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THE COMPARATIVE EFFECTS OF HEXOSAMINES IN THE INHIBITION OF ARTICULAR CARTILAGE DEGRADATION

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ABSTRACT

THE COMPARATIVE EFFECTS OF HEXOSAMINES IN THE INHIBITION OF ARTICULAR CARTILAGE DEGRADATION

By

David Michael Mello

In this study the capacities of two hexosamines, glucosamine and mannosamine, to inhibit chemically induced bovine and equine articular cartilage breakdown were compared. Lipopolysaccharide (LPS) (10 μg/ml) was added to explant cultures of bovine and equine articular cartilage to stimulate tissue degradation. Glucosamine or mannosamine was added to cultures of bovine cartilage at doses of 0.5, 1.0, 2.5, 5.0, or 10.0 mg/ml. Equine cartilage received glucosamine at 2.5, 5.0, or 10 mg/ml or mannosamine at 0.5, 1.0, 2.5, or 10 mg/ml. Culture media were analyzed for the presence of markers of cartilage catabolism, nitric oxide (NO) and proteoglycans (PG). Bovine cartilage explants were analyzed for gelatinase activity. Chondrocyte viability was also assessed. Mannosamine, at lower doses than glucosamine, inhibited the accumulation of NO and PG in culture media. LPS had no effect on gelatinase activity. Hexosamines did not elicit cell death at doses that were protective for cartilage degeneration. In conclusion, mannosamine appears to be a more potent inhibitor of selected indices of articular cartilage degradation than glucosamine *in vitro*.

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DEDICATION

I dedicate this thesis to my parents in gratitude for their unconditional love, support and encouragement.

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Images in this thesis are presented in color.

LIST OF ABBREVIATIONS

CO_2 - Carbon Dioxide

FBS – Fetal Bovine Serum

GlcN – Glucosamine

INOS – Inducible Nitric Oxide Synthase

LPS – Lipopolysaccharide

ManN – Mannosamine

MMP – Matrix Metalloproteinase

NO – Nitric Oxide

OA- Osteoarthritis

PG - Proteoglycan

Introduction

Osteoarthritis (OA) is a disease characterized by the loss of basic articular cartilage function. It affects the entire joint, ultimately ending in the full thickness loss of articular cartilage (Flores and Hochberg, 1998). OA is of particular concern to the horse industry as it is a leading cause of decreased performance in athletic horses (Baxter, 1992). Similarly, OA is a major source of disability among humans, particularly for the elderly population (Creamer and Hochberg, 1997). In the United States alone, 20.7 million adults are affected (Lawrence et al., 1998).

The degeneration of the articular cartilage matrix that is observed in OA is hypothesized to be a result of proteolytic articular cartilage matrix degradation exceeding matrix synthesis. Currently, the precise mechanisms through which this balance is lost are not understood; however, multiple factors can contribute to the development of OA. Among these are age (Sandy and Lark et al., 1998) and obesity (Creamer and Hochberg, 1997), as well as mechanical stresses of abnormal intensity, duration, and frequency placed on the joint (Pool, 1996). In addition, there appears to be a genetic predisposition to the development of OA (Knowlton et al, 1990; Horton et al., 1998). Due to a lack of nerve endings in articular cartilage, detection of OA may be delayed until it has progressed to the advanced stages of the disease. Progression of OA can result in permanent joint pain and dysfunction.

Commonly used treatments of OA are aimed at decreasing pain. Traditional therapies include non-pharmacologic methods such as light exercise to increase joint mobility, as well as pharmacologic methods including nonsteroidal anti-inflammatory drugs (NSAIDs) and intra-articular corticosteroids (Hochberg et al., 1995a; Hochberg et al., 1995b). Although light to moderate exercise helps improve muscle strength to support the joint and provides some pain relief (Buckwalter and Lane, 1997); NSAIDs and intra-articular corticosteroids are more frequently utilized to manage pain. Despite the popularity of these pharmacologic methods, the analgesic benefits of prolonged use can be outweighed by adverse side effects that may contribute to the loss of articular cartilage. Consequently, emphasis has been placed on the development of alternative therapies for OA that are not only analgesic, but that may also prevent the degeneration of articular cartilage.

Glucosamine, a six-carbon amino sugar, has shown promise as an alternative therapy for OA. Clinical trials of glucosamine have revealed that glucosamine alleviates the pain of OA (Crolle and D'Este, 1980; Pujalte et al., 1980), while both *in vivo* (Reginster et al., 2001) and *in vitro* (Piperno et al., 2000; Bassleer et al., 1998; Fenton et al., 2000a; Fenton et al., 2000b) studies have demonstrated the potential of glucosamine to inhibit articular cartilage degradation. Consequently, scientists have begun to examine the efficacy of related amino sugars in the prevention of articular cartilage breakdown.

Mannosamine has recently earned attention for its potential therapeutic benefits. Mannosamine is an aminomonosaccharide and an isomer of glucosamine that differs in structure only by an inversion of the amino group at the C2 position. In *in vitro* studies, mannosamine inhibits articular cartilage matrix breakdown (Sandy et al., 1999; Bryson et al., 2000), as well as proteolytic enzyme activity, to a greater degree than glucosamine (Sandy et al., 1999). Consequently, further investigation of the effects of mannosamine on articular cartilage appears to be merited.

In these studies, the capacities of glucosamine and mannosamine to inhibit experimentally induced articular cartilage degeneration were compared. In the first of two studies, a bovine model was used to obtain a general understanding of the potency of these two amino sugars in the inhibition of articular cartilage breakdown. In the second study, an equine model was used to compare the abilities of glucosamine and mannosamine to inhibit articular cartilage degradation in a species that is commonly afflicted with OA. The hypothesis tested was that mannosamine is a more potent inhibitor of articular cartilage degradation than glucosamine.

Chapter 1

Literature Review

Articular Cartilage

Composition and Structure

Articular cartilage is found at the end of long bones and serves as a shock absorber for the underlying subchondral bone, as well as providing a smooth surface on which joints can bend and articulate. It contains a fibrillar network of collagen, predominantly type II with smaller quantities of types IX and XI present (Mendler et al., 1989; Poole et al., 1996). Collagen fibrils provide tensile strength to cartilage (Broom and Silyn-Roberts, 1990; Bader et al., 1992; Zhu et al., 1993). Type XI collagen is found within the type II fibril (Mendler et al., 1989), while type IX collagen is covalently attached to type II collagen at the nonhelical telopeptide region (Eyre et al., 1987; van der Rest et al., 1988). A fourth type of collagen, type VI, is also found in the cartilage matrix adjacent to the chondrocytes where it is proposed to help chondrocyte attachment to the matrix (Buckwalter and Lane, 1997).

Interwoven within the collagen network are proteoglycan (PG) molecules of which the largest, aggrecan, predominates (Heinegard et al., 1998). Aggrecan consists of three globular domains (G1, G2 and G3) separated by two chondroitin sulfate binding domains, CS1 and CS2 (Heinegard et al., 1998). The first globular domain (G1) allows

aggrecan to be incorporated into the extracellular matrix through binding to hyaluronic acid (Heinegard et al., 1998), a chondrocyte-produced linear glycosaminoglycan consisting of repeating disaccharide units of glucuronic acid and N-acetyl-D-glucosamine. The stable complex formed by interactions between aggrecan and hyaluronic acid helps maintain cartilage stability (Buckwalter et al., 1984). The negatively charged chondroitin sulfate side chains of aggrecan cause the repulsion of aggrecan molecules and contribute to its osmotic activity, yielding a swelling stress against the tensile properties of collagen. These counteracting forces impart the tissue with compressive stiffness (Maroudas, 1976).

Within the articular cartilage matrix are chondrocytes, which are responsible for the synthesis and degradation of extracellular matrix components. They only constitute 1% of the tissue volume (Buckwalter and Lane, 1997). In mature healthy cartilage, chondrocytes synthesize sufficient components of articular cartilage to maintain the cartilage matrix (Bullough, 1992). As chondrocytes fail to make contact with one another, intracellular signaling is mediated through cellular connections to the extracellular matrix (Bullough, 1992). Consequently, alterations in the extracellular matrix organization and composition provide signals to alter chondrocyte metabolism. Given that articular cartilage is not vascular, chondrocytes also depend on extracellular matrix integrity for the diffusion of nutrients and metabolites (Woo et al., 1992). As a

result, maintenance of a properly functioning and organized extracellular matrix is imperative to chondrocyte signaling and survival.

Articular cartilage consists of four distinct zones, which can be differentiated based on chondrocyte shape and matrix organization (Buckwalter and Mankin, 1997). These are the superficial, transitional, radial, and calcified cartilage zones. In the superficial zone, chondrocytes appear flat and oval in shape with a random distribution within the matrix. Chondrocytes of the transitional zone are more round in shape and randomly distributed throughout the matrix. The radial zone consists of round chondrocytes in an organized columnar arrangement. In the zone of calcified cartilage, the chondrocytes are round and visibly smaller than those of the other zones (Buckwalter et al., 1988). Similarly, the orientation of the collagen matrix changes from one zone to the next. Collagen fibrils of the superficial zone lie parallel to the articular surface. In the transitional zone, collagen fibrils take on a somewhat random orientation, while in the radial zone they are arranged perpendicular to the articular surface. In the deepest zone, the calcified cartilage, collagen surrounding the chondrocytes has become hard and calcified through a chondrocyte-mediated process (Bullough et al., 1992).

Function

In a healthy joint, articular cartilage will deform under the application of mechanical stress to achieve proper stress transmission in order to resist destruction (Broom and Silyn-Roberts, 1990). The degree of deformation in articular cartilage subjected to mechanical load is dependent on the tensile properties of collagen and the osmotic pressure generated by its PG content (Mizrahi et al., 1986). Loss of PG from the matrix loosens the collagen network and decreases the compressive stiffness, or pre-stress of the tissue, making the cartilage more susceptible to deformations that may lead to articular cartilage degeneration (Zhu et al., 1993). Likewise, decreases in tensile strength and compressive stiffness may lead to mechanical damage (i.e. fibrillation of the cartilage) in tissue subjected to compressive loading (Bader et al., 1992). Tensile strength is particularly important in preventing the erosion of the surface layer of the tissue and the subsequent disruption of the deeper layers (Aydelotte and Keuttner, 1988). Consequently, a structurally intact collagen network and the maintenance of PG within the matrix is key for articular cartilage to perform its role of absorbing and transmitting shock, as well as protecting the subchondral bone (Broom and Silyn-Roberts, 1990).

Osteoarthritis

Osteoarthritis (OA) is a disease characterized by the loss of basic articular cartilage function. It affects the entire joint including the articular cartilage, subchondral bone, ligaments, capsule, synovial membrane, and periarticular muscles, ultimately ending in full thickness loss of the joint surface (Flores and Hochberg, 1998). The pain associated with OA and its prevalence causes it to be a major source of disability for people, particularly the elderly population (Creamer and Hochberg, 1997). As of 1990, 15% of the U.S. population (37.9 million) was estimated to suffer from arthritis, with the projected percentage reaching 18.2% (59.4 million) by 2020 (Centers for Disease Control, 1994). Of the different forms of arthritis, OA is the most prevalent, (Creamer and Hochberg, 1997; Lawrence et al., 1998) affecting approximately 20.7 million US adults (Lawrence et al., 1998). Similarly, OA is of particular concern to the horse industry as it is a leading cause of decreased performance in athletic horses (Baxter, 1992). The magnitude of the occurrence of OA among horses was illustrated by a random evaluation of 72 equine joints at necropsy, in which it was revealed 35% of the joints showed obvious cartilage damage (Morris and Treadwell, 1994).

Etiology

Although the precise mechanisms initiating OA are not entirely understood, several factors contribute to the disease process. Among these are age (Sandy and Lark, 1998), obesity (Creamer and Hochberg, 1997), and mechanical stress of abnormal intensity, duration, and frequency placed on the joint (Pool, 1996). In addition, some people have a genetic predisposition to the development of some forms of OA. A study by Horton et al. (1998) demonstrated an association between a human aggrecan gene polymorphism and the occurrence of bilateral hand OA in white men over 60 years of age. Another study linked mutations in the type II collagen allele and susceptibility to OA (Knowlton et al., 1990).

Pathology

The degenerative nature of OA results in the formation of cracks or fissures at the articular cartilage surface with partial or complete loss of the tissue (Meachim and Emery, 1974; Mow and Setton, 1998). Additionally, changes in the structure and composition of PGs and the collagen network within the cartilage matrix are associated with OA. Specifically, there is increased collagen degradation and decreased PG content in OA cartilage. Osteoarthritis also results in a reduced cartilage content of other matrix molecules including hyaluronan, thereby limiting PG aggregation and increasing subsequent PG loss from the cartilage matrix (Manicourt and Pita, 1988). Initial lesions

include increased cartilage thickness, quite possibly as an anabolic response by chondrocytes to maintain normal amounts of the extracellular matrix in the presence of increased proteolytic activity (Brandt et al., 1991). Such efforts by the chondrocytes are often fruitless as the destruction of the collagen network precludes the ability of newly synthesized matrix molecules, i.e. PGs, to be retained within the matrix. Consequently, degradation prevails leading to the progressive destruction of the tissue (Heinegard and Saxne, 1991).

The effects of OA are not confined to articular cartilage as the formation of osteophytes, as well as thickening and stiffening of subchondral bone, are often associated with the disease. Changes in the subchondral bone often lead to the abnormal distribution of forces over the joint surface that potentially perpetuate cartilage degradation (Radin et al., 1973; Radin et al., 1978; Dedrick et al., 1993).

Diagnosis

Typically, diagnosis of OA is made by the presence of joint pain, changes in gait, radiographically detectable joint space narrowing, and osteophytes. Since articular cartilage lacks nerve endings (Kuettner and Hascall, 1992), detection of the aforementioned diagnostic features of OA may be delayed (Heinegard et al., 1998). Progression to the advanced stages of OA results in complete articular cartilage destruction and permanent joint pain and dysfunction (Baxter, 1992).

