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OSTEOARTHRITIS and CARTILAGE

Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro

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Summary

Objective: This study investigated the *in-vitro* effects of a crystalline glucosamine sulfate (GS) preparation on DNA synthesis and on proteoglycan (PG) and type II collagen (coll II) production by human articular chondrocytes isolated from human osteoarthritic articular cartilage in a 3-dimensional culture system for 4, 8, and 12 days.

Materials and Methods: Human articular chondrocytes from osteoarthritic femoral heads were isolated from their matrix by collagenase digestion and then cultured in suspension. Under constant agitation, cells aggregated and formed a cluster within a few days. The effects of GS (1–100 µg/ml) on chondrocytes were determined by quantifying DNA synthesis (by measurement of [^{3}H]-thymidine uptake) as well as PG and coll II production using radioimmunoassays (RIAs) specific for coll II and to human human cartilage PG. Cross-reaction with GS in the RIAs was not detected. Moreover, PG size distribution was determined by exclusion chromatography under associative conditions to determine the association of PG monomers with hyaluronic acid (HA) to form large molecular weight PG aggregates.

Results: Under the above conditions, PG production in culture media and chondrocyte clusters was increased by GS (10–100 µg/ml). DNA synthesis and coll II production were not modified by GS. In addition, GS did not modify the physico-chemical form of PG produced by cells during culture.

Conclusions: Glucosamine sulfate did not affect DNA synthesis nor coll II production but caused a statistically significant stimulation of PG production by chondrocytes from human osteoarthritic cartilage cultured for up to 12 days in 3-dimensional cultures.

Keywords: Glucosamine sulfate, Human chondrocytes, Chondrocyte, Glycosaminoglycan, Proteoglycan, Osteoarthritis.

Introduction

Osteoarthritis (OA), a common disease that induces pain and impairs quality of life [1], is caused by a degenerative process primarily affecting articular cartilage. Articular cartilage is a connective tissue composed of chondrocytes embedded in a matrix essentially constituted by proteoglycans (PGs) and type II collagen (coll II) [2]. Cartilage is subject to anabolic processes (chondroformation) and catabolic processes (chondroresorption). In healthy chondrocytes, a dynamic equilibrium of these processes is maintained. However, articular diseases such as OA are characterized by a disturbance in this equilibrium. Catabolic processes exceed anabolic processes, leading to a net decrease of matrix [3]. Much research into the causes of this net decrease has focused on hormones, growth factors, precursors of matrix elements, and cytokines, such as interleukin-1 (IL-1) [4].

Current OA treatment includes physical, pharmacological, and surgical approaches. Pharmacological treatments have been divided into three categories [5], (1) analgesics and non steroidal anti-inflammatory drugs (NSAIDs); (2) symptomatic slow-acting drugs for osteoarthritis; and (3) chondroprotective or disease modifying drugs. The agent investigated in this study, glucosamine sulfate (GS), falls under the second category.

GS is an amino-monosaccharide and one of the basic components of the disaccharide units of articular cartilage glycosaminoglycans. Recent

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attention about the symptomatic relief afforded by GS for OA has spurred new basic scientific investigation into the agent's effects on cartilage. Clinically, GS has been developed according to the international Osteoarthritis Research Society and GREES clinical trial guidelines [6]. A comparative study has demonstrated that GS relieves knee OA symptoms to an extent similar to that of NSAIDs [7].

Research from as early as 1971 demonstrated that exogenous glucosamine increased the synthesis of glycosaminoglycans in cartilage cultures [8–10]. *In vivo*, animal pharmacokinetic studies have shown that radiolabeled GS was selectively incorporated into articular cartilage after systemic administration [11].

The aim of this study was to assess *in vitro* the effects of GS on human osteoarthritic articular chondrocytes maintained for as long as 12 days in a 3-dimensional culture. After a few days of culture in suspension, human chondrocytes (obtained from osteoarthritic femoral heads) aggregate and form a cluster. In 3-dimensional culture, chondrocytes maintain their differentiated phenotype both morphologically (round shape, surrounded by a newly synthesized matrix) and biosynthetically (by production of a matrix composed of cartilage PGs and coll II) [12]. Chondroformation was studied by measuring DNA synthesis and the production of specific components of the cartilage matrix (PGs and coll II) in the culture media (CM) and in the chondrocyte clusters (CCs).

