Effects of glucosamine and chondroitin sulfate on bovine cartilage explants under long-term culture conditions

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Objective—To determine effects of glucosamine (GLN) and chondroitin sulfate (CS) on expression of genes encoding putative mediators of osteoarthritis in bovine cartilage explants cultured for 2 weeks.

Sample Population—Articular cartilage explants harvested from carpal joints of 4 Holstein steers after slaughter.

Procedures—Cartilage disks were treated as follows: fetal bovine serum only (control treatment), human recombinant interleukin (IL)-1β (50 ng/mL; IL-1 treatment), GLN (5 μg/mL) with addition of CS (20 μg/mL; GLN-CS treatment), and human recombinant IL-1β (50 ng/mL) with addition of GLN and CS (IL-1–GLN-CS treatment). Media were analyzed for nitric oxide and prostaglandin E_2 (PGE_2) release. Explants were subjected to quantitative real-time PCR analysis; expressions of mRNA for inducible nitric oxide synthase, cyclooxygenase-2, microsomal prostaglandin E synthase 1, matrix metalloproteinase (MMP)-3 and -13, aggrecanase-1 and -2, tissue inhibitor of metalloproteinase (TIMP)-3, type II collagen, and aggrecan were assessed.

Results—IL-1–GLN-CS and GLN-CS treatments decreased nitrite release, compared with IL-1 treatment; IL-1–GLN-CS treatment decreased IL-1–induced PGE_2 release. Expressions of inducible nitric oxide synthase, cyclooxygenase-2, and microsomal prostaglandin E synthase 1 mRNA were abrogated by GLN-CS and IL-1–GLN-CS treatments. Interleukin-1–induced mRNA expressions of proteolytic enzymes were diminished by IL-1–GLN-CS treatment. Compared with control treatment, GLN-CS treatment decreased MMP-3 and aggrecanase-2 mRNA expression. Transcripts of TIMP-3 were increased by IL-1–GLN-CS treatment, compared with IL-1 treatment. Genes encoding type II collagen and aggrecan on day 14 were upregulated by GLN-CS and IL-1–GLN-CS treatments, compared with control treatment.

Conclusions and Clinical Relevance—Treatment with GLN and CS consistently downregulated mRNA expression for inflammatory mediators and matrix degrading enzymes while increasing TIMP-3 transcripts. (*Am J Vet Res* 2007;68:709–715)

Osteoarthritis is a chronic disorder that plagues humans and other species. Progressive and permanent articular cartilage degeneration leading to the loss of extracellular matrix components, mainly Col II and aggrecan, necessary to transmit forces placed on the joint is a hallmark of osteoarthritis. Consequences of articular cartilage destruction are pain, swelling, mild inflammation, joint stiffness, and ultimately, remodeling and destruction of affected joints. Although the exact underlying causes of osteoarthritis are unknown, several factors contribute to the development of osteoarthritis.

Catabolic mediators of osteoarthritis identified to date include matrix proteolytic enzymes such as MMPs and aggrecanases, as well as inflammatory mediators such as nitric oxide and PGE_2. An inflammatory response that is similar to that found in naturally occurring osteoarthritis is mounted by the cytokine IL-1. It is capable of inducing the production of nitric oxide, PGE_2, MMPs, and aggrecanases and, at the same time, decreasing the production of TIMPs.[1-5] The inducible enzyme responsible for nitric oxide production (ie, iNOS) and enzymes that catalyze the formation of PGE_2 (ie, COX-2 and mPGEs1) are increased in articular cartilage stimulated with IL-1.[6-8]

Conventional treatments available for osteoarthritis are basically only effective for symptomatic relief. These are primarily nonsteroidal anti-inflammatory

Abbreviations

Col II	Type II collagen
MMP	Matrix metalloproteinase
PGE_2	Prostaglandin E_2
IL	Interleukin
TIMP	Tissue inhibitor of metalloproteinase
iNOS	Inducible nitric oxide synthase
COX	Cyclooxygenase
mPGEs1	Microsomal prostaglandin E synthase 1
FBS	Fetal bovine serum
GLN-CS	Glucosamine-chondroitin sulfate

Received October 2, 2006.
Accepted January 2, 2007.
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Supported by Grayson-Jockey Club Research Foundation, Nutramax Laboratories Incorporated, and the Michigan Agricultural Experiment Station.
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drugs such as ibuprofen and naproxen, corticosteroids, and COX-2 inhibitors, which are analgesic and anti-inflammatory. They often can relieve pain and restore joint function. However, they can cause serious adverse effects.[9,10]

