Effect of glucosamine and chondroitin sulfate on regulation of gene expression of proteolytic enzymes and their inhibitors in interleukin-1–challenged bovine articular cartilage explants

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Objective—To determine the effects of glucosamine (GLN) and chondroitin sulfate (CS), at concentrations attainable in vivo, on expression of genes encoding proteolytic enzymes, enzyme inhibitors, and macromolecules of articular cartilage in interleukin-1(IL-1)–challenged bovine cartilage explants.

Sample Population—Articular cartilage explants harvested from 9 steers.

Procedures—Cartilage explants were exposed to media containing 10% fetal bovine serum (FBS) only, IL-1 (50 ng/mL), IL-1 with GLN (5 µg/mL), IL-1 with CS (20 µg/mL), or IL-1 with GLN and CS for 24 and 48 hours. Cartilage was frozen, and RNA was extracted. Gene expression of matrix metalloproteinases (MMPs)-2, -3, -9, -13, and -14; aggrecanases (Aggs)-1 and -2; tissue inhibitors of metalloproteinases (TIMPs)-1, -2, and -3; and type II collagen and aggrecan were assessed with quantitative real-time polymerase chain reaction.

Results—Upregulated MMP-3, MMP-13, and Agg-1 transcripts at 24 hours were repressed by the GLN and CS combination by at least approximately 6-fold. Glucosamine was effective in suppressing IL-1–induced mRNA expression of MMP-13, Agg-1, and Agg-2, whereas CS was effective in decreasing IL-1–induced MMP-13 transcript at 24 hours. At 48 hours, GLN and CS added separately and in combination significantly abrogated Agg-1 and Agg-2 gene induction. The combination also decreased IL-1–stimulated MMP-13 transcript.

Conclusions and Clinical Relevance—GLN and CS, at concentrations that are within the range measured in synovial fluid and blood after oral administration, may regulate expression of matrix degrading enzymes and their inhibitors at the transcriptional level, providing a plausible mechanism for their purported chondroprotective properties. (*Am J Vet Res* 2005;66:1870–1876)

Progressive and permanent articular cartilage degeneration is the hallmark characteristic of **osteoarthritis (OA)**. Biological and mechanical factors uncouple the normal balance between articular cartilage degradation and synthesis. Cartilage destruction primarily results from an imbalance between synthesis and degradation of the extracellular matrix, particularly aggrecan and **type II collagen (Col II)**. The expression and activity of proteolytic enzymes, such as **matrix metalloproteinases (MMPs)** and **aggrecanases (Aggs)**, exceed that of endogenous inhibitors like **tissue inhibitors of metalloproteinases (TIMPs)**.[1] Excess production of matrix-degrading enzymes is in large part induced by the release of **interleukin-1 (IL-1)**.[2]

Interleukin-1 is widely accepted as one of the proinflammatory cytokines that plays a critical role in OA pathogenesis.[3] It upregulates proteolytic enzymes and retards anabolic activities of the chondrocyte, leading to declines in synthesis of collagen and proteoglycan.[4,5] Degradation of the extracellular matrix in vivo and in vitro with IL-1 stimulation is reported in the human, bovine, porcine, equine, and lapine species.[6]

The nutraceuticals, **glucosamine (GLN)** and **chondroitin sulfate (CS)**, have been used over the past 30 years for treatment of OA and have reduced articular cartilage degeneration in animal and human clinical trials. In humans, both compounds have demonstrated therapeutic effects on symptoms of OA by alleviating pain and improving mobility.[7-9] In animals, the combination reduces lameness in dogs and horses and is effective in managing pain in cats.[10-12] Despite several studies with positive results, use of these nutraceuticals is still not widely accepted as a result of the small sample sizes and short-term nature of the trials.