Materials and methods

CULTURE OF HUMAN OA CHONDROCYTES

Using the method of Bassleer *et al.* [12], cartilage was taken from three human osteoarthritic femoral heads (mean age: 72 years) immediately after surgery for total hip prosthesis. Macroscopically, the collected cartilage was not altered, but histological study of representative samples showed typical osteoarthritic changes such as the presence of chondrocyte clusters, loss of metachromasia, and fibrillation. Cartilage was digested with 1 mg/ml clostridial collagenase (Boehringer Mannheim, GmbH, Germany) in a carbonate–bicarbonate buffer (120 mM $CaCl_2$, 5 mM KH_2PO_4, 25 mM $NaHCO_3$, 2 mM glucose and 30 mM HEPES, pH 7.4) for 24 h. Chondrocyte viability was examined by trypan blue exclusion. After six successive washings and centrifugations, the cells were suspended in glass flasks (Sovirel flask) at a density of 10^6 cells/2 ml culture medium. The culture medium was DMEM-Dulbecco's Modification of Eagle's Medium (ICN, Brussels, Belgium) containing Ultroser G 1% (Gibco, Ghent, Belgium) and 50 µg ascorbate/ml. Cultures were maintained on a gyratory shaker at 37°C in a 95% air–5% CO_2 atmosphere.

TREATMENT OF CHONDROCYTE CULTURES WITH GS

The preparation of crystalline GS (MW:573.30) (Dona®, Rotta Research Laboratorium, Monza, Italy) consists of two molecules of glucosamine combined with one molecule of sulfate. Crystalline GS contains 79.6% (w/w) GS (M.W. 456.42), i.e. 62.5% GS base (M.W. 179.17). Crystalline GS (0.56×10^{-5}, 5.6×10^{-5}, 5.6×10^{-4} M, i.e. 0, 1, 10, 100 µg/ml GS base) was present in the culture media (CM) for 4, 8, or 12 days; CM was renewed each 4 days. In other experiments, chondrocytes were further cultured in the absence of GS from days 12 to 16. These periods of culture are based on previous experiments demonstrating varied effects of GS on chondrocyte biosynthetic activities. For each concentration of GS and for the corresponding controls, four flasks were used; each flask contained a chondrocyte cluster obtained from 10^6 isolated chondrocytes.

PG and coll II produced by human chondrocytes were assayed in conditioned CM and CCs employing specific radioimmunoassays (RIA). There was no cross-reaction between PG synthesized by the chondrocytes and GS present in the cultures, even at 10 mg/ml, indicating that the RIA did not detect GS epitopes. At the end of the various culture periods, the CCs were prepared for fluorimetric DNA assay, for measurements of [^{3}H]-thymidine incorporation and for RIAs as follows: CCs were washed three times with phosphate-buffered saline (PBS: PO_4 0.05 M, NaCl 0.15 M, pH 7.4) and homogenized in PBS containing a mixture of protease inhibitors [13], sodium azide 6.7×10^{-3} M, and superoxide dismutase (200 U/ml) by ultrasonic dissociation (power: 200 W/cm^2) for two 30-sec intervals at 4°C. The mixture of protease inhibitors included 0.1 M 6-amino-hexanoic acid, 0.001 M EDTA, 0.05 M benzamidine chlorhydrate, and 5×10^{-8} M trypsin inhibitor.

ANALYSIS OF [^{3}H]-THYMIDINE INCORPORATION

Human chondrocytes were incubated in media containing GS (0–100 µg/ml) during the entire culture duration (4, 8, or 12 days) and 2 µCi/ml (5 Ci/mM) methyl [^{3}H]-thymidine (Amersham, Brussels, Belgium) during the last 24 h of the experiment (i.e. from day 3–4, from day 7–8, or from

day 11–12). In order to remove non-incorporated [^{3}H]-thymidine, the CCs were washed repeatedly with PBS containing unlabeled thymidine (100 μg/ml) (3×20 min) and transferred to PBS containing protease inhibitors as described above. At that point, CCs were homogenized by ultrasonic dissociation for 2×30 seconds at 4°C (power 200 W/cm^2) in the same PBS buffer described above. Incorporated radioactivity was measured with a beta-counter (Packard tri-Carb) as described previously [14, 15].

