


Differential Response of Bovine Mature Nucleus Pulposus and Notochordal Cells to Hydrostatic Pressure and Glucose Restriction

CARTILAGE
2020, Vol. 11(2) 221–233
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DOI: 10.1177/1947603518775795
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Abstract

Objective. The nucleus pulposus of the human intervertebral disc contains 2 cell types: notochordal (NC) and mature nucleus pulposus (MNP) cells. NC cell loss is associated with disc degeneration and this process may be initiated by mechanical stress and/or nutrient deprivation. This study aimed to investigate the functional responses of NC and MNP cells to hydrostatic pressures and glucose restriction. **Design.** Bovine MNP and NC cells were cultured in 3-dimensional alginate beads under low (0.4–0.8 MPa) and high (1.6–2.4 MPa) dynamic pressure for 24 hours. Cells were cultured in either physiological (5.5 mM) glucose media or glucose-restriction (0.55 mM) media. Finally, the combined effect of glucose restriction and high pressure was examined. **Results.** Cell viability and notochordal phenotypic markers were not significantly altered in response to pressure or glucose restriction. MNP cells responded to low pressure with an increase in glycosaminoglycan (GAG) production while high pressure significantly decreased ACAN gene expression compared with atmospheric controls. NC cells showed no response in matrix gene expression or GAG production with either loading regime. Glucose restriction decreased NC cell TIMP-1 expression but had no effect on MNP cells. The combination of glucose restriction and high pressure only affected MNP cell gene expression, with decreased ACAN, Col2 α 1, and ADAMTS-5 expression. **Conclusion.** This study shows that NC cells are more resistant to acute mechanical stresses than MNP cells and provides a strong rationale for future studies to further our understanding the role of NC cells within the disc, and the effects of long-term exposure to physical stresses.

Keywords

hydrostatic pressure, glucose, notochordal cells, alginate culture, extracellular matrix

Introduction

The nucleus pulposus develops from the embryonic notochord.^{1–3} However, during aging and disease, notochordal (NC) cells are gradually replaced by “chondrocyte-like” mature nucleus pulposus (MNP) cells.^{1,3–5} The ratio of these cell types in the adult intervertebral discs (IVDs) varies with species, age, and disease.^{6–8} Humans retain a small proportion of NC cells, typically less than 20% of the total NP cell population into adulthood.^{5,9,10} While the adult human NP is a fibrous and opaque tissue, species that retain higher proportions of NC cells, such as rats, rabbits, and pigs, have a proteoglycan-rich, gelatinous, and translucent nucleus.^{6,11}

Differences in extracellular matrix (ECM) composition across species and disease states are believed to underlie susceptibility to structural failure.^{1,8,12–14} It has therefore been hypothesized that loss of NC cells is an initiating factor of disc degeneration. Previous studies have suggested that mechanical stress or nutrient restriction in large animal discs initiate the death or differentiation of NC cells.^{15–18} *In vivo*,

nucleus pulposus cells are exposed to dynamic hydrostatic pressures, ranging from 0.1 to 2.3 MPa for normal activity.¹⁹ *In vivo* disc-clamp and *ex vivo* studies have shown that in addition to a denser and more fibrous ECM, the proportion of NC cells decreases under excessive mechanical stress.^{17,20–22} Although numerous studies have investigated the effect of mechanical stimulation on nucleus pulposus cells, these studies have used cells separately isolated from either NC or MNP cell-rich tissues. **Table 1** summarizes the findings from *in vitro* hydrostatic pressure studies using different animal

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Table 1. A Summary of Studies That Examined the Effects of Hydrostatic Pressure on Nucleus Pulposus Cells *In Vitro* from a Range of Species.

Authors	Animal (Predominate Cell Type)	Pressures (MPa) Range Applied	Summary of Results	
			Collagen ^a	Proteoglycan ^b
Kasra <i>et al.</i> (2003) ³⁰	Rabbit (NC)	0.75-3	No change 0.75-2.5 MPa ↓ 3 MPa	Not measured
Hutton <i>et al.</i> (2001) ³²	Dog (NC)	0.35 and 1	↑ 0.35 MPa ↓ 1 MPa	↓ 0.35 MPa ↑ 1 MPa
Gokorsch <i>et al.</i> (2004) ²⁸	Pig (NC)	0.4, 3.4, and 6.0	↑ 0.4 MPa ↓ 6 MPa	↑ 0.4 MPa ↓ 3.4 and 6 MPa
MacLean <i>et al.</i> (2004) ³¹	Rat (NC)	0.2 and 1	No change at 0.2 or 1 MPa	↑ 1 MPa
Purmessur <i>et al.</i> (2013) ³³	Pig (NC)	0.5-2	No change	↑ PG staining No change in aggrecan gene expression
Wang <i>et al.</i> (2007) ²⁹	Rabbit (NC)	0.5 and 1	↑ 0.5 MPa ↑ 1 MPa	↑ 0.5 MPa ↑ 1 MPa
Sowa <i>et al.</i> (2011) ²⁷	Rabbit (NC)	0.7, 2, and 4	Not measured	↑ 0.7 MPa No change at 2 or 4 MPa
Neidlinger-Wilke <i>et al.</i> (2006) ²²	Human (MNP)	0.25 and 2.5	No change	↓ 2.5 MPa
Neidlinger-Wilke <i>et al.</i> (2006) ²²	Bovine (MNP)	0.25 and 2.5	↑ 0.25 MPa ↓ 2.5 MPa	↑ 0.25 MPa ↓ 2.5 MPa
Ishihara <i>et al.</i> (1996) ²³	Bovine (MNP)	2.5-10	Not measured	↓ PG with increasing pressure (2-10 MPa)
Le Maitre <i>et al.</i> (2008) ²⁵	Human (healthy and degenerate)	0.8-17	No change	↑ Healthy ↓ Degenerate

MNP = mature nucleus pulposus; NC = notochordal; PG = proteoglycan.

