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

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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

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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

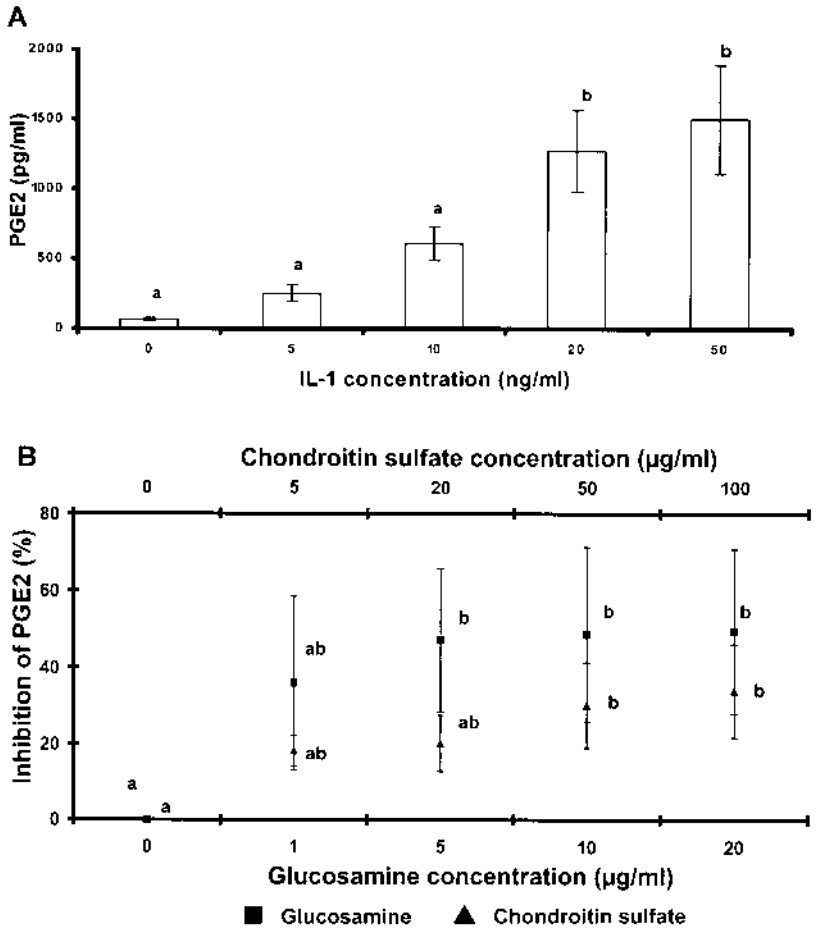

Figure 1. A. Prostaglandin E_2 (PGE_2) release (± SE) in conditioned medium with increasing IL-1 concentration. B. Percentage inhibition of PGE_2 release (± SE) from 0 μg/ml glucosamine (GLN) or chondroitin sulfate (CS) in conditioned medium with 15 ng/ml IL-1 and increasing GLN or CS concentration.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

