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The effects of a single acute load on an equine articular cartilage explant system and further studies on the ability of glucosamine and chondroitin sulfate to inhibit cytokine-induced cartilage degradation

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THE EFFECTS OF A SINGLE ACUTE LOAD ON AN EQUINE ARTICULAR CARTILAGE EXPLANT SYSTEM AND FURTHER STUDIES ON THE ABILITY OF GLUCOSAMINE AND CHONDROITIN SULFATE TO INHIBIT CYTOKINE-INDUCED CARTILAGE DEGRADATION

By

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ABSTRACT

THE EFFECTS OF A SINGLE ACUTE LOAD ON AN EQUINE ARTICULAR CARTILAGE EXPLANT SYSTEM AND FURTHER STUDIES ON THE ABILITY OF GLUCOSAMINE AND CHONDROITIN SULFATE TO INHIBIT CYTOKINE-INDUCED CARTILAGE DEGRADATION

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Trauma to a joint can initiate of equine osteoarthritis (OA) by damaging the cartilage and increasing the synthesis of catabolic molecules. Oral treatment of OA is also becoming more popular within the equine industry. Glucosamine (GLN) and chondroitin sulfate (CS) may slow cartilage degeneration associated with OA and improve lameness in the horse. Equine articular cartilage explants were used to evaluate the effects of loading (15 or 30 MPa) compared to cytokine-stimulation (LPS). LPS-treated explants had the highest nitric oxide (NO) and prostaglandin E_2 (PGE_2) production over all treatments, while loading explants at 30 MPa resulted in the highest proteoglycan (PG) release, and second highest keratan sulfate (KS) degradation and PGE_2 production. Explants loaded at 15 MPa did not differ from the control in NO, PG, or PGE_2 production, but had the highest KS loss of any treatment. The same explant system was used to evaluate if GLN (0.2-0.5 mg/ml) in combination with CS (0.125 mg/ml) are effective in inhibiting cytokine-induced cartilage degradation. NO and PGE_2 production and matrix metalloproteinase (MMP) activity were evaluated. NO production was lowered from 0.3 to 0.5 mg/ml GLN, while PGE_2 production was decreased at 0.4 and 0.5 mg/ml. Matrix metalloproteinase-9 activity was decreased at 0.5 mg/ml and tended to be decreased at 0.4 mg/ml.

I would like to dedicate this thesis to my loving family, especially my parents Randall and Janet Schlueter. Without their strong support, this work would not have been possible.

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LIST OF ABBREVIATIONS

Acetylsalicylic Acid	ASA
Bovine Basal Media	BBM
Bovine Viral Diarrhea Virus	BVDV
Carprofen	CAR
Chondroitin Sulfate	CS
Computed Radiology	CR
Computed Tomography	CT
Cyclooxygenase	COX
Cyclooxygenase-2	COX-2
Degenerative Joint Disease	DJD
Dexamethasone	DEX
Dimethylmethylene Blue	DMB
Distal Interphalangeal	DIP
Dulbecco's Modified Eagles Medium	DMEM
Extracellular Matrix	ECM
Enzyme-Linked Immunosorbent Assay	ELISA
Fetal Bovine Serum	FBS
Fibronectin Fragments	Fn-f
Flunuxin	FNX
Glucosamine	GLN
Glycosaminoglycan	GAG

Hyaluronan	HA
Insulin Transferrin Sodium Selenite	ITS
Insulin-Like Growth Factor 1	IGF-1
Interleukin-1	IL-1
Ketoprofen	KET
Keratan Sulfate	KS
Leukotriene	LTB
Linoleic Acid Albumen	LLA
Lipopolysaccharide	LPS
L-Thyroxine	T
Magnetic Resonance Imaging	MRI
Matrix Metalloproteinases	MMP
mega Pascal	MPa
Meloxicam	MEL
Metacarpalphalangeal	MCP
Middle Carpal Joint	MCJ
Nitric Oxide Synthase	NOS
Nitric Oxide	NO
Nonenzymatic Glycation	NEG
Non-Steroidal Anti-Inflammatory Drugs	NSAIDs
Nuclear Medicine	NM
Osteoarthritis	OA
Phenylbutazone	PBZ

Polysulfated Glycosaminoglycans	PSGAGs
Prostaglandin E_2	PGE_2
Proteoglycan	PG
Proximal Articular Surface	PAS
Proximal Phalanx	P1
Serum Free	SF
Thromboxane	TXB
Tissue Inhibitor of Matrix Metalloproteinases	TIMP
Ultrasound	US

INTRODUCTION

Osteoarthritis (OA) is characterized by deterioration of the articular cartilage, accompanied by changes in the bone and soft tissues of the joint, including subchondral bone sclerosis and marginal osteophyte formation.[1] Considerable attention has been given to OA, because it is one of the most common causes of lameness in horses. Due to its degenerative effect, OA leads to significant financial loss in the equine industry.[2] Horses most affected by OA are western performance horses, racehorses, jumping horses, and other sport horses involved in highly athletic events.[2-4] As athletic events have become more competitive, so has the breeding and training of the horses, because exceptional athletes are needed to compete in the industry today.[3] The demand for increased athletic ability and rigorous training predisposes these types of horses to athletic injury.

Mechanical loading, or trauma to the joint, is a significant cause of OA in the horse. Athletic horses undergo extensive and intensive training regimens, which result in repetitive loads being placed on the tissues of the joints.[5] Because the tissues of articulating joints are subject to impact loading, cartilage must be capable of resisting and redistributing the forces arising during joint movement. Research investigating the in vivo effects of loading on articular cartilage has shown that it adapts to load.[6-8] *In vitro* studies of loaded bovine cartilage explants have indicated changes in the extracellular matrix (ECM), which include cell death, loss of proteoglycans (PGs), and increased nitric oxide

(NO) production.[9] Little research has been done on the effects of mechanical impact on equine cartilage explants. The biochemical changes that occur following loading, which predispose the horse to OA, require further investigation.

Increased application of modern technology, including anesthesia and imaging techniques, has facilitated more accurate and earlier diagnosis of joint disease.[10] However, a lack of information about the basic pathogenesis of OA has hindered progress in developing innovative treatments. Because OA is considered to be a chronic irreversible disease, noninvasive treatments to protect articular cartilage and prevent further damage are being sought. Glucosamine (GLN) and chondroitin sulfate (CS) are amino sugars that have a beneficial effect in the treatment of OA.[11-17] Both of these agents are endogenous to chondrocytes, which produce the structural components of the ECM, and are considered chondroprotective.[18]

My research consists of two objectives. The first objective combines the use of an equine explant system[14,15] and a method previously designed in our lab to mechanically load articular cartilage explants,[19] and evaluate the effects of mechanical loading on equine explants. The second objective was to determine the effectiveness of glucosamine at a lower concentration, in combination with chondroitin sulfate, in inhibiting degradation of articular cartilage. In my first experiment, I applied two separate single acute loads (15 and 30 MPa) to equine articular cartilage explants and compared their biological responses to cytokine stimulation with LPS. In the second experiment I determined the lower dose at

which GLN HCl, in combination with a fixed dose of CS, is effective in inhibiting in NO and PGE_2 production and MMP activity.

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CHAPTER 1

LITERATURE REVIEW

1. The Importance of OA in the Equine Industry

The equine industry is a growing industry that encompasses a diverse discipline ranging from sport horse to work horse, and has a sizeable share in the U.S. economy. The American Horse Council Foundation (Barents Group LLC, 1996) reported that there were 6.9 million horses in the U.S. in 1996; 725,000 horses were involved in racing, 1,974,000 in showing, 2,970,000 in recreation, and 1,262,000 in other activities. In addition, the equine industry directly provided 338,500 full-time jobs. The report also determined that in 1996 the horse industry produced goods and services valued at $25.3 billion, and the total contribution to the U.S. Gross Domestic Product was $112.1 billion. Lameness is a major cause of wastage in horses and adversely affects the horse industry, because one of the main factors determining a horse's value is soundness, especially in athletic horses.[1-3] Jeffcott[1] assessed the wastage in Thoroughbred race horses from conception to four years of age, and determined that lameness was the most significant factor responsible for failure to race, outweighing respiratory problems, colic, or limited racing ability. The study also indicated that in 140 Thoroughbred 2-year-olds evaluated, only 34 (23%) did not show any signs of lameness. Similar results were found in a study determining the wastage of racehorses between 1982 and 1983.[2] These authors found that the

greatest number of days lost to training was caused by lameness (68%); among 314 horses examined, 53% were lame at some period during the racing season.

OA is one of the most common causes of lameness, and is of particular concern in horses because their value is closely tied to their soundness. Lameness that results from OA is a major cause of poor performance and early retirement of equine athletes.[4-6] Table 1 lists equine events and movements associated with OA.

TABLE 1: EQUINE EVENTS AND ACTIVITIES GENERALLY ASSOCIATED WITH OA[7]

Event	Movement	Type of OA
Dressage	Increased joint flexion	Early arthritis of the tarsal joints
	Collection; extension	Fetlock arthritis (synovitis/capsulitis)
Reining	Fast spins	Early arthritis of the tarsal joints
Cutting	Lateral driving with forelimbs	Early arthritis of the tarsal joints
Roping	Hard stops and abrupt change in direction	Arthritis of the tarsal joints
Western Pleasure	Repetitive slow jogging	Early arthritis of the tarsal joints
TB and QH Racing	Fatigue	Fetlock arthritis Arthritis of the tarsal joints
SB racing	Extended and fast pacing and trotting	Fetlock arthritis Arthritis of the tarsal Joints
Barrel racing, pole bending	Speed and turning Torque and twist	Fetlock arthritis Arthritis of the inter-phalangeal joints

TB, Thoroughbred; QH, Quarter Horse; SB, Standardbred.

A survey performed at a veterinary school found that 33% of all equine patients had intra-articular lesions related to OA.[8] Tew and Hackett[9] randomly evaluated 72 equine joints at necropsy and discovered that 35% of the joints had evidence of grossly visible cartilage damage. Not only is this degenerative disease found in domestic horses, but it naturally occurs in the joints of wild horses.[10] Despite the huge economic importance of joint disease and OA in horses, our understanding of the pathophysiological mechanisms involved in joint degeneration in this species is very poor.[11] Whether OA is a single disease or is caused by several disorders with a similar final common pathway remains unclear.[12]

2. Osteoarthritis

Osteoarthritis, often referred to as degenerative joint disease (DJD) in the horse, is characterized by deterioration of articular cartilage, accompanied by changes in the bone and soft tissues of the joint. The end stage of OA results in a net loss of articular cartilage, causing pain, deformity, loss of motion, and decreased function. Horses have naturally occurring OA, which is similar to that of humans, and are often used as models to investigate the pathogenesis and treatment of OA.

Synovial joints are the joints usually associated with lameness in the horse. These joints have two major functions: 1) to enable movement, and 2) to transfer load. Synovial joints consist of the articulating surfaces of bone, covered by articular cartilage, secured by a joint capsule and ligaments, and have a cavity

containing synovial fluid. Articular cartilage is an avascular complex structure, which serves as a shock absorber for bone and has a frictionless surface bathed in synovial fluid. This tissue consists of sparsely scattered chondrocytes (cartilage cells) intermeshed within the ECM. The ECM provides cartilage with its compressive strength and is primarily composed of type II collagen and PGs. Collagen forms a fibrous network giving cartilage its tensile strength, while large hydrophilic aggregating PGs (aggrecans) hydrate the collagen network and provide the tissue with viscoelastic properties and the ability to resist mechanical compression.[13] When the joint capsule is disrupted, proteolytic enzymes are secreted into the synovial fluid and break down PGs and collagen, the main components of the ECM.[14,15] In an attempt to repair structural changes in the ECM, chondrocytes proliferate and stimulate synthesis of these components. However, over time, the metabolic activity of chondrocytes shifts toward a state where the breakdown of matrix constituents outweighs new matrix synthesis,[12,16] beginning the gradual process of ECM degeneration and thus the loss of articular cartilage. Articular cartilage degradation on the joint surface is a common feature of OA.

Subchondral bone, the bone underlying the articular cartilage, can also be affected by OA. Because it remodels rapidly, subchondral bone is responsible for changing the shape and congruity of the joint. Mechanical stimulation of subchondral bone often leads to microdamage, which may result in 1) normal remodeling; 2) excessive remodeling, leading to bone sclerosis; or 3) accumulation of microdamage, leading to gross fracture.[6] Subchondral bone

thickening is a normal response in exercising horses. However, an increase in the degree of subchondral bone sclerosis corresponds to greater degrees of generalized OA in joints (i.e. fetlock).[17] Sclerosis of the subchondral bone can lead to the development of chip fractures, focal lesions of traumatic osteochondritis dissecans, and slab fractures. Articular cartilage covering sites of subchondral bone sclerosis is predisposed to the development of OA because the cartilage is no longer supported by healthy subchondral bone.[18] Soft tissues of the joint include the intraarticular ligaments, joint capsule, menisci, and synovial membrane. Damage to intraarticular ligaments, which provide support for the joints and distribute normal surface stresses, can stimulate an inflammatory response and change the loading characteristics of the joint surface. An example of this phenomenon is shown by mechanical instability of the joint after transection of the cranial cruciate ligament. This surgical procedure produces joint laxity, loss of joint congruency, and abnormal cartilage weight-bearing forces and trauma that can directly and indirectly induce abnormal cartilage wear.[19-22] Degenerative joint disease often develops in humans following meniscal injury.[20] Increased stress across the knee joint induced by performing surgical meniscectomies stimulates OA in humans, rabbits, and guinea pigs.[20,23-25] Chronic disease of the equine joint capsule, capsulitis, can lead to formation of scar tissue and increased stiffness, leading to instability of the joint by changing its surface stresses .[6] Acute synovitis and capsulitis may cause significant clinical compromise of the joint, and also contribute to the degenerative process by the release of enzymes, inflammatory

mediators, and cytokines.[26] While the cause of acute primary synovitis has never been determined, the development of an acutely inflamed joint is prevalent in a racing and training Thoroughbreds and Standardbreds.[27]

2.1 Pivotal molecules associated with OA

Enzymatic degradation of articular cartilage precedes morphologic breakdown and plays a central role in equine joint disease.[17] Biochemical degradation is considered to represent an imbalance of the normal homeostatic processes within the matrix of the articular cartilage. This imbalance causes an inflammatory cascade, which takes control and is responsible for the majority of pain in the equine joint. Several inflammatory mediators such as MMPs, interleukins (Ils), prostaglandins, lysozyme, and free radicals have been incriminated in contributing to this cascade.

Matrix metalloproteinases are the primary class of enzymes responsible for ECM degradation, and their increased activity plays a crucial role in the progression of OA. Although all classes of proteinases may be involved in the degeneration of the ECM, the MMPs are considered to play the most pivotal role in cartilage destruction.[28] These enzymes are characterized by a requirement for Zn^2+ in their active site. Calcium is also required for the expression of full activity but does not reside in the active site. Overall, the MMPs are capable of degrading the major cartilage matrix components, such as collagen, aggrecan, link protein, and cartilage oligomeric protein.[28] This growing family of proteolytic enzymes has been divided into four main classes: collagenases (MMP-1, –8, and -13), gelatinases (MMP-2 and –9), stromelysins (MMP-3 and –10), and

membrane-type (MMP-14, -15, and –17). Matrix metalloproteinases are inhibited by a group of tissue inhibitors called tissue inhibitors of MMPs (TIMPs).

Relatively few studies have been conducted determining the activity of MMPs in equine OA. Matrix metalloproteinase activity increases in equine osteoarthritic joints, and as age increases, MMP activity decreases.[29] Specifically, MMPs -2 and –9 have been found in synovial fluid from diseased equine joints.[30-32] The activity of both of these MMPs has been found to be upregulated in normal equine cartilage and synovial fluid following stimulation with interleukin-1β (IL-1β).[33] Stimulation of cartilage explants with IL-1 also induced the synthesis of MMP-3 in young and adult horses.[34] Caron[35] found that MMP-13 expression was significantly stimulated by human recombinant IL-1 (rhIL-1), and is produced by equine chondrocytes.

Interleukin-1 is a protein secreted by stimulated cells of macrophages, and has a number of important biologic activities.[36] This protein has been implicated in the induction and augmentation of the pathologic processes involved in inflammatory joint disease. Morris[36] was the first to identify IL-1 in the equine osteoarthritic joint, and found that equine IL-1 has many of the characteristics of IL-1 isolated from other species. Interleukin-1 stimulates chondrocytes and synovial cells to release enhanced amounts of PGE_2, PGs, MMPs, such as collagenases and stromelysin, and increases NO production.[17,37-39] Stimulation by IL-1 creates an inflammatory response that is similar to naturally occurring OA. As a result, IL-1 is often used as a model *in vivo* to stimulate an inflammatory response in chondrocytes.

Interleukin-1 has widely been used to study the pathogenesis of OA in equine articular cartilage. Equine explants stimulated with IL-1 have demonstrated an increase in the release of GAGs from the ECM.[40-43] Decreased PG synthesis and increased MMP-3 activity have been reported in equine explants following stimulation with IL-1,[34] while recombinant human interleukin-1β (rhIL-1β) induces the expression of MMP-13 in equine chondrocytes in monolayer culture.[44] Interleukin-1 also induces IL-6 synthesis in human cartilage from normal controls, patients with OA, and patients with rheumatoid arthritis.[45] Increased PGE_2 and IL-6 concentrations were found in the synovial fluid of equine joints injected with IL-1β.[42] A subsequent study done by Simmons[22] injected rhIL-1β into the metacarpalphalangeal (MCP) joints of horses, and also found an increased concentration of IL-6.

Prostaglandins are widely distributed in the body and mediate or modulate a variety of physiologic and pathophysiologic processes in many organ systems and tissues, including the hematopoietic, cardiovascular, and reproductive systems.[46] They are believed to bind to receptors on the sensory nerve endings, promoting the discharge of impulses and consequently causing an increase in pain. [47]

Prostaglandins (primarily E group) are produced in inflamed joints and can cause a decrease in the PG content of the cartilage matrix.[12,17] Actions of PGE_2 in joints include vasodilation, enhancement of pain perception, degradation of PGs and inhibition of PG synthesis from cartilage, bone demineralization, and promotion of plasminogen activator secretion. Cyclooxygenase-2 (COX-2) is one

of the enzymes responsible for the production of PGE_2 from cell membrane phospholipids.[48] IL-1 stimulates the synthesis of PGE_2, and increased concentrations of PGE_2 in affected joints suggests a causal link of this inflammatory mediator to the pathophysiologic events of OA.[49,50] Equine synovial cells and chondrocytes increased PGE_2 production after stimulation with reIL-1β and LPS.[50,51] Exposure of equine chondrocytes to reIL-1β also resulted in enhanced expression of COX –2.[48] In addition, equine articular cartilage explants incubated with LPS[52] or IL-1[53,54] had an increase of PGE_2 released into the culture medium. In vivo, significantly higher PGE_2 production has been reported in the medium of explants originating from horses with moderate OA when compared to normal joints.[55] These researchers also saw a similar increased PGE_2 content in the synovial fluid of equine osteoarthritic joints when compared to normal joints.[56,57] Prostaglandin E_2 may also have an effect on the expression of MMP activity by inhibiting IL-1 expression through a negative feedback mechanism in articular cartilage degradation. Exogenous PGE_2 significantly reduced reIL-1β-induced expression of MMP1, MMP3, MMP13, and tissue inhibitor of MMP-1 (TIMP-1) in equine chondrocytes.[50]

Nitric oxide is another important physiologic mediator that is thought to be involved in the pathogenesis of OA. This uncharged free radical is released from various tissues and cells, and is the product of a reaction between L-arginine and oxygen. Nitric oxide has one unpaired electron and readily reacts with oxygen, superoxide radicals, or transition metals, which may generate further destructive species.[58,59] Stadler[60] first showed that articular chondrocytes have the ability to

generate large amounts of NO. Nitric oxide is a major component of the inflammatory response, and may mediate the suppression of cartilage matrix synthesis occurring in response to intraarticular cytokines.[61,62] Nitric oxide activates MMPs,[63] suppresses PG synthesis,[64,65] and induces apoptosis in human articular chondrocytes.[66] Chondrocyte cell death from NO occurs under conditions where other reactive oxygen species are generated.[67]

An increased interest to determine the extent of the effect of NO production on equine OA has developed. Although NO is generally thought to be an important mediator of the inflammatory response, it may have an anabolic function in inhibiting articular cartilage catabolism. Nitric oxide inhibited aggrecan degradation in equine explant cultures, suggesting that NO has an anticatabolic role in PG degradation.[62] These researchers suggest that this mechanism may be mediated by the regulation of aggrecanase activity. Explant cultures of equine synovial membrane and articular cartilage released significantly higher amounts of NO in cartilage explants originating from horses with OA.[55] Simmons[22] injected rhIL-1β intra-articularly into the MCP joints of 6 horses, and measured nitric oxide synthase (NOS) in the synovial fluid of injected joints 6 hours post treatment. Although intensity and extent of inflammation was significantly greater in the IL-1β exposed specimens when compared to healthy specimens, no significant increase in the inducible isoform of nitric oxide synthase (iNOS) was found between the control and the IL-1β exposed joints. Synovial cell expression of iNOS varies among species, and horses have very limited iNOS expression. These researchers propose that a longer challenge of

IL-1β alone, treatment with a combination of cytokines, or a greater challenge may be necessary to induce an effect from synovium. Increased NO synthesis occurs in chondrocytes and synoviocytes in response to LPS and IL-1 within a 48 hour incubation period.[68-70] In addition, LPS or IL-1β dramatically increase NO synthesis relative to non-stimulated controls in equine explants.[54,71] In general, the bulk of information investigating the role of NO on articular cartilage degradation has demonstrated that it has a negative effect on cartilage metabolism.