^aCollagen measured by gene expression or collagen protein assay

^bProteoglycan measured by aggrecan gene, glycosaminoglycan assay, or radiolabeled proteoglycan synthesis assay.

models. While the studies differ in the magnitude and duration of loading, studies using predominately MNP cell-rich human or bovine NP cells, consistently show that high pressure causes a decrease in proteoglycan expression.²³⁻²⁵ In contrast, the effect of hydrostatic pressure on NC cells remains unclear with reports that both low and high pressure loading can increase proteoglycan expression.²⁶⁻³¹

In addition to the high-pressure environment, nucleus pulposus cells reside in a nutrient-restricted environment.¹⁶ Because of the avascular nature of the IVD, nutrients and waste are transported in and out by diffusion and convection.¹⁶ The nucleus pulposus cells rely on nutrients supplied through the cartilaginous endplates. The glucose concentration gradient in a healthy adult human disc ranges from 5.5 mM at the endplates to 0.5 mM or lower in the center of the nucleus pulposus.^{16,34,35} Glucose concentration is primarily determined by the distance from capillaries; however, other factors such as endplate porosity can affect glucose supply.^{16,36-38} With age and disc degeneration, endplates calcify and glucose concentrations decrease.^{16,36,39} Previous work comparing bovine-MNP and porcine-NC cells found that porcine NC cells had a greater decrease in cell viability in low glucose culture conditions, compared with bovine MNP

cells, suggesting that glucose restriction may be one of the causes of NC cell death *in vivo*.⁴⁰

While several previous studies have investigated the effects of mechanical loading or nutrient restriction on MNP and NC cells, these studies have all used cell populations isolated from different species.^{17,24,32,33,40} Using our recently published method for isolating enriched fractions of NC cell-clusters (with an intact pericellular matrix) together with MNP cells from the same bovine discs,⁴¹ the present study aimed to examine how each cell type responded to different levels of hydrostatic intermittent pressure and glucose restriction. Our hypothesis was that high pressure and glucose restriction would be more detrimental to NC cells compared to MNP cells. Each cell fraction was cultured under dynamic low and high physiological range hydrostatic pressure for 24 hrs in a custom-built hydrostatic pressure vessel. Second, cells were cultured in either physiologically normal (5.5 mM) or glucose-restricted (0.55 mM) media under atmospheric or high hydrostatic pressure loading for 24 h. Following each treatment, cells were assessed for changes in viability, notochordal phenotypic markers and extracellular matrix production and gene expression.

Methods

Culture Media

To mimic the physiochemical environment of nucleus pulposus tissue, culture media was adjusted to 400 mOsm and pH 7.0. Media containing serum was used for cell isolation and all experimental media was serum-free and supplemented with glucose where appropriate. Ten percent serum media: 5.5 mM glucose Dulbecco's modified Eagle medium (DMEM; cat no. D2902 Sigma-Aldrich, Auckland, New Zealand) was supplemented with 1% v/v penicillin/streptomycin (Life Technologies, Auckland New Zealand), 0.5% v/v fungizone (Life Technologies), 85 μ M ascorbic acid (Sigma-Aldrich), 3.7 g/L NaHCO₃, 10% v/v fetal bovine serum (FBS), and 19 g/L mannitol (Sigma-Aldrich). Serum-free control media: 5.5 mM glucose DMEM (cat no. D2902 Sigma-Aldrich), was supplemented with 1% v/v penicillin/streptomycin, 0.5% v/v fungizone, 85 μ M ascorbic acid, and 19 g/L mannitol. Serum-free, glucose-restricted media: No glucose DMEM (cat no. D5030 Sigma-Aldrich) was supplemented with 100 mg/L (0.55 mM) glucose, 1% v/v penicillin/streptomycin/glutamine (Life Technologies), 0.5% fungizone, 85 μ M ascorbic acid, 19 g/L mannitol.

Cell Isolation

Nucleus pulposus cell subpopulations were isolated from bovine caudal discs of 18-30-month-old animals. Bovine tails were obtained from the local slaughterhouse and isolation of NC cell- and MNP cell-rich fractions were performed as described previously.⁴¹ Briefly, NP tissue was separated from the surrounding annulus and finely minced. The tissue was then placed in a large volume of media and mechanically digested for 5 minutes to release the NC cell clusters. The mixture was then strained through a stainless steel mesh sieve (pore size 0.5 mm) to collect the NC cell-rich fraction. The remaining tissue was enzymatically digested in pronase solution (8 units/mL; Sigma-Aldrich) for 45 minutes, then in collagenase (245 units/mL; Life Technologies) for 2.5 hours at 37°C to isolate the MNP cells. The isolated cells were seeded in 1.2% w/v alginate solution (Sigma-Aldrich) at a density of 0.3×10^6 cell/mL for the NC cells and 4×10^6 cells/mL for the MNP cell fraction to retain the *in situ* cell densities.^{41,42} Cell fractions from two caudal discs from three tails were pooled for each experiment, that is, $n = 6$ discs for each of the 3 biological replicates ($N = 3$). Cell-seeded alginate beads were cultured in 10% serum media overnight at 37°C and 5% CO₂.

Hydrostatic Pressure

Following overnight culture, cell-seeded alginate beads were transferred to plastic pouches made from high-density polyethylene film, filled with 5-mL serum-free media.

The air was removed from the plastic pouches and the edges sealed with a heat-sealer. This created a cell-culture system compatible with hydrostatic pressure as illustrated in **Figure 1A**. A custom-built hydrostatic pressure rig consisted of a 1-L capacity stainless steel pressure vessel (**Fig. 1B**), connected by a hose to a hydraulic piston (**Fig. 1C**). This was attached to an Instron Electropuls E3000 materials testing machine (Norwood, MA, USA). The cyclic compressive forces generated by the Instron were translated via a fluid-filled piston into an oscillating hydrostatic pressure within the water-filled pressure vessel. Instron Blue Hill 2 and Wave Matrix software were used to produce the loading regimes detailed in **Table 1**. An analogue pressure gauge in the lid of the pressure vessel was used to monitor pressure throughout the experiment. The pressurization experiment had 2 phases to mimic a diurnal cycle; an 8-hour rest phase at 0.2 MPa and a 16-hour exercise phase. For the purposes of this study, a regime of 0.4 to 0.8 MPa at 1 Hz, was termed "low pressure" since it approximates the loads experienced during walking. The pressure regime of 1.6 to 2.4 MPa at 1 Hz, was termed "high pressure" since this represents the higher end of physiologically relevant pressure.¹⁹ For the pseudo-static rest phase of the experiment, a load-controlled compression protocol was used to maintain the vessel at 0.2 ± 0.1 MPa. A trimodal testing protocol was used to generate a cyclic loading regime for the dynamic 1 Hz oscillating pressure phase. All pressures are stated as gauge pressure; that is, atmospheric pressure = 0 MPa gauge pressure.

Cells were exposed to 24 hours of dynamic pressure as detailed in **Table 2**. Atmospheric control samples were placed in a water-filled 1-L glass bottle. Both the pressure and control vessel were maintained at 37°C in a water-bath. Each experimental condition was conducted on a separate occasion with a paired control sample (5.5 mM glucose at atmospheric pressure). Following treatment, the pouches were removed from the pressure vessel; the media was collected with a needle and syringe, the pouches opened, and the cell-alginate beads were removed for analysis.

Oxygen concentration in both the pressure vessel and control glass bottle was measured before and after the loading period using a Neofox oxygen probe (Ocean Optics, Auckland, New Zealand). The oxygen concentrations remained stable over the loading regime (6 mg/mL).

