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THE EFFECTS OF GLUCOSAMINE ON EQUINE ARTICULAR CARTILAGE DEGRADATION

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THE EFFECTS OF GLUCOSAMINE ON EQUINE ARTICULAR CARTILAGE DEGRADATION

By

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ABSTRACT

THE EFFECTS OF GLUCOSAMINE ON EQUINE ARTICULAR CARTILAGE DEGRADATION

By

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Osteoarthritis (OA), a progressive degradation of articular cartilage, is a common cause of lameness and decreased performance for athletic horses. Biochemical changes leading to OA cause an imbalance in the normal extracellular matrix (ECM) turnover process with degradation exceeding synthesis. Increased proteolytic enzyme activity is a major factor that is responsible for ECM degradation. Oral supplementation of compounds that prevent cartilage degradation and/or joint injury is an attractive potential solution for lameness. Glucosamine is a potential anti-arthritic compound currently being marketed. It is a naturally occurring, non-toxic molecule that decreased pain and improved mobility in osteoarthritic joints in a number of human studies. However, the biochemical basis to support its potential as an anti-arthritic agent is not well documented. Therefore, the objectives of this work were to 1) develop an equine explant culture system and 2) determine whether glucosamine and its derivatives (glucosamine sulfate and N-acetyl-glucosamine) could inhibit experimentally induced cartilage degradation in explant culture.

Articular cartilage was obtained from the weight bearing region of the antebrachio-carpal and middle joints of horses (2-8 years old) sacrificed for

reasons unrelated to lameness. Cartilage discs (3.5mm) were collected and maintained in a modified version of media without serum 2 days prior to the start of 4 treatment days (media were exchanged daily and the recovered media stored at 4°C). On days 1 and 2 lipopolysaccharide (LPS, 10 μg/ml) or recombinant human interleukin-1ß (rhIL-1ß, 50 ng/ml) were added to induce cartilage degradation. To test the potential protective effects of glucosamine, the compound was added in three concentrations (0.25, 2.5, or 25 mg/ml) and treatments were performed in triplicate. To test the effects of the glucosamine derivatives, equimolar concentrations of glucose-3-sulfate and N-acetyl-glucosamine were added. Controls included wells without LPS, rhIL-1ß, or glucosamine. Controls for the sugar moiety included glucose and glucose-3-sulfate. Nitric oxide, proteoglycan and matrix metalloproteinase (MMP) released into conditioned media and tissue proteoglycan synthesis were measured as indicators of cartilage metabolism. Maximal nitric oxide production, proteoglycan release, and MMP activity were detected 1 day after the addition of LPS or rhIL-1ß to the media. The addition of 25 mg/ml of glucosamine HCl prevented the increase in nitric oxide production, proteoglycan release and MMP activity induced by LPS or rhIL-1. Glucosamine sulfate consistently inhibited cartilage degradation in a manner similar to glucosamine HCl, while the effects of N-acetyl-glucosamine were highly variable and generally did not inhibit cartilage degradation. These data substantiate anecdotal *in vivo* observations and suggest a mechanism through which glucosamine may possess chondroprotective properties in equine articular cartilage.

This dissertation is dedicated to my husband and family. Without their love and support this would not have been possible.

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LIST OF ABBREVIATIONS

Extracellular Matrix	ECM
Osteoarthritis	OA
Matrix Metalloproteinases	MMP
Proteoglycans	PG
Glycosaminoglycan	GAG
Keratan Sulfate	KS
Chondroitin Sulfate	CS
Dermatin Sulfate	DS
Heparin Sulfate	HS
Hyaluronic Acid	HA
Interleukin-1	IL-1
Recombinant Equine Interleukin-1	reIL-1
Insulin Like Growth Factor-1	IGF-1
Tumor Necrosis Factor Alpha	TNF-α
Tissue Growth Factor-Beta	TGF-β
Polysulfated Glycosaminoglycans	PSGAG
N-acetyl-Glucosamine	GlcNac
Fetal Bovine Serum	FBS
Glucosamine	GLCN
Lipopolysaccharide	LPS
Twice Daily	BID

INTRODUCTION

Osteoarthritis (OA), a common cause of lameness in horses, is characterized by the progressive degradation of articular cartilage. Trauma, physical forces, and inadequate chondrocyte response to stress can induce this degradation.

Although *in vitro* models of cartilage degradation have been developed, the degradative process is still not clearly understood. In culture, equine chondrocytes recently have been shown to produce nitric oxide in response to two arthritogenic molecules, lipopolysaccharide (LPS) and interleukin-1 (IL-1). Synovial fluid from diseased joints has increased concentrations of nitric oxide and IL-1. *In vitro* data also suggest that nitric oxide activates cartilage-degrading proteinases, specifically metalloproteinases (MMP). Overproduction and/or chronic activation of MMPs eventually will damage articular cartilage by degrading the extracellular matrix proteins, including proteoglycans and collagens. These proteins function to absorb compressive forces and provide tensile strength.

Oral supplementation of compounds that may prevent cartilage degradation due to joint injury is an attractive potential solution for lameness. Glucosamine is one of the potential anti-arthritic supplements currently being marketed. It is a naturally occurring, non-toxic compound that was shown to decrease pain and improve mobility in human osteoarthritic joints in a number of studies. Results of single *in vitro* study suggested that glucosamine increases the synthetic activity of chondrocytes [1], potentially alleviating the imbalance

between synthesis and degradation characteristic of OA. These results have neither been confirmed nor refuted. Furthermore, the biochemical basis in support of glucosamine as a potential anti-arthritic agent is not well documented.

Therefore, the hypothesis of the studies reported herein was that glucosamine or its derivatives will prevent experimentally induced equine articular cartilage degradation *in vitro*. However, no standardized in vitro methodology for studying equine articular cartilage degradation has been developed. Therefore the objectives of this research were to: 1) standardize the methodology for an in vitro equine explant cartilage system; 2) develop methods for the induction of cartilage degradation in the standardized equine explant cartilage system; 3) determine if and how glucosamine protects articular cartilage from damage and 4) to determine the effects of glucosamine derivatives on equine articular cartilage degradation.

The following chapters of this dissertation describe *in vitro* research projects that address these objectives. Chapter I (literature review) provides background information necessary to understand the subsequent chapters describing the research projects. In chapter 2, I describe methodology for the in vitro projects and show that glucosamine inhibits either LPS or IL-1 induced cartilage degradation. In Chapter 3, I present data that indicates the actions of glucosamine may be dependent on the derivative studied (glucosamine HCL vs glucosamine-3-sulfate vs N-acetyl-glucosamine). In Chapter IV, I use a recently available recombinant equine IL-1 to induce cartilage degradation and show that glucosamine also inhibits cartilage degradation induced by this compound. In

conclusion, I discuss the effects of glucosamine observed in these projects and present potential mechanisms of action of glucosamine.

Chapter 1

LITERATURE REVIEW

Introduction

Osteoarthritis (OA) or degenerative joint disease causes progressive and permanent degeneration of articular cartilage. It is a common cause of lameness in racing horses and horses that perform in other athletic events [2]. Articular cartilage, found on the ends of long bones, absorbs compressive forces imposed on the joint. The biomechanical properties of cartilage are related directly to its structure. The extracellular matrix of articular cartilage is composed primarily of collagens, which provide tensile strength, and proteoglycans (PGs), which absorb compressive forces. When OA develops, PG loss from the extracellular matrix of articular cartilage is one of the first detectable biochemical changes. Oral glucosamine supplementation has been proposed to prevent this loss; however, the mechanisms by which this prevention might occur are poorly understood.

Articular Cartilage

Articular cartilage provides a wear-resistant and low-friction cover over the joint surface of many bones. It combines the properties of stiffness and elasticity allowing it to distribute loads and absorb compressive, tensional and shearing

forces. The cartilage is comprised of cells called chondrocytes, which synthesize, organize and regulate the extracellular matrix (ECM) surrounding them (Figure 1). The ECM is designed for water attraction and displacement to allow the cartilage to respond to physiologic loads [3]. It consists of approximately 70-80% water by volume [4]. On a dry weight basis, articular cartilage contains about 50% collagen, 35% PG, 10% non-collagenous proteins, 3% minerals and 1% lipids.

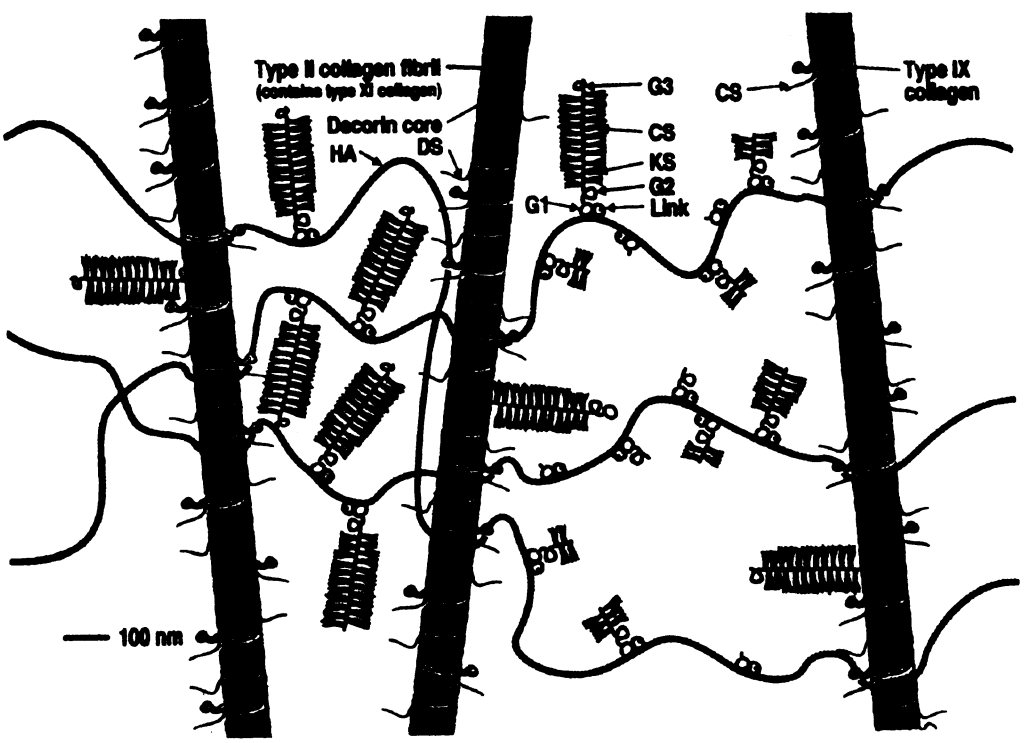

Figure 1. Organization of the major extracellular matrix proteins found in articular cartilage (Poole, 1993).

Tensile stiffness and strength is provided by collagen fibrils [5, 6]. The primary collagen types found in articular cartilage are types II (95%), IX and XI (Table 1) [7, 8]. Type II is the principal constituent of collagen fibrils and provides tensile strength. Type XI is present in small amounts and is associated with type II in collagen fibrils. During cartilage development, type XI fibers may influence the formation of type II collagen and limit collagen fibril size [9]. Type IX (1-2%) is found crosslinked to type II on the surface of these collagen fibrils and limits diameter growth of fibrils, interacts with other matrix components (such as PGs), and prevents fibril-fibril interaction [9]. Intermolecular crosslinks between and within the fibrils stabilize the structure and prevent most proteolytic cleavage of collagen [10, 11]. Types VI, X, XII, and XIV are also present in small amounts but little is known about their function in articular cartilage [7, 8]. Type VI collagen is located in the pericellular matrix of articular cartilage and may form an anchoring network linking the cells to the collagen fibrils in the ECM [12]. Enrichment with type VI collagen was observed in human osteoarthritic cartilage [13]. Type X collagen is almost exclusively associated with hypertrophic chondrocytes in growth plate cartilage. In articular cartilage, type X is located pericellularly in chondrocytes in the zone of calcified cartilage below the tidemark [14]. However, in human osteoarthritic cartilage a marked increase in type X expression, restricted to fibrillated osteoarthritic cartilage and repair cartilage, was observed [15]. Types XII and XIV are associated closely with collagen fibrils, but their function in adult

Table 1. Collagen types found in articular cartilage

Collagen Type	Comments
II	Major collagen of articular cartilage; forms principal molecule of collagen fibrils
IX	Located on surface of major collagen fibrils; potentially 1) limit growth in diameter of fibrils 2) interact with other matrix components 3)prevent fibril-fibril interaction
XI	Probably located within same fibrils as type II collagen; function unknown
X	Present in hypertrophic cartilage during development and in deep calcified zone of articular cartilage; function unclear but appears to be localized with the collagen fibrils in noncalcified region of hypertrophic cartilage
VI	Present in mammalian articular cartilage; forms microfibrils that may serve linking function between larger collagen fibrils and cell surface
XII	Structurally related molecules present in small amounts in mammalian articular cartilage and in many other connective tissue
XIV	Related structurally to type IX collagen but of unknown function

From: Mayne, R. R.G. Brewton. Extracellular Matrix of Cartilage,1993

articular cartilage is unknown.

Proteoglycans are responsible for hydration, swelling pressure and compressive strength of articular cartilage [16]. Aggrecan (Figure 2), the primary PG in cartilage, is a protein with regions rich in specific glycosaminoglycan side chains (GAGs) including keratan sulfate (KS), chondroitin sulfate (CS), dermatan sulfate (DS), and heparin sulfate (HS). The protein core of aggrecan consists of three globular domains, G1, G2 and G3. The G1 domain associates with hyaluronic acid (HA, another GAG) [17]. Each attachment is stabilized by link protein, a non-collagenous protein [18]. This association occurs at specific intervals throughout HA and forms a network of PGs. Between the G2 and G3 domains, there is an area of GAG attachment regions [19]. Adjacent to the G2 domain, the KS attachment region is found. Between this region and the G3 region CS chains are highly concentrated [19]. GAGs are synthesized within the cell (golgi) and HA is synthesized in a compartment associated with the cell membrane and elongated into the ECM [20]. HA then associates with aggrecan and link protein outside the cell, creating part of the ECM [21, 22].

PGs have GAG highly sulfated disaccharide chains that attract water into the tissue for cushion and support. GAGs can absorb up to 50 times their weight in water. The tensile strength of collagen constrains this hydration. The GAGs are composed of the following specific sugar repeats (Figure 3):

HA- N-acetyl-D-glucosamine (GlcNac) and glucuronic acid

CS/DS- n-acetyl-D-galactosamine and glucuronic acid

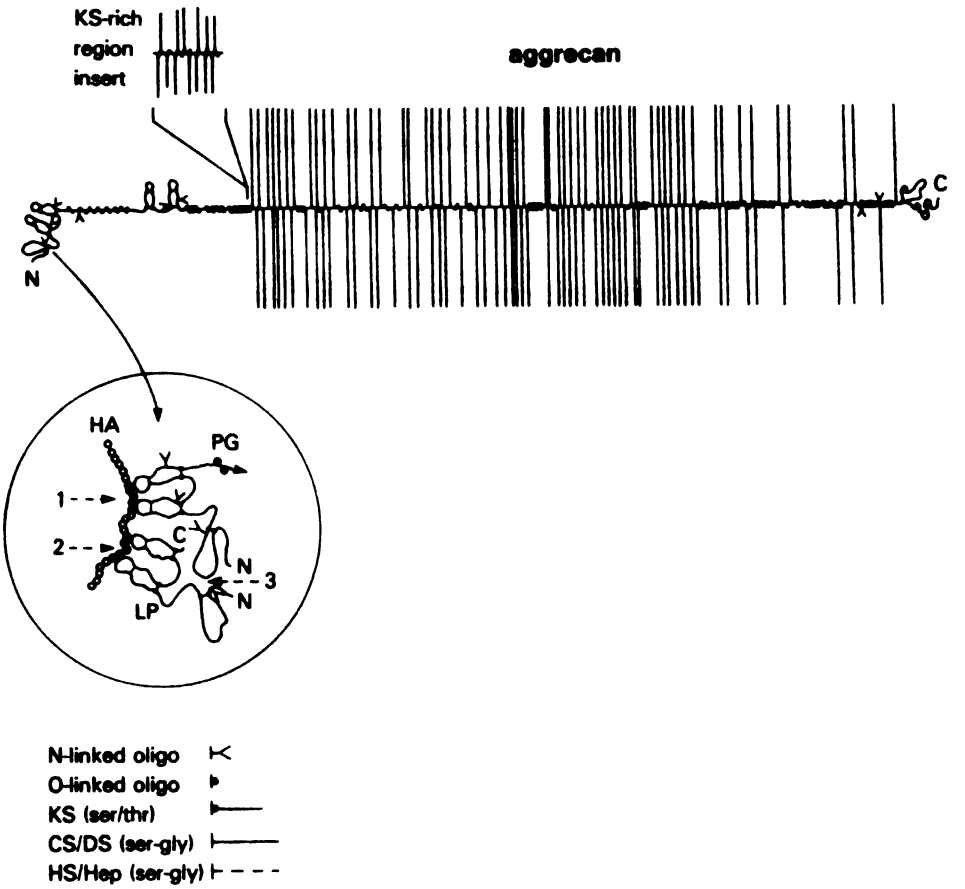

Figure 2. Schematic drawing of the mature proteoglycan aggrecan. The protein core and associated glycosaminoglycan side chains are illustrated. The link protein interaction with HA is also indicated in the circle. (From: Fife, R.S. and K.S. Brandt. Extracellular matrix of cartilage: Glycoproteins. 1993)

KS- GlcNac and galactose

HS- GlcNac and glucuronic acid.

The primary function of non-collagenous proteins is to regulate the interaction of chondrocytes with the ECM. These proteins include chondronectin, fibronectin, anchorin, 59kDa protein, 54kDa protein, and thrombospondin [23] (Table 2). Link protein is a non-collagenous protein that binds to PGs, stabilizing aggregates and protecting PGs from proteolysis [22].

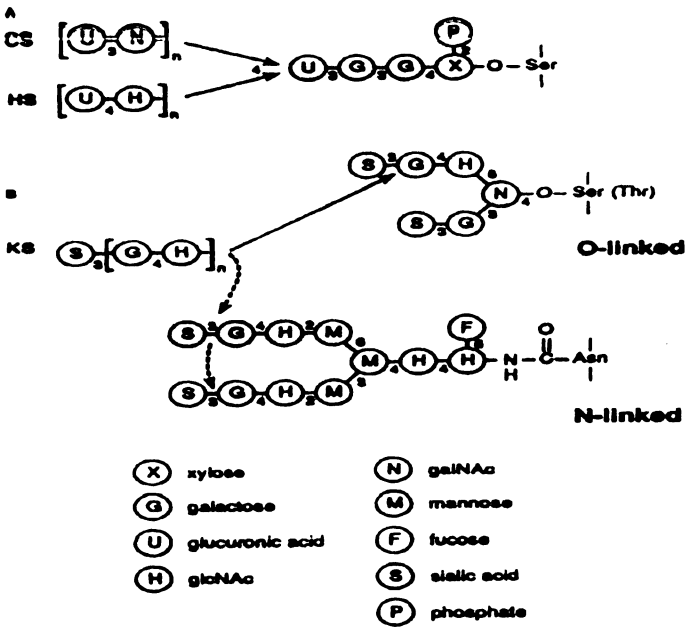

Figure 3. Panel A shows the linkage common to all CS/DS and HS side chains and KS side chains (Panel B). The sugars are indicated in the key. (From: Fife, R.S. and K.S. Brandt. Extracellular matrix of cartilage: Glycoproteins. 1993)

Table 2. Noncollagenous proteins found in articular cartilage

Protein	Function
Link protein	Binds to proteoglycans and hyaluronan, stabilizes aggregates, protects proteoglycans from proteolysis
Chondronectin	Adhesion of chondrocytes to type II collagen surfaces
Fibronectin	Adhesion of cells to molecules, surfaces
Thrombospondin	Adhesion, binds calcium
Anchorin CII	Binds type II collagen
Structural Proteins	
69,000-Da protein	Binds hydroxyapatite, (?) mineralization
59,000-Da protein	Binds collagen
54,000-Da protein	Binds type II collagen
36,000-Da protein	Unknown
Chondrocyte membrane receptors	C-terminus of type II collagen, calcification of growth plate

From: Fife, R.S. and K.S. Brandt. Extracellular matrix of cartilage: Glycoproteins. 1993

Turnover of ECM

In normal cartilage, ECM proteins are degraded and synthesized at a balanced rate. Collagen turnover rate in adult articular cartilage is estimated at 150-350 years [24] depending on species, as opposed to 300 days for proteoglycans [25]. Collagen stability is related to the covalent intermolecular cross-links in and among the collagen fibrils. These cross-links prevent enzymatic cleavage. Chondrocytes control the ECM turnover by the production of specific proteinases, proteinase inhibitors, and growth factors. Proteinases include the metalloproteinases and serine, cysteine and aspartic proteinases (Table 3).