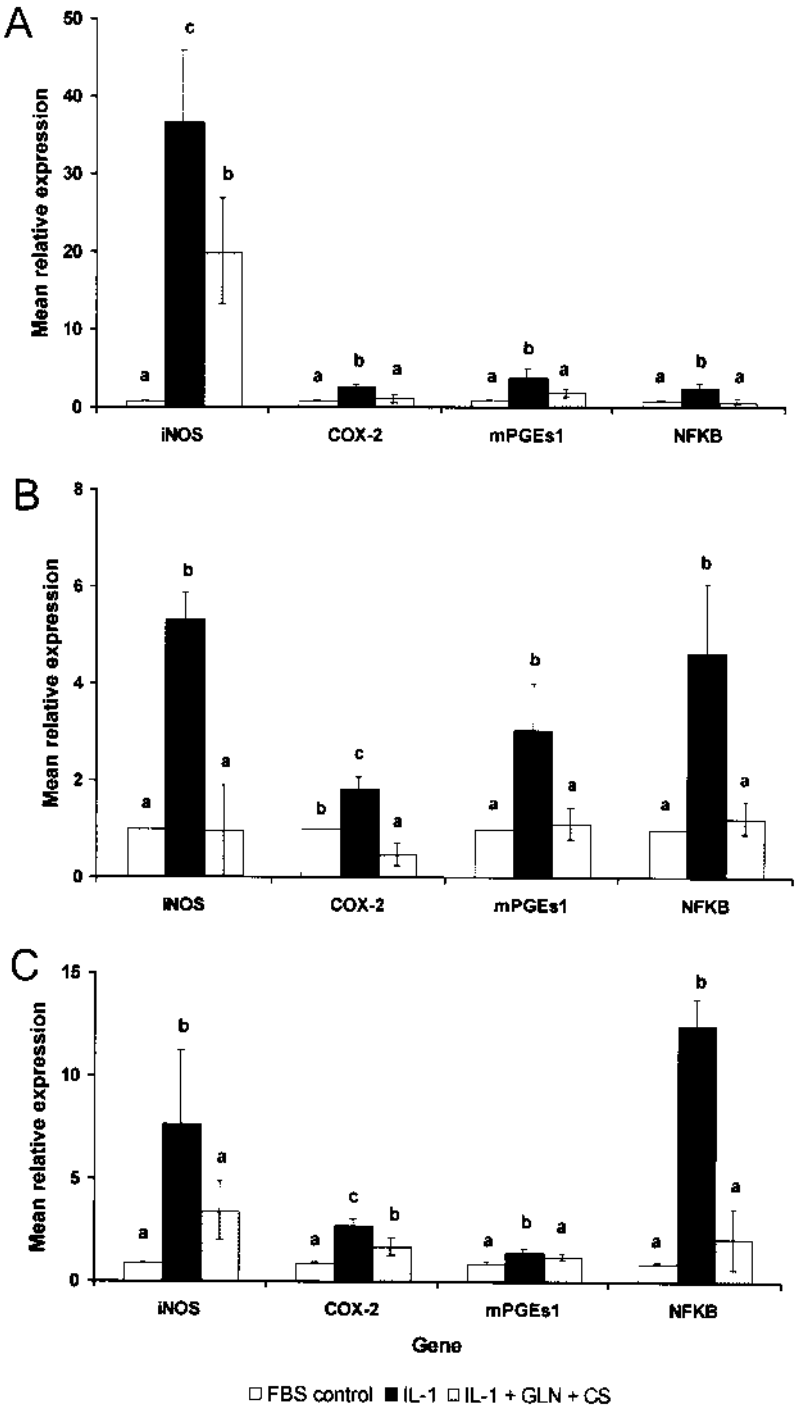

Figure 2. Mean relative gene expression (± SE) of inflammatory mediators at 8 (A), 16 (B), and 24 hours (C) post-stimulation. Different letters for values at each gene indicate significant ($p < 0.05$) differences between treatments. iNOS: inducible NO synthase; COX-2: cyclooxygenase-2; mPGEs1: microsomal 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*

