OsteoArthritis and Cartilage (2006) **14,** 250–257

doi:10.1016/j.joca.2005.10.001

Osteoarthritis and Cartilage

International Cartilage Repair Society

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Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants[1]

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Summary

Objective: To investigate the effect of glucosamine (GlcN) in a human osteoarthritic explant model on expression of genes involved in anabolic and catabolic activities of chondrocytes.

Methods: Human osteoarthritic explants, obtained during knee arthroplasty surgery, were pre-cultured (3 days) and treated with glucosamine-hydrochloride (GlcN-HCl) or glucosamine-3-sulphate (GlcN-S) at 0.5 mM and 5 mM (4 days). RNA was isolated from the explants and real time RT-PCR was performed. Additionally, total matrix metalloproteinase (MMP) activity was measured in culture medium.

Results: Addition of 5 mM GlcN led to significant down-regulation of aggrecan (2.65–7.73-fold) and collagen type II (7.75–22.17-fold) gene expression, indicating inhibited anabolic activity. Considering catabolic activities, 5 mM GlcN significantly down-regulated aggrecanase-1 and MMP3 and 5 mM GlcN-S additionally down-regulated aggrecanase-2 and tissue inhibitor of MMP gene expression significantly. Gene expression was not significantly altered by 0.5 mM GlcN. Total MMP activity in culture medium was only significantly reduced after addition of 5 mM GlcN-HCl.

Conclusion: The effects of GlcN on gene expression in a human osteoarthritic explant model suggest that enzymatic breakdown of the extracellular matrix might be reduced by the addition of 5 mM GlcN. Additionally, restoration of already damaged cartilage is not to be expected, because gene expression of anabolic genes is also down-regulated. We suggest that chondroprotective properties of GlcN *in vivo* may be based on inhibiting further degradation due to catabolic activities, rather than on the ability to rebuild cartilage.

Key words: Glucosamine, Explant, Cartilage, Human, Gene expression.

Introduction

Glucosamine (GlcN) is becoming increasingly popular as an alternative treatment for osteoarthritis (OA). There is evidence in the literature that GlcN is equally effective or even better in decreasing pain in patients with knee OA, as compared to low dose Non-Steroidal Anti-Inflammatory Drug (NSAID) use[1,2]. Furthermore, two publications showed less joint space narrowing in people with knee OA who took GlcN compared to placebo, over a period of 3 years[3,4]. Both articles conclude that GlcN could delay the progression of knee OA. The authors speculate that GlcN might have an effect on the chondrocytes due to stimulation of anabolic activities and depression of catabolic activities. Some *in vitro* studies indeed showed a positive effect of GlcN on glycosaminoglycan (GAG) production in human chondrocyte cell cultures and the same anabolic effect was found on bovine and rat explants[5–9]. Controversially, others found no effect or even an inhibition on GAG production following GlcN addition[10–16]. Inconsistent results were also found for the effect of GlcN on catabolic activities. These *in vitro* studies used interleukin-1 (IL-1), lipopolysaccharide or retinoic acid to mimic a degenerative environment for chondrocytes in culture and thereby induce the synthesis of catabolic factors such as matrix metalloproteinase (MMP) and aggrecanase. Considering the final result of catabolic activities, i.e., degradation of GAG, studies using IL-1 as a model for degeneration found that GlcN decreased GAG degradation in some cases, but in other cases no such effect was found[6,11,12,17,18]. In the studies using lipopolysaccharide or retinoic acid as a model for degeneration, treatment with GlcN consistently led to a decreased GAG degradation in all cases[12,14,17,19–21]. Since GAG degradation finally is the result of enzymatic breakdown, several studies investigated the effects of GlcN on extra-cellular matrix degrading enzymes in a culture system using IL-1, lipopolysaccharide or retinoic acid. Most of these studies found that addition of

[1]Grant supporter: Translational research project, Erasmus MC, University Medical Center Rotterdam.

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Received 6 June 2005; revision accepted 4 October 2005.

GlcN led to less MMP activity and aggrecanase activity[6,11,16,18,20,22]. One study observed no effect on MMP activity and another study found no changes in aggrecanase activity after GlcN addition[14,21].

The majority of the *in vitro* studies testing GlcN have been performed with chondrocytes from different animal species (bovine, equine, rat, dog and mouse chondrocytes). We found only eight studies in the literature that used human chondrocytes and none of these studies used the physiologically relevant human osteoarthritic cartilage explant culture system[5–7,10,23–27].

The aim of our study was to investigate the expression of genes, involved in both anabolic and catabolic activities of chondrocytes, in response to GlcN treatment in a human OA explant model. In addition to gene expression screening we also studied whether changes in the transcription led to altered overall enzymatic activity as well.

