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# Glucosamine Hydrochloride but Not Chondroitin Sulfate Prevents Cartilage Degradation and Inflammation Induced by Interleukin-1 $\alpha$ in Bovine Cartilage Explants

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## Abstract

**Objective.** Glucosamine hydrochloride (GH) and chondroitin sulfate (CS) are commonly used for the treatment of osteoarthritis (OA). The aim of this study was to assess their effects, alone and in combination, on preventing aggrecan degradation and inflammation in an *in vitro* model of OA. **Design.** To test the effects of GH and/or CS as a preventative treatment, cartilage explants were pretreated with the compound(s) using concentrations that showed no detrimental effect on chondrocyte viability. Interleukin-1 $\alpha$  (IL-1 $\alpha$ ) was added to induce cartilage degradation, supernatant and explants were analyzed for proteoglycan degradation products, aggrecanase mRNA expression and activity, and for the release of inflammatory markers. **Results.** Following treatment with IL-1 $\alpha$ , 2 mg/mL dose of GH pretreatment was associated with a reduction of glycosaminoglycan release, reduced generation of the pathological interglobular domain aggrecan catabolites, decreased mRNA levels of ADAMTS-4 and -5 and reduced activity of ADAMTS-4. In contrast, CS alone did not have a significant effect on IL-1 $\alpha$ -induced cartilage degradation and the addition of 0.4 mg/mL CS to 2 mg/mL GH did not further inhibit IL-1 $\alpha$ -induced activity. Pretreatment with 2 mg/mL GH also reduced the release of inflammatory markers, prostaglandin E<sub>2</sub> and nitric oxide induced by IL-1 $\alpha$  while CS did not have a significant effect. **Conclusions.** The results suggest that GH prevents cartilage degradation mediated by aggrecanases ADAMTS-4 and -5, and may also reduce inflammation. This could be part of the mechanisms by which GH is effective in maintaining joint integrity and function, and preventing or delaying early symptoms of OA.

## Keywords

glucosamine, chondroitin, osteoarthritis, aggrecanase

## Introduction

Osteoarthritis (OA) is a degenerative disease of the joints characterized by a loss of articular cartilage and remodeling of subchondral bone. More than 8.75 million people are affected in the United Kingdom and about 27 million people in the United States.<sup>1,2</sup> Conventional treatments, which mainly include analgesics and nonsteroidal anti-inflammatory drugs, can cause serious side-effects. With no cure for OA, agents that not only reduce the pain but also prevent or at least slow down the progression of the disease are desirable and in this study we are interested in testing the 2 commonly used nutraceuticals, glucosamine and chondroitin, to prevent or delay the pathological changes in cartilage that manifest during the early stages of the disease.

Aggrecan consists of a core protein with 100 to 150 glycosaminoglycan (GAG) chains attached to it. Along with type-II collagen, aggrecan forms a major structural component of articular cartilage. The loss of aggrecan from articular cartilage is an early event in the development of OA and continued loss of aggrecan leads to the susceptibility of the collagen network to proteolysis and irreversible tissue damage.<sup>3–5</sup> Metalloproteinases, namely

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ADAMTS-4 and ADAMTS-5 (A Disintegrin And Metalloproteinase with Thrombospondin motifs) also referred to as aggrecanase 1 and 2, are the key enzymes involved in the degradation of aggrecan in normal and osteoarthritic cartilage.<sup>6-8</sup> Prevention of early loss of aggrecan during the disease process is likely to contribute to a slowing in the progression of the disease and therefore to improved joint health.

Glucosamine, an amino monosaccharide, and chondroitin, a polysaccharide macromolecule, are both present naturally in cartilage. In clinical trials, consumption of either glucosamine or chondroitin sulfate (CS) has been associated with a reduction in joint pain, improvement of joint function and reduction of joint space narrowing in patients with OA.<sup>9-22</sup> However, there is conflicting evidence as to the effectiveness of such treatments.<sup>23-30</sup> *In vitro* studies using glucosamine alone have found positive effects such as decreased interleukin-1 (IL-1) induced expression of matrix metalloproteinases, MMP-3 and MMP-13, cyclooxygenase-2 (COX-2), and nitric oxide synthase, reduced aggrecan degradation, and increased synthesis of aggrecan core protein<sup>31-36</sup> while others have found a decrease in prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production induced by IL-1.<sup>36,37</sup> Studies using CS alone have shown that CS has anti-inflammatory and chondroprotective actions.<sup>38-40</sup>

The aim of this research was to determine whether glucosamine hydrochloride (GH) and CS, individually and in combination, could inhibit the cytokine-induced catabolism of aggrecan *in vitro* and to elucidate mechanisms specific to each compound or to the combination. While clinical trials and most *in vitro* studies have focused on the effects of glucosamine and CS after the appearance of the symptoms or after the induction of OA-like pathology *in vitro*, the present study focuses on the preventive effects.

## Methods

### *Viability of Chondrocyte Monolayer Cell Cultures*

Chondrocytes from the metacarpophalangeal joint of 18 month-old oxen showing no visible signs of cartilage degeneration were isolated as previously described.<sup>41</sup> Chondrocyte monolayer cultures were established in sterile 12-well plates (Corning Incorporated) at  $1 \times 10^6$  cells per well in 2 mL basal medium: Dulbecco's modified Eagle medium (DMEM) containing 50 µg/mL gentamicin and 1% Insulin-Transferrin-Selenium-X supplement (ITS, Invitrogen). Cells were incubated overnight and medium was then replaced with 2 mL basal medium with GH or CS in a range of 0 to 4 mg/mL, or with a combination of 2 mg/mL GH and 0.4 mg/mL CS. This dose combination of GH and CS was based on the formulation of an emulsified product available on the market (VeryWise Nutrition) where the proportion of the 2 compounds was kept consistent (GH:CS, 5:1, w/w). GH and CS (from bovine cartilage) were supplied by Obsidian Research Ltd. Cultures were maintained and tested for cell

viability using the thiazolyl blue tetrazolium bromide (MTT) assay.<sup>43</sup> Chondrocyte monolayer cultures were generated from articular cartilage collected and pooled from 3 different joints. Each experimental condition was replicated 3 times ( $n = 3$ ).

