Hydrostatic pressure mimicking diurnal spinal movements maintains anabolic turnover in bovine nucleus pulposus cells in vitro

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Abstract

Current treatment strategies for progressive intervertebral disc degeneration often alleviate pain and other symptoms. With the goal of developing strategies to promote regeneration of nucleus pulposus (NP), this study tries to identify the effects of certain hydrostatic (HP) and osmotic pressures on the biology of the NP cells. We hypothesized that a repetitive regimen of cyclic HP followed by constant HP in high-osmolality medium would increase anabolic molecules in NP cells. Bovine NP cells/clusters were enclosed within semi-permeable membrane pouches and incubated with a regimen of cyclic HP for 2 days followed by constant HP for 1 day, repeated six times over 18 days. NP cells increased expression of some anabolic genes significantly over time: aggrecan, chondroitin sulfate N-acetylgalactosaminyltransferase 1, hyaluronan synthase 2, and collagen type-2 (P<0.05); and suppressed some catabolic or degenerative genes: matrix metalloproteinase-13, and collagen type-1; cellular characteristic genes: proliferating cell nucleic antigen, and E-cadherin. The amount of accumulated sulfated glycosaminoglycan increased significantly at day 18 compared to that at day 3 (P<0.01). Immunohistological staining revealed deposition of extracellular matrix molecules and localization of other specific molecules corresponding to their genetic expression. With a better understanding of how the cells respond to physicochemical stresses, we will be much better armed with biologically treating the degenerating disc with either cell or gene-based therapies and other potential matrix enhancing therapies. Efforts to apply these tissue engineering and regenerative medicine strategies will need to consider these important physicochemical stresses that may have major impact on the survivability of such treatments.

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Key words: extracellular matrix, nucleus pulposus, hydrostatic pressure, osmotic pressure, metabolic turnover, in vitro model.

Running title: Hydrostatic pressure in nucleus pulposus
INTRODUCTION

Intervertebral disc (IVD) degeneration is irreversible because of the low cellularity and avascularity in nucleus pulposus (NP) tissue and manifests clinically as chronic pain in the neck and lower back (Alvin et al., 2014). Current first-line treatments are targeted to pain management, e.g., analgesics and physiotherapy. Patients with late-stage disease and pain refractory to medication are treated surgically by discectomy, spinal fusion, or total disc replacement (Makhni et al., 2016; Snider et al., 1999). However, these interventions cannot restore normal biomechanical disc function and instead accelerate degenerative changes within adjacent IVDs (Alentado et al., 2016; Senteler et al., 2017). Therefore, regenerative therapies with the potential to restore spinal function and to offer relief from back pain are of great interest (Sakai and Schol, 2017; Smith et al., 2018; Thorpe et al., 2016). The latest concept for cell-based therapy is to implant therapeutic cells, which then produce typical NP extracellular matrix (ECM) into the space left after removal of degenerated NP, ultimately promoting regeneration of NP tissue (Detiger et al., 2016; Rosenzweig et al., 2018). An important element of this strategy is that the implanted therapeutic cells and resident cells are exposed to dynamic compressive stresses: hydrostatic pressure (HP), deviatoric stress, and intradiscal pressure (Sato et al., 1999; Wilke et al., 1999, Takeoka et al., 2020).

Since the NP consists of substantial fluid, NP cells are exposed to high HP due to diurnal spinal loading. A number of studies investigating the effects of HP on NP cells have been conducted using custom-made pressure culture systems (Zvicer and Obradovic, 2017). Most of these studies demonstrated that HP stimulated anabolic gene expression or production of typical ECMs of NP. However, few studies have addressed innovative therapeutic strategies for IVD regeneration (Le Maite et al., 2008, 2009; Shah and Chahine, 2018). We believe that investigations of the effects of changes in physicochemical stresses on cellular behavior and metabolic turnover require repetitive stress regimens and longer study duration with multiple biomarkers, based in part on previous studies (Mizuno et al., 2019). In addition, a unique characteristic of NP tissue is the accumulation of highly sulfated glycosaminoglycan (S-GAG), which generates higher intratissue osmotic pressure compared to other tissues (Wuertz et al., 2007). Furthermore, NP cells are exposed to intratissue fluid movement by compressive loading, which causes the NP to exude fluid (Ayotte et al., 2001; Vergroesen et al., 2014), and by off-loading, which makes the NP absorb fluid (Vergroesen et al., 2016), resulting in swelling (Neidlinger-Wilke et al., 2012). Thus, to develop regenerative therapies for IVD degeneration, it is important to understand cellular behavior and metabolic turnover in NPs under diurnal spinal loading (Chan et al., 2011; Haschtmann et al., 2006; Tyrrell et al., 1995).