Because joint diseases such as osteoarthritis can be a long-term problem, nutritional supplements that are safe and effective would be ideal. The 2 most popular are glucosamine and chondroitin sulfate. These nutraceuticals are typically marketed as a combination, are reported in clinical studies on humans and other species to be efficacious in alleviating signs of joint pain, and are suggested to be chondroprotective.[11-14] In a large clinical trial[15] on humans, the mixture of glucosamine and chondroitin sulfate was deemed effective in reducing pain scores in a subgroup of patients with moderate to severe knee joint pain. They also provided a synergistic effect in reducing cartilage lesions in vivo, were complementary and additive in repressing catabolic mediators, and were more effective together in suppressing gene expression of putative osteoarthritis mediators in vitro.[16-20]

Limitations pertaining to experimental designs and the unresolved mechanism of actions of the nutraceuticals have contributed to limited acceptance of glucosamine and chondroitin sulfate as a viable treatment for osteoarthritis. Few in vitro mechanistic studies have used the combination and concentrations of nutraceuticals that are attainable in vivo. Even fewer have assessed their effects over longer-term culture conditions. In the present study, we expand on previous studies[18,19] by performing a 2-week culture of cartilage explants supplemented with the nutraceutical combination at concentrations that approximate those measured in previous pharmacokinetic studies.[21-25] The effect of glucosamine (5 µg/mL) and chondroitin sulfate (20 µg/mL) in combination, at concentrations attainable in vivo, on gene expression of IL-1–stimulated bovine articular cartilage explants, and on the release of nitric oxide and PGE_2 in the media were assessed. Genes of interest included were those of iNOS, COX-2, mPGEs1, MMP-3, MMP-13, aggrecanase-1, aggrecanase-2, TIMP-3, Col II, and aggrecan.

Materials and Methods

Explant cultures—Articular cartilage was isolated from left and right carpal joints of Holstein steers (n = 4; 18 to 24 months old) obtained from a local abattoir within 3 hours of slaughter. Cartilage disks (6 mm in diameter) were biopsied from the articular surface. They did not include the calcified layer of the tissue or cartilage with gross characteristics of osteoarthritis. Two explant disks (approx 60 mg of total wet weight) were selected at random and cultured in each well of a 24-well culture plate[a] that contained a 1:1 combination of Dulbecco modified Eagle medium–Hams F-12 nutrient mixture,[b] as previously described.[26] The medium was supplemented with amino acids, ascorbic acid (50 µg/mL), and penicillin-streptomycin[b] (100 U/mL).[26] Cartilage explants were maintained in a humidified incubator at 37°C with 7% CO_2.

Explants were maintained in media without serum for 24 hours before the addition of treatments. Media in the wells were exchanged every other day. After equilibration, all treatments included 10% FBS.[b] Four treatments per experiment were studied as follows: FBS only (control treatment); human recombinant IL-1β[c] (50 ng/mL; IL-1 treatment); glucosamine (5 µg/mL) with the addition of chondroitin sulfate (20 µg/mL; GLN-CS treatment); and human recombinant IL-1β (50 ng/mL) with the addition of glucosamine and chondroitin sulfate (IL-1–GLN-CS treatment). For treatments that included human recombinant IL-1β, it was added at a concentration of 50 ng/mL on days 2 and 10 to induce cartilage catabolism. To examine the effects of glucosamine and chondroitin sulfate, glucosamine hydrochloride[d] and low molecular weight chondroitin sulfate[e] were added to the wells at the same time as FBS. Concentrations of glucosamine and chondroitin sulfate chosen were 5 and 20 µg/mL, respectively, similar to previous experiments.[18,19] Each treatment consisted of 24 wells (48 disks) containing cartilage specimens from 1 steer for an experiment. Media were collected and replaced every other day starting from day 0. Technical replicates for media for each treatment were 24 wells prior to day 6. After day 6, the technical replicates were 12 wells. Cartilage explants for each treatment were collected on day 6 (first 12 wells, 24 disks) and day 14 (last 12 wells, 24 disks) after stimulation, frozen in liquid nitrogen, and stored at −80°C until RNA isolation. The experiment was repeated 4 times, each time with cartilage specimens from 1 steer.