Fibronectin, a noncollagenous protein in articular cartilage that appears to be important in chondrocyte-matrix interactions and cell adhesion, is another component of cartilage affected in OA. In OA, fibronectin content is markedly increased in the altered matrix because of an increased synthesis by the chondrocytes and accumulation in the ECM;[72] however, the role of fibronectin in OA has been controversial. Fibronectin localization at sites of cartilage degeneration and fibrillation was evident in the carpal joints of both gently and strenuously exercised 2-year-old Thoroughbreds.[73] Fibronectin was released into the matrix by chondrocytes, and distribution between the two exercise groups was similar. Stimulation of bovine articular cartilage explant cultures with fibronectin fragments (Fn-f) results in enhanced release of MMP's, rapid cartilage PG degradation and loss, and decreased PG synthesis.[74-76] In addition, general protein synthesis is suppressed following Fn-f incorporation.[77] Fibronectin fragments also stimulated cytokine release in human knee cartilage explants,

resulting in an immediate peak release of tumor necrosis factor-alpha (TNF-α) and IL-1β, and an early release of IL-6.[78]

3. Causes of OA

Cartilage damage due to trauma, impact injuries, abnormal joint loading, excessive wear, or as part of an aging process can lead to changes in the composition, structure, and material properties of the tissue.[12,79,80] These changes can compromise cartilage function in the strenuous mechanical environment normally found in weight-bearing joints. Regardless of the specific cause, the initial injury is usually mechanical in nature, with an imbalance between the load applied and the tissues' capacity to withstand that load.[46] Trauma to the joint, immobilization of the joint, poor conformation, improper shoeing, and age are often preliminary factors that contribute to the onset of OA in the horse. Figure 1 identifies the factors involved in articular OA.[17]

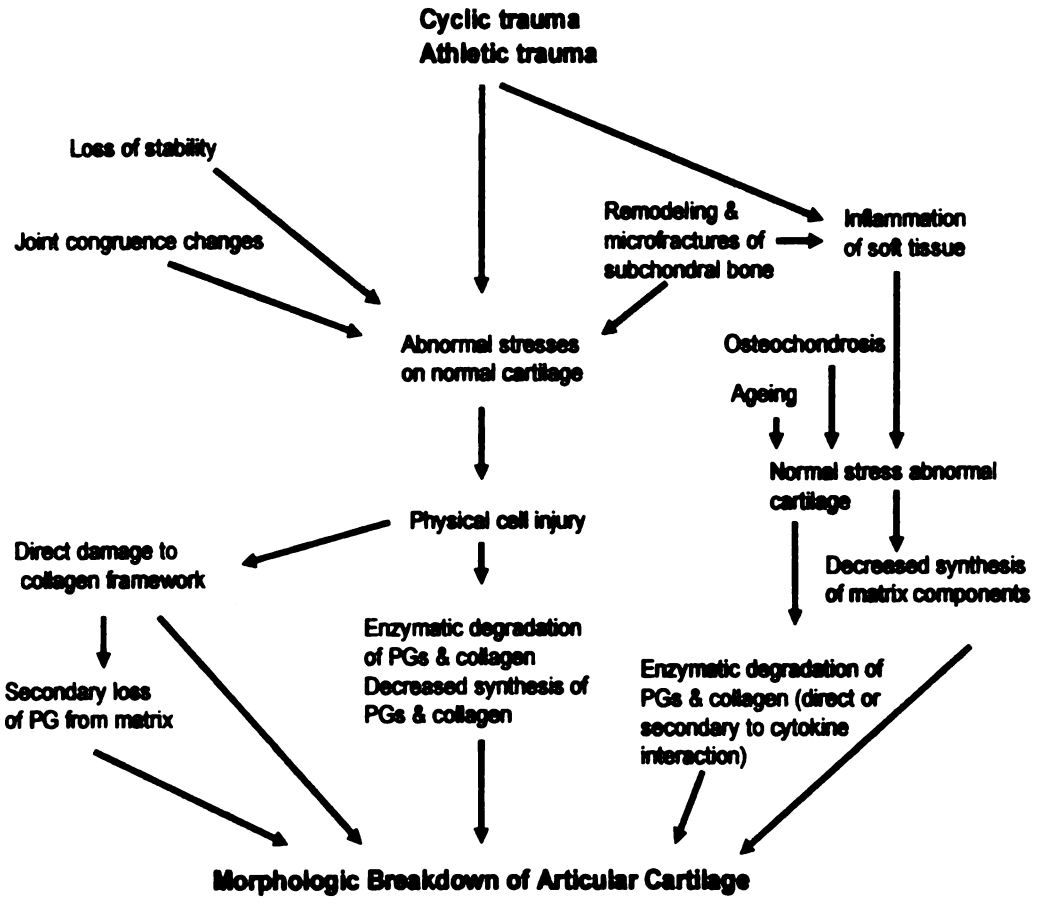

Figure 1. Factors involved in articular cartilage degradation in equine OA.[17]

3.1 Trauma

Trauma to the joint is believed to be the primary cause of OA in the horse. Mackay-Smith[4] referred to use-trauma, or trauma occurring from normal use of the joint, as a precurrent factor of OA that had been ignored in previous literature. Very strenuous exercise injures articular cartilage by increasing fibrillation of the cartilage and reducing proteoglycan content and quality. Cartilage no longer responds with improved biomechanical properties, and overload results

from such factors as extensive and intensive exercise, fatigue, speed, and poor conformation or footing.[46] For example, a racehorse's pace generates millions of foot-pounds of force per mile, and the wear and tear produced on the joints during a race can be severe.[4]

Most lameness occurs in the forelimbs, because they carry 60 to 65% of the horse's weight and are subjected to higher load rates when compared to the hindlimbs.[7,81,82] The hindlimbs propel the horse, while the forelimbs receive the shock of landing. However, this may vary among breed and performance event. Different areas of joints and joint surfaces in both the forelimb and hindlimb are subjected to different types of loading, such as low level constant loading during weight bearing, intermittent loading during locomotion, and very high and sudden loading during training or racing.[83,84] The carpal, fetlock, proximal interphalangeal, and distal intertarsal/tarsometatarsal joints are most frequently effected by OA.

The fetlock joint of the foreleg has the largest number of unique degenerative and traumatic lesions of any limb joint in racing horses.[85] Brama[86] topographically mapped contact areas and pressure distributions on the proximal articular surface (PAS) of the proximal phalanx (P1) under various clinically relevant loading conditions in the forelimbs of 13 horses. These authors found that certain areas of the PAS of the P1 are permanently loaded in the standing horse, and as the load was increased to mimic the walk or trot, the contact area enlarged in the dorsal, dorsolateral and dorsomedial direction. The joint pressures in the continuously loaded central area of the equine fetlock joint are

relatively low in the standing horse, but may increase up to 6-fold when loads are applied that can be expected during athletic performance.

Articular cartilage degeneration of the dorsal joint margins of the carpal bones in race horses may be the direct result of trauma.[85] Repetitive exercise may induce the replacement of normal subchondral bone by sclerotic bone, therefore contributing to the pathogenesis of OA. Research into the effects of exercise on PG metabolism in the carpal joints has produced conflicting results. Palmer[87] assessed the relevance of site and the influence of exercise on articular cartilage PG synthesis and metabolism on third carpal articular cartilage in 16 horses. PG synthesis was increased in exercised horses relative to nonexercised horses at the end of a 6-week period. However, the increase in newly synthesized PG was not reflected in endogenous PG within the matrix at different sites on the third carpal bone. A significant correlation of site on endogenous PG was evident, with a greater concentration of PG located in the palmar aspect of the radial facet compared to the sites located on the dorsal aspect of the radial facet or all sites on the intermediate facet. Total PG content on sites of the middle carpal joint (MCJ) increased in untrained Thoroughbreds with short-term exercise.[88] Proteoglycan content was greater at palmar sites overall, and dorsal sites of the high-intensity trained group had 12% higher PG when compared to those of the low-intensity trained group. A contradictory study to those previously described evaluated the effect of strenuous versus moderate exercise on the metabolism of PGs in the articular cartilage from different weight-bearing regions in the equine third carpal bone.[11] PG synthesis was reduced in

both exercise groups, and greater PG loss was found in the different joint regions of the strenuously trained animals. No change in PG size or ability to aggregate in different regions of any articular cartilage site was found in this study.

Low-motion joints such as the proximal interphalangeal, distal intertarsal, and tarsometatarsal joints are vulnerable to the development of OA, because they have a relatively smaller area of joint surface that must sustain the same weight-bearing load for a relatively longer period of time during joint movement.[85] Both ring-bone and bone spavin can produce crippling lameness in horses. Although the etiopathogenesis of ring-bone and bone spavin is undetermined, the cause is probably trauma to the periarticular soft tissues including the joint capsule insertions and periosteum.[89]

Ring-bone is a term used to describe DJD of the proximal and distal interphalangeal joint. This disorder most commonly occurs in horses forced to make quick turns and abrupt stops, such as western performance horses, polo ponies, and jumpers.[90] Ellis and Greenwood[91] evaluated six cases of ring-bone in young Thoroughbreds ranging from the age of three months to four years. All cases except one had other pre-existing or concurrent bone disease, which could have consequently placed abnormal weight on the interphalangeal joint resulting in DJD. Ring-bone has been the most serious cause of wasting in Norwegian Döle horses.[92]

Bone spavin is the most common cause of hindlimb lameness of athletic horses, and involves the distal intertarsal, tarsometatarsal, and occasionally the proximal intertarsal joints.[7,93-95] This degenerative disorder has been found in a

variety of breeds including Quarter Horses, Thoroughbreds, Standardbreds, and Icelandic horses. Wyn-Jones and May[93] treated 30 horses and ponies for lameness due to bone spavin, and found that 25 of the 30 horses were lame in both hindlegs and lameness varied from slight to severe. Twenty-three percent of Icelandic horses radiographically evaluated (379 total) had signs of bone spavin, suggesting a predispostion to the disease.[96]

3.2 Immobilization

Reduced loading or immobilization, due to lack of exercise, can lead to atrophy or degeneration of articular cartilage. While excessive forces may lead to articular cartilage loss, removal of all mechanical stimulation leads to atrophy. When cartilage is subjected to high-pressure loads, PGs are compressed and water is expressed from the cartilage.[46] Cartilage then expands as it is rehydrated upon alleviation of pressure. Physiologic loading and motion are therefore essential to maintain the normal nutrition and metabolism of articular cartilage provided by exchange with synovial fluid.

Although several immobilization studies have been conducted, few have been done on horses. An early study investigating changes in the metabolism of proteoglycans in immobilized limbs of sheep, showed a decrease in glycosaminoglycan (GAG) content of the non-loadbearing joint.[97] Proteoglycans isolated from the immobilized limb were smaller than those isolated from load-bearing joints. Instability of the MCP joint was surgically performed in six horses by transecting the collateral and lateral sesamoidean ligaments.[22] This procedure induced OA in all horses, which resulted in lameness, increased joint

circumference, decreased joint range of motion, and increased new synthesis of PG production. Horses immobilized with fiberglass casts from the proximal portion of the metacarpus down to the hoof tended to have lower hexosamine concentrations in articular cartilage biopsied from their casted joints.[98] The contralateral limbs of each horse served as a mobilized control and the control articular cartilage tended to gain hexosamine during the 30-day trial. These researchers saw little change in the GAG synthesis of the casted joints, while the largest significant change occurred in the control. Similar results have been found in the rabbit.[99] Thus, contralateral limbs are unsuitable for controls in immobilization studies because of their biological response to increased weight bearing. Palmer[87] found a lower concentration of newly synthesized PGs in nonexercised horses when compared to exercised horses. Exercised horses had a noticeable increase in the early PG peak of newly synthesized PGs, while this did not occur in the sites of the non-exercised group. Immobilization studies performed with canine and rabbit limbs have indicated a depletion of PGs, defective aggregation of PGs, and accumulation of water in the tissue.[99-101] These problems may be reversed after remobilization.

3.3 Conformation

Conformation is defined by the physical appearance and outline of a horse, which is dictated primarily by bone and muscle structures. Certain conformational traits can predispose the horse to lameness. Conformation defects, such as "calf knees", "knocked knees" (carpus valgus), "bowed knees" (carpus varus), or "bench knees", cause the animal to load its carpus abnormally,

and OA can result.[102] In the rear legs, horses that are extremely straight in angulation of the stifle and hock or are obviously sickle- or cow-hocked are predisposed to conformationally induced lameness.[103] Certain breeds' characteristic conformation magnifies their risk of developing OA. Icelandic horses with sickle hocks had a prevalence of radiographic signs of bone spavin of 42%, which was significantly higher than that of horses with straight (20%) or normal (19%) conformation.[96] In addition, the prevalence of bone spavin was found in 19% of horses with a light skeletal type, whereas lesions were identified in 23% of those with intermediate and in 24% of those with heavy skeletal type. A more recent study confirmed this finding, and indicated that the prevalence of radiographic signs of DJD in the distal tarsus of Icelandic horses increased in horses with a smaller tarsal angle.[104] Upright pasterns, base narrow front limbs, and a rectangular shaped P1 in the Norwegian Döle horse are conformation defects that contribute to the development of ring-bone.[92] Quarter Horses are often more prone to OA because they have a relatively large body mass, poor carpal conformation, small feet, and short upright pasterns.[105]

3.4 Shoeing

Since the hoof capsule is malleable, the manner in which it is trimmed and shod can have a marked effect on performance and soundness of the equine athlete. The hoof of the horse must be balanced to absorb high impact vibrations when it is exposed to the repetitive trauma incurred during performance events and normal use. Maximum energy dissipation depends on proper hoof

preparation and shoeing.[84] Good shoeing is an art and maintenance of the natural angle and balance of the hoof is critical.

Improper shoeing can change the limb configuration of the horse, resulting in a modification of the forces placed on the joint surfaces.[106] Increased abnormal wear and loading on the joint surface due to improper shoeing can contribute to degeneration of articular cartilage. The typical long toe/low heel conformation commonly seen in Thoroughbred racehorses can accentuate hyperextension type injuries in the fetlock and carpus and cause direct injury to the foot in the form of OA in the distal interphalangeal (DIP) joint.[107]

Corrective trimming and shoeing alters the hoof shape or angle to affect stance or stride and breakover in order to help the horse achieve a more normal movement. Altered foot orientation, which could result from trimming and shoeing, influences intra-articular pressure in the articular contact area of the DIP.[108] When a hoof is being actively re-formed, the change in shape during one trimming may be dramatic. Types of shoes and shoeing devices can alter the traction of the hoof. For instance, sliding plates and wide web shoes are often used on reining horses. These types of shoes provide inadequate traction for the horse, and can result in strained tendons or sprained ligaments. Traction devices, such as toe grabs, heel calks, and borium can provide too much traction. Excess torque on the limb and joints resulting from using these devices can lead to strain or sprain and may contribute to the development of OA. Horses shod with hoof caulks had altered joint angles, which could change the forces placed on the joint surfaces, or the soft tissue structures in the lower

limb.[106] A study evaluating the effects of shoeing horses with wedges (angle 3.7 and 5°) showed that an increased elevation of the heel delayed unloading of the heel and an increased elevation of the toe advanced unloading.[109] These results suggested that the horse is unable to compensate for an acute foot imbalance by redistributing the load under the foot. Increased joint pressure has been implicated in the progression of OA.[110] An *in vitro* study evaluating the intra-articular pressure in the DIP showed that elevating the heels by 5° significantly increased DIP pressure.[108]

3.5 Age

Advancing age is the most significant risk factor for OA in humans.[111] In horses, however, OA is known to develop in animals as young as two years of age. Young performance horses are most likely to develop OA early in life, because of the emphasis on racing and showing young horses in futurities and other events. Training horses at a young age may precipitate damage to joints unable to withstand the extreme forces they are subjected to during training and competition.[102] Racing and training may accelerate the naturally occurring age-related changes. In addition, some horses may be genetically predisposed to developing OA due to either age or training, while other horses may never be prone to the disease.[10]

Pathologic and arthroscopic examinations have shown that OA is commonly observed in the joints of older horse.[7,10,85,112] Naturally occurring OA also becomes more severe with age in untrained wild horses.[10] Increased severity of lesions is correlated with subchondral bone sclerosis and ossicles with

increasing age. Age is also a significant cause for the prevalence of OA in Icelandic Horses.[96,113] Many studies have described surgical treatment of horses diagnosed with OA ranging from the age of one year up to the age of 21.

Similar to humans, as the horse ages, the biochemical properties of articular cartilage change. Several recent studies have investigated the effect of age on the biochemical characteristics of equine articular cartilage. Variations in biochemical characteristics of cartilage in relation to site and age showed no significant change in cartilage collagen between horses ranging from age 4 to 30 years old, but indicated that nonenzymatic crosslinking was higher in older horses and was linearly related to age.[114] A steady increase in pentosidine crosslinking increased with age from 5 years onward resulting in a 10-fold increase up to the age of 30 years. Crosslinking of articular cartilage by nonenzymatic glycation (NEG) is expected to result in stiffer, more brittle tissue that is more vulnerable to damage by mechanical loading. Nonenzymatic crosslinking during aging may predispose older horses to development of OA.

The biochemical characteristics of articular cartilage in mature cartilage differ from those of immature cartilage at different sites on the joint surface. No significant differences in water content and hydroxylysylpyrodinoline crosslinks were found at two different sites of the MCP joint in neonatal, 5-month-old, and 1-year old horses.[83] However, differences in DNA, GAG, collagen and hydroxylysine content between sites paralleled those shown in the mature horse.[114] In a more recent study, the same researchers investigated the influences of age and exercise on the biochemical characteristics of articular

cartilage.[115] Neonatal foals showed no site specific biochemical heterogeneity in contrast to mature horses. The process of formation of site differences was almost completed in exercised foals at age 5 months, but was delayed in those deprived of exercise. These researchers concluded that the functional adaptation of articular cartilage to mechanical loading occurs during the first 5 months postpartum, and that a certain amount of exercise is required to sustain this adaptation. Joints of horses less than two years of age had significantly higher cell numbers, total collagen, and DNA content, and lower PG content relative to mature horses ranging in age from 2 to 20 years old.[116] No significant difference in these measurements was found within the mature age groups. Another study has reported no significant difference in collagen or GAG content in cartilage derived from horses 2 to 5 years of age.[117]

Two specific GAGs, which are the main GAGs found in aggrecan, have been linked to age-related changes in equine articular cartilage. Chondroitin sulfate, the most abundant GAG in aggrecan, and keratan sulfate (KS), the most widely distributed GAG in aggrecan, have both been reported to change with age.

The sulfation patterns in CS chains affect specific properties and functions of these molecules. Cartilage degeneration in the MCP joints of racehorses was accompanied by deposition of CS chains with altered sulfation patterns.[118] Six-sulfation of internal and terminal CS residues increased with age. The same phenomenon has also been reported in human studies.[119]

High KS concentrations were reported in foals from 1 week after birth to 3 months of age.[120] These values decreased rapidly from 3 to 5 months, and gradually reached adult values between the ages of 5 to 18 months. This pattern also has been reported in children[121] and puppies.[122] Todhunter[123] had a similar finding, and reported a significant relationship between age of foals and plasma KS concentration. Mean plasma KS concentration peaked when foals were 10 weeks old. Age affected KS concentration in the synovial fuid of 32 clinically normal horses. However, no significant effect of age on plasma KS concentration was seen in normal adult horses with a mean age of 65 months. An earlier study also reported no age-related changes in synovial fluid KS concentrations in mature horses ranging in age from 8 to 30 years old.[124]

4. Diagnosis

The clinical signs of OA in the horse vary with the type and degree of OA as well as with the amount of acute inflammation.[125] The ultimate goal in properly treating horses with joint disease is to make an accurate and early diagnosis, institute appropriate treatment, and prevent ongoing deterioration of joint tissues.[17]

Clinically, OA is characterized by pain and disfunction of the joint. There have been various interpretations of OA in the horse; as a result, it has been found necessary to divide equine OA into 3 different classifications.[17] Table 2 describes the classifications of OA in the horse. Originally, McIlwraith[125] classified OA into 5 different types; however, this author later found it more

appropriate to combine 2 of the original types while eliminating another. Type 1 OA typically affects young racehorses in highly mobile joints (carpal or MCP), and also involves high-load-low-motion joints such as the interphalangeal and intertarsal joints in both mature and young horses (ring-bone and bone spavin). Cases that develop secondarily to primary joint problems have been classified under Type 2 OA.

