

# Effects of glucosamine and chondroitin sulfate on mediators of osteoarthritis in cultured equine chondrocytes stimulated by use of recombinant equine interleukin-1 $\beta$

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**Objective**—To determine whether glucosamine and chondroitin sulfate (CS) at concentrations approximating those achieved in plasma by oral administration would influence gene expression of selected mediators of osteoarthritis in cytokine-stimulated equine articular chondrocytes.

**Sample Population**—Samples of grossly normal articular cartilage obtained from the metacarpophalangeal joint of 13 horses.

**Procedure**—Equine chondrocytes in pellet culture were stimulated with a subsaturating dose of recombinant equine interleukin (reIL)-1 $\beta$ . Effects of prior incubation with glucosamine (2.5 to 10.0  $\mu$ g/mL) and CS (5.0 to 50.0  $\mu$ g/mL) on gene expression of matrix metalloproteinase (MMP)-1, -2, -3, -9, and -13; aggrecanase 1 and 2; inducible nitric oxide synthase (iNOS); cyclooxygenase (COX)-2; nuclear factor  $\kappa$ B; and c-Jun-N-terminal kinase (JNK) were assessed by use of a quantitative real-time polymerase chain reaction assay.

**Results**—Glucosamine at a concentration of 10  $\mu$ g/mL significantly reduced reIL-1 $\beta$ -induced mRNA expression of MMP-13, aggrecanase 1, and JNK. Reductions in cytokine-induced expression were also observed for iNOS and COX-2. Chondroitin sulfate had no effect on gene expression at the concentrations tested.

**Conclusions and Clinical Relevance**—Concentrations of glucosamine similar to those achieved in plasma after oral administration in horses exerted pretranslational regulation of some mediators of osteoarthritis, an effect that may contribute to the cartilage-sparing properties of this aminomonosaccharide. Analysis of results of this study indicated that the influence of CS on pretranslational regulation of these selected genes is limited or lacking. (*Am J Vet Res* 2005;66:1861–1869)

**O**steoarthritis (OA) remains an important and expensive cause of lameness in affected horses. Although a number of factors can initiate the disease process, all articular tissues are ultimately affected. The hallmark of OA is the degeneration of the articular cartilage matrix, which is attributed to an excess produc-

tion of proinflammatory cytokines.<sup>1</sup> Interleukin (IL)-1 $\beta$  is widely accepted as one of the cytokines that plays a pivotal role in the pathophysiologic processes of OA.<sup>2,3</sup> This cytokine induces a number of catabolic events in synoviocytes and chondrocytes, including induction of genes of matrix degrading proteinases, such as the metalloproteinases (MMPs) and aggrecanases, as well as a number of other inflammatory mediators, including inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2.<sup>3-7</sup> Many of the effects of IL-1 $\beta$  on mediators of osteoarthritis pathophysiologic processes are mediated through the activation of transcription factors, such as nuclear factor  $\kappa$ B (NF $\kappa$ B) and activator protein (AP)-1.<sup>8,9</sup>

Oral administration of nutraceuticals containing glucosamine, chondroitin sulfate (CS), or both has received considerable attention as a palliative treatment of humans and domestic animals with OA. Ongoing research in this area indicates that these nutraceuticals may possess chondroprotective or cartilage-sparing properties. For example, a chondroprotective effect has been supported by 2 studies<sup>10,11</sup> in which investigators indicated that glucosamine prevented narrowing of the knee joint space in patients with OA during a 3-year period. Similarly, in a randomized, double-blind, controlled study,<sup>12</sup> CS was found to be protective against radiographic progression of OA of the finger joints.

Certain effects of these substances on cellular metabolism have received attention. Glucosamine acts as a substrate for and enhances the production of proteoglycans and glycosaminoglycans.<sup>13</sup> Furthermore, glucosamine prevents the repression of galactose  $\beta$ -1,3-glucuronosyltransferase I, a key biosynthetic enzyme in glycosaminoglycan synthesis.<sup>14</sup> Glucosamine inhibits proteoglycan loss, prostaglandin production, iNOS induction, and MMP activity in equine cartilage explants stimulated with lipopolysaccharide or human and equine recombinant IL-1.<sup>15-18</sup>

Although purported to have favorable effects on chondrocyte metabolism, CS has been less well characterized than glucosamine with respect to its anticatabolic functions. In an in vitro culture system that used

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human chondrocytes in clusters, CS was protective against IL-1-induced deleterious effects on sulfated proteoglycan and inhibited synthesis of type II collagen.<sup>19</sup> Chondroitin sulfate stimulates production of hyaluronic acid by synoviocytes.<sup>20</sup>

It has been suggested that at least some of the anti-catabolic effects of glucosamine are exerted at the pre-translational level. Modulation of cytokine- and endotoxin-stimulated induction of MMPs and aggrecanases in chondrocytes has been documented *in vitro*.<sup>18,21,22</sup> Specifically, glucosamine reduced expression of MMP-1, MMP-3, and MMP-13 in monolayer cultures of equine chondrocytes<sup>18</sup> and MMP-3 in rat chondrocytes<sup>14</sup> and chondrocytes of humans with OA.<sup>22</sup> Analysis of data suggests that inhibition of IL-1-induced synthesis of inflammatory mediators and matrix degrading proteinases by glucosamine may take place via a reduction of the activity of certain cell-signaling pathways. For example, concentrations of the transcription factors NF $\kappa$ B and AP-1 are increased in osteoarthritic cartilage, compared with concentrations in normal specimens, and IL-1-induced activity of NF $\kappa$ B and AP-1 is associated with enhanced transcription of the genes encoding the aforementioned mediators of cartilage degradation.<sup>23,24</sup> Glucosamine inhibits binding of NF $\kappa$ B in a dose-dependent manner and prevents IL-1-induced translocation of p50 and p65 subunits of NF $\kappa$ B to the nucleus in osteoarthritic human cartilage.<sup>25</sup>