Matrix Metalloproteinases and Osteoarthritis

The cartilage degeneration of OA is hypothesized to be a result of proteolytic articular cartilage matrix degradation overwhelming matrix synthesis (Sandy and Lark, 1998). Once degradation is initiated and arrangement of the supporting fibers has been disrupted, cartilage lacks the ability to repair itself (Kuettner and Hascall, 1992) and is considered to be a self-destructive process (Pool et al., 1996). Although the pathogenesis of OA is not completely understood, cytokines, including interleukin-1 (Il-1) (Martel-Pelletier et al., 1999), induce the expression of specific members of a large family of matrix degrading enzymes, the matrix metalloproteinases (MMPs) (Murphy et al., 1991). Most of the MMPs are secreted as inactive zymogens that are subsequently cleaved and activated in the extracellular matrix. The majority of the MMPs are secreted, however, the membrane type MMPs (MT-MMP) remain bound to the cell membrane through a transmembrane domain (Nagase and Woessner, 1999). Matrix metalloproteinase activity is inhibited by the tissue inhibitors of metalloproteinases (TIMPs), which reversibly bind to the MMPs in a one to one ratio. However, in OA tissue the ratio of MMP to TIMP is shifted in favor of the MMPs (Ishiguro et al., 1999).

Several studies support the involvement of members of the MMP family in OA. Caron et al. (1996) showed that Il-1 receptor antagonist treatment of experimentally induced canine OA inhibited development of lesions, including osteophyte formation and cartilage erosion, and reduced expression of collagenase-1 (MMP-1) mRNA, hence,

suggesting a potential role for Il-1 mediated stimulus of MMPs in joint destruction in this model system. Recently, another collagenase, MMP-13 (collagenase-3), has also received much attention for its potential role in the breakdown of collagen in OA. MMP-13 mRNA abundance was greater in OA cartilage (Mitchell et al., 1996) than in normal tissue (Reboul et al., 1996). Protein synthesis of MMP-13 is approximately 9.5 fold greater in OA tissue compared to normal tissue and its mRNA expression and protein synthesis are inducible by cytokines, such as Il-1 and tumor necrosis factor -α, that are associated with OA (Reboul et al., 1996). Moreover, MMP-13 has a five to ten- fold (Reboul et al., 1996; Mitchell et al., 1996) greater rate of type II collagen breakdown than MMP-1. Further evidence for the role of MMP-13 in articular cartilage degeneration stems from a study using an inhibitor that blocked MMP-8 and MMP-13, but not MMP-1 (Dahlberg et al., 2000). Type II collagen degradation was inhibited despite the activity of MMP-1. These data support the theory that MMP-13 is an important mediator in the collagen degradation seen in OA (Reboul et al., 1996). Other reports indicate that there may be regional roles for MMPs in matrix destruction. In OA cartilage both MMP-1 and MMP-13 are present, but MMP-1 localized predominantly in the superficial layers, and MMP-13 is restricted to the deeper zones. Thus, these two enzymes may have different functions in the disease process (Fernandes et al., 1998).

Other members of the MMP family may also be involved in OA. Subchondral bone of affected joints exhibits increased expression of MMP-2 compared to normal and

osteoporotic bone tissue (Mansell et al., 1997). Elevations in MMP-2 may reflect an attempt of bone to remodel in response to altered mechanical loads across the joint in the presence of damaged overlying cartilage (Mansell et al., 1997). Studies on equine tissue have revealed that both MMP-2 and MMP-9 are produced by various articular cell types including chondrocytes and synovial fibroblasts (Clegg et al., 1997a). Furthermore, increased activity of the gelatinases (MMP-2 and MMP-9) appear in the synovial fluid of equine joints afflicted with aseptic joint diseases, i.e. OA, compared to healthy joints. (Clegg et al., 1997b). Given that MMP-2 can cleave type II collagen, and that both MMP-2 and MMP-9 have high affinity for degraded collagen, their increased activities in diseased joints suggest a pathogenic role for these two enzyme in OA (Clegg et al,, 1997a; Clegg et al., 1997b). Additionally, increased MMP-9 activity is present in equine joints with gross cartilage changes characteristic of OA (Jouglin et al., 2000). Studies of human articular cartilage revealed the presence of MMP-9 mRNA, protein, and enzyme activity in OA tissue while MMP-9 appears to be absent in normal articular cartilage (Mohtai et al., 1993). Moreover, mRNA expression of MMP-2, MMP-3, and tissue collagenase was detected in both normal and OA tissue; however, the level of expression was increased with OA (Mohtai et al., 1993). Expression of MMP-9 was also associated with fibrillated cartilage suggesting that it may be a marker of progressive articular cartilage degradation (Mohtai et al, 1993).

In addition to collagen destruction, members of the MMP family participate in the release of PG from the cartilage matrix (Buttle et al., 1993). Flannery et al. (1992) demonstrated that MMP-3 cleaves aggrecan at a specific site within the molecule, between Asn341 and Phe342. Epitopes resulting from this cleavage accumulate in articular cartilage with aging and OA (Flannery et al., 1992).

Aggrecanase and Osteoarthritis

While the MMPs are implicated in collagen and PG catabolism in OA, a related enzyme, aggrecanase, demonstrates particularly high catalytic activity toward PG (aggrecan) breakdown (Sandy et al., 1999). Aggrecanase is a member of the a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS) family of proteins. As such, aggrecanase contains a metalloproteinase domain, a disintegrin domain, a cysteine-rich region, an EGF repeat and a thrombospondin motif. The thrombospondin domain allows for the tight association of aggrecanase with extracellular matrix molecules (Tang and Hong, 1999). Within the aggrecan molecule a cleavage site in the interglobular (G1-G2) domain is located between Glu373-Ala374 that is specific for aggrecanase (Sandy et al., 1990). Experiments using bovine articular cartilage explants indicate that aggrecanase specific cleavage products are generated upon Il-1 stimulation (Lark et al., 1995). Moreover, through the use of N-terminal sequencing, synovial fluid from human patients with OA was shown to contain aggrecan fragments predominantly generated by

cleavage between Glu373-Ala374 (Lohmander et al., 1993). These results suggest an important role for aggrecanase in the chondrocyte mediated cartilage matrix damage observed in OA (Lohmander et al., 1993). Likewise, antibodies directed toward aggrecan epitopes generated by aggrecanase and MMPs revealed that in late stage OA, aggrecanase activity was elevated compared to normal tissue (Little et al., 1999). A similar increase in MMP activity, as detected by the presence of epitopes of aggrecan generated by cleavage between Asn 341-Phe342, was not observed in late stage OA tissue (Little et al., 1999). Collectively, this suggests that aggrecan degradation in OA is due primarily to aggrecanase and not MMP activity (Little et al., 1999).

Nitric Oxide and Osteoarthritis

The free radical, nitric oxide (NO), is proposed to be involved in articular cartilage breakdown (Martel-Pelletier et al., 1999). NO levels are highly elevated in OA cartilage compared to normal tissue (Pelletier et al., 1996) as a consequence of increased inducible NO synthase (iNOS) activity (Grabowski et al., 1997). Furthermore, nitrite, a stable byproduct of NO metabolism, accumulates in the synovial fluid of patients with OA (McInnes et al., 1996).

Nitric oxide contributes to decreased articular cartilage matrix integrity through the inhibition of matrix molecule synthesis, including the PGs (Taskiran et al., 1994; Hauselmann et al., 1994). Nitric oxide also promotes MMP activity, contributing to

cartilage breakdown (Murell et al., 1995). Furthermore, it decreases the synthesis of the soluble IL-1 receptor antagonist that may also lead to enhanced cartilage catabolism (Martel-Pelletier et al., 1999). Administration of iNOS inhibitors is protective for cartilage lesions (Pelletier et al., 1998) and results in decreased MMP activity *in vitro* (Murrell et al., 1995).

In contrast to the catabolic effects of NO, an *in vitro* study using equine articular cartilage explant cultures demonstrated NO to be capable of decreasing aggrecanase activity (Bird et al., 2000). In this study, exogenous NO led to a decrease in the presence of aggrecanase generated cleavage products in culture medium. Collectively, these data suggest that this free radical may have both catabolic and anti-catabolic roles in OA through its mediation of aggrecanase activity (Bird et al., 2000).

Traditional Treatments for Osteoarthritis

Commonly used therapies for the management of OA in humans include non-pharmacologic methods such as light exercise to increase joint mobility, as well as pharmacologic methods including nonsteroidal anti-inflammatory drugs (NSAIDs) and intra-articular corticosteroids (Hochberg et al., 1995a; Hochberg et al., 1995b). Although light to moderate exercise helps improve muscle strength to support the joint (Buckwalter and Lane, 1997) and provides some pain relief (Hochberg et al., 1995a; Hochberg et al., 1995b), NSAIDs and intra-articular corticosteroids are frequently necessary to provide

pain relief and improve joint mobility. Despite the popularity of these pharmacologic methods, particularly the NSAIDs (Hochberg et al., 1995a), the analgesic benefits can be outweighed by deleterious side effects associated with prolonged use.

Prolonged use of NSAIDs may result in the development of disorders such as gastrointestinal ulcers (Hochberg et al., 1995a; Towheed and Hochberg, 1997; Towheed and Hocherg, 1997b; Gabriel et al., 1991; Griffin, 1998; Rodriguez, 1998). The damaging effects of NSAIDs are most pronounced in the elderly population, with thousands of deaths being attributed to NSAID use each year (Griffin, 1998). Furthermore, the combined use of NSAIDS with corticosteroids greatly increases the risk of developing gastrointestinal ulcers compared to NSAID therapy alone (Gabriel et al., 1991). Similarly, the most commonly used NSAID for horses, phenylbutazone (May and Lees, 1996), can lead to the formation of oral cavity and gastric ulcers, inappetance, weight loss, and diarrhea (Traub et al., 1983). Despite the risks associated with phenylbutazone it is widely used primarily due to its low cost (May and Lees, 1996). Beyond the risk of gastrointestinal disorders, some NSAIDS decrease both glycosaminoglycan (GAG) (Brandt and Palmoski, 1984; deVries et al., 1985; McKenzie et al., 1976; Heman et al., 1986) and protein (Herman et al., 1986) synthesis in articular cartilage. Therefore, NSAIDS may promote degradation and inhibit the reparative processes of the diseased tissue (Herman et al., 1986). Moreover, the analgesic effects of

NSAIDs may contribute to the disease process as a result of over-loading the diseased tissue (Brandt, 1997).

Frequent injections of intra-articular corticosteroids can lead to progressive tissue damage in weight-bearing joints (Hochberg et al., 1995b). In an experimental model of OA, intra-articular corticosteroid injections caused a decrease in type-II procollagen mRNA expression (MacLeod et al., 1998). Considering the central role type II collagen plays in the tensile stiffness of articular cartilage, decreases in type II procollagen mRNA expression may be detrimental to cartilage integrity over time (MacLeod et al., 1998). Furthermore, frequent intra-articular corticosteroid injections lead to diminished PG content of equine articular cartilage, as detected by decreased safrinin O staining (Trotter et al., 1991), as well as increased chondrocyte necrosis (Shoemaker et al., 1992). The analgesic effects of corticosteroids have similar drawbacks to those of NSAIDs in that they may result in excessive loading of the diseased tissue (Baxter, 1992). Nevertheless, the use of intra-articular corticosteroids continues in both humans (Hochberg et al., 1995a; Hochberg et al., 1995) and horses (Trotter, 1996). Consequently, a great deal of importance has been placed on developing therapies for OA that not only alleviate the symptoms of the disease, but also reverse the disease process in a nontoxic manner.

Glucosamine and Osteoarthritis

Glucosamine is an aminomonosaccharide that has earned considerable attention as a therapeutic agent in the management of OA (Hungerford et al., 2000). Glucosamine is obtained primarily from two sources, either the exoskeletons of insects and shellfish, or synthesized in the laboratory (Barclay et al., 1999). This amino sugar is reported to be non-toxic (Setnikar et al., 1986) and localizes to the articular cartilage after oral administration (Setnikar et al., 1991; Deal and Moskowitz, 1999). Currently, glucosamine is marketed to consumers as pure glucosamine or salts of glucosamine i.e., glucosamine sulfate and glucosamine HCl (Deal and Moskowitz, 1999). The sulfate group of glucosamine sulfate may be important to its therapeutic effects in OA (Deal and Moskowitz, 1999). Glucosamine salts are easily absorbed by the gut, with greater than 90% efficiency, whereas the large majority of non-conjugated glucosamine cannot be efficiently absorbed (Deal and Moskowitz, 1999). Multiple short-term human clinical trials have demonstrated glucosamine sulfate to decrease the pain and stiffness associated with OA (Crolle and D'Este, 1980; Pujalte et al., 1980; Barclay et al., 1998; da Camara and Dowless , 1998). Furthermore, a three-year clinical trial demonstrated OA patients treated with glucosamine sulfate have decreased joint space narrowing and joint pain compared to patients that received a placebo (Reginster et al., 2001). A 16-week human clinical trial of glucosamine HCl demonstrated the capacity of this glucosamine salt to decrease the pain associated with knee OA (Houpt et al., 1999). Additionally, studies of

experimental OA revealed glucosamine HCl to inhibit the formation of mild to moderate lesions compared to controls that did not receive glucosamine (Lippiello et al., 2000).

Glucosamine is also marketed in combination with other dietary supplements thought to be protective of cartilage, such as chondroitin sulfate (CS) (Hungerford et al., 2000; Deal and Moskowitz, 1999). *In vivo* studies on the effects of combination therapies of glucosamine HCl and CS on experimental OA, suggest such treatments are effective in slowing OA progression compared to controls or animals treated with the individual components (Lippiello et al., 2000). Veterinary clinical trials using glucosamine HCl and CS combinations, report symptomatic improvement in treated animals (Anderson et al., 1999; Hanson et al., 1997). Interestingly, a study using a similar combination, revealed that pretreatment with this combination therapy can inhibit the onset of joint inflammation associated with synovitis, demonstrating a prophylactic effect of glucosamine and/or chondroitin sulfate (Canapp et al., 1999).