determination of PG content in CM and CC

PGs released into CM or present in the CC were radioimmunologically assayed as described in detail by Gysen and Franchimont [16]. For the assay, human cartilage PGs were extracted according to the methods of Roughley *et al.* [17] and Bayliss and Venn [18]. The human cartilage PG antiserum was raised in rabbits as described by Vaitukaitis *et al.* [19]. The incubation solution consisted of 0.1 ml tracer solution (15 000–20 000 cpm ^{125}I-PG labeled by the chloramine-T method) [20]; 0.1 ml anti-PG antiserum diluted 1:5000 in PBS (as above) with 5 g/L BSA (incubation buffer); and 0.2 ml serial dilutions of either conditioned CM, cluster extracts, or unlabelled antigen ranging from 0.1 to 500 ng/tube (reference curve). After 4 days at 4°C, the labeled PG-antibody complexes were separated by double precipitation [21]. The analytical sensitivity of the RIA was 0.6 ng/tube, and intra- and inter-assay coefficients of variation were less than 10% and 20%, respectively, along the linear range of the curve. Separate glycosaminoglycan digestion experiments and the absence of cross-reactivity with isolated glycosaminoglycans indicated that the antibodies were specifically directed against the antigenic determinants of the PG core protein. There was no interference of coll II, fibronectin, chondroitin sulfate, GS, or hyaluronic acid with the assay [16].

gel filtration chromatography of PGs produced by chondrocytes

The PGs in CM and in CCs were chromatographed on a Sepharose Cl-2B column. CM were directly chromatographed on the column. PGs were extracted from CC after various culture durations (4, 8 or 12 days) employing 4M-guanidine HCl, (0.2 M sodium acetate, pH 5.8), containing protease inhibitors [13], and maintained for 24 h at 4°C. The cluster extracts were then dialyzed against distilled water and 0.5 M sodium acetate, pH 7.0. The extracts employed for chromatographic analyses were not submitted to sonication. For analytical purposes, a column (0.8×60 cm) was packed with Sepharose Cl-2B. The column was equilibrated with 0.05 M sodium acetate, pH 7.0, containing the protease inhibitors previously described [13]. Flow rate was 5 ml/hour and 1 ml fractions were collected. PG aggregates (MW > 20.106) and [^{3}H]-methyl thymidine were used to determine the excluded (Vo) and total (Vs) column volumes, respectively. PG immunoreactivity was determined in each elution fraction [16].

coll II assay

Coll II was assayed radioimmunologically as described by Henrotin *et al.* [22]. In this assay coll II extracted from human articular cartilage [23] is used as immunogen, tracer, and reference preparation. A polyclonal antiserum was obtained in guinea pigs. The sequential assay first utilized a mixture of 0.1 ml of incubation buffer (PBS; 0.3 M NaCl, pH 7.4), 0.1 ml of coll II reference solution (1 to 500 ng), CM or cluster extracts, and 0.1 ml guinea pig antiserum (1:5000). Then 0.1 ml ^{125}I-labelled coll II, which was iodinated using the Iodogen method [24] and diluted to obtain 20 000 cpm, was added. After 18 h at 4°C, the labelled PG-antibody complexes were separated by double precipitation [21]. Cartilage PG, fibronectin, laminin, type I or type III collagen, GS and glycosaminoglycans did not interfere in the assay. The assay's detection limit was 20 ng/ml. Intra- and inter-assay coefficients of variation were 8 and 15%, respectively.

DNA assay

Cluster DNA content was assayed according to the fluorimetric method of Labarca and Paigen [25]. This method is based on the principle of a fluorescent emission when the reactive fluorochrome–bis–benzimidazol (Calbiochem-Behring, La Jolla, CA, USA; Hoechst dye 33258) is bound to DNA.

statistical analysis

The results were expressed as PG amounts in CM or CC per μg of DNA. Total PG and coll II production was also calculated by adding the amounts present in CM and in the corresponding clusters. The mean ± SD of each variable ([^{3}H]-thymidine incorporation, and coll II and PG amounts in CM or in CC) was calculated.

