NUCLEUS PULPOSUS CELLS SYNTHESIZE A FUNCTIONAL EXTRACELLULAR MATRIX AND RESPOND TO INFLAMMATORY CYTOKINE CHALLENGE FOLLOWING LONG-TERM AGAROSE CULTURE

Lachlan J. Smith, Joseph A. Chiaro, Nandan L. Nerurkar, Daniel H. Cortes, Sarena D. Horava, Nader M. Hebela[1], Robert L. Mauck, George R. Dodge and Dawn M. Elliott

Department of Orthopaedic Surgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

[1]Department of Veterans Affairs Medical Center, Philadelphia, PA, 19104, USA

Abstract

Intervertebral disc degeneration is characterized by a cascade of cellular, biochemical and structural changes that may lead to functional impairment and low back pain. Interleukin-1 beta (IL-1β) is strongly implicated in the etiology of disc degeneration, however there is currently no direct evidence linking IL-1β upregulation to downstream biomechanical changes. The objective of this study was to evaluate long-term agarose culture of nucleus pulposus (NP) cells as a potential *in vitro* model system to investigate this. Bovine NP cells were cultured in agarose for 49 days in a defined medium containing transforming growth factor-beta 3, after which both mechanical properties and composition were evaluated and compared to native NP. The mRNA levels of NP cell markers were compared to those of freshly isolated NP cells. Glycosaminoglycan (GAG) content, aggregate modulus and hydraulic permeability of mature constructs were similar to native NP, and aggrecan and SOX9 mRNA levels were not significantly different from freshly isolated cells. To investigate direct links between IL-1β and biomechanical changes, mature agarose constructs were treated with IL-1β, and effects on biomechanical properties, extracellular matrix composition and mRNA levels were quantified. IL-1β treatment resulted in upregulation of a disintegrin and metalloproteinase with thrombospondin motifs 4, matrix metalloproteinase-13 and inducible nitric oxide sythase, decreased GAG and modulus, and increased permeability. To evaluate the model as a test platform for therapeutic intervention, co-treatment with IL-1β and IL-1 receptor antagonist (IL-1ra) was evaluated. IL-1ra significantly attenuated degradative changes induced by IL-1β. These results suggest that this *in vitro* model represents a reliable and cost-effective platform for evaluating new therapies for disc degeneration.

Key Words: Intervertebral disc, nucleus pulposus, agarose, interleukin-1 beta, interleukin-1 receptor antagonist; biomechanical properties.

*Address for correspondence:
Lachlan J Smith
Department of Orthopaedic Surgery
Perelman School of Medicine
University of Pennsylvania
424 Stemmler Hall
36th and Hamilton Walk
Philadelphia, PA, 19104 USA

Telephone Number +1 215-898-8653
FAX Number: +1 215-573-2133
E-mail: lachlans@mail.med.upenn.edu

Introduction

Intervertebral disc degeneration is characterized by a cascade of cellular, biochemical and structural changes that may ultimately lead to functional impairment and low back pain. This cascade begins in the central nucleus pulposus (NP), where decreasing proteoglycan content and an associated reduction in hydrostatic pressure impair the ability of the NP to perform its most critical role: the even distribution and transfer of compressive loads between the vertebral bodies (Adams *et al.*, 1996; Roughley, 2004).

Inflammatory cytokines play key roles in the etiology of disc degeneration (Freemont, 2009). Interleukin-1 beta (IL-1β) in particular is highly expressed by cells in the degenerate NP (Le Maitre *et al.*, 2005), and has been shown to be associated with increased activity of downstream catabolic enzymes, including those that degrade proteoglycans and collagen (Le Maitre *et al.*, 2004; Le Maitre *et al.*, 2007c; Hoyland *et al.*, 2008). While previous studies have demonstrated that IL-1β induces a catabolic response by NP cells at the molecular level, to date no studies have reported a direct link between these molecular changes and altered biomechanical function. One reason for this is that there is currently a lack of appropriate mimetic model systems that facilitate simultaneous evaluation of cellular events, and molecular, compositional and biomechanical changes associated with disc degeneration.

The molecular and biosynthetic characteristics of NP cells have been studied extensively in three-dimensional culture (Yang and Li, 2009). The majority of such studies have used alginate, a hydrogel that preserves the NP cell phenotype, however there is evidence that NP cells are not able to assemble a functional extracellular matrix in alginate (Baer *et al.*, 2001). A smaller number of studies have used agarose, but have been of relatively short duration, and have not evaluated mechanical properties (Horner and Urban, 2001; Sato *et al.*, 2001; Shen *et al.*, 2003; Gokorsch *et al.*, 2004; Zeiter *et al.*, 2009; Fernando *et al.*, 2011). Long-term agarose culture has been extensively used to study the behavior of articular cartilage chondrocytes *in vitro* (Buschmann *et al.*, 1992; Mauck *et al.*, 2000; Lima *et al.*, 2008; Huang *et al.*, 2010). In the presence of specialized chemically defined media, chondrocytes cultured in agarose synthesize mechanically robust, cartilage-like constructs. Due to phenotypic similarities with chondrocytes (Sive *et al.*, 2002), it was hypothesized that mature NP cells would respond in a similar way when cultured under the same conditions.

The first objective of this study, therefore, was to engineer an *in vitro*, biological nucleus pulposus-like

construct which reflects the characteristics of the native tissue, including biochemical composition, biomechanical properties and mRNA levels, using long term culture in agarose and a chemically defined medium. The second objective was to investigate effects of IL-1β treatment on the biomechanical properties, extracellular matrix composition and mRNA levels of functionally mature NP cell-seeded agarose constructs. Interleukin 1 receptor antagonist (IL-1ra) has shown early promise as a potential therapy for disc degeneration, by inhibiting IL-1β activity (Le Maitre *et al.*, 2007a). The final objective of this study was to examine the ability of IL-1ra to attenuate functional matrix degradation induced by IL-1β.

