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The Effects of Glucosamine Sulfate on Intervertebral Disc Annulus Fibrosus Cells *in Vitro*

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Abstract

Background context—Glucosamine has gained widespread use among patients, despite inconclusive efficacy data. Inconsistency in the clinical literature may be related to lack of understanding of the effects of glucosamine on the intervertebral disc, and therefore, improper patient selection.

Purpose—The goal of our study was to investigate the effects of glucosamine on intervertebral disc cells *in vitro* under the physiological conditions of inflammation and mechanical loading.

Study Design—Controlled *in vitro* laboratory setting

Methods—Intervertebral disc cells isolated from the rabbit annulus fibrosus were exposed to glucosamine sulfate in the presence and absence of interleukin-1 β and tensile strain. Outcome measures included gene expression, measurement of total glycosaminoglycans, new proteoglycan synthesis, prostaglandin E₂ production, and matrix metalloproteinase activity. The study was funded by NIH/NCCAM and the authors have no conflicts of interest.

Results—Under conditions of inflammatory stimulation alone, glucosamine demonstrated a dose dependent effect in decreasing inflammatory and catabolic mediators and increasing anabolic genes. However, under conditions of mechanical stimulation, although inflammatory gene expression was decreased, PGE₂ was not. In addition, MMP-3 gene expression was increased and aggrecan expression decreased, both of which would have a detrimental effect on matrix homeostasis. Consistent with this, measurement of total glycosaminoglycans and new

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proteoglycan synthesis demonstrated detrimental effects of glucosamine under all conditions tested.

Conclusions—These results may in part help to explain the conflicting reports of efficacy, as there is biological plausibility for a therapeutic effect under conditions of predominate inflammation, but not under conditions where mechanical loading is present or in which matrix synthesis is needed.

Introduction

In a CDC report from 2004 on complementary and alternative medicine use among adults, low back pain was identified as the most common condition for which patients sought alternative treatments[1], highlighting the need for additional research in this area. Among adults using a non-vitamin, non-mineral supplement, 15% were using glucosamine with or without chondroitin, with over 5 million respondents using glucosamine within a previous 12 month period. Clinical evidence for the efficacy of oral supplements exists for articular cartilage [2, 3], as well as intervertebral disc degeneration [4]. However, in a randomized controlled trial of glucosamine for non-specific low back pain, no difference was noted in pain or pain related disability after 6 months of glucosamine supplementation [5]. With the inconsistency in the literature due to variability in study design, patient inclusion, and formulation of compounds, one can conclude that it is possible that subgroups of patients may derive benefit from glucosamine supplementation. However, identification of the patient characteristics which may be responsive to this treatment requires an understanding of the basic biological action of glucosamine to identify any potential biologic plausibility for a clinical effect, if one exists. Importantly, it has recently been shown that orally administered glucosamine has a detrimental effect on matrix homeostasis in an animal model of injury induced degeneration[6]. The current *in vitro* study aims to investigate the effects on each of the contributors to disc matrix homeostasis, namely, inflammatory, catabolic, and anabolic expression. Without knowledge of the mechanism of action of a particular intervention, clinical trials cannot be targeted toward the appropriate patient population. For example, it is likely that a trial with an agent/intervention that has a predominantly anti-inflammatory effect will have little effect if the chosen patient population has minimal inflammation. Similarly, an intervention stimulating the mechanical signaling pathways but not altering inflammatory pathways may have little efficacy in a purely inflammatory process. Because all patients with low back pain do not have the same pathophysiology and pain generators, understanding the pathways affected by glucosamine would allow for prediction of which patients may respond to the treatment, facilitating more directed clinical trials with greater chance of demonstrating efficacy, which was the rationale for the current *in vitro* study.

The purpose of the current study was to explore the effects on glucosamine on key mediators of matrix homeostasis under physiologic conditions of inflammation and tensile strain in intervertebral disc cells to evaluate any biologic plausibility for an effect in intervertebral disc degeneration.

