

EFFECTS OF ORAL GLUCOSAMINE SULFATE SUPPLEMENTATION ON GAIT
PARAMETERS AND BLOOD OXIDATIVE STATUS IN THE AGED HORSE

by

ANDY NICHOLAS CARDEN

(Under the Direction of Kari Turner)

ABSTRACT

Degraded articular cartilage resulting from osteoarthritis and oxidative stress can affect both humans and animals. Glucosamine (GLN) sulfate is readily absorbed and distributed throughout the body. Glucosamine sulfate has been shown to have anti-inflammatory properties in animal models, regulate mRNA of matrix metalloproteinase1, and support the growth of the proteoglycans *in vitro*. Additionally, glucosamine sulfate has been shown to reduce induced oxidative stress *in vitro*. This study's purpose is to determine the effects of oral glucosamine sulfate supplementation on gait parameters and blood oxidative levels in the aged horse. Greater front fetlock dorsi flexion was observed in glucosamine supplemented horses as compared to controls. No differences were observed in plasma thiobarbituric acid reactive substances levels between control and treatment horses. Oral supplementation of glucosamine sulfate resulted in modest changes in biomechanical parameters measured. Differences in blood oxidative levels between treatments were not observed.

INDEX WORDS: Equine, glucosamine sulfate, glucosamine, anti-inflammatory, gait parameters, oxidative stress, osteoarthritis

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DEDICATION

I dedicate this work to my friends and colleagues Kylee Duberstein Ph.D., Kari Turner Ph.D., and Randy Eggleston DVM, Dip ACVS. Also I thank the University of Georgia's Department of Animal and Dairy Science for their support.

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CHAPTER 1

NORMAL ARTICULAR CARTILAGE

Introduction

Osteoarthritis (OA) is a potentially crippling degradation of the cartilage within the joint capsule that can affect both humans and animals including horses. In today's equine industry the horse is becoming more specialized in each unique discipline. From racing and jumping to reining cow horse and roping, the horse is continually developing into an intense athlete. These healthy and talented individuals are expected to be long-term athletes and companions, often performing well into their aged years. Unfortunately, OA's potentially crippling results can force an active equine athlete into retirement. Therefore, continued research in the area of equine osteoarthritis is important to the equine industry.

Cartilage Structure

The joint is made up of subchondral bone, cartilage, intracapsular ligaments, and the synovial fluid which is contained within the synovial membrane. Essentially, cartilage acts as a spacer between bone ends enabling the joint to absorb concussion and provide pain free, flexible motion (Caron, 2003). Joint stability is achieved by cartilage's tendency to swell and expand thus creating two opposing forces trying to push the bone ends apart. Joint stability is achieved when the muscles and ligaments prevent the separation (Freemont, 2006; Lorenz and Richter, 2006; Pearle *et al.*, 2005; Revell, 1998; Todhunter, 1996). This tension ultimately holds the joint together. Joint instability caused from cartilage loss can lead to joint disease and is the initial stage of osteoarthritis (Freemont, 2006).

Cartilage is made up of three types: hyaline, fibrocartilage, and elastic tissues. Of main concern, hyaline cartilage is located on the articulating surfaces of moveable joints. For this reason hyaline cartilage is also referred to as articular cartilage. Healthy articulating joints surfaces should appear firm and smooth (Caron, 2003; Lorenz and Richter, 2006; Todhunter, 1996). This tissue is avascular and is composed of mainly proteoglycans and fibrous collagen proteins. As a result of its avascularity, articular cartilage has poor regenerative ability and low metabolic activity (Hall, 1998; Pearle *et al.*, 2005). Therefore, damage to cartilage is extremely difficult to repair and in some instances is permanent. Another form of cartilage is fibrocartilage which is associated with dense connective tissue. Fibrocartilage is not involved in articulating surfaces but, it is found in the intervertebral discs of the spine, ligaments that attach to bone, and the pubic symphysis. Elastic cartilage is the third form of cartilage and appears as pliable, flexible fibers; hence, the name elastic. This cartilage is found mainly in structures such as the ear. Although not involved with articulating surfaces, these cells closely resemble those in articular cartilage as they are comprised of proteoglycans and collagens in addition to elastins (Hall, 1998).

There are four zones that form articular cartilage: the superficial or tangential zone, the middle, deep, and calcified zones (Caron, 2003; Hall, 1998; Lorenz and Richter, 2006; Pearle *et al.*, 2005; Todhunter, 1996). The superficial zone's main purpose is to provide a smooth gliding surface and withstand shearing forces related with joint motion. Collagen proteins comprise this zone and are oriented in a tangent fashion to help protect and lubricate the joint. This parallel orientation of the proteins to the joint surface allows for less friction on the joint surface. The superficial zone makes up 10 to 20 percent of the total cartilage volume. The middle zone comprises 40 to 60 percent of the total cartilage volume, and is composed mainly of hydrated

proteoglycans. It functions to distribute the majority of compressive forces produced by joint loading. Its collagen is oriented in a interwoven or “basket weave” (Hall, 1998; Todhunter, 1996) pattern to the joint surface. This pattern gives lateral strength and flexibility to the entire joint. The deep zone is thirty percent of total cartilage volume. It is mainly comprised of low water concentrated or hydrophobic proteoglycans. Its purpose is for distributing significant load bearing forces. Its collagen (type II) orientation is perpendicular to the joint surface and integrates through the “tide mark” into the calcified region (Freemont, 2006; Hall, 1998; Lorenz and Richter, 2006; Pearle *et al.*, 2005). This integration binds the cartilage to the bone surface increasing the strength of the joint (Freemont, 2006). The calcified cartilage zone is separated from the non-mineralized cartilage by the tide mark. The calcified cartilage zone rests directly on the subchondral bone (Hall, 1998; Pearle *et al.*, 2005). Subchondral bone is thinner than cortical bone and its haversian systems are oriented parallel to the joint surface rather than parallel to the long axis of the bone. Organization of the subchondral cancellous bone varies between joints according to the biomechanical forces the joint experiences. The flexibility of the subchondral region is greater than the epiphyseal region and plays a significant role in force attenuation. Stiffening of the subchondral region is an indication of late stage disease (Caron, 2003).

Joint Capsule

The joint capsule is a thick fibrous structure providing stability and protection by enclosing the joint (Caron, 2003; Ilic *et al.*, 2000; Todhunter, 1996). Collateral ligaments are intracapsular and aid in providing stability. The articular surface is comprised of hyaline cartilage that covers the underlying subchondral bone plate. The joint capsule is lined with synovium which is responsible for the production of synovial fluid. The synovium is a

specialized tissue which is found at the boundary between solid and liquid connective tissues and lacks a basement membrane. The subsynovium is an incomplete layer of synoviocytes and contains the only blood vessels and nerves within the lining of the joint (Caron, 2003; Freemont, 2006; Ilic *et al.*, 2000). Synoviocytes possess both secretory and phagocytic properties. The synovial lining's rich blood supply enables it to produce synovial fluid, facilitate the exchange of nutrients, dispose of metabolic waste, and provide nutrients by diffusion to the avascular articular cartilage (Caron, 2003). Molecules as large as 65kDa are able to penetrate normal articular cartilage by diffusion. Absorption of solutes may be achieved through simple diffusion or by compression-relaxation cycles (Caron, 2003).

The synovial fluid is contained within the joint space and forms a barrier between the articular surfaces providing the necessary lubrication. It visually appears as a clear or pale yellow viscous liquid. The thin subsynovium serves as a barrier between intravascular and interstitial fluid and the synovial fluid. Diffusion of nutrients is easily achieved through the subsynovium resulting from the absence of a basement membrane. Fluid exchange is accomplished through hydraulic, hydrostatic, and osmotic pressures between the plasma and synovial fluid. Normal intrasynovial pressure is subatmospheric which may aid in stabilizing the joint. In the canine, several factors affect the intraarticular pressure including: joint size, synovial fluid volume, the position of the joint in regards to flexion and extension, periarticular tissue and joint anatomy, membrane permeability, capsular compliance, and movement of fluid into and out of the joint (Todhunter, 1996).

The synovial lining produces concentrated hyaluronan, which is secreted into the synovial fluid (Todhunter, 1996). Synovial fluid hyaluronan is a large molecule ranging from 3×10^2 to 2×10^3 kDa. Normal human hyaluronan concentration ranges from 2 to 3 mg/ml while

equine concentration is approximately 0.5 mg/ml. The structure of hyaluronan appears as a stiff coil and is highly hydrophilic. High viscosity is maintained by the mucopolysaccharide's overlapping and entangling nature. The viscosity allows the joint to resist momentary shear forces generated by motion. This non-sulfated glycosaminoglycan also binds to opposing articular cartilage surfaces, preventing the direct contact of these surfaces when under compression. In addition, hyaluronan will experience thixotropy (thinning) when the synovial fluid experiences higher rates of stress (Todhunter, 1996).

Chondrocytes

Chondrocytes are a small percentage of the extracellular matrix, but are fundamental in the support of the musculoskeletal system and are the building blocks of the cartilage. Chondrocytes are used in manufacturing, exporting, and degrading the connective tissue or extracellular matrix (ECM) of the joint (Caron, 2003; Freemont, 2006; Hall, 1998; Pearle *et al.*, 2005; Todhunter, 1996; Trumble, 2005). Chondrocytes are responsible for producing collagen and proteoglycans. They are the basis of support for the musculoskeletal system in distributing load bearing forces and resisting mechanical forces. Chondrocytes are enclosed within a lacunae which is a protective shell comprised of collagen type VI (Freemont, 2006). The lacunae and the interstitial fluid provide the necessary aqueous environment where chondrocytes synthesize all forms of connective tissue macromolecules. Nutrients are diffused to chondrocytes from synovial fluid through this aqueous tissue. Density of the ECM does not prevent diffusion of nutrients and molecules as large as 65kDa can penetrate normal articular cartilage (Caron, 2003). The interstitial fluid is comprised of negatively charged acidic proteoglycans anions which attract cations from the synovial fluid. This attraction results in water absorption causing the tissue to inflate due to osmoregulation within the matrix. Water content varies with age but can

be upwards of 80% (Caron, 2003). Water is freely exchanged between the synovial fluid and the matrix maintaining joint osmotic pressure, absorbing and distributing compressive load forces, and lubricating the joint (Caron, 2003). In addition, chondrocytes adapt to produce a balance within the ECM in response to various mechanical forces from physical and chemical changes to environment. These above abilities to change and adapt allows for a sustainable extracellular matrix. It is this response to these factors that determine the characteristics of the matrix (Hall, 1998; Pearle *et al.*, 2005).

Understanding the normal functional properties of the chondrocytes is necessary in comprehending a rationale for treatment of disease processes. It is important to be open minded to prophylactic supplementation in order to prevent permanent cell loss. Extensive damage to the cells is not usually repaired; therefore, the afflicted cartilage adds more burden to surrounding matrix and thus the entire joint (Hall, 1998; Lorenz and Richter, 2006; Pearle *et al.*, 2005). This in turn negatively effects the normal matrix turnover and indicates early stages of disease (Freemont, 2006). As disease progresses normal matrix synthesis is overtaken by the rate of degrading cartilage resulting in arthritis progression (Caron, 2003; Lorenz and Richter, 2006).

Proteoglycans

Proteoglycans are the main cartilage protein among the others (decorin, biglycan, and fibromodulin) which forms the ECM. They are composite molecules consisting of protein and glycosaminoglycan components. Some collagens can be classified as proteoglycans. The most common proteoglycan of articular cartilage is aggrecan which interacts with hyaluronan to form multimolecular aggregates. Hyaluronan and aggrecan are non-covalently bonded but are stabilized by a link protein of equal affinity. Aggrecan's increased hydrated state plays a

significant role in resisting compression within the middle zone of cartilage (Caron, 2003; Todhunter, 1996). Aggrecan's hydrophilic property enables the molecule to absorb water which allows more absorption of biomechanical stress. Water is used in lubricating the joint during movement. When the joint experiences loading, water is unable to pass through subchondral bone and thus is secreted onto the joint surface aiding in lubrication. When the compressive force is released the cartilage expands and allows the water to return to middle cartilage zone. Aggrecan is catabolized by aggrecanases, in particular disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), which is part of the metalloproteinase family (Ilic *et al.*, 2000). In addition, chondrocytes have hyaluronan receptors which help bind proteoglycans. Therefore, hyaluronan is key to providing a place for chondrocytes to bind and aids in constructing a strong ECM (Hall, 1998).

Other non-proteoglycan proteins that help construct cartilage are anchorin, fibronectrin, and thrombospondin-5 (COMP). Anchorin is located on the surface of chondrocytes and possesses a great affinity to collagen type II fibrils. These properties suggest that anchorin acts as a mechanoreceptor, which provides information in changes of stress experienced by the ECM. Fibronectrin contributes to the cartilage network by aiding the in the assembly of the ECM. Elevated fibronectrin levels have been associated with osteoarthritis and may assist in catabolic events in cartilage. Thrombospondin-5 may regulate cell growth within newly synthesized cartilage (Caron, 2003).

Collagen

Collagen is a three dimensional fibrillar network that helps with the tissue volume, shape, and tensile strength of the ECM. Its rate of turnover in articular cartilage is very limited especially compared to proteoglycans (Caron, 2003; Hall, 1998; Todhunter, 1996). Collagen

provides cartilage with structural and functional support by interacting with other matrix components. In particular, Type I is the most prevalent collagen in connective tissues such as ligaments and tendons (Todhunter, 1996). Within the cartilage layers, collagen fibrils are oriented parallel to the joint surface in the cartilage superficial zone acting as a protective layer. Deeper into the cartilage layers, collagen is oriented radially and anchors the cartilage to the subchondral bone. Collagen's fibrils are not uniform in size but larger fibrils are found within the middle and deep cartilage zones. This larger size and location indicates a supportive role in absorbing biomechanical forces (Caron, 2003; Todhunter, 1996).

Several types of collagen are found in the ECM; however, type II collagen is the most abundant accounting for 90% of the fibrillar network in articular cartilage. All fibril-forming collagens (types I, II, III, V, and XI) are similar in structure. Type II in addition to all fibrillar collagen molecules are assembled in alpha chains which are three identical amino acid chains arranged in a single uninterrupted triple helix. The helix is stabilized by hydroxyproline cross-links (Caron, 2003; Todhunter, 1996). These crosslinks or bonds create a higher breaking point making collagen stronger. Type XI is a fibrillar collagen found within type II. Its function is not understood but may affect Type II's assembly and organization. Type VI is a microfibrillar collagen which acts as a link between collagen and other matrix components. Type IX stabilizes Type II by covalently bonding to its surface. Other types of collagen of unclear functions include Types XI, XII, and XIV (Caron, 2003).