Methods

Cell isolation and agarose culture

Four caudal intervertebral discs were obtained from each of four fresh adult bovine tails (16 discs total), purchased from a local slaughterhouse according to Institutional guidelines. NP tissue was isolated, placed in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing 2 % penicillin/streptomycin/fungizone (PSF) and incubated overnight at 37 °C to verify no bacterial growth. Each sample was then digested for 1 h in 2.5 mg/mL pronase (66 PUK/mg solid), followed by 4 h in 0.5 mg/mL collagenase (>125 CDU/mg solid), both at 37 °C, then filtered through a 70 μm cell strainer. Three aliquots ($2x10^5$ cells) of these 'freshly isolated' cells were retained for comparative mRNA experiments. The remaining isolated cells were expanded in monolayer in high glucose DMEM containing 10 % fetal bovine serum (FBS) and 1 % PSF. Passage 2 cells from all donor animals were combined to minimize the effects of inter-donor biological variation, and suspended in a chemically defined medium comprised of high glucose DMEM supplemented with 1 % PSF, 0.1 μM dexamethasone, 50 mg/mL ascorbate 2-phosphate, 40 mg/mL L-proline, 100 mg/mL sodium pyruvate, ITS Premix (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenious acid), 1.25 mg/mL bovine serum albumin (BSA), 5.35 μg/mL linoleic acid and 10 ng/mL transforming growth factor-beta 3 (TGF-β3). This chemically defined medium has been demonstrated previously to facilitate enhanced matrix deposition over serum alone containing media by both NP cells and articular chondrocytes in three-dimensional culture (Mauck *et al.*, 2006; Reza and Nicoll, 2010b). The cell solution was mixed with an equal volume of 4 % sterile, low gelling temperature agarose at a temperature of 49 °C such that the final seeding density was $2.0x10^7$ cells/mL. While this cell density is higher than the native adult NP (Maroudas *et al.*, 1975), it was necessary to achieve sufficient levels of functional matrix deposition that would facilitate measurable changes in mechanical properties following cytokine treatments. Gels were cast between 2 glass plates to obtain a slab 2.25 mm thick and individual constructs 4 mm in diameter were then cut using a biopsy punch. Constructs were cultured for 49 days. Agarose (catalogue no. A4018, batch no. 109K1232), collagenase, dexamethasone, L-proline, ascorbate 2-phosphate, linoleic acid and BSA were purchased from Sigma-Aldrich (St Louis, MO, USA); DMEM, PSF (catalogue no. 15240), sodium pyruvate and FBS (lot #769376) from Invitrogen (Carlsbad, CA, USA); pronase from Merck (Darmstadt, Germany); TGF-β3 from R&D Systems (Minneapolis, MN, USA); ITS Premix from BD Biosciences (Bedford, MA, USA); and sodium pyruvate from Mediatech Inc (Manassas, VA, USA).

Mechanical properties, biochemical composition, histology and mRNA levels were evaluated after 14 and 49 d, and compared with day one properties and native tissue properties.

IL-1β and IL-1ra treatment

For samples undergoing cytokine treatments, TGFβ-3 was removed from the media 7 d prior to commencing treatments (i.e., at day 42 of agarose culture). Constructs (n = 16 per group) were treated with defined media (without TGFβ-3) supplemented with a single dose of recombinant human IL-1β (10 ng/mL), IL-1ra (100 ng/mL), or both IL-1β and IL-1ra together (R&D Systems). Dosages were selected based on the results of previous studies (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007a) and our own pilot studies. Samples were harvested after 3 d. Mechanical properties, biochemical composition and mRNA levels were evaluated and compared to untreated controls as described below.

Mechanical testing

Constructs (n = 5) at 1, 14 and 49 d of culture, and from each treatment group were tested in confined compression. The testing system consisted of an acrylic chamber fixed above a porous, stainless steel platen (10 μm pore size, 50 % void ratio) within a testing bath filled with culture media (without TGFβ-3). To account for variability in sample diameter following agarose culture, six confinement chambers were constructed ranging in diameter from 4.00 to 4.50 mm. Prior to testing, the diameter of each construct was measured using digital calipers and matched to a confinement chamber. Compression was applied using an impermeable ceramic indenter, size-matched to the confinement chamber, attached to a mechanical testing system fitted with a 5 N load cell (Instron; Norwood MA, USA). Samples were initially subjected to a 0.02 N preload held for 500 s, followed by a stress relaxation test. This consisted of 10 % strain, calculated based on the sample thickness following preload, applied at a rate of 0.05 % per second, followed by relaxation to equilibrium for 10 min. Aggregate modulus (H_A) was calculated as the final, equilibrium stress (equilibrium force/sample area) divided by the applied strain. Hydraulic permeability (k_0) was calculated from the relaxation data using linear biphasic theory, assuming material isotropy, as described previously (Soltz and Ateshian, 1998).

Biochemical composition

Following mechanical testing, samples were weighed and digested overnight in papain at 60 °C. Digests were assayed for DNA content using the PicoGreen assay (Invitrogen), sulfated GAG content using the dimethylmethylene blue (DMMB) technique, and collagen (following acid hydrolysis) using the p-diaminobenzaldehyde/

Table 1: PCR primer sequences.

Primer	Accession Number	Direction (5'->3')	Sequence	Tm (°C)	Product Length	Source
ACAN	NM_173981.2	F	CCTGAACGACAAGACCATCGA	54.29	101	(Fitzgerald *et al*., 2006)
		R	TGGCAAAGAAGTTGTCAGGCT	54.48		
ADAMTS4	NM_181667.1	F	AACTCGAAGCAATGCACTGGT	54.96	149	
		R	TGCCCGAAGCCATTGTCTA	53.16		
COL1A2	NM_174520.2	F	GGTAGCCATTTCCTTGGTGGTT	54.84	102	
		R	AATTCCAAGGCCAAGAAGCATG	53.85		
COL2A1	NM_001113224.1	F	AAGAAGGCTCTGCTCATCCAGG	56.09	124	
		R	TAGTCTTGCCCCACTTACCGGT	57.04		
COL6A2	NM_001075126.1	F	CTGGAGAGCCTGGACAGAAG	53.64	95	(Dimicco *et al.*, 2007)
		R	GCCTTTGAAACCAGGAACAC	51.77		
GAPDH	NM_001034034.1	F	ATCAAGAAGGTGGTGAAGCAGG	54.61	101	(Fitzgerald *et al*., 2006)
		R	TGAGTGTCGCTGTTGAAGTCG	55.17		
INOS	NM_001076799.1	F	GTAACAAAGGAGATAGAAACAACAGG	52.30	146	This paper
		R	CAGCTCCGGGCGTCAAAG	55.52		
KRT8	NM_001033610.1	F	CCGAGTCCTCTGATGTCCTGTCCA	59.23	86	This paper
		R	GCTCCATCTGCAAGGAGCCAATGA	59.29		
MMP13	NM_174389.2	F	TGGTCCAGGAGATGAAGACC	52.51	80	(Fitzgerald *et al*., 2006)
		R	TGGCATCAAGGGATAAGGAA	50.22		
SOX9	XR_083993.1	F	TGAAGAAGGAGAGCGAGGAG	52.72	128	This paper
		R	CTTGTTCTTGCTCGAGCCGTTGA	57.90		

chloramine-T technique for hydroxyproline. Collagen content was calculated assuming a ratio of hydroxyproline to collagen of 1:10 (Nimni, 1983). DNA content was normalized per construct, and GAG and collagen were normalized to construct wet weight. For treatment groups, media aliquots ($n = 5$) were also analyzed for GAG content (normalized per construct) using the DMMB assay.

mRNA levels

Nine constructs from each culture and treatment group pooled into three groups of three, and the three samples of freshly isolated cells, were used for mRNA analyses. RNA was isolated *via* two sequential extractions in TRIZOL/chloroform (Invitrogen) and spectrophotometrically quantified (ND-1000; Nanodrop Technologies, Wilmington, DE, USA). Reverse transcription was performed on 1 µg of RNA with random hexamers using a Superscript II kit (Invitrogen) in a 20 µL volume.