Viability Assay

Cell viability was assessed within 1 hour of the cessation of loading using an Apoptotic, Necrotic, and Healthy Cells Quantification Kit (Biotium, Fremont, CA, USA). Cell-alginate beads were rinsed once in PBS, then incubated in binding buffer, followed by incubation in a solution of FITC-annexin antibody (green), ethidium homodimer (red), and Hoechst (blue) according to the manufacturer's

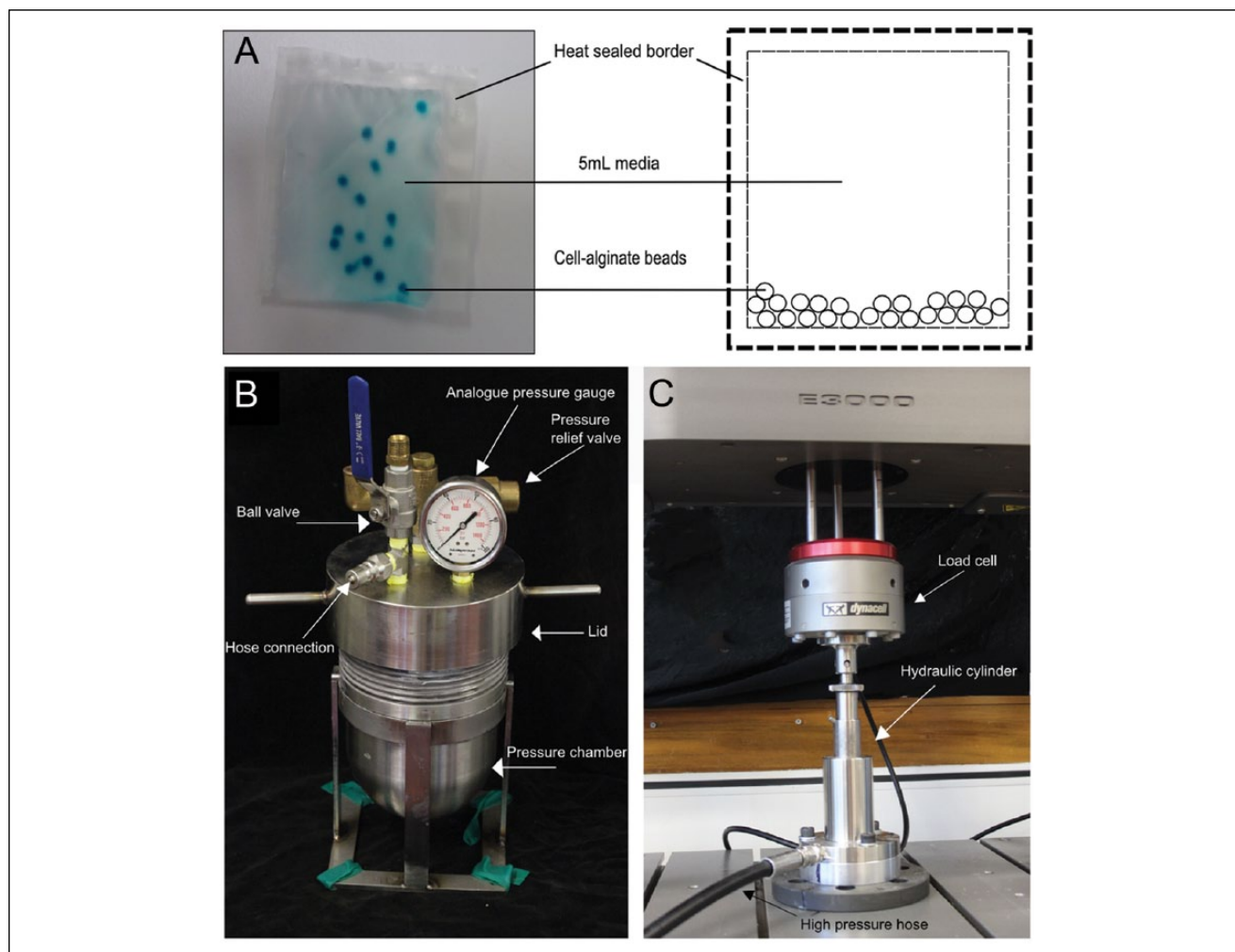


Figure 1. (A) Pressure compatible cell culture system comprising alginate beads in media-filled plastic pouch (4 cm × 5 cm). (B) Custom-built hydrostatic pressure vessel. (C) Hydraulic piston attached to Instron.

Table 2. Culture, Glucose Concentrations and Hydrostatic Pressure Conditions Used in This Study.

Group	Glucose (mM)	Pressure (MPa)	Frequency	Time (h)
Pressure studies				
Control	5.5	Atmospheric	Static	24
Low pressure	5.5	0.2, followed by 0.4-0.8	Static 1 Hz	8 + 16
High pressure	5.5	0.2, followed by 1.6-2.4	Static 1 Hz	8 + 16
Glucose-restriction studies				
Control	5.5	Atmospheric	Static	24
Glucose-restricted	0.55	Atmospheric	Static	24
High pressure, glucose-restricted	0.55	0.2, followed by 1.6-2.4	Static 1 Hz	8 + 16

instructions. The beads were then rinsed and imaged immediately on a Nikon TE2000 widefield fluorescence microscope using a 20× objective. Cells from 3 alginate beads (≥100 cells) per group were examined using Image J

software grid counter. To determine cell viability, cells labeled with Hoechst only (blue) were counted as alive, while cells labeled annexin V (green) or ethidium (red) were counted as dead cells.

Table 3. Taqman Gene Expression Assays Used in This Study.

Protein	Gene	Taqman Assay Reference
Collagen type I	COL1 α 1	Bt03225322_m1
Collagen type II	COL2 α 1	Bt03251861_m1
Aggrecan	ACAN	Bt03212186_m1
ADAMTS-5	ADAMTS5	Bt04230785_m1
Tissue inhibitor of metalloproteinases 1	TIMP1	Bt03213713_m1
Matrix metalloproteinase 3	MMP3	Bt04259490_m1
Brachyury	T	Bt04313978_m1
Ribosomal RNA 18s ^a	r18s	4319413E / X03205.1
Tyrosine activation protein 14-3-3 ^a	YWHAZ	Bt03216375_g1

^aIndicates reference genes.

GAG/DNA Assays

The 1,9-dimethylmethylene blue (DMMB) dye binding assay was used to measure total sulfated glycosaminoglycan (GAG) content in cell-alginate beads, and a SYBR green-based plate assay was used to determine DNA content as previously described.⁴¹ GAG content was normalized to DNA content within each sample, then each condition was normalized to its paired atmospheric control.

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) was used to assess change in gene expression of the notochordal phenotypic marker T (brachyury) as well as key ECM relevant genes. Following treatment, approximately 25 beads per group were removed and preserved in RNA Later (Life Technologies) at -20°C . RNA was extracted using the TRIzol-chloroform method and real-time PCR was performed as described previously.⁴¹ Real-time PCR was performed using Taqman gene expression assays (Life Technologies) detailed in **Table 3**. The tyrosine activation protein, YWHAZ, and ribosomal 18S were used as reference genes. Relative expression was calculated from mean expression of r18S and YWHAZ in each sample.

