The catabolic effect of TNFα on bovine nucleus pulposus intervertebral disc cells and the restraining role of glucosamine sulfate in the TNFα-mediated up-regulation of MMP-3

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Running title: Glucosamine protects intervertebral disc cells

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Abstract

Glucosamine is an endogenous amino monosaccharide naturally occurring in the cartilage. We have recently shown that glucosamine sulfate promotes the biosynthesis of glycosaminoglycans in intervertebral disc cells. Here we assessed the role of glucosamine sulfate in the response of bovine nucleus pulposus cell monolayers to TNFα that constitutes an early signal of disc degeneration. TNFα was not found to affect nucleus pulposus cells' viability, while it resulted in a ~2.5-fold increase of the intracellular ROS levels, a rapid transient phosphorylation of p38 MAPK and a ROS-dependent activation of JNKs. In addition, TNFα had a prominent inflammatory effect on nucleus pulposus cells by up-regulating *mmp*-3 expression that was reversed when inhibiting the kinase activity of p38 MAPK. Glucosamine sulfate also diminished the increased by TNFα *mmp*-3 mRNA levels, but this was unrelated to the p38 MAPK or ROS-mediated JNK activation. Even though the mode of action of glucosamine towards TNFα remains to be elucidated, to the best of our knowledge, this is the first report providing evidence for the protective role of glucosamine against this early mediator of disc degeneration that could support the potential usage of this molecule as a treatment for preventing disc degenerative disorders.

Keywords: glucosamine sulfate; TNFα; intervertebral disc; MMP-3; inflammatory response

Low back pain is a very common disorder in modern industrial societies with an enormous social and financial impact stemming directly from health care costs for treatment and indirectly from decreased productivity and lost workdays. [1-3] Given the great socioeconomic toll of the disease, attempts have been made for the understanding of its aetiology and the development of approaches in order to cure it before it becomes chronic or even to prevent it. [4,5] Although all

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causal factors of back pain are not yet fully elucidated, an association with intervertebral disc degeneration has been firmly established. [6,7]

Disc degeneration is characterized by a disturbance in the dynamic equilibrium between synthesis and degradation of the extracellular matrix (ECM) due to a reduced production of its structural components, as well as to an increased expression of degrading enzymes. [8-11] This catabolic phenotype has been attributed to the secretion of inflammatory mediators, such as interleukins (IL) and tumor necrosis factor (TNF)α. [12-14] TNFα and its receptors have been reported to be expressed in healthy and degenerated discs [15,16] and it has been shown that this cytokine promotes ECM destruction by down-regulating aggrecan and collagen type II gene expression, while increasing mRNA levels and/or activity of matrix metalloproteinases (MMPs) and ADAMTSs in bovine nucleus pulposus cells. [17,18]

Glucosamine is an endogenous amino monosaccharide that naturally occurs in the cartilage, [19] while it has been recently shown to promote the biosynthesis of glycosaminoglycans in intervertebral disc cells. [20] It has been broadly administered as a supplement with potential chondroprotective effects in cases of osteoarthritis, but its possible use as an alternative drug that acts by directly targeting the causes of the disease and not by just relieving its symptoms is still under investigation. [21] Intradiscal injection of glucosamine has been also suggested as a treatment for low back pain. [22] Given that discogenic low back pain has been hitherto connected to the presence of pro-inflammatory molecules, [23] any evidence for a potential anti-inflammatory activity of glucosamine in intervertebral disc cells is of great interest. Still, there has been only one report that shows alterations in the production of inflammatory molecules in IL-1-stimulated rat intervertebral disc cells after treatment with glucosamine. [22] However, dealing with the disease at the early stages of its manifestation would be much more effective. Thus, since TNFα is known to play a role in the initiation of degeneration before the establishment of a pathological

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situation in the disc by the prolonged action of IL-1β, [13] our goal in this study is to explore the possible protective effect of glucosamine towards the TNFα-induced inflammatory response of nucleus pulposus cells which, if existing, could provide clinicians with a valuable tool in their design for preventive strategies against disc degeneration.

