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The Effects of Circular and Linear Exercise on Articular Cartilage in Sheep

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THE EFFECTS OF CIRCULAR AND LINEAR EXERCISE ON ARTICULAR CARTILAGE IN SHEEP

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ABSTRACT

Excessive exercise may induce osteoarthritis (OA), or degeneration, of articular cartilage, which is a leading cause of lameness and decreased use in horses. The purposes of this study were to utilize a sheep model to determine the effects of circular and linear exercise on the macroscopic and microscopic characteristics of articular cartilage in the metacarpal-phalangeal (MCP) joint and to evaluate biochemical changes in serum and synovial fluid compared to non-exercised control lambs.

Twenty lambs were randomly assigned to three groups: circular exercise (C, 8.5m diameter; n = 8), straight-line exercise (S, treadmill; n = 8) and non-exercised control (CON, n = 4). Lambs (C and S) were exercised at 1.3 m/s over a 6- to 8-wk period. Serum and synovial fluid (SF) from the MCP joint was collected throughout the study. Upon euthanasia, MCP joints were collected and fixed for gross morphology and histological analysis. Serum was analyzed for total protein (STP), collagen Type II cleavage ¾ fragments (C2C) and lysyl oxidase (LOX) activity. Synovial fluid was analyzed for total protein (SFTP) and LOX activity for each MCP separately.

Circularly-exercised sheep had the most severe lesion development on the lateral condyle of the distal metacarpus compared to linearly-exercised and non-exercised control sheep. Histological morphology indicated the linearly-exercised sheep had more severe histology OA scores than the circularly-exercised and the non-exercised control lambs. Biochemically, STP was not different among treatment groups over the course of the study. There were no differences among groups for serum C2C concentrations or LOX over the course of the study. There was a polynomial response to serum LOX over

time, with peak LOX specific activities being reached at Wk 9 and then decreasing throughout the duration of the study. There were no differences in SFTP or SF LOX specific activities among groups throughout the study. However, SF LOX specific activity did increase over time for all treatment groups.

Results from this study indicate that early forced exercise (1.3 m/s) in juvenile lambs for 6 to 8 wks is capable of altering gross and microscopic morphology of articular cartilage in the MCP joint. However, the biomarkers utilized in this study did not directly correlate to these physical changes.

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CHAPTER ONE

INTRODUCTION

Importance of Osteoarthritis in the Horse Industry

Joint disease is the most prevalent cause of lameness in the horse (Todhunter, 1992). The distal limb joints are at the highest risk due to increased forces and rotation, and the metacarpophalangeal (MCP) joint is the most frequently affected with joint disease (MacDonald et al., 2002). Osteoarthritis (OA) is a group of diseases that manifest as a loss of extracellular matrix (both collagen and proteoglycan) in articular cartilage; fibrillation, wear lines and erosions to full thickness defects or lesions of the cartilage; and changes in chondrocyte cell conformation, orientation, number and synthetic properties (McIlwraith, 1996). In advanced stages of OA, radiological changes are evident such as joint space narrowing, subchondral bone changes and osteophyte development; however, these changes are poorly correlated to clinical signs (Trotter and McIlwraith, 1996; Kidd et al., 2001).

The incidence of OA anecdotally appears to be increasing and is a major contributor to reduced performance, loss of use, and economic losses in the horse industry. Earlier detection of OA would be useful in order to attenuate its complications. In addition, understanding the training techniques that may contribute to the "wear and tear" development of OA and manipulating these training practices to lessen their role in disease progression would be very useful. There is much anecdotal belief that certain training techniques used on non-racing performance horses are "hard" on horses' joints; however, very little data exists to support these beliefs. To examine common training

practices utilized in the horse industry and their implications on cartilage health and the possible development of OA, we have used a sheep model. The purpose of this research is to test the effects of straight line exercise versus circular exercise versus non-exercised controls on the articular cartilage of the metacarpophalangeal (MCP) joint in juvenile sheep. This research may provide the preliminary evidence necessary to warrant further investigation and perhaps elucidate clinical, non-invasive diagnostics for OA in horses.

The Research Question

In the horse industry, circular exercise is used extensively in the majority of saddle breaking and training processes with yearling, 2- and 3-year-old horses. This exercise may come in the form of longeing, round penning, or 20-m circles under saddle used to teach a horse balance, suppleness and self carriage. While there are no data quantifying the degree of increased pressure or force that this exercise may place on the distal limb, there are some data discussing this phenomenon in other species. Usherwood and Wilson (2005) found that racing greyhounds experience increased force on a 22.4 m bending turn compared to the first or second straight away portions. The dogs did not slow during the turn compared to the straight away, and the centripetal acceleration through the turn resulted in an increase in the effective weight of the dogs by 71%. Further, during the bending turn, all four limbs experienced a 64.5% increase in peak force compared to straight running. Usherwood and Wilson (2005) concluded that despite the increased force placed on the dogs' limbs during the turn, they were able to continue top speeds. These data support the premise that horses working on a continual

bending turn, such as during longeing, may experience increased force on their limbs compared to straight line exercise.

van den Bogert and colleagues (1999) studied humans who had undergone hip replacement surgery and the strain placed on the hip during various exercise protocols. Patients were analyzed performing a walk, run, alpine (down-hill) skiing and cross-country skiing. The alpine skiing incorporated four different protocols: flat slope with either a sweeping (long radius) or tight (short radius) turn or a steep slope with both radii. The cross-country skiing was both classic and skating techniques. The short radius, steep slope down hill skiing generated force similar to running, and the short radius, flat slope generated the second most severe force, with all other exercises generating less severe force on the patients. It was concluded that running and alpine skiing on the short radius, steep slope generated force that was too excessive for patients with hip replacement. Admittedly, the hip joint in the human is considerably different than in sheep or horses, as these data may not be clinically significant to the MCP joint; however, it may be deduced that the sharper turns, with the associated increase in centripetal force, generates excessive force on the joints and articular cartilage. The same phenomenon may be experienced by horses that are working on circles which generate increased centripetal forces compared to straight line exercise.

Excessive exercise may induce OA in horses through increased wear and tear. Osteoarthritis, or degeneration of articular cartilage, is a leading cause of lameness and decreased use in horses. In much of the horse industry, young horses are trained to longe (round pen) and to be ridden in circles. There is anecdotal belief, but a paucity of

scientific data, relating to training methods used on young horses that may contribute to an increased incidence of OA. The purpose of this study was to utilize a sheep model to determine the effects of circular and linear exercise on the macroscopic and microscopic characteristics of articular cartilage in the MCP joint and the biochemical changes in serum and synovial fluid compared to non-exercised sheep. A sheep model was employed due to affordability, accessibility and acceptability for euthanasia to collect joint surfaces for macroscopic and histological analysis, despite its obvious conformational and locomotive differences from horses. Data from this research project may be useful in identifying potential training techniques that may increase the incidence of OA in horses, identify useful, non-invasive biomarkers of OA, and provide the necessary preliminary data to warrant further research in the area of forced exercise in juvenile animals, especially horses.

CHAPTER TWO

LITERATURE REVIEW

Composition and Histology of Articular Cartilage

Articular cartilage covers the surface of the articulating joints, and provides cushioning during normal physical use. It also reduces the coefficient of friction and attenuates peaks of tensile and compressive stress by virtue of fluids within the joint. Articular cartilage has a zonal architecture (Glaser and Putz, 2002), and is divided into three non-mineralized zones. The most superficial zone (nearest the lumen of the synovial space) is the tangential or gliding zone (10% of the thickness), which has a high tensile strength, and is attributed to the high collagen fiber content (approximately 50% dry weight; Buckwalter, 1983) and increased fibronectin and water content compared to other layers (arranged parallel to the surface of the tissue). Chondrocytes within this superficial layer are flatter and are parallel to the articular surface (Glaser and Putz, 2002, Temenoff and Mikos, 2000, Todhunter, 1996). The intermediate zone is known as the transitional zone (20-50% of the cartilage thickness), which has random alignment of the fibers. The chondrocytes within the transitional zone are more spherical and have larger endoplasmic reticula, Golgi bodies, and mitochondria. Further, the extracellular matrix in this zone has more proteoglycan and less collagen and water (Glaser and Putz, 2002, Temenoff and Mikos, 2000, Todhunter, 1996). The deepest layer is the radial zone (40-70% of the cartilage thickness), and has fiber orientation perpendicular to the joint surface. Chondrocyte cells are spherical to columnar and stacked in columns perpendicular to the surface. These cells have a very high synthesis rate. The radial zone

provides an anchor for the calcification junction of bone and cartilage (Buckwalter, 1983; Glaser and Putz, 2002, Temenoff and Mikos, 2000; Todhunter, 1996). The demarcation of the radial zone of cartilage and the underlying calcified cartilage is known as the tidemark, and is microscopically observable (Mainil-Varlet et al., 2003). Deep to the calcified cartilage is the subchondral boney plate. The variation of thickness for the zones of articular cartilage is dependent on age, species and the specific joint. The microscopic analyses of these characteristics have been utilized to develop a histological assessment of cartilage damage and repair during traumatic or developmental arthritis (Mainil-Varlet et al., 2003).

There are three types of cartilage: hyaline, elastic, and fibrocartilage. For the purposes of this review only hyaline cartilage will be discussed due to its relevance to articular cartilage. Hyaline cartilage is the most common form and appears pearly white, glassy, and translucent. Hyaline cartilage exists in the articular surfaces of the joints and in parts of the ear, nose, larynx, trachea and bronchi and walls of the upper respiratory tract. Further, hyaline cartilage is the tissue of the future bones in fetal tissue and persists postnatally in the epiphyseal plates of long bones until skeletal maturity.

Cells

The cells within the articular cartilage are chondrocytes. Chondrocytes are from mesenchymal stem cell (MSC) lineage and make up approximately 5% of the hyaline cartilage volume (Buckwalter, 1983; Temenoff and Mikos, 2000). Chondrocytes are responsible for replacing degraded matrix to maintain the articular cartilage thickness and mechanical properties. The chondrocyte cells produce structural macromolecules, such

as collagens and proteoglycan (PG) and secrete them into the extracellular matrix. These cells have an extensive endoplasmic reticulum and Golgi apparatus, and store lipid and glycogen in secretory vesicles for fuel. Further, some chondrocytes have cilia that allow the cell to communicate with the extracellular matrix and are believed to sense the mechanical environment of the cell, since chondrocytes are known to modify matrix properties in response to mechanical loading such as exercise (Poole et al., 2001; McGlashan et al., 2007). Chondrocytes in the deepest radial zone secrete proteins that are important in calcification of the matrix, and the periphery chondrocytes secrete collagen and matrix molecules to produce hyaline cartilage. Mature articular chondrocytes are unable to proliferate and appear rounded and completely encased in matrix (Buckwalter, 1983; Temenoff and Mikos, 2000). During skeletal growth, the chondrocytes proliferate rapidly and have a high rate of matrix synthesis. After maturity, proliferation slows and cell numbers decrease (Buckwalter, 1983).

Extracellular Matrix (ECM)

Because there is relatively low cell volume in articular cartilage, the extracellular matrix must assume the major role in cartilage function. The fibril molecules (collagen) of articular cartilage constitute approximately 50% of the dry weight and give the cartilage its tensile strength and shape (Buckwalter, 1983). Articular cartilage contains collagen Types II, VI, IX, X, XI, XII, and XIV, where Type II is the most prominent form accounting for approximately 90% of the collagen in the matrix (Temenoff and Mikos, 2000; Todhunter, 1996). Type II collagen has a high content of proteoglycan bound to the fibers, allowing more interaction with water than other types of collagen.