Immunocytochemistry

Cell morphology and phenotype was assessed by cytokeratin 8 and vimentin immunolabeling.⁴¹ Alginate beads were cross-linked in 100 mM BaCl_2 for 10 minutes then fixed in 4% (w/v) paraformaldehyde for 20 minutes at 37°C . Beads were permeabilized in 0.5% (v/v) Triton X-100 (Sigma-Aldrich) for 10 minute, washed in PBS containing 0.1% (w/v) bovine serum albumin and 10 mM CaCl_2 then incubated in 5% donkey serum (Sigma Aldrich) for 30 minutes for blocking. Finally, beads were incubated in goat polyclonal vimentin primary antibody (1:200; Santa Cruz Biotechnology Inc.) overnight at 4°C . The following day,

beads were washed, incubated in donkey anti-goat Alexa-488 at room temperature for 2.5 hours (1:500; Life Technologies), washed again, blocked in 5% goat serum (Sigma Aldrich), and incubated overnight with mouse monoclonal cytokeratin-8 primary antibody (1:200; Acris Germany) at 4°C . The following day, beads were washed and incubated in goat anti-mouse Alexa-594. Finally, beads were counterstained with Hoechst (1:500; Sigma-Aldrich) for 15 minutes, washed and mounted by firmly pressing onto a slide under a coverslip with Prolong Gold (Life Technologies). A minimum of 100 cells per replicate were imaged using a Leica DMR fluorescence microscope and the number of CK8-positive cells was expressed as percentage of total cells.

Statistical Analysis

GAG measurements were normalized to DNA content within each sample to yield GAG/DNA ($\mu\text{g}/\text{ng}$) ratio. GAG/DNA ratios were then normalized to paired control samples (5.5 mM glucose atmospheric pressure) to calculate the relative change in response to each treatment condition. Statistical differences were calculated compared to control using a 2-sample *t*-test. Differences between treatment groups were compared using Sidak's multiple comparisons test.

Gene expression data were analyzed using the Livak *et al.* $2^{-\Delta\Delta\text{Ct}}$ method where target gene expression is normalized to averaged reference gene expression and normalized again to the paired controls (5.5 mM glucose atmospheric pressure), to allow the data to be expressed as fold-change from control. Differences between treatment groups were compared using Sidak's multiple comparisons test.

For cell viability and cytokeratin 8 expression, data were expressed as mean \pm standard deviation for all groups. Differences between groups were analyzed using Tukey's multiple comparison test. A *P* value of <0.05 was considered statistically significant. All statistical analysis was performed using GraphPad Prism statistical software (v 6.01).

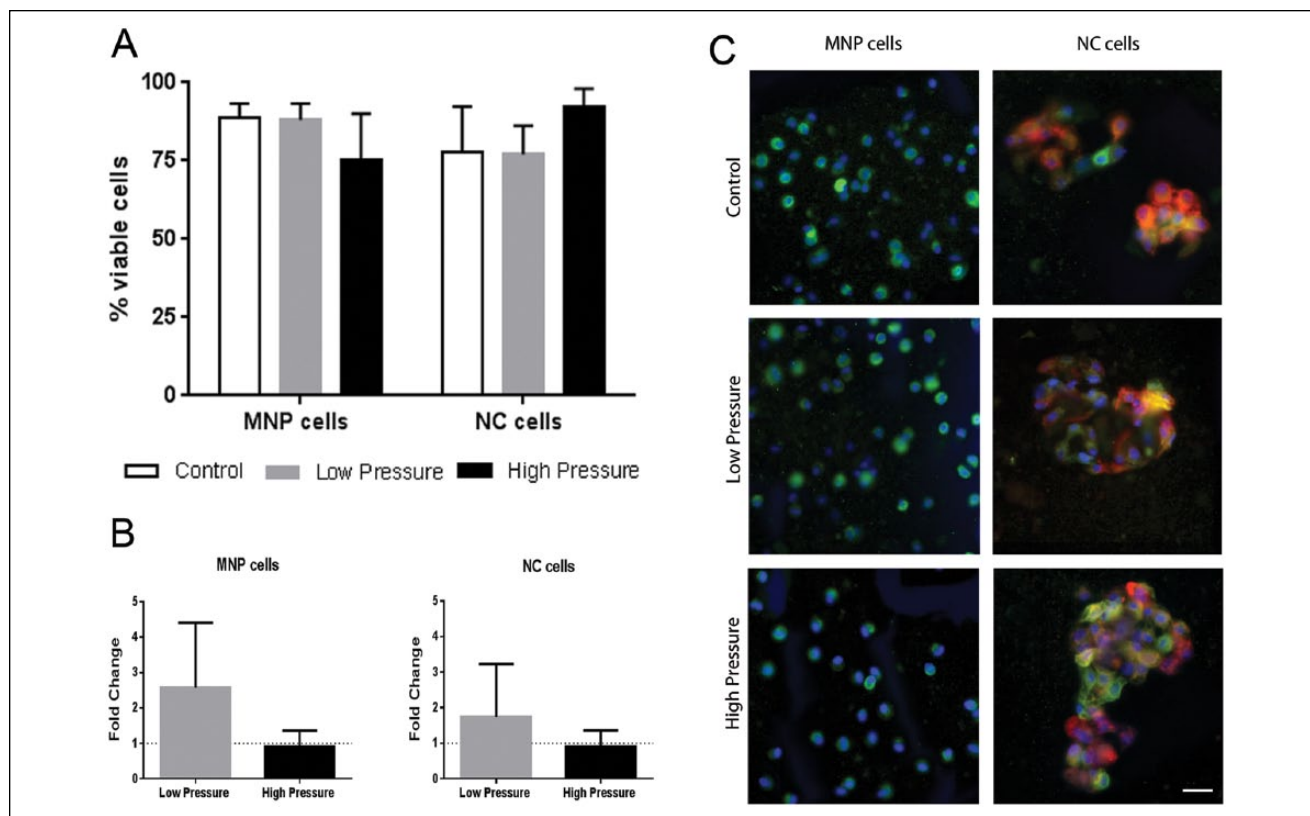


Figure 2. (A) Viability of mature nucleus pulposus (MNP) and notochordal (NC) cells following 24-hour culture under control (atmospheric), low-, and high-pressure loading. (B) T (brachyury) gene expression relative to paired atmospheric control ($\gamma = 1$). (C) MNP and NC cells labeled against vimentin (green) and cytokeratin 8 (red) counterstained with Hoechst (blue), scale bar = 20 μm (mean \pm SD, $N = 3$) (For interpretation of the references to colours in this figure legend, refer to the online version of this article).