Matrix metalloproteinases degrade ECM at neutral pH and play a major role in ECM turnover. The MMP family of proteinases includes collagenases 1 and 3 (MMP-1, MMP-13), gelatinases (MMP-2 and -9) and stromelysin (MMP-3). These proteinases have specific inhibitors, tissue inhibitor metalloproteinases (TIMPs), which are secreted to regulate degradation [26]. In normal articular cartilage there is a slight excess of TIMP relative to metalloproteinases [27]. Collagenase and stromelysin are the MMPs of primary interest in articular cartilage degradation. Collagenase-1 is specific to collagen and cleaves types II, VII, and X but does not cleave types VI, IX and XI [28, 29]. However, collagenase-3 has been shown to cleave collagen types I, II, and III [30] and the PG aggrecan [31]. Stromelysin cleaves a wide variety of substrates including

proteoglycans [32], link protein [33], and collagen types II, IX and XI [34]. Stromelysin also activates the proenzyme form of collagenase [35].

The serine, cysteine, and aspartic proteinases play a relatively smaller but still important role in ECM turnover. Serine proteinases are thought to activate MMPs [36]. In addition, elastase and cathepsin G possess potent PG degrading activity [37] and inhibition of PG synthesis [38]. Two cysteine proteinases, cathepsin B and L, can cleave collagen cross-links [39], PGs [37] and link protein [40]. Calpain, another cysteine proteinase, has also been shown to degrade PG's [41].

Two factors known to increase the rate of ECM turnover are joint load and cytokines. Joint loads including weight-bearing and running exercise increase cartilage stiffness and thickness [42]. Moderate load increases PG and protein synthesis but heavy loads inhibit PG and protein synthesis [43, 44]. The cytokines IL-1 and TNF-α upregulate metalloproteinase secretion by chondrocytes [45, 46], whereas IGF-1 and TGF-β growth factors can offset the effects of IL-1 [47, 48]. The delicate balance of synthesis and degradation is critical for cartilage maintenance; any change can be detrimental to the integrity of the matrix and induce disease.

Table 3. Proteinases of significance, their substrate, and inhibitors in the degradation of articular cartilage.

Enzyme	Substrate	Inhibitor
Tissue Collagenase (MMP-1)	Types I, II, III, VII, X collagen (not IX, XI)	TIMP, TIMP-2
Gelatinase (MMP-2)	Denatured type II collagen Collagen type XI, X	TIMP, TIMP-2
Gelatinase (MMP-9)	Collagen IV, V	TIMP, TIMP-2
Stromelysin (MMP-3)	Aggrecan, fibronectin, types IX, XI collagen, procollagens, link protein, decorin, elastin	TIMP
Serine Proteinases		
Plasmin	MMPs	α_2-antiplasmin
Neutrophil elastase	Types I, II, IX, X, XI collagen, aggrecan	α_1-proteinase inhibitor (PI)
Cathepsin G	TIMP, aggrecan, elastin, type II collagen	PI
Cysteine Proteinases		
Cathepsin B	Procollagenase, type II collagen, aggrecan, link protein	Cystatin
Calpain	Proteoglycan	Calpastatins
Aspartic Proteinases		
Cathepsin D	Aggrecan, denatured type II collagen	α_2-macroglobulin

From: Poole, A.R. Cartilage in Health and Disease. 1993.

Osteoarthritis

Osteoarthritis is characterized by progressive and permanent degeneration of articular cartilage. Specifically, biochemical changes occur that cause an apparent imbalance in the normal ECM turnover process; degradation is greater than synthesis [49, 50]. The major factor appears to be increased proteolytic enzyme activity, which is responsible for ECM degradation [51, 52]. Specific changes include a decreased PG content [53], changes in PG structure [54], and increased water content [55, 56]. In addition, collagen fibers become fragmented [19]. This degradation of articular cartilage results in decreased compressive and tensile stiffness [3].

OA commonly develops in previously normal joints that are damaged by a variety of factors. These factors can include developmental disorders such as osteochondrosis [57], sudden [58] and repetitive injury to a joint or cartilage surface [59, 60], joint infections, and/or medications that alter cartilage integrity. The onset of structural degradation can begin at any age (after damage) and the factors discussed earlier likely influence the rate of cartilage degradation.

Initially, in diseased cartilage, matrix synthesis increases in an attempt to compensate for the increased degradation [61]. As the disease progresses, the synthesis eventually decreases [53]. IL-1, which is elevated in OA cartilage and synovial fluid, increases matrix degradation and inhibited PG synthesis [62]. IL-1 may mediate this process by up-regulating a variety of enzymes including inducible nitric oxide synthase [63], which increases the production of nitric oxide

(NO), by chondrocytes [64]. NO, in turn, can depress the synthesis of PGs [65] and may activate MMPs [66].

Upon gross inspection, OA cartilage may exhibit dimples or linear infoldings, fibrillation, and finally yellowish discoloration, dullness and ulcerations as the disease progresses [12]. The clinical manifestations of the structural and biochemical changes associated with OA are primarily joint pain and decreased mobility [67]. In elderly humans, OA is the primary lower extremity disease [68]; the estimated cost of OA in the US alone is $15.5 billion [69]. The symptoms of OA are also the primary reason for decreased equine performance, although the magnitude of the pain and the severity of the disease may not be correlated.

Equine OA has been classified into three types. Type 1 is associated with synovitis and capsulitis (common in high motion joints such as the carpus); type 2 is associated with other identifiable injuries or problems; and type 3 is due to incidental or nonprogressive erosion [70]. Potential causes of OA include physical forces and biomaterial failure, failure of chondrocyte response to insult, and extra-articular injuries that cause articular cartilage problems secondarily. OA is a common cause of lameness in racing horses and horses that perform in other athletic events. The greatest number of days lost to race training was caused by lameness, a large portion of which is due to joint disease [2].

Treatments

Adult equine articular cartilage has little potential for adequate repairing the original tissue [43, 71]. However, many factors can affect the repair process including the location and depth of the injury, the size, depth and location of the resulting lesion, and the age of the animal [72, 73]. Logically, large defects, full thickness lesions and lesions in the weight bearing region are less likely to heal. Under these conditions, the biochemical composition of the repair tissue is not normal. For example, the new tissue may be composed of more type I than type II collagen [43]. Because collagen type I is not normally found in cartilage, it does not possess properties suitable for cartilage function. In addition, decreased GAG content is observed [74]. Such structural alterations of the tissue make it unsuitable as replacement because it lacks durability [72]. For these reasons, emphasis has been placed on finding a surgical, chemical, or nutritional way to treat or prevent OA. Past treatments have included rest (natural repair), surgical joint re-surfacing, intra-articular or intramuscular injections of corticosteroids, non-steroidal anti-inflammatory agents and polysulfated glycosaminoglycans (PSGAGs), and nutritional supplements such as PSGAGs and glucosamine.

In most cases, natural repair is not an effective treatment of OA [75]. Instead, attention has turned to joint resurfacing and/or use of anti-inflammatory drugs. Surgical resurfacing is costly, risky, and its validity as a treatment has not yet been established. Most attempts at resurfacing appear successful initially but

ultimately fail [76]. Even if surgery is successful, the new tissue can be soft and lack some of the properties of normal cartilage [77, 78]. Anti-inflammatory drugs such as steroids have been used to treat the symptoms associated with OA but have not been documented to aid in repairing the joint surface. In addition, the drugs are also costly, require administration by medical or veterinary professionals, and the long-term use of steroids has many side effects unrelated to joint health [79]. Injections of PSGAGS have been reported to result in side-effects which include local hematomas, blood pressure changes and hair loss [80].

Evidence suggests that loss of up to 30% of the visible articular surface will not compromise return of horses to racing [81]. Therefore, attention has turned to the use of oral nutritional supplements to aid in the prevention of further matrix loss due to OA or the complete prevention of the onset of OA. Oral administration of these supplements is cheaper and easier for horse owners than previously discussed methods. These compounds include PSGAGs and glucosamine which have been used in human medicine for the treatment of OA. PSGAGs supplements consist of primarily chondroitin sulfate. These compounds are just beginning to be investigated for their potential equine uses.

Oral absorption of most PSGAGs supplements have not been studied, however oral absorption of chondroitin sulfate has been studied and the results are conflicting [82, 83]. Baici *et al.* [84] evaluated the oral administration of chondroitin sulfate and found that neither intact nor depolymerized chondroitin sulfate was absorbed. Oral supplementation of PSGAGs in rats enhanced

cartilage proteoglycan biosynthesis and maintenance [85]. In horses, PSGAGs decreased PG synthesis in osteoarthritic [86] and normal cartilage explants [87]. More recently, the use of an oral supplement containing chondroitin sulfate and glucosamine HCl (Cosequin) is being marketed. Based on the questionable absorption of chondroitin sulfate and its effects on cartilage, attention has turned to the potential use of glucosamine as a treatment for osteoarthritis.

Glucosamine

Glucosamine is an amino sugar formed by the transfer of the amide nitrogen of glutamine to the C-2 carbon of fructose. The formation of glucosamine is thought to be self-regulated through inhibition of the amidotransferase enzyme, which is the rate-limiting step specific to this pathway. The rate-limiting enzyme is glutamine: 6-P-fructose amino transferase (GFAT). High GFAT activity is present in rabbit epiphyseal cartilage, corneal epithelium, rat liver, beef and calf lung, beef kidney cortex and rabbit intestine. Costal and tracheal cartilage show only low GFAT activity, and many tissues show no measurable activity. Available evidence concerning the localization of GFAT indicates that glucosamine is formed at the site of its occurrence. Therefore, it is unlikely that glucosamine is transported from a central site of metabolism.

Glucosamine is not found in the free form in nature. It is either modified by acetylation to N-acetyl-D-glucosamine (GlcNac) or sulfation to glucosamine-sulfate. In culture, free glucosamine is rapidly converted to intracellular UDP-N-acetyl-glucosamine. N-acetyl-glucosamine is found in articular cartilage and

formed from glucosamine by the following reaction: glucosamine-6-phosphate→GlcNac-6-phosphate↔GlcNac-1-phosphate→UDP-GlcNac→GlcNac. The UDP-GlcNac serves as a substrate for protein glycosylation and is essential for the synthesis of HA , KS and HS. The UDP is cleaved and the GlcNac is added to the growing disaccharide of the GAG. GlcNac is also a component of N- and O-linked oligosaccharides found on aggrecan. They are also important for the elongation of KS on aggrecan and the formation of oligosaccharides.

Glucosamine can be purchased in one of three forms: glucosamine HCl; glucosamine sulfate; and N-acetyl-D-glucosamine. The HCl and sulfate forms both dissolve in the stomach and are readily absorbed in the small intestine. Setnikar *et al.* [88] administered radioactive glucosamine to dogs and followed its distribution. It diffused rapidly from the blood stream into the body and was actively taken up by the liver, kidney and articular cartilage. Oral dosing with glucosamine thus seems an appropriate method of therapeutic administration at least in monogastric species.

Glucosamine as potential treatment of OA

Companies marketing products containing of glucosamine claim that oral administration of this sugar relieves the symptoms of OA. They suggest that glucosamine stimulates articular cartilage regeneration or repair because it is a building block for articular cartilage tissue. During the diseased state there may be an increased demand for "building blocks" of cartilage metabolism.

Supplemental glucosamine may also simply bypass rate limiting steps in glucosamine metabolism and lead to an increase in available "building blocks" for extracellular matrix molecules. In addition, it has been hypothesized that altered glucosamine metabolism may lead to OA.

Yet, the knowledge needed to justify the biochemical basis for the use of glucosamine as a treatment of OA is limited. To date, evidence in support of these claims is limited to a few published reports. When given orally to human OA patients, glucosamine decreased joint pain and improved mobility [89]. In addition, drug tolerance to glucosamine sulfate was similar when control groups were given either ibuprofen [90, 91] or a lactose placebo [92]. One difference between ibuprofen and glucosamine sulfate treatment was that it took only two weeks for ibuprofen to alleviate pain compared to four weeks for glucosamine sulfate [91]. However, the pain returned during the trial with ibuprofen treatment. In horses suffering from clinical signs of OA, an orally administered glucosamine-chondroitin sulfate compound appeared to alleviate symptoms more rapidly than expected [93]. However, this study is limited in its conclusions because no horses with OA served as untreated controls. *In vitro*, glucosamine was shown to have favorable effects on cartilage metabolism, including a reduction in articular cartilage breakdown and stimulation of synthesis of matrix components by chondrocytes [1]. However, information regarding the efficacy of this supplement for the prevention of OA is non-existent, especially in the case of horses.

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Chapter 2

GLUCOSAMINE REDUCES EQUINE ARTICULAR CARTILAGE DEGRADATION IN EXPLANT CULTURE

Summary

Objective. To determine whether glucosamine inhibits experimentally induced degradation of equine articular cartilage explants.

Materials and Methods. Articular cartilage was obtained from the antebrachio-carpal and middle joints of horses (2-8 years old) sacrificed for reasons unrelated to lameness. Cartilage discs were harvested from the weight-bearing region of the articular surface and cultured. Media were exchanged daily and the recovered media stored at 4°C. Explants were maintained in basal media 2 days prior to the start of 4 treatment days. On days one through four lipopolysaccharide (LPS, 10 μg/ml) or recombinant human interleukin-1ß (rhIL-1ß, 50 ng/ml) were added to induce cartilage degradation. To test the potential protective effects of glucosamine, the compound was added in three concentrations (0.25, 2.5, or 25 mg/ml) and treatments were performed in triplicate. Controls included wells without LPS, rhIL-1ß, or glucosamine. Nitric oxide, proteoglycan and matrix metalloproteinases (MMP) released into conditioned media and tissue proteoglycan synthesis were measured as indicators of cartilage metabolism.

Results. Maximal nitric oxide production, proteoglycan release, and MMP activity were detected one day after the addition of LPS or rhIL-1ß to the media.

The addition of 25 mg/ml of glucosamine prevented the increase in nitric oxide production, proteoglycan release and MMP activity induced by LPS or rhIL-1ß.

Conclusions. These data indicate that glucosamine can prevent experimentally induced cartilage degradation *in vitro*.

Introduction

In elderly humans, osteoarthritis (OA) is the primary lower extremity disease [1] with estimated cost in the US alone of $15.5 billion annually [2]. The symptoms of OA are also a principal cause of lameness and decreased performance in horses. OA is characterized by progressive and permanent degeneration of articular cartilage and commonly develops in previously normal joints that are damaged by a variety of factors. These factors can include developmental disorders [3], sudden [4] and repetitive injury to a joint or cartilage surface [5,6], joint infections, and/or medications that alter cartilage integrity.

OA is the result of biochemical changes which cause an imbalance in normal cartilage extracellular matrix turnover with degradation exceeding synthesis [7,8]. Increased proteolytic enzyme activity is a major factor responsible for matrix degradation [9,10]. Specific changes include a decreased proteoglycan (PG) content [11], changes in PG structure [12], and increased water content [13,14]. In addition, collagen fibers become fragmented [15]. This degradation of articular cartilage results in decreased compressive and tensile stiffness [16].

Initially, in diseased cartilage, matrix synthesis increases in an attempt to compensate for increased degradation [17]. However, as the disease progresses, matrix synthesis eventually decreases. Interleukin-1 (IL-1), a cytokine present in elevated amounts in OA cartilage and synovial fluid, is an important mediator of increased matrix degradation and reduced PG synthesis [18]. IL-1 mediates this process by a number of processes including up-regulating enzymes such as inducible nitric oxide synthase [19], and matrix metalloproteinases (MMPs). Enhanced production of nitric oxide (NO) by chondrocytes [20] depresses the synthesis of PGs [21] and may activate MMPs [22]. Equine chondrocytes have recently been shown to produce nitric oxide in response to two arthritogenic molecules, lipopolysaccharide (LPS) or recombinant interleukin-1ß (rhIL-1ß) [23].

Oral supplements that prevent cartilage degradation and/or joint injury are an attractive potential solution for the treatment of OA in humans and domestic animals. Glucosamine is a potential anti-arthritic compound currently being marketed. It is a naturally occurring, non-toxic compound that when given orally has been shown to decrease pain and improve mobility in osteoarthritic joints in a number of studies in humans [24]. In addition, drug tolerance to glucosamine sulfate was similar when control groups were given either ibuprofen [25, 26] or a lactose placebo [27]. One difference between ibuprofen and glucosamine sulfate treatment was, that ibuprofen alleviated pain after only 2 weeks compared with 4 weeks for glucosamine sulfate. However, the ibuprofen treated group experienced renewed symptoms during the trial period [25]. Controlled clinical

studies of the efficacy of glucosamine in horses are lacking. However, in horses suffering from clinical signs of OA, an orally administered glucosamine-chondroitin sulfate compound appeared to alleviate OA symptoms more rapidly than expected [28]. The majority of clinical studies have evaluated the ability of glucosamine to relieve pain and have not evaluated its ability to inhibit cartilage degradation. Admittedly this is difficult to accomplish *in vivo*.

In vitro, glucosamine reduced articular cartilage extracellular matrix degradation and stimulated of synthesis of matrix components by chondrocytes [29], potentially alleviating the imbalance between synthesis and degradation characteristic of OA. Recently, glucosamine was shown to inhibit aggrecanase, a suspected MMP, activity *in vitro* [30]. Largely anecdotal support for clinical efficacy of glucosamine, paralleled by limited biochemical studies, suggest a need for further study of the effects of this molecule. In the present study, we tested whether glucosamine inhibits experimentally induced cartilage degradation as indicated by NO production, PG release, MMP production and PG synthesis in equine articular cartilage explants. We report that glucosamine inhibits the release of these indicators of cartilage degradation. Our results support the hypothesis that glucosamine has the potential to prevent or reduce articular cartilage degradation.

Materials and Methods

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

EXPLANT CULTURES

Articular cartilage was obtained from the antebrachio-carpal and middle carpal joints of horses (2-8 years old) sacrificed for reasons other than joint problems. Cartilage discs (3.5mm) were biopsied from the weight-bearing region of the articular surface. Randomly selected explant discs (3 per well, 40-60 mg total) were cultured in a 24-well Falcon culture plate (Fisher Scientific; Pittsburgh, PA) with a modified version of Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Ham) (1:1) (Gibco; Grand Island NY) [31]. The media was supplemented with 10% fetal bovine serum (FBS, Gibco), 50 μg/ml ascorbate and 100 units/ml penicillin/streptomycin (Gibco). The explants were maintained in culture in a humidified incubator with 7% CO_2 at 37°C.

Explants were maintained in media without serum for 2 days prior to the first of 4 treatment days. The nine treatments and three controls established are outlined in table 4. Conditioned media was removed and replaced daily and stored at 4°C until analyzed for indicators of degradation. LPS (10 μg/ml) or rhIL-1 (50 ng/ml) (R&D Systems Inc; Minneapolis, MN) was added to induce cartilage degradation. To evaluate the effects of glucosamine, varying

Table 4. Detailed description of components of explant culture treatments*

Treatment	FBS	LPS	rhIL-1	Glucose	Glucosamine (GLCN)
FBS-Control 1	10%				
LPS-Control 2	10%	10 μg/ml			
rhIL-1-Control 3	10%		50 ng/ml		
Treatment 1	10%	10 μg/ml		0.21 mg/ml	
Treatment 2	10%	10 μg/ml		2.1 mg/ml	
Treatment 3	10%	10 μg/ml		21.0 mg/ml	
Treatment 4	10%	10 μg/ml			0.25 mg/ml
Treatment 5	10%	10 μg/ml			2.5 mg/ml
Treatment 6	10%	10 μg/ml			25.0 mg/ml
Treatment 7	10%		50 ng/ml		0.25 mg/ml
Treatment 8	10%		50 ng/ml		2.5 mg/ml
Treatment 9	10%		50 ng/ml		25.0 mg/ml

*FBS=fetal bovine serum; LPS=lipopolysaccharide; rhIL-1=recombinant human interleukin-1

concentrations of glucosamine HCl (0.25, 2.5, or 25 mg/ml) or glucose control (0.21, 2.1 or 21.0 mg/ml; equimolar basis to glucosamine) were added to the cultures. Treatments were performed with triplicate wells using tissue from one animal donor. Experiments were repeated at least five times, each time using tissue from a different animal donor.

LACTATE DEHYDROGENASE ASSAY

Lactate dehydrogenase activity in the conditioned media was measured using a commercial cytotoxicity detection kit (Boehringer Mannheim; Indianapolis, IN). Lactate dehydrogenase was measured as an indicator of chondrocyte viability during culture [32].

PROTEOGLYCAN ASSAY

PG release into conditioned media was measured using a dimethylmethylene blue assay as previously described [33]. Briefly, PG content was determined by measuring sulfated glycosaminoglycan content compared to a chondroitin sulfate standard and expressed as μg PG/well. Tissue PG content was determined following papain digest (1μg papain/ mg tissue) of the tissue. Tissue PG content was expressed as μg PG/mg tissue wet weight.

NITRIC OXIDE ASSAY

Nitrite, a stable end-product of nitric oxide metabolism, was measured in conditioned media using the Greiss reaction and sodium nitrite as a standard [34]. Absorbance at 540 nm was determined using the SpectraMax 300 plate reader (Molecular Devices, Sunnyvale, CA).