Methods

Tissue Isolation and Cell Culture

The lumbar intervertebral discs of 6-9 month-old female New Zealand White rabbits were excised immediately after sacrifice. All protocols were approved by the institutional animal care and use committee at the University of Pittsburgh. The annulus fibrosus tissue was separated, minced, and digested in 0.2% pronase followed by 0.02% collagenase to liberate cells from their extracellular matrix. Cells were cultured in Ham's F12 medium (Cellgro, Mediatech), 10% fetal bovine serum (Hyclone, Logan, UT), 1% penicillin/streptomycin (Cellgro, Mediatech), 2mM glutamine (Gibco, Invitrogen), and grown at 5% CO₂, 37°C. First passage cells were used for all experiments.

Concentration Testing of Glucosamine

First passage fibrochondrocytes isolated as above were grown in six well culture plates and supplemented with increasing concentrations (0, 0.1 ng/mL, 0.5 ng/mL, 1 ng/mL, 2.5 ng/mL or 5 ng/mL) of D -glucosamine 6-sulfate (Sigma-Aldrich, St. Louis, MO, lot 075K1569, free base synthetic, >=99% purity by TLC). Target concentrations were chosen at, above and below previously observed intradiscal concentrations in response to oral administration to most closely mimic the in vivo condition[6]. We chose this formulation over commercially available supplements to ensure purity and stability, as the pure salt is not stable for oral dosing. In addition, we chose the sulfated form over the hydrochloride salt due to the potential for greater bioavailability[7]. The compound utilized in all experiments was reviewed and approved by the Product Integrity Work Group, National Center for Complementary and Alternative Medicine, National Institutes of Health. All experiments were completed with the use of compound from a single lot, which was stored at 4°C per the manufacturer's instructions.

Mechanical Stimulation of Annulus Fibrosus Cells

First passage fibrochondrocytes derived from annulus fibrosus tissue were plated onto six-well culture plates with a flexible, collagen I coated silicone membrane forming the floor of each well. Approximately 50,000 cells were plated per well, and they were allowed to grow to 95% confluence. After 72 hours, F12 media containing 1% fetal bovine serum and 1% penicillin/streptomycin was used to minimize the interference of serum derived biologically active proteins with media assessments. There were eight conditions examined: control (C), interleukin-1 β (IL), glucosamine sulfate (G), glucosamine sulfate and interleukin-1 β (GIL), tensile strain (S), tensile strain and interleukin-1 β (SIL), tensile strain and glucosamine sulfate (SG), tensile strain and glucosamine sulfate and interleukin-1 β (SGIL). Three wells were dedicated to each condition.

Cells in the glucosamine groups were pre-incubated with 2.5ng/ml of D-glucosamine 6-sulfate (Sigma-Aldrich, St. Louis, MO, lot 075K1569) for 30 minutes. Immediately prior to induction of mechanical strain, cells in the interleukin containing groups were stimulated by the addition of human recombinant interleukin-1 β (CalBiochem) to the culture media at a final concentration of 1ng/ml. Cells in the tensile strain group were subjected to tensile strain via the use of a Flexercell Strain Unit FX-4000 (Flexcell International Corp.,

Hillsborough, NC). Cells adherent to the membrane were exposed to circumferential strain at 6% at a rate of 0.1 Hz for 24 hours. This mechanical strain regimen was selected based on previous data demonstrating a catabolic effect of 24 hours of strain at this magnitude and frequency[8]. After 24 hours, cells were removed from the Flexercell Unit, and outcome measures, including gene expression, new proteoglycan synthesis, total glycosaminoglycan content, prostaglandin E₂ levels (PGE₂), and matrix metalloproteinase (MMP) activity as described below were performed.

mRNA Isolation

After the appropriate condition, cells were washed with phosphate-buffered saline, and RLT lysis buffer (Qiagen, Valencia, CA) containing 1% β -mercaptoethanol. Cells were then detached from the membrane by mechanical disruption. The cellular lysate from all three wells per condition were pooled and passed through a Qias shredder. mRNA was extracted using the Qiagen mRNA extraction kit including a DNase-I step to remove genomic DNA.