Results

Effect of Pressure

Viability and Phenotype. Cell viability was not significantly affected by either low or high pressure loading for either cell type compared to atmospheric controls. For MNP cells, the mean viability was 89%, 88%, and 75% for atmospheric control, low-, and high-pressure groups, respectively. The NC cell groups had a mean viability of 78%, 77%, and 92% for atmospheric control, low-, and high-pressure groups, respectively (Fig. 2A).

The gene expression of the notochordal marker T (brachyury) was measured and found to be present in both cell-rich fractions. In accordance with our previous findings NC fractions expressed 4-fold more T than MNP fractions and these levels did not change in response to low or high pressure for either cell type (Fig. 2B).

Cell-alginate beads were immunolabeled against the mesenchymal marker vimentin and the notochordal marker cytokeratin-8, these abundantly expressed cytoskeletal proteins are most suited to clearly differentiate cell phenotype (Fig. 2C). Following pressurization, both cell types maintained their distinctive cell morphologies. MNP cells were

seen as small individual spherical cells, which only expressed vimentin. NC cells were present as dense clusters which expressed both vimentin and CK8. The MNP fraction contained 2% to 3% CK8-positive cells across all conditions. The NC cell fractions had a frequency of $\geq 89\%$ CK8-positive cells across all conditions.

GAG Production. Change in GAG/DNA ratio was calculated relative to paired control (atmospheric pressure) samples. Following low-pressure loading, the MNP cells had a 1.4-fold increase in GAG/DNA ratio ($P = 0.0250$), while high-pressure loading had no significant effect on GAG/DNA ratio (Fig. 3A). Neither low- nor high-pressure loading significantly altered GAG/DNA ratio in the NC cells (Fig. 3B).

Gene Expression. For MNP cells under low pressure, there was a nonsignificant increase in gene expression compared to atmospheric pressure for all genes except ADAMTS-5 (Fig. 4A). High-pressure treatment resulted in a significant decrease in aggrecan gene expression for MNP cells compared with atmospheric control, $P = 0.0478$ (Fig. 4A). Col2 $\alpha 1$ expression was nonsignificantly decreased, $P = 0.0600$. The NC cells had no significant change in gene

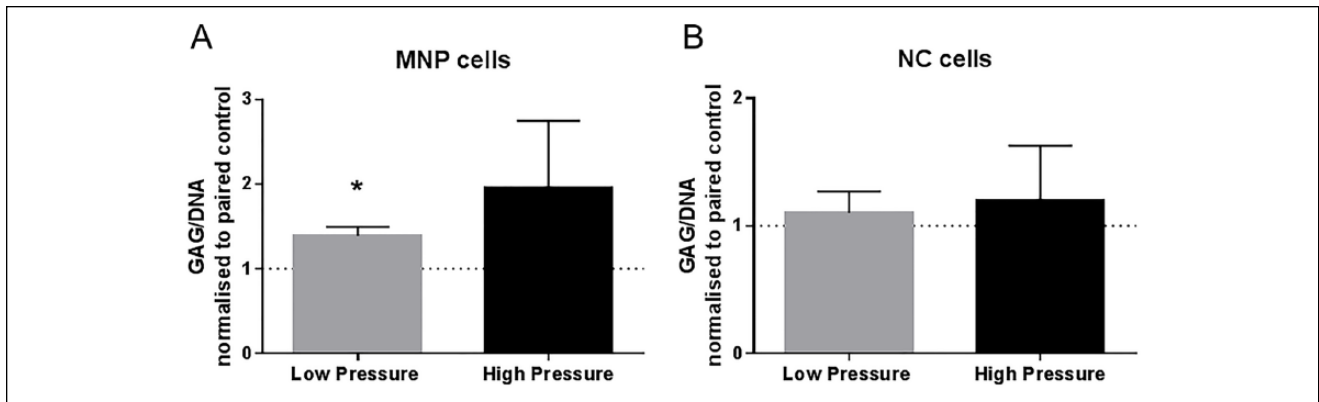


Figure 3. GAG/DNA ratio normalized to paired controls (atmospheric pressure) for (A) mature nucleus pulposus (MNP) and (B) notochordal (NC) cells (mean \pm SD, $N = 3$).

expression for any of the target genes studied when comparing pressurized with atmospheric controls (Fig. 4B).

Effect of Glucose Restriction

Viability and Phenotype. Cell viability was not significantly reduced for either cell type under glucose restriction. MNP cell viability remained high ranging from 75% to 91% across all conditions (Fig. 5). NC cells showed greater variability, ranging from 63% in the 0.55 mM group to 92% in the 5.5 mM + HP group (Fig. 5). Despite these fluctuations, neither glucose concentration nor high pressure had a statistically significant effect on cell viability for NC cells compared with control conditions. NC cells remained in clusters expressing both vimentin and CK8, while the MNP cells remained as single cells expressing vimentin. Less than 3% of cells in the MNP fraction expressed CK8, while 90% of the cells in the NC fraction expressed CK8. There was no significant change in the frequency of CK8+ cells in either cell fraction under any of the tested conditions (Fig. 5).

GAG Production. GAG/DNA ratios did not significantly change for either cell type in response to glucose restriction or the combination of glucose restriction and high hydrostatic pressure (Fig. 6).

Gene Expression. MNP cells showed no significant change in gene expression in response to 0.55 mM glucose alone, however, in combination with high hydrostatic pressure, there was a significant decrease in the expression of collagen type II ($P = 0.0274$), aggrecan ($P = 0.0251$), and a highly significant decrease in ADAMTS-5 expression ($P = 0.0087$) compared with the 5.5 mM glucose atmospheric pressure controls (Fig. 7A). Glucose restriction induced a significant decrease in TIMP-1 expression in NC cells compared with paired control ($P = 0.0198$). However, in the combined 0.55 mM glucose and high-pressure group, TIMP-1 expression

levels returned to control levels. All other genes did not change in response to either treatment (Fig. 7B).

Discussion

To examine the functional differences between NC and MNP cells, without the interference of species-derived differences, our study isolated both cell types from the same discs. Since mechanical stress and nutrient deprivation have both been hypothesized to cause NC cell loss,¹⁵⁻¹⁸ the effects of both of these factors were investigated. Both cell types were cultured under physiologically relevant hydrostatic pressures and glucose concentrations for 24 hours and subsequently assessed for changes in viability, NC phenotypic markers and ECM-related gene expression.

Response to Low and High Pressure

In vivo disc-clamp studies, which have loaded NC cell-rich discs over days or weeks, have reported changes in ECM composition and an accompanying decrease in the proportion of NC cells.^{17,18,20} In contrast, *in vitro* studies, which exposed isolated NC cells to pressure (between minutes and hours), reported both anabolic and catabolic changes in ECM expression in response to hydrostatic pressure (see Table 1).^{28-30,32} In the present study, we used physiologically relevant magnitudes and frequencies of loading¹⁹ to compare the response of bovine MNP and bovine NC cells in 3-dimensional culture. We found no decrease in cell viability for either cell-type compared to controls. Additionally, we found no evidence of cell differentiation in response to mechanical stress. The MNP cell-rich fraction had a small fraction (2%-3%) of CK8-positive cells and an extremely low T gene expression (Ct = 37-40) across all conditions, presumably due to contaminating NC cells.⁴¹ There was no significant change in these phenotypic markers following 24 hours of low- or high-pressure dynamic loading.