Table I
PG content of culture media from control or GS treated chondrocytes

Culture period (days)	Control	GS (1 μg/ml)	GS (10 μg/ml)	GS (100 μg/ml)
0–4	19.5 ± 2.0	22.6 ± 0.6	27.3 ± 0.9*	30.1 ± 2.6*
4–8	3.7 ± 0.1	3.7 ± 0.5	5.0 ± 0.4*	5.8 ± 0.2*
8–12	1.0 ± 0.1	0.9 ± 0.1	1.3 ± 0.1*	1.5 ± 0.1*

Isolated human OA articular cartilage chondrocytes were cultured as described in Materials and Methods. The amounts of PG were measured in culture medium (μg/μg DNA 96 h) for each period of culture in samples from chondrocytes incubated without GS (controls) or in presence of GS (1, 10, 100 μg/ml) (*:$P < 0.025$ v control; U-test from Mann–Whitney).

Comparison of mean values was performed using the Mann–Whitney U-test.

Results

[^{3}H]-thymidine incorporation

DNA synthesis, as measured by [^{3}H]-thymidine uptake, was similar in the four groups examined (controls, 1, 10 or 100 μg GS/ml).

PG and coll II production

Tables I and II show the amounts of PG measured in CM and in CC, as a function of culture duration. In absence of treatment, these results are similar to the results obtained with human osteoarthritic chondrocytes cultured under similar conditions in previous studies [12]. GS (10 or 100 μg/ml) induced a significant increase of the amount of PG measured in CM (Figure 1) and in CC (Figure 2) at each period of the culture. The total PG production (sum of the amounts of PG measured in CM and in the corresponding cluster) after 12 days of culture demonstrated that GS increased total PG production significantly (38 and 54% for 10 and 100 μg/ml GS, respectively). However, 96% of the newly synthesized PG was released in the CM while only 4% remained in CC. When cells were cultured during the first 12 days in the presence of GS (10 or 100 μg/ml) and in the absence of GS during the following four days, a significant increase of the amount of PG in CC and CM continued to be observed in cultures previously incubated with 100 μg of GS, as compared to untreated controls (Table III). Comparisons between cells treated with GS for 12 days with continued culture through 16 days without GS and untreated controls demonstrated a significant increase in PGs of the cells treated with GS until day 12 during days 12–16 (following removal of GS at the day 12) as shown in Table III. GS did not affect coll II production in CM and CC, as compared to untreated controls.

Gel filtration chromatography of PG from CM or CC

Chromatographic profiles of PG extracted from CC after 4, 8, or 12 days of culture in the presence of 0, 10, or 100 μg GS/ml performed under associative conditions showed that GS (10 or 100 μg/ml) did not modify the PG physico-chemical forms in CC, as compared to untreated controls (data not shown). Similarly, GS (10 or 100 μg/ml) did not affect the PG physico-chemical forms in CM, as compared to untreated controls (data not shown). In samples from both control and GS-treated cultures the majority of PG appeared in the region of intact PG complexes and PG monomers. Measurements of PG content in the chromatographic fractions corroborated the results shown in Tables I and II since greater amounts of PG were present in the fractions obtained with equal volume samples from GS-treated cultures compared to untreated cultures (data not shown).

Table II
PG content of chondrocyte clusters from control and GS treated cultures

Period of culture (days)	Control	GS (1 μg/ml)	GS (10 μg/ml)	GS (100 μg/ml)
0–4	394 ± 42	420 ± 56	513 ± 52*	558 ± 4*
0–8	504 ± 57	584 ± 99	660 ± 69*	814 ± 10*
0–12	1050 ± 64	1100 ± 49	1335 ± 97*	1481 ± 12*

Isolated human OA articular cartilage chondrocytes were incubated as described in Materials and Methods. The amounts of PG were measured in chondrocyte clusters (ng/μg DNA) for each period of culture, in samples from chondrocytes incubated without GS (controls) or in presence of GS (1, 10, 100 μg/ml) (*:$P < 0.025$ v control; U-test from Mann–Whitney).