MATERIALS AND METHODS

Chemical reagents

Tumor necrosis factor α (Biochrom AG) was used at a final concentration of 10 ng/ml unless otherwise stated. The p38 MAPK and JNK inhibitors SB203580 and SP600125, as well as the free radical scavenger N-acetyl-L-cysteine (NAC) were supplied by Sigma (St. Louis, MO, USA) and were used at final concentrations of 10 μM, 15 μM and 10 mM, respectively. Glucosamine sulfate (Taizhou Candorly Sea Biochemical & Health Products. Co., Ltd., Zhejiang, China) was kindly offered by Uni-Pharma Kleon Tsetis Pharmaceuticals Laboratories S.A. (Athens, Greece) and was used at 1 and 2.5 mM. Cells were pre-incubated with SB203580 and SP600125 for 1 h, with NAC for 16 h and with glucosamine sulfate for 2 or 24 h before treatment with TNFα. The time-periods of treatment with the chemical reagents and the general experimental setup of each assay are presented in Supplementary Table 1.

Isolation of bovine nucleus pulposus intervertebral disc cells and cell culture conditions

Bovine caudal discs have been proposed as a suitable biological and biomechanical model for the study of the human lumbar disc. [24] Cultures of nucleus pulposus intervertebral disc cells were established as described previously, [20,25-27] routinely cultured in monolayers in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) (all from Biochrom AG, Berlin, Germany) and 10% (v/v) FBS (Gibco BRL, Invitrogen, Paisley, UK) and maintained in a humidified atmosphere of 5% CO_2 at 37°C. When confluent, cells were subcultured using a trypsin/citrate (0.25%/0.30%, w/v) solution.

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Estimation of cell viability

Cytotoxicity of TNFα was assessed by the MTT assay using concentrations from 0 to 100 ng/ml for 24 and 48 h and the assay was performed as reported previously. [20,28] OD_{550} values of treated samples were divided by the respective values of the untreated samples and data are presented as a % ratio of the results. Cell viability was also measured by direct cell counting. In brief, cells treated or not with a selected concentration of TNFα (25 ng/ml) for 48 h were detached by trypsinization and cell number was measured using a Beckman Coulter Z1 Dual Cell and Particle Counter (Nyon, Switzerland).

Measurement of intracellular levels of reactive oxygen species

Intracellular levels of reactive oxygen species (ROS) were calculated using the DCFH-DA assay. [28,29] In details, nucleus pulposus cells were plated in a 96-well plate in DMEM supplemented with 10% (v/v) FBS until confluence. TNFα was added along with 10 μM of DCFH-DA (Sigma) and cells were further incubated at 37°C. Measurements (excitation wavelength: 485 nm, emission wavelength: 520 nm) were taken at several time points up to 48 h using a microtiter-plate photometer (Infinite 200, Tecan Trading AG, Switzerland). ROS production was expressed as a % ratio of the untreated control.

Western blot analysis

Protein extraction was performed as previously reported in a Laemmli sample buffer with protease and phosphatase inhibitors (Sigma). [25-27] Antibodies used were against the following proteins: phospho-p38 (Thr180/Tyr182), p38, phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, phospho-Akt (Ser473), Akt (all purchased from Cell Signaling Technology, Hertfordshire, UK), phospho-ERK1/2 (Thr202/Tyr204) and panERK (both supplied by BD Transduction Laboratories, Bedford, MA, USA). Secondary horseradish peroxidase-conjugated antibodies (goat anti-mouse and goat anti-rabbit) were obtained from Sigma, as well. Immune

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complexes were visualized using an ECL reagent (Amersham Biosciences, Buckinghamshire, UK).

RNA extraction and real-time PCR experiments

RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions and the concentration was measured by a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). First-strand cDNA synthesis was performed using the PrimeScript RT Reagent Kit (Takara, Tokyo, Japan), while real-time PCR for the MMP-3 expression profile was accomplished with the KAPA SYBR universal fast master mix (KAPA Biosystems, Woburn, MA) in a MX3000P cycler (Stratagene, La Jolla, CA). Estimation of the mRNA levels for each gene was carried out with the $2^{-\Delta\Delta Ct}$ method [30] using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. The primers used were designed with Beacon Designer 7.0 (PREMIER Biosoft International, Palo Alto, CA) and are presented in Table 1.

Statistical analysis

Data presented are the results of at least three independent experiments, while the numerical values are the means ± standard deviations. Statistical significance was estimated by the Student's *t* test for $p<0.05$.