Many of the commercially available nutraceutical products contain GLN and CS. Glucosamine and CS were synergistic in protecting articular damage in vivo,[13] and findings in another study[14] suggested they were complementary. Thus, the chondroprotective properties of each molecule may increase when taken together, and combining them for explant studies is important. Most in vitro studies have used concentrations of the nutraceuticals that exceed those generally found in synovial fluid and blood.[15] The concentrations of GLN in synovial fluid and blood after oral and IV administration range from 0.05 to 20 µg/mL.[15-19] Depending on the route of administration, species, and source and molecular weight of CS, the concentration of CS in serum ranges from 5 to 200 µg/mL.[18-20] Thus, in the present study, we determined the effect of GLN (5 µg/mL) and CS (20 µg/mL) individually and in combination, at concentrations attainable in vivo, on gene expression of IL-1–stimulated bovine articular cartilage explants. The genes of interest included were MMP-2, MMP-3, MMP-9, MMP-13, MMP-14, Agg-1, Agg-2, TIMP-1, TIMP-2, TIMP-3, Col II, and aggrecan.

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Materials and Methods

Explant cultures—Articular cartilage was isolated from the carpal joints of 3 Holstein steers (age, 18 to 24 months old) obtained from a local abattoir within 3 hours of slaughter. Cartilage disks (diameter, 6 mm) were biopsied from the articular surface and pooled into 1 Petri dish. They did not include the calcified layer of the tissue and cartilage with gross characteristics of OA. Two explant disks (approx 60 mg in 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.[21] Media were supplemented with amino acids, ascorbic acid (50 µg/mL), and penicillin-streptomycin[b] (100 U/mL).[21] Cartilage explants were maintained in a humidified incubator at 37°C with 7% CO_2.

Explants were maintained in media without serum for 48 hours before the addition of treatments. Media in the wells were exchanged daily. After equilibration, all treatments received 10% **fetal bovine serum (FBS)**.[b] **Human recombinant IL-1 beta (rhIL-1β)**[c] was added at a concentration of 50 ng/mL to induce cartilage catabolism. To examine the effects of GLN and CS, GLN hydrochloride[d] and low–molecular-weight CS[e] were added to the wells at the same time as FBS and rhIL-1β. The concentrations of GLN and CS chosen were 5 and 20 µg/mL, respectively. These concentrations were well within the range of concentrations attainable in the blood after oral and IV administration (1 to 20 µg/mL for GLN and 5 to 200 µg/mL for CS).[15-20] There were 5 treatments/experiment as follows: an FBS control (control treatment), rhIL-1β (50 ng/mL; IL-1 treatment), rhIL-1β (50 ng/mL) with the addition of GLN (5 µg/mL; IL-1 + GLN treatment), rhIL-1β (50 ng/mL) with the addition of CS (20 µg/mL; IL-1 + CS treatment), and rhIL-1β (50 ng/mL) with the addition of GLN and CS (IL-1 + GLN + CS treatment; **Appendix 1**). Each treatment consisted of 12 wells (24 disks) containing pooled samples from 3 steers for an experiment. Cartilage explants were collected 24 and 48 hours after stimulation, frozen in liquid nitrogen, and stored at –80°C until RNA isolation. An experiment was repeated a total of 3 times for each time point, each time with pooled cartilage samples from 3 steers.

Total RNA isolation—Total RNA was extracted from cartilage explants following a modified protocol.[22] Briefly, cartilage was homogenized in Trizol reagent,[f] 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), and RNA was precipitated with an equal volume of 70% ethanol. Total RNA was then purified further with minicolumns[g] and quantified by UV spectrophotometry.[h] Total chondrocyte RNA was resolved on 1.2% agarose gel to validate spectrophotometric determination and RNA integrity.

cDNA synthesis—For each sample, 2 µg of total RNA was treated with DNAse I[i] to degrade contaminating single- and double-stranded DNA. Treated RNA was converted to single-stranded cDNA by use of a reverse transcriptase,[j] as recommended by the manufacturer. Single-stranded cDNA was quantified by UV spectrophotometry[h] and diluted with RNAse-free water to 50 ng/µL.

