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Effects of glucosamine sulfate on intracellular UDP-hexosamine and UDP-glucuronic acid levels in bovine primary chondrocytes[1]

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Summary

Objective: To analyze the effects of exogenously added glucose (Glc), glucosamine (GlcN) and glucosamine sulfate (GS) on the intracellular UDP-hexoses (UDP-Hex), UDP-*N*-acetylhexosamines (UDP-HexN) and UDP-glucuronic acid (UDP-GlcA) levels in bovine primary chondrocytes.

Methods: Chondrocytes were incubated with different concentrations of Glc, GlcN and GS either in high- or low-glucose DMEM for up to 120 min to analyze the intracellular levels of UDP-Hex, UDP-GlcA and UDP-HexN by a reversed-phase high-performance liquid chromatography–electrospray ionization mass spectrometry analysis. Glycosaminoglycan (GAG) synthesis rate and aggrecan mRNA expression levels were quantified using ^{35}S-sulfate incorporation assay and quantitative real-time RT-PCR, respectively. The cells were cultivated for 2 days or 8 days before UDP-sugar analysis.

Results: Levels of UDP-HexN and UDP-GlcA were unchanged at 10 μM concentration of GS in low-glucose DMEM, while addition of 1 mM GlcN or GS in low-glucose DMEM for 10 min increased UDP-HexN level. The highest intracellular level of UDP-HexN was reached at 30 min after addition of 1 mM GS to the cells. The intracellular contents of UDP-HexN and UDP-GlcA related to UDP-Hex were higher after prolonged cultivation of chondrocytes for 8 days compared with 2-day-old cultures. Aggrecan mRNA expression and GAG synthesis remained at control level after the cells were treated with 10, 100 μM or 1 mM of GS for 24 h.

Conclusion: Physiologically relevant level of GS could not increase the intracellular UDP-HexN and UDP-GlcA levels in bovine primary chondrocyte, while longer-time culture itself appeared to increase the intracellular UDP-HexN and UDP-GlcA levels.

Key words: Glucosamine sulfate, UDP-sugar, Proteoglycan, Osteoarthritis.

Introduction

Intracellular nucleotide-activated sugars are required for cellular energy metabolism and the assembly of several cellular structures. They serve as substrates for glycosyltransferases that are involved in the synthesis of carbohydrate structures of numerous glycoproteins, glycolipids and glycosaminoglycan (GAG) chains of proteoglycans. Glucosamine (GlcN) is a basic structural unit of chondroitin sulfate (CS) and hyaluronan of cartilage proteoglycan aggregates. Therefore, oral administrations of GlcN, glucosamine sulfate (GS) or CS, either alone or in combination[1–5], are widely used as nutraceuticals to treat osteoarthritis (OA), and they are also prescribed as drugs in some European countries. It has been suggested that besides having a symptom-modifying effect they are also structure-modifying agents[4], even though a number of recent clinical trials have not uniformly shown that either GlcN or GS would be more effective than placebo in the treatment of OA[6–10].

It has been reported that GlcN or GS stimulates CS synthesis in a rat model[11] and in human OA chondrocytes[12]. Glucosamine also appeared to inhibit degradation of cartilage aggrecan[13], and inflammatory responses[14]. Although these results have raised promises of the disease-modifying effects of GlcN or GS, the cellular mechanisms behind these proposed effects are not clear. The bioavailability of GlcN and GS has been confirmed. However, the level of the sugar reaching the cartilage after oral administration is very low, approximately 10 μM[15]. Even if there would be a substantial uptake of GlcN in the digestive system its availability is remarkably declined during the passage by the portal system and through the liver. Therefore, it has been questioned whether the low level of GlcN achieved in serum and synovial fluid after oral administration would be sufficient to affect GAG synthesis in chondrocytes[15].