MATRIX METALLOPROTEINASE ASSAY

MMP activity was detected in the conditioned media and cartilage extracts using the EnzChek Collagenase/Gelatinase Assay Kit (Molecular Probes; Eugene, OR). Conditioned media or cartilage extracts were incubated with DQ-gelatin in reaction buffer (0.5 *M* Tris-HCl, 1.5 *M* NaCl, 50 m*M* $CaCl_2$, 2 m*M* Sodium azide, pH 7.6) for 1 hour at room temperature. Enzymatic release of fluorescent signal from DQ-gelatin by either gelatinase or collagenase was quantitated using the Cytofluor 4000 fluorescent plate reader (PerSeptive Biosystems; Framingham, MA). Inhibition of gelatinase/collagenase activity was performed by the addition of 10 m*M* 1,10-phenanthroline to the reaction buffer. Collagenase from *Clostridium histolyticum* was used as a standard. Tissue gelatinase/collagenase content was extracted with 1M guanidine-HCl, 50 mM Tris and 10 mM Calcium chloride [35], dialyzed into reaction buffer and gelatinase/collagenase activity of the sample was determined as described above. Results were presented as units of enzyme activity/ml/hr.

QUANTIFICATION OF PROTEOGLYCAN SYNTHESIS

PG synthesis was quantified by measuring the incorporation of radioactive sulfur into PGs. Media containing treatments and 25 μCi of [^{35}S]-sulfate/ml (ICN, Costa Mesa, CA) were added to explants and cultured for 24 hours. PGs were extracted from explant tissue at 4°C for 72 hours with 4 M guanidine hydrochloride including proteinase inhibitors [36]. Proteoglycans were isolated using cetylpyridinium chloride (CPC) precipitation [37, 38]. Briefly, 100 μl of conditioned media or tissue extract was spotted onto chromatography paper and incubated with 0.1% CPC and 0.3 M NaCl. Chromatography paper was dried and assayed for radioactivity, using a liquid scintillation system. Results were presented as counts per minute per milligram of cartilage wet weight (cpm/mg).

STATISTICAL ANALYSIS

Data for indicators of degradation (NO production, PG release and MMP activity) were analyzed using the repeated measure option of the SAS (1996) statistical software PROC MIXED [39]. No interaction of day was present so all data were represented as the average response of the 3 treatment wells at 24 hours after treatment addition. Treatments were compared using the Bonferroni procedure. Synthesis data were normalized by an inverse transformation and analyzed using PROC GLM [39]. Treatments were compared using the least

squares difference procedure. Statistical significance was considered at $p<0.05$ unless noted.

Results

A successful equine explant culture system was developed using discs of articular cartilage and a modified media designed for chondrocytes [31]. Lack of LDH activity indicated that chondrocytes were viable throughout the culture period. Both LPS (10 μg/ml) and rhIL-1 (50 ng/ml) induced cartilage degradation. Addition of either LPS or rhIL-1 to the culture system resulted in a significant increase in NO production, PG release, and gelatinase/collagenase activity relative to the control medium. The effect of glucosamine or glucose control was tested using this degradative system.

Effect of glucosamine on LPS induced cartilage degradation

Glucosamine was added at three doses (0.25, 2.5 25.0 mg/ml) to the LPS-induced degradative system. When tested against LPS treatment, glucosamine significantly inhibited NO production at 2.5 and 25.0 mg/ml (Figure 4), and also inhibited PG release at all three doses (Figure 5). The highest dose of glucosamine (25.0 mg/ml) significantly inhibited PG release compared to the two lower doses (0.25 and 2.5 mg/ml) (Figure 5). Glucosamine (25.0 mg/ml) also significantly inhibited tissue PG synthesis compared to all other treatments

(Figure 6). At the same glucosamine dose, tissue PG content was significantly increased (Figure 7). All doses of glucosamine significantly decreased gelatinase/collagenase activity; activity was completely inhibited at 25.0 mg/ml glucosamine using the described method (Figure 8). MMP activity was not detected in tissue extracts.

Glucose, a sugar moiety control, was added at 3 doses (0.21, 2.1 and 21.0 mg/ml) to the LPS induced degradative system. Glucose did not significantly inhibit LPS induced NO production (Figure 4) or PG release (Figure 5) at any dose. In addition, glucose did not alter PG synthesis when compared to FBS (Figure 6).

EFFECT OF GLUCOSAMINE ON RHIL-1 INDUCED CARTILAGE DEGRADATION

Glucosamine was added at three doses (0.25, 2.5 25.0 mg/ml) to the rhIL-1 induced degradative system. Glucosamine significantly inhibited NO production at 2.5 and 25 mg/ml when compared to rhIL-1; the low dose (0.25 mg/ml) did not influence NO production (Figure 9). Glucosamine also inhibited PG release at 25.0 mg/ml when compared to rhIL-1 and the two lower doses did not inhibit PG release (Figure 10).

Discussion

These data provide biochemical evidence supporting the purported chondroprotective properties of glucosamine and also suggest disease-modifying characteristics of the compound. A chondroprotective agent can be defined as a substance that increases chondrocyte anabolic activity while simultaneously suppressing the effect of mediators (cytokines, prostaglandins, and proteinases) on cartilage degradation [29]. Proteolytic degradation of cartilage matrix is generally considered the hallmark of OA. A disease-modifying drug such as glucosamine should reduce degradation either by inhibiting proteinases or by inhibiting the activity of mediators such as IL-1. Using indicators of chondrocyte metabolism (NO production, PG release, PG synthesis and MMP activity), we have shown that glucosamine (25mg/ml) inhibits *in vitro* cartilage degradation induced by either LPS or the cytokine IL-1. We feel confident that these responses were due to glucosamine and were not an artifact of high sugar moieties in the media. Compared to the LPS treatment, glucose treatment at any dose did not decrease NO production, PG release, MMP activity or PG synthesis.

Several explanations may account for the poorly understood action of glucosamine. One mechanism by which glucosamine may prevent cartilage matrix degradation is inhibition of NO production. Nitric oxide may be an important regulatory molecule in the cartilage matrix degradation cascade, by mediating some of the inhibitory effects of cytokines on matrix synthesis. The presence of increased NO concentrations in the synovial fluid of diseased joints

indicates that NO is involved in articular cartilage degradation. In addition, NO is up-regulated in human OA cartilage [34]. This increased NO level has been proposed to regulate matrix degradation by inducing chondrocyte apoptosis [34], inhibiting cell proliferation [40], and activating MMPs [22]. In our system, cartilage degradation induced by either LPS or rhIL-1 resulted in increased NO production and MMP activity, both inhibited by glucosamine. NO production may have been inhibited through a variety of mechanisms including regulation of the production or activity of NO synthase. Alternatively, supplemental glucosamine may simply quench increased NO or act as an antioxidant and prevent MMP degradation of the cartilage matrix. The latter explanation is similar to that reported by Homandberg *et al.* (1997), who found that the antioxidant N-acetylcysteine suppressed IL-1 mediated damage to articular cartilage and promoted PG repair *in vitro*.

A second mechanism by which glucosamine may affect the onset or progression of OA is through the direct inhibition of MMPs. MMPs are implicated in the OA process as the main family of enzymes responsible for cartilage matrix degradation. The importance of MMPs in OA is evidenced by their elevated activity in OA cartilage. We have shown that glucosamine is a potent inhibitor of LPS and rhIL-1 induced gelatinase/collagenase activity at all doses (0.25, 2.5 25.0 mg/ml) tested. The mechanism remains unclear, but inhibition of expression, synthesis or activity of MMPs are all possible. Sandy *et al.* (1998) have shown that glucosamine, like mannosamine, is an effective inhibitor of aggrecanase, a suspected MMP. Both are effective inhibitors of

glycosylphosphatidylinositol anchor synthesis and the authors suggest that the aggrecanase catabolic unit may possess a glycosylphosphatidylinositol-linked component responsive to glucosamine. Tetracyclines, by interfering with MMP ability to bind divalent cations, have also been shown to beneficially affect cartilage and bone loss by inhibiting MMP activity [42]. These drugs may also reduce the production of MMPs by inhibition of NO synthase [43].

Alteration of chondrocyte metabolism by glucosamine was also evidenced by changes in PG metabolism. Glucosamine inhibited both PG degradation and synthesis. At first, the reduced rate of PG synthesis may appear detrimental to the matrix of articular cartilage. However, we observed a slight increase in tissue PG content at the highest dose of glucosamine compared to all other treatments. These data indicated that, although synthesis was decreased, turnover was maintained or PG accretion was favored. PG synthesis might not have been stimulated because growth factors such and IGF-1 and FGF were not proteolytically released from the surrounding extracellular matrix. Thus, if PG degradation is not occurring, PG synthesis is not up-regulated. Most *in vitro* studies in which glucosamine increased PG synthesis used OA cartilage. In our study, however, we used normal cartilage, which may explain the disparity between our results and others.

Finally, in damaged cartilage, glucosamine production by chondrocytes may be limited and supplementation of high levels of glucosamine may restore normal metabolism. Some have suggested that in OA joints the demand for "building block" molecules might exceed the tissues' ability to produce them [44].

Providing glucosamine exogenously could bypass rate-limiting steps in the synthesis of molecules that inhibit degradation or restore normal metabolism. To date, this hypothesis has not been tested.

Although compounds other than glucosamine can inhibit cartilage degradation, most are not practical for preventing or treating OA. For example, N-acetylcysteine and DMSO were shown by Homandberg *et al.* (1997) to inhibit cartilage degradation. However, if taken orally these compounds would be toxic. Glucosamine possesses many qualities that make it an ideal disease-modifying or chondroprotective agent. These qualities include: wide availability, relatively low cost, absence of known side-effects, and high oral absorption rate and ready transfer into cartilage. Although the most effective dose tested in this study (25mg/ml) may be a pharmacological dose, glucosamine is transported via the glucose transport system and has been shown to accumulate in articular cartilage. Over extended oral dosing it may be possible to achieve high concentrations *in vivo*. Because it is difficult to study cartilage *in vivo*, biochemical evidence supporting the action of glucosamine is limited. Our *in vitro* data substantiate anecdotal *in vivo* observations. Further research to elucidate the mechanism through which glucosamine may exert chondroprotective properties in articular cartilage is under investigation.

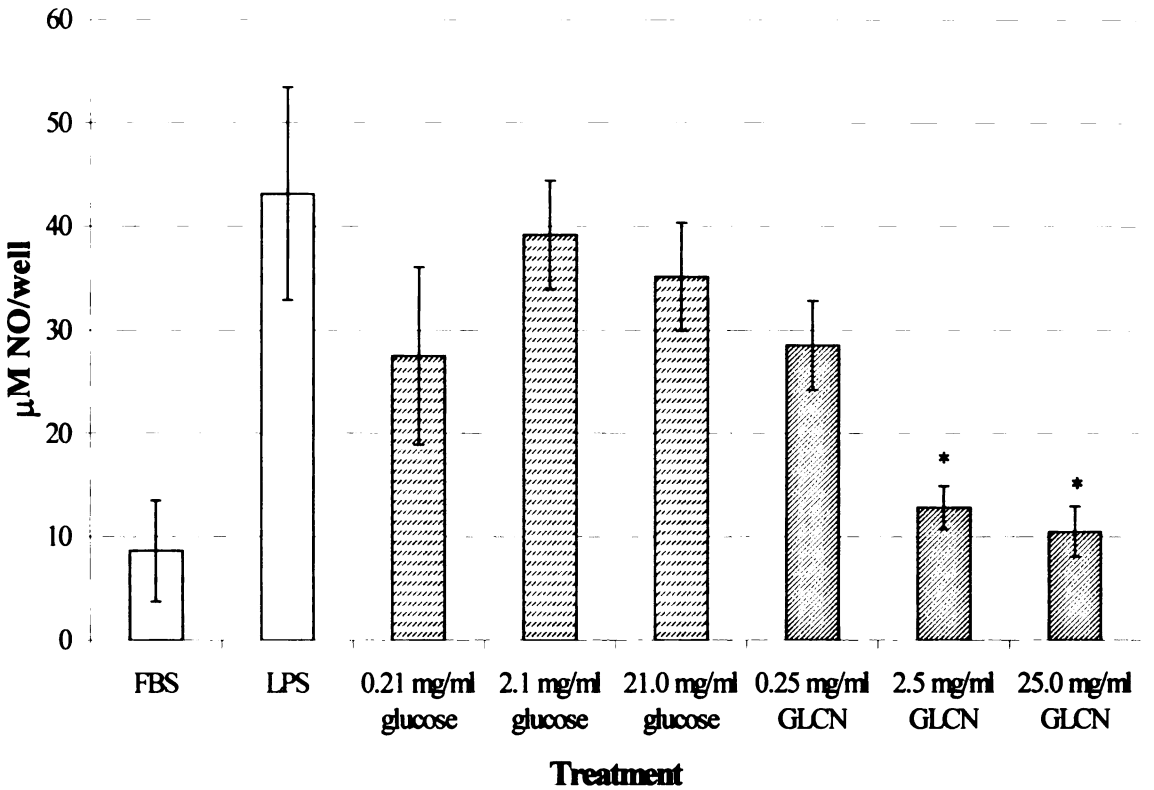

Figure 4. Mean (± SD) nitric oxide production released into the conditioned media 24 hours after LPS induced degradation treated with glucose or glucosamine. * indicates statistical significance at $p \leq .05$ compared to LPS and 0.25 mg/ml glucosamine (GLCN). FBS=fetal bovine serum; LPS=lipopolysaccharide

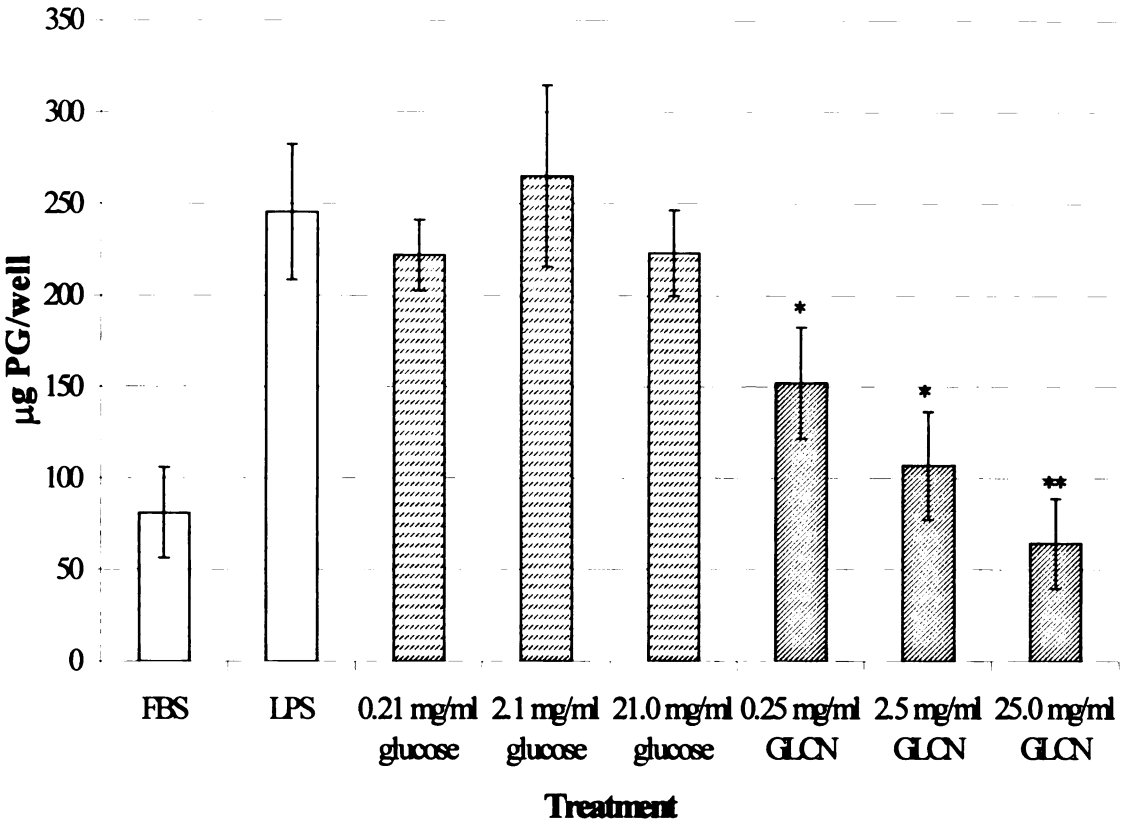

Figure 5. Mean (± SD) proteoglycan production released into the conditioned media 24 hours after LPS induced degradation treated with glucose or glucosamine. * indicates statistical significance at p≤.05 compared to LPS. ** indicates statistical significance at p≤.05 compared to other doses of glucosamine (GLCN). FBS=fetal bovine serum; LPS=lipopolysaccharide

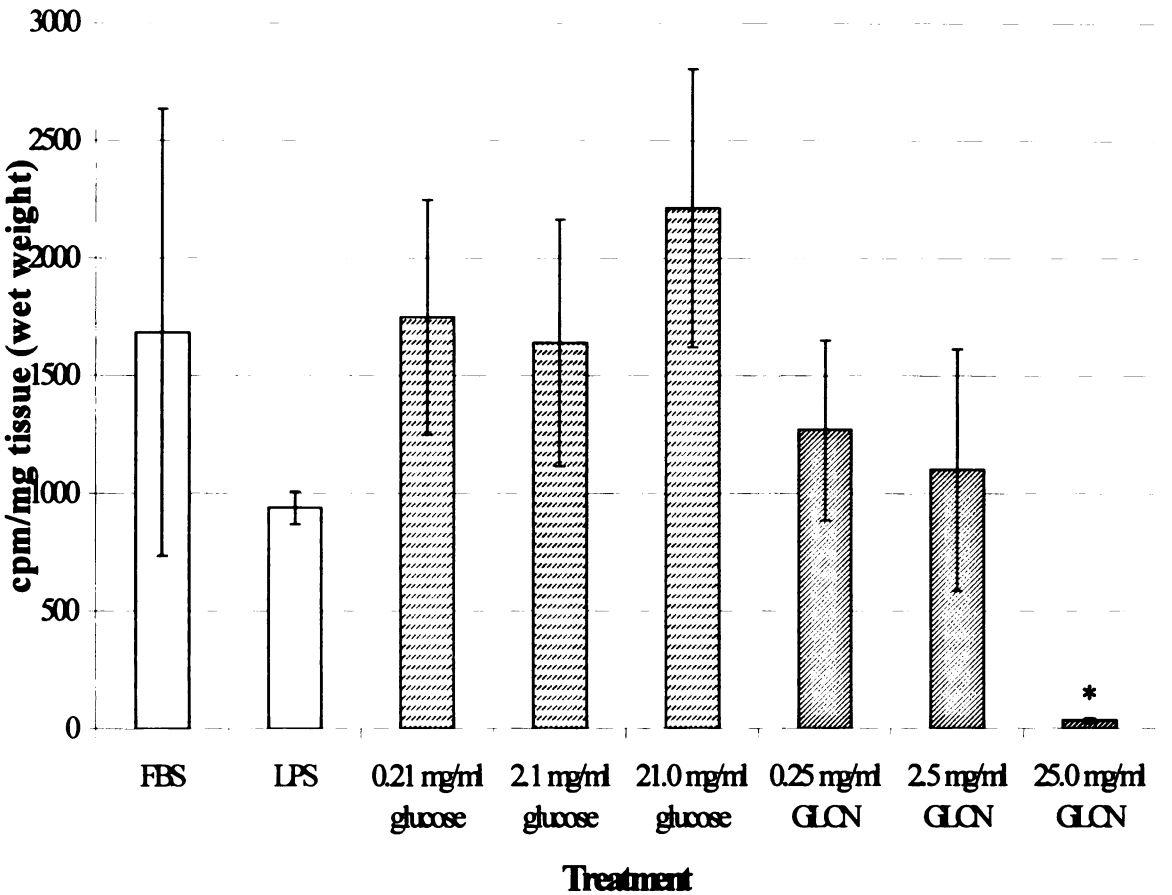

Figure 6. Mean (± SD) tissue proteoglycan synthesis as indicated by counts per minute/mg tissue (wet weight) 24 hours after LPS induced degradation treated with glucosamine. * indicates statistical significance at $p \leq .001$. FBS=fetal bovine serum; LPS=lipopolysaccharide

