Short-term Gene Expression Changes in Cartilage Explants Stimulated with Interleukin 1ß plus Glucosamine and Chondroitin Sulfate

POOI-SEE CHAN, JOHN P. CARON, and MICHAEL W. ORTH

ABSTRACT ***Objective.*** To determine the short-term effects of glucosamine (GLN) and chondroitin sulfate (CS) on expression of genes encoding inflammatory mediators and matrix enzymes in bovine cartilage explants stimulated with interleukin 1 (IL-1).

Methods. Dose-response experiments were conducted for IL-1, GLN, and CS to select concentrations of each optimized for detecting treatment effects on cartilage explants. Based on the dose-response experiments, treatments included fetal bovine serum (FBS) control, 15 ng/ml IL-1, and 15 ng/ml IL-1 with the addition of 10 μg/ml GLN and 20 μg/ml CS. Media were measured for nitric oxide (NO) and prostaglandin E_2 (PGE_2) while explants were frozen for RNA extraction at 8, 16, and 24 hours. Gene expression relative to FBS control for inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), microsomal PGE synthase-1 (mPGEs1), nuclear factor-κB p65 subunit (NF-κB), matrix metalloproteinase (MMP)-3 and 13, aggrecanase (Agg)-1 and 2, and tissue inhibitor of metalloproteinase-3 (TIMP-3) were assessed by quantitative real-time polymerase chain reaction (RT-PCR). In a separate study using incubation of explants with the same treatments for 48 hours, proteoglycan release was measured with dimethylmethylene blue assay and TIMP-3 protein was evaluated with Western blots.

Results. The GLN and CS combination abrogated IL-1-induced gene expression of iNOS, COX-2, mPGEs1, and NF-κB at all timepoints. NO, PGE_2, and proteoglycan release were reduced with the combination. The abundance of stimulated MMP-13, Agg-1, and Agg-2 mRNA was repressed, whereas TIMP-3 was upregulated by the combination at all timepoints. The abundance of TIMP-3 protein was increased by the combination relative to IL-1 at 48 hours.

Conclusion. GLN and CS in combination suppress synthesis and expression of genes encoding inflammatory mediators and proteolytic enzymes while upregulating TIMP-3. This provides a plausible mechanism for the purported mild antiinflammatory and chondroprotective properties of GLN and CS. (J Rheumatol 2006;33:1329–40)

Key Indexing Terms:

GLUCOSAMINE | CHONDROITIN SULFATE | ARTHRITIS
MATRIX METALLOPROTEINASE | INFLAMMATORY MEDIATORS | GENE EXPRESSION

Glucosamine (GLN) and chondroitin sulfate (CS) are the predominant nutraceutical supplements marketed for improving joint health. GLN is an amino monosaccharide and a major building block of proteoglycans. CS, a complex glycosaminoglycan, is a major component of aggrecan. A number of clinical trials with the nutraceuticals have been performed in humans. The majority of these trials show that GLN administered orally was effective in decreasing pain and improving joint mobility in patients with osteoarthritis (OA)[1,2]. Beneficial effects of CS include improved joint mobility, reduced rate of joint space narrowing, and a reduction of erosive OA[2-4].

GLN is commonly combined with CS in many commercially available nutraceutical products. The combination was efficacious in reducing pain, improving joint function, and halting or reversing joint degeneration in humans with mild to moderate OA of the knee[5]. Severe cartilage lesions in an *in vivo* rabbit instability model of OA were prevented with GLN and CS supplementation[6]. However, shortcomings in the design of earlier studies include small sample size, lack of placebos and randomization, and the short-term design of the trials. In spite of this, GLN trials are stronger than nonsteroidal antiinflammatory drug (NSAID) trials qualitatively[7]. Some studies also report negative outcomes, where GLN-treated patients experienced no additional analgesic effects compared to placebo[8,9]. A large placebo-controlled randomized controlled clinical trial with GLN and CS, funded by the US National Institutes of Health (NIH), has been completed[10]. Initial results from this trial demonstrated only a tendency for

From the Bone and Joint Center, Henry Ford Hospital; and Department of Large Animal Clinical Sciences and Department of Animal Science, Michigan State University, East Lansing, Michigan, USA.

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P.S. Chan, PhD, Bone and Joint Center, Henry Ford Hospital; J.P. Caron, DVM, Professor, Department of Large Animal Clinical Sciences, Michigan State University; M.W. Orth, PhD, Associate Professor, Department of Animal Science, Michigan State University.

Address reprint requests to Dr. M.W. Orth, Department of Animal Science, Room 2209F, Anthony Hall, Michigan State University, East Lansing, MI 48824. E-mail: orthm@msu.edu

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patients with OA to respond positively to the combination, compared to placebo. A floor effect contributed by patients with mild OA limiting the ability to detect a response was the reason given for this unexpected finding. When patients were evaluated by degree of pain, significant improvement in pain relief was reported for patients with moderate to severe OA taking the GLN and CS combination relative to placebo. Further analysis of this independent study and similar good quality trials are needed before the nutraceuticals can be recommended with confidence as an alternative OA therapy.

Very few *in vitro* studies to determine the mode of action of these nutraceuticals have employed the GLN/CS combination, although clinical studies have reported symptomatic improvement with coadministration of GLN and CS[5,11]. Despite several studies reporting favorable results, the exact mechanism of action of these nutraceuticals remains to be resolved. The applicability of many *in vitro* mechanistic studies has been questioned because concentrations of the nutraceuticals used greatly exceed those generally found in blood and synovial fluid[12,13]. The concentrations of GLN in blood and synovial fluid after oral and intravenous (IV) administration range from about 0.05 to 20 μg/ml[13-16]. Depending on the route and frequency of administration, the species, and the source and molecular weight of CS, the concentration of CS in serum ranges from 5 to 200 μg/ml[12,14,17,18].

In some studies, cartilage explants or cell cultures stimulated with interleukin 1 (IL-1) demonstrated suppression of nitric oxide (NO) and prostaglandin E_2 (PGE_2) release to the media with supplementation of GLN and CS in concentrations of 0.1 to 10 mg/ml[19-28]. Two other studies employed concentrations of GLN and CS that are attainable *in vivo*, and showed that these nutraceuticals were able to prevent a decline in proteoglycan synthesis typically induced by catabolic agents[29,30]. Recently, we provided preliminary data demonstrating that biologically relevant concentrations of GLN (5 μg/ml) and CS (20 μg/ml) regulated both gene expression and protein synthesis of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in addition to a reduction in the microsomal PGE synthase-1 (mPGEs1) gene in 24-hour incubations[31]. These concentrations of GLN and CS were also able to repress major cartilage proteolytic enzymes implicated in OA pathogenesis at the pretranslational level[32].

Most *in vitro* studies have used IL-1 as a catabolic agent in the range of 1 to 10 ng/ml[25,30,33-35]. Our objective was to further characterize the effects of biologically relevant concentrations of these nutraceuticals using a subsaturating IL-1 induction of bovine cartilage explants. Specifically, we investigated the effect of GLN and CS in combination on IL-1-induced proteoglycan degradation, gene expression, and protein synthesis on an array of inflammatory mediators and cartilage matrix-degrading enzymes and one of their inhibitors in short-term explant cultures.

MATERIALS AND METHODS

Explant cultures. Articular cartilage was isolated from the carpal joints of Holstein steers (age 18–24 mo) obtained from a local abattoir within 3 h of slaughter. Cartilage discs (6 mm diameter) were biopsied from the articular surface and did not include the calcified layer of the tissue (based on macroscopic observation) or cartilage with characteristics of OA. Two explant discs (roughly 60 mg total wet weight) were selected at random and cultured in each well of a 24-well Falcon culture plate (Fisher Scientific, Pittsburgh, PA, USA) containing 1 ml of 1:1 modified Dulbecco's modified Eagle's medium:F-12 nutrient mixture (Ham; Gibco, Grand Island, NY, USA), as described[20]. The medium was supplemented with 50 μg/ml ascorbic acid, 100 units/ml penicillin-streptomycin (Gibco), and 20 amino acids (Sigma, St. Louis, MO, USA). Concentrations of amino acids added were 50% of those previously reported[36]. Cartilage explants were maintained in a humidified incubator at 37°C with 7% CO_2.

