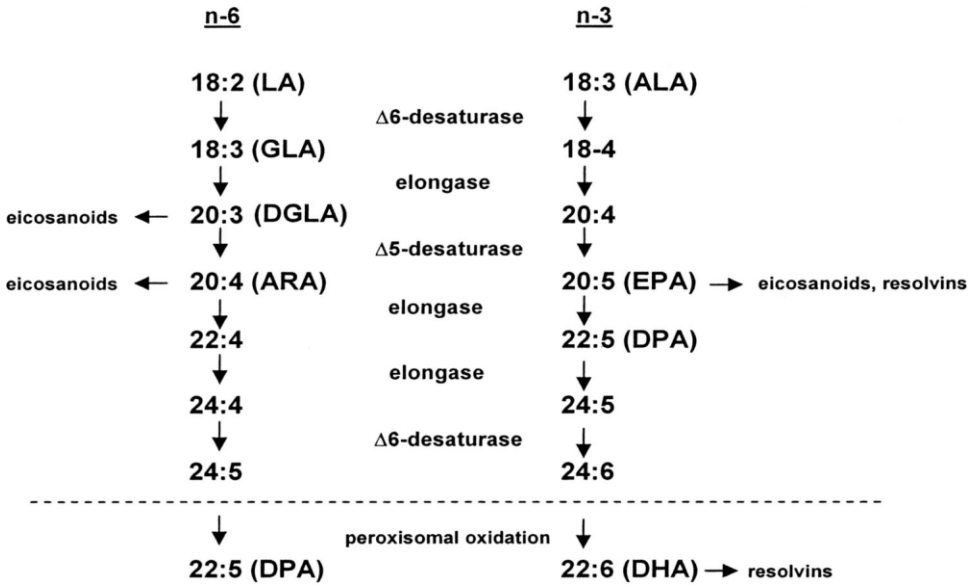

Figure 1.5 Biochemical pathways for the interconversion of n-6 and n-3 fatty acids. ALA, alpha-linolenic acid; ARA, arachidonic acid; DGLA, dihomo--linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, -gamma-linolenic acid; LA,linoleic acid. (Reproduced from Arterburn, L.M., and H. Oken. Distrubution, interconversion and dose response of n-3 fatty acids in humans. 2006. *Am J Clin Nutr*. 83:S1467-1476)

Anti-inflammatory properties of n-3 LCPUFA

The investigation of dietary long chain polyunsaturated fatty acids (LCPUFA) in nutritional research has grown in popularity in recent years, with particular interest in the role of the omega 3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in modifying inflammation. When incorporated in appropriate amounts, these essential fatty acids likely alter the cell membrane lipid bilayer, influencing protein function and signaling (Min and Crawford, 2004). Arachidonic acid derivatives include PGE_2, thromboxane A_2 (TXA_2) and leukotriene B_4, all part of a family of very effective signaling molecules responsible for inflammation and platelet aggregation. Eicosapentaenoic acid derivatives, prostaglandin H_3 (PGH_3) and thromboxane A_3 (TXA_3) have similar steriochemistry to AA-derived molecule but may function in different ways (Calder 2009). Excess ARA can lead to the increase in the formation of prostaglandin E_2, its n-6 derived eicosanoid. At certain levels, PGE_2 is instrumental in many biological processes; however, excess amounts can be detrimental, exacerbating inflammatory responses (Calder, 2002). If provided in adequate amounts in the diet, EPA and DHA can have inhibitory effects on the production of AA-derived inflammatory biomolecules via competitive action on cyclooxygenase and lipoxgenases pathways (Figure 1.6), thus the basis for their anti-inflammatory properties.

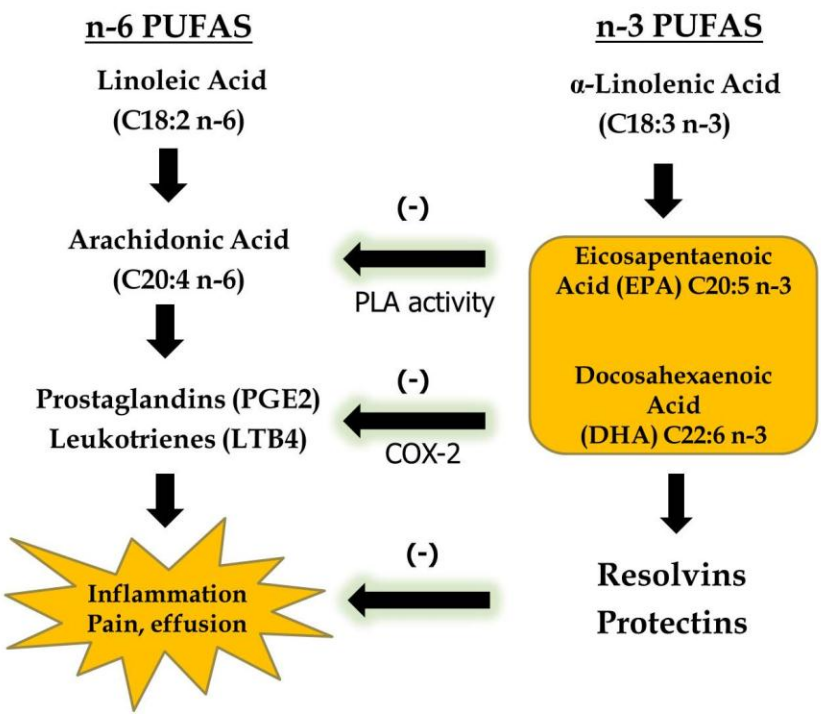

Figure 1.6 Mechanisms of anti-inflammatory action of long chain n-3 fatty acids (Reproduced from Goldberg and Katz; Pain 2007;129:210-223)

Recently, a novel class of n-3 PUFA-derived anti-inflammatory mediators have been recognized, termed E-series and D-series resolvins; formed from EPA and DHA, respectively (Calder, 2009).While information is limited, it appears n-3 PUFA derived resolvins are heavily involved in the resolution of inflammation; a detailed cellular process that is equally important as the initiation of inflammation (Kohli and Levy, 2009).

Proposed mechanisms of EPA and DHA on inflammation

Several mechanisms have been proposed regarding the processes by which EPA and DHA act on the inflammatory response in tissues. It is well established that these lipids act on both a direct (by alteration of eicosanoid production via cyclooxygenase and lipoxygenase

pathways) and indirect (modification of gene transcription) mechanism (Calder, 2006). Direct modification of prostaglandin and leukotriene synthesis was outlined previously. Regarding influence of gene transcription, it is hypothesized that particular fatty acids, such as EPA and/or DHA may modify transcription factors in the nucleus and thus influence cytokine and eicosanoid production at the level of gene expression. Another theory is that n-6 (i.e. ARA) and n-3 (i.e. EPA and DHA) fatty acids modify protein synthesis of inflammatory mediators via modification of cell surface receptors on lipid rafts or within the cell by suppression of nuclear receptor activation (Figure 1.7) (Chapkin et al., 2009). Additional regulation comes in the form of peroxisome proliferator-activated receptors (PPAR); key nuclear receptors that regulate transcription of gene through ligand binding with a variety of lipophilic metabolites, having a high affinity for PUFAs, in particular DHA (Stulnig, 2003). PPARs usually have three isoforms; α, β, γ and are believed to be potent regulators of adipocyte function as well as immune molecules such as lymphocytes and macrophages (Marx et al., 2002) influencing downstream transcription of inflammatory cytokines.

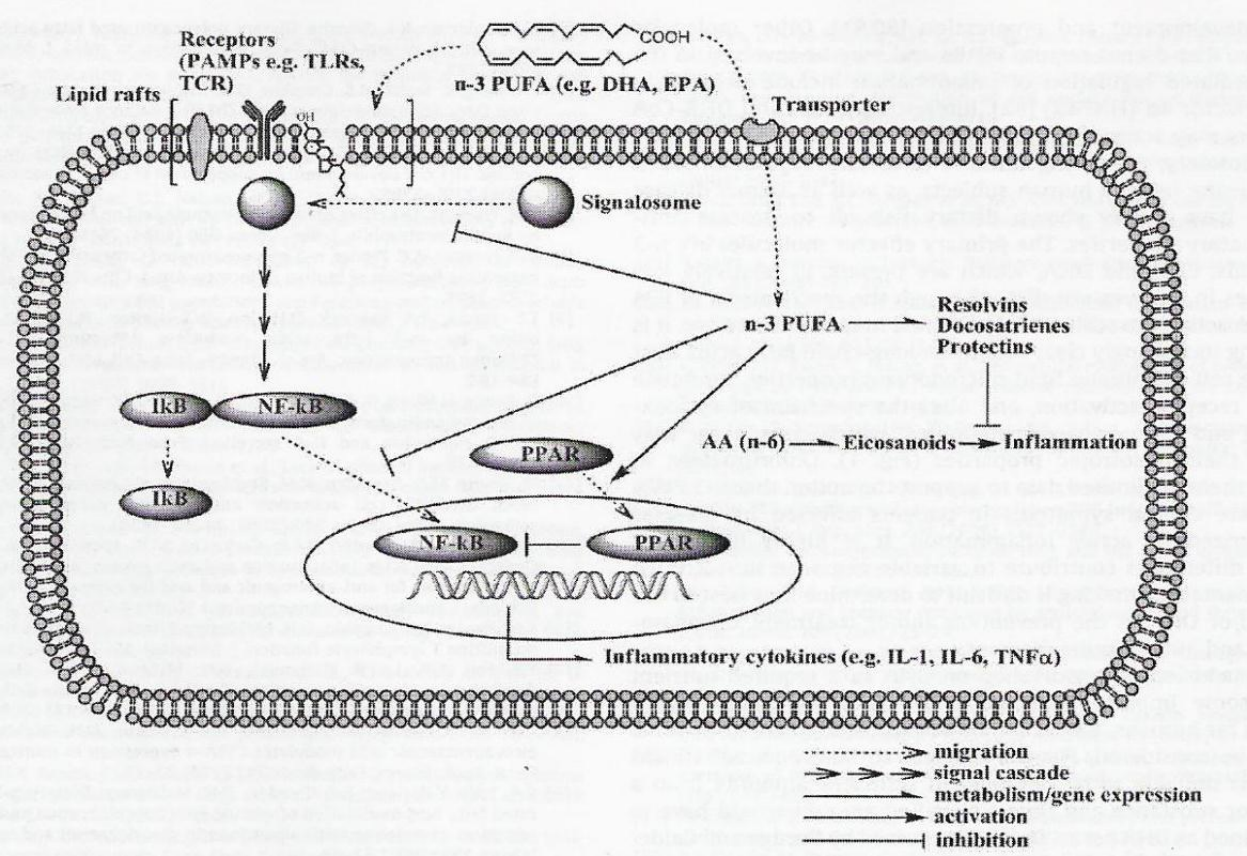

Figure 1.7 Proposed models of EPA and DHA and inflammation. (Reproduced from Chapkin et al., 2009 Prostgland Leukot Essent Fatty Acids (81):189

Fatty acid composition of mammalian joint tissues and fluids

Minimal information exists regarding the fatty acid composition of synovial tissue and fluid and relevant roles in joint health. Cleland and colleagues (1995) reported age-related differences in fatty acid levels of human articular cartilage. Arachidonate (ARA) levels were significantly higher ($P<0.01$) in fetal articular cartilage when compared to healthy, mature cartilage (15 % vs 0.9% of fatty acids, respectively). Additionally, total n-3 fatty acid content was higher ($P<0.01$) in fetal cartilage samples (~3% of fatty acids) compared to adult cartilage (1.3% of fatty acids) (Cleland et al., 1995). It is likely the essential fatty acids ARA, EPA and DHA are in higher demand during fetal development compared to a non-growing adult.

Additionally, articular cartilage samples from young adults (age <40 yrs) were significantly lower in ARA compared to older adult cartilage (age >65 yrs) (Cleland et al., 1995). The increase in ARA present in the cartilage may be due to age-related physiological changes in the joint and decreases in other fatty acids. Interestingly, there were no differences in fatty acid composition between mature human articular cartilages obtained from arthritic or non-arthritic joints (Cleland et al., 1995).

Mammalian studies- LCPUFAs as treatment of OA

In-vitro studies

An in-vitro study utilized bovine chrondrocytes from 7-day old calves to the investigate effects of ALA, EPA, DHA and AA in culture. Chondrocytes incubated for 8 hours in 30 µg/ml of EPA, 30 µg/ml of DHA, 30 µg/ml of ALA and 30 µg/ml of AA resulted in detectable chondrocyte uptake of all fatty acids, respectively (Zainal et al., 2009). When chondrocytes were stimulated with 10 ng/ml of IL-1α, EPA cultures had significantly lower mRNA expression of ADAMTS-4 and 5, MMP-3 and 13, COX-2, IL-1 and TNF-α compared to control chondrocytes (Zainal et al., 2009). Concentrations of 0.1 and 1 µm EPA were effective in prolonged modulation of IL-1β stimulated sulfated glycosaminoglycan loss in bovine cartilage explants as well as reduce IL-1β induced gene expression of ADAMTS-4, ADAMTS-5 and COX-2 (Wann et al., 2009). Additionally, EPA but not ARA significantly reduced IL-1β stimulated COX-2 gene expression and subsequent protein concentrations of PGE_2 in both bovine and human chondrocytes (Hurst et al., 2009). These results indicate, that the level of gene expression, n-3 polyunsaturated fatty acids may modify the synthesis of catabolic proteins directly associated with progression of osteoarthritis.

In-vivo studies

Information gathered from in-vivo studies support the healthful benefits of dietary supplementation of EPA and DHA. When incorporated in appropriate amounts, these essential fatty acids likely alter the cell membrane lipid bilayer, influencing protein function and signaling (Min and Crawford, 2004). In addition, data suggests these lipids have a regulatory role in the inflammatory process, mediating cytokine production in both arthritic human subjects (Kremer et al., 1987) and healthy men and women (Endres et al., 1989, Meydani et al., 1991) after at least 4 weeks of diet change. A high n-3 fatty acid diet (17g/kg fish oil) attenuated OA progression in an OA-prone strain of guinea pigs through increased cartilage GAG content and reduced pro and activated MMP-2 after 20 weeks of supplementation (Knott et al., 2011).

In dogs suffering an acute cranial cruciate ligament injury, 64 days of fish oil supplementation (90 mg/d) resulted in a reduction of pro-MMP 2 and 9 and significant increase in TIMP-2 in the *non-affected limb* compared to a control group of dogs (Hansen et al., 2008). Therefore, in the Manhart 2009 study, it is possible that pre-existing conditions may have masked the anti-inflammatory effects of EPA/DHA supplementation. Hansen and others (2008) concluded that the dose of fish oil (~ 2% of energy provided) was likely insufficient in reducing extreme inflammation (affected joint) cytokine and MMP levels but reduced MMP production in mild and moderate inflammation (non-affected joint). They also mentioned that a higher dosage of fish oil (>%2 of dietary energy) may cause a more consistent, robust inhibitory effect on inflammatory markers (Hansen et al., 2005). Regarding this issue, it is also possible that the assays used to detect concentration levels of cytokines and prostaglandins are not sensitive enough to detect subtle yet significant changes that may be influenced by n-3 fatty acids.

Bioactive lipid mediators act locally at nanomolar levels through processes on cell surface receptors (Jump and Clarke, 1999); however, with the inability to detect minute changes, it would be reasonable to conclude that no differences between treatment groups exist.

Essential fatty acids are readily absorbed by the horse, with measureable increases in plasma (Vineyard et al., 2007, Manhart et al., 2009 and Hall et al., 2004) and red blood cells (King et al., 2005) occurring in a dose-dependent manner. To date, only one study has investigated oral supplementation of a protected n-3 fatty acid supplement in horses diagnosed with osteoarthritis. Manhart and others (2009) supplied 24.8g of EPA/DHA daily for approximately 90 days to a group of mature horses (n=6) with at least one arthritic carpal joint. Fluid biomarkers were compared against a control group of arthritic horses (n=6) that did not receive n-3 supplementation. Blood PGE_2 and synovial white blood cell count were significantly lower in the supplemented group compared to control group. These results indicate that a daily oral EPA/DHA supplement provided an anti-inflammatory effect in horses with pre-existing arthritic conditions.

The findings of Manhart and colleagues are novel and important; however, there are some notable limitations of the experiment. Plasma and synovial fluid markers reflected a reduction in inflammatory molecules in supplemented horses. However, there were no differences in force plate analysis (a measure of weight bearing capability) between groups indicating dietary treatment did not modify OA associated pain; an important goal in managing equine OA. Additionally, no other synovial fluid markers beyond cytology analysis were conducted, leaving the question of the oral supplementation's effect on prostaglandin production within the joint.

Use of experimental models to evaluate pathogenesis of OA

Given the importance of synovitis in the pathogenesis of OA, various equine experimental models for inducing joint inflammation have been developed. Experimental *in vivo* models provide valuable information for understanding disease progression and provide an avenue for evaluating putative therapies. Models of synovitis include amphotericin (Peloso et al., 1993), carrageenan (Owen et al., 1995), Freund's Complete Adjuvant (Toutain and Cesser, 2004), and polyvinyl alcohol foam particles (Cornelissen et al., 1998) with the endotoxin lipopolysaccharide (LPS) being the primary model used. All of these models have proven effective for inducing equine synovitis, however, the response may be reflective of bacterial or sepsis, rather than a reaction to injury or use-trauma (low grade synvoitis associated with athletic training/competition). A literature review identified 48 previous *in vivo* studies that used intra-articular injection of an agent to induce inflammation in horse joints (Table 1.2). Studies that specifically used LPS or IL-1β (Tables 1.3-1.4) were further evaluated for experimental design and results.

Table 1.2 Journal articles of in-vivo experimental models for inducing joint inflammation

Stimulatory agent	Number of articles
Lipopolysaccharide (LPS)	18
Human Interleukin-1β (rhIL-1β)	1
Equine Interleukin-1β (reIL-1β)	1
Freund's Adjuvant (CFA)	5
Amphotericin B	8
Surgical induction	5
Other	7

Lipopolysaccharide-induced Arthritis Models

Lipopolysaccharide has been previously validated as an appropriate model for inducing rapid, reversible joint inflammation in the horse, causing localized synovial changes in absence of a systemic response (Palmer and Bertone, 1994). Lipopolysaccharide is a constituent of the outer cell wall of gram-negative bacteria. Endogenous LPS synthesis is part of normal gram negative bacteria growth with neglible amounts of LPS circulating in the body due to turnover of growing/decaying bacteria (Morris, 1991). Presence of bacteria alone in the joint may directly stimulate cartilage degradation (Jasin, 1983), however, the immune response to the presence of bacteria causes a cytokine-mediated inflammatory response that can further activate catabolic activity.

Of the research conducted using an *in vivo* experimentally-induced inflammation model (Table 1.3), the primary objectives were either evaluation of inflammatory response (Todhunter et al., 1996; Smith et al., 1998; Firth et al., 1987; Gottschalk et al., 1998; Hawkins et al., 1993; de Graw et al., 2009; Jacobsen et al., 2006; Berg et al., 2008; Khumsap et al., 2004; Verde et al., 2001) or treatment methods for synovitis (Santos et al., 2009; Meulyzer et al., 2009; Kay et al., 2008; Morton et al., 2005; Campebell et al., 2004; Todhunter et al., 1998; Verde et al., 2001). In these studies, the variables that were measured most frequently were changes in synovial fluid total protein and leukocyte production (De Puy et al., 2007; Palmer and Bertone, 1994; Morris, 1991; Santos et al., 2009; Meulzyer et al., 2009; Kay et al., 2008; Morton et al., 2005; Smith et al., 1998). One third of the studies included information regarding cytokine and eicosanoid production (Santos et al., 2009; Meulyzer et al., 2009; Firth et al., 1987; Gottschalk et al., 1998; Campebell et al., 2004) while only two identified changes in cartilage biomarkers (Smith et al., 1998; deGrauw et al., 2009). Synovial fluid concentrations of PGE_2, cytokine IL-1, total protein

and nucleated cell count consistently increased from baseline values indicating an inflammatory response to LPS.

Table 1.3. In-vivo equine models using LPS for inducing joint inflammation; article summaries

Authors/# of horses	LPS dosage	Action on SF, SM or cartilage	Treatment or OA eval	Control group included
Santos et al., 2009 n=12; mature, free of joint abnormalities	0.5 ng/dl *dosage did not induce systemic response	SF: ↑ RBC, ↑TNC,↑ neutrophils and↓in pH	Treatment eval: morphine and ropivacaine	n=3
Meulyzer et al., 2009 n=4; mature, healthy	0.5 ng or 0.125 ng	SF: WBC and TP ↑ in a dose-dependent manner; WBC peaked at PIH 6; TP at PIH 12	Pilot study to eval SF response to LPS dosage	n=2
Kay et al., 2008 n=18; mature	0.0002 µg/kg BW 3 injections; d0, d5, d10	LPS induced lameness after each injection SF:WBC and TP ↑ with peak concentration at PIH 12 after each injection. SF WBC was highest after 2nd LPS at PIH 12; SF TP was elevated, decreasing after each LPS injection.	Treatment; Eval of trimicinolone acetonide w/ or w/o mepivacaine.	n=6 Contra-lat joint as control
Morton et al., 2005 n=18 mature; healthy	0.5 ng/dl	SF: ↑ WBC at PIH 6, 24; peak at PIH 6 ↑ PGE_2	Treatment; eval of NSAIDs; COX inhibitors	n=6 Contra-lat joint a CONT
Khumsap et al., 2003 n=4; two year olds	5µg (from 10 µg/ml LPS solution)	Gait analysis; altered stride phases Mild increase in rectal temperature *Evaluated tarsal joints*	Gait evaluation	No
Verde et al.,2001 N=6 ; mature, skeletally healthy	0.01 µg/kg (1 µg/1 ml solution)	Joint reaction to LPS injections not reported	Treatment; eval of ketoprofen	n=6 crossover study
Todhunter et al., 1998 *n*=10 ponies (2-3 yrs old)	0.5 ng/dl every 48 hr; 4 injections total	Carpal effusion; SF:WBC and TP ↑ Moderate lameness Changes in PG synthesis in culture *In-vivo & in-vitro model*	Treatment: methylprednisolone acetate (MPA)	n=5 all received LPS injections
Smith et al., 1998 n=6 ; mature, skeletally healthy	0.0125ng/dl	Topically administered DMSO able to penetrate into SF; ↓ inflammation *All horses were euthanized on day 8*	Treatment: DMSO evaluation	Double Blind; crossover study
Todhunter et al., 1996 n= 12 ponies	0.02 µg/kg BW + 0.1 mg/kg of morphine	Immunochemical analysis: ↑ in cytokines IL-1, TNFα & gene receptors in SM and cartilage	Immuno-chemical analysis of processes of synovitis	n=6

Palmer & Bertone, 1994 n=6 ; mature, healthy	Varied: 5000 ng, 25 ng, 0.5 ng, 0.25 ng, 0.17 ng, 0.125 ng	Dosage over 0.5 ng caused clinical signs of endotoxemia; SF:TNC and TP ↑ linearly with doses up to 0.5 ng	Evaluation of exp-induced synovitis for model in acute synovitis	n=6 Did each horse undergo each dosage? Not well explained
Hawkins et al., 1993 n=4; mature, skeletally healthy	3µg	Use of intra-articular catheter to take SF samples PIH 0-12. Increase in rectal temp; mild lameness PIH1-10 Increase in cytokines and eicosanoids.	Use of exp-model to eval clinical signs of OA and changes in SF values over 144 hrs	n=4
Firth et al., 1987 n=5 ponies	o.1µg/kg BW dosage range among ponies: 25-45 µg	Clinical signs (fever, edema, inappetite, depression) occurred soon after injection. Similar to bacterial infection. SF: TP, WBC and Alk P increased w/in 2 hrs, remained elevated over baseline through PIH 36.	Evaluation of septic synovitis using experimental model	No control group
de Grauw et al., 2009 n=6; mature, skeletally healthy	0.5 ng	Sharp ↑ in PGE_2 at PIH 8; substance P, bradykinin and MMP ↑ delayed until PIH 8 & 24. GAG content ↑ and peaked at PIH 24; CPII marker peaked at PIH 24, remained high through PIH 168	Evaluation of processes in SF and cartilage w/ exp-induce synovitis model	No control group
Jacobsen et al., 2005 n=4; mature, skeletally healthy		Systemic/localized response to inflammation; detectable SF SAA isoforms; role in inflammatory arthritis?	Evaluation of role of SF SAA	n=4
Gottschalk et al., 1998 n=12; mature, skeletally healthy	4 treatment groups: LPS, LPS + hyperimmune antiendotoxin, antiendotoxin and saline solution	Changes in SF measured PIH 0,4,8,24 and 72. Anti-LPS stimulated joints in same manner as LPS; repeated arthrocentesis (saline group) caused mild inflammation	Eval of endotoxin on inflam response	n=3 (4 trt groups)
Campebell et al., 2004 n=18 (2-3 yrs of age)	1.5 ng + 0.9% saline	No systemic response or loss of appetite reported. Joint effusion and heat increased SF: ↑ in leukocytes and TP by PIH 3 – PIH 36 compared to baseline values.	Treatment evaluation: Use of lidocaine to measure inflammatory response in LPS model.	n=6 for control group (no treatment); contra-lateral joint also control
Berg et al., 2008 n=33; age n/a, skeletally healthy	5 horses induced with either 1 µg or 3µg of LPS	SF: ↑ in CD-RAP in LPS joint compared to control joint Differences were significant at PIH 12 through PIH 144	Synovitis processes: Evaluation of CD-RAP in the horse	n=5 for control group (no trt); contra-lateral joint also control joint

Lipopolysaccharide has been validated as a reliable method for inducing reversible joint inflammation; however, it remains unclear if the reaction is more related to septic arthritis than non-septic arthritis. As suggested with IL-1β models, future research using an *in vivo* LPS model should include more thorough investigations of cytokine and eicosanoid production in the synovium, in addition to cartilage degradation markers such as aggrecanase and MMPs.