To date, most *in vitro* research in this area has been performed with concentrations of glucosamine and CS that exceed those obtained in plasma after oral administration.<sup>15-18,21,25-27</sup> Depending on the species and molecular weight of CS, concentrations of CS in plasma after oral administration range from 19 to 208  $\mu$ g/mL, with a cumulative effect after multiple doses.<sup>28-31</sup> Glucosamine concentrations in plasma after oral administration are in the range of 1.25 to 20  $\mu$ g/mL, with concentrations up to 350  $\mu$ g/mL possible after IV administration.<sup>28-31</sup> Thus, the objective of the study reported here was to determine the effect of concentrations of glucosamine (2.5 to 10.0  $\mu$ g/mL) and CS (5.0 to 50.0  $\mu$ g/mL) that more closely approximate those achieved in plasma after oral administration. Effects were measured as gene expression of a number of mediators of cartilage catabolism in OA by use of equine chondrocytes in pellet cultures stimulated with recombinant equine (re)IL-1 $\beta$ .

## Materials and Methods

**Sample population**—Specimens of grossly normal articular cartilage were obtained from the metacarpophalangeal joint of 13 horses immediately after the horses died or were euthanized. Horses were between 2 and 8 years of age and died or were euthanized for reasons other than joint disease.

**Pellet culture**—Isolation and propagation of chondrocytes in pellet culture were conducted as described elsewhere,<sup>18,32</sup> with minor modifications. Briefly, cartilage was dissected from the subchondral bone. After incubation at 25°C for 1 hour in medium containing penicillin (500 U/mL) and streptomycin<sup>a</sup> (500 mg/mL), chondrocytes were isolated by sequential digestion with pronase<sup>b</sup> (1 mg/mL; digested for 1 hour) and collagenase<sup>c</sup> (0.3 mg/mL; digested for 18 hours).

After digestion, the cells were separated by sequential centrifugation (300  $\times$  g for 10 minutes, repeated 3 times), washed, and resuspended in 20 mL of Dulbecco modified Eagle medium:nutrient mixture Ham F-12 (1:1).<sup>d</sup> The medium was supplemented with insulin-transferrin-sodium selenite supplement<sup>e</sup> (insulin, 5  $\mu$ g/mL; transferrin, 5  $\mu$ g/mL; and sodium selenite, 5 ng/mL), L-ascorbic acid<sup>f</sup> (50  $\mu$ g/mL), amino acids,<sup>g</sup> lactalbumin hydrolysate<sup>h</sup> (2  $\mu$ g/mL), linoleic acid<sup>i</sup> (5  $\mu$ g/mL), thyroxine<sup>j</sup> (40 ng/mL), and 100 U of penicillin-streptomycin<sup>k</sup>/mL. The concentration of amino acids was 50% of that reported in another study.<sup>33</sup>

Cell concentration was determined by use of a hemacytometer, and aliquots of 6  $\times$  10<sup>6</sup> cells were transferred to 15-mL polypropylene centrifuge tubes in 1 mL of the aforementioned supplemented serum-free medium. After centrifugation (300  $\times$  g for 5 minutes), pellets were incubated by use of standard cell culture conditions (37°C, 95% relative humidity, and 5% carbon dioxide). Medium was exchanged every 3 days. Pellet cultures were maintained in serum-free medium that was not supplemented with insulin-transferrin-sodium selenite supplement, linoleic acid, thyroxine, and L-ascorbic acid for 2 days before the start of an experiment for equilibration. At the conclusion of experiments, pellets were collected for isolation of total RNA.

**Isolation of total RNA**—Total RNA was extracted by use of a commercial extraction preparation<sup>l</sup> and RNA isolation kit<sup>m</sup>; the extraction was conducted in accordance with the manufacturer's instructions, with minor modifications.<sup>34</sup> Briefly, medium was removed from each pellet, and 1 mL of extraction preparation was then added to each pellet. Pellets were incubated (25°C for 5 minutes), mixed on a plate shaker (25°C for 5 minutes), and transferred to microcentrifuge tubes. After centrifugation (10,000  $\times$  g for 10 minutes), 200  $\mu$ L of chloroform was added to extract total RNA, followed by agitation and a second incubation (25°C for 2 minutes). The solution was centrifuged (12,000  $\times$  g for 15 minutes at 4°C); the aqueous phase containing RNA was then collected and RNA precipitated by addition of an equal volume of 75% ethanol. The RNA was further purified by the use of columns contained in the commercial isolation kit. Pellets were resuspended in RNase-free water (0.1% diethylpyrocarbonate) and centrifuged (10,000  $\times$  g for 1 minute) to elute RNA. The RNA was analyzed by electrophoresis through 1% agarose gels containing ethidium bromide (10  $\mu$ g/mL) in 1 $\times$  3-(N-morpholino) propanesulfonic acid to validate spectrophotometric determination and RNA integrity. The RNA was quantified by use of UV spectrophotometry<sup>n</sup> and adjusted by the addition of RNase-free water to achieve solutions with a final concentration of 1  $\mu$ g/ $\mu$ L.

**Quantitative real-time polymerase chain reaction assays**—Two micrograms of each RNA sample was treated with DNase I<sup>o</sup> to degrade contaminating single- and double-stranded DNA. Treated RNA was converted to single-stranded cDNA by use of reverse transcriptase<sup>p</sup> in accordance with recommendations of the manufacturer. The cDNA was quantified by use of UV spectrophotometry and adjusted by the addition of RNase-free water to achieve a final concentration of 25 ng/ $\mu$ L.