Although the precise mechanisms by which glucosamine operates are not known, several *in vitro* studies have provided information regarding some of the potential pathways of action. Glucosamine increases protein synthesis and inhibits collagenolytic activity in human OA cartilage (Piperno et al., 2000), while it increases PG synthesis by human OA chondrocytes in culture (Bassleer et al., 1998). Glucosamine also inhibits PG release from lipopolysaccharide (LPS) stimulated equine articular cartilage (Fenton et al., 2000a; Fenton et al., 2000b). Glucosamine inhibits the production of NO in LPS

stimulated equine articular cartilage (Fenton et al., 2000a; Fenton et al., 2000b). Specifically, glucosamine may inhibit NO synthase by decreasing the bioavailability of NADPH, a cofactor required for the activity of the NO producing enzyme (Wu et al., 2001). Furthermore, MMP activity is inhibited in LPS stimulated equine articular cartilage treated with glucosamine HCl as compared with non-glucosamine treated samples (Fenton et al., 2000b). Studies also demonstrate the ability of glucosamine to inhibit aggrecanase activity in both rat chondrosarcoma cells and articular cartilage from one to two week old calves stimulated with interleukin-1 or retinoic acid (Sandy et al., 1998). These findings are consistent with the properties of a disease-modifying drug for OA. Specifically, these properties are that it prevents, retards the progression of, or reverses morphologic changes of OA (Brandt, 1995).

Mannosamine and Articular Cartilage

Since the demonstration of positive effects of glucosamine, scientists have begun to examine the therapeutic effects of related hexosamines in the prevention of articular cartilage degeneration. Of these hexosamines, mannosamine has recently earned attention for its potential therapeutic benefits. Mannosamine is an aminomonosaccharide and an isomer of glucosamine that differs in structure only by an inversion of the amino group at the C2 position. *In vitro*, mannosamine inhibits the activity of aggrecanase at one-tenth the dose of glucosamine in retinoic acid and interleukin-1 stimulated rat

chondrosarcoma cells and bovine articular cartilage explants (Sandy et al., 1999). Mannosamine also inhibits interleukin-1 induced reductions in tissue modulus, dynamic stiffness, streaming potential, and hydraulic permeability (Patwari et al., 2000), suggesting a role for mannosamine in the preservation of the mechanical properties of articular cartilage. Mannosamine inhibits the release of PGs from interleukin-1, retinoic acid, and tumor necrosis factor-α stimulated bovine nasal and articular cartilage, as well as from retinoic acid stimulated human cartilage (Bryson et al., 2000).

The mechanism by which mannosamine effects occur is not clear. Mannosamine inhibits the incorporation of glycosylphosphatidylinositol (GPI) anchors into proteins (Lisanti et al., 1991). Mannosamine may inhibit aggrecanase activity through the prevention of GPI anchor incorporation into proteins involved in signaling cascades leading to aggrecanase activity (Sandy et al., 1999). However, more recent reports suggest that mannosamine inhibits markers of articular cartilage degeneration at doses lower than what is required to inhibit the incorporation of GPI anchors (Bryson et al., 2000). Despite the uncertainties regarding the mechanism of action, the high potency and protective effects of mannosamine promotes interest in this amino sugar as a potential future therapy for OA.

Bovine *in vitro* models of Osteoarthritis

Due to limited availability and inconsistency in results obtained with human tissue in the study of disease, animal models are ideal alternatives for the study of disease processes (Moskowitz, 1984). Through the use of *in vitro* animal models scientists can investigate disease pathophysiology in a controlled environment, and therefore gain larger quantities of data in a reproducible manner. The challenge of using *in vitro* animal models is the selection of the most appropriate model to answer questions surrounding human disease. In the study of OA scientists have used several animal models, among the more commonly used is the bovine. Studies examining healthy articular cartilage have demonstrated similarities between the biomechanical properties of bovine and human knee (Athanasiou et al., 1991) and hip (Athanasiou et al., 1995) articular cartilage. Furthermore, human and bovine articular cartilage exhibit similar patterns of degradation in the presence of both mechanical (Lane et al., 2000; Ostendorf et al., 1994) and chemical (Bryson et al., 2000) stimuli. Therefore, use of bovine *in vitro* models of cartilage degradation appears to be valid for the study of the pathobiology and treatment of OA.

Rationale for experiments

Osteoarthritis is a debilitating disease that affects both humans and horses. Given the potential adverse side effects of prolonged use of traditional treatments for OA,

emphasis has been placed on the development of alternative therapies that are non-toxic and retard or reverse disease progression. Glucosamine is an alternative therapy that has gained attention for both its analgesic effects, as well as its ability to inhibit articular cartilage degradation. Consequently, scientists have begun to examine the potential chondroprotective effects of related hexosamines. Of these hexosamines, *in vitro* studies have shown mannosamine to be a potent inhibitor of markers of chemically induced articular cartilage degeneration. As a result, there has been increasing interest in the use of mannosamine as a potential future therapy for OA.

The purpose of these studies was to compare the abilities of glucosamine and mannosamine to inhibit selected markers of chemically induced articular cartilage degradation. The hypothesis tested was that mannosamine inhibits articular cartilage degradation at lower doses than glucosamine.

In the first of two studies, a bovine articular cartilage explant system was used to gain a general understanding of the potencies of glucosamine and mannosamine to inhibit articular cartilage degeneration. The chondroprotective effects of these amino sugars were assessed via the analysis of selected markers of articular cartilage degeneration including chondrocyte viability, NO production, PG release, and gelatinolytic activity of protein extracts. In the second study, through the use of an equine articular cartilage explant culture system, the capacities of glucosamine and mannosamine to inhibit articular cartilage degeneration were compared in a species that is commonly afflicted

with OA. The comparative chondroprotective effects of these two hexosamines were determined through the analysis of chondrocyte viability, NO production, and PG release. The results of these studies indicate both glucosamine and mannosamine inhibit selected markers of articular cartilage degradation. However, mannosamine exerts its chondroprotective effects at lower doses than glucosamine.

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Chapter 2

The acute effects of glucosamine and mannosamine on bovine articular cartilage degradation

Abstract

Objective: To compare the effects of glucosamine and mannosamine on the inhibition of selected indices of articular cartilage degradation.

Design: Articular cartilage was collected from weight bearing regions of bovine carpi and cultured in basal medium for 48 hours. Tissues were exposed for 24 hours to medium containing 10% FBS, 10 μg/ml LPS, and 0.5, 1.0, 2.5, 5.0 or 10.0 mg/ml glucosamine or mannosamine. n=6 wells per treatment per replicate. Experiments were replicated three times. Culture medium was changed daily and analyzed for nitric oxide (NO) and proteoglycan (PG) content as indicators of cartilage catabolism. Cartilage extracts were analyzed by zymography to detect gelatinolytic activity. At the end of the experiment, tissue samples were analyzed for chondrocyte viability.

Results: Addition of LPS increased NO production and PG release, but did not increase gelatinolytic activity. The presence of glucosamine and mannosamine at doses as low as 0.5 mg/ml inhibited NO production. Glucosamine inhibited PG release at 0.5 mg/ml, while mannosamine elicited a similar effect on PG release at one half the dose of glucosamine. Zymography revealed that LPS did not induce gelatinolytic activity. Doses

of glucosamine up to and including 5.0 mg/ml did not adversely affect chondrocyte viability. However, at 10.0 mg/ml glucosamine, cell death was present throughout the tissue. Mannosamine exhibited a toxic effect at 5.0 mg/ml, with pronounced chondrocytes death at a dose of 10.0 mg/ml.

Conclusion: The data presented in this study confirm the ability of two hexosamines, glucosamine and mannosamine, to inhibit selected indices of bovine articular cartilage degradation at doses that do not hinder chondrocyte viability. Moreover, we have shown a differential capacity of these two hexosamines to exert their protective effects, with mannosamine being a more potent inhibitor of articular cartilage degradation than glucosamine.

Introduction

Osteoarthritis (OA) is a disease characterized by derangement of basic articular cartilage structure and function that ultimately ends in full thickness loss of the cartilage and loss of joint function (Flores and Hochberg, 1998). In humans, OA is major cause of morbidity and disability, as well as a burden on health care resources, particularly for the elderly population (Creamer and Hochberg, 1997). In the United States alone, OA affects approximately 20.7 million people (Lawrence et al., 1997).

OA progresses by the slow degradation of the articular cartilage matrix, which is hypothesized to be a consequence of proteolytic degradation overwhelming cartilage

matrix synthesis (Sandy and Lark, 1998). In OA tissue, diminished PG content leads to decreased compressive stiffness of the articular cartilage (Armstrong and Mow, 1982; Grushko et al., 1989). Additionally, collagen degradation results in decreased tensile strength of the articular surface (Venn and Maroudas, 1977). Associated with OA are pro-inflammatory cytokines that induce high levels of nitric oxide (NO) production by articular chondrocytes (Stadler et al., 1991). Elevated NO production is a consequence of increased activity of inducible nitric oxide synthase (iNOS). Nitric oxide is thought to contribute to the weakening of the articular cartilage matrix through inhibition of PG synthesis (Taskiran et al., 1994) and by increasing the activity of matrix metalloproteinases (MMP), enzymes implicated in the progression of OA (Murrell et al., 1995). Ultimately, decreases in both tensile strength and compressive stiffness render articular cartilage unable to perform its main function of stress distribution and frictionless joint motion.

Contributing factors to the development of OA include age (Sandy and Lark, 1998), obesity (Creamer and Hochberg, 1997), mechanical stresses of abnormal duration frequency and intensity (Pool, 1996), and genetic predisposition (Horton et al., 1996; Knowlton et al., 1990). Due to a lack of nerve endings in articular cartilage, detection and diagnosis of OA is often delayed until the disease has progressed to the advanced stages. As osteoarthritic cartilage is not capable of adequate self-repair once breakdown

of collagen fibrils ensues (Kuettner et al., 1992), the development of therapies that slow or reverse the disease process are of great interest.

Currently, common treatments for OA include non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular corticosteroids. While these treatments are successful at alleviating the pain and stiffness associated with OA (Hochberg et al., 1995a; Hochberg et al., 1995b), prolonged use, particularly at high doses, has deleterious effects on articular cartilage (Herman et al., 1986; Trotter et al., 1991; Shoemaker et al., 1992). Analgesic effects that result in excessive loading of the diseased tissue may also contribute to adverse effects (Brandt, 1997; Baxter, 1992).

In recent years, glucosamine, an aminomonosaccharide, has received increasing popularity as a potential therapy for OA (Hungerford et al., 2000). Appealing qualities of glucosamine are that it is non-toxic (Setnikar et al., 1986) and localizes to the articular cartilage after oral administration (Setnikar et al., 1991; Deal and Moskowitz, 1999). Furthermore, glucosamine is naturally present in almost all tissues and can be readily incorporated into PG molecules (da Camara and Dowless, 1998).

Multiple short-term human clinical trials have demonstrated glucosamine sulfate (Crolle and D'Este, 1980; Pujalte et al., 1980; Barclay et al., 1998; da Camara and Dowless, 1998) and glucosamine HCl (Houpt et al., 1999) decrease the pain of OA. Additionally, a three-year clinical trial reported OA patients treated with glucosamine sulfate have decreased joint space narrowing and joint pain compared to patients

receiving a placebo (Reginster, 2001). Consequently, glucosamine may have beneficial effects that can be sustained over long periods of time.

Recently, attention has been given to the *in vitro* evaluation of related aminomonosaccharides as potential therapies for OA (Fenton et al., 2000a). Of these, mannosamine is a particularly potent inhibitor of articular cartilage degradation. Mannosamine, an isomer of glucosamine with an inversion of the C2 amine group, inhibits aggrecanase activity in both rat chondrosarcoma cells and bovine articular cartilage at one tenth the dose of glucosamine (Sandy et al., 1999). Furthermore, mannosamine inhibits PG release from articular cartilage, as well as inhibits chemically induced decreases in the mechanical properties of articular cartilage (Patwari et al., 2000).

The purpose of this study was to compare the effects of glucosamine and mannosamine in the inhibition of articular cartilage degradation. Through the analysis of markers of cartilage catabolism and proteolytic enzyme activity, we tested the hypothesis that mannosamine is a more potent inhibitor of articular cartilage degradation than glucosamine.

Materials and methods

Unless otherwise stated, chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Explant Cultures

Articular cartilage disks (3.5 mm) were biopsied from the weight bearing regions of the carpi of bovine (Holstein) steers collected from a local abbatoir. Biopsies of articular cartilage did not include the calcified layer of the tissue. Three explant discs were selected at random and cultured in each well of a 24-well Falcon tissue culture plate (Fisher Scientific; Pittsburgh, PA, U.S.A.). Cultures were maintained in 1:1 Dulbecco's Modified Eagles Medium: nutrient mixture F-12 (Ham) (Gibco, Grand Island, NY) supplemented with amino acids, trace minerals, 50 µg/ml ascorbate (Gibco), 100 units/ml penicillin/streptomycin (Gibco), and 10% fetal bovine serum (FBS) (Gibco). Cultures were maintained in a humidified chamber at 37 °C with 7 % CO_2 .