Interleukin-1β-induced Arthritis Models

Most research investigating the involvement of IL-1 in joint disease has used *in vitro* experimental models. For example, cartilage samples that were stimulated in culture with IL-1 showed an increase in PGE_2 and NO synthesis, an increase in GAG released from the cartilage, and a decrease in proteoglycan synthesis (Pearson et al., 2008).

Human IL-1 isoforms have been evaluated in equine synovial cell cultures and human IL-1 stimulated PGE_2 production in a dose dependent manner (May et al., 1992b). However, there was no detectable change in neutral metalloproteinases (specifically stromelysin and collagenase) indicating human IL-1 may be a competitive antagonist of equine IL-1 mediated MMP production (May et al., 1992b). Two forms of interleukin-1 in the horse have been identified as equine IL-1α and IL-1β (May et al., 1990). Both have been cloned and evaluated for stimulatory effect in an *in vitro* model, with equine IL-1β being more potent for stimulating PGE_2 than equine IL-1α (Takafuji et al., 2002). Interleukin-1β has been identified as the more potent cytokine of the two, resulting in a 4 fold increase in PGE_2 production, compared to cultures stimulated with IL-1α (Takafuji et al., 2002; May et al., 1992a). *In vitro* studies used significantly smaller dosages of equine IL-1 [0.1ng/ml] to evoke a similar inflammatory response compared to human IL-1 [4-10 ng/ml] (Takafuji et al., 2002).

To date, only three studies (Table 1.4) have used an IL-1 model for inducing inflammation in the live horse. Intra-articular injection of recombinant human IL-1β (rhIL-1β) (175ng per 500 kg horse) caused significant increases in synovial neutrophils, PGE_2, IL-1 and IL-6, with a reported decrease in cartilage proteoglycan content (Hardy and Bertone, 1998). A dose of 100 ng of rhIL-1β resulted in a measureable increase in synovial PGE_2, glycosaminoglycan (GAG) concentration, total nucleated protein and neutrophil production over a 24 hour period (Pearson et al., 2009). The use of recombinant equine IL-1β resulted in significant increases in synovial total protein and white blood cell count within 24 hours of induction (De Puy et al., 2007). Neither study reported measureable changes in hematological parameters, indicating IL-1β, applied as an exogenous stimulator, acts on local tissue.

The importance of IL-1β in the joint's inflammatory response and subsequent activation of catabolic processes on articular cartilage has been well demonstrated. The lack of research using an equine-specific isoform of IL-1, particularly for in vivo evaluations, justifies the need for future inquiries to focus in this area.

Table 1.4 In-vivo equine models using IL-1β for inducing joint inflammation; article summaries

Reference/# of horses	Product/Dosage	Results	Comments
Hardy and Bertone, 1998 n=6	**Human recombinant IL-1 β** Intra-articular injection: 0.35 mg/kg/BW i.e., 175 ng per 500 kg horse	↑ SF neutrophils and synovium neutrophilic leukocytes ↑in SF substance P, PGE_2, IL-1, IL-6; however, IL-1 was resolved by PIH 5.5 IA injection of rhIL-1β caused no endogenous IL-1 production Cartilage PG ↓ at PIH 24 and 48	4 treatment groups (n=6 each); control, control-denervated; inflamed and inflamed-denervated
Pearson et al., 2009 n=5	**Human recombinant IL-1 β** Intra-articular injection: 10 ng IL-1β on day 0 100 ng of IL-1β on day 1	IL-1β vs. saline caused: ↑ PGE_2 ↑ GAG Initial ↑in NO, though short term ↑ in TP, TCC initially. No sign change in joint circumference b/t IL-1 β & saline injections	Horses supplemented with dietary nutraceutical. 14 days prior to exposure and then throughout experiment through day 29. Authors commented on the need of evaluating PGE_2 more consistently over the 8 hours post-injection
DePuy et al., 2007 n=2	**Recombinant equine IL-1β** (100 ng) Saline (0.9%) 1 ml injected into each carpal joint on Day 0 Day 14: 100 ng of IL-1β injected into one random carpal joint; contralateral carpal injected with 1 ml saline (0.9%) Day 15: horses euthanized	SF values were normal at baseline (data not shown) Total protein (SF) on day 15: 7.3 g/dl Total WBC (SF) on day 15: 106,400/μl Histology: synovium extracted on day 15, presented evidence of acute inflammation (authors did not state what the evidence was)	Pilot study to evaluate use of BoNT-A (botulinum toxin type A) as a treatment for acute synovitis Post mortem collection: SF, synovium and full thickness cartilage; analysis of SF and histology conducted though data not shown.

Rationale for research

Extensive research in multiple species supports the anti-inflammatory properties of dietary n-3 fatty acids. Little information is available, however regarding ability of these fats to alleviate equine synovitis. Our laboratory recently reported the detection of EPA and DHA in the synovial fluid of horses receiving an n-3 fatty acid supplement (Ross et al., 2010), warranting further research into whether these fatty acids can modulate equine synovitis. Investigating the in-vivo (via intra-articular chemo-stimulation) inflammatory response in non-arthritic horses receiving an oral supplementation of n-3 fatty acids would be helpful in evaluating potential alternative treatment methods in horses with osteoarthritis.

Hypotheses and Objectives

We hypothesized that the intra-articular injection of reIL-1β would cause a temporary, reversible episode of synovitis in the horse. We further hypothesize that dietary supplementation of the n3 PUFA's, EPA and DHA will reduce the inflammatory response, in the equine joint under experimentally-induced conditions Three specific study objectives are outlined below to further clarify these hypotheses:

Objective 1: Determine if an oral supplementation of the n3 PUFA's eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) will result in detectable levels of EPA and DHA in the synovial fluid.

Objective 2: Compare two temporary, acute, non-septic, synovitis models; reIL-1β and LPS and validate the use of re-IL-1β as a model for experimentally-induced synovitis, designed to emulate early processes of OA in the horse.

Objective 3: Using a reversible model of synovitis, determine if an oral supplementation of the n3 PUFA's eicosapentaenoic acid (EPA) and docosahexaenoic acid

(DHA) reduces synovial inflammatory biomolecules and gene expression of synovium and articular cartilage-derived degradation markers.

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Chapter II

Evaluation of synovial fluid in horses fed two different sources of n-3 long chain polyunsaturated fatty acids: a pilot study

Overview

Objective: To determine if dietary n-3 long chain polyunsaturated fatty acid (LCPUFA) and prostaglandin E_2 (PGE_2) concentrations are altered in the synovial fluid of mature horses fed one of two dietary n-3 PUFA treatments compared to a non-supplemented, control group.

Methods: Twenty mature mixed breed mares were randomly assigned into one of three dietary treatment groups. Group 1 (MARINE) received 38 g of the long chain n-3 polyunsaturated fatty acids alpha linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) via a marine-derived n-3 (PUFA) supplement daily; Group 2 (FLAX) received 38 gof n-3 ALA via a flaxseed supplement daily and Group 3 (CONT) did not receive additional n-3 PUFA supplementation. On day 90 of supplementation, approximately 3 ml of synovial fluid was extracted from the right carpus of each horse. Fluid was analyzed for fatty acid concentration and PGE_2 concentrations.

Results: Synovial fluid samples from the MARINE group exhibited EPA and DHA in their synovial fluid, whereas the FLAX and CONT groups did not express detectable concentrations. Prostaglandin E_2 concentrations did not differ among horses; however, the MARINE group tended to have lower synovial PGE_2 concentrations compared to CONT horses.

Discussion: The absence of EPA and DHA in the synovial fluid collected from the FLAX-fed group indicates a possible inefficient conversion of these fatty acid metabolites from ALA.

Furthermore, PGE_2 concentrations in the MARINE group tended to be lower compared to CONT group, indicating dietary treatment may reduce inflammatory prostaglandin production. The presence of DHA and EPA in synovial fluid may potentially modify inflammatory processes in the joint.

Introduction

Studies utilizing dietary alpha linolenic acid (ALA) and its metabolic derivatives, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), indicate a potential regulatory role in reducing joint inflammation by mediating cytokine production in arthritic human patients (Cleland et al., 2003) and plasma prostaglandin levels in arthritic horses (Manhart et al., 2009). Elevating these fatty acids in mammalian diets may down regulate inflammatory processes in the joint (Proudman et al., 2008) and has been shown to reduce joint tenderness in patients suffereing from rheumatoid arthritis (Lau et al., 1993). One hypothesized mechanism is the potential of n-3 long chain polyunsaturated fatty acids (LCPUFA) to reduce the production of potent inflammatory eicosanoids (Chapman et al., 2009), primarily prostaglandin E_2 (PGE_2). Little to no information exists regarding oral n-3 supplementation and effects on inflammatory markers common in the equine joint. Therefore, mature horses were fed dietary n-3 PUFA for ninety (90) days to determine if oral supplementation alters synovial fatty acid levels and concentrations of PGE_2.

Methods

Animals and dietary treatments

Twenty mature mixed breed mares with no history of joint disease and free of lameness were blocked by age, weight and body condition score and randomly assigned into one of three

dietary treatment groups. Diets were formulated to provide either a marine-derived source of n-3 PUFA (MARINE); a plant-derived source of n-3 LCPUFA (FLAX) or no additional n-3 LCPUFA (CONT) supplementation. The MARINE (n=7) received 38 g of the n-3 polyunsaturated fatty acids alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) via a commercially available supplement (Magnitude™; JBS United, Sheridan, IN) daily; Group 2 The FLAX (n=6) received 38 g of the n-3 polyunsaturated fatty acids alpha linolenic acid (ALA) via a flaxseed supplement (Nutra-Flax™) daily and the CONT(n=7) received no supplementation. Dietary treatments also contained alfalfa/bromegrass mix hay, commercially-available rolled barley grain (Ranch-Way Feeds, Inc., Fort Collins, CO) (Table 2.1). All diets were formulated to be similar in energy, protein and fatty acid (n6:n3) ratios (Table 2.2). Supplements (MARINE or FLAX) were separately weighed, mixed with respective barley grain ration (1.1 g/kg BW) and fed to each horse individually, once daily, using a canvas nose bag. Any feed offered via the nosebag that was not consumed was weighed and recorded as a refusal. Alfalfa/grass hay was group fed and while individual intake was not measured; estimated consumption was approximately1.6% of the horses' body weight on a dry matter basis. Total diet composition of n-3 LCPUFA were approximately 143.5 mg/kg BW (MARINE), 142.5 mg/kg BW (FLAX) and 78.7 mg/kg BW (CONT), respectively.

Table 2.1 Nutritional analysis and fatty acid content of feedstuffs

	Feed components			
Item	Alfalfa/Grass Hay	Barley	MARINE[1]	Flaxseed[2]
DM, %	91.20	89.98	95.30	93.20
DE, Mcal/kg	2.14	3.65	3.40	4.90
CP, %	20.60	12.65	12.30	21.80
ADF, %	33.40	7.65	9.00	17.10
NDF, %	42.80	19.27	14.90	25.20
WSC, %	7.70	7.05	5.40	3.80
NFC, %	25.40	64.42	45.70	23.50
Fat, %	1.40	2.59	22.50	39.00
Fatty acid, % of fat				
LA	16.98	53.60	5.74	15.69
ALA	35.92	4.25	2.18	56.52
ARA	-	-	-	-
EPA	-	-	7.94	-
DPA	-	-	1.72	-
DHA	-	-	27.64	-

NFC: non-fibrous carbohydrates; LA: linoleic acid; ALA: alpha linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; and DHA: docosahexaenoic acid.[1]Magnitude (JBS United, Sheridan, IN).[2]Nutra Flax (Horsetech, Scottsdale, AZ).

Table 2.2 Estimated daily average dietary intake for treatment groups

	Treatments		
Item	CONT (n=7)	MARINE (n=7)	FLAX (n=6)
DE, Mcal	24.65	24.71	24.64
CP, kg	1.935	1.937	2.075
Starch, kg	0.941	0.891	0.579
Fat, g	256.0	402.9	360.2
Fatty acids, mg/kg BW			
LA	61.91	61.84	66.08
ALA	78.15	80.97	149.78
ARA	-	-	-
EPA	-	13.06	-
DHA	-	45.51	-

CONT: basal control diet; MARINE: algae and fish oil supplement added to basal diet; and FLAX: flaxseed meal added to basal diet. LA: linoleic acid; ALA: alpha linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; and DHA: docosahexaenoic acid.

On day 90 of supplementation, approximately 3 ml of synovial fluid was extracted from a random carpal joint of each horse. Gross observation of synovial fluid was immediately conducted with color and clarity subjectively recorded based on a grading scale (Frisbie et al., 2007); fluid viscosity was graded normal, decreased or markedly decreased (Steel, 2008) and

total protein (g/dl) was determined using a refractometer. Fluid was then placed into a 5 ml tube containing an anti-coagulant (EDTA). Samples were immediately centrifuged at 2500 rpm for 10 minutes and supernatant was aliquoted in 500 ml volumes into individual microcentrifuge tubes. All samples were stored at -20°C until laboratory analysis was conducted.

Statistical analysis

Data were analyzed using SAS © 9.1 (Cary, N.C.) statistical software and procedures for ANOVA. Significant differences ($P \leq 0.05$) were further analyzed using Fisher's protected least squared means (LSD) test. All results are expressed as mean $\pm$ 95% confidence intervals.

Fatty acid determination

Feedstuff and synovial fluid total lipid concentrations were determined using a modified procedure of the Folch Lipid Extraction method (Folch et al., 1957). Synovial fluid was thawed and 100 µl of fluid and 80 µl of an internal standard (C23:1-Tricosenoic Acid; NuCheck, Inc., MN, USA.) was transferred into a 12 ml glass screw cap tube. Each synovial fluid sample was processed in duplicate. Two ml of $CHCI_3$:MeOH was added to each tube and vortexed until solution was thoroughly mixed. Samples were gravity-filtered through a S&S (#1) filter into a new, clean 12 ml glass screw cap tube. Original tube was flushed with 1 ml of $CHCI_3$:MeOH into second tube. Approximately 375 µl of 0.88% potassium chloride (KCl) was added to each sample. Tubes were vortexed for 30 seconds and then centrifuged at 2500 g for 10 minutes. Top layer of solution was aspirated and discarded. Lower layer ($CHCI_3$) was evaporated using a light flow of nitrogen until dried. Samples were reconstituted with 500 µl of hexane, vortexed for 30 seconds and 500 µl of 0.5M of potassium hydroxide (KOH) was added and samples were again vortexed for 30 seconds. Tubes were placed on a heat block (70°C) for 10 minutes, removed and allowed to cool. Once cool, 3 ml of boron trifluoride (BF_3) was added, tubes were vortexed for

30 seconds and placed in a second heat block (100°C) for 90 minutes. Once cooled, 500 µl of hexane and 1 ml of distilled H_2O were added to each tube. Tubes were vortexed and centrifuged at 2500g for 1 minute to allow for phase separation. Upon centrifugation, there were obvious layers; a non-polar (upper layer) and a polar (lower layer). The non-polar layer was pipetted into individual gas chromatography vials and evaporated to dryness using a light nitrogen stream. Samples were reconstituted with 100 µl of hexane and placed on gas chromatography machine for analysis. Hexane extracts of fatty acid methyl esters (FAME) were separated by gas chromatography on a 30 m×0.25 mm×0.2 µm film thickness DB-225 column Agilent (Palo Alto, CA) using an Agilent model 6890 GC equipped with autosampler, Chemstation and flame ionization detection. Individual fatty acids detected in synovial fluid are presented as a percentage of total fatty acids (% total FA).

Synovial Prostaglandin E_2

Synovial PGE_2 levels were determined using a two-step process. Synovial fluid samples were first subjected to solid-phase extraction procedures. Synovial samples (500µl) were added to 500 µl of 80% ethanol and 10µl of glacial acetic acid, vortexed, and incubated at room temperature for 5 minutes. The samples were spun at approximately 3100g using a Spectrafuge microcentrifuge (National Lab Net Co., Edison, NJ) for 8 minutes. The sample supernatant was transferred to C2 ethyl minicolums (SampliQ™; Agilent Technologies, Wilmington, DE) that had been washed twice in 1 ml of 10% ethanol under vacuum. Each column (containing synovial samples) was further washed (under vacuum) once with 1 ml of deionized water and 1 ml of hexane. PGE_2 was eluted into collection tubes using two 750 µl washes of ethyl acetate under vacuum. Prior to analysis, ethyl acetate was evaporated from the samples using a multiple-port gas evaporation system (Savant Speed Vac Plus, Thermo Savant, Holbrook, NY). The samples

were evaluated in triplicate using a commercially available PGE_2 ELISA kit (Assay Designs; Ann Arbor, MI) in accordance with manufacturer's instructions. Prostaglandin E_2 concentrations are presented in picograms per milliliter (pg/ml).

Results

Feed intake data and plasma, red blood cell and muscle cell fatty acid results have been previously reported (Hess et al., 2012). Synovial fluid was easily collected from each horse and arthrocentesis procedures did not result in lameness. Synovial fluid color, clarity, viscosity and total protein levels were all within normal parameters and were not significantly different among dietary groups (results not shown).

Synovial fatty acid concentrations

Synovial fluid fatty acid concentrations of alpha linolenic acid (ALA), arachidonic acid (ARA) and docosapentanoic acid (DPA) did not differ among groups (Table 2.3). Synovial fluid linoleic acid (LA) % were lower in synovial fluid obtain from CONT horses at day 90 compared to both MARINE and FLAX synovial fluid. Samples at day 90 of dietary supplementation collected from the MARINE group presented EPA and DHA in their synovial fluid, whereas the FLAX and CONT groups did not express detectable concentrations.

Table 2.3 Fatty acid composition (% of total fatty acids) of synovial fluids on day 90

[2]Fatty acid	[1]Treatment (n = 6-7 horses)			*P*-value
	CONT	MARINE	FLAX	TRT
LA				
Mean	9.61 +/-2.73[a]	14.91 +/-2.35[b]	16.14 +/-2.54[b]	0.004
ALA				
Mean	0.64 +/-0.47[a]	0.99 +/-0.4[a]	1.36 +/-0.41[a]	0.080
ARA				
Mean	2.14 +/-0.58[a]	2.01 +/- 0.48[a]	1.91 +/-0.52[a]	0.821
EPA				
Mean	0.18 +/-0.05[a]	1.32 +/- 0.25[b]	ND[a]	0.027
DPA				
Mean	1.46 +/-0.55[a]	1.16 +/- 0.55[a]	0.87 +/-0.56[a]	0.439
DHA				
Mean	0.22 +/-0.22[a]	1.47 +/-0.53[b]	0.39 +/-0.56[a]	0.008

Values expressed as % of total fatty acids ± 95% confidence interval. Values within a row lacking a common superscript differ, $P \leq 0.05$. ND: Not detectable. [1]CONT: control diet; MARINE: algae and fish oil supplement added to basal diet; FLAX: flaxseed meal added to basal diet; TRT: treatment. [2]LA: linoleic acid; ALA: alpha linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid and DHA: docosahexaenoic acid.

Synovial Prostaglandin E_2 concentrations

Synovial PGE_2 levels at day 90 (Figure 2.1) were within concentration ranges reported previously for values obtained from healthy equine joints (Kirker-Head et al, 2000). There was a trend (P=0.07) for PGE_2 concentrations to be lower in MARINE group (21.26 +/- 4.01 pg/ml) compared to CONT group (32.23 +/- 4.01 pg/ml). A ninety (90) day supplementation of the n-3 fatty acid ALA resulted in no difference in PGE_2 concentrations (28.20 +/- 4.9 pg/ml) when compared to MARINE (P=0.30) or CONT (P=0.54) horses.

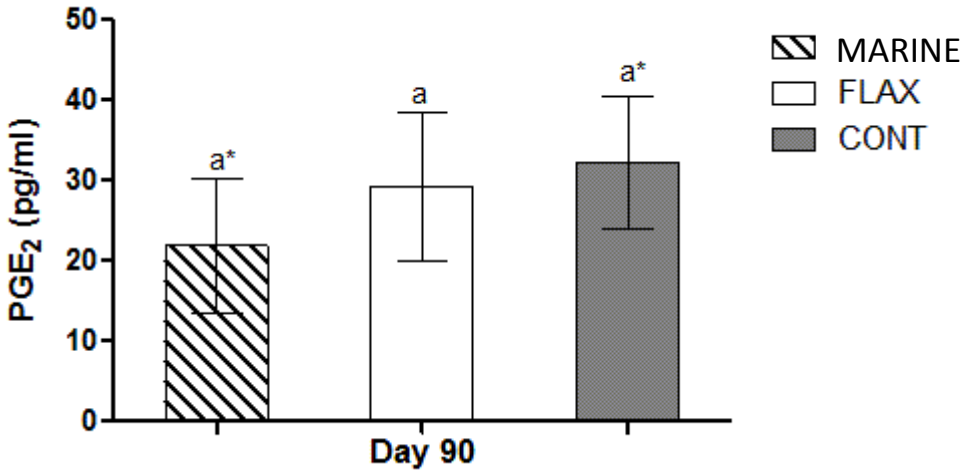

Figure 2.1 Effect of dietary treatment on synovial fluid prostaglandin E_2 concentrations. Columns lacking common letters differ $P \leq 0.05$. *Indicates trend for difference between MARINE and CONT (P=0.07) Data are displayed as means ± 95% confidence intervals. (CONT=control diet; MARINE=algae/fish oil diet; FLAX=flaxseed diet)

Discussion

To the best of our knowledge, this is the first report of synovial fluid fatty acid evaluation in the horse after dietary fatty acid supplementation. A 90 day supplementation of a protected EPA/DHA supplement resulted in significant changes in fatty acid composition. Synovial fluid fatty acid levels for EPA and DHA were significantly higher in the MARINE group compared to either CONT or FLAX groups, indicating that direct supplementation of EPA and DHA are required if higher fluid concentrations of those fatty acids are desired. While both EPA and DHA represented a minor percentage of the total lipid composition within synovial fluid, their mere presence as a result of dietary treatment reflects a potential for biological activity within the joint.