Types IX and XI, along with Type II, form a mesh that provides the tensile strength to the tissue and physically entraps other macromolecules. Type X is found only near calcified matrix in an adult and in the hypertrophic portion in growing animals (Temenoff and Mikos, 2000; Todhunter, 1996).

Also in the ECM are proteoglycans (PG), which are approximately 95% polysaccharide and 5% protein, and account for 40% of the dry weight of articular cartilage. Proteoglycans exist as monomers or aggregates. Monomers have a thin, central protein core with glycosaminoglycan (GAG) side chains that protrude much like the bristle of a brush. These GAG chains are unbranched polysaccharides made from disaccharides of an amino sugar (glucosamine or galactosamine) and another sugar (hexuronic acid or galactose) and are covalently attached to the core protein. At a minimum, one of the disaccharides has a negatively charged sulfate or carboxylate group, causing the GAGs to repel one another and other anions, but attract cations and water. Some GAGs of importance in articular cartilage are hyaluronic acid, chondroitin sulfate, keratin sulfate, dermatan sulfate and heparin sulfate (Buckwalter, 1983; Temenoff and Mikos, 2000; Todhunter, 1996). Aggregating PG, known as aggregans, are composed of keratin sulfate and chondroitin sulfate GAGs attached to the protein core, with link proteins connecting many monomers to a hyaluronic acid chain. Aggregate PGs are the primary form of PG in the ECM of articular cartilage. Aggregans fill most of the interfibrillar space of the ECM and provide much of the strength to the articular cartilage because of the attraction to water, which provides stiffness to the tissue, as fluids are largely incompressible. The aggregation holds the PG in the matrix during loading since

no chemical bonds exist between the PG and collagen fibers. Smaller PGs include decorin, biglycan and fibromodulin, which have shorter protein cores and fewer GAG side chains. They are believed to help with chondrocyte cell function, such as adhesion, multiplication, differentiation and migration, and organization of the collagen matrix (Buckwalter, 1983; Buckwalter and Mankin, 1998; Temenoff and Mikos, 2000; Todhunter, 1996).

The noncollagenous glycoproteins anchorin CII and cartilage oligomeric protein, which are glycoproteins with a small amount of oligosaccharide on a protein core, help anchor the chondrocytes to the surrounding matrix. These polypeptides aid in chondrocyte-ECM interactions. There are also fibronectin and tenascin in articular cartilage, which are believed to act similarly as the noncollagenous glycoproteins (Temenoff and Mikos, 2000; Buckwalter and Mankin, 1998).

Fluid within the ECM of the cartilage tissue comprises 65 - 80% of the wet weight volume of the cartilage. Within this fluid are water, gases, metabolites and cations that are used to balance the negatively charged GAGs. The interaction of the matrix water and the macromolecules is critical to retain the water in the tissue. These interactions allow for structural and nutritive support to the cartilage itself. The water within the cartilage exchanges with the synovial fluid and provides nutrients and oxygen via diffusion (concentration gradients) to the avascular cartilage tissue. The fluid avoids compression and can return to the original shape after a decompressive load cycle (i.e. exercise) and helps to provide the tissue with stiffness (Buckwater, 1983; Buckwalter and Mankin, 1998; Temenoff and Mikos, 2000).

Synovial Fluid

Synoviocyte cells originate from the same MSC pool as the chondrocyte cells, and in various pathological conditions can behave with chondrogenic potential (Hunziker, 2001). Synoviocytes form the synovial membrane lay in an incomplete layer, one to four cells thick, with no basement membrane. These cells have both a secretory (Type B) and phagocytic (Type A) function (Todhunter, 1996). Synoviocytes (Type B) produce hyaluronan, a very large GAG (10^4 KD) with no protein core, and lubricin, a lubricating glycoprotein, into the synovial fluid (Palmer and Bertone, 1994; Todhunter, 1996).

Synovial fluid is an ultrafiltrate of serum (Todhunter, 1996); most of the ions and molecules from synovial fluid are also found in plasma. The synovial fluid contains mononuclear cells, such as synovial lining cells, monocytes and lymphocytes (90% of total cells), and the remainder are polymorphonuclear leukocytes. Equine synovial fluid contains less than 500 nucleated cells/µl (Kraus, 2006; Todhunter, 1996). Fluid exchange between plasma and synovial fluid is under hydraulic or hydrostatic pressure, and a colloid osmotic pressure differential (Levick and Knight, 1988; Todhunter, 1996). Glucose enters the synovial fluid through facilitated diffusion, and oxygen and carbon dioxide freely diffuse in and out of the fluid, passing between and through the synoviocytes (Todhunter, 1996). This synovial fluid is responsible for bringing nutrients to the chondrocytes within the articular cartilage (Buckwalter and Mankin, 1998).

Chondrogenesis and Cartilage Turnover

During skeletal development, the chondrocytes produce new tissue to allow the articulating joint surface to grow and remodel. At skeletal maturity, the volume of cartilage does not change, but is rather degraded and replaced (remodeled) through matrix metalloproteinase activity and subsequent matrix synthesis by chondrocyte cells. Hyaline cartilage develops from undifferentiated mesenchymal cells that later become chondrocytes. These chondrocytes synthesize collagen, PG, and noncollagenous protein. An accumulation of matrix then separates the cells and they assume a spherical shape. As the long bones have completed growth, the chondrocytes no longer divide, but continue to produce the macromolecules of collagen, PG and the noncollagenous proteins. Enzymatic activity of the chondrocytes degrades the matrix macromolecules to aid in remodeling of the cartilage. As an animal or human matures, the capacity of the cells to synthesize PGs and their response to stimuli decrease. This may contribute to the development of degeneration of the cartilage, which is known as osteoarthritis (Buckwalter, 1983). Osteoarthritis can be diagnosed by a variety of methods, which may include biochemical protein markers, such as pro-inflammatory cytokines or ECM components, in serum or synovial fluid, arthroscopy, radiographs, magnetic resonance imaging or ultrasound.

Cartilage Turnover

Cartilage matrix synthesis and degradation is adjusted during growth and maturation to allow for growth, remodeling, or homeostasis. Collagen turnover has been estimated to be around 120 years in dogs and 350 years in mature humans (Akizuki et al., 1987). This slow turnover is believed to be a result of the high degree of covalent cross-

links between collagen proteins, which limits the number of sites available for enzymatic cleavage. Matrix metalloproteinases (MMPs) are the enzymes responsible for degrading collagen within the ECM, with MMP-1, -2 and -3 being the most critical in cartilage turnover. In comparison, PGs have a higher rate of turnover, as they are not protected like collagen. Adult rabbit and dog cartilage PG turnover is 300 days and in the human hip joint, approximately 1,800 days (Marouda, 1980). Aggrecan is fairly easily degraded between the G_1 and G_2 domains near the linkage between the PG monomer and its connection to the hyaluronan core protein. With moderate cleavage of PG, it can reincorporate into the ECM; however, injection of 2.0 mg of chymopapain, an enzyme that degrades non-collagenous protein, into rabbit knees caused more than 50% loss of PG and degeneration of the joint (Chen, 1993) indicating that there is a point where loss of PG becomes irreversible and joint degeneration occurs.

Factors Involved in Turnover of Articular Cartilage

Regulation of the turnover of cartilage matrix is not fully understood, but does seem to be accelerated by excessive load and cytokine release from the synoviocytes and chondrocytes. The interleukin (IL) family and tumor necrosis factor α (TNF α) are responsible for up-regulating the synthesis and release of MMP enzymes from the chondrocytes. These degradative activities are reversed through the action of MMP inhibitors and by the release of growth factors, which enhance matrix synthesis.

Matrix metalloproteinases degrade the cartilage matrix at neutral pH and are inhibited by tissue inhibitors of metalloproteinase (TIMP). In healthy articular cartilage, there is normally a slightly higher concentration of TIMP than MMP, therefore allowing

for homeostasis or growth (Davidson et al., 2006; Dean et al., 1989). There are two MMPs of particular relevance to articular cartilage turnover and net loss: MMP-1, collagenase and MMP-3, stromelysin. Collagenase produces a three-quarter length N-terminal fragment and a one-quarter length C-terminal fragment upon degradation of collagen. These fragments can then be further degraded by other enzymes. Stromelysin degrades aggrecan, link protein, and collagen Type II, and also upregulates the conversion of MMP-1 from the latent form to the active form (Todhunter, 1996). The products produced by such enzymatic activities either become endocytosed or are cleared from the cartilage into the synovial space and subsequently can be detected in the blood. Finally, MMPs are also activated by plasmin, a serine proteinase. Plasmin is regulated by chondrocyte and synoviocyte synthesis of plasminogen activator (PA). The activity of PA, and consequently the presence of plasmin, is regulated by PA inhibitors (PAI-1, PAI-2). Plasmin, PA, and PAI are all present in healthy cartilage (Hamilton, 1991) to maintain homeostasis and for normal tissue remodeling.

Cytokines are responsible for the loss of PG concentrations within the ECM. This has been demonstrated *in vitro* whereby IL-1 increases degradation and decreases synthesis of PG. Interleukin-1 receptors have been found in normal cartilage in humans (McCollum et al., 1991), rabbits (Chin and Horuk, 1990), pigs (Bird and Saklatvala, 1986), and horses (May et al., 1992). Interleukin-1 (α and β) are considered pro-inflammatory cytokines that up-regulate MMP gene expression. These cytokines also attenuate any compensatory synthetic attempts by the chondrocytes to bolster ECM (Fernandes et al., 2002). In OA, there is also a decreased production rate of the receptor

antagonist of IL-1 (IL-1Ra). These compounded effects may lead to further degradation of the cartilage tissue (Fernandes et al., 2002). Another pro-inflammatory cytokine that is responsible for similar catabolic effects on cartilage is TNF-α. Human Recombinant TNF-α increased degradation and decreased synthesis of PGs in cartilage explants (Saklatvala, 1986). Both IL-1 and TNF-α are produced by the synoviocytes and chondrocytes as well as from a systemic immune response by mononuclear cells (Fernandes et al., 2002). Therapeutic efforts employing the use of IL-1Ra are currently being researched in horses (Haupt et al., 2005). Additionally, glucosamine therapy has received much attention, and *in vitro* has been shown to reduce IL-1-induced catabolic effects on cartilage explants (Fenton et al., 2002). Finally, insulin-like growth factor-1 (IGF-1) has also been studied as a cartilage-sparing agent during matrix catabolism (Frisbie and Nixon, 1997; Haupt et al., 2005).

Eicosanoids are a third group that is implicated in cartilage degeneration. Eicosanoids are produced via the cyclooxygenase (COX) pathway either constitutively (COX-1) or are induced (COX-2) and the lipoxygenase pathway to produce either prostaglandins or leukotrienes, respectively (Gosset et al., 2006). Prostaglandins and leukotrienes are formed from arachidonic acid, via the inflammatory cascade. Prostaglandin E_2 is one of the key eicosanoids involved in cartilage degradation (Palmer and Bertone, 1994), chondrocyte apoptosis (Laufer, 2003) and works synergistically with MMPs, IL-1 and TNF-α in the horse (Palmer and Bertone, 1994). Interleukin-1ß was found to up-regulate Prostaglandin E_2 synthase (Masuko-Hongo et al., 2004), and

therefore the cytokine response and eicosanoid pathways appear to play a role in the pathogenesis of OA.