RESULTS

TNFα does not affect the viability and leads to an increase in the intracellular ROS levels of nucleus pulposus intervertebral disc cells

The effect of TNFα on the viability of nucleus pulposus intervertebral disc cells was assessed using the MTT assay. As shown in Fig. 1A, TNFα not only was not lethal for nucleus pulposus cells even at the concentration of 100 ng/ml, but it also resulted in higher OD_{550} values in comparison to the untreated control, especially after exposure of the cells to the cytokine for 48 h,

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when these differences were estimated to be statistically significant. When cells were treated with a moderate selected TNFα concentration (25 ng/ml) for 48 h and were directly counted, we found that TNFα had neither a positive nor a negative effect on cell number, indicating that the molecule is not cytotoxic or cytostatic for nucleus pulposus cells, without though stimulating cellular proliferation (Fig. 1B). Since no inhibitory concentration of TNFα on nucleus pulposus cells was detected by the MTT assay up to 100 ng/ml, we decided on the concentration that would be used in the rest of our experiments based on bibliographic data. We chose 10 ng/ml, concentration at which TNFα has been reported to exert its inflammatory action in human chondrocytes and nucleus pulposus intervertebral disc cells. [13,31]

We followed by examining the role of TNFα on the intracellular ROS levels in our cell model (Fig. 2). TNFα led to an approximately 2.5-fold increase in the production of ROS, already detected in the first 30 min of treatment. Intracellular ROS levels remained elevated in the cells until 48 h (Fig. 2).

TNFα activates the MAPK pathways

In an attempt to determine the molecular pathways that are implicated in the TNFα-mediated responses of nucleus pulposus cells, we investigated the phosphorylation of proteins that are known to be activated by stress (Fig. 3). We found that p38 MAPK and JNKs are phosphorylated after 15 min of TNFα treatment, while this phosphorylation returned to basal levels for both kinases after exposure to the stimulus for 1 h. No differences in the phosphorylation status of ERKs and Akt were observed in accordance with our findings from the MTT and direct cell counting experiments, which showed that TNFα does not affect the viability and proliferation potential of nucleus pulposus cells.

To explore the possibility that p38 MAPK and JNKs activation was related to the oxidative stress provoked by the ROS increase due to TNFα, the scavenger of free radicals NAC

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was used. As presented in Fig. 4, pre-treatment of nucleus pulposus cells with NAC did not abolish the TNFα-mediated phosphorylation of p38 MAPK, but inhibited to an extent the phosphorylation of JNKs (basal and induced by TNFα), suggesting that increased intracellular ROS participate in the activation of the JNK pathway in nucleus pulposus cells.

TNFα leads to a p38 MAPK-dependent inflammatory response in nucleus pulposus intervertebral disc cells

Given the prominent role of TNFα as an inflammatory mediator in several tissues including the intervertebral disc, we examined the action of the molecule on the transcription of the *mmp-3* gene, a direct and renowned marker of inflammation. MMP-3 mRNA levels were indeed higher in nucleus pulposus cells exposed to TNFα for 24 h (Fig. 5).

When inhibiting the p38 MAPK or the JNK pathways that were shown to be activated by TNFα, we found that only the presence of the p38 MAPK inhibitor SB203580 abolished the increase of *mmp-3* mRNA levels (Fig. 5). This finding indicates that the former kinase promotes the TNFα-induced inflammatory response in nucleus pulposus cells. NAC also resulted in the partial reduction of the increased by TNFα *mmp-3* mRNA levels (data not shown), although by a JNK-independent mechanism.

Glucosamine sulfate possesses a putative anti-inflammatory role in nucleus pulposus cells

To assess the potential protective role of glucosamine sulfate, nucleus pulposus cells were pre-treated with the molecule for 2 h before being exposed to the inflammatory stimulus. The role of glucosamine was unambiguous regarding the direct manifestation of inflammation in nucleus pulposus cells, since even at the lowest concentration used (1 mM), the molecule was active against TNFα by moderating the cytokine-mediated increase in the *mmp*-3 mRNA levels (Fig. 6A). Nevertheless, none of the two different concentrations of glucosamine sulfate had any inhibitory action towards the TNFα-driven activation of p38 MAPK and JNKs (Fig. 6B). The

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same results were obtained if cells were pre-incubated with glucosamine sulfate for 24 h before treatment with TNFα (data not shown).

DISCUSSION

Intervertebral discs are the joints of the spine with the capability of absorbing vibrations due to motion, thus providing protection and flexibility to the body. The main part of this tissue consists of ECM, in which a limited number of intervertebral disc cells are embedded. [8] Based on our previously published observations regarding the stimulation of glycosaminoglycan synthesis in nucleus pulposus cells by glucosamine, [20] in this work we studied its role in inflammation that leads to adverse degenerative effects in disc cells. TNFα, which is normally expressed in the intervertebral disc and participates in the launching of catabolic processes *in vivo*, [13,15,16] was used as the inflammatory stimulus.