Though levels did not differ between treatments, the MARINE group tended to have the lowest levels of synovial PGE_2 in the current study, which may be due to having higher

concentrations of EPA and DHA in the fluid. Prostaglandin E_2 concentrations have been reported to be higher in joints of horses with existing osteoarthritis compared to levels in normal, healthy joints (Kirker-Head et al., 2000). Additionally, synovial fluid PGE_2 levels have been positively correlated to degree of lameness and joint effusion in dogs (Trumble et al., 2004), indicating usefulness as an indicator of joint inflammation and as a therapeutic target. Osteoarthritic horses receiving oral n-3 fatty acid supplementation for 90 days had lower plasma PGE_2 and synovial white blood cell concentrations when compared to arthritic horses receiving no n-3 fatty acid supplementation (Manhart et al., 2009), demonstrating a potential benefit of oral n-3 fatty acids for reducing severity of pain and inflammation. The lack of significant difference in synovial fluid PGE_2 concentrations in the current study may be due to all horses being healthy and free of existing joint inflammation or disease. Inducing experimental inflammation in healthy animals receiving a dietary n-3 LCPUFA supplement may cause measurable differences in eicosanoid levels.

Data presented here indicate a possible inhibition of inflammatory eicosanoid prostaglandin E_2 production in equine synovial fluid by oral supplementation of the long chain polyunsaturated fatty acids EPA and DHA. Further research is needed to determine if oral n-3 LCPUFA supplementation can be therapeutically advantageous in horses experiencing joint inflammation.

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Chapter III[1]

Evaluation of the inflammatory response in experimentally-induced synovitis in the horse: A comparison of recombinant equine interleukin 1 beta and lipopolysaccharide

Overview

Objective: To compare two transient models of synovitis in horses by characterizing biological changes in synovial fluid and joint tissue.

Methods: Twelve skeletally-mature mares were utilized in a block design. Synovitis was induced by an intra-articular injection of 100 ng recombinant equine interleukin 1 beta (reIL-1β) or 0.5 ng lipopolysaccharide (LPS) into a middle carpal joint in 1 ml volumes. One ml of saline was injection into the contra-lateral control joint. Lameness evaluations were conducted through post injection hour (PIH) 8, and at PIH 240. Arthrocentesis collection of synovial fluid occurred between PIH 0 and 48. An arthroscopic examination at PIH 8 included synovium and articular cartilage biopsies for gene expression analysis.

Results: Synovial fluid analysis indicated that single injections of reIL-1β or LPS increased synovial white blood cell, neutrophil count, total protein, prostaglandin E_2 concentrations and general matrix metalloproteinase activity relative to control joints through PIH 8. Injections of either reIL-1β or LPS increased mRNA expression for MMP-1 and ADAMTS-4 in synovium and for MMP-1, ADAMTS-4, and ADAMTS-5 in articular cartilage collected at PIH 8 compared to saline injections.

[1] Portions of this chapter have been accepted for publication (Ross et al., 2012).

Conclusions: Injections of reIL-1β into equine carpal joints resulted in a transient inflammatory response that was similar in severity to the LPS injection, causing increased expression of certain deleterious mediators in joint tissues at 8 hours. Given that IL-1β is a known critical mediator of traumatic arthritis and osteoarthritis (OA), this humane and temporary model may be useful in evaluating therapeutics that act against early stages of joint disease.

Introduction

Synovitis is a key factor in the pathophysiology of osteoarthritis (OA) in human (Sellam et al., 2010) and horses (McIlwraith, 2005). The development of OA associated with synovitis is likely due to persistent up-regulation of mediators that contribute to articular cartilage degradation including metalloproteinases (MMPs), a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) and prostaglandin E_2 (PGE_2) (McIlwraith, 2005). The cytokine interleukin-1β (IL-1β), which is prevalent in equine (McIlwraith, 2005; Frisbie et al., 2002) and human (Fernandes et al., 2002; Malcolm et al., 1997; Blom et al., 2007) OA, stimulates production of MMPs and PGE_2 (Fernandes et al., 2002). Interleukin-1β's importance in the OA cascade has been demonstrated by its inhibition significantly reducing OA in well-established equine osteochondral fragment (Frisbie et al., 2002) and canine anterior cruciate ligament (Pelletier et al., 1997) models.

There are several similarities between horses and humans when considering OA. Both experience naturally-occurring OA, due primarily to traumatic injury. The ability of inflammation to produce early signs of OA was demonstrated in horses over 30 years ago (McIlwraith et al., 1979). Given the importance of synovitis in the pathogenesis of OA, equine experimental models for inducing joint inflammation, including amphotericin, carrageenan, polyvinyl alcohol foam particles and blood, have been evaluated. The endotoxin

lipopolysaccharide (LPS) from E-coli bacteria is known to induce acute synovitis resembling septic arthritis, and has been used as a model (Todhunter et al., 1996; Firth et al., 1987; Palmer et al., 1994; Hawkins et al., 1993; deGrauw et al., 2009) to evaluate treatments for acute synovitis (Santos et al., 2009; Kay et al., 2008; Morton et al., 2005; Smith et al., 1998; Gottschalk et al., 1998; Campebell et al., 2004; Verde et al., 2001). The role of IL-1β in the natural inflammatory cascade makes it a more appropriate model for evaluating the pathobiology of OA; however, IL-1β use in *in vivo* experimental modeling has been minimal. Two studies have induced inflammation using intra-articular injections of recombinant human IL-1β (rhIL-1β) (Hardy et al., 1998; Pearson et al., 2009) reporting increased synovial white blood cells and total protein levels.. Also, gene expression changes in rat joints following intra-articular injection of rat IL-1β have been reported (Scott et al., 2009). While these studies suggest intra-articular injection of IL-1β is suitable for evaluating OA pathobiology, a comprehensive profile of changes in both synovial fluid and articular tissue has not been done. Additionally, the effect of recombinant equine IL-1β (reIL-1β) has not been evaluated *in vivo* despite *in vitro* studies demonstrating a potent degradative response in articular cartilage explants at concentrations as low as 0.1ng/ml (Takafuji et al., 2002). Therefore, we hypothesized that a single injection of reIL-1β causes, 1) inflammatory changes in equine synovial fluid that are similar to LPS, and 2) gene expression changes in joint tissue that closely mimic early clinical OA.

Methods

Experimental design and induction of temporary carpal synovitis

Twelve healthy mares with clinically normal carpal joints, determined by radiographic evaluation, were blocked by age (mean=9.8 years old), weight (mean=540 kg) and body condition score (Henneke et al., 1983) (mean=7), and randomly assigned into LPS (n=6) or reIL-

1β (n=6) treatment groups. With the exception of TNR, all investigators and staff were blinded to the study. All study horses underwent routine health examinations, a diagnostic lameness exam and radiographic evaluation to eliminate pre-existing health or skeletal abnormalities. Research protocols were approved by the Colorado State University's Institutional Animal Care and Use Committee.

At post-injection hour (PIH) 0, localized inflammation was induced in the dorsal side of a random intercarpal joint of each horse using sterile arthrocentesis procedures. Horses assigned to LPS group received an intra-articular injection of 1 ml of 0.5 ng/ml lipopolysaccharide (LPS from Escherichia coli 055:B5; catalog number L5418; Sigma-Aldrich) diluted in sterile phosphate buffered saline (PBS). Horses assigned to IL-1β group received 1 ml of 100 ng/ml recombinant equine IL-1β (catalog number 3340-EL; R& D Systems) diluted in sterile PBS. In addition, each horse received a 1 ml injection of sterile PBS into the contra-lateral control joint. Immediately prior to aseptic administration of either LPS, reIL-1β or PBS solutions, baseline (PIH 0) synovial fluid samples were taken.

Evaluation of clinical response to treatment

Lameness evaluations and joint circumference measurements were conducted at PIH 0, 4 and 8 and 240. A subjective lameness evaluation was conducted by a veterinarian with scoring based on the American Association of Equine Practitioners grading scale: Grade 0-sound, no lameness; Grade 1-normal at the walk with mild lameness at a trot; Grade 2 –mild lameness at a walk with consistent lameness at a trot; Grade 3-consistent lameness at a walk with pronounced lameness at a trot; Grade 4-severe lameness at a walk and trot; Grade 5-non-weight bearing lameness (Anon, 1991). Joint circumference measurements were recorded at PIH 0, 4, 8, 24 and 48 and conducted by same individual (TNR). Joint circumference measures were recorded as a

subjective measurement taken at the plane of the accessory carpal bone using a fabric measuring tape and expressed in centimeters (cm).

Horses were monitored throughout the experimental period for signs of distress or anxiety. Temperature, pulse and respiration were measured and recorded daily for one week prior to beginning experimental period to accustom horses to procedures and establish a baseline reference. At initial arthrocentesis (PIH 0), every 2 hours through PIH 12 and then every 6 hours through PIH 48, horses were evaluated for signs of discomfort, temperature, respiration and pulse.

Blood and synovial fluid collection

Blood was collected via jugular venipuncture at PIH 0, 4, 8, 24, and 48 just prior to sedation for arthrocentesis. A volume of 5 ml of blood was placed into EDTA tubes with remaining volume (10 ml) distributed in plain tubes. Synovial fluid was aseptically aspirated at hour 0 and PIH 4, 8, 24 and 48. Gross observation of synovial fluid was conducted at time of collection with color and clarity subjectively recorded based on a grading scale (Frisbie et al., 2007). Viscosity of synovial fluid was graded normal, decreased or markedly decreased (Steel, 2008). Of the volume of synovial fluid was collected, 0.5 ml was placed in EDTA tubes and immediately processed for routine cytology (white blood cell count, cell differential and total protein); the remaining volume was transferred to plain tubes for processing for later analysis. Plasma, serum and synovial fluid supernatant were collected upon centrifugation of fluid and aliquoted into 1.5 ml eppendorf tubes and stored at -80°C until analyzed.

Synovial fluid analysis

Synovial fluid WBC and total protein concentrations were determined using a Coulter T6-60 automated cell counter and refractometry, respectively. Differential WBC values were evaluated using a cytospin and direct smear analysis.

Assays were performed on each synovial fluid sample to determine prostaglandin E_2, general matrix metalloproteinase activity and glycoaminoglycan concentrations. All samples were analyzed in triplicate. Synovial prostaglandin E_2 levels were determined using a two-step process. Synovial fluid samples were first subjected to solid-phase extraction procedures. Synovial samples (500µl) were added to 500 µl of 80% ethanol and 10µl of glacial acetic acid, vortexed and incubated at room temperature for 5 minutes. The samples were spun at approximately 3100g (using a Spectrafuge microcentrifuge; National Lab Net Co., Edison, NJ) for 8 minutes. Sample supernatant was transferred to C2 ethyl minicolums (SampliQ™; Agilent Technologies, Wilmington, DE) that had been washed twice in 1 ml of 10% ethanol under vacuum. Each column (containing synovial samples) were further washed (under vacuum) once with 1 ml of deionized water and 1 ml of hexane. A twice washing of columns with 750 µl of ethyl acetate (under vacuum) eluted PGE_2 into collection tubes. Samples were storage at -20°C until assayed. Prior to analysis, ethyl acetate was evaporated from samples using a multiple-port gas evaporation system (Savant Speed Vac Plus, Thermo Savant, Holbrook, NY). Samples were evaluated using a commercially available PGE_2 ELISA kit (Assay Designs; Ann Arbor, MI) in accordance with manufacturer's instructions. Prostaglandin E_2 levels were expressed in pg/ml.

Total matrix metalloproteinase (MMP) activity was determined using fluorimetric assay methods based on cleavage of substrate FS-6 (Calbiochem, San Diego, CA) , a technique that has been validated for use with equine synovial fluid (de Grauw et al., 2009). A negative control to

inhibit MMP activity was created used a 10mM ethylenediaminetetraacetic acid (EDTA) solution. An EDTA-free protease inhibitor (cOmplete™, Roche Diagnostics GmbH; Mannheim, Germany) was used as a positive control to eliminate non-MMP enzymatic activity. Samples, blanks and controls were aliquoted into black 96-well flat bottom microplates (Corning #3915) and fluorescence was read at excitation$_{max}$ 324 and emission$_{max}$ 400 every five minutes for a total of 20 minutes. General MMP activity was determined by the rate of substrate turnover (linear slope of increase in fluorescence with time) and expressed as a rate of FS-6 conversion per second (RFU/s).

Synovial proteoglycan content was determined by measuring glycoaminoglycan (GAG) concentrations using a 1,9-dimethylmethylene blue (DMMB) dye-binding assay. Synovial samples (100 µl) were added to 100 µl of digestion solution (0.4 M sodium acetate, 10mM disodium EDTA, 2.42 mg cysteine/ml, 20 µl papain/ml, 1 ml deionized water [pH 5.8]) and incubated for 18 hours at 60°C. Immediately after incubation, the digestion was stopped by adding 4 µl of 4mM iodoacetamide. Digested samples were further diluted (1:10) with deionized water, and then pipetted (15µl) into clear, flat-bottom 96-well microplates. A stock solution of shark chondroitin sulfate C (0-100 µg/ml) was used to establish standard values. 185 µl of dimethylmethylene blue (DMMB) solution (40.55mM NaCl, 40.50 mM glycine, 16 µg/ml DMMB) was added to each well and the concentration of GAG was determined spectrophotometrically using a plate reader set at 540 nm. Results were expressed in units of µg/ml.

Arthroscopic biopsy procedure

At post-injection hour 8, all horses underwent surgical arthroscopy to obtain synovium and articular cartilage samples. In short, a 3mm incision was created medial to the extensor carpi radialis tendon at the plane of the third carpal bone in the middle carpal joint. A small closed, concave curette was used to collect cartilage samples (6 mg wet weight) and a Ferris-Smith rongeur instrument was used to extract synovial membrane (6 mg wet weight) tissue. Tissue samples were placed in a 1.5 ml collection tube containing Trizol, immediately flash frozen in liquid nitrogen and then stored at -80°C until analyzed.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of joint tissues

Cartilage samples were thawed, added to TRIzol reagent, and then pulverized. Synovial membrane samples were homogenized on ice for 30 seconds and mixed with TRIzol. To isolate RNA, a chloroform extraction procedure, previously used for joint tissue (Kisiday et al., 2010), was performed. Nucleic acid concentrations were determined using the ND1000 spectrophotometer at 260/280nm. Samples with 260/280 ratios less than 1.7 were further purified using the RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to manufacturer's instructions. RNA was reverse transcribed to cDNA using Superscript III cDNA synthesis kit (Invitrogen, Carlsbad, CA). . Primer/probes were mixed with TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) and cDNA, and semi-quantitative real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Primer/probes for ADAMTS-4, ADAMTS-5, MMP-1, MMP-13, IL-1β, TNF-α and GAPDH (Table 3.1) were designed and validated at the authors' institution. Primer/probes for COX-2 were purchased from the University of California-Davis's Lucy Whitter Molecular Core Facility (Davis, CA). Samples were analyzed in duplicate with expression normalized to the

housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression of each gene was calibrated to the average expression in the LPS control joints and reported as fold change relative to LPS controls.

Table 3.1 Primers and probes used for semi-quantitative real-time polymerase chain reaction.

Target Gene	PCR primers	PCR probe
GAPDH	Forward-AAGTGGATATTGTCGCCATCAAT Reverse-AACTTGCCATGGGTGGAATC	TGACCTCAACTACATGGTCTACATGTTTCA
ADAMTS4	Forward-TGTGATCGTGTCATTGGCTCC Reverse-TGTTTGCTGCAGCTAGAACCATC	AGTTTGACAAGTGCATTGGTGTGCGGT
ADAMTS5	Forward-AAGGTGACTGATGGGACCGAATGT Reverse-TTTGAGCCAATGATGCCGTTCACAG	AGGCCATACAGTAATTCCGTCTGCGT
MMP-1	Forward-ACTGCCAAATGGACTTCAAGCTGC Reverse-TCTTCACAGTGCTAGGAAAGCCG	CAGGATGTGCTCTACGGATACCCCAAGGAC
MMP-13	Forward-TGATGAAACTTGGACAAGCAGTTCC Reverse-CCTTGGAGTGGTCGAGACCTAAG	AGGCTACAACTTGTTTCTTGTCGCTGCACAC
TNF-α	Forward-AGTGACAAGCCTGTAGCCCATGTT Reverse-ATGAGGTACAGCCCATCCAATGGT	AATGGCGTGAAGCTGACAGACAACCA
IL-1β	Forward-AGTCTTCAGTGCTCAGGTTTCTGA Reverse-TGCCGCTGCAGTAAGTCATC	CAGCCATGGCAGCAGTACCCGA

Statistical analysis

Sample size was determined using SAS 9.2© statistical software (SAS Institute, Inc., Carey, NC) and data from previous research that measured synovial fluid total protein from horses that received LPS (Firth et al., 1987) , IL-1β or saline (Pearson et al., 2009). A sample size of 6 per treatment group provided a statistical power of 0.90 (α=0.05) as calculated using one-way ANOVA. White blood cell, neutrophil cell count, and PGE_2 data were log transformed to standardize variance. All clinical and synovial fluid data were compared using analysis of variance for repeated measures. Between-subject effects were treatment and joint with time being the repeated measures effect. Significance level was set at $P \leq 0.05$; differences were further analyzed using Fisher's least squares means test. The data are presented as mean $\pm$ 95% confidence interval.

Results

Clinical response to intra-articular injection

All horses were free of lameness (Figure 3.1) prior to injection. By PIH 4 lameness was higher in all treated limbs (reIL-1β: 3.7 ± 0.39; LPS: 3.8 ± 0.39) compared to baseline measurements (P<0.001), which continued through PIH 8. Injections of PBS in the contra-lateral control joints did not result in lameness. Lameness evaluations were not performed at PIH 24 and 48 due to surgery, although all horses were bearing weight on limbs and walking comfortably. All horses were clinically sound 240 hours post injection.

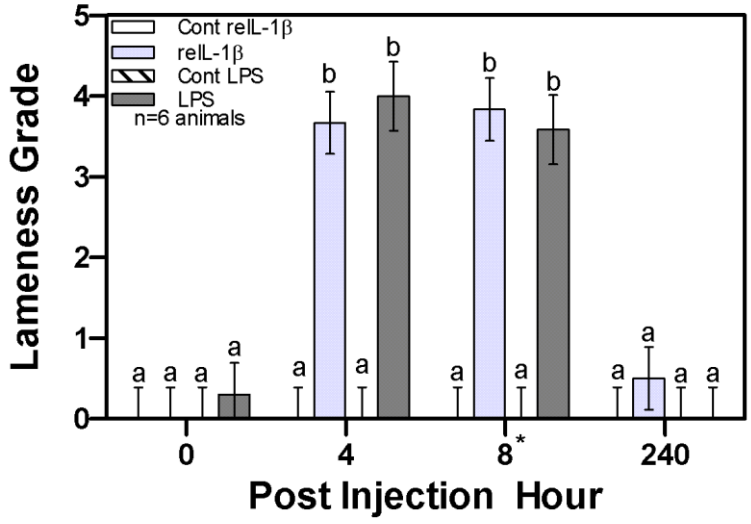

Figure 3.1 Clinical response to intra-articular injection of reIL-1β (100ng), LPS (0.5 ng) or PBS on lameness grade. Columns lacking common letters indicate significant differences across both treatments (reIL-1b, LPS) and time points (P<0.0001) *Indicates time point when arthroscopic biopsy was performed. Data are displayed as means ± 95% confidence intervals.

Temperature and respiration did not significantly increase from baseline (PIH 0) through PIH 8 (Fig 3.2). A significant time effect in temperature and respiration were observed beginning at PIH 12 and PIH 16. Overall, respiration rate was higher in reIL-1β treated horses (19. 9 ± 1.3

bpm) compared to LPS treated horses (15.5 ± 1.3 bpm) (P=0.0339). The heart rate for the majority of the study was within normal range as described by the AAEP (30-42 bpm). A modest increase in heart rate occurred at PIH 4 in all horses (47.1 ± 2.8 bpm). Heart rates remained elevated above baseline values from PIH 8 thorough PIH16, and then returned to pre-treatment levels by PIH 24. Average heart rates throughout the experimental period (PIH 0-PIH 48) were higher in LPS-treated horses (44.1 ± 1.2 bpm) than in reIL-1β treated horses (40.2 ±1.2 bpm) (P=0.045). The attitudes and appetites of all horses were normal throughout the experimental period.