To determine the degree to which the NP cells recovered and/or maintained their phenotype in three-dimensional culture following monolayer expansion, the mRNA levels of aggrecan (ACAN), collagen II (COL2A1), collagen I (COL1A2), collagen VI (COL6A2), SOX9 and cytokeratin 8 (KRT8) after 1, 14, and 49 d of culture in agarose were measured and compared to the mRNA levels of freshly isolated NP cells. ACAN, COL2A1 and SOX9 are general phenotype markers that NP cells share with cartilage chondrocytes, while KRT8 is an NP cell specific marker (Minogue *et al.*, 2010). For treatment groups, we additionally examined mRNA levels of matrix metalloproteinase 13 (MMP13), a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4), and inducible nitric oxide synthase (iNOS). Primer sequences are provided in Table 1. Primer specificities were confirmed by performing conventional polymerase chain reaction (PCR) and determining amplicon size on agarose gel electrophoresis of the products on a 3 % w/v agarose gel stained with ethidium bromide. Optimal annealing temperatures were determined by performing a temperature gradient from 60 °C to 50 °C using conventional PCR as above. Quantitative PCR was subsequently performed on an Applied Biosystems 7500 system using SYBR Green reagents (Applied Biosystems, Carlsbad, CA, USA). Absolute mRNA levels were calculated from standard curves, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and expressed as a ratio to freshly isolated cells (1, 14 and 49 d agarose culture groups) or untreated constructs (treatment groups).

Histology

To assess uniformity of the distribution of the synthesized extracellular matrix, 2 samples from the 14 and 49 d culture groups were fixed in 10 % buffered formalin immediately at harvest and processed for histology in paraffin. 7 µm thick sections were stained with either Alcian blue or picrosirius red to demonstrate GAG or collagen respectively.

Native bovine NP samples

Native bovine NP samples were obtained and properties compared with those of engineered constructs. Samples for biomechanical testing ($n = 4$) were trimmed on a freezing stage microtome to a uniform thickness of approximately 2.5 mm. A biopsy punch was then used to core a central

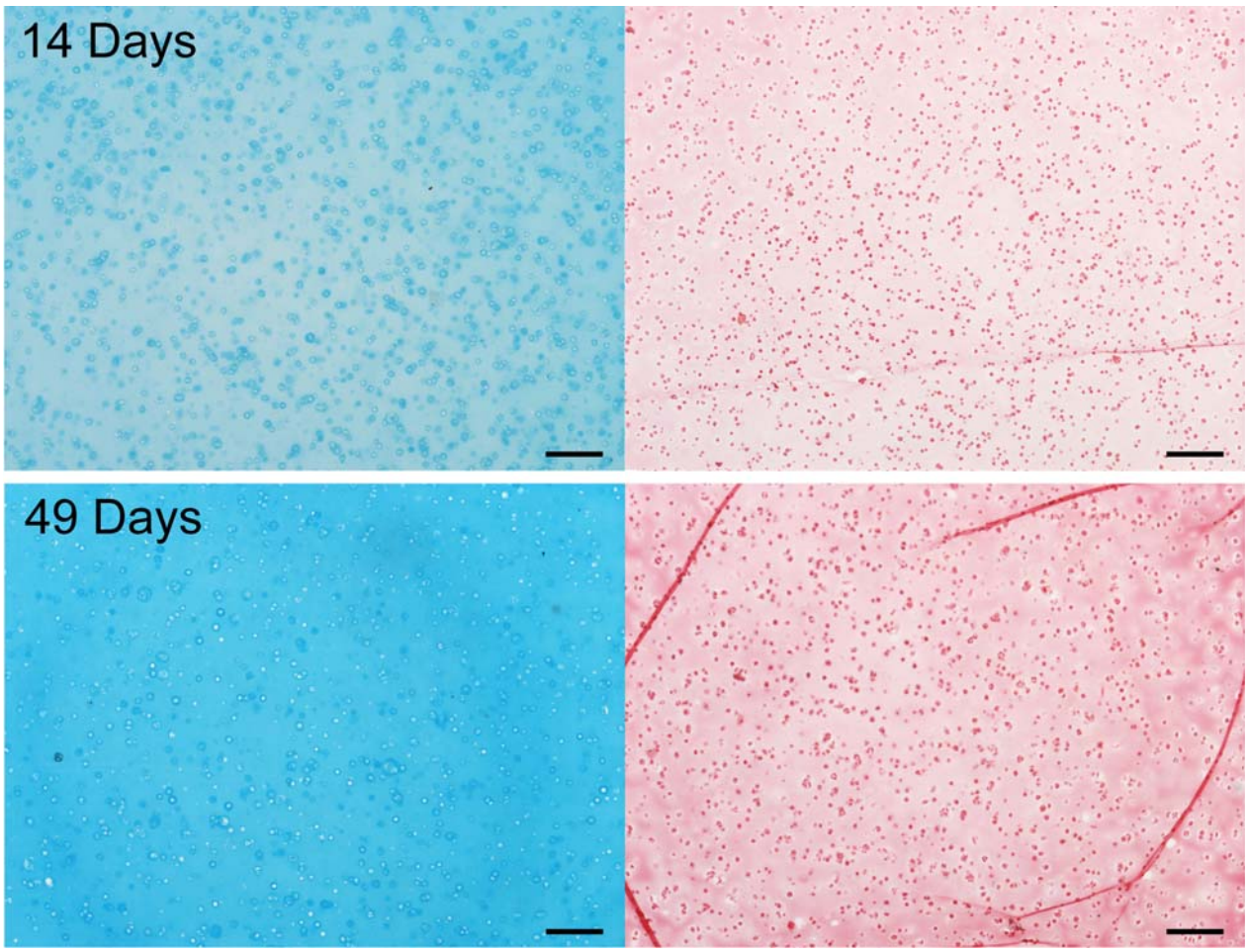

Fig. 1. Alcian blue (left) and picrosirius red (right) staining after 14 and 49 d of agarose culture showing progressive accumulation of GAG and collagen. Scale bars = 200 μm.