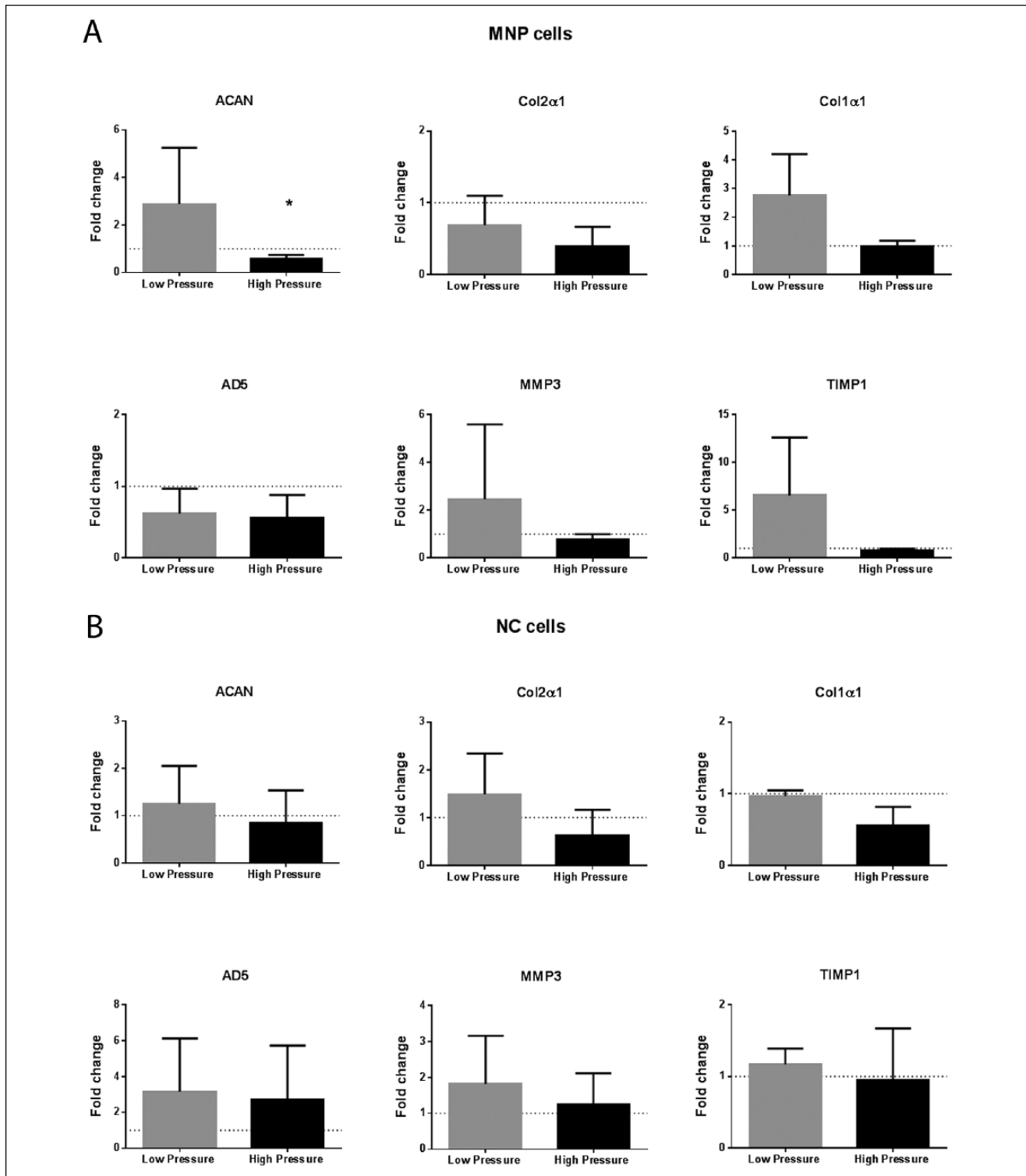


Figure 4. Extracellular matrix (ECM) gene expression. Fold change in gene expression relative to paired atmospheric control for **(A)** mature nucleus pulposus (MNP) cells and **(B)** notochordal (NC) cells in response to low- and high-pressure loading (mean \pm SD, $N = 3$).

Using a mixed MNP and NC population of bovine as well as human cells *in vitro*, Neidlinger-Wilke *et al.*²⁴ showed that low-range hydrostatic pressure (0.25 MPa) promoted ECM synthesis, while high-magnitude pressure

(2.5 MPa) decreased anabolic gene expression. In the present study, we found that MNP cells had a small but significant increase in GAG/DNA ratio in response to low-pressure loading. Also, in agreement with Neidlinger-Wilke *et al.*,²⁴