Quantitative real-time polymerase chain reaction—Primers for **glyceraldehyde phosphate dehydrogenase (GAPDH)**, which is used as a housekeeping gene, and other molecules were designed by use of a software program (**Appendix 2**).[k] Nucleotide sequences used for primer design were obtained from public databases (Genbank[l]). Optimal concentrations of each set of primers were determined with a primer matrix (ie, lowest SD with no change in **cycle to threshold [C_T]**). **Quantitative real-time polymerase chain reaction (q-RT-PCR)** was performed with 50-ng cDNA templates in 96-well plates by use of a detection system.[m] The cDNA templates were combined with optimal concentrations of primers and PCR dye mix[n] 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 and 10 minutes at 95°C, followed by 40 cycles of extension at 95°C for 15 seconds and 1 minute at 60°C; data were collected during the last 30 seconds. Threshold lines were adjusted to intersect amplification lines in the linear portion of the amplification curves. The software automatically recorded the C_T. The analysis of each sample was performed in duplicate, and an SD of ≤ 0.5 between replicates was set as a criterion for inclusion of data. The GAPDH gene was used as an endogenous

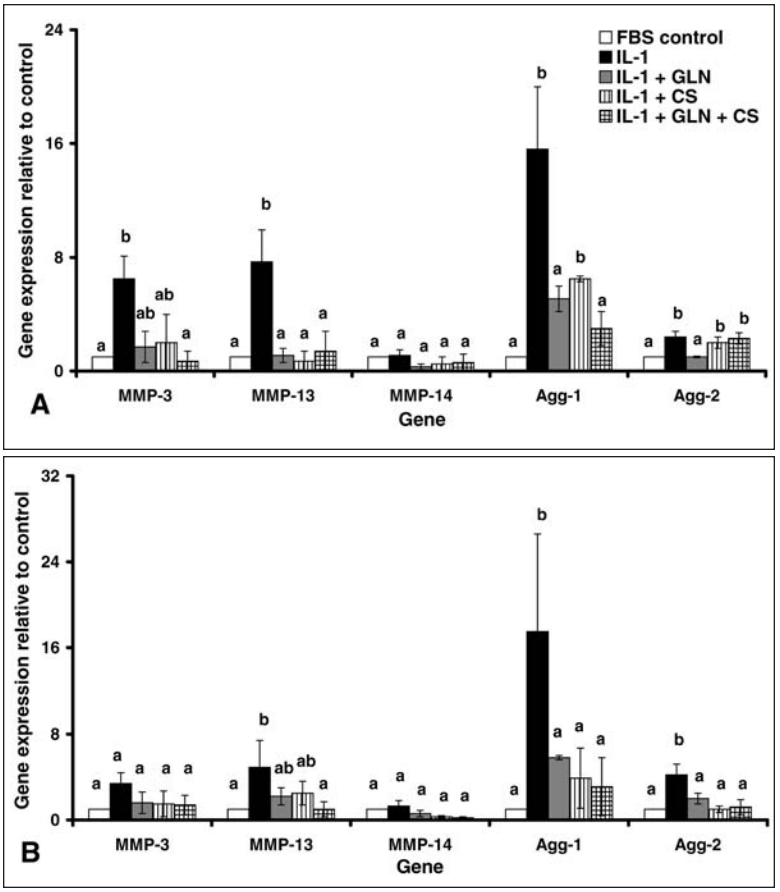

Figure 1—Mean ± SE expression of genes encoding proteolytic enzymes relative to control for cartilage explants stimulated for 24 hours (A) and 48 hours (B). FBS control = Fetal bovine serum control treatment. IL-1 (interleukin-1) = Human recombinant IL-1 beta (rhIL-1β; 50 ng/mL). IL-1 + GLN (glucosamine) = rhIL-1β (50 ng/mL) and GLN (5 µg/mL). IL-1 + CS (chondroitin sulfate) = rhIL-1β (50 ng/mL) and CS (20 µg/mL). IL-1 + GLN + CS = rhIL-1β (50 ng/mL) and GLN and CS. MMP = Matrix metalloproteinase. Agg = Aggrecanase. [a,b]Different letters for a gene indicate significant ($P < 0.05$) differences between treatments.

control and run together with the samples for each amplification reaction to allow for normalization of different samples for RNA loading, cDNA synthesis, and amplification efficiencies and for comparison of samples run at different times. The FBS control treatment was used as a calibrator (ie, the fold change for control is 1.0). Replicated data were normalized with GAPDH, and the fold change in gene expression relative to FBS control was calculated by use of the $2^{(-\Delta\Delta CT)}$ method.[23]

Statistical analyses—Relative gene expression data were obtained with the q-RT-PCR assay and analyzed by use of a software program[o] and the nonparametric ANOVA approach of Friedman. The *P* values of gene-specific analyses were corrected for a false discovery rate of 5% as reported by Benjamini and Hochberg.[24] Values of $P < 0.05$ were considered significant.

