Glucosamine and chondroitin sulfate regulate gene expression and synthesis of nitric oxide and prostaglandin E₂ in articular cartilage explants

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Summary

Objective: Glucosamine (GLN) and chondroitin sulfate (CS) are widely used to alleviate symptoms of osteoarthritis (OA). However, the mechanism(s) of action of these nutraceuticals remains unresolved. In the present study, we determined the effect of physiologically relevant concentrations of GLN and CS on gene expression and synthesis of nitric oxide (NO) and prostaglandin E₂ (PGE₂) in cytokine-stimulated articular cartilage explants.

Methods: Using bovine articular cartilage explants in culture stimulated with IL-1, the effects of physiologically relevant concentrations of GLN and CS on gene expression of inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGEs1) were assessed with quantitative real-time polymerase chain reaction (Q-RT-PCR). The production of NO and PGE₂ was also quantified.

Results: CS and the GLN and CS combination at concentrations attainable in the blood down-regulated IL-1 induced mRNA expression of iNOS at 24 and 48 h post-culture. Up-regulated iNOS expression at 24 h by IL-1 was also suppressed by GLN. GLN and CS transiently repressed the cytokine-stimulated mPGEs1 transcript. Synthesis of NO was reduced with CS alone and the combination after 24 h of culture. Repression of COX-2 transcripts by GLN and CS was accompanied by concomitant reduction in PGE₂.

Conclusion: Our results indicate that physiologically relevant concentrations of GLN and CS can regulate gene expression and synthesis of NO and PGE₂, providing a plausible explanation for their purported anti-inflammatory properties.

Key words: Osteoarthritis, Interleukin-1, Nitric oxide, Prostaglandin, Glucosamine, Chondroitin sulfate.
studies to be beneficial in reducing articular cartilage degeneration, pain, and inflammation. In animals, the combination reduces lameness in dogs and horses and is effective in managing pain in cats. GLN and CS used alone or in combination can decrease joint space narrowing, alleviate pain, and improve joint mobility in humans affected with knee OA.

Elucidating the mechanism(s) of action of GLN and CS in vivo is an active area of research. Pain amelioration with GLN and CS may be attributed to a reduction in inflammatory mediators. In vitro, cytokine-stressed cartilage explants treated with GLN demonstrated a decline in iNOS transcript and NO release into the media. Parallel with the inhibition of COX-2, PGE2 production and release was also inhibited with GLN. CS possesses anti-inflammatory properties.

To date, the majority of in vitro research has been performed with concentrations of GLN and CS that exceed those likely to be obtained by oral administration. This was one of the reasons given by rheumatologists in their objection to recommending GLN to patients. The concentrations of GLN in serum after oral and intravenous administration range from 1 to 20 μg/ml while CS concentrations are in the 5–200 μg/ml range depending on the route of administration, species, and the molecular weight of CS. Thus, the aim of the present study was to determine the effect of concentrations of GLN (5 μg/ml) and CS (20 μg/ml) that have been attained in blood on mRNA expression of iNOS, eNOS, COX-2 and mPGEs1 and the production of NO and PGE2 in articular cartilage explants stimulated with IL-1, a cytokine associated with cartilage catabolism.

Materials and methods

EXPLANT CULTURES

Articular cartilage was isolated from the carpal joints of Holstein steers (18–24 months old) obtained from a local abattoir within 3 h of slaughter. Cartilage discs (6 mm) were biopsied from the articular surface and did not include cartilage with gross characteristics of OA and calcified cartilage. Two explant discs (approximately 60 mg total wet weight) were randomly picked and cultured in each well of a 24-well Falcon culture plate (Fisher Scientific, Pittsburgh, PA, USA) containing 1 ml of 1:1 modified version of Dulbecco’s modified Eagle’s medium:nutrient mixture F-12 (Ham) (Gibco, Grand Island, NY, USA), as previously described.

TOTAL RNA ISOLATION

The extraction of RNA from explants was performed following a modified protocol. Briefly, cartilage was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), chloroform was added to extract total RNA followed by vigorous agitation and a 2 min incubation. The aqueous phase containing RNA was collected after centrifugation and the RNA was precipitated with ethanol. Total RNA was then purified further with RNasy 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 determinations and RNA integrity.