We first used the MTT assay to examine the possibility of any cytotoxic effect of TNFα on nucleus pulposus cells, which was actually revealed to be stimulating. In order to exclude the possibility of erroneous results due to TNFα interference with mitochondrial activity in the MTT assay, cell viability was also measured by counting the number of the cells. Direct cell counting did not show any difference between treated and untreated nucleus pulposus cells, indicating that TNFα at the concentrations used was neither cytotoxic nor cytostatic for nucleus pulposus intervertebral disc cells. At the same time, TNFα led to a mild oxidative stress in nucleus pulposus cells by increasing intracellular ROS levels by a factor of ~2.5 in comparison to basal ROS production. TNFα-mediated oxidative stress seemed to be a signal for the activation of the JNK pathway, since phosphorylation levels of JNKs were decreased when cells were pre-incubated with NAC. This is in accordance with previous findings in osteoblastic cells, in which TNFα has been reported to have a pro-oxidant action and to increase JNK phosphorylation, effect that was also attenuated by NAC. [32]

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Subsequently, we investigated the MAPK intracellular pathways that are implicated in the inflammatory and catabolic signaling in the intervertebral disc. [33] In parallel to the aforementioned phosphorylation of JNKs, TNFα led to a rapid and transient activation of p38 MAPK. p38 MAPK and JNKs have been very often associated with inflammatory stress in several cell types. They have been reported to be phosphorylated by IL-1β in human chondrocytes, [34-36] human primary synovial [37] and human skin [38] fibroblasts, as well as by TNFα in human endothelial [39], human colonic epithelial [40] and HaCaT cells. [41] In some of these cases, ERKs were also found to be activated in contrast to our results. [34-38,41] This discrepancy could be attributed to species- or cell-specific differences (bovine vs. human tissue, nucleus pulposus cells vs. chondrocytes, fibroblasts, etc.), to the diverse inflammatory mediators used in each study (e.g. IL-1β vs. TNFα) or even to a different experimental design using other concentrations of the same stimulus. Nevertheless, the fact that ERKs were not activated by TNFα was in accordance with the unaltered phosphorylation profile of Akt and both observations were expected since these kinases mainly regulate cell proliferation that was not shown to be affected by the presence of TNFα in our cell model.

As mentioned earlier, the catabolic effect of inflammatory mediators is characterized by the down-regulation of ECM molecules and the up-regulation of MMPs. Seguin and co-workers have shown a dose-dependent decrease in aggrecan and type II collagen gene expression and a dose-dependent increase in MMP-1, MMP-3, and MMP-13 gene expression in nucleus pulposus cells obtained from bovine distal caudal spines exposed to TNFα. [17] In addition, Millward-Sadler et al. have shown that in human intervertebral disc cells TNFα had its highest stimulating action on MMP-3 expression in comparison to other MMPs. [13] Furthermore, MMP-3 has been found to be over-expressed by several cytokines in different cell types. [13,35-37] Accordingly, for this study we selected MMP-3 expression as a marker in order to assess the possible anti-catabolic effect of

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glucosamine in nucleus pulposus cells. We demonstrated that TNFα led to a p38 MAPK-dependent up-regulation of the *mmp*-3 transcript in bovine nucleus pulposus cells in agreement with the previous observations in the human intervertebral disc. [13] Congruently, inhibition of p38 MAPK activity has been reported to markedly decrease synovial MMP-3 gene expression in rat models with arthritis [42] and has already been suggested as a potential target for treating intervertebral disc degeneration. [43]

Given the aforementioned discussed anti-inflammatory role of glucosamine in several experimental models, we employed glucosamine sulfate as an alternative way to restrain inflammation provoked by TNFα in nucleus pulposus cells. The molecule was used in concentrations that did not exceed 2.5 mM, since according to our previous study above this limit it has a significant cytotoxic effect. [20] Glucosamine sulfate partly inhibited *mmp*-3 up-regulation after TNFα treatment even at 1 mM, concentration that has not been found to exert cytotoxicity in these cells. [20] An inhibitory effect of glucosamine against IL-1-mediated MMP-3 up-regulation has been reported in the past for other cell types [37,44] and for rat intervertebral disc cells, [22] but this is the first time such an effect of the molecule is demonstrated in intervertebral disc cells exposed to TNFα. The anti-inflammatory action conferred by glucosamine sulfate in nucleus pulposus cells could not be associated with the activation of MAPKs, since it was not able to abolish the TNFα-triggered phosphorylation of neither p38 MAPK nor JNKs up to 2.5 mM.