Reactive oxygen species (ROS) have also been found to be a contributing factor in OA. These ROS have been reported to serve as an intracellular secondary messenger that aids in up-regulating the gene expression of some of the mentioned compounds, including cytokines and MMPs (Fay et al., 2006; Henrotin et al., 2005). Chondrocytes produce nitric oxide and superoxide anion radical in cartilage that is pathological for OA. Nitric oxide is formed by NO synthase (NOS), which has two constitutively expressed isoforms and one inducible form. Chondrocytes express both a constitutive and inducible form of NOS, where the latter seems to be expressed as a result of both mechanical and chemical stimulation (Tomiyama et al., 2007; Henrotin et al., 2005). TNF-α and IL-1β also appear to stimulate the inducible form of NOS (iNOS) while transforming growth factor-β (TGF-β) inhibits iNOS (Henrotin et al., 2005).

In concert, the metalloproteinase, cytokine, ecosanoid and ROS activities yield net degradation of articular cartilage. These reactants can be useful tools to determine the incidence (or severity) of OA development through biochemical markers. Additionally, the breakdown products of collagen and PG or enzymes utilized in synthesis or degradation of these macromolecules can be determined in biological fluids (synovial fluid or serum) by employing the use of immunoassays, such as those used for the research presented in this dissertation.

Pathogenesis of Osteoarthritis: Joint Changes and Diagnostic Tools

Macroscopic and Microscopic Changes

Osteoarthritis is characterized as a chronic progressive disorder of diarthrodal synovial joints with degradation of the articular cartilage; fibrillation and thickening of the synovial lining; loss of ECM (collagen and PG); and subchondral bone changes. More specifically, gross pathological changes to the articular surface include three main patterns. The first pattern is the development of wear lines, which are lines that run parallel to the plane of movement for the joint. A second pathological sign is randomly distributed defects or pitting that can be observed over the entire surface or just one portion of the joint surface. Finally, the more traditional deterioration of OA can be noted as yellowish discoloration, dullness, fibrillation, ulceration, and lesion development. These lesions become more progressive until the cells, after a futile attempt at repair, die and the matrix disappears. The progressive development of OA can lead to subchondral bone defects with gross changes including the development of osteophytes, or bone spurs. Other visible changes to the synovial joint exist with the synovial lining. In severe cases of OA, chronic hypertrophy in villous-like protrusions can be noted. The synovium becomes very thickened, hyperplastic and discolored and nodules of cartilage tissue known as synovial chondromas can develop within the synovium (Pool, 1996).

The Osteoarthritis Research Society International (OARSI) has developed and adopted a scoring system for osteoarthritis cartilage histopathology, which includes grading and staging the degree of damage to articular cartilage (Pritzker et al., 2006). This scoring system is based on a system of visual patterns rather than previously described written descriptions such as the Mankin Scoring System. The repair of "wear

and tear" or trauma to articular cartilage involves modest cell replication, up-regulation of ECM synthesis, and reorganization of the matrix by the cells (Salter, 1989). At low power magnification, microscopic detection of morphological characteristics can be made. The OARSI grading and staging scoring system is based on histological features of OA progression. The histopathology of OA is broken down into grades, or severity, of OA detection. A Grade 0 joint surface is intact and the cartilage morphology is normal, whereby the matrix and cells have the architecture described above. Grade 1 damage has an intact surface and superficial matrix, but may have superficial abrasion or fibrillation or focal superficial matrix condensing; and some of the cells may have died, proliferated in clusters, or appear hypertrophic in the superficial zone. Grade 2 damage demonstrates surface discontinuity, deep fibrillation of the superficial zone, clusters of cells in the transitional zone and disorientation of matrix columns in the deeper zone; some cells have died (empty lacunae), proliferated into chondrone clusters or appear hypertrophic. Grade 3 damage includes vertical fissures (clefts) into the middle transitional zone, decreased matrix staining; and cell death, hypertrophy and clusters. Grade 4 damage encompasses cartilage erosion, with matrix loss, delamination of the superficial layer, mid layer cyst formation in the matrix and loss of matrix through the transitional zone. Grade 5 is denudation of the cartilage, with sclerotic bone or other reparative tissue such as fibrocartilage in the denuded region. Microfractures of the subchondral bone may be evident. The most severe grade is Grade 6, which is deformation of the cartilage and bone, with extensive bone remodeling, osteophyte formation, microfractures, and fibrocartilage repair (Pritzker et al., 2006). The histopathology of OA can further be

defined by its stage, or horizontal extent of cartilage involvement, within the joint surface. A Stage 0 demonstrates no OA activity on the joint surface. A Stage 1 represents less than 10% involvement of the joint surface. Stage 2 includes 10 to less than 25% of the joint surface demonstrating OA progression. Stage 3 represent 25 to 50% involvement. The most severe stage, Stage 4, includes greater than 50% of the joint surface in OA progression. The result OARSI histopathology OA score is derived by multiplying the Grade and Stage for scores ranging from 0 to 24, with 24 being the most severe (Pritzker et al., 2006). This is the scoring system utilized in this research, as it describes the degree of microscopic damage to the articular cartilage.

Once cartilage is damaged or has progressed with OA, the reparative attempts of articular cartilage are either intrinsic or extrinsic, from mesenchymal elements of the underlying subchondral bone. The intrinsic repair is generated from a limited mitotic effort by the chondrocyte cells. These chondrocytes also yield a futile attempt to increase collagen and PG production. The extrinsic repair typically results in fibrocartilage development due to metaplasia into cartilage-like elements. A third mechanism of repair is known as matrix flow, where the cartilage forms ridges around the perimeter of the lesion and then fills in towards the center with new matrix (McIlwraith, 1996). The fibrocartilage that typically results during these reparative efforts is structurally inferior to the original hyaline cartilage, and can not support mechanical loading placed on the articular surface. With continued load placed on this metaplastic tissue, the fibrocartilage is mechanically unsuitable in weight-bearing regions of the joint surface and may fail (McIlwraith, 1996). There are many factors that impact the degree of repair, including

depth or severity of injury, size of lesion, location (lateral, medial; weight-bearing vs. non), and age of animal. Ultimately, the shear forces placed upon this inferior tissue will lead to continued damage, and the damage to the innervated underlying bone will produce pain for the animal, inflammation of the joint and propagation of degeneration of the joint (McIlwraith, 1996).

In 2-year-old race horses (n=14) that underwent a 4-week initial saddle breaking period and a 13-week flat race training period, the metacarpal phalangeal (MCP) joints had observable wear lines and high lesion scores upon gross analysis. These gross defects were not coupled with previous clinical evidence of lameness or joint swelling. Histologically, 3-7 mm wide lesions and 5 mm deep crevices were observed in 8 of the 14 horses. Further, chondrocyte morphology differences existed, but no clusters or chondrones were evident. The most severe macroscopic defects did not correlate with increased joint effusion or lameness scores. No horse with severe post-mortem gross pathological changes scored above a Grade 1 when trotting on a hard surface (American Association of Equine Practioners lameness scoring system) or Grade 2 for flexion tests (Firth et al., 2004). These results indicate that while macroscopic or microscopic changes may be severe, the defects to the synovial joint do not always lead to clinical evidence of pain, inflammation, effusion or lameness. Alternatively, these results could indicate that an adequate clinical indicator for macroscopic and microscopic defects is not available.

Biochemical Changes

During OA, chondrocytes release cytokines, which up-regulate MMPs and ROS. These molecules will lead to degradation of collagen and PG, and the fragments of these

proteins will be released from the ECM into the fluid exchange within the joint. Upon load, these fragments may be released into the lymphatic system and eventually into circulation. Therefore, synovial fluid, serum or urine may be analyzed to quantify degradative fragments indicative of OA. Release of MMP-1 cleaves collagen Type II into two portions between Gly^{794} and Leu^{795}, yielding the ¾-length fragment, and ¼-length fragment. It is these by-products of the enzymatic degradation by MMPs that have been utilized to develop enzyme-linked immunosorbent assays (ELISAs) to detect degradation products in both serum and synovial fluid, which can indicate the degree of degradation of collagen in OA and rheumatoid arthritis (RA). In this novel research project, the ¾-length fragment of Type II collagen was utilized to assay for systemic evidence of increased degradation of articular cartilage and the collagen within its ECM as detected in serum. Increases in the ¾-length fragment have been detected in animal RA models and in SF in humans affected with RA (Elsaid and Chichester, 2006).

Lysyl Oxidase (LOX) is an enzyme responsible for post-translational modification of collagen and elastin. The enzyme catalyzes the oxidative deamination of lysine and hydroxylysine into aldehyde cross-links and therefore is partly responsible for the maturation of collagen by cross-linking (Romero-Chapman et al., 1991). In humans with increased fibrosis as a result of progressive disease or trauma, LOX is up-regulated and is responsible for the increased fibrotic connective tissue. Lysyl oxidase activity appears to be impacted by a variety of things, including nutritional state, particularly dietary copper, as copper is a co-factor for this enzyme (Romero-Chapman et al., 1991). Lysyl oxidase may also be influenced by testosterone (Bronson et al., 1987) and growth

factors that impact collagen formation and turnover (Tang et al., 1989). Research presented here utilized a fluorometric assay for LOX as an indicator of changes in collagen maturation or fibrosis.

Other biochemical markers have been utilized with varying success as indicators of either earlier OA or to augment later stage changes found by radiographic, ultrasounographic or magnetic resonance imaging. Keratan sulfate, a GAG sidechain of aggrecan, has been quantified in both RA and OA models. Keratan sulfate in both SF and sera were elevated in equine OA joints. Further, horses with septic arthritis had high levels of keratan sulfate in the SF (Alwan et al., 1990). Serum keratan sulfate concentrations were also elevated in adult human OA patients (Thonar et al., 1985) and in SF of knee joints in OA patients (Campion et al., 1991). In the research presented in this dissertation, keratan sulfate was not measured due to difficulty in obtaining antibodies in sheep, reagents and controls as well as questionable assay reliability. Aggrecan contains an 846 epitope of chondroitin sulfate GAG sidechains, and is increased during cartilage turnover due to a reparative attempt by chondrocyte cell synthesis. Increases in the 846 epitope have been found in OA cartilage in SF and serum (Poole, 2002), which may prove this a useful diagnostic tool in OA detection.

Cytokine and eicosanoid quantification may also be useful in diagnosing the presence of early OA. In a clinical study utilizing four groups of horses (Bertone et al., 2001), SF concentrations of TNF-α, IL-1β, IL-6, Prostaglandin E_2 (PGE_2), Thromboxane B_2 (TXB_2), Prostaglandin F1-α (PGF_1-α), and Leukotrienes B_4 (LTB_4) were compared among horses with no evidence of disease, and those with acute or chronic disease or just

cartilage damage with no other disease signs. Differences existed among groups for the various markers of inflammation. Cytokines TNF-α and IL-1β were both strong indicators of disease, and IL-6 was an excellent predictor. Eicosanoids PGE_2, TXB_2, and PGF_1-α were strong predictors, and LTB_4 tended to increase in severe cases of acute joint disease. These data indicate that these inflammatory signaling molecules may be very useful in detecting early signs of OA development. Further, MMP quantification in SF has also been found to significantly differ between horses with joint disease and those with normal joints (Trumble et al., 2001). These enzymes, particularly MMP-1, -13 and -3 may also be very useful in the diagnosis of OA.