Dose-response experiment with IL-1. Explants were maintained in medium without serum for 48 h before the addition of treatments. Medium in the wells was exchanged daily. After equilibration, all wells received 10% fetal bovine serum (FBS; Gibco) and varying concentrations (0, 5, 10, 20, and 50 ng/ml) of recombinant human IL-1ß (rhIL-1ß; R&D Systems, Minneapolis, MN, USA) for 24 h to determine the subsaturating concentration of IL-1 that would result in stimulation of NO and PGE_2 release. There were 8 wells per IL-1 concentration. Conditioned media collected at 24 h were stored at 4°C for NO and PGE_2 analysis. Experiment was repeated a total of 3 times, each time using tissue from a different animal.

Dose-response experiments with GLN and CS. Similar to the methods above, after a 48 h equilibration with serum-free medium, all explants were treated with 10% FBS, 15 ng/ml IL-1 (approximated subsaturating concentration of IL-1), and varying concentrations of glucosamine HCl (FCHG49®; Nutramax Laboratories, Edgewood, MD, USA) for 24 hours. The concentrations of GLN were 0, 1, 5, 10, and 20 μg/ml. These concentrations were within the range of levels attainable in blood after oral and IV administration[12-16]. Conditioned media collected at 24 h were stored at 4°C for NO and PGE_2 analysis. There were 8 wells for each GLN concentration. The experiment was repeated a total of 3 times, each time using tissue from a different animal. These procedures for GLN were repeated for CS. Concentrations of low molecular weight (16.9 kDa) CS (TRH122®, Nutramax Laboratories) were 0, 5, 20, 50, and 100 μg/ml[12,14,17,18,37].

GLN and CS in combination. Explants were maintained in medium without serum for 48 h prior to addition of treatments. After equilibration, all treatments received 10% FBS and 15 ng/ml IL-1 for 8, 16, and 24 h to induce inflammatory mediators and cartilage catabolism. For examination of the effects of GLN and CS, they were added to the wells at the same time as FBS and IL-1. The concentrations of GLN and CS were 10 μg/ml and 20 μg/ml, respectively. There were 3 treatments per experiment: an FBS control, 15 ng/ml IL-1 (IL-1), and 15 ng/ml IL-1 with the addition of 10 μg/ml GLN and 20 μg/ml CS (IL-1 + GLN + CS). Each treatment consisted of 12 wells (24 discs) per timepoint. Cartilage explants were collected at 8, 16, and 24 h after stimulation, frozen in liquid nitrogen, and stored at –80°C until isolation of RNA. Conditioned media were collected at all timepoints and stored at 4°C for NO analysis (all timepoints) and PGE_2 analysis (only the 24-h samples). The experiment was repeated a total of 3 times, each time using cartilage from a different animal.

Proteoglycan and tissue inhibitor of metalloproteinase-3 (TIMP-3) study. Explants were maintained in medium without serum for 48 h prior to addition of treatments. After equilibration, all treatments received 10% FBS. To examine the effects of GLN and CS on proteoglycan release (an indicator of metalloproteinase and aggrecanase activity) and TIMP-3 protein, they were added to the wells at the same time as FBS and IL-1. There were 4 treatments per experiment: an FBS control, 15 ng/ml IL-1 (IL-1), 10 μg/ml GLN and 20 μg/ml CS (GLN + CS), and 15 ng/ml IL-1 with the addition of 10 μg/ml GLN and 20 μg/ml CS (IL-1 + GLN + CS). Each treatment consisted of 6 wells (12 discs). Medium was collected and replaced daily. Cartilage explants were collected 48 h after stimulation, frozen in liquid nitrogen, and stored at –80°C

until protein extraction. Conditioned media were collected at 24 and 48 h post-stimulation and stored at 4°C for dimethylmethylene blue (DMB) analysis. The experiment was repeated a total of 3 times, each time using cartilage from a different animal.

NO assay. Nitrite was measured in conditioned media using the Griess reagent and sodium nitrite as standard[38]. Briefly, 150 µl medium was incubated with 150 µl of 1.0% sulfanilamide, 0.1% N-1-napthylethylenediamide hydrochloride, and 25% phosphoric acid at room temperature for 5 min. Due to some precipitation of reagents with CS, 96-well plates were spun at 1000 g for 3 min at 4°C. The remaining supernatant was transferred to a new plate. Absorbance was measured at 540 nm using a Spectromax 300 plate reader (Molecular Devices, Sunnyvale, CA, USA).

PGE_2 assay. PGE_2 release into conditioned medium was quantified using a commercial competitive ELISA kit according to manufacturer's instructions (R&D Systems). Conditioned media samples were stabilized with indomethacin (10 µg/ml) and stored at –20°C until analysis.

DMB assay. Proteoglycan release into conditioned medium was measured by DMB assay as described[39]. Proteoglycan concentration was determined by measuring sulfated glycosaminoglycan (GAG) content compared to a CS standard. Absorbance was measured at 530 nm with a wavelength correction set at 590 nm using a Spectromax 300 plate reader. For samples containing medium with 20 µg/ml CS (GLN + CS and IL-1 + GLN + CS), GAG content was subtracted, with DMB readings from medium measured only with 20 µg/ml CS.

Total RNA isolation. Total RNA was extracted from cartilage explants following a modified protocol[40]. Briefly, cartilage was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and chloroform was added to extract total RNA followed by vigorous agitation and 2-min incubation. The aqueous phase containing RNA was collected after centrifugation (4°C, 12,000 g, 15 min) and RNA was precipitated with an equal volume of 70% ethanol. Total RNA was then purified further with RNeasy mini columns (Qiagen, Valencia, CA, USA) and quantified by UV spectrophotometry (Beckman Coulter, Fullerton, CA, USA). Total chondrocyte RNA was resolved on 1.2% agarose gel to validate spectrophotometric determination and RNA integrity.

cDNA synthesis. For each sample, 2 µg total RNA was treated with DNase I (Invitrogen) to degrade contaminating single- and double-strand DNA. Treated RNA was converted to single-strand cDNA using Superscript II reverse transcriptase (Invitrogen) as recommended by the manufacturer. Single-strand cDNA was quantified by UV spectrophotometry, and diluted with RNase-free water to 10 ng/µl.

Quantitative real-time polymerase chain reaction (RT-PCR). Glyceraldehyde phosphate dehydrogenase (GAPDH) was validated as an appropriate housekeeping gene (not upregulated by IL-1). Primers for GAPDH and target genes (Table 1) were designed using Primer Express software version 2.0 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). These genes were chosen from studies that described significant induction with higher concentrations of IL-1[31,32]. Nucleotide sequences used for primer design were obtained from public databases (GenBank). Optimal concentrations of each set of primers were determined with a primer matrix [lowest standard deviation with no change in cycle to threshold (C_T)]. Quantitative real-time PCR was performed with 50 ng cDNA templates in 96-well plates (Perkin-Elmer) using the ABI Prism 7000 sequence detection system (Perkin-Elmer)[31]. The FBS control treatment was used as a calibrator (i.e., 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 using the $2^{(-\Delta\Delta CT)}$ method[41].

Protein extraction. Protein was extracted from cartilage explants using a modified protocol[42]. Explants were rinsed with sterile phosphate buffer solution (PBS) and homogenized. Pulverized explants were placed in microcentrifuge tubes with stir bars and 10 µl extraction buffer (50 mmol/l Tris HCl, 10 mmol/l $CaCl_2$, 2 mol/l guanidine HCl, 0.05% Brij-35, pH 7.5) per mg tissue. The mixture was stirred overnight at 4°C and then centrifuged at 18,000 g for 30 min at 4°C. The supernatant was dialyzed for 24 h against assay buffer (50 mmol/l Tris HCl, 10 mmol/l $CaCl_2$, 0.2 mol/l NaCl, 0.05% Brij-35, pH 7.5) using Spectrapor 3 dialysis tubing with a 3.5-kDa cutoff (Spectrum Medical Industries, Los Angeles, CA, USA). Dialysis was continued for 24 h with distilled water.

