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IL-1 and *iNOS* gene expression and NO synthesis in the superior region of meniscal explants are dependent on the magnitude of compressive strains

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Summary

Objective: Partial meniscectomy is known to cause osteoarthritis (OA) of the underlying cartilage as well as alter the load on the remaining meniscus. Removal of 30–60% of the medial meniscus increases compressive strains from a maximum of approximately 10% to almost 20%. The goal of this study is to determine if meniscal cells produce catabolic molecules in response to the altered loading that results from a partial meniscectomy.

Method: Relative changes in gene expression of *interleukin-1* (*IL-1*), *inducible nitric oxide synthase* (*iNOS*) and subsequent changes in the concentration of nitric oxide (NO) released by meniscal tissue in response to compression were measured. Porcine meniscal explants were dynamically compressed for 2 h at 1 Hz to simulate physiological stimulation at either 10% strain or 0.05 MPa stress. Additional explants were pathologically stimulated to either 0% strain, 20% strain or, 0.1 MPa stress.

Results: *iNOS* and *IL-1* gene expression and NO release into the surrounding media were increased at 20% compressive strain compared to other conditions. Pathological unloading (0% compressive strain) of meniscal explants did not significantly change expression of *IL-1* or *iNOS* genes, but did result in an increased amount of NO released compared to physiological strain of 10%.

Conclusion: These data suggest that meniscectomies which reduce the surface area of the meniscus by 30–60% will increase the catabolic activity of the meniscus which may contribute to the progression of OA.
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Key words: Fibrochondrocyte, Osteoarthritis, Mechanotransduction, Unconfined compression.

Introduction

Partial meniscectomy is a common treatment for meniscal tears which do not heal due to the lack of vascularity in the inner two-thirds of the tissue. Partial meniscectomy has been shown to lead to osteoarthritis (OA) in the long term[1,2]. The amount of tissue removed has a bearing on the severity of the onset of OA[2], the loads carried by the meniscus[3], and the cellular environment of fibrochondrocytes within the tissue[4]. Hellio Le Graverand *et al.* [5] used ACL transection as an experimental model of OA to capture early changes in meniscal tissue and articular cartilage. This study showed that extracellular matrix deterioration and changes in cell and collagen distribution occurred in the menisci prior to any changes in articular cartilage. This suggests that meniscal tissue degeneration may be a precursor to the development of OA. Although the mechanism by which articular cartilage degrades has been widely studied, the mechanism of meniscal tissue degeneration and its association with OA are not clear. However, two biomolecules, nitric oxide (NO)[6,7] and interleukin-1 (IL-1)[8–10], are believed to regulate catabolic activity in both articular cartilage and meniscus.

NO is an important intracellular and extracellular messenger serving different functions in a variety of tissues[11]. NO causes tissue degeneration[12,13] and apoptotic cell death in cartilage and meniscus[6,7]. NO is produced by a family of enzymes called nitric oxide synthases (NOSs), two of which, NOS1 and NOS3, are constitutive forms and the third, NOS2 or inducible nitric oxide synthase (iNOS) is an inducible form. NO production within cartilaginous tissues such as articular cartilage and meniscus has been previously shown to be primarily of the inducible form and is regulated by chemical and mechanical stimuli[11–15,18]. While it has previously been shown that meniscal cells can make both NOS2 and NOS3[19], it is the inducible form of NOS that can be activated by inflammatory mediators such as IL-1 and tumor necrosis factor[11].

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IL-1, an inflammatory cytokine, has been shown to increase the production of NO in meniscal and articular cartilage[11,12] and also reduce synthesis of the major articular cartilage matrix protein (type II collagen), independently of NO at a transcriptional or post-transcriptional level[12]. IL-1 also causes a decrease in the incorporation of proteins specific to collagen[13]. Exogenous IL-1 has been shown to regulate the production of prostaglandin E_2 (PGE_2). PGE_2 is an inflammatory mediator which inhibits synthesis of collagen [15], *via* an NO dependant pathway and decreases proteoglycan synthesis[15,17]. Taken together, this suggests a catabolic role for IL-1 as collagen and proteoglycans constitute the majority of the solid constituents of meniscal tissue.