Glucosamine is produced intracellularly from glucose (Glc), and it is one of the basic sugar structures used for the synthesis of CS through conversion of fructose-6-phosphate to glucosamine-6-phosphate (GlcN-6-P) by the enzyme glutamine:fructose-6-phosphate aminotransferase (GFAT, Fig. 1). GlcN-6-P is then rapidly converted into the

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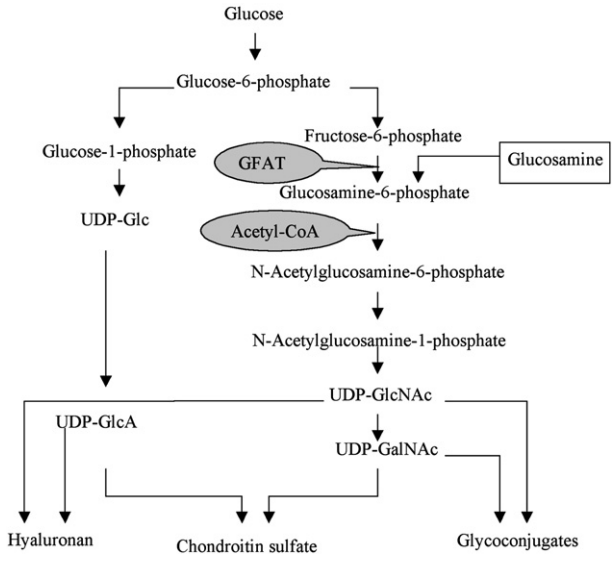

Fig. 1. The biosynthetic pathway of CS from glucose or glucosamine. UDP-Glc, UDP-glucose; Acetyl-CoA, acetyl-coenzymeA; UDP-GlcNAc, UDP-*N*-acetylglucosamine; UDP-GalNAc, UDP-*N*-acetylgalactosamine; GFAT, glutamine:fructose-6-phosphate aminotransferase; UDP-GlcA, UDP-glucuronic acid.

N-acetylglucosamine-6-phosphate (GlcNAc-6-P) by acetyl-CoA:glucosamine-6-phosphate *N*-acetyltransferase (Fig. 1). Exogenous GlcN or GS supplemented to the cultured cells can enter this metabolic pathway *via* conversion into GlcN-6-P.

N-Acetylglucosamine-6-phosphate is further converted *via* *N*-acetylglucosamine-1-phosphate into UDP-GlcNAc, and by epimerase into UDP-GalNAc. These nucleotide-activated sugars, together with UDP-glucuronic acid (UDP-GlcA), are exploited in the assembly of GAG chains (Fig. 1). CS polysaccharide chain is composed of GlcA and GalNAc, and keratan sulfate of galactose and GlcNAc, while hyaluronan consists of GlcA and GlcNAc (Fig. 1). It has been shown that supplemental GlcN can affect cellular metabolism. In adipocytes, high exogenous concentration (2 mM) of GlcN increased UDP-GlcNAc levels at 14–18 min of treatment by increasing the levels of glucose-6-phosphate or GlcN-6-P, while GlcN concentration lower than 250 μM elevated the levels of UDP-GlcNAc without increasing GlcN-6-P[16]. However, the report did not show how the levels of UDP-GlcA and UDP-GalNAc behaved or if GlcN at concentrations available after oral administration of GS have effect on the cultivated cells.

In the present study, we used a reversed-phase high-performance liquid chromatography–electrospray ionization mass spectrometry (RP-HPLC/ESI-MS) method to analyze the intracellular levels of UDP-hexoses (UDP-Hex, UDP-Glc is the predominant form, however, may contain other UDP-hexoses), UDP-GlcA, and UDP-hexosamines (UDP-HexN, the pool of the UDP-GlcNAc and UDP-GalNAc) in bovine primary chondrocytes after treatment with different concentrations of Glc, GlcN and GS (including physiologically obtainable concentrations) following 2- and 8-day-long cell cultures. Aggrecan mRNA expression and GAG synthesis were also analyzed after the cells were treated with different concentrations of GS for 24 h.