Western blots. The amount of protein in the explant extract was quantified using the Pierce Micro BCA Protein Assay (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard. Protein extracts (80 µg/lane) were loaded on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels following denaturation by boiling for 5 min in SDS loading buffer, and electrophoresed. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Protein transfer and size determinations were validated using prestained protein markers. Membranes were blocked with 5% nonfat dry milk in TTBS (10 mmol/l Tris-HCl, pH 8, 0.05% Tween-20, 150 mmol/l NaCl) for 1 h at room temperature, and subsequently probed with antibodies directed against TIMP-3 (Chemicon International, Temecula, CA, USA). Signals were detected using horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence detection kit (ECL, Amersham Biosciences, Piscataway, NJ, USA). Blots were developed (Futura Model E film processor), stripped (62.5 mmol/l Tris-HCl, pH 6.8, 100 mmol/l ß-mercaptoethanol, and 2% SDS; 50°C for 30 min), and reprobed with anti-ß-actin antibody (Abcam Inc., Cambridge, MA, USA). Films were scanned after development and the density of each band quantified using computer-aided densitometry. Amounts of TIMP-3 proteins were normalized relative to amounts of ß-actin detected in each sample.

Table 1. Forward and reverse primer sequences (5'→3') of genes of interest used for quantitative RT-PCR.

Gene	Genbank Accession No.	Forward Primer	Reverse Primer
iNOS	AF333248	CCC GCA TGC AAC TCC AA	TCG TAA GTC ATG AAC TGC CAC TTC
COX-2	AF004944	GCA CAA ATC TGA TGT TTG CAT TC	GGT CCT CGT TCA AAA TCTG TCT TG
mPGEs1	NM_174443	GTA CGT GGT GGC CGT CAT C	GGG TTG GCA AAA GCC TTC TT
NF-κB	X61499	GCC AAG GCA GGC AGT TAC C	AGA CGA GGT TTC ACG CTG TTG
MMP-3	AF135232	TAC GGG TCT CCC CCA GTT TC	TCG GGA GGC ACA GAT TCC
MMP-13	NM_174389	GCA GAG AGC TAC CTG AAA TCA TAC TAC T	AAT CAC AGA GCT TGC TGC AGT TT
Agg-1	AF516915	CTG GGC CAT GTC TTC AGC AT	GGC GGG AGG TGC TCT CA
Agg-2	AF192771	TTT CGG CTC CAC GGA AGA	GGG TTT GGA TGC GTC AAT G
TIMP-3	NM_174473	CGC GTT CTG CAA CTC AGA CA	CCC CTC CTT CAG CAG TTT CTT
GAPDH	AB098979	GCA TCG TGG AGG GAC TTA TGA	GGG CCA TCC ACA GTC TTC TG

iNOS: inducible NO synthase; COX-2: cyclooxygenase-2; mPGEs1: microsomal prostaglandin E synthase-1; NF-κB: nuclear factor-κB p65 subunit; MMP: matrix metalloproteinase; Agg: aggrecanase; TIMP: tissue inhibitor of metalloproteinase; GAPDH: glyceraldehyde phosphate dehydrogenase.

Statistical analysis. Data for NO, PGE_2, inhibition of NO and PGE_2 (expressed as percentage inhibition relative to negative controls), and proteoglycan release into conditioned medium were analyzed using a linear mixed-effects model, including the fixed effect of concentration/treatment and the random effect of steer. Concentration/treatment effects were compared within each timepoint using the multiple comparisons approach of Tukey. The computations were performed using the Mixed procedure of SAS[43]. Relative gene expression data determined using RT-PCR and densitometry measurements from Western blots were analyzed using the nonparametric ANOVA approach of Friedman using SAS[43]. Differences were declared statistically significant when $p < 0.05$, unless otherwise noted. Spearman's rank correlations (r) between RT-PCR data and data for NO and PGE_2 release were computed using the Corr procedure of SAS[43].

RESULTS

Effect of increasing IL-1 concentration on NO and PGE_2 release. There were dose-dependent increases in NO and PGE_2 release with increasing IL-1 concentration. Nitrite release increased from 12.84 μM to 65.95 μM with increasing IL-1 concentrations from 0 ng/ml to 50 ng/ml, respectively (data not shown). The release of PGE_2 increased from its basal level of 65.56 pg/ml in the absence of IL-1 to 1501.03 pg/ml with 50 ng/ml IL-1 (Figure 1A). Based on these data, a subsaturating concentration of IL-1 (15 ng/ml) was selected for subsequent experiments.

Effect of increasing GLN and CS concentrations on NO and PGE_2 release. Increasing GLN concentration abrogated IL-1-stimulated release of PGE_2. The release of PGE_2 was inhibited by about 36%, 47%, 49%, and 50% with 1, 5, 10, and 20 μg/ml GLN, respectively (Figure 1B). Increasing CS concentration inhibited PGE_2 release from 18%, 20%, 30%, and 34% with 5, 20, 50, and 100 μg/ml CS, respectively (Figure 1B). There was no significant effect on NO of GLN or CS at any concentration (data not shown). Numerically, maximal reduction of NO occurred with 10 μg/ml GLN (16%) and 20 μg/ml CS (11%). Based on these results, we selected 10 μg/ml of GLN and 20 μg/ml of CS for the subsequent experiments.

The GLN/CS combination suppressed inflammatory mediators. IL-1-stimulated increases in iNOS, COX-2, mPGEs1, and NF-κB transcripts at all timepoints were downregulated by the GLN and CS combination (Figures 2A, 2B, 2C). Nitrite release was not different between treatments at 8 h post-stimulation. Release of both NO and PGE_2 was significantly

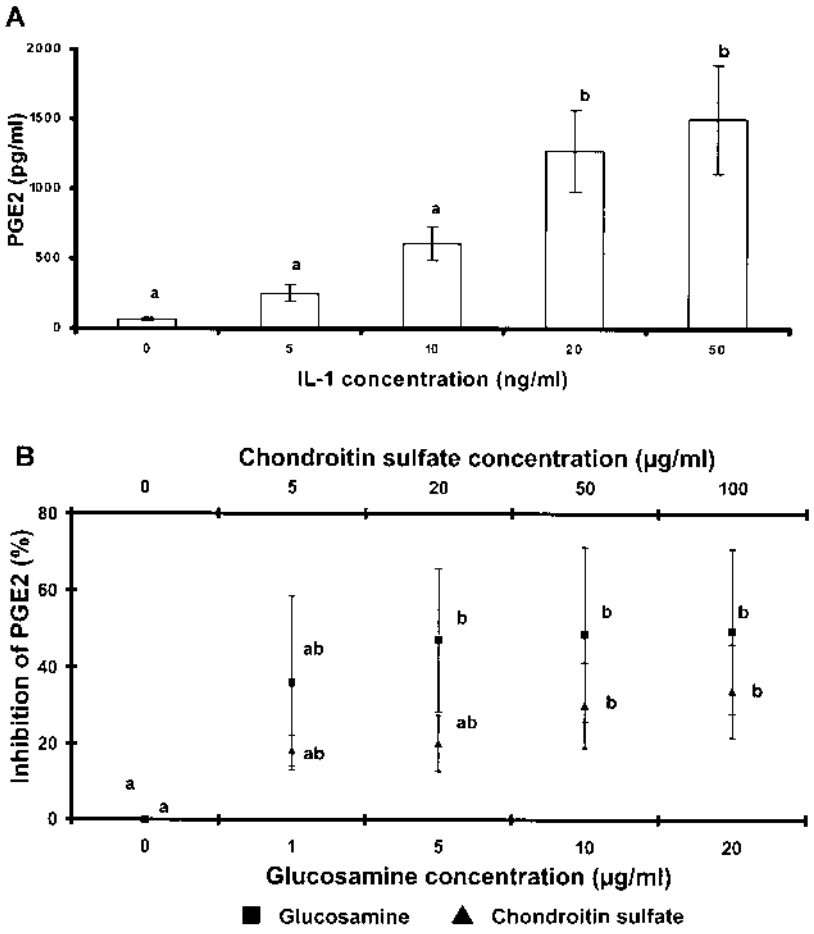

Figure 1. A. Prostaglandin E_2 (PGE_2) release (± SE) in conditioned medium with increasing IL-1 concentration. B. Percentage inhibition of PGE_2 release (± SE) from 0 μg/ml glucosamine (GLN) or chondroitin sulfate (CS) in conditioned medium with 15 ng/ml IL-1 and increasing GLN or CS concentration. Different letters for values at each marker indicate significant ($p < 0.05$) differences between concentrations of IL-1, GLN, or CS.