Nitric oxide assay—Nitrite was measured in conditioned media by use of the Griess reagent and sodium nitrite as the standard.[27] Briefly, 150 µL of medium was incubated with 150 µL of 1.0% sulfanilamide, 0.1% N-1-naphthylethylenediamine hydrochloride, and 25% phosphoric acid at room temperature (approx 21°C) for 5 minutes. Because of some precipitation of reagents with chondroitin sulfate, 96-well plates were centrifuged at 1,000 × *g* for 3 minutes at 4°C. The remaining supernatant was transferred to a new plate. Absorbance was measured at a wavelength of 540 nm by use of a spectrophotometric plate reader.[f]

PGE_2 assay—The release of PGE_2 into day-4 and day-12 conditioned media was quantified in units of picograms per milliliters by use of a commercially available competitive ELISA kit according to manufacturer's instructions.[c] Conditioned media samples were stabilized with indomethacin (10 µg/mL) and stored at −20°C until analysis.

Total RNA isolation—Total RNA was extracted from cartilage explants following a modified protocol.[28] Briefly, cartilage was homogenized in Trizol reagent,[g] and chloroform was added to extract total RNA followed by vigorous agitation and a 2-minute incubation. The aqueous phase containing RNA was collected after centrifugation (4°C, 12,000 × *g*, 15 minutes); the RNA was then precipitated with an equal volume of 70% ethanol. Total RNA was then purified further with mini columns[h] and quantified by UV spectrophotometry.[i] To validate spectrophotometric determination and RNA integrity, RNA from chondrocytes was resolved on 1.2% agarose gel.

cDNA synthesis—For each sample, 2 µg of total RNA was treated with DNase I[j] to degrade contaminating

single-and double-stranded DNA. Treated RNA was converted to single-stranded cDNA by use of a reverse transcriptase[k] as recommended by the manufacturer. Single stranded cDNA was quantified by UV spectrophotometry[i] and diluted with nuclease-free water to 10 ng/μL.

Quantitative real-time PCR assay—Glyceraldehyde phosphate dehydrogenase was validated as an appropriate housekeeping gene. Primers for glyceraldehyde phosphate dehydrogenase and target genes were designed by use of a software program[l] (**Appendix**). These genes were chosen on the basis of findings in other studies[18,19] that have determined induction with IL-1. Nucleotide sequences used for primer design were obtained from public databases.[m] Optimal concentrations of each set of primers were determined with a primer matrix (lowest SD with no change in cycle to threshold). Quantitative real-time PCR assay was performed with 50 ng of cDNA templates in 96-well plates by use of a sequence detection system[n] as previously described.[18,19] The control treatment was used as a calibrator (ie, the fold change for control is 1.0). Replicated data were normalized with glyceraldehyde phosphate dehydrogenase, and the fold change in gene expression compared with the control treatment was calculated by use of the $2^{-\Delta\Delta C_T}$ method.[29]

Statistical analysis—Data for nitric oxide (expressed as cumulative values) and PGE_2 (expressed as logarithmic values) release into conditioned media were analyzed by use of a linear mixed-effects model, including the fixed effect of treatment and the random effect of steer. Treatment effects were compared within each time point by use of the multiple-comparisons approach of Tukey. Computations were performed by use of a software program.[30] Relative gene expression data were obtained with quantitative real-time PCR assay and analyzed by use of a software program[30] and the nonparametric ANOVA approach of Friedman. The *P* values of the gene-specific analyses were corrected for a false discovery rate of 5% as discussed by Benjamini and Hochberg.[31] Values of $P < 0.05$ were considered significant.

Results

Effect of glucosamine and chondroitin sulfate on nitric oxide—Treatment with IL-1–GLN-CS suppressed cumulative nitrite release, compared with IL-1 treatment, by 66%, 52%, 50%, 51%, 51%, 51%, and 50% on days 2, 4, 6, 8, 10, 12, and 14 of culture, respectively (**Figure 1**). In addition, GLN-CS treatment significantly decreased nitrite release, compared with control treatment, from days 8 to 14.