Materials and methods

MATERIALS

Glucosamine hydrochloride was purchased from Sigma (Steinheim, Germany, purity ≥99%), and sodium sulfate from Merck (Darmstadt, Germany). Glucosamine hydrochloride and sodium sulfate salts were dissolved in DMEM (sulfate concentration 0.81 mM, Euroclone, Pero, Italy) at known concentrations, thus, the added GS contained glucosamine, sulfate and chloride (but not covalently N-linked glucosamine sulfate) in the dissociated forms. Eurozol reagent was obtained from Euroclone, and [^{35}S] sulfate from New England Nuclear (Boston, MA, USA). Dimethylhexylamine (DMHA) was purchased from Aldrich (Milwaukee, WI, USA). *N*,*N*-Methyladenosine 5′-triphosphate (AppCp), uridine 5′-diphosphoglucose (UDP-Glc), uridine 5′-diphosphoglucuronic acid (UDP-GlcA) and uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) were from Sigma Chemical Co. (St. Louis, MO, USA). Methanol was HPLC grade and purchased from Labscan Ltd. (Dublin, Ireland). Water was purified and deionized in a Milli-Q purification system from Millipore (Bedford, MA, USA).

PREPARATION OF ISOLATED CHONDROCYTE

Isolated primary chondrocytes were obtained from the articular cartilage of bovine (estimated age of animals 13–22 months) femoral condyles by collagenase digestion[17]. After digestion, the isolated chondrocytes were washed twice with PBS, counted and plated in monolayer cultures at 5×10^5 chondrocyte per well of a 6-well plate. Cultures were maintained in DMEM culture medium (1.0 g of glucose/l, or 5.0 g of glucose/l) supplemented with 10% FCS (PAA, Linz, Austria), penicillin (100 U/ml, Euroclone), streptomycin (100 μg/ml, Euroclone) and 2 mM L-glutamine (PAA), and maintained in a humidified incubator at 37°C with 5% CO_2 atmosphere. The primary chondrocytes were cultured for 2 or 8 days.

TREATMENT OF CHONDROCYTE CULTURES WITH GLUCOSE, GLUCOSAMINE OR GLUCOSAMINE SULFATE

In the first set of experiment, Glc, GlcN and GS at 10, 100 μM and 1 mM concentration were added to the regular medium described above, and 1.4 ml of this medium was changed into the cell cultures after the initial 2- or 8-day-long cultivation on the 6-well plates. The cells were then cultured for 10 min in an incubator before collection of the samples for UDP-sugar analysis. The cells were also treated with 1 mM of GS in high-glucose (5.0 g/l) DMEM media for 10 min. Both experiments were repeated with cells obtained from three animals.

In the next experiment, the cells were treated with 10 μM and 1 mM concentrations of GS in low-glucose DMEM medium for 10, 20, 30, 60 and 120 min to investigate how GS affects the UDP-HexN and UDP-GlcA levels in chondrocytes. The experiment was repeated with cells from two animals. Maximal responses were observed 30–60 min after the addition of exogenous GS. Therefore, we performed additional experiments in 10 μM concentration of GS in low-glucose DMEM for 30 and 60 min. These experiments were repeated with cells from nine animals.

The cells were also treated with 10, 100 μM and 1 mM concentrations of GS for 24 h after prior 8-day-long cell cultures to analyze the GAG synthesis and aggrecan mRNA expression. These experiments were repeated with cells from 12 animals.

UDP-SUGAR ANALYSIS

After treatment, the plates were placed on ice, and the mixture of the cells and medium were collected in a 1.5 ml microcentrifuge tubes. The tubes were centrifuged at 380 × g (2000 rpm) for 5 min at 4°C, and the pellets were washed once with ice-cold 1× PBS. The cell pellet was extracted with 300 μl of cold acetonitrile with a subsequent addition of 200 μl of cold H_2O within 2 min. The samples were mixed with vortex, and then centrifuged at 16,060 × g (13,000 rpm) for 1 min at 4°C. The supernatant was transferred to a new tube, and stored frozen at −20°C for later analysis. The acetonitrile was vaporized using vacuum centrifuge and dissolved by vortex mixing in 150 μl of Milli-Q water containing 5 μM AppCp as an internal standard prior to analysis.