Clinical Examination and Imaging Techniques for Diagnosis

Horses exhibiting signs of pain, effusion, heat, and/or reduced range of motion or flexion may all manifest into lameness in a horse with OA. Nerve or joint blocking via regional or intrasynovial anesthesia can isolate the location of the lameness to a specific joint(s). Joint space narrowing or uneven joint surfaces can be observed radiographically. Other imaging techniques include arthroscopy, whereby an arthroscope camera is inserted into the joint space to determine if vascularization has occurred, villous formation of the synovium is present, pitting on the cartilage surface is detected or other complications associated with OA or other joint disease are observed. Ultrasonography and magnetic resonance imaging are also useful to diagnose advanced OA. Sampling the SF can be used clinically to determine pathology. Synovial fluid can be analyzed for color, consistency, precipitation, and viscosity as well as for the presence of total protein and white blood cell concentrations (Trotter and McIlwraith, 2003; Park et al., 2003).

Research Intent

Data and techniques presented above will be utilized to study the effects of circular and linear exercise on articular cartilage in sheep. Gross morphological, histology, and biochemical markers will be studied to further understand how early forced exercise in a sheep model impacts OA pathology. This dissertation research is presented in two journal articles (Chapters 3 and 4), and a summary of the research and ideas for future study complete this text.

CHAPTER THREE

FORCED EXERCISE IN JUVENILE SHEEP ALTERS BOTH MACROSCOPIC AND MICROSCOPIC CHANGES TO ARTICULAR CARTILAGE

Introduction

Joint disease is the most prevalent cause of lameness in the horse (Todhunter, 1992). The incidence of osteoarthritis (OA) anecdotally appears to be increasing and presenting clinical signs at an earlier age in horses, and is a major contributor to reduced performance, loss of use and loss of money in the horse industry. Understanding training techniques in young horses that may contribute to the "wear and tear" development of OA and manipulating these training practices to lessen their role in disease progression would be useful, financially beneficial and potentially increase the performance career of an equine athlete.

The distal limb joints are at the highest risk for OA development due to increased forces and rotation. The metacarpophalangeal (MCP) joint is the most frequently affected with traumatic or degenerative joint disease (MacDonald et al., 2002; Pool, 1986; Rossdale et al., 1985). There is considerable speculation that various training techniques used on non-racing performance horses are "hard" on horses' joints; however, very little scientific data exists to support or refute these beliefs. In the horse industry, circular exercise is used extensively in saddle breaking and training processes with yearling, 2- and 3-year-old horses. This exercise may come in the form of longeing, round penning, or 20-m circles under saddle used to teach a horse balance, suppleness and self carriage. While there are no data quantifying the degree of increased pressure or force that these

exercises may place on the distal limb, there are some data addressing this phenomenon in other species.

Usherwood and Wilson (2005) found that racing greyhounds experienced increased force on a 22.4 m bending turn compared to the first or second straight away portions. The dogs did not slow during the turn compared to the straight away, and the centripetal acceleration through the turn resulted in an increase in the effective weight of the dogs by 71%. Further, during the bending turn, all four limbs experienced a 64.5% increase in peak force compared to straight running. Usherwood and Wilson (2005) concluded that despite the increased force placed on the dogs' limbs during the turn, they were able to continue top speeds. These data suggest that horses working on a continual bending turn, such as during longeing, may experience increased force on their limbs compared to straight-line exercise.

van den Bogert and colleagues (1999) studied humans who had undergone hip replacement surgery and the strain placed on the hip during various exercise protocols. A short radius, steep slope down-hill skiing exercise generated force similar to running, and the short radius, flat slope generated the second most severe force; all other exercises generated less severe force on the patients. It was concluded that running and alpine skiing on the short turning radius, steep slope generated force that was too excessive for patients with hip replacement. It may be deduced that the sharper turns, with the associated increase in centripetal force, generates excessive force on the joints and articular cartilage. The same phenomenon may be experienced by horses that are

working on circles, which generates increased centripetal forces compared to straight-line exercise.

To examine the effects of circular exercise on cartilage health and the possible development of OA, a sheep model was utilized in this study due to affordability, accessibility, acceptability for euthanasia to collect joint surfaces for macroscopic and histological analysis, and to facilitate repeat joint synovial fluid sampling. Certainly, it is recognized that sheep are artiodactyles, whereas horses are perissodactyles, and have different locomotive patterns than horses. However, sheep have been used as a model for human and dog exercise studies and were appropriate for this study. The purpose of this study was to test the effects of straight-line exercise versus circular exercise on the articular cartilage surfaces of the MCP joint. We hypothesize that early forced exercise will induce cartilage damage both macroscopically and histologically compared to non-exercised controls, and that circular exercise will cause more severe damage than linear exercise.

Methods

Twenty lambs (ewes and wethers; ages 5 mo, avg 28 kg BW – start; ages 8 mo, 50 kg BW - end) were utilized in this study. Lambs were group housed in pens (4.5 m x 4.5 m; 6 per pen, treatment groups mixed together) and fed *ad libitum* Coastal Bermuda hay and 0.9 kg of a finishing ration (Southern States, Starter & Grower - BVT) per day per lamb (group fed 5.4 kg concentrate daily). Following a 4-wk acclimation period, sheep were randomly assigned to three groups: circular exercise (C; 8.5 m circle; n = 8), straight-line exercise (S, n = 8) and non-exercised control (CON, n = 4). Circular- and

straight line-exercised lambs were exercised at 1.3 m/s for 6 d per wk for 10 min increasing by 5 min increments to 30 min daily over a 6-wk period. Eight sheep per treatment were exercised linearly on a treadmill (S) or clockwise circularly (C) in an 8.5 m round pen. Four of the eight sheep per group were continued on exercise protocols for 30 min/d for an additional 2 wks. Two controls were euthanized at the beginning and two age-matched controls were euthanized at the end of the research trial. At the end of either six or eight weeks, lambs were euthanized via captive bolt, and MCP joints were collected and tissues were fixed in 4% buffered formalin. This research was approved by the Institutional Animal Care and Use Committee of Clemson University (AUP #50114).

Gross Analysis

Articular cartilage surfaces of the distal metacarpus (left and right legs, medial and lateral toes) were analyzed (grossly) by one individual blinded to treatment for wear lines, lesions or observable color differences. Scores for the Location of Lesion (from dorsal surface), Area of Lesion (mm^2) and Severity of Lesion on the lateral condyle were analyzed. Severity scores were determined as follows: 0 = No marks, discolorations, or lesions; 1 = Wear line; 2 = Superficial dot or dimple; 3 = Superficial dot or dimple with pink, red or yellow color; 4 = Medium depth lesion; 5 = Medium depth lesion with pink, red or yellow color; 6 = Deep lesion; 7 = Deep lesion with pink, red or yellow color. Data were analyzed using ANOVA (SAS, 2007). The complete randomized model was analyzed using the PROC GLIMMIX procedure of SAS and included treatment differences in the model. Data were reported either collectively for all toes for animals assigned to each of the three treatment groups for the above three parameters or

individually (single toe surface) only if statistical differences ($P \leq 0.05$) or trends ($P > 0.05, \leq 0.10$) were noted. Non-significant individual joint surfaces were not reported.

Histological Analysis

The palmar surface of the distal metacarpus was isolated for histological analysis using a tabletop hobby band saw. Samples were held by padded blocks when sawing bones to prevent damage to the articular surface. The epiphysis of the metacarpus was separated distally from the growth plate using the band saw, and the resultant triangular wedge of bone and articular cartilage was stored in buffered formalin. Each bone sample was decalcified using a 10% ethylene diamine tetraacetic acid, 2% paraformaldehyde (10.2 pH – adjusted with NaOH) for approximately 3 weeks or until 1-mm sections could easily be cut from the sample using a razor blade. Once the samples were decalcified, the 1-mm samples were dehydrated using a graduated, increasing ethanol concentration protocol in preparation for embedding and histological analysis. After the samples were dehydrated fully in 100% ethanol, the fat was cleared using xylene and samples were replaced into 100% ethanol. Samples were then infiltrated with catalyzed Immunobed™ Resin (Polysciences, Inc., Warrington, PA; 1.25 g benzoyl peroxide/100 mL Immunobed monomer A). The infiltration protocol was graded with a 50:50 ethanol:resin for a minimum of 8 hrs, followed by 100% resin overnight. Tissue samples were then embedded into plastic molds using an accelerated resin (0.4 mL Accelerator to one 10-mL aliquot of catalyzed Resin A) to allow for adequate polymerization. Polymerized blocks were stored in a dessicator under vacuum until sectioning.

Sectioning of tissues was completed on a microtome (Leica RM 2165, Bannockburn, IL) using a glass knife suitable for the block size. Samples were trimmed and then captured at 1.5 to 3 μm. Sections were placed onto a slide and stained with Azure II to provide contrast. Slide covers were adhered using slide mount glue (Poly-Mount, Polysciences, Inc., Warrington, PA) and stored until analysis. Each block was labeled with an identification code to allow for blinded histological analysis. Samples were scored by two individuals familiar with the scoring system who remained blinded to sample treatments. Samples were scored using the Osteoarthris Research Society International (OARSI) for Grade and Stage (Pritzker et al., 2006). OARSI score is calculated by determining the Grade (depth of damage from 0 to 6, where 0 = morphology intact and 6 = deformation and denudation) and the Stage (degree of surface area affected from 0 to 4, where 0 is no OA activity and 4 involves over 50% of the surface) and multiplying these two factors together for the total score. The OARSI score indicated any potential damage or deviations from normal physiology. Total OARSI histology scores were analyzed using an ANOVA with SAS software (SAS, 2007). The complete randomized model for the PROC GLIMMIX procedure of SAS included treatment for total histology scores, and trends were reported with $\alpha > 0.05, \leq 0.10$.

Results

Macroscopic Analyses

Mean Distance to Lesion, Area of Lesion and Severity of Lesion data for lesions present on the distal metacarpal epiphysis for the lateral condyle are shown in Table 3.1. Data for the individual joint surfaces for the above three parameters that were

significantly different among means or showed a trend for differences are presented in Table 3.2. All other joint surfaces (not presented) showed no significant differences for the three parameters measured macroscopically. Circled sheep had lesions on 12 surfaces on the medial condyle, while only one S and no CON sheep had lesions. The mean Distance to Lesion for lesions on the medial condyle of C sheep was 59.7 (% from dorsal surface); the Area of Lesion was 1.4 mm^2 and the Severity of Lesion was 3.1. These medial lesions were located at the same distance along the medial condyle as the lateral condyle lesions. No medial condyle lesions were present without a corresponding lateral lesion.

Table 3.1. Means ± SEM for Distance to Lesion (% from dorsal surface), Area of Lesion (mm^2) or Severity of Lesion (0 to 7) among circular (C; n = 8), linear (S; n = 8) and non-exercised control (CON; n = 4) groups of lambs with lesions on the lateral condyle of the MCP joint surfaces.

Parameter	**Circular Exercise**	**Linear Exercise**	**Control (Non-exercised)**	**P-value among means**
Distance to Lesion (% from dorsal surface)	$61.4^{b} \pm 1.0$	$65.4^{a} \pm 1.0$	$55.7^{b} \pm 2.8$	0.002
Area of Lesion (mm^2)	1.6 ± 0.3	1.4 ± 0.3	1.2 ± 0.4	0.704
Severity of Lesion (0 to 7)	$2.9^{a} \pm 0.3$	$1.7^{b} \pm 0.3$	$0.8^{b} \pm 0.5$	0.001

[a,b,c] Means with different superscripts are different ($P \leq 0.05$)

Table 3.2. Means ± SEM for Distance to Lesion (% from dorsal surface) or Severity of Lesion (0 to 7) among circular (C; n = 8), linear (S; n = 8) and non-exercised (CON; n = 4) groups of lambs for the lateral condyle of individual MCP joint surfaces.