4 mm diameter plug, and samples were tested in confined compression using the protocol described. Samples for biochemistry were processed identically to agarose constructs and assayed for GAG and collagen.

Statistical analysis

All results are presented as mean ± standard deviation. Properties of samples after 14 and 49 d of agarose culture were compared to day one properties using unpaired Student's *t*-tests. 1, 14 and 49 d properties were compared to native NP properties using unpaired Student's *t*-tests. To confirm that removal of TGFβ-3 at day 42 had no effect, properties of samples cultured under these conditions were compared to those of samples cultured with TGFβ-3 for the full 49 days using unpaired Student's *t*-tests. The mRNA levels for 1, 14 and 49 d samples were expressed as a ratio to freshly isolated cells for statistical comparisons. Properties of treated samples were compared to those of untreated samples using unpaired Student's *t*-tests. The mRNA levels for treated samples were expressed as a ratio to untreated for statistical comparisons. Significance was defined as two-tailed $p < 0.05$.

Results

The intensity of GAG and collagen staining increased from 14 to 49 d of culture in agarose (Fig. 1).

Mean aggregate modulus of constructs increased progressively with culture time, and was significantly greater at 14 d (35 kPa) and 49 d (115 kPa) than at 1 d (2 kPa) (Fig. 2A). At day 1 of culture, aggregate modulus of constructs was 2 % of the native NP modulus ($p < 0.05$), 63 % of native after 14 d culture (not significantly different) and 207 % of native after 49 d ($p < 0.05$) (Fig. 2A). Hydraulic permeability of constructs decreased progressively with culture time, and was significantly lower at 14 d (0.76 $mm^4/N.s \times 10^{-12}$) and 49 d (0.04 $mm^4/N.sx10^{-12}$) than at 1 d (0.76 $mm^4/N.sx10^{-12}$) (Fig. 2B). At day 1, hydraulic permeability of constructs was 2,432 % of the native NP permeability ($p < 0.05$), 401 % of native after 14 d culture ($p < 0.05$) and 102 % of native after 49 d (not significantly different) (Fig. 2B). Removal of TGFβ-3 from the culture medium at 42 d had no significant effect on either aggregate modulus (Fig. 2A) or hydraulic permeability (Fig. 2B) at 49 d compared with constructs cultured for the full 49 d with TGFβ-3.

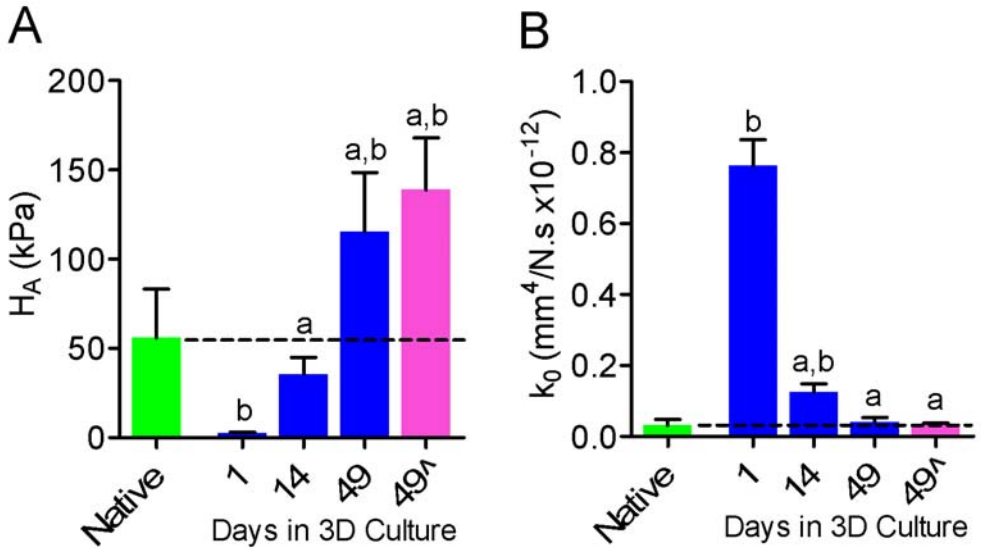

Fig. 2. Mechanical properties of NP cell-seeded agarose constructs with culture duration and in comparison to native bovine NP ($n = 5$). (**A**) Aggregate modulus, H_A. (**B**) hydraulic permeability, k_0; [a]Significantly different from day 1, $p < 0.05$; [b]significantly different from native NP, $p < 0.05$. Dashed lines represent mean native values. 49^ = TGF-β3 removed from media at day 42.