The sugar nucleotides were analyzed by RP-HPLC/ESI-MS measurement. Briefly, measurements were carried out with a Finnigan Surveyor MS Pump and a Finnigan Surveyor autosampler (Thermo Electron Corporation, San Jose, CA, USA) with a 50 μl loop. The RP column used was Phenomenex Gemini C18 (50× 2.00 mm, particle size 5 μm), flow rate 200 μl/min, and injection volume 45 μl. Eluents were 20 mM DMHA formate (pH adjusted to 7.0 with formic acid, A), and 80% methanol containing 2 mM DMHA formate (B). The HPLC gradient was 2 min of eluent A, 0−100% of eluent B in 3 min, 100% eluent B for 4 min, and subsequent stabilization of the column with eluent A for 4 min.

A Finnigan LTQ quadrupole ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) equipped with an electrospray ionization source was used, and the instrument was operated on a negative ion mode. The quantitation was based on multiple reaction (MRM) of the most intense fragment ions. The followed ions were *m/z* 579 → 403 for UDP-GlcA, *m/z* 606 → 385 for UDP-GlcNAc, *m/z* 565 → 323 for UDP-Glc, and *m/z* 504 → 406 for the internal standard AppCp. To create a standard curve, the sugar nucleotide standards were diluted in Milli-Q water over a concentration range of 0.5−10 μM for UDP-GlcA and UDP-Glc, and 0.5−30 μM for UDP-GlcNAc, and the chromatographic peak area ratios of the standards and the internal standard were plotted against the concentrations of standards. The values in pmols were normalized against DNA content of the samples.

The RP-HPLC/ESI-MS measurements used in the present study could not distinguish between the different UDP-Hex, or UDP-HexN because they have the same mass. Thus, UDP-Hex refers to the pool of UDP-Glc, UDP-mannose and UDP-galactose, and UDP-HexN to the pool of UDP-GlcNAc, and UDP-GalNAc.

RNA EXTRACTION AND REAL-TIME QUANTITATIVE RT-PCR

Total RNA extractions from bovine primary chondrocytes were performed with EUROzol according to the manufacturer's instruction after the cells were treated with different concentrations of GS for 24 h following an 8-day period of cell culture. The concentration of total RNA was determined by spectrophotometric measurement at 260 nm.

Total RNA (10 μg) was treated with recombinant DNase I (Ambion, Houston, TX, USA). Purity and integrity of RNA was determined by spectrophotometry, and by gel electrophoresis before reverse transcription (RT). For 20 μl of RT reaction, 0.5 μg of DNA-free total RNA from each group, random primers, and ABsolute™ MAX QRTase Blend reverse transcriptase (ABsolute™ MAX QRTase kit, ABgene, Epsom, Surrey, UK) were used. The RT reaction was incubated for 60 min at 42°C, for 10 min at 75°C, and for 2 min at 4°C in an MJ Research PTC-200 device (Waltham, MA, USA). The cDNA was stored at −20°C prior to use. The cDNA was checked with gel electrophoresis before real-time quantitative RT-PCR performance.