CHAPTER 2

OSTEOARTHRITIS

Osteoarthritis Defined

According to Arden and Cooper (2006), there are two basic types of arthritis: atrophic and hypertrophic. Atrophic results from viral infection or auto immune deficiencies and includes diseases such as Rheumatoid Arthritis and septic arthritis. These diseases usually involve synovial inflammation, cartilage, and bone erosion. Hypertrophic is a broad category including spontaneous arthritis more commonly known as osteoarthritis (Arden and Cooper, 2006). A current definition of the disease describes it as a reaction of the progressively aging joint to continuous heavy loading or injury. Osteoarthritis is defined radiographically by observation of joint narrowing, osteophytes, subchondral sclerosis, cyst formation, and abnormal bone contour (Pool, 1996)

Articular Cartilage and Osteoarthritis

Osteoarthritis, also known as degenerative joint disease, can be defined as a disease of cartilage degeneration. It is the progressive deterioration of articular cartilage and the generation of new bone at the joint surface (Caron, 2003; Surapaneni and Venkataramana, 2007). Early stages of osteoarthritis begin with proteoglycan (aggrecan) loss (Freemont, 2006; Ilic *et al.*, 2000; Lorenz and Richter, 2006; Pearle *et al.*, 2005). This results in the loss of cartilage causing narrowing of the joint space and creating joint instability. The osmotic pressure within the joint is disrupted by the imbalance of proteoglycans and type II collagen fibers. Cartilage begins losing its stiffness and becomes soft.

This early stage of OA has a chance of repair. However, permanent damage can occur in later stages when the type II fibers are affected. Damage manifests as superficial splits oriented parallel and at right angles to the joint surface which travel deep and laterally towards the bone (Freemont, 2006; Lorenz and Richter, 2006). The focal damage is matched on both opposing articular surfaces. The damage continues to worsen as permanent loss of cartilage occurs and bone is exposed. This results in bone rubbing against each other causing eburnation (Freemont, 2006). At this late stage, chondrocytes do attempt to repair the damage. However when repaired, the newly synthesized cartilage is much more cellular than the original resulting in unevenly distributed chondrocytes throughout the matrix. The new tissue has difficulty integrating into the old tissue resulting in the inability to endure loading. Increased levels of proteoglycans are present in the synovial fluid as they have decreased binding ability to the defective collagen fibers. Fibrocartilage is formed from the exposure of bone marrow which may develop into a complete tissue to cover the eburnated bone (Freemont, 2006).

Some studies show that the initial stages of OA begin with the thickening and increased density of subchondral bone (Freemont, 2006; Pearle *et al.*, 2005). This can interfere with the transport of nutrients to the cartilage from bone marrow. The balance between osteoblasts and osteoclasts is interrupted, causing an increase in bone cell activity. Mainly an excess of osteoblastic deposition is seen and bone sclerosis and osteophytes form (Freemont, 2006). This is a feature which differentiates OA from other arthritic diseases (Lorenz and Richter, 2006). Joint instability is the main cause of osteophyte formation which forms to stabilize the joint by increasing the articulating surface. Bone necrosis occurs from the pressure of communicating eburnated bone which prevents the underlying marrow from repairing itself. Once this occurs, synovial fluid can leak into the marrow. This is a particular feature of advanced OA and results

in cysts within the bone. In addition, the synovium becomes inflamed resulting from perivascular edema and increased blood flow to the area. Leaking synovial capillaries increase the volume of synovial fluid. As a sign of disease progression, the synovial fluid contains more debris from the deteriorating articular surfaces and joint lining (Freemont, 2006; Lorenz and Richter, 2006; Pearle *et al.*, 2005).

Pro-inflammatory markers

Joint inflammation is the precursor and is among the first indicators of joint degradation and OA. Certain pro-inflammatory cytokines can be detected and measured. One such cytokine is Interleukin-1beta (IL-1 β) which is up regulated by prostaglandin in the cyclooxygenase inflammatory mechanism. Interleukin-1 is responsible for a catabolic chain reaction of events such as the up regulation of metalloproteinases, nitric oxide synthase, cyclo-oxygenase 2, IL-6 genes, and apoptotic pathway in human chondrocytes (Afonso *et al.*, 2007; Aghazadeh Habashi and Jamali, 2011; Caron, 2003; Valvason *et al.*, 2008). In a bovine joint capsule study, IL-1 β stimulated aggrecan catabolism by the stimulation of ADMTS aggrecanase-1 and aggrecanase-2 (Ilic *et al.*, 2000). Interleukin-1 causes the inhibition of heme oxygenase-1(HO-1) which is a rate limiting enzyme in the oxidative degradation of heme to biliverdin, free iron, and carbon monoxide. Heme oxygenase-1 is thought to be a mechanism specially adapted to protect against injury caused by oxidative stresses. Interleukin-1 stimulates nitric oxide production which in turn forms peroxynitrite which is involved in guanine repeats in DNA telomeres. Telomeres are involved in protecting the ends of chromosomes from deterioration. This indicates oxidative stress and is linked with telomere erosion causing damage to DNA (Afonso *et al.*, 2007). Moreover, nitric oxide synthase has directly been linked with chondrocytes in development or progression of OA. Over production of nitric oxide has been detected in articular cartilage and

synovial fluid in patients with OA (Valvason *et al.*, 2008). It is responsible for inflammation and articular degeneration by enhancing cytokine production, elevating MMP's, and suppressing collagen and proteoglycan syntheses (Afonso *et al.*, 2007; Valvason *et al.*, 2008).

Collagen and Osteoarthritis

In normal disease free cartilage, type II collagen is uniformly distributed throughout the non-mineralized cartilage zones. In initial stages of OA, type II synthesis is down regulated in the upper cartilage zones. During this time, ECM repair mechanisms and regeneration efforts are observed in the deeper zones. Collagen type II continues to degrade in the lower cartilage zones in late stage OA. Collagen type I and III are produced in advanced OA. Type VI is up regulated during moderate stages of OA in the lower middle and upper deep zones (Lorenz and Richter, 2006). Type X collagen is found around chondrocyte clusters in advanced staged OA. Type X is normally absent in normal articular cartilage (Caron, 2003).

CHAPTER 3

OXIDATIVE STRESS

Overview of Oxidative Stress

The aging process exposes the cartilage matrix to different alterations in structure, molecular composition, and mechanical properties. One of these processes is oxidation. According to Kirschvink, oxidants play an vital role by inactivating and destructing microorganisms through peroxidation and destabilization of their lipid membranes, oxidation and inactivation of their proteins acting as receptors or enzymes, and oxidation of the nuclear material (Kirschvink *et al.*, 2008). Oxidants play a major role in signaling within intra-cellular pathways by acting as mediators of cellular regulation such as proliferation, apoptosis, and stimulating inflammation. Normally, oxidants are isolated by specialized proteins in order to prevent stress and superoxide anion production (Afonso *et al.*, 2007). However, stress occurs when oxidant volume overwhelms antioxidant volume and its ability to defend the body. Defined, oxidative stress is the imbalance between oxidants and antioxidants within the circulatory system (Kirschvink *et al.*, 2008). It can be caused by an overabundance of reactive oxygen species (oxidants) or a deficiency in antioxidants (Kirschvink *et al.*, 2008).

Horses are exposed to numerous different oxidants that are placed into two categories: exogenous and endogenous. Exogenous oxidants are a major factor in respiratory research (Kirschvink *et al.*, 2008). This study focuses on endogenous oxidants affecting cell make up and the immune system. Of three sources of oxidative stress, one major source is the formation of superoxide in the mitochondria during electron transfer. This source is significantly important

during exercise where oxygen consumption can increase approximately forty times in horses. A second source is considered oxidants produced from enzymes such as xanthine oxidase, membrane oxidases, and nitric oxide synthases. A third and perhaps a more relevant source to this study, is superoxide anion produced under inflammatory conditions by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Deger *et al.*, 2009; Kirschvink *et al.*, 2008; Valvason *et al.*, 2008).

The oxidation of proteins is important to the integrity of the joint capsule (Afonso *et al.*, 2007). Increased levels of oxidative stress in the blood can directly cause protein breakdown within the joint. This damaging process starts with the degradation of cartilage cells. More specifically, high levels of reactive oxygen species (ROS) cause the breakdown or oxidation of membrane proteins, nucleic acids, proteoglycans, collagen, lipids and carbohydrates (Afonso *et al.*, 2007; Kirschvink *et al.*, 2008; Valvason *et al.*, 2008). This is accomplished by oxidative stress inducing enzyme malfunction and cell membrane lipid peroxidation initiating the breakdown of cell integrity (Deger *et al.*, 2009; Surapaneni and Venkataramana, 2007).

Reactive oxygen species are defined as oxygen containing molecules that are more reactive than the oxygen present in the atmosphere (Kirschvink *et al.*, 2008). Reactive oxygen species encompasses both free radicals and reactive compounds without unpaired electrons in their outer orbit (Afonso *et al.*, 2007; Kirschvink *et al.*, 2008). Such non-radical oxidants include superoxide anion (O_2^-), hydrogen peroxide, peroxyxynitrite, and hypochlorous acid (Kirschvink *et al.*, 2008). Free radicals are defined as significantly increased reactive molecules or molecular fragments which contain one or more unpaired electrons in their atomic or molecular orbits. The radicals include nitric oxide, superoxide, and hydroxyl radical (Kirschvink *et al.*, 2008; McIlwraith, 1996). As stated, free radicals are generated by NADPH oxidase integrating into the

cell membrane of metalloproteinases and macrophages (McIlwraith, 1996). These molecules are then released from the cell and are free to cause damage to neighboring cells. Since free radicals are capable of cleaving proteoglycans (Kirschvink *et al.*, 2008; McIlwraith, 1996), these released species can cause direct damage to joint structures (Afonso *et al.*, 2007). In particular, the oxidation of superoxide anion to nitric oxide causes cartilage damage (Afonso *et al.*, 2007). The final electron transport from NADPH to heme and molecular oxygen in the NADPH oxidase complex can be converted to other products such as hydrogen-peroxide (H₂O₂). These toxic products oxidize several aromatic compounds and generate reactive nitrogen species from nitric oxide. *In vitro*, hydrogen peroxide in particular has been shown to depolymerize proteoglycan aggregates assisting in the degradation of hyaluronan and destabilizing aggregates. Collagenase shows a direct effect on the destruction of the matrix (Afonso *et al.*, 2007; Kirschvink *et al.*, 2008; Surapaneni and Venkataramana, 2007).

Antioxidants

As a result of oxidation, the body's defense system produces antioxidants used in combating ROS and free radicals. Antioxidants include systems that prevent, alter, or inactivate oxidant and ROS generation, and allow repair of oxidative damage (Deger *et al.*, 2009; Kirschvink *et al.*, 2008; Surapaneni and Venkataramana, 2007). They compete successfully with other oxidizable substrates. Their major role is the inactivation or transformation of oxidants into less reactive or stable forms (Afonso *et al.*, 2007; Kirschvink *et al.*, 2008). They can be categorized as hydrophilic or hydrophobic. Antioxidants can also be categorized by their role played in oxidation. One such role is to prevent the generation of free radicals and the other is intercepting any generated free radicals (Surapaneni and Venkataramana, 2007). Antioxidant enzymes of significant importance are glutathione-peroxidase, superoxide dismutase (SOD), and

catalase (Afonso *et al.*, 2007; Deger *et al.*, 2009; Kirschvink *et al.*, 2008; Surapaneni and Venkataramana, 2007). Glutathione as well as others such as uric acid, ascorbic acid, thiols, proteoglycans, and hyaluronic acid are considered antioxidants that protect against lipid peroxidation. Lipid peroxidation is the cellular damage resulting from the deterioration of lipids when free radicals take electrons from cell membranes. Superoxide dismutase (SOD) (Afonso *et al.*, 2007; Deger *et al.*, 2009; McIlwraith, 1996), catalase (Deger *et al.*, 2009; Kirschvink *et al.*, 2008; Surapaneni and Venkataramana, 2007), and glutathione peroxidase are enzymes protecting the cells from accumulating hydrogen peroxide by using it as an oxidant or by dismutating it into oxygen and water (Afonso *et al.*, 2007). In addition, superoxide dismutase has shown to produce greater clinical improvements in arthritis patients than traditional anti-inflammatories (Afonso *et al.*, 2007; Surapaneni and Venkataramana, 2007). Superoxide dismutase plays a major role in the make-up of antioxidants that aid in protecting the extracellular matrix from superoxide anion by dismutating it into dioxygen (O₂) and hydrogen peroxide (Afonso *et al.*, 2007; Surapaneni and Venkataramana, 2007). Three isoforms of SOD are found within humans: SOD1 is found in the cytoplasm, Superoxide dismutase-2 is found in the mitochondria, and SOD3 is found in the extracellular matrix (Afonso *et al.*, 2007). SOD2 is activated by proinflammatory cytokines such as interleukin-1, 4, 6 and tumor necrosis factor (TNF)- α . According to Afonso *et al.* (2007), the promoter region involved in SOD2 activation contains sites that bind to transcription factors belonging to NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), C/EBP (enhancer-binding protein), and NF-1(nuclear factor) families (Afonso *et al.*, 2007). Superoxide dismutase-3 is strongly attracted to proteoglycans in the extracellular matrix. Its main location is within synovial fluid. Much like SOD2, this isoform protects cells against inflammation from superoxide anion generated active neutrophils. Proteases released by these inflammatory cells

cleave the SOD3 from the ECM. This act exposes the matrix to degrading ROS and in turn increases the volume of the dismutase isoform. Genetically deficient SOD3 mice showed increased collagen-induced arthritis and an increase in proinflammatory cytokines. In humans, SOD3 levels correlate negatively with disease activity (Afonso *et al.*, 2007). In addition, glutathione – S – transverases (GST) are reported as a multifunctional protein family that significantly aids in the detoxification of electrophiles and the removal of harmful hydrophobic compounds from the blood by the liver (Surapaneni and Venkataramana, 2007). Surapaneni found levels of GST were increased in patients with OA compared against its controls.

Surapaneni and Venkataramana (2007) suggests incorporation of antioxidants into OA therapy alongside conventional drugs. Treatments with antioxidants in early stages of OA potentially prove useful as a secondary therapy in the prevention of cartilage and other musculoskeletal degeneration (Surapaneni and Venkataramana, 2007). Figure 1 demonstrates how antioxidants are produced through the oxidative pathways.