We recently evaluated metabolic turnover in bovine NP (bNP) cells/clusters in response to changes in HP and osmolality in 12 systematic combinations (Mizuno et al., 2019). bNP cells showed upregulation of anabolic molecules, forming ECM in response...
to changes in cyclic HP and in high-osmolality (HOSm) culture medium (450 mOsm/kg H₂O). However, newly accumulated ECM showed gaps (void spaces) with high osmolality, but appeared more dense with low osmolality (LOsm, 320 mOsm/kg H₂O). Since we associated the upregulation of anabolic molecules with dense accumulation of ECM, these results were contradictory. In another study, however, we found that a regimen of cyclic HP in HOSm followed by constant HP in HOSm prevented formation of gaps in ECM (Takeoka et al., 2020). To further explain these results, we sought to reproduce the anabolic turnover in NP cells under repetitive regimens of changes in HP over multiple days, mimicking the circadian rhythm of spinal loading (Sato et al., 1999; Wilke et al., 1999). We hypothesized that a repetitive regimen of cyclic HP followed by constant HP in HOSm medium reproduces anabolic turnover in NPs. To test this hypothesis, we seeded primary bNP cell/clusters within semipermeable membrane pouches and incubated them under a repetitive regimen of cyclic HP at sinusoidal 0.2 – 0.7 MPa, 0.5 Hz followed by constant HP at 0.3 MPa in HOSm for 18 days. We intended the cyclic HP to mimic active upright loading and the constant HP to recapitulate intradiscal pressure in the recumbent position. We evaluated the regenerative capability of NP cells (defined as anabolic turnover) and degenerative capability (defined as catabolic turnover) in NP cells via gene expression levels and immunohistology of the relevant molecules.

MATERIALS AND METHODS

Isolation of NP cells/clusters

Bovine tails (from two- to three-week-old cows) were purchased from a local USDA-certified slaughterhouse. Caudal NPs with the adequate margin from annulus fibrosus were harvested from IVDs using surgical blades (#15 and #22, Bard-Parkers, Aspen Surgical, Caledonia, MI). If any extra fragment of annulus fibrosus remained attached to the NP, it was carefully removed with scissors. The technique for harvesting NP was validated histologically and standardized (Fig. 1a). The harvested NPs were digested in sterilized 0.15% collagenase (CLS-1, Worthington Biochemical, Lakewood, NJ) dissolved in Ham’s F12 medium (Life Technologies, Carlsbad, CA) at 37°C overnight on a rotator at 8 cycles/min. Usually, five NP tissue samples were harvested from one tail and digested in 14 ml of the collagenase using a 15-ml conical tube (Falcon®, Franklin Lakes, NJ). A minimal volume of air space within the conical tube allowed gentle agitation. The NP cells/clusters, were then rinsed in Dulbecco’s phosphate-buffered saline (D-PBS, Life Technologies) twice, seeded onto 1.5% agarose (1.0 ml, cell culture grade, Sigma-Aldrich)-coated 6-well plates (Falcon®), and incubated in 8 ml of Dulbecco’s Minimal Eagle Medium (DMEM)/Ham’s F - 12 (1:1) with 10% fetal bovine serum, penicillin 100 U/mL, and streptomycin 100 µg/mL (Life Technologies) at 37°C and 5% CO₂ in air for 48 hours. The NP cells/clusters from one tail were seeded onto 3 wells of a 6-well plate. During
incubation, the suspended NP cells/clusters aggregate or connect to each other (we designated these connected cells and cell aggregations seen in the digested tissue as “cell clusters”), and the non-digested debris or erythrocytes (if any) settle down on the agarose. This method also allows cell clusters in the NP tissue to remain in culture. After 24 hours’ incubation, debris (non-digested matrices) were removed piece-by-piece with a 200-µl pipette under a dissection microscope. After 48 hours’ incubation, the NP cells and cell clusters were collected and rinsed in Dulbecco’s phosphate-buffered saline (D-PBS) by centrifugation at 800 rpm for 5 min, twice.

Preparation of semipermeable membrane pouches and cell seeding

The number of cells in suspended NP cells/clusters collected at 48 hours was determined according to DNA concentration, since the NP cells/clusters were often aggregated and cannot be separated into individual cells for cell count. The amount of DNA was measured with fluorescent dye (Hoechst 33258, Molecular Probe) using a fluorometer (TBS-380, Turner Biosystems, Sunnyvale, CA) (Kim et al., 1988). Calf thymus DNA (Sigma-Aldrich) was used as the DNA standard. The correlation between the amount of DNA and the number of cells was validated in independent experiments using bovine NP cells and given 1 x 10^5 cells as 195 ± 35 ng (Mean ± SD, n = 4).