Materials and methods

EXPLANT PREPARATION

Human osteoarthritic cartilage was obtained during total knee replacement surgery (12 patients; age 51–79 years, Kellgren and Lawrence grade 2.9 ± 0.67 (mean $\pm$ SD)). Pre-operative treatment regimes were not considered. For each patient, the experimental condition was compared to the control condition. Explants were taken from areas of macroscopically normal cartilage (with knowledge that this cartilage is affected by the disease process) from both the femoral condyles and the tibial plateau using a 4 mm diameter dermal biopsy punch, and freed from the underlying bone by dissection with a scalpel. After dissection, the explants were all pooled in a Petri dish. For each condition, six explants were randomly taken from the Petri dish and cultured in a six-well plate with 3 ml low glucose (1000 mg/l, 5.55 mM) Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY), supplemented with 10% foetal calf serum (FCS), 50 μg/ml gentamicin, 1.5 μg/ml fungizone and freshly added 25 μg/ml L-ascorbic acid-2-phosphate. The amount of glucose present in the FCS added was less than 0.5 mM and is neglected. We only started an explant culture when the amount of cartilage was enough to make at least 12 explants and we were thus able to culture experimental and one control conditions.

CULTURE EXPERIMENTS

After an initial 3-day pre-culture period (days 0–3), experimental reagents were added for 4 days (days 3–7). For the experimental conditions, culture medium (see above) was supplemented with 0.5 mM and 5 mM glucosamine-hydrochloride (GlcN-HCl; Sigma, St Louis, MO) or glucosamine-3-sulphate (GlcN-S; Sigma, St Louis, MO). As a control, culture medium and culture medium supplemented with 5 mM glucose (Gluc; Sigma, St Louis, MO; final Gluc concentration 10.5 mM) were used. Culture medium with or without supplements was refreshed once. Conditioned medium from all time points of refreshment and when the explants were harvested was stored at −20 °C for analysis. Explants were harvested after a total of 7 days of culture.

GENE EXPRESSION ANALYSIS

At harvesting, six explants were collected and snap frozen in liquid nitrogen. The wet weight per sample was determined and the frozen cartilage was then processed using the Mikro-Dismembrator S® (B. Braun Biotech International GmbH, Melsungen, Germany). RNA was extracted using RNA-Bee™ (TEL-TEST, Inc; Friendswood, TX, USA) according to manufacturer's guidelines and subsequently precipitated with 2-propanol. RNA was further purified using RNeasy Micro Kit (Qiagen, Venlo, The Netherlands) with on-column DNA-digestion. Total RNA was quantified accurately using Ribogreen™ reagent (R-11490, Molecular Probes Europe BV, Leiden, The Netherlands) according to manufacturer's instructions and 500 ng total RNA of each sample was reverse transcribed into complementary DNA (cDNA) using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). Primers and probe sets were designed using PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA, USA) to meet TaqMan® requirements and were designed to bind to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA. BLASTN search was used to ensure gene specificity of all oligo-nucleotide sequences. The primer and probe nucleotide sequences can be found in Table I. Collagen II (COL2) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assays were adopted from Martin *et al.*[28]. Both anabolic genes (encoding for extra-cellular matrix components) and catabolic genes (involved in degradation of the extra-cellular matrix) were studied. TaqMan® assays were performed on an ABI 7700 as described earlier and data are presented as relative expression normalized to GAPDH ($2^{-\Delta\Delta C_t}$) according to Mandl *et al.*[29] and Livak and Schmittgen[30].

TOTAL MMP ASSAY

The stored culture medium was used to determine general MMP activity as described earlier[31]. General MMP activity was measured using 5 μM (all concentrations are final) fluorogenic substrate TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys[Fluorescein]-Ala-Lys-NH_2) in the presence or absence of 12.5 μM BB94 (a general MMP inhibitor). Medium samples were diluted (final dilution 1/2) in MMP buffer (50 mM Tris [pH 7.5], 5 mM $CaCl_2$, 150 mM NaCl, 1 μM $ZnCl_2$, 0.01% Brij-35, 0.02% NaN_3) containing the general proteinase inhibitor (Complete, EDTA-free, one tablet in 10 ml). The MMP activity in each sample was calculated as the difference in the initial rate of substrate conversion (linear increase in fluorescence in time, expressed as relative fluorescence units per second) between samples with and without BB94 addition. Fluorescence was measured for 6 h at 30 °C using a Cytofluor 4000 (Applied Biosystems, Foster City, CA, USA). This assay is considered to represent overall MMP activity.