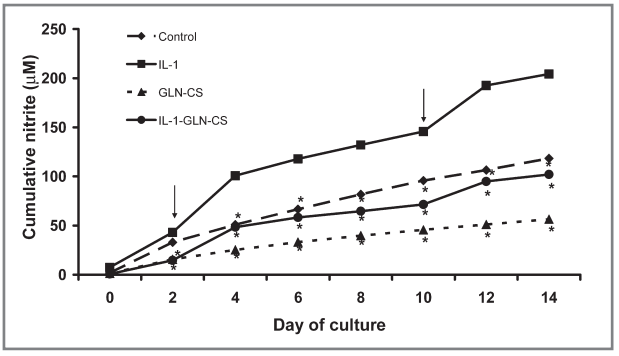

Figure 1—Mean cumulative nitrite release into conditioned media as measured every other day up to day 14 of culture. Notice the addition of IL-1 (arrows) to treatments containing the cytokine. Control = Treatment with FBS only. IL-1 = Treatment with human recombinant IL-1β (50 ng/mL). GLN-CS = Treatment with glucosamine (5 μg/mL) with the addition of chondroitin sulfate (20 μg/mL). IL-1–GLN-CS = Treatment with human recombinant IL-1β (50 ng/mL) with the addition of glucosamine (5 μg/mL) and chondroitin sulfate (20 μg/mL). *Value differs significantly ($P <$ 0.05)from value for IL-1 treatment.

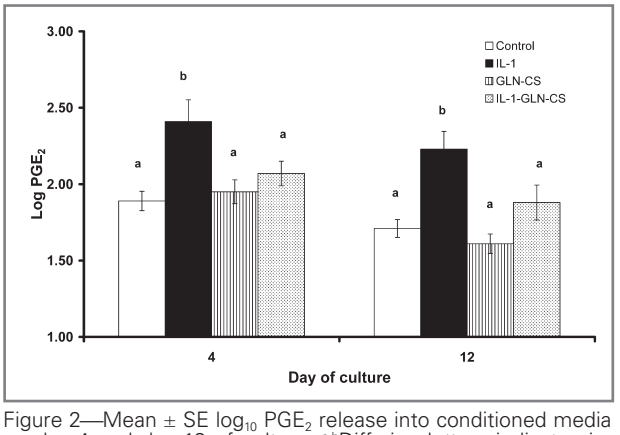

Figure 2—Mean ± SE $\log_{10}$ PGE_2 release into conditioned media on day 4 and day 12 of culture. [a,b]Differing letters indicate significant ($P < 0.05$) difference within day among treatments. *See* Figure 1 for remainder of key.

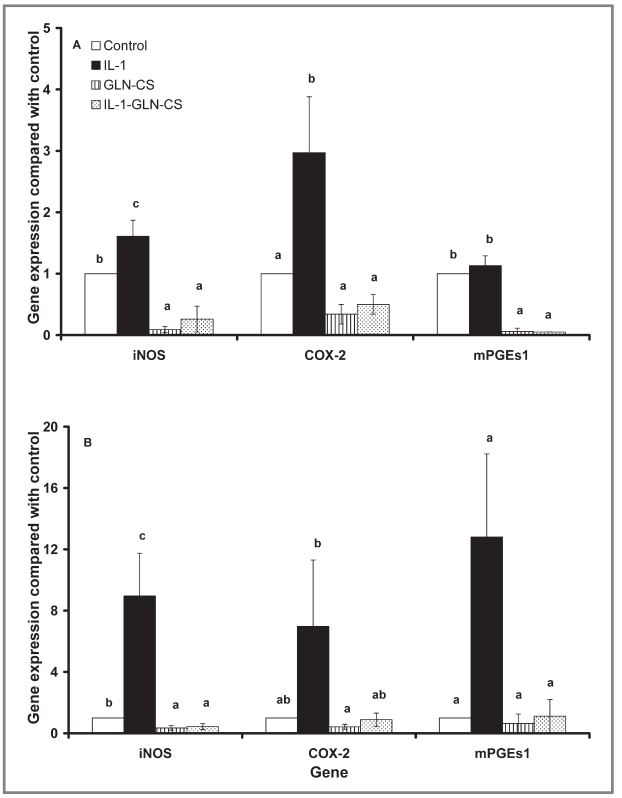

Figure 3—Mean ± SE expression of genes encoding inflammatory mediators, compared with control treatment, for cartilage explants stimulated for 6 days (A) and 14 days (B). All treatments included 10% FBS. [a-c]Differing letters indicate significant ($P < 0.05$) differences among treatments for expression of a gene. *See* Figure 1 for remainder of key.