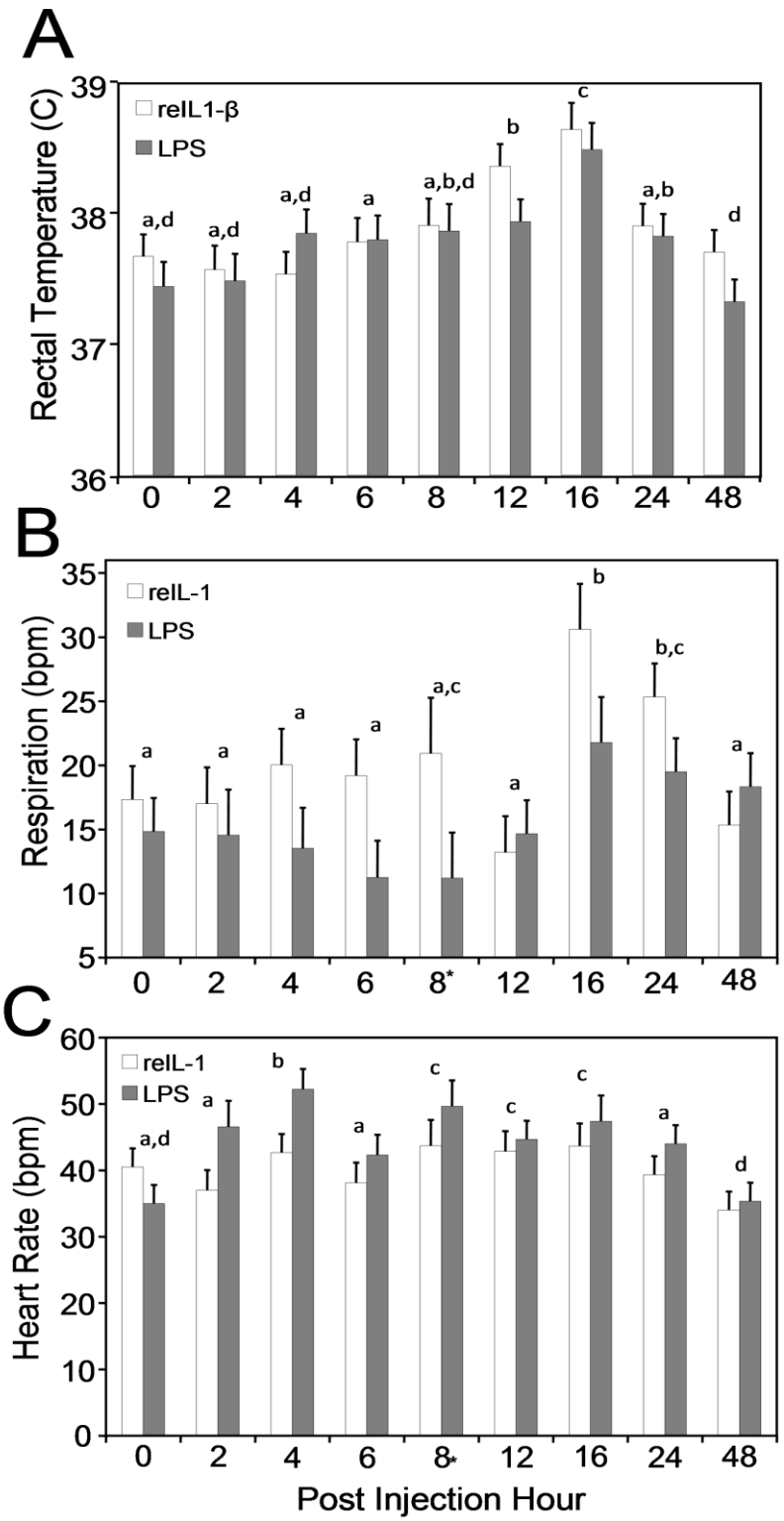

Figure 3.2 Clinical responses to intra-articular injection. Columns lacking common letters indicate significant differences across both time points (P<0.05). *Indicates time point when arthroscopic biopsy was performed. Data are displayed as means ± confidence intervals

Synovial fluid analysis

The volume of aspirated synovial fluid was significantly higher ($P<0.0001$) in treated joints (4.49 ± 0.46 ml) than in control joints (2.87 ± 0.46 ml). Baseline measurement of synovial fluid WBCs (Figure 3.3A) were similar among all horses and within normal ranges (McIlwraith, 2002). Neutrophil values (Figure 3.3B) at baseline differed amongst horses; therefore, data were adjusted with baseline values as covariates. At baseline, two horses had significantly higher neutrophil values in treated joints compared to the remaining treated joints; however, baseline values were less than 1% of values obtained following treatment at PIH 4. In control joints, WBC concentrations were significantly different between reIL-1β (12,800 ± 21,447 cells/µl) and LPS joints (1000 ± 21447 cells/µl) at PIH 8 ($P=0.001$); Injections of reIL-1β or LPS resulted in a significant increase in WBC count at PIH 4 (reIL-1β: 134,300 ± 19,581 cells/µl; LPS:143,283 ± 19,582 cells/µl) and 8 (reIL-1β: 170,700 ± 37,581 cells/µl; LPS: 234,833 ± 19,582 cells/µl), respectively when compared to PBS control joints ($P<0.001$) at similar time points (Figure 3.3A). At PIH 24 and 48, WBC concentrations in treated joints were not significantly different between reIL-1β and LPS. A rise in neutrophils occurred in all joints above baseline values, with treated joints having significantly higher values compared to control joints ($P<0.01$) (Figure 3.3B). Total protein (Figure 3.3C), within normal levels (Todhunter et al., 1996) prior to IA injections, was elevated significantly over baseline values at PIH 4 (reIL-1β: 5.11 ± 0611 g/dl; LPS: 5.45 ± 0.61 g/dl), PIH 8 (reIL-1β: 6.1 ± 0.61 g/dl; LPS: 6.32 ± 0.61 g/dl), PIH 24 (reIL-1β: 5.96 ± 0.61 g/dl; LPS: 6.38 ± 0.61 g/dl)and PIH 48 (reIL-1β: 4.96 ± 0.61 g/dl; LPS: 5.08 ± 0.61 g/dl) in all treated joints ($P<0.05$). Synovial fluid total protein levels in control joints, regardless of treatment, increased over time with concentrations peaking at PIH 24 (reIL-1β: 5.28 ± 0.61 g/dl; LPS: 5.66 ± 0.61 g/dl).

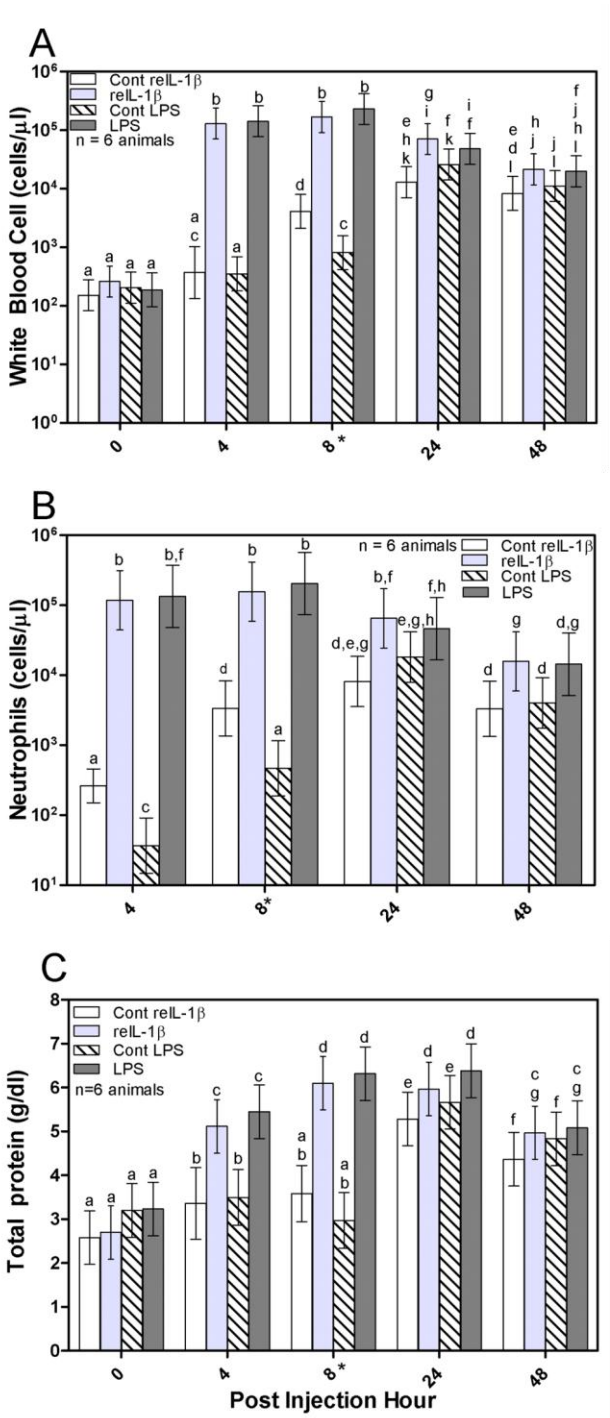

Figure 3.3 Cytology evaluation of synovial response to intra-articular injection of reIL-1β (100ng), LPS (0.5 ng) or PBS. For each outcome, columns lacking common letters indicate significant differences across treatments (reIL-1b, LPS), joints (treated, control), and time points. (A) White blood cells (P<0.01). (B) Neutrophils (P<0.01). Neutrophil values at baseline differed among groups; therefore data were adjusted with PIH 0 values as covariates. (C) Total protein (P<0.0001). *Indicates time point when arthroscopic biopsy was performed. Data are displayed as means ± 95% confidence intervals.

Synovial fluid biomarkers

Synovial PGE_2 concentrations (Figure 3.4A) did not differ among horses at PIH 0. Injection of reIL-1β or LPS increased PGE_2 concentrations (reIL-1β: 1,952 ± 357 pg/ml; LPS: 2,244 ± 450 pg/ml) over control joints (reIL-1β: 37 ± 357 pg/ml; LPS: 33 ± 330 pg/ml) by PIH 4 ($P<0.01$). PGE_2 levels remained elevated through PIH 48 in all treated joints. Joints treated with reIL-1β had significantly higher PGE_2 levels than LPS-treated joints at PIH 24 ($P=0.011$) (reIL-1β: 748 ± 329 pg/ml; LPS: 316 ± 329 pg/ml) and PIH 48 (reIL-1β: 220 ± 329 pg/ml; LPS: 73 ± 329 pg/ml), respectively ($P=0.001$).

Synovial MMP activity (Figure 3.4B) was similar among all horses at PIH 0. Repeated fluid aspiration did not significantly change MMP activity in control joints. Synovial fluid from both treated groups demonstrated significantly higher MMP activity (reIL-β: 0.37 ± 0.13 Δ RFU/s; LPS: 0.44 ± 0.13 Δ RFU/s) compared to control joints at PIH 4 (reIL-β: 0.06 ± 0.14 Δ RFU/s; LPS: 0.06 ± 0.13 Δ RFU/s) ($P<0.0001$). Levels were also significantly higher in treated joints (reIL-β: 0.61 ±0.13 Δ RFU/s; LPS: 0.53 ± 0.13 Δ RFU/s) compared to control joints (reIL-β: 0.07 ± 0.13 Δ RFU/s; LPS: 0.11 ± 0.13 Δ RFU/s) at 8 hours post-injection ($P<0.0001$). There were no significant differences between reIL-1β and LPS groups at any time point.

Synovial fluid GAG levels (Figure 3.4C) were similar in all joints prior to injections, and remained similar through PIH 8. However, GAG levels, regardless of joint or treatment, increased at PIH 24, and remained elevated through PIH 48 ($P<0.001$).

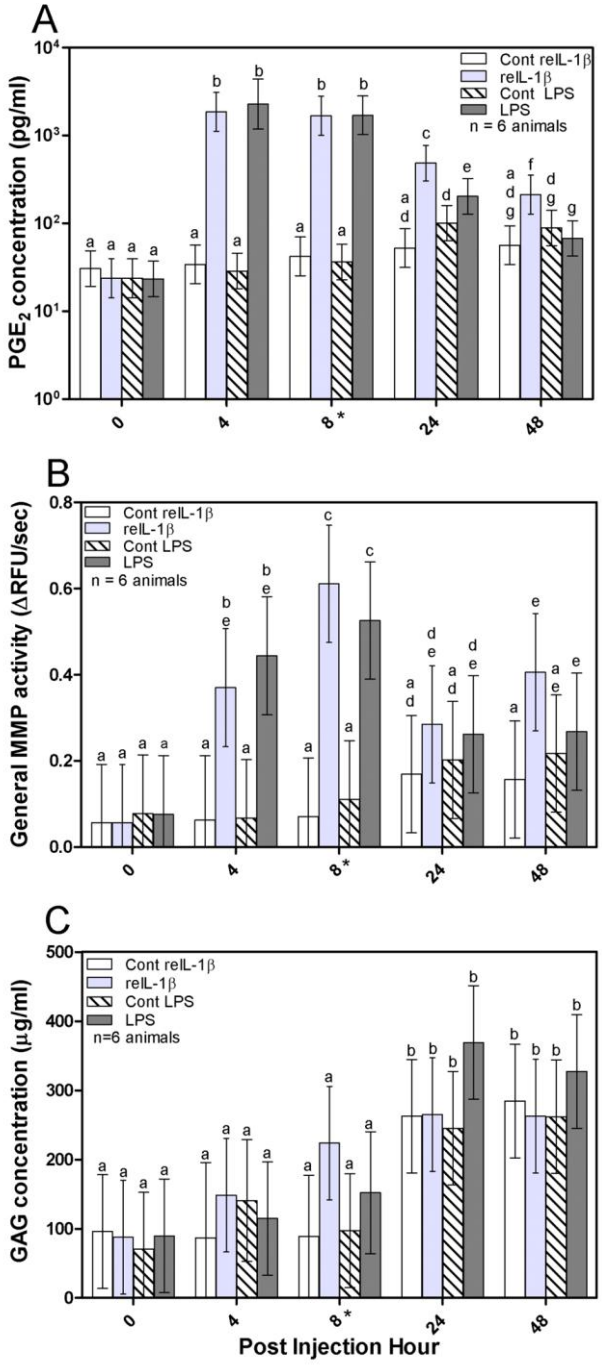

Figure 3.4 Synovial fluid response to intra-articular injection of reIL-1β (100ng), LPS (0.5 ng) or PBS. (A) PGE_2 concentration: columns lacking common letters indicate significant differences across treatments (reIL-1b, LPS), joints (treated, control), and time points. (P<0.01). (B) General MMP activity: columns lacking common letters indicate significant differences across joints (treated, control), and time points (P<0.0001). (C). Glycoaminoglycan concentration: columns lacking common letters indicate significant differences across time points (P<0.0001); significant difference over time across joints (P=0.051). *Indicates time point when arthroscopic biopsy was performed. Data are displayed as means ± 95% confidence intervals.

Gene expression in joint tissues at post injection hour 8 (Synovium)

Synovial tissue gene expression of MMP-13, ADAMTS-5, TNF-α, and COX-2 did not differ significantly among treated and control joints. There was a significant difference between LPS and reIL-1β tissues compared to controls for MMP-1 (59 fold increase) (P=0.011) and ADAMTS-4 (13 fold increase) (P<0.0001) (Figures 6A & 6B). Expression of synovium IL-1β mRNA (Figure 3.5C) in treated joints was 5.5-fold higher than control tissues (P=0.003) with LPS treated joints having a 2-fold higher expression than reIL-1β (P=0.038).

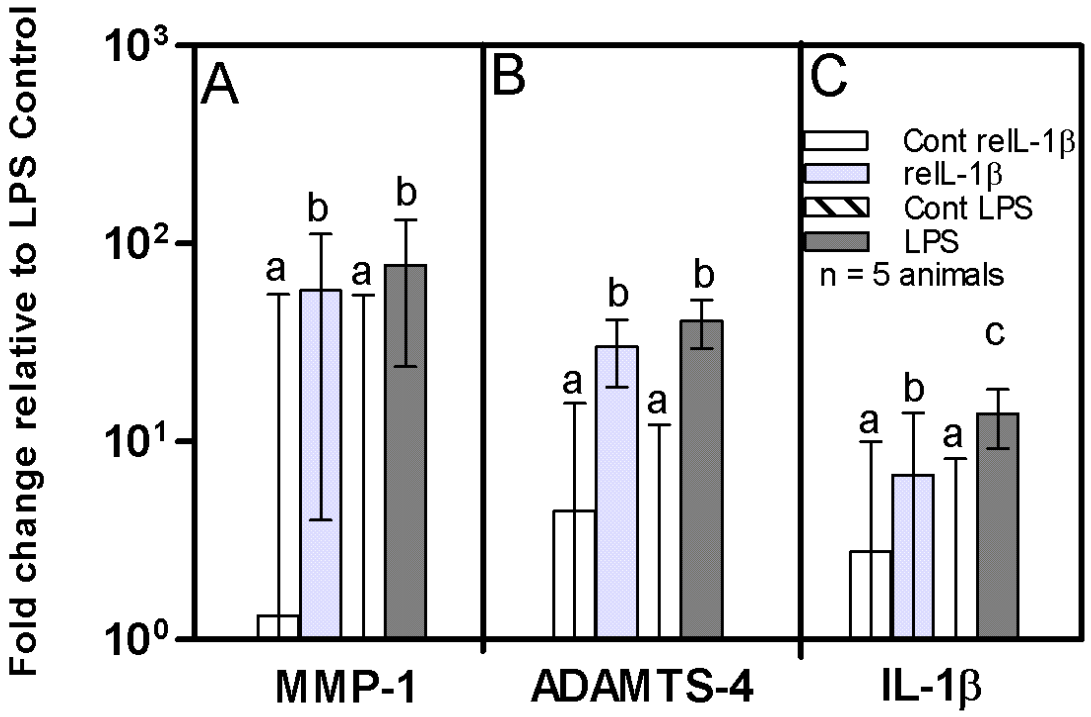

Figure 3.5. Effect of single intra-articular injection of reIL-1b (100 ng), LPS (0.5 ng) or PBS on synovium gene expression at 8 hours post injection. Values expressed as fold difference relative to LPS controls. (A) MMP-1 expression: columns lacking common letters indicate a significant difference between joints (P=0.011). (B) ADAMTS-4 expression: columns lacking common letters indicate a significant difference between joints (P<0.0001). (C) IL-1b gene expression: columns lacking common letters indicate significant difference between joints (P=0.003) and treatments (P=0.038). IL-1b= Interleukin 1 Beta. MMP-1=Matrix Metalloproteinase 1 (collagenase 1). ADAMTS-4=A disintegrin and metalloproteinase with a thrombospondin type motif class 4 (aggrecanase 1). Data are displayed as means ± 95% confidence intervals.

Gene expression in joint tissues at post injection hour 8 (Articular cartilage)

Gene expression of MMP-13 and TNF-α was not significantly different among treated and control joints (P=0.18). There was a significant difference between LPS and reIL-1β tissues compared to controls for MMP-1 (223 fold increase) (P=0.002) and ADAMTS-5 (3 fold increase) (P=0.002) (Figures 3.6A & 3.6C). Expression of ADAMTS-4 (Figure 3.6B) was 2.9-fold higher in LPS treated cartilage versus reIL-1β (P=0.054) with treated joints 20-fold higher compared to control joints (P<0.0001). Expressions of IL-1β and COX-2 in control cartilages were not consistently within the detectable limit of the assay; therefore, these data were not analyzed. In cartilage from LPS joints, expression of IL-1β and COX-2 were 3.4 and 3.9 fold higher than reIL-1β, respectively.

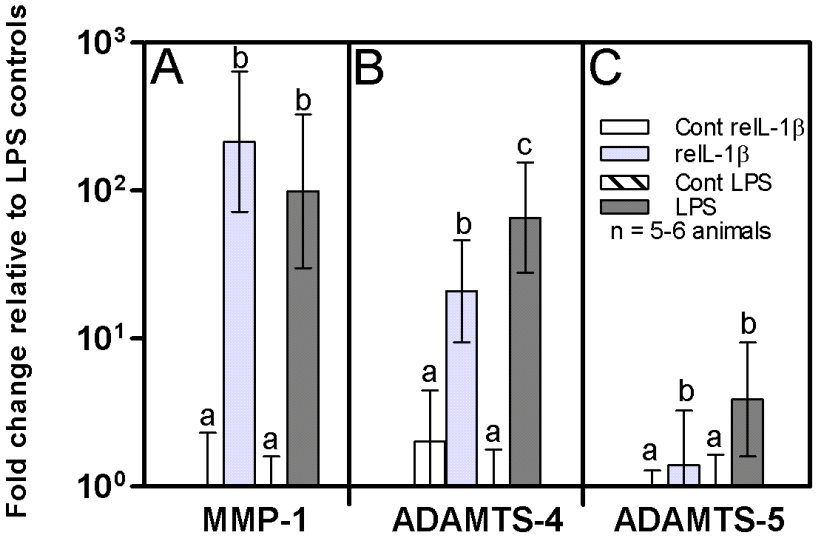

Figure 3.6 Effect of single intra-articular injection of reIL-1b (100 ng), LPS (0.5 ng) or PBS (controls) on cartilage gene expression at 8 hours post injection. Values expressed as fold difference relative to LPS controls. (A) MMP-1: columns lacking common letters indicate a significant difference between joints (P=0.002). (B) ADAMTS-4: columns lacking common letters indicate significant difference between joints (P<0.0001) and treatments (P=0.054). (C) ADAMTS-5: columns lacking common letters indicate significant difference between joints (P=0.002). MMP-1=Matrix Metalloproteinase 1 (collagenase 1). ADAMTS-4=A disintegrin and metalloproteinase with a thrombospondin type motif class 4 (aggrecanase 1). ADAMTS-5= A disintegrin and metalloproteinase with a thrombospondin type motif class 5 (aggrecanase 2). Data are displayed as means ± 95% confidence intervals.

Discussion

The development of reversible *in vivo* models of synovitis is valuable for studying OA pathophysiology, and testing therapeutic modalities. The horse is an excellent translational model for evaluating OA treatments (Frisbie et al., 2002) with outcomes relevant to human OA. The use of the endotoxin LPS has been an effective model for inducing joint inflammation (Palmer and Bertone, 1994; Hawkins et al., 1993; Kay et al., 2008) and has been the primary agent used for *in vivo*, non-terminal equine studies. Comparative studies with other models are lacking, and little information is available regarding reIL-1β's ability to induce predictable inflammation in the joint. The reIL-1β product has only recently become available and the *in vivo* use of intra-articular reIL-1β has only been reported once (DePuy et al., 2007) with results limiting in defining inflammatory effects. Therefore, this study was aimed at comparing reIL-1β to LPS as an *in vivo* synovitis model and exploring the potential of reIL-1β as a reversible model of OA.

The AAEP lameness scoring system (Anon, 1991) is a clinical standard for classifying degrees of equine musculoskeletal injury and pain. The absence of lameness in the PBS-injected joints illustrated that intra-articular injection of LPS or reIL-1β induced lameness. Lameness in horses treated with LPS is consistent with other studies that utilized a single dose of 0.5 ng LPS (Palmer and Bertone, 1994; deGrauw et al., 2009). Furthermore, 100 ng reIL-1β and 0.5 ng LPS resulted in a similar degree of lameness, indicating reIL-1β is equally sufficient as LPS for inducing clinical signs of lameness.

A single injection of 100 ng of recombinant equine IL-1β or 0.5 ng of LPS into a random metacarpal joint of horses resulted in observable signs of acute synovitis, yet caused only mild, temporary discomfort. Previous studies administering 100 ng of reIL-β (Kisiday et al., 2010) or

0.5 ng LPS (McIlwraith et al., 1979; Firth et al., 1987) reported no systemic response (in terms of respiration, temperature or heart rate). The results in the current study were similar in regards to temperature and respiration; no changes were observed from baseline measurements through PIH 8. We report an increase in temperature at PIH 12 and 16 and respiration at PIH 16 and 24, which are likely due to recovering from the arthroscopic biopsy procedure (and general anesthesia) and not the effect of either reIL-1β or LPS injections. Average heart rates were overall higher in LPS-treated horses compared to reIL-1β treated horses; however, there were differences between horses at individual time points. While the average for the LPS horses (44.12 +/- 1.24 bpm) was slightly higher than the range (30-44 bpm) established by the AAEP, the influence of surgery and environment needs to be considered.

A strong intra-articular inflammatory response in treated joints was demonstrated by significant increases in synovial WBC, neutrophil counts and total protein between PIH 4-48, which was consistent with previous studies using exogenous LPS (Palmer and Bertone, 1994; Hawkins et al., 1993; deGrauw et al., 2009), rh IL-1β (Hardy et al., 1998; Pearson et al., 2009) or reIL-1β (Smith et al., 1998). In control joints, a significant increase in WBC concentrations at PIH 8, neutrophil counts at PIH 4, and total protein at PIH 4 are considered to be reflective of repeated arthrocentesis, which is consistent with other studies (Hawkins et al., 1993; Hardy et al., 1998). The response to PBS was much less profound than that of LPS and reIL-1β. Notably, at PIH 8, 6 ml of synovial fluid was easily aspirated from joints injected with LPS or reIL-1β, and it was difficult to get more than 1 ml from control joints.

Synovial PGE_2 concentrations were elevated from intra-articular injections of reIL-1β or LPS but not PBS. These results are consistent with previous studies using LPS (Hawkins et al.,

1993; deGrauw et al., 2009) or rhIL-1β (Hardy et al., 1998; Pearson et al., 2009). In addition, PGE_2 levels remained elevated in treated joints through PIH 48 when compared to baseline values; these findings differ from others using similar concentrations of LPS (deGrauw et al., 2009) or rhIL-1β (Pearson et al., 2009). Hawkins and others (1993) reported a comparable prolonged elevation in PGE_2 (beyond 8 hours post-injection) to what was reported here; however, the LPS dosage (1.5 μg) was higher than in the present study. Although one could expect the sustained PGE_2 concentrations in our study to reflect a secondary response due to surgery at PIH 8, PGE_2 levels in reIL-1β control joints did not change significantly from baseline post-surgery (PIH 24-48) with surgical manipulation, indicating the act of flushing the joint during the surgery may have caused an anti-inflammatory effect. We conclude that a single 1 ml, intra-articular injection of 100 ng of reIL-1β is adequate in stimulating *in vivo* PGE_2 production to levels that reflect an inflammatory response.