DATA ANALYSES

From the material obtained from each patient, one control culture and at least one experimental culture were performed. The C_t values of each control and experimental condition were normalized to GAPDH. Hereafter, each experimental condition was expressed relative to the corresponding control condition of the same patient, according to the $2^{-\Delta\Delta C_t}$ method. The resulting number of this calculation indicates whether there is an up- or down-regulation of gene expression in an experimental condition compared to its paired untreated control. Now for each subset of experimental conditions (e.g., GlcN-HCl 5 mM, GlcN-S 0.5 mM), the median of these numbers was calculated. For graphical display purposes, only the $2^{-\Delta\Delta C_t}$ values were expressed as a ^{10}LOG. Because of the sampling size we used box–whisker plots, with the box representing the middle two quartiles (25–75) and the whiskers the highest and lowest

Table I
Primer and probe nucleotide sequences of the tested genes

	Accession no.	Primer	Probe
COL2	NM_033150	Fw: GGCAATAGCAGGTTCACGTACA Rv: CGATAACAGTCTTGCCCCACTT	CCGGTATGTTTCGTGCAGCCATCCT
AGC1	NM_001135	Fw: TCGAGGACAGCGAGGCC Rv: TCGAGGGTGTAGCGTGTAGAGA	ATGGAACACGATGCCTTTCACCACGA
MMP1	NM_002421	Fw: CTCAATTTCACTTCTGTTTTCTG Rv: CATCTCTGTCGGCAAATTCGT	CACAACTGCCAAATGGGCTTGAAGC
MMP2	NM_004530	Fw: TCAAGTTCCCCGGCGAT Rv: TGTTCAGGTATTGCACTGCCA	TCGCCCCCAAAACGGACAAAGA
MMP3	NM_002422	Fw: TTTTGGCCATCTCTTCCTTCA Rv: TGTGGATGCCTCTTGGGTATC	AACTTCATATGCGGCATCCACGCC
MMP9	NM_004994	Fw: TGAGAACCAATCTCACCGACAG Rv: TGCCACCCGAGTGTAACCAT	CAGCTGGCAGAGGAATACCTGTACCGC
MMP13	NM_002427	Fw: AAGGAGCATGGCGACTTCT Rv: TGGCCCAGGAGGAAAAGC	CCCTCTGGCCTGCGGCTCA
MMP14	NM_004995	Fw: TGCCTGCGTCCATCAACACT Rv: CATCAAACACCCAATGCTTGTC	AAGACGAATTTGCCATCCTTCCTCTCGT
TIMP1	NM_003254	Fw: TGCCGCATCGCCGAGAT Rv: ATGGTGGGTTCTCTGGTG	CCAGCGCCCAGAGAGAC
TIMP2	NM_003255	Fw: ATGGTGGGTTCTCTGGTG Rv: CGGTACCACGCACAGGA	CCTGCATCAAGAGAAGTGAC
TIMP3	NM_000362	Fw: AGGACACATTTTGCCCGATG Rv: TGCACATGCTCGCCCA	CCACCCCCAGGACGCCTTCTG
ADAMTS1	NM_006988	Fw: GGACAGGTGCAAGCTCATCTG Rv: TCTACAACCTTGGGCTGCAAA	CAAGCCAAAGGCATTGGCTACTTCTTCG
ADAMTS4	NM_005099	Fw: CAAGGTCCCATGTGCAACGT Rv: CATCTGCCACCACCAGTGTCT	CCGAAGAGCCAAGCGCTTTGCTTC
ADAMTS5	NM_007038	Fw: TGTCCTGCCAGCGGATGT Rv: ACGGAATTACTGTACGGCCTACA	TTCTCCAAAGGTGACCGATGGCACTG

Fw: forward; Rv: reverse.

values, with exclusion in case of outlier variables. Total MMP activity was calculated as relative fluorescence units per second and presented as mean and standard deviation relative to the untreated control, which was set at 100%.

For statistical analysis, a Friedman test with *post hoc* Wilcoxon signed ranks test was performed on the normalized C_t values (gene expression) and the relative fluorescence units per second (total MMP activity) using SPSS 11.0.1 (SPSS Inc., Chicago, IL). A P-value ≤ 0.05 was considered to indicate statistically significant differences.

Results

ANABOLIC ACTIVITIES

Collagen type II expression (Fig. 1) was down-regulated by addition of 5 mM GlcN-HCl (7.75-fold; $P = 0.005$, $N = 10$) and by 5 mM GlcN-S (22.17-fold; $P = 0.005$, $N = 10$). Aggrecan gene expression (Fig. 2) decreased 2.65-fold by addition of 5 mM GlcN-HCl ($P = 0.012$, $N = 8$) and 7.73-fold by 5 mM GlcN-S ($P = 0.008$, $N = 9$). Gene expression was not significantly altered by addition of 0.5 mM GlcN-HCl or 0.5 mM GlcN-S, but showed a trend similar to that observed with the 5 mM concentration. No effect of addition of 5 mM Gluc was found.