Mechanical forces such as tension and compression are essential for maintaining tissue homeostasis by balancing the catabolic and anabolic activities of biomolecules[20]. Mechanically loading cartilaginous tissue has been shown to both inhibit[15,18] and upregulate[16,17] NO production in articular cartilage and meniscus. This discrepancy may be due to a dose dependent response of the tissue to loading or tissue type. Although mechanical loading is a potent mediator of matrix synthesis and degeneration, it is not clear which magnitudes of mechanical loading are catabolic and which are anabolic in their overall effect on meniscal tissue. It is our hypothesis that there exists an optimal level of compression that will cause the lowest production of IL-1 and NO and hence be least damaging to meniscal tissue. This level may be ~10% compression which is predicted to be the upper limit of the healthy range of strains within intact meniscus[3]. We also hypothesize that superior and deep tissues will respond differently to the same loading by producing different amounts of NO and IL-1. These hypotheses will be tested by measuring *iNOS* and *IL-1* gene expression and NO production *in vitro* in response to unconfined compression at levels below physiological loading (0%), physiological levels (~10%) and supraphysiological loading (20%).

Methods

COMPRESSION TESTING

Stifle joints (knees) were obtained from 18-week-old pigs 24 h after death (Mayo Foundation, Rochester, MN) giving a sample size of six animals. For a given animal, both the medial and lateral menisci were excised using sterile techniques and 24 explants (six from each of the four menisci in an animal) measuring 6 mm in diameter and 5 mm in height were cored using a biopsy punch (6 mm Biopunch, Fray Products Corp., Buffalo, NY). The bottom face of each explant was trimmed to be parallel to the superior surface of the explants. The explants were cored perpendicular to the superior surface of the meniscus and were taken primarily from the outer region. Explants were then allowed to equilibrate for 48 h at 37°C with 5% CO_2 in media constituting 44.5% Dulbecco's modified Eagle medium (DMEM), 44.5% Ham's F12, 10% fetal bovine serum (FBS) and 1% penicillin streptomycin. Media were changed after 24 h of incubation.

Mechanical compression was achieved with a previously validated bioreactor[21]. Briefly, the bioreactor was designed to test six explants simultaneously in unconfined compression. Six frictionless Delrin© (DuPont, Willmington, DE) compression rods were driven by a stepper motor to compress explants in individual wells while housed in a sterile casing. A static pre-stress of 0.0076 MPa was applied prior to dynamic compression. Dynamic tests were then carried out at 37°C at 0%, 5%, 10%, 15% or 20% strain and also at 0.05 MPa or 0.1 MPa stress for 2 h at 1 Hz. Therefore, 0% strain actually refers to the amount of dynamic strain superimposed on top of the static 0.0076 MPa of pre-stress, and 0 MPa stress is actually no dynamic stress superimposed on top of the static 0.0076 MPa of pre-stress. The bioreactor used in the current study was previously presented with details of the stress and strain waveforms[21]. Tables I and II present a brief summary of the stress and strain tests. Neither confined or unconfined compression exactly replicates the *in vivo* environment of meniscal tissue. While previous computational modeling has documented the strain levels in the normal and meniscectomized menisci, unconfined compression to similar strain levels leads to abnormally large stresses on the tissue explant. Therefore, since the loading history is quite different between a stress controlled test and a strain controlled test, stress controlled experiments were also completed to determine differences between these two different mechanical effects. The stress levels were chosen such that they represent approximately the equilibrium values found during stress relaxation studies of meniscal tissue. For the 10% stress relaxation studies, equilibrium stress was approximately 0.05 MPa. For 20% stress relaxation studies, 0.1 MPa was the equilibrium stress. Hence, 0.05 MPa is considered to be physiologically normal, whereas 0.1 MPa is considered to be supraphysiological, or overloading[21].