Cultures were maintained in the absence of serum for 48 hr prior to experimentation. Conditioned media were exchanged daily and stored at 4°C until analyzed for indicators of cartilage degradation. Cultures were subsequently exposed for 24 hr to the same medium containing 10% FBS (Gibco), and cartilage degradation was stimulated by the addition of 10 µg/ml lipopolysaccharide (LPS). To examine the effects of glucosamine and mannosamine on LPS induced cartilage degradation either glucosamine HCl or mannosamine HCl was added to explants at concentrations of 0.5, 1.0, 2.5, 5.0, or 10.0 mg/ml. Controls for these experiments included tissue exposed only to FBS (FBS, no LPS) or tissue exposed only to FBS and LPS (FBS, LPS). Neither

hexosamine was added to the controls. All treatments were run with six wells in each of a total of three experiments.

Proteoglycan Assay

The dimethylmethylene blue assay was employed, as previously described (Chandrasekhar, 1987), to measure proteoglycan (PG) release into the conditioned media. Briefly, PG content was determined by measuring sulfated glycosaminoglycan content compared to a chondroitin sulfate standard and expressed as μg PG/well.

Nitric Oxide Assay

To measure nitric oxide, nitrite, a stable end product of nitric oxide metabolism was quantified using the Greiss reaction. Sodium nitrite was used as a standard. Absorbance at 450 nm was determined using the SpectraMax 300 plate reader (Molecular Devices, Sunnyvale, CA) and results expressed as μM NO/well.

Cell Viability Assay

Upon completion of the culture period, chondrocyte viability was assessed using a commercially available kit (Molecular Probes, Eugene, OR). Briefly, cartilage explants were cut into 1 mm thick sagital sections and exposed to a combination of non-fluorescent calcein AM (2 μM) and ethidium homodimer (4 μM). Tissue sections were

maintained in this mixture for 30 min and rinsed three times in 1X PBS. Cartilage sections were subsequently viewed under a fluorescence microscope at 100X. Calcein AM is plasma membrane permeable and reacts with intracellular esterase to produce fluorescent green calcein to indicate live cells. Ethidium enters the cells through damaged cell membranes and fluoresces red to indicate cell death.

Matrix Metalloproteinase Extraction

Extraction of MMP was carried out as previously described (Fernandes et al., 1995). Briefly, glucosamine-treated cartilage, not used for the chondrocyte viability assay, was frozen in liquid nitrogen, pulverized, and placed in extraction buffer (50mM Tris HCl, 10 mM $CaCl_2$, 2M guanidine HCl, 0.05% Brij-35, pH 7.5). The mixture was stirred overnight at 4°C and then centrifuged (18,000g for 30 minutes at 4°C). The supernatant was dialyzed for 24 h at 4°C against assay buffer (50 mM Tris HCl, 10 mM $CaCl_2$, 0.2 M NaCl, 0.05% Brij-35, pH 7.5) followed by dialysis against ddH_2O for 48 hours at 4°C. Dialysis was carried out using Spectra/Por Molecularporous Membrane tubing with a 12 to14-kd cutoff (Spectrum, Laguna Hills, CA). Protein concentrations were determined using the Micro BCA Protein Assay (Pierce, Rockford, IL). Absorbance at 562 nm was determined using the SpectraMax 300 plate reader (Molecular Devices, Sunnyvale, CA).

Gelatin Zymography

This method allows for the detection of matrix metalloproteinases (MMPs) that can degrade gelatin (Kleiner et al., 1994). Protein extracts from explant cultures were diluted in 4X sample buffer consisting of 40% glycerol, 4% SDS, and 0.25 M Tris (pH 6.8). Equal amounts of protein were loaded into each well of 8% polyacrylamide gels containing 0.05% swine skin gelatin. Polyacrylamide gel electrophoresis (PAGE) was carried out at 150 V for 60 minutes. The gel was then washed in equilibration buffer containing 2.5% Triton X-100 for two washes, each lasting 30 minutes, and then incubated in incubation buffer (50mM Tris (pH 7.5), 200 mM NaCl, 10 mM $CaCl_2$, 10 μM $ZnCl_2$, 0.02% Brij 35) overnight at 37°C. Gels were stained with Coomassie R-250 for 60 minutes. Areas of digestion were viewed as non-staining regions of the gel. In this study, zymograms are presented as negative images so the non-staining areas appear as black bands. Conditioned media from serum starved BHK cells (kindly provided by Dr. Jenifer Fenton, East Lansing, MI) that over-expresses MMP-2 (activity detected at 72 kd) was used as a positive control. Incubation of gels with 1,10 phenanthroline (10 mM) served as a negative control for these experiments.

Statistical Analysis

Data were analyzed using SAS statistical software PROC MIXED. Treatments were compared using the Tukey procedure. Statistical significance was considered at $P<0.05$. Trends towards significance were considered at $P<0.1$.

Results

HEXOSAMINES AND CHONDROCYTE VIABILITY

Glucosamine and mannosamine were added to explant cultures at doses of 0.5, 1.0, 2.5, 5.0 or 10.0 mg/ml. Exposure of the articular cartilage explants to the different culture conditions resulted in minimal cell death for both the LPS-positive and LPS-negative controls. Similarly, treatment of cartilage explants with glucosamine at doses up to 5.0 mg/ml or mannosamine up to 2.5 mg/ml resulted in little or no chondrocyte death (Figures 1A and 1B). However, mannosamine (5.0 mg/ml) resulted in greater cell death than did glucosamine at the same dose (Figures 1C and 1D). At a dose of 10 mg/ml cell death occurred throughout the cartilage with both hexosamines (Figure 1E and 1F). Chondrocytes appeared to be more sensitive to mannosamine treatment than to treatment with glucosamine.

ACUTE EFFECTS OF HEXOSAMINES ON INDICES OF LPS-INDUCED BOVINE ARTICULAR CARTILAGE DEGENERATION

Treatment of bovine articular cartilage explants with LPS for 24 hours significantly increased both NO production (P=0.0001) and PG release (P=0.0001) compared to the negative control.

Bovine articular cartilage explant cultures were exposed to glucosamine at doses of 0.5, 1.0, 2.5, 5.0 or 10.0 mg/ml. Nitrite content in 24 h conditioned media was significantly lowered by glucosamine at all of the doses tested (Figure 2). However, only glucosamine at 10.0 mg/ml reduced nitrite accumulation in 24 h conditioned media to the level of the LPS-negative control. Significant reductions in PG release were observed for glucosamine at concentrations of 1.0, 2.5, 5.0, and 10.0 mg/ml (Figure 3). Glucosamine at 5.0 mg/ml inhibited PG release to levels comparable to those of the LPS- negative control.

Bovine articular cartilage explant cultures were exposed to mannosamine at doses of 0.5, 1.0, 2.5, 5.0 or 10.0 mg/ml. All doses of mannosamine tested significantly inhibited nitrite accumulation in the 24 h conditioned media compared to the LPS-positive control (Figure 2). Mannosamine at 5.0 mg/ml inhibited the accumulation of nitrite in the 24 h conditioned media to a level comparable to that of the LPS-negative control. Nitrite content in the 24 h conditioned media was similar for the 0.5 mg/ml mannosamine and 2.5 mg/ml glucosamine treatment groups. All the doses of

mannosamine tested inhibited PG release from bovine articular cartilage explants (Figure 3). The 1.0 mg/ml dose of mannosamine inhibited PG release to a level comparable to that of the LPS-negative control. Mannosamine at 0.5 mg/ml inhibited PG release to a degree that was intermediate to the inhibition observed for glucosamine at doses of 2.5 and 5.0 mg/ml.

THE ACUTE EFFECTS OF HEXOSAMINES ON GELATINOLYTIC ACTIVITY IN LPS-INDUCED BOVINE ARTICULAR CARTILAGE DEGENERATION

Upon completion of the culture period, protein extracts from hexosamine treated cartilage explants were prepared as indicated in the materials and methods section. Gelatin zymograms were employed to detect gelatinolytic activity of protein extracts from the different treatment groups (Figures 4a and 4b). Treatment of bovine articular cartilage with LPS for 24 hours did not result in a detectable increase in gelatinolytic activity.

Discussion

Through the use of selected markers of articular cartilage catabolism, NO production and PG release, we have demonstrated that mannosamine inhibits LPS-induced articular cartilage degeneration at lower concentrations than glucosamine. Of the doses of hexosamine examined in this study, glucosamine up to, and including, 5.0

mannosamine tested inhibited PG release from bovine articular cartilage explants (Figure 3). The 1.0 mg/ml dose of mannosamine inhibited PG release to a level comparable to that of the LPS-negative control. Mannosamine at 0.5 mg/ml inhibited PG release to a degree that was intermediate to the inhibition observed for glucosamine at doses of 2.5 and 5.0 mg/ml.

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mg/ml appeared to be non-toxic to chondrocytes, while cell death was observed with mannosamine at 5.0 mg/ml. However, at 10.0 mg/ml, both glucosamine and mannosamine elicited marked chondrocyte death. The toxic effects exerted by these hexosamines occurred at doses in excess of what was required to inhibit selected markers of cartilage catabolism. The precise cause of cytotoxicity is not known; however, as the hexosamines in this study were HCl salts, it is possible that an excess of Cl^- ion in the culture media contributed to this toxic effect. Furthermore, mannosamine (Onoda et al., 1982) and glucosamine (Friedman and Skehan, 1980) are toxic to tumor cells. The toxicity observed for both hexosamines is hypothesized to be a result of hexosamine-induced alterations in cell plasma-membrane integrity (Friedman and Skehan, 1980; Onoda et al., 1982). As both glucosamine and mannosamine are very similar is structure; the only difference being an inversion of the amino group at the C2 position, the difference in chondrocyte sensitivity to these amino sugars is unclear. Glucosamine and mannosamine uptake by chondrocytes occurs through the same glucose transporter system (Bryson et al., 2000). Consequently, mannosamine may be more readily taken up by chondrocytes than glucosamine, thereby eliciting toxic effects at lower doses.

In the current study, zymography revealed a single band of activity that corresponded to MMP-2 (72 kd). Inhibition of band formation with the addition of 1,10 phenanthroline provided further evidence that the gelatinolytic activity was that of MMP-2 (data not shown). Our data showing a lack of induction of MMP-2 activity in response

to LPS is consistent with previous reports demonstrating an absence of increased MMP-2 activity in synovial fluid of cows with septic arthritis compared to normal joints (Arican et al., 2000). However, compared to normal joints, Arican et al. (2000) did observe augmented MMP-9 activity in bovine joints with septic arthritis. In our study, MMP-9 activity was not detected. These findings are contrary to previous reports that showed increased MMP activity in equine articular cartilage explants after 24 h exposure to LPS, while glucosamine treatment inhibited MMP activity (Fenton et al., 2000a; Fenton 2000b). The reason for the difference in the results presented here compared to the results of other studies is not known. However, studies by Fenton et al. (2000) used kinetic assays of gelatinolytic activity rather gelatin zymography. Consequently, assay differences may account for the conflicting results between the two studies. Furthermore, species differ in their biological response to LPS (Galanos et al., 1990). Recent studies conducted in our laboratory indicate that a combination of glucosamine and chondroitin sulfate inhibit equine MMP-9 activity to a greater extent than MMP-2 (unpublished findings).

Both hexosamines inhibited NO production and PG release at doses lower than those that elicited cell death. However, mannosamine inhibited NO production and PG release at one-fifth the dose of glucosamine. Based on these data, mannosamine is a more potent inhibitor of selected markers of LPS-induced articular cartilage degradation than glucosamine.

Although the information presented in this study lends additional support to the role of hexosamines as inhibitors of articular cartilage degeneration, the mechanism of action of these aminomonosaccharides remains to be determined. However, due to similarity in structure, cellular uptake, and actions on markers of cartilage catabolism, different mechanisms of action probably do not account for the differences in potency between glucosamine and mannosamine. *In vitro* studies have suggested glucosamine can decrease the cellular availability of NADPH, a cofactor required for iNOS activity (Wu et al., 2001), the enzyme responsible for NO production by chondrocytes (Grabowski et al., 1997). Meanwhile, other *in vitro* and *in vivo* studies have reported that glucosamine inhibits NO production by inhibiting iNOS protein synthesis, while having no effect on cellular NADPH levels (Meininger et al., 2000). Further investigation of the effects of glucosamine on NO production will hopefully clarify this matter. However, as the data presented here are the first to show the inhibitory effects of mannosamine on NO production in bovine articular cartilage.

How these hexosamines work to inhibit PG release was not addressed in this study. However, both glucosamine and mannosamine inhibit aggrecanase activity (Sandy et al., 1999), the enzyme largely responsible for PG breakdown in OA (Little et al., 1999). Sandy et al. (1999) showed mannosamine to inhibit aggrecanase activity at one-tenth the dose of glucosamine, while mannosamine inhibits PG release from bovine cartilage explants at doses that are ineffective for glucosamine (Bryson et al., 2000).

Another hypothesis is that glucosamine inhibits equine articular cartilage degradation in part via the inhibition of MMP activity (Fenton et al., 2000b), hence preserving the cartilage matrix. Glucosamine also inhibits collagenolytic activity in human OA cartilage (Piperno et al., 2000). Which of these functions of glucosamine and mannosamine is most important to preventing cartilage breakdown remains unknown.

Alternatively, glucosamine may be protective of the cartilage matrix due to its stimulatory effects on matrix synthesis (Bassleer et al. 1998; Piperno et al., 2000). Glucosamine is known to increase protein synthesis in human OA cartilage (Piperno et al., 2000) and increase PG production from human OA chondrocytes (Bassleer et al., 1998). Furthermore, a recent report showed glucosamine to reverse the inhibitory effects of Il-1β on mRNA expression and catalytic activity of glucuronosyltranferase (GclAT-1), an enzyme involved in glycosaminoglycan synthesis (Gouze et al., 2001). As interest in mannosamine as a chondroprotective agent is recent, future experiments will determine whether or not mannosamine has similar stimulatory effects on matrix synthesis.