The levels of mRNA encoding for bovine aggrecan and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified with real-time quantitative RT-PCR employing Mx3000P™ Real-Time PCR System (Stratagene, La Jolla, CA, USA). The 25 μl of RT-PCR reaction contained 4 μl of cDNA, 12.5 μl of ABsolute™ QPCR SYBR Green Mix, 0.5 μl of ROX reference dye, and 300 nM (GAPDH) or 100 nM (aggrecan) forward and reverse primers. The conditions of real-time RT-PCR was a single cycle of enzyme activation for 15 min at 95°C, followed by 40 amplification cycles for 30 s at 95°C denaturation, 1 min at 60°C annealing, and 30 s at 72°C extension. Sequences of the primers used in real-time RT-PCR were the following: GAPDH forward primer: 5′ TTC AAC GGC ACA GTC AAG G 3′, reverse primer: 5′ ACA TAC TCA GCA CCA GCA TCA C 3′; aggrecan forward primer: 5′ CAC TGT TAC CGC CAC TTC CC 3′, reverse primer: 5′ GAC ATC GTT CCA CTC GCC CT 3′. The experiments were repeated in samples from 12 animals.

^{35}S-SULFATE INCORPORATION ANALYSIS

On the eighth cultivation day, the media were changed with the one containing the different concentrations (0, 10 μM and 1 mM) of GS. ^{35}S-sulfate (5 μCi/ml) was added into each well of the 6-well plates, then the cultures were incubated in a humidified incubator at 37°C with 5% CO_2 for 24 h. The supernatants were collected, and the amounts of incorporated and free ^{35}S-sulfate were analyzed as in our previous study[17]. The experiment was performed with the cells from 12 individual animals. The increase of sulfate concentration in the medium caused by the addition of GS was taken into account when the results were calculated on a molar basis.

STATISTICAL ANALYSIS

The statistical significance of the differences between the control and treated groups were tested using non-parametric Wilcoxon Signed Ranks Test. A difference was considered statistically significant when *P*-value was less than 0.05.

Results

EFFECTS OF GLUCOSAMINE, GLUCOSAMINE SULFATE AND GLUCOSE ON INTRACELLULAR UDP-HEXOSES, UDP-GLUCURONIC ACID AND UDP-*N*-ACETYLHEXOSAMINES

The effect of GlcN and GS on the intracellular UDP-sugar levels at different concentrations was examined first, both in low- and high-glucose DMEM. The content of UDP-HexN increased after 10 min of treatment at 1 mM concentrations of GlcN (Fig. 2A) and GS (Fig. 2B) in low-glucose DMEM, while the content of UDP-GlcA slightly decreased. Similar changes were found in the ratio of UDP-HexN/UDP-Hex and UDP-GlcA/UDP-Hex. However, no changes were found at 10 and 100 μM concentrations of GlcN and GS 10 min after addition of the sugars (Fig. 2A, B). In high-glucose DMEM, 1 mM GS did not affect the UDP-sugar

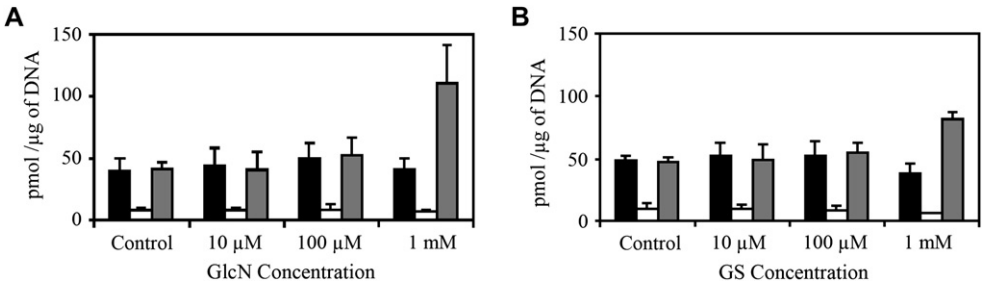

Fig. 2. The levels of UDP-Hex (black bar), UDP-GlcA (white bar) and UDP-HexN (grey bar) analyzed by RP-HPLC/ESI-MS after bovine primary chondrocytes were treated with 0, 10, 100 µM and 1 mM of glucosamine (GlcN) (A) and glucosamine sulfate (GS) in low-glucose DMEM (B) for 10 min. The experiments were performed with cell cultures from three different animals.

levels (data not shown). Glucose at 10, 100 µM and 1 mM concentrations in low-glucose DMEM did not change UDP-sugar levels (data not shown). Consequently, the following experiments were performed only in low-glucose DMEM.