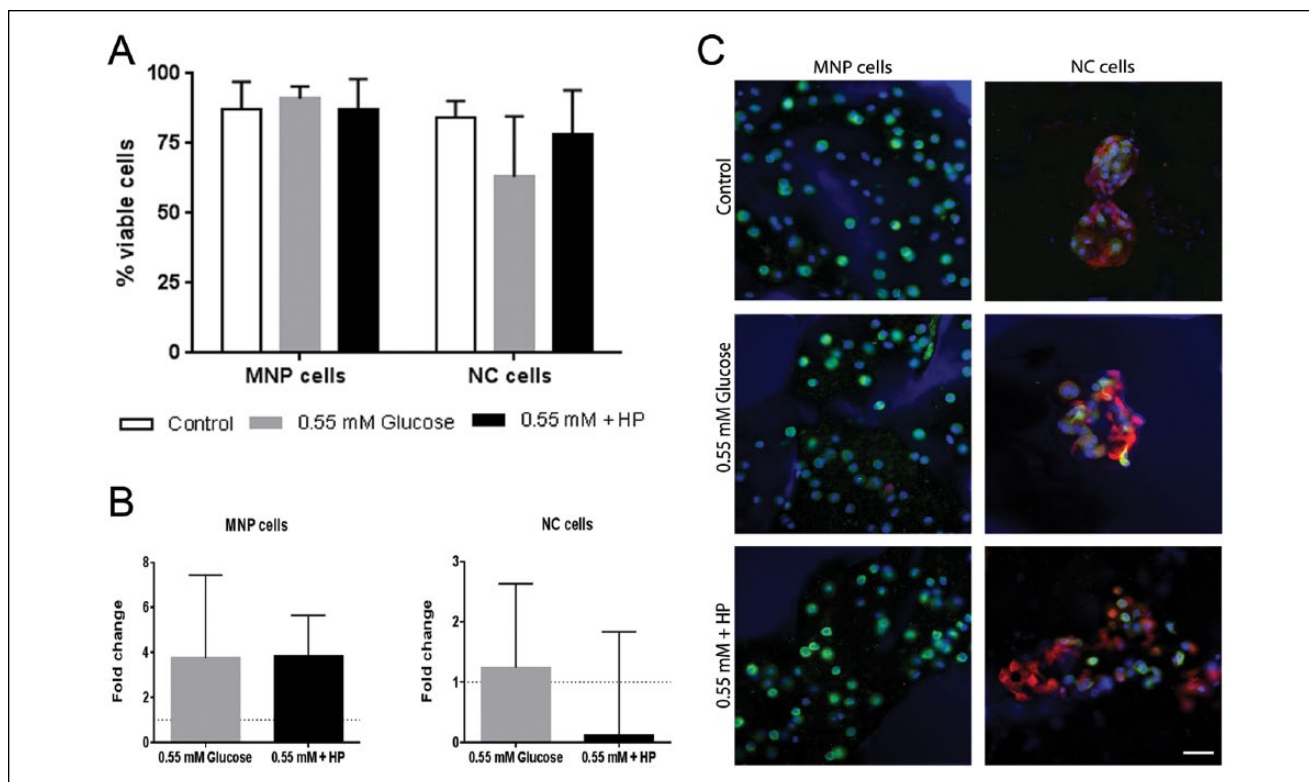


Figure 5. (A) Viability of mature nucleus pulposus (MNP) and notochordal (NC) cells following 24-hour culture under control (5.5 mM glucose), 0.55 mM glucose and 0.55M glucose + HP (high pressure) loading. (B) T (brachyury) gene expression relative to paired atmospheric control ($\gamma = 1$) (mean \pm SD, $N = 3$). (C) MNP and NC cells labeled against vimentin (green) and cytokeratin 8 (red) counterstained with Hoechst (blue), scale bar = 20 μ m (For interpretation of the references to colours in this figure legend, refer to the online version of this article).

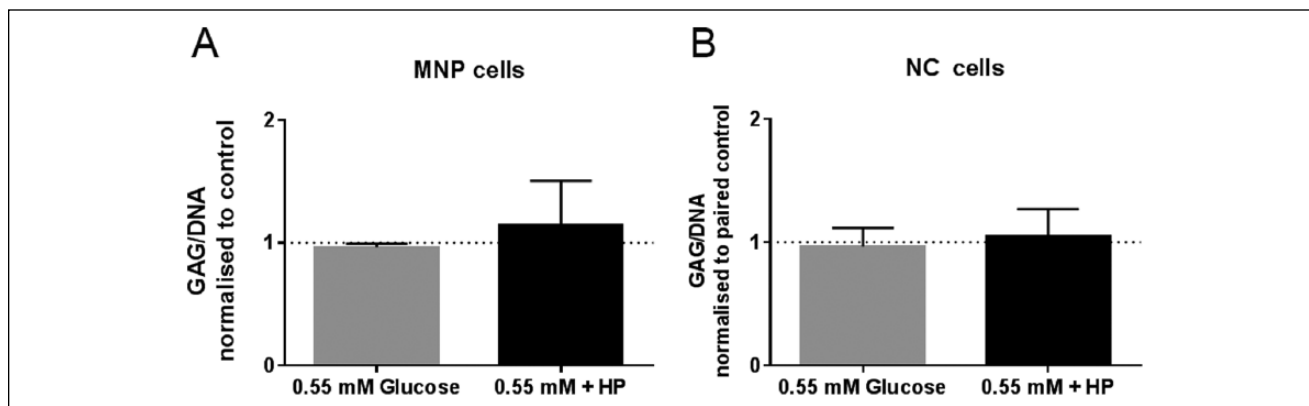


Figure 6. GAG/DNA ratio normalized to paired 5.5 mM glucose atmospheric controls for (A) mature nucleus pulposus (MNP) cells and (B) notochordal (NC) cells in response to 0.55 mM glucose and 0.55 mM glucose + HP (high pressure) loading (mean \pm SD, $N = 3$).

we found that high pressure resulted in a significant decrease in ACAN expression and a nonsignificant decrease in Col2a1 expression. Low-pressure loading did not significantly alter the expression of any of the genes examined.

Notably, NC cells showed no change in GAG/ DNA ratio or ECM related gene expression in response to either the low- or high-pressure loading conditions used in this study. While this finding does not prove that NC cells are

mechanically insensitive, it does indicate that NC cells behave differently to their MNP counterparts and are possibly less sensitive to acute mechanical stress. Additionally, our finding may be species specific since no previous pressure study has used NC cells isolated from bovine samples. Since both cattle and humans naturally lose NC cells with age, the bovine model may be a more relevant model for testing medical interventions.