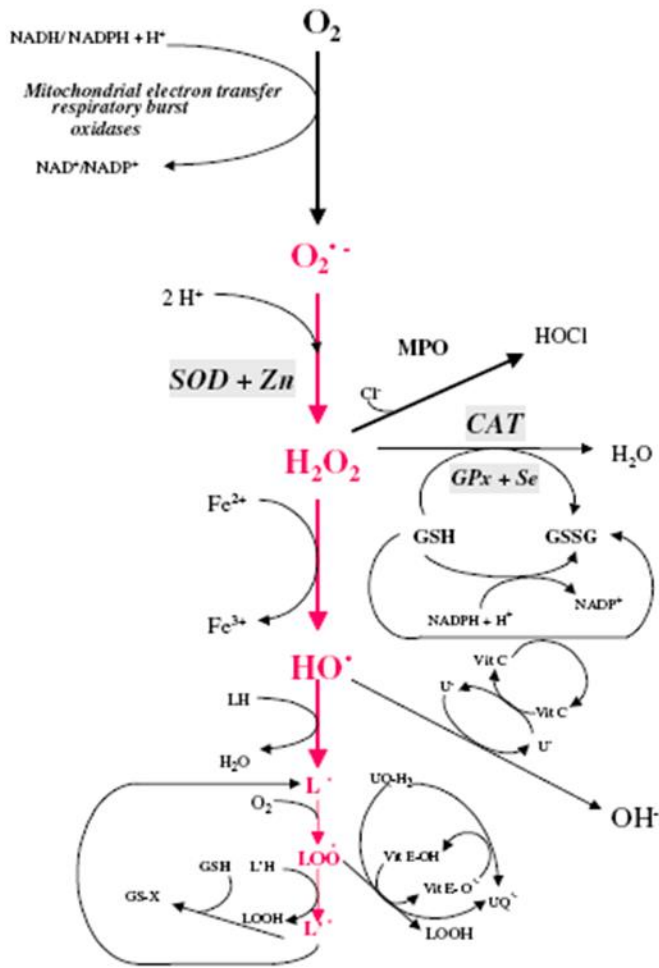


Figure 1. The oxidative pathway - demonstrates how living organisms transform or inactivate oxidants into antioxidants (Kirschvink *et al.*, 2008)

Oxidant/Anti-oxidant Levels in Osteoarthritis

Molecules that have undergone oxidation have the potential to serve as measurements for oxidative stress. However, only a few studies have focused on oxidant markers, oxidative stress, and their relationship of joint disease in horses (Kirschvink *et al.*, 2008). Oxidant markers such as protein carbonyls and lipid peroxidation (8-isoprostane) were found in synovial fluid (Kirschvink *et al.*, 2008), while others like nitric oxides increased resulting from OA formation in cartilage and subchondral bone (Valvason *et al.*, 2008). *In vitro*, spin trapping techniques

(free radical detection technique) reveal that when exposed to repeated cycles of anoxia/re-oxygenation, synoviocytes increase ROS formation leading to osteoarthritis (Kirschvink *et al.*, 2008). As aforementioned, oxidized proteins can induce enzyme malfunction and lipid membrane peroxidation initiating chain reactions which compromises cell integrity (Kirschvink *et al.*, 2008; Surapaneni and Venkataramana, 2007). One study found that blood malondialdehyde (MDA) levels significantly increased in human patients experiencing OA (Surapaneni and Venkataramana, 2007). Malondialdehyde levels serve as an index of the extent of lipid peroxidation. It is suggested that the increase in MDA levels is due to a greater presence of ROS resulting from excessive cellular oxidative damage. In addition, decreases in the antioxidants glutathione, ascorbic acid, and plasma vitamin E was observed in these patients. A possible cause for the decrease in antioxidants may be due to over demand in preventing oxidative damage. Furthermore, this study suggested that decreased catalase activity supports the higher oxidative stress hypothesis in osteoarthritis (Surapaneni and Venkataramana, 2007). Catalase serves as an enzyme involved in down regulating oxidative stresses. Nitrotyrosine resulting from the nitration of tyrosine is oxidized in the presence of nitrous oxide (NOO), which can serve as a marker *in vivo* for oxidative damage. Presence of nitrotyrosine is associated with aging and with osteoarthritis, suggesting oxidative stress degrades cartilage in the aging process (Afonso *et al.*, 2007).

Measurements of Oxidative Stress

Caution is needed when planning and selecting oxidant/antioxidant equilibrium assessments due to the sensitive nature of the process. Determining target markers for measurement is crucial in deducing meaning of the system's equilibrium. Kirschvink advises a broader spectrum to account for all classes of target molecules and antioxidants (Kirschvink *et*

al., 2008). Of relative importance to this study, glutathione (GSH) and malondialdehyde (MDA) were selected as markers of oxidant/antioxidant equilibrium. Glutathione is used in measuring non-enzymatic antioxidant parameters. Malondialdehyde measured as thiobarbituric acid reactive substances (TBARS) serve as an indicator of the level of lipid peroxidation damage (Deger *et al.*, 2009; Surapaneni and Venkataramana, 2007). One study found MDA levels increased significantly in patients with OA (Surapaneni and Venkataramana, 2007). This study determined the rise in MDA levels was due to increased ROS generation related to excessive oxidative damage. In addition, decreased levels of GSH, ascorbic acid, and plasma vitamin E were observed in patients with OA when compared against controls. This study suggested the decrease in levels was possibly due to the increased turnover for preventing oxidative damage which suggests an increased defense against oxidative damage in OA patients (Surapaneni and Venkataramana, 2007).

Rapid processing of blood samples is essential as oxidative processes continue or increase due to exposure to ambient oxygen, ambient temperatures, and ultra violet light. Immediate cooling of collected material is recommended. Processing, stabilization, and or centrifugation are encouraged within two hours of collection. Long term storage varies from -4°C to -80°C depending upon the selected marker (Kirschvink *et al.*, 2008).

CHAPTER 4

KINEMATICS

Kinematics and Osteoarthritis

Osteoarthritis can have a significant negative impact causing a decrease in horse joint range of motion. According to Palmer (1996), “Kinematic analysis evaluates movement of the limbs or the body without references to the forces acting on the subject or the mass of the subject (Pg. 116).” Unavoidable forces and movement of the horse, marker placement, and expense are large variables commonly seen in kinematic studies and makes detailed gait analysis difficult (Forsyth *et al.*, 2006; Palmer and Bertone, 1996).

The joint experiences three perpendicular planes of transitional motion: medial to lateral, dorsal to palmar or plantar, and proximal to distal, with the possibility of rotational motion in each joint. Research has proven the distal leg experiences a wide range of motion through protraction and retraction of the limb (Palmer and Bertone, 1996). Normally, the range of motion in the fetlock joints increase by 65 to 75% when the horse transitions from a walk to a trot whereas the carpus and tarsus joints only increase slightly (McIlwraith, 1996; Palmer and Bertone, 1996). This large range of motion requires the shoulder and coxofemoral joints to experience all three planes of motion. Smaller ranges of motion in the shoulder, elbow, coxofemoral, and stifle joints cause the larger movements in the distal limb. In addition, the distal limb’s greater range of motion requires the distal joints to be well stabilized with soft tissues. These soft tissues including tendons, ligaments, cartilage, and synovial fluid are essential to the longevity of the horse.

Training, fatigue, and inflammation may influence joint range of motion. Of concern to this study, inflammation has been shown to significantly decrease maximum flexion angle of the carpus as well as the maximum dorsiflexion of the fetlock (Palmer and Bertone, 1996). The stance phase has also been shown to shorten in horses with acute inflammation. Inflammation decreases the stride duration and creates a quick legged, mechanical, altered gait. As a result of the altered gait, abnormal stresses occur by slight rotation in the proximal limb in the joints predisposing the cartilage, ligaments, and synovial capsule to damage (Palmer and Bertone, 1996).

Hoof and Shoeing Influences

The conformation, balance, breakover, length, and angulation of the hoof can affect the way the horse moves (Back, 2001). The hoof wall grows at approximately 1cm every six weeks with the hoof wall at the toe growing faster than in the heels. This growth influences how the hoof lands and what forces it experiences resulting from the dorsal hoof wall angulation (Back, 2001). Continual growth of the hoof wall will change the way the hoof lands and impacts the ground. Larger dorsal hoof wall angulations will increase the strain of the hoof wall at the toe. In addition, more upright hoof angles will cause the hoof to possess an exaggerated heel first landing. More acute angles of the dorsal hoof wall will cause the horse to land toe first with the ground. Acute hoof angulation is associated with undesirable effects such as tripping or stumbling (Back, 2001). Both upright and acute hoof wall angulations put more stress on associated tendons and joints of the distal limb. Comparisons of the trot in horses with more normal upright angles to horses with more acute angles showed no difference in stride length or suspension. In addition, this comparison showed the angles had no influence on the flight arc of

these horses. However, acute angulation did extend the duration of breakover, which is the terminal part of the stance phase (Back, 2001).

In addition, hoof wall length can influence stride parameters. Longer hooves are associated with prolonged stride duration, swing duration, and breakover (Back, 2001). However, longer hooves do not change stride length and duration. Hoof length does influence the flight arc by it peaking earlier and higher in the swing phase (Back, 2001).

As a result of hoof wall growth influencing dorsal hoof wall angulation and hoof wall length, it is necessary to trim the hoof on a routine basis. Regular routine trimming will decrease the stresses on tendons and joints when dorsal hoof angles and hoof wall lengths are within normal ranges. Routine trimming will also decrease the horse from experiencing prolonged stance times and irregular flight arcs (Back, 2001).

The repetitive nature of the hoof impacting the ground during movement increases the likelihood of arthritis formation in the horse (Back, 2001). Applications of shock absorbing pads or specialized shoes may decrease the impact the horse experiences when the hoof contacts the ground. A study found that using a full roller motion shoe may beneficially affect horses with arthritis of the distal interphalangeal joint. This shoe was able to reduce stress and pain from the ligaments and the joint capsule by adapting to the breakover of the particular horse (Back, 2001).

CHAPTER 5

TREATMENTS FOR INFLAMMATION

Traditional Treatments for Inflammation

Non-steroidal anti-inflammatory drugs (NSAIDs) are classified as substances other than steroids that are involved in blocking or suppressing the inflammatory responses. Drugs included in this classification pertain to aspirin associated compounds, but; they also can incorporate intramuscular substances such as hyaluronan and polysulfated glycosaminoglycan. A more appropriate NSAID classification is drugs used in inhibiting cyclooxygenase in the arachidonic acid cascade. The cyclooxygenase (COX) is used in converting arachidonic acid to prostaglandin PGG₂ and ending with its reduced form prostaglandin PGE₂. Other pro-inflammatories include cytokines, metalloproteinases, and tumor necrosis factor (TNF). Certain drugs targeting cytokines can block eicosanoid and enzyme production and modify connective tissue remodeling. Drugs blocking COX strictly affects prostaglandin production (May and Lees, 1996).

Problems can exist when treating with NSAIDs. Drugs such as phenylbutazone and flunixin meglumine are competitive antagonists of cyclooxygenase, therefore; their effect depends on the continuing presence of the drug. Aspirin is an irreversible antagonist which deactivates the COX by acetylation. In addition, aspirin affects blood platelets by increasing the template bleeding time by reducing platelet aggregation. In addition, many NSAIDs like aspirin are more successful in inhibiting COX-1 than COX-2. Cyclooxygenase-1 is an important enzyme which carries out normal, physiological production of prostaglandins. Cytokines and

bacterial lipopolysaccharides induce the production of COX-2 which is involved in inflammation. NSAIDs inhibiting COX-1 interfere more with normal physiological processes than inflammation processes (May and Lees, 1996). Therefore, selecting a NSAID based on individual method of action is significant in preventing toxicity. One common side effect is gastric and colonic ulceration. Prostaglandin PGGs have been shown to provide protection of gastric mucosa against damage from acids. Therefore, NSAIDs may indirectly cause gastric ulceration by preventing PGGs from protecting the mucosa. In addition to the aforementioned problems, NSAIDs also affect proteoglycan synthesis by influencing the anabolism of cartilage. Aspirin and sodium salicylate have been shown to inhibit proteoglycan synthesis. In established diseased joints, salicylate is more profound in proteoglycan suppression (May and Lees, 1996).

NSAIDs are a useful group of drugs for the humane treatment of pain and inflammation in the horse. Phenylbutazone is a common and widely used long term anti-inflammatory as it is inexpensive. However, care should be used in preventing toxic effects such as gastric ulceration. In addition, phenylbutazone has been shown to be less effective in the reduction of edema and leukocyte infiltration into inflamed sites (May and Lees, 1996). For arthritis, newer and safer alternatives should be considered in combination with preventive drugs such as glucosamine.

Polysulfated Glycosaminoglycan

Polysulfated glycosaminoglycan (PSGAG) is considered a disease modifying osteoarthritis drug (DMOAD). It has been shown to prevent, delay, or reverse the damage to cartilage and other ECM components of OA (Verde *et al.*, 2010). Like NSAIDs, PSGAGs are able to inhibit prostaglandins in the arachidonic acid cascade and inactivate cytokines. They also possess chondroprotective properties as well as promoting the metabolic chondrocyte processes. Other PSGAG properties include: anti-apoptotic effects, provide the basis in proteoglycan

production, reduce metalloproteinase activity, and reduces nitric oxide synthesis and gene expression (Verde *et al.*, 2010). Verde *et al.* (2010) found that PSGAGs are highly efficacious on treatment of chemically induced arthritis in the horse.

Glucosamine

The use of traditional anti-inflammatory long-term drugs has shown to be problematic. In addition they do not aid in the reconstruction of the ECM and thus possibly helps the progression of joint cartilage degradation by alleviating only symptoms. As a result, incorporation of prophylactic treatments is necessary. Glucosamine hydrochloride and chondroitin (GHCL/CS) are considered preventative drugs (Forsyth *et al.*, 2006). According to Forsyth, the combination of GHCL and CS show more effectiveness in the prevention of cartilage degradation, GAG stimulation, and contributes to joint stability than glucosamine alone. Glucosamine, used as a chondroprotective agent, has been shown to relieve disease progress and symptoms of osteoarthritis (Ilic *et al.*, 2008). In a rat study, GLN inhibited the development of adjuvant arthritis (Aghazadeh Habashi and Jamali, 2011). Different forms of glucosamine are available: hydrochloride, sulfate, N-acetyl, and chlorohydrate salt (Hoffer *et al.*, 2001). One study reported that the nature of the salt does not influence the bioavailability of GLN administered orally (Aghazadeh Habashi and Jamali, 2011). A number of studies on rabbits treated with GLN Hydrochloride (HCl) showed various beneficial effects including site specific disease modifying effect, improved subchondral bone turnover and mineralization, chondroprotective properties, reduced MMP's, and enhanced synthesis of Collagen Type II. In addition, a rat study has shown that GLN HCl is bioequivalent to GLN sulfate in horses (Aghazadeh Habashi and Jamali, 2011). Moreover, oral administration of glucosamine sulfate was shown to be more bioavailable than GLN HCl in the horses (Meulyzer *et al.*, 2008).

Effective concentrations of GLN are still debated but are centered on its pharmacokinetics. Industry biases make determining an effective dose difficult and should be considered in evaluating research. Minimum effective plasma concentrations of GLN both in humans and animals remain unknown. In human studies, 1500 mg/day is a common but possibly an ineffective dosage (Aghazadeh Habashi and Jamali, 2011). A rat study found that repeated 300mg/kg dose of GLN completely inhibits emergence of clinically induced arthritis (Aghazadeh Habashi and Jamali, 2011). Future experiments using high doses and bioavailable formulations is needed (Aghazadeh Habashi and Jamali, 2011). Another study on the pharmacokinetics of glucosamine demonstrated that administered glucosamine sulfate via nasogastric intubation at a concentration of 20mg/kg showed increase levels of glucosamine in synovial fluid in the horse (Meulyzer *et al.*, 2008).