The levels of UDP-sugars during a time course of 2 h were also studied. The highest level of UDP-HexN was reached at 30 min after the addition of 1 mM GS treatment (Fig. 3A). Although the absolute level of UDP-HexN appeared to decrease after 30 min, UDP-Hex level also dropped so that the ratio of UDP-HexN/UDP-Hex tended to increase during the time course of the treatment in 1 mM GS treatment. However, the level of UDP-GlcA still remained at the control levels. Previously, the maximum level of GlcN in serum was found to be in the range of 1.9−11.5 µM after oral administration of GS[18]. Therefore, we treated the chondrocytes with 10 µM concentration of GS in low-glucose DMEM for 30 and 60 min to investigate whether exogenous, physiologically relevant concentration of GS could increase the intracellular UDP-HexN and UDP-GlcA levels. The experiment was repeated with chondrocyte cultures from nine animals. However, no changes were noticed in the contents of UDP-HexN and UDP-GlcA after different time points (Fig. 3B).

Primary chondrocytes are often cultivated for several days before experimental procedures which may affect the metabolic balance of the cells. Thus, we investigated whether the duration of the culture time has impact on the responses of chondrocytes to GS. The cells were treated with 10 µM and 1 mM concentrations of GS for 30 min after prior 2- and 8-day-long cell cultures. After the 2-day culture period, the content of UDP-HexN increased ($P < 0.05$) after the addition of 1 mM concentration of GS for 30 min, while the contents of UDP-GlcA and UDP-Hex decreased ($P < 0.05$) (Fig. 4A). The ratio of UDP-HexN/UDP-Hex was significantly higher in 1 mM GS treatment for 30 min compared with that of the controls, while the ratio of UDP-GlcA/UDP-Hex was significantly decreased ($P < 0.05$). However, treatment with 10 µM concentration of GS for 30 min did not affect the contents of UDP-sugars.

After the 8-day-long culture period of chondrocytes, the content of UDP-HexN was significantly increased ($P < 0.05$) after the cells were treated with 1 mM of GS for 30 min (Fig. 4B), but no changes could be found in the content of UDP-GlcA (Fig. 4B). Like after the 2-day culture period, addition of 10 µM of GS did not have any effect on the UDP-sugar levels of chondrocytes (Fig. 4B). The level of UDP-GlcA had clearly increased during the longer culture period and, as a consequence, the ratios of the UDP-HexN/UDP-Hex and UDP-GlcA/UDP-Hex were much higher after the 8-day culture period than after the 2-day culture period.

The DNA content remained at a constant level when the cells were treated with Glc, GlcN or GS for different time periods (data not shown). Thus, none of the treatments had apparent cytotoxic effects on the cell cultures.

EFFECTS OF GLUCOSAMINE SULFATE ON GAG SYNTHESIS AND AGGRECAN mRNA EXPRESSION

In the present study, the cells were treated with 10 µM and 1 mM of GS in low-glucose (1.0 g/l) DMEM for 24 h. Parallel experiments with cells from 12 different individual animals showed that on an average GS did not increase the GAG synthesis (Table I), or aggrecan mRNA expression after normalization against GAPDH (Table I). However,