CATABOLIC ACTIVITIES

ADAMTS1 [Fig. 3(A)] showed no significant alteration in gene expression for both GlcN derivatives ($N = 10$). Aggrecanase-1 (ADAMTS4) [Fig. 3(B)] was down-regulated 6.38-fold by 5 mM GlcN-HCl ($P = 0.005$, $N = 10$) and 7.83-fold by 5 mM GlcN-S ($P = 0.005$, $N = 10$). Aggrecanase-2 (ADAMTS5) [Fig. 3(C)] revealed a significant down-regulation only upon addition of 5 mM GlcN-S (median 4.51-fold; $P = 0.005$, $N = 10$).

GlcN had no statistically significant effect on the expression of the majority of the MMP genes that were tested (i.e., MMP1, 2, 9, 13 and 14). MMP3 (Fig. 4) was the only MMP that showed a significant down-regulation of gene expression in response to both 5 mM GlcN-HCl (2.02-fold; $P = 0.005$, $N = 10$) and 5 mM GlcN-S (2.66-fold; $P = 0.005$, $N = 10$).

The MMP activity assay (Fig. 5) showed an activity for 5 mM GlcN-HCl of $79.5 \pm 15.6\%$ (mean $\pm$ SD, $N = 10$) and $91.1 \pm 17.1\%$ (mean $\pm$ SD, $N = 10$) for 5 mM GlcN-S, as compared to the total MMP activity of the untreated control which was set at 100%. This reduction in total MMP activity was only statistically significant for GlcN-HCl ($P = 0.047$). For both 0.5 mM concentrations of the GlcN derivatives, total MMP activity was not reduced as compared to the untreated control.

In order to obtain a broad view of the catabolic potential, expression of the natural tissue inhibitors of MMPs (TIMPs) was also studied. The gene expression of the TIMPs (Fig. 6) was only slightly down-regulated by the addition of 5 mM GlcN-HCl and 5 mM GlcN-S. The only statistically significant alteration was a 3.07-fold down-regulation of TIMP3 gene expression after addition of 5 mM GlcN-S

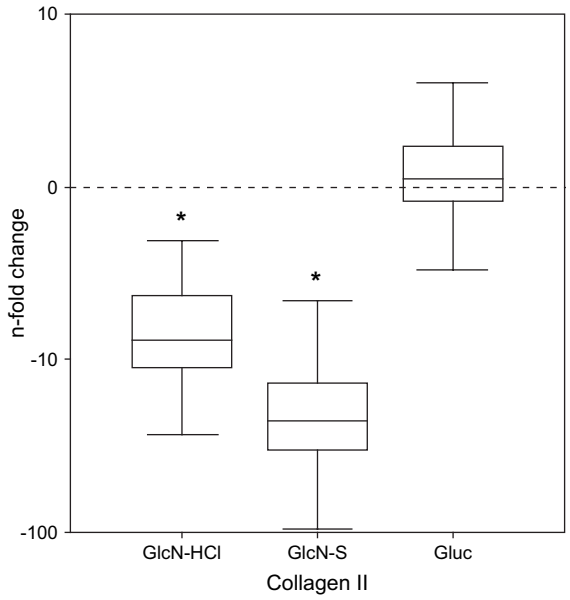

Fig. 1. Change in collagen type II gene expression in human osteoarthritic cartilage after culture with GlcN. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 5 mM GlcN-HCl ($N=10$), 5 mM GlcN-S ($N=10$) or 5 mM Gluc ($N=9$). The *n*-fold change normalized to GAPDH and relative to the untreated control (indicated by the dotted line) is displayed on the vertical axis. Negative values indicate down-regulation and positive values indicate up-regulation of gene expression. *Indicates a P-value ≤ 0.05.

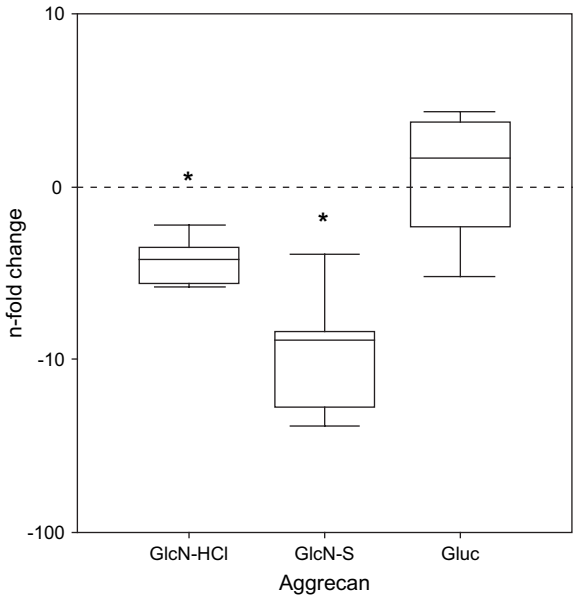

Fig. 2. Change in aggrecan gene expression in human osteoarthritic cartilage after culture with GlcN. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 5 mM GlcN-HCl ($N=8$), 5 mM GlcN-S ($N=9$) or 5 mM Gluc ($N=7$). The *n*-fold change normalized to GAPDH and relative to the untreated control (indicated by the dotted line) is shown on the vertical axis. Negative values indicate down-regulation and positive values indicate up-regulation of gene expression. *Indicates a P-value ≤ 0.05.