Following mechanical loading, explants were removed from the bioreactor, cut into half to distinguish between superior and deep portions, weighed and incubated for 24 h in 1 ml of media similar in composition to that used for equilibration but containing 2% FBS. Explants were then removed from the media and stored in 200–300 μl of RNALater (Ambion Inc., Austin, TX) at 4°C for 1 day. RNALater was then removed and the samples were stored at −20°C. Post-compression media were stored in 1.5 ml vials at −80°C for subsequent analysis.

Table I
Change in pressures over the duration of meniscal tissue stress–relaxation tests. Data represent mean ± standard deviation. n = 6 *for all groups*

Strain (%)	Pressure (MPa)	
	Start	End
5	0.166 ± 0.108*,†	0.038 ± 0.010†
10	1.141 ± 0.103†	0.046 ± 0.010†
15	2.185 ± 0.827	0.035 ± 0.026†
20	3.548 ± 0.429	0.128 ± 0.020

*Significantly different than 15% ($P < 0.05$).
†Significantly different than 20% ($P < 0.05$)[21].

RNA ISOLATION

Total RNA was isolated from superior and deep sections of the explants using a commercial kit (SV Total RNA Isolation System, Promega, Madison, WI). Three explants were combined and crushed to a fine powder using liquid nitrogen. Preliminary data showed that combining three explants provided sufficient RNA for subsequent polymerase chain reaction (PCR) analyses. The powder was immediately added to lysis buffer and then stored at 4°C. A homogenizer was used to lyse the cells further. RNA was extracted and DNase-treated on the column according to the manufacturer's instructions. RNA was eluted in 100 μl of nuclease free water, analyzed with a spectrophotometer and run on a 1.5% native agarose gel to check for integrity by observing the 18S rRNA and 28S bands.

PRIMERS DESIGN

Specific primers were design based on *Sus scrofa* sequence (either partial or complete mRNA) available from the National Center for Biotechnology Information (NCBI). Amplicon sizes ranged from 150 bp to 260 bp and primers were approximately 20 bp long (Table III). PCR was performed using gene-specific primers and the products were verified by sequencing.

SEMI-QUANTITATIVE REVERSE TRANSCRIPTION-PCR (RT-PCR)

RT-PCR was performed using a two step process. First-strand RT was synthesized using Superscript II (Invitrogen Corporation, CA) and random primers. PCR was carried out for 28 cycles at an annealing temperature of 58°C using cDNA equivalent to 300 ng of RNA. cDNA used for amplifying *18S rRNA* was diluted 1/40 to allow both *iNOS* and *18S rRNA* to be amplified to similar levels, within the linear range of amplification, using the same cycle number and annealing temperature. PCR products were run on a 2.5% agarose gel and a digital image was taken. MATLAB (The MathWorks Inc.,

Table II
Change in strains over the duration of meniscal tissue creep tests. Data represent mean ± standard deviation. n = 6 *for all groups*

Pressure (MPa)	Strain (%)	
	Start	End
0.05	2.6 ± 0.53	11.6 ± 1.36
0.1	3.0 ± 0.12	20.7 ± 1.45*

*Significantly different than 0.05 MPa ($P < 0.05$)[21].

Table III
Sequences of designed primers for all genes

Gene	Forward (5′−3′)	Reverse (5′−3′)
iNOS	ACGCTCAGCTCATCCGGTAT	CACTTCAGCTCCAGCTCCTG
IL-1α	AGACACCCAAAACCATCAAAG	TCACAGGTAAGTAGACACCAG
*IL-1β**	GCTGAAGGCTCTCCACCTCC	TAAGGTCACAGGTATCTTGTTGTTGC
18S rRNA	GCAAATTACCCACTCCCGAC	CGCTCCCAAGATCCAACTAC

*Primers from literature [41].