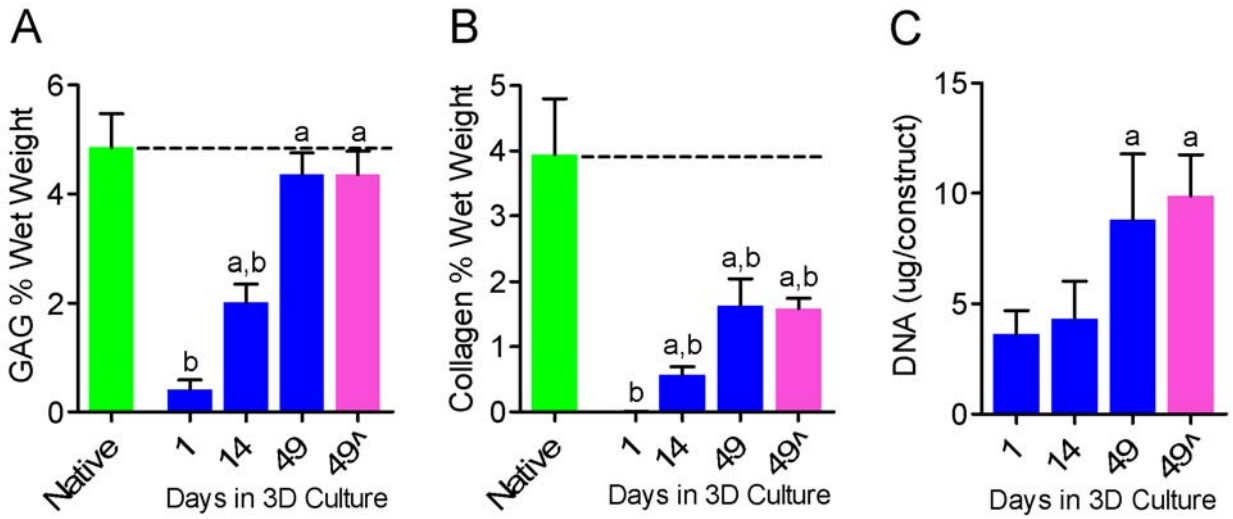

Fig. 3. Biochemical composition of NP cell-seeded agarose constructs with culture duration and in comparison to native bovine NP ($n = 5$). (**A**) GAG content, (**B**) Collagen content, (**C**) DNA content. [a]Significantly different from day 1, $p < 0.05$; [b]significantly different from native NP, $p < 0.05$. Dashed lines represent mean native values. 49^ = TGF-β3 removed from media at day 42.

Both GAG and collagen content of constructs increased progressively with culture time, and were significantly greater at 14 d (2.0 and 0.6 % wet weight respectively) and 49 d (4.4 % and 1.6 % wet weight respectively) than at day 1 (0.4 and 0.002 % wet weight respectively) (Figs. 3A,B). At day 1, GAG content of constructs was 8 % of the native NP GAG content ($p < 0.05$), 42 % of native after 14 d culture ($p < 0.05$) and 90 % of native after 49 d (not significantly different) (Fig. 3A). At day 1, collagen content of constructs was 0.05 % of the native NP collagen content ($p < 0.05$), 14 % of native after 14 d culture ($p < 0.05$) and 41 % of native after 49 d ($p < 0.05$) (Fig. 3B). DNA content of agarose constructs increased progressively with culture time, and was significantly greater at 49 d than at 14 d or day 1 (Fig. 3C). Removal of TGF-β3 from the culture medium at 42 d had no significant effect on either GAG, collagen or DNA content (Figs. 3A, B, C) at 49 d compared with constructs cultured for the full 49 d with TGFβ-3.

The level of ACAN mRNA at day 1 of agarose culture was 23 % of that of freshly isolated cells ($p < 0.05$), 43 % after 14 d ($p < 0.05$) and 171 % after 49 d (not significantly different) (Fig. 4A). The level of COL2A1 mRNA at day 1 of agarose culture was 0.6 % of that of freshly isolated cells ($p < 0.05$), 9 % after 14 d ($p < 0.05$) and 47 % after 49 d ($p < 0.05$) (Fig. 4B). The level of COL1A2 mRNA expression

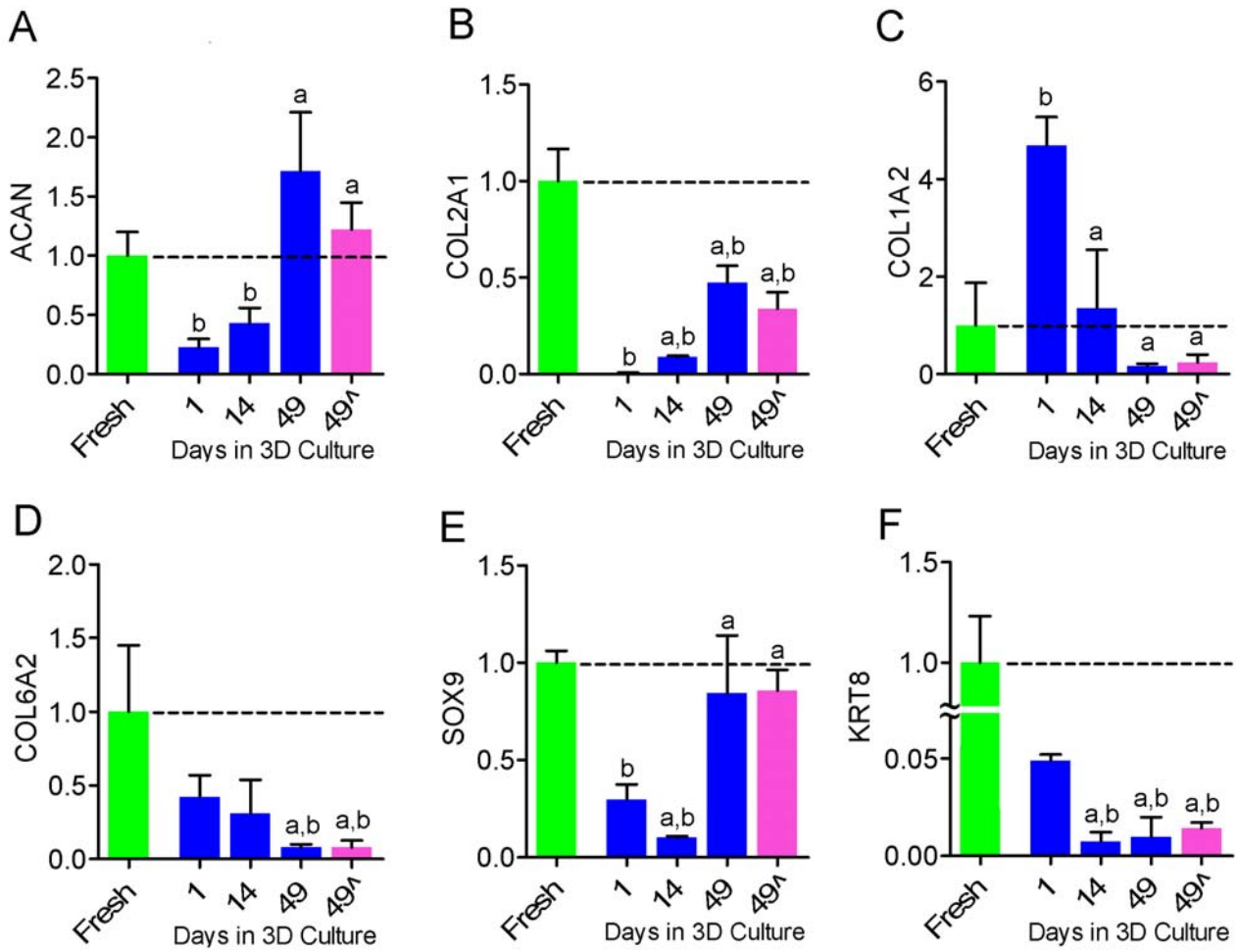

Fig. 4. mRNA levels for NP cell-seeded agarose constructs with culture duration and in comparison to freshly isolated cells ($n = 3$). (**A**) ACAN, (**B**) COL2A1, (**C**) COL1A2, (**D**) COL6A2, (**E**) SOX9, (**F**) KRT8. Values are expressed as a ratio to fresh (dashed line). [a]Significantly different from day 1, $p < 0.05$; [b]significantly different from fresh, $p < 0.05$; 49^ = TGF-β3 removed from media at day 42.