($N=10$). Gene expression was not significantly altered by addition of 0.5 mM GlcN-HCl or 0.5 mM GlcN-S, but showed a trend similar to that observed with the 5 mM concentration. No effect of addition of 5 mM Gluc was found.

Catabolic gene expression was not substantially influenced by 5 mM Gluc, with the exception of a 1.54-fold up-regulation of the MMP14 gene expression compared to control ($P=0.021$, $N=9$).

GLcN-HCl vs GlcN-S

To explain some of the literature differences regarding the *in vitro* effects of GlcN, we also tested if there was a difference in alteration of gene expression between treatment with 5 mM GlcN-HCl and 5 mM GlcN-S. We found that expression of ADAMTS5 ($P=0.008$, $N=9$), TIMP3 ($P=0.008$, $N=9$) and aggrecan ($P=0.012$, $N=8$) was significantly more down-regulated by 5 mM GlcN-S than by 5 mM GlcN-HCl.

Discussion

Addition of GlcN derivatives (both the sulphate and the hydrochloride salt) at a concentration of 5 mM to osteoarthritic cartilage *in vitro* leads to a down-regulation of genes that encode for anabolic (e.g., COL2 and aggrecan) and catabolic (ADAMTS enzymes and MMPs) processes. For the MMPs, this was also shown at protein activity level.

From all published studies that found an inhibition of proteoglycan production by the addition of GlcN, four were performed using a cartilage explant model[12,14,17,19]. Two of these studies used equine cartilage and the other two used bovine cartilage. In all these studies, IL-1, lipopolysaccharide or retinoic acid was used to induce a catabolic response and mimic OA. The GlcN concentrations that significantly reduced proteoglycan production varied from 5 mM to 116 mM. Lower GlcN concentrations used in these studies did not lead to a significant decrease of proteoglycan production. This is in agreement with our finding that 5 mM concentrations of both GlcN derivatives led to a significant down-regulation of aggrecan gene expression, while the 0.5 mM concentrations did not. Although it was not a subject of our investigation, it might be expected that a down-regulation of aggrecan gene expression as we have found ultimately will lead to less GAG production.

Two earlier studies using human cell clusters found no effect on COL2 production after addition of crystalline GlcN-S (Dona®) at concentrations up to 0.56 mM[5,7]. In our data we could not find a significant effect for this concentration as well. In contrast, for the 5 mM concentration we found a significant down-regulation of COL2 gene expression. In osteoarthritic cartilage, expression of MMPs, especially, MMP1, 2, 3, 9, 13 and 14, has been demonstrated[32]. In the present study we show that addition of GlcN to culture medium inhibits MMP activity. In the assay we used, only active MMPs were measured. While pro-MMP levels only provide information on the potential of the system to breakdown matrix, active MMPs are the molecular forms of the enzyme that causes the actual tissue breakdown. On gene expression level, only MMP3 (stromelysin-1) was significantly down-regulated. At this point we do not have an explanation for this. We can speculate that it probably has to do with differences in regulation of gene expression of the different MMPs. In our view, this apparent rather selective inhibition of MMP3 may even be an advantage. If GlcN would down-regulate all MMPs (as if it were a broad-spectrum MMP inhibitor) normal tissue turnover in other connective tissues