Natick, MA) was used to measure the intensities of each band on the gel. Relative changes (increases or decreases) in the concentration of *iNOS* in each sample were recorded as intensities of the bands of *iNOS* normalized to the intensities of the corresponding bands of *18S rRNA.* To validate a semi-quantitative PCR for *iNOS*, PCR was first conducted for 26−30 cycles, and annealing temperatures up to 63°C were checked. Sequencing was completed on the gel to ensure proper product.

REAL-TIME PCR

Real-time PCR with SYBR Green mix (Absolute QPCR SYBR Green Mix, ABgene Inc., NY) detection was used to quantify the expression of both types of *IL-1*: α and β. The efficiency of amplification was calculated for each gene from a standard curve. Average values (82.7% − *IL-1α*, 87% − *IL-1β*, 88.25% − *18S rRNA*) of efficiency were used in further analysis. Since the Pfaffl[22] method was used to analyze the results, a calibrator was run with each test for both genes of interest and the housekeeping gene (*18S rRNA*). Each sample was run for 40 cycles (95°C/15 s, 59°C/45 s, 72°C/40 s) and each 25 µL reaction contained 15 ng of cDNA and 1 µL of carboxy-X-rhodamine (ROX) reference dye (Absolute QPCR SYBR Green Mix, ABgene Inc., NY) to normalize the fluorescence. Primer concentrations were 2.5 µM, 5 µM and 10 µM for *IL-1α*, *IL-1β* and *18S rRNA*, respectively. ROX was diluted to 0.25%. All samples were run in duplicate. A dissociation curve was run with each plate setup to confirm regularity of the tests. Samples were analyzed using the Pfaffl method by which the ratio of the target gene to the housekeeping gene is quantified with respect to the calibrator using the following formula:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta C_{\text{t}}\text{target(control−treated)}}}{(E_{\text{ref}})^{\Delta C_{\text{t}}\text{ref(control−treated)}}}$$

where C_t − cycle number in the linear range of amplification, E − efficiency of the process for each gene, target − gene of interest, ref − housekeeping gene, control − calibrator, treated − sample.

All samples were normalized to controls (either 0% strain or 0 MPa stress) from each animal.

NO QUANTIFICATION

NO release was quantified using a commercially available kit (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical Company, Ann Arbor, MI). Since the final products of NO *in vivo* are nitrite (NO_2^-) and nitrate (NO_3^-), the best quantification of total NO production is the sum of the concentration of each. Hence, this assay measured total NO concentration in two steps. In the first step nitrate was converted to nitrite utilizing nitrate reductase. In the second step nitrite was converted into a deep purple azo compound through a Griess reaction. Absorbance of colored azo was measured and then converted to total NO concentration (µM) using a standard curve. All measurements were taken in duplicate. The final concentration was normalized by wet weight (g) of the explants. Since proteins are known to interfere with the Griess reaction, conditioned media were filtered using a 10,000 molecular weight cut-off filters before it was assayed (Millipore Microcon YM-10, Bedford, MA).

DATA ANALYSIS

All data are presented as mean ± standard error. A one way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) testing was used to measure statistical differences ($p < 0.05$ was considered significant). Paired *t* tests were used at each stress or strain level to determine differences between superior and deep zones.

Results

RELATIVE *iNOS* GENE EXPRESSION

Superior explants tested at 20% strain showed the largest *iNOS* gene expression compared to other strain levels [Fig. 1(A)]. Compared to the uncompressed samples, 20% compression significantly upregulated *iNOS* gene expression. Samples from the superior zone expressed slightly greater amounts of *iNOS* compared to deep regions for each compression level (statistical significance at 0% and