### *Articular Cartilage Explants and Treatments*

Articular cartilage was dissected from the metacarpophalangeal joints of 18-month-old oxen showing no visible signs of cartilage degeneration. Cartilage explants (15-60 mg wet weight) were maintained in DMEM containing 10% (v/v) fetal calf serum and 50 µg/mL gentamicin for 3 days.<sup>44</sup> Cartilage explants were then maintained individually in 1 mL of basal medium or 1 mL of basal medium with test concentrations of either GH or CS, or a combination of both. Following 24-hour incubation, human IL-1 $\alpha$  (PeproTech Inc.) was added to appropriate wells at 10 ng/mL final concentration such that the following experimental conditions were obtained and maintained for a further 24 or 72 hours:

*CS and GH individually:* (a) basal medium (control); (b) basal medium + IL-1; (c) basal medium + 0.2 mg/mL CS or GH; (d) basal medium + 0.2 mg/mL CS or GH + IL-1; (e) basal medium + 2 mg/mL CS or GH; (f) basal medium + 2 mg/mL CS or GH + IL-1

*GH and CS combination:* (a) basal medium (control); (b) basal medium + IL-1; (c) basal medium + 2 mg/mL GH; (d) basal medium + 2 mg/mL GH + IL-1; (e) basal medium + 0.4 mg/mL CS; (f) basal medium + 0.4 mg/mL CS + IL-1; (g) basal medium + 2 mg/mL GH + 0.4 mg/mL CS; (h) basal medium + 2 mg/mL GH + 0.4 mg/mL CS + IL-1.

A series of explants were treated for each condition, using cartilage taken from 3 different joints ( $n = 3$ ). Explants and culture supernatant were harvested and stored at  $-80^\circ\text{C}$  prior to analysis.

### *Measurement of Sulfated-Glycosaminoglycans*

Sulfated-glycosaminoglycan (s-GAG) release from cartilage explants into the culture supernatant was measured at 72 hours after the addition of IL-1 $\alpha$ , using the 1,9-dimethylmethylene blue (DMMB) assay.<sup>45</sup> Where appropriate, s-GAG released into the culture supernatant was calculated by subtracting the s-GAG measured in basal media containing CS at the appropriate concentration. This calculation allowed for the subtraction of s-GAG in the CS added to the basal medium prior to incubation with the cartilage explant. The effect of treatments on the release of s-GAG was expressed as micrograms (µg) GAG per milligram (mg) wet weight of cartilage explant.

### Western Blot Analysis of Interglobular Domain (IGD) Aggrecan Catabolite ARGSVIL

Western blotting with monoclonal antibody BC-3 recognizing the new N-terminal neoepitope ARGSVIL on aggrecanase-generated interglobular domain (IGD) fragments was used to assess the effect of GH and CS on the generation of the pathological aggrecan catabolic products after IL-1 $\alpha$  stimulation.<sup>46</sup> BC-3 antibody<sup>42</sup> was provided in-house (Cardiff University). Samples of culture supernatants (equivalent of 2 mg wet weight of tissue) collected 72 hours after the addition of IL-1 $\alpha$  were deglycosylated, dialyzed, and lyophilized as previously described.<sup>41,47</sup> Samples were reconstituted and separated on 4% to 12% Novex Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Invitrogen). After transfer to nitrocellulose membranes the BC-3 antibody was used to recognize the N-terminal ARGSVIL generated by aggrecanase cleavage of the bovine aggrecan core protein at the Glu373-Ala374 bond.<sup>41,42</sup> Membranes were developed using alkaline phosphatase (AP) substrate. The density of the bands was measured and expressed as the area under the signal intensity curve (AUC, in arbitrary units) using the ImageJ software (NIH Image).

### Measurement of mRNA Expression of ADAMTS-4 and ADAMTS-5

Total RNA was isolated from the cartilage explants collected 24 hours after the addition of IL-1 $\alpha$ . Explants were snap-frozen in liquid nitrogen and reduced to a fine powder using a Mikro-Dismembrator (Braun Biotech International). RNA was extracted using Tri reagent and was then isolated using Qiagen RNeasy mini-columns and reagents according to the manufacturer's protocol. RNA was eluted in sterile RNAase free water and samples were stored at -80°C. Complementary DNA (cDNA) was synthesized from 500 ng RNA by reverse transcription using Moloney Murine Leukemia Virus (MuLV) reverse transcriptase (New England BioLabs). Quantitative real-time polymerase chain reaction (PCR) amplification was performed using an Mx qPCR System (Agilent Technologies). Sequence of specific oligonucleotide primers corresponding to the genes of interest, ADAMTS-4 and ADAMTS-5, are listed in Table 1. Serial dilutions from  $1 \times 10^7$  copies/ $\mu$ L to  $1 \times 10^1$  copies/ $\mu$ L of a plasmid containing the corresponding genes were used as standards to quantify the expression of ADAMTS-4 and ADAMTS-5. Brilliant SYBR Green qPCR Master Mix (Stratagene) was used for the qPCR reactions with 1  $\mu$ L cDNA or plasmid. The amplification cycle used was as follows: initial denaturation for 30 seconds at 95°C (1 cycle), 30 seconds at 95°C followed by 45 seconds at annealing temperature and 1 minute, elongation at 72°C (40 cycles), and a final extension for 5 minutes at 72°C (1 cycle). Melting curves were evaluated for each gene and the fold change in gene expression relative to the control (no

treatment, no IL-1 $\alpha$ ) was expressed using the copy number calculated from the plasmid standards.

### Measurement of ADAMTS-4 Activity

ADAMTS-4 activity was measured in culture supernatant collected 72 hours after IL-1 $\alpha$  stimulation, using the SensoLyte 520 Aggrecanase-1 fluorimetric assay kit from AnaSpec. Results were expressed as relative fluorescence normalized to tissue wet weight (in milligrams).

**Table 1.** Primers Used for Quantitative Real-Time Polymerase Chain Reaction (PCR).

Target Gene	PCR Primer Sequence (5'-3')	Annealing Temperature (°C)	Product Size (bp)
ADAMTS-4	AAGTTCGACAAGTGC	57	215
	ATGGTG		
	TATTCACCGTTGAGG GCATAG		
ADAMTS-5	CAAATGTGGCGTCTG	57	254
	TGGAGG		
	TCCCGTTGATGTCGA TGATGG		

### Measurement of Prostaglandin E<sub>2</sub>

PGE<sub>2</sub> was measured in tissue culture supernatant collected at 72 hours after the addition of IL- $\alpha$ , using a competitive ELISA kit (R&D Systems) with standards of PGE<sub>2</sub> (0-2500 pg/mL). Results were expressed as picograms of PGE<sub>2</sub> per milligram tissue wet weight.

### Nitric Oxide Assay

Nitric oxide (NO) was measured in tissue culture supernatant collected at 72 hours after the addition of IL- $\alpha$ , using the Griess reaction and standards of sodium nitrite (0-100  $\mu$ M) from the Griess Reagent System (Promega) following the manufacturer's protocol. Absorbance was measured at 540 nm and results were expressed as nanomoles NO per milligram tissue wet weight.