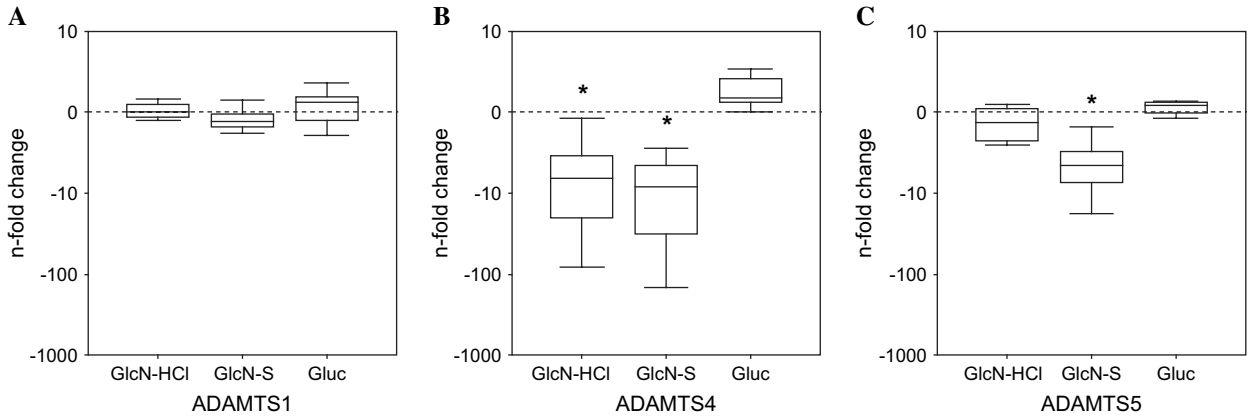

Fig. 3. Change in aggrecanase gene expression in human osteoarthritic cartilage after culture with GlcN. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 5 mM GlcN-HCl ($N = 10$), 5 mM GlcN-S ($N = 10$) or 5 mM Gluc ($N = 9$). The *n*-fold change for ADAMTS1 (panel A), ADAMTS4 (panel B) and ADAMTS5 (panel C) normalized to GAPDH and relative to the untreated control (indicated by the dotted line) is displayed on the vertical axis. Negative values indicate down-regulation and positive values indicate up-regulation of gene expression. *Indicates a P-value ≤ 0.05.

than cartilage may also be suppressed, causing unwanted side effects. The apparent selective MMP3 inhibition may block the excess proteolytic activity that is inherent to the OA disease state, due to its pivotal role in the MMP activation cascade[33], but may leave normal tissue turnover unaffected. We have found that addition of 5 mM GlcN-HCl or 5 mM GlcN-S leads to a significant down-regulation, when compared to the untreated control. This significant down-regulation is in accordance with the findings by Gouze *et al.*[16], who used rat chondrocytes in monolayer culture stimulated with IL-1β to mimic OA. The addition of GlcN in the same concentration as Gluc to the culture medium, led to significant lower MMP3 mRNA levels. In contrast, another study using human osteoarthritic cells in cell suspension culture, showed no effect on MMP3 mRNA levels with GlcN-S concentration up to 0.2 mM[6]. The latter being in agreement with our study, as we could not find a significant effect of the 0.5 mM concentrations on MMP3 gene expression either. Lower MMP activity in the culture medium as we observed upon the addition of 5 mM GlcN was also described in two studies in which GlcN was added to lipopolysaccharide treated equine chondrocytes[17,22]. Thus, our results suggest that addition of GlcN *in vitro* leads to less MMP-mediated extra-cellular matrix degradation.

Next to MMP3, our results showed that ADAMTS4 expression is down-regulated by GlcN-HCl and GlcN-S and that ADAMTS5 expression is significantly down-regulated by 5 mM GlcN-S. This confirms the results of Sandy *et al.*[11] who found less aggrecanase activity in a rat chondrosarcoma cell line and bovine explants after addition of GlcN in a dose-dependent manner. This down-regulation of ADAMTS4 and ADAMTS5 by addition of GlcN might lead to preservation of the extra-cellular matrix, since these two aggrecanases are significantly up-regulated in OA, when compared to normal cartilage[34]. Addition of GlcN had no effect on expression of ADAMTS1, which was also identified as an aggrecanase[35]. Since up-regulation of ADAMTS1 expression has not been reported in OA, its aggrecan-degrading activity seems of less importance[36].

To obtain a broad view on MMP activation, the expression of TIMPs was studied. TIMP1, 2 and 3 are important regulators of the proteolytic activity of MMPs by endogenous inhibition[37]. Addition of GlcN did not affect the expression of TIMP1 and TIMP2. Apart from inhibitory effects on MMPs, TIMP3 was also shown to be a potent endogenous inhibitor of aggrecanases and therefore protects against aggrecanase-mediated cartilage degeneration[38–40]. Since TIMP3 was significantly down-regulated by 5 mM GlcN-S, aggrecanase activity might be less inhibited as expected purely based on the down-regulation of ADAMTS4 and ADAMTS5 gene expression upon adding 5 mM GlcN-S *in vitro*.