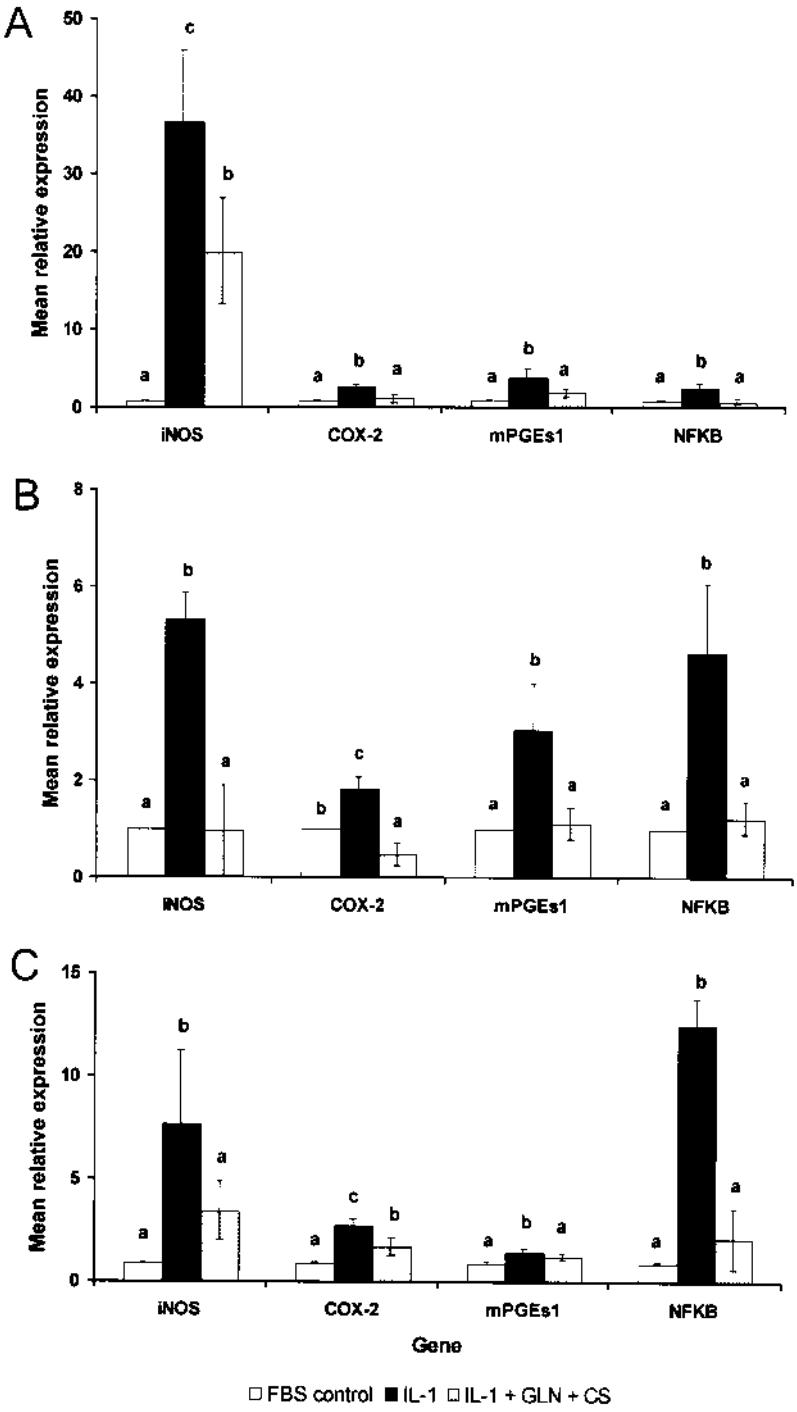

Figure 2. Mean relative gene expression (± SE) of inflammatory mediators at 8 (A), 16 (B), and 24 hours (C) post-stimulation. Different letters for values at each gene indicate significant ($p < 0.05$) differences between treatments. iNOS: inducible NO synthase; COX-2: cyclooxygenase-2; mPGEs1: microsomal prostaglandin E synthase-1; NFKB: nuclear factor-κB p65 subunit; FBS: fetal bovine serum; IL-1: 15 ng/ml recombinant human interleukin 1ß (rhIL-1ß); IL-1 + GLN + CS = 15 ng/ml rhIL-1ß with addition of 10 μg/ml GLN and 20 μg/ml CS.

induced by IL-1 at 16 h (NO only) and 24 h post-culture. The elevation in these inflammatory mediators was effectively reduced by the GLN and CS combination (Table 2).

The GLN/CS combination repressed gene expression of matrix enzymes and reduced proteoglycan release. There was significant upregulation in abundance of MMP-3 mRNA by about

Table 2. Nitrite and PGE_2 release from explants at 8, 16, and 24 hours post-stimulation.

Variable	Treatment		
	FBS Control	IL-1	IL-1 + GLN + CS
Nitrite release (μM ± SE) at 8 h post-stimulation	1.80 ± 0.37[a]	7.67 ± 1.25[a]	5.82 ± 0.44[a]
Nitrite release (μM ± SE) at 16 h post-stimulation	5.56 ± 0.99[a]	28.17 ± 3.86[c]	15.61 ± 1.87[b]
Nitrite release (μM ± SE) at 24 h post-stimulation	8.04 ± 0.94[a]	52.60 ± 5.55[c]	22.54 ± 2.31[b]
PGE_2 release (pg/ml ± SE) at 24 h post-stimulation	70.03 ± 9.11[a]	1159.16 ± 248.09[c]	675.29 ± 106.84[b]

FBS: fetal bovine serum; IL-1: 15 ng/ml recombinant human interleukin-1ß; IL-1 + GLN + CS: 15 ng/ml rhIL-1ß with the addition of 10 μg/ml glucosamine and 20 μg/ml chondroitin sulfate; PGE_2: prostaglandin E_2. Different superscripts for values within a row (i.e., one variable) denote significant ($p < 0.05$) differences between treatments.

4-fold and 42-fold at 8 and 24 h after IL-1 stimulation, respectively. GLN and CS suppressed IL-1-induced expression of the MMP-3 gene by 35% at 24 h. Cytokine-induced mRNA expression of MMP-13, Agg-1, and Agg-2 was repressed at all timepoints by the combination (Figures 3A, 3B, 3C). Induction of proteoglycan release with IL-1 was significantly ($p < 0.01$) reduced by the GLN/CS combination at 24 and 48 h post-stimulation (Figure 4A).

The GLN/CS combination upregulated TIMP-3. Gene expression of TIMP-3 was elevated with the GLN/CS combination relative to IL-1 at all timepoints and relative to FBS control at 8 and 24 h post-stimulation (Figures 3A, 3B, 3C). TIMP-3 protein was increased by the combination relative to IL-1 at 48 h after culture (Figures 4B, 4C).

DISCUSSION

We performed our investigation to expand on previous studies[31,32] involving the effects of GLN and CS on the expression and synthesis of putative mediators of OA. Specifically, in these experiments, GLN and CS were employed at concentrations achievable in blood after oral and IV administration based on available pharmacokinetic studies reported for the GLN and CS combination in animals. Moreover, a subsaturating dose of cytokine was used in this study to ensure the possibility of a 2-tailed response to the arthritogenic stimulus, and gene expression was examined at additional timepoints following stimulation and treatment. Pooling of animals in this experiment was avoided to prevent masking of outliers that might contribute to large variation in the results. Our results indicate that biologically relevant concentrations of this nutraceutical combination retain cartilage-sparing effects in this model.

As expected, IL-1 induced significant expression of the iNOS gene at all timepoints, an event that has been associated with cartilage degradation and suppression of synthesis of extracellular matrix[44-46]. Hence, limiting NO synthesis may be critical to retarding progression of OA; this is supported in the documented beneficial effects of iNOS inhibitors in a canine OA model[42]. In our experiment, GLN and CS suppressed iNOS mRNA and NO release. Individually, neither compound significantly decreased NO production, suggesting the 2 had a synergistic effect[6]. At concentrations 10-fold or greater than those employed in our study, GLN and CS alone or in combination are capable of repressing iNOS mRNA expression and reducing cytokine-induced release of NO[20,21,24-28,33]. Our findings complement reports supporting coadministration of GLN and CS[6,28].