Real-Time RT-PCR

The mRNA expression was measured by quantitative real time RT-PCR using custom designed and validated primers (see Table 1) for mediators of inflammation (inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2)), mediators of catabolic activity (matrix metalloproteinase-1 (MMP-1, collagenase), matrix metalloproteinase-3 (MMP-3, stromolysin), and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1)), and mediators of anabolism (collagen I, collagen II and aggrecan) with the SYBRTM green detection system, using an annealing temperature of 62°C and melting temperature of 95°C. Thirty ng mRNA was analyzed in duplicate via real-time polymerase chain reaction in iQ SYBR Green supermix (25- μ l volume), using a Biorad iCycler IQ thermocycler (Biorad, Valencia, CA). Melting curve analyses were then performed to assure amplicon specificity. Glyceraldehyde phosphate dehydrogenase (GAPDH) is a constitutively expressed housekeeping gene shown to not be affected by mechanical loading [9], and was therefore amplified under the same conditions with each sample to correct for any small differences in starting amounts of mRNA. After normalization using GAPDH, the relative gene expression was reported as relative fold expression compared with control, which was set at one using the delta-delta Ct method as previously described [10].

Proteoglycan Synthesis

New proteoglycan synthesis was measured via incorporation of ³⁵S. Cells were pulse labeled with 5 μ Ci/ml of ³⁵S for 6 hours. Cells were then homogenized with a solution containing 200mM NaCl, 50mM NaOAc, 0.1% Triton X-100, and 10mM EDTA at 4°C for one hour. Proteoglycans were extracted with a guanidine hydrochloride extraction buffer containing 8M guanidine hydrochloride, 50mM NaOAc, and 10mM EDTA for four hours at 4°C. Sulfated proteoglycan in 100 μ l of the liquid sample was complexed with 600 μ l of a 0.2% alcian-blue solution for one hour. The solution was then filtered through mixed cellulose ester membrane filters to retain the radio-labeled proteoglycans complexed with alcian blue dye. Membrane filters were washed to remove free radiolabel and then dissolved with 2ml of a 4M guanidine hydrochloride and 33% isopropanol solution for one hour. Radioactive count was amplified with 2ml of scintillation fluid and passed through a scintillation counter

to provide the final radioactive count per minute(CPM). Raw CPM values were normalized to DNA content using a Quant-iT PicoGreen ds DNA Assay Kit (Invitrogen, Eugene, Oregon).

Glycosaminoglycan Content

Total glycosaminoglycan content was measured via digestion with a solution containing 50mM NaOAc, 5mM EDTA, 5mM L-cysteine, 300µg/ml papain, 55mM citric acid, and 150mM NaCl for 20 hours at 40°C, followed by detection using dimethylmethylene blue dye. Absorbance was measured at 595nm. Raw absorbance values were normalized to DNA content using a Quant-iT PicoGreen ds DNA Assay Kit (Invitrogen, Eugene, Oregon).

Prostaglandin E₂ production

Prostaglandin E₂ released into cell media was quantified using the Parameter™ PGE₂ ELISA Assay per the manufacturer's instructions (R&D Systems, Minneapolis, MN).

MMP activity assay

Culture media was assayed for MMP activity using flourophore labeled peptides. Peptide substrates 25uM MMP1 substrate (Calbiochem #444219) or 20uM MMP3 substrate (Calbiochem #444223) was added to 200ul of freshly harvested cultured media. Flourescence, resulting from cleaved substrate liberating the fluorophore, was measured with excitation at 355nm and emission at 460nm on a VictorX plate reader (Perkin Elmer) at 10 minute intervals for 4 hours. Maximum slope of the initial linear portion of the reaction curve (V_{max}) under conditions of substrate excess, with autoflourescence correction, was calculated using Workout software (Perkin Elmer).