### Statistical Analyses

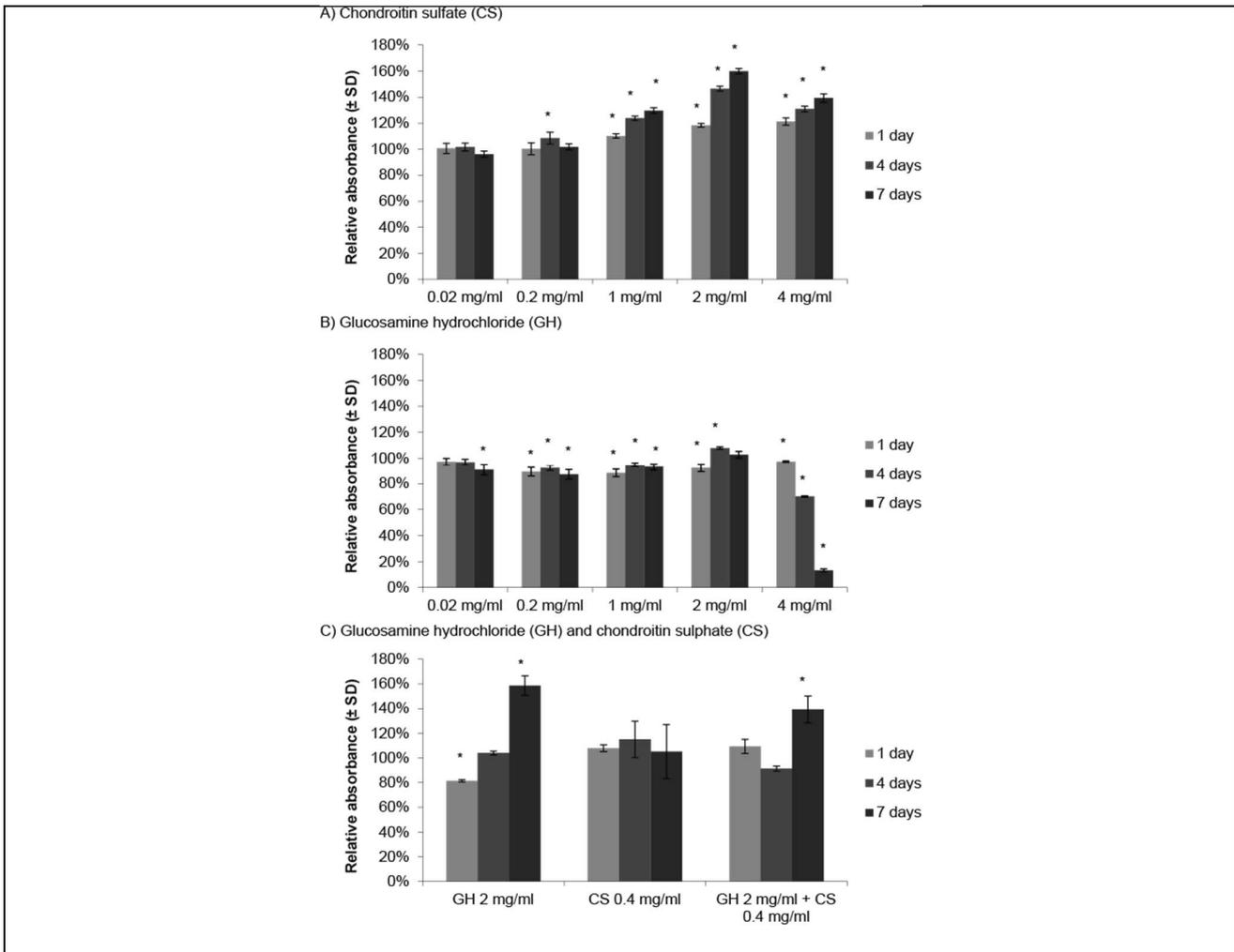
Each condition was carried out using cartilage explants (n = 3) from 3 individual joints. Analyses were carried out on the 3 replicate samples (cartilage explant or media supernatant) from each of the individual joints. Results were expressed as mean ( $\pm$  standard deviation [SD] or standard error of the mean [SEM]) of the 3 samples from the 3 joints. SD was applied to the results from cell viability experiments and SEM was applied to the results of all experiments using explants. One-

sample Student *t* test was used to compare means. Differences were considered significant at  $P < 0.05$ .

At concentrations of 0.02 to 4 mg/mL, CS had little effect or increased the metabolic activity of the chondrocytes above that seen in controls (**Fig. 1A**). GH in a range of

## Results

### *Effects of Chondroitin Sulfate and Glucosamine Hydrochloride on Chondrocyte Viability*



**Figure 1.** Cellular metabolic activity of chondrocytes with chondroitin sulfate (**A**) and glucosamine hydrochloride (**B**), assessed individually and in combination (**C**) using MTT assay. Values represent the mean relative absorbance to the control without treatment  $\pm$  SD,  $n = 3$ . One-sample Student *t* test was used. \*Indicates  $P < 0.05$  for a comparison with the control without glucosamine hydrochloride (GH) and/or chondroitin sulfate (CS).

0.02 to 2 mg/mL resulted in no major change in cellular metabolic activity except at 4 mg/mL there was a large decrease in metabolic activity, with 70% and 13% average relative absorbance after 4 and 7 days in culture, respectively (**Fig. 1B**), suggesting that high concentrations of GH have toxic effects on chondrocyte viability and/or metabolism. The combination 2 mg/mL GH and 0.4 mg/mL CS showed no detrimental effect on chondrocyte viability (**Fig. 1C**). Unlike the first experiment (**Fig. 1B**), GH at 2