Results

Effect of GLN and CS on gene expression of proteolytic enzymes—Explants cultured with rhIL-1β for 24 hours resulted in significant upregulation of MMP-3, MMP-13, Agg-1, and Agg-2 relative to control (**Figure 1**). Glucosamine repressed mRNA expression of MMP-13, Agg-1, and Agg-2 at 24 hours after induction. Chondroitin sulfate suppressed a 7.7 times higher resting-level induction of MMP-13 by IL-1 to control concentrations. The GLN and CS combination was effective in downregulating IL-1–induced gene expression of MMP-3, MMP-13, and Agg-1. There were no treatment effects for MMP-2, MMP-9 (data not shown), and MMP-14 at 24 and 48 hours after culture.

The induction of MMP-13, Agg-1, and Agg-2 mRNA expression by IL-1 at 24 hours was sustained for up to 48 hours after stimulation (Figure 1). Glucosamine and CS added alone or in combination for 48 hours significantly reduced IL-1–stimulated Agg-1 and Agg-2 mRNA transcripts. At 48 hours, the GLN and CS combination decreased IL-1–induced MMP-13 from 4.9- to 1.0-fold. No treatment effects were found for MMP-3 at the 48-hour time point.

Effect of GLN and CS on gene expression of inhibitors of proteolytic enzymes—At 24 hours after stimulation, the GLN and CS combination resulted in upregulation of TIMP-3 transcript relative to CS, but this finding was not significant (**Figure 2**). Treatment effect was not found for TIMP-1 and TIMP-2 (data not shown) at the 24- and 48-hour time points and for TIMP-3 at 48 hours.

Effect of GLN and CS on gene expression of cartilage macromolecules—No treatment effect was found for expression of genes encoding both Col II and aggrecan at all time points.

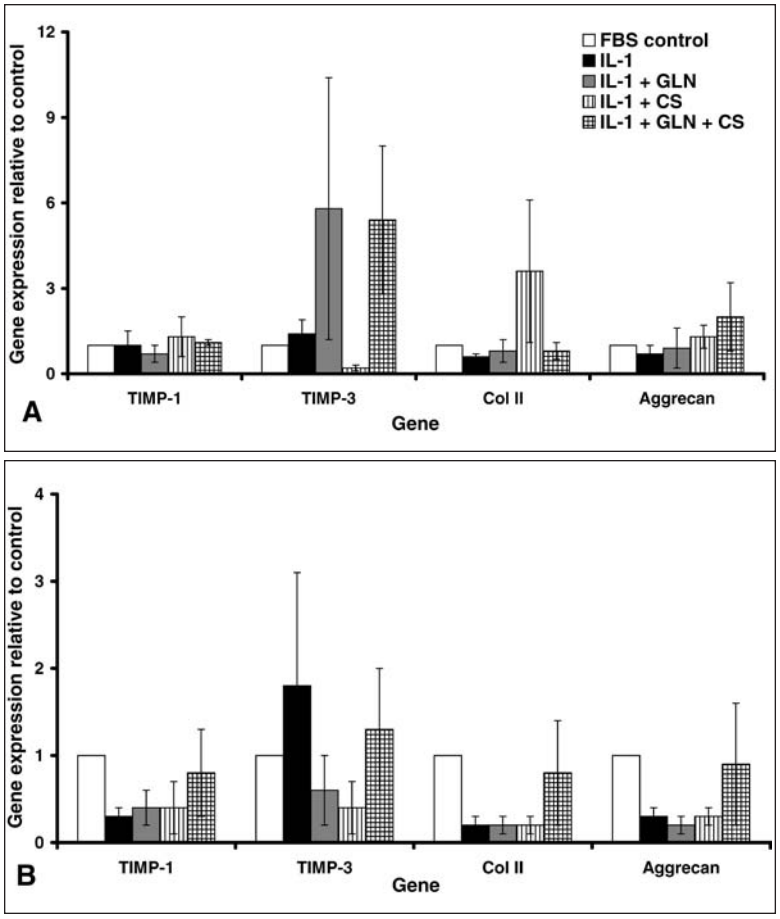

Figure 2—Mean ± SE expression of genes encoding inhibitors of proteolytic enzymes and cartilage macromolecules relative to control for cartilage explants stimulated for 24 (A) and 48 hours (B). TIMP = Tissue inhibitor of metalloproteinase. Col II = Type II collagen. *See* Figure 1 for remainder of key.