Statistical Analysis

Values represent the average of 3-5 trials from independent cell isolations from separate rabbits +/- standard error, with 95% confidence intervals calculated to determine statistical significance at p<0.05 level. The confidence intervals were calculated based on the t-distribution because of the small sample size using Microsoft Excel. This statistical approach has been validated for use in calculations of relative gene expression by real-time PCR data using the delta-delta Ct method [11].

Results

Dose response to glucosamine in the presence of inflammatory stimuli

Cells treated with IL-1beta demonstrated expected changes in gene expression compared to control, untreated cells. Relative gene expression of IL-1beta stimulated cells was found to be 239.21 for iNOS, 10.45 for COX-2, 0.98 for collagen I, 0.16 for collagen II, 0.47 for aggrecan, 1889.78 for MMP-3, 1.2 for MMP-1 and 0.99 for TIMP-1. In cells stimulated with interleukin-1beta, glucosamine supplementation demonstrated a dose dependent effect in decreasing the expression of iNOS, COX-2, and MMP-3, as well as increasing expression of TIMP-1 as the concentration was increased to 2.5 ng/mL (Figure 1). Increasing doses also demonstrated increased gene expression of collagen I, II, and aggrecan. Though 5 ng/mL

demonstrated a trend toward increased anabolic gene expression, greater variability in cellular responses was noted and these changes did not reach statistical significance. In addition, increase in COX-2 and MMP-1 gene expression was also noted at this concentration. We therefore chose 2.5 ng/mL glucosamine for the remainder of the studies, which demonstrated increase in anabolic gene products with consistent and statistically significant inhibition of inflammatory and catabolic gene products.

Response to glucosamine under conditions of mechanical strain

Gene Expression of mediators of inflammation (See Figure 2)—Glucosamine supplementation (G) did not decrease gene expression of iNOS or COX-2 in unstimulated cells compared to control cells (C), contrary to the effect noted in cells stimulated with interleukin-1beta above, where decreased iNOS and COX-2 gene expression was noted. Under conditions of tensile strain, glucosamine supplementation (SG) showed statistically significant decrease in iNOS and COX-2 expression, with decreased iNOS (by 49%) and COX-2 (by 39%) compared to cells stimulated with tensile strain alone (S). Under conditions of combined inflammatory and tensile stimulation, glucosamine supplementation (SGIL) showed a trend toward decreased iNOS (by 58%) and COX-2 (by 37%) gene expression compared to cells treated with inflammatory and tensile stimulation alone (SIL). Thus, glucosamine decreased expression of these two major inflammatory mediators, COX-2 and iNOS, under mechanically stimulated conditions.

Gene expression of mediators of catabolism (See Figure 3)—Glucosamine supplementation (G) in unstimulated cells did not change gene expression of MMP-1 or MMP-3 compared to control cells (C). Under conditions of tensile strain, glucosamine supplementation (SG) resulted in statistically significant increase in MMP-3 gene expression, with 26% increase compared to cells treated with tensile strain alone (S). Under combined conditions of inflammatory and tensile strain, glucosamine supplementation (SGIL) did not change gene expression compared to cells treated with inflammatory and tensile strain alone (SIL). Glucosamine supplementation did not alter gene expression of TIMP-1 under conditions of tensile strain or combined inflammatory and tensile strain. Therefore, the only significant change induced by glucosamine was increased MMP-3 expression under conditions of tensile strain.