Matrix metalloproteinase activity in synovial fluid followed a similar pattern as PGE_2; joints treated with reIl-1β or LPS exhibited increasing levels to PIH 8, and decreasing by PIH 24. While an increase in MMP activity was observed at PIH 48 in reIL-1β treated joints, this increase was not at the magnitude seen at PIH 4, nor was it significantly different from LPS-treated joints at same time. Synovial fluid collection did not resume until 16 hours after surgery; therefore, it is unknown if the difference in MMP activity in treated joints is a result of treatment, effect of surgery or lag in time between collections. This was the first time arthroscopic biopsy had been introduced to either model so this requires further evaluation. Repeated aspiration did not alter MMP activity as evident by lack of significant change in control joints throughout the experimental period. These findings were in contrast to van den Boom and others (van den Boom et al., 2005) who reported subsequent arthrocentesis increased MMP activity in unstimulated

joints within 12 hours. However, in that study, half of the horses were subjected to daily exercise; horses used in the current study were sedentary. Despite multiple collections, an intra-articular injection of 1 ml of PBS and repeated aspiration did not cause a significant change in MMP activity in control joints. The data presented here are the first to demonstrate reIL-1β's ability to instigate general MMP activity in the live animal, indicating reIL-1β potential as a method for investigating inflammatory processes associated with early stages of OA.

In the current study, the arthroscopic surgical procedure at PIH 8 may have influenced MMP activity and GAG concentrations measured post-surgery. An increase in GAG concentrations in control and treated joints is speculated to be a consequence of the biopsy procedure rather than repeated aspiration or treatment application. It is unknown whether the overall change in GAG concentration is a reflection of increased synthesis or increased degradation. This could be clarified with the evaluation of additional anabolic and catabolic markers.

Synovial membrane and articular cartilage biopsies showed that intra-articular injection of LPS or reIL-1β upregulated gene expression of certain catabolic enzymes associated with OA. The increase in MMP-1 expression with LPS or reIL-1β is consistent with *ex vivo* stimulation of chondrocytes (Bau et al., 2002). To our knowledge, this study is the first to report *in vivo* upregulation of MMP-1 expression in equine joint tissue.

Intra-articular injection of reIL-1β or LPS induced greater expression of ADAMTS-4, but not ADAMTS-5, in synovial membrane compared to control joints. These findings are consistent with ADAMTS-4 and ADAMTS-5 expression in human OA synovium (Bondeson et al., 2006). Articular cartilage expression of ADAMTS-4 and ADAMTS-5 is consistent with other equine (Busschers et al., 2010) and human (Bondeson et al., 2008) OA models, yet differs from murine

OA models in which ADAMTS-5 was the primary aggrecanase in cartilage degradation (Stanton et al., 2005). Discrepancies between human and murine models have been reported (Bondeson et al., 2008) presenting a challenge in comparing findings in those species. However, significant expression of ADAMTS-5 in cartilage, but not in synovial tissue in the present equine study is similar to what has been reported in human studies (as reviewed) (Fosang et al., 2008) indicating potential similarities in expression of aggrecanases between these two species.

The increase in IL-1β expression in LPS treated synovium compared to reIL-1β treated joints may indicate an LPS-stimulated endogenous production of IL-1β. It is unclear if the lower expression of IL-1β in reIL-1β treated synovium is due to a lack of or minimal endogenous IL-1β production at PIH 8 that follows reIL-1β injection. Previous *in vitro* studies have reported LPS-induced IL-1β production in human OA synovial tissue and cartilage (Shirazi et al., 2001) ; however, measurements were of protein concentrations and not gene expression. Exogenous IL-1β is rapidly cleared from the joint and does not stimulate endogenous production of IL-1β (Hardy et al., 1998), possibly allowing for a more representative evaluation of mediator response to IL-1β. Concentrations of IL-1β in synovial fluid were not evaluated in the present study; therefore, it is unknown if and to what extent endogenous IL-1β production may have occurred in treated joints.

Upregulation of chondrocyte expression of ADAMTS-4 in LPS joints compared to reIL-1β joints may be due to endogenous cytokine production stimulated by LPS injection; however, synovial fluid biochemical analysis did not differ between LPS and reIL-1β.

The inflammatory response to LPS is characterized by an initial release of the cytokines IL-1 and TNF-α (Fernandes et al., 2002, Blom et al., 2007, Matsukawa et al., 1993) and is followed by a release of PGE_2 (Hawkins et al., 1993; deGrauw et al., 2009; Morton et al., 2005)

and MMPs (deGrauw et al., 2009) . The two forms of interleukin-1 found in the horse are IL-1α and IL-1β (May et al. 1990). Both have been cloned and evaluated for stimulatory effect *in vitro*, and act through the same receptor, but equine IL-1β is more potent for stimulating PGE_2 than equine IL-1α (Takafuji et al., 2002). The present study is the first to demonstrate that an injection of exogenous reIL-1β resulted in significant increases in PGE_2 and general MMP activity.

To our knowledge, we report the first *in vivo* investigation of equine joint tissues in response to LPS or reIL-1β injections. The tissue amounts collected through biopsy procedures were sufficient for evaluating mRNA expression without causing significant joint damage nor requiring euthanasia of the horses; a practice often required in other animal models (Scott et al., 2009). These findings further validate the role of synovitis in the generation of MMPs and aggrecanases that are associated with OA, and that MMP-1 and ADAMTS-4 may be therapeutic targets.

A limitation to this reIL-1β synovitis model is that the injection of reIL-1β overrides potential upstream regulators of IL-1β synthesis in the natural disease process. However, given that inhibitors of IL-1β have shown a strong ability to modulate cartilage degradation in experimental osteoarthritis models (Matsukawa et al., 1993; Pelletier et al., 1997) we feel that this model has great promise for identifying therapeutics that can significantly impact the progression of osteoarthritis. The absence of protein measurements for synovial IL-1β restricts our interpretation of LPS-stimulated cytokine activity, although based on gene expression analysis it appears LPS could stimulate IL-1β protein synthesis. The inflammatory response was similar between reIL-1β and LPS, as expected. Lipopolysaccharide activates IL-1β which further stimulates eicosanoid production and MMP activity. Additionally, the reaction by the synovial

membrane is non-specific (McIlwraith, 2005). Synovitis is related to acute stimulus rather than a specific cause.

We administered 100 ng of reIL-1β as we believed that this dose would induce a robust inflammatory response that was temporary and not severe enough to require rescue analgesic, and had been the reIL-1b dose used in a previous *in vivo* study (DePuy et al., 2007). While a reIL-1β dose of 0.1 ng/ml was reported to stimulate GAG release from equine cartilage explants (Takafuji et al., 2002*) in vitro*, a six-fold increase in PGE_2 release was reported in the same study for 10 ng/ml of reIL-1β. Given that PGE_2 release is an important outcome measure for models of synovitis, our dose was intended to stimulate robust PGE_2 release based on an estimated joint volume of 10 ml, resulting in a reIL-1β concentration of 10 ng/ml upon injection. We confirmed lameness scores consistent with a robust yet humane inflammatory response. Additional studies would be needed to determine if a reIL-1β dose less than 100 ng/ml could stimulate an inflammatory response that can be detected by changes in fluid biomarkers and tissue gene expression.

In this study, we characterized changes in synovial fluid and tissue following intra-articular injection of reIL-1β that are consistent with known pathogenesis of OA, and are similar to the cellular response of acute synovitis commonly reported in equine OA. While the degree of inflammation may be reflective of sepsis or human rheumatoid arthritis, the response was short and temporary. The use of the contra-lateral joint as a control is valid, given the lack of significant change in inflammatory markers. The methods introduced in this study produced a synovitis response in horses that were temporary and reflective of OA, supporting the use of the horse as a translational model for studying osteoarthritis.

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Chapter IV

Evaluation of oral n-3 long chain polyunsaturated fatty acid supplementation effects on experimentally-induced synovitis in horses

Overview

Objective: To determine if dietary supplementation of n-3 long chain polyunsaturated fatty acids (LCPUFA) to horses reduce inflammatory molecules in the synovial fluid and gene expression degradation markers in the synovium and articular cartilage relative to non-supplemented horses.

Materials and Methods: Twelve skeletally-mature, healthy mares that were free of lameness were randomly assigned a diet supplemented with 40 g/day of a marine-derived n-3 LCPUFA (N3FA), or a control diet (CONT) without n-3 LCPUFA supplementation. All horses were individually fed these diets for 90 days. Blood samples collected on day 0, 30, 60 and 91, and synovial fluid collected on day 0 and 91, were processed of fatty acid composition. On day 91, inflammation was experimentally-induced in a single carpal joint of each horse using recombinant equine IL-1beta (IL-1β). Synovial fluid samples were repeatedly taken at post injection hour (PIH) 0, 4, 8 and 24, and were analyzed for inflammatory biomarker prostaglandin E_2 (PGE_2), general matrix metalloproteinase activity and routine cytology. Synovium tissue and articular cartilage was collected at PIH 8 via arthroscopic biopsy procedures and analyzed for gene expression of matrix metalloproteinases 1 and 13; the cytokines tumor necrosis factor alpha

(TNF-α) and interleukin 1-beta (IL-1β); cyclooxygenase 2 (COX-2) and the aggrecanases, ADAMTS-4 and ADAMTS-5.

Results: A 90 day supplementation of daily n-3 LCPUFAs resulted in significantly higher serum phospholipid and synovial fluid lipid composition of the LCPUFA eicosapentaneoic acid (EPA) and docosahexaneoic acid (DHA) compared to non-supplemented horses. Serum arachidonic acid (ARA) was higher in N3FA horses than CONT horses through days 30-90 of the dietary treatment period. Synovial fluid levels of ARA were significantly lower at day 91 compared to baseline values (day 0), regardless of dietary treatment. On day 91, a single injection of reIL-1β increased synovial white blood cell and neutrophil count, total protein; prostaglandin E_2 concentrations and general matrix metalloproteinase activity, although each were not significantly different between CONT and N3FA horses. Injection of reIL-1β stimulated mRNA expression for MMP-1, COX-2 and IL-1β in synovium, with no difference between dietary treatments. Horses receiving the N3FA supplement had significantly lower expression of synovium ADAMTS-4 compared to CONT horses. Gene expression for MMP-13, IL-1β, ADAMTS-4, and ADAMTS-5 was higher in treated cartilage samples compared to saline injections, regardless of dietary treatment.

Discussion: A 90 day dietary n-3 LCPUFA supplementation significantly increased serum phospholipid and synovial fluid lipid concentrations of EPA and DHA. The significantly higher amounts of serum ARA phospholipids in N3FA horses likely reflects a small, yet measureable amount of ARA in the n-3LCPUFA supplement. The administration of reIL-1β (100 ng) in equine carpal joints resulted in a robust, yet temporary inflammatory response characterized by significant increases in cytology parameters, biological markers of inflammation and gene

expression of cartilage degradative enzymes. However, differences between dietary treatment groups were not significant for clinical response (lameness), white blood cell counts, eicosanoid production, and matrix metalloproteinase activity. We postulate that the lack of significant differences between CONT and N3FA groups is due to the high dose of reIL-1β that overwhelmed any anti-inflammatory effects of n-3 LCPUFA supplementation. The discovery of significantly lower expression of ADAMTS-4 in the N3FA synovium warrants further investigation of n-3 LCPUFAs (in particular, EPA and DHA) potential as therapies for equine OA.

Introduction

Osteoarthritis (OA) is a leading cause of joint pain and lameness in a variety of species, including horses and humans. Joint inflammation and effusion are common sequelae of the disease, with uncontrolled inflammation, leading to cartilage degradation. Osteoarthritis (OA) accounts for 60% of equine lameness cases in the United States (Caron and Genovese, 2003) with original diagnosis and subsequent treatment costing horse owners millions of dollars each year. While often associated with the natural processes of aging, OA may occur in younger animals due to direct or cyclic trauma that is associated with synovitis or capsulitis (McIlwraith, 2005). Performance horses undergo rigorous training protocols; therefore joint inflammation is common with consequent mediator release resulting in the progression of OA (McIlwraith, 2005). In addition to the health risks of these animals, OA results in a significant annual economic loss for the equine industry.

Inflammation with joint effusion occurs immediately after an insult, and is associated with the progression of joint disease. After the initial episode of acute inflammation, irreversible

changes such as thickening of the joint capsule and degradation of the articular cartilage can occur in progressive stages of OA (McIlwraith, 1979). Clinical signs may become apparent only after tissue degradation has initiated; therefore, early detection is necessary to minimize tissue damage.

Chronic joint inflammation can cause progressive degradation of articular cartilage in joints. The primary mediator of the inflammatory response is interleukin-1β (IL-1β). Interleukin-1 acts as an inter-cellular messenger that can stimulate the production of MMPs, aggrecanase and PGE_2. (McIlwraith, 2005). In most cases of equine joint disease the process begins with synovial inflammation producing IL-1β which stimulates the production of matrix metalloproteinases (MMP), PGE_2, free radicals and aggrecanase. Aggrecanases, potent catabolic enzymes that are instrumental in normal cartilage turnover, cleave aggrecan molecules (aggregating proteoglycans) of the extracellular matrix, contributing to cartilage degradation (Nagase and Kashiwagi, 2003). Aggrecanases have been recognized as the most significant enzymes in aggrecan breakdown during early stage of OA (Bondeson et al., 2008). Specific inhibitors of aggrecanases regulate their activity and it has been shown *in-vitro* that dietary n-3 fatty acids may have inhibitory properties (Hurst et al., 2009).

Research in human nutrition has shown that supplementation of n-3 long chain polyunsaturated fatty acids in the diet can have beneficial effects on inflammation by direct competition with pro-inflammatory mediators produced from the n-6 class of LCPUFAs. Arachidonic acid (ARA) the primary n-6 LCPUFA involved in inflammation can be converted to several products including PGE_2, thromboxane A_2 (TXA_2) and leukotriene B_4 (LTB_4). These ARA derivatives are part of two families of very effective signaling molecules known as

eicosanoids and leukotrienes, and they contribute to natural immune responses by stimulating neural pain receptors, vasodilation and platelet aggregation during inflammation (Funk 2001). The n-3 LCPUFAs eicosapentaenoic acid (EPA) and docosahexenoic acid (DHA) are believed to exert less potent inflammatory eicosanoids and leukotrienes and are found in the highest quantities in marine fish oil. Eicosapentaenoic acid derivatives, such as prostaglandin H_3 (PGH_3) and thromboxane A_3 (TXA_3), are regarded as less potent substrates when compared to ARA derivatives. It has been well documented that EPA competes with ARA for metabolism by the cyclooxygenase and lipoxygenase pathways (Lee et al., 1991); therefore, when available in adequate quantities, EPA can depress the production of the pro-inflammatory metabolites of ARA, through its own production of the less inflammatory eicosanoids and leukotrienes.

Due to the documented health benefits of EPA and DHA and their abundance in fish oil, researchers have tested the use of fish oil as a dietary supplement for horses. Unfortunately, the 'fishy' odor of the oil affects palatability and the instability of the LCPUFAs can cause rapid oxidation, preventing long-term storage of such products. The recent development of marine-based feed supplements containing antioxidants and algae-derived n-3 LCPUFAs have enhanced product quality and improved acceptability by horses. Studies utilizing these types of n-3 LCPUFA supplements have reported a potential for pain reduction, as indicated by a tendency of increased stride length in exercising horses (Woodward et al., 2007) and a significant decrease in synovial white blood cells and plasma PGE_2 in arthritic horses (Manhart et al., 2009). Our laboratory recently reported the detection of EPA and DHA in the synovial fluid of horses receiving an n-3 fatty acid supplement (Ross et al., 2010). These data support further investigation into the use of oral n-3 LCPUFA for equine joint health. The recent development of an experimental model for inducing temporary synovitis in the horse (see Chapter III) provides

researchers with a useful tool for evaluating inflammatory processes associated with natural-occurring OA in the horse. Investigating the response to experimentally-induced synovitis in non-arthritic horses receiving an oral n-3 LCPUFA supplement would be helpful in evaluating alternative treatments for equine OA.

Methods

Animals

Skeletally-mature mares, between 5 and 14 years of age, were evaluated for pre-existing joint disease by undergoing a diagnostic lameness exam and radiographic evaluation prior to being selected for the study. Selected horses were free of lameness and radiographic evidence of joint disease. All horses were clinically healthy prior to beginning of trial and were housed at the Colorado State Equine Teaching and Learning Center. All horses were vaccinated and de-wormed prior to the start of the feeding trial and were maintained on a routine schedule for de-worming and feet trimming throughout the duration of the study. Horses were managed according to procedures as stated in the Animal Use Protocol approved by the Institutional Animal Care and Use Committee.

Sample size determination

The experimental sample size was determined using SAS 9.2© statistical software (SAS Institute, Inc., Carey, NC). Data from previous research that evaluated the fatty acid composition of synovial fluid in horses receiving a daily EPA/DHA supplement (Ross et al., 2010) was used for the power analysis for paired t-test calculation. A sample size of n=6 per treatment group provided a statistical power of .90 (α=0.05).

Experimental diet

Twelve (12) skeletally-mature mares, free of lameness and radiographic evidence of joint disease were blocked by age, body weight, body condition score and serum total lipid concentrations and randomly assigned into one of two treatment groups: CONTROL (n=6) or N3FA (n=6). All horses were first group fed timothy grass hay at 1.2% of average body weight for fourteen (14) days prior to start of experimental feed trial. Timothy grass hay and a commercial oat/molasses grain mix (Ranch-Way Feeds, Inc; Fort Collins, CO) were allocated as the study's basal diet (Table 4.1). Daily feed rations were formulated based on NRC requirements for the mature, idle horse (NRC, 2007) and amount fed was based on percentage of each individual horse's body weight. Timothy grass hay was provided at approximately 1.1% BW for CONT horses and 1.1 % of BW for N3FA horses. The amount of oat/molasses mix (0.4% of BW for CONT; 0.35% of BW for N3FA) was adjusted to keep diets isonitrogenous and isocaloric and to serve as a vehicle to deliver the n-3 LCPUFA fatty acid supplement. Mares assigned to the N3FA group received the basal diet, along with a marine-derived n-3 LCPUFA fatty acid supplement (Magnitude^{TM}) that was formulated to provide approximately 19.5 mg of EPA and 53.5 mg of DHA per kg of body weight. CONTROL mares received only the basal diet; no addition supplementation was given. Horses received their dietary treatments for 90 days. Individual refusals for each feedstuff were weighed after each feeding and included, if applicable, into daily feed intake calculations. Nutrient analysis for each feedstuff was determined by collecting small (~30 grams) sub-samples of each hay bale, concentrate bag and n-3 LCPUFA supplement pail as they were opened and fed during the dietary trial. Sub-samples were placed into large collection bags and separated by three distinct feeding periods; day0-day 30, day 31-day 60 and day 61-day 90. Samples from each feeding period were then thoroughly

mixed. Approximately 1 lb of each feedstuff from each feeding period was ground using a Wiley Feed Mill with a 2 mm screen. Ground samples were then divided into two equal portions (~ ½ lb) with one set reserved for fatty acid composition and the second set analyzed for nutrient content by an independent contractor (Mid-West Laboratories, Omaha, NE).

Table 4.1 Nutritional analysis[1] of research diet; fatty acids[2] as a percentage of fat in feedstuff

	Feedstuff		
Nutrient value	Timothy Hay	Oats with molasses[3]	Magnitude ™ [4]
Dry Matter (%)	89.57	86.59	95.49
Crude Protein (%)	7.33	11.29	12.67
Crude Fat (%)	2.18	4.70	21.55
Acid Detergent Fiber (%)	34.28	13.92	6.57
Total Digestible Nutrients (%)	63.98	86.62	100.83
Digestible Energy (Mcal/lb)	1.23	1.73	2.11
Phosphorous (%)	0.17	0.42	1.37
Calcium (%)	0.49	0.13	0.39
Copper (ppm)	5.02	6.62	5.50
Zinc (ppm)	18.71	36.01	31.42
Fatty acid, g/100g of total fatty acids			
C18:2 Linoleic Acid	19.14	42.32	7.43
C18:3 Alpha Linolenic Acid	47.66	1.94	1.85
C20:4 Arachidonic Acid	-	-	0.67
C20:5 Eicosapentaeonic Acid	-	-	9.35
C22:5 Docosapentaenoic Acid	-	-	1.84
C22:6 Docosahexaenoic Acid	-	-	25.56
Other fatty acids[4]	33.2	55.72	53.30

[1]Analysis conducted by MidWest Laboratories; Omaha, NE.[2]Analysis completed at Food Science & Human Nutrition Laboratories, Colorado State University; Fort Collins, CO. [3]Ranch-Way Feeds; Fort Collins, CO. [4]JBS United; Sheridan, IN. [4]C14:0, C16:0, C16:1, C18:0, C18:1n9, C20:3n6

Body Weights and Body Condition Scoring

Horses were weighed bi-weekly using an electronic livestock scale and diets were adjusted accordingly to maintain a consistent body weight throughout the dietary treatment period. Individual horse's body condition was determined using a 1-9 scale based on the Henneke body condition scoring system (Henneke et al., 1983). Body condition scores (BCS)

was determined by TNR and by two other trained persons who were blinded to the study. Evaluations were conducted every 14 days and the three reported scores were averaged.

Blood and synovial fluid collection for fatty acid compositions

Blood samples (approximately 10 ml) collected on day 0, 30, 60 and 91 of dietary treatment were obtained from the jugular vein into sterile, non-heparinized tubes and left at room temperature for approximately 20 minutes to allow sample to clot. Samples were then centrifuged at 2500 rpms for 10 minutes to separate serum and red blood cells. Serum supernatant was collected and aliquoted into 1. 5 ml eppendorfs in 500 µl volumes and immediately stored at -80° C until analyzed. Synovial fluid (average of 3 ml) was collected from both front mid-carpal joints of each horse on day 0 and day 91 of dietary treatment using sterile arthrocentesis techniques. Of the volume collected, approximately 0.5 ml was placed into an EDTA tube and immediately processed for routine cytology (white blood cell count, differential neutrophil count and total protein). The remaining volume of synovial fluid was transferred into plain sterile tubes and processes as described for the serum samples.

Induction of temporary carpal synovitis

On day 91, horses were moved to the Equine Orthopaedic Research Center where temporary synovitis was induced in a random carpal joint of each horse. At post injection hour (PIH) 0, horses were sedated and prepped for arthrocentesis. A single dose (100 ng) of recombinant equine interleukin-1 β (reIL-1β) in 1 ml of phosphate buffered saline (PBS) was given via intra-articular injection into a middle carpal joint (treated) to induce temporary inflammation. The contra-lateral joint (control) was injected with an equivalent volume (1 mL) of PBS. Vital signs (temperature, pulse and respiration) and comfort scores were taken every 2 hours starting at post-injection hour (PIH) 0 through PIH 24. Lameness evaluations and joint

circumference measurements were taken at PIH 0, 4, 8, 24 and 240. Blood samples and synovial fluid samples (from both treated and control joints) were taken at hours 0, 4, 8 and 24 post injection using techniques described in previous section. At hour 8, an arthroscopic biopsy was conducted and synovium and cartilage samples were collected from the treated and control joints of each horse. Arthroscopic biopsy procedures have previously been described (Chapter III). After the final fluid collection (hour 24 post injection) horses were moved back to the Equine Center where they underwent a 10 day recovery period.

Synovial fluid analysis

Methods for synovial fluid analysis for gross appearance, cytology (white blood cell count, differential neutrophil and total protein), prostaglandin E_2 concentrations, glycosaminoglycan levels and general MMP activity have previously been described (Chapter III); procedures were carried out in an identical matter in present study.

Synovial fluid, serum phospholipid and feedstuff fatty acid composition

Samples of serum, synovial fluid and feedstuff samples were processed in duplicate and serum phospholipid composition and synovial fluid and feedstuff total lipid profiles were determined using a modified procedure of the Folch Lipid Extraction method (Folch et al., 1957).