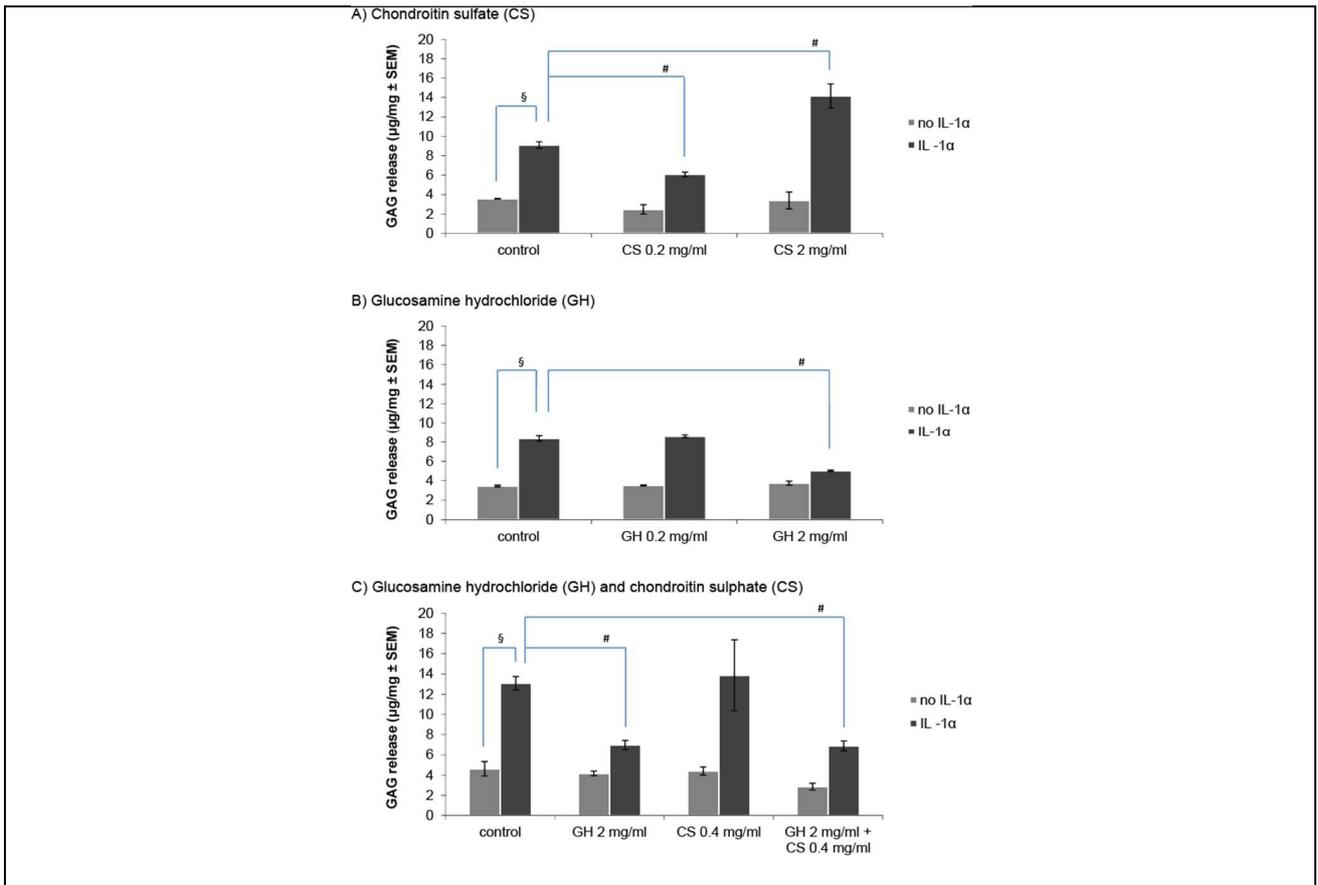
mg/mL showed an increase in chondrocyte cellular metabolic activity after 7 days in culture (**Fig. 1C**). As expected, CS at 0.4 mg/mL showed no substantial change in metabolic activity (**Fig. 1C**). Interestingly, the combination of GH and CS resulted in an increase in cellular metabolic activity following 7 days in culture, with 139% relative absorbance (**Fig. 1C**). Concentrations of GH and CS that did not appear detrimental to chondrocyte viability over a period of 7 days were used to test their

potential effects in preventing cartilage degradation induced by IL-1 $\alpha$ .

### Glucosamine Hydrochloride Alone or with Chondroitin Sulfate Suppresses Proteoglycan Degradation Induced by IL-1 $\alpha$

Stimulation of cartilage explants in culture with 10 ng/mL IL-1 $\alpha$  significantly induced the release of s-GAG

from the extracellular matrix ( $P < 0.05$ , **Fig. 2**). CS 0.2 mg/mL decreased s-GAG release induced by IL-1 $\alpha$  significantly ( $P < 0.001$ , **Fig. 2A**) while CS at 2 mg/mL increased s-GAG release further when compared with



**Figure 2.** Sulfated glycosaminoglycan (s-GAG) release measured using DMMB assay in tissue culture supernatant at 72 hours poststimulation with interleukin-1 $\alpha$  (IL-1 $\alpha$ ), in the presence of chondroitin sulfate (**A**) and glucosamine hydrochloride (**B**) individually, and with a combination (**C**). Values represent the mean GAG release in  $\mu\text{g}$  per mg of tissue  $\pm$  SEM,  $n = 3$ . One-sample Student  $t$  test was used. §Indicates  $P < 0.05$  for a comparison with the control without IL-1 $\alpha$  stimulation. #Indicates  $P < 0.05$  for a comparison with the control with IL-1 $\alpha$  stimulation.

control with IL-1 $\alpha$  ( $P < 0.05$ , **Fig. 2A**). GH 0.2 mg/mL had no significant effect on s-GAG release induced by IL-1 $\alpha$  (**Fig. 2B**), while GH at 2 mg/mL prevented s-GAG release induced by IL-1 $\alpha$  significantly ( $P < 0.05$ , **Fig. 2B**). Consistently, GH at 2 mg/mL as well as GH at 2 mg/mL + CS 0.4 mg/mL significantly inhibited the release of endogenous aggrecan loss from the matrix of articular cartilage explants stimulated with 10 ng/mL IL-1 $\alpha$  compared with control with IL-1 $\alpha$  ( $P = 0.002$  for both, **Fig. 2C**). However, 0.4 mg/mL CS alone did not inhibit this loss of aggrecan (**Fig. 2C**).