CDNA SYNTHESIS

For each sample, 2 μg of RNA was treated with DNase I (Invitrogen) to degrade contaminating single and double...
stranded DNA. Treated RNA was converted to single stranded cDNA using Superscript II reverse transcriptase (Invitrogen) as recommended by the manufacturer. Single stranded cDNA was quantified with UV spectrophotometry (Beckman Coulter) and diluted to 50 ng/μl.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (Q-RT-PCR)

Primers for glyceraldehyde phosphate dehydrogenase (GAPDH, used as a housekeeping gene) and molecules from Table II were designed using the Primer Express software version 2.0 (Applied Biosystems, Foster City, CA, USA). Optimal concentrations of each set of primers were determined by a Primer Matix (lowest standard deviation with no change in cycle to threshold (Ct)). Q-RT-PCR was performed with 50 ng cDNA templates in 96-well plates using the ABI PRISM 7000 sequence detection system (Applied Biosystems). The analysis of each sample was performed in duplicate. The cDNA templates were combined with optimal concentrations of primers and SYBR Green PCR dye mix (Applied Biosystems) in a total volume of 50 μl and the amplification conducted as recommended by manufacturer.

The PCR conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles of extension at 95°C for 15 s and 1 min at 60°C, and data collected during the last 30 s. Threshold lines were adjusted to intersect amplification lines in the linear portion of the amplification curves. The software automatically recorded the Ct. The GAPDH gene was used as an endogenous control and run together with the samples for each amplification reaction to allow for normalization of different samples for RNA loading, cDNA synthesis, and amplification efficiencies and for comparison of samples run at different times. The FBS control 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 treatment was calculated using the delta delta Ct method.

STATISTICAL ANALYSIS

Data for NO and PGE₂ release into conditioned media were analyzed using a linear mixed effects model, including the fixed effect of treatment and the random effects of pool, the interaction between treatment and pool, and repeated measures within each treatment and pool combination. Treatment effects were compared within each time point using the multiple comparisons approach of Tukey. The computations were performed using the MIXED procedure of SAS. Relative gene expression data determined using Q-RT-PCR were analyzed considering the nonparametric ANOVA approach of Friedman. Differences were declared statistically significant when \( P < 0.05 \), unless otherwise noted. Spearman’s rank correlations \( (r) \) between gene expression data and biochemical data were computed using the CORR procedure of SAS.

Results

THE EFFECT OF GLN AND CS ON GENE EXPRESSION

Bovine articular cartilage explants cultured with 50 ng/ml rhIL-1β for 6, 24 and 48 h resulted in significant \( (P < 0.01) \) up-regulation of iNOS expression relative to control (Table III). The large standard error for both IL-1 and IL-1 plus CS treatments at 6 h was attributed to variation between pools of animal tissue and not technical error within a replication. GLN (5 μg/ml) down-regulated iNOS mRNA expression from 11.1-fold to 4.5-fold in explants cultured for 24 h (Table III). CS (20 μg/ml) added alone or in combination with GLN for 24 h was as effective as GLN in suppressing iNOS expression to control levels. After 48 h, cytokine-stimulated explants treated with CS or GLN and CS repressed iNOS mRNA expression to control levels (Table III). The iNOS transcript of explants treated with GLN alone at the 48 h time point was not different from IL-1 stimulated explants (Table III).

Cartilage explants stimulated with 50 ng/ml rhIL-1β demonstrated significant elevation in COX-2 mRNA expression relative to control by 30-fold for 6 h post-stimulation (Table III). Like iNOS mRNA expression at 6 h, variability of COX-2 level of transcript at this time point was due to differences between replications. At the 6 h time point, GLN or CS used individually and used in combination