Parameter	Circular Exercise	Linear Exercise	Control (Non-ex)	P-value among means
Right Leg, Medial Toe **Distance to Lesion**	63.4 [a] ± 1.8	65.6 [a] ± 0.9	41.7 [b] ± 0.0	0.001
Left Leg, Lateral Toe **Distance to Lesion**	62.6 ± 2.3	67.7 ± 1.2	62.1 ± 0.0	0.070
Right Leg, Lateral Toe **Distance to Lesion**	58.4 [b] ± 2.1	63.5 [a] ± 0.5	N/A	0.037
Right Leg, Lateral Toe **Severity of Lesion**	3.3 ± 0.9	1.8 ± 0.5	0.5 ± 0.3	0.087

[a,b] Means with different superscripts are different ($P \leq 0.05$). Trends for differences are included at ($P > 0.05, \leq 0.10$). All other joint surfaces were not statistically different.

Histological Analyses

There were no differences among groups for the right leg, lateral toe (RL); right leg, medial toe (RM); or left leg, lateral toe (LL). There was a trend ($P = 0.078$) for the left leg, medial toe (LM) to have overall treatment effects (Table 3.3). When all joint surfaces were combined (Table 3.3), there was an overall trend for differences among groups ($P = 0.0695$), with the linearly exercised group (S) having a higher OARSI score than the circle (C) or control (CON).

Table 3.3. Mean histological score [c] ± SEM among treatment groups for the distal metacarpophalangeal joint individual toe surfaces and for all toes combined in exercised and non-exercised lambs.

Surface	Circular (C) Means ± SEM	Linear (S) Means ± SEM	Control (Con) Means ± SEM	P – value among means
Individual mean joint surface (toes) scores [c]				
RL	3.67 ± 1.39	6.75 ± 1.65	5.00 ± 4.00	0.43
RM	3.58 ± 1.05	3.00 ± 0.67	3.50 ± 2.50	0.90
LL	4.13 ± 1.17	7.25 ± 1.64	3.00 ± 0.00	0.38
LM	3.10^{b} ± 0.46	5.63^{a} ± 1.46	2.38^{b} ± 0.69	0.078
Total score [c] for all toes combined				
Total Score ± SEM	3.59^{b} ± 0. 64	5.50^{a} ± 0.66	3.28^{b} ± 0.98	0.0695

[ab] Treatment groups with different letters tend to be statistically different ($P \leq 0.1$).
[c] OARSI scoring system - (grade x stage); Pritzker et al., 2006.
RL = Right leg, lateral toe; RM = Right leg, medial toe; LL = Left leg, lateral toe; LM = Left leg, medial toe.

Discussion

The severity of wear on the exercised sheep (S and C) indicated that the exercise protocol was adequate to test the hypothesis and to induce macroscopic changes on the distal metacarpal surface articular cartilage. A very severe lesion was present on one of the age-matched control sheep (right leg, medial toe), which was uncharacteristic of all other lambs, being much more dorsal on the lateral condyle and the most severe of all lesions. It is possible that this lamb injured itself at some point prior to or during the study. Due to low statistical power in the control group, this potential outlier was not removed. However, without this data point, it would further distinguish an exercise effect on the articular cartilage.

Circled lambs had more severe lesions on the lateral and medial condyles of the distal metacarpal epiphysis macroscopically. This may indicate that the circular exercise led to increased wear on the MCP joint of lambs exercised on an 8.5 m circle. Sheep may provide a helpful model to study exercise effects on articular cartilage, allow for invasive and repeated data collection, and for providing preliminary data for equine exercise physiology research.

The location and nature of lesion development in these exercising lambs were very similar to those found in racing thoroughbreds. Racehorses develop traumatic osteochondrosis lesions on both condyles of the MCP, which can lead to condylar fractures and failure of the bone. These lesions manifest typically as a result of overload. Commonalities in these lesions are a flattened area on the condyles where the proximal seasamoid bone provides force upon loading, which often leads to sclerotic subchondral bone, focal cracks or indentations of the articular cartilage, wear lines, or increased vascularization. Nordin and colleagues (1998) studied racehorses that were euthanized at racetracks as well as horses who were exercised on treadmills which were not previously raced to determine effects of exercise on articular cartilage. Of the 16 total horses studied, one did not demonstrate lesions, seven had mild superficial cartilage fibrillation and erosions, 4 had indentations of the articular cartilage, 2 demonstrated wear lines and 6 had 3 – 5 mm pits and infolded cartilage indicating osteochondrosis. These lesions were all on the distal metacarpal or metatarsal at or behind the transverse ridge where the distal end of the opposing sesamoid bone contacts the articular surface (Nordin et al., 1998). Histologically, subchondral sclerosis was increased in most horses and alterations

in vascularization in the subchondral bone and changes in the cartilage morphology were noted (Nordin et al., 1998). The macroscopic evidence from the current study on lamb MCP articular cartilage indicate similar alterations varying from increased vascularization and wear line development to infolding and pitting of the cartilage. The alterations in both macroscopic and microscopic morphology to the distal metacarpal articular cartilage in this study are very similar to the traumatic injuries sustained by athletic horses (Pool, 1996). Similarities of lesions in the exercised sheep to those seen in horses further support sheep as a useful model to study the pathogenesis of OA due to exercise and overload.

Trends for histological differences were noted specifically on the left leg, medial toe, where the linearly-exercised group had more severe histological damage than the circled or non-exercised groups. This was an unexpected finding, and perhaps a statistical artifact given that the treadmills utilized to administer the linear exercise were level. Despite the fact that variation for all treatment groups was considerable, possibly due to low animal numbers, trends for histological differences between groups existed. When all joint surfaces are combined together, there was an overall trend for the linear treadmill group to have more severe histological OA damage than the circled or control groups. It is interesting that the linearly-exercised sheep indicated the highest degree of histological damage when compared to the macroscopic data, which indicated that the circularly-exercised group had more severe damage. Regardless, both exercise groups had more severe macroscopic damage (numerically, but not statistically for linear) compared to control sheep. Microscopic changes of the MCP joint, such as furrowing,

thinning, infolding and changes in chondrocyte architecture, have been observed in horses that received forced exercise (Firth et al., 2004). In horses undergoing either intense simulated race training or hand walk, significantly more fibrillation was demonstrated histologically in the carpal joints of the intensely-exercised group compared to the lightly-exercised group (Murray et al., 1999). Additionally, in the areas of fibrillation, chondrocyte clustering was observed in approximately one-third of the studied sites, with the intensely-exercised group showing higher incidences of chondrocyte clustering (Murray et al., 1999). These data suggest that forced exercised in the horse does result in microscopic changes compared to non-exercised or lightly-exercised individuals. Similarities were seen in the current study, whereby either circularly- or linearly-exercised lambs did demonstrate increased macroscopic evidence of wear on the articular cartilage of the MCP joints compared to controls, but only the linearly-exercised group demonstrated microscopic changes. There is no direct or linear relationship between macroscopic and microscopic severity scores in this study.

Given the observable differences macroscopically and microscopically among circular-exercised, linearly-exercised and non-exercised controls, further research studying the effects of forced exercise styles on articular cartilage is warranted. Identifying the diameter or camber of circle or velocity at which alterations to the articular cartilage occur will be useful. A similar study using horses should be completed to compare to the results found in sheep. Such a study may aid in establishing sound horse training practices. Certainly, it appears that any forced exercise on a juvenile sheep at the given volume used in this study has a negative effect on articular cartilage,

resulting in both macroscopic lesion development and histological damage in both grade (depth) and stage (percent of surface area affected). A further understanding of linear and circular exercise and the ramifications on an animal's MCP joint may help provide guidance when developing training protocols for horses. Ultimately, a non-invasive biochemical marker detected in serum or synovial fluid that could suggest changes in cartilage due to various exercise regiments would be useful. Future research avenues may be to modify this exercise protocol to elicit further or different responses, include a recovery period from the exercise, change the camber, velocity or diameter of circle utilized in this protocol or perhaps to test dietary supplements that may attenuate the observations noted in this study.

Conclusions

There was an increased number and severity of lesions observed in sheep exercised on an 8.5m circle at 1.3 m/s for either 6 or 8 wks compared to linearly-exercised and non-exercised controls. However, these macroscopic changes did not correlate with microscopic differences. The linearly-exercised group sustained more severe microscopic damage, but there was evidence for microscopic changes seen in all treatment groups. The microscopic damage may not be severe enough to induce macroscopic changes. This research suggests that forced exercise in the juvenile sheep is capable of producing alterations in the articular cartilage on the palmar region along the condylar groove, and that microscopic damage also exists. Relating these alterations to biochemical markers would prove useful in detecting OA development earlier in exercising animals. Further research in the area of forced exercise and its effects on

articular cartilage, particularly in horses, will be useful in developing sound training techniques or OA diagnostics that promote and monitor joint health and longevity in the equine athlete.

CHAPTER FOUR

THE EFFECTS OF FORCED EXERCISE ON COLLAGEN TYPE II FRAGMENTS (C2C), LYSYL OXIDASE CONCENTRATIONS AND TOTAL PROTEIN CONCENTRATIONS IN SERA AND SYNOVIAL FLUID OF LAMBS

Introduction

In the horse industry, osteoarthritis (OA) leads to significant loss in training time, reduced performance ability, and loss of money. Joint disease is the most prevalent cause of lameness in the horse (Todhunter, 1992), and the incidence of OA anecdotally appears to be increasing and presenting clinical signs at an earlier age in horses. Clinical diagnosis of OA is often difficult in early stages, as definitive analyses typically involve radiologic, arthroscopic or ultrasound imaging only after a horse has presented with decreased performance, lameness or discomfort. The use of biological markers in serum or synovial fluid may prove incredibly useful to detect early changes in articular cartilage metabolism and turnover. Several biomarkers were utilized in this study to determine the effects of forced exercise on Type II collagen turnover (C2C) as well as general collagen maturation (lysyl oxidase). Further, total serum and synovial fluid protein concentrations were determined to estimate any immune responses due to the forced exercise.

The extracellular matrix of articular cartilage is composed of a fibril network of collagen (predominantly Type II) and non-collagenous proteins, such as aggrecan, the largest sulfated proteoglycan bound to the non-sulfated hyaluronan proteoglycan in articular cartilage. Type II collagen is comprised of three α subunits of collagen which forms a triple helix (Buckwalter, 1983; Temenoff and Mikos, 2000; Todhunter, 1996). The chondrocyte cells secrete this in a pro form, which undergoes extracellular

processing including enzymatic cleavage of the telopeptides and enzymatic cross-linking of hydroxylysine and lysine by lysyl oxidase. The extracellular matrix of articular cartilage, particularly the collagen, turns over very slowly. Matrix metalloproteinases (MMPs) degrade various components of this matrix. Collagenase, or Matrixmetalloproteinase-1, produces a three-quarter length N-terminal fragment and a one-quarter length C-terminal fragment upon degradation of collagen (Todhunter, 1996), and can be quantified to indicate the degree of degradation. In healthy tissue, the turnover is balanced by tissue inhibitors of these metalloproteinases (tissue inhibitor metalloproteinases or TIMPs; Davidson et al., 2006; Dean et al., 1989); however, in OA, Type II collagen is destroyed because of an up-regulation of the MMPs without a further up-regulation of TIMPs. Therefore, increases in the ¾-length fragment of Collagen Type II (C2C), as a result of MMP-1 degradation, have been detected in animal rheumatoid arthritis models and in synovial fluid in humans affected with rheumatoid arthritis (Elsaid and Chichester, 2006).