OA is a leading cause of morbidity and mortality (Creamer and Hochberg, 1997). Unfortunately, traditional treatments of OA possess deleterious side effects and have therefore prompted the development of alternative therapies that are both non-toxic and that potentially stop OA progression. The data presented in this study confirm the ability of two hexosamines, glucosamine and mannosamine, to inhibit bovine articular cartilage degradation at doses that do not hinder chondrocyte viability. Moreover, we have shown

a differential capacity of these two hexosamines to exert their protective effects, with mannosamine being a more potent inhibitor of articular cartilage degradation than glucosamine. As investigation into alternative therapies for the treatment of OA continues to develop, a better understanding of the mechanisms of action of glucosamine and mannosamine should clarify the differences in their potencies as chondroprotective molecules. Such studies could provide needed information that will aid in the development of improved disease therapies that can inhibit disease progression, as well as increase the quality of life for OA patients.

Figure 1 (Next page). Representative sections of bovine articular cartilage demonstrating chondrocyte viability 24 hours after LPS induced cartilage degradation and hexosamine treatment. A) 2.5 mg/ml glucosamine B) 2.5 mg/ml mannosamine C) 5.0 mg/ml glucosamine D) 5.0 mg/ml mannosamine E) 10.0 mg/ml glucosamine F) 10.0 mg/ml mannosamine. Arrowheads point to the superficial layers of the cartilage. Arrows point to the basal layers of the cartilage. Green indicates live cells. Red indicates dead cells.

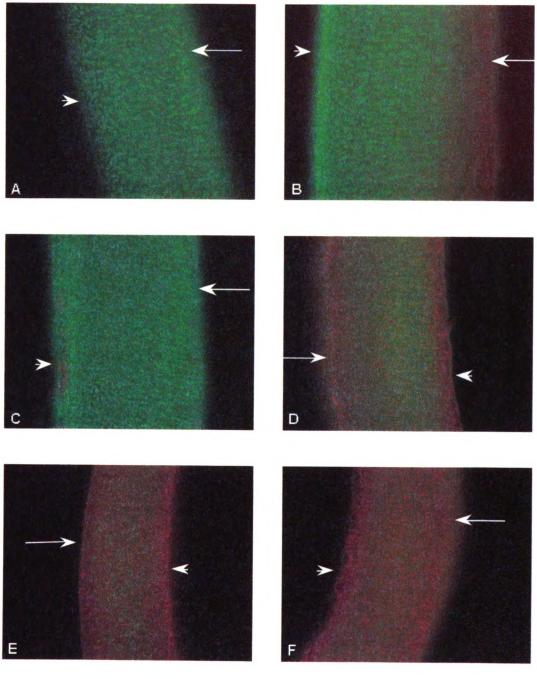
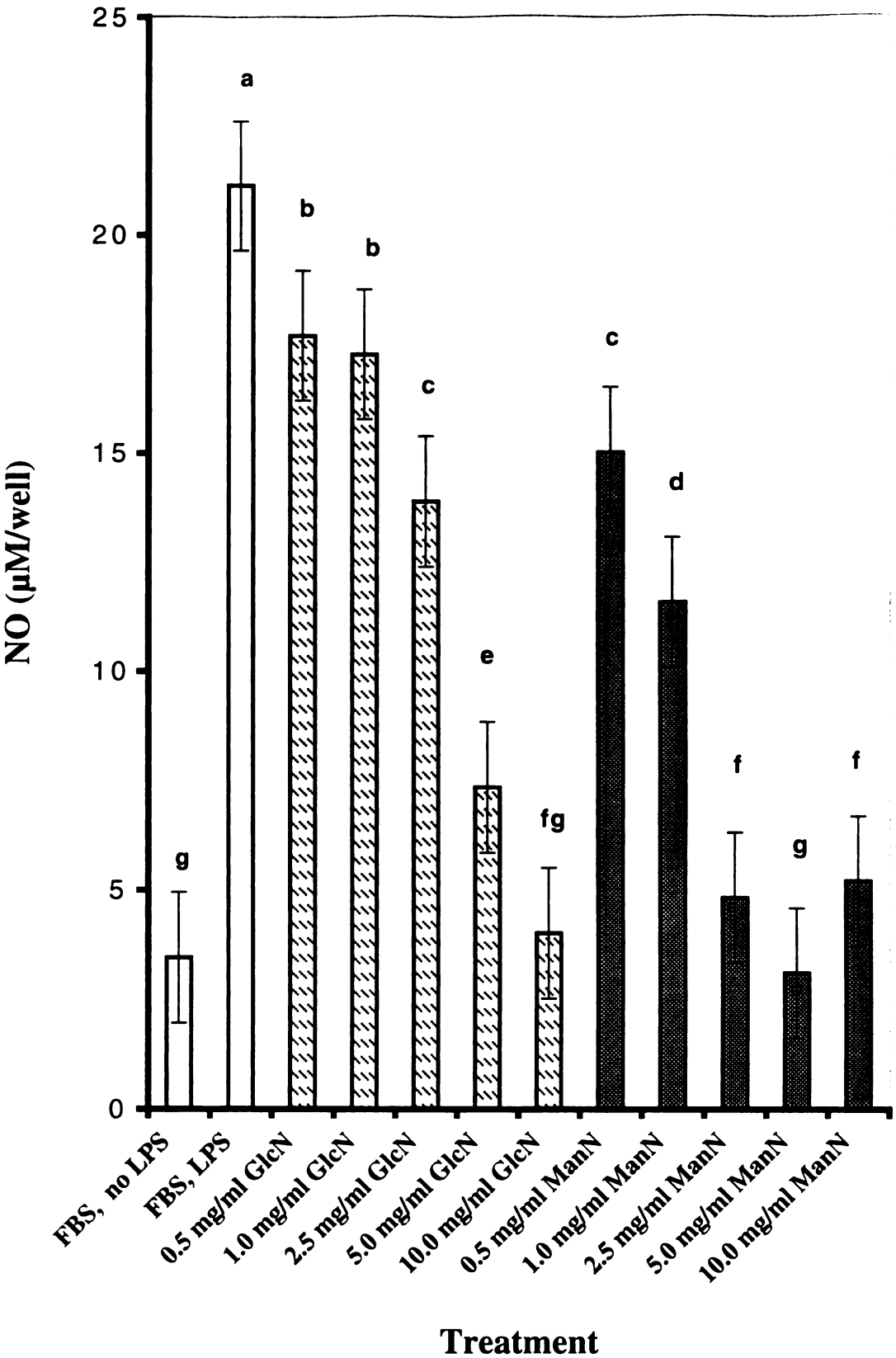

A
B
C
D
E
F

Figure 2 (Next page). Mean (± SE) nitric oxide production from bovine articular cartilage explants 24 hours after LPS induced degradation and treatment with glucosamine or mannosamine. Treatments with different superscripts are significantly different at $P<0.05$. FBS, no LPS = LPS-negative control; FBS, LPS = LPS-positive control; GlcN = glucosamine; ManN = mannosamine.


25
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NO (µM/well)
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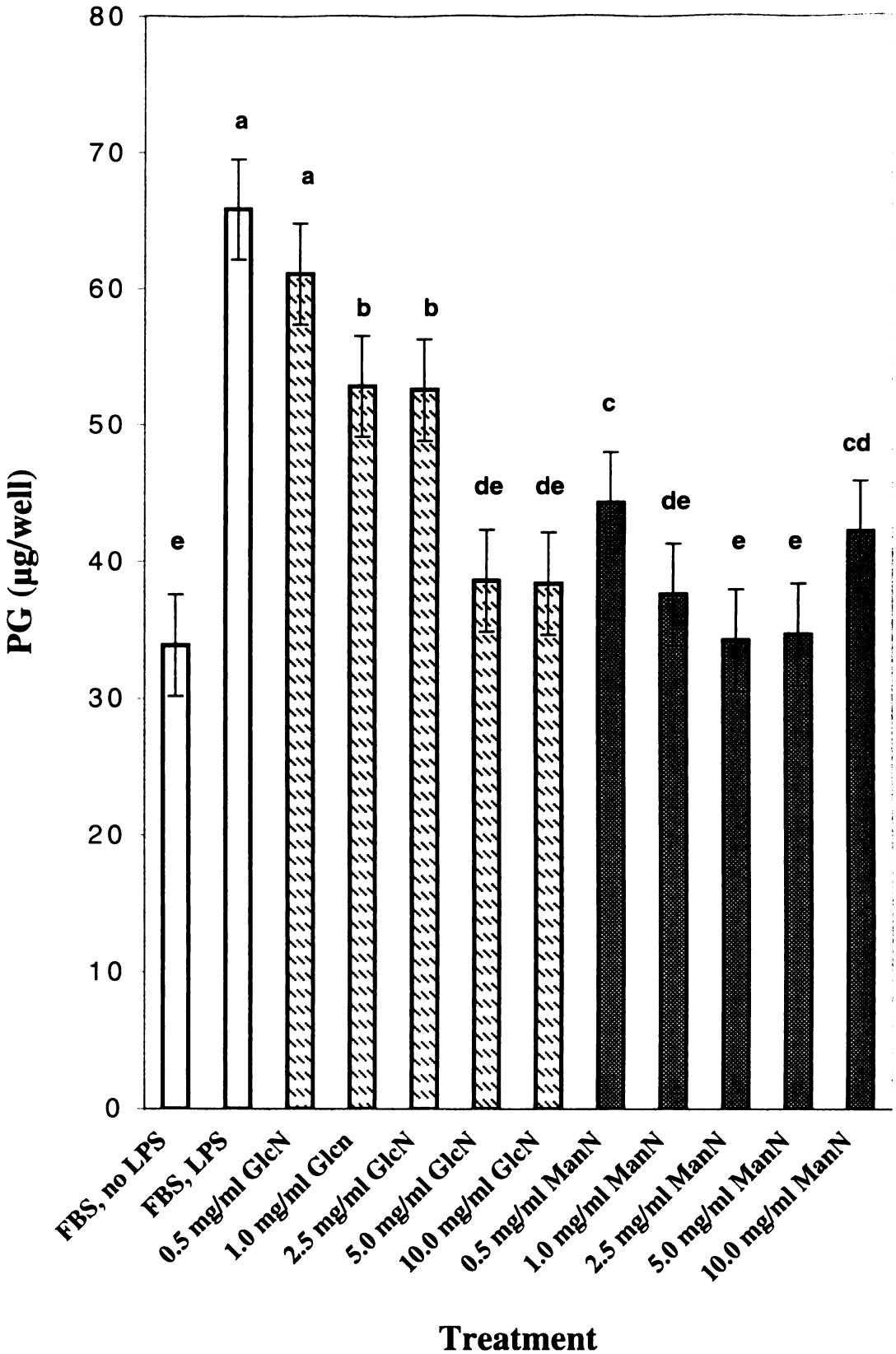
FBS, no LPS
FBS, LPS
0.5 mg/ml GlcN
1.0 mg/ml GlcN
2.5 mg/ml GlcN
5.0 mg/ml GlcN
10.0 mg/ml GlcN
0.5 mg/ml ManN
1.0 mg/ml ManN
2.5 mg/ml ManN
5.0 mg/ml ManN
10.0 mg/ml ManN
Treatment

Figure 3 (Next page). Mean (± SE) proteoglycan release from bovine articular cartilage explants 24 hours after LPS induced cartilage degradation and treatment with glucosamine or mannosamine. Treatments with different superscripts are significantly different at $P<0.05$. FBS, no LPS = LPS-negative control; FBS, LPS = LPS-positive control; GlcN = glucosamine; ManN = mannosamine.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70
60
50
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30
20
10
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PG (µg/well)
a
a
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b
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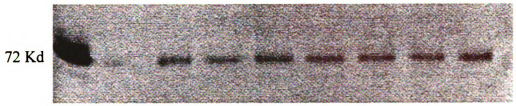
de
e
e
FBS, no LPS
FBS, LPS
0.5 mg/ml GlcN
1.0 mg/ml Glcn
2.5 mg/ml GlcN
5.0 mg/ml GlcN
10.0 mg/ml GlcN
0.5 mg/ml ManN
1.0 mg/ml ManN
2.5 mg/ml ManN
5.0 mg/ml ManN
10.0 mg/ml ManN
Treatment

Figure 4a (Next page). Representative gelatin zymogram of protein extracts from bovine articular cartilage explants stimulated with LPS and treated with glucosamine for 24 hours. Below the zymogram is a histogram indicating the mean band density for each of the treatments. The histogram values represent the mean band density per treatment for two experiments. Lane 1) MMP-2 positive control; Lane 2) blank; Lane 3) LPS-negative control; Lane 4) LPS-positive control; Lane 5) 0.5 mg/ml glucosamine; Lane 6) 1.0 mg/ml glucosamine; Lane 7) 2.5 mg/ml glucosamine; Lane 8) 5.0 mg/ml glucosamine; Lane 9) 10.0 mg/ml glucosamine. FBS, no LPS = LPS-negative control; FBS, LPS = LPS-positive control; GlcN = glucosamine.


72 Kd
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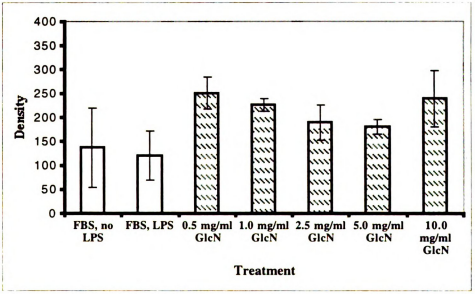
Density
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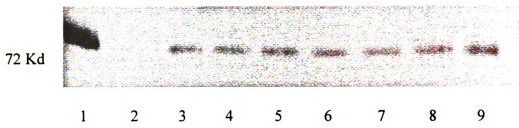
350
300
250
200
150
100
50
0
FBS, no LPS
FBS, LPS
0.5 mg/ml GlcN
1.0 mg/ml GlcN
2.5 mg/ml GlcN
5.0 mg/ml GlcN
10.0 mg/ml GlcN
Treatment

Figure 4b (Next page). Representative gelatin zymogram of protein extracts from bovine articular cartilage explants stimulated with LPS and treated with mannosamine for 24 hours. Below the zymogram is a histogram indicating the mean band density for each of the treatments. The histogram values represent the mean band density per treatment for two experiments. Lane 1) MMP-2 positive control; Lane 2) blank; Lane 3) LPS-negative control; Lane 4) LPS-positive control; Lane 5) 0.5 mg/ml mannosamine; Lane 6) 1.0 mg/ml mannosamine; Lane 7) 2.5 mg/ml mannosamine; Lane 8) 5.0 mg/ml mannosamine; Lane 9) 10.0 mg/ml mannosamine. FBS, no LPS = LPS-negative control; FBS, LPS = LPS-positive control; ManN = mannosamine.