In conclusion, in this work we showed that TNFα has an inflammatory action on nucleus pulposus intervertebral disc cells *in vitro* by promoting the up-regulation of MMP-3 that was found to be connected to the p38 MAPK activation. On the other hand, glucosamine sulfate was shown to attenuate the inflammatory action of TNFα in nucleus pulposus cells, but this effect was p38 MAPK-independent. Thus, the pathway(s) through which glucosamine sulfate exerts its protective action on TNFα-treated nucleus pulposus cells need(s) further investigation. Even

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though glucosamine has been tested in the past for its anti-inflammatory efficiency, it was mainly towards IL-mediated effects. To the best of our knowledge this is the first report assessing the role of the molecule against an early inflammatory mediator of disc degeneration. Research on new molecules with possible anti-catabolic effects is of great importance, especially if they are considered to be supplements with no reported adverse effects. Furthermore, glucosamine has been reported to be bioavailable after oral administration, [45] approach that is certainly less interventional than intradiscal injections. With our findings we believe that we provide some evidence for the potential clinical use of glucosamine, possibly in combination with other drugs or supplements, in anti-inflammatory therapies designed against disc degenerative disorders.

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LEGENDS TO FIGURES

Figure 1

Effect of TNFα on the viability of nucleus pulposus intervertebral disc cells, as estimated by the MTT assay (A) or by direct cell counting (B). Asterisks represent statistically significant differences of samples in comparison to their respective untreated control (Student's *t*-test, $p<0.05$). .

Figure 2

Estimation of intracellular ROS production in nucleus pulposus intervertebral disc cells after exposure to TNFα. Asterisks represent statistically significant differences of treated samples when compared to the untreated ones (Student's *t*-test, $p<0.05$).

Figure 3

Kinetics analysis of the activation of the MAPK and Akt pathways in nucleus pulposus intervertebral disc cells treated with TNFα. Blots for the non-phosphorylated forms of the kinases

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served as loading controls. Representative blots of three independent experiments are depicted here.

Figure 4

Effect of ROS production on the TNFα-mediated phosphorylation of p38 MAPK and JNKs. The non-phosphorylated forms of the proteins were used as loading controls. Experiments were repeated three times and representative blots are shown.

Figure 5

TNFα exerts a p38 MAPK-dependent inflammatory effect on nucleus pulposus intervertebral disc cells. Data are presented as a ratio of the values of treated/untreated samples and the means ± standard deviations are shown here. Statistically significant differences in comparison to the respective untreated controls are represented by an asterisk (Student's *t*-test, $p<0.05$).

Figure 6

Effect of glucosamine sulfate on the TNFα-induced *mmp-3* up-regulation (A) and on the p38 MAPK and JNKs activation (B) in nucleus pulposus intervertebral disc cells. In (A) results are expressed as ratios to the untreated control (with no glucosamine sulfate and TNFα added) and are the means ± standard deviations of three separate experiments. Asterisks represent statistically significant differences of the treated with glucosamine sulfate and TNFα samples in comparison to the sample that was treated only with TNFα (Student's *t*-test, $p<0.05$). In (B) blots are representative of three independently performed experiments. The non-phosphorylated levels of the kinases were analyzed in order to confirm equal loading.

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Table 1. Sequences of primers used in the real-time PCR experiments

Target gene	Primers
mmp-3	ACA-ATG-GAC-AAA-GGA-TAC-ATC-AGG
	TTC-GGT-TGA-GTG-CTG-GAG-AC
gapdh	GCC-ATC-ACT-GCC-ACC-CAG-AA
	GCG-GCA-GGT-CAG-ATC-CAC-AA