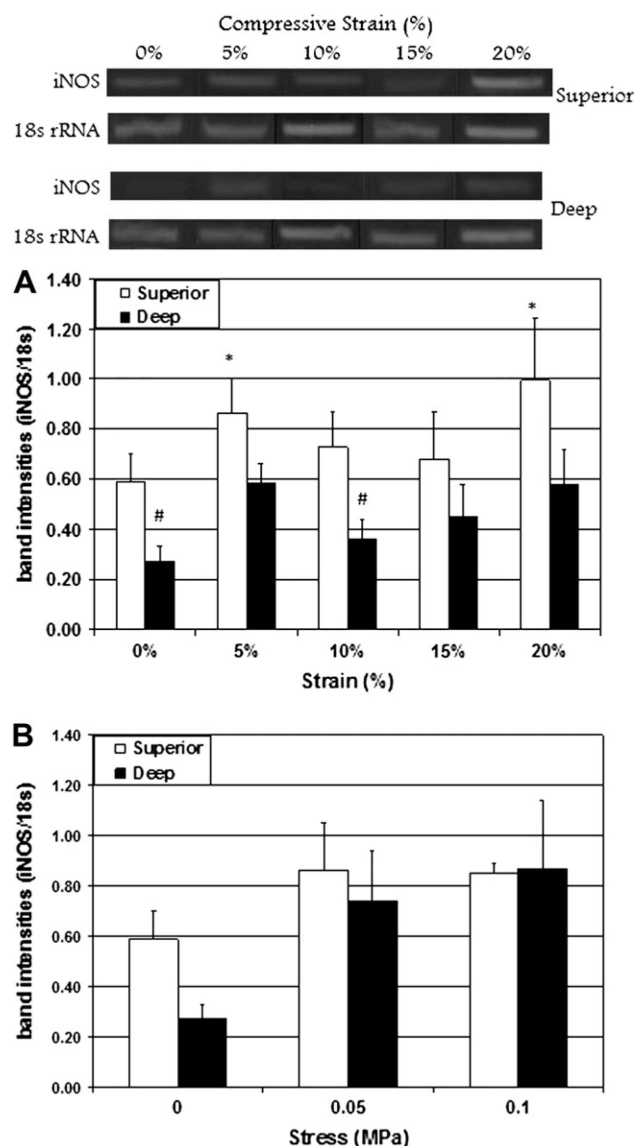

Fig. 1. Relative *iNOS* gene expression in superior (white) and deep (black) zones of meniscal explants after unconfined compression to 0%, 5%, 10%, 15% or 20% (A) and 0.0 MPa, 0.05 MPa or 0.1 MPa (B). *Significantly different than 0% compression superior zone. #Significantly different than superior zone for the same strain.

10% compression) [Fig. 1(A)]. No differences between stress levels were noted for *iNOS* expression [Fig. 1(B)].

RELATIVE *IL-1α* EXPRESSION

A significant increase in *IL-1α* gene expression was seen for explants strained to 20% compared to other tested levels for both superior and deep zones [Fig. 2(A)]. There were no significant differences between superior and deep regions for *IL-1α* [Fig. 2(A) and (B)]. As with *iNOS* expression, no significant differences between stress levels were seen. The greatest expression of *IL-1α* occurred with 0.05 MPa of stress from the deep zone [Fig. 2(B)].

RELATIVE *IL-1β* EXPRESSION

While the superior and deep zones behaved similarly for expression of *IL-1α*, the expression of *IL-1β* is different between zones. No statistical differences were noted in the superior zones across the different strain levels. *IL-1β* gene expression was significantly upregulated with 10% dynamic compressive strain compared to 0% dynamic compressive strain in the deep zone explants [Fig. 3(A)]. The only significant difference between superior and deep zone explants was for 10% dynamic compressive strain, with the deep zone expressing more than double that of the superior zone [Fig. 3(A). For the stress experiments, *IL-1β* follows the same trend as *iNOS*, and *IL-1α* with expression following 0.05 MPa stress being the highest [Fig. 3(B)].