at day 1 of agarose culture was 468 % of freshly isolated cells ($p < 0.05$), and 135 % and 20 % of fresh at days 14 and 49 respectively (both not significantly different) (Fig. 4C). The ratio of COL1A2 to COL2A1 was 0.06 for freshly isolated cells, and 135, 1.07 and 0.06 for 1, 14 and 49 d of agarose culture. The level of COL6A2 mRNA at days 1 and 14 of agarose culture was 42 % and 31 % of freshly isolated cells respectively (both not significantly different), and 8 % after 49 d ($p < 0.05$) (Fig. 4D). The level of SOX9 mRNA at day 1 of agarose culture was 30 % of that of freshly isolated cells ($p < 0.05$), 10 % after 14 d ($p < 0.05$) and 84 % after 49 d (not significantly different) (Fig. 4E). The mRNA level of the NP cell specific marker KRT8 at day 1 of agarose culture was 5 % of that of freshly isolated cells ($p < 0.05$), and 1 % after both 14 d and 49 d ($p < 0.05$, Fig. 4F). Removal of TGFβ-3 from the culture medium at 42 d had no significant effect on mRNA levels of ACAN, COL2A1, COL1A2, COL6A2 SOX9 or KRT8 (Figs. 4A-D) at 49 d compared with samples cultured for the full 49 d with TGF-β3.

For constructs treated with IL-1β, aggregate modulus was significantly lower and hydraulic permeability was significantly higher than for untreated controls (67 % and 141 % of untreated, respectively, Figs. 5A and B). For constructs treated with both IL-1β and IL-1ra, or IL-1ra alone, neither of these properties was significantly different from those of untreated constructs.

For constructs treated with IL-1β, GAG content was 73 % of untreated ($p < 0.05$, Fig. 5C) and significantly more GAG was released into the media (Fig. 5D). For constructs treated with both IL-1β and IL-1ra, or IL-1ra alone, GAG content was not significantly different from untreated. Neither collagen nor DNA contents were significantly different from untreated for any of the 3 treatment groups.

ACAN and COL2A1 mRNAs were both down-regulated by IL-1β treatment (23 % ($p < 0.05$) and 8 % (not significant) of untreated respectively, Figs. 6A,B). ACAN and COL2A1 mRNAs were also lower than untreated for samples treated with both IL-1β and IL-1ra, and IL-1ra alone, but the differences were not statistically significant. COL1A2, COL6A2, SOX9 and KRT8 mRNA levels were not significantly affected by any treatment (not shown). ADAMST4 and iNOS mRNAs were significantly up-regulated by IL-1β treatment (12,730 % and 38,330 % of untreated, respectively, Figs. 6C,E, $p < 0.05$). MMP13 mRNA levels were elevated for IL-1β treated samples,

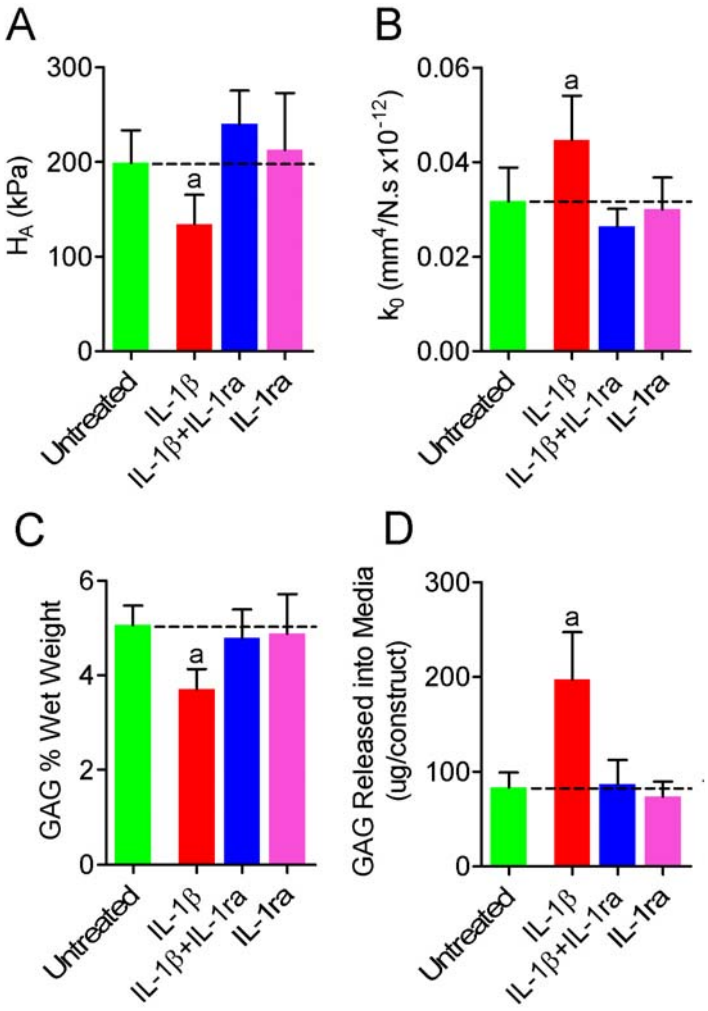

Fig. 5. Mechanical properties and biochemical composition of NP cell-seeded agarose constructs following treatment with IL-1β and IL-1ra ($n = 5$). (**A**) Aggregate modulus, H_A, (**B**) Hydraulic permeability, k_0, (**C**) GAG content, (**D**) GAG released into the media. [a]Significantly different from untreated, $p < 0.05$; dashed lines represent mean untreated values.

but not significantly (Fig 6D). For samples treated with both IL-1β and IL-1ra, and IL-1ra alone, ADAMTS4 and MMP13 mRNA levels were not significantly different from untreated (Figs. 7C,D), while INOS mRNA was elevated with marginal significance (1,500 % ($p = 0.05$) and 1,110 % ($p = 0.06$) of untreated respectively, Figure 6E).