Anabolic Gene Expression (See Figure 4)—When comparing glucosamine supplemented cells (G) to control cells (C) under unstimulated conditions, type I collagen and aggrecan expression was unchanged, and type II collagen gene expression was statistically significantly lowered (by 54%) by glucosamine supplementation. Under conditions of tensile strain, the addition of glucosamine (SG) to culture medium showed a trend toward decreased collagens I and II, and aggrecan gene expression compared to cells treated with tensile strain alone (S). The decrease in aggrecan expression under conditions of tensile strain was statistically significant. Under conditions of combined inflammatory and tensile strain (SGIL), glucosamine treatment did not change types I and II collagen expression compared to cells treated with inflammatory and tensile strain alone (SIL), but decreased aggrecan gene expression by 24%. Thus, glucosamine was found to decrease the

expression of the important matrix constituent aggrecan under both mechanical and inflammatory stimulation.

Proteoglycan Synthesis (See Figure 5)—The outcome of the decreased expression of aggrecan was further explored by measuring new proteoglycan synthesis. Glucosamine supplementation decreased synthesis of new proteoglycans under normal conditions (G vs. C), inflammatory conditions (GIL vs IL), tensile strain (SG vs S), and under conditions of inflammation and tensile strain combined (SGIL vs SIL).

Glycosaminoglycan Content (See Figure 6)—Similarly, glucosamine supplementation resulted in decreased total glycosaminoglycan content in all of the experimental conditions examined (G vs C, GIL vs IL, SG vs S, and SGIL vs SIL).

Prostaglandin E₂ Levels (See Figure 7)—Glucosamine supplementation decreased prostaglandin E₂ levels under unstimulated (G vs C) and inflammatory (GIL vs IL) conditions. However, under conditions of tensile strain, this effect of glucosamine was lost (SG vs S and SGIL vs SIL).

MMP activity (See Figure 8)—Glucosamine supplementation demonstrated no significant effect on MMP 1 and MMP 3 enzymatic activity, though MMP3 showed a trend toward increased activity in response to glucosamine under conditions of tensile strain (SG vs S and SGIL vs SIL).

Discussion

Our data demonstrating a dose dependent anti-inflammatory action of glucosamine sulfate is similar to previous studies on chondrocytes suggesting anti-inflammatory activity [12], as well as in an in vivo animal model of inflammatory arthritis [13]. However, this anti-inflammatory effect was lost under conditions of mechanical stimulation. Because the disc exists in a mechanically active environment, this suggests that any potential anti-inflammatory effects of glucosamine may not be realized in vivo, and in fact glucosamine treatment under conditions of combined mechanical and inflammatory stimuli may be detrimental to overall disc matrix homeostasis. In fact, it is this interaction of inflammation and mechanical stress that most likely represents the in vivo mechanism of matrix loss associated with disc degeneration, underscoring the importance of this finding.

Although the addition of glucosamine showed potentially positive effects on anabolic gene expression under inflammatory conditions, detrimental effects were demonstrated under mechanical or combined inflammatory and mechanical stimuli. In addition, glucosamine actually demonstrated negative effects on total glycosaminoglycan and new proteoglycan synthesis under inflammatory as well as mechanical conditions. This raises concern over the use of glucosamine as a potentially therapeutic supplement in the intervertebral disc due to the potentially detrimental effects on matrix homeostasis.

Importantly, these data are similar to a previously reported study on articular chondrocytes, which demonstrated decreased GAG in response to glucosamine sulfate *in vitro*. [14].

However, this study by Uitterlinden also demonstrated protection against extracellular matrix breakdown with glucosamine supplementation. This differs from the current study in which catabolic enzymatic activity was unchanged in response to glucosamine. Other studies in chondrocytes have demonstrated upregulation of collagen II and aggrecan gene expression in response to glucosamine, as well as increases in collagen and total glycosaminoglycans [15]. However, glucosamine HCl was used in these experiments, at concentrations of 2-15 mM, much higher than the concentrations used in our study, which used 0.4-20 nM (0.1-5 ng/mL). The concentrations used in our study represent a more physiologically relevant dose [16],[17]. Likely the intervertebral disc, in its relatively avascular environment, is exposed to even lower concentrations. In fact, our previous work demonstrated that oral glucosamine was bioavailable in an animal model, with average intra-discal concentrations at 2.3 ng/mL[6], similar to the concentrations used in the current study. Nevertheless, the beneficial effects on matrix observed previously in cartilage were not observed in the current study, and therefore the potential benefit from an anti-inflammatory effect may not be realized as it relates to overall disc matrix homeostasis. In fact, *in vivo* studies have demonstrated negative net effects on intervertebral disc matrix[6]. This demonstrates that important differences exist between articular cartilage and intervertebral disc cartilage.