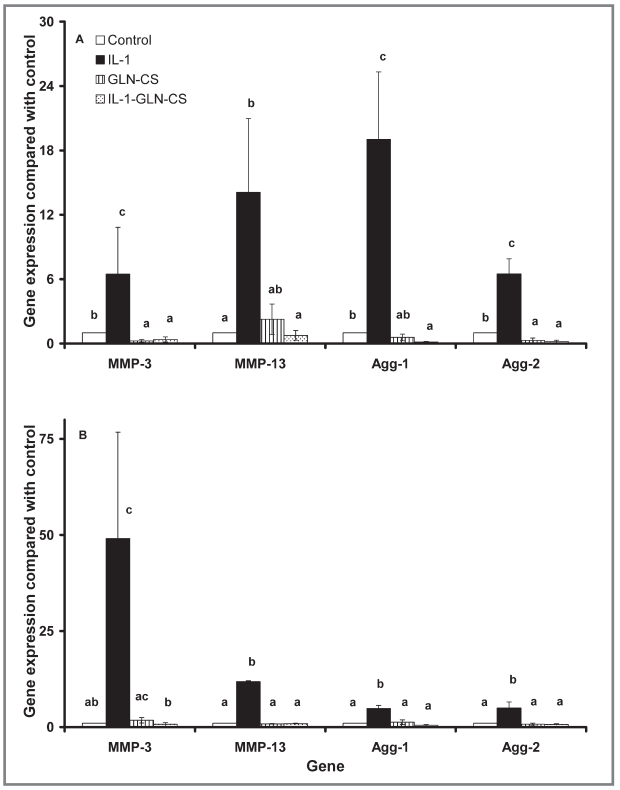

Figure 4—Mean ± SE expression of genes encoding proteolytic enzymes, compared with control treatment, for cartilage explants stimulated for 6 days (A) and 14 days (B). All treatments included 10% FBS. Agg = Aggrecanase. *See* Figures 1 and 3 for remainder of key.

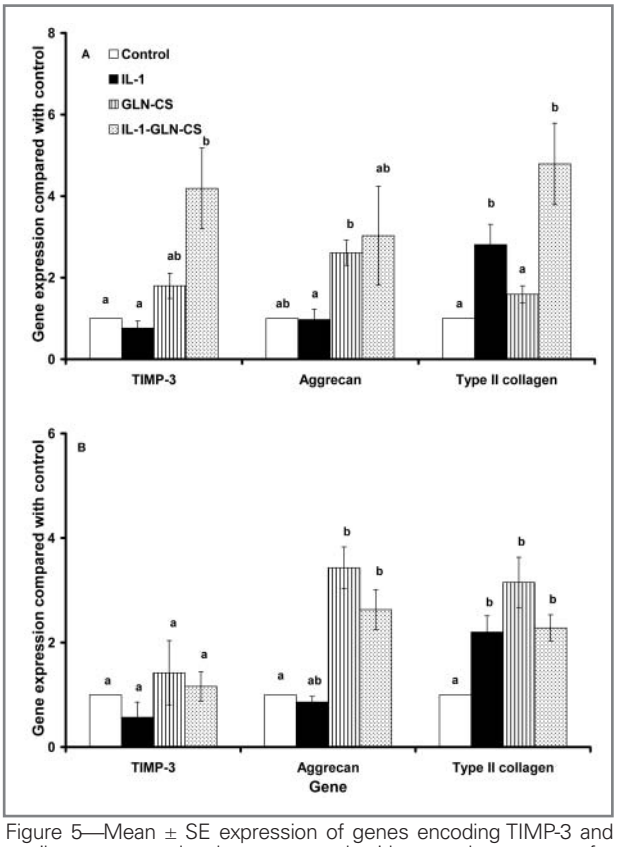

Figure 5—Mean ± SE expression of genes encoding TIMP-3 and cartilage macromolecules, compared with control treatment, for cartilage explants stimulated for 6 days (A) and 14 days (B). All treatments included 10% FBS. *See* Figures 1 and 3 for remainder of key.

Effect of glucosamine and chondroitin sulfate on PGE_2—The GLN-CS treatment had no effect on PGE_2 release (**Figure 2**), compared with control treatment, on days 4 and 12 of culture. Treatment with IL-1–GLN-CS repressed IL-1–induced PGE_2 release by 70%, similar to control treatment concentrations, on day 4 of culture; reductions in IL-1–induced PGE_2 release by IL-1–GLN-CS treatment were also observed on day 12, but this finding was not significant.