PGE_2 is the most abundant prostanoid found in diseased joints[47]. Formed from the arachidonic acid cascade, PGE_2 mediates synoviocyte proliferation and inflammatory and pain responses. Rate-limiting enzymes responsible for making PGE_2 include the cyclooxygenases and PGE synthases. The inducible forms of these enzymes are COX-2 and mPGEs1, respectively. Synthesis of PGE_2 correlates well with the elevation of COX-2 transcripts ($r = 0.93$, $p < 0.0002$) and mPGEs1 transcripts ($r = 0.95$, $p < 0.0001$), in agreement with other studies[31,48,49]. The concomitant regulation of COX-2, mPGEs1, and PGE_2 in our study parallels other reports[31,49]. At biologically relevant concentrations, GLN and CS in combination effectively decreased IL-1-induced gene expression of COX-2 and mPGEs1 at all timepoints, and eventually PGE_2 synthesis. A significant difference observed for 24 h post-culture in our study compared to the findings of Chan, *et al*[31] was that marginal reduction in mPGEs1 transcript with GLN and CS was detected. These findings of modulation of iNOS, COX-2, and mPGEs1 activities may explain the analgesic effects of GLN and CS[5,11].

The expression and activity of catabolic enzymes such as MMP and aggrecanases exceeds those of endogenous inhibitors like TIMP in OA[50]. The ability of GLN and CS in combination to inhibit cartilage erosion[6] and prevent proteoglycan release in our study (Figure 4) may be partly attributed to regulation of these enzymes. Concentrations of MMP are elevated in OA cartilage specimens and they are localized at the site of OA lesions[51,52]. The nutraceutical combination demonstrated effectiveness in mitigating IL-1 elevation of MMP-3 only at 24 h post-culture, consistent with a report where there was no treatment effect at the 6 h timepoint[32]. Experiments with GLN at higher concentrations showed suppression of MMP-3 gene expression and enzyme activity[21,23,25,53]. Suppression of the MMP-13 transcript with the combination at 24 h also agrees with the findings of Chan, *et*

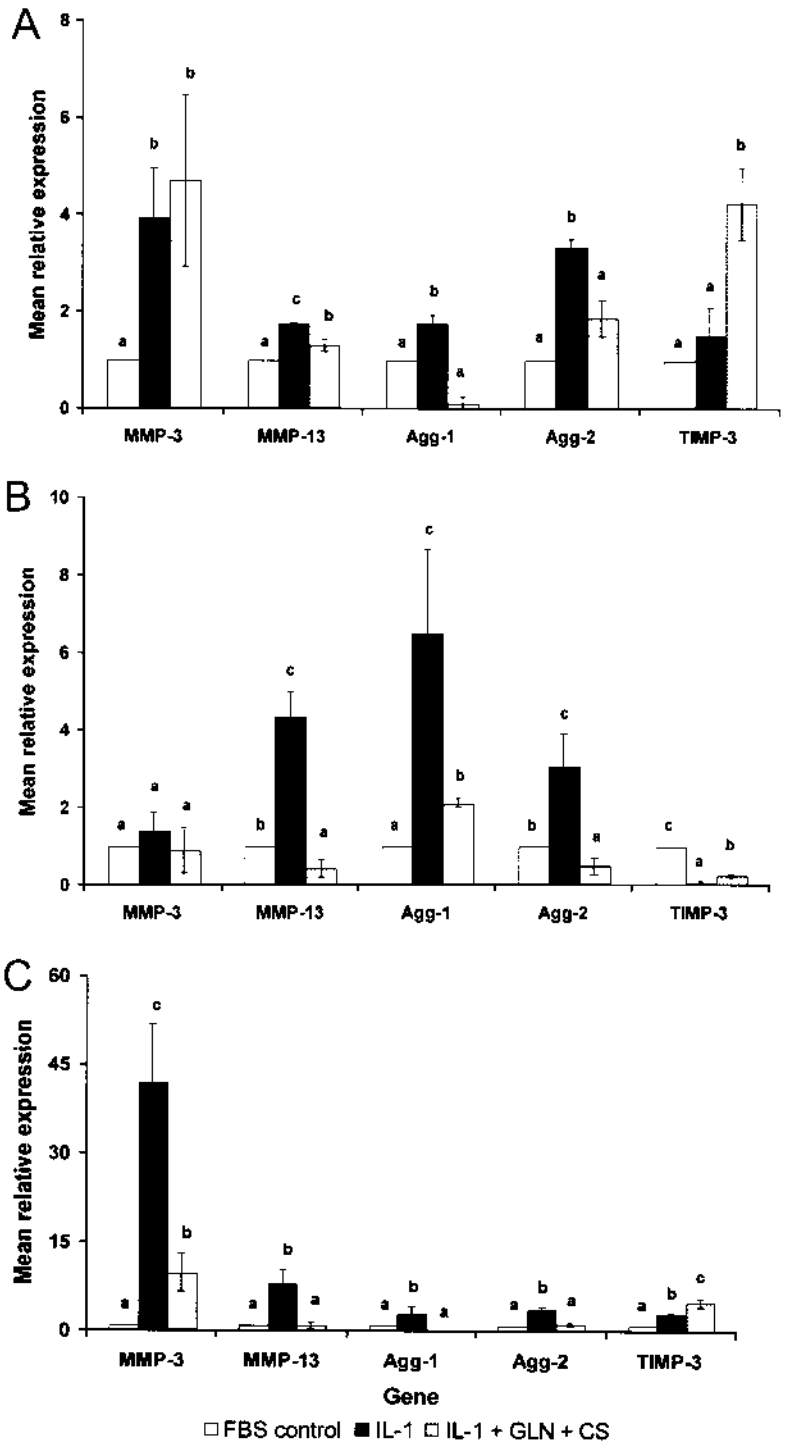

Figure 3. Mean relative gene expression (± SE) of enzymes at 8 (A), 16 (B), and 24 hours (C) post-stimulation. Different letters for values at each gene indicate significant ($p < 0.05$) differences between treatments. MMP: matrix metalloproteinase; Agg: aggrecanase; TIMP: tissue inhibitor of metalloproteinase; FBS: fetal bovine serum; IL-1: 15 ng/ml recombinant human interleukin 1ß (rhIL-1ß); IL-1 + GLN + CS = 15 ng/ml rhIL-1ß with addition of 10 μg/ml GLN and 20 μg/ml CS.

al[32]. However, at higher IL-1 concentration, the ability of GLN and CS to reduce expression of the MMP-13 gene at 6 h was not detected. In our study, induction of MMP-13 occurred at all timepoints, and was suppressed at all timepoints by the combination. The findings on MMP-13 are also in agreement with studies that reported a decrease in MMP-13 protein and