TABLE 2: CLASSIFICATION OF OSTEOARTHRITIS IN THE HORSE[17]

Type 1	Associated with synovitis and capsulitis (common in carpus, fetlock, and distal tarsal and distal interphalangeal joints)
Type 2	Associated with (and usually secondary to) other identified injuries or problems including intra-articular fractures, traumatic articular cartilage ligamentous injuries, osteochondritis, subchondral bone injury and disease, subchondral cystic lesions, septic arthritis, and fragmentation of distal patella
Type 3	Incidental or nonprogressive articular cartilage erosion

This includes intraarticular fractures, unresolved osteochondrosis, tarsal bone collapse, distal palmar metacarpal lesions, and septic arthritis.[17] Type 1 and 2 can overlap. Type 3 OA may be recognized during routine necropsies, and includes a series of changes in the cartilage that are of questionable clinical significance. These changes do not always correlate with clinical problems, and

can be associated with degeneration of the articular cartilage with age or trauma to the joint, which results from continued use.

Clinical signs of OA in high-motion joints with acute inflammation include lameness, heat, swelling of the joint, pain on flexion, and decreased motion. Pain is detected either as a painful withdrawal response on joint palpatation or joint flexion, or as lameness when the horse is jogged in-hand or exercised. Horses affected with OA usually show greater lameness when worked on a hard surface or in circles, and often elicit pain upon flexion and rotation of the digits. However, even in clinically affected joints, a relationship between the amount of lameness and the cartilage degeneration is not easily established.[126] Joint enlargement, which is associated with fibrous tissue deposition or bone enlargement, also occurs in more chronic cases of OA, but acute inflammatory signs may persist to various degrees. The most prominent signs of OA in low motion joints are joint enlargement and exacerbation of the lameness with flexion.[7]

4.1 Imaging

An increased understanding of clinical findings, appreciation of diagnostic local anaesthesia and improvements in technology have facilitated more accurate and earlier diagnosis of OA in the horse. Imaging plays a major role in the diagnosis, and is a critical diagnostic tool for identifying the cause, location, extent, and severity of lesions associated with OA. Radiography was the earliest and is currently the most widely used diagnostic imaging modality for detection of equine OA.[127] There have been several reports on the incidence of degenerative

joint disease of the tarsal,[93,128,129] carpal,[130,131] and pastern joints[132-134] as assessed radiographically. Radiologic signs of OA include a narrowed joint space, widened joint space (when there is destruction of the subchondral bone plate), periarticular osteophytes, and soft tissue swelling. For most lamenesses, the traditional methods of careful examination, selective local anesthesia, and radiography provide sufficient diagnostic information for prognostic and treatment purposes.[126] Unfortunately, a significant group of horses develop an obscure, mild to moderate impairment of locomotory performance for which the traditional methods of examination are unreliable. A poor correlation between the radiographic and arthroscopic assessment of degenerative joint disease has been reported.[130,131,135] Therefore, combining a number of newer imaging methods with conventional radiography may greatly improve diagnostic accuracy and provide considerable insight into the pathogenesis of OA.[126,135,136]

Ultrasound (US), nuclear medicine (NM), thermography, computed radiology (CR) and tomography (CT), and magnetic resonance imaging (MRI) offer the potential for a more accurate and detailed diagnosis of OA. Choosing a particular imaging procedure requires not only an understanding of the relative strengths of available techniques, but also an appreciation of the clinical criteria needed to chose the appropriate imaging technique for a particular case most efficiently.[126]

Ultrasound is a useful noninvasive imaging modality that provides images of periarticular soft tissue structures, such as the joint capsule and articular surface. It has been used extensively in the evaluation of equine lameness. A

major advantage that US has over other imaging procedures is its real time dynamic capability.[137] Comparison views can be taken immediately, and, if necessary, treatment decisions can be made without delay.

The field of equine NM is developing rapidly.[138] This technique involves administering a radioactively labeled substance to a patient, and quantifying the emitted radiation outside of the body by a scintillation (or gamma) camera. The resulting image is a record of the differential uptake of labeled substance in various parts of the skeleton including soft tissue.

Thermography is an imaging modality that uses infrared radiation emitted from the skin to produce a visual image. The skin surface temperature reflects changes in circulation and deeper tissues such as those resulting from synovitis. Joint inflammation produces characteristic thermal patterns and studies are underway to attempt to correlate the inflammatory response with the gross damage to the joint.[139] Thermography may be able to locate inflammation before clinical signs are evident, and as a result, training programs can be changed to reduce stress on the inflamed area.

Computed radiography produces a radiographic image from digital information similar to that of conventional radiography, while CT uses digital information to provide an image in the third dimension. An advantage of CR is the ability to alter the imaging algorithm and produced improved edge enhancement and wide contrast latitude.[140] Computed tomography scanning has excellent contrast resolution and demonstrates subtle differences in bone or soft-

tissue density displayed in cross-section, which cannot be attained by radiography.[126,141]

Magnetic resonance imaging is a noninvasive effective technique that provides very good soft tissue and intraarticular information, information which is presently only available using arthroscopy.[7,136,140,141] Magnetic resonance images are proton images, mainly of hydrogen nuclei, with high contrast and spatial resolution. This technique provides exceptionally good anatomic, pathoanatomic, and pathophysiologic information of intraarticular and periarticular structures. Both bone and soft tissues can be imaged with high contrast and sections can be made in any plane.[141] The development of this technique for use in equine limbs is required in order to validate its cost and use in providing a beneficial technique for clinically defining OA.[7,126,136,140,142,143]

4.2 Arthroscopy

Development of equine arthroscopy in the late 1970s was one of the major revolutions in equine surgery, and has been referred to as the 'gold standard' for detecting articular derangement.[136,144] Arthroscopy is a useful diagnostic technique that, when used in conjunction with traditional diagnostic methods enables the examiner to make a more accurate and detailed diagnosis of OA.

The acceptance of arthroscopy as a primary method of treatment of articular disease in the horse has resulted from its use in removing osteochondral fragments and the treatment of articular lesions via minimally invasive arthroscopic portals. This technique enables evaluation of the nonosseous

tissues of the joint, including synovial membrane and associated villi, articular cartilage, intraarticular ligaments, and menisci. In addition, the use of the arthroscope in the horse is used to make an assessment of articular cartilage when radiographic signs are equivocal or nonexistent.[145,146] The principal advantages of arthroscopy as a diagnostic surgical tool are: 1) the greatly increased accuracy with which a joint can be evaluated, and 2) all types of surgical manipulations can be performed through the small stab incisions with decreased postoperative morbidity, decreased convalescence time and improved performance.[147]

Diagnostic arthroscopy is a relatively atraumatic technique and can be used on multiple joints during a single period of anesthesia. Initially, arthroscopy was restricted to the carpus and fetlock joints, but in later years, it has been used to investigate and treat the shoulder, elbow, DIP (coffin joint), hock, and stifle joints.[143,148]

The arthroscopic procedure consists of introducing an arthroscope into the fibrous joint capsule through a cannula placed in a 5 mm skin incision.[149] Interior examination of the joint is performed using the arthroscope and the aid of light source, lens, and video technology. Introducing the arthroscope into the proper site is critical, because if it is inserted in the wrong area, its maneuverability can become limited and the arthroscope or the instruments may be placed through sites or structures of undesirable penetration.

4.3 Synovial Fluid

Synovial fluid analysis in combination with clinical and radiologic examinations is a valuable tool in determining the cause and probable duration of OA in the horse.[7,150-152] Although analysis of the synovial fluid alone will not render a specific diagnosis, it can give you an indication of the degree of synovitis and metabolic derangement within the joint.

Synovial fluid has an essential role in the nutrition and lubrication of articular cartilage.[153] This protein-containing dialysate of blood plasma has hyaluronan (HA) widely distributed throughout it, which acts as a boundary lubricant of the synovial membrane and gives synovial fluid its viscosity.[125,151,154] Alterations in the concentration of HA in the synovia may indicate a functional abnormality of the synovial membrane. Hyaluronan content in horses with OA is similar to values found in normal synovial fluid; however, animals with septic or infectious arthritis have a reduced hyaluronan concentration.[151,154,155]

Routine examination of synovial fluid should also include determination of appearance, volume, clot formation, sugar content, microscopic character of the sediment, and cytologic properties.[151] Normal synovial fluid in the horse is pale yellow, clear, and free of flocculent material. Horses with OA have clear to pale yellow and opaque colored fluid in conjunction with signs of flocculent material in the fluid sample. Synovitis leads to increased formation and decreased absorption of synovial fluid, resulting in increased synovial fluid volume and distention of the joint capsule.[156] Total volume of aspirated synovial fluid from joints with synovitis is generally increased. Fluid volume will vary in direct

proportion to the size of the joint, and younger horses often contain a proportionally greater volume of fluid than mature horses.[151] Although normal equine synovial fluid does not clot, joints affected with acute to subacute traumatic arthritis clot rapidly. Synovial fluid sugar content in horses with OA decreases with increased inflammation. In addition, cartilaginous fragments increase in fluid from joints affected with OA or traumatic arthritis.[9,151,156] A marked increase in protein is shown in acute synovitis, but this increase may only be slight in chronic OA. Increased synovial membrane permeability and altered joint metabolism are reflected by an increase in serum enzymes in the synovial fluid.[156] Enzymes generally affected are alkaline phosphatase, lactate dehydrogenase, aspartate aminotransferase, and alkaline dehydrogenase.

Additional analysis of synovial fluid includes the use of synovial fluid to assess GAG concentration, synovial fluid cytokines, and eicosanoids as markers of joint disease of the horse. Horses with OA have increased synovial fluid GAG levels when compared to normal horses.[87,157] High levels of GAGs in the synovial fluid of horses with OA presumably reflects the rate of PG loss from the articular cartilage during the disease process.[157,158] In addition, a large increase of specific GAGS such as CS may reflect alterations in the synthetic or degradative process of both the articular cartilage and synovial membrane since CS is a ground substance GAG common to both tissues.[87] Furthermore, high levels of KS indicate the rate of aggrecan degradation in articular cartilage within the joint.[159] Bertone[160] evaluated the value of various synovial fluid cytokines and eicosanoids in the diagnosis of joint disease. The results of this study

indicated that IL-6 was the most sensitive and specific for joint disease when compared other factors such as tumor necrosis factor-α (TNF-α), IL-1β, PGE_2, thromboxane$_2$ (TXB_2), prostaglandin F1-α (PGF_1-α), and leukotriene B_4 (LTB_4). Prostaglandin E_2 levels were also found to be useful as a functional screening test for the presence of joint disease.

5. Therapy

In recent years, significant advances have been made in the ability to diagnose arthropathies in the horse. Although successful treatment of established equine OA has not progressed to the same extent, a rapid advancement in knowledge of articular structure, physiology and pathology has facilitated development of effective OA therapies. Osteoarthritis is the result of a number of different pathologic processes and the choice of treatment and its effectiveness depends on the stage of the disease and the degree of active inflammation present.[112] The goals of any treatment for OA include: 1) decrease pain; 2) improve function and/or range of motion; and 3) minimize or reverse the progression of the OA process.[161] Several of the primary methods of treatment of equine OA will be discussed below.

5.1 Rest

It is well recognized that rest, limited motion and physical therapy are the most obvious and perhaps most essential contributors to the normal return to function without further cartilaginous damage to the equine joint.[27] Rest, either alone or in conjunction with other forms of therapy, has long been proposed as a

treatment for OA.[27,125,162,163] However, the economic limitations of the racing and showing industries often prevent or limit this option.

Complete joint immobilization has been shown to promote articular cartilage degeneration.[21,99,100,164] However, discontinuance of hard work is essential in the management of OA. Richardson and Clark[98] demonstrated that normal equine joints can tolerate at least 4 weeks of cast immobilization without significant irreversible change to the articular cartilage.

The extent to which rest has on the resolution of cartilage erosion or bony proliferation is uncertain.[7] Stall rest is the most common form of rest recommended in horses. The amount of stall rest depends on the degree of articular cartilage damage; however, 60 days is the minimum amount of recovery time for the repair of soft tissues and the return of a horse to training.[165] Hand walking is often recommended in conjunction with stall rest, and paddock rest following stall rest is the traditional method used before an arthritic horse is returned to active training.

5.2 Arthroscopy Surgery

Arthroscopic surgery has revolutionized equine joint surgery and has become a routine method of joint surgery for nearly all conditions, including OA.[149] Surgery consists of the removal of bone chips and debri, deep curettage of the cartilaginous defect into subchondral bone in order to encourage a good healing response from the specific region, production of a defect with vertical rather than sloping edges, and conservative smoothing and removal of new bone from the dorsal surface of the bone.[46] Although arthroscopy originally started as

a diagnostic tool, techniques for doing surgery under arthroscopic visualization developed and virtually all joint surgery is now done arthroscopically.[131]

Several studies have documented arthroscopic removal of chip fractures and cartilage fragments. Debris from osteochondral or cartilage fragments can cause mechanical damage to the articular surface in addition to initiating an inflammatory response in the affected joint.[166-168] Severe compromise of the articular surface leads to instability, as does tearing of fibrous joint capsule and ligaments.[17] Surgical removal of these fragments is a means of preserving the economic value of the horse, and may allow a rapid and successful return to performance.

Osteochondral chip fractures of the carpus and the proximal phalanx are primarily a problem in racehorses.[131,168-172] Arthroscopic removal of chip fractures decreases lameness and increases the likelihood of racehorses to resume racing. Chip fractures are considered to cause pain through tugging on synovial membrane attachment, inducing synovitis due to release of debris, and by causing damage to the apposing articular surface (kissing lesions).[173] All of these factors lead to the cyclic process of OA.

After arthroscopic surgery, 63% percent of 87 Standardbred racehorses returned to preoperative racing levels after surgical removal of osteochondral fragments of the proximal phalanx,[170] while 74% of 176 returned to racing following carpal chip fragments.[172] Houttu[174] operated on 45 horses of which 42 were Standardbred trotters. This researcher reported that 51% of the horses returned to speed training in 3 months and 91% returned to speed training in 6

months. Additionally, a study demonstrating removal of chip fractures in Standardbreds and Thoroughbreds resulted in 80% of treated horses returning to racing, and 68% of these horses raced at a level of competition equal to or better than the pre-injury level.[167]

Studies in Quarter Horses and Thoroughbreds have also shown promising results. Eighty-nine percent of Thoroughbreds raced after arthroscopic removal of fractures of the proximal phalanx and 82% did so at the same or higher class.[168] An Australian study reported that 76% of Thoroughbreds returned to racing following surgery, however, 48% failed to earn in more than $1,000 in post-operative races.[171] Carpal fracture removal in Quarter and Thoroughbred horses resulted in a 68% return to racing that was at a level equal to or better than their pre-injury level.[131] The severity of articular cartilage damage also had a detrimental effect on horses' return to racing. This study also evaluated degradation of the articular cartilage and separated damage into 4 grades, classifying grade-1 as the least severe OA and grade-4 the most severe; 71.1% of grade-1 damage, 75% of grade-2 damage, 53% of grade-3 damage, and 54% of grade-4 damage horses returned to racing at a level equal to or better than their pre-injury level.

5.3 Joint lavage and synovectomy

Two treatments associated with synovitis and septic arthritis are joint lavage and synovectomy. As noted above, cartilage breakdown products can promote or induce synovitis. Both of these treatments have potential benefits of

lowering the level of deleterious factors such as MMPs, PGE_2, cytokines, and free radicals.[112]

The pathologic changes associated with many arthroses and the accompanying mechanical destruction of the articular surfaces produce significant debris within the joint capsule.[9] Joint lavage was initially proposed to remove cartilaginous debris that caused synovitis.[125] This technique is often used in conjunction with arthroscopic surgery to remove debris from the joint following surgery.

The rational for synovectomy is to remove the inflamed, hypertrophic synovial membrane in an attempt to decrease the production and stimulation of cartilage destructive enzymes.[27] Removing the synovium is advantageous in severe inflammation, but removal of synoviocytes may be detrimental to synovial physiology and may alter and induce changes in articular chondrocyte metabolism.[175,176]

5.4 Arthrodesis

Arthrodesis is a technique that was first described for the treatment of bone spavin by Adams,[128] although May and Larsen had used a similar technique with success prior to this point.[177] At this time, numerous types of therapy to treat bone spavin had failed, and horses affected with bone spavin remained lame and resistant to all methods of therapy. Bone spavin may later result in ankylosing arthritis of the involved joint, which is a slow progress and may not establish complete immobility of the joint.[128] Treatment by surgical arthrodesis is an internal fixation of the joint that is aimed at eliminating any

motion of the joint thereby decreasing pain and lameness. The arthrodesis procedure involves removal of the articular cartilage, internal fixation with screws, or with a bone plate, followed by external immobilization with a cast for 3 to 5 weeks. Although this procedure is most commonly used to treat bone spavin,[93,94,178,179] it also is effective in the fixation of the pastern joint,[132,134] coffin joint[180,133] and carpus.[173]

Arthrodesis of the joint is often accompanied by a prolonged convalescent period that can require up to 1 year before soundness can be evaluated.[134] Success rate for horses returning to performance following treatment with surgical arthrodesis has varied. Caron[179] showed that the success rate for return to serviceability following arthrodesis of the proximal interphalangeal joint was approximately 46% for the forelimbs and 83% for the hind limbs. A more recent study of the proximal interphalangeal joint in 34 horses indicated a successful outcome in 85% of forelimbs and 89% of hindlimbs.[181] Surgical arthrodesis in 30 horses for the treatment of OA of the proximal intertarsal, distal intertarsal and tartsometatarsal joints revealed that 78% of horses treated in the distal intertarsal and tarsometatarsal and 55% in the proximal intertarsal became sound following surgery.[93] Surgery of the distal intertarsal and tarsometatarsal joints in 17 racing Standardbreds and Thoroughbreds resulted in a 71% success rate as determined by clinical examination or communication with clients.[94] All Standardbreds and 67% of Thoroughbreds in this study had successful outcomes.

Complications of arthrodesis as reviewed by McIlwraith[112] include implant breakage or loosening, bent screws, fracture of the proximal phalanx, infection, laminitis, toe elevation, DJD of the distal interphalangeal joint, and navicular abnormalities. The definition of successful outcomes also vary from study to study, and depending on the opinion of the author, certain degrees of improvement may or may not be classified as a successful outcome.

5.5 Conventional Medications

Medications used to treat joint disease can provide relief by decreasing pain, acting as anti-inflammatory agents, and contributing chondroprotection. The term chondroprotection implies the maintenance, or restoration of normal homeostatic mechanisms to protect cartilage from injury.[182] Many drugs have been termed chondroprotective by their function of either enhancing cartilage repair or retarding cartilage degradation. Non-steroidal anti-inflammatory drugs (NSAIDS), corticosteroids, HA, polysulfated glycosaminoglycans (PSGAGs), and glucosamine and chondroitin sulfate have all been used in the treatment of OA. These drugs have been extensively reviewed by McIlwraith and Trotter in Joint Disease in the Horse,[183] and recent investigations of the effects of these chondroprotective drugs will briefly be described here.

NSAIDS

Nonsteroidal anti-inflammatory drugs provide anti-inflammatory and analgesic effects to acutely inflamed joints. The mechanism by which NSAIDS exhibit their anti-inflammatory effect is through inhibition of COX.[184,185]

Cyclooxygenase is the first of a series of enzymes that convert arachidonic acid to prostaglandins, and all NSAIDs inhibit COX activity to some degree.