Prior to cell seeding, semipermeable hollow fiber membrane tubing (polyvinylidene fluoride, 1 mm ID, 1.2 mm OD, and 500 KD cut-off molecular weight, Repligen, Rancho Dominguez, CA) was cut into 35-mm lengths (Fig. 1b, 1c). The pieces of tubing were immersed in 100% ethyl alcohol for 30 minutes, then autoclaved in D-PBS at 121°C for 15 minutes (Mizuno and Ogawa, 2011; Mizuno et al., 2019).

Twenty-five microliters of the NP cells/clusters suspension (5 x 10^5 cells/25 µl) was slowly aspirated with a 200-µl tip inserted to the tubing. The tubing was sealed at each end with a stainless-steel clip to form a semipermeable membrane pouch (Fig. 1a, b). Actual volume of the seeded cell suspension was approximately 25 µl, since 2 – 3 mm margin at each end of the tubing was needed for closure with a stainless-steel clip (1.0 mm in width).

Incubation of NP cells/cluster

The pouches were divided into six groups (Fig. 2). LOsm Group: low-osmolality medium at 320mOsm/kg H_2O for 18 days; HOsm Group: high-osmolality medium at 450 mOsm/kg H_2O for 18 days; HOsm/HP Group: cyclic HP at 0.2 - 0.7 MPa, 0.5 Hz for two days in HOsm followed by constant HP at 0.3 MPa for 1 day in HOsm, repeated 6 times for 18 days; HOsm/cyHP Group: cyclic HP at 0.2 - 0.7 MPa, 0.5 Hz in HOsm for 18 days; HOsm/coHP Group: constant HP at 0.3 MPa in HOsm for 18 days; H-LOsm/HP Group: cyclic HP at 0.2 - 0.7 MPa, 0.5 Hz in HOsm for 2 days followed by constant HP at 0.3 MPa in LOsm for 1 day, repeated 6 times for 18 days.
The pouches were placed in a pressure chamber (Fig. 1e, 1f), which was installed in a pressure/perfusion culture system (TEP-2, PURPOSE, Shizuoka, Japan, Fig. 1g), to which cyclic or constant HP was applied, with medium replenishment at 0.1 ml/min, 3% O\textsubscript{2} and 5% CO\textsubscript{2} in air, mimicking diurnal HP loading in NPs. Briefly, the culture chamber has a backpressure valve/regulator, which precisely regulates backpressure and outlet medium flow (Fig. 1d). At the set maximum HP, the amount of replenished culture medium can be ejected precisely. A piston pump can send culture medium into the culture chamber and simultaneously the backpressure valve releases that precise amount of medium. This piston pump can send pressurized fluid at up to 5.0 MPa. The mechanism of this pressurized fluid control is the same as that of high-performance liquid chromatography (HPLC). For the experimental groups without HP, the pouches were placed in a stainless-steel mesh basket held in 100 ml medium with a stirrer at five spins/second to maintain sufficient mass transfer through the semipermeable membrane.

The culture medium at high osmolality was prepared with supplemented sodium chloride at 4.6 g/ml for 450 mOsm/kg H\textsubscript{2}O. The osmolality of the medium was measured using a freeze point osmometer (OMET 5004, Precision System, Natick, MA). If the osmolality was not between 448 and 450 mOsm/kg H\textsubscript{2}O, additional sodium chloride was added.

Evaluation of gene expression

The pouches were harvested for RNA extraction, immunohistology, and S-GAG and DNA assays at 3, 12, and 18 days. To quantify gene expression, the NP cells/clusters were harvested by flushing the pouches with a guanidine isothiocyanate-based extraction buffer (Buffer RLT, RNeasy kit\textsuperscript{®}, Qiagen, Valencia, CA) containing 1% β-mercaptoethanol for lysing cells prior to RNA isolation. The NP cells/clusters were homogenized using a handheld homogenizer pestle (Fisher Scientific). Total RNA was extracted in accordance with the manufacturer's instructions of the RNeasy kit\textsuperscript{®}. The amount of RNA was determined with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Wilmington, DE) and the samples were kept at -80\textdegree C until reverse transcription polymerase chain reaction (RT-PCR) was performed. The RNA samples were amplified with a RT-PCR using a high-capacity cDNA reverse transcription kit (Life Technology) to synthesize cDNA. This cDNA was mixed with TaqMan\textsuperscript{TM} gene expression master mix\textsuperscript{®} (Life Technology) and the fluorescent-labeled probe of the desired molecule (TaqMan\textsuperscript{TM} probe, Life Technology), followed by measuring gene expression with real-time PCR (QuantStudio\textsuperscript{TM}, Applied Biosystems, Foster City, CA). TaqMan gene expression assays were aggrecan core protein, Acan: Bt03212189_m1; chondroitin sulfate N-acetylgalactosaminyltransferase 1, Csgalnact1: Bt03272948_m1; hyaluronan synthase 2, Has2: Bt03212694_g1; collagen-type II, Col2a1: Bt03251837_mH; collagen-type I, Col1a1:Bt03225358_g1; connective tissue
growth factor, Ctgf: Bt03212492_m1; matrix-metalloproteinase-13, Mmp13: Bt03214051_m1; proliferating cell nuclear antigen, Pcna: Bt03211154_g1; N-cadherin, Cdh2; Bt04298958_m1; integrin-V, ItgaV: Bt04299013_g1, and glyceraldehyde 3-phosphate dehydrogenase, Gapdh: Bt03210919_g1 (Life Technology). We used Expression Suite Software v.1.0.4 to convert from ΔCt to relative quantity (RQ).