Reference amplicons were developed for each mRNA. Specific primers (Appendices 1 and 2) were designed by use of commercial software<sup>q</sup> and synthesized by commercial facilities.<sup>5</sup> Nucleotide sequences used for primer design were obtained from public databases.<sup>1</sup> Full- or partial-length equine sequences were available for 18S ribosomal subunit (18S),  $\beta$ -actin,  $\beta_2$ -microglobulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), iNOS, MMP-1, MMP-2, MMP-3, MMP-13, aggrecanase 1, aggrecanase 2, COX-2,

ribosomal protein L19 (RPL19), and ubiquitin. When an equine sequence was not available (ie, c-Jun-N-terminal kinase [JNK], NFκB, and MMP-9), equine expressed sequence tag sequences corresponding to the target gene were used on the basis of similarity to the corresponding bovine, human, or mouse sequence. Searches for all of the primer and amplicon sequences were conducted by use of specially designed software<sup>u</sup> to ensure gene specificity. Optimal concentrations of each set of primers were determined by use of a primer matrix (lowest SD with no change in number of cycles to threshold [ $C_T$ ]).

Fifty nanograms of sample cDNA primed each real-time polymerase chain reaction (PCR) assay. All analyses were conducted in a sequence detection system.<sup>v</sup> The cDNA templates were combined with optimal concentrations of primers and PCR dye mix<sup>w</sup> in a total volume of 50 μL, and the amplification was conducted as recommended by the manufacturer. The PCR conditions were 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of extension at 95°C for 15 seconds, and 1 minute at 60°C. Threshold lines were adjusted to intersect amplification lines in the linear portion of the amplification curves. The software automatically recorded the  $C_T$ . Analysis of each sample was performed in duplicate, and an SD < 0.5 between replicates was set as a criterion for inclusion of data. Reference amplicons for each gene and primer set were verified on the basis of test amplifications that used equine cartilage RNA-derived cDNA. Replicated data were adjusted on the basis of the geometric mean of 3 endogenous control samples (ie, 18S, GAPDH, and β<sub>2</sub>-microglobulin),<sup>35</sup> and the fold-change in gene expression relative to serum-free control samples was calculated by use of the  $2^{-\Delta C_T}$  method.<sup>36</sup>

**Determination of a subsaturating dose of reIL-1β**—The reIL-1β was purified as described elsewhere<sup>3</sup> and suspended in supplemented serum-free medium. Chondrocyte pellet cultures were established as described previously. After an establishment period of 8 to 12 days, reIL-1β was added in incremental doses to achieve concentrations ranging from 0 to 20 ng/mL, and cultures were incubated for 6 hours. Pellets were then harvested and RNA isolated for quantitative real-time (qRT) PCR assays, with data adjusted on the basis of results for GAPDH. Effects on expression were quantified for MMP-13, iNOS, COX-2, and aggrecanase 1. This was repeated by use of tissues from 5 horses.

**Selection of housekeeping genes for use as endogenous control samples in subsequent analyses**—Six housekeeping genes were selected as potential control samples for loading on the basis of the availability of full- or partial-length equine sequences for commonly used endogenous control samples (Appendix 1). Validation of the effect of experimental treatment on the expression of each housekeeping gene was determined for each horse by use of the  $2^{-\Delta C_T}$  method, where,

$$\Delta C'_T = C_{T(\text{treatment})} - C_{T(\text{serum-free control sample})},^{36}$$

with the fold change ( $2^{-\Delta C'_T}$ ) expressed as a typical fold change from the mean and the SD expressed as the maximum fold change (maximum variability).<sup>37</sup> Absolute  $C_T$  values were used as an indicator of the amount of gene expression. Overall median expression (ie,  $C_T$ )

and deviation from the median for each housekeeping gene were used as an additional measure of gene stability.

To determine the efficiency of gene amplification, cDNA was serially diluted (10 to 100 ng/mL) and the mean  $C_T$  calculated for each gene of interest and each housekeeping gene. The  $\Delta C_T$  (ie,  $C_{T(\text{gene of interest})} - C_{T(\text{housekeeping gene})}$ ) was determined and plotted as a function of the logarithm of cDNA dilution. The value of the slope of the regression was a measure of the difference in amplification efficiencies between the gene of interest and the housekeeping gene. A difference in efficiency of < 0.1 was determined for appropriate adjustment of each gene of interest to a particular housekeeping gene.<sup>36</sup> Selection of the 3 endogenous control genes to be used for adjustment was determined on the basis of amplification efficiency, lowest variability, and amount of expression.

**Effect of glucosamine and CS on gene expression**—Pellet cultures were placed in fresh supplemented serum-free medium described previously. After an incubation period of 5 to 7 days to establish stable metabolism, glucosamine<sup>x</sup> (2.5,