Interestingly, greater changes in gene expression were noted for collagen II than collagen I, despite the greater overall proportion of collagen I in the annulus fibrosus. However, it is relevant to note that upon progressing from the outer to the inner annulus, the level of type I collagen declines while type II collagen increases[18]. Because this cell culture experiment was not designed to examine differential effects of inner vs. outer annulus, it is possible that different effects may be observed in different regions of the annulus, and suggests that more significant changes in response to glucosamine may be observed in the inner, more collagen II rich region.

Our results, demonstrating a blunting of IL-1 beta induced inflammation, are consistent with a previous study demonstrating a glucosamine induced decrease in IL-6 and TNF-alpha production in annulus cells stimulated with interleukin-1 [19]. However, this study also demonstrated reduced annulus cell viability in response to glucosamine HCl. Therefore, the potential negative effect on cell viability, coupled with our results demonstrating a loss of the anti-inflammatory effect under conditions of mechanical stimulation and detrimental effects on anabolism, call in to question the potential therapeutic utility of glucosamine for intervertebral disc degeneration.

An important limitation of the study relates to the use of annulus fibrosus cells isolated from rabbit tissue. This was done to ensure phenotypic homogeneity of the sample to determine the effects of glucosamine without confounders such as degree of degeneration, medications, gender, age, etc. that would be present in a study on human cells. Nevertheless, these results need to be confirmed in human cells to determine the clinical relevance. In addition, our study focused on the effects of glucosamine on annulus fibrosus cells. It is possible that different effects would be observed in cells from the phenotypically distinct nucleus pulposus. It is also possible that different concentrations outside of the ranges tested in this study may have different effects, and this point warrants further study. In fact, at higher

doses of glucosamine in chondrocytes, any beneficial effect on matrix production was lost[15]. This suggests a relatively narrow therapeutic window, which would be less than desirable for an over-the-counter supplement. Finally, the changes observed in gene expression may not be equivalent to changes in protein expression. However, this study focused on studying end products of the gene expression pathways, such as PGE2, enzyme activity, and total and new proteoglycan synthesis because this reflects protein activity, which are likely more functionally relevant than protein levels.

Conclusion

The current study demonstrates a potentially anti-inflammatory effect of glucosamine under non-mechanically active cell culture conditions. This effect was lost under conditions of mechanical stimulation, and was accompanied by a negative effect on matrix constituents. These results may in part help to explain the conflicting reports of efficacy, as there is biological plausibility for a therapeutic effect under conditions of predominate inflammation. However, under conditions where mechanical signaling predominates or is coupled with inflammatory stimuli, these results suggest a negative biologic response on matrix. Therefore, the potential anti-inflammatory effects of glucosamine may not be realized in the clinical setting, where the potential exists for detrimental effects on matrix. Further studies are needed to determine the net effects of glucosamine supplementation under *in vivo* conditions, which should precede, and may inform, future clinical studies.

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Figure 1.

Relative gene expression of annulus fibrosus cells exposed to increasing concentrations of glucosamine sulfate under inflammatory conditions (ie stimulation with 1 ng/mL IL-1 beta). All values are expressed as relative gene expression compared to control cells stimulated with IL-1 beta alone. Values represent average of $n=3 \pm$ standard error. *statistical significance compared to control, unstimulated cells; # statistically significant comparison between conditions.