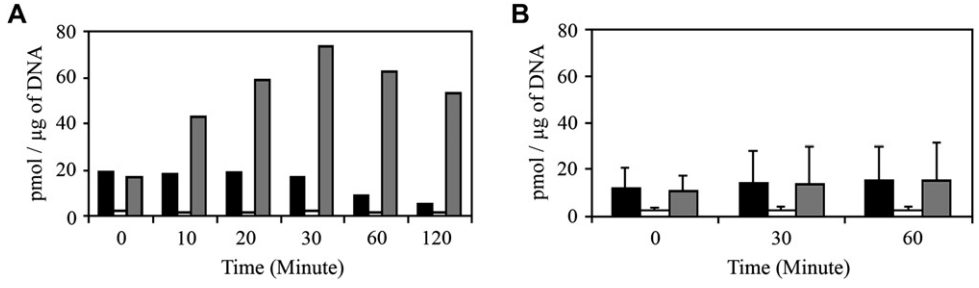

Fig. 3. The levels of UDP-Hex (black bar), UDP-GlcA (white bar) and UDP-HexN (grey bar) analyzed by RP-HPLC/ESI-MS measurements after the chondrocytes were treated with 1 mM of GS in low-glucose DMEM (A) for 0, 10, 20, 30, 60 and 120 min, and with 10 µM of GS in low-glucose DMEM for 30 and 60 min (B). The experiments were performed with cell cultures from two and nine different animals, respectively.

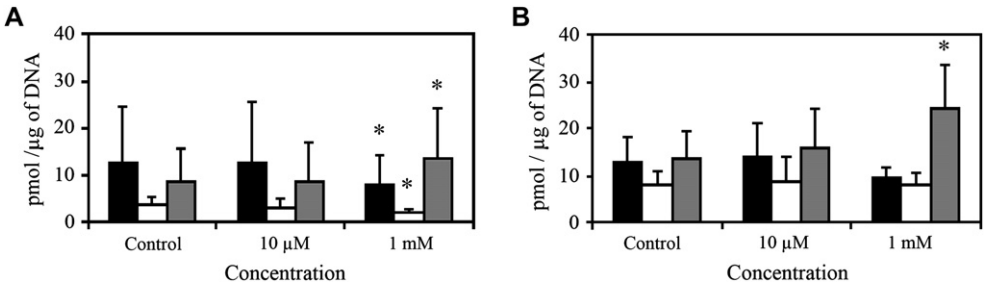

Fig. 4. The effects of different concentrations of GS on the intracellular levels of UDP-Hex (black bar), UDP-GlcA (white bar) and UDP-HexN (grey bar) analyzed by RP-HPLC/ESI-MS measurements after the bovine primary chondrocytes were treated with 0, 10 μM and 1 mM of GS for 30 min following 2-day-long cell culture period (A) or 8-day-long cell culture period (B) after cell isolation. *$P < 0.05$. The experiments were repeated with chondrocytes from six different animals.

one individual cellular isolate (1/12) revealed increase in GAG synthesis at 10 μM or 1 mM concentration of GS, respectively, and increased aggrecan mRNA expression was observed at 1 mM concentration of GS in one individual cellular isolate (1/12). The chondrocyte cultures from four different individual animals were also treated with 100 μM of GS for 24 h, nevertheless, there were no changes found in the levels of aggrecan mRNA expression (data not shown).

Discussion

The daily dose of the commercial GS product used to treat OA patients is 1500 mg, which is equal to about 1175 mg of GlcN, or 6.5 mmol. If this amount of GlcN could be totally and rapidly distributed in the extracellular and intracellular fluid of a person weighing 100 kg, the concentration of GlcN would be approximately 136 μM. However, in practice this molar concentration is reduced due to absorption, metabolism and clearance of GlcN inside the body before reaching the synovial fluid and the cartilage. Thus, the concentration of GlcN in cartilage can never attain 100 μM after oral administration of 1500 mg GS. In fact, the serum GlcN concentration was shown to reach 1.9−11.5 μM after ingestion of 1500 mg of GS[15].