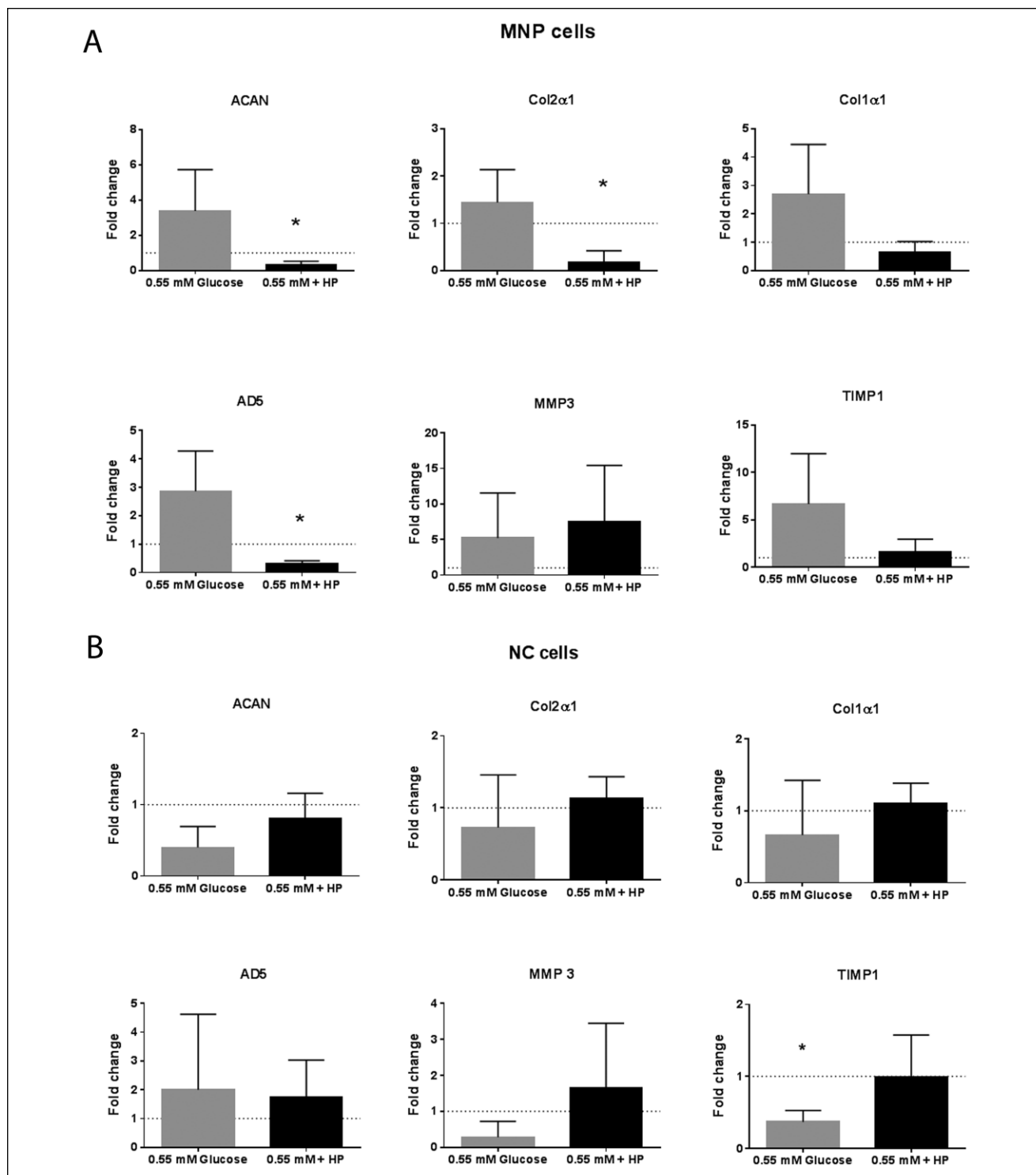


Figure 7. Extracellular matrix (ECM) gene expression. Fold change in gene expression relative to paired 5.5 mM glucose paired atmospheric control for **(A)** mature nucleus pulposus (MNP) cells and **(B)** notochordal (NC) cells in response to 0.55 mM glucose and 0.55 mM glucose + high pressure loading (mean \pm SD, $N = 3$).

Response to Glucose Restriction

Since NC cells in this model showed no significant response to pressure, we next investigated the effect of glucose restriction alone and in combination with high-pressure loading. The MNP cells retained high cell viability with glucose restriction while the NC cells had a nonsignificant decrease in viability. Bibby *et al.*² reported a decrease in MNP cell viability only when cultured in 0 mM glucose, while Guehring *et al.*⁴⁰ found a decrease in porcine NC viability in response to 0.5 mM glucose after 3 days. With a longer culture duration, we may have also seen a significant decrease. Alternatively, the discrepancy in NC viability could be due to species differences since Guehring *et al.*⁴⁰ used porcine NC cells and bovine MNP cells, while our cells were both isolated from bovine discs. Additionally, we found no change in cell morphology or phenotype for either cell-type under glucose restriction or glucose restriction combined with high pressure.

Glucose restriction alone did not alter MNP cell ECM gene expression; however, glucose restriction combined with high pressure decreased ACAN, Col2a1 and ADAMTS 5. This result is an exacerbation of the pattern of ACAN and Col2a1 gene expression decrease following high pressure alone and is in line with the findings of Neidlinger-Wilke *et al.*²⁴ for bovine NP cells. The reduction in ADAMTS 5 was unexpected, however, since the absolute expression was relatively low (Ct > 30), we believe this reduction would have a significant effect on secreted matrix composition. In the degenerated disc, glucose restriction and high pressure loading often occur together, and our results predict this would lead to a cumulative effect *in vivo*. Aggrecan and collagen II are the most abundant anabolic proteins of the nucleus pulposus; reduced gene expression for these proteins would substantially disrupt ECM homeostasis.

The NC cells had a nonsignificant decrease in viability under glucose restriction as well as a significant decrease in the anti-catabolic factor TIMP-1, suggesting that glucose restriction has a detrimental influence on NC cells. However, in the combined glucose restricted and high hydrostatic pressure group, TIMP-1 expression levels returned to control levels. This result suggests that pressure is inhibiting the effects of glucose restriction in NC cells.

The interaction between pressure and glucose restriction could be due to differential glucose transporter (GLUT) expression. Hydrostatic pressure has been shown to reduce glucose uptake in chondrocytes by inhibiting GLUT function,⁴³ whereas osmotic pressure increases glucose uptake in skeletal muscle,⁴⁴ hepatocytes,⁴⁵ and adipocytes.⁴⁶ Glucose transporters GLUT-1, -3, and -9 have been identified in adult human nucleus pulposus⁴⁷ and only GLUT-1 in the NC cell-rich rat nucleus pulposus⁴⁸; however, their precise function in IVD homeostasis still remains unclear.

While the present study examined the differences between NC and MNP cell populations *in situ*, NC and MNP cells coexist. While the majority of co-culture studies have focused on NC cell signaling and its influence on MNP cells, signaling is likely bidirectional. Gantenbein-Ritter and Chan⁴⁹ cultured porcine NC cells and bovine MNP cells separated by a membrane under standard conditions and found that in a 50:50 NC:MNP ratio, NC cells stimulated increased GAG production in MNP cells. In contrast, MNP cells stimulated aggrecan and collagen II expression at culture ratios of 25%, 50%, and 75%, indicating robust MNP cell to NC cell signaling at a wide range of cell concentrations. Therefore, extensive co-culture studies are needed to understand if the cell-specific response to loading and nutrient deprivation is influenced by cell signaling and how this response changes as cell ratios change from development through to disease states.

While *in vivo* disc-clamp studies report substantial changes in cell populations due to mechanical stress, these studies require devices to be inserted into the vertebrae; where the applied mechanical stress causes changes to the bone, vertebral endplates and other tissues. It is possible that these changes in the surrounding structures influence the loss of NC cells in *in vitro* models.^{17,50}

Conclusion

Using a same-species *in vitro* cell model, this study found that MNP and NC cells respond differently to hydrostatic pressure and glucose restriction. These functional differences could explain why species and individuals that retain a higher NC:MNP cell ratio into adulthood are less likely to develop disc degeneration. Additionally, our findings underline the importance of species and, as such, cell type selection in IVD biological research.

Authors' Note

All work reported in this article was conducted at the University of Auckland.

Acknowledgments and Funding

We thank the support of Auckland Medical Research Foundation and the staff at the Biomedical Imaging Research Unit. The work was funded by an Auckland Medical Research Foundation Doctoral Scholarship (TS), the School of Medical Sciences, University of Auckland Graduate Student Fund and the University of Auckland's Doctoral PReSS account.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Animal Welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

Ethical Approval

Ethical approval was not sought for the present study because animal tissue was obtained from a local met processing plant.

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