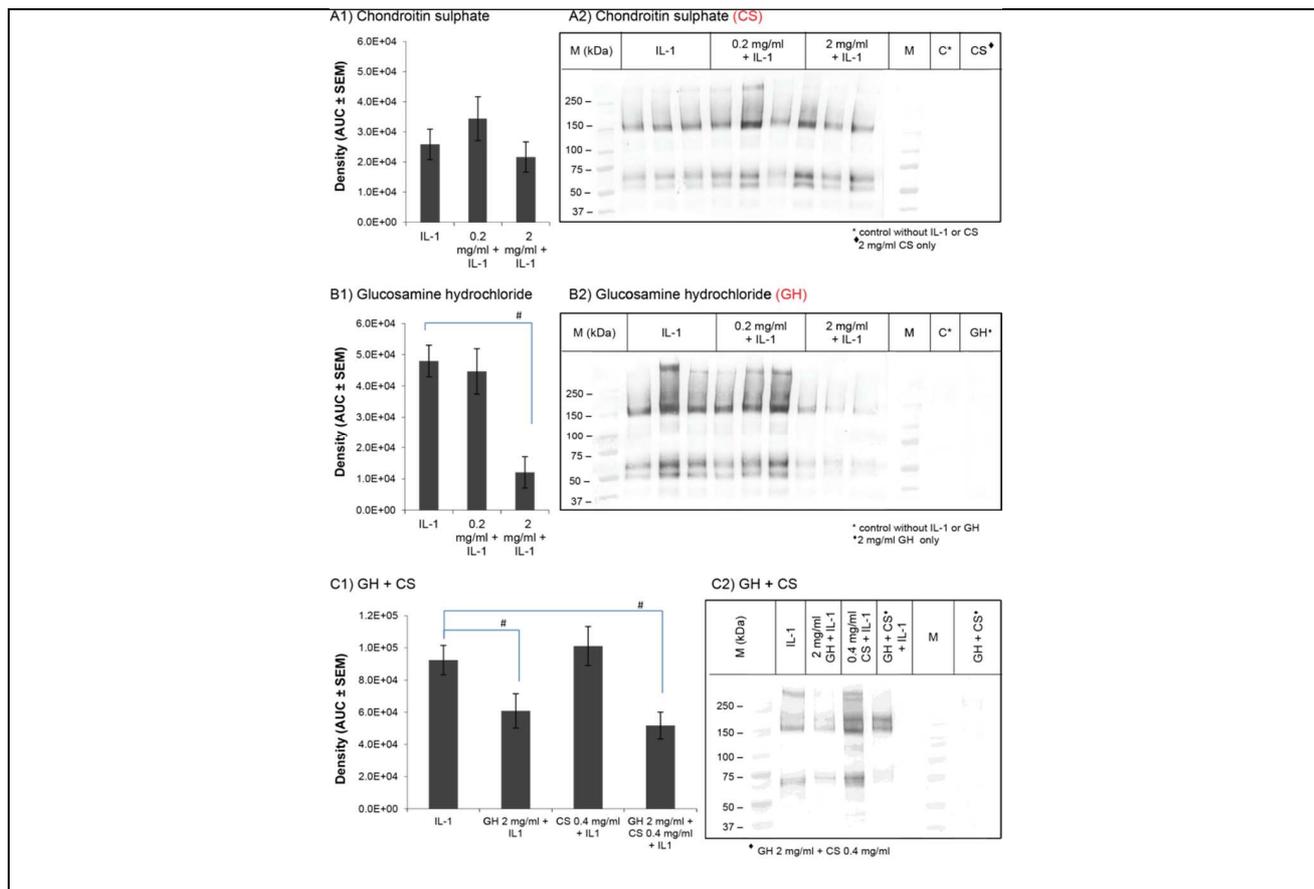
IL-1 $\alpha$ -treated cartilage explants released BC-3 positive interglobular domain (IGD) fragments into the culture media, typically generating molecular mass fragments of around 50, 60, and 150 kDa (**Fig. 3A2-3C2**; IL-1). Reduction in staining for BC-3 positive catabolites was seen in cultures pretreated with GH at 2 mg/mL (**Fig. 3B2 and 3C2**) and GH at 2 mg/mL + CS 0.4 mg/mL (**Fig. 3C2**), without further decrease with the addition of CS at 0.4 mg/mL and there was no reduction in staining for BC-3 positive bands in cultures pretreated with CS alone (**Fig. 3A2**). Densitometric analysis of samples from 3 separate

experiments showed a significant reduction of aggrecan loss induced by IL-1 $\alpha$  in GH 2 mg/mL only or GH 2 mg/mL + CS 0.4 mg/mL pretreated cultures ( $P < 0.05$ , **Fig. 3B1** and **3C1**).

### *Glucosamine Hydrochloride Suppresses IL-1 $\alpha$ -Induced Gene Expression of ADAMTS-4 and ADAMTS-5*

In control explants without treatment, the mRNA expression of ADAMTS-4 was low and stimulation with

IL-1 $\alpha$  increased the expression of ADAMTS-4 mRNA, but the increase was not statistically significant ( $P = 0.076$ , **Fig. 4A**). An increase in ADAMTS-4 mRNA expression was also seen in cultures treated with GH 2 mg/mL alone but was not statistically significant ( $P = 0.095$ , **Fig. 4A**), and this effect was negated with the addition of CS 0.4



**Figure 3.** Western bolt analysis of tissue culture supernatant using BC-3 antibody to assess the effect of chondroitin sulfate (**A**) and glucosamine hydrochloride (**B**) individually and in combination (**C**) on the level of aggrecanase products at 72 hours poststimulation with interleukin-1 $\alpha$  (IL-1 $\alpha$ ). (1) Densitometry was used to measure the overall density of the bands. Values represent the mean area under the curve (AUC) in arbitrary units  $\pm$  SEM,  $n = 9$ . #Indicates  $P < 0.05$  for a 1-sample Student  $t$  test comparison with the control with IL-1 $\alpha$  stimulation. (2) Representative BC-3 Western blots. M: Molecular mass marker.

mg/mL (**Fig. 4A**). In cultures stimulated with IL-1 $\alpha$ , in the presence of GH 2 mg/mL or GH 2 mg/mL + CS 0.4 mg/mL, a reduction of ADAMTS-4 mRNA expression was seen compared with the control with IL-1 $\alpha$  but was not statistically significant ( $P = 0.077$  and  $P = 0.138$ , respectively, **Fig. 4A**). Pretreatment with CS 0.4 mg/mL only had no significant effect on the expression of ADAMTS-4 mRNA (**Fig. 4A**).