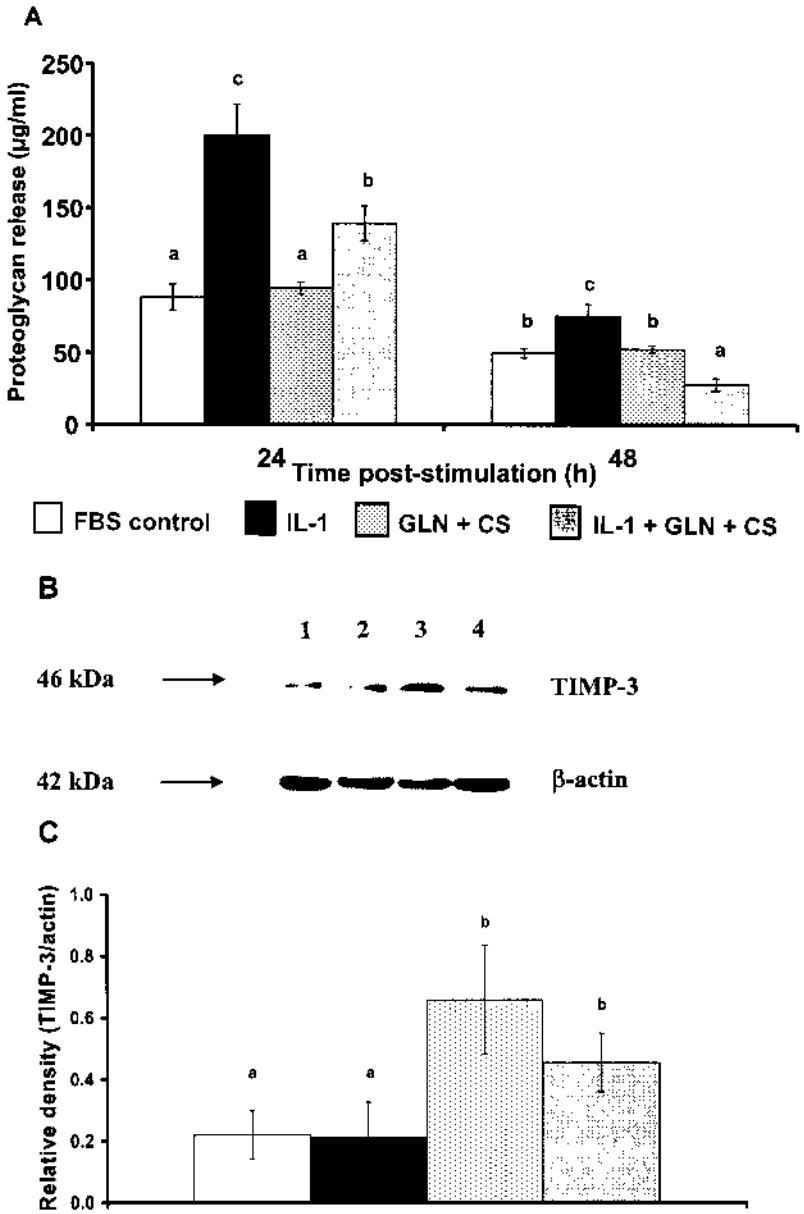

Figure 4. Proteoglycan release (± SE) in conditioned medium at 24 and 48 h (A) post-stimulation. Different letters for values at each timepoint indicate significant ($p < 0.01$) differences between treatments. Representative Western blots of TIMP-3 and ß-actin protein expression at 48 h post-culture (B). Mean relative abundance of TIMP-3 proteins (± SE), as determined by densitometry (C). Different letters indicate significant ($p < 0.05$) differences between treatments. FBS: fetal bovine serum; IL-1: 15 ng/ml recombinant human interleukin-1ß (rhIL-1ß); GLN + CS: 10 µg/ml GLN and 20 µg/ml CS; IL-1 + GLN + CS: 15 ng/ml rhIL-1ß with addition of 10 µg/ml GLN and 20 µg/ml CS.

activity in equine cartilage with the nutraceutical combination[28] and with GLN alone[23]. Concentrations used in our study were at least 30 times lower for GLN and 6 times lower for CS than those reported in previous *in vitro* studies.

The aggrecanases have been implicated as the primary proteins responsible for initiating aggrecan release from OA and injured joints[54]. Our study demonstrated that GLN and CS in combination repressed IL-1 upregulation of Agg-1 and Agg-2 at all timepoints. Our findings roughly parallel those of Chan, *et al*[32]. The effect of GLN and CS in combination on aggrecanases has not been studied extensively. GLN alone suppressed IL-1-stimulated aggrecanase activity at concentrations that were at least 40 times higher than in our study[34,55]. CS used by itself in culture inhibited aggrecanase activity[56].

The TIMP have the potential to reduce proteoglycan

destruction. Localized in the extracellular matrix, TIMP-3 exhibits potent inhibitory activities against MMP and aggrecanases[57]. IL-1-stimulated GAG release via aggrecanase was reversed by TIMP-3[58]. We detected elevation in the TIMP-3 transcript with the combination relative to IL-1 throughout the 24 h culture period. Simultaneous suppression of MMP and aggrecanases coupled with upregulation of TIMP-3 with GLN and CS supplementation may represent an effective way to protect matrix components from being degraded, as evidenced by the decline in proteoglycan release.

IL-1 is upstream of the activation of a number of phosphorylation-dependent signaling pathways leading to the nuclear translocation of transcription factors and activation of proteins participating in translation of mRNA. NF-κB stimulates expression of iNOS and COX-2 and their endproducts, which contribute to the inflammatory process in arthritis[59]. There was simultaneous expression of NF-κB with iNOS ($r = 0.48$, $p < 0.01$) and COX-2 ($r = 0.55$, $p < 0.003$) in our study. Downregulation of certain MMP with GLN and CS is perhaps also a consequence of the repression of IL-1 signaling molecules. NF-κB is one of 2 early response genes needed for MMP transcription[60]. Activation of MMP-3 and MMP-13 relies on NF-κB[61,62]. The mRNA expression of NF-κB in our study is highly correlated with MMP-13 ($r = 0.76$, $p < 0.0001$), and IL-1 induction transcripts of both NF-κB and MMP-13 increased significantly ($p < 0.05$) with time of stimulation. Glucosamine at 1 mg/ml prevented IL-1-induced IκB degradation, NF-κB activation, and nuclear translocation of p50 and p65 NF-κB subunits and PGE_2 release in human chondrocytes[19]. In rat chondrocytes, GLN at concentration 450 times higher than in our study decreased NF-κB activation[22]. Our study shows the ability of GLN and CS in combination to repress IL-1-stimulated mRNA abundance of the p65 NF-κB subunit. This is essential, since NF-κB is able to regulate its own gene expression whereby IL-1-induced increases in NF-κB translocation to the nucleus stimulate continuous synthesis of NF-κB to replace those that were translocated[63]. Thus, the effect of GLN and CS on genes of iNOS, COX-2, and the MMP could be explained at least in part by the inhibition of NF-κB. Further studies are needed to substantiate the effect of these nutraceuticals on signaling events in chondrocytes.

The range of doses used in our study was based on concentrations of GLN and CS derived from pharmacokinetic studies in animals fed 40 mg/kg to 125 mg/kg of the nutraceutical combination[12,14]. The compounds employed in our study were GLN hydrochloride and low molecular weight CS, as in the cited pharmacokinetic studies. The typical dose of GLN given to humans is 20 mg/kg. Higher doses were required in previous pharmacokinetic experiments due to the detection limits of the bioanalytical methods used. Assays with increased detection sensitivity, such as high performance liquid chromatography with mass spectrometry, are now available. Pharmacokinetic studies on ingestion of a regular dose of GLN in humans[64,65] were not available until after the completion of our study. The form of GLN used in these reports was GLN sulfate. One study used GLN hydrochloride and investigated the concentration of GLN in horses after oral and IV administration[13]. No pharmacokinetic data exist to date for the ingestion of CS alone or the combination of GLN and CS in humans. These recent studies have reported that GLN concentration in serum centers around 1 to 2 μg/ml.

Our study was conducted with 10 μg/ml of GLN, a concentration derived from our dose-response study with bovine explants aimed at optimized changes in gene expression. The higher concentration used in our study compared to recent pharmacokinetic reports may limit extrapolation of our *in vitro* data to clinical benefits. However, from the dose-response data on PGE_2 production, 1 μg/ml and 5 μg/ml GLN did reduce it by 36% and 47%, respectively. A different study also reported suppression of PGE_2 release with 5 μg/ml GLN[31]. *In vivo* concentrations of GLN higher than 1 to 2 μg/ml in the blood may be attainable with the typical dose since the concentration of GLN among individuals was variable, and there is a possibility of accumulation of endogenous GLN levels for patients who have taken the nutraceutical for a prolonged period of time[65]. The available pharmacokinetic studies usually measure from several hours up to 2 days post-administration of the nutraceutical. Further, about 2 μg/ml GLN sulfate was reported to decrease IL-1-stimulated gene expression of COX-2, iNOS, MMP-3, Agg-2, and p65 NF-κB subunit by 50% *in vitro*[66]. Our study, with 10 μg/ml GLN in combination with 20 μg/ml CS, also demonstrated repression of these genes.

Persiani and colleagues stated that synovial fluid concentration of GLN in humans can achieve the same concentration found in serum[64]. In horses, on the other hand, synovial concentrations of GLN were only 5% to 12% of the level found in serum[13]. Because of this, the nutraceuticals may exert their effects in other tissues[13]. Studies are needed to explore the effects of the nutraceuticals on tissues other than cartilage, such as the synovium. More pharmacokinetic studies on concentrations of GLN and CS found in the synovial fluid and even articular cartilage are necessary. Additional experiments with lower and attainable concentrations of GLN and CS after administration of a typical dose are needed before the suppression of putative OA catabolic mediators can begin to explain the beneficial effects seen clinically.