Glucosamine Sulfate is simply the structure of glucose with an amino acid group and sulfate functional group attached. Nutritional supplements of GLN are usually made from marine exoskeleton. Glucosamine is normally synthesized by chondrocytes and is a foundation in the structure of proteoglycans (Koh and Dietz, 2005). When supplemented orally, glucosamine sulfate has shown to increase aggrecan production and decrease the levels of matrix degrading enzymes (Arden and Cooper, 2006; Trumble, 2005). Moreover, supplemented glucosamine has shown to cause stimulation and protection to chondrocytes in osteoarthritic cartilage (Trumble, 2005). Glucosamine is naturally synthesized by chondrocytes which is incorporated into proteoglycans and hyaluronan from glucose. Physiological benefits of GLN are most likely due to the increase bioavailability of glucuronic acid and N-acetyl-galactosamine during digestion (Bassleer *et al.*, 1998; Trumble, 2005). These monosaccharides are the building blocks of the glycosaminoglycan (GAG) which is essential to chondroitin sulfate,

hyaluronan, and cartilage growth (Trumble, 2005). Glycosaminoglycan production has shown to be directly proportionate to the severity of joint disease (Forsyth *et al.*, 2006). Glucosamine has a minimal effect on collagen type II synthesis (Trumble, 2005).

Some human studies have shown that glucosamine sulfate is readily absorbed and distributed throughout the body while others state there is no benefit (Aghazadeh Habashi and Jamali, 2011; Koh and Dietz, 2005). In humans it takes up to four to eight weeks to show any effect. Trumble (2005) found in human and animals models, glucosamine has an approximate 90% absorption when orally administered. This subsequently allows incorporation into plasma proteins and is made available to the body by the liver (Trumble, 2005). Another study using radioactive labeling has found glucosamine to possess good distribution into articular cartilage (Trumble, 2005). There have been no known toxic effects documented as late as three years post supplementation (Trumble, 2005). Glucosamine as a molecule is completely water soluble and is mediated as a glucose transporter within the body. A review of nutraceuticals in horses found glucosamine sulfate possesses anti-inflammatory properties and inhibited cartilage degeneration (Trumble, 2005).

However, researchers do not agree on whether glucosamine directly influences cartilage. According to conflicting evidence, sulfate alone may be more responsible for increasing levels of serum sulfate in humans than other forms of glucosamine (Hoffer *et al.*, 2001). This study showed similar sulfate levels both in serum and synovial fluid. This indicates that changes in serum sulfate will match sulfate changes in synovial fluid. In addition, Hoffer's study showed that serum sulfate increased as early as three hours after the ingestion of 1g of glucosamine sulfate. Hoffer suggests that these changes offer evidence that sulfate is significantly involved in therapeutic effects of glucosamine sulfate. Interestingly, as a side note, Hoffer showed that

beneficial effects of GLN sulfate were reversed when patients ingested acetaminophen. This should be considered when used therapeutically and in future studies. In addition, conflicting research suggests that GLN being a larger molecule may not incorporate into cartilage but rather on nonarticular tissues (Hoffer *et al.*, 2001; Trumble, 2005). As aforementioned, absorption of nutrients in the joint is achieved through the synovial fluid and interstitial fluid to the articular cartilage, intraarticular ligaments, and proteoglycans. GLN may not be able to reach the synovium as joint capsule's endothelium prevents large molecules from leaving the synovial capillaries. Molecules less than 10kDa usually equilibrate between plasma and synovial fluid by simple diffusion (Todhunter, 1996).

Instead of a direct osteoarthritic preventive factor, glucosamine sulfate has also been linked to indirect benefits to the joint. Studies in equine and humans have showed that GLN decreases prostaglandin (PGE₂) in the arachidonic acid cascade inhibiting cyclo-oxygenase (COX-2) gene expression (Forsyth *et al.*, 2006; Trumble, 2005). Therefore, GLN aids in reducing inflammation. In addition, *in vitro* studies show that GLN down regulates mRNA of matrix metalloproteinases (MMP) and therefore supports the growth of the proteoglycans of the matrix. In particular, exogenous GLN helps prevent the degradation of the matrix caused by aggrecanase (Bassleer *et al.*, 1998; Trumble, 2005). Part of this effect can be linked to GLN reducing the transcription factors of intracellular signaling of the proinflammatory cytokine interleukin 1. This further regulates the proteinases by preventing this cytokine's production. Interleukin 1 is up regulated by prostaglandins in the inflammation response and has been shown to increase in incidences of OA. Bryon showed *in vitro* that the addition of glucosamine prevented the repression of glucuronosyltransferase I, an enzyme that is used in

glycosaminoglycan biosynthesis (Byron *et al.*, 2003). This enzyme has shown repression by interleukin-1 in rat models.

The mechanism by which the body uses glucosamine is still largely unknown (Aghazadeh Habashi and Jamali, 2011; Byron *et al.*, 2003; Hoffer *et al.*, 2001; Ilic *et al.*, 2008). Possibilities may lie in the genetic processes that lead to premature MMP mRNA degradation (Byron *et al.*, 2003) or affect aggrecanase gene expression and activation (Ilic *et al.*, 2008). Minimum effective dosages for animals remain unknown as well as associated plasma concentrations (Aghazadeh Habashi and Jamali, 2011). One study states that there is no evidence of efficient hepatic metabolism for GLN rather it is absorbed in the gut (Aghazadeh Habashi and Jamali, 2011).

Glucosamine and Oxidative Stress

Glucosamine's mechanism of action is not fully understood, but; it can be categorized as a scavenger for free radicals. In addition, supplementation of antioxidants such as GLN sulfate may protect cellular components against oxidation by restoring the oxidative signaling associated in the process of inflammation (Kirschvink *et al.*, 2008; Surapaneni and Venkataramana, 2007; Valvason *et al.*, 2008). A human study found a possible effective concentration of glucosamine sulfate at 10mmol/liter did protect against oxidative stress (Valvason *et al.*, 2008). This study determined heme oxygenase (HO-1) production by use of a commercial enzyme-linked immunosorbent assay. Results showed this concentration demonstrated restoring heme oxygenase-1 production in human chondrocytes which increases protein production (Valvason *et al.*, 2008). The mechanism of HO-1 is unknown. However, HO-1 has been shown to protect against damage caused by cellular stress (Valvason *et al.*, 2008) as well as possessing anti-apoptotic and anti-inflammatory properties. One study found HO-1 reduced clinical scores and

incidence of collagen-induced arthritis in mice (Devesa *et al.*, 2005). At a concentration of 10 mmol/liter, glucosamine sulfate may restore HO-1 gene expression controls damaged by IL-1 β . It has demonstrated approximately 30% inhibition of IL-1 β induced iNOS expression. As a result of its ability to restore the HO-1 gene and protein production, conclusions are drawn that glucosamine sulfate is effective in counteracting cytokines (Valvason *et al.*, 2008).

CHAPTER 6

PURPOSE OF THE STUDY

Many horses experience inflammation and pain from osteoarthritis. Incidences of the disease increase as horses age. Many owners experience significant financial loss when battling joint disease. Proactive and preventive intervention can greatly decrease the progression of the disease. In addition, further study of antioxidants in preventing inflammation responses is necessary. Glucosamine is a popular choice among human patients as evident from its global sale of greater than two billion dollars in 2009 (Aghazadeh Habashi and Jamali, 2011). Many joint supplements claim success in extending a horse's useable life span. However, most of these supplements are not regulated. Therefore, this study's purpose is to determine the effects of oral glucosamine sulfate supplementation on gait parameters and blood oxidative levels in the aged horse. The objectives of this study were to determine the efficacy of oral glucosamine sulfate on the quality of gait parameters, on the associated levels of oxidants in the circulatory system of the horse, and to provide better understanding of prophylactic use of alternative therapies on the aged horse. It was hypothesized that oral supplementation of glucosamine sulfate would show greater range of motion on selected gait parameters, that blood oxidative levels would decrease resulting in reduced joint inflammation.

CHAPTER 7

MATERIALS AND METHODS

Animals

Twelve horses, six mares and six geldings, between the ages of 9 to 24 years old were used. All horses were from stock horse, thoroughbred, or warm blood bloodlines. No gaited horses were used. Six horses were obtained from the University of Georgia's Women's Varsity Equestrian Team (UGA ET). Six others were obtained from the University of Georgia's Animal and Dairy Science Department's (ADS) Equine Teaching Unit horsemanship herd. The health of all horses was evaluated prior to the start of experiment. Gait evaluation for soundness was visually assessed and only sound horses were used. Selected horses had not received joint injections and supplements within one year. All animal procedures were conducted within the guidelines of and approved by the University of Georgia's Animal Care and Use Committee.

Diets, Housing, Shoeing

All horses were shod on a regular six week interval prior to and during evaluation. Toe length and hoof angle was recorded prior to and post trimming to account for dorsal hoof wall angulation and length consistency. The six UGA Equestrian Team horses received a commercial pelleted 12% crude protein concentrate. The six ADS horses received a textured grain 12% crude protein concentrate produced by the UGA Animal and Dairy Science Department. All horses were given ad libitum water. Bermuda mix hay was supplemented to pasture as needed. Concentrates and hay were adjusted in order to maintain body score conditions from 4.5 to 6.5. All horses were pastured a significant part of each day (>8 hrs) at each particular farm.

Exercise

The six horses obtained from the UGA Equestrian Team received routine exercise in a daily lesson program. UGA ADS horses were ridden four to five days per week in a lesson program. All horses were ridden in a regularly maintained and groomed dirt arena. Each horse's exercise consisted of walking, trotting, and cantering for the duration of 1 to 1.5 hours. All horses were worked on the flat. No horses were jumped or used in more athletic maneuvers such as sliding stops in the reining discipline.

Drug, Dosage, Administration

The study was executed using a double blind with repeated measures over time design. The selected horses were split into 2 groups: 6 control horses and 6 treatment horses. The horses were also paired by age, sex, location (UGA ET or ADS), and type of use. During the 90 day treatment period, all the horses were given a visually identical daily oral supplementation in the form of a treat. The treatment group received a supplement containing: 10 grams of 100% pharmaceutical grade glucosamine sulfate powder, sugar, molasses, flour, and peppermint coffee syrup. Glucosamine sulfate dosage was determined by a study which showed 20mg/kg of oral glucosamine sulfate increased levels of glucosamine in synovial fluid in the horse (Meulyzer *et al.*, 2008). The control group was fed a placebo containing identical ingredients except for the glucosamine sulfate powder. The treats were made one week in advance, wrapped in wax paper and refrigerated until time of feeding. The glucosamine sulfate was obtained through NutraBulk, Inc. (Phoenix, AZ).

Gait Parameter Assessment

Video footage was recorded using two high speed Ethernet GigE uEye™ cameras (IDS Imaging Development Systems, Obersulm, Germany) placed perpendicular to the line of travel

on either side of the horse. Cameras were synchronized and controlled through GigEye computer drivers and set to record at a frame rate of 70 frames per second. A calibration measurement was recorded for each horse using the distance from the marker at the carpal joint to the marker at the metacarpophalangeal joint. Each horse was walked and trotted in hand with the use of a halter and lead rope. The same handler was used throughout all video recording sessions for each horse to eliminate handler variability. Horses were allowed to travel at their own pace and only video recorded if no pressure was applied to their head and neck with the lead rope. The recording frame of the experimental setup was set at 3.00 meters in length. It was preceded by a warm up distance of 6.00 m and followed by an additional distance of 6.00 m in order to capture footage where the horse was in a consistent gait. Cameras were installed 9.00 meters away from the center of the recording path and perpendicular to the line of travel, and the height of the tripods were fixed at 86.00 cm. Horses first walked the set distances for warm up for two repetitions and then trotted the same set distances a total of six repetitions.

Horses were video recorded 10, 5, and 1 day(s) prior to treatment and then again at days 30, 60, and 90 during the supplementation period. There were a total of 36 video recordings for each horse. All horses were evaluated on each given day. Uniformly sized 3.81 cm reflective three dimensional markers were strategically placed at the center of joints on the right and left forelimbs. Marker placements were determined by palpation of anatomical land marks. To account for consistency of marker placement, the hair was clipped using electrical clippers from the determined anatomical land marks. In addition, an adjustable measuring stick was used to record the distances between markers. These distances and clipped areas were used in consistent marker placement for all recordings. The following anatomical land marks were determined: dorsal point of scapula (distances were measured from the occiput of the skull to the dorsal

scapula and the height of scapula); scapulohumeral joint (measured the height of the joint and the distance from scapula marker); elbow joint (measured the height of joint and the distance from scapulohumeral joint marker); carpal joint (measured the height of joint, the distance from elbow marker, and the distance from front of carpal); metacarpophalangeal joint (measured the height of joint, the distance from carpal marker, and the distance from front of metacarpophalangeal joint). Uniformly sized reflective three dimensional markers were also placed at the center of rotation of the following joints on the left and right hind limb: tuber coxae of the pelvis, greater trochanter of the coxofemoral joint, lateral condyle of the tibia where it articulates with the femur (stifle), articulation of the fourth tarsal bone and metatarsus (hock), and the metatarsalphalangeal joint.

Equine Tec (Monroe, GA, USA) software was used in analyzing all video recordings. All parameters were manually tracked through each frame. Each of the 30 gait parameters was isolated and measured in degrees, time, velocity, length, or distance. All researchers used in analyzing the video recordings were blinded to the study. The front leg markers were used in measuring the following eight front leg joint parameters: minimum elbow angle (measured the angle created during the mid-swing phase from the scapulohumeral joint to the elbow joint to the carpal joint); maximum elbow angle (measured the angle created during the late retraction phase from the scapulohumeral joint to the elbow joint to the carpal joint); elbow angle range of motion (measured the amount of change between minimum and maximum elbow angles); minimum carpal angle (measured the angle created during the mid-swing phase from the elbow joint to the carpal joint to the metacarpophalangeal joint); maximum carpal height (measured during the swing phase as the distance perpendicular from the carpal joint to the ground); minimum fetlock angle (measured the extension of the metacarpophalangeal joint during the stance phase by

obtaining the angle measured from the carpal to the metacarpophalangeal joint to the toe); minimum fetlock height (measured during the stance phase as the distance perpendicular from the metacarpophalangeal joint to the ground); and maximum fetlock height (measured during the swing phase as the distance perpendicular from the metacarpophalangeal joint to the ground). In addition the front leg markers were used in measuring the following eight front stride parameters: leg protraction angle (measured the angle between the perpendicular line drawn to the ground at the scapula marker to the line drawn by connecting the scapula marker to the toe during the maximum protraction phase); leg protraction distance (measured from the perpendicular line drawn from the ground, which was isolated at mid-point between retraction and protraction, to where the toe landed at the end of the protraction phase); leg retraction angle (measured the angle between the perpendicular line drawn to the ground at the scapula marker to the line drawn by connecting the scapula marker to the toe during the maximum retraction distance); leg retraction distance (measured from the perpendicular line drawn from the ground, which was isolated at mid-point between retraction and protraction, to the toe at the maximum retraction phase); stride length (measured the distance from where the toe left the ground in the retraction phase to where the same toe landed during the protraction phase); stride velocity (measured the combined time over distance of the swing and stance phases); swing time (measured the amount time the hoof was suspended in the air); and stance time (measured the amount of time the hoof contacted the ground to where the hoof left the ground).