The most common NSAIDs approved for use in the horse include phenylbutazone (PBZ), flunixin melamine (FNX), and ketoprofen (KET).[48] Other NSAIDs that have been evaluated for their anti-inflammatory properties in the horse include carprofen (CAR), meloxicam (MEL), diclofenac, acetylsalicylic acid (ASA), naproxen, and indomethacin. Phenylbutazone has been shown to decrease PGE_2 concentration in equine articular cartilage explants and chondrocytes,[48,49] suppress PG loss,[186] and function as a more potent inhibitor of COX-1 than COX-2.[180] Carprofen was the weakest inhibitor of COX-2 when compared to PBZ, FNX, KET, diclofenac, indomethacin, and MEL in the whole blood of horses, dogs and cats, while PBZ and FLX were more potent overall in inhibiting COX-1 than COX-2.[180] This is in agreement with Tung[49] who demonstrated that PBZ had no effect on the expression of COX-2 in equine chondrocytes; however, PBZ did inhibit COX-2 activation. Phenylbutazone had no effect on inhibiting the expression of iNOS or influencing nitrite concentrations in cytokine-stimulated equine chondrocyte cultures.[187] Diclofenac, ASA, indomethacin, MEL, and naproxen profoundly differ in their ability to modulate proteolytic activities by articular chondrocytes.[37] All of these NSAIDS were effective in inhibiting collagenase activity in IL-1 stimulated bovine chondrocytes; however, only indomethacin, MEL, and naproxen reduced transcript levels of MMP-1.[28] In addition, indomethacin and MEL specifically reduced proteoglycanase activity; however, no NSAID had an effect on TIMP activity or

TIMP-1 biosynthesis. Neither PBZ or FLX have been shown to regulate MMP-2 or –9.[31]

Corticosteroids

Synthetic analogs of natural corticosteroids possess potent anti-inflammatory activity and are commonly injected intra-articularly for local relief of inflammatory lesions in performance horses.[184] However, they are also anti-catabolic, which can delay healing of injured tissue. Corticosteroids prevent inflammation through an interaction in which they bind to steroid-specific receptors in the cellular cytoplasm of steroid-responsive tissues. The corticosteroid binds to the receptor, resulting in a change in the allosteric nature of the receptor-steroid complex, and ultimately, directing the synthesis of new proteins.[185] Similar to NSAIDS, corticosteroids predominantly function by inhibiting prostaglandin production.[38,48,49] Additionally, their beneficial effects may be mediated by inhibiting cartilage MMPs, cytokines such as IL-1 and TNF-α, and by enhancing synthesis and release of TIMPs.[31,185,188,189]

Although corticosteroids seem beneficial in preventing the inflammatory response involved in OA, the deleterious effects of these drugs on articular cartilage have been questioned. Detrimental effects of intra-articular corticosteroids on articular cartilage include: 1) decreased cartilage elasticity and GAG content with progressive degeneration of the cartilage; 2) formation of calcium deposits on the hyaline surface; 3) thinning and fissuring of the cartilage, and 4) decreased viscosity and HA content of synovial fluid.[184] The doses of corticosteroids achieved in a joint after intra-articular administration are often

many orders of magnitude greater than required to produce a cellular effect in vitro.[190] This leads to the question whether inappropriately high doses of these drugs are used in joints and if previously reported deleterious articular effects of corticosteroids may, in part, be due to incorrect dosing.[185,189,190]

The best corticosteroid drug and the appropriate dose rate remain to be elucidated. Corticosteroids approved for intra-articular use in the horse include cortisol, methylprednisolone, triamcinolone acetonide, betamethisone, isoflupredone, and flumethasone.[184] Recent *in vivo* studies on corticosteroids in equine articular cartilage have focused on their effects on PGE_2 synthesis and gene expression. Betamethasone, dexamethasone, and methylprednisolone have been evaluated for their possible inhibitory effect on the activity of MMP-2 and –9 in equine synovial fibroblasts and peripheral blood neutrophils.[31] Betamethasone had no inhibitory activity on either MMP, while higher concentrations of dexamethasone and methylprednisolone significantly inhibited MMP-2 activity. In spite of this, the high doses used in this study are unlikely to be achieved *in vivo* for any length of time. High concentrations of methyprednisolone and betamethasone inhibited PG synthesis; however, synthesis was increased with a lower concentration of methylprednisolone, while it remained unchanged with a lower concentration of betamethasone.[38,189] Methylprednisolone has been shown to have similar effects on PGE_2 synthesis. Prostaglandin E_2 production was suppressed by low concentrations of methylprednisolone, while higher concentrations had no effect.[48] In addition, methyprednisolone acetate decreases type-II procollagen synthesis.[188]

Dexamethasone (DEX), another potential anti-inflammatory corticosteroid, significantly reduces PGE_2 production in equine chondrocytes.[49] It is also involved in pre-translational regulation of COX-2 and iNOS gene in equine chondrocytes.[49,187] Lower concentrations of dexamethasone have also been found to reduce transcript levels of MMP-1, decrease the expression of MMP-3, and inhibit collagenase and proteoglycanase activity.[37]

Hyaluronic Acid

Hyaluronic acid belongs to the category of local anti-inflammatory drugs. It is a weak acid, nonsulfated polysaccharide that is synthesized by special cells of the synovial layer of the joint capsule and is responsible for the high viscosity of the synovial fluid. Hyaluronate was first isolated in 1939 from bovine synovial fluid.[156] Large quantities of HA are obtained for experimental and commercial use from umbilical cords, rooster combs, and bacteria. Administration of purified HA restores the permeability barrier restricting the flow of leukocytes and active plasma components into the joint, and reduces the effusive component of the inflammation process.[191]

Intra-articular injection of HA (HYVISC®) resulted in the highest clinical improvement (87%) in horses diagnosed with OA compared to arthritic horses treated with rest, intra-articular corticosteroid injections, NSAIDs, systemic anti-inflammatory agents, physical therapy, or counterirritation.[191] Lameness was greatly improved showing 54% of HA treated horses having a lameness score of zero post-treatment, while only 1% of these horses had been diagnosed with a lameness score of zero pretreatment. A study using amphotericin B to induce

synovitis had conflicting results to the previous study, and indicated that intra-articular Hylan® had no effect on lameness grade, total nucleated cell count, total protein, or polymorphonuclear cell count in the synovial fluid.[192] However, horses injected with HA following surgically induced chip fractures to produce lameness, had a highly significant increase in weight bearing on the treated limb.[193,194] Horses with unilateral front limb lameness also showed improved bilateral weight bearing.[194] *In vitro*, IL-1 stimulated canine explants had reduced PG degradation following treatment with HA compared to untreated IL-1 explants.[195] A more recent study showed that HA had no effect on the expression of iNOS or nitrite content in cultured equine chondrocytes.[187] In addition, HA treated equine chondrocytes showed no significant effect in the reduction of the gene expression of COX-2.[49] However, high concentrations of HA inhibited PGE_2 synthesis in LPS stimulated equine synoviocytes.[196]

Hyaluronic acid has also proved to be effective in rabbit models in vivo. Rabbit knees pre-treated with HA followed by Fn-f injection had significantly less decrease in PG content compared to Fn-f injection alone.[197] Similar results were found in bovine articular cartilage explants.[198] Intraarticular hyaluronan down regulated MMP-3 and IL-1β,[199] and suppressed NO production[200] in New Zealand rabbits.

Polysulfated GAGs

Polysulfated GAG agents are a group of biosynthetic compounds composed of repeating units of hexosamine and hexuronic acid designed to re-establish normal joint conditions via their inhibition of lysosomal and MMP

enzymes, stimulation of HA synthesis, and proposed chondroprotective effect of diffusing into the articular cartilage superficial matrix and acting as a replacement GAG.[201] The routes by which PSGAGs are administered include intraarticular, intramuscular, and intravenous injection. Studies investigating the effects of PSGAGs on inhibiting articular cartilage degradation have been controversial, and their effect on chondrocyte metabolism may be explained by the lack of consistency between different experimental designs.[44,202,203]

A survey completed by equine practitioners to evaluate the perceived efficacy of PSGAG, discovered that practitioners found PSGAG to be more effective than sodium hyaluronate for the treatment of subacute degenerative joint disease.[204] In addition, practitioners found the efficacy of PSGAG for incipient and chronic forms of degenerative disease compared favorably to sodium hyaluronate. Polysulfated GAG was not proven to be chondroprotective in equine joints of ponies injected with methylprednisolone[201] or equine explants in culture obtained from mildly osteoarthritic joints.[205] However, a previous study indicated increased rates of cartilage-specific type-II collagen and CS-rich GAG synthesis following treatment with PSGAGs.[206] The varying outcomes of these studies may have been due to the use of different experimental designs to determine the efficacy of PSGAG. Prostaglandin E_2 synthesis was decreased in LPS stimulated equine synoviocytes by concentrations of PSGAG similar to those estimated to be obtainable by intra-articular injection.[196] Polysulfated GAG also reversed the concentration-related suppression of PG synthesis induced by IL-1β in equine chondrocytes.[38] In addition, keratan sulfate concentration was

found to be decreased in synovial fluid from non-exercised ponies with chymopapain induced arthritis following treatment with PSGAG.[207] This indicated that, in nonexercised joints, medication with PSGAG may have decreased either release of KS from the articular cartilage into the synovial fluid or inhibited synthesis of KS. Although intra-articular PSGAG combined with exercise in ponies with OA induced a more fibrous repair tissue than tissue from nonmedicated joints,[208] PSGAG ameliorated the soft tissue swelling and bony changes of OA in the exercised joints of ponies.[209] It also significantly reduced iNOS gene expression and nitrite concentrations in cytokine-stimulated cultures.[187] Polysulfated GAG did not, however, have any effect on inhibiting the activity of MMP-2 or –9.[210]

Glucosamine and chondroitin sulfate

The combined use of the nutraceuticals GLN and CS in the treatment of OA has become an extremely popular supplementation protocol in arthritic conditions of joints. Glucosamine is an amino-monosaccharide that is biosynthesized endogenously by animals and humans from glucose and glutamine.[211] It plays an important role in the biochemistry of cartilage as it is a fundamental molecule for the synthesis of GAGs and HA which make up the foundation of aggrecan.[212] Absorption of GLN is very high (about 90% in humans), and articular cartilage concentrates glucosamine to a greater extent than any other structural tissue.[213] Chondroitin sulfate is a long chain polymer of a repeating disaccharide unit, and is the predominant GAG found in articular cartilage. The metabolic fate of CS is complex due to the variability in molecular

weight, chain length, electrical charge distribution, location of sulfate groups, and percentage of similar disaccharide residues.[213] However, disaccharides formed specifically from the breakdown of CS have been found in plasma samples of horses following oral dosing of CS.[214,215]

Oral GLN decreases pain and improves mobility in humans with OA;[216-218] however, the use of GLN as an alternative treatment for OA requires more attention in the scientific community. Glucosamine sulfate is commonly used in the treatment of OA[219] and recently has been found to inhibit IL-1β-induced NFκB activation in human osteoarthritic chondrocytes *in vitro*.[220] Small-animal practitioners evaluated a GLN/CS product and rated it good or excellent for the treatment of OA in small animals and found the product to be safe with minimal side effects.[221] Both GLN and CS were chondroprotective in a rabbit knee instability model.[222] In addition, prior treatment with GLN and CS reduced lameness in dogs with induced synovitis.[10]

Studies performed in our laboratory have evaluated the effects of GLN HCL, GLN sulfate, n-acetylglucosamine, and CS in inhibiting cytokine induced cartilage degradation in equine explants. Glucosamine HCL and glucosamine sulfate, but not N-acetylglucosamine, prevented LPS and rhIL-1 induced cartilage degradation in equine explants.[223,224] The addition of GLN HCL prevented increased NO production, PG release, and MMP activity induced by LPS or rhIL-1 in equine explants.[224] A similar study using equine IL-1β-stimulated equine explants demonstrated that GLN HCL inhibited cartilage catabolic responses in a dose-dependent manner. Stromelysin activity was suppressed at a dose of 0.25

mg/ml, while collagenase/gelatinase activity was inhibited at 2.5 mg/ml.[69] In addition, a GLN HCL concentration of 25 mg/ml prevented IL-1β-induced increases in NO production, PGE_2 production, and PG release into the media. Glucosamine inhibited aggrecanase activity *in vitro* in rat and bovine cartilage explants.[225] An evaluation of the combined effects of GLN HCL and CS on LPS stimulated equine cartilage explants determined that a low concentration of GLN (1 mg/ml) alone was capable of decreasing NO production relative to LPS stimulated cartilage, while the addition of CS at either .25 or .5 mg/ml in combination with GLN did not further inhibit NO production.[54] Furthermore, GLN HCL inhibited PGE_2 production, whereas CS had no effect on PGE_2. The combination of GLN HCL/CS decreased MMP-9 gelatinolytic activity, but had no effect on MMP-2. Most recently, it was found that the response of bovine explants from aged animals to GLN/CS under simulated conditions of stress was significantly greater than that seen in nonstressed or young tissue.[226] *In vivo,* lameness in horses with OA was reversed after supplementation with the GLN/CS product Cosequin®.[227] Twenty-five horses with natural OA induced lameness showed significant improvement in lameness grade, flexion test, and stride length within 2 weeks following oral supplementation with Cosequin®.

The mechanism by which GIN and CS inhibit mediators of articular cartilage degradation remains unclear. It has yet to be determined whether the combination of these two molecules provides an additive or synergistic effect. Our research suggests that GLN specifically could be regulating cell signaling molecules, such as NO and PGE_2, while the combination suppresses proteolytic

activity.[54,69] It has been hypothesized from results of *in vitro* research that GLN and CS may work by stimulating the synthesis of matrix macromolecules, specifically type II collagen, aggrecan and HA; decreasing the activity of MMPs and aggrecanases; and decreasing the production of cell signaling molecules such as NO and PGE_2. However, the mechanism of action of GLN and CS *in vivo* is unknown. Furthermore, the *in vivo* pharmacological concentration and dosage of the combination of GLN and CS most effective in preventing articular cartilage degradation and maintaining cartilage metabolism remains to be elucidated.

Because GLN and CS are considered nutritional supplements, they are not subject to the same stringent requirements for quality manufacturing as are pharmaceutical products. An analysis of the GLN and CS content in oral joint supplement products revealed that only 5 out of the 11 products claiming to contain either GLN, CS, or a combination of both contained GLN only, while 6 out of 11 contained CS only.[228] The amount of GLN found in the product was different from that suggested by the label, and ranged from as low as 62.6% to 112.2% of label claims. The amount of CS was also differed from the content suggested on the product label, and ranged from 22.5% to over 155.7% of label claims. Similar results have been produced in a study analyzing 32 products for their GLN and CS content.[229] In general, both of these studies found that the least expensive products were most likely to be seriously deficient in meeting the label claim.

6. Focus of my research

The use of equine joint tissues provides a valuable *in vitro* model for investigation of OA in all species, and tissue from horses of all ages, levels of joint disease, and athletic performance are available for investigation.

Mechanical loading or trauma to a joint can initiate osteoarthritis (OA) in horses. The biochemical changes that occur following loading, which predispose the horse to OA, require further investigation. Therefore, my first objective is to determine the effect of a single acute load *in vitro* on an equine articular cartilage explant model, and compare its biological response to that of a cytokine (LPS) stimulated explant model. I will measure the following parameters to identify whether loading of equine explants results in distinct biochemical changes to those resulting from LPS stimulation: NO production, PGE_2 production, PG release, MMP-2 and -9 activity, and KS content. The lower dose at which GLN, in combination with CS, inhibits LPS-induced NO production, PGE_2 production, and MMP activity in equine explant culture is yet to be elucidated. Hence, my second objective is to determine the lower dose at which GLN and CS combined are effective in inhibiting cytokine induced OA in equine explants. I will investigate this relationship by measuring NO and PGE_2 production in conditioned media surrounding the explants, and analyzing explants for MMP-2 and -9 activity.

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CHAPTER 2

THE EFFECTS OF A SINGLE ACUTE LOAD ON AN EQUINE ARTICULAR CARTILAGE EXPLANT SYSTEM

Abstract

Trauma to a joint is a major initiator of OA. Research studying the effects of trauma on equine cartilage in vitro is limited. Thus, our aim was to evaluate the effects of mechanical loading on equine articular cartilage explants. Six mm diameter cartilage discs were extracted from the knee joints of horses. Explants were equilibrated for 2 days. At the start of the experiment, 2 groups of explants were subjected to a single load of either 15 or 30 MPa within 50 ms, while a third group was treated with LPS (10 μg/ml) to induce cartilage degradation. An additional untreated group of explants served as a control. Nitric oxide and total PG released into the media were measured on days 1 and 2, while the concentrations of PGE_2 and KS in the media and MMP activity in tissue and media were measured on day 1. Experiments were replicated 4 times with cartilage from 4 horses. LPS significantly increased NO production and PG release similar to the 30 MPa loaded treatment on day 1, had the highest NO on day 2, and increased PGE_2 production the highest over all treatments except the 30 MPa treatment. The 15 MPa treatment was lower in NO and total PG release on day 2, had lower PGE_2 production on day 1 than both the LPS and 30 MPa treatment, but had the highest KS loss of any treatment. The 30 MPa loaded treatment had the highest NO and PG release among treatments on day 1, PGE_2

production similar to LPS, and higher KS loss relative to the LPS and control treatments. Minimal changes were seen in MMP activity. We have confirmed that acute trauma does initiate a catabolic response in equine cartilage explants, and differs to some degree from cytokine stress.

Introduction

Wastage due to lameness results in a significant loss of performance and productivity in the horse industry.[1-3] Osteoarthritis is a major cause of lameness in the horse. Lameness resulting from OA is an principal cause of poor performance and early retirement of equine athletes.[4,5] The deteriorating effects of OA are frequently seen in western performance horses, racehorses, jumping horses, and other sport horses involved in highly athletic events.[4,6,7] Not only is this disease prevalent in domestic horses, but it also occurs naturally in the joints of wild horses.[8] Although OA significantly contributes to financial loss in the equine industry and remains a problem in the horse, our understanding of the pathophysiological mechanisms involved in this disease is poor.[9]

Osteoarthritis is characterized by deterioration of articular cartilage accompanied by changes in the bone and soft tissues of the joint. The end stage of OA results in a net loss of articular cartilage, causing pain, deformity, loss of motion, and decreased function. Enzymatic degradation of articular cartilage precedes morphologic breakdown and plays a central role in OA. Several

inflammatory mediators such as IL-1,[10,11] PGE_2,[12,13] NO,[14,15] and matrix MMPs[16,17] may contribute to the cascade of events leading to OA in the horse.

Although factors such as increasing age, poor conformation, and improper shoeing have been shown to contribute to the onset of OA in the horse, trauma to the joint is believed to be the primary source of equine OA.[18,19] Proteoglycans are one of the main components of the ECM of articular cartilage, and provide cartilage with its ability to withstand the compressive deformation associated with loading. The PG molecule has GAG highly sulfated disaccharide chains that attract water into the tissue for cushion and support. Very strenuous exercise injures articular cartilage by increasing fibrillation of the cartilage and reducing PG content and quality. The properties of the cartilage become altered and overload results, which may lead to the development of OA.

Within equine joints, cartilage adapts to load by altering the biochemical composition of cartilage early in life.[20-22] Topographical and exercise-related differences in fibronectin distribution,[23] GAG, collagen and DNA content, and chondrocyte density exist in the composition of equine carpal articular cartilage.[20,24] Changes in these parameters in a load bearing region may predispose this site to clinical injury such as OA. Decreased PG synthesis has been observed in the articular cartilage of strenuously exercised horses compared to that of moderately exercised horses.[25] Severe loading due to overload trauma may occur in horses that are subjected to extensive and intensive exercise or speed, experience fatigue, or have poor conformation or footing. For example, joint pressures in the continuously loaded area of the

fetlock joint are relatively low in the standing horse, but may increase up to 6-fold when loads are applied that can be expected during athletic performance.[26]

Synovitis is often present following trauma to the joint, and may be the initial sign of injury in situations of 'wear and tear'.[27] A cytokine-induced *in vivo* model, using IL-1 or LPS, targeted at stimulating an inflammatory response similar to that of synovitis in the joint, has frequently been used to study the pathogenesis of OA in the horse. Cytokine stimulation induces changes in the ECM of cartilage similar to that of OA, including PG loss, increased NO and PGE_2 release, and synthesis of MMPs. Several experimental *in vitro* studies employing an articular cartilage explant model to investigate the effect of trauma on articular cartilage have been conducted in various species; however, little research has studied the effects of trauma on equine cartilage. Mechanically loaded articular cartilage explants studies have indicated that loaded explants have altered biochemical and physical characteristics including increased cell death and matrix damage,[28] PG loss,[29,30] decreased PG synthesis, and increased NO production and swelling of the tissue.[31]

The objectives of this study were to (1) determine the effect of trauma on equine explants, and (2) compare these catabolic effects to selected indices of cartilage degradation induced by cytokine stimulation. A recent study, researching the combined effects of injurious compression and cytokine stimulation on articular cartilage explants, suggests that these two processes may induce cartilage catabolism through distinct pathways.[31] Therefore, we hypothesize that the biologic response of equine articular cartilage explants

subjected to a single acute load will differ from that of explants stimulated with LPS.

Materials and Methods

Experimental design

Articular cartilage was obtained from the antebrachio-carpal and middle carpal joints of 4 horses (1-8 years old) euthanized for reasons other than lameness. Four separate experiments were conducted using tissue from each of the 4 horses. Cartilage discs (6.0 mm in diameter) were obtained from the load bearing region of the joint and were washed for a total of 3 washes in Dulbecco's Modified Eagles Medium (DMEM): nutrient mixture F-12 (Ham) (1:1).[a] Two randomly-selected discs (approximately 40 mg of cartilage) were placed in each well of a 24 well Falcon culture plate.[b] Each well consisted of 1 ml of DMEM/F-12 (Ham) (1:1) serum free media supplemented with linoleic acid (5 µg/ml),[c] L-thyroxine (40 ng/ml),[c] insulin-transferrin-sodium selenite supplement (ITS) (1 µl/ml),[d] 50 µg ascorbic acid, 100 units/ml penicillin/streptomycin,[a] and all 20 amino acids.[c] The explants were maintained in culture in a humidified incubator with 7% CO_2 at 37°C.

