Effects of clinically relevant concentrations of glucosamine on equine chondrocytes and synoviocytes in vitro

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Objective—To evaluate the effects of glucosamine on equine articular chondrocytes and synoviocytes at concentrations clinically relevant to serum and synovial fluid concentrations.

Sample Population—Articular cartilage and synovium with normal gross appearance from metacarpophalangeal and metatarsophalangeal joints of 8 horses (1 to 10 years of age).

Procedures—In vitro chondrocyte and synoviocyte cell cultures from 8 horses were treated with glucosamine (0.1 to 20 µg/mL) with or without interleukin-1 (IL-1; 10 ng/mL) for 48 hours. Negative control cultures received no glucosamine or IL-1, and positive control cultures received only IL-1. Cultures were assayed for production of proteoglycan (via media containing sulfur 35 (35S)—labeled sodium sulfate and Alcian blue precipitation), prostaglandin E2 (PGE2; via a colorimetric assay), cyclooxygenase-2 (via real-time reverse-transcriptase PCR assay), microsomal PGE2 synthase (mPGEs; via real-time reverse-transcriptase PCR assay), and matrix metalloproteinase (MMP)-13 (via a colorimetric assay).

Results—Glucosamine had no impact on proteoglycan production or MMP-13 production under noninflammatory (no IL-1) or inflammatory (with IL-1) conditions. Glucosamine at 0.1 and 0.5 µg/mL significantly decreased IL-1–stimulated production of mPGEs by chondrocytes, compared with that of positive control chondrocytes. Glucosamine at 0.1 and 5 µg/mL significantly decreased IL-1–stimulated production of mPGEs and PGE2, respectively, compared with that of positive control synoviocytes.

Conclusions and Clinical Relevance—Glucosamine had limited effects on chondrocyte and synoviocyte metabolism at clinically relevant concentrations, although it did have some anti-inflammatory activity on IL-1–stimulated articular cells. Glucosamine may have use at clinically relevant concentrations in the treatment of inflammatory joint disease. (Am J Vet Res 2008;69:1129–1134)

Osteoarthritis is one of the most common performance-limiting maladies of athletic horses.1,2 Traditional osteoarthritis treatment regimens have focused on the use of NSAIDs. Although these drugs have been effective in reducing arthritis-related pain, NSAIDs do not reliably arrest progression of joint disease and have been implicated in the development of clinically important adverse effects such as gastrointestinal ulceration.3 They may also speed progression of osteoarthritis by allowing increased use of damaged joints, thereby increasing cartilage destruction.4 Intra-articular injection of corticosteroids has been an economical and efficacious treatment; however, certain preparations and doses may cause undesirable morphologic changes in articular cartilage, leading to progression of osteoarthritis.5–8 Other promising treatments have included polysulfated glycosaminoglycans and hyaluronic acid; however, rigorous randomized controlled clinical trials proving efficacy are scant.9

Received October 30, 2007.
Accepted December 12, 2007.
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AJVR, Vol 69, No. 9, September 2008 1129

ABBREVIATIONS

| IL-1 | Interleukin-1 |
| MMP | Matrix metalloproteinase |
| mPGEs | Microsomal prostaglandin E2 synthase |
| PGE2 | Prostaglandin E2 |

Interest in alternative treatments for osteoarthritis to improve patient mobility, reduce clinical pain, relieve financial burden of treatment, improve ease and convenience of treatment, and reduce adverse effects of traditional treatments has been growing. The 6-carbon amino sugar glucosamine has been used as an alternative treatment for arthritis. Some support is found within the peer-reviewed literature, suggesting that glucosamine may possess benefits as a palliative reliever of osteoarthritis pain and may confer beneficial metabolic effects on articular chondrocytes.10–12 In vitro research has elucidated several mechanisms of glucosamine that lend support to its role in osteoarthritis treatment. Proteoglycans are a major component of cartilage matrix, and their degradation is an important contributor to the pathophysiologic processes of osteoarthritis. Glucosamine prevents degradation of cartilage proteoglycans in vitro13,14 and upregulates production of proteoglycan by chondrocytes.15 These effects are likely secondary to...
downregulation of aggreganase enzymes and upregulation of aggrecan gene expression.\textsuperscript{16-19} Inflammation is an important contributor to osteoarthritis, and glucosamine downregulates the inflammatory agents nitric oxide and PGE\textsubscript{2}.\textsuperscript{17,19} Additionally, MMPs, a family of degradative enzymes responsible for cartilage extracellular matrix damage, are also downregulated by glucosamine.\textsuperscript{11,16,17} Taken together, these findings support a role for glucosamine in the protection of cartilage matrix and chondrocyte metabolism, suggesting a possible mechanism whereby glucosamine may help alleviate clinical signs and retard progression of osteoarthritis.