The hind leg markers were used in measuring the following six hind leg joint parameters: minimum fetlock angle (measured the extension of the metacarpophalangeal joint during the stance phase by obtaining the angle measured from the hock to the metacarpophalangeal joint to the toe); maximum fetlock height (measured during the swing phase as the distance

perpendicular from the metacarpophalangeal joint to the ground); minimum fetlock height (measured during the stance phase as the distance perpendicular from the metacarpophalangeal joint to the ground); minimum hock angle (measured during the most flexed position of the swing phase from the stifle joint to the hock joint to the metacarpophalangeal joint); maximum hock angle (measured during the most retracted stance phase from the stifle joint to the hock joint to the metacarpophalangeal joint); hock angle range of motion (measured the amount of change between minimum and maximum hock angles). In addition the hind leg markers were used in measuring the following eight hind stride parameters: leg protraction angle (measured the angle between the perpendicular line drawn to the ground at the tuber coxae marker to the line drawn by connecting the tuber coxae marker to the toe during the maximum protraction phase); leg protraction distance (measured from the perpendicular line drawn from the ground, which was isolated at mid-point between retraction and protraction, to where the toe landed at the end of the protraction phase); leg retraction angle (measured the angle between the perpendicular line drawn to the ground at the tuber coxae marker to the line drawn by connecting the tuber coxae marker to the toe during the maximum retraction distance); leg retraction distance (measured from the perpendicular line drawn from the ground, which was isolated at mid-point between retraction and protraction, to the toe at the maximum retraction phase); stride length (measured the distance from where the toe left the ground in the retraction phase to where the same toe landed during the protraction phase); stride velocity (measured the combined time over distance of the swing and stance phases); swing time (measured the amount time the hoof was suspended in the air); and stance time (measured the amount of time the hoof contacted the ground to where the hoof left the ground).

Blood Collection

Blood via jugular puncture was collected using a BD Vacutainer K3 EDTA 12mg purple top tube the morning after video recording days 10, 5, and 1 day(s) prior to supplementation and days 30, 60, and 90 post supplementation. Samples were immediately stored in a cooler in ice during the collection time period to slow the oxidative process to protect them from ambient temperatures. The cooler also protect the blood samples from ultra violet light. Also, blood was protected from exposure to atmospheric oxygen via the purple top vacutainers. In the lab, the blood was centrifuged for 10 minutes at 3,000 rpms and the plasma was pipetted into three individual micro-tubule samples. Enough plasma was collected for three micro-tubules per sample. These tubules were kept in a -80°C freezer for later analysis.

Thiobarbituric acid reactive substances (TBARS): Centrifugation Method Assay

For determining levels of malonaldehyde (MDA), the TBARS procedure was conducted according to the methods established by Ahn et al. (1998) and Ahn and Jo (1998). Pre-testing of the TBAR procedure by preparation of TEP (1,1,3,3 – Tetraethoxypropane) standards was performed extensively prior to sample testing. This pre-testing period ensured consistency of laboratory methods during sample testing.

A solution of Thiobarbituric Acid/ Trichloroacetic acid (TBA/TCA) was prepared ahead of analysis procedure. This was to allow the TBA to go into solution. The solution was prepared by adding 500 ml of 20 mM TBA into 15% TCA solution. The TBA/TCA solution was then kept on a stir plate for later use. In addition, a solution of Butylated Hydroxy Toluene (BHT) was required. This BHT solution was prepared by adding 3.6 grams of BHT in 50 ml of ethanol (95%). This solution was stored at room temperature for later use.

On a total of two separate sample analysis days, half of the plasma tubules were randomly chosen and then were thawed on ice. Each sample was tested in triplicate including the standards for accuracy and consistency. During the thawing period TEP (1,1,3,3 – Tetraethoxypropane) standards were prepared for comparison against samples. The additional following steps were taken to complete the TBARS procedure: In tubes, ten TEP standards were prepared using the daily TEP standard. This required a weekly $1 \times 10^{-3} \text{M}$ TEP standard to be made. This was accomplished by adding 23.94 μl of concentrated TEP to 100ml of deionized water. The weekly TEP was stored in a cooler. The weekly TEP was diluted again to create a daily working $1 \times 10^{-5} \text{M}$ TEP standard. This was done by adding the $1 \times 10^{-3} \text{M}$ TEP to 100mls of deionized water into a 100ml volumetric flask. Once thawed, each plasma sample was added at a 25% concentration to tubes. This was done by adding 0.25 ml to 0.75 ml of deionized water. Fifty μl of BHT solution was then added to all tubes. Two ml of TBA/TCA solution as added to all tubes. All tubes were then vortexed. Tubes were then incubated in a preheated (90 °C) water bath for 15 minutes. Tubes were allowed to cool in tap water for 10 minutes. Tubes were centrifuged at room temperature for 15 minutes at 3000 x g (4000rpm). Three samples of each time point of each horse were analyzed using a spectrometer. Absorbance of supernatant was read at a wavelength of 531 nanometers against a blank. Data is reported in moles per milliliter of MDA.

Statistical Analysis

Data for both kinematic and blood variables were analyzed using SAS version 9.3 Proc GLM (SAS Institute Inc., Cary, NC, Proprietary Software 9.3 (TS1MO)) with repeated measures over time to determine the effects of treatment, time, and treatment by time interactions. Right and left side data for all pretreatment times (pre 1, pre 2, and pre 3) was averaged for a final

pretreatment average for analysis. Post treatment data of both the right and left sides was averaged and pretreatment average was subtracted from each post treatment average (30d post, 60d post, 90d post) to give three time points used for statistical analysis (post 1, post 2, and post 3). This adjusted data was run in SAS 9.3 to determine any significance in treatment over time. Gait symmetry was determined and prepared by taking the difference of left and right side at each of each gait parameter. Treatment differences were analyzed over four time points (pretreatment average, 30d post, 60d post, 90d post) for changes in gait symmetry. In addition, outlier tests were performed and all gait parameters were measured against three standard deviations. Outliers were eliminated and data were reanalyzed with the same previously mentioned data preparations and SAS 9.3 codes. Hoof measurements were analyzed using treatment by time SAS code. TBAR data was prepared by calculating a final average for each time point of all pretreatment averages. Data was then prepared by taking the post treatment average minus the pretreatment final average for each time point. This data was enter into SAS and used the same treatment over time comparison codes. Significance was determined by $P < 0.05$ and trends were determined by $P < 0.10$.

CHAPTER 8

RESULTS

Twelve horses and 6 time periods (3 pretreatment and 3 post supplementation) created 72 gait data collections. At each time point, the video recordings were analyzed for gait parameters. Blood was taken the following morning of each pretreatment and post treatment time points which yielded 72 blood collections for analysis. All horses remained healthy and maintained good weight for the duration of the study.

Initial analysis that includes outlier data (Tables 1-8) showed significance was seen between treatments at certain time points for the following gait parameters: front fetlock minimum height showed differences between treatment at d30 ($P=0.0451$, Table 1) to d60 ($P=0.0058$, Table 1); hind leg protraction angle increased for treatment horses at d90 ($P=0.0478$, Table 7); hind leg retraction angle decreased in treatment horses by d60 ($P=0.0294$, Table 7). Additional significance was found in the following symmetry parameters: front leg fetlock minimum height ($P=0.0373$, Table 2) was more symmetrical in comparison to control at d30; front leg retraction distance ($P=0.0168$, Table 4) was smaller in comparison to control at d60; and both front leg stride length ($P=0.0202$, Table 4) and hind stride length ($P=0.0195$, Table 8) were more symmetrical in comparison to control and decreased over time at d60. The outlier tests revealed two horses with data entries beyond three standard deviations from the mean of the particular parameter. One outlier was found in the pretreatment data (10 days prior to supplementation) for the front fetlock minimum height parameter on the right side. Another

outlier was found at d90 in the hind fetlock minimum angle parameter on the left side. Both horses' data was completely eliminated from analysis at those time points.

After eliminating outliers, the prepared data were again analyzed using the same treatment by time and symmetry SAS codes as previous analysis (Tables 9-16). Significant differences were seen at certain time points for the following gait parameters: front fetlock minimum height ($P=0.0072$, Table 9) decreased over time for treatment horses at d60 and hind leg retraction angle ($P=0.0154$, Table 15) decreased over time for treatment horses by d60.

Although no significance was found when directly comparing control horses to treatment horses at each time point, the front fetlock minimum angle averages (Table 9, Figure 4) showed a steady decrease from treatment d30 to d90 ($P=0.0176$) for glucosamine supplemented horses, but not for control horses. A trend ($P=0.0794$) was observed in glucosamine supplemented horses to have more retraction of the front leg (Table 11) when comparing d30 to d90. In addition, the hock minimum angle (Table 13) averages showed no difference comparing control against treatment at any time point. However, a P -value of 0.0519 was observed as the angle decreased over time when comparing d30 to d90 for glucosamine supplemented horses (Figure 6). In the front and hind leg stride parameters (Table 11 and 15), velocity as well as stride length in both the control and treatment increased over the supplementation time period of 30, 60, and 90 days.

Significance was found in the following symmetry parameters: front leg retraction distance in treatment horses ($P=0.0178$, Table 12) became more symmetrical from pretreatment time points to d30 post supplementation; front stride length ($P=0.0219$, Table 12) became more symmetrical for treatment horses from pretreatment time points to d30; and hind stride length ($P=0.0211$, Table 16) became more symmetrical from pretreatment average to d30.

No differences were found between treatment and control horses in malondialdehyde levels (as measured by TBARS assay) at any time point (Table 17). Dorsal hoof wall angles and dorsal hoof wall toe lengths were collected at each shoeing interval using an aluminum hoof gauge and tape measurer. Each horse was shod on a six week rotation and was shod at least one rotation prior to pretreatment. Figure 2 demonstrates that all the dorsal hoof wall length for all the horses remained consistent during the study period. Dorsal hoof wall angles remained constant expect during the late supplementation period (Figure 3). All hoof angles averages were within 5° from the most upright angle to the most acute angle. No statistical differences were found in the hoof measurements over time.

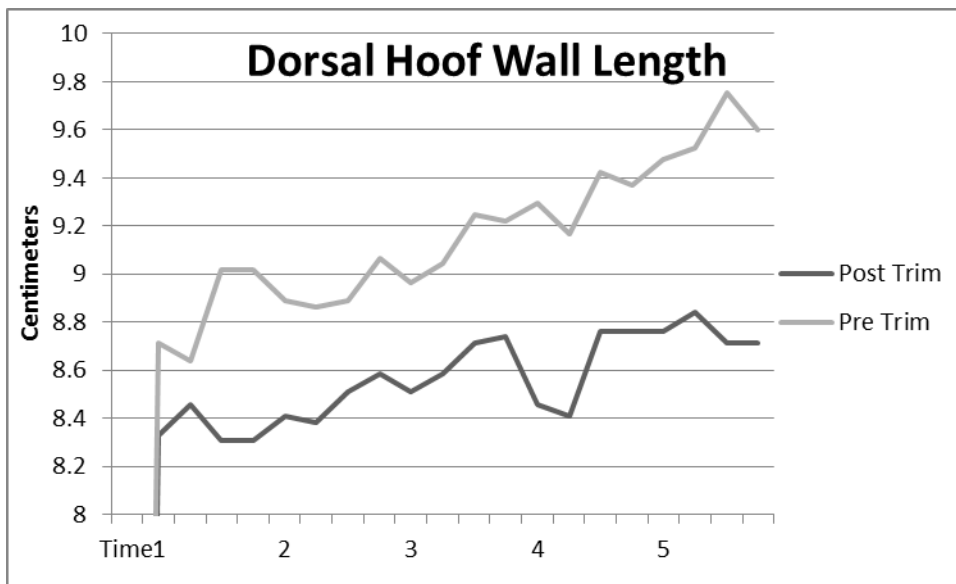


Figure 2 – Dorsal Hoof Wall Length over Time

– Time indicates dorsal hoof wall length prior to pretreatment and post supplementation time points

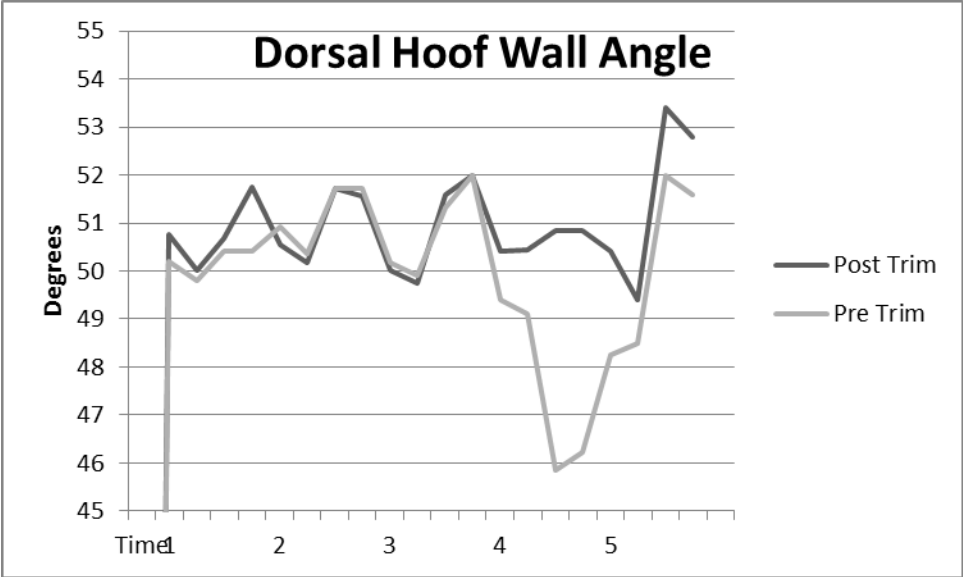


Figure 3 – Dorsal Hoof Wall Angle over Time
 – Time indicates dorsal hoof wall length prior to pretreatment and post supplementation time points