Explants were equilibrated in media 2 days prior to the first of 2 treatment days. There were 6-8 wells per treatment, depending on the amount of tissue obtained from each horse, and each experiment had an equal number of wells randomly assigned to 1 of 4 treatment groups: (1) control- no impact, no LPS, (2) impact- single acute load of 15 MPa within 50 msec, (3) impact- single acute

load of 30 MPa within 50 msec, (4) LPS. An Instron (model 1331, Canton MA) was used to deliver a peak pressure of either 15 or 30 MPa to impacted explants. Briefly, specimens were placed between two highly polished stainless steel plates of the servo-hydraulic testing machine in an unconfined compression, where peak load, time to peak, and maximum displacement were recorded for each experiment. After loading, explants were washed 3 times in fresh media (10 min/wash) and were placed in pre-assigned wells of 24 well culture plates. Lipopolysaccharide-stimulated explants received 10 μg/ml of LPS on days 1 and 2 of treatment. Conditioned media were collected and replaced with new media daily and stored at 4°C until analysis.

Nitric oxide analysis- Nitric oxide (NO) was measured indirectly in the conditioned media on days 1 and 2 as described previously.[32] Nitrite (NO_2), a stable end-product of NO metabolism, was quantified using the Greiss reaction and sodium nitrite as a standard. Briefly, 75 μl conditioned medium was incubated with 75 μl 1.0% sulfanilamide, 0.1% N-1-naphthylethylenediamide dihydrochloride in 25% phosphoric acid at room temperature for 5 min. Absorbance at 540 nm was determined using the Spectromax 300 plate reader.[e] Results are expressed as nmol NO_2/well.

Proteoglycan analysis

Proteoglycan (PG) release into conditioned media was measured on days 1 and 2 as previously described[33] using a dimethylmethylene blue assay (DMB). Briefly, PG content was determined by measuring sulfated GAG content

compared to a chondroitin sulfate standard. Results are expressed as μg PG/well.

Keratan sulfate analysis

The DMB assay measures both degraded and newly synthesized PG. Thus, we also measured KS levels in the conditioned media as a specific indicator of proteoglycan degradation. KS released into conditioned media was measured on day 1 using a previously described enzyme-linked immunosorbent assay (ELISA) with an inhibition step using a monoclonal antibody[c] specific for KS.[34] Media was incubated with an anti-KS monoclonal antibody to bind the antigen to the antibody. A secondary antibody, goat anti-mouse IgG HRP-conjugated,[f] was then added to the antigen-coated plate. Color development was initiated using O-phenylenediamine[c] and stopped with 2 M sulfuric acid and read at 490 nm on a Spectromax 300 plate reader. Intensity is inversely proportional to the amount of KS antigen present in the sample. Samples are compared to a KS standard (kindly provided by the laboratory of Dr. Eugene Thonar, Rush University,Chicago, Illinois) and are expressed as μg/ml.

Cartilage extraction

Matrix metalloproteinases were extracted from articular cartilage using a modified protocol.[35] Following treatment on day 2, explants from each well were placed in a cold stainless steel mortar apparatus and snap froze with liquid nitrogen. They were then powdered immediately using a stainless steel pestle and hammer. Powdered explants were placed in microcentrifuge tubes with stir bars and 1 ml extraction buffer (50 mmol/l Tris HCl, 10 mmol/l $CaCl_2$, 2 mol/l

guanidine HCl and 0.05% Brij-35: pH 7.5) was added to 2 powdered explants from each well. Samples were stirred overnight at 4°C and then centrifuged at 18,000 g for 30 min at 4°C. Supernatant was dialysed (24 h) against assay buffer (50 mmol/l Tris HCl, 10 mmol/l $CaCl_2$, 0.2 mol/l NaCl, 0.05% Brij-35: pH 7.5) using Spectrapor 2 dialysis tubing with a 12-kd cutoff.[g] Dialysis was continued for 48 h with distilled water. The amount of protein in the extracts was determined using the Pierce Micro BCA Protein Assay[h] with bovine serum albumen as the standard. Extractions were stored at 4°C and immediately analyzed for MMP activity.

Gel zymography

Matrix metalloproteinase activity in conditioned media and tissue was detected by gel zymography. Extracts of articular cartilage samples containing 7 to 8 μg of protein (each gel received the same amount of protein) were applied without reduction to an 8% polyacrylamide gel with 1 mg/ml gelatin incorporated as the substrate. Samples were diluted with 4x sample buffer and were electrophoresed at room temperature. Conditioned media samples from day 1 of treatment were prepared for gel zymography by diluting samples 1:4 with 4x sample buffer. A gelatinase A (MMP-2) molecular weight standard served as the gelatinase control. Following electrophoresis, gels were then incubated in 2.5% (v/v) Triton X-100 for 1h and then overnight at 37°C in 50 mmol/l Tris (pH 7.5) containing 200 mmol/l NaCl, 10 mmol/l $CaCl_2$, 10 μmol/l $ZnCl_2$ and 0.02% Brij-35. The gels were stained with Coomassie Blue R250 for 1 h at room temperature

and enzyme activity was measured by scanning densitometry (Gel Doc 2000),[i] using Quantity One 4.0.1 software.

Prostaglandin E_2 immunoassay

Prostaglandin E_2 (PGE_2) was measured using a commercially available competitive enzyme immunoassay kit.[j] Indomethacin (10 μg/ml) was added to conditioned media samples after 1 day of treatment and samples were then stored at -20°C until analysis. Samples were diluted 10-fold in provided assay buffer and run in duplicate. Multiple washes were performed to remove excess conjugate and unbound sample from the plate. Substrate solution was then added to determine bound enzyme activity and the absorbance was read at 405 nm with a wavelength correction set at 590 nm. A four parameter logistic curve ranging from 39-5,000 pg/ml PGE_2 was used to determine sample concentrations. All samples from each PGE_2 microplate had acceptable total activity, nonspecific binding, maximum binding and substrate blanks.

Statistical analysis

Data for indicators of degradation were analyzed using the repeated measure option of the SAS statistical software PROC MIXED (2001).[k] Data was combined by pooling wells from all 4 horses according to treatment. Each group of treatments using tissue from one animal was considered a replicate (n=4). Experimental effects of treatment, treatment*day, and treatment*horse were assessed for the pooled treatments. Differences between effects were compared using difference of least square means and Tukey's multiple comparison

procedure. All data were presented as mean ± SEM, and statistical significance was considered at $P<0.05$ unless noted.

Results

Loaded and LPS-treated explants had higher media content of NO_2 than untreated controls on day 1, however, this difference was statistically significant for only the 30 MPa loaded specimens ($P<0.05$). In contrast, NO_2 content on day 2 was higher in the LPS and 30 MPa treatment groups than the control and 15 MPa group, but this difference was only significant for LPS treated explants ($P<.05$). The 30 MPa loaded and LPS-treated groups had higher PGE_2 production than the control and 15 MPa loaded explants ($P<0.05$) (Figure 3), whereas loading at 15 MPa did not differ from the control. Specimens from the LPS-treated group produced two fold greater PGE_2 than the 30 MPa loaded group.

30 MPa loaded specimens had the highest total PG release among treatments on day 1 ($P<0.05$) (Figure 4). In contrast, on day 2 of culture, the 30 MPa loaded and LPS-treated explants had higher PG release than the control and 15 MPa loaded groups ($P<0.05$). Loaded explants had the highest KS concentrations 24 hours post-treatment relative to the control and LPS groups ($P<0.05$) (Figure 5). Lipopolysaccharide-treated specimens did not differ in KS loss from the control group.

No differences between treatment groups were detected in MMP-2 activity in the media (Figure 6) or tissue (Figure 7). Similarly, there was no difference in MMP-9 activity in the media (Figure 8) or tissue (Figure 9).

Discussion

Under normal physiological circumstances, articular cartilage is exposed to a complex and diverse array of mechanical stresses and strains due to loading of the joint.[36] The results in this study confirm that a single acute load on equine articular cartilage explants induces catabolic effects as it had in other *in vitro* models. We applied two separate loads, a lower load of 15 MPa, and a higher load of 30 MPa to equine articular cartilage explants. Previous work in our laboratory has shown that loading at 15 MPa causes slight fissuring and cell death in the superficial tangential zone of articular cartilage.[37] The macroscopic damage seen following loading at 15 MPa is similar to the damage observed over time in *in vivo* trauma models. Loading at 30 MPa causes more extensive fissuring in the superficial layer and cell death up to 40% throughout the superficial tangential and middle zone of cartilage.[38] Stimulating equine cartilage explants with 10 µg/ml of LPS induces a catabolic response in cartilage that includes increased NO and PGE_2 production, increased KS loss, and an up-regulation of MMPs.[39,40]

Nitric oxide is a major component of the inflammatory response, possibly mediating pro-inflammatory cytokines.[15] Nitric oxide activates MMPs,[41] suppresses PG synthesis,[42,43] and induces apoptosis in human articular

chondrocytes. *In vitro*, equine articular cartilage chondrocytes[44] and explants[39,40] produce significant amounts of NO following stimulation of LPS. In several studies, NO production was also significantly increased in chondrocytes following mechanically loading. Bovine chondrocytes exposed to dynamic compressive strain had decreased NO_2 production in response to increasing load, while LPS stimulated chondrocytes had increased production 2.5-fold greater than the lowest compressive strain.[45] Bovine chondrocytes subjected to fluid-induced shear force also had a five-fold increase in NO release relative to a non-treated control.[46] This study demonstrated a decrease in GAG synthesis in response to shear stress was blocked by NO synthase inhibitors. A similar study showed a four fold increase in NO release in human chondrocytes subjected to the same type of force.[47] Both static and intermittent compression induced an up-regulation of NO production, while only intermittent compression increased nitric oxide synthase (NOS), the enzyme that catalyzes the reaction producing NO.[48] Mechanical compression of articular cartilage increases NO synthesis in a manner dependent on the magnitude of stress.[45,46,49] This proved to be true both days post-impact, 15 MPa specimens consistently had less production of NO compared to the 30 MPa treatment group. Loading at 30 MPa was not different from LPS on day 1; however, LPS was greater than both loading groups by almost two-fold on day 2. Our results agree with previous studies that suggest that mechanical loading influences and increases NO production. Different types of loading, the duration of loading, and imposed strain likely elicit different levels of NO production. In this study, a single acute load of 15 MPa was not different

from LPS on day 1, but was distinctly different from cytokine-stimulated explants on day 2. In general, LPS-stimulated NO response peaks on day 2 in our equine explant cultures, thus the response elicited in LPS on day 1 may have not been great enough to differ from loading at 15 MPa. On the other hand, loading at 30 MPa had the highest NO response on day 1, and continued to be elevated similar to LPS on day 2. The trauma incurred by loading at higher loads such as 30 MPa, which includes increased cell death, does induce a higher level of NO production. Thus, a single acute traumatic load may cause an increase in NO production that is similar to cytokine induction, whereas loading at a lower load may not induce the cell signaling necessary to stimulate significant NO production.

Lipopolysaccharide and 30 MPa treated explants had the highest PGE_2 production overall. Dramatically increased production of PGE_2 has been demonstrated in equine chondrocytes[12,50] and explant cultures[39,40,51] following stimulation with LPS. This trend was demonstrated in 3 out of the 4 horses used in our study; however, due primarily to an increased response of PGE_2 production in the 30 MPa loaded group in one horse, this group was not different from LPS. Similar to NO production on day 1, PGE_2 production was greater in the 30 MPa and LPS groups compared to the control and 15 MPa groups. Nitric oxide can inhibit or stimulate PGE_2 production due to interactions between NO and prostanoid production.[52] Mechanical compression of articular cartilage does increase PGE_2 production through a NO-dependent pathway.[36] Nitric oxide may have a role in intracellular signaling induced PGE_2 production in chondrocytes.[53]

The similarity in our NO and PGE_2 data supports this proposed mechanism. Little work has investigated the effect of loading on PGE_2 production; however, fluid-induced shear force was reported to induce a 10-20-fold increase in PGE_2 production relative to a non-loaded control in bovine[54] and human[47] chondrocytes. Our PGE_2 data could suggest that similar to the NO response, PGE_2 may be strain dependent. The up-regulation of PGE_2 in acute loaded explants in this study indicates that mechanically induced PGE_2 production potentially may play a role in the physiological or pathophysiological regulation of chondrocyte metabolism.

Glycosaminoglycan (GAG) release[28-31] PG synthesis are increased and decreased respectively in loaded bovine explants.[46] Our results indicating increased PG release from loaded explants, measured by two different methods, is consistent with results from loading bovine explants similarly.[55] The slight differences between the results of these two methods could be due to the fact that the DMB assay measures total PG release, both synthesized and degraded, while KS is a measurement of PG degradation or synthesis without aggregation. D'Lima[50] found that after 48 hours of culture, explants receiving a load of 14 or 23 MPa had increased PG loss relative to unloaded explants or those receiving a load of 7 MPa. Additionally, these loads showed higher cellular apoptosis relative to all treatments. Cell viability experiments conducted in our lab (results not published) have detected very little cell death in LPS-stimulated explants. A single acute load on explants caused increased PG turnover, as indicated by an up-regulation of total PG and KS loss, which may be caused by mechanical

deformation of the matrix versus the regulation of catabolic mediators as commonly seen in cytokine degradation. This study agrees with others demonstrating that mechanical loading affects PG turnover, and further indicates that loading equine explants induces greater total PG loss and degradation relative to LPS.

Matrix metalloproteinase activity measured in the tissue or media did not differ among treatments. Equine explants stimulated with LPS have increased MMP-2 and –9 activity;[39] however, LPS stimulation did not up-regulate either of the MMPs in this study. Little work has reported the effects of loading on MMP expression in cartilage explants; however, both MMP-2 and –9 have increased expression following cyclical loading at 0.1-0.5 MPa for a period of 1-16 h in bovine explants.[56] Shear stress of 1.6 MPa for 30 min to 24 h also induced MMP-9 expression in rabbit chondrocytes.[57] One study has indicated that porcine explants subjected to cyclic dynamic or static loading over a time period of 10 min did not change the expression of MMP -1,-3, -13, or 14, or the tissue inhibitors TIMP 1 and –3.[58] The few studies showing increased MMP-2 or –9 in loaded explants used different loading models than that used in our study and our method used to measure MMP activity was semi-quantitative and this may have had a direct effect on our results. An acute load may increase proteolytic activity through other enzymes such as aggrecanase, or MMP-3 or –13. Further investigations are needed to determine whether the effects of loading on matrix loss are mechanical via loosening or destabilizing the matrix, or if loading actually induces an enzymatic response through up-regulating MMPs or aggrecanase.

Elucidating the mechanism by which trauma to the joint contributes to OA in the horse is important in defining the pathogenesis of this degenerative disease. This study has provided biochemical evidence confirming that acute trauma does initiate a catabolic response in equine cartilage explants; however, the response (especially at 15 MPa) differs somewhat from LPS-stimulated explants, suggesting at least some unique cell signaling pathways. In agreement with this, a recent study reported that IL-1 caused a synergistic loss of PG from mechanically injured bovine and human cartilage.[31] During a traumatic injury *in vivo*, the synovial tissue, a significant producer of IL-1, will also likely be affected. Thus, an *in vitro* model employing the use of both mechanical loading and cytokine stimulation may be more beneficial in understanding how trauma to a joint induces a catabolic response in articular cartilage in the horse, so that more effective treatment protocols can be established.

[a]Gibco, Grand Island, New York, USA.
[b]Fisher Scientific, Pittsburgh, Pennsylvania, USA.
[c]Sigma Chemical, St Louis, Missouri, USA.
[d]RocheDiagnostics Corporation, Indianapolis, Indiana, USA.
[e]Molecular Devices, Sunnyvale, California, U.S.A.
[f]ICN Pharmaceuticals Inc, Costa Mesa, California, USA.
[g]Spectrum Medical industries, Los Angeles, California, USA.
[h]Pierce, Rockford, Illinois, USA.
[i]BioRad, Hercules, California, USA.
[j]R&D Systems, Minneapolis, Minnesota, USA.
[k]SAS Institute, Inc., Cary, North Carolina, USA.

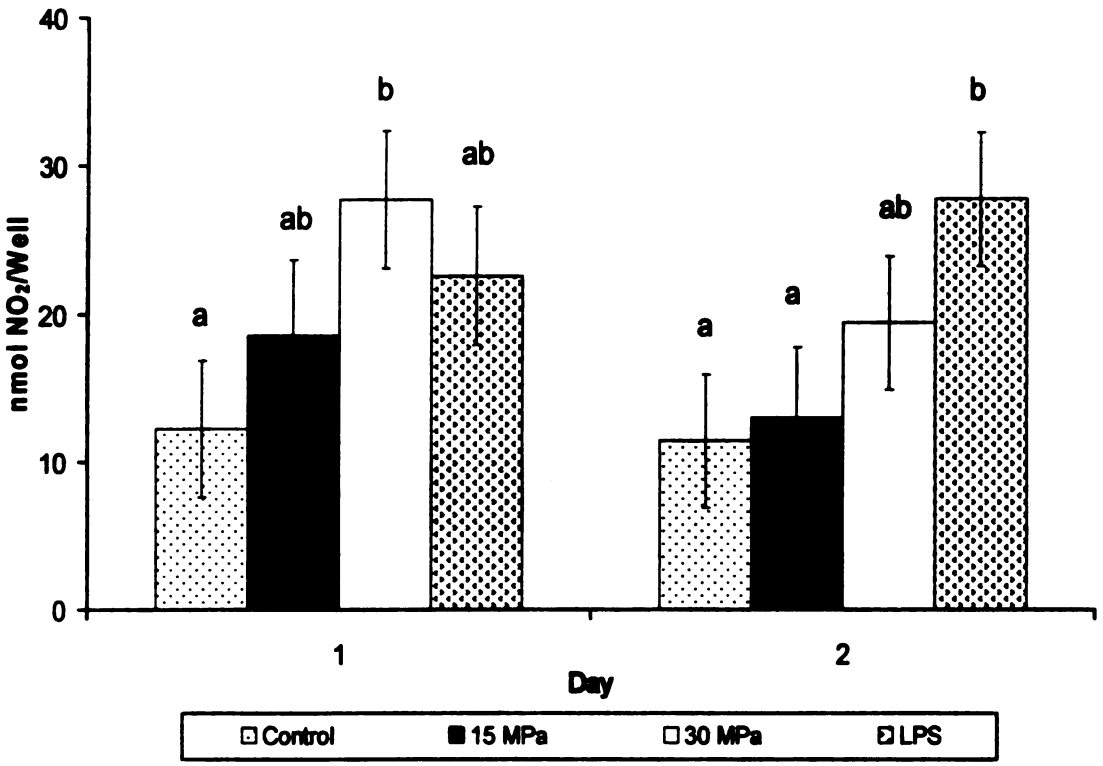

Figure 2. Mean nitric oxide (NO_2) (± SEM) released into the media per well each day post-treatment for control, loading at 15 MPa (15 MPa), loading at 30 MPa (30 MPa), and lipopolysaccharide (LPS). NO_2 concentration was quantified using an assay employing the Greiss reaction. Different superscripts indicate significant differences ($P<0.05$) between treatment groups.

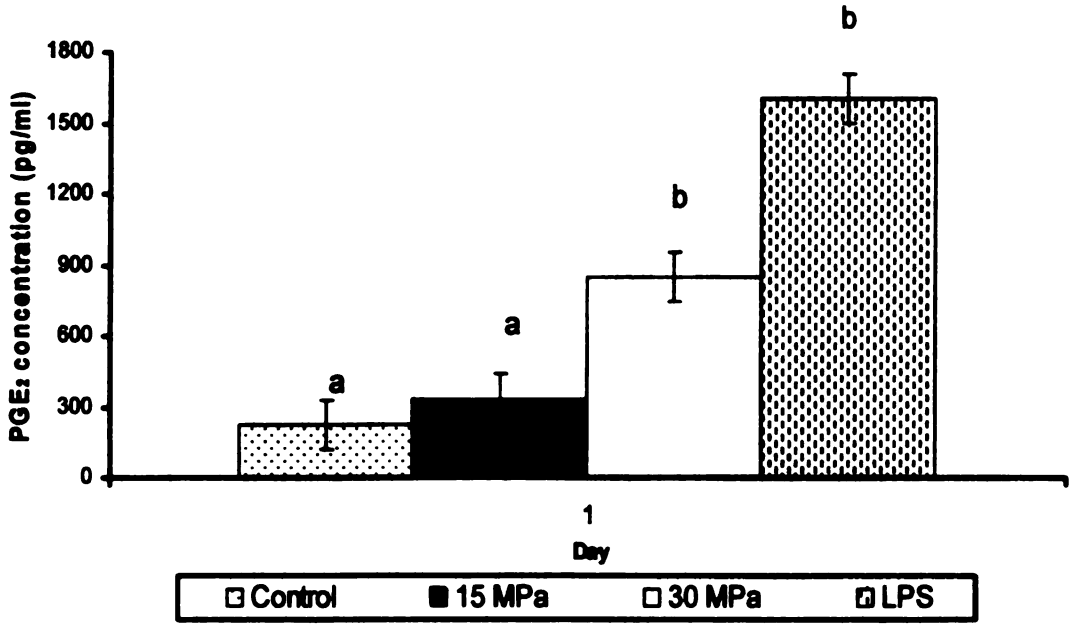

Figure 3. Mean prostaglandin E_2 (PGE_2) (± SEM) released into the media per well 24 hours post-treatment for control, loading at 15 MPa (15 MPa), loading at 30 MPa (30 MPa), and lipopolysaccharide (LPS). PGE_2 concentration was determined by means of a commercially available competitive enzyme immunoassay kit. Different superscripts indicate significant differences ($P<0.05$) between treatment groups.