Pharmacokinetic evidence has recently been presented that suggests the majority of in vivo research has been conducted with glucosamine concentrations well in excess of those attained in vivo after oral administration.\textsuperscript{20} Serum glucosamine concentrations reach 2 \(\mu\)g/mL in humans after oral administration.\textsuperscript{20} These data agree well with findings in horses, with serum glucosamine concentrations reaching between approximately 1 and 10 \(\mu\)g/mL after oral administration, depending on the dose given.\textsuperscript{21,22} Some existing evidence suggests that glucosamine retains beneficial effects at similar concentrations.\textsuperscript{16,17,19} However, synovial fluid glucosamine concentrations in horses reach only 10% of those found in serum.\textsuperscript{23} If the beneficial actions of glucosamine are attributed to local effects within the joint environment, then support should be found for metabolic actions at these low concentrations.

Synoviocytes are important modulators of articular cartilage damage.\textsuperscript{24} Synoviocytes are likely exposed to plasma glucosamine concentrations 10-fold higher than the synovial fluid concentrations that chondrocytes are exposed to because of their proximity to the periarticular blood supply. Given the importance of synoviocytes in the pathogenesis of osteoarthritis and their exposure to potentially higher concentrations of glucosamine than chondrocytes, synoviocytes may be a target for therapeutic activity.

The objective of the study reported here was to evaluate the effects of glucosamine on equine articular chondrocytes and synoviocytes at concentrations clinically relevant to plasma and synovial fluid concentrations. Our hypothesis was that glucosamine, at clinically achievable tissue concentrations, would reduce inflammatory mediator release, reduce degradative enzyme release, and improve production of proteoglycan by cultured equine chondrocytes and synoviocytes.

**Materials and Methods**

**Tissue sources**—Articular cartilage and synovium with normal gross appearance from metacarpophalangeal and metatarsophalangeal joints were obtained from 8 horses (1 to 10 years of age) that had died or had been euthanatized with an overdose of pentobarbital for reasons other than joint disease. Cartilage was dissected free of underlying subchondral bone, and synovium was dissected free from the outer layer (or subintima). Both tissues were incubated separately at 25°C for 1 hour in physiologic saline (0.9% NaCl) solution containing 1% penicillin and streptomycin.\textsuperscript{4} Chondrocytes and synoviocytes were isolated separately by 16-hour digestion at 37°C with 0.13% collagenase\textsuperscript{6} in Dulbecco modified Eagle medium.\textsuperscript{7} Following digestion, cells were counted by use of a hemocytometer and viability was determined via trypan blue stain exclusion.\textsuperscript{3} Chondrocytes and synoviocytes were separated by centrifugation (300 \(\times\) g for 5 minutes), washed, and resuspended in Dulbecco modified Eagle medium supplemented with 1% penicillin and streptomycin, 4.5 g of glucose/l, 1% l-glutamine,\textsuperscript{8} 50 \(\mu\)g of ascorbic acid/ml,\textsuperscript{9} and 10% fetal bovine serum.\textsuperscript{6}

**Cell cultures**—Chondrocyte pellets (5 \(\times\) 10\textsuperscript{4} cells) were formed by centrifugation (300 \(\times\) g for 5 minutes) in 15-mL polypropylene centrifuge tubes and incubated under standard cell culture conditions (37°C in 95% relative humidity with 5% carbon dioxide). Synoviocytes were seeded at a density of 5 \(\times\) 10\textsuperscript{4} cells in culture flasks and grown as monolayers to confluence in identical conditions. Medium was renewed every 2 to 3 days. Cells were deprived of serum for 3 days prior to the start of an experiment. On day 7, cultures were supplemented with glucosamine\textsuperscript{3} (0 to 20 \(\mu\)g/mL) with or without IL-1 (10 ng/mL)\textsuperscript{5} and incubated for an additional 48 hours. Twenty-four hours prior to the end of the experiment, chondrocyte pellets were radiolabeled with media containing sulfur 35 ((35S)–labeled sodium sulfate (10 \(\mu\)Ci/mL).\textsuperscript{11} Cells and media were collected and stored at \(-80^\circ\)C.