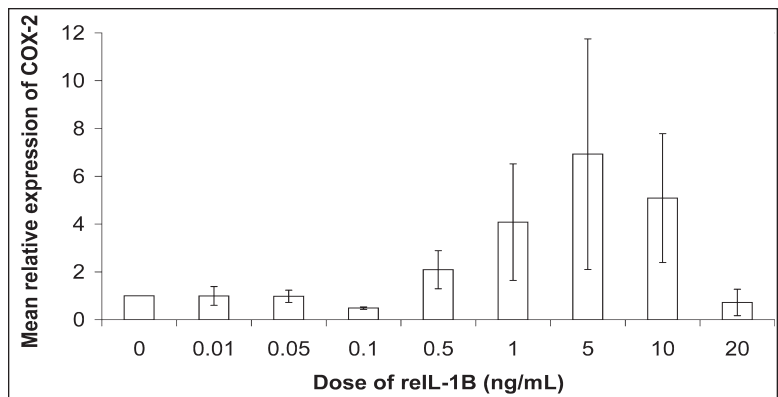


Figure 1—Mean ± SEM relative expression of cyclooxygenase (COX)-2 in response to graduated doses of recombinant interleukin (reIL)-1β. Pellet cultures of chondrocytes ( $3 \times 10^6$  cells/pellet) were incubated with graduated concentrations (0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 20 ng/mL) of reIL-1β for 6 hours, which was followed by RNA isolation and quantitative real-time polymerase chain reaction (PCR) assays conducted by use of specific primers for COX-2. Approximately half-maximal stimulation was evident at 0.5 ng/mL. A qualitatively similar response was observed for aggrecanase (Agg-1), inducible nitric oxide synthase (iNOS), and matrix metalloproteinase (MMP)-13 (data not shown).

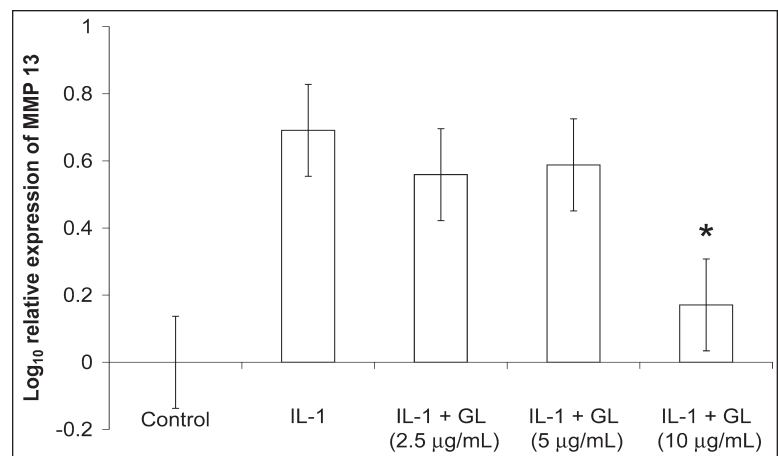


Figure 2—Mean ± SEM values for reIL-1β-induced expression of MMP-13 in response to graduated concentrations of glucosamine hydrochloride (GL). Values reported represent results of experiments conducted by use of tissues from 3 horses. Relative expressions were calculated by use of the  $2^{-\Delta C_T}$  method with 18S, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β<sub>2</sub>-microglobulin as the reference genes. \*Value differs significantly ( $P < 0.05$ ) from the value for the control treatment.

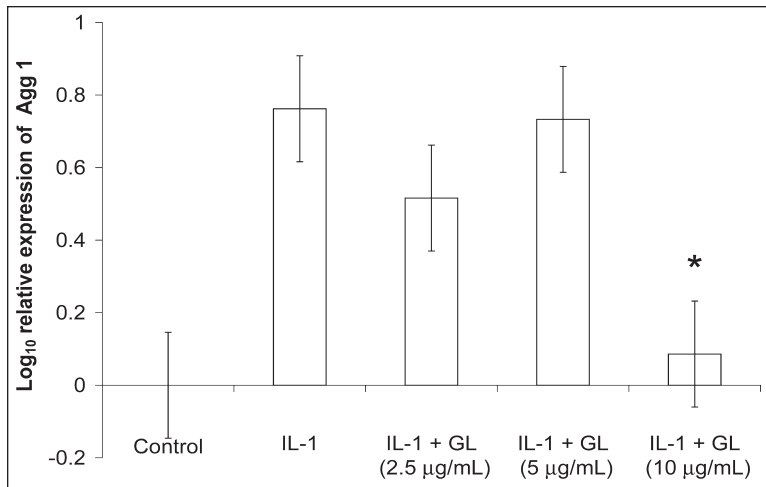


Figure 3—Mean  $\pm$  SEM values for reIL-1 $\beta$ -induced expression of Agg 1 in response to graduated concentrations of GL. Values reported represent results of experiments conducted by use of tissues from 4 horses. Relative expressions were calculated by use of the  $2^{-\Delta\Delta C_T}$  method with 18S, GAPDH, and  $\beta_2$ -microglobulin as the reference genes. See Figure 2 for remainder of key.

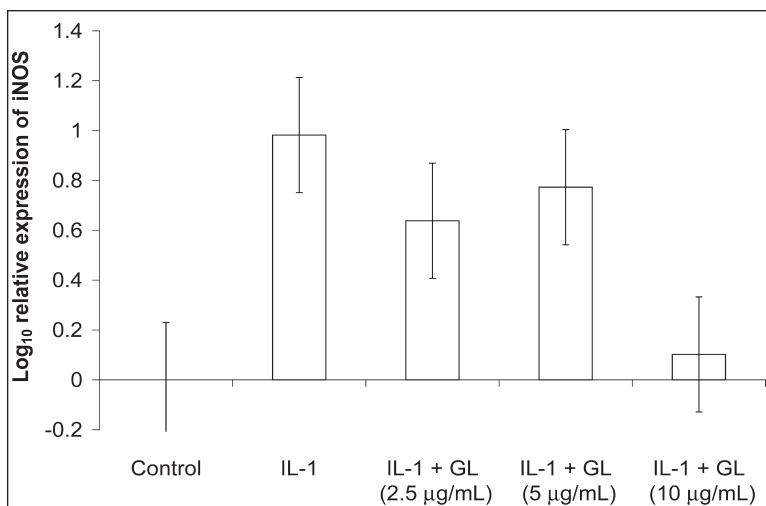


Figure 4—Mean  $\pm$  SEM values for reIL-1 $\beta$ -induced expression of iNOS in response to graduated concentrations of GL. Values reported represent results of experiments conducted by use of tissues from 3 horses. Relative expressions were calculated by use of the  $2^{-\Delta\Delta C_T}$  method with 18S, GAPDH, and  $\beta_2$ -microglobulin as the reference genes. The effect of treatment was not significant ( $P = 0.06$ ).