Lysyl oxidase is biologically important for biosynthesis of functional extracellular matrix and is the enzyme used in the final step of collagen and elastin cross-linking through oxidative deamination and condensation of the amino group of hydroxylysine and lysine residues (Kagan and Trackman, 1991; Todhunter, 1996). Articular cartilage extracellular matrix is composed predominately of Type II collagen, which utilizes lysyl oxidase to mature into the functional matrix protein. This final step is critical to yield the tensile strength necessary to prevent failure of the cartilage and the development of OA and other degenerative problems. A large increase in lysyl oxidase concentrations may

indicate a reparative mechanism due to overuse, trauma or general OA, and is often present in patients with fibrotic tumors (Woznick et al., 2005). Moderate increases in lysyl oxidase may be a simple biological response to increased turnover of collagen and net formation, as in growth. Suppressed lysyl oxidase concentrations may lead to decreased cross-linking of Type II collagen, and therefore less mature, weaker collagen fibers via poor crosslinking. Concentrations of lysyl oxidase were quantified in both serum and synovial fluid to determine its usefulness as an early biomarker indicator of OA in this current study.

In this study, lambs were utilized as a model to test the effects of circular exercise versus linear exercise. All exercised lambs worked on either 8.5-m circles or linearly on a treadmill to determine if these exercises altered the concentration of C2C in serum and lysyl oxidase (LOX) in serum and synovial fluid compared to non-exercised controls. The purpose of the study was to determine if circle exercise would cause more degradation of the articular cartilage and therefore elicit elevated C2C and LOX concentrations compared to a linear-exercise group. Further, it was expected that both types of exercise would have higher C2C and LOX than the non-exercised controls.

Methods

Animal Exercise Protocol

Twenty lambs (ewes and wethers; ages 5 mo, avg 28 kg BW – start; ages 8 mo, 50 kg BW - end) were utilized in this study. Lambs were group housed in pens (4.5 m x 4.5 m; 6 per pen, treatment groups mixed together) and fed ad libitum Coastal Bermuda hay and 0.9 kg of a finishing ration (Southern States, Starter & Grower-BVT) per day per lamb (group fed 5.4 kg concentrate daily). Following a 4-wk acclimation period, sheep were randomly assigned to three groups, circular exercise (C, 8.5 m, n = 8), straight-line exercise (S, n = 8) and non-exercised control (CON, n = 4). Lambs were exercised at 1.3 m/s for 6 d per wk for 10 min increasing by 5 minute increments to 30 min daily over a 6-wk period. Four of the 8 sheep per group continued to exercise for 30 min/d for an additional 2 wks (8 wks). Two controls were euthanized at the beginning and two age-matched controls were euthanized at the end of the research trial. Blood was collected weekly via jugular venipuncture throughout the trial and synovial fluid was collected using aseptic technique from the metacarpophalangeal (MCP) joint pre-trial and upon euthanasia. At the end of either 6 or 8 wks of exercise, lambs were euthanized via captive bolt, and MCP joints were collected and fixed using 4% buffered formalin as part of another portion of this research project. This research was approved (AUP #50114) by the Institutional Animal Care and Use Committee of Clemson University. All sheep completed the exercise protocol and no observable lameness was noted.

Total Protein for Serum and Synovial Fluid

Total protein for serum and synovial fluid was determined using a BioRad protein Bradford assay. Gamma globulin (IgG) – mouse (standard curve for synovial fluid), bovine serum albumin (BSA; standard curve for serum) and BioRad Bradford Dye were purchased from Sigma Aldrich (Saint Louis, MO). Standards were made fresh in the following concentrations for synovial fluid: 0.5, 0.25, 0.125, 0.0625, and 0.0313 mg/mL (IgG); and for serum: 1.0, 0.5, 0.25, 0.125, 0.0625 mg/mL (BSA). Standards, blank and unknown samples were run in triplicate. Unknown samples of synovial fluid were diluted 1:70 with 0.05M Borate Buffer. Unknown samples of serum were diluted 1:150 with deionized water. In each well of a 96-well assay plate, 10 µL of standard, blank or unknown were added, and 200 µL of BioRad Bradford dye was added to each well using a multichannel pipetter. The plates were then shaken for 5 min on a Biotek Synergy Spectrophotometer and were further incubated for an additional 5 min (total incubation 10 min) and the absorbance measured at 570 nm. Serum and synovial fluid total protein concentrations (mg/mL) were recorded.

Total serum and synovial fluid protein concentrations were analyzed using an ANOVA for repeated measures with SAS software (SAS, 2007). The complete randomized model for the PROC GLIMMIX procedure of SAS included treatment, time, and treatment by time interactions for repeated measures of total protein concentrations, and differences were reported with $\alpha = 0.05$. Serum total protein was analyzed for each wk (total of 13 wks with 5 wks acclimation and 8 wks total exercise) and time points for

synovial fluid included pre-trial (during acclimation period) and the final wk (either Wk 11 or 13) of the formal exercise protocol.

LOX Assay

Reagents

Resorufin, cadaverine dihydrochloride, and β-aminoproprionitrile fumarate (BAPF) and Amplex Red™ were purchased from Sigma Aldrich (Saint Louis, MO).

Stock Solutions

Stock solutions were prepared and stored in 0.5-mL (Amplex Red and BAPF) or 1.0-mL (Cadaverine) aliquots at -20ºC. Amplex Red (10 μM) was prepared by dissolving 0.64 mg of the dye in a small amount of borate buffer (0.05 M, pH 9) and brought to 25-mL volume with the same buffer. Stock cadaverine dihydrochloride (10 mM) was made by adding 175.2 mg to 5 mL of water to yield a 2X stock solution, which was diluted when ready to assay with 1 mL of 0.1 M borate buffer (pH 9). The diluted cadaverine was used immediately. Horseradish peroxidase (HRPO) was made fresh for each assay by adding 5 units HRPO/mL of 0.05M borate buffer. β-Aminoproprionitrile fumarate (500 μM) was prepared by adding 6.4 mg BAPF/5 mL of water to yield a 2X stock, which was diluted when ready to assay by adding equal volume of 0.1 M borate buffer to yield 125 nmol BAPF/50 μL of 0.05 M borate buffer.

Assay Protocol and Sample Preparation

Lysyl oxidase was quantified in this study using a fluorescence assay modified from Palamakumbura and Trackman (2002). A 96-well black/clear bottom plate (BD Biosciences Falcon; Ref # 353293; Franklin Lakes, NJ) was used to perform the LOX

assay. Unknown sera samples were diluted 1:2 and unknown synovial fluid samples were diluted 1:10 with 0.05 M borate buffer. A standard curve of resorufin was made in duplicate on each assay plate, and consisted of 0, 0.0125, 0.025, 0.5, 1 and 2 μM. In each well of the assay plate, 25 μL of Amplex Red and diluted cadaverine dihydrochloride and 50 μL of 0.05 M borate buffer were added. The diluted unknown samples (100 μL) were then added. In a parallel series, 50 μL of borate buffer was replaced with 50 μL of diluted BAPF. The BAPF completely inhibits the lysyl oxidase activity in the unknown sample and the difference between the unblocked and BAPF-blocked fluorescence units yields the degree of lysyl oxidase fluorescence activity in the sample. The standard curve was utilized to determine the specific activity of the LOX in the samples. The final step was to add 50 μL of HRPO to all wells with unknown samples. Samples were incubated for 10 min and then fluorescence units were determined on a Biotek Synergy HT™ fluorescence spectrophotometer with excitation wavelength at 563 nm and emission wavelength at 587 nm.

Mean fluorescence units (FUs) for each group were recorded; specific activity was determined and analyzed for differences using an ANOVA with SAS statistical software (SAS, 2007). The complete randomized model for the PROC GLIMMIX procedure of SAS included treatment, time, and treatment by time interactions for repeated measures of LOX specific activity (units of activity/min/mg total protein), and differences were reported with $\alpha = 0.05$. As there were no gender effect on treatment or treatment by time interactions, gender was removed from the final model for these statistical evaluations. Time points were from initial acquisition of the animals, through

the acclimation period and the eight wks of formal exercise protocol, totaling 13 wks for serum LOX and just prior to formal exercise and upon euthanasia for synovial fluid LOX analyses.

To evaluate the relationship between LOX and bone/cartilage growth in juvenile sheep, nine additional lambs of similar genetics were utilized to develop a LOX profile during the first 4.5 mo of life. Blood samples were taken weekly via jugular venipuncture from lambs beginning at 2 wks until 17 wks of age. Serum was assayed for LOX specific activity as above and repeated measures of the data were analyzed using the PROC GLIMMIX procedure of SAS. Time differences were reported with $\alpha = 0.05$.

C2C Assay

The Collagen Type II Cleavage ELISA (C2C) was a kit purchased from IBEX (Montreal, Canada). The assay measures any neoepitopes at the C terminus of the ¾-length cleavage product that is generated when collagenases degrade Type II collagen. In various arthritis models, this neoepitope increases in serum.

Reagents

The kit included all reagents: C2C Standard Stock (10 µg/mL) was used to make a standard curve of 0, 10, 50, 100, 200, 500 and 1000 ng/mL; a pre-coated C2C ELISA plate; a protein-based assay buffer for dilution of primary antibody; a buffer III for dilution of the standards and secondary antibody; the C2C primary antibody (murine IgG); goat anti-mouse IgG-horseradish peroxidase (GAM-HRP) conjugate (secondary antibody); wash buffer; tetramethylbenzidine (TMB; the chromophore) and a sulfuric acid stop solution.

Assay Protocol and Sample Preparation

The assay was conducted according to the standard protocol for the kit. Briefly, the assay was completed at room temperature. Serum samples were diluted 1:2 with Buffer III. 50 µL of standard and unknown sample were plated onto a mixing plate, followed by 50 µL/well of the diluted (in assay buffer) primary C2C antibody. The mixing plate was incubated on a high speed plate shaker at approximately 650 rpm for 30 min and then 80 µL of the antigen-antibody mixtures from each well were transferred to the corresponding wells on the coated ELISA plate. This ELISA plate was then incubated on a plate shaker for 1 hr, and then washed three times. 100 µL of the diluted (with Buffer III) GAM-HRP conjugate was then added to each well and the plate was further incubated on a plate shaker for 30 min, and washed again six times. Finally, 100 µL of TMB was added to each well and the plate received a final incubation for 30 min. Color development was monitored, and 100 µL of stop solution was added per well. The absorbences were measured on a Biotek spectrophotometer (PowerWaveX™) at 450 nm. The OD_{450} is inversely proportional to the amount of collagen Type II ¾-length fragment neoepitope present in the unknown sample. The standard curve was plotted using a spline fit, OD results were recorded and mean concentrations of C2C neoepitope were analyzed using an ANOVA in SAS statistical software, and differences were reported with $\alpha = 0.05$.