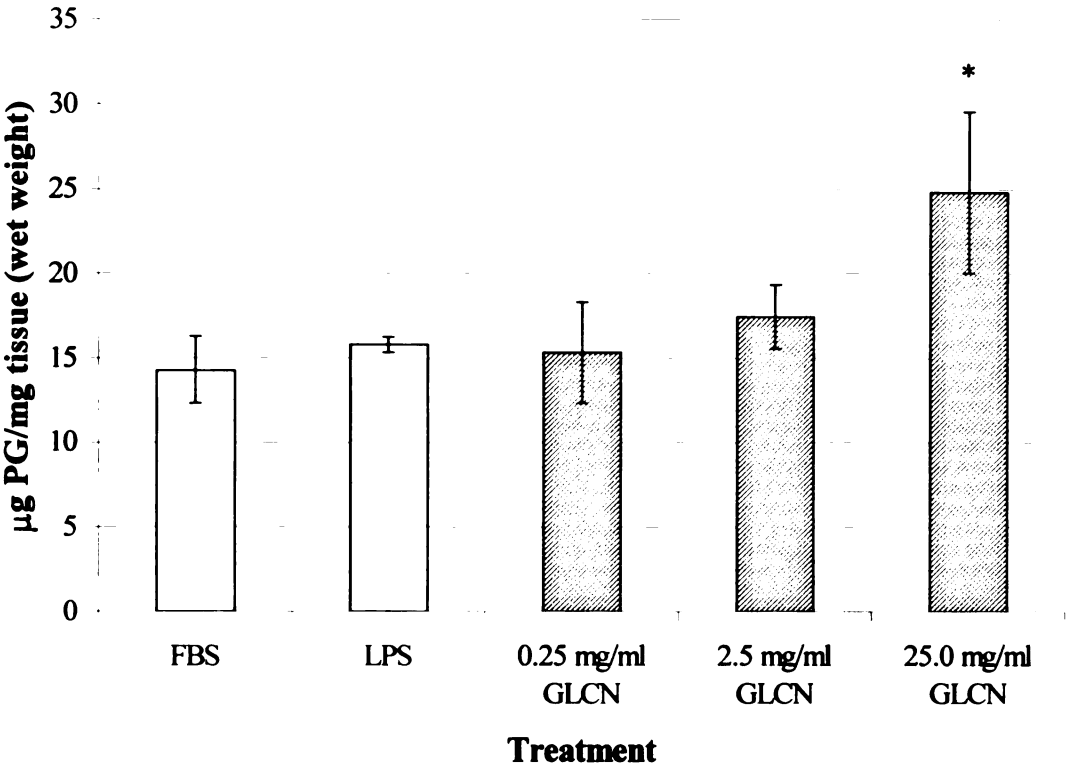

Figure 7. Mean (± SD) proteoglycan content of tissue remaining upon completion of culture period. Tissue samples were papain digested prior to quantification of proteoglycans. * indicates statistical significance at $p \leq .05$ compared to other treatments. GLCN=glucosamine; FBS=fetal bovine serum; LPS=lipopolysaccharide

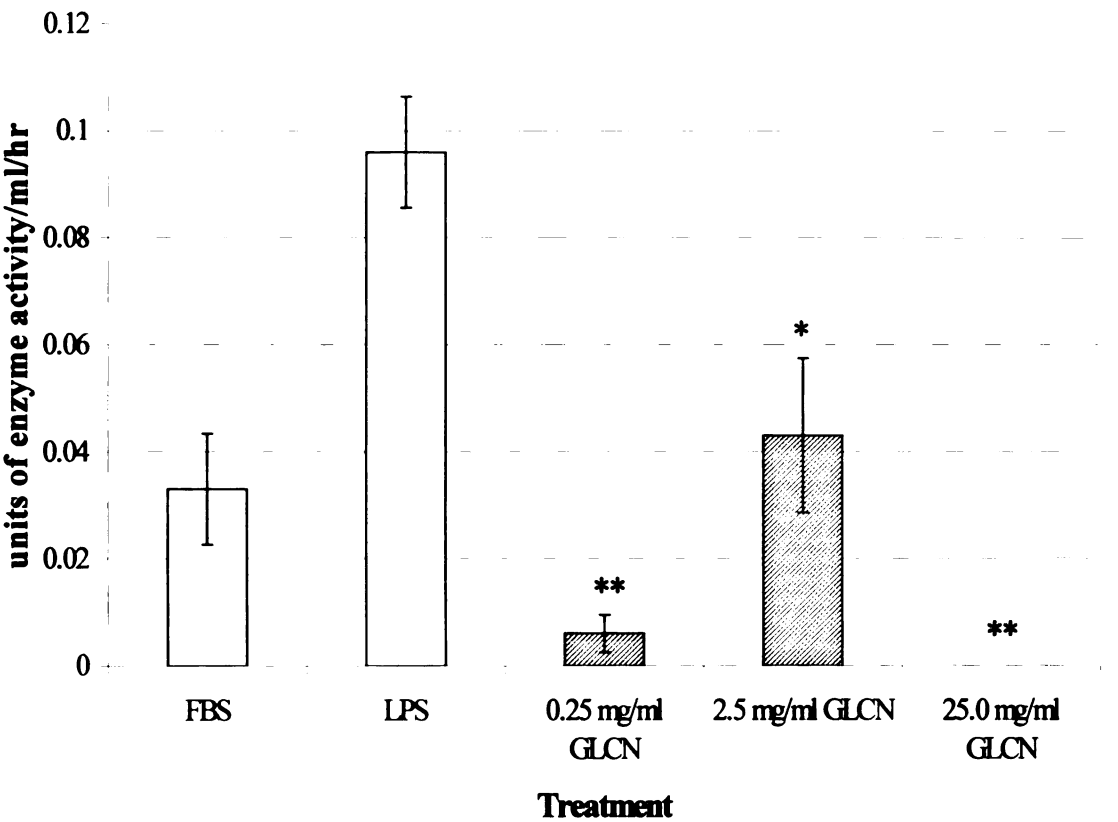

Figure 8. Mean (± SD) gelatinase/collagenase activity released into the conditioned media 24 hours after LPS induced degradation treated with glucosamine. * indicates statistical significance at p≤.05 compared to LPS. ** indicates statistical significance at p≤.001 compared to other doses of glucosamine (GLCN).

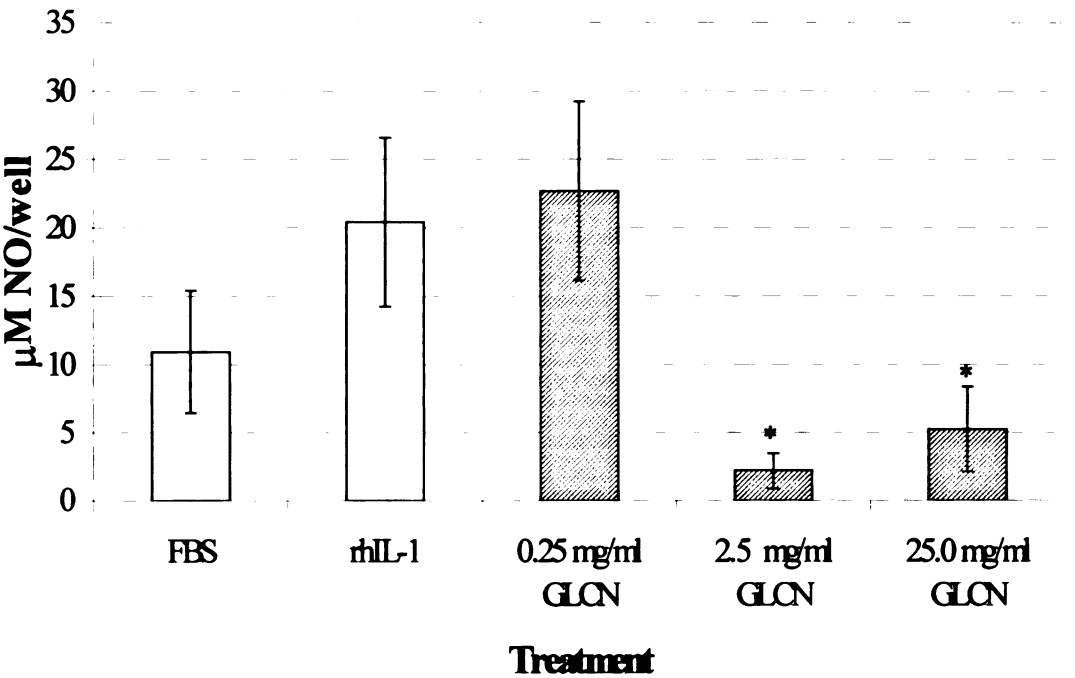

Figure 9. Mean (± SD) nitric oxide production released into the conditioned media 24 hours after rhIL-1 induced degradation treated with glucosamine. * indicates statistical significance at $p \leq .05$ compared to rhIL-1 and 0.25 mg/ml glucosamine (GLCN). FBS=fetal bovine serum; rhIL-1=recombinant human interleukin-1

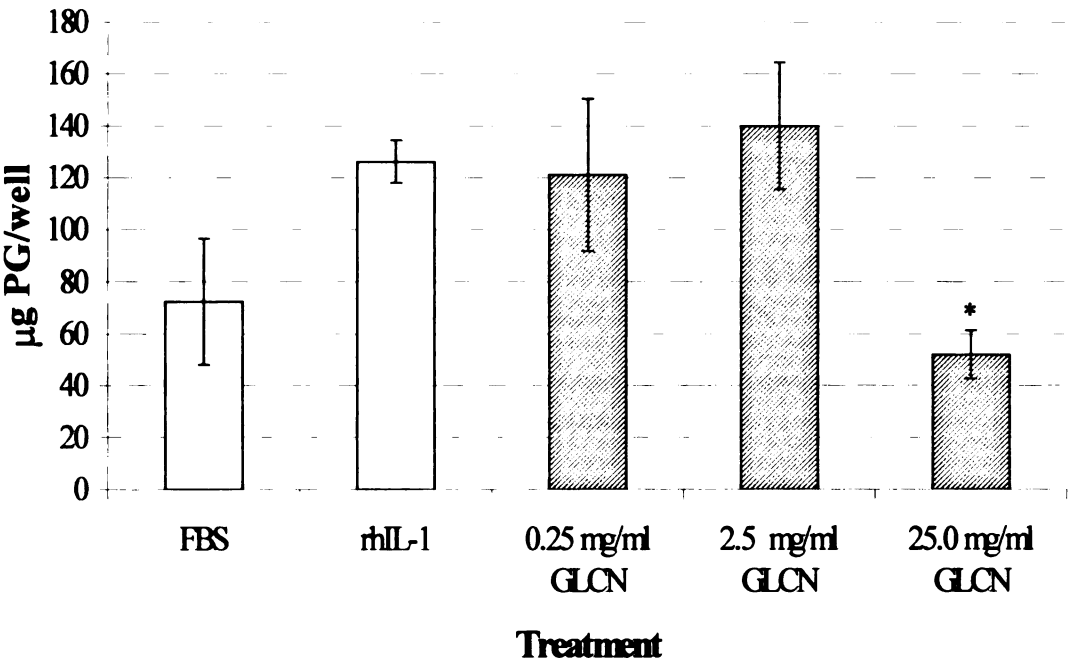

Figure 10. Mean (± SD) proteoglycan production released into the conditioned media 24 hours after rhIL-1 induced degradation treated with glucosamine. * indicates statistical significance at $p \leq .05$ compared to rhIL-1 and other doses of glucosamine (GLCN). FBS=fetal bovine serum; rhIL-1=recombinant human interleukin-1

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Chapter 3

THE EFFECTS OF GLUCOSAMINE DERIVATIVES ON EQUINE ARTICULAR CARTILAGE DEGRADATION IN EXPLANT CULTURE

Summary

Objective. To determine whether glucosamine-3-sulfate, glucose-3-sulfate (control) and N-acetyl glucosamine inhibit experimentally induced degradation of equine articular cartilage explants similar to glucosamine HCl.

Materials and Methods. Articular cartilage was obtained from the antebrachio-carpal and middle joints of horses (2-8 years old) sacrificed for reasons unrelated to lameness. Cartilage discs were harvested from the weight-bearing region of the articular surface and cultured. Media were exchanged daily and the recovered media stored at 4°C. On days 1 and 2 lipopolysaccharide (LPS, 10 μg/ml) was added to induce cartilage degradation. To evaluate the effects of different sources of glucosamine (on an equal molar basis), varying concentrations of glucosamine HCl (0.25, 2.5, or 25 mg/ml), glucosamine-3-sulfate (0.304, 3.04, or 30.4 mg/ml), or N-acetyl-glucosamine (0.256, 2.56, or 25.6 mg/ml) were added to the cultures. The glucose-3-sulfate control was added at 0.3075, 3.075 or 30.75 mg/ml. Nitric oxide and proteoglycan released into conditioned media and tissue proteoglycan synthesis and total tissue PG content were measured as indicators of cartilage metabolism.

Results. Glucosamine-3-sulfate consistently inhibited cartilage degradation in a manner similar to glucosamine HCl, while the effects of N-

acetyl-glucosamine were highly variable and did not inhibit cartilage degradation. Glucose-3-sulfate did not inhibit cartilage degradation.

Conclusion. Our results indicate that glucosamine sulfate also has the potential to prevent or reduce articular cartilage degradation similar to glucosamine HCl *in vitro*. The amine group at the carbon-2 position appears important for the effectiveness of the glucosamine derivative. The therapeutic value of N-acetyl-glucosamine remains questionable.

Introduction

Osteoarthritis (OA), characterized by the progressive degradation of articular cartilage, is a debilitating lower extremity disease in both humans and animals. Degradation of cartilage in joints can cause joint inflammation, pain, decreased mobility, and reduced performance of athletic humans, horses and dogs. Once clinical signs of OA are detectable, the disease is considered irreversible. Therefore, medical attention has turned to means of preventing OA, treating symptoms, and slowing the progression of the disease. Traditional treatments including surgery, non-steroidal anti-inflammatory drugs, or corticosteroids have had limited success and are often accompanied by significant side-effects preventing long-term use. The medical/veterinary community has thus been forced to explore nontraditional treatments of this disease including many nutritional supplements purported to ameliorate the symptoms of OA.

Recently, glucosamine has become a popular nutritional supplement for the treatment of OA in humans. It is a naturally occurring, non-toxic compound that when given orally has been shown to decrease pain and improve mobility in osteoarthritic joints of humans [1]. In horses suffering from clinical signs of OA, an orally administered glucosamine HCl-chondroitin sulfate compound appeared to alleviate OA symptoms [2]. Similar findings have been observed in dogs treated with an oral glucosamine HCl-chondroitin sulfate supplement [3]. Most recently, our lab has shown that glucosamine HCl can prevent equine articular cartilage degradation *in vitro* (see concurrent paper).

Glucosamine is commercially available in one of three forms: glucosamine HCl; glucosamine sulfate; and N-acetyl-D-glucosamine. The HCl and sulfate forms both dissolve in the stomach and are readily absorbed in the small intestine. Once in the blood stream, each form is equally bioavailable independent of origin. Setnikar *et al.* [4] administered radioactive glucosamine HCl to dogs and followed its distribution; it diffused rapidly from the blood stream into the body and was actively taken up by the liver, kidney and articular cartilage. In humans, the pharmacokinetics of glucosamine are similar to those of dogs and rats [5]. Therefore, oral dosing with glucosamine seems an appropriate method of therapeutic administration.

The majority of clinical studies on oral glucosamine supplementation have been performed with glucosamine sulfate in humans [6, 7] and glucosamine HCl in animals (horse and dog primarily). Both sources elicit favorable responses and lack significant side-effects. Glucosamine HCl is even being included in

sporting dog food for "good joint health". N-acetyl-glucosamine is found in many commercially available glucosamine complex supplements but has not been studied clinically. Most commercially available supplements contain a combination of the three sources of glucosamine even though glucosamine sulfate is the only source clinically studied in humans. We used our recently developed equine cartilage degradation system to study these compounds *in vitro*. The objective of this research was to evaluate the relative effectiveness of the glucosamine derivatives on preventing cartilage degradation *in vitro*. We report that glucosamine sulfate consistently inhibited cartilage degradation in a manner similar to glucosamine HCl, while the effects of N-acetyl-glucosamine were highly variable and generally did not inhibit cartilage degradation.

Materials and Methods

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

EXPLANT CULTURES

Articular cartilage was obtained from the antebrachio-carpal and middle carpal joints of horses (2-8 years old) sacrificed for reasons other than joint problems. Cartilage discs (3.5mm) were biopsied from the weight-bearing region of the articular surface. Randomly selected explant discs (3 per well, 40-60mg

total) were cultured in a 24-well Falcon culture plate (Fisher Scientific; Pittsburgh, PA) with a modified version of Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Ham) (1:1) (Gibco; Grand Island NY)[8]. The media was supplemented with 10% fetal bovine serum (FBS, Gibco), 50 μg/ml ascorbate and 100 units/ml penicillin/streptomycin (Gibco). The explants were maintained in culture in a humidified incubator with 7% CO_2 at 37°C.

Explants were maintained in media without serum for 2 days prior to the first of 4 treatment days. Conditioned media was removed and replaced daily and stored at 4°C until analyzed for indicators of degradation. Lipopolysaccharide (LPS) was added (10 μg/ml) to induce cartilage degradation on day 0. The effect of glucosamine-3-sulfate and N-acetyl-D-glucosamine on LPS induced cartilage degradation compared to glucosamine HCl was evaluated. Varying concentrations of glucosamine HCl (0.25, 2.5, or 25 mg/ml), glucosamine-3-sulfate (0.3075, 3.075 or 30.75 mg/ml; equimolar basis to glucosamine) and N-acetyl-D-glucosamine (0.256, 2.56 or 25.6 mg/ml; equimolar basis to glucosamine) were added to the cultures. In a concurrently published study we showed that a glucose control did not inhibit cartilage degradation. In this study, glucose-3-sulfate (0.327, 3.27 or 32.7 mg/ml) was tested as a control for the glucose moiety of glucosamine-3-sulfate and to evaluate the importance of the amine group of glucosamine. Treatments were performed with triplicate wells using tissue from one animal donor. Treatments are listed in table 5.

Table 5. Detailed description of components of explant culture treatments*

Treatment	FBS	LPS	Glucose-3-sulfate	Glucosamine-3-sulfate	N-acetyl-glucosamine
FBS-Control 1	10%				
LPS-Control 2	10%	10 μg/ml			
Treatment 1	10%	10 μg/ml	0.327 mg/ml		
Treatment 2	10%	10 μg/ml	3.27 mg/ml		
Treatment 3	10%	10 μg/ml	32.7 mg/ml		
Treatment 4	10%	10 μg/ml		0.3075 mg/ml	
Treatment 5	10%	10 μg/ml		3.075 mg/ml	
Treatment 6	10%	10 μg/ml		30.75 mg/ml	
Treatment 7	10%	10 μg/ml			0.256 mg/ml
Treatment 8	10%	10 μg/ml			2.56 mg/ml
Treatment 9	10%	10 μg/ml			25.6 mg/ml

*FBS=fetal bovine serum; LPS=lipopolysaccharide

Experiments were repeated at least three times, each time using tissue from a different animal donor.

PROTEOGLYCAN ASSAY

Proteoglycan (PG) release into conditioned media was measured using a dimethylmethylene blue assay as previously described [9]. Briefly, PG content was determined by measuring sulfated glycosaminoglycan content compared to a chondroitin sulfate standard and expressed as μg PG/well. Tissue PG content was determined following papain digest (1μg papain/ mg tissue) of the tissue. Tissue PG content was expressed as μg PG/mg tissue wet weight.

NITRIC OXIDE ASSAY

Nitrite, a stable end product of nitric oxide (NO) metabolism, was measured in conditioned media using the Greiss reaction and sodium nitrite as a standard [10]. Absorbance at 540nm was determined using the SpectraMax 300 plate reader (Molecular Devices, Sunnyvale, CA).

QUANTIFICATION OF PROTEOGLYCAN SYNTHESIS

PG synthesis was quantified by measuring the incorporation of radioactive sulfur into PGs. Media containing treatments and 25 μCi of [^{35}S]-sulfate/ml (ICN,

Costa Mesa, CA) were added to explants and cultured for 24 hours. PGs were extracted from explant tissue at 4°C for 72 hours with 4 M guanidine hydrochloride including proteinase inhibitors [11]. Proteoglycans were isolated using cetylpyridinium chloride (CPC) precipitation [12, 13]. Briefly, 100 μl of conditioned media or tissue extract was spotted onto chromatography paper and incubated with 0.1% CPC and 0.3 M NaCl. Chromatography paper was dried and assayed for radioactivity, using a liquid scintillation system. Results were presented as counts per minute per milligram of cartilage wet weight (cpm/mg).

STATISTICAL ANALYSIS

Data for indicators of degradation (NO production, and PG release) were analyzed using the repeated measures option of the SAS statistical software PROC MIXED [14]. No interaction of day was present so all data were represented as the average response of the 3 treatment wells at 24 hours after treatment addition. Treatments were compared using the Bonferroni procedure. Synthesis data were normalized by an inverse transformation and analyzed using PROC GLM [14]. Treatments were compared using the least squares difference procedure. Statistical significance was considered at $p<0.05$ unless noted.

Results

Preliminary experiments indicated that the maximal release of NO and PG into the conditioned media was 24 hours after the addition of LPS. Therefore, all data are represented as 24 hours after treatment. In a concurrently published article, we have shown that glucosamine HCl inhibits LPS induced cartilage degradation and the glucose control did not.

EFFECT OF GLUCOSAMINE SULFATE ON LPS INDUCED CARTILAGE DEGRADATION

Glucosamine-3-sulfate was added at three doses (0.3075, 3.075 30.75 mg/ml) to the LPS-induced cartilage degradative system. When tested against LPS treatment, glucosamine-3-sulfate significantly inhibited NO production at 3.075 and 30.75 mg/ml (Figure 11a), and also inhibited PG release at the highest dose (Figure 11b). Additionally, glucosamine-3-sulfate (30.75 mg/ml) significantly inhibited tissue PG synthesis compared to all other treatments (Figure 11c) consistent with glucosamine HCl treatment (see concurrently published paper for data).