Methods for determining serum phospholipid composition were as follows: 500 µl of serum was added to a new screw cap 10 ml tube. A 4 ml solution of 2:1 $CHCI_3$:MeOH was added to the sample and vortexed for 60 seconds. Sample was then filtered through a 2V grade qualitative 12.5 cm Whatman filter into a clean screw cap 10 ml tube. Enough 2:1 $CHCI_3$:MeOH solution was added to new tube to bring volume to 4 ml. One ml of double distilled water (ddH_2O) was added to separate the lipid layer. Sample was vortexed for 30 seconds before being

centrifuged for 10 minutes at 2500 rpm. The top layer (non-lipid) was removed and discarded. The lower layer (containing lipids) was evaporated to dryness using a light nitrogen flow. Upon dryness sample was res-suspended in 500 µl of hexane and vortexed for 30 seconds. The entire sample was then transferred using a glass bulb pipette onto a 20cm X 20cm thin-layer chromatography plate. The plate was then placed in a 70:30:1 hexane:ethyl ether:acetic acid solution for one hour to separate out the phospholipid fraction. The band of phosphoplipids that remained at the site of origin was removed using a clean razor blade by scaping into a new 10 ml screw cap tube. A 500 µl volume of hexane was added to the tube to dissolve the sample. Samples underwent a methylation procedure as follows: approximately 500 µl of 0.5 N potassium hydroxide (KOH) was added to the sample and vortex for 30 seconds. The sample was then placed in a heating block set at 70°C for 10 minutes. After 10 minutes, the sample was removed from the heating block and allowed to cool to room temperature. Once the sample had cooled, 2 ml of boron trifluoride (BF_3) was added and the sample was vortexed for 30 seconds. The sample was then returned to the heating block, now set at 100°C and incubated for 30 minutes. The sample was then removed and once it cooled to room temperature, 500 µl of hexane and 0.67 ml of ddH_2O were added. The sample was then vortexed for 30 seconds and followed by centrifugation at 2500 rpm for approximately 1 minute to allow for phase separation. Upon centrifugation, there were obvious layers; a non-polar (upper) layer and a polar (lower) layer. The non-polar layer was pipetted into individual gas chromatography vials and evaporated to dryness using a light nitrogen stream. Samples were reconstituted with 500 µl of hexane and placed on gas chromatography machine for analysis.

Synovial fluid was thawed and 100 µl of fluid and 80 µl of an internal standard (pure C23:1-Tricosenoic Acid; NuCheck, Inc., MN, USA.) was transferred into a 12 ml glass screw cap tube. Two ml of $CHCI_3$: MeOH was added to each tube and vortexed until solution was thoroughly mixed. Samples were gravity-filtered through a S&S (#1) filter into a new, clean 12 ml glass screw cap tube. The original tube was flushed with 1 ml of $CHCI_3$:MeOH into second tube. Approximately 375 µl of 0.88% potassium chloride (KCl) was added to each sample. Tubes were vortexed for 30 seconds and then centrifuged at 2500 g for 10 minutes. The top layer of solution was aspirated and discarded while the lower layer ($CHCI_3$) was evaporated using a light flow of nitrogen until dried. The sample was reconstituted with 500 µl of hexane, vortexed for 30 seconds and 500 µl of 0.5M KOH was added and the sample were again vortexed for 30 seconds. Tubes were placed on a heat block (70^0C) for 10 minutes, removed and allowed to cool. Once cool, 2 ml of BF_3 was added, the sample was vortexed for 30 seconds and placed in a second heat block (100^0C) for 30 minutes. Once cooled, 500 µl of hexane and 1 ml of dd H_2O were added to the tube. The sample was vortexed and centrifuged at 2500g for 1 minute to allow for phase separation. Upon centrifugation, there were obvious layers; a non-polar (upper layer) and a polar (lower layer). The non-polar layer was pipetted into individual gas chromatography vitals and evaporated to dryness using a light nitrogen stream. Samples were reconstituted with 100 µl of hexane and placed on gas chromatography machine for analysis.

Feed samples were processed in identical matter as synovial fluid samples yet without an internal standard.

Hexane extracts of fatty acid methyl esters (FAME) were separated by gas chromatography on a 30 m×0.25 mm×0.2 µm film thickness DB-225 column Agilent (Palo Alto,

CA) using an Agilent model 6890 GC equipped with autosampler, Chemstation and flame ionization detection. Amounts of individual fatty acids (C14:0-C22:6) were expressed as percent of total area of all fatty acid peaks detected by gas chromatography.

Serum samples were presented as percentage (%) of total phospholipids and synovial fluid and feedstuff samples were presented as percentage (%) of total lipids.

Arthroscopic biopsy procedure

At post-injection hour 8, all horses underwent surgical arthroscopy to obtain synovium and articular cartilage samples. In short, a 3mm incision was created medial to the extensor carpi radialis tendon at the plane of the third carpal bone in the middle carpal joint. A small closed, concave curette was used to collect cartilage samples (42 mg wet weight) and a Ferris-Smith rongeur instrument was used to extract synovial membrane (101 mg wet weight) tissue. Tissue samples were placed in a 1.5 ml collection tube containing Trizol, immediately flash frozen in liquid nitrogen and then stored at -80°C until analyzed.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of joint tissues

Cartilage and synovial tissue samples obtained by arthroscopic biopsy procedures were analyzed for gene expression of MMP-1, MMP-13, IL-1β, TNF-alpha, COX-2, ADAMTS-4 and ADAMTS-5. Methods for tissue processing and analysis of qRT-PCR have been previously described in Chapter III's materials and methods. Protocols for determining gene expression in cartilage and synovium samples were similar in present study.

Statistical analysis

The effects of dietary treatment (CONT, N3FA) on body weight, body condition score, nutrient intake, and serum and synovial fluid fatty acid compositions were determined using

PROC MIXED procedures of SAS 9.2©. Between-subject effects were diet with time being the repeated measured measures effect. White blood cell, neutrophil cell count, PGE_2 and GAG data were log transformed to standardize variance. All clinical and synovial fluid data were compared using analysis of variance for repeated measures. Between-subject effects were treatment and joint with time being the repeated measures effect. Significance level was set at $P \leq 0.05$; differences were further analyzed using Fisher's least squares means test. The data are presented as mean $\pm$ 95% confidence interval. Model residuals were plotted to verify normality and assumption of independence; any variables not meeting these assumptions were log transformed, reanalyzed and presented in log scale.

Results

Feed intake

While all horses were started on their respective dietary treatments on day 0, there was a notable adaptation period of approximately 3 days for the N3FA group to reach complete consumption of the offered amount of the marine-derived n-3 LCPUFA supplement. Average refusal of n-3 fatty acid supplement during the 3 day period was 29.92 grams or 1.5% of n-3 fatty acid supplement offered during same period. Overall, average refusal of n-3 fatty acid supplement was 3.6 grams or 0.72% of amount offered. The average refusal of total feed offered (hay, concentrate and/or supplement) throughout the 90 day feed trial was 1.6% for the CONT group and 2.5% for the N3FA group; the majority being hay refusals (1.5% for CONT; 1.2% for N3FA).

Average daily intake (Table 4.2) of dietary energy (DE) and grams of protein (CP) were similar between groups. Intake of crude fat differed between the CONT and N3FA group due to

the inclusion of the n-3 fatty acid supplement in the N3FA diet. There was a significant time effect ($P<0.01$) on average daily intake among all horses (data not shown); total intake was lower during the first 30 days compared to the remaining 60 days of treatment.

Table 4.2 Average daily dietary intake for treatment groups[1,2] (based on DMI)

	Treatments	
Item	CONT	N3FA
Animals	n=6	n=6
BW (kg)	498.33+/- 35.92[a]	494.57 +/- 35.91[a]
DMI (%BW)	1.43 +/- 0.15[a]	1.41 +/- 0.15[a]
DMI (%$BW^{0.75}$)	6.73 +/- 0.60[a]	6.65 +/- 0.60[a]
DE, Mcal	20.79 +/- 1.29[a]	21.29 +/- 1.29[a]
CP, g	588.17 +/-37.96[a]	597.96 +/-37.45[a]
Fat, g	165.27 +/-13.41[a]	248.46 +/-13.41[b]
Fatty acids, mg/kg BW		
LA	112.0 +/- 2.10[a]	116.3 +/-1.70[a]
ALA	89.67 +/- 5.10[a]	85.72 +/- 4.34[a]
ARA	ND[a]	1.43 +/- 0.01[b]
EPA	ND[a]	19.93 +/-0.16[b]
DPA	ND[a]	3.93 +/-0.04[b]
DHA	ND[a]	54.26 +/-0.49[b]
Total n6 fatty acids, g/100 kg BW	11.20 +/- 0.21[a]	11.77 +/- 0.18[a]
Total n3 fatty acids, g/100 kg BW	8.96 +/- 0.51[a]	16.78 +/- 0.50[b]
n6:n3 fatty acid ratio	1.25 to 1[a]	0.70 to 1[b]

Values expressed as mean +/- 95% confidence intervals.[1]CON: basal control diet; N3FA: algae and fish oil supplement added to basal diet.[2]LA: linoleic acid; ALA: alpha linolenic acid; ARA: arachidonic acid; BW: body weight; CP: crude protein; CF: crude fat; EPA: eicosapentaenoic acid; DE: dietary energy; DPA: docosapentaenoic acid and DHA: docosahexaenoic acid. DMI: dry matter intake. ND: non-detectable. Rows without common letters are significantly different ($P<0.001$)

Body weights and Body Condition Scores

There were no significant differences within and between treatment groups for body weight (BW) or body condition scores (BCS) (Table 4.3).

Table 4.3 Treatment Group Mean Body Condition Score (1-9) and Body Weight (kg)

TRT	Day	BCS (range)	Day	BW (range)
CONT	0	5.5 +/- 0.5[a]	0	507.6 +/- 31.7[a]
	14	5.4 +/- 0.6[a]	15	501.7 +/- 32.5[a]
	28	5.4 +/- 0.6[a]	30	496.3 +/- 32.5[a]
	42	5.4 +/- 0.6[a]	45	495.2 +/- 32.4[a]
	56	5.6 +/- 0.6[a]	60	497.1 +/-32.5[a]
	70	5.4 +/- 0.6[a]	75	498.1 +/-32.4[a]
	86	5.6 +/-0.6[a]	90	502.4 +/-30.4[a]
N3FA	0	5.6 +/- 0.6[a]	0	494.1 +/-31.7[a]
	14	5.3 +/- 0.5[a]	15	491.8 +/-32.5[a]
	28	5.3 +/- 0.5[a]	30	492.9 +/-32.4[a]
	42	5.3 +/- 0.5[a]	45	491.2 +/-32.5[a]
	56	5.4 +/- 0.5[a]	60	494.9 +/-32.5[a]
	70	5.3 +/- 0.5[a]	75	493.7 +/-32.5[a]
	86	5.5 +/- 0.6[a]	90	497.9 +/-32.5[a]

Values presented as mean +/- 95% confidence intervals. BCS=body condition score; BW= body weight. Columns lacking a common superscript differ, $P \leq 0.05$.

Serum phospholipid fatty acid incorporation

Serum samples taken on day 0, 30, 60 and 90 reflected a dietary effect on phospholipid concentrations (% total fatty acids [FA]). Serum phospholipid linoleic acid (LA) % was similar in all horses (CONT: 34.54 +/-1.92 % total FA; N3FA: 36.14 +/- 1.61 % total FA) prior to the start of dietary treatment (day 0) (Figure 4.1A). Compared to day 0, horses receiving the control diet (CONT) had significant increases (P<0.0001) in serum phospholipid LA% by day 30 (40.59 +/- 1.64 % total FA) and remained elevated through day 90 (41.05 +/- 1.62 % total FA). Horses receiving EPA/DHA supplementation (N3FA) had a significant decrease (P=0.008) in serum LA phospholipid % at day 30 (33.63 +/- 1.62% total FA); however, levels increased to baseline values by day 60 and remained consistent through day 90. Serum alpha linolenic acid (ALA) % (Figure 4.1B) was similar among all horses at day 0 (CONT: 0.88 +/- 0.12 % total FA; N3FA: 0.82 +/- 0.11 % total FA) and decreased significantly in all horses, regardless of dietary

treatment, by day 30, remaining lower than baseline levels through day 90 (P<0.0001). Throughout the duration of the feed trial (day 0-day 90), N3FA horses had significantly lower (P=0.04) serum phospholipid ALA% (0.46 +/- 0.08 % total FA) compared to CONT horses (0.58 +/- 0.08% total FA). Serum phospholipid arachidonic acid (ARA) % (Figure 4.1C) did not differ between horses at day 0 (CONT: 1.41 +/- 0.2 % total FA; N3FA: 1.47+/- 0.2 % total FA). Phospholipid ARA % increased significantly in all horses at days 30 and 60, returning to baseline values in CONT horses by day 90 but remained elevated in N3FA horses. Serum phospholipid ARA % was significantly higher (P=0.0007) in N3FA horses compared to CONT horses at day 30 (CONT: 1.67 +/- 0.2 % total FA; N3FA: 2.27 +/- 0.2 % total FA), 60 (CONT: 1.67 +/- 0.2 % total FA; N3FA: 2.29 +/- 0.12 % total FA) and 90 (CONT: 1.58 +/- 0.2 % total FA; N3FA: 2.26 +/- 0.2 % total FA). Eicosapentaenoic acid (EPA) phospholipid % (Figure 4.2A) was minimal (0.20 +/- 0.20 % total FA) and similar in all horses at day 0. Only N3FA horses exhibited significant (P<0.0001) changes in serum phospholipid EPA%; increasing by day 30 (2.84 +/- 0.29 % total FA), with the highest % observed at day 90 (3.23 +/- 0.30 % total FA). Serum phospholipid % for docosapentaenoic acid (DPA) (Figure 4.2B) followed a similar pattern as EPA; no change in CONT horses from baseline values (0.26 +/- 0.08 % total FA) when compared to days 30-90 and only N3FA horses exhibited significant increases (P<0.0001) over the duration of the study, reflecting a dietary effect. Docosahexaenoic acid (DHA) % (Figure 4.2C) was similar among all horses (CONT: 2.85 +/-0.74% total FA; N3FA: 2.81 +/- total FA) prior to the beginning of the feed trial (day 0). Only N3FA horses had significant (P<0.0001) changes in serum DHA %; levels increased by day 30 (6.14 +/- 0.73 % total FA) and remained elevated above baseline through day 90 (5.31 +/- 0.74 % total FA).

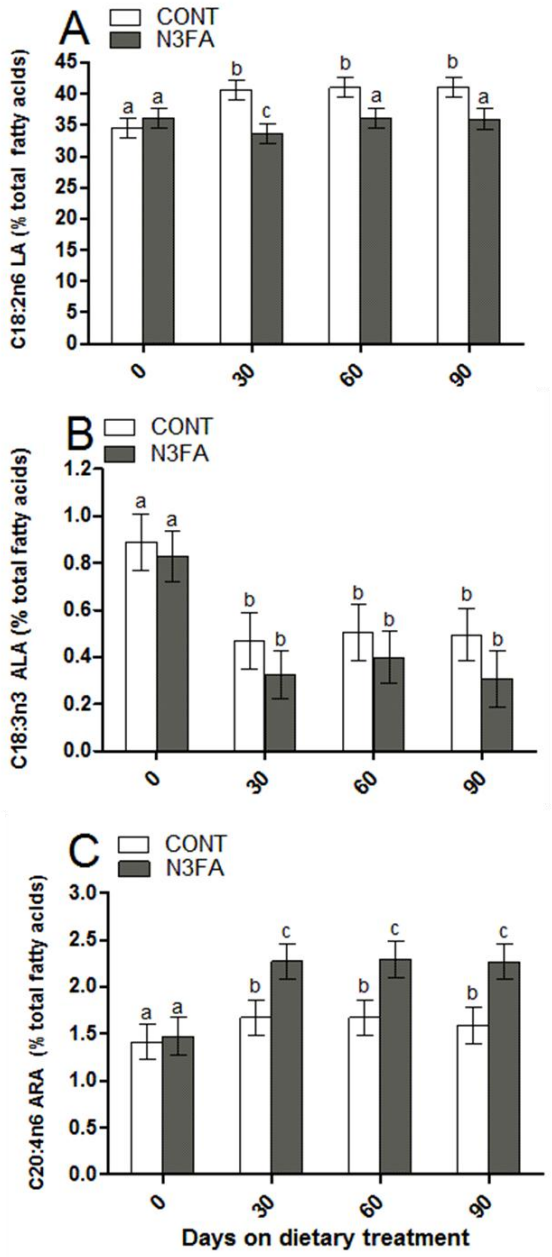

Figure 4.1 Effect of dietary treatment on serum phospholipid compositions. (A) Linoleic Acid: columns lacking common letter indicate a treatment X time effect (P<0.0001). (B) Alpha Linolenic Acid: columns lacking common letters indicate a time effect (P<0.0001). N3FA group had overall lower ALA composition (P=0.003). (C). Arachidonic Acid: columns lacking common letters indicate treatment X time effect (P<0.0001). Data are displayed as means ± 95% confidence intervals. (CONT=control diet; N3FA=algae/fish oil diet)

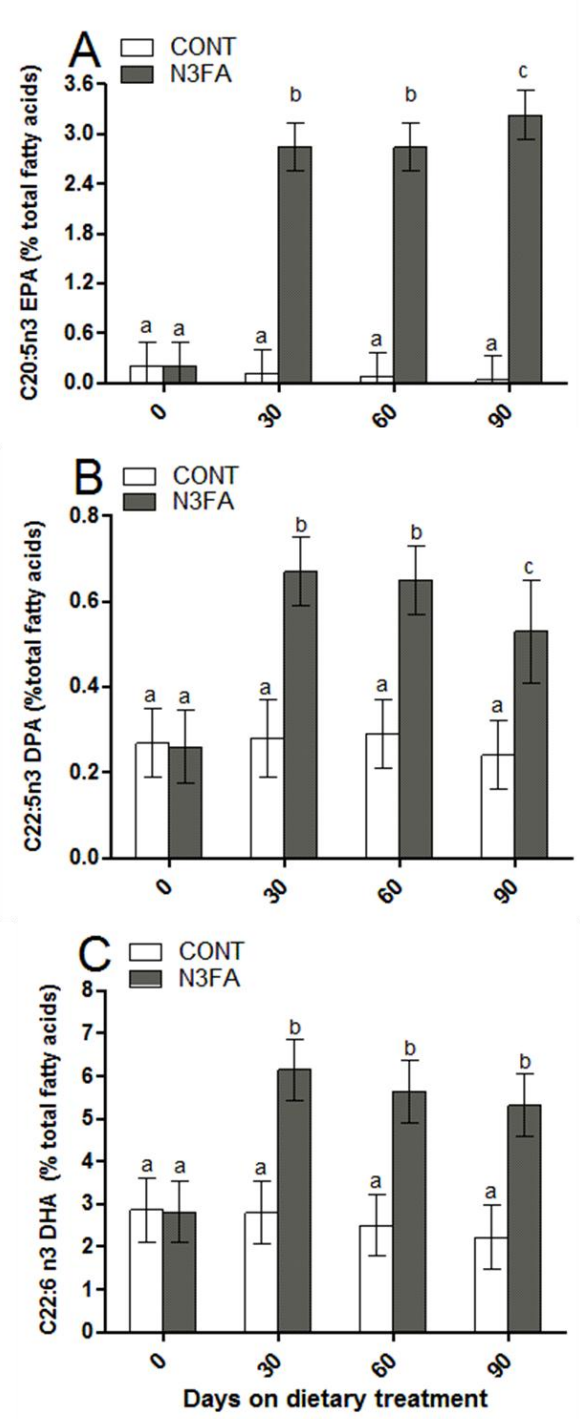

Figure 4.2 Effect of dietary treatment on serum phospholipid compositions. (A) Eicosapentaenoic Acid: columns lacking common letter indicate a treatment X time effect (P<0.0001). (B) Docosapentaenoic Acid: columns lacking common letter indicate a treatment X time effect (P<0.0001). (C). Docosahexaenoic Acid: columns lacking common letter indicate a treatment X time effect (P<0.0001). Data are displayed as means ± 95% confidence intervals. (CONT=control diet; N3FA=algae/fish oil diet)

Synovial fluid fatty acid incorporation

Synovial fluid total fatty acid percentages for LA, ALA, ARA, EPA, DPA and DHA did not differ among horses prior to the start of dietary treatment (day 0). Significant (P=0.011) decreases in synovial fluid LA% (Figure 4.3A) occurred in both groups of horses from day 0 (CONT: 34.87+/-3.46 % total FA; N3FA: 38.04 +/- 3.46 % total FA) to day 90 (CONT: 32.60 +/- 3.45 % total FA; N3FA: 30.69 +/-3.45 % total FA); however, there were no differences between dietary treatments. Alpha linolenic acid % (Figure 4.3B) in synovial fluid did not differ among treatment groups (CONT or N3FA) at any time point; however, there was a significant (P<0.0001) decrease in ALA% from baseline (CONT: 0.89 +/- 0.20 % total FA; N3FA: 1.047 +/- 0.20 % total FA) compared to day 90 (CONT: 0.48 +/- 0.20 % total FA; N3FA: 0.35 +/- 0.20 % total FA). Synovial fluid ARA % total FA (Figure 4.3C) also decreased significantly (P=0.014) in both dietary groups from day 0 (CONT: 1.49 +/-0.25 % total FA; N3FA: 1.63 +/-0.21 % total FA) to day 90 (CONT: 1.16 +/-0.21 % total FA; N3FA: 1.48 +/-0.21 % total FA) of feeding; however, N3FA horses tended to have higher %ARA overall (P=0.06). Eicosapentaenoic acid (Figure 4.4A) was non-detectable in the synovial fluid of any horse prior to treatment (day 0) or in the synovial fluid of CONT horses at day 90 of feeding. Horses receiving the EPA/DHA supplement had measureable levels of synovial fluid EPA% (1.49 +/- 0.04 % total FA) after 90 days of dietary supplementation. Synovial fluid DPA % was detectable and did not differ among horses at baseline (CONT: 0.41 +/- 0.06 % total FA; N3FA: 0.39 +/- 0.06 % total FA). These percentages significantly decreased from day 0 to day 90 (P<0.001) with a more significant decrease (P=0.005) at day 90 in CONT horses (0.17 +/-0.06 % total FA) compared to N3FA horses (0.31 +/- 0.06 % total FA). Docosahexaenoic acid levels in synovial fluid (Figure 4.4C)

were similar at day 0, regardless of dietary treatment; yet only N3FA horses exhibited significant increases ($P<0.0001$) after 90 days of dietary supplementation (1.82 +/- 0.14% total FA).