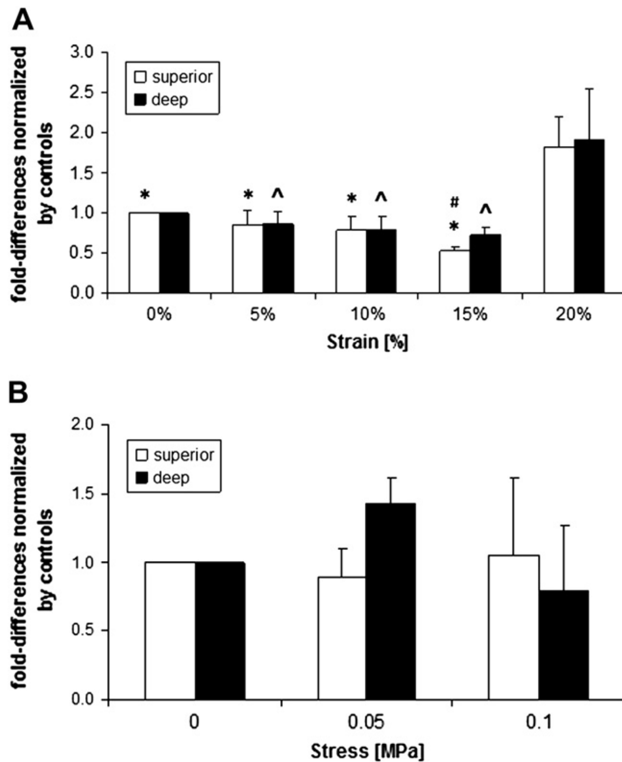

Fig. 2. Relative (to *18S rRNA*) changes in *IL-1α* after compression to (A) 0%, 5%, 10%, 15% or 20% strain and (B) 0.05 MPa or 0.1 MPa. *Significantly different than 20% strain superior zone, ^significantly different than 20% strain deep zone, #significantly different than 0% superior zone.

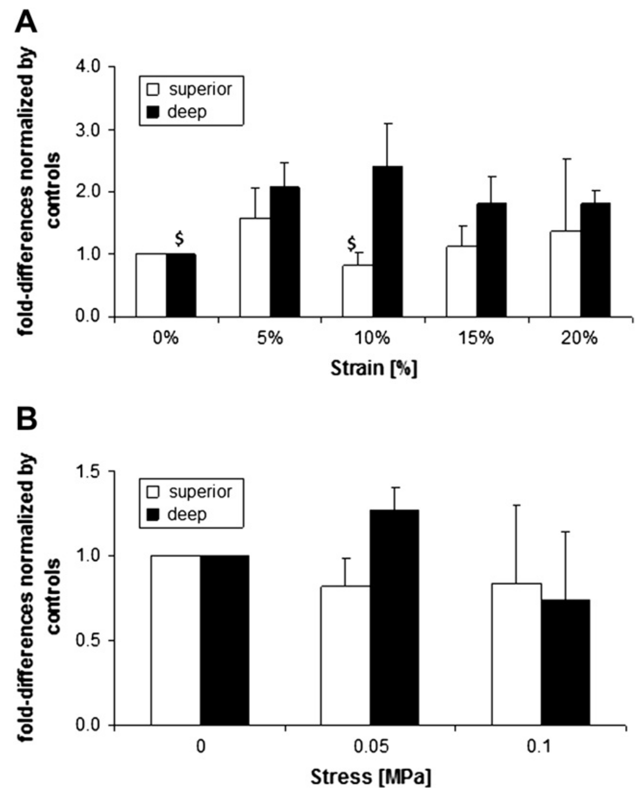

Fig. 3. Relative (to *18S rRNA*) changes in *IL-1β* after compression to (A) 0%, 5%, 10%, 15% or 20% and (B) 0.05 MPa or 0.1 MPa. $Significantly different than 10% compressive strain deep zone.

NO RELEASE

The lowest release of NO was seen in superior samples strained to 10% compression compared to 0%, 5%, 15% and 20% compressive strains [Fig. 4(A)]. There were no statistical differences between superior and deep zones samples or the stress control samples [Fig. 4(A) and (B)].