Glucose-3-sulfate, a sugar moiety control, was added at three doses (0.327, 3.27 and 32.7 mg/ml) to the LPS-induced degradative system. Glucose-3-sulfate did not significantly inhibit LPS induced NO production (Figure 12a) and PG release (Figure 12b) consistent with glucose treatment (see concurrently published paper for data). At the highest dose of glucose-3-sulfate, the increase

in NO and PG was statistically significant. Therefore, total tissue PG content was measured after glucose-3-sulfate treatment to determine if there was a significant degradation of tissue PG's. At the highest dose of glucose-3-sulfate there was a trend ($p<.1$) for decreased PG content (Figure 12c).

EFFECT OF N-ACETYL-GLUCOSAMINE ON LPS INDUCED CARTILAGE DEGRADATION.

N-acetyl-glucosamine was added at three doses (0.256, 2.56 25.6 mg/ml) to the LPS induced degradative system. N-acetyl-glucosamine did not inhibit NO production (Figure 13a), or PG release (Figure 13b). Although not significantly different, NO and PG release appeared to slightly increase with dose. Therefore, tissue PG content was determined to further indicate degradation. Tissue PG content did not change as a result of N-acetyl-glucosamine treatment (Figure 13c). PG synthesis was not determined due to lack of significant changes in other indicators of cartilage metabolism.

Discussion

This study is the first to compare the three common commercially available sources of glucosamine (glucosamine-3-sulfate, N-acetyl-glucosamine and glucosamine HCl) using an *in vitro* model for cartilage degradation. In a concurrently published article, we have shown that glucosamine HCl inhibits LPS or interleukin-1 induced cartilage degradation as indicated by decreased PG and

NO release and matrix metalloproteinase (MMP) activity, while the glucose control did not and discussed potential mechanisms. Using that *in vitro* model we show that glucosamine-3-sulfate consistently inhibits cartilage degradation in a manner similar to glucosamine HCl, while the effects of N-acetyl glucosamine were highly variable and did not inhibit cartilage degradation. The glucose-3-sulfate control also did not inhibit cartilage degradation.

Comparing these five compounds (glucosamine-3-sulfate, N-acetyl glucosamine, glucosamine HCl, glucose and glucose-3-sulfate) indicates the importance of the free amine group related to the bioactivity of glucosamine. Glucosamine-3-sulfate and glucose-3-sulfate, as well as glucosamine (HCl) and glucose differ only by the $-NH_2$ group substitution for the –OH on the second carbon of the six-carbon backbone (Figure 14). In both glucosamine-3-sulfate and glucosamine HCl the amine group exists in a free form. However, in nature the amine group does not exist in this free form: it is usually acetylated or sulfated. Interestingly, the acetylated form of glucosamine, N-acetyl glucosamine, did not inhibit cartilage degradation in our system suggesting the importance of the reactive amine group for bioactivity of glucosamine. Future research will focus on other amine sugars to determine if this bioactivity is unique only to glucosamine.

In the concurrently published paper we discuss some possible mechanisms of action of glucosamine. Among these is the possibility that glucosamine may simply quench small signal molecules including NO and oxygen radicals that can damage articular cartilage [15]. This explanation seems

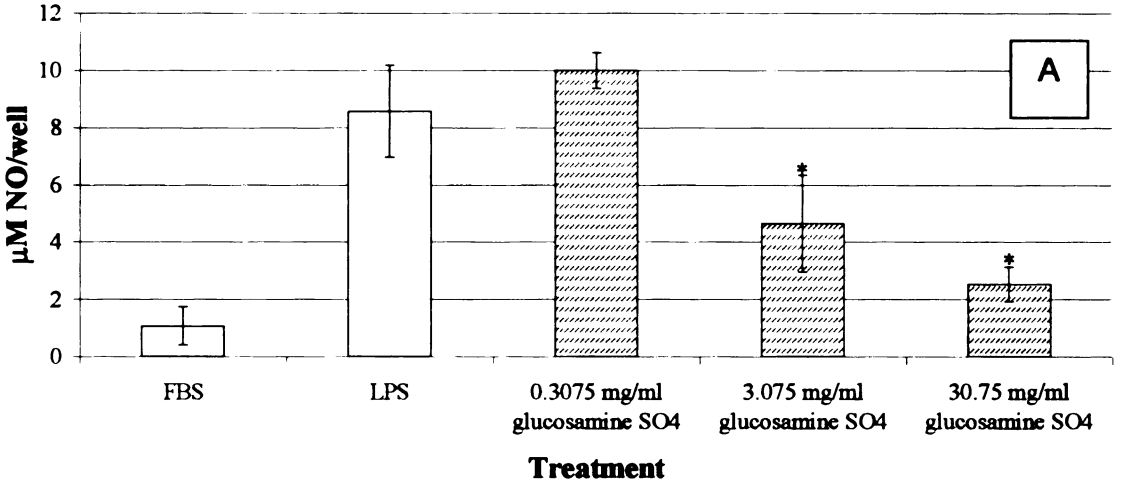
plausible due to the lack of inhibition of cartilage degradation observed by N-acetyl glucosamine. Glucosamine may also mediate matrix metalloproteinase degradation of cartilage, often thought of as the hallmark of OA. Glucosamine has been shown to inhibit aggrecanase activity, a putative MMP, in IL-1 stimulated bovine cartilage explants [16]. This inhibition was apparently not due do to any cytotoxicity or interference with IL-1 signaling, since protein synthesis and lactate production were not altered by glucosamine. In agreement with this conclusion, we were not able to detect any decrease in cell viability as measured by lactate dehydrogenase (data not shown).

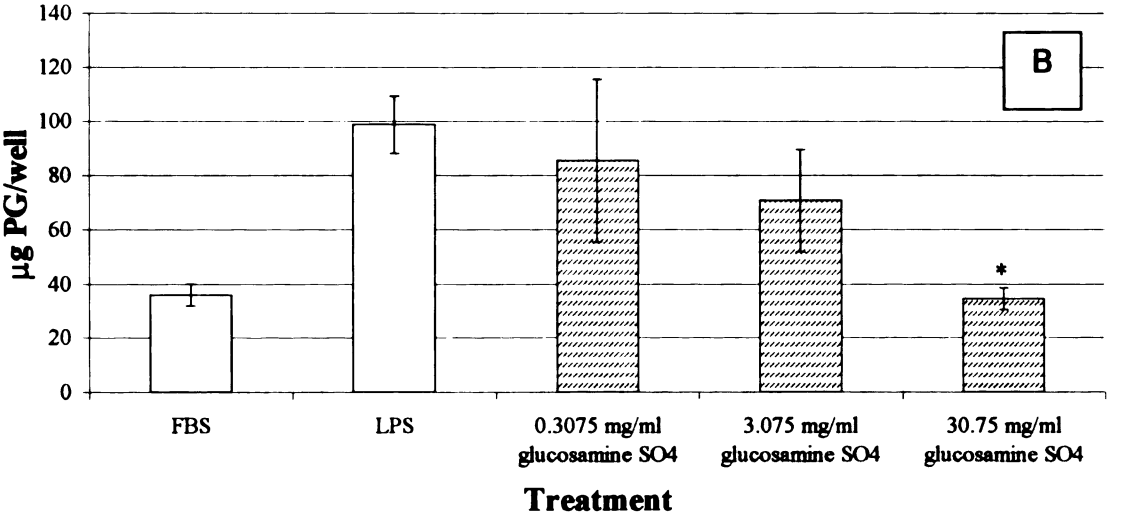
During the diseased state there may be an increased demand for "building blocks" of cartilage metabolism. Supplemental glucosamine may also simply bypass rate limiting steps in glucosamine metabolism and lead to an increase in available "building blocks" for extracellular matrix molecules. Addition of glucosamine to cell culture resulted in an increase in intracellular UDP-N-acetylglucosamine [17]. This compound is critical in the formation of glycosaminoglycans found in cartilage including hyaluronic acid, heparin sulfate, and keratan sulfate. Supplemental glucosamine may act to reduce symptoms of OA by increasing the production of hyaluronic acid in synovial fluid [18]. Intra-articular injection of hyaluronic acid has been shown to alleviate the symptoms of OA [19]. HA has also been shown to prevent fibronectin mediated cartilage injury *in vivo* [20] and *in vitro* [21].

Our *in vitro* data support the reported therapeutic value of glucosamine sulfate and glucosamine HCl. However, we do not show any effect of N-acetyl

glucosamine on the prevention of cartilage degradation. Lack of FDA regulation of compounds classified as nutrients allows for a variety of sources of glucosamine in nutritional supplements whether or not they are proven effective or have been tested clinically. Although investigations of glucosamine HCl and glucosamine sulfate conducted so far indicate beneficial effects, the medical community has not embraced this as a treatment for OA. Many of the studies have serious flaws [22] and limited data are available regarding the adverse effects or potential drug interactions of glucosamine. Further research to elucidate its mode of action and to evaluate its long-term efficacy in humans and animals such as horses is necessary to validate its preventative and therapeutic value in OA. Several combinations of glucosamine and its derivatives are commercially available. Our research suggests that their relative "effectiveness" in improving joint health could depend on the composition of the supplements, with glucosamine HCl and glucosamine sulfate being more important than N-acetyl-glucosamine.

Figure 11 (on next page). Panel **A** depicts mean (± SD) nitric oxide production released into the conditioned media 24 hours after LPS induced degradation treated with glucosamine sulfate. Panel **B** depicts mean (± SD) proteoglycan production released into the conditioned media 24 hours after LPS induced degradation treated with glucosamine sulfate. Panel **C** depicts mean (± SD) tissue proteoglycan synthesis as indicated by counts per minute/mg tissue (wet weight) 24 hours after LPS induced degradation treated with glucosamine sulfate. * indicates statistical significance at $p \leq .05$ compared to LPS. FBS=fetal bovine serum; LPS=lipopolysaccharide, SO4=sulfate.


A
µM NO/well
12
10
8
6
4
2
0
FBS
LPS
0.3075 mg/ml glucosamine SO4
3.075 mg/ml glucosamine SO4
30.75 mg/ml glucosamine SO4
Treatment


B
µg PG/well
140
120
100
80
60
40
20
0
FBS
LPS
0.3075 mg/ml glucosamine SO4
3.075 mg/ml glucosamine SO4
30.75 mg/ml glucosamine SO4
Treatment

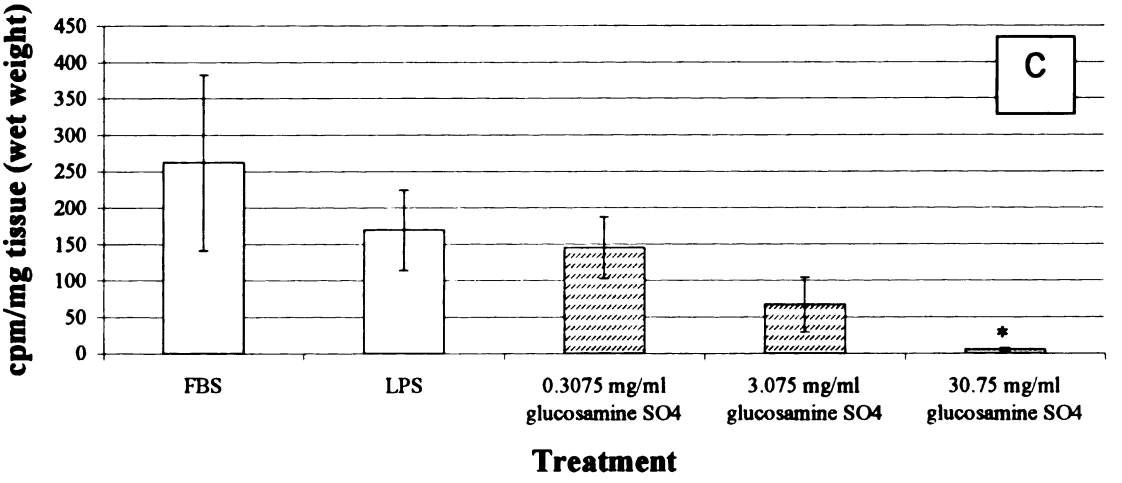
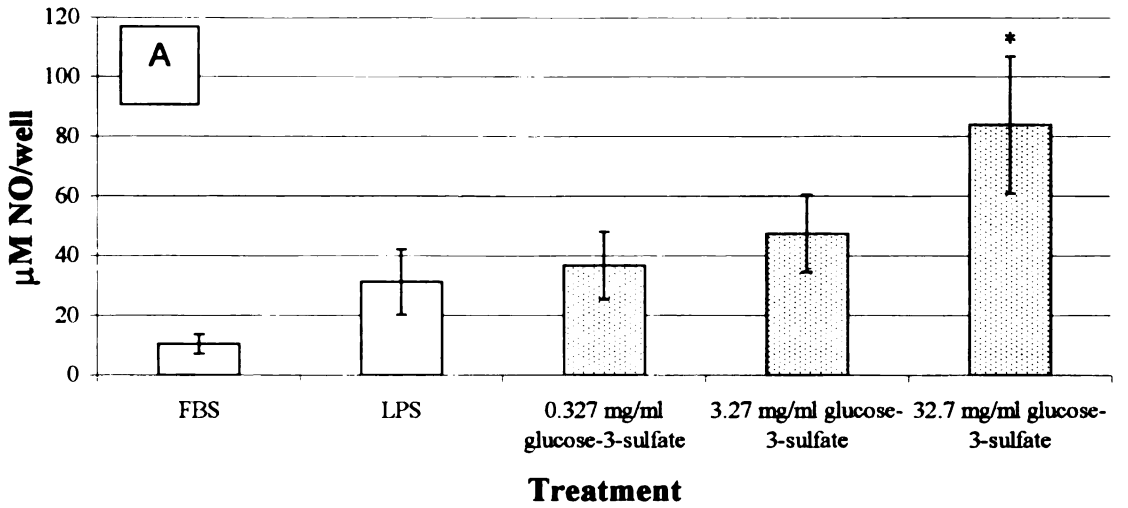

C
cpm/mg tissue (wet weight)
450
400
350
300
250
200
150
100
50
0
FBS
LPS
0.3075 mg/ml glucosamine SO4
3.075 mg/ml glucosamine SO4
30.75 mg/ml glucosamine SO4
Treatment

Figure 12 (on next page). Panel **A** depicts mean (± SD) nitric oxide production released into the conditioned media 24 hours after LPS induced degradation treated with glucose-3-sulfate. Panel **B** depicts mean (± SD) proteoglycan production released into the conditioned media 24 hours after LPS induced degradation treated with glucose-3-sulfate. Panel **C** depicts mean (± SD) total tissue proteoglycan content per mg tissue (wet weight) 24 hours after LPS induced degradation treated with glucose-3-sulfate. * indicates statistical significance at $p \leq .05$ compared to LPS. FBS=fetal bovine serum; LPS=lipopolysaccharide.


A
120
100
80
60
40
20
0
µM NO/well
*
FBS
LPS
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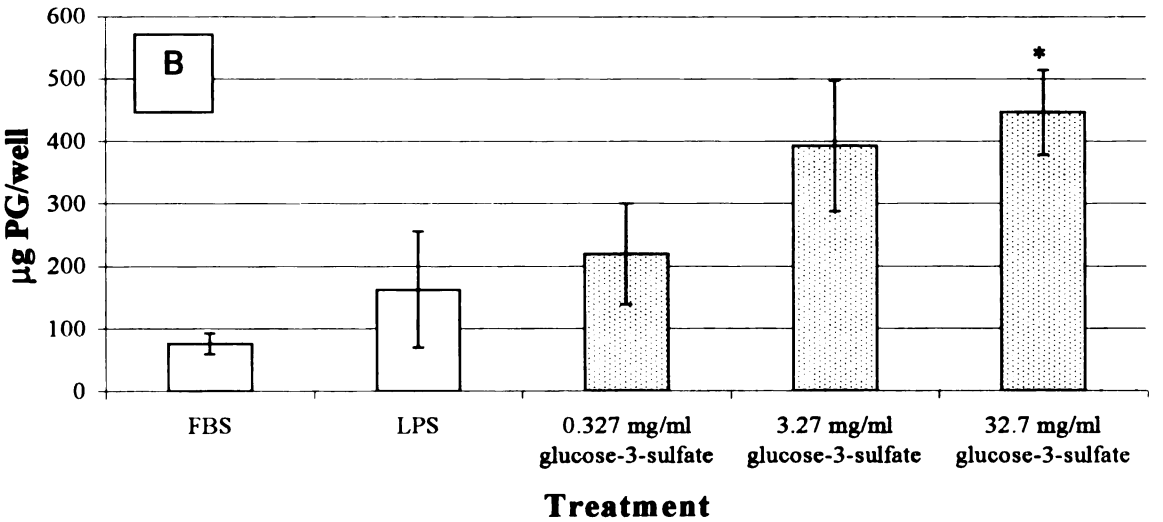
3.27 mg/ml glucose-3-sulfate
32.7 mg/ml glucose-3-sulfate
Treatment


B
600
500
400
300
200
100
0
µg PG/well
*
FBS
LPS
0.327 mg/ml glucose-3-sulfate
3.27 mg/ml glucose-3-sulfate
32.7 mg/ml glucose-3-sulfate
Treatment

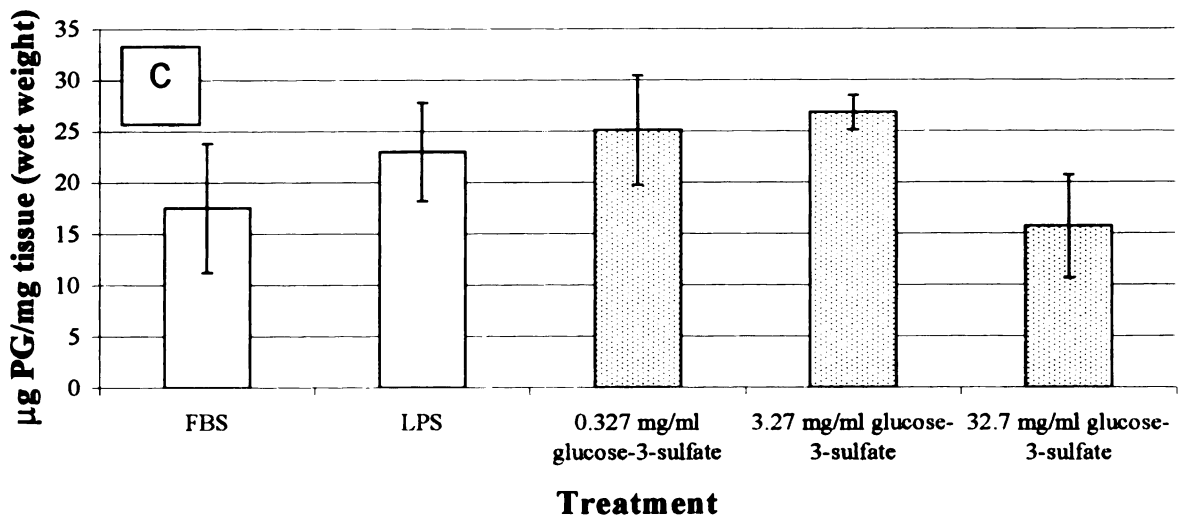
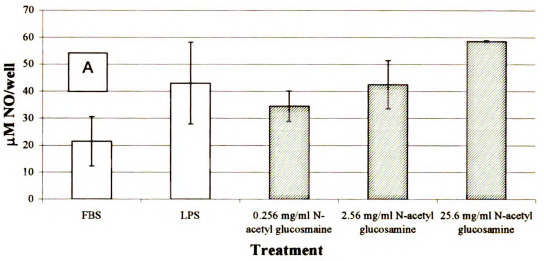
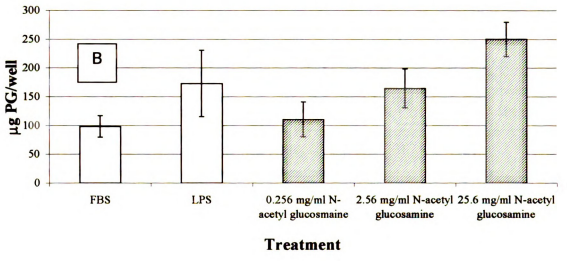
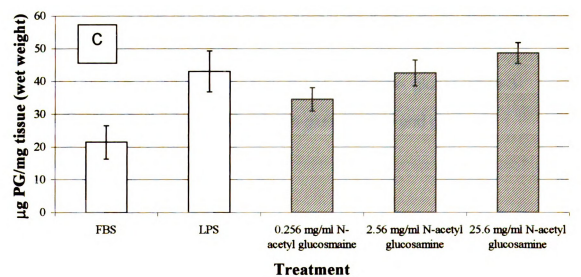

C
35
30
25
20
15
10
5
0
µg PG/mg tissue (wet weight)
FBS
LPS
0.327 mg/ml glucose-3-sulfate
3.27 mg/ml glucose-3-sulfate
32.7 mg/ml glucose-3-sulfate
Treatment

Figure 13 (on next page). Panel **A** depicts mean (± SD) nitric oxide production released into the conditioned media 24 hours after LPS induced degradation treated with N-acetyl glucosamine. Panel **B** depicts mean (± SD) proteoglycan production released into the conditioned media 24 hours after LPS induced degradation treated with N-acetyl-glucosamine. Panel **C** depicts mean (± SD) total tissue proteoglycan content per mg tissue (wet weight) 24 hours after LPS induced degradation treated with N-acetyl glucosamine. * indicates statistical significance at $p \leq .05$ compared to LPS. FBS=fetal bovine serum; LPS=lipopolysaccharide.