**Proteoglycan**—Radiolabeled pellets were papain digested (150 \(\mu\)g of papain/2mL) at 65°C for 24 hours. Precipitation with Alcian blue dye\textsuperscript{6} and scintillation of the explant digests and media were used to determine new proteoglycan production as a result of incorporation of \(^{(35)}\)S into the cartilage matrix.\textsuperscript{12} Results were expressed as counts per minute per chondrocyte pellet.

**MMP-13**—Stored media from chondrocyte and synoviocyte cultures were assayed for MMP-13 and pro–MMP-13 by means of a commercially available kit\textsuperscript{13} (in accordance with the instructions of the manufacturer). Briefly, 100 \(\mu\)L of prepared standard and test media (diluted 1:4) were incubated at 25°C for 2 hours in assay wells coated with anti–MMP-13 antibody. Wells were washed with the supplied phosphate buffer and incubated at 25°C for 1 hour with 100 \(\mu\)L of anti–MMP-13 peroxidase conjugate. Wells were washed and incubated at 25°C for precisely 30 minutes with 100 \(\mu\)L of 3,3′,5′-tetramethylbenzidine-hydrogen peroxide in 20% (wt/vol) dimethylformamide. The reaction was stopped by the addition of 1.0M sulfuric acid, and optical density was measured at 450 nm.\textsuperscript{13}

**PGE\textsubscript{2}**—The concentration of PGE\textsubscript{2} in spent media from chondrocyte and synoviocyte cultures was determined by use of a commercial colorimetric assay\textsuperscript{14} following the directions of the manufacturers. Briefly, 10-fold dilutions of the stored media were incubated in assay buffer containing a fixed amount of competitive alkaline phosphatase–labeled PGE\textsubscript{2}. Assay wells were washed, and p-nitrophenyl phosphate was added as a substrate for alkaline phosphatase. Absorbance was measured at 405 nm,\textsuperscript{14} and PGE\textsubscript{2} concentrations were determined by comparison to a standard curve.
Quantitative real-time reverse-transcriptase PCR assay—Total RNA was isolated from chondrocytes and synoviocytes by Trizol® extraction, following the instructions of the manufacturer. First-strand cDNA was synthesized by use of dNTP mix® and reverse transcriptase® at 42°C for 50 minutes. Quantitative real-time reverse-transcriptase PCR assay was performed for cyclooxygenase-2 and mPGEs by use of elongation factor-1α as a normalization standard in an automated PCR detection system.

Statistical analysis—Data were compared by use of repeated-measures ANOVA with a statistical software program.* Values of $P < 0.05$ were considered significant. Nonparametric data were compared by use of repeated-measures ANOVA on ranks. Post hoc analysis was accomplished by use of the Student-Newman-Keuls method.

Results

Proteoglycan and MMP-13—Treatment with glucosamine at any concentration had no effect on production of proteoglycan by chondrocytes as measured in chondrocyte pellets or media under noninflammatory (no IL-1) or inflammatory (with IL-1) conditions. Treatment with glucosamine at any concentration had no effect on production of MMP-13 by chondrocytes or synoviocytes under noninflammatory or inflammatory conditions.

PGE₂—Treatment with glucosamine had no effect on production of PGE₂ by chondrocytes at any concentration studied under noninflammatory (no IL-1) or inflammatory (with IL-1) conditions. Treatment with glucosamine at 5 µg/mL significantly decreased production of PGE₂ by synoviocytes under inflammatory (with IL-1) conditions, compared with that of positive control synoviocytes (ie, with IL-1 and without glucosamine; Figure 1).