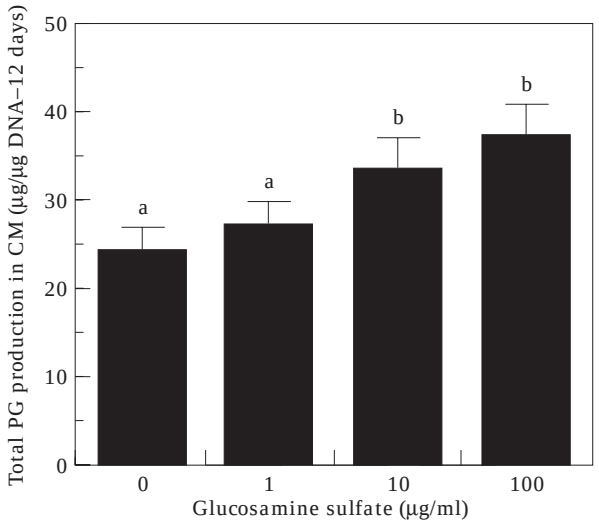

FIG. 1. Total amount of PG measured in culture medium (CM)(µg/µg DNA) after 12 days of culture in chondrocytes cultured under control conditions or in the presence of GS (1, 10, 100 µg/ml). Different letters (a ≠ b) indicate significantly different values ($P < 0.05$).

Discussion

This study demonstrates that treatment of human articular cartilage chondrocytes with GS (10–100 µg/ml) *in vitro* induces a significant increase of PG amounts measured in clusters and in CM. Moreover, when GS (100 µg/ml) was added to the chondrocytes during the first 12 days of culture and removed from the CM for days 12–16, a significant increase of the amount of PG in CM and in CC was still observed at day 16 of culture.

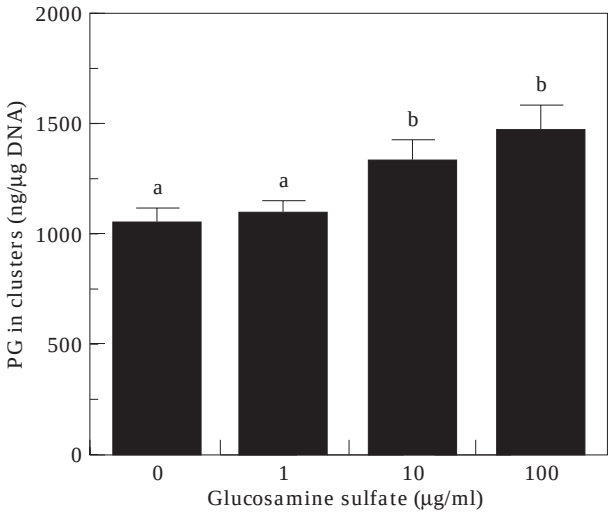

FIG. 2. Amount of PG measured in chondrocyte clusters (CC) (ng/µg DNA) after 12 days of culture in chondrocytes cultured under control conditions or in presence of GS (1, 10, 100 µg/ml). Different letters (a ≠ b) indicate significantly different values ($P < 0.05$).

Thus the stimulatory effect persisted at least 4 days after the withdrawal of GS.

The PG cluster content at day 12 was approximately 1 µg/µg DNA. The total PG production in CM (sum of the PG amounts measured in CM during the 12 days of culture) was $\pm$ 25-fold the PG amounts measured within the clusters. Indeed, only 4% of PG produced by chondrocytes remained in the clusters, surrounding the cells. This may be explained by the fact that a large cluster surface was in direct contact with the nutrient medium (2 ml). Moreover, the cluster volume (2–3 mg fresh weight/µg DNA) was substantially smaller than the 2 ml CM volume.

Our results showing a GS-induced increase of PG production indicate that GS caused a positive balance between PG synthesis and PG degradation. Several former experiments have reported that GS was able to stimulate the $^{35}SO_4$ uptake (marker of GAG synthesis) *in vitro* [8, 9] and *ex vivo* [10]. This study suggests that GS stimulates PG core protein production, because in the RIA the PG antiserum is directed against the human cartilage PG core protein [16].

GS appears to affect specifically the synthesis of monomeric PG capable of assembling into large molecular weight PG aggregates, since lower size PG identified by gel filtration on Sepharose Cl-2B only represent a minor portion of the total immunoreactive PG. These findings are supported by the recent work of Jimenez *et al.* [26], which demonstrated that in normal human adult chondrocytes *in vitro*, GS increases aggrecan protein mRNA. In addition, Hellio *et al.* [27] recently observed that GS induced an increase of protein synthesis in human OA chondrocytes *in vitro*. Based on these results, we suggest that GS acts through an increase of sulfated GAG synthesis, which, in turn, induces PG core protein production by human chondrocytes. Such an explanation is supported by the fact that GS stimulates $^{35}SO_4$ uptakes in rat articular cartilage *in vitro*. Similar results were also achieved in *ex vivo* experiments following treatment of animals for 14 days with oral GS [9, 28]. Analyzed together, these experiments suggest that GS is able to stimulate GAG production and the synthesis of PG.