<table>
<thead>
<tr>
<th>Gene</th>
<th>Treatment</th>
<th>IL-1</th>
<th>IL-1 + GLN</th>
<th>IL-1 + CS</th>
<th>IL-1 + GLN + CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>Mean fold change (±SE) in gene expression relative to control 6 h post-stimulation</td>
<td>87.7 ± 73.6a</td>
<td>4.1 ± 1.0b</td>
<td>26.7 ± 24.9a</td>
<td>4.1 ± 2.8a</td>
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<tr>
<td>COX-2</td>
<td>30.0 ± 13.3b</td>
<td>1.5 ± 0.9b</td>
<td>2.7 ± 2.5a</td>
<td>3.3 ± 3.1a</td>
<td></td>
</tr>
<tr>
<td>mPGEs1</td>
<td>8.7 ± 2.8b</td>
<td>4.5 ± 1.0a</td>
<td>5.4 ± 1.5a</td>
<td>4.9 ± 1.5a</td>
<td></td>
</tr>
<tr>
<td>GLN</td>
<td>Mean fold change (±SE) in gene expression relative to control 24 h post-stimulation</td>
<td>11.1 ± 3.1b</td>
<td>4.5 ± 1.9b</td>
<td>4.4 ± 0.6a</td>
<td>3.0 ± 0.8a</td>
</tr>
<tr>
<td>COX-2</td>
<td>7.7 ± 0.6b</td>
<td>3.2 ± 2.0a</td>
<td>7.1 ± 0.6b</td>
<td>3.4 ± 0.6b</td>
<td></td>
</tr>
<tr>
<td>mPGEs1</td>
<td>0.7 ± 0.1a</td>
<td>0.4 ± 0.1a</td>
<td>1.0 ± 0.3a</td>
<td>0.6 ± 0.2a</td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>Mean fold change (±SE) in gene expression relative to control 48 h post-stimulation</td>
<td>9.4 ± 2.7b</td>
<td>3.1 ± 1.4b</td>
<td>2.3 ± 1.3a</td>
<td>1.2 ± 1.0a</td>
</tr>
<tr>
<td>COX-2</td>
<td>12.6 ± 4.0b</td>
<td>3.4 ± 0.7b</td>
<td>2.8 ± 1.0a</td>
<td>1.0 ± 0.8a</td>
<td></td>
</tr>
<tr>
<td>mPGEs1</td>
<td>4.8 ± 2.0b</td>
<td>1.6 ± 0.8a</td>
<td>1.5 ± 0.8a</td>
<td>0.8 ± 0.3a</td>
<td></td>
</tr>
</tbody>
</table>
| IL-1      | IL-1 = 50 ng/ml rhIL-1β; IL-1 + GLN = 50 ng/ml rhIL-1β and 5 μg/ml GLN; IL-1 + CS = 50 ng/ml rhIL-1β and 20 μg/ml CS; IL-1 + GLN + CS = 50 ng/ml rhIL-1β, 5 μg/ml GLN and 20 μg/ml CS. Different superscripts for values within a row (i.e., one gene) denote a significant difference \( (P < 0.05) \) between treatments.
resulted in a decline of COX-2 transcripts to control levels (Table III). The COX-2 gene for explants treated with 50 ng/ml rhIL-1β was significantly up-regulated by 8-fold and 13-fold for 24 and 48 h post-stimulation, respectively (Table III). At both time points, GLN suppressed the activation of the COX-2 gene to control levels (Table III). Physiologically relevant concentration of CS at 20 μg/ml did not repress COX-2 transcript increased by IL-1 until 48 h (Table III). The GLN and CS combination decreased COX-2 expression to 3.4-fold and 1.0-fold change for 24 and 48 h, respectively (Table III). No effect on mPGEs1 expression was found for any of the treatments at 24 and 48 h (Table III).

The transcript for mPGEs1, the terminal enzyme involved in PGE2 synthesis was elevated by about 9-fold (P = 0.0008) relative to control, in explants cultured with 50 ng/ml rhIL-1β over a 6-h period (Table III). At this time point, GLN alone and the nutraceuticals combination repressed mPGEs1 mRNA expression to control levels (Table III). No effect on mPGEs1 expression was found for any of the treatments at 24 and 48 h (Table III).

THE EFFECT OF GLN AND CS ON NO AND PGE2 SYNTHESIS

NO released into conditioned media at 24 h after the addition of treatment was increased with 50 ng/ml rhIL-1β to 21.39 μM from 5.98 μM for FBS control [Fig. 1(A)]. The addition of CS or GLN plus CS suppressed NO release to 16.93 and 17.27 μM, respectively [Fig. 1(A)]. At 48 h, the elevation in NO release with 50 ng/ml rhIL-1β was not reduced by either nutraceutical [Fig. 1(B)].

Explants stimulated with 50 ng/ml rhIL-1β released significantly more PGE2 into media when compared to control. After 24 h stimulation, PGE2 release was elevated to 1986 pg/ml compared with basal PGE2 release at 169 pg/ml [Fig. 2(A)]. The addition of GLN and CS, or in combination reduced PGE2 release induced by cytokine to control levels [Fig. 2(A)]. The basal PGE2 release into media 48 h post-treatment was 171 pg/ml [Fig. 2(B)]. This level was elevated to 1122 pg/ml when 50 ng/ml rhIL-1β was added [Fig. 2(B)]. GLN decreased PGE2 release due to IL-1 by 68% [Fig. 2(B)]. Likewise, the combination resulted in a 76% decline in PGE2 release induced by IL-1 [Fig. 2(B)]. CS addition to the explants in vitro tended (P < 0.10) to decrease PGE2 release [Fig. 2(B)].