5.0, or 10.0  $\mu\text{g/mL}$ ) or CS<sup>r</sup> (5.0, 10.0, 20.0, or 50.0  $\mu\text{g/mL}$ ) was added to all pellet cultures, except the positive and negative control samples. One hour later, reIL-1 $\beta$  was added (final concentration, 0.5 ng/mL) to all pellet cultures, except negative control samples. After incubation for 12 hours in standard cell culture conditions, RNA was isolated for qRT-PCR assay. Genes evaluated included MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, aggrecanase 1, aggrecanase 2, iNOS, COX-2, NF $\kappa$ B, and JNK. This was repeated by use of tissues from 8 horses.

**Statistical analysis**—Means for relative expression of each gene of interest included only those horses for which the subsaturating dose of reIL-1 $\beta$  resulted in a  $\geq 2$ -fold upregulation of expression from that of control samples. Distributions were tested for normality by use of the method of Kolmogorov-Smirnov. When required, means were logarithmically transformed, which was followed by analysis with a 2-way ANOVA (blocked by horse). Post hoc testing was

conducted by use of the Duncan multiple range test. Statistical testing was conducted by use of a commercially available statistical program.<sup>2</sup> Values of  $P < 0.05$  were considered significant.

## Results

**Determination of a subsaturating dose of reIL-1 $\beta$** —Exposure of pellet cultures to graduated concentrations of reIL-1 $\beta$  resulted in dose-dependent saturable induction of expression of aggrecanase 1, MMP-13, iNOS, and COX-2 (Figure 1). The dose of reIL-1 $\beta$  corresponding to approximately half-maximal induction of expression of these genes varied from 0.1 to 1.0 ng/mL. For this reason, the intermediate dose of 0.5 ng/mL was selected for use in subsequent experiments. This concentration of reIL-1 $\beta$  resulted in approximately 2- to 5-fold increases in expression of the 4 genes. On the basis of sizable variability in data in the subsequent experiments, an analogous dose-response protocol was repeated with cartilage from 4 horses; there was an incubation period of 12 hours before RNA isolation and amplification with primers for COX-2, which yielded comparable results.

**Selection of housekeeping genes for use as endogenous control samples for subsequent analyses**—Amount of gene expression for available housekeeping genes varied substantially, and there were substantial differences in stability of various housekeeping genes among horses. Amplification efficiency varied for each housekeeping gene, with GAPDH being the most suitable for comparison of 6 of 11 genes evaluated. Three housekeeping genes (18S, GAPDH, and  $\beta_2$ -microglobulin) were subsequently chosen on the basis of the amount of expression, amplification efficiency, and lowest variability (overall and for each horse).

**Effect of glucosamine and CS on gene expression**—Glucosamine at a concentration of 10  $\mu\text{g/mL}$  significantly reduced reIL-1 $\beta$ -induced mRNA expression of MMP 13 and aggrecanase 1 (Figures 2 and 3). A nonsignificant reduction in cytokine-induced expression was also observed for iNOS ( $P = 0.06$ ) and COX 2 ( $P = 0.08$ ; Figures 4 and 5). Pellets treated with glucosamine at a concentration of 10.0  $\mu\text{g/mL}$  had significantly reduced expression of JNK, compared with expression for positive (reIL-1 $\beta$ ) control samples; however, for JNK, the expression of positive and negative control samples was comparable. Glucosamine at a concentration of 10.0  $\mu\text{g/mL}$  also caused a nonsignificant reduction in IL-1 $\beta$ -induced stimulation of aggrecanase 2 ( $P = 0.13$ ) and NF $\kappa$ B ( $P = 0.11$ ). Recombinant equine IL-1 $\beta$  failed to significantly upregulate expression of MMP-9. Chondroitin sulfate had no significant effect on gene expression at the concentrations tested.

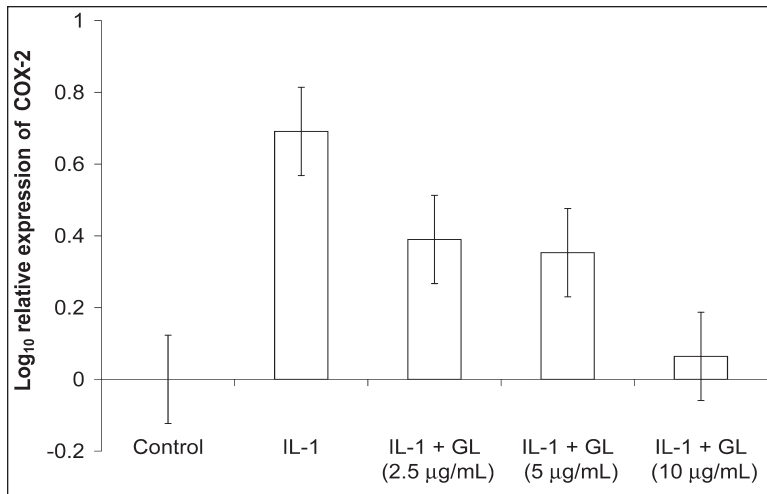


Figure 5—Mean ± SEM values for reIL-1β-induced expression of COX-2 in response to graduated concentrations of GL. Values reported represent results of experiments conducted by use of tissues from 3 horses. Relative expressions were calculated by use of the  $2^{-\Delta\Delta C_T}$  method with 18S, GAPDH, and  $\beta_2$ -microglobulin as the reference genes. The effect of treatment was not significant ( $P = 0.08$ ).