72 Kd
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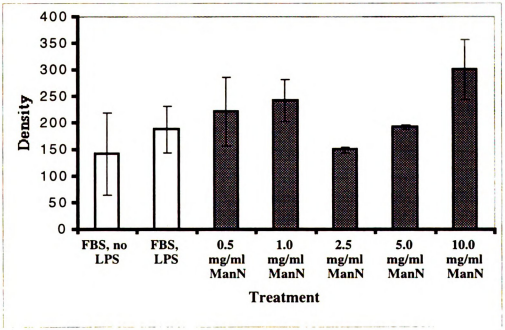
Density
400
350
300
250
200
150
100
50
0
FBS, no LPS
FBS, LPS
0.5 mg/ml ManN
1.0 mg/ml ManN
2.5 mg/ml ManN
5.0 mg/ml ManN
10.0 mg/ml ManN
Treatment

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Appendix

Preliminary studies on the comparative effects of glucosamine and mannosamine on the inhibition of equine articular cartilage degradation

Abstract

Objective: To compare the abilities of glucosamine and mannosamine to inhibit equine articular cartilage degradation.

Design: Articular cartilage was collected from weight bearing regions of healthy equine carpi (2 to 8 years old) and cultured in basal medium for 48 h. Tissues were subsequently exposed for 48 h to medium containing 10% fetal bovine serum (FBS), 10 μg/ml lipopolysaccharide (LPS), and 2.5, 5.0 or 10.0 mg/ml glucosamine or 0.5, 1.0, 2.5, or 10.0 mg/ml mannosamine. All treatments were run in triplicate in each of a total of two experiments. Culture medium was changed daily and analyzed for nitric oxide (NO) production and proteoglycan (PG) release to indicate cartilage catabolism. On the final day of culture, tissue samples were collected and cell viability was assessed.

Results: LPS treatment increased NO production and PG release. After 24 h, glucosamine inhibited NO production only at 5.0 mg/ml, while mannosamine inhibited NO production at all doses tested. After 48 h both hexosamines inhibited NO production at all doses tested. At 24 h, only mannosamine at 2.5 mg/ml inhibited PG release. However, at 48 h, no dose of glucosamine inhibited PG release, while mannosamine at

0.5, 1.0, and 2.5 mg/ml inhibited PG release. Both hexosamines were toxic to chondrocytes at 10 mg/ml, with cell death present throughout the tissue. Hexosamines at the lower doses did not result in chondrocyte death.

Conclusions: Both glucosamine and mannosamine are protective of equine articular cartilage. However, as mannosamine exhibited its protective effects at lower doses than glucosamine, mannosamine appears to be a more potent inhibitor of equine articular cartilage degeneration.

Introduction

Osteoarthritis (OA) is a disease that is characterized by the loss of basic articular cartilage function that results in full thickness of loss of joint cartilage. OA poses a significant problem to the horse industry as it is a major cause of decreased performance in athletic horses (Baxter, 1992). Although there are multiple causes of OA, the main causes in athletic horses during training and racing are stresses of abnormal intensity, duration and frequency placed on the joint (Pool, 1996). Consequently, horses that perform at high speeds are at particularly high risk of developing OA due to the application of loads to the joint that are in excess of the maximal force the tissue can withstand (Baxter, 1992). Due to a lack of nerve endings in articular cartilage, OA frequently goes undetected until it has reached the advanced stages of the disease (Keuttner and Hascall, 1992).

Traditionally, OA in the horse is treated with intra-articular corticosteroids and oral administration of non-steroidal anti-inflammatory drugs (NSAIDs). Unfortunately, prolonged use of these therapies may have deleterious consequences. Intra-articular corticosteroids may decrease cartilage proteoglycan (PG) content (Trotter et al., 1991) and increase chondrocyte necrosis (Shoemaker et al., 1992). Phenylbutazone, the most commonly used NSAID for horses, may lead to gastric ulcer formation, inappetance, weight loss and diarrhea (Traub et al., 1983). As a result, emphasis has been placed on alternative therapies that are non-invasive and nontoxic.

Glucosamine is a non-toxic (Setnikar et al., 1986), naturally occurring, six-carbon amino sugar that has shown beneficial results in the management of cartilage degeneration both *in vivo* and *in vitro*. In human clinical trials, glucosamine provided analgesic benefits (Crolle and D'Este, 1980; Pujalte et al., 1980; Barclay et al., 1998; da Camara and Dowless, 1998; Reginster et al., 2001) and inhibited joint space narrowing in OA patients (Reginster et al., 2001). In a veterinary clinical trial, a combination of glucosamine and chondroitin sulfate markedly improved lameness grade, flexion test scores, and stride length in horses suffering from OA (Hanson et al., 1997). *In vitro* studies using equine articular cartilage demonstrated glucosamine to inhibit selected markers of cartilage degeneration, including proteoglycan (PG) release and nitric oxide (NO) production (Fenton et al., 2000a; Fenton et al., 2000b). Consequently, glucosamine is marketed heavily to horse owners as a dietary supplement that is protective of articular

cartilage. The positive results obtained for glucosamine have spurred the investigation of related glucose derivatives that may be more potent chondroprotective agents (Fenton et al., 2000a).

Mannosamine is an isomer of glucosamine that differs only by an inversion of the amino group located at the C2 position. *In vitro*, mannosamine inhibits aggrecanase activity, the enzyme largely responsible for PG breakdown in OA (Little et al., 1999), in bovine cartilage and rat chondrosarcoma cells at one-tenth the dose of glucosamine (Sandy et al., 1999). Additionally, mannosamine preserves the mechanical integrity of bovine articular cartilage subjected to chemical degradation *in vitro* (Patwari et al., 2000). Investigation of the effects of mannosamine on articular cartilage has been limited to *in vitro* studies. Consequently, mannosamine is not yet marketed to consumers as an agent that maintains joint health.

To date, no studies have been conducted that examine the effects of mannosamine on experimentally induced articular cartilage degradation utilizing an equine model. The purpose of this research is to compare the abilities of two hexosamines, glucosamine and mannosamine, to inhibit equine articular cartilage degradation. We tested the hypothesis that mannosamine is a more potent inhibitor of equine articular cartilage degeneration than glucosamine.

Materials and methods

Unless otherwise stated, chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Explant Cultures

Articular cartilage discs (3.5 mm) were biopsied from the weight bearing regions of the carpi of two horses (2 to 8 years of age). Horses were killed for reasons other than joint disease. Biopsies of articular cartilage did not include the calcified layer of the tissue. Three explant discs were selected at random and cultured in each well of a 24-well Falcon tissue culture plate (Fisher Scientific; Pittsburgh, PA, U.S.A.). Cultures were maintained in 1:1 Dulbecco's Modified Eagles Medium: nutrient mixture F-12 (Ham) (Gibco, Grand Island, NY) supplemented with amino acids, trace minerals, 50 μg/ml ascorbate (Gibco), 100 units/ml penicillin/streptomycin (Gibco), and 10% fetal bovine serum (FBS) (Gibco). Cultures were maintained in a humidified chamber at 37 °C with 7% CO_2.

Cultures were maintained in the absence of serum for 48 h prior to the addition of treatment. Conditioned media were changed daily and stored at 4°C until analyzed for indicators of cartilage degradation. Cultures were subsequently exposed for 48 h to the same medium containing 10% FBS (Gibco), and cartilage degradation was stimulated by the addition of 10 μg/ml lipopolysaccharide (LPS). To examine the effects of glucosamine and mannosamine on LPS-induced cartilage degradation, either

glucosamine HCl at doses of 2.5, 5.0, or 10.0 mg/ml or mannosamine HCl at doses of 0.5, 1.0, 2.5, or 10.0 mg/ml was added to explants. Controls for these experiments included tissue exposed only to FBS (FBS, no LPS) or tissue exposed only to FBS and LPS (FBS, LPS). Hexosamines were not added to the controls. n = 6 wells per treatment per replicate. Experiments were replicated two times.

Proteoglycan Assay

The dimethylmethylene blue assay was employed, as previously described (Chandrasekhar, 1987), to measure PG release into the conditioned media. Briefly, PG content was determined by measuring the sulfated glycosaminoglycan content compared to a chondroitin sulfate standard. Results were expressed as μg PG/well.

Nitric Oxide Assay

To measure nitric oxide, nitrite, a stable end product of nitric oxide metabolism was quantified using the Greiss reaction. Sodium nitrite was used as a standard. Absorbance at 540 nm was determined using the SpectraMax 300 plate reader (Molecular Devices, Sunnyvale, CA). Results were expressed as μM of NO/well.

Cell Viability Assay

Upon completion of the culture period, chondrocyte viability was assessed using a commercially available kit (Molecular Probes, Eugene, OR). Briefly, cartilage explants were cut into 1 mm thick sagital sections and exposed to a combination of non-fluorescent calcein AM (2 μM) and ethidium homodimer (4 μM). Tissue sections were maintained in this mixture for 30 minutes and rinsed three times in 1X PBS. Cartilage sections were subsequently viewed under a fluorescence microscope at 100X. Calcein AM is plasma membrane permeable and reacts with intracellular esterase to produce green fluorescent calcein indicating live cells. Ethidium enters the cells through damaged cell membranes and fluoresces red to indicate cell death.

Statistical Analysis

Data were analyzed using SAS statistical software PROC MIXED. Treatments were compared using the Tukey procedure. Statistical significance was considered at $P<0.05$. Trends towards significance were considered at $P<0.1$.

Results

After 24 h, LPS significantly increased nitrite accumulation in conditioned media ($P=0.001$) and PG release ($P=0.03$) from equine articular cartilage explants compared to

the LPS-negative control. After 48 h, LPS significantly elevated nitrite accumulation in the conditioned media (P=0.0004), while it failed to augment PG release (P=0.4) compared to the LPS-negative control tissue.

HEXOSAMINES AND CHONDROCYTE VIABILITY

After 48 h-exposure to the different culture conditions, both the LPS-positive and LPS- negative control tissue exhibited little or no cell death (data not shown). Similarly, explants treated with glucosamine at doses up to, and including, 5.0 mg/ml, and tissue treated with mannosamine up to, and including, 2.5 mg/ml demonstrated little or no cell death (Figure 5A, 5B, and 5C). Cartilage treated with glucosamine or mannosamine at 10 mg/ml exhibited high cell death (Figure 5D and 5E). At 10.0 mg/ml chondrocyte death was observed throughout the tissue for both hexosamines. Mannosamine (10 mg/ml) showed the greatest amount of cell death of all the different treatment groups. Due to the toxic effect of these hexosamines at 10 mg/ml, no further studies were conducted using these treatment groups.

EFFECTS OF GLUCOSAMINE ON LPS-INDUCED CARTILAGE DEGRADATION

Equine articular cartilage explants were exposed to glucosamine at doses of 2.5 or 5.0 mg/ml. Nitrite content in 24 h conditioned media was significantly reduced by glucosamine at 5.0 mg/ml (Figure 6). However, after 48 h, both doses of glucosamine reduced nitrite content in the conditioned media (Figure 7). PG release was not significantly inhibited by glucosamine at 2.5 or 5.0 mg/ml at either the 24 h or 48 h time point (Figures 8 and 9). At the 48 h time point, glucosamine (2.5 mg/ml) exhibited the greatest level of PG release of all the treatment groups, including the LPS-positive control (Figure 9).

EFFECTS OF MANNOSAMINE ON LPS-INDUCED CARTILAGE DEGRADATION

Equine articular cartilage explants were exposed to mannosamine at doses of 0.5, 1.0, or 2.5 mg/ml. All three doses of mannosamine significantly inhibited nitrite accumulation in both the 24 and 48 h conditioned media (Figures 6 and 7). After 24 h, there was no significant difference between the nitrite content of conditioned media for 0.5 mg/ml mannosamine and 5.0 mg/ml glucosamine. Inhibition of nitrite accumulation in 48 h conditioned media was similar for mannosamine at 0.5 mg/ml and glucosamine at 2.5 mg/ml. Mannosamine at 2.5 mg/ml inhibited PG release from cartilage explants after

24 h (Figure 8). However, after 24 h, there was also a trend towards a significant reduction in PG release for 1.0 mg/ml mannosamine (P=0.07). After 48 h, all three doses of mannosamine tested significantly inhibited PG release from the articular cartilage explants (Figure 9).

Discussion

Prolonged use of traditional treatments of OA, including NSAIDs and intra-articular corticosteroids, are accompanied by deleterious side effects that may perpetuate OA progression. Consequently, emphasis has been placed on developing alternative treatments that are analgesic, non-toxic, and potentially slow the progression of OA. Previous findings showing glucosamine to inhibit joint space narrowing (Reginster et al., 2001), as well as to increase protein (Bassleer et al., 1998; Fenton et al., 2000 a and b; Pipero et al., 2000) and PG (Bassleer et al., 1998) synthesis, are consistent with the properties of a disease modifying drug for OA. Specifically, these properties are that the drug must prevent, retard the progression of, or reverse morphologic changes of OA (Brandt, 1995). Such results have prompted the investigation of the potential use of related hexosamines as more potent inhibitors of OA progression.