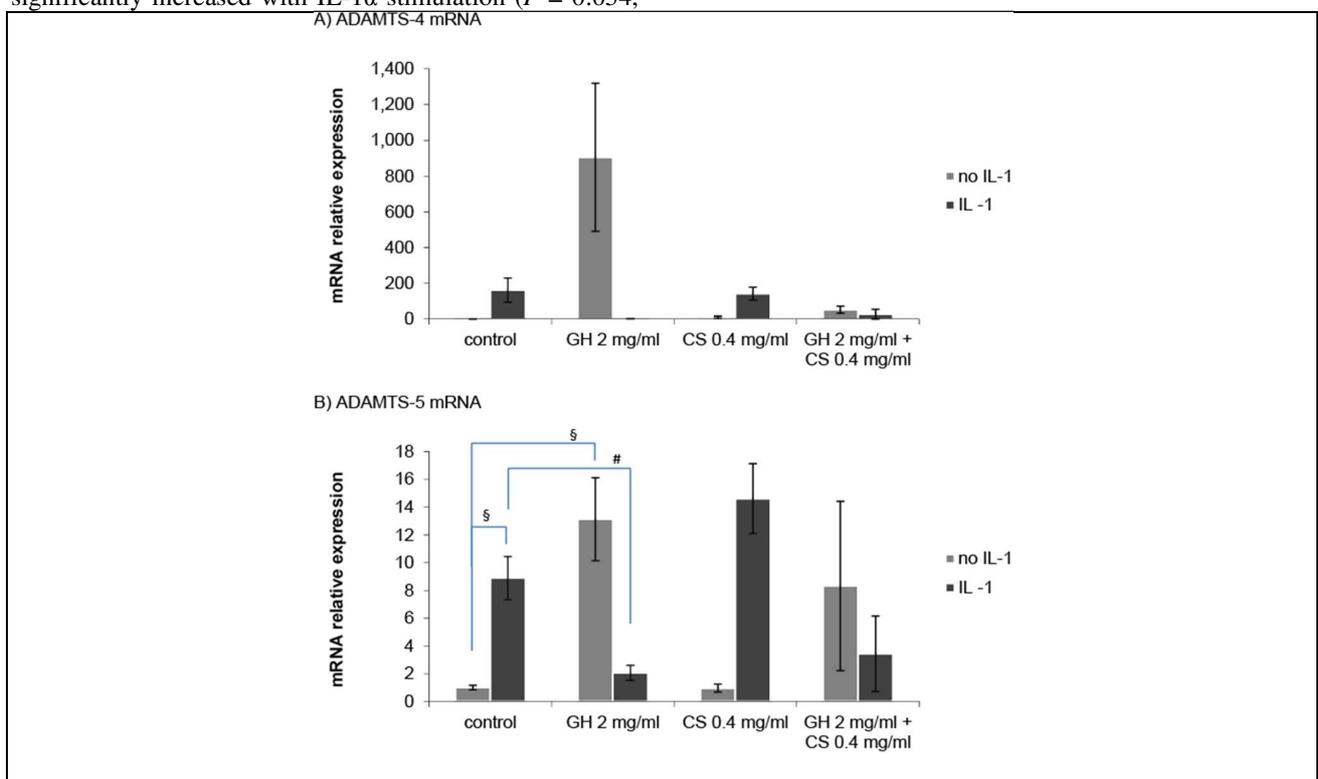
ADAMTS-5 mRNA was constitutively expressed in control cartilage explants and stimulation with IL-1 $\alpha$  significantly increased expression of ADAMTS-5 ( $P = 0.007$ , **Fig. 4B**). As observed for ADAMTS-4, the mRNA expression of ADAMTS-5 was also significantly increased in cultures containing GH 2 mg/mL alone ( $P = 0.015$ , **Fig. 4B**), and this effect was lessened with the addition of CS 0.4 mg/mL (**Fig. 4B**). Pretreatment with GH 2 mg/mL or GH 2 mg/mL + CS 0.4 mg/mL suppressed the expression of

ADAMTS-5 mRNA induced by IL-1 $\alpha$ . The effect was significant with GH 2 mg/mL alone but not with GH 2 mg/mL + CS 0.4 mg/mL compared with control with IL-1 $\alpha$  ( $P = 0.014$  and  $P = 0.157$ , respectively, **Fig. 4B**). As observed for ADAMTS-4, pretreatment with CS 0.4 mg/mL had no significant effect on the expression levels of ADAMTS-5 mRNA (**Fig. 4B**).

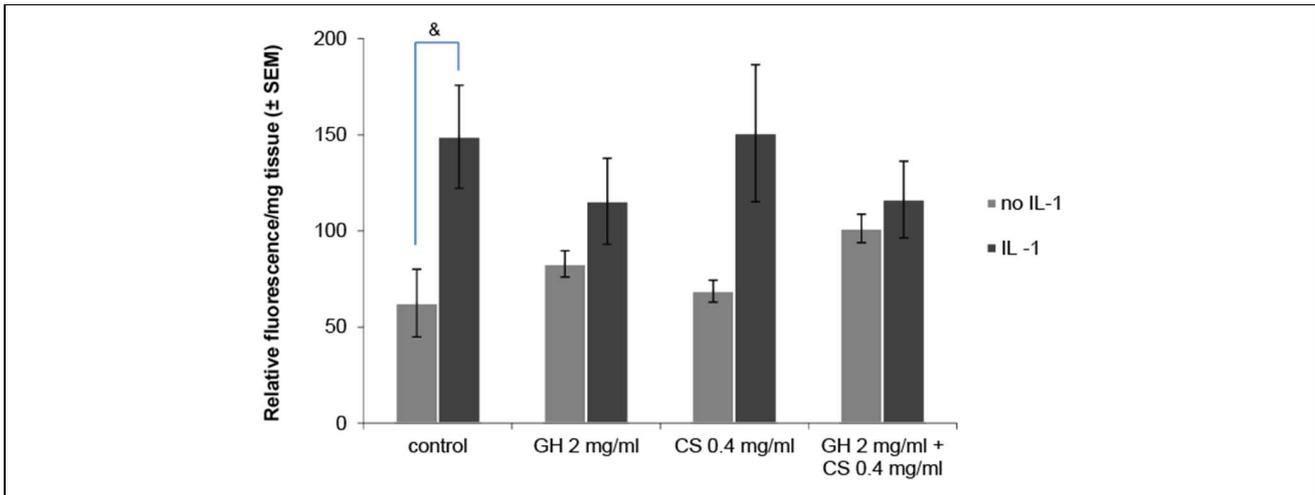
#### *Glucosamine Hydrochloride Only or with Chondroitin Sulfate Suppresses IL-1 $\alpha$ -Induced Activity of ADAMTS-4*

The mean relative activity of ADAMTS-4 in controls was significantly increased with IL-1 $\alpha$  stimulation ( $P = 0.054$ ,