THE EFFECT OF GLN AND CS ON ENOS GENE EXPRESSION

Since the biochemical data on NO release conflicted with the Q-RT-PCR data on iNOS gene expression profile, we decided to investigate the expression of the eNOS gene, another isoform of the NO synthase enzyme. Only a transient pattern of eNOS regulation by CS was witnessed. The IL-1 mediated increase in eNOS transcript of 3.3-fold at 24 h was significantly (P < 0.05) reduced to 0.3-fold with 20 μg/ml CS. No significant differences were seen with the other treatments (data not shown).

![Fig. 1](image1.png)

![Fig. 2](image2.png)

**Fig. 1.** Mean (± SE) nitrite release into conditioned media 24 h (A) and 48 h (B) after treatments are added. Ctrl = FBS control; IL-1 = 50 ng/ml rhIL-1β; IL-1 + GLN = 50 ng/ml rhIL-1β and 5 μg/ml GLN; IL-1 + CS = 50 ng/ml rhIL-1β and 20 μg/ml CS; IL-1 + GLN + CS = 50 ng/ml rhIL-1β, 5 μg/ml GLN and 20 μg/ml CS. Different superscripts indicate significant differences at P < 0.05.

**Fig. 2.** Mean (± SE) PGE2 release into conditioned media 24 h (A) and 48 h (B) after the addition of treatments. Ctrl = FBS control; IL-1 = 50 ng/ml rhIL-1β; IL-1 + GLN = 50 ng/ml rhIL-1β and 5 μg/ml GLN; IL-1 + CS = 50 ng/ml rhIL-1β and 20 μg/ml CS; IL-1 + GLN + CS = 50 ng/ml rhIL-1β, 5 μg/ml GLN and 20 μg/ml CS. Different superscripts indicate significant differences at P < 0.05. * Tended to be different than the IL-1 treatment (P < 0.10).
Discussion

To our knowledge, the present study is the first to describe the effect of GLN and CS attained in blood on gene expression and synthesis of inflammatory mediators using a cartilage explant culture. The concentration chosen for GLN was 5 µg/ml while that for CS was 20 µg/ml. These concentrations were at the low end of the range found in blood after oral or intravenous administration. Previous trials from our laboratory used concentrations which are higher than the range found in the plasma of animals administered GLN and CS. Another novelty of this research lies in the nutraceutical combination investigated. Few in vitro studies have been conducted with the GLN and CS combination although they are frequently marketed as a combination. One study reported that they are synergistic while another suggested they may be complementary.

NO is implicated in the pathogenesis of arthritis. Normal cartilage explants produce little NO. On the contrary, chondrocytes and the synovial membrane in OA and rheumatoid arthritis (RA) patients produce NO abundantly and so does cytokine-induced cartilage. Constitutive form of eNOS was initially thought to be one of the mechanisms of action of these compounds may be the suppression of NO production with chronic inflammatory arthritides. One of the mechanisms of action of these compounds may be the suppression of NO production with chronic inflammatory arthritides. COX-2 is a constitutive enzyme involved in the formation of PGE2 which is responsible for pain and inflammatory responses. In OA cartilage cultured in vitro, large amounts of NO are released into the medium. This inorganic free radical also mediates the degradation of matrix components via activation of MMPs. Besides cartilage destruction, NO has also been implicated as a mediator of inflammatory responses. Thus, the inhibition of NO synthesis may be effective as a therapeutic route for OA.

GLN and CS have been used successfully for treating chronic inflammatory arthritides. One of the mechanisms of action of these compounds may be the suppression of NO production. Previous work from our laboratory using explants from horses indicates decreased NO production with 250 µg/ml CS and with the combination of 500 µg/ml GLN and 250 µg/ml CS. Inhibition of NO with GLN occurred at concentrations greater than 1 ng/ml in equine explants and human chondrocytes. Suppression of NO synthesis with GLN was associated with iNOS mRNA and protein repression. Suppression of NO synthesis with GLN was associated with iNOS mRNA and protein repression. In the current trial, CS alone and in combination with GLN at physiological concentrations were effective in reducing NO synthesis induced by IL-1 at 24 h. These levels of NO in conditioned media may be explained by transcriptional regulation of genes pertaining to the enzymes responsible for synthesizing NO. Although the levels of NO were significantly reduced at 24 h, these were marginal declines. Since NO and PGE2 can modulate the activity of the other, even transient regulation of NO may affect the anti-inflammatory response to some extent.