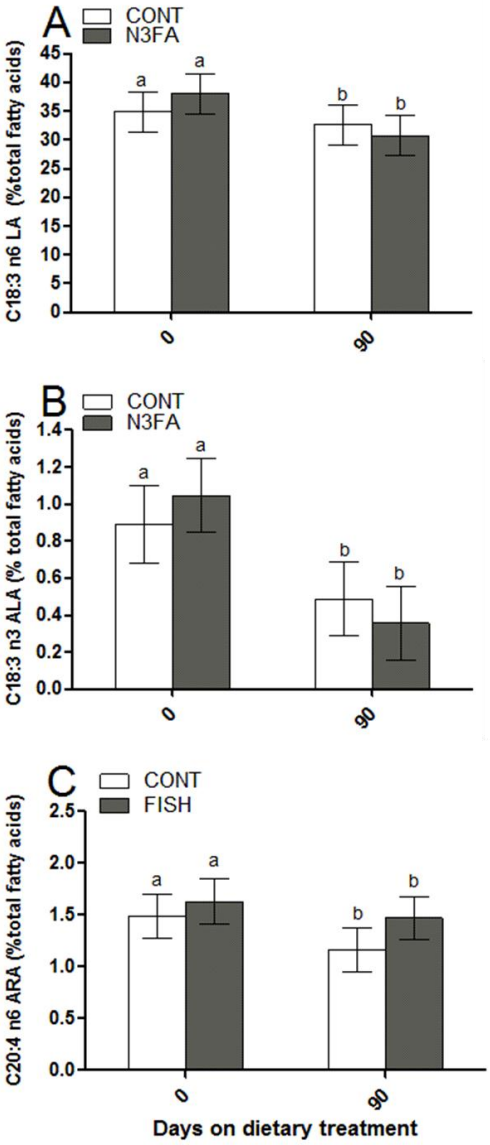

Figure 4.3 Effect of dietary treatment on synovial fluid lipid concentrations. (A) Linoleic Acid (LA): columns lacking common letter indicate a time effect (P=0.001). (B) Alpha Linolenic Acid (ALA): columns lacking common letters indicate a time effect (P<0.0001). (C). Arachidonic Acid (ARA): columns lacking common letters indicate time effect (P=0.014). Overall, N3FA group tended to have higher % ARA (P=0.068). Data are displayed as means ± 95% confidence intervals. (CONT=control diet; N3FA=algae/fish oil diet)

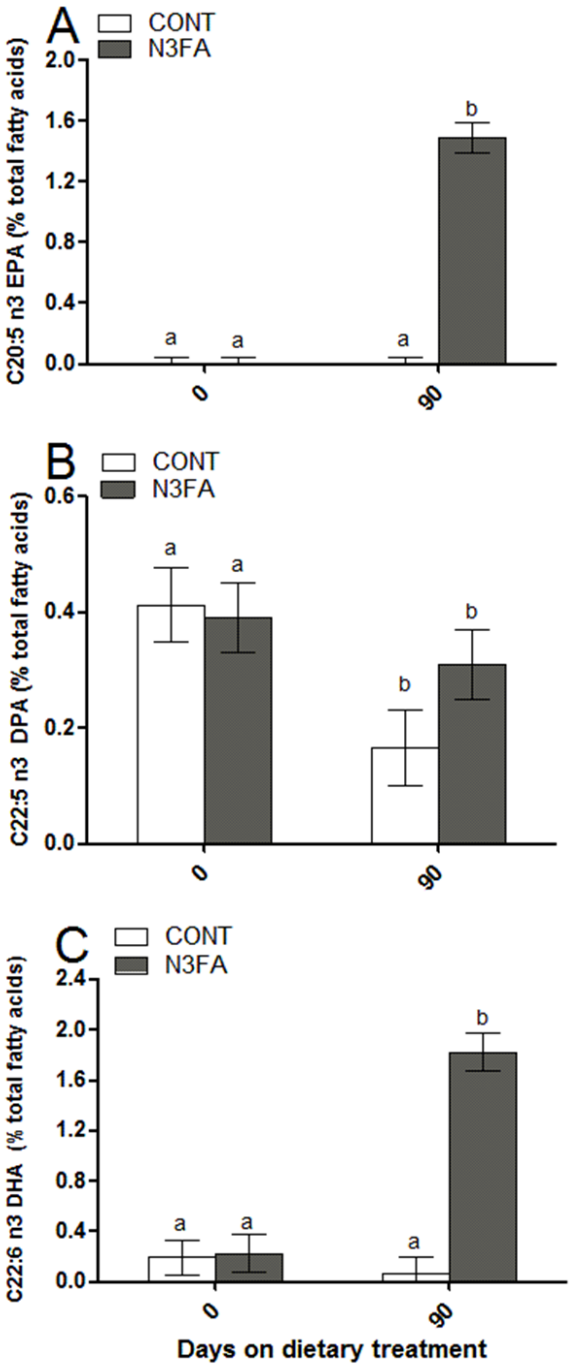

Figure 4.4 Effect of dietary treatment on synovial fluid lipid concentrations. (A) Eicosapentaenoic Acid (EPA): columns lacking common letter indicate a treatment X time effect (P<0.0001). (B) Docosapentaenoic Acid (DPA): columns lacking common letter indicate a time effect (P=0.005). (C). Docosahexaenoic Acid (DHA): columns lacking common letter indicate a treatment X time effect (P<0.0001). Data are displayed as means ± 95% confidence intervals. (CONT=control diet; N3FA=algae/fish oil diet)

Clinical response (vital signs) to intra-articular injections

All horses exhibited vital measurements that were within normal, resting ranges at PIH 0. Respiration, heart rate and temperature values exhibited significant time effects; however, there were no differences between dietary treatment groups. Average temperatures (Figure 4.5A) were elevated at PIH 12 when compared to PIH 0, peaking at PIH 16 and remaining above baseline values through PIH 24. Respiration (Figure 4.5B) was elevated significantly from baseline (PIH 0) at PIH 14 through PIH 22, returning to baseline values by PIH 24. Heart rates (Figure 4.5C) were significantly higher at PIH 10-22 compared to resting values at PIH 0-6. While there were significant time effects with each parameter, all values were within normal ranges for healthy horses and were unchanged from baseline values through PIH 10.

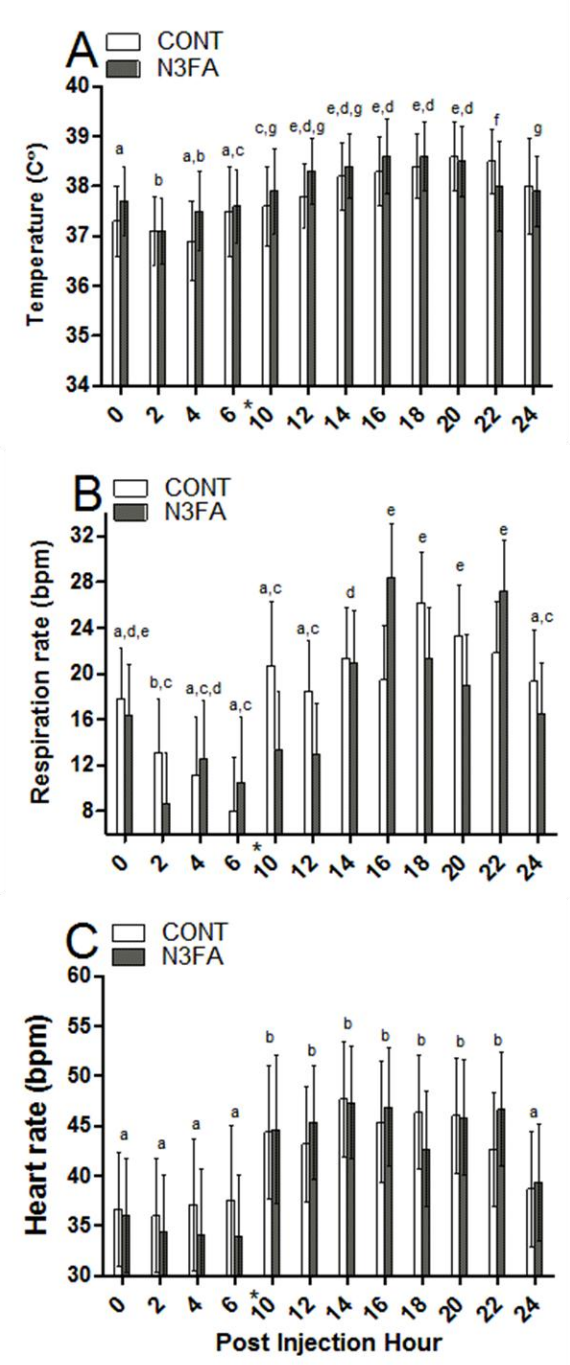

Figure 4.5 Clinical responses to intra-articular injections. Columns lacking common letters indicate significant differences across time points (P<0.05). *Indicates time point when arthroscopic biopsy was performed. Data are displayed as means ± 95% confidence intervals.

Clinical response (lameness) and synovial fluid analysis to intra-articular injections

Clinical signs (lameness evaluation) and fluid biomarkers (WBC, total protein, PGE_2, and general MMP activity) did not differ between day 0 (prior to beginning dietary treatment) and day 91 (prior to start of experimental synovitis model) (Table 4.4). Prostaglandin E_2 concentrations in synovial fluid collected from N3FA horses tended to be lower (P=0.08) on day 91 compared to day 0, while there were no difference for CONT horses (P=0.84). Synovial GAG levels did not differ among horses; however, concentrations were significantly lower (P=0.03) on day 91 (70.57 +/- 6.33 µg/ml) compared to day 0 (62.15 +/- 6.33 µg/ml).

Table 4.4 Synovial fluid biomarkers Day 0 vs. Day 91 of dietary treatment

	Treatment Groups[1]	
Item[2]	**CONT**	**N3FA**
Lameness (1-5 scale)		
Day 0	0 (ND)[a]	0 (ND)[a]
Day 91	0 (ND)[a]	0 (ND)[a]
WBC (cells/µl)		
Day 0	255.01 +/- 95.89[a]	239.20 +/- 102.32[a]
Day 91	183.33 +/- 91.52[a]	216.70 +/- 95.98[a]
Total Protein (g/dl)		
Day 0	2.81 +/- 0.32[a]	3.15 +/- 0.43[a]
Day 91	2.56 +/- 0.44[a]	3.04 +/- 0.46[a]
PGE_2 (pg/ml)		
Day 0	34.10 +/- 9.95[a]	43.17 +/- 9.21[a]
Day 91	35.25 +/- 7.54[a]	29.41 +/- 7.54[a]
General MMP (ΔRFU/sec)		
Day 0	0.06 +/- 0.03[a]	0.10 +/- 0.04[a]
Day 91	0.04 +/- 0.04[a]	0.07 +/- 0.04[a]
GAG (µg/ml)		
Day 0	68.63 +/- 8.48[a]	72.52 +/- 9.39[a]
Day 91	67.15 +/- 8.48[a]	57.14 +/- 9.39[a]

[1]CONT: basal control diet; N3FA: algae and fish oil supplement added to basal diet.
[2]WBC: white blood cells; PGE_2: prostaglandin E_2; MMP: matrix metalloproteinase; ND: non-detectable; RFU: relative fluorescence units; GAG: glycosaminoglycan. No significant difference between joints; therefore, values were pooled. Values expressed as mean +/- 95% confidence interval. Rows and columns for each biomarker: different superscripts indicate significant difference (P<0.05)

All horses were free of lameness prior to start of experimental synovitis model (PIH 0). Lameness (Figure 4.6) was detected by PIH 4 in all reIL-1β injected joints, with significantly higher scores observed at PIH 8. Lameness in all horses subsided by PIH 24 and returned to PIH 0 values by PIH 240.

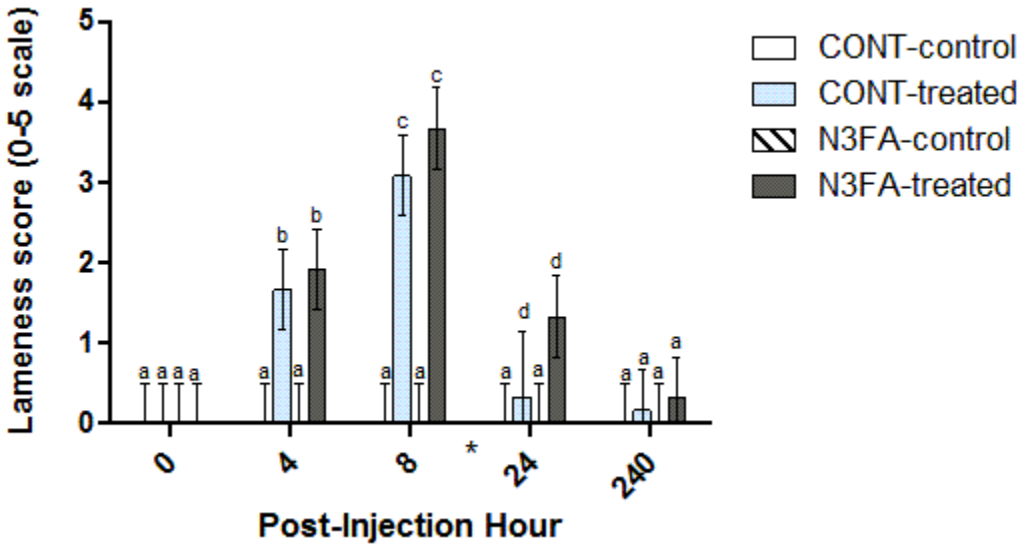

Figure 4.6 Clinical response to intra-articular injection of reIL-1β (100ng) [treated] or PBS [control]on lameness grade. Columns lacking common letters indicate a time X joint effect (P<0.0001). *Indicates time point when arthroscopic biopsy was performed. Data are displayed as means ± 95% confidence intervals.

White blood cell (WBC) (Figure 4.7A) and neutrophil counts (Figure 4.7B) increased significantly in all reIL-1β treated joints (regardless of diet) starting at PIH 4 through PIH 24 when compared to baseline values. Control joint white blood cell counts exhibited modest, yet significant increases at PIH 4 and 8 and remained elevated through PIH 24. Neutrophil levels in control joints were significantly higher at PIH 4 through PIH 24 compared to PIH 0. There were no significant differences in WBC or neutrophil counts between dietary treatments; however,

there was a trend for horses receiving the N3FA diet to have higher neutrophil counts (P=0.059) when all time points were considered. Synovial fluid total protein concentrations (Figure 4.7C) increased in all joints (regardless of dietary treatment) at PIH 4 and remained elevated through PIH 24. Horses receiving an EPA/DHA supplement exhibited significantly higher (P=0.003) levels of total protein (4.85 +/- 0.34 g/dl) when compared to CONT horses (4.02 +/- 0.34 g/dl). All synovial fluid biomarkers in reIL-1 β treated joints remained higher than PIH 0 levels through PIH 24 (16 hours post-surgery) and contra-lateral joints injected with saline remained unchanged from PIH 0 through PIH 8, with significantly higher levels detected at PIH 24.

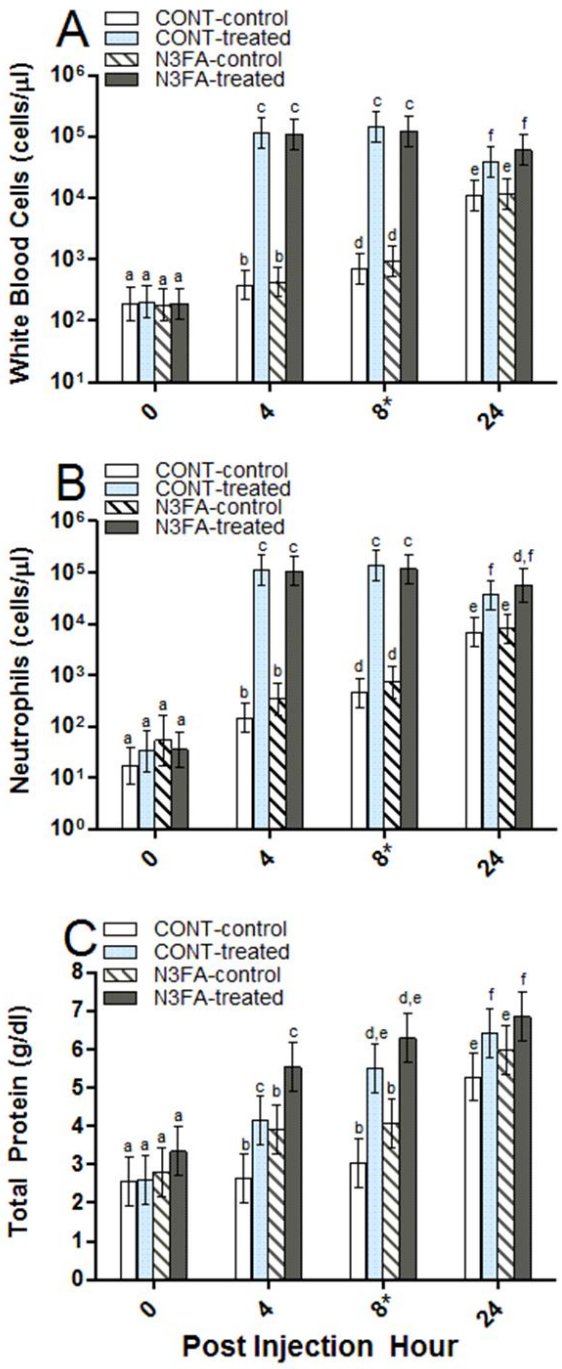

Figure 4.7 Cytology evaluation of synovial response to intra-articular injection of reIL-1β (100ng) [treated] or PBS [control]. For each outcome, columns lacking common letters indicate significant differences between joints (treated, control), and time points. (A) White blood cells (P<0.001). (B) Neutrophils (P<0.001). When including all time points, N3FA tended to have higher neutrophils (P=0.059) (C) Total protein (P<0.0001). When including all time points, N3FA group had higher synovial total protein level (P=0.003). *Indicates time point when arthroscopic biopsy was performed. Data are displayed as means ± 95% confidence intervals.

Synovial prostaglandin E_2 concentrations (Figure 4.8A) were within normal values and did not differ between horses or joints prior to an intra-articular injection of reIL-1β. There were significant increases ($P<0.001$) in PGE_2 in all reIL-1β treated joints from PIH 4-PIH 24 compared to baseline values (PIH 0) regardless of dietary treatment. All saline-injected joints expressed mild, yet significant increases ($P<0.001$) in PGE_2 concentrations above baseline (PIH 0) values; however, PGE_2 concentrations did not differ between PIH 4, 8 and 24. When considering all time points there was a trend ($P=0.08$) for N3FA horses to have lower synovial PGE_2 (702.35 +/- 393.58 pg/ml)) compared to horses receiving no supplementation (950.92 +/- 388.78 pg/ml).

Synovial fluid general MMP activity was similar among all horses and did not differ among joints prior to intra-articular injection of either reIL-1β or PBS (PIH 0). After injection, general MMP activity did not differ between CONT or N3FA horses at any time point. MMP activity increased ($P<0.001$) in all treated joints by 4 hours post injection (CONT: 0.14 +/-0.09 RFU/s; N3FA: 0.21 +/- 0.08 RFU/s), remaining elevated over PIH 0 values through PIH 24 (CONT: 0.37 +/- 0.107 RFU/s; N3FA: 0.46 +/- 0.12 RFU/s). Treated joint MMP activity was significant higher ($P=0.003$) than control joint MMP activity at PIH 4-24.

Glycosaminoglycan (GAG) concentrations (Fig 4.8C) were consistent among all horses and joints prior to injections (PIH 0). A 90 day dietary N3FA supplementation resulted in significantly lower synovial GAG concentrations compared to pre-dietary treatment (day 0) ; however, there were no differences between CONT and N3FA concentrations at day 90. Additionally, dietary treatment had no effect on GAG concentrations during the experimental bout of synovitis. Both PBS- injected and reIL-1β-injected joints demonstrated similar and

significantly higher (P<0.001) GAG concentrations at PIH 4 (CONT-control: 237 +/- 119 µg/ml; CONT-treated: 235 +/- 106 µg/ml; N3FA-control: 219 +/- 125 µg/ml; N3FA-treated: 218 +/- 106 µg/ml), peaking at PIH 24 (CONT-control: 500 +/- 106 µg/ml; CONT-treated: 269 +/- 129 µg/ml; N3FA-control: 371 +/- 116 µg/ml; N3FA-treated: 394 +/- 129 µg/ml).

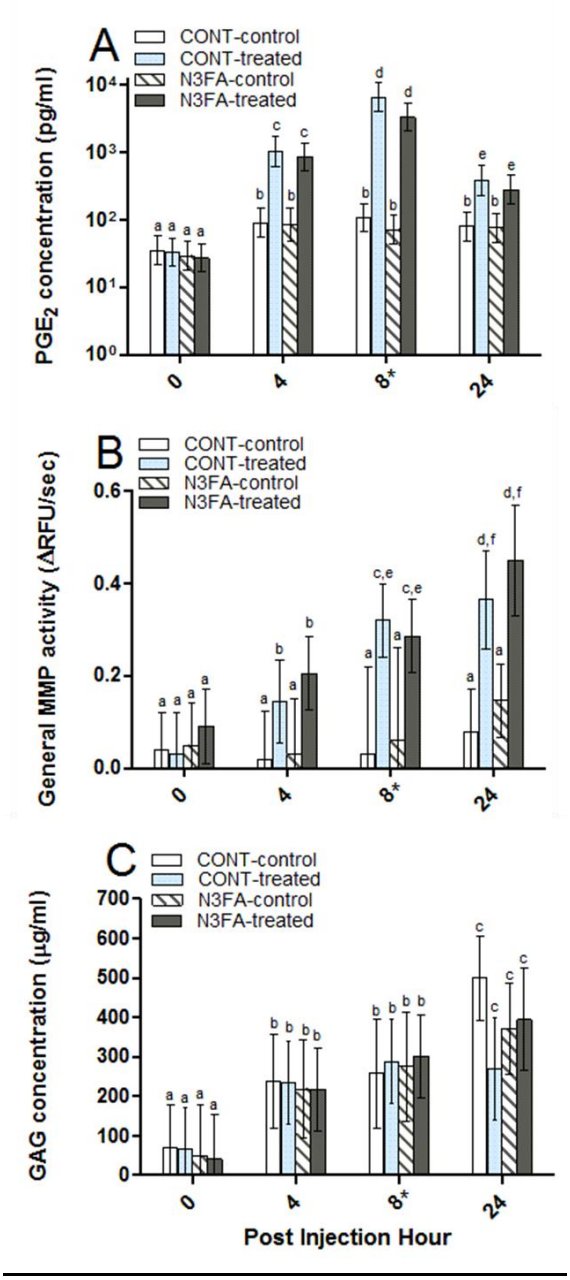

Figure 4.8 Synovial fluid response to intra-articular injection of reIL-1β (100ng) [treated] or PBS [control]. (A) PGE_2 concentration: columns lacking common letters indicate differences between joints (treated, control), and time points. (P<0.001). N3FA group tended to have overall lower synovial PGE_2 levels (P=0.08). (B) General MMP activity: columns lacking common letters indicate significant differences across joints and time points (P<0.001). (C). Glycosaminoglycan (GAG)concentration: columns lacking common letters indicate significant differences between joints and time points (P<0.001); *Indicates time point when arthroscopic biopsy was performed. Data are displayed as means ± 95% confidence intervals.

Gene expression in joint tissues at post injection hour 8 (Synovium)

Synovium expression of TNF-α and ADAMTS-5 was significantly higher (P<0.01) in reIL-1β treated joints (TNF-α: 3.2 fold higher; ADAMTS-5: 3.3 fold higher) compared to control joints (Figure 4.9); regardless of dietary treatment. There was significantly higher (P<0.001) expression of MMP-1 (175 fold higher); COX-2 (33.3 fold higher) and IL-1β (21 fold higher) in reIL-1β treated joints compared to control joints (Figure 4.10A-C). Gene expression of synovium ADAMTS-4 (Figure 4.10D) in reIL-1β treated joints was 55-fold higher than in control tissues (P<0.0001) with CONT horses having significantly higher (2 fold increase) expression than N3FA horses (P=0.03).