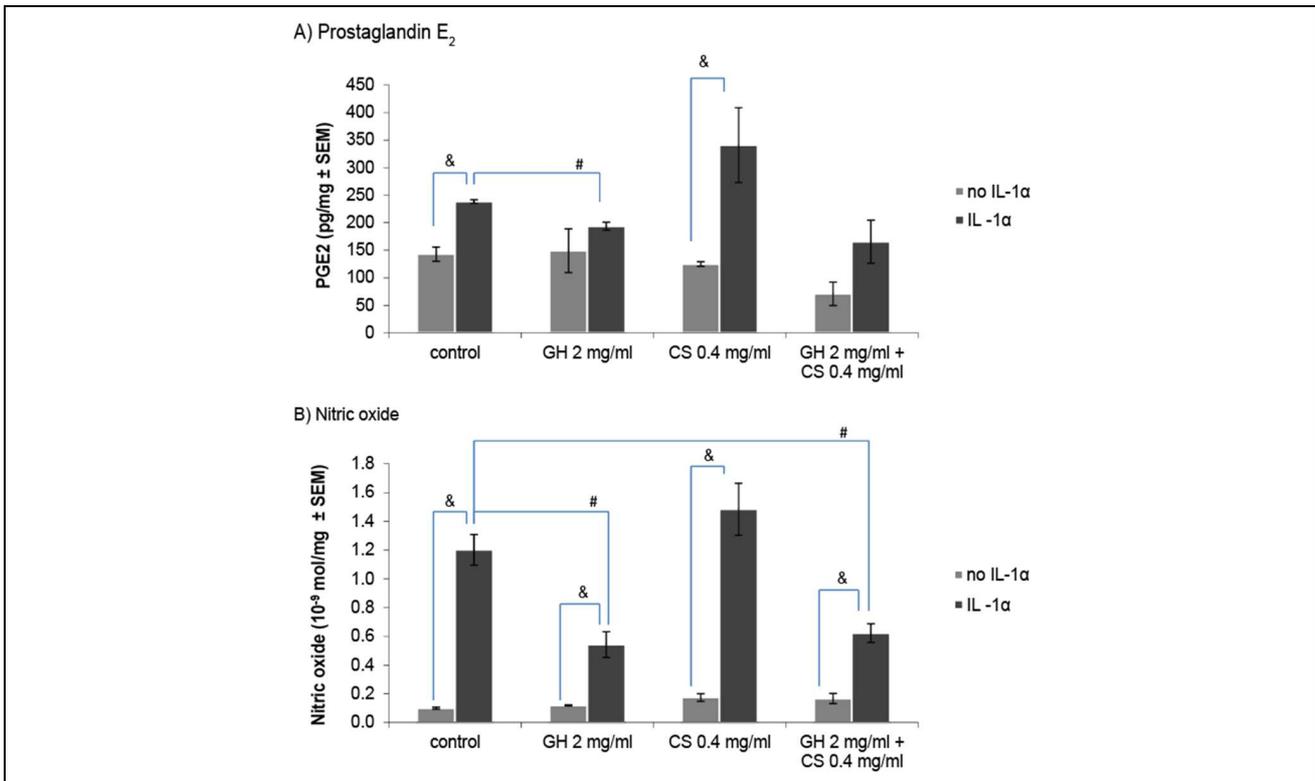
**Fig. 5**). With GH 2 mg/mL or GH 2 mg/mL + CS 0.4 mg/mL and IL-1 $\alpha$  stimulation, the mean ADAMTS-4 activity was decreased compared with that of the control with IL-1 $\alpha$  stimulation, but this was not statistically significant (**Fig. 5**). When compared with the samples without IL-1 $\alpha$  stimulation, GH 2 mg/mL or GH 2 mg/mL + CS 0.4 mg/mL prevented a significant increase of the relative activity of ADAMTS-4 induced by IL-1 $\alpha$  ( $P = 0.236$  and  $P = 0.518$ , respectively, **Fig. 5**).



**Figure 4.** ADAMTS-4 mRNA expression (**A**) and ADAMTS-5 mRNA expression (**B**) at 24 hours poststimulation with interleukin-1 $\alpha$  (IL-1 $\alpha$ ), with chondroitin sulfate (CS) and glucosamine hydrochloride (GH) pretreatment. Values represent the mean fold change in expression relative to the control without IL-1 $\alpha$   $\pm$  SEM,  $n = 3$ . One-sample Student  $t$  test. §Indicates  $P < 0.05$  for a comparison with the control without IL-1 $\alpha$  stimulation. #Indicates  $P < 0.05$  for a comparison with the control with IL-1 $\alpha$  stimulation.



**Figure 5.** Effect of glucosamine hydrochloride (GH) and chondroitin sulfate (CS) on ADAMTS-4 activity at 72 hours poststimulation with interleukin-1 $\alpha$  (IL-1 $\alpha$ ). Values represent the mean relative fluorescence per mg tissue  $\pm$  SEM,  $n = 3$ . One-sample Student  $t$  test was used. Check symbol indicates  $P < 0.05$  for comparison between no-IL-1 $\alpha$  and IL-1 $\alpha$  control samples.



**Figure 6.** Effect of glucosamine hydrochloride (GH) and chondroitin sulfate (CS) on prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production (A) and on nitric oxide (NO) production (B) at 72 hours poststimulation with interleukin-1 $\alpha$  (IL-1 $\alpha$ ). (A) Values represent the mean PGE<sub>2</sub> level expressed in pg per mg tissue  $\pm$  SEM,  $n = 3$ . (B) Values represent the mean NO level expressed in nanomoles per mg tissue  $\pm$  SEM,  $n = 3$ . One-sample Student  $t$  test was used. Check symbol indicates  $P < 0.05$  for comparison between no-IL-1 $\alpha$  and IL-1 $\alpha$  samples with the same treatment. #Indicates  $P < 0.05$  for a comparison with the control with IL-1 $\alpha$  stimulation.

### Glucosamine Hydrochloride Only or with Chondroitin Sulfate Decreases IL-1 $\alpha$ -Induced Release of Prostaglandin E<sub>2</sub> and Nitric Oxide

In control cultures or with CS 0.4 mg/mL, the level of PGE<sub>2</sub> was significantly increased by IL-1 $\alpha$  ( $P = 0.002$  and  $P = 0.034$ , respectively, **Fig. 6A**). In the presence of GH 2 mg/mL or the combination GH 2 mg/mL + CS 0.4 mg/mL, PGE<sub>2</sub> average concentration was reduced when compared with the IL-1 $\alpha$ -treated control, significantly with GH alone ( $P = 0.005$ , **Fig. 6A**).

A significant increase in NO concentration was seen in all cultures stimulated with IL-1 $\alpha$  ( $P < 0.01$ , **Fig. 6B**). In GH 2 mg/mL and GH 2 mg/mL + CS 0.4 mg/mL pretreated cultures, the increase in NO generated by stimulation with IL-1 $\alpha$  was significantly suppressed when compared with control with IL-1 $\alpha$  ( $P = 0.009$  and  $P = 0.010$ , respectively, **Fig. 6B**).

## Discussion

Our results support the hypothesis that GH can partially prevent the degradative and pro-inflammatory effects of IL-1 $\alpha$  in a dose-dependent manner, minimizing cartilage degradation by down-regulating aggrecanase activity and partially preventing inflammation by reducing the release of mediators of inflammation (PGE<sub>2</sub>, NO).