Using markers of cartilage catabolism, NO production and PG release, we have demonstrated mannosamine to inhibit equine articular cartilage degradation, while glucosamine only inhibited NO production. The results of this study indicate that, of the

doses of hexosamine tested, glucosamine up to 5.0 mg/ml and mannosamine up to 2.5 mg/ml inhibited articular cartilage degradation in a manner that was non-toxic to chondrocytes. To the contrary, high cell death was observed with hexosamine treatment at 10.0 mg/ml. Further study of this toxic effect, examining doses that are closer in spread, will determine the dose at which the cytotoxic effects can be delineated from the chondroprotective effects. The precise cause of chondrocyte mortality is not clear; however, it may be attributed to an excess of Cl^- ion in the culture medium resulting from the HCl component of the hexosamine salts used in this study. Moreover, glucosamine (Friedman and Skehan, 1980) and mannosamine (Onoda et al., 1982) are toxic to tumor cells as a result of hexosamine-induced alterations in cell membrane integrity. Consequently, a similar mechanism may account for the toxic effects of high doses of glucosamine and mannosamine treatment on chondrocytes.

Although both hexosamines inhibited selected markers of cartilage catabolism, mannosamine appears to be more potent as it inhibited both NO production and PG release from equine cartilage explants. Inspection of the PG release data reveals that at 48 h, glucosamine at 2.5 mg/ml exhibited the greatest PG release of all the treatment groups, including the controls. Moreover, at 48 h, mannosamine inhibited PG release from the articular cartilage explants at all three doses examined in this study.

The data presented here are consistent with other studies demonstrating the capacity of glucosamine to inhibit NO production from LPS-stimulated articular cartilage

(Fenton et al., 2000a; Fenton et al., 2000b). However, the results of this study are contrary to those of Fenton et al. (2000), which demonstrated glucosamine to inhibit PG release from LPS-stimulated equine articular cartilage. Although the reason for the response to glucosamine treatment in this study is not clear, in the initial stages of OA there is an anabolic response by chondrocytes to maintain normal amounts of extracellular matrix components (Brandt et al., 1991). Furthermore, glucosamine can be incorporated into glycosaminoglycans (Barclay et al., 1998) and increase PG synthesis from OA chondrocytes (Bassleer et al., 1998). Consequently, the increase in PG release observed after 48 h exposure to glucosamine and LPS may be a result of both an anabolic response by chondrocytes, combined with a stimulus for PG synthesis. Alternatively, this difference may be attributed to the lower number of experiments run in this study compared to those conducted by Fenton et al. (2000).

Our findings regarding the inhibitory effects of mannosamine are in agreement with those of Bryson et al. (2000) that also showed mannosamine to inhibit PG release from chemically induced cartilage degradation. However, these data are the first to show the inhibitory effects of mannosamine on NO production in equine articular cartilage explants.

Although the precise mechanism behind the chondroprotective effects of these amino sugars is not known, glucosamine and mannosamine appear to inhibit similar markers of articular cartilage catabolism. Mannosamine, as glucosamine, inhibits

aggrecanase activity (Sandy et al., 1999) and PG release (Bryson et al., 2000) by chemically degraded bovine cartilage *in vitro*. However, mannosamine inhibits aggrecanase activity at one-tenth the dose of glucosamine (Sandy et al., 1999), while mannosamine inhibits PG release from bovine cartilage explants at doses that are ineffective for glucosamine (Bryson et al., 2000). Additionally, both glucosamine and mannosamine enter chondrocytes through the same glucose transporter systems (Bryson et al., 2000). Collectively, this information suggests that the difference in potencies observed for these two hexosamines may occur for reasons other than mechanism of action. For example, as the rate of uptake of different hexosamines varies at glucose transporters (Foley et al., 1980), mannosamine may be preferentially taken up over glucosamine, hence eliciting its effects at lower doses.

Given that joint health is crucial to athletic performance in horses, horse owners will continue to seek out alternative therapies for OA. Of particular interest are therapies that are non-invasive and with low risk of adverse side effects. Glucosamine has been, and continues to be, marketed to members of the horse industry as an oral dietary supplement that protects against joint damage. However, as OA continues to hinder equine performance, emphasis remains on improving OA treatment. The current study demonstrates glucosamine and a related hexosamine, mannosamine, to inhibit equine articular cartilage degradation in vitro. Moreover, we have shown mannosamine to be a more potent inhibitor of articular cartilage degradation, exerting its protective effects at

doses at which glucosamine was ineffective. The results of this study merit future investigation of the mechanisms employed by mannosamine to elicit these protective effects on articular cartilage. Understanding the mechanisms by which different hexosamines work to prevent cartilage degeneration will aid in the development of therapies for OA that prolong the athletic careers of equine athletes

Figure 5 (Next page). Representative equine articular cartilage sections demonstrating chondrocyte viability 24 hours after LPS induced articular cartilage degradation and treatment with glucosamine or mannosamine. A) 2.5 mg/ml glucosamine B) 2.5 mg/ml mannosamine C) 5.0 mg/ml glucosamine D) 10.0 mg/ml glucosamine E) 10.0 mg/ml mannosamine. Arrowheads indicate the superficial layers of cartilage. Arrows indicate the basal layers of cartilage. Green indicates live cells. Red indicates dead cells.

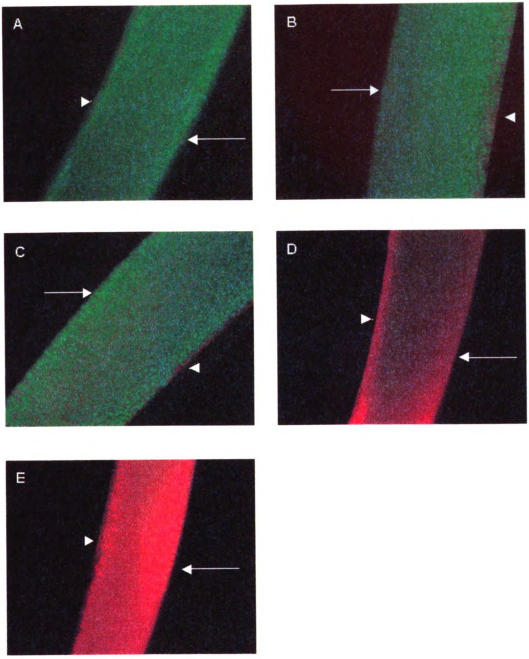
A
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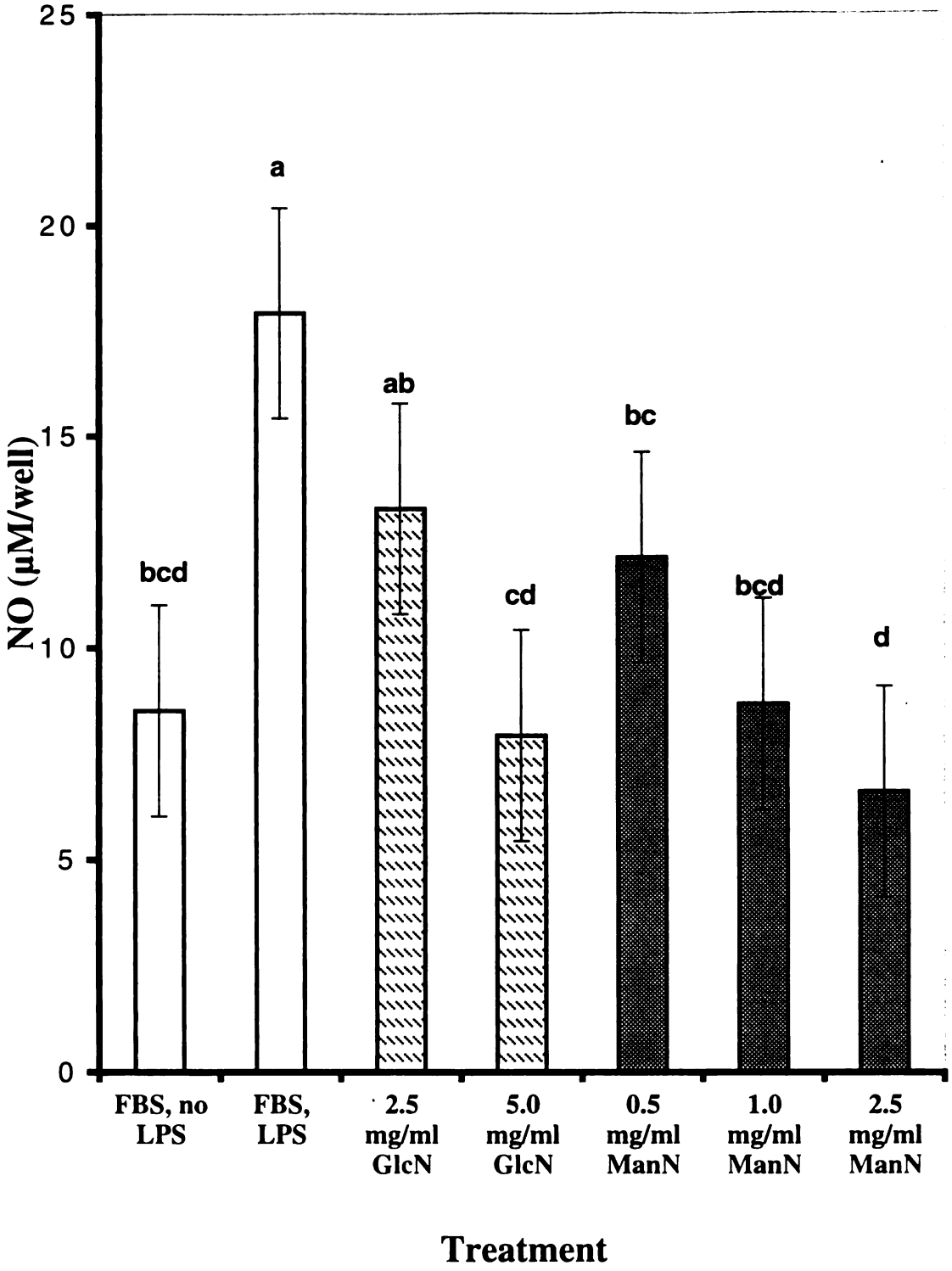
Figure 6 (Next page). Mean (± SE) nitric oxide production by equine articular cartilage explants 24 hours after LPS induced degradation and treatment with glucosamine or mannosamine. Treatments with different superscripts are significantly different at $P<0.05$. FBS, no LPS = LPS-negative control; FBS, LPS = LPS-positive control; GlcN = glucosamine; ManN = mannosamine.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NO (µM/well)
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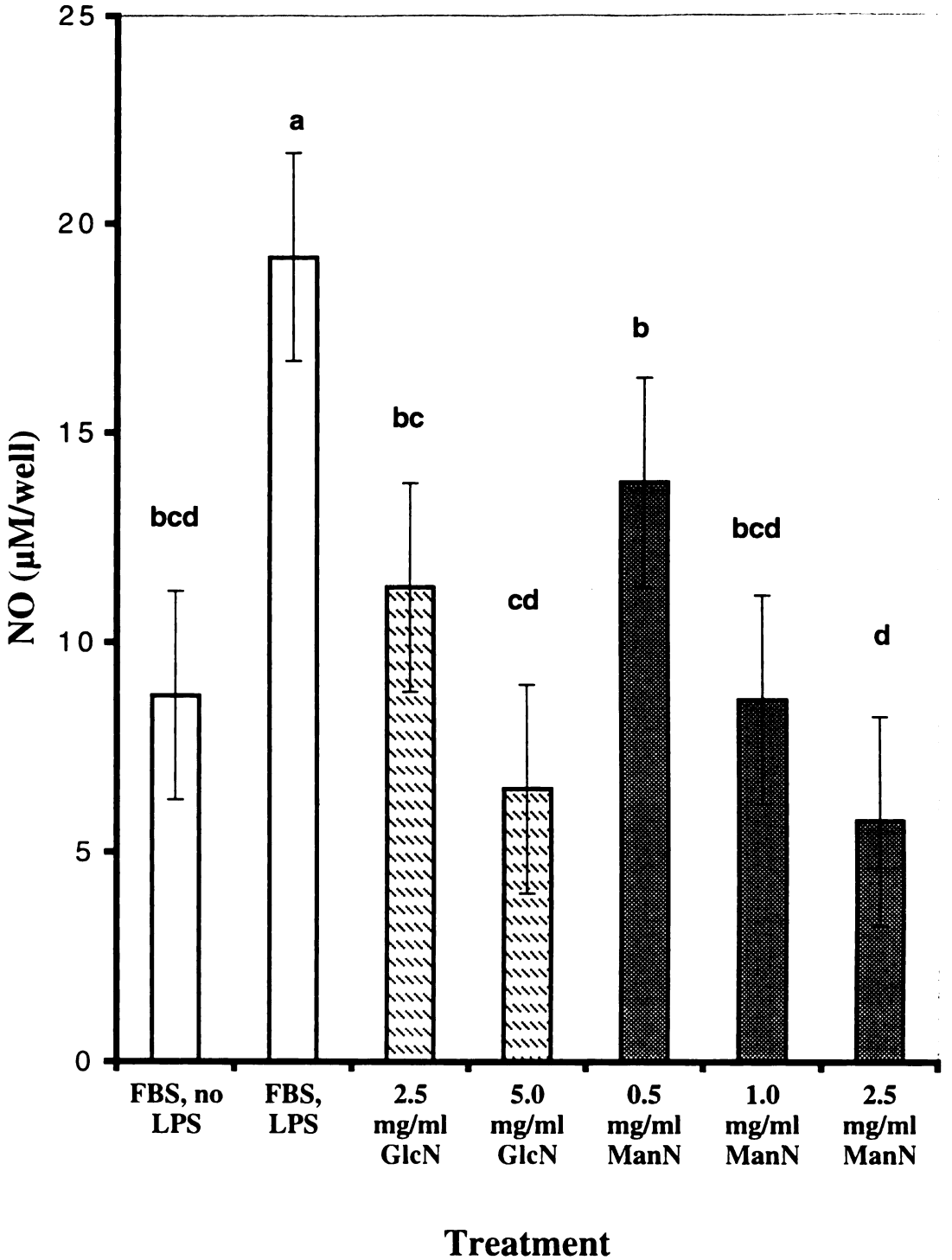
FBS, no LPS
FBS, LPS
2.5 mg/ml GlcN
5.0 mg/ml GlcN
0.5 mg/ml ManN
1.0 mg/ml ManN
2.5 mg/ml ManN
Treatment