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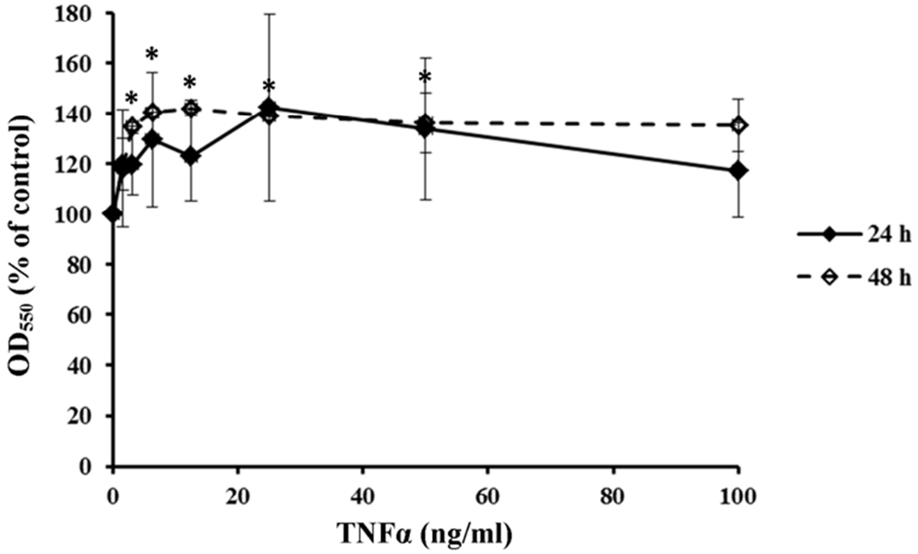

Figure 1A

Effect of TNFα on the viability of nucleus pulposus intervertebral disc cells, as estimated by the MTT assay
141x107mm (300 x 300 DPI)

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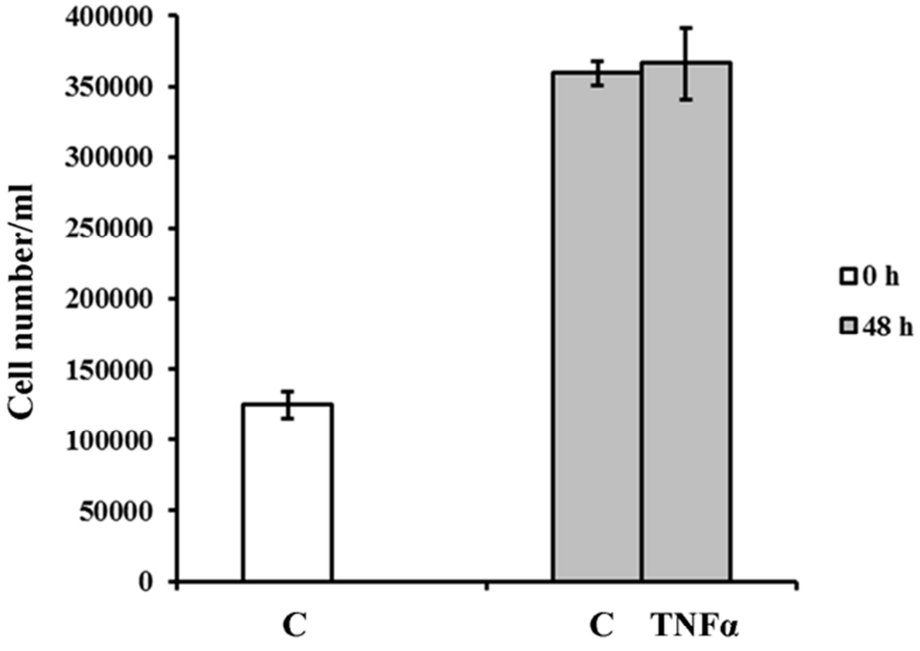

Figure 1B

Effect of TNFα on the viability of nucleus pulposus intervertebral disc cells, as estimated by direct cell counting
120x105mm (300 x 300 DPI)

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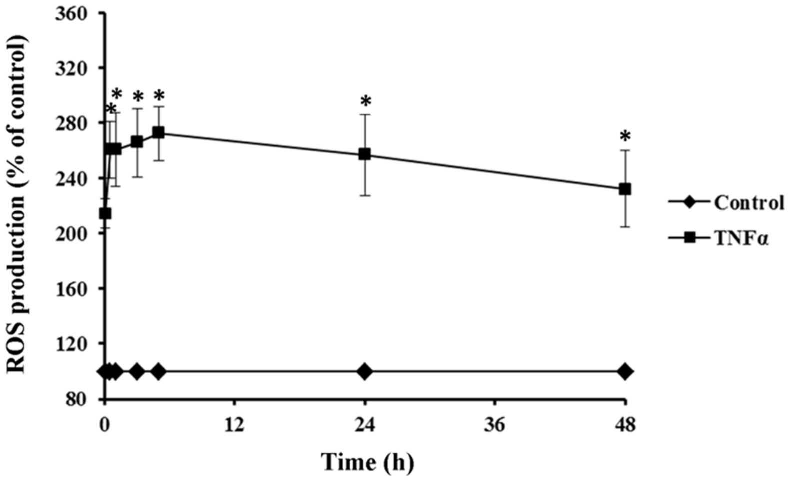