Considering the adverse effects elicited by NSAID and COX-2 inhibitors[67,68], availability of other compounds that can relieve joint pain is essential. The benefits of GLN and CS for symptomatic relief of OA have been documented[1-5,11]. These nutraceuticals are safer alternatives, judging from the paucity of adverse events[10,69]. Results of clinical studies with the nutraceuticals were questioned since they were not well controlled and may have been tainted by publication bias. A recent independent study funded by the NIH with 1258 patients demonstrated a tendency for the combination of GLN

and CS to reduce OA knee pain[10]. When the data were isolated and analyzed by different groups of pain severity, GLN and CS given together significantly alleviated knee pain in patients with moderate and severe pain, while celecoxib showed only a tendency toward treatment effect. No effect on pain variables was reported for the individual nutraceuticals. Notably, the CS used in our studies was the same product used in the NIH trial. Other studies, both *in vivo* and *in vitro*, have suggested that GLN and CS were synergistic[6] or complementary[28] or even additive[70]. Our studies have shown that the combination affected gene expression more than the individual compounds[31,32]. More controlled clinical studies are needed to substantiate the therapeutic effects of GLN and CS.

Suppression of NO and PGE_2 production by GLN and CS in combination may partially contribute to the antiinflammatory effects experienced by OA patients consuming these compounds. The purported cartilage-protective feature of GLN and CS[6] and the ability of these nutraceuticals to prevent IL-1-induced proteoglycan degradation and decrease proteoglycan synthesis[29,30] is attributed, at least in part, to suppression of catabolic matrix enzymes and upregulation of TIMP-3, an important enzyme inhibitor. GLN and CS may regulate signaling pathways upstream of the production of inflammatory mediators and matrix enzymes, which translates into beneficial effects. GLN and CS may also positively influence interactions between all these molecules that are associated with OA pathogenesis. Further experiments are planned to confirm these findings *in vivo*.

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REFERENCES

1. Reginster JY, Deroisy R, Rovati LC, et al. Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001;357:251-6.
2. McAlindon TE, LaValley MP, Gulin JP, Felson DT. Glucosamine and chondroitin for treatment of osteoarthritis: a systematic quality assessment and meta-analysis. JAMA 2000;283:1469-75.
3. Uebelhart D, Thonar EJ, Delmas PD, Chantraine A, Vignon E. Effects of oral chondroitin sulfate on the progression of knee osteoarthritis: a pilot study. Osteoarthritis Cartilage 1998;6 Suppl A:39-46.
4. Rovetta G, Monteforte P, Molfetta G, Balestra V. Chondroitin sulfate in erosive osteoarthritis of the hands. Int J Tissue React 2002;24:29-32.
5. Leffler CT, Philippi AF, Leffler SG, Mosure JC, Kim PD. Glucosamine, chondroitin, and manganese ascorbate for degenerative joint disease of the knee or low back: a randomized, double-blind, placebo-controlled pilot study. Mil Med 1999;164:85-91.
6. Lippiello L, Woodward J, Karpman R, Hammad TA. In vivo chondroprotection and metabolic synergy of glucosamine and chondroitin sulfate. Clin Orthop 2000;381:229-40.
7. Towheed TE. Current status of glucosamine therapy in osteoarthritis. Arthritis Rheum 2003;49:601-4.
8. Hughes R, Carr A. A randomized, double-blind, placebo-controlled trial of glucosamine sulphate as an analgesic in osteoarthritis of the knee. Rheumatology Oxford 2002;41:279-84.
9. Rindone JP, Hiller D, Collacott E, Nordhaugen N, Arriola G. Randomized, controlled trial of glucosamine for treating osteoarthritis of the knee. West J Med 2000;172:91-4.
10. Clegg DO, Reda DJ, Harris CL, Klein MA. Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. N Engl J Med 2006;23:795-808.
11. Das A Jr, Hammad TA. Efficacy of a combination of FCHG49 glucosamine hydrochloride, TRH122 low molecular weight sodium chondroitin sulfate and manganese ascorbate in the management of knee osteoarthritis. Osteoarthritis Cartilage 2000;8:343-50.
12. Adebowale A, Du J, Liang Z, Leslie JL, Eddington ND. The bioavailability and pharmacokinetics of glucosamine hydrochloride and low molecular weight chondroitin sulfate after single and multiple doses to beagle dogs. Biopharm Drug Dispos 2002;23:217-25.
13. Laverty S, Sandy JD, Celeste C, Vachon P, Marier JF, Plass AH. Synovial fluid levels and serum pharmacokinetics in a large animal model following treatment with oral glucosamine at clinically relevant doses. Arthritis Rheum 2005;52:181-91.
14. Du J, White N, Eddington ND. The bioavailability and pharmacokinetics of glucosamine hydrochloride and chondroitin sulfate after oral and intravenous single dose administration in the horse. Biopharm Drug Dispos 2004;25:109-16.
15. Setnikar I, Palumbo R, Canali S, Zanolo G. Pharmacokinetics of glucosamine in man. Arzneimittelforschung 1993;43:1109-13.
16. Liang Z, Leslie J, Adebowale A, Ashraf M, Eddington ND. Determination of the nutraceutical, glucosamine hydrochloride, in raw materials, dosage forms and plasma using pre-column derivatization with ultraviolet HPLC. J Pharm Biomed Anal 1999;20:807-14.
17. Du J, Eddington N. Determination of the chondroitin sulfate disaccharides in dog and horse plasma by HPLC using chondroitinase digestion, precolumn derivatization, and fluorescence detection. Anal Biochem 2002;306:252-8.
18. Volpi N. Oral absorption and bioavailability of ichthyic origin chondroitin sulfate in healthy male volunteers. Osteoarthritis Cartilage 2003;11:433-41.
19. Largo R, Alvarez-Soria MA, Díez-Ortego I, et al. Glucosamine inhibits IL-1 beta-induced NF kappa B activation in human osteoarthritic chondrocytes. Osteoarthritis Cartilage 2003;11:290-8.
20. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. Glucosamine HCl reduces equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:258-65.
21. Fenton JI, Chlebek-Brown KA, Caron JP, Orth MW. Effect of glucosamine on interleukin-1-conditioned articular cartilage. Equine Vet J Suppl 2002;34:219-23.
22. Gouze JN, Bianchi A, Bécuwe P, et al. Glucosamine modulates IL-1-induced activation of rat chondrocytes at a receptor level, and by inhibiting the NF-kappa B pathway. FEBS Lett 2002;510:166-70.
23. Byron CR, Orth MW, Venta PJ, Lloyd JW, Caron JP. Influence of glucosamine on matrix metalloproteinase expression and activity in lipopolysaccharide-stimulated equine chondrocytes. Am J Vet Res 2003;64:666-71.
24. Meininger CJ, Kelly KA, Li H, Haynes TE, Wu G. Glucosamine inhibits inducible nitric oxide synthesis. Biochem Biophys Res Commun 2000;279:234-9.
25. Nakamura H, Shibakawa A, Tanaka M, Kato T, Nishioka K. Effects of glucosamine hydrochloride on the production of prostaglandin E_2, nitric oxide and metalloproteases by chondrocytes and synoviocytes in osteoarthritis. Clin Exp Rheumatol 2004;22:293-9.
26. Mello DM, Nielsen BD, Peters TL, Caron JP, Orth MW. Comparison of inhibitory effects of glucosamine and mannosamine on bovine articular cartilage degradation in vitro. Am J Vet Res 2004;65:1440-5.
27. Schlueter AE, Orth MW. Further studies on the ability of glucosamine and chondroitin sulphate to regulate catabolic mediators in vitro. Equine Vet J 2004;36:634-6.