## Discussion

Although the potential cartilage-sparing role of glucosamine and CS has been studied by various methods, the specific mechanism or mechanisms of action and the minimally effective concentrations remain to be established. We conducted a study based on results of qRT-PCR assays to characterize the effects on gene expression for concentrations of glucosamine and CS that correspond to concentrations that approximate those achieved in plasma after oral administration in monogastrics.<sup>28–31</sup> Our data support the hypothesis that glucosamine at a concentration of 10 µg/mL is capable of pretranslational regulation of reIL-1β-induced stimulation of at least some of the proteins implicated in the process of cartilage degradation. Specifically, glucosamine led to a significant reduction of cytokine-stimulated expression of MMP-13 and aggrecanase 1 and a nonsignificant pattern of reduced expression for iNOS and COX-2. These findings supplement those of other studies<sup>17,18,21,22</sup> conducted by our laboratory group and by other investigators that were conducted with concentrations higher than those found in the plasma of animals orally administered glucosamine and CS. The observations for the study reported here parallel those in other publications and provide additional evidence in support of a cartilage-sparing effect of glucosamine.

In contrast to our findings for MMP-13 and aggrecanase 1, reIL-1β significantly upregulated the expression of MMP-1, MMP-3, and aggrecanase 2, but this effect was not significantly influenced by treatment with glucosamine. Although the effect was not significant because of variation among horses, glucosamine at a concentration of 10 µg/mL reduced expression of aggrecanase 2 to values approximately 25% those of the cytokine control sample. The lack of an effect of glucosamine for MMP-1 and MMP-3 is in contrast to other reports<sup>15,18,38</sup> in which investigators used higher doses of glucosamine; however, our results parallel those of a report<sup>38</sup> in which glucosamine at 10 µg/mL

failed to significantly inhibit IL-1-induced MMP production. Differences in responses among the MMPs may have been attributable to experimental design or the variability inherent in these particular experiments. For example, prior incubation of rat chondrocytes with glucosamine (albeit at a higher dose) downregulates MMP-3 gene expression.<sup>14</sup> The potential for differential regulation of MMPs by glucosamine should be considered on the basis of documentation in other studies.<sup>17,22,27</sup> For example, investigators in 1 study<sup>22</sup> reported that glucosamine was able to modulate activity of MMP-3 but not activity of MMP-1. In addition, temporal and dose-dependent differential regulation of MMPs and aggrecanases has been documented *in vitro*,<sup>8,39–41</sup> and coordinated regulation of MMPs and aggrecanases has been postulated.<sup>42</sup>

In contrast to the other MMPs examined, significant upregulation of MMP-2 and MMP-9 by reIL-1β was not observed. This is in keeping with results of other studies<sup>43–45</sup> in chondrocytes in which investigators detected no change or mild increases in expression induced by IL-1. It may also relate to differences between normal and OA chondrocytes, reflecting the differential expression patterns of MMPs in cartilage from early OA, compared with expression patterns for cartilage from late OA.<sup>46</sup>

Cyclooxygenase-2 and iNOS are both implicated in the pathophysiologic process of cartilage degeneration. For both, a nonsignificant reduction in cytokine-induced synthesis was observed. The ability of glucosamine to regulate expression of these genes and their respective inflammatory mediators in equine cartilage has been reported.<sup>15–17,27</sup> We attribute the lack of a clearly detectable protective effect of glucosamine to the inherent variability in the design of our study because experiments conducted by use of bovine explants revealed that addition of glucosamine at a concentration of 5 µg/mL resulted in significant inhibition of prostaglandin E<sub>2</sub> by recombinant human IL-1β.<sup>4a</sup>

The most common form of AP-1 is as a heterodimer of 2 proteins, c-Jun and c-Fos.<sup>47</sup> The JNK phosphorylates and hence activates c-Jun, which then translocates to the nucleus and dimerizes with c-Fos.<sup>48</sup> The expression of JNK increases in osteoarthritic cartilage,<sup>49</sup> with increased expression preceding the expression of degradative enzymes, such as the MMPs, along with clinical signs of OA.<sup>50</sup> Because of inconsistent induction of the JNK transcript by reIL-1β in the study reported here, the statistical analysis was limited to data for tissues from only 2 horses. Thus, despite what appears to be a dramatic inhibition of cytokine-induced expression of this protein for glucosamine at a concentration of 10 µg/mL, categorical conclusions cannot be drawn. The inconsistent induction of JNK by IL-1β in this study may be a dose-related phenomenon because maximal induction of JNK activity required recombinant human IL-1 at a concentration of

10 ng/mL for stimulation of rabbit articular chondrocytes.<sup>31</sup> In contrast to our findings, investigators in 2 other studies<sup>25,26</sup> used much higher doses of glucosamine but did not detect an influence on binding of AP-1 DNA or AP-1 activity despite a concurrent suppression of NFκB. Nonetheless, the possibility that glucosamine influences JNK synthesis warrants additional investigation and could provide an additional mechanism by which glucosamine can inhibit IL-1-mediated effects in osteoarthritic cartilage.