Sulfate is an essential substrate in the synthesis of cartilage GAG and its availability is a limiting factor in this synthesis [29–33]. In other experimental conditions, *in vitro* studies have demonstrated that GS is a substrate for GAG synthesis. For instance, Roden *et al.* [34], showed that GS stimulates the $^{35}SO_4$ uptake by cartilage while it inhibits the uptake of labeled glucose (which is usually used for these syntheses). A possible

Table III
Total PG content of chondrocyte cultures following withdrawal of GS

Previous treatment with GS from day 0–12 16 (ng/μg DNA)	PG in CC at day 16 (ng/μg DNA)	PG in CM from day 12–
0 μg/ml	420 ± 60	784 ± 89
10 μg/ml	440 ± 50	796 ± 78
100 μg/ml	580 ± 70*	985 ± 113*

The conditions of culture were as described in Materials and Methods. The amounts of PG were measured in culture medium (CM) and in chondrocyte clusters (CC) (ng/μg DNA) between day 12 and day 16 in control cultures and in cultures previously treated from day 0 to day 12 with GS (10, 100 μg/ml).
(*: $P < 0.025$ v control; U-test from Mann–Whitney).

explanation is that GS enters a metabolic pathway that is more favorable for the chondrocytes and requires less energy than the one starting from glucose. GS may be then transformed into its derivatives N-acetyl-glucosamine or galactosamine, which are precursors of hyaluronic acid, chondroitin sulfate and keratan sulfate, the cartilage GAG [35]. GS is likely to be incorporated in the cytoplasm of the chondrocytes, integrated into GAG and PG and deposited in the matrix. This metabolic process was illustrated previously using [^{14}C]-glucosamine incorporation in cartilage tissue, as well as in human chondrocytes in three-dimensional culture [12].

Many factors and substances modulate the biosynthesis and the ultimate physico-chemical forms of the PG macromolecule, thus altering the physical properties of articular cartilage [36–42]. Although there were no detectable differences between control or GS treated samples, Sepharose Cl-2B profiles of PG in CM and PG extracted from CC demonstrate that there is more immunoreactive material corresponding to the complexes PG-HA-link protein in CC than in CM. Conversely, there was a greater proportion of small PG and PG fragments in CM than in CC. These data indicate that PGs in clusters are functionally capable of binding to HA (hyaluronic acid) stabilized by link protein, whereas PGs in CM represent either molecules with incomplete structures (without HA-binding region) or complexes of small PG bound to HA. Furthermore, our experiment showed that PG extracted from the matrix of human chondrocyte clusters are comparable to native cartilage PG in terms of molecular size and ability to participate in complex formation with HA.

In this work, we found that GS did not affect cell proliferation nor coll II production by human chondrocytes cultivated in clusters. Our results agree with other studies showing that GS did not change the level of production of type II procollagen in another type of culture [26].

The stimulatory effects of GS on PG production *in vitro* are supported by a favorable pharmacokinetic behavior. It was, in fact, demonstrated that after systemic administration (oral or parenteral) of uniformly labeled [^{14}C]-GS, to rats and dogs the compound appeared in plasma and then rapidly diffused into several organs and tissues, including the articular cartilage [43–44]. Our results, showing a stimulatory effect of GS on the biosynthetic activity of human chondrocytes are also in agreement with previous experiments reporting that GS exerts a protective action in animal models of experimental OA [45]; that GS counteracts the metabolic and morphologic damages induced on chondrocytes by dexamethasone [46] or on cartilage by some NSAIDs [11]; and that its medium- or long-term effect in patients with knee or spine OA compares favorably to that of NSAIDs [47–50].

In conclusion, the GS-induced increase of PG production by OA chondrocytes *in vitro* may represent a step towards normalization of impaired chondrocyte metabolism that is characteristic of OA and thus a step to cartilage repair.

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