Figure 2

Estimation of intracellular ROS production in nucleus pulposus intervertebral disc cells after exposure to TNFα
165x112mm (300 x 300 DPI)

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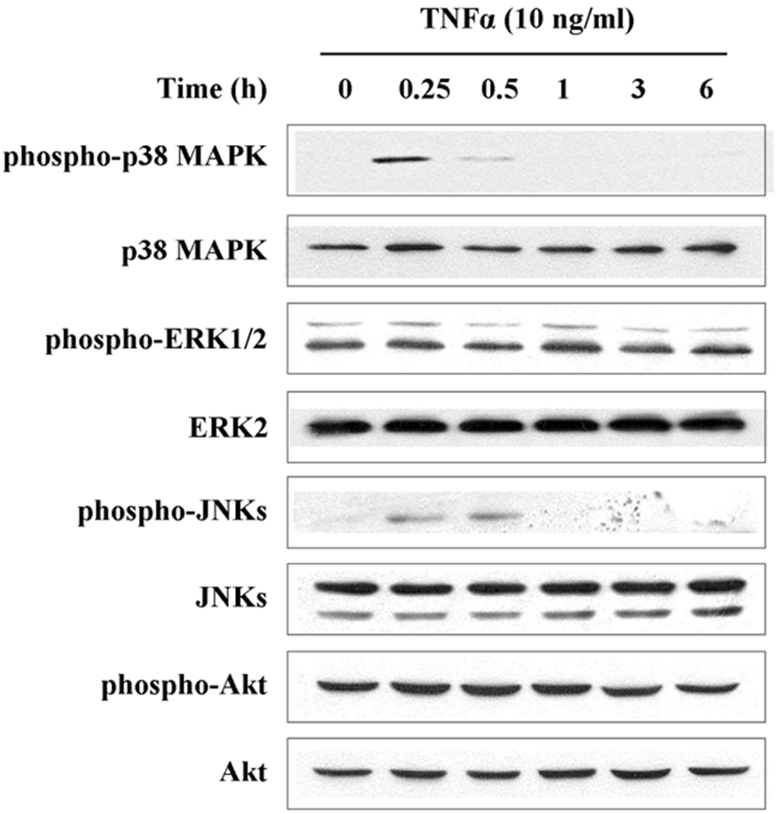

Figure 3

Kinetics analysis of the activation of the MAPK and Akt pathways in nucleus pulposus intervertebral disc cells treated with TNFα
141x146mm (300 x 300 DPI)

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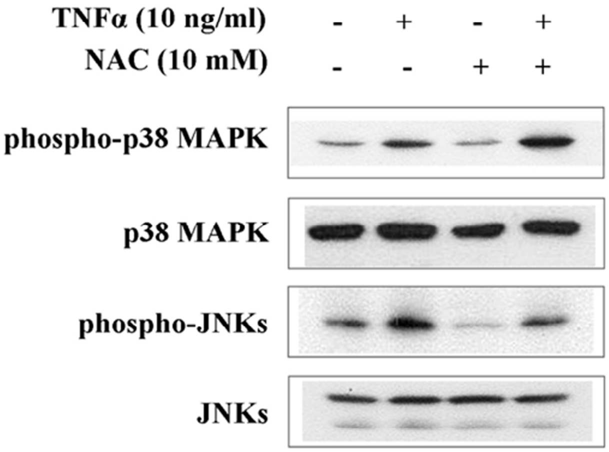

Figure 4

Effect of ROS production on the TNFα-mediated phosphorylation of p38 MAPK and JNKs
141x100mm (300 x 300 DPI)