Discussion

In this study we present an engineered three-dimensional model of the nucleus pulposus that exhibits similar composition and mechanical properties to the native tissue. Aggregate modulus and hydraulic permeability of native bovine NP tested in confined compression were of a similar magnitude to that reported previously (Perie *et al.*, 2006), as was the aggregate modulus of acellular 2 % agarose (Mauck *et al.*, 2000). As far as the authors are aware, this is the first study to report mechanical properties of engineered NP tested in confined compression.

Monolayer expansion has been demonstrated previously to affect the phenotype of NP cells. Serial passaging of NP cells has been shown to result in a reduction in the expression of type collagen II and aggrecan (Wang *et al.*, 2001; Kluba *et al.*, 2005). The results of the current study show that these key anabolic markers, and the transcription factor SOX9, are significantly reduced after two-dimensional culture. Even after 14 d of three-dimensional culture expression levels are still lower than those of freshly isolated cells. Supportive of our model, the phenotype of the agarose-cultured NP cells was restored only after 49 d of culture in chemically defined media, with levels of aggrecan and SOX9 mRNA similar to levels for freshly isolated cells. Even at this later time point, however, collagen II expression levels remained significantly lower. These mRNA expression level findings complement the findings for extracellular matrix composition, which showed that after 49 d, GAG content was similar to that of native NP, while collagen content was still significantly lower. The ratio of collagen I

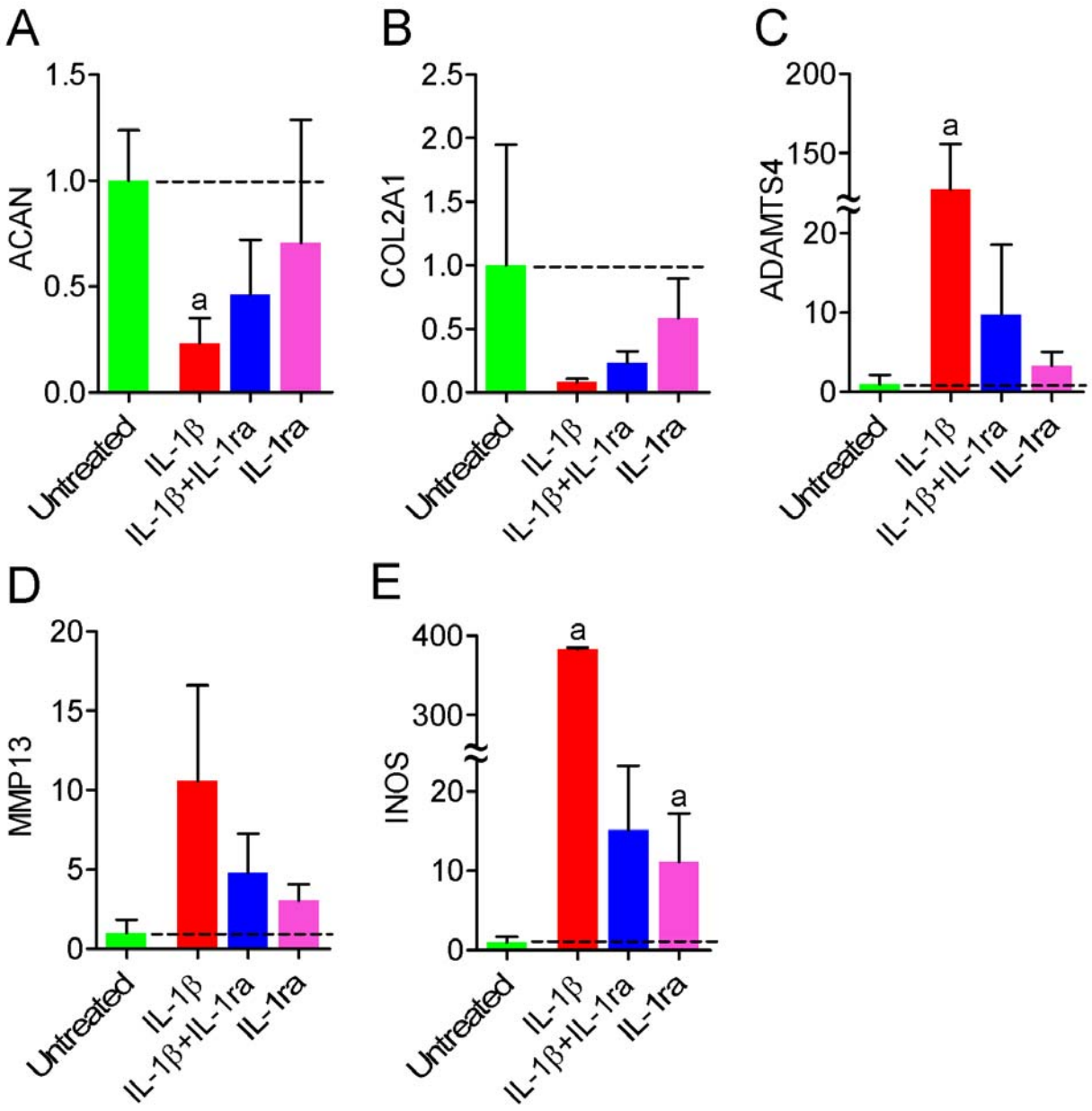

Fig. 6. mRNA levels of phenotypic and catabolic markers in NP cell-seeded agarose constructs following treatment with IL-1β and IL-1ra ($n = 3$). (**A**) ACAN (**B**) COL2A1, (**C**) ADAMTS4, (**D**) MMP13, (**E**) iNOS. Values are expressed as a ratio to untreated (dashed line). [a]Significantly different from untreated, $p < 0.05$.

to collagen II initially increased relative to freshly isolated cells at the beginning of agarose culture, but returned to native levels after 49 d. This finding is consistent with cells that have de-differentiated towards a fibroblastic phenotype in monolayer culture, but re-differentiated upon return to a 3D environment with extended culture time. While KRT8 expression was significantly lower at day 1 of agarose culture than for freshly isolated cells, expression continued to decrease with extended 3D culture time. KRT8 is more highly expressed by notochordal-like NP cells than mature chondrocyte-like NP cells (Minogue *et al.*, 2010). This suggests that our model may promote differentiation towards a more mature-NP cell like phenotype.