Discussion

Samples taken from both the superior and deep zones support the hypothesis of this study showing the lowest NO release for samples compressed to 10%. Additionally *IL-1β* gene expression was the lowest at 10% compression for superior explants. In previous studies, the positive effects of compression on protein and proteoglycan incorporation (measures of matrix synthesis) were eliminated by the addition of IL-1 but restored in the presence of IL-1 once NO was blocked[17]. This may indicate that compression acts on IL-1, which then mediates the production of NO, which in turn mediates tissue degeneration[23]. While others have studied the iNOS/NO response of meniscal tissue to loading[24,16], this study is novel in that a loading scheme has been chosen that can be related to the consequences of partial meniscectomy.

Compressive strains of 20% resulted in the highest expression/release of *iNOS*, *IL-1α* and NO. Similar results were obtained in a previous study that measured the release of glycosaminoglycans (GAG) into the media following dynamic compression[21]. 20% strain is considered pathological and removing 30–60% of the meniscus during

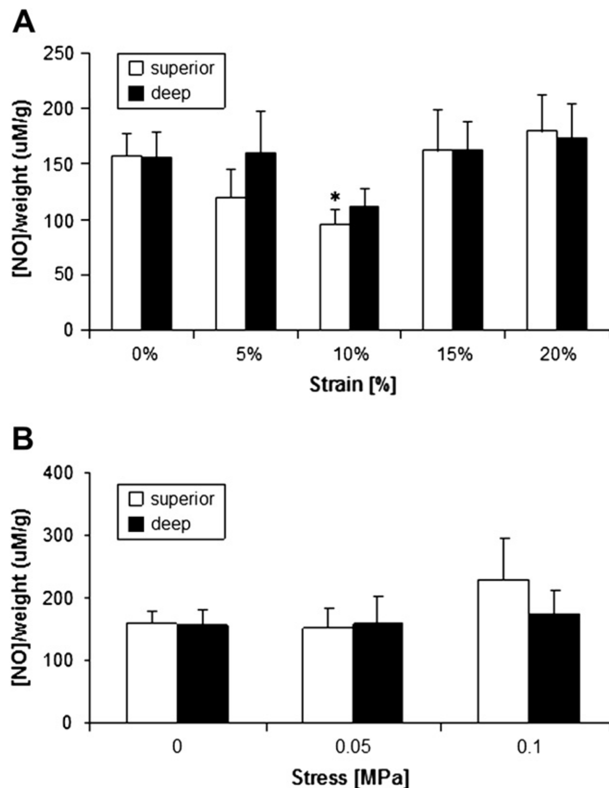

Fig. 4. NO released into media measured as concentrations of nitrite for explants compressed to 0%, 5%, 10%, 15% or 20% strain (A) and 0.0 MPa, 0.05 MPa or 0.1 MPa (B). Results are standardized with respect to the wet weight of the tissue. *10% superior is significantly different than 20%.

a partial meniscectomy increases the strains to approximately 20%[3]. This upregulation of catabolic gene expression may be a precursor to meniscal degeneration and subsequent OA.

Kobayashi *et al.*[7] showed that cells from superior zones of the meniscus produced more NO compared to cells from the deep zone following partial meniscectomy. This agrees with our finding in that superior sections of explants showed some significant differences in NO release at varying compression levels perhaps indicating that the superficial zone is more sensitive to variations in the mechanical environment compared to the deep zone. Cells from the superior zone of meniscal tissue are thought to be more elliptical or fibroblastic compared to cells from the deeper regions that are more spherical or chondrocytic. A finite element model of a chondron within meniscal tissue showed that fibroblastic cells experience larger strains and stresses than chondrocytic cells[4]. Inhomogeniety between the material properties of superior and deep tissues[25–27] may cause cells in the superior region to be more sensitive to compression regardless of morphology. In this study we did not characterize cell populations following division. This may be a reason for the lack of statistical differences between superior and deep samples.