Nevertheless, the mechanism of action of GlcN in the treatment of OA was initially suggested to stimulate the cartilage GAG synthesis because GlcN is a basic component of cartilage GAGs. However, it must be borne in mind that exogenous GlcN is not essential for the biosynthesis of CS in cartilage proteoglycan. Some previous studies *in vitro* and *in vivo* have shown that the addition of GlcN or GS to culture medium increases the proteoglycan synthesis by the chondrocytes[19–24]. However, the concentrations of GS or GlcN used in these studies have been so high that these concentrations cannot be reached physiologically *in vivo* after oral administration. Consequently, we investigated whether exogenous Glc, GlcN or GS would enhance CS synthesis in chondrocytes by increasing the intracellular levels of UDP-GlcA and UDP-GalNAc. No changes in the contents of UDP-HexN and UDP-GlcA could be detected at either 10 or 100 μM concentration of GS in low-glucose DMEM. It is important to notice that even when the level of UDP-HexN increased at 1 mM concentration of GlcN or GS, the level of UDP-GlcA appeared to remain at the control level or even decreased. This finding raises a question whether increase in UDP-HexN can actually accelerate GAG synthesis rate if the intracellular content of one of the two essential components required for the GAG assembly remains unchanged. Our present finding that low concentration of GS did not stimulate CS synthesis rate via increased intracellular UDP-HexN and UDP-GlcA levels is in line with the previous studies performed with mouse and human chondrocytes[25,26]. It has been calculated that the chondrocytes can produce a sufficient level of GlcN for CS synthesis from endogenous Glc, and that the exogenous GlcN will be diluted by the GlcN formed from endogenous Glc[25–27]. It was also determined that only 9% of galactosamine involved in the synthesis of CS derives from exogenous GlcN in human chondrocyte, when the concentration of exogenous radioactively labeled GlcN was 102 μM (at maximum) in mouse and human chondrocyte cultures[25,26].

Interestingly, the intracellular levels of UDP-HexN and UDP-GlcA were remarkably higher after the 8-day culture period than after the 2-day culture period, and it seems possible that the level of UDP-GlcA can be the rate-limiting factor of GAG synthesis in newly isolated chondrocytes. Low concentration of GS did not affect the UDP-sugar levels either in 2- or in 8-day-old primary chondrocyte cultures.

Aggrecan is a large CS proteoglycan present in the cartilage, which provides osmotic resistance for the cartilage to

Table I
GAG synthesis and aggrecan mRNA expression evaluated by ^{35}S-sulfate incorporation assay and real-time quantitative RT-PCR, respectively

Treatment	Relative rate of ^{35}S-sulfate incorporation (mean ± S.D.), $n = 12$	Relative quantity of aggrecan mRNA expression (mean ± S.D.), $n = 12$
Control	1.00 ± 0.0	1.00 ± 0.11
10 μM	1.16 ± 0.04	1.04 ± 0.13
1 mM	1.15 ± 0.05	1.15 ± 0.14

Bovine primary chondrocytes were treated with 0, 10 μM and 1 mM of GS in low-glucose DMEM for 24 h. The difference between the control (untreated group) and treated group was evaluated with non-parametric two related-sample tests (Wilcoxon Signed Ranks Test). No significant changes were observed ($P > 0.05$).

absorb compressive loads. Our previous study showed that GlcN could not increase GAG synthesis or aggrecan mRNA expression after the chondrocytes were treated with GlcN in low- (1.0 g/l) or high-glucose (5.0 g/l) DMEM[17]. High concentration of glucose in the DMEM media might have a profound effect on the cellular viability. However, in the present study, GS at concentration of 10 μM and 1 mM in low-glucose DMEM did not increase GAG synthesis and aggrecan mRNA expression in bovine primary chondrocyte. It further confirms our previous results[17], and the results performed with MC615 mouse chondrocytes[25] and human chondrocytes[26], even if donor-dependent variation in response to GS of chondrocytes has been shown[23]. In this study, two out of 12 different animals showed increased aggrecan mRNA level or GAG synthesis rate in GS-treated cell cultures, however, there were no statistically significant changes when the whole data were analyzed.

In conclusion, our results indicate that exogenous, physiologically relevant level of GS did not increase the intracellular UDP-HexN and UDP-GlcA levels in bovine primary chondrocytes. However, a prolonged cell culture period could affect the intracellular UDP-HexN and UDP-GlcA levels.

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