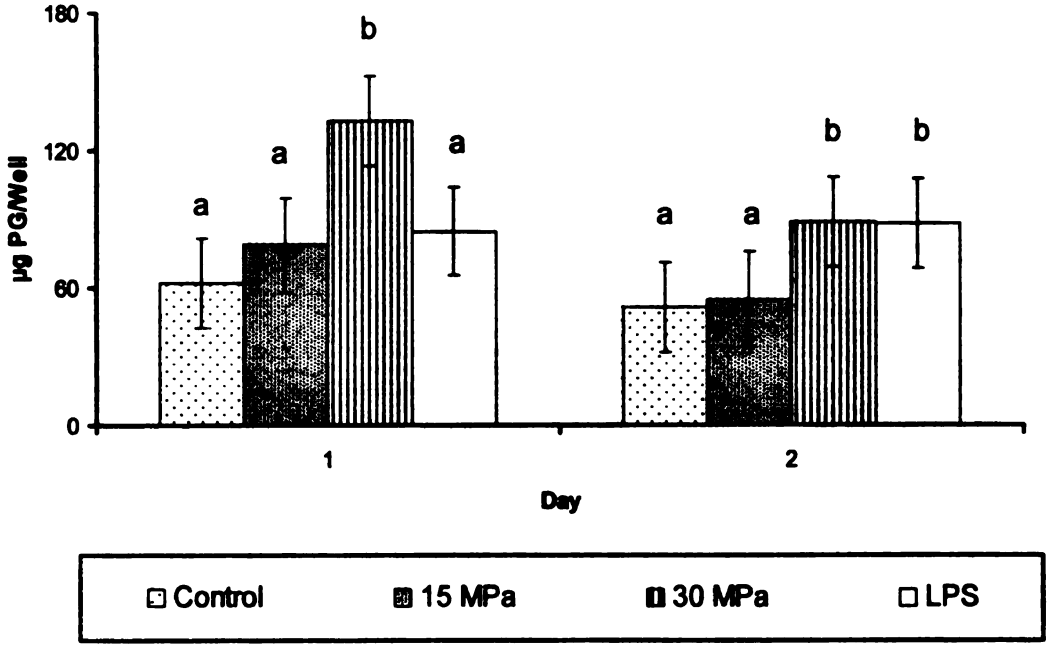

Figure 4. Mean proteoglycan (PG) (± SEM) released into the media per well each day post-treatment for control, loading at 15 MPa (15 MPa), loading at 30 MPa (30 MPa), and lipopolysaccharide (LPS). Total PG released into the media was quantified using a dimethymethylene blue (DMB) assay. Different superscripts indicate significant differences ($P<0.05$) between treatment groups.

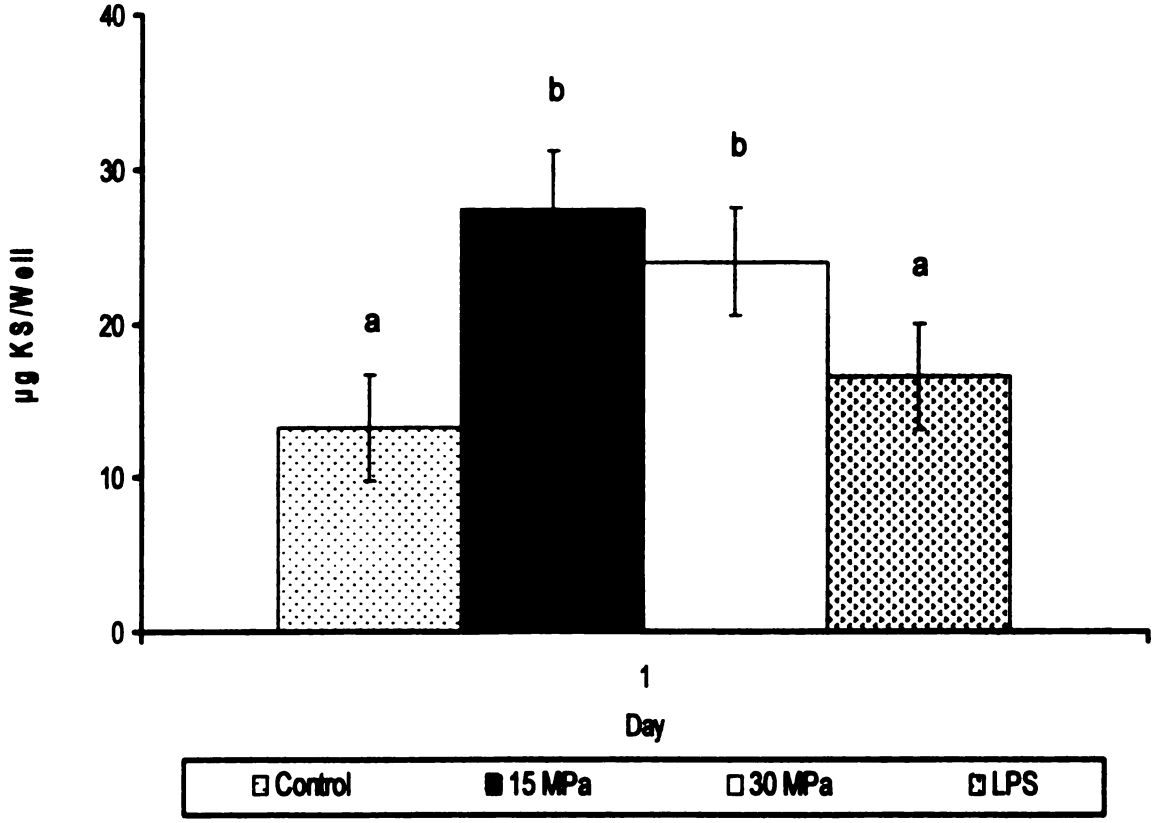

Figure 5. Mean keratan sulfate (KS) (± SEM) released into the media per well 24 hours post-treatment for control, loading at 15 MPa (15 MPa), loading at 30 MPa (30 MPa), and lipopolysaccharide (LPS). KS loss in the media was quantified using an ELISA with a monoclonal antibody specific for KS. Different superscripts indicate significant differences ($P<0.05$) between treatment groups.

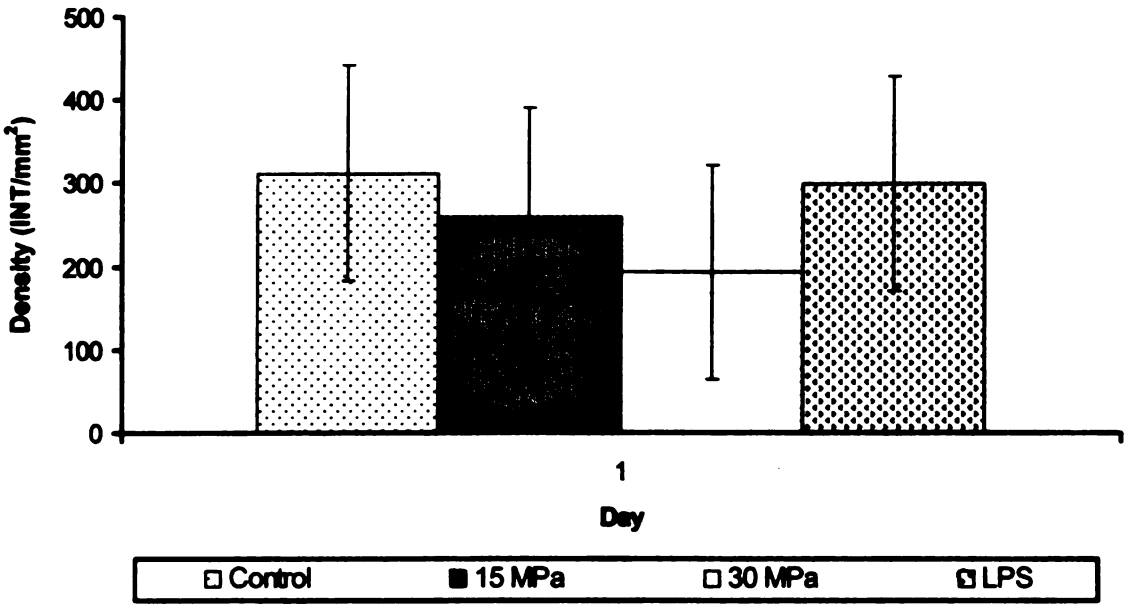

Figure 6. Mean matrix metalloproteinase-2 (± SEM) activity measured in the media per well 24 hours post-treatment for control, loading at 15 MPa (15 MPa), loading at 30 MPa (30 MPa), and lipopolysaccharide (LPS). MMP-2 activity was determined by gel zymography. MMP-2 activity was not significantly different between groups ($P>0.05$).

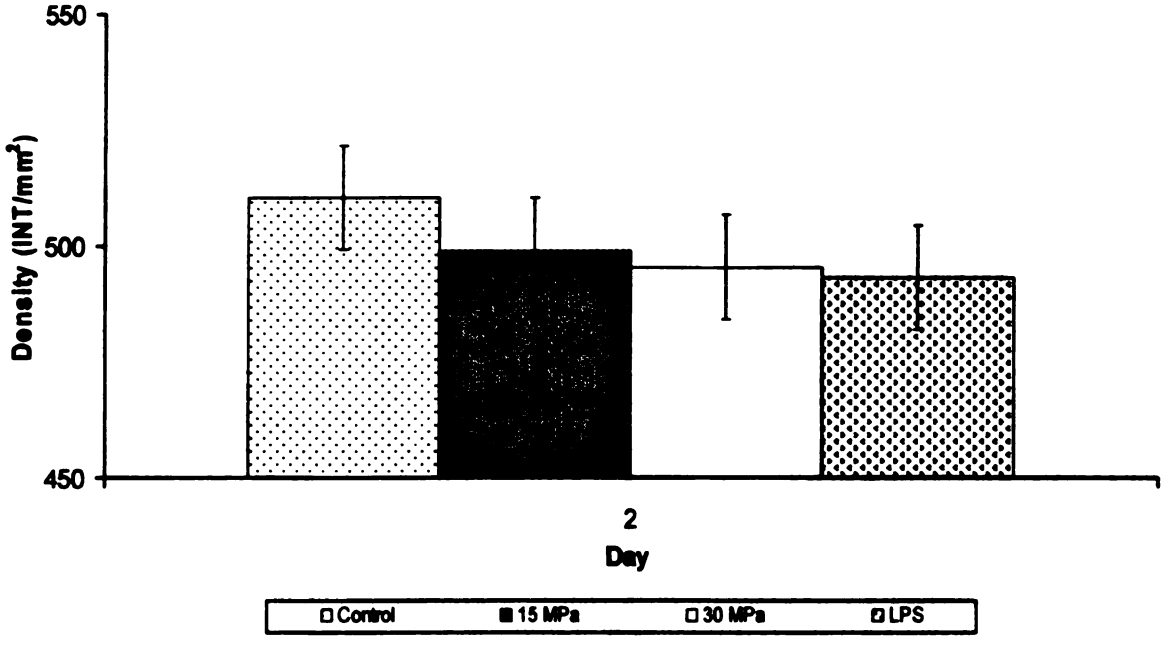

Figure 7. Mean matrix metalloproteinase-2 (± SEM) activity measured in the tissue per well 2 days post-treatment for control, loading at 15 MPa (15 MPa), loading at 30 MPa (30 MPa), and lipopolysaccharide (LPS). MMP-2 activity was determined by gel zymography. MMP-2 activity was not significantly different between groups ($P>0.05$).

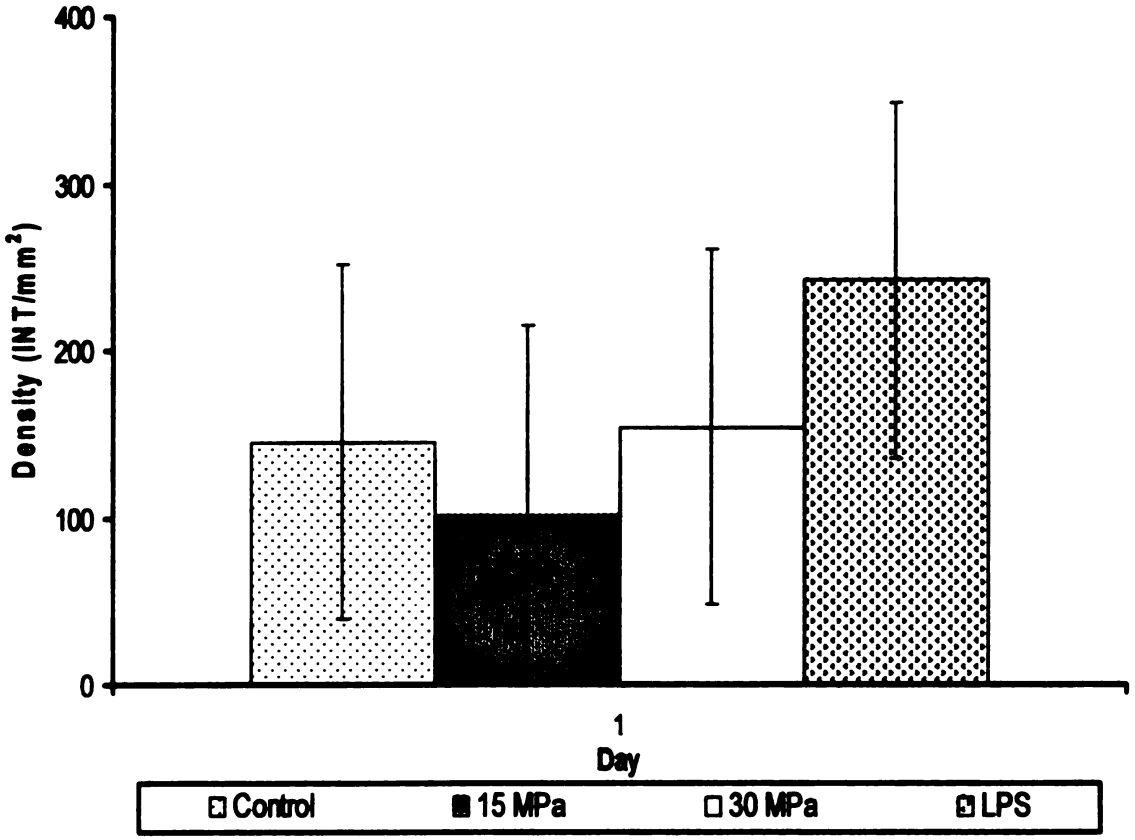

Figure 8. Mean matrix metalloproteinase-9 (± SEM) activity measured in the media per well 24 hours post-treatment for control, loading at 15 MPa (15 MPa), loading at 30 MPa (30 MPa), and lipopolysaccharide (LPS). MMP-9 activity was determined by gel zymography. MMP-9 activity was not significantly different between groups ($P>0.05$).

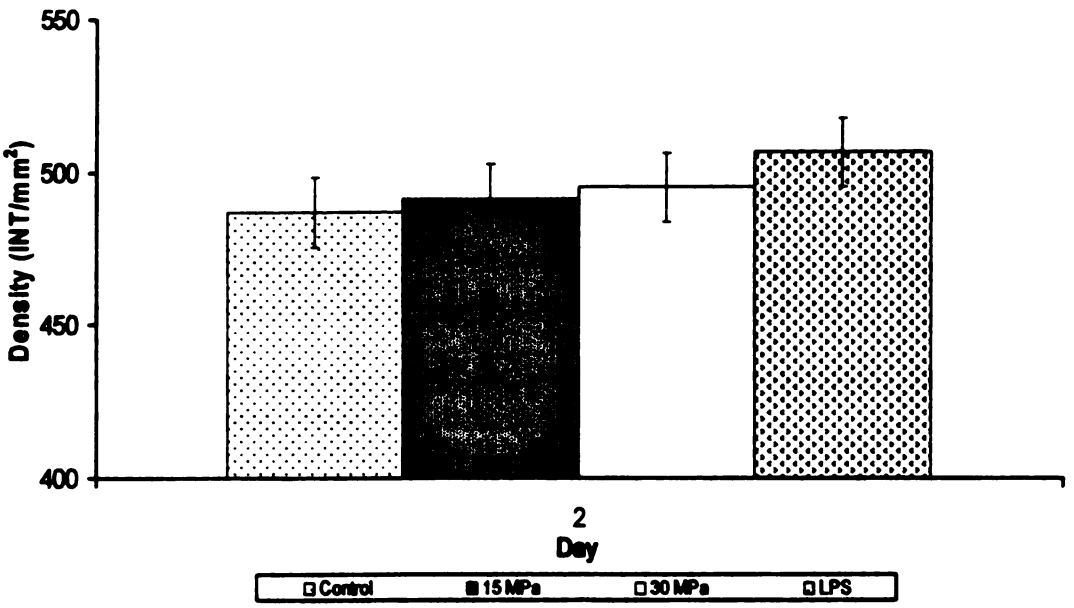

Figure 9. Mean matrix metalloproteinase-9 (± SEM) activity measured in the tissue per well 2 days post-treatment for control, loading at 15 MPa (15 MPa), loading at 30 MPa (30 MPa), and lipopolysaccharide (LPS). MMP-9 activity was determined by gel zymography. MMP-9 activity was not significantly different between groups ($P>0.05$).

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CHAPTER 3

FURTHER STUDIES ON THE ABILITY OF GLUCOSAMINE AND CHONDROITIN SULFATE TO INHIBIT CYTOKINE-INDUCED CARTILAGE DEGRADATION

Introduction

Osteoarthritis, also known as DJD, is a permanent condition that can cause lameness in horses at all levels of performance. Increased athletic ability and rigorous training predispose many horses to the degenerative effects of OA. Preliminary factors often involved in contributing to OA in the horse include trauma to the joint, instability of the joint, fatigue, and increased age. As a rule, the earlier the disease is diagnosed and properly treated, the greater the likelihood of improvement in the affected joint.

The outcome of OA generally results in decreased motion, pain, and dysfunction of the joint. The inflammatory response caused by this degenerative disease up regulates inflammatory mediators such as NO, PGE_2, and MMPs. In humans, NO activates MMPs, suppresses PG synthesis, and induces apoptosis in articular chondrocytes.[1,2] Increased production of NO may mediate the suppression of cartilage matrix synthesis occurring in response to intra-articular cytokines. Explants of equine synovial membrane and articular cartilage released significantly higher amounts of NO in tissue originating from horses with OA.[3] Prostaglandin E_2 is produced in inflamed joints, and can regulate PG degradation and inhibit PG synthesis.[4] Increased PGE_2 production occurred after cytokine stimulation in equine synovial cells and chondrocytes in vitro,[5,6] and was upregulated in cartilage explants from horses with moderate OA.[3]

Exogenous PGE_2 significantly reduced reIL-1β induced expression of MMP-1, -3, -13, and TIMP 1 in equine chondrocytes.[5] Matrix metalloproteinases are the primary class of enzymes that are considered to play the most pivotal role in destruction of the cartilage ECM. Specifically, MMPs –2 and –9 have been found in synovial fluid from diseased equine joints, and are up-regulated in equine cartilage and synovial fluid following stimulation with IL-1β.[7,8]

Oral non-invasive treatment of this disease with alternative therapeutic agents is becoming more popular within the equine industry. Two nutraceuticals (nutritional compounds added to the diet that have pharmacological-like effects) shown to slow the progression of OA are GLN and CS. In combination, these compounds suppress NO production, PGE_2 release, and MMP-2 activity in equine cartilage explants.[9-11] Horses diagnosed with OA and supplemented with GLN and CS orally had significantly improved lameness scores.[12,13] The same effects have been seen in humans and other animals. A combination of GLN and CS relieved symptoms of knee OA in military personnel,[14] and in patients with radiographically diagnosed mild to moderate OA.[15] Joint improvement after intercarpal injection with the combination has been seen in dogs with chemically induced OA.[16] Furthermore, GLN and CS, in combination with manganese ascorbate, was effective in reducing OA-induced lesions in rabbits.[17]

The lowest doses at which GLN and CS are most effective in inhibiting the degenerative effects of OA *in vitro* remain to be elucidated. High concentrations of GLN (6.5, 25 mg/ml) have been found to be toxic to bovine articular chondrocytes *in vitro*; however, these doses are extremely high, and the

actual dose in vivo is considered significantly less.[18] We have recently reported that the combination of GLN (1 mg/ml) and CS (0.25 mg/ml) inhibited the synthesis of several mediators of cartilage degradation in equine articular cartilage explants.[11] These results were at least 2.5-fold lower than concentrations in our previously reported work.[19] This research project was designed to study even lower concentrations of GLN HCl, in combination with a fixed concentration of CS, to determine if they would also inhibit NO production, PGE_2 release, and MMP activity in equine explants *in vitro*.