The inducible form of the NO synthases is iNOS and there are two constitutive isoforms, eNOS and nNOS. The constitutive form of eNOS was initially thought to be confined to endothelial cells while nNOS was localized to the central and autonomic nervous systems. However, eNOS and nNOS have increasingly been identified in a lot of different cell types including chondrocytes. eNOS may also be inducible since human eNOS gene contains an activator protein-1 (AP-1) site in its promoter region. The mitogen-activated protein kinase (MAPK) pathway is activated by cytokines leading to AP-1 phosphorylation and up-regulation of a number of genes associated with cartilage catabolism. Human OA chondrocytes express elevated levels of nNOS. To date, only the genes for iNOS and eNOS and not nNOS in the bovine species have been cloned and sequenced. Since eNOS mRNA levels were not markedly affected at 48 h, the lack of treatment effect on NO production could be in part due to the activity of eNOS and potentially nNOS.

PGE2, another catabolic mediator in the pathogenesis of OA is formed from a series of enzymatic reactions termed the arachidonic acid cascade. It mediates synovioocyte proliferation and is responsible for pain and inflammatory responses. In OA cartilage cultured in vitro, large amounts of PGE2 are synthesized by chondrocytes that are exposed to IL-1 in vivo. Bovine explant cultures induced with IL-1 demonstrated significant increase in PGE2 from basal levels. Elevated levels of PGE2 depleted matrix components from intact articular cartilage cultured in vitro.

The induction of COX-2 is largely responsible for elevated concentrations of PGE2. Cox-2 expression is elevated in cartilage specimens of OA and RA patients. Findings from the present study demonstrated that COX-2 is a key enzyme involved in the pathogenesis of arthritis. In clinical studies, GLN and CS have been consistently beneficial in improving the pain scores and inflammatory responses of patients affected with OA. In vitro results from previous work in our laboratory and others demonstrated declines in the inflammatory mediator, PGE2, with GLN and CS supplementation. In the present study, GLN, CS alone or in combination reduced IL-1 induced PGE2 levels. The ability of GLN and CS to reduce PGE2 synthesis in the present study can be explained by their effect on COX-2 gene expression. Since there is a lag time of about 12–18 h from the time the gene is expressed to the time it is translated into protein, the COX-2 mRNA data at 6 h does correspond to the PGE2 levels at 24 h while mRNA data at 24 h has a similar profile with PGE2 levels at 48 h. The ability of GLN to suppress COX-2 mRNA expression by about 2-fold at 24 h post-stimulation parallels the work by Largo et al. in human chondrocytes. However, the concentration of GLN used in their study was 1 mg/ml which is 200 times the concentration used in our study. The present study substantiates the claim that repression of COX-2 transcripts and eventually PGE2 is at least one of the mechanisms GLN alone or together with CS exert their analgesic effects.

The effect of these chondroprotective agents on terminal PGE enzymes involved in the formation of PGE2 has not been reported. In fact, we only know of one abstract that
has investigated this enzyme in chondrocytes. There are reports of concomitant regulation of COX-2, mPGEs1 and PGE2 in cartilage. Transcripts of COX-2 and mPGEs1 are correlated in the present study (r = 0.43, P < 0.05). However, differences in the timing for COX-2 and mPGEs1 induction may be due to dissimilar regulatory sequences in these two genes. Explants treated either with GLN alone or in combination with CS repressed IL-1 induced mPGEs1 expression at 6 h post-culture (Table III). Further studies on the contribution of this brief regulation on mPGEs1 in chondrocytes by the nutraceuticals are warranted.

Determining the mechanism of action behind GLN and CS is essential to elucidating the beneficial effects elicited by these compounds in combating arthritic symptoms. Understanding how these agents may regulate NO and PGE2 is an active area of research. Besides being proinflammatory, NO and PGE2 have the ability to increase proteoglycan loss and decrease proteoglycan synthesis. Our results suggest a transient modulation of proteoglycan loss and decrease proteoglycan synthesis is prevented in vitro.

Results from the present study indicate that GLN and CS may regulate the expression and synthesis of NO and PGE2, providing a plausible explanation for the symptomatic relief attributed to the administration of these compounds.

Acknowledgments

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References

8. Tyler JA, Benton HP. Synthesis of type II collagen is decreased in cartilage cultured with interleukin 1 while the rate of intracellular degradation remains unchanged. Coll Relat Res 1988;8:393–405.
22. Leffler CT, Philipp AF, Leffler SG, Mosure JC, Kim PD. Glucosamine, chondroitin, and manganese ascorbate for degenerative joint disease of the knee or low back:


36. Mansson JJ, Rahman A. This house believes that we should advise our patients with osteoarthritis of the knee to take glucosamine. Rheum Oxf 2004;43:100–1.


53. Mansson JJ, Rahman A. This house believes that we should advise our patients with osteoarthritis of the knee to take glucosamine. Rheum Oxf 2004;43:100–1.