The complete randomized model included treatment, time, and treatment by time interactions. There were no effects of gender on treatment or treatment by time interactions, so gender was removed from the final model for these estimates. Time

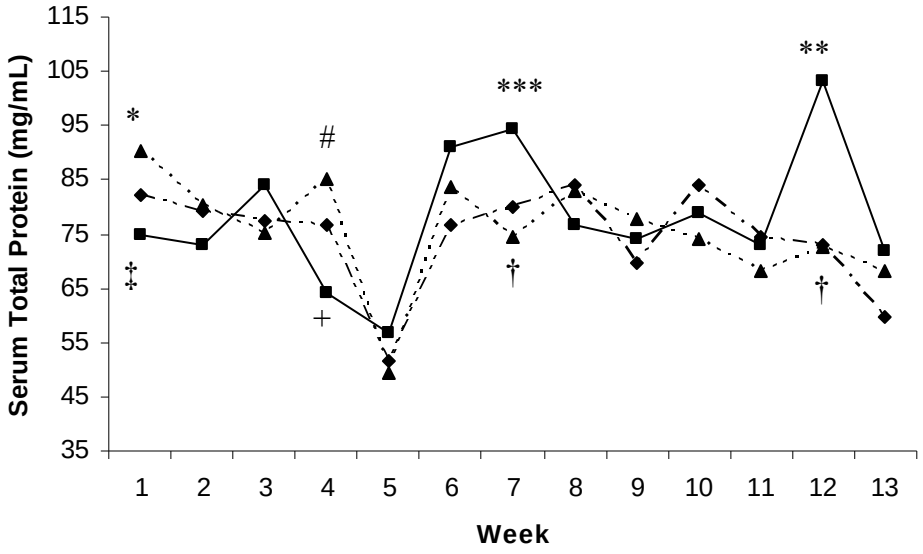
points included the first week (Wk 5) and the final week (Wk 11 or 13) of the formal exercise protocol.

Results

Serum Total Protein (STP)

There were no overall treatment differences for STP concentrations among groups over the length of the study (Table 4.1). There was an overall difference for STP over time during the length of the study ($P < 0.0001$) for all treatment groups combined and a trend for a treatment by week interaction ($P = 0.068$). Treatment by time differences for individual weeks for STP concentrations are represented in Figure 4.1.

Figure 4.1. Mean time by treatment interactions for serum total protein concentrations for lambs that were exercised on a circle (-·-◆-·-), linearly (- - -▲- - -) or non-exercised (—■—).



*‡ Week 1 – Linear exercise group STP tended to be higher than controls ($P < 0.10$)
#+ Week 4 – Linear exercise had higher STP than controls ($P < 0.05$)
***† Week 7 - Control group had higher STP than linear exercise group ($P < 0.05$)
**† Week 12 - Exercise groups (circle and linear) had lower STP than controls ($P < 0.05$)
Week 5 began formal exercise protocol

Serum C2C

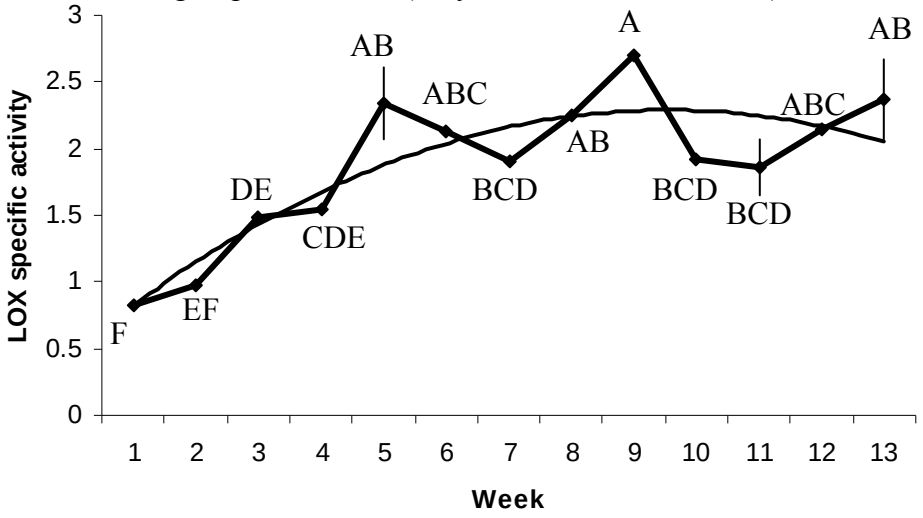
The overall fixed effects for treatment (Table 4.1), time, and treatment by time interactions for serum C2C concentrations were not different.

Serum LOX

There were no differences among treatment groups over the length of the study for serum LOX specific activity (Table 4.1). The main effect of time for all treatments combined for serum LOX activity concentrations was different ($P < 0.0001$; Figure 4.2). The main effect for serum LOX activity treatment by time interaction was not statistically significant ($P = 0.497$). Differences between the interaction of treatment and time for specific weeks are represented in Figure 4.3 and denoted by symbols.

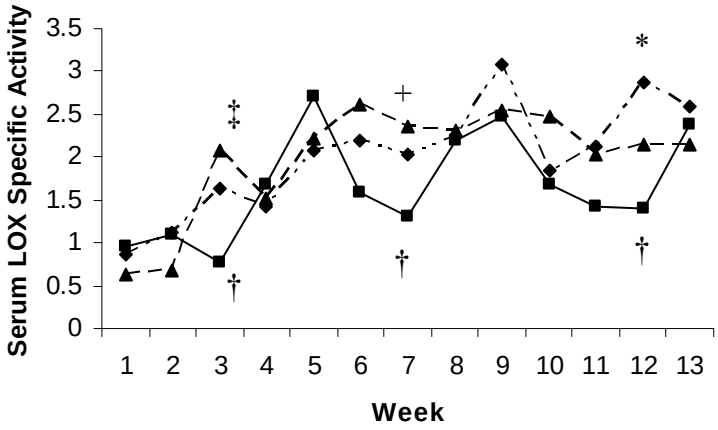
Data from the 9 additional lambs utilized to generate a LOX growth curve are represented in Figure 4.4. There was a main effect of time ($P < 0.0001$), where LOX specific activity increased over the course of the 15 weeks studied.

Figure 4.2. Differences among weeks for serum LOX specific activities (—◆—) in lambs for all treatment groups combined (Polynomial trend line ——).



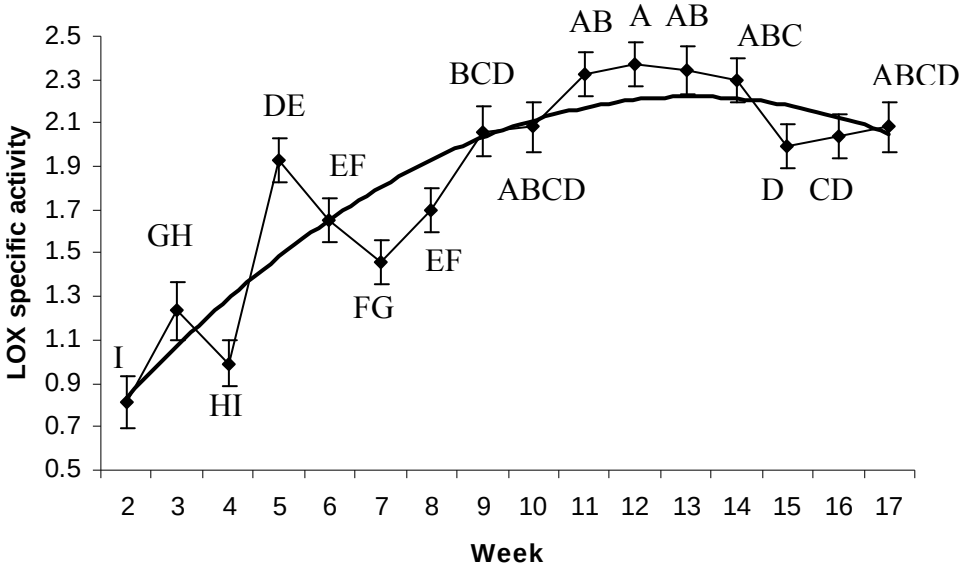
Weeks with different letters are significantly different ($P \leq 0.05$)
Week 5 began formal exercise protocol
Trend Line $y = -0.0201x^2 + 0.3838x + 0.4604$
Trend Line $R^2 = 0.7464$

Figure 4.3. Differences among least square means for the interaction of treatment and time (wk) for serum lysyl oxidase (LOX) specific activity in lambs that were exercised in a circle (- -◆- -), linearly (- - -▲- - -) or non-exercised (—■—).



‡† Week 3 – Linear exercise group had higher LOX than control group ($P < 0.05$)
+† Week 7 – Linear exercise group had a trend for higher LOX than control group ($P < 0.10$)
*† Week 12 – Circle exercise group had higher LOX than control non-exercise group ($P < 0.05$)
Week 5 began formal exercise protocol

Figure 4.4. LOX specific activity growth curve for nine additional lambs of similar genetics to illustrate the increase in LOX (—◆—) during growth in lambs aged 2 through 17 weeks (Polynomial trend line ——).



Weeks with different letters are significantly different ($P \leq 0.05$)
Trend Line $y = -0.0112x^2 + 0.272x + 0.5738$
Trend Line $R^2 = 0.8309$

Table 4.1. Serum analysis results for total protein, LOX specific activity, and C2C (Means ± SEM) for lambs exercised on a circle, linearly or not exercised.

Analysis	Circular Exercise (n =8)	Linear Exercise (n = 8)	Non-Exercised Control (n = 4)	p-value among means
Total Protein (mg/mL)	74.6 ± 1.8	78.1 ± 1.7	75.4 ± 1.7	0.66
LOX (spec. activity*)	2.01 ± 0.08	1.96 ± 0.08	1.67 ± 0.15	0.15
C2C (ng/mL)	134.4 ± 8.9	131.2 ± 14.3	118.6 ± 8.7	0.44

*Specific Activity = units of activity/min/mg protein

Synovial Fluid Total Protein (SFTP)

Synovial fluid total protein concentrations were analyzed separately for each leg (right versus left). There were no differences among treatments (Table 4.2), over time or as a treatment by time interaction for SFTP for the right MCP joint. For the left leg, there

were no differences among treatments (Table 4.2) or in a time by treatment interaction, but there were time differences when all treatments were combined over the course of the study (beginning – 19.43 ± 1.29 mg/mL; end – 15.77 ± 1.53 mg/mL; P = 0.047).

Synovial Fluid LOX

The synovial fluid LOX specific activity was analyzed separately for each individual MCP joint. There were no treatment (Table 4.2), time or time by treatment fixed effects for synovial fluid LOX specific activity for the right MCP. For the left MCP, there were no differences among treatment groups for synovial fluid LOX specific activity (Table 4.2) nor any time by treatment effects; however, there were differences over time when all groups were combined (beginning – 2.07 ± 0.24; end – 3.48 ± 0.30; P = 0.001).

Table 4.2. Results of the metacarpophalangeal joint synovial fluid analyses for total protein and LOX specific activity (Means ± SEM) in lambs that were exercised on a circle, linearly or not exercised.