Materials and Methods

Experimental design

Articular cartilage was obtained from the antebrachio-carpal and middle carpal joints of 4 horses (2-14 years old) euthanized for reasons other than lameness. Four separate experiments were conducted using tissue from each of the 4 horses. Cartilage discs (3.5 mm in diameter) were biopsied from the load bearing region of the joint and were washed for a total of 3 washes in Dulbecco's Modified Eagles Medium (DMEM): nutrient mixture F-12 (Ham) (1:1).[a] Three randomly selected discs (approximately 40 mg of cartilage) were placed in each well of a 24 well Falcon culture plate.[b] Each well consisted of 1 ml of DMEM: F12 media supplemented with 10% fetal bovine serum,[a] 10 µl/ml ascorbic acid, 100 units/ml penicillin/streptomycin,[a] and all 20 amino acids[c].[20] The explants were maintained in culture in a humidified incubator with 7% CO_2 at 37°C.

Explants were equilibrated in media for 2 days. Lipolysaccharide[c] (10 µg/ml) was added to induce cartilage degradation in the presence or absence of GLN-HCl (FCHG49)[d] in combination with CS (TRH122)[d] (see Table 3) both days of treatment. Conditioned media were collected and replaced with new media daily and stored at 4°C until analysis. Treatments and controls of each experiment were identical and treatment groups are summarized in Table 3.

Table 3: Description of treatment groups in equine articular cartilage explant experiments

Treatment	FBS	LPS	GLN	CS
FBS-Control 1	10%			
LPS-Control 2	10%	10 µg/ml		
Treatment 1	10%	10 µg/ml	0.50 mg/ml	0.125 mg/ml
Treatment 2	10%	10 µg/ml	0.40 mg/ml	0.125 mg/ml
Treatment 3	10%	10 µg/ml	0.30 mg/ml	0.125 mg/ml
Treatment 4	10%	10 µg/ml	0.20 mg/ml	0.125 mg/ml

*FBS = fetal bovine serum; LPS = lipopolysaccharide; GLN = glucosamine HCl; CS = chondroitin sulfate

Nitric oxide analysis

Nitric oxide (NO) was measured indirectly in the conditioned media as described previously.[1] Nitrite (NO_2), a stable end-product of NO metabolism, was quantified using the Greiss reaction and sodium nitrite as a standard. Briefly, 75 µl conditioned medium was incubated with 75 µl 1.0% sulfanilamide, 0.1% N-1-naphthylethylenediamide dihydrochloride in 25% phosphoric acid at room temperature for 5 min. Due to some precipitation of reagents with CS, plates were spun at 950 g at a temperature of 4°C. Following centrifugation, the

remaining supernatant was transferred to a new 96-well plate, and absorbance at 540 nm was determined using a Spectromax 300 plate reader.[e] Results are expressed as nmol NO_2/well.

Cartilage extraction

Matrix metalloproteinases were extracted from articular cartilage using a modified protocol.[21] Explants from each well were placed in a cold stainless steel mortar apparatus and snap frozen with liquid nitrogen. They were then powdered immediately using a stainless steel pestle and hammer. Powdered explants were placed in microcentrifuge tubes with stir bars and 600 µl extraction buffer (50 mmol/l Tris HCl, 10 mmol/l $CaCl_2$, 2 mol/l guanidine HCl and 0.05% Brij-35: pH 7.5) was added to 3 powdered explants from each well. Samples were stirred overnight at 4°C and then centrifuged at 18,000 g for 30 min at 4°C. Supernatant was dialysed (24 h) against assay buffer (50 mmol/l Tris HCl, 10 mmol/l $CaCl_2$, 0.2 mol/l NaCl, 0.05% Brij-35: pH 7.5) using Spectrapor 2 dialysis tubing with a 12-kd cutoff.[f] Dialysis was continued for 48 h with distilled water. The amount of protein in the extracts was determined using the Pierce Micro BCA Protein Assay[g] with bovine serum albumen as the standard. Extractions were stored at 4°C and immediately analyzed for MMP activity.

Gel zymography

Gelatinase activity was detected by gel zymography. Extracts of articular cartilage samples containing 7 or 8 µg of protein (each gel received the same amount of protein) were applied without reduction to an 8% polyacrylamide gel with 1 mg/ml gelatin incorporated as the substrate. Samples were diluted

with 4x sample buffer and samples were electrophoresed at room temperature. A gelatinase A (MMP-2) molecular weight standard served as the gelatinase control. Following electrophoresis, gels were then incubated in 2.5% (v/v) Triton X-100 for 1h and then overnight at 37°C in 50 mmol/l Tris (pH7.5) containing 200 mmol/l NaCl, 10 mmol/l $CaCl_2$, 10 µmol/l $ZnCl_2$ and 0.02% Brij-35. The gels were stained with Coomassie Blue R250 for 1 h at room temperature and enzyme activity was measured by scanning densitometry (Gel Doc 2000),[h] using Quantity One 4.0.1 software.

Prostaglandin E_2 immunoassay

Prostaglandin E_2 (PGE_2) was measured using a commercially available competitive enzyme immunoassay kit.[i] Indomethacin (10 µg/ml) was added to conditioned media samples after 1 day of treatment and samples were then stored at -20°C until analysis. Samples were diluted 10-fold and run in duplicate. Briefly, the sample competes with a fixed amount of alkaline phosphatase-labeled PGE_2 for sites on a mouse monoclonal antibody. The antibody becomes bound to the goat anti-mouse antibody coating the microplate. Excess conjugate and unbound sample were removed from the plate through multiple washes. Absorbance was read at 405 nm with a wavelength correction set at 590 nm. A four parameter logistic curve ranging from 39-5,000 pg/ml PGE_2 was used to determine sample concentrations.

Statistical analysis

Data for indicators of degradation were analyzed using the repeated measure option of the SAS statistical software PROC MIXED (2001).[j] Data were

combined by pooling wells from all 4 horses according to treatment. Each group of treatments using tissue from one animal was considered a replicate (n=4). Data were normalized using log transformation due to variation in the level of response between horses. Experimental effects of treatment, and treatment by day effect were assessed for the pooled treatments. Differences between effects were compared using difference of least square means and Tukey's multiple comparison procedure. Statistical significance was considered at $P<0.05$ unless otherwise noted.

Results and Discussion

The concentrations of GLN and CS used in this study were developed from a previously published observation[11] indicating that concentrations of GLN as low as 0.50 mg/ml and CS as low as 0.125 mg/ml were beneficial in inhibiting some catabolic mediators of cytokine-induced cartilage degradation.

Equine cartilage explants demonstrated decreased NO production (Figure 10) with supplementation of GLN between the range of 0.30 and 0.50 mg/ml ($P<0.05$). Previously, we have reported that concentrations of GLN as low as 0.50 mg/ml inhibited NO production in equine explants.[11] Nitric oxide is a major component of the inflammatory-like response generally seen in OA, and has been implicated as a mediator of some of the effects of the pro-inflammatory cytokines LPS and IL-1 in equine articular chondrocytes.[22,23] Blocking NO production can prevent cartilage degradation in animal models.[24]

We have previously shown that GLN alone,[9,19] and in combination with CS[11] is effective in inhibiting the NO response.

Prostaglandin E_2 production (Figure 11) also tended to decrease with increased GLN concentration, and the minimum amount of GLN required to significantly inhibit production was 0.40 mg/ml ($P<0.05$). Concentrations of GLN that proved to be effective in this study were similar to the efficacy of GLN at 0.50 mg/ml in our previous studies.[9,11] Prostaglandin E_2 is an important cellular signal in both normal and pathological joint metabolism. Significantly greater concentrations of PGE_2 were detected in synovial fluid from equine joints affected with OA.[25-27] Cytokine stimulated explants and chondrocytes have enhanced levels PGE_2 production.[5,6,28] Blocking PGE_2 production is an important strategy to decrease symptoms of OA in humans as evidenced by the development of COX-2 inhibitors.

In agreement with our previous study,[11] GLN and CS had no effect on MMP-2 activity (Figure 12). In addition, LPS-stimulated explants did not display an up-regulation of MMP-2 as demonstrated in the prior study. Matrix metalloproteinase-9 activity was suppressed, however, at a concentration of 0.50 mg/ml GLN ($P<0.05$) and tended to be decreased at 0.40 mg/ml GLN ($P=0.08$) (Figure 13). This is in agreement with our previous study demonstrating that the combination of GLN and CS down-regulate MMP-9 activity.[11] However, this study used a concentration of 1.0 mg/ml GLN and 0.25 mg/ml CS. We have shown that concentrations even lower than this may be effective in suppressing MMP-9 activity. The differences between the two MMPs could be that in articular

cartilage, MMP-2 is constitutively expressed while MMP-9 is induced by pro-inflammatory cytokines such as IL-1.[29] MMP activity has been reported to be nearly twice as high in equine joints with OA compared to normal joints, and most likely reflects matrix destruction.[30] Thus, if GLN and CS could inhibit at least some proteolytic activity, this could prove beneficial to horses with OA.

Our results have provided further evidence that GLN and CS in combination are chondroprotective and may serve as an effective treatment for inhibiting the catabolic response of articular cartilage degradation found in OA. The combination may suppress cartilage catabolism through regulating cell signaling molecules, such as NO and PGE_2, which regulate MMPs. Most recently, GLN alone appeared capable of pre-translational, and possibly also translational, regulation of MMP expression.[31] Both NO and PGE_2 are up-regulated in equine synovial membrane and cartilage obtained from osteoarthritic joints.[3] Weinberg[32] has suggested that inhibiting the production of NO and PGE_2 simultaneously would provide potent anti-inflammatory effects. The concentrations of GLN and CS used in this study are still somewhat higher than values measured in blood, although the concentration of CS (0.125 mg/ml) is closer to the higher end of plasma concentrations seen in dogs[33] and horses.[34] Further studies investigating even more physiological concentrations of GLN and CS in combination are ongoing. Elucidating the mechanism of action of these compounds should further increase our understanding of how to maintain and prevent OA in athletic and performance horses.

[a]Gibco, Grand Island, New York, USA.
[b]Fisher Scientific, Pittsburgh, Pennsylvania, USA.
[c]Sigma Chemical, St Louis, Missouri, USA.
[d]Nutramax Laboratories, Edgewood, Maryland, USA.
[e]Molecular Devices, Sunnyvale, California, U.S.A.
[f]Spectrum Medical Industries, Los Angeles, California, USA.
[g]Pierce, Rockford, Illinois, USA.
[h]BioRad, Hercules, California, USA.
[i]R&D Systems, Minneapolis, Minnesota, USA.
[j]SAS Institute, Inc., Cary, North Carolina, USA.

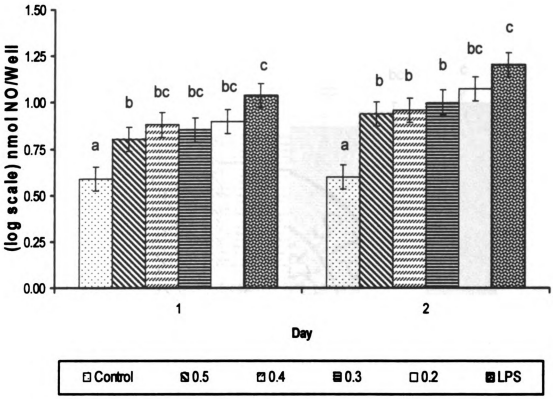

Figure 10. Mean nitric oxide (NO) (± SEM) released into the media each day post-treatment for all 4 horses (36-48 wells per treatment). Values are shown as log transformed. Treatments: control = no glucosamine (GLN), chondroitin sulfate (CS), or lipopolysaccharide (LPS); 0.5 = 0.5 mg/ml GLN + 0.125 mg/ml CS; 0.4 = 0.4 mg/ml GLN + 0.125 mg/ml CS; 0.3 = 0.3 mg/ml GLN + 0.125 mg/ml CS; 0.2 = 0.2 mg/ml GLN + 0.125 mg/ml CS; LPS = 10 µg LPS. ^{bc} means not sharing the same superscript differ ($P<0.05$).

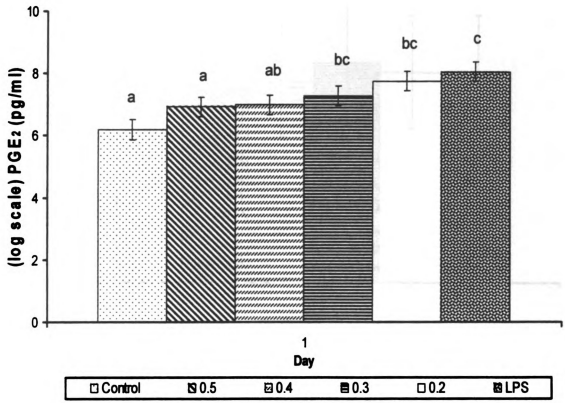

Figure 11. Mean prostaglandin E_2 (PGE_2) (± SEM) released into the media each day post-treatment for all 4 horses (36-48 wells per treatment). Values are shown as log transformed. Treatments: control = no glucosamine (GLN), chondroitin sulfate (CS), or lipopolysaccharide (LPS); 0.5 = 0.5 mg/ml GLN + 0.125 mg/ml CS; 0.4 = 0.4 mg/ml GLN + 0.125 mg/ml CS; 0.3 = 0.3 mg/ml GLN + 0.125 mg/ml CS; 0.2 = 0.2 mg/ml GLN + 0.125 mg/ml CS; LPS = 10 µg LPS. ^{ab} and ^{bc} means not sharing the same superscript differ ($P<0.05$).

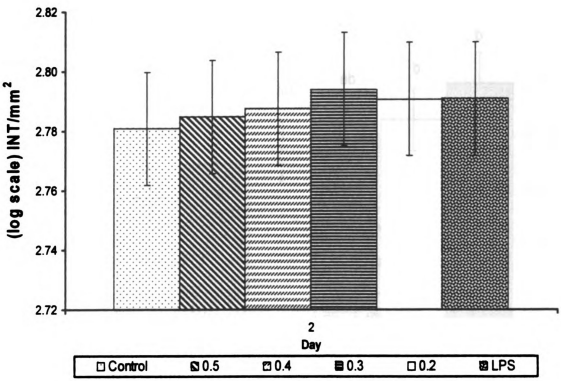

Figure 12. Mean matrix metalloproteinase-2 (MMP-2) activity (± SEM) in the tissue 2 days post-treatment for all 4 horses (36-48 wells per treatment). Values are shown as log transformed. Treatments: control = no glucosamine (GLN), chondroitin sulfate (CS), or lipopolysaccharide (LPS); 0.5 = 0.5 mg/ml GLN + 0.125 mg/ml CS; 0.4 = 0.4 mg/ml GLN + 0.125 mg/ml CS; 0.3 = 0.3 mg/ml GLN + 0.125 mg/ml CS; 0.2 = 0.2 mg/ml GLN + 0.125 mg/ml CS; LPS = 10 µg LPS. MMP-2 activity was not significantly different between groups (P>0.05).

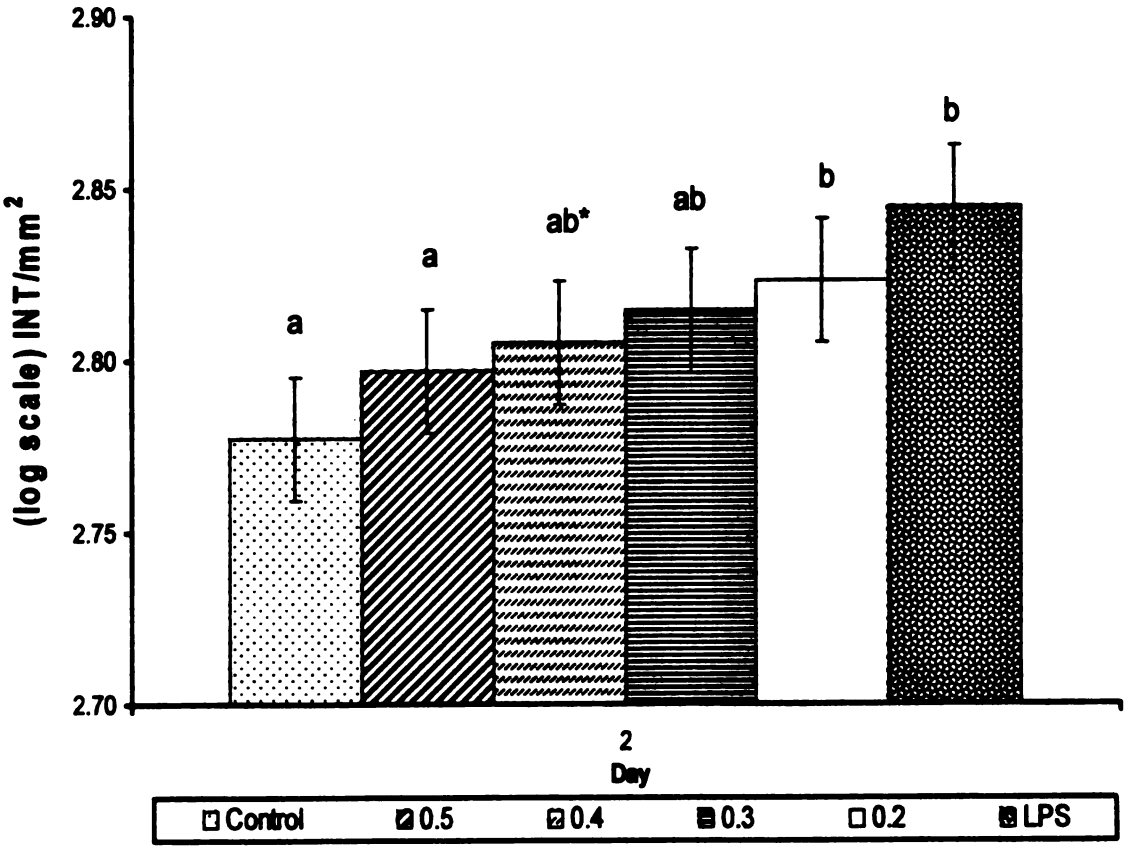

Figure 13. Mean matrix metalloproteinase-9 (MMP-9) activity (± SEM) in the tissue 2 days post-treatment for all 4 horses (36-48 wells per treatment). Values are shown as log transformed. Treatments: control = no glucosamine (GLN), chondroitin sulfate (CS), or lipopolysaccharide (LPS); 0.5 = 0.5 mg/ml GLN + 0.125 mg/ml CS; 0.4 = 0.4 mg/ml GLN + 0.125 mg/ml CS; 0.3 = 0.3 mg/ml GLN + 0.125 mg/ml CS; 0.2 = 0.2 mg/ml GLN + 0.125 mg/ml CS; LPS = 10 µg LPS. ^{ab} means not sharing the same superscript differ ($P<0.05$). * indicates a trend ($P<0.08$) to differ from LPS.

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CONCLUSION

The previous chapters have demonstrated the differences between applying a single acute load or cytokine stimulation to equine articular cartilage explants, and have further investigated whether reduced concentrations of GLN (in combination with CS) are effective in inhibiting cytokine-induced cartilage degradation. Several catabolic measurements of articular cartilage were used to assess degradation, which included NO and PGE_2 production, PG release, KS degradation, and MMP activity. These factors are up-regulated in OA, and can contribute to irreversible cartilage damage in a joint.

Evidence is accumulating that direct damage to the cartilage is not the only route by which trauma contributes to OA, and that fibrillation and fissuring of the cartilage surface is not necessarily the primary indicator of cartilage degradation. Our study employing two different levels of loading on cartilage explants agrees with similar mechanical loading studies demonstrating increased NO and PGE_2 production, and increased PG release. We have shown that acute trauma causes distinct biochemical differences in cartilage catabolism compared to cytokine-induced degradation. This evidence is supported by gene expression studies performed in our lab indicating that loading explants significantly increased MMP-3 expression, but had little effect on other MMP expression; whereas IL-1 stimulated explants had an increase in MMP-3, -13, and -14.

Biochemical analysis revealed that loaded explants peaked in nitric oxide production on day 1; whereas cytokine treated explants peaked on day 2.

Proteoglycan release was significantly higher overall in the 30 MPa loaded explants, and both loaded groups demonstrated the greatest KS degradation. Although MMP-2 and -9 activity were not significantly different across any treatment, LPS treated explants has the highest MMP-9 activity among treatments. Other studies have indicated up-regulation of MMP-9 following LPS stimulation, and increasing the number of animals and replication in this study may have resulted in a significant up-regulation in LPS treated explants. Contrary to cytokine treated explants, loaded explants showed little difference, if any, from the control explants. Results from previous studies employing different models of mechanical loading to study proteolytic activity have had conflicting results. Further investigations to determine whether the loss of PGs in models of injury to cartilage is due to mechanical disruption or dislodgement of the matrix rather than cell-mediated enzymatic degradation would be helpful. Induction of cytokines following an *in vitro* injury to articular cartilage synergistically increases the loss of GAGs from the tissue. This finding, combined with the work of our study, suggests that factors external to cartilage, such as cytokines in the synovial fluid, could play an important role in the development of cartilage degradation after acute joint trauma. Future studies observing the effects of cytokine stimulation following loading could further aid in explaining the trauma-induced OA commonly seen in horses.