A
μM NO/well
70
60
50
40
30
20
10
0
FBS
LPS
0.256 mg/ml N-acetyl glucosmaine
2.56 mg/ml N-acetyl glucosamine
25.6 mg/ml N-acetyl glucosamine
Treatment


B
μg PG/well
300
250
200
150
100
50
0
FBS
LPS
0.256 mg/ml N-acetyl glucosmaine
2.56 mg/ml N-acetyl glucosamine
25.6 mg/ml N-acetyl glucosamine
Treatment


C
μg PG/mg tissue (wet weight)
60
50
40
30
20
10
0
FBS
LPS
0.256 mg/ml N-acetyl glucosmaine
2.56 mg/ml N-acetyl glucosamine
25.6 mg/ml N-acetyl glucosamine
Treatment

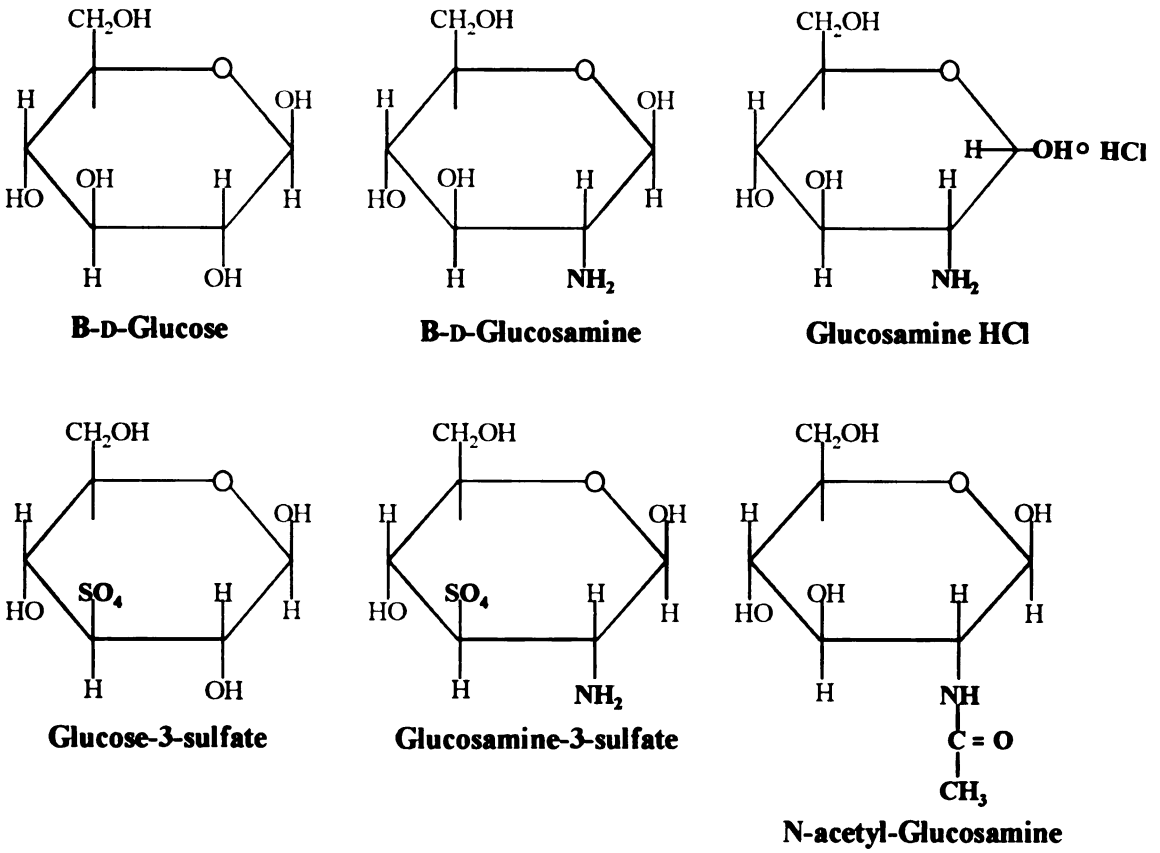


Figure 14. Diagram of the structure N-acetyl-glucosamine, glucosamine-3-sulfate, and glucose-3-sulfate compared to glucosamine and glucose.

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21. Homandberg G, Hui F, Wen C, Kuettner K, Williams J: Hyaluronic acid suppresses fibronectin fragment mediated cartilage chondrolysis: I. In vitro. Osteoarthritis Cartilage 1997; 5(5): 309-19.

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Chapter 4

THE EFFECTS OF RECOMBINANT EQUINE INTERLEUKIN-1 ON EQUINE ARTICULAR CARTILAGE DEGRADATION IN EXPLANT CULTURE

Summary

Objective. To 1) use a newly available recombinant equine IL-1 to better mimic equine cartilage degradation *in vitro* and determine if glucosamine inhibits this reIL-1 induced equine articular cartilage damage as indicated by NO and PG production, gelatinase/collagenase activity as previously shown with rhIL-1 and LPS and 2) examine the effects of glucosamine on reIL-1 induced stromelysin activity and prostaglandin E_2 production.

Materials and Methods. Articular cartilage was obtained from the antebrachio-carpal and middle joints of horses (2-8 years old) sacrificed for reasons unrelated to lameness. Cartilage discs were harvested from the weight-bearing region of the articular surface and cultured. Media were exchanged daily and the recovered media stored at 4°C. Explants were maintained in basal media 2 days prior to the start of 4 treatment days. On days 1 through 4 lipopolysaccharide (10 μg/ml) was added to induce cartilage degradation. To test the potential protective effects of glucosamine, the compound was added in three concentrations (0.25, 2.5, or 25 mg/ml) and treatments were performed in triplicate. Controls included wells without LPS or glucosamine. Nitric oxide, prostaglandin E_2, proteoglycan, stromelysin and gelatinase/collagenase activity

released into conditioned media and total tissue PG content were measured as indicators of cartilage metabolism.

Results. The addition of 25 mg/ml of glucosamine prevented the increase in nitric oxide production, prostaglandin E_2 and proteoglycan release and MMP activity induced by LPS.

Conclusion. These data indicate that glucosamine can prevent equine articular cartilage degradation experimentally induced by recombinant equine interleukin-1 *in vitro*.

Introduction

Osteoarthritis (OA) is a debilitating lower extremity disease in both humans and animals and is characterized by the progressive degradation of articular cartilage. OA is commonly diagnosed as a significant health problem to the equine industry and is a major cause of lameness for athletic horses. Results of a veterinary school survey found that intra-articular lesions account for 33% of all diagnosed equine conditions [1]. Because the disease is widely considered irreversible once clinical signs have been observed, medical attention has turned to means of preventing OA, treating symptoms, and slowing the progression of the disease. Traditional treatments including surgery, non-steroidal anti-inflammatory drugs, or corticosteroids have had limited success and are often accompanied by significant side-effects preventing long-term use. The medical/veterinary community has thus been forced to explore nontraditional

treatments of this disease including many nutritional supplements purported to ameliorate the symptoms of OA.

Many compounds are commercially available which claim to prevent or alleviate the symptoms of OA such as chondroitin sulfate (CS), glucosamine, methylsulfonylmethane, grape extract, vitamin C, and shark cartilage. By far the most commonly used compounds are glucosamine and CS. The CS molecule is not likely absorbed intact but rather an enzymatically degraded metabolite accounts for its biological activity making it difficult to study its mechanism of action [2]. However, glucosamine is rapidly absorbed into the blood stream and has been shown to concentrate in articular cartilage [3]. It is a naturally occurring, non-toxic compound that when given orally has been shown to decrease pain and improve mobility in osteoarthritic joints of humans [4]. In horses suffering from clinical signs of OA, an orally administered glucosamine HCl-chondroitin sulfate compound appeared to alleviate OA symptoms more rapidly than expected [5]. Similar findings have been observed in dogs treated with an oral glucosamine HCl-chondroitin sulfate supplement [6]. However, evaluating the biochemical mechanisms by which glucosamine may prevent or treat OA *in vivo* is difficult due to the nature of the tissue. Our lab has developed an equine explant cartilage system for use as a model for studying equine OA and we have shown that glucosamine HCl can prevent experimentally induced equine articular cartilage degradation *in vitro* (submitted for publication).

Currently, most equine articular cartilage explant systems use lipopolysaccharide (LPS) or recombinant human interleukin-1 (rhIL-1) to induce

cartilage degradation. LPS is an endotoxin produced by gram-negative bacteria that has been shown to induce cartilage degradation and is present in the joint during septic arthritis. Horses are at least 10 times more sensitive to endotoxin than dogs or most other mammals [7]. Equine chondrocytes may be more responsive to LPS than that of other species [8] and predispose cartilage to rapid degradation not necessarily consistent with OA. Therefore, the use of LPS to model OA in equine tissue is questionable.

IL-1 is a cytokine that is present in high levels in synovial fluid of diseased joints [9] and has been shown *in vitro* to induce changes in the ECM of articular cartilage similar to OA cartilage. These changes include PG release, increased NO and prostaglandin E_2 (PGE_2) release and induction of matrix metalloproteinases (MMPs). Matrix metalloproteinases are a family of metal dependent proteases including gelatinases, collagenases and stromelysins (regarded as the predominant enzyme involved in cartilage degradation of PG's). Equine chondrocytes have been shown to produce these proteases in response to IL-1. Prostaglandins are involved in inflammation and pain, the primarily clinical symptoms associated with OA. However, the use of human IL-1 in equine tissue has been questioned as a model and acquisition of equine IL-1 could reveal different results [10]. May *et al.* reported that species restrictions on the activity of human IL-1 on equine cells exists [11]. Recently, a recombinant form of equine IL-1 has become available for use in our equine explant system.

Our laboratory has shown that glucosamine can inhibit LPS or rhIL-1 induced NO and PG production and gelatinase/collagenase activity. Yet, we

have not examined the effect of glucosamine on stromelysin. A fluorescent stromelysin assay has been modified to examine stromelysin activity in conditioned media from equine articular cartilage explants. Additionally, we have not examined the potential of glucosamine to inhibit prostaglandins.

Therefore, the objectives of this research were to 1) use a newly available recombinant equine IL-1 to better mimic equine cartilage degradation *in vitro* more consistent with OA and to determine if glucosamine inhibits reIL-1 induced equine articular cartilage damage as indicated by NO and PG production, gelatinase/collagenase activity as previously shown with rhIL-1 and LPS and 2) examine the effects of glucosamine on reIL-1 induced stromelysin activity and PGE_2 production.

We have shown that reIL-1 induced cartilage degradation was inhibited by glucosamine in our *in vitro* system. Further, we demonstrate that glucosamine is also able to inhibit stromelysin activity and PGE_2 production by chondrocytes in equine explant culture.

Materials and Methods

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

EXPLANT CULTURES

Articular cartilage was obtained from the antebrachio-carpal and middle carpal joints of horses (2-8 years old) sacrificed for reasons other than joint problems. Cartilage discs (3.5mm) were biopsied from the weight-bearing region of the articular surface. Explant discs (3 per well, 40-60mg total) were cultured in a 24-well Falcon culture plate (Fisher Scientific; Pittsburgh, PA) with a modified version of Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Ham) (1:1) (Gibco; Grand Island NY) [12]. The media was supplemented with 10% fetal bovine serum (FBS, Gibco), 50 μg/ml ascorbate and 100 units/ml penicillin/streptomycin (Gibco). The explants were maintained in culture in a humidified incubator with 7% CO2 at 37°C.

Explants were maintained in basal media for 2 days prior to the first of 4 treatment days. The three treatments and two controls established are outlined in table 6. Conditioned media was removed and replaced daily and stored at 4°C until analyzed for indicators of degradation. Recombinant equine IL-1 (50 ng/ml) (generously donated by J.P. Caron) was added to induce cartilage degradation. To evaluate the effects of glucosamine, varying concentrations of glucosamine HCl (0.25, 2.5, or 25 mg/ml) were added to the cultures. Treatments were performed with triplicate wells using tissue from one animal donor. Experiments were repeated at least three times, each time using tissue from a different animal donor.

PROTEOGLYCAN ASSAY

PG release into conditioned media was measured using a dimethylmethylene blue assay as previously described [13]. Briefly, PG content was determined by measuring sulfated glycosaminoglycan content compared to a chondroitin sulfate standard and expressed as μg PG/well. Tissue PG content was determined following papain digest (1μg papain/ mg tissue) of the tissue. Tissue PG content was expressed as μg PG/mg tissue wet weight.

NITRIC OXIDE ASSAY

Nitrite, a stable end product of nitric oxide metabolism, was measured in conditioned media using the Greiss reaction and sodium nitrite as a standard [14]. Absorbance at 540nm was determined using the SpectraMax 300 plate reader (Molecular Devices, Sunnyvale, CA).

MATRIX METALLOPROTEINASE ASSAYS

Gelatinase/collagenase activity was detected in the conditioned media and cartilage extracts using the EnzChek Collagenase/Gelatinase Assay Kit according to the manufacturer (Molecular Probes, Eugene, OR). 50 μl conditioned media or cartilage extracts were incubated with DQ-gelatin in reaction buffer (0.5 *M* Tris-HCl, 1.5 *M* NaCl, 50 m*M* $CaCl_2$, 2 m*M* Sodium azide,

pH 7.6) for 1 hour at room temperature. Enzymatic release of fluorescent signal from DQ-gelatin by either gelatinase or collagenase was quantitated using the Cytofluor 4000 fluorescent plate reader (PerSeptive Biosystems; Framingham, MA). Inhibition of gelatinase/collagenase activity was performed by the addition of 10 m*M* 1,10-phenanthroline to the reaction buffer. Collagenase from *Clostridium histolyticum* was used as a standard. Results were presented as units of enzyme activity/ml/hr.

Stromelysin activity was detected in the conditioned media and cartilage extracts using the EnzChek Protease Assay Kit according to the manufacturer (Molecular Probes, Eugene, OR). Conditioned media was dialyzed in buffer (50 mM Tris, 5mM $CaCl_2$, pH 7.5) to remove phenol red and 50 µl of the dialyzed sample was incubated with casein-BODIPY in reaction buffer (0.5 *M* Tris-HCl, 1.5 *M* NaCl, 50 m*M* $CaCl_2$, 2 m*M* Sodium azide, pH 7.6) for 4 hours at room temperature. Enzymatic release of fluorescent signal from casein-BODIPY by stromelysin was quantitated using the Cytofluor 4000 fluorescent plate reader (PerSeptive Biosystems; Framingham, MA). Inhibition of stromelysin activity was performed by the addition of 10 m*M* 1,10-phenanthroline to the reaction buffer. Recombinant stromelysin kindly donated Dr. M. Smith (University of Missouri) was used as a standard. One unit of activity was defined as the ability to change the flourescence by 1000 units. Results were presented as units of enzyme activity/ml/hr.

Tissue MMP content was extracted with 1M guanidine-HCl, 50 mM Tris and 10 mM Calcium chloride [15], dialyzed into reaction buffer and

gelatinase/collagenase activity of the sample was determined as described above.

PROSTAGLANDIN E_2 IMMUNOASSAY

Prostaglandin E_2 release into conditioned media was determined using a commercially available ELISA (R&D systems, Minneapolis, MN). An aliquot of the conditioned media was collected from culture wells and 10 μg/ml indomethacin was added to inhibit prostaglandin synthase. Samples were stored at -20°C until analysis. Media samples were diluted 1:15 and assayed according to the manufacturer instructions.

STATISTICAL ANALYSIS

Data for indicators of degradation were analyzed using the repeated measure option of the SAS (1996) statistical software PROC MIXED [16]. No interaction of day was present so all data were represented as the average response of the 3 treatment wells at 24 hours after treatment addition. Treatments were compared using the Bonferroni procedure. Statistical significance was considered at $p<0.05$ unless noted.

Results

Effect of glucosamine on reIL-1 induced cartilage degradation

Glucosamine was added at three doses (0.25, 2.5 25.0 mg/ml) to the reIL-1-induced cartilage degradative system. When tested against reIL-1 treatment, glucosamine significantly inhibited NO production at 25.0 mg/ml (Figure 15a), and also inhibited PG release at the highest dose (Figure 15b). Glucosamine did not alter total tissue PG content compared to all other treatments (Figure 15c). Gelatinase/collagenase activity was significantly inhibited by glucosamine at 2.5mg/ml ($p \leq .01$) and there was no detectable activity at 25.0mg/ml ($p \leq .001$) (Figure 16a). Glucosamine also significantly inhibited stromelysin activity at 0.25 and 2.5mg/ml ($p \leq .01$) and there was no detectable activity at 25.0 mg/ml ($p \leq .001$) (Figure 16b). No MMP activity was detectable in tissue extracts. Prostaglandin E_2 was significantly inhibited by glucosamine at 2.5 and 25.0mg/ml ($p \leq .001$) (Figure 17).

Discussion

In vivo investigation of equine articular cartilage is relatively difficult due to the nature of the tissue, the expense of the animals, and the emotional response it elicited working with horses. As with any *in vivo* experiment, determining which chemical signals are emanating from the cartilage itself or from other tissues and

influencing chondrocyte metabolism is difficult. *In vivo* chondrocytes exist in an avascular environment with limited oxygen present and require the diffusion of metabolites from the synovial cavity. Therefore, articular cartilage can be cultured as full thickness tissue with the extracellular matrix surrounding the chondrocytes intact. This intact ECM is considered important in regulating chondrocyte and matrix metabolism. However, the ECM of articular cartilage can vary by joint, region, area and zonal depth of the cartilage. Accounting for these limitations, *in vitro* culture of articular cartilage can provide an excellent experimental model for studying equine articular cartilage responses to different compounds.

Studying osteoarthritic cartilage *in vitro* can be complicated. Diseased articular cartilage is typically grossly altered by erosions or loss of matrix components and is highly variable throughout the joint. Therefore, regulatory factors implicated in the disease process have been isolated and used to experimentally induce cartilage degradation in explant culture. The regulatory factors most commonly studied include interleukin-1 and LPS. As previously described, both induce cartilage changes relatively consistent with early OA. Until reIL-1 became available recently, equine systems used a human form of this protein to induce degradation. This research has been criticized because of the potential differential response of equine tissue to human proteins. Some evidence exists to support this criticism. Equine cells have been shown to respond differently to human IL-1 [11]. In addition, the amino acid sequence of equine interleukin-1 beta showed only 66.7% homology to human interleukin-1

beta [17]. This could be enough of a difference to cause decreased binding to the receptor and an attenuated response. We have developed an equine articular cartilage explant culture system and characterized changes induced by reIL-1. The current research examines the effect of glucosamine on reIL-1 induced cartilage degradation.

Recently, our lab has shown that glucosamine HCl inhibits LPS or rhIL-1 induced cartilage degradation as indicated by decreased PG and NO release and matrix metalloproteinase (MMP) activity, while the glucose control did not (in press). Some possibile mechanisms of action discussed include that glucosamine may simply quench small signal molecules including NO and oxygen radicals that can damage articular cartilage [18]. Glucosamine may also mediate matrix metalloproteinase degradation of cartilage, often thought of as the hallmark of OA. Glucosamine has been shown to inhibit aggrecanase activity, a putative MMP, in IL-1 stimulated bovine cartilage explants [19]. This inhibition was apparently not due do to any cytotoxicity or interference with IL-1 signaling, since protein synthesis and lactate production were not altered by glucosamine. In agreement with this data, we showed that glucosamine inhibited reIL-1 induced gelatinase/collagenase and stromelysin activity and we were not able to detect any decrease in cell viability as measured by lactate dehydrogenase (data not shown). These data provide biochemical evidence supporting the limited *in vivo* studies indicating that glucosamine may prevent or attenuate equine osteoarthritis.