Discussion

Glucosamine and CS have been used for over 3 decades to treat OA. Results of clinical trials indicate that GLN and CS used individually or in combination are effective in improving the symptoms of knee and hip OA.[7-9,25,26] Their benefits also extend to animals with clinical signs of OA, which is a common and natural disease occurrence in dogs, horses, and cats.[10-12] Although GLN and CS have been reported to be beneficial, their exact mechanism of action remains to be resolved. Previous experiments have provided some clues about the chondroprotective properties of the nutraceuticals. However, one major limitation to these studies is that concentrations of the nutraceuticals used have been quite high, ranging from 0.1 to 10 mg/mL.[14,21,27-36] These concentrations are most probably not attainable in vivo in the blood or synovial fluid after administration of the nutraceuticals via the oral or IV routes. This is one reason given by rheumatologists for not advocating the use of these nutraceuticals.[37] The current study represents an improved approach to study the effects of GLN and CS in that the nutraceuticals were used at concentrations that have been reported in blood after oral and IV administration. Now that we have determined the beneficial effects at these concentrations, further experiments will be performed to look at a range of concentrations within the reported values. We also studied the effect of GLN and CS as a com-

bination because they are increasingly marketed as one entity and the combination has a putative synergistic effect in decreasing the severity of cartilage lesions.[13]

The predominate groups of enzymes responsible for cartilage breakdown are MMPs and Aggs. The MMPs are a group of zinc-containing proteinases that play a central role in the degeneration of the extracellular matrix. During matrix breakdown, the normal balance between MMPs and TIMPs is disrupted towards an increase in activity of MMPs and a decrease in TIMPs.[1] Chondrocytes and synoviocytes in OA specimens produce MMPs, particularly MMP-3 and MMP-13.[38] Cytokines stimulate the expression of MMP-3 and MMP-13[39-42] in agreement with the findings in our study. Matrix metalloproteinase-3 has a broad range of substrate specificity and is deemed as one of the major enzymes responsible for matrix proteolysis.[43] Matrix metalloproteinase-13 plays a critical role in OA by breaking down Col II preferentially.[44] Matrix metalloproteinase-14 belongs to the membrane-type MMPs. It hydrolyzes collagens and is capable of activating latent MMP-2.[43] Thus, suppressing the expression of these enzymes will likely be beneficial to OA patients.

The ability of GLN and CS to protect cartilage from being degraded pathologically[13] may be explained, at least in part, by the regulation of MMP synthesis. Specifically, the GLN and CS combination inhibited the cytokine-induced expression of a number of the MMPs examined. Glucosamine and CS in combination exerted a transient influence on the MMP-3 transcript in that a significant reduction in cytokine-induced expression was observed at 24 hours but not at 48 hours. Previous reports[27,29,31,34] have demonstrated suppression of MMP-3 gene expression and enzyme activity by GLN. However, the concentration of GLN used was at least 100 µg/mL, far exceeding the concentration used in our study. One study[45] that used GLN sulfate at low concentrations (approx 200 ng/mL) on human OA chondrocytes did show a decline in MMP-3 activity and production but not on mRNA expression. We observed that GLN and CS used alone were effective in downregulating MMP-13 for at least 24 hours and that the repression was prolonged by the combination. These findings supplement previous studies that reported a decrease in MMP-13 protein and activity with the nutraceutical combination[14] and with GLN alone at higher concentrations than those we used.[27]

To date, the effects of GLN and CS on MMP-14 mRNA expression have not been investigated. Neither the nutraceutical combination nor IL-1 had a significant influence on its expression. The absence of an effect of IL-1 on MMP-14 and MMP-2 is supported by their reported constitutive expression in adult human cartilage.[42,46] Lack of an effect with GLN and CS on the expression and activity of MMP-2 paralleled previous studies.[14,33,36] Expression patterns of MMP-2 also coincided with MMP-14 as MMP-14 activated it. The expression of MMP-9 was strongly inducible by inflammatory cytokines.[47] The activity of MMP-9 was, however, significantly reduced by 1 mg/mL GLN in equine cartilage explants.[14] The reason for an absence of IL-1 effect on MMP-9 mRNA expression in our study is not known.