Analysis	**Circular Exercise (n =8)**	**Linear Exercise (n = 8)**	**Non-Exercised Control (n = 4)**	**p-value among groups**
Total Protein Right MCP (mg/mL)	19.82 ± 1.35	19.78 ± 1.33	19.01 ± 2.14	0.94
Total Protein Left MCP (mg/mL)	18.78 ± 1.58	17.88 ± 1.64	16.13 ± 2.56	0.68
LOX activity* Right MCP	1.66 ± 0.16	1.81 ± 0.15	1.50 ± 0.27	0.59
LOX activity* Left MCP	2.79 ± 0.26	2.86 ± 0.26	2.68 ± 0.44	0.93

*Specific Activity = units of activity/min/mg protein

Discussion

The STP was not significantly different among treatment groups over the length of the study. There were certain time points throughout the study where there was a time by treatment difference in STP. In Wk 1 and Wk 4, the straight-line exercise group had higher STP than the controls, but this was during the acclimation period and therefore would not be a result of the exercise protocol. In Wk 7, the control group had higher STP than the straight-line group, and in Wk 12, the control was higher than both exercise groups. This finding at Wk 12 (Wk 7 of their formal exercise protocol) was likely not attributed to the exercise protocol as all animals reached their maximum exercise regime at Wk 11 and therefore were not being worked any more intensively than the previous or subsequent weeks. Additionally, no animals were sick at this time and ambient temperature, while 35°C and above, was no different than previous weeks. Additionally, there were no changes in feed concentrations or source during the entire length of the formal exercise protocol. As evident by the SFTP concentrations, the exercise treatments did not alter the protein concentration within the synovial fluid. The time differences that existed for the left MCP in synovial fluid total protein concentrations are interesting but difficult to explain.

Lysyl oxidase has not been previously reported as a biomarker for OA development. van de Lest and colleagues (2003) analyzed lysyl oxidase activity in foals that were reared in various management conditions: pasture, box stalls or stalled with forced gallop training. The foals that were housed in a box stall had lower LOX than the other two groups throughout the study, which corresponded to the number of

hydroxylysylpyridinoline and lysylpyridinoline cross-links (van de Lest et al., 2003). This decrease in LOX activity, and subsequent decrease in cross-linking of collagen, was likely due to increased bone resorption due to confinement rearing. Suppressed metabolism of bone and a decrease in bone quality has been correlated to confinement rearing (Hiney et al., 2004a, 2004b; Shackelford et al., 2004; Weiler et al., 2006).

In this study, forced exercise did not alter the specific activity of LOX compared to non-exercise controls over the course of the study (acclimation and 8 wks of exercise) as evident in both serum and synovial fluid analyses. Increased LOX activity was expected in this study due to increased turnover of collagenous tissue, both bone and cartilage, which contain Type I and Type II collagen respectively. Forced exercise has been shown to increase bone turnover in a variety of species, including horse (Hiney et al., 2004a) and bull calves (Hiney et al., 2004b). Research on the effects of forced exercise on cartilage turnover using biomarkers and the effects of exercise on cartilage in sheep models is limited. Billinghurst and workers (2003) investigated the effects of exercise-related changes in serum concentrations of a number of collagenous and non-collagenous biomarkers for collagen and cartilage metabolism in horses. They found that forced training transiently suppressed serum Type II collagen synthesis marker CPII compared to pasture-reared horses but recovered by five months. Of the eight markers evaluated in the study, the authors were not able to identify significant differences among groups, and so a meaningful biomarker for cartilage metabolism was elusive (Billinghurst et al., 2003).

van de Lest and colleagues (2003) utilized lysyl oxidase to determine the effects of exercise on immature equine subchondral bone. Horses were either pasture reared or maintained in box stalls with or without forced exercise from birth until weaning at five months of age. After weaning, all foals were housed in a loose box with access to a paddock to determine if exercise-induced effects could be reversed. Lysyl oxidase concentrations were suppressed in the confined group (box stall with no exercise) and all LOX data corresponded to the number of collagen crosslinks measured. During the reversal phase of the study, LOX concentrations remained lower in the former confined animals and compensation was not reached (van de Lest et al., 2003). In the current study, although the control sheep received no forced exercise, the pens did allow for adequate movement, and the control sheep were allowed out of the pen daily to prevent suppressed bone metabolism. Therefore, no negative effects from the lack of forced exercise in the control sheep were expected in the current study.

The increase in LOX concentrations for all groups over time (both serum and synovial fluid) is supported by the concept that as an animal ages, the connective tissues modify and mature to provide tensile strength and compressive stiffness. In an aged individual, these collagenous tissues can become brittle and stiff and therefore susceptible to failure. Avery and Bailey (2005) reviewed enzymatic and non-enzymatic cross-linking mechanisms in relation to turnover of collagen. The Type II collagen in articular cartilage has a high activity of hydroxylation reactions, which forms cross-links that provide structural integrity. The turnover of this cartilage is much slower compared to bone, skin or tendon, which have predominantly Type I collagen fibers, with an estimated

half-life of about 100 years. So, as an animal ages, it continues to utilize the enzymatic reactions of lysyl oxidase to mature this collagen. This enzymatic reaction appears to be confined to growth and maturation, whereas non-enzymatic changes occur throughout the lifetime of the individual (Avery and Bailey, 2005). Sheep are typically skeletally mature between 10 and 12 mos, when the growth plates of the "break joint" (distal end of the metacarpal bone) have fused. Ho and colleagues (1989) found that several breeds of sheep (Finn x whiteface crosses - Rambouillet, Targhee and Columbia; and Suffolk x whiteface crosses) had fused spool joints (MCP) between 459 and 557 days. Therefore, it is possible that skeletal maturity is not reached until well into the first year of life. The sheep in this study began the experiment at approximately 5 months of age, and were euthanized at approximately 8 months of age. It is possible, that the enzymatic activity of lysyl oxidase began to taper in the final month of the study due to maturation of the collagenous tissues. It would not appear that the changes to LOX over time were related to the exercise protocol (circular or treadmill) or lack of exercise (control), as there were no overall treatment differences. In lambs of similar genetics, LOX specific activity increased from ages 2 through 17 weeks supporting the concept that the increase in LOX over time for all sheep (circular and linear exercised as well as non-exercised controls) in the exercise study was not due to treatment effects, but rather growth. Further investigation into the effects of non-enzymatic cross-linking and glycation may be warranted to understand the problems associated with exercise in a mature animal.

The LOX marker may be an indicator as to the quality of collagens in general, and there have been limited previously reported studies investigating the correlation of LOX

to cartilage quality. When coupled with other markers for cartilage synthesis or degradation, LOX may prove useful in early detection of OA. The fluorometric method used in this study was extremely sensitive for detection of LOX in serum and synovial fluid. Further investigation of the role of LOX in cartilage degradation and extracellular matrix destruction is justified. Utilizing LOX coupled with other clinical diagnoses may assist pathologists and researchers in earlier diagnosis of osteoarthritis and lead to improved management of this orthopedic disease.

There were no differences among groups for serum C2C concentrations. The C2C fragment is released as a result of increased MMP activity, and therefore can also be considered an indirect measure of MMP activity. Similar markers, such as the 234CEQ assay, have been utilized to determine the collagenase-derived ¾-length fragment of Type II collagen (Billinghurst et al., 2003). Billinghurst and colleagues (2003) found no differences in the 234CEQ marker among groups of horses that were confined to box stalls, kept on pasture, or were in box stalls but received forced exercise. It is possible that the exercise regimen for the sheep in the current study or the horses in the Billinghurst et al. (2003) study was not sufficient enough to elicit systemic indices of collagen Type II breakdown using biomarkers. Further investigation into early forced exercise, various types of exercise and overall volume of exercise and its effects on cartilage development is warranted. Further research by this group using the same sheep revealed both macroscopic and microscopic changes in the articular cartilage surface of the MCP joint of exercised sheep compared to control sheep, indicating that the exercise protocol was sufficient to induce localized alterations to the cartilage. Continuing to

identify systemic biomarkers that would be useful in determining cartilage metabolism would prove valuable to determine which types of exercise could be damaging to young animals and perhaps be modified to ameliorate such damage.

Conclusion

There were no differences among treatment groups for serum or synovial fluid total protein, C2C, or LOX for sheep exercised at 1.3 m/s on either a treadmill or in a clockwise circle compared to non-exercised controls exercised for a total of 8 wks. In a companion study, there were macroscopic differences among groups with circled sheep developing the most severe lesions, linear-exercised sheep developing moderate lesions, and control sheep developing minimal wear. However, in this companion study, histological evidence revealed that linear-exercised sheep had statistically higher microscopic damage compared to circled sheep or non-exercised controls. It would appear that the exercise protocol was sufficient to induce structural alterations to the articular surface, but biochemical markers in this study failed to demonstrate localized (synovial fluid) or systemic (serum) changes between the two exercised groups. At the volume of exercise utilized in this study, circle exercise did not appear to be any more severe than linear exercise in juvenile lambs. Further research utilizing a different exercise protocol or biochemical markers may be revealing into the effects of various forced exercise techniques in juvenile animals.

Lambs were an effective model in this study and further research using horses may be warranted to investigate appropriate biochemical markers for determining the early signs of OA. Efforts to increase the longevity of a horse's athletic career are still an

important issue in the horse industry. Continued efforts to test various exercise protocols and to discover a meaningful and non-invasive tool for determining the early signs of OA development in horses would prove financially beneficial and useful in maintaining health and wellbeing.

CHAPTER FIVE

SUMMARY

The results of this study indicate that early forced exercise does alter both macroscopic and microscopic characteristics of the articular cartilage of the distal metacarpus. Circle-exercised lambs had more severe gross lesion development on the lateral condyles of the joint surface, and a higher incidence of lesion development on the medial condyles compared to both linear- and non-exercised lambs. Linear-exercised lambs had moderate severity lesion development located almost entirely on the lateral condyle of each joint surface (toe) compared to the other treatment groups. Further, the lesions that developed were located on the palmar portion of the joint surface. The type and location of lesions found in this study were similar to those found in racing Thoroughbreds that often lead to traumatic osteochondrosis. Interestingly, the results of the histological analyses indicated that the linear-exercised group had more severe OA progression using the OARSI scoring system for grade and stage compared to the other treatment groups. Therefore, the exercise protocol utilized in the study was sufficient to induce both macroscopic and microscopic alterations to the articular cartilage in both circular and linear exercise, and indicate that early forced exercise (volume - 1.3 m/s; 10 to 30 min/d; 6 d/wk; 6 to 8 wks) is damaging to the articular cartilage in the MCP joint in the juvenile animal.

The biomarkers utilized in this study did not correlate to the results of the gross lesion development and histological changes. Serum and synovial fluid total protein and LOX as well as serum C2C failed to demonstrate differences among treatment groups.

Regardless, these markers provided valuable information relating to systemic and localized inflammatory responses, collagen maturation and MMP activity through collagen cleavage fragments. Further research into alternative biomarkers should be pursued in an effort to correlate the morphological changes to cartilage. Ideally, a non-invasive biomarker (either derived from serum or synovial fluid) that could serve as an early indicator of OA progression would be incredibly useful in preventing and/or treating OA in performance horses and other species.

This study provides valuable preliminary information as to the effects of circular exercise compared to linear exercise in a juvenile animal. The horse industry's utilization of circular exercise in the form of longeing, round penning and riding is extensive. Further investigation into circular exercise is warranted. Future research may include investigating various diameters of circles, altering the camber (slope) of the footing of the circle, and the effects of the duration or speed of exercise bouts. Additionally, the results of this study pose further questions into early forced exercise in juvenile animals in general regardless of how it is delivered. Certainly, identifying the total volume of exercise (duration, distance and/or intensity) that would minimize OA progression or that which would be deemed too excessive would provide clarity for horse trainers who aim to protect the articular cartilage in their equine athletes. Further, investigation into a recovery period after early forced exercise to determine if OA progression could be attenuated is warranted. Improving the way we train and exercise horses may lead to decreased incidence of OA, which would improve the welfare of the horse and improve the financial health of the horse industry as a whole.

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