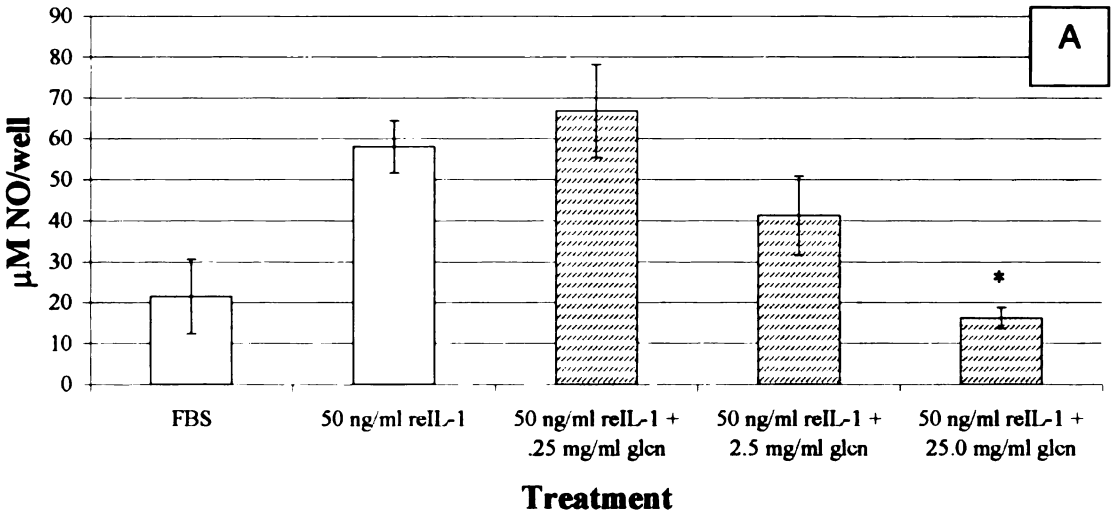
Many compounds are commercially available which claim to "improve joint health". Systems must be developed to study the effects of these compounds on the joint (negative or positive). Studies like ours using equine tissue and equine proteins are critical for screening and providing biochemical evidence supporting or refuting the claims of commercially available nutritional supplements. Recognizing that *in vitro* data should be cautiously applied to the *in vivo* situation, this study still provides valuable information regarding the effects of glucosamine on equine articular cartilage.

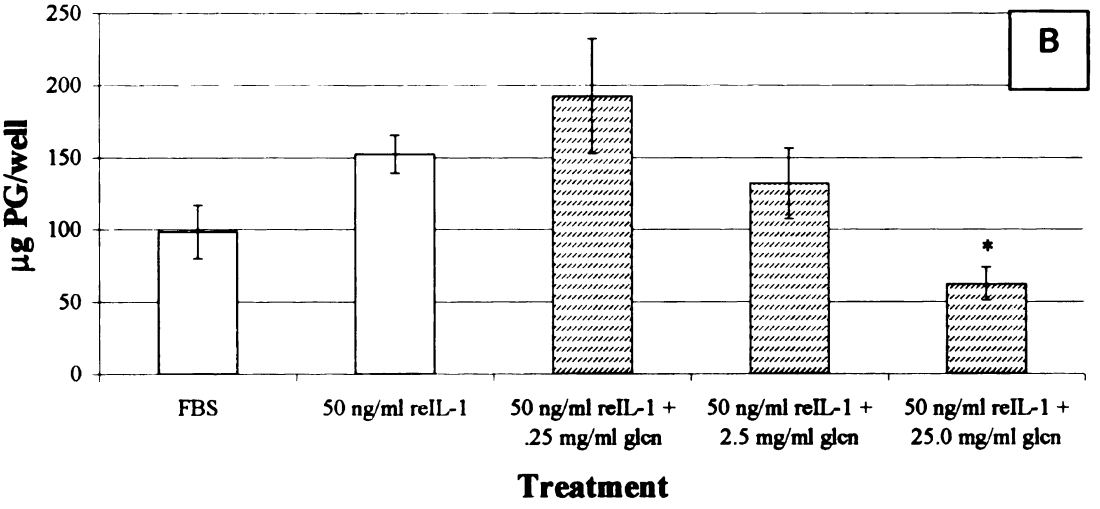
Table 6. Detailed description of components of explant culture treatments*

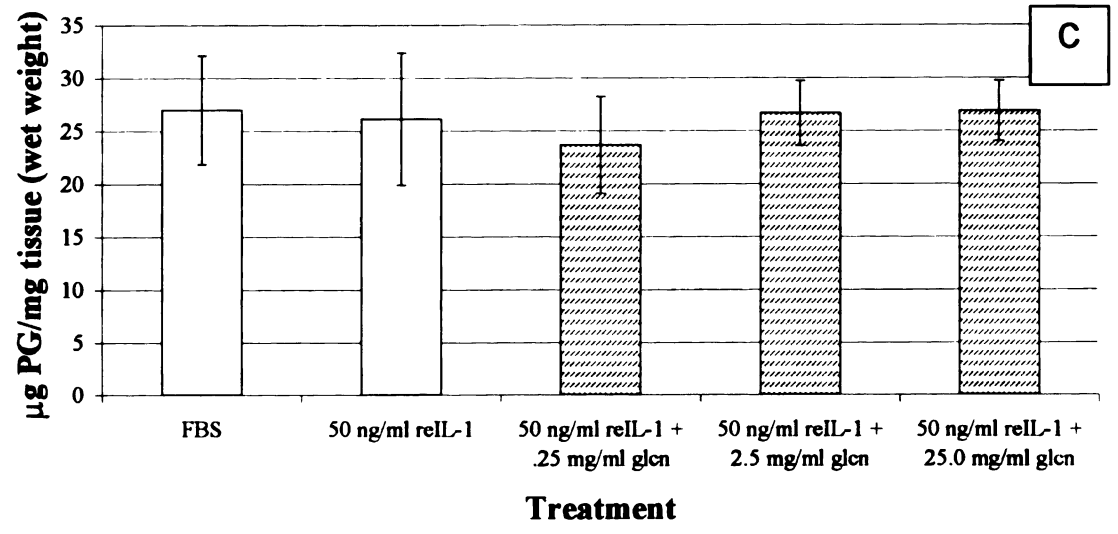
Treatment	FBS	reIL-1	Glucosamine (glcn)
FBS-Control 1	10%		
reIL-1-Control 2	10%	50 ng/ml	
Treatment 1	10%	50 ng/ml	0.25 mg/ml
Treatment 2	10%	50 ng/ml	2.5 mg/ml
Treatment 3	10%	50 ng/ml	25.0 mg/ml

*FBS=fetal bovine serum; reIL-1=recombinant equine interleukin-1

Figure 15 (next page). Panel **A** depicts mean (± SD) nitric oxide production released into the conditioned media 24 hours after reIL-1 induced degradation treated with glucosamine. Panel **B** depicts mean (± SD) proteoglycan released into the conditioned media 24 hours after reIL-1 induced degradation treated with glucosamine. Panel **C** depicts mean (± SD) total tissue proteoglycan content (wet weight) 24 hours after reIL-1 induced degradation treated with glucosamine. * indicates statistical significance at $p \leq .01$ compared to reIL-1. FBS=fetal bovine serum; reIL-1=recombinant equine interleukin-1; glcn=glucosamine.


A
90
80
70
60
50
40
30
20
10
0
µM NO/well
*
FBS
50 ng/ml reIL-1
50 ng/ml reIL-1 + .25 mg/ml glcn
50 ng/ml reIL-1 + 2.5 mg/ml glcn
50 ng/ml reIL-1 + 25.0 mg/ml glcn
Treatment


B
250
200
150
100
50
0
µg PG/well
*
FBS
50 ng/ml reIL-1
50 ng/ml reIL-1 + .25 mg/ml glcn
50 ng/ml reIL-1 + 2.5 mg/ml glcn
50 ng/ml reIL-1 + 25.0 mg/ml glcn
Treatment


C
35
30
25
20
15
10
5
0
µg PG/mg tissue (wet weight)
FBS
50 ng/ml reIL-1
50 ng/ml reIL-1 + .25 mg/ml glcn
50 ng/ml reIL-1 + 2.5 mg/ml glcn
50 ng/ml reIL-1 + 25.0 mg/ml glcn
Treatment

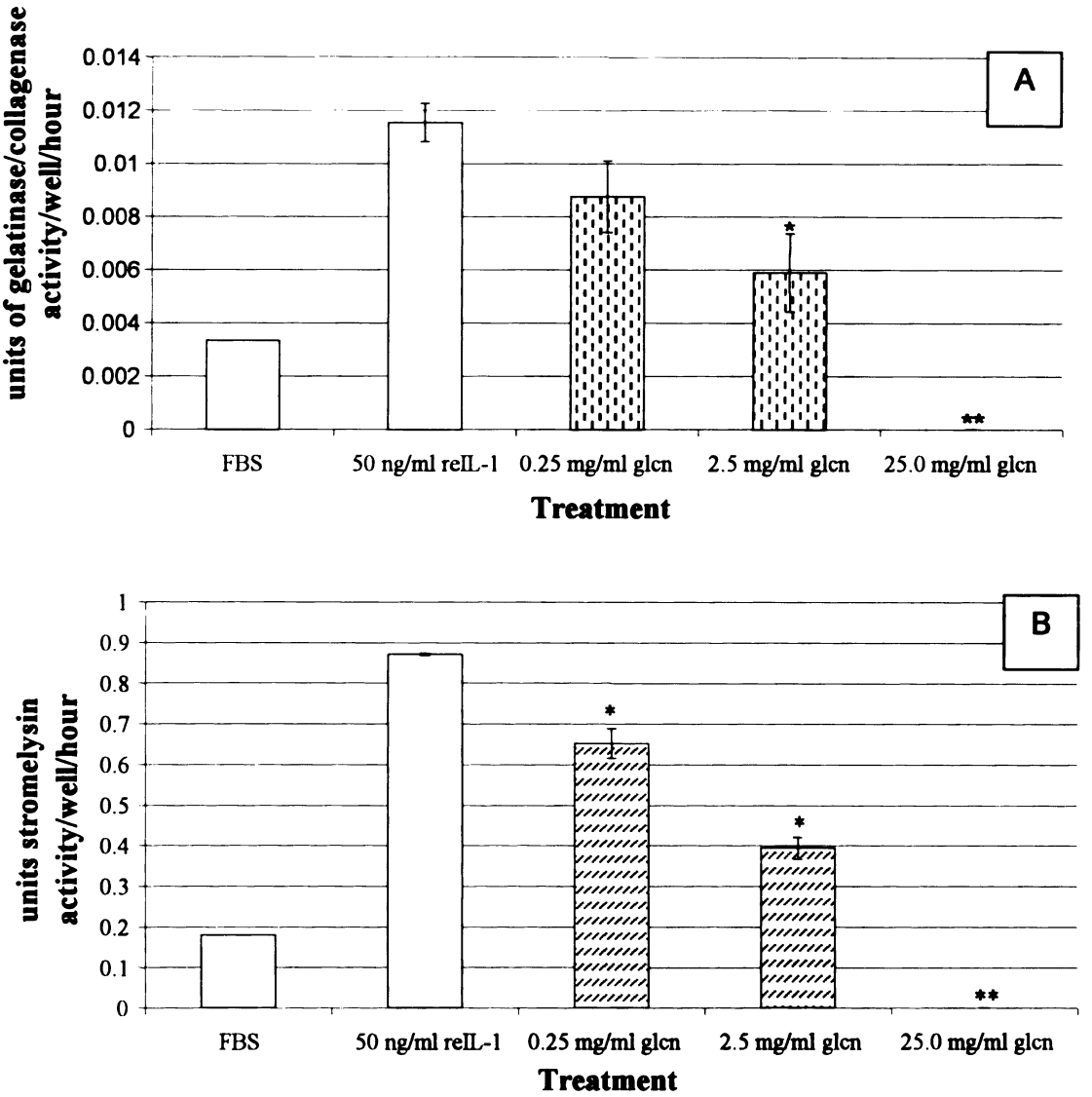


Figure 16. Panel **A** depicts mean (± SD) gelatinase/collagenase activity released into the conditioned media 24 hours after reIL-1 induced degradation treated with glucosamine. Panel **B** depicts mean (± SD) stromelysin activity released into the conditioned media 24 hours after reIL-1 induced degradation treated with glucosamine. * indicates statistical significance at $p \leq .0$, ** indicates statistical significance at $p \leq .001$ compared to reIL-1. FBS=fetal bovine serum; reIL-1=recombinant equine interleukin-1; glcn=glucosamine.

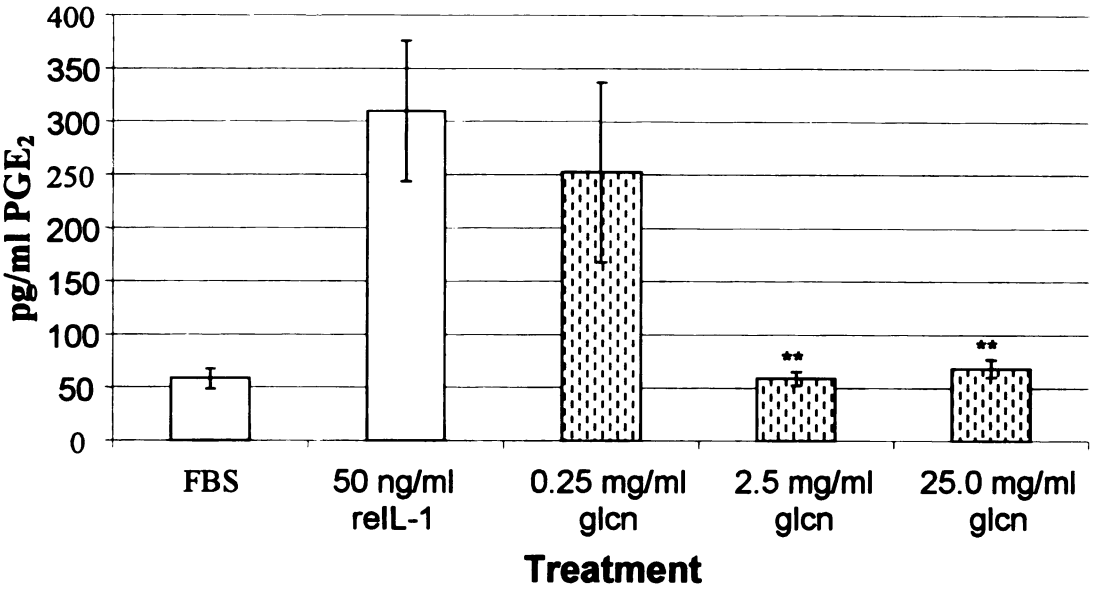


Figure 17. Mean (± SD) prostaglandin E_2 released into the conditioned media 24 hours after relL-1 induced degradation treated with glucosamine. ** indicates statistical significance at $p \leq .001$ compared to relL-1. FBS=fetal bovine serum; relL-1=recombinant equine interleukin-1; glcn=glucosamine.

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Chapter 5

CONCLUSION

The previous chapters present data that provide biochemical evidence supporting the purported chondroprotective properties of glucosamine HCl and glucosamine sulfate and also suggest disease-modifying characteristics of the compound. A chondroprotective agent can be defined as a substance that increases chondrocyte anabolic activity while simultaneously suppressing the effect of mediators (cytokines, prostaglandins, and proteinases) on cartilage degradation. Proteolytic degradation of cartilage matrix is generally considered the hallmark of OA. A disease-modifying drug such as glucosamine should reduce degradation either by inhibiting proteinases or by inhibiting the activity of mediators such as IL-1. Using indicators of chondrocyte metabolism (NO production, PG release, PG synthesis and MMP activity), we have shown that glucosamine HCl and glucosamine sulfate (25mg/ml) inhibit *in vitro* cartilage degradation induced by either LPS or rhIL-1. However, the therapeutic value of N-acetyl-glucosamine remains questionable; we were not able to show that it inhibited cartilage degradation in our system. In addition, we have shown that glucosamine HCl is also able to inhibit reIL-1 induced cartilage degradation.

We feel confident that these responses were due to glucosamine and were not an artifact of high sugar moieties in the media. Compared to the LPS treatment, glucose or glucose sulfate treatment at any dose did not decrease NO production, PG release, MMP activity or PG synthesis, indicating the importance

of the amine group on glucosamine. The amine group at the carbon-2 position appears important for the effectiveness of the glucosamine derivative.

Some possibile mechanisms of action discussed include that glucosamine may simply quench small signal molecules including NO and oxygen radicals that can damage articular cartilage. Nitric oxide may be an important regulatory molecule in the cartilage matrix degradation cascade, by mediating some of the inhibitory effects of cytokines on matrix synthesis or MMP activity. Glucosamine may also mediate matrix metalloproteinase degradation of cartilage, often thought of as the hallmark of OA. Glucosamine has been shown to inhibit aggrecanase activity, a putative MMP, in IL-1 stimulated bovine cartilage explants. This inhibition was apparently not due do to any cytotoxicity or interference with IL-1 signaling, since protein synthesis and lactate production were not altered by glucosamine. In agreement with these data, we showed that glucosamine inhibited reIL-1 induced gelatinase/collagenase and stromelysin activity and we were not able to detect any decrease in cell viability as measured by lactate dehydrogenase (data not shown). The mechanism remains unclear, but inhibition of expression, synthesis or activity of MMPs are all possible.

Alteration of chondrocyte metabolism by glucosamine was also evidenced by changes in PG metabolism. Glucosamine inhibited both PG degradation and synthesis. At first, the reduced rate of PG synthesis may appear detrimental to the matrix of articular cartilage. However, we observed a slight increase in tissue PG content at the highest dose of glucosamine compared to all other treatments. These data indicated that, although synthesis was decreased, turnover was

maintained or PG accretion was favored. Thus, if PG degradation is not occurring, PG synthesis is not up-regulated. Most *in vitro* studies in which glucosamine increased PG synthesis used OA cartilage. In our study, however, we used normal cartilage, which may explain the disparity between our results and others.

During the diseased state there may be an increased demand for "building blocks" of cartilage metabolism. Supplemental glucosamine may also simply bypass rate limiting steps in glucosamine metabolism and lead to an increase in available "building blocks" for extracellular matrix molecules. Addition of glucosamine to cell culture resulted in an increase in intracellular UDP-N-acetylglucosamine. This compound is critical in the formation of glycosaminoglycans found in cartilage including hyaluronic acid, heparin sulfate, and keratan sulfate. Supplemental glucosamine may act to reduce symptoms of OA by increasing the production of hyaluronic acid in synovial fluid. Intra-articular injection of hyaluronic acid has been shown to alleviate the symptoms of OA. HA has also been shown to prevent fibronectin mediated cartilage injury *in vivo* and *in vitro*.

These data provide biochemical evidence supporting the limited *in vivo* studies indicating that glucosamine may prevent or attenuate equine osteoarthritis. This biochemical evidence is summarized diagrammatically and depicts the proposed mechanisms of the action of glucosamine (Figure 18). Many compounds are commercially available which claim to "improve joint health". Systems must be developed to study the effects of these compounds on

the joint (negative or positive). Studies like ours using equine tissue and equine proteins are critical for screening and providing biochemical evidence supporting or refuting the claims of commercially available nutritional supplements. Recognizing that *in vitro* data should be cautiously applied to the *in vivo* situation, this dissertation still provides valuable information regarding the effects of glucosamine on equine articular cartilage.

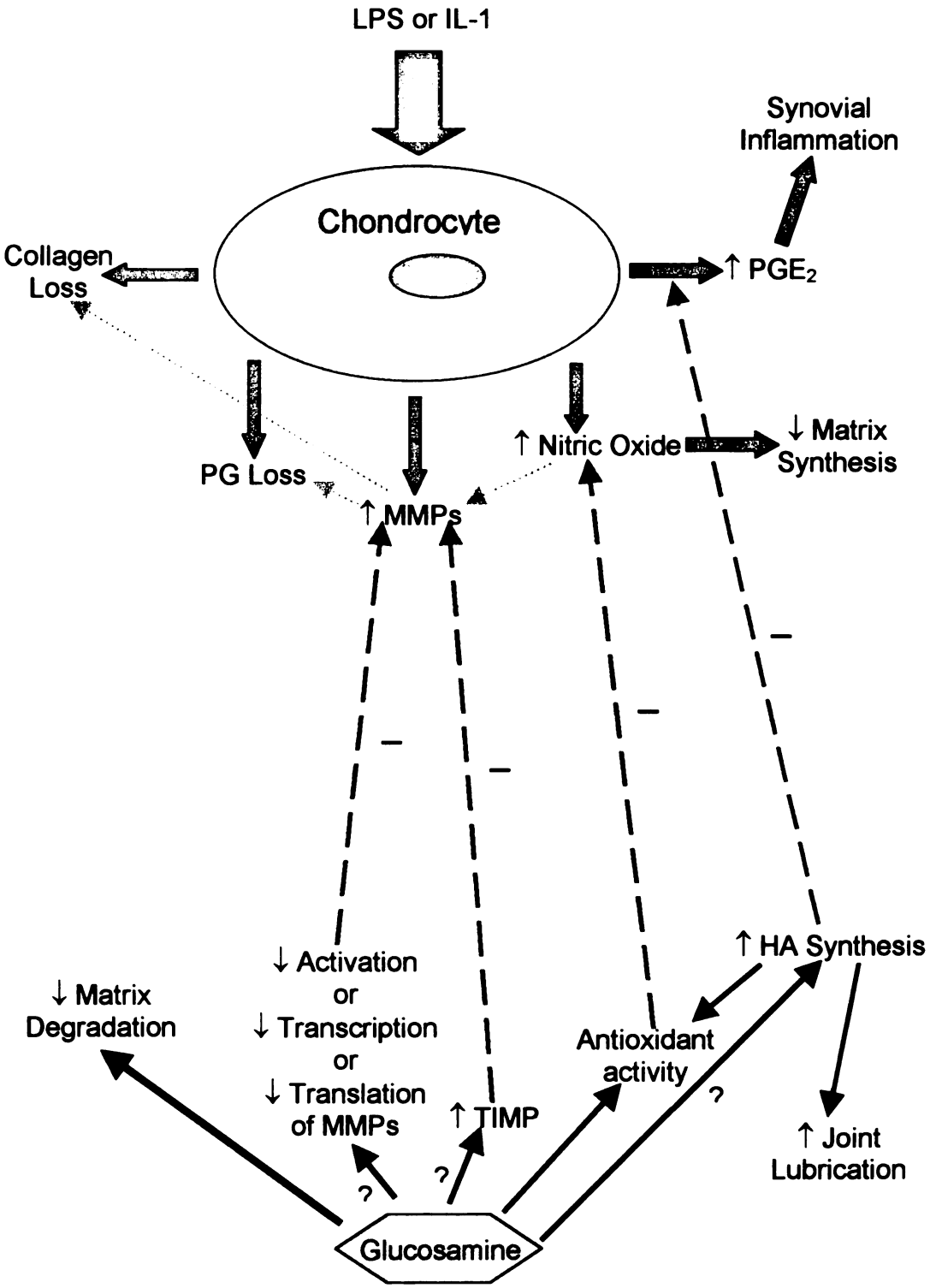

Figure 18. Proposed mechanisms for glucosamine inhibited cartilage degradation as indicated by available data and our in vitro results.