Aggrecanases play a pivotal role in normal and pathologic turnover of aggrecan in cartilage.[48] Together with MMPs, Agg-1 and Agg-2 hydrolyze the major proteoglycan of articular cartilage and are induced by IL-1.[28,42,49,50] The suppression of IL-1–stimulated Agg activity with GLN has been reported by Sandy et al[28] and Ilic et al.[51] The concentration of GLN used in our study is about 80 and 200 times lower than that used in studies by Sandy et al[28] and Ilic et al,[51] respectively. Chondroitin sulfate and the nutraceutical combination at pharmacologic concentrations successfully repressed Aggs by 48 hours after stimulation. Chondroitin sulfate inhibited Agg activity in culture.[30] Simultaneous suppression of MMPs and Aggs with GLN and CS supplementation may represent an effective way to protect matrix components from catabolic processes.

Synthetic inhibitors of MMPs, TIMPs, and agents that increased TIMPs are effective toward reversing or halting catabolic activities associated with OA.[52] Addition of TIMP-1 and TIMP-2 reduced MMP-3 concentrations in canine chondrocyte culture.[53] Tissue inhibitor of metalloproteinase-3 retarded IL-1–stimulated proteoglycan release, which is mediated by Agg.[54] Thus, upregulation of TIMPs may be one of many events to counter catabolic activity in OA pathogenesis. Tissue inhibitor of metalloproteinase-3 was upregulated 2.4-fold by the nutraceutical combination relative to IL-1 at 6 hours of culture (data not shown). In a different study conducted in our laboratory that used GLN (10 µg/mL in combination with CS (20 µg /mL, it was found that TIMP-3 was significantly elevated relative to IL-1 at 8 hours after stimulation (unpublished observation). Studies are ongoing in our laboratory to verify whether TIMP-3 transcript upregulation is accompanied by an elevation in TIMP-3 protein when explants are supplemented with the nutraceutical combination.

Glucosamine and CS at these pharmacologic concentrations did not affect the expression of gene encoding aggrecan and Col II. Glucosamine sulfate at concentrations of 10 to 100 µg/mL had no effect on collagen production in vitro.[55,56] Horses affected with OA had no association in MMP mRNA expression with aggrecan and Col II gene expression.[57] In normal human chondrocytes, GLN had no effect on proteoglycan synthesis.[58] Our findings on aggrecan contrast the study by Dodge and Jimenez,[45] who reported a dose-dependent increase in aggrecan mRNA with GLN (from 200 ng/mL to 30 µg/mL). The contrasts may be attributed to the difference in species (bovine vs human), type of tissue (normal vs OA), source of GLN (GLN hydrochloride vs GLN sulfate), type of culture used (explants vs monolayers), different concentrations of IL-1 (50 vs 5 ng/mL), time length of culture (a maximum of 48 vs 72 hours), and absence versus the presence of preincubation with GLN. Lack of adequate stress may also prevent changes seen in macromolecules with GLN and CS supplementation.[58,59] The synthesis of these major macromolecules of cartilage is catalyzed by a number of enzymes. These nutraceuticals may exert their effects on mediators of aggrecan and collagen synthesis or affect macromolecule synthesis at a translational or posttranslational level.

Glucosamine and CS increased sulfate incorporation in stressed cartilage,[59] and GLN prevented IL-1–mediated inhibition of galactose-β1, 3-glucuronosyltransferase I, an enzyme that catalyzes the addition of the initial glucuronic acid residue in glycosaminoglycan synthesis.[29] Both GLN and CS also increase hyaluronic acid synthesis.[60,61]

With the recent controversy surrounding cyclooxygenase-2 inhibitors in humans and the deleterious effects of nonsteroidal anti-inflammatory drugs on matrix homeostasis, the use of alternative treatments for OA needs to be explored further. Because GLN and CS have good therapeutic efficacy, remarkable tolerability, and safety coupled with effective relief from pain for humans and animals,[7-12,25,26,62] their continued use looks promising. Results of our study indicate that GLN and CS at concentrations that are biologically relevant prevented some of the changes in gene expression associated with IL-1. The inhibition of proteoglycan degradation with GLN and CS[55,59] may be in part mediated by the repression of genes associated with some major cartilage-degrading enzymes and, possibly, the upregulation of metalloproteinase inhibitors. The combination was more effective in antagonizing some gene expression than when GLN and CS were used individually. Further experiments should be conducted to determine whether these changes in gene expression can be replicated in models of OA in animals.