INITIAL ANALYSIS TABLES

Table 1- Front leg joint parameters (treatment over time) ¹

- include outlier data
- 1- Values reported as differences in means (post time point – pretreatment average)
- 2- Times 1,2,3 indicate day 30, 60, 90 during supplementation period
- 3- Control horses received placebo cookie daily
- 4- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std. Error</u>	<u>P value</u>
Elbow angle (minimum) (degrees)	1	-0.39	0.91	1.9	0.4998
	2	0.02	1.82	2.0	0.3124
	3	0.50	1.82	1.9	0.4911
Elbow angle (maximum) (degrees)	1	-0.39	0.91	1.9	0.4998
	2	0.02	2.07	2.0	0.3124
	3	0.50	1.82	1.9	0.4911
Elbow angle (range of motion) (degrees)	1	-1.01	-0.75	1.7	0.8759
	2	-1.28	-1.89	1.8	0.4934
	3	0.67	-1.77	1.7	0.1577
Knee angle (minimum) (degrees)	1	-1.54	-0.78	1.9	0.6994
	2	-2.52	-1.91	1.9	0.755
	3	-0.72	0.88	1.8	0.3935
Knee height (maximum) (cm)	1	0.39	1.34	2.8	0.7401
	2	1.68	4.59	3.0	0.337
	3	7.40	7.64	2.8	0.9353
Fetlock angle (minimum) (degrees)	1	0.12	0.41	1.3	0.8326
	2	-1.15	-1.17	1.4	0.9833
	3	-0.74	-3.00	1.3	0.0948
Fetlock height (minimum) (cm)	1	-2.36	1.57	1.8	0.0451*
	2	-3.72	2.14	2.0	0.0058**
	3	-2.51	0.06	1.8	0.1113
Fetlock height (maximum) (cm)	1	0.33	-0.05	4.1	0.8499
	2	-0.15	1.86	4.1	0.6296
	3	-2.85	0.27	3.8	0.4345

Table 2 – Front leg joint parameters (symmetry) ¹

- include outlier data

1- Values reported as differences in means (post time point – pretreatment average)

2- Times 1,2,3,4 indicate pretreatment averages and day 30, 60, 90 during supplementation period

3- Control horses received placebo cookie daily

4- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
Elbow angle (minimum) (degrees)	1	4.12	4.22	1.9	0.9583
	2	4.92	5.09	1.9	0.9258
	3	4.52	3.42	2.0	0.5813
	4	5.08	3.63	1.9	0.4416
Elbow angle (maximum) (degrees)	1	4.34	4.07	2.0	0.8929
	2	3.31	5.44	2.0	0.2968
	3	4.02	3.64	2.1	0.8578
	4	4.72	5.45	2.0	0.7176
Elbow angle (range of motion) (degrees)	1	1.77	1.82	1.0	0.9635
	2	2.94	4.09	1.0	0.2794
	3	2.53	2.14	1.1	0.7209
	4	1.56	2.79	1.0	0.2472
Knee angle (minimum) (degrees)	1	2.74	2.27	2.2	0.8294
	2	0.88	2.54	2.5	0.5184
	3	4.50	4.83	2.3	0.8879
	4	5.62	3.12	2.2	0.2996
Knee angle (maximum) (degrees)	1	2.72	1.56	1.4	0.403
	2	4.49	3.02	1.4	0.2897
	3	2.54	1.07	1.4	0.2267
	4	2.16	2.81	1.4	0.6350
Fetlock angle (minimum) (degrees)	1	1.50	1.33	1.3	0.7806
	2	2.47	1.80	1.3	0.5977
	3	2.35	2.29	1.3	0.9657
	4	4.25	2.89	1.3	0.2920
Fetlock height (minimum) (cm)	1	7.10	2.05	2.3	0.0373*
	2	3.80	3.15	2.3	0.7851
	3	2.54	4.00	2.5	0.5554
	4	1.42	2.93	2.3	0.5240
Fetlock height (maximum) (cm)	1	5.03	4.39	3.8	0.8679
	2	6.95	14.32	3.8	0.0593
	3	6.19	10.02	3.8	0.319
	4	6.96	7.01	3.6	0.9907

Table 3 – Front leg stride parameters (treatment over time) ¹

- include outlier data

1- Values reported as differences in means (post time point – pretreatment average)

2- Times 1,2,3 indicate day 30, 60, 90 during supplementation period

3- Control horses received placebo cookie daily

4 - Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
Leg protraction angle (degrees)	1	0.24	0.15	0.5	0.4437
	2	0.40	0.32	0.5	0.8803
	3	0.99	0.33	0.5	0.1913
Leg protraction distance (cm)	1	8.91	3.37	6.4	0.3864
	2	8.73	3.96	6.6	0.4754
	3	11.54	13.01	6.4	0.8167
Leg retraction angle (degrees)	1	0.01	-0.05	0.4	0.8809
	2	-0.54	-0.05	0.4	0.2311
	3	-0.93	-0.35	0.4	0.136
Leg retraction distance (cm)	1	3.97	2.18	3.6	0.6063
	2	-1.13	-0.32	3.6	0.8233
	3	0.95	6.47	3.6	0.1199
Stride length (cm)	1	20.40	7.04	26.2	0.6148
	2	26.61	14.23	27.4	0.6563
	3	54.97	48.56	26.2	0.8087
Stride velocity (m/s)	1	0.24	0.04	0.22	0.356
	2	0.24	0.06	0.23	0.4287
	3	0.31	0.25	1.2	0.7619
Swing time (s)	1	0.00	0.01	0.01	0.3357
	2	0.01	0.01	0.01	0.7957
	3	0.01	0.01	0.01	0.8461
Stance time (s)	1	-0.02	-0.01	0.01	0.2887
	2	-0.02	-0.01	0.01	0.7594
	3	-0.02	-0.02	0.01	0.593

Table 4 – Front leg stride parameters (symmetry) ¹

- include outlier data

1-Values reported as differences in means (post time point – pretreatment average)

2-Times 1,2,3,4 indicate pretreatment averages and day 30, 60, 90 during supplementation period

3-Control horses received placebo cookie daily

4-Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
Leg protraction angle (degrees)	1	0.79	0.86	0.65	0.9131
	2	1.99	1.61	0.65	0.5635
	3	1.21	0.97	0.68	0.7272
	4	1.41	1.16	0.65	0.6979
Leg protraction distance (cm)	1	8.81	7.30	5.8	0.8017
	2	15.76	9.06	5.8	0.2683
	3	18.93	14.98	6.4	0.531
	4	10.14	17.17	5.8	0.2452
Leg retraction angle (degrees)	1	1.08	0.59	0.65	0.4633
	2	2.31	1.17	0.65	0.879
	3	1.22	1.03	0.68	0.7805
	4	1.73	1.19	0.65	0.4099
Leg retraction distance (cm)	1	7.56	8.40	5.3	0.8774
	2	21.75	8.26	5.3	0.0168*
	3	10.12	7.65	5.3	0.6641
	4	16.34	9.40	5.3	0.2058
Stride length (cm)	1	36.03	25.82	18.0	0.5750
	2	67.79	24.09	18.0	0.0202*
	3	34.66	23.62	18.8	0.5627
	4	43.18	30.23	18.0	0.4772
Stride velocity (m/s)	1	0.14	0.13	0.08	0.9278
	2	0.24	0.21	0.08	0.7238
	3	0.11	0.08	0.08	0.7039
	4	0.15	0.10	0.08	0.4921
Swing time (s)	1	0.01	0.01	0.00	1.0
	2	0.01	0.01	0.00	0.6427
	3	0.01	0.00	0.00	0.7905
	4	0.00	0.01	0.00	0.3555
Stance time (s)	1	0.00	0.01	0.00	0.1387
	2	0.00	0.01	0.00	0.1387
	3	0.01	0.01	0.00	0.297
	4	0.00	0.01	0.00	0.6172

Table 5 – Hind leg joint parameters (treatment over time)¹

- include outlier data

1- Values reported as differences in means (post time point – pretreatment average)

2- Times 1,2,3 indicate day 30, 60, 90 during supplementation period

3- Control horses received placebo cookie daily

4- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
Fetlock angle(minimum)	1	1.55	0.91	1.4	0.6420
(degrees)	2	0.92	-1.13	1.4	0.1595
	3	2.25	-0.35	1.4	0.0651
Fetlock height (maximum) (cm)	1	4.94	2.99	3.3	0.5572
	2	5.87	5.33	3.6	0.8781
	3	3.61	4.07	3.3	0.8893
Fetlock height (minimum) (cm)	1	2.16	1.15	2.0	0.6169
	2	-0.30	0.62	2.1	0.6611
	3	0.32	1.21	2.0	0.6616
Hock angle (minimum)(degrees)	1	-0.53	-0.42	1.6	0.9489
	2	-0.82	-1.31	1.6	0.7699
	3	-1.62	-3.73	1.6	0.1899
Hock angle (maximum)(degrees)	1	-0.87	-0.45	1.4	0.7754
	2	1.16	0.13	1.5	0.5016
	3	1.8	-0.73	1.4	0.0899
Hock angle range of motion	1	-0.30	-0.09	1.6	0.8957
(degrees)	2	2.02	1.37	1.7	0.7105
	3	3.46	2.95	1.6	0.7564

Table 6 – Hind leg joint parameters (symmetry) ¹

- include outlier data

1- Values reported as differences in means (post time point – pretreatment average)

2- Times 1,2,3,4 indicate pretreatment averages and day , 30, 60, 90 during supplementation period

3- Control horses received placebo cookie daily

4- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
Fetlock angle (minimum)	1	2.56	2.48	1.8	0.966
(degrees)	2	3.17	2.64	1.8	0.7715
	3	4.64	2.97	1.9	0.3779
	4	6.47	4.31	1.8	0.2340
Fetlock height (maximum) (cm)	1	1.25	1.64	3.3	0.9037
	2	3.81	5.69	3.3	0.5608
	3	8.64	7.35	3.3	0.7026
	4	7.65	8.70	3.3	0.7433
Fetlock height (minimum) (cm)	1	2.63	1.84	1.4	0.5675
	2	1.45	3.03	1.4	0.2608
	3	4.11	2.31	1.5	0.2213
	4	5.16	2.69	1.4	0.0810
Hock angle (minimum) (degrees)	1	4.12	4.22	1.9	0.9584
	2	4.92	5.09	1.9	0.9261
	3	7.65	6.80	2.0	0.6664
	4	4.11	6.08	1.9	0.2992
Hock angle (maximum) (degrees)	1	4.34	4.07	2.2	0.9027
	2	3.31	5.43	2.2	0.3428
	3	4.96	4.43	2.3	0.8231
	4	2.86	4.06	2.2	0.5942
Hock angle range of motion	1	1.77	1.82	1.2	0.9681
(degrees)	2	2.94	4.09	1.2	0.344
	3	3.19	3.03	1.3	0.8999
	4	2.86	3.92	1.2	0.3841

Table 7 - Hind leg stride parameters (treatment over time) ¹

- include outlier data

1- Values reported as differences in means (post time point – pretreatment average)

2- Times 1,2,3 indicate day 30, 60, 90 during supplementation period

3- Control horses received placebo cookie daily

4- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
Leg protraction angle	1	0.44	0.12	0.45	0.4842
(degrees)	2	-0.92	0.76	0.45	0.0795
	3	0.11	1.04	0.45	0.0478*
Leg protraction distance (cm)	1	-0.41	1.47	5.8	0.7517
	2	-2.76	8.04	5.8	0.0902
	3	5.98	7.41	5.8	0.8086
Leg retraction angle (degrees)	1	0.72	0.25	0.58	0.4266
	2	1.11	-0.25	0.61	0.0294*
	3	1.32	0.59	0.58	0.2246
Leg retraction distance (cm)	1	2.95	2.54	7.4	0.9563
	2	6.95	-1.11	7.9	0.3098
	3	10.56	2.34	7.4	0.2783
Stride length (cm)	1	17.96	9.88	26.9	0.7658
	2	20.16	14.67	28.2	0.8469
	3	46.27	44.70	26.9	0.9537
Stride velocity (m/s)	1	0.23	0.09	0.24	0.5578
	2	0.19	0.08	0.25	0.6339
	3	0.31	0.24	0.25	0.7943
Swing time (s)	1	0.00	0.01	0.01	0.452
	2	0.00	0.01	0.01	0.1891
	3	-0.01	0.00	0.01	0.3485
Stance time (s)	1	-0.02	-0.02	0.01	0.4566
	2	-0.01	-0.01	0.01	0.3
	3	-0.01	-0.02	0.01	0.4566

Table 8 – Hind leg stride parameters (sidedness) ¹

- include outlier data

1- Values reported as differences in means (post time point – pretreatment average)

2- Times 1,2,3,4 indicate pretreatment averages and day 30, 60, 90 during supplementation period

3- Control horses received placebo cookie daily

4- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

Parameter	Time ²	Control ³	Treatment ⁴	Std Error	P value
Leg protraction angle (degrees)	1	0.91	0.84	0.43	0.8745
	2	1.50	2.30	0.43	0.0691
	3	1.00	0.98	0.45	0.9684
	4	0.55	1.08	0.43	0.2235
Leg protraction distance (cm)	1	8.15	5.79	5.6	0.6821
	2	15.86	14.94	5.6	0.8731
	3	12.20	8.479	6.1	0.5382
	4	11.71	13.53	5.6	0.7517
Leg retraction angle (degrees)	1	0.78	0.69	0.70	0.8927
	2	0.89	1.72	0.70	0.2398
	3	1.47	1.48	0.73	0.9928
	4	1.37	1.59	0.70	0.7584
Leg retraction distance (cm)	1	13.54	5.44	6.9	0.2402
	2	8.61	16.55	6.9	0.2495
	3	14.22	26.16	7.1	0.1016
	4	17.92	25.66	6.9	0.2614
Stride length (cm)	1	39.26	29.08	18.8	0.5919
	2	72.97	27.11	18.8	0.0195*
	3	42.09	21.03	19.8	0.2925
	4	51.96	33.54	18.8	0.3338
Stride velocity (m/s)	1	0.10	0.14	0.08	0.6167
	2	0.27	0.18	0.08	0.2983
	3	0.07	0.10	0.08	0.7757
	4	0.20	0.73	0.08	0.4122
Swing time (s)	1	0.01	0.01	0.01	0.5105
	2	0.01	0.01	0.01	1.0
	3	0.01	0.01	0.01	0.1913
	4	0.01	0.01	0.01	0.7416
Stance time (s)	1	0.01	0.01	0.00	0.394
	2	0.01	0.02	0.00	0.0927
	3	0.00	0.01	0.00	0.1083
	4	0.00	0.01	0.00	0.2037

FINAL ANALYSIS TABLES

Table 9- Front leg joint parameters (treatment over time) ¹

- exclude outlier data

^{a,b} - Means within column within parameter differ (P<0.10)

1- Values reported as differences in means (post time point – pretreatment average)