APPENDICES

Appendix A

EFFECT OF LONGEING AND GLUCOSAMINE SUPPLEMENTATION ON SERUM MARKERS OF BONE AND JOINT METABOLISM IN YEARLING QUARTER HORSES

Summary

Objective. The effect of longeing and glucosamine supplementation on known biological markers of joint disease was studied in yearling Quarter Horses.

Materials and Methods. Twenty-one yearling Quarter Horses were randomly assigned to one of four treatments: 1) longeing (longeing 20 minutes once per day) supplement control (LN); 2) longeing/glucosamine (LG); 3) walking (mechanical walker for 120 minutes per day (WN)); and 4) walking/glucosamine (WG). Oral glucosamine was administered at 5.5 g BID weeks 1-4, 3.5 g BID during week 5-6, and 2.0 g BID during week 7-8. Serum was obtained weekly for eight weeks and analyzed for keratan sulfate and osteocalcin concentrations.

Results. Walked horses receiving glucosamine showed slight elevation in serum keratan sulfate compared to controls ($p=0.04$). Glucosamine or longeing exercise had no significant effect ($p \geq 0.08$) on serum osteocalcin concentrations.

Conclusion. Under these conditions, longeing and/or glucosamine supplementation did not significantly alter serum concentrations of keratan sulfate or osteocalcin.

Key Words: Glucosamine, Longeing, Osteocalcin, Keratan Sulfate

Introduction

Osteoarthritis (OA) is a degenerative disorder of articular tissues that can arise from a number of causes. While disease can occur after a joint experiences a single traumatic event, repeated trauma of relatively low magnitude is regarded as a more common factor in the development of lesions (1). The nature of the mechanical stimuli that initiate arthritis is variable and probably depends on a combination of factors including age, joint conformation, and the duration and type of activity. Physical activity can place impressive loads on joint tissues; when horses run at speed through a turn, the size of the load bearing surfaces is reduced by half, with a corresponding increase in load per unit area experienced by the load-bearing portion of the joint (2). Particularly in growing animals, activities leading to repeated episodes of uneven compression and loading may result in bone and articular cartilage damage and predispose joints to OA (3). Longeing (exercise in a circle on a long line) is a common practice in the training of many types of horses. This form of exercise is increasing in popularity in the Quarter Horse industry and is used as a means of showing young horses before they are trained to ride. Although speculative, there is concern that excessive longeing may contribute to cartilage damage and OA.

Despite the potential deleterious impact on articular tissues, conditioning growing horses for athletic use is essential. As a result, interest in the identification of pharmacological means to protect bone and cartilage from the

effects of use trauma is increasing. A number of oral feed supplements have been formulated that contain substances purported to have beneficial effects on joint metabolism. A popular constituent of these preparations is glucosamine, a non-toxic, bioavailable, naturally-occurring component of the cartilage matrix (4). Glucosamine has been shown to have a number of favorable effects on cartilage metabolism in vitro, including a reduction in articular cartilage breakdown and stimulation of synthesis of matrix components by chondrocytes (5). When given orally to human OA patients, glucosamine decreases joint pain and improves mobility (6). Information regarding the efficacy of this treatment for prevention of OA in horses is limited.

Joint metabolism can be monitored by measuring biological markers in serum. Keratan sulfate (KS) is a sensitive indicator of cartilage metabolism. This glycosaminoglycan, confined primarily to articular cartilage, can be detected in very small amounts in serum after its enzymatic release from the proteoglycan (PG) aggrecan. Thus, increased serum KS concentrations can be correlated with early cartilage degradation (7). Concentrations of KS rise before the onset of clinical symptoms of OA.

Active osteoblasts synthesize osteocalcin, a vitamin K dependent protein that regulates bone mineralization (8). Osteocalcin present in serum reflects the portion of newly synthesized protein that is not incorporated into the mineral phase of bone and is released into circulation. Antibodies have been developed that detect only intact osteocalcin in serum, and not proteolytic fragments that are released during bone resorption. Therefore, serum osteocalcin concentrations

can be used to monitor the rate of bone formation (9). This study examined the effects of longeing and oral glucosamine supplementation on serum KS and osteocalcin concentrations in yearling Quarter Horses.

Materials and Methods

Management of Animals

Twenty-one yearling Quarter Horses (mean age 529 d, range 435 to 625d) were separated by sex and then randomly assigned to four treatment groups in a 2 x 2 factorial arrangement: longeing/no supplement (LN), longeing/glucosamine (LG), walking/no supplement (WN) and walking/glucosamine (WG). Longed horses trotted 10 circles (diameter = 18 m) and loped 20 circles in one direction, and then repeated the sequence in the opposite direction. The longeing exercises lasted 20 minutes. The non-longed horses walked on a mechanical walker for 2 hours per day. LG and WG were fed 11.0 g/d of glucosamine (Sigma Chemical, St Louis, MO) during weeks 1 through 4, 7.0 g/d during weeks 5 and 6, and 4.0 g/d during weeks 7 and 8 with the amount divided equally between two feedings each day. This approximates a dose of 29, 18 and 10 mg/kg respectively. The dose and frequency of supplementation used in this trial were similar to those recommended by the manufacturer of the commercial product Cosequin (Nutramax Laboratories Inc, Baltimore, MD). Blood samples

were obtained prior to the start of the trial and then weekly for an 8-week period. Serum was collected and stored at -20°C until analyzed.

ELISA FOR QUANTIFICATION OF SERUM KERATAN SULFATE

Serum KS was quantified using a previously described enzyme-linked immunosorbent assay (ELISA) with an inhibition step (10,11). Incubation of the serum with the anti-KS monoclonal antibody (ICN Pharmaceuticals Inc, Costa Mesa, CA) was performed at pH 5.3 as previously described (11,12). The detection process required incubation with the secondary antibody, Goat Anti-Mouse IgG HRP-conjugated (ICN Pharmaceuticals Inc, Costa Mesa, CA), and color development was initiated using o-phenylenediamine (Sigma Chemical, St Louis, MO). All concentrations of KS reported in the text refer to equivalents of an international standard (generously provided by Dr. R.J. Todhunter, Cornell University).

QUANTIFICATION OF SERUM OSTEOCALCIN

Serum concentrations of osteocalcin were quantified using a commercially available ELISA (Novocalcin®) (Metra Biosystems, Mountain View, CA) according to manufacturer's instructions (13). Data obtained with this kit, which was designed for humans, were comparable to published reference values for equine samples.

STATISTICAL ANALYSES

Statistical analyses were performed using the repeated measure option of the SAS (1996) statistical software PROC MIXED (14). The first-order auto regressive model was used to model the correlation between repeated measurements within each subject based on the plausibility that residuals involving measurements made at neighboring times are more closely correlated than measurements further apart. The residual variance and autocorrelation were estimated using restricted maximum likelihood, which is generally the method of choice for unbalanced data and the default method in PROC MIXED. Statistical significance was assumed at $p < 0.05$.

Results

Initial KS concentrations for all treatments differed prior to the start of the study (Figure 18). Therefore, beginning with day seven, all KS concentrations were expressed and analyzed as deviations from day zero concentrations (Figure 19). Changes in serum KS concentrations did not differ between longed and walked horses ($p=0.1$; -8.82 vs -0.296). However, there was a trend for glucosamine treatment to have elevated treated horses serum KS concentration when compared to controls ($p=0.08$; 20.663 vs –30.075). Within the exercise treatments, the walked group receiving glucosamine showed slightly elevated KS

concentrations compared to the walked horses not receiving supplementation ($p = 0.04$; Figure 19).

Neither exercise nor diet significantly affected serum osteocalcin concentrations. The pattern of decreasing osteocalcin over time was similar for all groups (Figure 20).

Discussion

The objective of this project was to determine the influences of longeing and oral glucosamine supplementation on serum markers of bone and cartilage metabolism. The project was conducted at a commercial Quarter Horse farm where yearlings were being prepared for fall sales. It was not possible to control the level of exercise any of the horses received prior to the onset of the trial; horses may have been longed for 0, 3 or 6 months. It is likely that treatment groups differed in initial average serum KS at least in part due to the duration of previous training and age. Therefore, changes in serum KS relative to baseline levels, rather than absolute KS values, were used to analyze treatment effects. Initially, serum KS decreased with increased amount of exercise (data not shown), but this decrease was confounded with normal effects of aging. Longeing as performed in this experiment did not elevate serum KS ($p = .1$), suggesting that cartilage degradation was not induced. However, longeing may still adversely affect cartilage integrity under different conditions. The speed at which horses are longed, the diameter of the longeing circle, and the duration of

longeing all need to be considered. Horses in this study were in training for longe-line classes and were therefore longed at a slow trot and cantered in a large circle. Increased speed in a smaller circle and/or prolonged exercise may be required to produce measurable damage.

Glucosamine may have had some effect on serum KS concentration ($p = .08$). Walked horses receiving glucosamine showed a slight elevation in KS concentration compared to those not receiving supplementation (Figure 19; $p = .04$; 21.01 vs –29.837). However, this increase could be explained as a result of increased cartilage turnover (increase in both synthesis and degradation) rather than increased cartilage degradation alone, similar to what is observed in early post-natal development (15). Todhunter *et al.* (1993) induced osteochondral defects in the radial carpal bones of 18 ponies and found that KS increased in exercised ponies administered polysulfated glycosaminoglycan (PSGAG) compared to exercised, non-medicated ponies. These authors hypothesized that exercise, when combined with medications such as PSGAG, may increase KS release from articular cartilage. Unlike the present study, Todhunter *et al.* induced damage prior to exercise. In our case, treatment effects were minimal and restricted to the walked, glucosamine-fed horses. The resulting small increase in serum KS is probably not biologically meaningful and could be attributed to a slight increase in turnover or may be due to the high sensitivity of the marker.

No differences between exercise or glucosamine treatment were observed for serum osteocalcin, although osteocalcin concentrations decreased over time

(Figure 20). This decrease is a normal occurrence as horses mature (16). In humans, serum osteocalcin, a marker specific for bone formation, increased as a result of exercise (17). Athletic subjects were interpreted to have had a higher turnover of bone status compared with non-athletic subjects. In horses, signaling for bone remodeling is based on strain, which is highly correlated with speed (2), and bone kinetics can be altered in response to varying exercise intensities (18). Apparently, the two exercise regimens used in this study were not significantly different in their ability to affect bone remodeling.

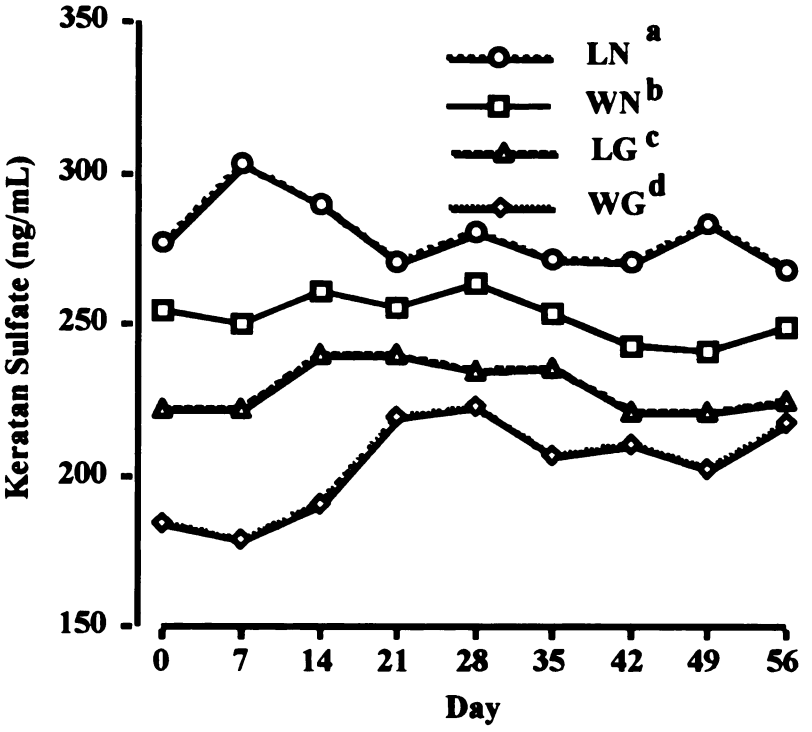

Figure 19. Mean changes in serum keratan sulfate throughout the treatment period. ^{abcd}Treatments lacking a common superscript differ ($P < 0.01$). LN=longeing/supplement control; LG=longeing/glucosamine; WN=walking/supplement control; WG=walking/glucosamine.

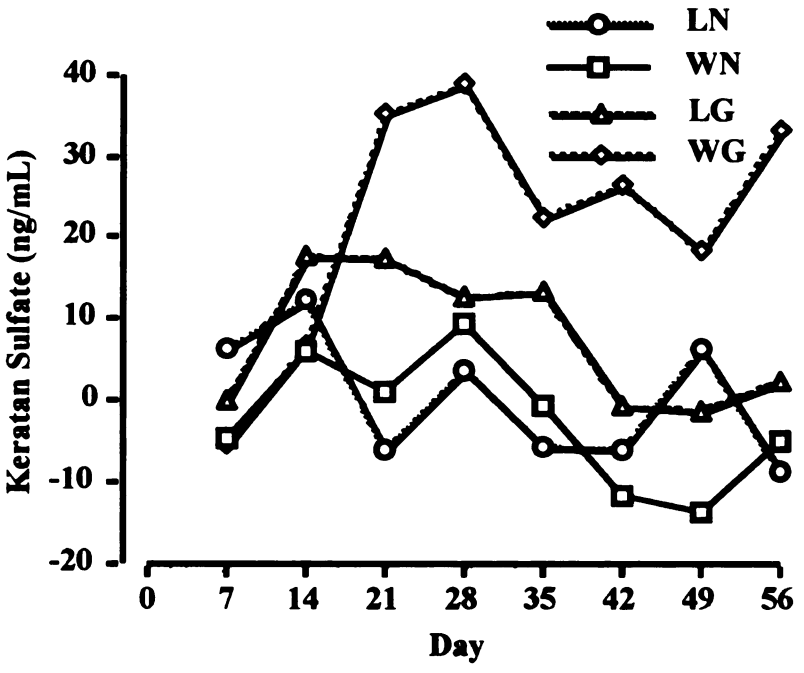

Figure 20. Mean serum keratan sulfate values transformed by taking the deviation from day 0. Treatments lacking common superscript differ ($P<0.05$). LN=longeing/supplement control; LG=longeing/glucosamine; WN=walking/supplement control; WG=walking/glucosamine.

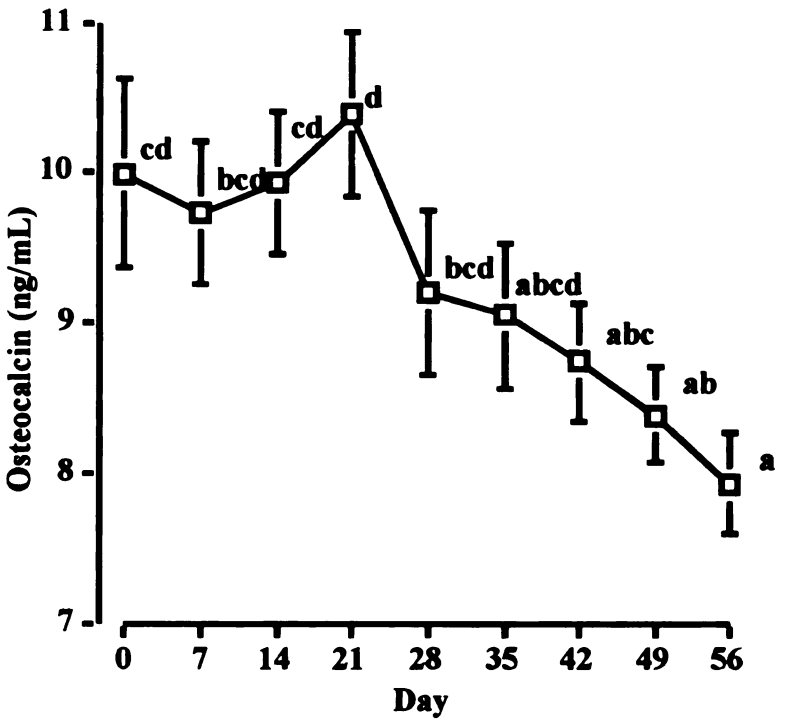

Figure 21. Mean change in serum osteocalcin throughout the treatment period.

^{abcd}Days lacking common superscript differ ($P < 0.05$).

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APPENDIX B

PEPSIN DIGEST OF EQUINE ARTICULAR CARTILAGE TREATED WITH LPS AND GLUCOSAMINE

Articular cartilage was obtained from the antebrachio-carpal and middle carpal joints of horses (2-8 years old) sacrificed for reasons other than joint problems. Cartilage discs (3.5mm) were biopsied from the weight-bearing region of the articular surface. Explant discs (3 per well, 40-60mg total) were cultured in a 24-well Falcon culture plate (Fisher Scientific; Pittsburgh, PA) with a modified version of Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Ham) (1:1) (Gibco; Grand Island NY). The media was supplemented with 10% fetal bovine serum (FBS, Gibco), 50 μg/ml ascorbate and 100 units/ml penicillin/streptomycin (Gibco). The explants were maintained in culture in a humidified incubator with 7% CO2 at 37°C.

Explants were maintained in basal media for 2 days prior to the first of 2 treatment days. Conditioned media was removed and replaced daily and stored at 4°C until analysis. Lipopolysaccharide was added to induce cartilage degradation. To evaluate the effects of glucosamine, varying concentrations of glucosamine HCl (0.25, 2.5, or 25 mg/ml) were added to the cultures.

Media collected from the cultures were digested with 1mg/ml pepsin for 72 hours at 4°C. The sample was then neutralized with 4N NaOH and dialyzed against TBSE for 48 hours (0.4M NaCl, 50mM Tris, 5mM EDTA). Treatments

digested included media, LPS and 25.0 mg/ml glucosamine on Days 0, 1 and 2. These digested samples were then run on 8% SDS PAGE gels either reduced or nonreduced.

Glucosamine significantly inhibited the amount of pepsin resistant protein released into the media on days 1 and 2 compared to LPS treatment as indicated by the band shown on the gels between 42 and 80KD (Figure 21). This was observed whether the samples were reduced (Figure 21 a) or not (Figure 21 b). Several pepsin resistant proteins fall within this size range, including collagen type VI or X and fibronectin. Further research should be focused on determining what protein this band represents.

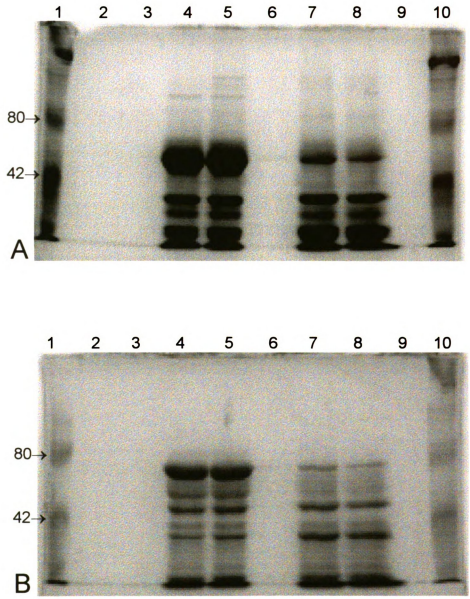

Figure 22. 8% SDS-page gels with media samples pepsin digested gel A) nonreduced B) reduced. Lanes: 1 and 10) std, 2) media Day 1, 3) lps Day 0, 4) lps Day 1, 5) lps Day 2, 6) lps + 25 mg/ml glcn Day 0, 7) lps + 25 mg/ml glcn Day 1, 8) lps + 25 mg/ml glcn Day 2.