Figure 7 (Next page). Mean (± SE) nitric oxide production by equine articular cartilage explants 48 hours after LPS induced degradation and treatment with glucosamine or mannosamine. Treatments with different superscripts are significantly different at $P<0.05$. FBS, no LPS = LPS-negative control; FBS, LPS = LPS-positive control; GlcN = glucosamine; ManN = mannosamine.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NO (µM/well)
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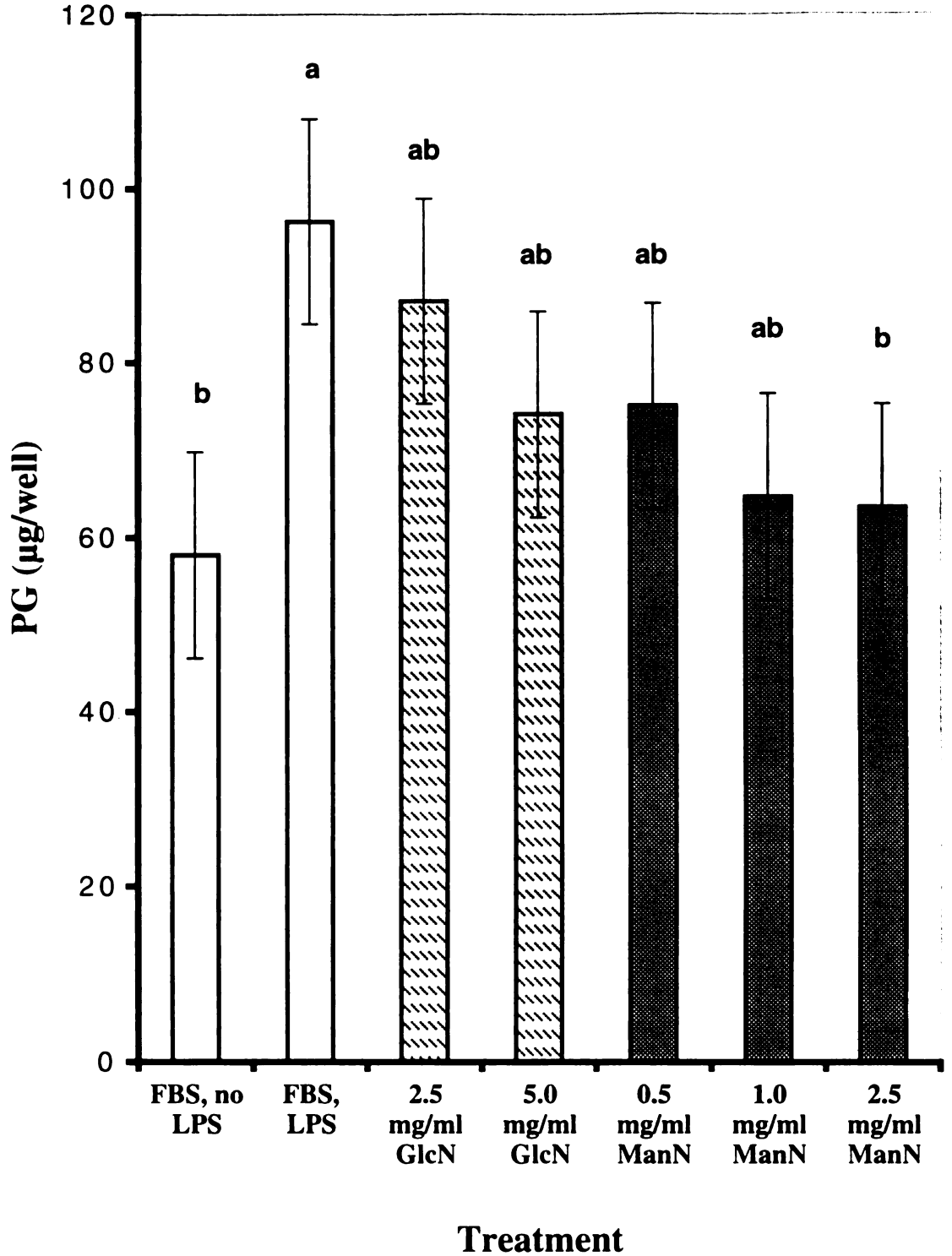
FBS, no LPS
FBS, LPS
2.5 mg/ml GlcN
5.0 mg/ml GlcN
0.5 mg/ml ManN
1.0 mg/ml ManN
2.5 mg/ml ManN
Treatment

Figure 8 (Next page). Mean (± SE) proteoglycan release from equine articular cartilage explants 24 hours after LPS induced degradation and treatment with glucosamine or mannosamine. Treatments with different superscripts are significantly different at $P<0.05$. FBS, no LPS = LPS-negative control; FBS, LPS = LPS-positive control; GlcN = glucosamine; ManN = mannosamine.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PG (µg/well)
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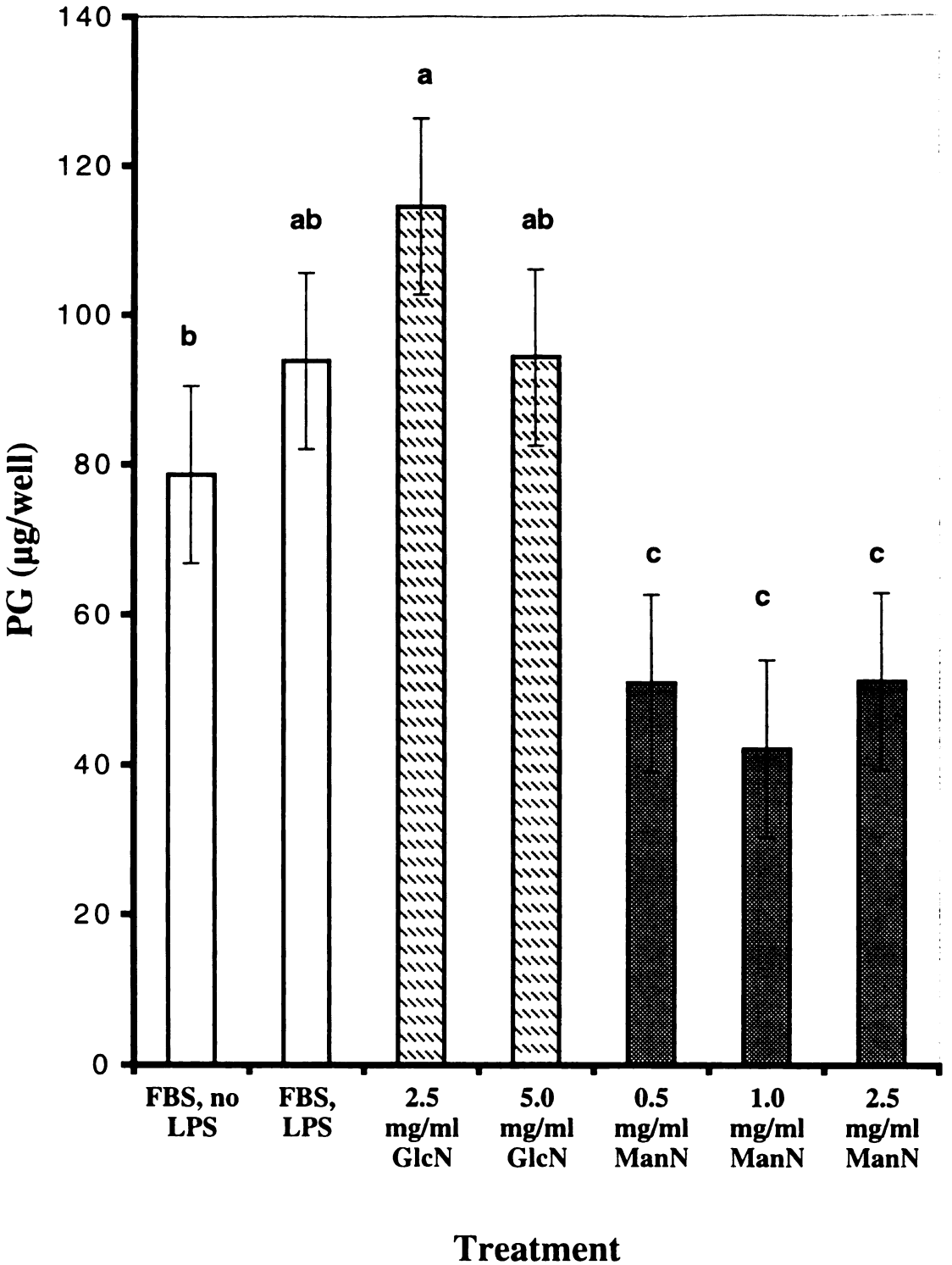
FBS, no LPS
FBS, LPS
2.5 mg/ml GlcN
5.0 mg/ml GlcN
0.5 mg/ml ManN
1.0 mg/ml ManN
2.5 mg/ml ManN
Treatment

Figure 9 (Next page). Mean (± SE) proteoglycan release from equine articular cartilage explants 48 hours after LPS induced degradation and treatment with glucosamine or mannosamine. Treatments with different superscripts are significantly different at $P<0.05$. FBS, no LPS = LPS-negative control; FBS, LPS = LPS-positive control; GlcN = glucosamine; ManN = mannosamine.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PG (µg/well)
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FBS, no LPS
FBS, LPS
2.5 mg/ml GlcN
5.0 mg/ml GlcN
0.5 mg/ml ManN
1.0 mg/ml ManN
2.5 mg/ml ManN
Treatment

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Chapter 4

Summary and Conclusion

The purpose of these studies was to compare the effects of glucosamine and mannosamine on the degeneration of articular cartilage. Use of bovine tissue served as a general model to compare the protective effects of these two aminomonosaccharides on chemically induced articular cartilage degradation. Through the use of an equine model the capacities of glucosamine and mannosamine to inhibit cartilage breakdown were specifically examined in a species that is commonly afflicted with OA.

An articular cartilage explant culture system was employed to accomplish the goals of this study. Lipopolysaccharide (LPS) (10 μg/ml) was added to explant cultures of bovine and equine articular cartilage to stimulate tissue degradation. At the same time, glucosamine or mannosamine was added to cultures of bovine tissue at doses of 0.5, 1.0, 2.5, 5.0, or 10.0 mg/ml. Equine tissue received glucosamine at doses of 2.5, 5.0, or 10 mg/ml or mannosamine at doses of 0.5, 1.0, 2.5, or 10 mg/ml. Culture media for both species were analyzed for the presence of markers of cartilage catabolism, nitric oxide (NO) and proteoglycans (PG). Protein extracts from bovine tissue were analyzed for matrix metalloproteinase (MMP) activity. Upon completion of the culture period, chondrocyte viability was assessed. Mannosamine, at lower doses than glucosamine, consistently inhibited the accumulation of NO and PG in the culture media to a similar or

greater degree than glucosamine. However, neither hexosamine had an effect on MMP activity. Notably, hexosamine treatment resulted in cell death only at doses in excess of what was required to inhibit cartilage degradation.

As the results of these studies demonstrated mannosamine to be a potent inhibitor of articular cartilage degradation, further study of the protective effects of mannosamine are merited. The study conducted on bovine tissue showed that neither LPS stimulation nor hexosamine treatment for 24 h had an effect on MMP activity. Consequently, it remains unclear how glucosamine and mannosamine elicit their protective effects. However, in the current study zymography revealed a single band that corresponded to that of MMP-2 (72 Kd). Consequently, if there were changes in activity of other MMP family members, they remained undetected. Therefore, future studies that utilize alternative techniques that assay for activity of specific MMPs that are known to be induced in OA may provide useful mechanistic data about the actions of these hexosamines. However, as it is unclear at which level these hexosamines may act to potentially inhibit MMP activity, future studies should not be limited to examining enzyme activity, but also gene expression and subsequent translation.

Investigation of MMP gene expression in response to hexosamine treatment will allow the specific determination of differences in transcription levels of genes encoding proteins that are believed to be important in OA progression. Among these genes of interest are MMP-1, MMP-9, and MMP-13. Although changes in gene expression are

not necessarily reflected by alterations in translation and subsequent activation, they can illustrate whether or not hexosamines are acting at the level of transcription. Determination of whether or not changes in protein levels mirror those observed for gene expression would provide additional information regarding the specific mechanisms of action of glucosamine and mannosamine. As a result, such studies would indicate where along the continuum from gene expression to protein activation these hexosamines act to inhibit articular cartilage degeneration.

In addition to looking at the effects of hexosamines on gene expression and protein synthesis, it may be meritorious to have a longer treatment period than what was used in these bovine experiments. In the current study, bovine cartilage was only exposed to the different treatment groups for 24 h. It is possible that 24 h treatment may have been insufficient to elicit a response in MMP activity from the bovine tissue. If the study had been conducted for an additional 24 h, LPS treatment may have increased MMP, while hexosamine treatment may have returned MMP activity back to basal levels.

Just as future studies should not be limited to the examination of enzyme activity, studies should also consider enzymes other than the MMPs that contribute to OA progression. As aggrecanase is responsible for the majority of PG breakdown in OA, the effects of glucosamine and mannosamine on aggrecanase may be important to their roles as inhibitors of cartilage degeneration. Although it has been previously demonstrated that glucosamine and mannosamine inhibit aggrecanase activity, it is unclear how this is