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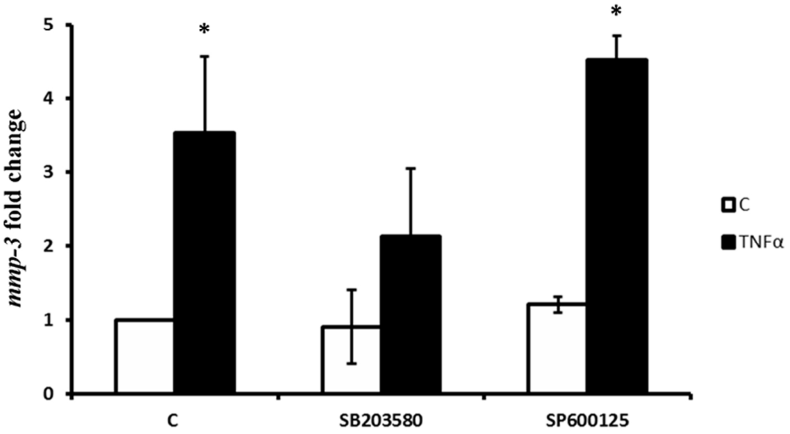

Figure 5

TNFα exerts a p38 MAPK-dependent inflammatory effect on nucleus pulposus intervertebral disc cells
171x110mm (300 x 300 DPI)

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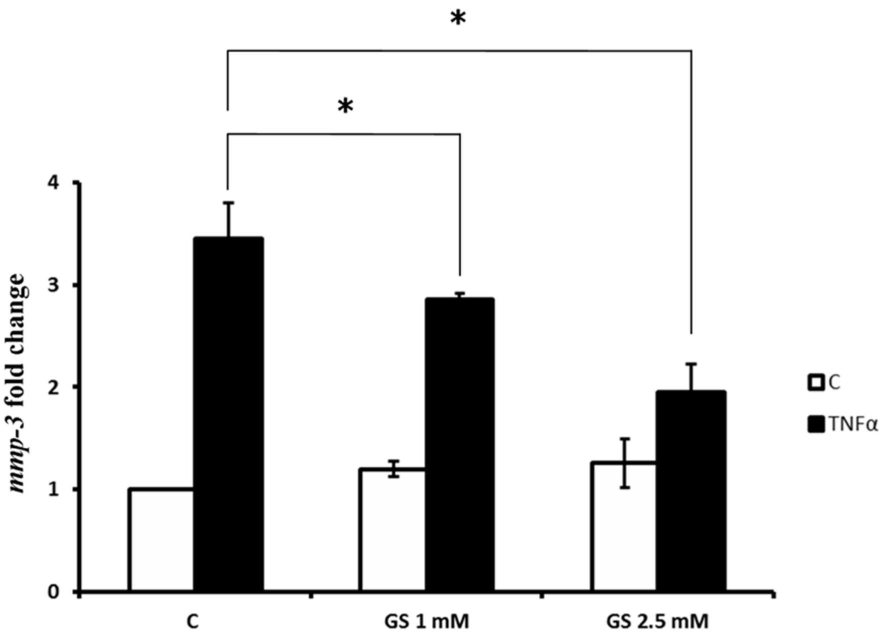

Figure 6A

Effect of glucosamine sulfate on the TNFα-induced mmp-3 up-regulation
154x117mm (300 x 300 DPI)

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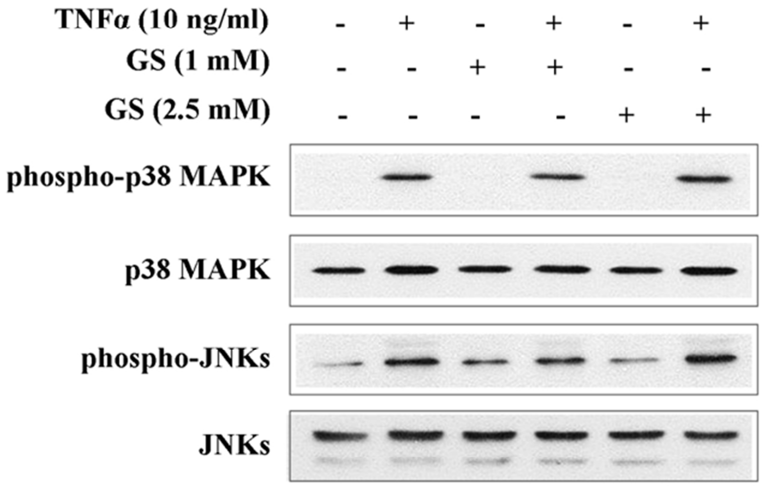

Figure 6B

Effect of glucosamine sulfate on the TNFα-induced p38 MAPK and JNKs activation
141x106mm (300 x 300 DPI)

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