Although we were unable to detect a significant ( $P = 0.11$ ) effect of glucosamine on induction of NFκB expression by reIL-1β, it remains possible that at least some of the effects of glucosamine are induced via this intracellular signaling pathway. Cartilage from osteoarthritic humans was used in a study<sup>25</sup> that revealed glucosamine sulfate significantly inhibits IL-1-induced binding of NFκB DNA, translocation of p50 and p65 subunits of NFκB to the nucleus, and prevention of IL-1-mediated degradation of the natural inhibitor of NFκB. However, doses used in that study were at least 100-fold greater than those used in our study, and doses equivalent to those used in the study reported here had no effect, suggesting that the influence of glucosamine on NFκB may be a dose-dependent event.

Regulation of gene expression was observed with glucosamine but not CS. The effect of glucosamine on pretranslational regulation of genes coding for proteins implicated in OA has been the subject of a number of studies; however, the potential effects of CS on gene expression have been examined less frequently. Nonetheless, results of the study reported here contradict results for studies<sup>17,19</sup> in which investigators used higher doses of CS. Specifically, CS at 20 μg/mL significantly reduced MMP-13 and aggrecanase 2 mRNA expression in explants of bovine cartilage.<sup>bb</sup> The specific reasons for this difference remain to be elucidated but may reflect differences in species (bovine vs equine), experimental design (pellets vs explants), the form of CS used (source, purity, and molecular weight), magnitude of the arthritogenic stimulus (recombinant human IL-1β [50 ng/mL] vs reIL-1β [0.5 ng/mL]), or the influence of timing of supplementation (before incubation vs concurrent with addition of IL-1). Conversely, the beneficial effects of CS may be principally related to anabolic processes, rather than the prevention of catabolic processes. For example, CS enhances the expression of genes, such as type II collagen, which suggests that the chondroprotective effects of CS may be attributable, in part, to stimulation of collagen and proteoglycan-glycosaminoglycan synthesis.<sup>19,20,52,53</sup>

Research on OA involving the use of in vitro methods of cartilage degradation incorporating lipopolysaccharide, crude mixtures of inflammatory mediators contained in conditioned media, or recombinant cytokines to induce cartilage degradation represents a popular and cost-effective means of investigating pathophysiologic events and the effects of putative therapeutic agents on the disease process. Glucosamine and CS have been investigated in this manner; however, many of the studies<sup>15-21,25-27</sup> conduct-

ed have used relatively large quantities of arthritogenic stimuli and nutraceuticals. The series of experiments reported here were planned to address these limitations of other studies. This design used a subsaturating dose of recombinant cytokine and physiologically relevant concentrations of the potential cartilage-sparing preparations. This series of experiments, although arguably more biologically relevant than some of the other studies conducted, was complicated by considerable variability from several sources. We endeavored to use a dose of reIL-1β to induce a half-maximal response in our genes of interest. The dose of 0.5 ng/mL was determined during preliminary experiments on 4 of our genes of interest by use of tissues from 5 horses in an attempt to address the substantial intrinsic variability among horses. Despite these efforts, the response to reIL-1β in the subsequent experiments varied widely among genes and among horses. As such, the prerequisite ≥ 2-fold upregulation of most genes was not uniformly achieved, resulting in inferential analyses disadvantaged by relatively small numbers.

The analysis of relative gene expression used in the study reported here was adapted from the  $2^{-\Delta\Delta C_T}$  method.<sup>36</sup> Validation of this technique is necessary for each experimental method because of the need to adhere to certain requirements, including similarity in amount of expression and amplification efficiencies of the target and reference (housekeeping) genes as well as stability of expression for the housekeeping genes. Variation in stability of housekeeping genes has been documented in other studies,<sup>35,54</sup> prompting recommendations to use more than 1 gene for the basis of adjustment. Because housekeeping genes have not been exhaustively compared for equine cartilage and considerable variability was observed during initial amplifications conducted by use of RNA isolated from glucosamine- and CS-treated pellets, we conducted a number of ancillary experiments to more fully characterize amplification efficiencies and stability of a number of standard reference genes, including 18S, β-actin, β<sub>2</sub>-microglobulin, GAPDH, RPL19, and ubiquitin. We observed that the suitability of each housekeeping gene varied among the genes of interest and among horses. Similar sizable interindividual differences have been documented in studies<sup>37,55</sup> conducted in other species. A geometric mean<sup>35</sup> for 18S, β<sub>2</sub>-microglobulin, and GAPDH was used in an attempt to incorporate requirements for amount of expression, amplification efficiency, and gene stability for particular genes of interest and each horse. However, inconsistent and irregular amplification behavior may have hampered our attempts to detect subtle treatment effects by use of this technique. Despite attempts to limit the effects of an observed lack of constitutive expression on the part of the housekeeping genes, important dispersion remained in the data set. For experiments of this type, provisions need to be made for greater replication than has been typical of experiments in which more potent stimulation and pharmacologic doses of therapeutic compounds were used.

- a. Penicillin-streptomycin solution, Sigma Chemical Co, St Louis, Mo.
- b. Protease, Sigma Chemical Co, St Louis, Mo.