2- Times 1,2,3 indicate day 30, 60, 90 during supplementation period

3- Control horses received placebo cookie daily

4- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
Elbow angle- minimum (degrees)	1	-0.16	0.91	2.0	0.6013
	2	0.02	2.07	2.0	0.3123
	3	1.07	1.82	2.0	0.7127
Elbow angle- maximum (degrees)	1	-.18	0.40	1.9	0.7571
	2	-0.49	0.51	1.9	0.5985
	3	1.83	0.39	1.9	0.4490
Elbow angle- range of motion (degrees)	1	-0.12	-0.75	1.7	0.7187
	2	-0.67	-1.89	1.7	0.4860
	3	0.48	-1.77	1.7	0.2054
Knee angle (minimum) (degrees)	1	-1.54	-0.78	1.9	0.6988
	2	-2.52	-1.91	1.9	0.7545
	3	-0.09	0.88	1.9	0.6166
Knee height (maximum) (cm)	1	0.55	1.34	2.5	0.7997
	2	1.68	4.59	2.5	0.3497
	3	8.07	7.62	2.5	0.888
Fetlock angle (minimum) (degrees)	1	-0.12	0.41 ^a	1.4	0.7114
	2	-1.15	-1.174 ^{a,b}	1.4	0.9838
	3	-0.90	-3.00 ^b	1.4	0.1481
Fetlock height (minimum) (cm)	1	-2.04	1.57	2.0	0.0844
	2	-3.72	2.14	2.0	0.0072**
	3	2.86	0.57	2.0	0.0999
Fetlock height (maximum) (cm)	1	-1.94	-0.46	4.1	0.7165
	2	-1.48	1.86	4.1	0.6061
	3	-4.61	0.27	4.1	0.2174

Table 10 – Front leg joint parameters (symmetry) ¹

- exclude outlier data

1- Values reported as differences in means (post time point – pretreatment average)

2- Times 1,2,3,4 indicate pretreatment averages and day 30, 60, 90 during supplementation period

3- Control horses received placebo cookie daily

4- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
Elbow angle (minimum) (degrees)	1	4.29	3.63	2.0	0.7466
	2	3.81	7.09	2.0	0.1016
	3	5.27	3.43	2.1	0.3940
	4	5.08	3.63	2.0	0.4622
Elbow angle (maximum) (degrees)	1	3.78	6.64	-2.9	0.2219
	2	4.11	5.30	-1.2	0.5888
	3	4.06	3.64	2.4	0.8619
	4	4.72	5.45	2.2	0.7388
Elbow angle (range of motion) (degrees)	1	3.14	2.89	1.6	0.8743
	2	3.14	4.67	1.5	0.3169
	3	2.39	2.13	1.7	0.8795
	4	1.56	2.79	1.5	0.4188
Knee angle (minimum) (degrees)	1	2.42	2.27	1.5	0.9464
	2	0.88	2.54	1.4	0.5184
	3	5.23	4.83	1.5	0.8879
	4	5.62	3.31	1.4	0.3046
Knee height (maximum) (cm)	1	7.46	3.96	3.8	0.3522
	2	11.41	7.66	3.6	0.2965
	3	7.41	10.96	3.8	0.3661
	4	5.47	7.15	3.6	0.6397
Fetlock angle (minimum) (degrees)	1	1.33	1.33	1.3	0.9958
	2	2.47	1.80	1.3	0.5968
	3	2.35	2.30	1.3	0.9656
	4	4.73	2.89	1.3	0.1744
Fetlock height (minimum) (cm)	1	4.84	2.05	2.2	0.2123
	2	3.80	3.16	2.1	0.7607
	3	2.54	4.00	2.2	0.5104
	4	1.60	2.93	2.2	0.5506
Fetlock height (maximum) (cm)	1	3.53	4.39	4.1	0.8293
	2	6.95	14.33	3.8	0.0607
	3	6.19	10.02	3.8	0.3213
	4	6.96	7.01	3.8	0.9903

Table 11 – Front leg stride parameters (treatment over time) ¹

- exclude outlier data

^{a,b} - Means within column within parameter differ (P<0.10)

1- Values reported as differences in means (post time point – pretreatment average)

2- Times 1,2,3 indicate day 30, 60, 90 during supplementation period

3- Control horses received placebo cookie daily

4 - Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

Parameter	Time ²	Control ³	Treatment ⁴	Std Error	P value
Leg protraction angle (degrees)	1	0.10	-0.15	0.51	0.6357
	2	0.40	0.32	0.51	0.8789
	3	0.77	0.33	0.51	0.3913
Leg protraction distance (cm)	1	8.93	3.37	6.6	0.4138
	2	8.73	3.96	6.6	0.4820
	3	9.34	13.01	6.6	0.5882
Leg retraction angle (degrees)	1	0.14	-0.05	0.40	0.6365
	2	-0.54	-0.05	0.40	0.2368
	3	-0.88	-0.35	0.40	0.1933
Leg retraction distance (cm)	1	4.58	2.18 ^{a,b}	3.8	0.5236
	2	-1.14	-0.32 ^a	3.8	0.8285
	3	1.07	6.47 ^b	3.8	0.1588
Stride length (cm)	1	20.40	7.04	24.9	0.5962
	2	26.61	14.23	26.2	0.6393
	3	38.83	48.56	26.2	0.7124
Stride velocity (m/s)	1	0.24	0.04	0.21	0.3511
	2	0.24	0.06	0.22	0.4240
	3	0.23	0.25	0.22	0.9289
Swing time (s)	1	0.00	0.012	0.01	0.3438
	2	0.01	0.014	0.01	0.7990
	3	0.01	0.005	0.01	0.9130
Stance time (s)	1	-0.02	-0.02	0.01	0.2841
	2	-0.15	-0.01	0.01	0.7571
	3	-0.02	-0.02	0.01	0.8906

Table 12 – Front leg stride parameters (symmetry) ¹

- exclude outlier data

1- Values reported as differences in means (post time point – pretreatment average)

2- Times 1,2,3,4 indicate pretreatment averages and day 30, 60, 90 during supplementation period

3- Control horses received placebo cookie daily

4- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

Parameter	Time ²	Control ³	Treatment ⁴	Std Error	P value
Leg protraction angle (degrees)	1	0.82	0.86	0.70	0.9590
	2	1.99	1.61	0.67	0.5735
	3	1.25	0.97	0.73	0.7026
	4	1.41	1.16	0.67	0.7053
Leg protraction distance (cm)	1	10.20	7.30	6.4	0.647
	2	15.76	9.06	6.1	0.2711
	3	20.88	14.98	6.1	0.3738
	4	10.14	17.17	6.1	0.2480
Leg retraction angle (degrees)	1	1.23	0.59	0.69	0.3574
	2	2.31	1.17	0.66	0.0898
	3	1.43	1.03	0.72	0.5778
	4	1.73	1.19	0.65	0.4125
Leg retraction distance (cm)	1	7.99	8.40	5.6	0.9439
	2	21.75	8.26	5.3	0.0178*
	3	12.11	7.65	5.8	0.4582
	4	16.34	9.39	5.3	0.2096
Stride length (cm)	1	36.03	25.82	18.3	0.5800
	2	67.79	24.09	18.3	0.0219*
	3	34.66	23.62	19.1	0.5678
	4	42.88	30.23	19.1	0.5136
Stride velocity (m/s)	1	0.14	0.13	0.08	0.9287
	2	0.24	0.21	0.08	0.7272
	3	0.11	0.08	0.08	0.7075
	4	0.15	0.09	0.08	0.4843
Swing time (s)	1	0.01	0.01	0.00	1.0000
	2	0.01	0.01	0.00	0.6467
	3	0.01	0.00	0.00	0.7930
	4	0.00	0.01	0.00	0.4327
Stance time (s)	1	0.00	0.01	0.00	0.1357
	2	0.00	0.01	0.00	0.1357
	3	0.01	0.01	0.00	0.2933
	4	0.00	0.01	0.00	0.3887

Table 13 – Hind leg joint parameters (treatment over time)¹

- exclude outlier data

^{a,b} - Means within column within parameter differ (P<0.10)

1- Values reported as differences in means (post time point – pretreatment average)

2- Times 1,2,3 indicate day 30,60,90 during supplementation period

3- Control horses received placebo cookie daily

4- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
Fetlock angle (minimum)	1	2.00	0.91	1.2	0.3708
(degrees)	2	0.84	-1.13	1.2	0.1102
	3	0.89	-0.35	1.2	0.3107
Fetlock height (maximum) (cm)	1	6.08	3.55	3.6	0.4773
	2	5.17	6.02	3.6	0.8130
	3	1.51	4.63	3.6	0.3805
Fetlock height (minimum) (cm)	1	2.73	1.15	2.2	0.4723
	2	-0.80	0.63	2.2	0.5181
	3	-1.10	1.20	2.2	0.2956
Hock angle (minimum) (degrees)	1	-0.42	-0.42 ^a	1.7	0.9994
	2	-0.82	-1.31 ^{a,b}	1.7	0.7776
	3	-1.51	-3.73 ^b	1.7	0.2042
Hock angle (maximum)(degrees)	1	-1.06	-0.45	1.5	0.6972
	2	1.16	0.13	1.5	0.5090
	3	2.24	-0.73	1.5	0.0643
Hock angle range of motion	1	-0.65	-0.09	1.8	0.7482
(degrees)	2	2.02	1.37	1.8	0.7163
	3	3.80	2.95	1.8	0.6329

Table 14 – Hind leg joint parameters (symmetry) ¹

- exclude outlier data

1-Values reported as differences in means (post time point – pretreatment average)

2-Times 1,2,3,4 indicate pretreatment averages and day 30, 60, 90 during supplementation period

3-Control horses received placebo cookie daily

4-Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
Fetlock angle (minimum)	1	2.42	2.48	1.9	0.973
(degrees)	2	3.17	2.64	1.8	0.777
	3	4.73	2.97	2.0	0.385
	4	6.47	4.31	1.8	0.246
Fetlock height (maximum) (cm)	1	1.26	1.64	3.6	0.9119
	2	3.81	5.69	3.3	0.5712
	3	8.63	7.35	3.6	0.7238
	4	7.65	8.70	3.3	0.7499
Fetlock height (minimum) (cm)	1	3.04	1.84	1.4	0.4027
	2	1.45	3.02	1.3	0.2517
	3	4.82	2.31	1.5	0.0993
	4	5.16	2.69	1.3	0.0756
Hock angle (minimum) (degrees)	1	4.09	4.22	2.0	0.9509
	2	4.92	5.09	1.9	0.9279
	3	7.85	6.80	2.1	0.6207
	4	4.11	6.08	1.9	0.3114
Hock angle (maximum) (degrees)	1	4.14	4.07	2.4	0.9747
	2	3.31	5.44	2.3	0.3500
	3	4.32	4.43	2.5	0.9621
	4	2.86	4.06	2.3	0.5996
Hock angle range of motion	1	2.04	1.82	1.3	0.8625
(degrees)	2	2.94	4.09	1.2	0.3488
	3	3.53	3.03	1.3	0.7097
	4	2.86	3.92	1.2	0.3888

Table 15 - Hind leg stride parameters (treatment over time) ¹

- exclude outlier data

1-Values reported as differences in means (post time point – pretreatment average)

2-Times 1, 2, 3 indicate day 30, 60, 90 during supplementation period

3-Control horses received placebo cookie daily

4-Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

<u>Parameter</u>	<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
Leg protraction angle (degrees)	1	0.99	0.84	0.47	0.7422
	2	1.50	2.30	0.47	0.0701
	3	0.84	0.98	0.47	0.7555
Leg protraction distance (cm)	1	-0.09	1.47	6.4	0.8092
	2	2.76	8.04	6.4	0.8761
	3	5.81	7.41	6.4	0.6121
Leg retraction angle (degrees)	1	0.69	0.25	0.54	0.4144
	2	1.12	-0.28	0.54	0.0154*
	3	0.80	0.59	0.54	0.7031
Leg retraction distance (cm)	1	4.18	2.54	7.4	0.8256
	2	6.95	-1.11	7.4	0.2824
	3	5.44	2.34	7.4	0.6771
Stride length (cm)	1	17.96	9.89	26.1	0.7598
	2	20.16	14.67	27.4	0.8429
	3	32.96	44.70	27.4	0.6721
Stride velocity (m/s)	1	0.23	0.09	0.23	0.5578
	2	0.19	0.08	0.23	0.6335
	3	0.23	0.24	0.23	0.9502
Swing time (s)	1	0.01	0.0	0.01	0.4591
	2	0.00	0.01	0.01	0.1961
	3	-0.00	0.00	0.01	0.4377
Stance time (s)	1	-0.02	-0.02	0.01	0.4597
	2	-0.01	-0.01	0.01	0.3034
	3	-0.01	-0.02	0.01	0.3487

Table 16 – Hind leg stride parameters (symmetry) ¹

- exclude outlier data

1-Values reported as differences in means (post time point – pretreatment average)

2-Times 1,2,3,4 indicate pretreatment averages and day 30, 60, 90 during supplementation period

3-Control horses received placebo cookie daily

4-Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

Parameter	Time ²	Control ³	Treatment ⁴	Std Error	P value
Leg protraction angle (degrees)	1	0.99	0.84	0.45	0.7422
	2	1.50	2.30	0.43	0.0701
	3	0.83	0.98	0.47	0.7555
	4	0.55	1.08	0.43	0.2248
Leg protraction distance (cm)	1	8.89	5.79	6.1	0.6164
	2	15.86	14.94	5.8	0.8761
	3	11.76	8.48	6.4	0.6121
	4	11.71	13.53	5.8	0.7574
Leg retraction angle (degrees)	1	0.83	1.37	0.75	0.8514
	2	0.89	1.72	0.72	0.2510
	3	1.57	1.48	0.78	0.9113
	4	1.37	1.59	0.72	0.7639
Leg retraction distance (cm)	1	15.32	5.44	7.2	0.1800
	2	8.62	16.55	6.9	0.2572
	3	13.44	26.16	7.5	0.1007
	4	17.92	25.66	6.9	0.2691
Stride length (cm)	1	39.26	29.08	19.1	0.5966
	2	72.97	27.11	19.1	0.0211*
	3	42.09	21.03	20.0	0.2987
	4	53.06	33.54	19.8	0.3351
Stride velocity (m/s)	1	0.10	0.14	0.08	0.6208
	2	0.27	0.18	0.08	0.3039
	3	0.40	0.10	0.09	0.7782
	4	0.21	0.73	0.08	0.3827
Swing time (s)	1	0.01	0.01	0.01	0.5160
	2	0.01	0.01	0.01	1.0000
	3	0.01	0.01	0.01	0.1971
	4	0.01	0.01	0.01	0.7563
Stance time (s)	1	0.01	0.01	0.00	0.4002
	2	0.01	0.02	0.00	0.0970
	3	0.00	0.00	0.00	0.1130
	4	0.00	0.01	0.00	0.2312

Table 17 – TBARS (over time)¹

* Indicates significant difference P<0.05

- 1- Values reported as differences in means (post time point – pretreatment average)
- 2- Times 1,2,3 indicate day 30, 60, 90 during supplementation period
- 3- Control horses received placebo cookie daily
- 4- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.
- 5- Data was measured in moles/ milliliter of malondialdehyde.