For Figures 2-4: All values are expressed as relative gene expression compared to control cells with no treatment, average of $n=5 \pm$ standard error. C=control; G= glucosamine at 2.5 ng/mL; S = mechanical strain at 6% 0.1 Hz; SG = glucosamine at 2.5 ng/mL added 30 minutes prior to initiation of mechanical strain at 6% 0.1 Hz; SIL = co-stimulation with 1 ng/mL interleukin-1 beta and 6% 0.1 Hz mechanical strain; SGIL = glucosamine at 2.5 ng/mL added 30 minutes prior to initiation of co-stimulation with 1ng/mL interleukin-1 beta and 6% 0.1 Hz mechanical strain. *statistical significance compared to control.

Figure 2.

Relative gene expression of iNOS and COX-2 of annulus fibrosus cells exposed to glucosamine with or without inflammatory (IL-1 beta) and/or mechanical (6% 0.1 Hz) strain for 24 hours.

Figure 3.

Relative gene expression of MMP-1, TIMP-1, and MMP-3 of annulus fibrosus cells exposed to glucosamine with or without inflammatory (IL-1 beta) and/or mechanical (6% 0.1 Hz) strain for 24 hours.

Figure 4.

Relative gene expression of collagen I, II, and aggrecan of annulus fibrosus cells exposed to glucosamine with or without inflammatory (IL-1 beta) and/or mechanical (6% 0.1 Hz) strain for 24 hours.

Figure 5.

Relative new proteoglycan synthesis, as measured by incorporation of ^{35}S after 24 hours of exposure to the test condition. All data are normalized to DNA content, and shown as relative incorporation compared to control (untreated) cells. Values are average of $n=3 \pm$ standard error.

Figure 6.

Relative total glycosaminoglycan, as measured by DMMB assay, after 24 hours of exposure to the test condition. All data are normalized to DNA content, and shown as relative incorporation compared to control (untreated) cells. Values are average of $n=3$ $\pm$ standard error.

Figure 7.

Concentration of PGE2 released into the media under each test condition. Values are average pg/mL in the media of n=3 +/- standard error.

Figure 8. Relative MMP activity measured using MMP-1 and MMP-3 specific substrates. Values represent maximum slope of the reaction kinetics, reported in fluorescent units per minute. Values represent average of n=3 +/- standard error.

Table 1

Custom designed primer sequences used for real time RT-PCR in all experiments.

Gene	Sequence
GAPDH	Rev: 5'-GCTGAGATGATGACCCTTTTGG-3' For: 5'-GATGCTGGTGCCGAGTAC-3'
Collagen I	Rev: 5'-GCCATCGACAAGAACAGTGTAAGT-3' For: 5'-ATGGATGAGGAACTGGCAACT-3'
Collagen II	Rev: 5'-CAGTCCCCGTGTCACAGACA-3' For: 5'-AAGAGGTATAATGATAAGGATGTGTGGAA-3'
Aggrecan	Rev: 5'-CGTAAAAGACCTCACCTCCAT-3' For: 5'-GCTACGGAGACAAGGATGAGTTC-3'
COX-2	Rev: 5'-CAGGCACCAGACCAAACTT-3' For: 5'-CACGCAGGTGGAGATGATCTAC-3'
iNOS	Rev: 5'-TCTGTGACGGCCTGATCTTTC-3' For: 5'-CCCCTTCAACGGCTGGTA-3'
MMP-1	Rev: 5'-GCCTGTCACTCGCAAACC-3' For: 5'-GACCTACGCACCCACACAC-3'
MMP-3	Rev: 5'-CCAGTGGATAGGCTGAGCAAA-3' For: 5'-AGCCAATGGAAATGAAAACCTCTTC-3'
TIMP-1	Rev: 5'-CCACAACTTGGCCCTGATG-3' For: 5'-AGCAGAGCCTGCACCTGTGT-3'