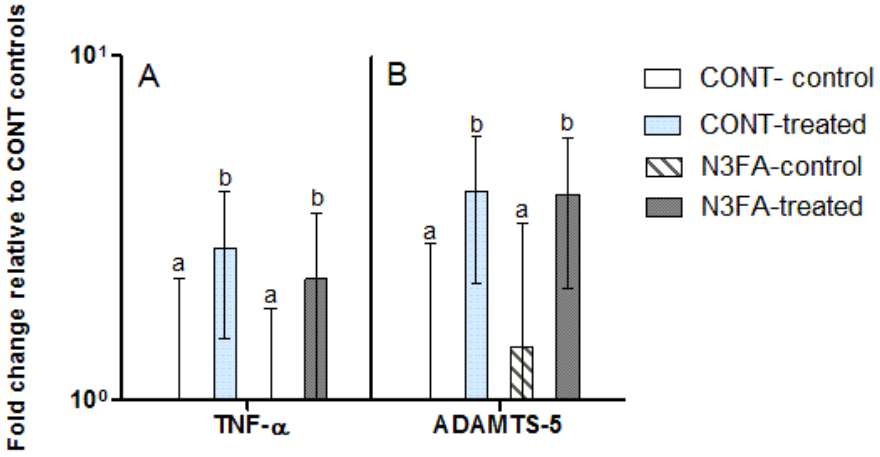

Figure 4.9 Effect of single intra-articular injection of reIL-1b (100 ng) [treated] or PBS [control] on synovium gene expression at 8 hours post injection. Values expressed as fold difference relative to CONT controls. (A) TNF-α gene expression: columns lacking common letters indicate a significant difference between joints (P=0.015). (B) ADAMTS-5 gene expression: columns lacking common letters indicate significant difference between joints (P=0.002). TNF-α=Tumor necrosis factor alpha. ADAMTS-5=A disintegrin and metalloproteinase with a thrombospondin type motif class 5 (aggrecanase 2). Data are displayed as means ± 95% confidence intervals.

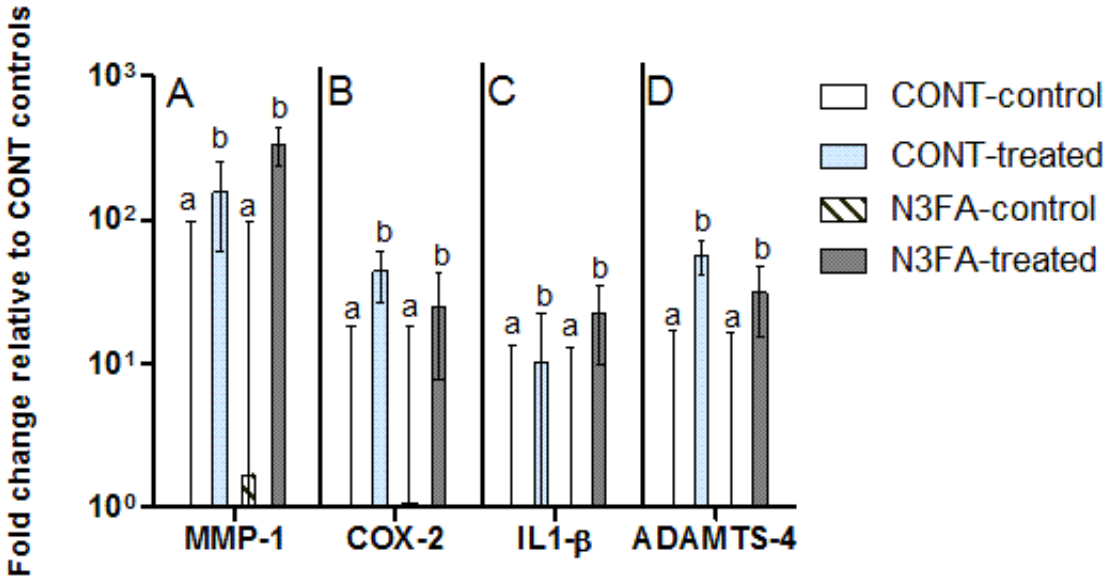

Figure 4.10 Effect of single intra-articular injection of reIL-1b (100 ng) [treated] or PBS [control] on synovium gene expression at 8 hours post injection. Values expressed as fold difference relative to CONT controls. (A) MMP-1 gene expression: columns lacking common letters indicate a significant difference between joints (P<0.0001). (B) COX-2 gene expression: columns lacking common letters indicate a significant difference between joints (P<0.001). (C) IL-1b gene expression: columns lacking common letters indicate significant difference between joints (P<0.001). (D) ADAMTS-4 gene expression: columns lacking common letters indicate significant difference between joints (P<0.0001). Overall, N3FA had lower expression of ADAMTS-4 (P=0.03). ADAMTS-4=A disintegrin and metalloproteinase with a thrombospondin type motif class 4 (aggrecanase 1). COX-2=cyclooxygenase II. IL-1β= Interleukin 1 Beta. MMP-1=Matrix Metalloproteinase 1 (collagenase 1). Data are displayed as means ± 95% confidence intervals.

Gene expression in joint tissues at post injection hour 8 (Articular cartilage)

A 90 day dietary treatment of an EPA/DHA rich supplement (N3FA) resulted in no significant difference in gene expression of cartilage MMP-1, MMP-13, IL-1β, ADAMTS-4, ADAMTS-5 or COX-2 compared to horses that did not receive EPA/DHA supplementation (CONT). Expression of TNF-α was beyond the detection limit of the assay for all cartilage samples. Gene expression of MMP-1 and COX-2 were also undetectable in control cartilage samples; therefore, those data were not included in the statistical analysis. Treated (reIL-1β) cartilage samples obtained from N3FA horses had 4.3 fold higher expression of MMP-1 and 1.7

fold higher expression of COX-2 compared to samples collected from CONT horses; however, level of expression was not statistically significant between groups. There was significantly higher (P<0.001) expression of MMP-13 (8.2 fold higher); IL-1β (8.1 fold higher); ADAMTS-4 (38.5 fold higher) and ADAMTS-5 (7.5 fold higher) in reIL-1β treated joints compared to control joints (Figure 4.11A-D), regardless of dietary treatment group.

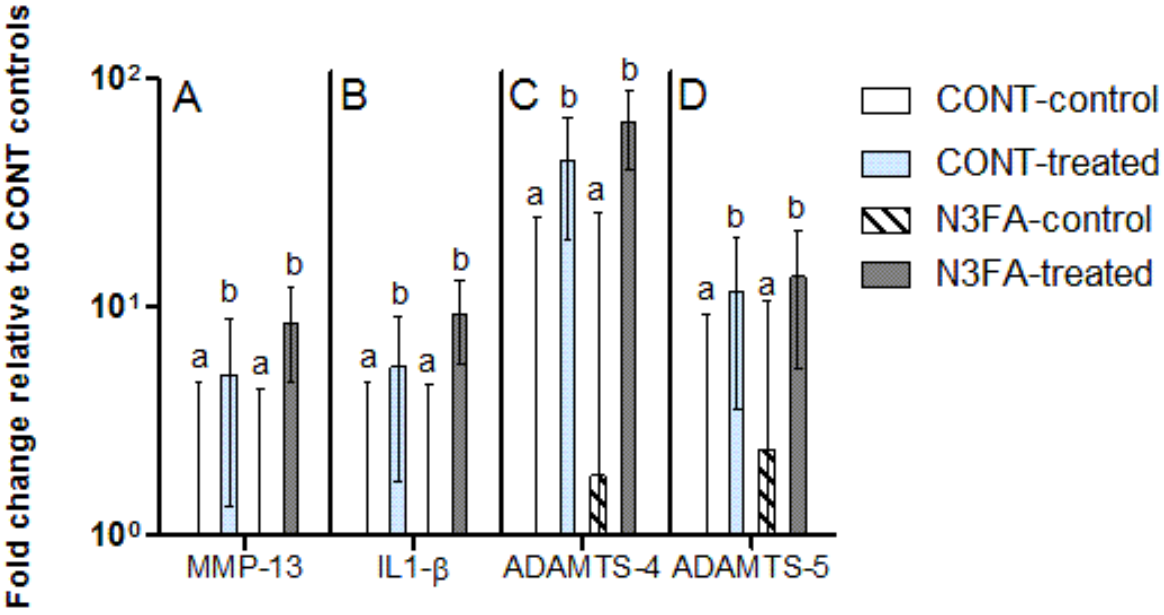

Figure 4.11 Effect of single intra-articular injection of reIL-1b (100 ng) [treated] or PBS [control] on cartilage gene expression at 8 hours post injection. Values expressed as fold difference relative to CONT controls. (A) MMP-13 gene expression: columns lacking common letters indicate a significant difference between joints (P=0.005). (B) IL-1b gene expression: columns lacking common letters indicate significant difference between joints (P=0.003). (C) ADAMTS-4 gene expression: columns lacking common letters indicate significant difference between joints (P<0.0001). (D) ADAMTS-5 gene expression: columns lacking common letters indicate significant difference between joints (P=0.001). ADAMTS-4=A disintegrin and metalloproteinase with a thrombospondin type motif class 4 (aggrecanase 1). ADAMTS-5=A disintegrin and metalloproteinase with a thrombospondin type motif class 5 (aggrecanase 2). IL-1β= Interleukin 1 Beta. MMP-13=Matrix Metalloproteinase 13. Data are displayed as means ± 95% confidence intervals.

Discussion

The use of oral n-3 polyunsaturated fatty acid supplementation to modify inflammatory responses has been documented in multiple species (Terano et al., 1986; Hansen et al., 2008) including the horse (Hall et al., 2004; Manhart et al., 2009). To the best of our knowledge, this is the first *in-vivo* investigation of oral n-3 LCPUFA supplementation and the influence on the inflammatory response to an experimental model of synovitis in the horse.

The observed higher feed refusal for the N3FA group was limited to a single horse; however, the refusal amount was insignificant to the outcome of the study as all fatty acid measurements were normally distributed within the N3FA group. The lower feed intake reported during the first 30 days compared to the remaining 60 days of dietary treatment was unexpected and believed to be due to variation in hay quality that presented palatability issues. While there was a notable statistical difference in feed intake, body weights and body condition scores did not differ between treatment groups or time points during the 90 day feeding trial. The lack of difference within and between treatment groups for BW and BCS was expected as diets were adjusted accordingly to ensure measurements were consistent throughout the study.

This study demonstrated that feeding an oral n-3 LCPUFA supplement was effective in increasing EPA and DHA in both serum phospholipids and synovial fluid lipid profiles. Serum and phospholipid compositions are assumed to reflect medium-term dietary fat intake while triglyceride fatty acid composition better illustrates short-term dietary fat intake (Riboli et al., 1987; Glatz et al., 1989). Significant increases at days 30-90 in serum phospholipid compositions of LA above baseline levels in the CONT horses, but not in the N3FA horses is unexpected as the amount of dietary LA was similar between diets. It is possible LA is being converted to ARA

in the N3FA horses to compensate for the presence of EPA and DHA in the diet. These findings are comparable to previously reported fatty acid analysis of fluid and tissue samples collected from horses receiving a similar amount of dietary EPA and DHA (Hess et al., 2012). Decreases in the serum phosphoplid levels for ALA in both CONT and N3FA horses during the 90 day feed trial are likely due to composition of ALA in the diets. The previous diets of the horses is unknown; however, they had unlimited access to hay prior to the 14 day adaptation where they were restricted and group fed approximately 1.% of their BW per day in timothy hay. The significant decrease in serum phospholipid % for ALA is likely due to less ALA in the experimental diets. Significantly higher serum phospholipid levels of ARA, EPA, DPA and DHA in the N3FA horses compared to controls may be due to a higher concentration these respective fatty acids in the N3FA supplement. A tracer study would be needed to compare exogenous sources to endogenous production of these fatty acids to further elucidate these findings. Previous equine studies utilizing a fish oil or algae source of EPA and DHA have reported higher levels of ARA in horses receiving n-3 PUFA supplementation compared to control horses (Hall et al., 2004a, Vineyard et al., 2009; King et al., 2008) due to the natural occurrence of a modest (0.67g/100g FA compared to non-detectable levels in the hay and concentrate), amount of ARA in the supplement. This is in contrast to what has been reported in humans where direct supplementation of EPA and DHA decreased concentrations of plasma ARA (Arterbrun et al., 2006). It should be noted that purified EPA and DHA were administered in the 2006 study; preventing an exogenous source of ARA in the dietary treatments. Additionally, it was been shown that human diets void of ARA resulted in lowered plasma concentrations of ARA despite sufficient dietary intake of LA, indicating a decreased capacity of conversion to ARA (Adam et al., 2003). This is the case in horses, especially in those consuming

only forage-based diets. Hess and others (2012) reported no detectable levels of ARA in a marine-derived n-3LCPUFA supplement, yet ARA was highest in the plasma and red blood cell of horses receiving the supplement for 90 days compared to horses receiving no supplementation or an ALA-supplemented diet. Increases in serum concentrations of EPA, DPA and DHA in the N3FA horses in the current study coincides with dietary supplementation and is consistent with previous reports of dose-dependent increases in the plasma of horses receiving either a marine-derived n-3 LCPUFA pellet or top-dressed fish oil (Hall et al., 2004a, O'Connor et al., 2004; Woodward et al., 2007; King et al., 2007; Manhart et al., 2009; Vineyard et al., 2010).

To the best of our knowledge, we report the first complete evaluation of synovial fluid lipid composition in response to dietary treatment in the horse. We previously reported (Ross et al., 2010) that synovial fluid lipid levels for EPA and DHA differed significantly among horses receiving various sources of n-3 PUFA supplementation for 90 days. In the present study, the significant increase from day 0 to day 91 in synovial fluid EPA (100 % increase) and DHA (727% increase) from day 0 to day 91 are consistent with the increased incorporation of lipids found in the parallel sampling of serum from N3FA horses. The decrease in synovial fluid ARA in all horses after 90 days of feeding potentially reflects the significant decrease in synovial LA in all horses during the same time period. Though not statistically different from the CONT group at day 91, the lesser change in synovial ARA in the N3FA horses may be due to presence of ARA in the N3FA diet.

A single injection of 100 ng of recombinant equine IL-1β into mid-carpal joints resulted in no significant change in vital signs in all horses during the first 8 hours post-injection. Changes in respiration, temperature and heart rate at PIH 10-22 are likely due to residual effects

of anesthesia from the arthroscopic biopsy and not a response to the reIL-1β intra-articular injection. Lameness in horses treated with reIL-1β was detected by 4 hours post-injection, which is similar to what our laboratory has previously reported (see Chapter III). In the current study, we observed an increase in lameness scores from PIH 4 (average grade 2) to PIH 8 (average grade 3.5) which subsided within 24 hours of injection. The decrease in lameness at 24 hours post injection is expected; probably from lavage of the joints during surgery and/or from the duration in time from previous sampling at PIH 8 (16 hour delay). Dietary treatment did not significantly influence the outcome of lameness among horses, indicating that the n-3 PUFA supplementation did not modify the clinical signs of joint inflammation.

In the present study, the acute yet temporary bout of synovitis that was induced by reIL-1β injection was consistent with our previous work using this model of joint inflammation. The inflammatory response, as measured by white blood cell concentrations, did not differ between dietary treatments despite daily intake of approximately 40g of EPA/DHA in the N3FA horses. This differs from previous research where horses receiving 34.8g of EPA/DHA daily exhibited significantly lower synovial WBC levels over 90 days of supplementation compared to non-supplemented horses (Manhart et al., 2009); however, those horses had late-stage OA and likely suffered from chronic, low-grade inflammation that was present prior to starting the study. The horses in the current study were all healthy animals that exhibited an acute response to experimentally-induced synovitis.

In the current study, horses receiving 90 days of n-3 LCPUFA supplementation tended to have higher synovial neutrophils counts and had significantly higher synovial total protein compared to non-supplemented horses. The mechanism for this effect is unclear; however, it is

possible EPA/DHA supplementation may influence macrophage TNF-α production during an inflammatory response, causing increased permeability of the surrounding tissue, leading to an increased concentration of proteins and neutrophils. This was a hypothesis present by Vineyard and others (2009) to explain a significantly higher inflammatory response in yearling horses receiving a protected EPA/DHA supplement (~27g EPA/DHA daily) compared to controls. Similarly, TNF-α production increased in bronchoalevolar lavage fluid cells obtained from horses that received either a fish oil supplementation (~49 g n3FA) for 8 weeks (Hall et al., 2004b). It is unknown if a similar effect occurred in the N3FA horses as TNF-α levels were not measured in the current study.

While the N3FA horses tended to have lower synovial PGE_2 concentrations compared to the CONT horses, the n-3 LCPUFA supplementation did not significantly modify PGE_2 levels in the reIL-1β treated joints. Research supports the effect of n-3 LCPUFAs in reducing PGE_2 production in multiple species (as reviewed by Cleland et al., 2003) including equine in-vitro (Munsterman et al., 2005; Hall et al., 2004b) and in-vivo models (Manhart et al., 2009). However; a 70 day feeding trial of EPA/DHA supplementation (27g/d) to healthy, yearling horses resulted in no change in LPS-stimulated PGE_2 production from equine peripheral blood mononuclear cells (Vineyard et al., 2009) nor was plasma PGE_2 production modified in horses receiving 4.6g of EPA/DHA daily for 75 days (Woodward et al., 2007). Synovial fluid fatty acid concentrations of EPA and DHA in the present study may have been insufficient to modify prostaglandin production during the induced episode of inflammation.

There are a few hypotheses as to the lack the ability of a dietary treatment to modify the inflammatory response of the current experimental model. While both EPA and DHA were

significantly higher in the synovial fluid of N3FA horses after 90 days of supplementation, the reported synovial fluid levels (as a % of total lipids) of these specific fatty acids may be insufficient to have a significant biological effect. For humans, a dosage of 2.6 g/day of EPA/DHA is recommended to provide symptomatic relief of rheumatoid arthritis (Cleland et al., 2005). This dietary amount of EPA corresponds to a 3.2% EPA plasma phospholipid composition; the minimum level of plasma EPA that is required to be effective in suppressing inflammatory cytokines (Cleland et al., 2003). If this recommended amount was used to extrapolate an equivalent dose in the horse, using an average of 70 kg of BW for a human, then a dosage of 18.6 g of EPA/DHA (11.4 g being EPA) for a 500 kg horse would be needed. The reported intake of 36.1 g of EPA/DHA (9.7g being EPA) in N3FA horses in the current study resulted in serum EPA phospholipid levels of 3.23% after 90 days of supplementation. It is unknown as to what amount of dietary EPA and DHA is needed to alter physiological responses in the equine joint, therefore it is difficult to determine if the percentage of synovial EPA and DHA we report are biologically appropriate. The near equivalent relative levels of ARA and EPA (1.47% and 1.49%, respectively) in the N3FA synovial fluid at day 91 may have resulted in EPA being unable to adequately suppress ARA-derived eicosanoid production. Additionally, cyclooxygenases have a stronger preference for ARA, especially during a robust inflammatory response or during OA (Marcouiller et al., 2005), while EPA may be a more effective substrate for lipoxygenases and leukotriene B_5 production (Terano et al., 1986). While not analyzed in the current study, incorporation of n-3 long chain fatty acids into the synovial membrane may have been limited. Henry and others (1991) reported that linseed oil (an ALA-rich plant oil) supplementation in horses failed to reduce endotoxin-stimulated eicosanoid production below levels of non-supplemented horses, and this was likely due to an inability of the n-3 long chain

fatty acids to be converted and incorporated into the membranes of cells responsible for eicosanoid production.

It is also plausible that the dose of reIL-1β used in the current study may have overwhelmed the anti-inflammatory properties of EPA and DHA. Control joints injected with a single dose of PBS (1 ml) demonstrated a low-grade inflammation response, evident by increased WBC and PGE_2 concentrations 4 hours post-injection, yet no difference between N3FA and CONT horses was reported.

One limitation of the current study is the analysis of one single eicosanoid: ARA-derived PGE_2. While this eicosanoid plays a vital role in inflammation and OA, a larger spectrum analysis of other eicosanoids, including thromboxanes would be helpful to determine what additional eicosanoids may be produced during synovitis and to what extent EPA-derived eicosanoids are manufactured. The recent reports of novel methods for detecting synovial fluid eicosanoids in horses (de Grauw et al., 2011) and phospholipids in equine synovial fluid and serum (Fuchs et al., 2008) may be valuable tools for monitoring joint response to inflammation and evaluating dietary therapeutics for OA.

Interleukin stimulation increased general MMP activity in treated joints, although no effect of diet was observed. These results differ from previous studies where EPA/DHA supplementation decrease in MMP-2 expression in dogs (Hansen et al., 2008) and OA-prone guinea pigs (Knott et al., 2011). In these two studies, inflammation was not induced; therefore, the absence of treatment difference for MMP activity in the current study may be due to the acuteness of the synovitis model.

The report of significantly lower synovial GAG concentrations in both CONT and N3FA horses at day 91 compared to day 0 is unknown as it was unrelated to dietary treatment. Multiple sampling of synovial fluid during the 90 day feed trial would have been needed to better evaluate the changes we reported. An in-vitro application of IL-1β induced sulfated-glycoaminoglycan release from bovine cartilage that was modified by EPA and DHA treatment (Wann et al., 2010); however, oral supplementation of EPA/DHA in the current model resulted in no difference in GAG concentrations when compared to CONT group. The significant increase in GAG in all joints, regardless of dietary treatment, by PIH 4 was unexpected; further evaluation of additional markers would be needed to determine if these values indicate GAG synthesis or degradation. The peaks of synovial GAG concentrations at PIH 24 are likely due to the arthroscopic biopsy.

We are the first to report the evaluation of dietary n-3 LCPUFA supplementation on in-vivo synovial tissue gene expression in the horse. Intra-articular injection of reIL-1β resulted in the upregulation of gene expression of catabolic enzymes in synovium and cartilage that is consistent with previously reported in-vivo stimulation of equine joint tissue (see Chapter III). The synovium expression of TNF-α and ADAMTS-5 was significantly higher in treated joints compared to controls; similar to what has been reported in human synovium (Bondeson et al.,2006), with levels being upregulated in the synovium collected from OA horses (Kamm et al., 2010). A significantly lower expression of ADAMTS-4 in N3FA synovium, compared to CONT synovium, was observed indicating 90 day supplementation of EPA/DHA modified ADAMTS-4 synovium gene expression. To the best of our knowledge, this is the first study to report decreased synovium aggrecanse expression due to dietary supplementation of n-3 LCPUFA.

Studies of in-vitro models utilizing bovine chondrocytes have shown significant reduction in IL-1β stimulated expression of COX-2 (Hurst et al., 2009; Zaninal et al., 2009; Wann et al., 2010), ADAMTS-4, ADAMTS-5, MMP-13 (Zaninal et al., 2009; Wann et al., 2010) and IL-1β and TNF-α (Zaninal et al., 2009) by n3 PUFA, with EPA being the most effective at abrogating expression. We report no difference in mRNA expression in reIL-1β treated cartilage obtained from CONT and N3FA horses. It is also plausible that the absence of treatment effect with EPA/DHA supplementation on protein levels of biological markers contributes to the lack of difference in cartilage gene expression.

In this study, a 90 day dietary treatment of n-3 LCPUFA significantly altered serum phospholipid and synovial fluid lipid concentrations of EPA and DHA. The administration of reIL-1β (100 ng) resulted in a robust, yet temporary inflammatory response characterized by significant increases in cytology parameters, biological markers of inflammation and gene expression of degradative enzymes associated with cartilage degradation. Differences between dietary treatment groups (N3FA vs. CONT) were not noted for clinical response (lameness), white blood cell counts, eicosanoid production, matrix metalloproteinase activity or cartilage gene expression of catabolic eznyzmes. While it is possible the 100 n g dose of reIL-1β may have overwhelmed EPA's and DHA's ability to moderate inflammation during the initial hours post-injection, synovial fluid collection and analysis beyond PIH 24 may have demonstrated a dietary n-3 LCPUFA effect on the inflammatory response to reIL-1β. Future inquiries should include a wider evaluation of ARA-derived and EPA-derived eicosanoids during inflammation. The discovery of significantly lower expression of ADAMTS-4 in N3FA synovium supports further inquiry into the capability of n-3 LCPUFAs, in particular, EPA and DHA as potential therapies for equine OA.

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