Cyclooxygenase-2—Treatment with glucosamine had no effect on production of cyclooxygenase-2 by chondrocytes or synoviocytes at any concentration studied under noninflammatory (no IL-1) or inflammatory (with IL-1) conditions. A general decrease in cyclooxygenase-2 production by chondrocytes was observed with increases in glucosamine concentrations under inflammatory conditions, although this effect was not significant (Figure 2).

mPGEs—Treatment with glucosamine at 0.1 and 0.5 µg/mL significantly decreased production of mPGEs by chondrocytes under inflammatory (with IL-1) conditions, compared with that of positive control chondrocytes (ie, with IL-1 and without glucosamine; Figure 3). Treatment with glucosamine at 0.1 µg/mL significantly decreased production of mPGEs by synovio-
cytes under inflammatory (with IL-1) conditions, compared with that of positive control synoviocytes (Figure 4).

Discussion

Results of this study indicate that glucosamine, at clinically relevant concentrations, does not impact production of proteoglycan by chondrocytes or the amount of MMP-13 released by chondrocytes and synoviocytes under noninflammatory or inflammatory conditions in this culture system. Glucosamine did have some limited effects on markers of inflammation at concentrations between 0.1 and 5 µg/mL. The hypothesis was therefore largely unsupported by these data.

Although glucosamine has been shown to increase the production of proteoglycan by chondrocytes,13 other researchers have failed to demonstrate any effect on production of proteoglycan or aggrecan at concentrations close to those achieved in vivo after oral administration.16,25 Evidence exists that beneficial effects of glucosamine on extracellular matrix gene expression may be enhanced by its use in combination with chondroitin sulfate and that these effects may not be observed in short-term culture.26 In addition, other researchers have shown that beneficial effects of glucosamine on matrix synthesis may occur within a narrow optimal concentration range.16,27 It is likely that the use of glucosamine alone at low concentrations under short-term culture conditions precluded discovery of any benefit to production of proteoglycan in the present study.

Glucosamine decreases the IL-1–induced up-regulation of MMP-13 transcription and translation.11 Clinically relevant concentrations of glucosamine have been shown to reduce inflammatory-induced MMP-13 mRNA expression.16,17 The lack of an effect by glucosamine on MMP-13 production in the present study, as with the proteoglycan data, is likely attributable to the use of low concentrations and short-term culture conditions. It is possible that the single time point at which MMP-13 was assayed may have contributed to the negative findings and that positive effects may be evident at other time points. Additionally, lack of treatment effect on certain markers in equine tissue,7 compared with findings in studies10,26 on bovine tissue, may be attributed to greater variability in responses in tissues from horses. The inherent genetic homogeneity in the bovine population may lend lower variability to metabolic treatment responses from these tissues.

Glucosamine has been shown to suppress PGE₂ production in chondrocytes and synoviocytes from osteoarthritic joints, although this effect was not evident at concentrations < 100 µg/mL.28 Other investigators have reported beneficial effects of glucosamine at clinically relevant concentrations on inflammatory mediators in IL-1–stimulated chondrocytes.19 Our findings lend some support to the contention that glucosamine is an effective mitigator of inflammatory mediator production and release at clinically relevant concentrations. Although cyclooxygenase-2 was not downregulated with any glucosamine treatment in either cell type, PGE₂ was abrogated in synoviocytes in inflammatory conditions at a concentration of 5 µg/mL. This concentration is realistic for what synoviocytes are exposed to in vivo, according to recent pharmacologic data.20–22 Furthermore, mPGEs, the terminal enzyme in PGE₂ synthesis, was downregulated under inflammatory conditions at concentrations of 0.1 and 0.5 µg/mL in chondrocytes and 0.1 µg/mL in synoviocytes. These data may be particularly germane because chondrocytes are likely exposed to glucosamine concentrations < 1 µg/mL in vivo after oral administration.22 To our knowledge, this is the first study to determine anti-inflammatory effects of glucosamine at concentrations encountered by chondrocytes in vivo. It is important to note, however, that these effects were not globally observed across the PGE₂ biosynthetic pathway. It is likely that the degree of anti-inflammatory activity by glucosamine may temporally fluctuate, as other investigators have reported this phenomenon when measuring degradative enzymes and proinflammatory agents.18,19 It is possible that the present study did not detect anti-inflammatory effects taking place earlier in the experiment.