The reduction of aggrecan degradation was associated with the higher concentration of GH (2 mg/mL) but not the lower (0.2 mg/mL), suggesting that the effect is dose dependent. No significant reduction of IL-1 $\alpha$ -induced aggrecan loss was observed in explant cultures with CS and the addition of CS to GH did not further decrease the loss of aggrecan observed with GH. Our results are in agreement with other studies where IL-1 induced GAG release in culture media of porcine cartilage explants was decreased in the presence of 20 to 80 mM GH in a dose-dependent manner.<sup>35</sup> Also, the release of aggrecanase products was reduced in the presence of 1.5 to 15 mM D-glucosamine in IL-1-induced rat chondrosarcoma cells and bovine cartilage explants.<sup>31</sup> In the study of Dechant *et al.*,<sup>48</sup> only the combination of glucosamine and CS at 250  $\mu$ g/mL (1:1 ratio) reduced IL-1-induced GAG release in equine cartilage explants, while the individual compounds did not have a significant effect.

mRNA expression and activity of catabolic enzymes such as ADAMTS-4 and ADAMTS-5 are reported to be significantly increased in OA.<sup>48</sup> The ability of 2 mg/mL GH (with or without 0.4 mg/mL CS) to inhibit cartilage degradation and prevent aggrecan release in the presence of IL-1 may be partly attributed to the downregulation of the mRNA for these enzymes. In line with the previous results, 2 mg/mL GH (with or without 0.4 mg/mL CS) reduced the increase of ADAMTS-4 and ADAMTS-5 mRNA levels induced by IL-1 $\alpha$ . More specifically, although trends were similar for both

enzyme genes, a significant ( $P = 0.014$ ) decrease in IL-1 induced mRNA expression was only obtained in the presence of GH for ADAMTS-5, suggesting that ADAMTS-5 may have a role in the degradation of aggrecan and that GH may prevent its activity through a reduction in production of the enzyme that is measurable by a reduction in mRNA. However, in the case of ADAMTS-4, we were able to demonstrate that the decreased IL-1-induced mRNA expression in the presence of GH correlated with a decrease in ADAMTS-4 activity, suggesting that ADAMTS-4 has a role in the degradation of aggrecan in this culture system. Surprisingly, mRNA expression for ADAMTS-4 and ADAMTS-5 was upregulated by 2 mg/mL GH alone. These results did not correlate with ADAMTS-4 activity or aggrecan degradation suggesting that there is another level of control downstream of mRNA expression.

It has been previously shown that ADAMTS-5 is secreted as active enzyme through the removal of a prodomain by furin cleavage so undergoing posttranslational modification to become active.<sup>49</sup> Indeed, a similar concentration of glucosamine as used in the present study impaired furin glycosylation compromising its conversion to active furin, which secondarily leads to failed activation of ADAMTS-5 zymogen.<sup>49</sup> This supports our speculation that ADAMTS-5 activity is regulated posttranslationally and this process is downregulated by glucosamine. In our study, glucosamine was shown for the first time to decrease levels of ADAMTS-4 and ADAMTS-5 aggrecan degradative products, together with its effects on mRNA expression and enzymatic activity of ADAMTS-4. The correlation between levels of degradative products with the enzymatic activity of ADAMTS-4 but not mRNA expression together with the findings of McCulloch *et al.*,<sup>49</sup> allow us to speculate that glucosamine affects mRNA expression of ADAMTS-4 but downregulates its activity at a posttranslation stage. In line with our results, 5 mM GH or glucosamine-3-sulfate significantly downregulated the mRNA expression of ADAMTS-4 and ADAMTS-5 in human osteoarthritic explants.<sup>50</sup> Also consistent with our findings, in equine chondrocytes, 10  $\mu$ g/mL glucosamine reduced IL-1-induced mRNA expression of ADAMTS-4 and ADAMTS-5 while 5 to 50  $\mu$ g/mL CS had no significant effect on the expression of these genes.<sup>34</sup> In bovine cartilage explants, 5  $\mu$ g/mL GH and 20  $\mu$ g/mL CS individually or in combination suppressed IL-1-induced mRNA expression of ADAMTS-4 and ADAMTS-5.<sup>33,51,52</sup> However, the effect of glucosamine alone on ADAMTS-5 mRNA expression and the increase in expression has only been previously shown by McCulloch *et al.*,<sup>49</sup> making this a new finding and adding to the understanding of ADAMTS-4 and ADAMTS-5 regulation in OA and by glucosamine.

To complement our investigation, we tested the effects of GH and CS on inflammation by measuring levels of PGE<sub>2</sub> and NO. PGE<sub>2</sub> is one of the most characteristic prostaglandins found in joints with OA.<sup>53</sup> Limiting its synthesis may possibly lessen inflammation and pain

involved in OA. NO synthesis is also associated with cartilage degradation and a reduction of proteoglycan synthesis. Therefore, limiting NO synthesis may be critical to delay the progression of OA and may also indicate a preventive effect of treatments. In our experiments, 2 mg/mL GH alone and with 0.4 mg/mL CS reduced the increase of both PGE<sub>2</sub> and NO synthesis caused by IL-1 $\alpha$ . These results show that, in addition to preventing aggrecanase activity and cartilage degradation, GH could also prevent inflammation processes involved in OA. Our results are in agreement with studies where glucosamine or a combination with CS inhibited the release of PGE<sub>2</sub> in cartilage explant induced by IL-1 or lipopolysaccharide.<sup>36,51,52</sup> However, results of the 2 compounds on NO release or of CS alone are mixed.<sup>51,52,54</sup> As IL-1 $\alpha$  promotes the phosphorylation of signaling proteins leading to the activation of transcription factors such as nuclear factor- $\kappa$ B (NF- $\kappa$ B), and NF- $\kappa$ B stimulates the expression of iNOS and COX-2 involved in the synthesis of NO and PGE<sub>2</sub> respectively, it is possible that in our study, the regulation of PGE<sub>2</sub> and NO production stimulated by IL-1 $\alpha$  and down-regulated by GH may have occurred via regulation of NF- $\kappa$ B.

Although this study has found some positive effects of GH in an *in vitro* model of OA, the validation of the effectiveness of this nutraceutical would require confirmation using normal human cartilage. In addition, future studies would require different treatment periods with GH and CS as well as prolonged time points of evaluation for the metrics tested, that is, cartilage catabolism at the pathological IGD site and the release of inflammatory mediators. A significant drawback within the current type of *in vitro* studies is the assessment of bioavailability of either GH or CS after oral ingestion of the compounds either individually or combined. It is possible that CS may be broken down into smaller molecules, including GH, which from our results could be advantageous. Although, the absorption and bioavailability of such dietary supplements have not been fully clarified, ingested chondroitin and glucosamine are partially absorbed and it has been documented that some reach the synovial fluid and cartilage.<sup>38,55-57</sup>

In conclusion, the results of this study, using an established model system, demonstrate that GH can partially prevent cartilage degradation generated by aggrecanases and reduce the release of inflammatory molecules. In contrast, and consistent with other studies, CS did not suppress the catabolism of aggrecan and the release of inflammatory factors following treatment with IL-1.<sup>34,54</sup> However, the beneficial effects of CS may be principally related to anabolic processes, such as its role in promoting collagen and proteoglycan synthesis,<sup>58</sup> rather than the prevention of catabolic processes.

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### Ethical Approval

This study was approved by our institutional review board. **IAQ1**

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