The agarose culture system used in this study has been used extensively for the study of articular chondrocytes (Buschmann *et al.*, 1992; Mauck *et al.*, 2000; Mauck *et al.*, 2003; Kelly *et al.*, 2009). Chondrocytes cultured under these conditions for an extended duration deposit an extracellular matrix with high GAG content and modulus. Furthermore, it has been shown that chondrocytes cultured in this defined medium will synthesize more extracellular matrix than those cultured in serum alone containing media (Mauck *et al.*, 2006). That NP cells and chondrocytes respond to this culture environment in a broadly similar way is in some respects not surprising. Mature NP cells and articular chondrocytes share phenotype similarities, reflected in their mutual high expression of aggrecan, collagen II and SOX9 (Sive *et al.*, 2002). These two cell types however, reside in unique biochemical and micromechanical environments *in vivo*, and have distinct developmental lineages (Smith *et*

al., 2011). As such, identification of unique markers which differentiate these cell types is being actively pursued (Sakai *et al.*, 2009; Minogue *et al.*, 2010; Rutges *et al.*, 2010; Stoyanov *et al.*, 2011). Transcription profiling of NP cells has suggested a handful of candidates, among them cytokeratin 8, a cytoskeletal protein that is also expressed by notochordal precursors (Gotz *et al.*, 1995; Minogue *et al.*, 2010). An important finding of the current study is that expression levels of cytokeratin 8, in contrast to aggrecan, collagen II and SOX9, are not restored to native levels in three-dimensional culture. This suggests that additional microenvironmental factors that are present *in vivo*, such as low oxygen tension, pH and glucose, mechanical loading, and molecular signaling from adjacent cell populations may be critical regulators of the NP cell phenotype (Stoyanov *et al.*, 2011). The influence of these factors on NP cell specific markers will be the subject of future investigations.

Nucleus pulposus cells from degenerate human discs express IL-1β both *in situ* and 3 dimensional culture (Le Maitre *et al.*, 2006a; Le Maitre *et al.*, 2007b). Furthermore, a positive correlation has been shown between severity of degeneration and levels of IL-1β expression. NP cells from degenerate human discs also express increased levels of catabolic enzymes, including MMPs -1, 3, 7, 13, and ADAMTS-4 (Le Maitre *et al.*, 2004; Le Maitre *et al.*, 2006a; Le Maitre *et al.*, 2007c). Treatment of human NP cells *in vitro* by IL-1β results in increased activity of these enzymes and INOS, an important intermodulatory mediator of inflammation (Le Maitre *et al.*, 2005; Zhao *et al.*, 2011), demonstrating an association between the IL-1β and these downstream factors. We have confirmed this association in the current study, demonstrating the treatment of NP cells in agarose cultured with IL-1β results in upregulation of key enzymes that degrade interstitial collagens and proteoglycans, as well as the cell stress marker, INOS. Our results highlight the clinical importance of IL-1β signaling in disc degeneration, by linking these associated downstream molecular events to functionally relevant compositional changes. While treatment with IL-1β also resulted in down regulation of anabolic matrix genes aggrecan and collagen II, SOX9 expression was not significantly affected, a finding supported by a previous study on human cells (Le Maitre *et al.*, 2005). Cytokeratin 8 expression was also unaffected.

While IL-1β is upregulated with disc degeneration, there is no concomitant upregulation of the endogenous inhibitor, IL-1ra (Le Maitre *et al.*, 2005). IL-1ra is currently used clinically for the treatment of rheumatoid arthritis and other inflammatory diseases (Gabay *et al.*, 2010), and it has shown early promise *in vitro* as a treatment for disc degeneration (Le Maitre *et al.*, 2006b; Goupille *et al.*, 2007; Le Maitre *et al.*, 2007a). Using the *in vitro* model developed in the current study, we have shown that not only does IL-1ra counteract the deleterious effects of IL-1β at the molecular level, but it effectively inhibits associated compositional and biomechanical changes. To enhance the clinical relevance of these important findings, ongoing work is investigating the effects of introducing IL-1ra to an environment with pre-existing inflammation.

In addition to biological therapies such as IL-1ra, NP tissue engineering is being actively pursued as an alternative strategy for restoring disc function (Chou *et al.*, 2009; Reza and Nicoll, 2010a). The results of the current study indicate that implantation of an engineered, biological construct into a disc space where there is substantial inflammation present will result in a significant reduction in the baseline mechanical properties of that implant. Integrating bioactive molecules, such as IL-1ra, into implant scaffolds may be an effective means of counteracting these effects.

In this study we describe a tissue-engineered model of the nucleus pulposus that closely mimics key functional, compositional and molecular characteristics of the native tissue. This model was used to demonstrate that exposure of NP cells to IL-1β leads to altered mechanical function, primarily due to loss of GAG. Furthermore, it was demonstrated that IL-1ra could effectively inhibit this cascade of catabolic events. In the future, this culture system could be further refined to incorporate additional features of the native NP microenvironment, including low oxygen, glucose and pH, and dynamic loading, all of which could reasonably be expected to further mediate cell behavior. Finally, the model described represents a stable and cost-effective platform for evaluating new therapies for disc degeneration, prior to translation to appropriate *in vivo* models and ultimately, human clinical use.

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Discussion with Reviewers

Reviewer I: Treatment with IL-1b caused a decrease in aggrecan and collagen-II expression as well as an increase in ADAMTS4 and iNOS expression. However, treatment with IL-1ra did not fully recover aggrecan/collagen-II expression and did not fully reduce levels of ADAMTS4/iNOS down to untreated levels. What do the authors think is the molecular reason for this? Which other pathways may be activated that IL-1ra treatment does not influence?
Authors: This is an interesting question. One possible explanation is that the effect of IL-1ra is dose dependent, and that higher dosages of IL-1ra are required to achieve complete inhibition of IL-1β at 10 ng/mL. Our preliminary studies, however, indicated that for an IL-1β concentration of 10 ng/mL, dosages of IL-1ra above 50 ng/mL had minimal additional benefit. A more likely explanation is that IL-1β induces native up-regulation of other inflammatory cytokines, such as IL-6 and TNFα, which then activate downstream proteases *via* independent pathways. Importantly, our functional and compositional outcome measures (modulus, permeability and GAG content) were not significantly different from untreated with IL-1ra treatment.

Reviewer II: Why are agarose and alginate (biomaterials from algae) still preferred over other artificial materials e.g. polyethylene glycol (PEG) hydrogels?
Authors: This is a good question. It is not clear why other materials have not been widely explored with regards to NP cell culture. It may be that the field of NP tissue engineering is less mature than cartilage tissue engineering, where PEG and other artificial materials are widely used. In this study, we chose agarose as it is well characterized in our group, and our preliminary findings pointed to its utility in first growing a mature construct, and then altering the construct with respect to inflammatory challenge. We are currently evaluating other materials (including a photo-crosslinkable hyaluronic acid hydrogel), and future studies relating maturation of these cells in a more 'biologic' context will be of great interest.