The use of glucosamine as the sole treatment in the present study was designed to isolate the effects of this single agent and to allow investigation at several concentrations within the anticipated achievable in vivo range. However, it is important to recognize that glucosamine is often administered in combination with other agents, most commonly chondroitin sulfate. Some support exists for this rationale because others have reported additive or synergistic effects when glucosamine and chondroitin sulfate are administered concurrently. Glucosamine and chondroitin sulfate in combination stimulate glycosaminoglycan synthesis in a synergistic manner, compared with administration of either agent alone.29 Likewise, combination treatment is more effective than each agent alone in mitigating IL-1–induced increases in proteolytic enzymes.30 Despite the support for combination treatment, we believe it is important to evaluate the effects of these agents individually to more fully determine the mechanism of action of each. Whether glucosamine and chondroitin sulfate are complementary through similar modes of action or through separate but interrelated systems remains to be determined.

Figure 4—Mean ± SE relative production of mPGEs by equine synoviocytes. Treatment included absence (−) and presence (+) of IL-1 with glucosamine. See Figure 1 for key.
Despite evidence that synoviocytes are an important contributor to the progression of osteoarthritis, few studies have investigated the effects of glucosamine on these cells. Our results support a role for synoviocytes in the progression of osteoarthritis through upregulation of MMP-13 and the PGE biosynthetic pathway. Higher concentrations of glucosamine than those used in the present study have been shown to suppress PGE$_2$ and MMP production by synoviocytes. Synoviocyte gene transfer experiments aimed at up-regulating the rate-limiting enzyme in endogenous cellular glucosamine synthesis, glutamine:fructose-6-phosphate amidotransferase, have similarly decreased IL-1–induced PGE$_2$ production.

To our knowledge, this is the first study that has investigated the response of synoviocytes to glucosamine treatment at clinically relevant concentrations.

Several treatments resulted in values with high variability in this study, particularly in the production of PGE$_2$. We attribute this high variability to genetic diversity of the tissues as well as to the wide age range of horses. An additional possibility is that varying glucosamine concentrations had an impact on cell growth or viability. We feel this possibility is less likely because of the low concentrations of glucosamine used as well as the short duration of exposure. Although glucosamine can have an impact on cell growth kinetics and viability, these effects have only been observed at much higher concentrations than those in the present report.

Results of the present study have supported a role for glucosamine in mitigating the proinflammatory effects of IL-1 on chondrocytes and synoviocytes, although this activity was not observed at all concentrations. Importantly, these effects occurred at glucosamine concentrations relevant to clinical usage for chondrocytes and synoviocytes. Glucosamine did not confer beneficial effects on proteoglycan or MMP-13 production in either cell type at the concentrations studied. It is likely that the observed effects are related to the ability of glucosamine to modulate transcription factors that are upregulated in osteoarthritis and in IL-1–stimulated cells. Transcription factors nuclear factor-κB and activator protein-1 have been implicated in osteoarthritis and are downregulated by glucosamine.

It is becoming clearer, as the body of work on glucosamine accumulates, that there is some basis for its purported positive effects as a treatment for osteoarthritis.

References

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e. L-glutamine, 200mM, Invitrogen, Carlsbad, Calif.
f. WAKO, Richmond, Va.
g. Gemini Bioproducts, Woodland, Calif.
h. Glucosamine hydrochloride, Sigma-Aldrich, St Louis, Mo.
i. rhIL-1, R&D Systems, Minneapolis, Minn.
j. Na$_2$SO$_4$, AP Biochemicals, Costa Mesa, Calif.
k. Sigma-Aldrich, St Louis, Mo.
l. MMP-13 ELISA, Amersham Biosciences Inc, Piscataway, N.J.
m. FLUOstar Optima, BMG LaTabech, Durham, N.C.
. N. PGE, ELISA, R&D Systems, Minneapolis, Minn.
o. Invitrogen Corp, Carlsbad, Calif.
p. Oligo d(T), Invitrogen Corp, Carlsbad, Calif.
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