a. 24-well Falcon culture plate, Fisher Scientific, Pittsburgh, Pa.
b. Gibco BRL, Grand Island, NY.
c. R & D Systems, Minneapolis, Minn.
d. FCHG49, Nutramax Laboratories, Edgewood, Md.
e. TRH122, Nutramax Laboratories, Edgewood, Md.
f. Trizol reagent, Invitrogen Corp, Carlsbad, Calif.
g. RNeasy, Qiagen Inc, Valencia, Calif.
h. Beckman Coulter Inc, Fullerton, Calif.
i. DNAse I, Invitrogen, Carlsbad, Calif.
j. Superscript II reverse transcriptase, Invitrogen, Carlsbad, Calif.
k. Primer Express software, version 2.0, Perkin-Elmer Applied Biosystems, Foster City, Calif.
l. Genbank database, National Center for Biotechnology Information, Bethesda, Md.
m. ABI PRISM 7000 sequence detection system, Perkin-Elmer Applied Biosystems, Foster City, Calif.
n. SYBR green PCR dye mix, Perkin-Elmer Applied Biosystems, Foster City, Calif.
o. SAS Institute Inc, Cary, NC.

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Appendices appear on the next page

Appendix 1

Description of components found in treatment media of explant cultures.

Treatment	FBS (%)	rhIL-1β (IL-1)	GLN	CS
FBS control	10	None	None	None
IL-1	10	50 ng/mL	None	None
IL-1 + GLN	10	50 ng/mL	50 μg/mL	None
IL-1 + CS	10	50 ng/mL	None	20 μg/mL
IL-1 + GLN + CS	10	5 μg/mL	5 μg/mL	20 μg/mL

FBS = Fetal bovine serum. rhIL-1β = Human recombinant interleukin-1 beta. IL-1 = Interleukin-1. GLN = Glucosamine. CS = Chondroitin sulfate.

Appendix 2

Forward and reverse primer sequences (5′→3′) of genes of interest used for quantitative real-time polymerase chain reaction.

Gene	Genbank accession No.	Forward primer	Reverse primer
MMP-2	NM_174745	AAGTTCCCCTTCCGGTTCA	TCGCTGCGGCCTGTGT
MMP-3	AF135232	TACGGGTCTCCCCCAGTTTC	TCGGGAGGCACAGATTCC
MMP-9	NM_174744	CGCACGACATCTTTCAGTACCA	GGAACTCACGCGCCAGTAG
MMP-13	NM_174389	GCAGAGAGCTACCTGAAATCATACTACT	AATCACAGAGCTTGCTGCAGTTT
MMP-14	AF144758	CCTCAACCCAGGACCACTTC	GGCCCGTAGGTGGGATTTT
Agg-1	AF516915	CTGGGCCATGTCTTCAGCAT	GGCGGGAGGTGCTCTCA
Agg-2	AF192771	TTTCGGCTCCACGGAAGA	GGGTTTGGATGCGTCAATG
TIMP-1	NM_174471	GCACATCACCACCTGCAGTT	CCGGCGCTGAGCAGAA
TIMP-2	NM_174472	GGGCTGTGAGTGCAAGATCA	CTCGTCCGGAGAGGAGATGTAG
TIMP-3	NM_174473	CGCGTTCTGCAACTCAGACA	CCCCTCCTTCAGCAGTTTCTT
Col II	X02420	GCATTGCCTACCTGGACGAA	CGTTGGAGCCCTGGATGA
Aggrecan	U76615	TTCACCTGTAAAAAGGGCACAGT	CAGGGCATTGATCTCGTATCG
GAPDH	AB098979	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG

MMP = Matrix metalloproteinase. Agg = Aggrecanase. TIMP = Tissue inhibitor of metalloproteinase. Col II = Type II collagen. GAPDH = Glyceraldehyde phosphate dehydrogenase.