Glucosamine and CS, in combination, are proposed to have chondroprotective properties, and have become a popular supplementation protocol in arthritic conditions of joints. The hypothesis that the combination of

these two molecules provides an additive or synergistic chondroprotective effect is not firmly established. Furthermore, the physiological concentrations of GLN and CS *in vivo* that are most effective in inhibiting cartilage degradation remain to be elucidated. Using indicators of cartilage metabolism, we have shown that GLN-HCL, in combination with CS (0.125 mg/ml), at concentrations ranging from 0.30 to 0.50 mg/ml is effective in inhibiting several inflammatory mediators of cytokine-induced cartilage degradation. The combination of GLN and CS decreased NO and PGE_2 production along with MMP activity. These molecules may suppress cartilage catabolism through regulating cell signaling molecules such as NO and PGE_2, which regulate MMPs. Although the concentrations of GLN and CS used in this study are still somewhat higher than values in the blood, the concentration of CS is close to higher values found in the plasma of dogs. One drawback to this study verses our previous loading study is that the horses used in this study were from a broad age group (4 to 14 years). Therefore, increased variation was observed between animals. However, this variable response may be helpful in understanding the pattern in efficacy of GLN and CS in inhibiting the inflammatory-like response of cartilage degradation in animals of different age groups. The biochemical evidence resulting from this study supports the limited *in vivo* research indicating that GLN and CS may prevent equine articular degradation.

An *in vitro* articular explant model may not be an ideal system to study the effects of articular degradation *in vivo*; however, it enables us to study possible pathways and mechanisms of action of contributing factors involved in OA. In

turn, this information can lead to a greater understanding of the physiological adaptations taking place during cartilage degeneration, without sacrificing the animal.

APPENDIX

APPENDIX A

DEVELOPMENT OF A SERUM-FREE MEDIUM FOR AN EQUINE ARTICULAR CARTILAGE EXPLANT SYSTEM

Various growth factors, hormones and proteins present in serum are presumed to be responsible for its growth-stimulating activity in culture media.[1] However, serum is a complex mixture, and many serum components are poorly characterized or have not been completely studied.[2] In addition, the concentrations of some of the components of serum vary drastically among several different serum batches. Fetal bovine serum is commonly used in articular cartilage tissue culture. Infection with viruses such as bovine viral diarrhea virus (BVDV) is frequent in the bovine population.[3] *In utero* infection leads to virus and antibody contamination of fetal and other serum used in cell culture production.

Serum substitute media eliminates the complex and variable effects of serum on cell growth. These substitutes can be mixtures of salts, amino acids, vitamins, glucose and various compounds such as nucleic acid and lipid precursors or antioxidative substances.[1] They are supplemented with hormones (insulin, growth factors, steroids), binding proteins (transferring, albumin) and trace elements, which replace the growth stimulating activities of serum with the appropriate serum substituents at the right concentrations.

Currently, the serum supplemented medium used in our laboratory limits our ability to detect certain known mediators of articular cartilage degradation in cultured media. Eliminating serum from our culture medium allows the simple

design and interpretation of experiments which would be difficult or impossible to carry out in serum-containing medium. Thus, my objective in this study is to develop a serum-free medium that sustains articular cartilage chondrocytes in cartilage explant culture.

Experiment 1

Materials and Methods

Experimental design

Articular cartilage was obtained from 1 pair of bovine forelegs (18 to 24 months of age) obtained from a local abattoir within three hours of slaughter. Cartilage discs (3.5 mm) were biopsied from the load-bearing region of the joint and were washed for a total of 3 washes in Dulbecco's Modified Eagles Medium (DMEM): nutrient mixture F-12 (Ham) (1:1).[a] Three randomly selected discs (approximately 40 mg of cartilage) were placed in each well of a 24 well Falcon culture plate[b] (42 wells total). Six wells from each plate were randomly assigned to one of 7 treatment groups shown in Table 4.

Table 4: Treatment groups for development of serum free culture medium

Treatment group	Treatments*
1	Serum-free media + 1 µl/ml ITS
2	Serum-free media + 10% FBS
3	Serum-free media + 1 µl/ml ITS + 10 µg/ml LPS
4	Serum-free media + 10% FBS + 10 µg/ml LPS
5	Bovine basal media + 1 µl/ml ITS
6	Bovine basal media + 1 µl/ml ITS + 10 µg/ml LPS
7	Bovine basal media + 10% FBS + 10 µg/ml LPS

*ITS = Insulin Transferrin Sodium Selenite Supplement, [c] FBS = Fetal bovine serum, [a] LPS = Lipopolysaccharide.[d]

The serum-free medium used in treatments 1 through 4 consisted of DMEM/F-12 (Ham) (1:1) serum free media supplemented with all 20 amino acids[d] (Gln 2.19 g/L, Gly 5.63 mg/L, His 5.26 mg/L, 25.17 mg/L, Leu 22.88 mg/L, Lys 27.63 mg/L, Met 6.38 mg/L, Phe 15.26 mg/L, Pro 8.64 mg/L, Ser 7.88 mg/L, Thr 20.88 mg/L, Trp 3.49 mg/L, Tyr 24.00 mg/L, Val 20.38 mg/L, 2.23 mg/L, 31.6 mg/L, 3.76 mg/L, 3.33 mg/L), ascorbic acid (50 µl/ml),[d] sodium bicarbonate (3.89 g/L),[b] lactalbumin hydrolysate (2 mg/L),[d] dexamethasone (100 µg/ml),[d] magnesium sulfate (16.9 mg/ml),[d] and penicillin/streptomycin (100 units/ml).[a] The bovine basal medium used in treatments 5 through 7 was identical to the recipe for the serum free medium listed prior, except it had twice the amounts of amino acids and 1 µg/ml sodium selenite[d] added to the medium.

The explants were maintained in culture in a humidified incubator with 7% CO_2 at 37°C. Explants from treatments 1 through 4 were equilibrated in serum-free media + ITS 2 days prior to the first of 4 treatment days, while explants from

treatments 5 through 7 were equilibrated in bovine basal media + 10% FBS prior to treatment. On day 1 of treatment, explants in groups 3, 4, 6, and 7 were incubated with 10 µg/ml LPS. Conditioned media were collected daily and stored at 4°C until analysis. In addition, one explant from one well of each treatment was saved to study cell viability of the explants.

Nitric oxide analysis

Nitric oxide (NO) was measured indirectly in the conditioned media as described previously (Blanco et al., 1995). Nitrite (NO_2), a stable end-product of NO metabolism, was quantified using the Greiss reaction and sodium nitrite as a standard. Briefly, 75 µl conditioned medium was incubated with 75 µl 1.0% sulphanilamide, 0.1% N-1-naphthylethylenediamide dihydrochloride in 25% phosphoric acid at room temperature for 5 min. Absorbance at 540 nm was determined using the Spectromax 300 plate reader.® Results are expressed as nmol NO_2/well.

Proteoglycan analysis

Proteoglycan (PG) release into conditioned media was measured as previously described (Chandrasekhar, 1987) using a dimethylmethylene blue (DMB) assay. Briefly, PG content was determined by measuring sulfated glycosaminoglycan (GAG) content compared to a chondroitin sulphate standard. Results are expressed as µg PG/well.

Cell viability

On the final day of tissue culture, one explant from each group was randomly chosen for a cell viability study. Explants were removed from their

assigned wells and sliced into approximately 0.5 mm sections using a scalpel blade. The sections were stained using a kit containing calcein and ethidium bromide homodimer (Live/Dead and Viability/Cytotoxicity).[f] All specimens were viewed in a florescence microscope (Leica DM LB)[g] (frequency: 50-60 Hz). Cell viability was determined by visual detection of dead (red) and viable (green) cells.

Results and Discussion

The serum-free medium + ITS treated control (treatment 1) and the bovine basil medium + ITS (treatment 5) were very similar in NO production on both days 1 and 2 (Figure 14). In addition, the serum-free LPS treated positive controls (treatments 4 and 7) for each of these mediums were very comparable in NO production on these two days. Proteoglycan release showed similar results to that of NO production for the different mediums (Figure 15). Treatments 1 and 2 comparing the ITS supplement to FBS in serum-free media had almost identical PG release, as did treatments 3 and 7 in both mediums with added LPS. Although there was a huge spike in PG release in treatment 4 on day 1, no visual differences are seen in the figure on day 2 when comparing the serum-free medium with FBS and LPS and the bovine basal media with FBS and LPS. This spike was also seen 1 day prior to treatment (data not shown), and thus was a result of factors other than addition of LPS. Cell viability was normal under serum-free culture conditions. Explants showed a small amount of cell death

around biopsied edges, but no abnormal cell death was observed upon detection with florescence.

Conclusion

The ITS supplement used in this experiment has been used with success in equine cartilage explant cultures.[4,5] I have also proved this to be true in our equine articular cartilage explant system in the current experiment. Insulin, transferrin, and selenium are important in cellular maintenance and are constituents that are often added to media.[2] Insulin regulates cell metabolism and promotes growth, transferrin works as an iron transporter, and selenium works as a component of the free-radical scavenger glutathione peroxidase, and as a nutrient for cell proliferation.[5] The results of this experiment indicate that incubating bovine articular cartilage in serum-free media containing ½ of the normally added amino acids in conjunction with the ITS supplement nourishes and helps chondrocytes to flourish similar to that of incubation with full amino acids and FBS. Although FBS treatments tended to have higher NO production and PG release, the ITS treated explants showed similar trends and had parallel results.

Experiment 2

Materials and Methods

Experimental design

Articular cartilage was obtained from 1 pair of bovine forelegs (18 to 24 mo of age) obtained from a local abattoir within three hours of slaughter. Cartilage discs (3.5 mm) were biopsied from the load-bearing region of the joint and were washed for a total of 3 washes in Dulbecco's Modified Eagles Medium (DMEM): nutrient mixture F-12 (Ham) (1:1). Four randomly selected discs (approximately 50 mg of cartilage) were placed in each well of a 24 well Falcon culture plate (42 wells total). Six wells from each plate were randomly assigned to one of 7 treatment groups shown in Table 5.

Table 5: Treatment groups for development of serum free culture medium

Treatment group	Treatments*
1	Serum-free media + 1 µl/ml ITS
2	Serum-free media + 1 µl/ml ITS + 10 µg/ml LPS
3	Serum-free media + 1 µl/ml ITS + 5 µg/ml LLA +10 µg/ml LPS
4	Serum-free media + 1 µl/ml ITS + 40 ng/ml Thy +10 µg/ml LPS
5	Serum-free media + 1 µl/ml ITS + 5 µg/ml LLA + 40 ng/ml Thy + 10 µg/ml LPS
6	Serum-free media + 10% FBS + 10 µg/ml LPS
7	Serum-free media + 10% FBS

*ITS = Insulin Transferrin Sodium Selenite Supplement, FBS = Fetal bovine serum, LPS = Lipopolysaccharide, LLA = Linoleic acid albumen,[d] T= Thyroxine.[d]

The serum-free medium used in treatments 1 through 7 was identical to that described in experiment 1 of this appendix (APPENDIX A). The explants were maintained in culture in a humidified incubator with 7% CO_2 at 37°C.

Explants from treatments 2 through 6 were equilibrated in serum-media + ITS and either LLA, T, or both (as indicated in Table 5) 2 days prior to the first of 1 treatment day. Explants in treatments 1 and 7 did not receive additional treatments except their maintenance supplement of ITS or FBS. On day 1 of treatment, explants in groups 2 through 6 were incubated with 10 µg/ml LPS. Conditioned media were collected daily and stored at 4°C until analysis.

Nitric oxide

Nitric oxide (NO) was measured indirectly in the conditioned media as described previously (Blanco et al., 1995) and in experiment 1 of this appendix (APPENDIX A).

Results and Discussion

Figure 16 shows the NO release for explants in experiment 2 for two days prior to treatment and the day of treatment. The addition of LLA by itself in treatment 3 shows a marked increase in NO production compared to treatment without LPS (treatment 1), with ITS + T + LPS (treatment 4), with FBS + LPS (treatment 5), and with FBS alone (treatment 6). Thyroxine alone with ITS and LPS (treatment 4) did not have the same effect; however, the combination of both T and LLA (treatment 5) showed the highest NO production over all treatments. The NO release seen in this treatment was also closer to the range of release typically seen in other experiments conducted in our lab using LPS treated bovine explants.

Conclusion

Linoleic acid, a prostaglandin precursor, has previously been used in equine articular cartilage explant cultures.[4,5] Thyroxine is a growth hormone that supports matrix assembly and hypertrophic expression in growth plate chondrocytes.[6] This experiment indicates that the combination of supplements, in conjunction with LPS, that causes chondrocytes to release NO in a manner most similar to culturing with FBS in serum-free medium is ITS + LLA + T. In the previous experiment, I confirmed the beneficial effect of supplementing ITS to cartilage explants in serum-free media. In the present experiment, I have shown that two addition supplements, LLA and T, in combination with ITS, have a positive effect on chondrocyte metabolism.

[a]Gibco, Grand Island, New York, USA.
[b]Fisher Scientific, Pittsburgh, Pennsylvania, USA.
[d]Sigma Chemical, St Louis, Missouri, USA.
[c]Roche Diagnostics Corporation, Indianapolis, Indiana, USA.
[e]Molecular Devices, Sunnyvale, California, U.S.A.
[f]Molecular Probes, Oregon, USA
[g]Lecia Mikroskopie und Systeme GmgH, Wetlzar, Germany

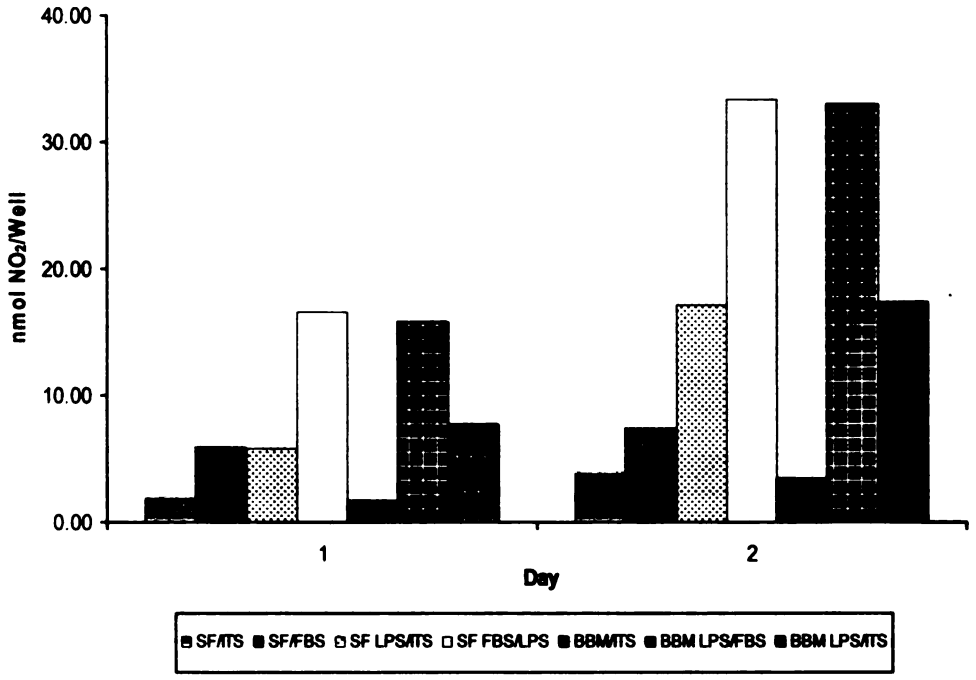

Figure 14. Mean nitrite (NO_2) released into serum-free (SF) or bovine basal media (BBM) 24 and 48 hours after treatment with ITS, FBS, and LPS. NO_2 concentration was quantified using an assay employing the Greiss reaction. ITS=insulin-transferrin-sodium-selenite supplement, FBS=fetal bovine serum, LPS=lipopolysaccharide.

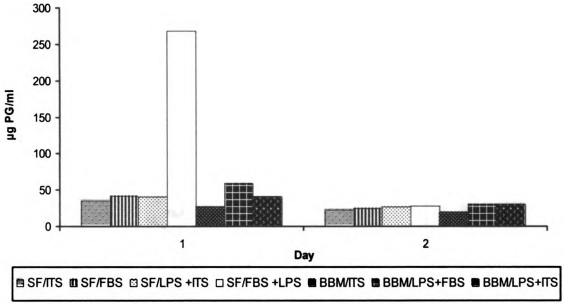

Figure 15. Mean proteoglycan (PG) released into serum-free (SF) or bovine basal media (BBM) 24 and 48 hours after treatment with ITS, FBS, and LPS. Total PG released into the media was quantified using a dimethymethylene blue (DMB) assay. ITS=insulin-transferrin-sodium-selenite supplement, FBS=fetal bovine serum, LPS=lipopolysaccharide.

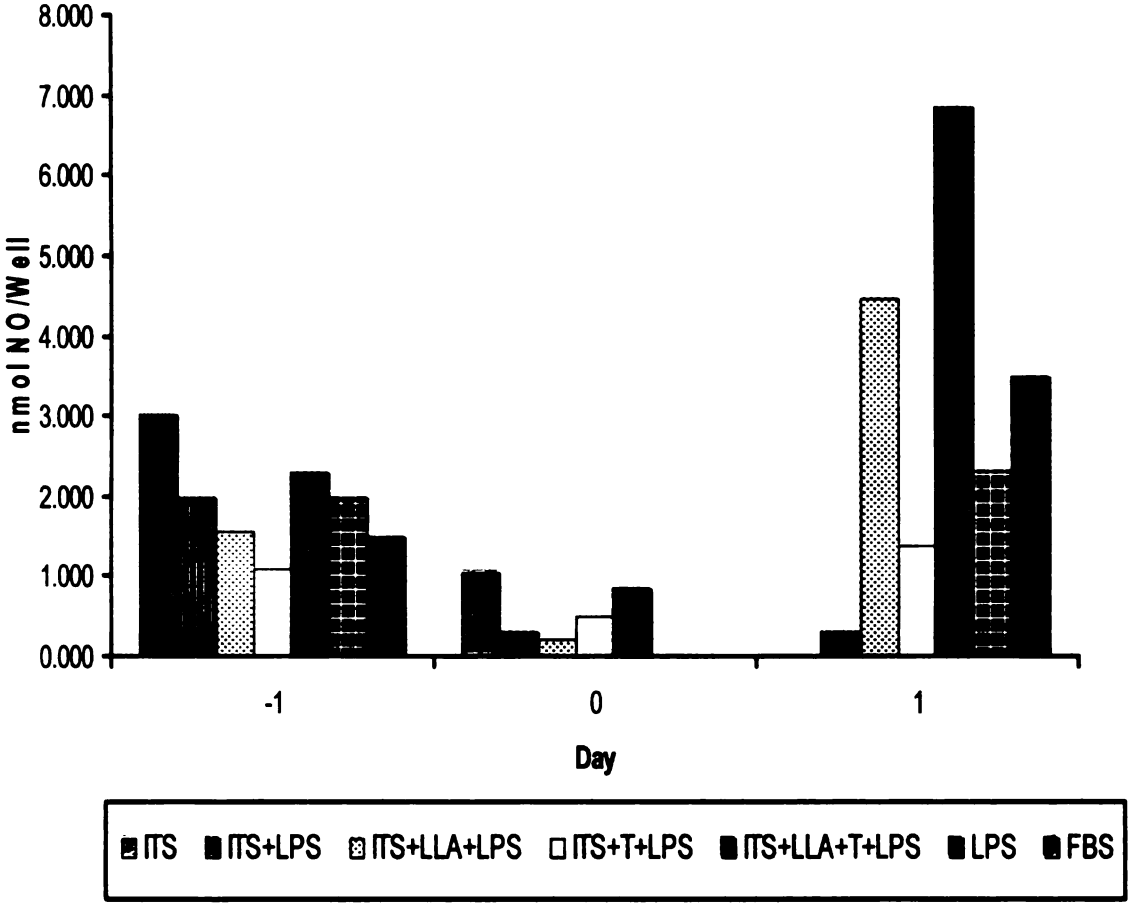

Figure 16. Mean nitrite (NO_2) released into serum-free (SF) media 24 and 48 hours prior to treatment with ITS, LLA, T, FBS, and LPS, and 24 hours following treatment. NO_2 concentration was quantified using an assay employing the Greiss reaction. ITS=insulin-transferrin-sodium-selenite supplement, LLA=linoleic acid albumen, T=thyroxine, FBS=fetal bovine serum, LPS=lipopolysaccharide.

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