Effect of glucosamine and chondroitin sulfate on gene expression of inflammatory mediators—The IL-1 treatment significantly increased expression of iNOS and COX-2 mRNA by approximately 1.6- and 3-fold, respectively, compared with control treatment, on day 6 of culture (**Figure 3**). These increases in iNOS and COX-2 mRNA expression were significantly abrogated by GLN-CS and IL-1–GLN-CS treatments. The GLN-CS and IL-1–GLN-CS treatments downregulated the expression of mPGEs1 mRNA on day 6. As for the last day of culture, only iNOS mRNA expression was significantly decreased by GLN-CS and IL-1–GLN-CS treatments, likely as a result of the high variation in gene expression by IL-1 treatment.

Effect of glucosamine and chondroitin sulfate on gene expression of proteolytic enzymes—The IL-1–induced mRNA expression of all proteolytic enzymes assessed was diminished by IL-1–GLN-CS treatment on day 6 (**Figure 4**). Repression of these genes by IL-1–GLN-CS treatment was also observed on day 14 of culture. Compared with control treatment, GLN-CS treatment decreased MMP-3 and aggrecanase-2 mRNA expression on day 6.

Effect of glucosamine and chondroitin sulfate on gene expression of TIMP-3 and cartilage macromolecules—The abundance of TIMP-3 transcript was significantly increased by IL-1–GLN-CS treatment, compared with IL-1 treatment, on day 6 by about 300% (**Figure 5**). The abundance of genes encoding Col II and aggrecan on day 14 were upregulated by GLN-CS and IL-1–GLN-CS treatments, compared with the control treatment.

Discussion

Results of our study supplement and support previous results obtained in short-term studies.[18,19,32] Treatment with glucosamine and chondroitin sulfate kept nitric oxide and PGE_2 synthesis in IL-1–stimulated explants comparable to that of control explants, likely as a result of decreased iNOS, COX-2, and mPGEs1 mRNA expression. Our findings agree with other published results.[32,33] A chronic overproduction of nitric oxide and PGE_2 may facilitate cartilage degeneration in joint diseases.[34] Thus, mitigating their production may help reduce their negative impact in the joint. As an example, glucosamine and chondroitin sulfate may prevent or reverse the negative effects of nitric oxide and PGE_2 on proteoglycan synthesis.[35]

Results of the study reported here regarding metalloproteinases and cartilage macromolecules parallel results of our short-term study,[18] in which glucosamine and chondroitin sulfate prevented the IL-1–induced increased expression of certain metalloproteinases genes. As in our short-term study,[18] no significant effect was seen on the gene expression of aggrecan and Col II by glucosamine and chondroitin sulfate in IL-1 treated explants. Expression of TIMP-3 mRNA was increased by glucosamine and chondroitin sulfate previously.[32] In the prevention of cartilage degeneration, TIMP-3 may be important as an inhibitor of aggrecanase activity.[36] In our study at both time points, IL-1 stimulated the synthesis of Col II mRNA expression, compared with control explants. This was somewhat surprising. However, a possible explanation is that the intermittent treatment with IL-1 initiated matrix damage that led to the release of growth factors sequestered in the matrix. The cartilage was collected 48 hours after the removal of IL-1, which may have provided enough time for growth factors to initiate a response. A similar type of effect has been observed with fibronectin fragments as the arthritogenic stimulus.[37]

A somewhat unique feature to our study is the treatment with glucosamine and chondroitin sulfate without the addition of IL-1. Thus, other than the transfer of cartilage from an in vivo to in vitro setting, no additional stressors were placed on the cartilage disks, although the addition of FBS may induce a slight inflammatory response. Compared with the control treatment, treatment with glucosamine and chondroitin sulfate alone decreased iNOS, mPGEs1, MMP-3, and aggrecanase-2 mRNA expression on day 6, as well as iNOS on day 14. The decreased accumulation of nitrite in the media correlates well with mRNA results for iNOS. In addition, on day 14, Col II and aggrecan gene expressions were increased, compared with control treatment. In a short-term study,[32] TIMP-3 protein synthesis was increased by treatment with glucosamine and chondroitin sulfate alone, compared with control treatment. Although the importance of this is unclear, it does suggest that glucosamine and chondroitin sulfate can have a beneficial effect on normal cartilage. Glucosamine and chondroitin sulfate may be more than biological stress modifiers.[38] Others have found that glucosamine had a more beneficial effect on normal chondrocytes than osteoarthritic chondrocytes.[39]