To evaluate cell death related effects, due to toxicity of the used GlcN concentration in our study, we compared the total amount of RNA per milligram wet weight of the

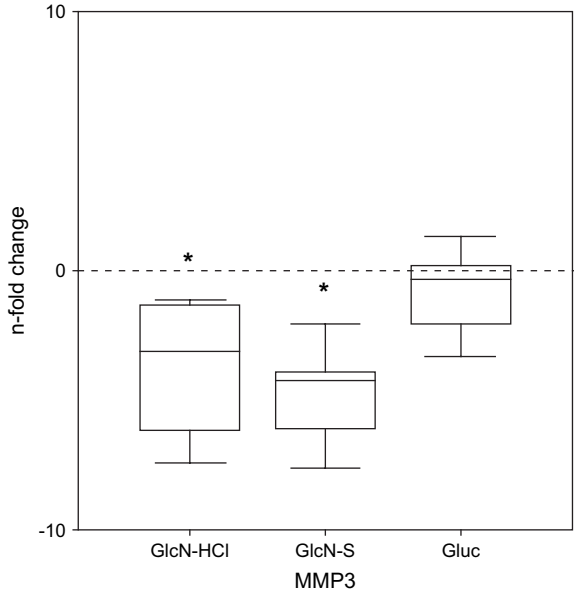

Fig. 4. Change in MMP3 gene expression in human osteoarthritic cartilage after culture with GlcN. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 5 mM GlcN-HCl ($N = 10$), 5 mM GlcN-S ($N = 10$) or 5 mM Gluc ($N = 9$). The *n*-fold change normalized to GAPDH and relative to the untreated control (indicated by the dotted line) is shown on the vertical axis. Negative values indicate down-regulation and positive values indicate up-regulation of gene expression. *Indicates a P-value ≤ 0.05.

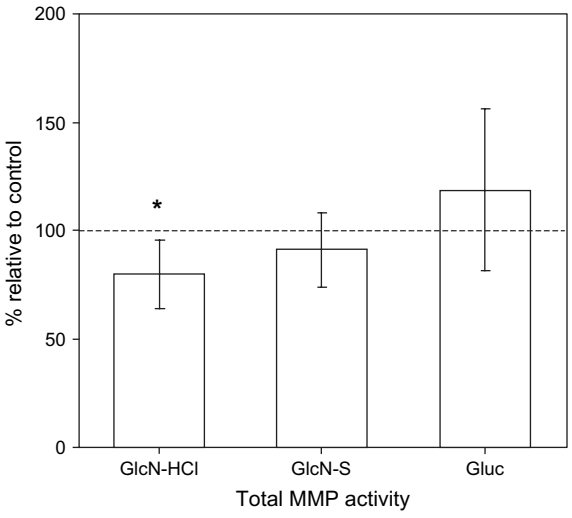

Fig. 5. Total MMP activity in the culture medium of human osteoarthritic cartilage explants after 3 days pre-culture, followed by 4 days of treatment with 5 mM GlcN-HCl ($N = 10$), 5 mM GlcN-S ($N = 10$) or 5 mM Gluc ($N = 9$). Total MMP activity is displayed as a percentage relative to the MMP activity in medium of the untreated control, which was set at 100% (indicated by the dotted line). *Indicates a P-value ≤ 0.05.

original tissue at the end of the culture period between control group and 5 mM GlcN treated conditions. No statistically significant difference was found. Furthermore, we found no significant difference in GAPDH expression between control group and all tested 5 mM concentrations. We thus believe that the effects we have found on gene expression are not based on cell death, but on actual regulatory effects of GlcN. Detrimental effects of high dose GlcN-HCl on bovine cartilage explants have been reported, but only with concentrations above 10 mM, which were twice as high as was used in this study[12]. The same group did not observe any effects on cell viability after 24 h culture with less than 10 mM GlcN-HCl.

In osteoarthritic knee-joint effusions, the mean Gluc concentration was previously shown to be 5.4 mM, with a range comparable to the reference range for serum[41]. With this in mind we decided to use medium with low glucose concentration (5.55 mM), since this comes closest to the physiological situation. In the experimental conditions, GlcN was added at concentrations equimolar with or 10 times lower than glucose concentration in medium. *In vitro*, exogenous GlcN was shown to be incorporated in newly formed chondroitin sulphate in cultured mouse chondrocytes and immortalized human chondrocytes when added at an equimolar concentration with Gluc in the culture medium[10,42]. When Gluc concentration became higher than the GlcN concentration, cells utilized less exogenous GlcN for the formation of chondroitin sulphate, but preferably incorporated GlcN that was endogenously formed from Gluc. These results suggest that not only the absolute concentration of GlcN but also the GlcN-to-Gluc ratio plays an important role in the utilization and therefore effectiveness of exogenously provided GlcN. This might explain why we did not find any significant results of the addition of 0.5 mM GlcN in culture medium with a 10 times higher glucose concentration.