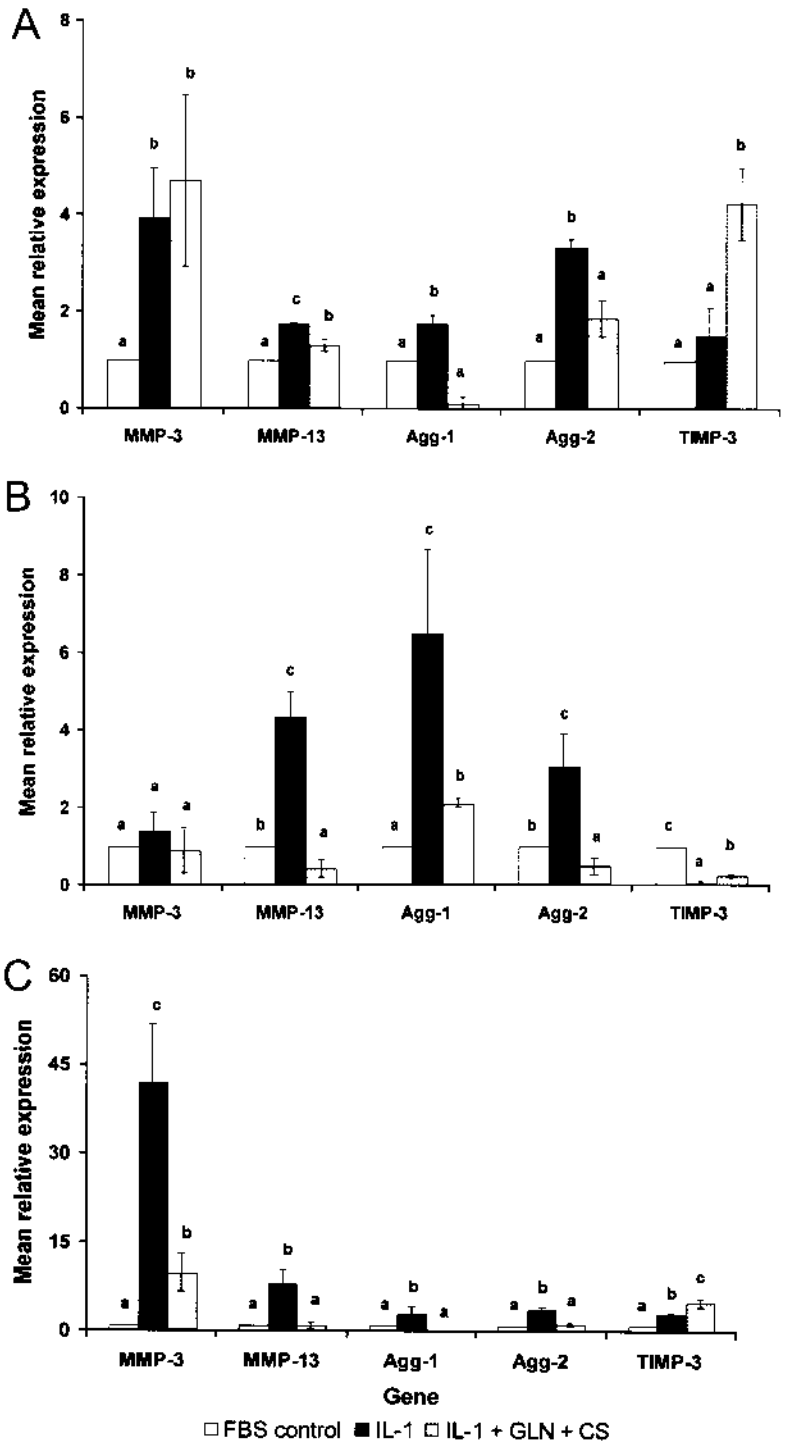

Figure 3. Mean relative gene expression (± SE) of enzymes at 8 (A), 16 (B), and 24 hours (C) post-stimulation. Different letters for values at each gene indicate significant ($p < 0.05$) differences between treatments. MMP: matrix metalloproteinase; Agg: aggrecanase; TIMP: tissue inhibitor of metalloproteinase; 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

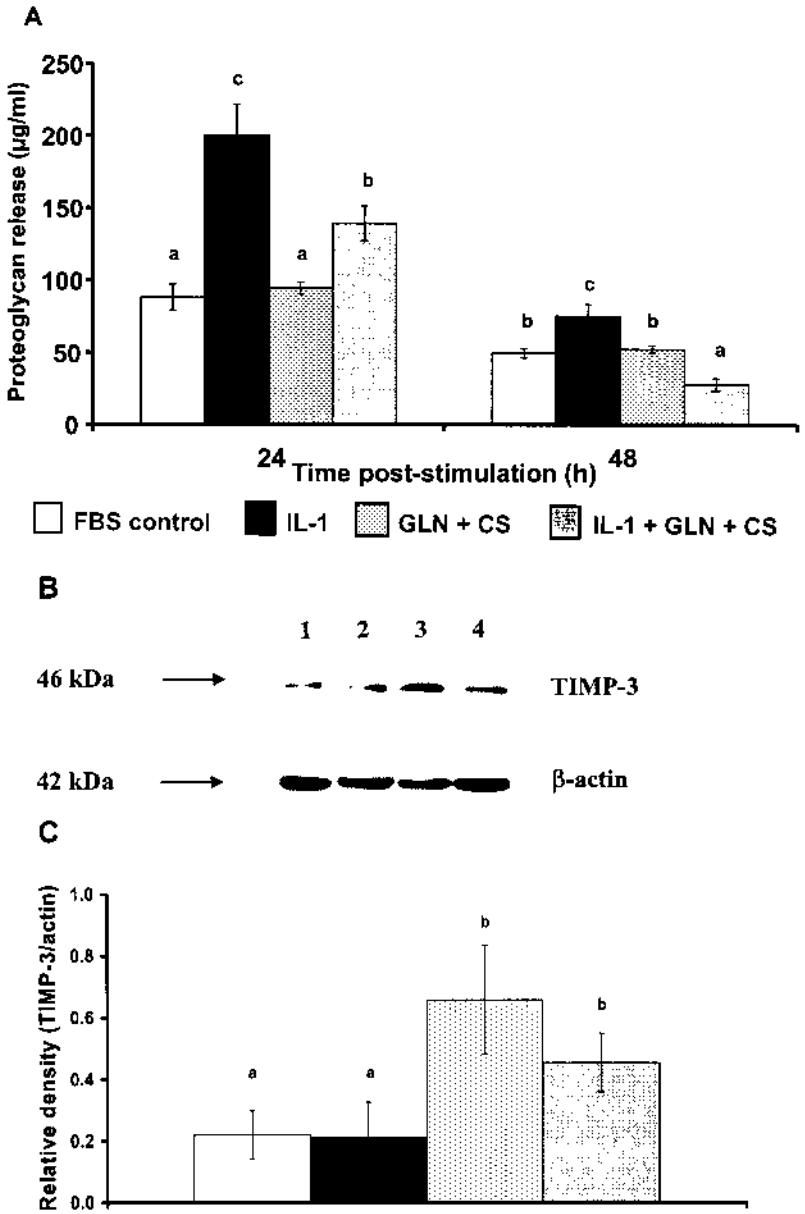

Figure 4. Proteoglycan release (± SE) in conditioned medium at 24 and 48 h (A) post-stimulation. Different letters for values at each timepoint indicate significant ($p < 0.01$) differences between treatments. Representative Western blots of TIMP-3 and ß-actin protein expression at 48 h post-culture (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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