28. Orth MW, Peters TL, Hawkins JN. Inhibition of articular cartilage degradation by glucosamine-HCl and chondroitin sulphate. Equine Vet J Suppl 2002;34:224-9.
29. Bassleer C, Rovati L, Franchimont P. Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. Osteoarthritis Cartilage 1998;6:427-34.
30. Lippiello L. Glucosamine and chondroitin sulfate: biological response modifiers of chondrocytes under simulated conditions of joint stress. Osteoarthritis Cartilage 2003;11:335-42.
31. Chan PS, Caron JP, Rosa GJM, Orth MW. Glucosamine and chondroitin sulfate regulate gene expression and synthesis of nitric oxide and prostaglandin E_2 in articular cartilage explants. Osteoarthritis Cartilage 2005;13:387-94.
32. Chan PS, Caron JP, Orth MW. Glucosamine and chondroitin sulfate regulate gene expression of proteolytic enzymes and their inhibitors in interleukin-1 induced bovine articular cartilage explants. Am J Vet Res 2005;66:1870-76.
33. Shikhman AR, Kuhn K, Alaaeddine N, Lotz M. N-acetylglucosamine prevents IL-1 beta-mediated activation of human chondrocytes. J Immunol 2001;166:5155-60.
34. Sandy JD, Gamett D, Thompson V, Verscharen C. Chondrocyte-mediated catabolism of aggrecan: aggrecanase-dependent cleavage induced by interleukin-1 or retinoic acid can be inhibited by glucosamine. Biochem J 1998;335:59-66.
35. Dodge GR, Jimenez SA. Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. Osteoarthritis Cart 2003;11:424-32.
36. Rosselot G, Reginato AM, Leach RM. Development of a serum-free system to study the effect of growth hormone and insulin-like growth factor-I on cultured postembryonic growth plate chondrocytes. In Vitro Cell Dev Biol 1992;28A:235-44.
37. Volpi N. Oral bioavailability of chondroitin sulfate (Condrosulf) and its constituents in healthy male volunteers. Osteoarthritis Cartilage 2002;10:768-77.
38. Blanco FJ, Ochs RL, Schwarz H, Lotz M. Chondrocyte apoptosis induced by nitric oxide. Am J Pathol 1995;146:75-85.
39. Chandrasekhar S, Esterman MA, Hoffman HA. Microdetermination of proteoglycans and glycosaminoglycans in the presence of guanidine hydrochloride. Anal Biochem 1987;161:103-8.
40. Reno C, Marchuk L, Sciore P, Frank CB, Hart DA. Rapid isolation of total RNA from small samples of hypocellular, dense connective tissues. Biotechniques 1997;22:1082-6.
41. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 2001;25:402-8.
42. Pelletier JP, Jovanovic DV, Lascau-Coman V, et al. Selective inhibition of inducible nitric oxide synthase reduces progression of experimental osteoarthritis in vivo: possible link with the reduction in chondrocyte apoptosis and caspase 3 level. Arthritis Rheum 2000;43:1290-9.
43. SAS Institute. SAS/STAT user's guide version 8.2. Cary, NC: SAS Institute Inc.; 2001.
44. Evans CH. Nitric oxide: what role does it play in inflammation and tissue destruction? Agents Actions Suppl 1995;47:107-16.
45. Murrell GA, Jang D, Williams RJ. Nitric oxide activates metalloprotease enzymes in articular cartilage. Biochem Biophys Res Commun 1995;206:15-21.
46. Taskiran D, Stefanovic-Racic M, Georgescu H, Evans C. Nitric oxide mediates suppression of cartilage proteoglycan synthesis by interleukin-1. Biochem Biophys Res Commun 1994;200:142-8.
47. Kirker-Head CA, Chandna VK, Agarwal RK, et al. Concentrations of substance P and prostaglandin E_2 in synovial fluid of normal and abnormal joints of horses. Am J Vet Res 2000;61:714-8.
48. Murakami M, Naraba H, Tanioka T, et al. Regulation of prostaglandin E_2 biosynthesis by inducible membrane-associated prostaglandin E_2 synthase that acts in concert with cyclooxygenase-2. J Biol Chem 2000;275:32783-92.
49. Stichtenoth DO, Thorén S, Bian H, Peters-Golden M, Jakobsson PJ, Crofford LJ. Microsomal prostaglandin E synthase is regulated by proinflammatory cytokines and glucocorticoids in primary rheumatoid synovial cells. J Immunol 2001;167:469-74.
50. Dean DD, Martel-Pelletier J, Pelletier JP, Howell DS, Woessner JF Jr. Evidence for metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. J Clin Invest 1989;84:678-85.
51. Dean DD. Proteinase-mediated cartilage degradation in osteoarthritis. Semin Arthritis Rheum 1991;20:2-11.
52. Tetlow LC, Adlam DJ, Woolley DE. Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. Arthritis Rheum 2001;44:585-94.
53. Gouze JN, Bordji K, Gulberti S, et al. Interleukin-1 beta down-regulates the expression of glucuronosyl-transferase I, a key enzyme priming glycosaminoglycan biosynthesis: influence of glucosamine on interleukin-1 beta-mediated effects in rat chondrocytes. Arthritis Rheum 2001;44:351-60.
54. Lohmander LS, Neame PJ, Sandy JD. The structure of aggrecan fragments in human synovial fluid. Evidence that aggrecanase mediates cartilage degradation in inflammatory joint disease, joint injury, and osteoarthritis. Arthritis Rheum 1993;36:1214-22.
55. Ilic MZ, Martinac B, Handley CJ. Effects of long-term exposure to glucosamine and mannosamine on aggrecan degradation in articular cartilage. Osteoarthritis Cartilage 2003;11:613-22.
56. Sugimoto K, Takahashi M, Yamamoto Y, Shimada K, Tanzawa K. Identification of aggrecanase activity in medium of cartilage culture. J Biochem Tokyo 1999;126:449-55.
57. Kashiwagi M, Tortorella M, Nagase H, Brew K. TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5). J Biol Chem 2001;276:12501-4.
58. Gendron C, Kashiwagi M, Hughes C, Caterson B, Nagase H. TIMP-3 inhibits aggrecanase-mediated glycosaminoglycan release from cartilage explants stimulated by catabolic factors. FEBS Lett 2003;555:431-6.
59. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-kappa B pathway in the treatment of inflammation and cancer. J Clin Invest 2001;107:135-42.
60. Vincenti MP, Brinckerhoff CE. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. Arthritis Res 2002;4:157-64.
61. Liacini A, Sylvester J, Li WQ, Zafarullah M. Inhibition of interleukin-1-stimulated MAP kinases, activating protein-1 (AP-1) and nuclear factor kappa B (NF-kappa B) transcription factors down-regulate matrix metalloproteinase gene expression in articular chondrocytes. Matrix Biol 2002;21:251-62.
62. Mengshol JA, Vincenti MP, Coon CI, Barchowsky A, Brinckerhoff CE. Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappa B: differential regulation of collagenase 1 and collagenase 3. Arthritis Rheum 2000;43:801-11.
63. Agarwal S, Deschner J, Long P, et al. Role of NF-kappa B transcription factors in antiinflammatory and proinflammatory actions of mechanical signals. Arthritis Rheum 2004;50:3541-8.
64. Persiani S, Roda E, Rovati LC, Locatelli M, Giacovelli G, Roda A. Glucosamine oral bioavailability and plasma pharmacokinetics after increasing doses of crystalline glucosamine sulfate in man. Osteoarthritis Cartilage 2005;13:1041-9.
65. Biggee BA, Blinn CM, McAlindon TE, Nuite M, Silbert JE. Low

levels of human serum glucosamine after ingestion of glucosamine sulphate relative to capability for peripheral effectiveness. Ann Rheum Dis 2006;65:222-6.
66. Piepoli T, Zanelli T, Letari O, Persiani S, Rovati LC, Caselli G. Glucosamine sulfate inhibits IL-1 stimulated gene expression at concentrations found in humans after oral intake [abstract]. Arthritis Rheum 2005;Suppl 52:S502.
67. Palmoski MJ, Brandt KD. Effects of some nonsteroidal antiinflammatory drugs on proteoglycan metabolism and organization in canine articular cartilage. Arthritis Rheum 1980;23:1010-20.
68. Bresalier RS, Sandler RS, Quan H, et al. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. N Engl J Med 2005;352:1092-102.
69. Jordan KM, Arden NK, Doherty M, et al. EULAR Recommendations 2003: an evidence based approach to the management of knee osteoarthritis: Report of a Task Force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCISIT). Ann Rheum Dis 2003;62:1145-55.
70. Chou MM, Vergnolle N, McDougall JJ, et al. Effects of chondroitin and glucosamine sulfate in a dietary bar formulation on inflammation, interleukin-1 beta, matrix metalloprotease-9, and cartilage damage in arthritis. Exp Biol Med 2005;230:255-62.