When trying to translate our *in vitro* results to clinical *in vivo* applicability of GlcN, the intra-articular GlcN concentration that can be reached after administration of this food additive to the patient is of concern. Since the first studies were performed on the effect of GlcN on articular cartilage there has been debate on this topic. When GlcN-HCl is administered to horses in a single dose, intravenously as well as orally, in a dosage per kilogram bodyweight at clinically relevant levels, GlcN concentrations in the synovial fluid ranged from 9 μM to 15 μM and from 0.3 μM to 0.7 μM, respectively[43]. Although these concentrations were less than 10% of the obtained serum concentrations at the same time, GlcN was still detectable in synovial fluids 6 h after it was nearly completely cleared from the serum. Several studies with radioactive labelled GlcN administered to animals have shown that articular cartilage has the capacity to accumulate and retain GlcN[44–46]. This is confirmed in a study with six healthy male volunteers who received a single dose ^{14}C labelled GlcN-S orally, intravenously or intramuscularly[47]. These studies indicate that due to its

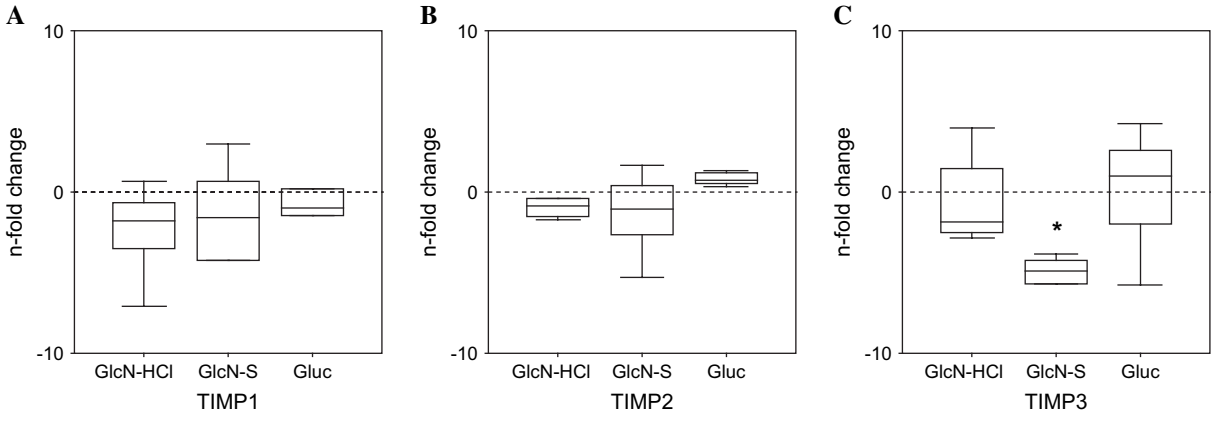

Fig. 6. Change in TIMP gene expression in human osteoarthritic cartilage after culture with GlcN. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 5 mM GlcN-HCl, 5 mM GlcN-S or 5 mM Gluc. The n-fold change for TIMP1 (panel A: GlcN-HCl, $N = 10$; GlcN-S, $N = 10$; Gluc, $N = 9$), TIMP2 (panel B: GlcN-HCl, $N = 9$; GlcN-S, $N = 9$; Gluc, $N = 8$) and TIMP3 (panel C: GlcN-HCl, $N = 10$; GlcN-S, $N = 10$; Gluc, $N = 9$) normalized to GAPDH and relative to the untreated control (indicated by the dotted line) is shown on the vertical axis. Negative values indicate down-regulation and positive values indicate up-regulation of gene expression. *Indicates a P-value ≤ 0.05. Missing whiskers in a graph are due to the exclusion of outliers, which results in the 25th and 75th percentile becoming the lowest value and the highest value, respectively.

special capacity to accumulate and retain GlcN, the concentrations of GlcN within the articular cartilage can actually be much higher than those found in the surrounding synovial fluid. It might however still be questioned whether 5 mM levels will ever reach the joint. In contrast with the previously mentioned synovial glucose concentration of 5 mM, Windhaber *et al.*[48] mentioned, based on measurements with microelectrodes, a Gluc concentration of 1 mM directly surrounding the chondrocytes. With the possible importance of the GlcN-to-Gluc ratio in mind, GlcN concentration perhaps does not have to be as high as 5 mM in order to make exogenous GlcN effective *in vivo*.

In conclusion, our results suggest that enzymatic breakdown of the extra-cellular matrix *in vitro* might be reduced by the addition of GlcN. This was suggested by down-regulation of transcript abundances and reduced MMP enzymatic activity, which preserves the cartilage matrix in the catabolic OA situation. On the other hand, on transcription level we showed that treatment with 5 mM GlcN-HCl or 5 mM GlcN-S led to a significant down-regulation of collagen type II and aggrecan expression. Whether this down-regulation of gene expression also results in less extra-cellular matrix production was not investigated in this study. However, restoration of already damaged cartilage is not to be expected. Taking this into consideration, our results indicate that chondroprotective properties of GlcN may be based on inhibiting further degradation due to catabolic activities, rather than on the ability to rebuild cartilage.

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