Few research studies have addressed the issue of whether glucosamine and chondroitin sulfate have a beneficial effect in apparently healthy animals. In 2 studies,[40,41] glucosamine did not have an effect on systemic bone or cartilage metabolism in young horses in training.[40,41] In rabbits with ligament transection, glucosamine and chondroitin sulfate at least partially protects normal cartilage from degenerating.[16] On the basis of the results of that study,[16] glucosamine and chondroitin sulfate may protect apparently healthy cartilage that is abnormally stressed. Anecdotally, many humans and other species are receiving glucosamine and chondroitin sulfate prophylactically, especially those physically active such as human runners and performance horses. Although our results provide some support for this, the prophylactic use of glucosamine and chondroitin sulfate has not been rigorously examined.

In summary, results of our study indicated that glucosamine and chondroitin sulfate in a 2-week culture can inhibit mRNA expression of several catabolic molecules in IL-1–stimulated cartilage while potentially increasing mRNA expression of some anabolic molecules. Results of another study on long-term culture, but with a different model, also suggest that glucosamine and chondroitin sulfate are beneficial.[35] As the amount of in vitro and in vivo research surrounding glucosamine and chondroitin sulfate increases, the rationale for its use may be solidified. Given the adverse impact that arthritis has in society with few other viable treatment options, the use of glucosamine and chondroitin sulfate may provide an alternative treatment with minimal risk.[42]

a. 24-well Falcon culture plate, Fisher Scientific, Pittsburgh, Pa.
b. Gibco, Grand Island, NY.
c. R & D Systems, Minneapolis, Minn.
d. FCHG49, Nutramax Laboratories, Edgewood, Md.
e. TRH122, Nutramax Laboratories, Edgewood, Md.
f. SpectraMax 300 plate reader, Molecular Devices, Sunnyvale, Calif.
g. Trizol reagent, Invitrogen, Carlsbad, Calif.
h. RNeasy, Qiagen, Valencia, Calif.
i. Beckman Coulter, Fullerton, Calif.
j. DNase I, Invitrogen, Carlsbad, Calif.
k. Superscript II reverse transcriptase, Invitrogen, Carlsbad, Calif.
l. Primer Express, version 2.0, Perkin-Elmer Applied Biosystems, Foster City, Calif.
m. Genbank Database, National Centre Biotechnology Information, National Institutes of Health, Bethesda, Md.
n. ABI PRISM 7000 sequence detection system, Perkin-Elmer Applied Biosystems, Foster City, Calif.

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Appendix appears on next page

Appendix

Forward and reverse primer sequences of genes of interest used for quantitative real-time PCR assay.

Gene	Genbank accession No.	Forward primer	Reverse primer
iNOS	AF333248	CCCGCATGCAACTCCAA	TCGTAAGTCATGAACTGCCACTTC
COX-2	AF004944	GCACAAATCTGATGTTTGCATTC	GGTCCTCGTTCAAAATCTGTCTTG
mPGEs1	NM_174443	GTACGTGGTGGCCGTCATC	GGGTTGGCAAAAGCCTTCTT
MMP-3	AF135232	TACGGGTCTCCCCCAGTTTC	TCGGGAGGCACAGATTCC
MMP-13	NM_174389	GCAGAGAGCTACCTGAAATCATACTACT	AATCACAGAGCTTGCTGCAGTTT
Agg-1	AF516915	CTGGGCCATGTCTTCAGCAT	GGCGGGAGGTGCTCTCA
Agg-2	AF192771	TTTCGGCTCCACGGAAGA	GGGTTTGGATGCGTCAATG
TIMP-3	NM_174473	CGCGTTCTGCAACTCAGACA	CCCCTCCTTCAGCAGTTTCTT
Col II	X02420	GCATTGCCTACCTGGACGAA	CGTTGGAGCCCTGGATGA
Agg	U76615	TTCACCTGTAAAAAGGGCACAGT	CAGGGCATTGATCTCGTATCG
GAPDH	AB098979	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG

Agg = Aggrecanase. GAPDH = Glyceraldehyde phosphate dehydrogenase.