Knee joint immobilization has been shown to decrease aggrecan gene expression in intact menisci which may lead to tissue degradation[28]. However, our study did not show increases in *iNOS*, *IL-1α* or *IL-1β* gene expression for underloaded samples (0% and 0 MPa). On the other hand, NO release was higher for uncompressed explants compared to explants compressed to 5% or 10%. This discrepancy between *iNOS* gene expression and NO release may be due to the duration of post-incubation. For example, Bonassar *et al.*[29] showed that the time taken for protein synthesis to occur in articular cartilage stimulated with insulin like growth factor-1 (IGF-1) vs mechanical compression was 12.2 h vs 2.9 h, respectively. Both *iNOS* gene expression and NO release were measured 24 h post-treatment in this study and disuse was modeled as the absence of loading.

It is also conceivable that a relatively lower level of *iNOS* gene expression may cause a relatively higher level of NO synthesis for unloaded explants compared to explants compressed to 5%. The stability of *iNOS* mRNA is important for iNOS induction[30]. Factors which control the translation of *iNOS* mRNA into iNOS enzyme and/or the synthesis of NO by the iNOS enzyme may be sensitive to loading. This may explain the discrepancy between the reduced *iNOS* gene expression at 0% compression and the increased NO release at 0% compression compared to 5% compression.

A comparison of the stress experiments vs the strain experiments highlights a few differences in the tissue response. Firstly, while it was expected that 0.1 MPa experiments and 20% strain experiments would yield similar results since both conditions result in overloading of the tissue, the stress experiments to 0.1 MPa did not result in a significant upregulation of *iNOS* compared to 0.0 MPa or 0.05 MPa of stress. This is likely due to the fact that the 20% strain explants were subjected to much larger stresses initially (3.5 MPa) whereas the stress control experiments were not. It is perhaps this large initial stress during the 20% strain control experiments that is responsible for the increased *iNOS* and *IL-1* gene expression.

Both IL-1α and IL-1β have been implicated in the pathophysiology of OA[31,32]. Others have shown that exogenous IL-1β added to chondrocytes increases NO production[33,23]. Exogenous IL-1α and IL-1β have both been shown to increase the production of PGE_2[34], decrease proteoglycan synthesis[35], and increase proteoglycan degradation[35]. When exogenous IL-1α was added to human articular cartilage a marked loss in proteoglycan was shown to occur[36]. A direct link between exogenously added IL-1α, iNOS and NO has been previously established[37]. The difference between these previous studies and the current study is that no exogenous IL-1α or IL-1β was added to the meniscal explants. The current study shows significant upregulation of *IL-1α* gene with pathological overloading (20% strain) for both the superior and deep zones. Approximately 10 molecules of IL-1β are needed to activate a signaling pathway[38]. Hence, biologically significant changes in *IL-1β* gene expression may have been masked by differences in gene expression between animals. To our knowledge, expression of *IL-1* by meniscal tissue following mechanical stimulation has not previously been studied.

This study is a step forward in understanding the mechanisms of compression induced changes in biochemical activity in meniscal tissue which may ultimately change its material properties.

Although this study shows that *IL-1* and *iNOS* gene expression are regulated by compression in a similar manner, further investigation is required to elucidate the interaction of these two genes. Although others have studied the effects of adding IL-1 to tissue exogenously[13,39,40,17], measurement of *iNOS* gene expression once IL-1 is blocked will clarify the dependence of *iNOS* gene expression on IL-1.

Additionally, this study has only documented gene expression for *IL-1*, thus it is not yet known if IL-1 protein is made or if the IL-1 has any activity. Future studies are directed toward studying the various zones of the tissue as well as blocking studies to determine the mechanism of response.

In conclusion, this study shows that catabolic activity, specifically *iNOS* and *IL-1α* gene expression and subsequent NO release, is dependent on the magnitude of mechanical loading *in vitro*. Results show an increase in *iNOS* and *IL-1α* gene expression and NO release when meniscal tissue is compressed to pathological overloading compared to physiological levels of load. The results of this study, taken together with studies[3], may indicate that degeneration of meniscal tissue will result from altered loading induced by 30% or more partial meniscectomy whereby leading to OA.

Conflict of interest

The authors have no conflict of interest.

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