- c. Collagenase type 4, Worthington Biochemical Corp, Lakewood, NJ.
- d. Dulbecco modified Eagle medium/nutrient mixture F-12 Ham, Sigma Chemical Co, St Louis, Mo.
- e. Insulin-transferrin-sodium selenite supplement, Roche, Indianapolis, Ind.
- f. L-Ascorbate, Sigma Chemical Co, St Louis, Mo.
- g. Amino acids standard solution, Sigma Chemical Co, St Louis, Mo.
- h. Lactalbumin enzymatic hydrolysate, Sigma Chemical Co, St Louis, Mo.
- i. Linoleic acid albumin, Sigma Chemical Co, St Louis, Mo.
- j. L-Thyroxine, Sigma Chemical Co, St Louis, Mo.
- k. Penicillin-streptomycin solution (10X), Gibco, Grand Island, NY.
- l. TRIzol, Invitrogen, Carlsbad, Calif.
- m. RNeasy mini kit, Qiagen, Valencia, Calif.
- n. ND 1000, Nanodrop Technologies, Wilmington, Del.
- o. Dnase I, Invitrogen, Carlsbad, Calif.
- p. SuperScript II, Invitrogen, Carlsbad, Calif.
- q. Primer Express, Perkin-Elmer Applied Biosystems, Foster City, Calif.
- r. Operon Technologies, Alameda, Calif.
- s. Macromolecular Structure, Sequencing and Synthesis Facility, Michigan State University, East Lansing, Mich.
- t. Genbank Database, National Center for Biotechnology Information, Bethesda, Md.
- u. BLASTN, National Center for Biotechnology Information, Bethesda, Md.
- v. ABI Prism 7000, Perkin-Elmer Applied Biosystems, Foster City, Calif.
- w. SYBR Green PCR dye mix, Perkin-Elmer Applied Biosystems, Foster City, Calif.
- x. Glucosamine hydrochloride, Sigma Chemical Co, St Louis, Mo.
- y. Chondroitin sulfate A, Sigma Chemical Co, St Louis, Mo.
- z. SigmaStat, version 2.0, Aspire Software International, Leesburg, Va.
- aa. Chan PS, Caron JP, Orth MW. Glucosamine and chondroitin sulfate regulate inflammatory mediators (abstr), in *Proceedings*. 9th World Cong Osteoarthritis Res Soc Int 2004;S133.
- bb. Chan PS, Caron JP, Orth MW. Glucosamine and chondroitin sulfate regulate IL-1 induced gene expression (abstr), in *Proceedings*. 9th World Cong Osteoarthritis Res Soc Int 2004;S133–S134.

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## Appendix 1

Sequences of forward and reverse primers of housekeeping genes used for quantitative real-time polymerase chain reaction assays.

Gene	Genbank accession No.	Forward primer	Reverse primer
18S	AJ311673	5'-CGC CGC TAG AGG TGA AAT TC-3'	3'-GCT TTC GTA AAC GGT TCT TAC-5'
β-actin	AF035774	5'-TCA CGG AGC GTG GCT ACA G-3'	3'-TT AGC ACG CAC TGT AGT TCC-5'
β <sub>2</sub> -microglobulin	X69083	5'-CCG CGT GTT CCG AAG GT-3'	3'-T TTC GGT TAA AAG GAC TTG ACG-5'
GAPDH	AF157626	5'-CCC ACC CCT AAC GTG TCA GT-3'	3'-CT TTC GAC GGT TTA TGC TAC TCT-5'
RPL19	AY246727	5'-CGA AAG CGA AGG GCA ATG T-3'	3'-TAG GTG TTC GGC TTC CGT-5'
Ubiquitin	AF506969	5'-CGA GGC CTT TTG GTG TGG TA-3'	3'-TA ATC GTC AAA GAA GCA CAG GCA-5'

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase. RPL19 = Ribosomal protein L19.



## Appendix 2

Sequences of forward and reverse primers of genes of interest used for quantitative real-time polymerase chain reaction assays.

Gene	Genbank accession No.	Forward primer	Reverse primer
Agg -1	AF368321	5'-TTT CCC TGG CAA GGA CTA TGA-3'	3'-TGA GTG CGG TAA CAG GCG-5'
Agg-2	AF368322	5'-AAA TGC ACC TCA GCC ACC AT-3'	3'-A ACG ATC TGG AGG GTG CGT-5'
COX-2	AB041771	5'-GGA GCT GTA TCC TGC CCT TCT-3'	3'-CCA TGG TCT CCC CGA AGA T-5'
iNOS	AY027883	5'-GGC CTT GGC TCC AGC AT-3'	3'-TGT AAC TGG TCT TCA ACA GAG TGG-5'
JNK	BM414648 (91% homology with human sequence)	5'-TTC CAA CTG GGC ATC ATA AAT TT-3'	3'-ACAA TCC CCA CTG CGT CCC-5'
MMP-1	AF148882	5'-GGG AGA TCA TCG TGA CAA TTC TC-3'	3'-AAG TCG CTG CGG GTG CAT-5'
MMP-2	AJ010314	5'-CCC TGA GAC GGT GGA TGA TG-3'	3'-GTG ATG CCA AAA GAG CGT AGG-5'
MMP-3	U62529	5'-TGT GGA GGT GAT GCA CAA ATC-3'	3'-A AGT GAT GTA AAG GAC CGT ACG-5'
MMP-9	BM734900 (86% homology with bovine sequence)	5'-CGG CGT GCC CTT GGA-3'	3'-TCG AAT GAA GAG GGT CCT GGC-5'
MMP-13	AF034087	5'-TGA AAT CAT ACT ACT ATC CCC TTA TCC T-3'	3'-A CCA ACT GTC CGA GGC TCT-5'
NFκB	BI960845 (84% homology with mouse sequence)	5'-CAT CGA GCA GAT AGC TCA CGT T-3'	3'-A ACA GTT AGA GTG GTT GGT GGA-5'

Agg = Aggrecanase. COX = Cyclooxygenase. iNOS = Inducible nitric oxide synthase. JNK = c-Jun-N-terminal kinase. MMP = Matrix metalloproteinase. NFκB = Nuclear factor κB.