<u>Time</u> ²	<u>Control</u> ³	<u>Treatment</u> ⁴	<u>Std Error</u>	<u>P value</u>
1	-12.092	-22.596	23.1	0.6547
2	-14.455	-23.018	23.1	0.7152
3	-15.403	-20.874	23.1	0.8155

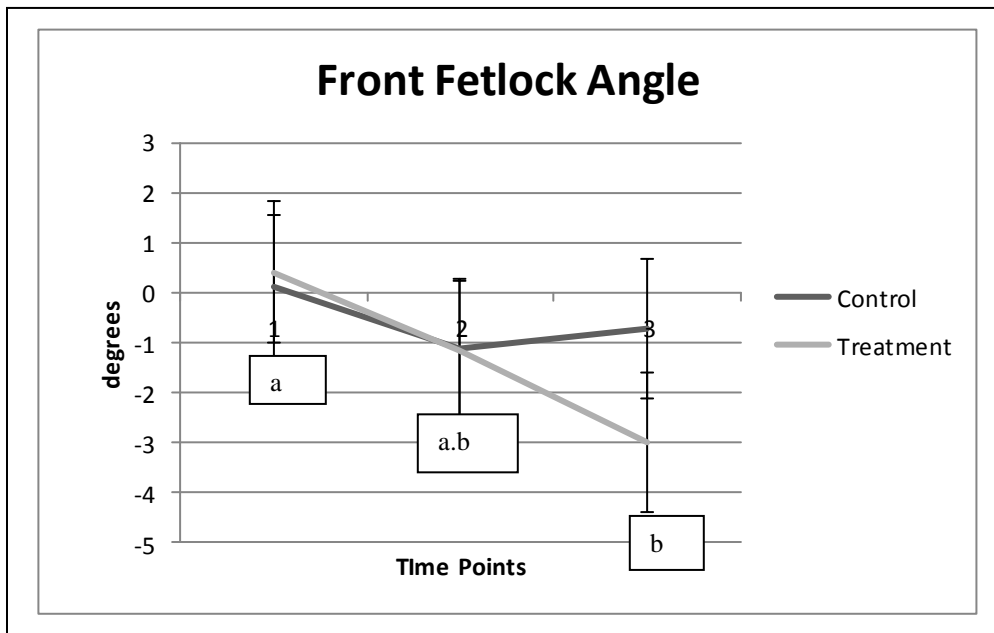


Figure 4 – Minimum Front Fetlock Angle over Time Points

^{a,b} Means within column within parameter differ (P<0.10)

- Times 1, 2, 3 represent differences in averages during the supplementation period at 30d, 60d, 90d respectively minus pretreatment average
- Control horses received placebo cookie daily
- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

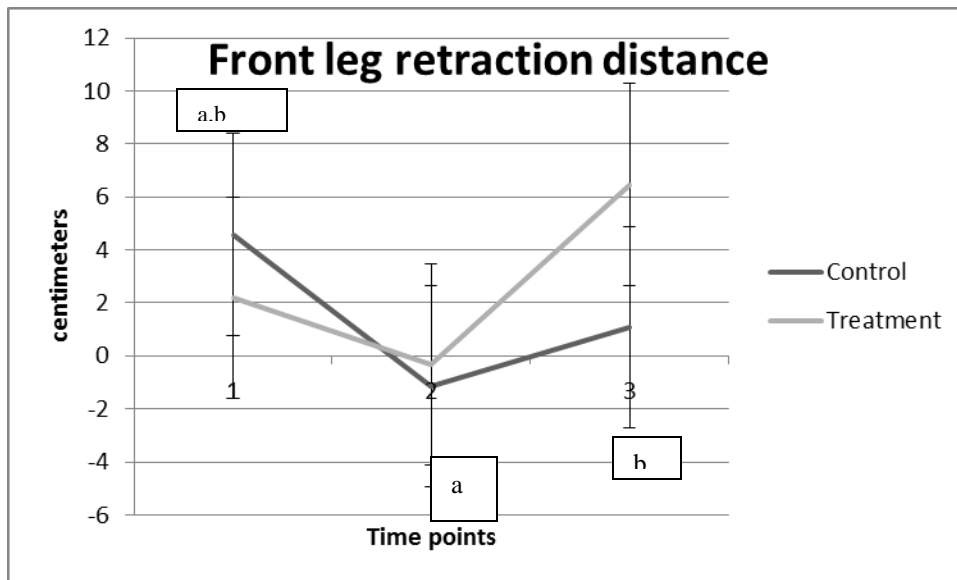


Figure 5 – Front Leg Retraction Distance over Time Points

^{a,b} Means within column within parameter differ ($P < 0.10$)

- Times 1, 2, 3 represent differences in averages during the supplementation period at 30d, 60d, 90d respectively minus pretreatment average
- Control horses received placebo cookie daily
- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

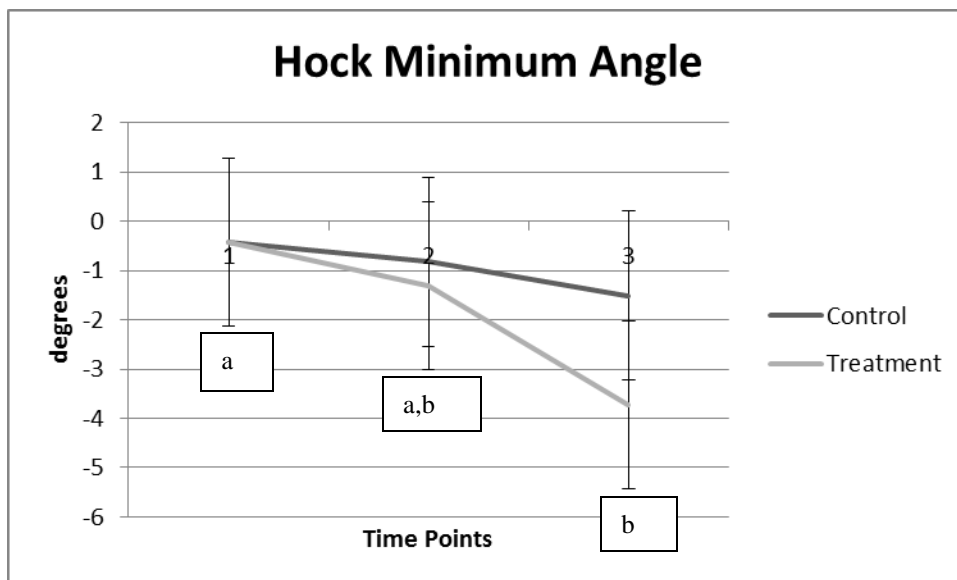


Figure 6 – Minimum Hock Angle over Time Points

^{a,b} Means within column within parameter differ ($P < 0.10$)

- Times 1, 2, 3 represent differences in averages during the supplementation period at 30d, 60d, 90d respectively minus pretreatment average
- Control horses received placebo cookie daily
- Treatment horses received orally a cookie containing 10 grams glucosamine sulfate daily.

CHAPTER 9

DISCUSSION

The present study demonstrated the positive effect of oral glucosamine sulfate supplementation on the movement of aged horses, by increasing the dorsi flexion of the front fetlock joint (seen as the decrease of angle in the front fetlock joint between supplementation d30 and d90 in Figure 4). Fetlock angle for control horses remained constant when comparing time points one and three. This supports the finding that glucosamine improved the fetlock dorsi flexion of the treatment horses. The smaller dorsal fetlock angle implies the horse retracted the front leg further and pushed off the ground at a later point. Supporting this statement, a trend ($P=0.0794$) was seen in the front leg retraction distance parameter by d90 (Figure 5). This parameter showed an increase in retraction distance which can be associated with a decreasing fetlock angle. A longer supplementation period might have revealed the front leg retraction distance as a significant gait parameter. *In vitro*, a study using cartilage from the distal end of the third metacarpal bone showed that hyaluronic acid-chondroitin sulfate-N-acetyl glucosamine had a beneficial effect on cartilage by reducing chondrocyte apoptosis (Henson *et al.*, 2012). In addition, study on polysulfated glycosaminoglycan showed a treatment effect in minimum and maximum fetlock angles (Stewart *et al.*, 2011).

Stride velocity may have influenced the retraction distance as velocity increased by d90 in both control and treatment horses. With retraction being part of the stride distance, it can be correlated that a longer retraction distance could also be caused by increased velocity (Barrey, 2001). Regardless, the decrease in fetlock angle and increase in retraction distance does suggest

that glucosamine sulfate has a beneficial effect on joint flexion allowing the horse more free flowing movement. In relation, a study on aged horses reveals that the combination of glucosamine hydrochloride and chondroitin improves kinematic parameters such as stride length and joint range of motion (Forsyth *et al.*, 2006).

Glucosamine HCl has been shown to be bioequivalent to GLN sulfate in the horse (Aghazadeh Habashi and Jamali, 2011). However, another study showed glucosamine sulfate is more bioavailable in comparison to GLN HCl (Meulyzer *et al.*, 2008). In a study by Forsyth (2006), glucosamine sulfate showed positive effects of increased range of joint motion which was determined to have caused increased stride length. This increase in stride length was associated with the increased swing time resulting from the horse protracting its limb further forward. This study concluded that these positive effects indicated that glucosamine improves horse comfort and wellbeing (Forsyth *et al.*, 2006).

Conducting outlier tests strengthened the overall analyzed data. As a result, previously significant gait parameters, such as the treatment over time hind leg protraction angle, were no longer significant. Treatment over time hind leg retraction angle and the symmetry front leg retraction distance, front and hind leg stride length were also no longer significant. However when compared to the controls, the treatment over time front fetlock minimum height and hind leg retraction angle, as well as, the symmetry hock minimum angle and leg retraction distance all showed significance. The control data resulted in an asymmetric pattern and therefore does not remain constant. The change of angle, distance, or height in the control time points decreases the significance in the aforementioned gait parameters as glucosamine may have not caused the significant differences seen in the treatment time points.

In addition, significance was approached in the treatment by time hock minimum angle ($P=0.0519$, Figure 6). There is a decrease within this parameter by d90. The decrease in angle indicates more flexion of the hock joint. A study on polysulfated glycosaminoglycan showed a decrease in hock range of motion after treatment (Stewart *et al.*, 2011). However, the current study showed an increase in the control's hock range of motion (Table 13) suggesting that the decrease in hock angle may not be due to glucosamine supplementation. The increase in hind leg velocity by d90 may also have caused the decrease in hock angle as a higher rate of speed requires more hock flexion. Between times d30 to d90 the hock range in motion increased as well. This greater range of motion coincides with the increase over time of the hind stride velocity. Therefore, this correlation indicates the horse may flex its hock more creating a smaller angle caused the increased velocity.

The gait parameter improvements seen within this study's 12 week treatment period may be due to the anti-inflammatory properties of glucosamine (Forsyth *et al.*, 2006; Valvason *et al.*, 2008). Inflammation is caused by the increased production of prostaglandin which up regulates interleukin-1 in the cyclooxygenase inflammatory mechanism. Interleukin-1 is responsible for a catabolic chain reaction of events such as the up regulation of metalloproteinases, nitric oxide synthase, cyclo-oxygenase 2, IL-6 genes, and apoptotic pathway in human chondrocytes (Afonso *et al.*, 2007; Aghazadeh Habashi and Jamali, 2011; Caron, 2003; Valvason *et al.*, 2008). A study has shown that glucosamine sulfate may restore heme oxygenase-1(HO-1) gene expression controls damaged by proinflammatory cytokines and proteinases. Restoring HO-1 helps protect against damage caused by these cytokines and proteinases (Valvason *et al.*, 2008). Therefore, actual joint improvement and repair of the extracellular matrix are possible as a result from glucosamine sulfate supplementation restoring HO-1.

No statistical differences were found in controls or treatments in the thiobarbituric acid reactive substances (TBARS) assay which measured serum malondialdehyde (MDA) levels in the blood. Thiobarbituric acid reactive substances assays have been widely used in as a biomarker of lipid peroxidation (Goranov, 2007). Malondialdehyde levels increase during lipid peroxidation which is considered one of the main pathways to cellular damage (Goranov, 2007). A study showed that MDA levels did increase in human patients experiencing osteoarthritis (Surapaneni and Venkataramana, 2007). In a canine study, MDA levels increased daily and the MDA concentration was consistently higher than baseline values. This study also reported MDA levels were correlated with the severity of arthritis. Significantly higher articular cartilage MDA concentrations were seen in rats with induced arthritis versus the controls (Goranov, 2007). In addition, glucosamine sulfate has shown to combat oxidative stress by restoring hemoxygase-1 (Valvason *et al.*, 2008). Treatment with antioxidants such as glucosamine have proven therapeutic in preventing oxidative stress (Surapaneni and Venkataramana, 2007).

A possible design improvement to this study is use of the combination glucosamine and chondroitin ingredients. A 12 week study using 2000mg of chondroitin sulfate, 5000mg glucosamine hydrochloride, and 500mg of N-acetyl-D-glucosamine in a daily feed ration showed improvement in joint range of motion (Forsyth *et al.*, 2006). This combination and concentration showed greater improvements in joint range of motion than the current study. In addition, the current study used 20mg/kg (10g) of glucosamine sulfate with no other drug combination. The current study's concentration of glucosamine sulfate was modeled after a study which showed oral glucosamine sulfate increased levels of glucosamine in synovial fluid (Meulyzer *et al.*, 2008). Future studies should consider using treatments that use glucosamine combined other ingredients such as chondroitin sulfate. However, the best combination of ingredients involving

glucosamine has not been determined and further research is needed (Aghazadeh Habashi and Jamali, 2011).

Dorsal hoof wall angles and length showed no statistical differences over time. All the horses were shod on a 6 week rotation by the same farrier. Dorsal hoof wall measurement started at two shoeing intervals before pretreatment gait assessment and continued until the end of the study. Dorsal hoof wall angle remained consistent, the averages varying approximately 5° between the most upright hoof angles to the most acute hoof angles. According to Back (2001), hoof angulation does not affect stride length, suspension, or flight arc; however, it may influence breakover time. According to Figure 3, a change in angle was seen during the supplementation period. This change may have resulted from measurement error while using a hoof gauge to obtain dorsal hoof wall angulation. Dorsal hoof wall length remained consistent throughout the study.

Many variables influenced the outcomes of this study. The increased velocity and stride length over time indicates the need to control these variables. Using velocity timers in future studies will eliminate or significantly prevent the effects of inconsistent velocity. Moreover, control data revealed inconsistent results. A chute system is needed to allow horses to freely move without a handler causing the horse to travel with its head turned or elevated. This design improvement would possibly make the horses travel with more symmetry between sides. The horse carrying its head at a more natural position would also increase the likelihood of symmetric results, consistent velocity, and stride parameters. In agreement with Forsyth (2006), the skin markers used were easily displaced and also moved slightly as a result of movement vibrations. A more in depth look of improving joint markers and ways of attaching them to the horse is needed.

CHAPTER 10

CONCLUSION

The significant findings of this study indicate that orally supplemented glucosamine sulfate had little effect on gait quality in aged horses. Some changes were observed in certain joints. Studies have shown prophylactic use of oral glucosamine supplementation reduces joint inflammation and improves joint range of motion in the horse. The current study's findings suggest glucosamine treatment has some ability to influence joint inflammation and cartilage. Future studies are needed for further research in developing alternative proactive treatments for osteoarthritis

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