Measurement of accumulated S-GAG and DNA

Newly synthesized S-GAG and DNA produced by bNP cells were measured biochemically. The experiments were conducted independently for quantitative evaluation of S-GAG and DNA. At days 3, 12, and 18, the pouches were harvested, and the sample was ejected from each pouch by replenishing 200 µl of 125 µg/ml papain solution (Sigma-Aldrich) into a 0.5 ml tube with a cap and digested at 60˚C for 18 h. The optical density of S-GAG was quantified with dimethylmethylene blue (Farndale et al, 1982) at 570 nm using a microplate reader (iMark™, Bio-Rad, Hercules, CA). Shark chondroitin sulfate (Sigma-Aldrich) was used for the standard. The same sample was used for DNA assay described in cell seeding section above.

Cell viability

Independent experiments were conducted to determine cell viability. At 18 days, pouches were harvested and samples ejected by injecting D-PBS (Fig 3a). One pouch was harvested from each group. Cell viability assays were conducted three times. The samples were minced with double-stacked shaver blades and incubated in 1 µM calcein AM (to detect live cells) and 2 µM ethidium homodimer-1 (to detect dead cells) diluted with D-PBS at room temperature for 30 minutes using a LIVE/DEAD® Assay kit (Life Technologies) (Papadopoulos et al., 1994). Then the samples were rinsed 3 times in D-PBS for 10 minutes and cover-slipped with mounting media with 4,6’-diamidino-2-phenylindole (DAPI, SlowFade™ Gold antifade reagent with DAPI, Invitrogen) and processed for imaging. The live cells were identified by illumination in green with a filter set (Ex 450-490 nm, Em 500-550 nm), dead cells were illuminated in red with another filter set (Ex 540-580 nm, Em 592-660 nm), and nuclei were illuminated in blue with a filter set (Ex 325-375, Em 435-485 nm) using an inverted fluorescence microscope (DMI 8, Leica Microsystem, Buffalo Grove, IL). The images were acquired with a 20x objective lens and a DFC 7000T camera (Leica Microsystems). Since the majority of cells were illuminated in green (live), cells illuminated in red and in blue (nuclei) were counted manually within the same defined square (150 x 150 pixels) to avoid overcounting live cells due to the thickness of the sample.

Immunohistological evaluation
NP cells/clusters were harvested at days 3, 12, and 18 by flushing them from the pouches with D-PBS. The samples were fixed in a 2% paraformaldehyde/0.1 M cacodylate buffer (pH 7.4) at 4°C. Since the samples flushed were fragile and a round-bar shape, the samples were immobilized within 3% agarose gel (Genetic analysis grade, Fisher Scientific) dissolved in water. These samples were trimmed, embedded in paraffin, and cut into 7-µm sections for immunostaining.

Dewaxed sections were rinsed with PBS, pretreated with 0.3% hydrogen peroxide for 20 min. For keratan sulfate (KS) immunostaining, the sections were incubated in 0.05 units/ml chondroitinase ABC (Sigma-Aldrich) dissolved in Tris-Base buffer (pH 7.4) with proteinase inhibitor cocktail (Roche Complete, Sigma Millipore) for 30 min at 37°C. For immunohistology with other antibodies, this chondroitinase ABC digestion was omitted. The sections rinsed three times in PBS were blocked with 3% normal horse serum (Vectastain™ ABC kit, Vector Laboratory, Burlingame, CA) in a humidified chamber for 30 minutes at room temperature, followed by incubation with a primary antibody against KS (1:200, mouse monoclonal antibody 4B3/D10, Santa Cruz Biotechnology, Dallas, TX). Mouse IgG (Sigma-Aldrich) was used for a negative control. For other immunostaining, the sections were incubated with a primary antibody: collagen type I (Col-1, rabbit anti-collagen type-I, 1:300, Abcam, Cambridge, MA), collagen type II (Col-2, rabbit anti-collagen type-II, 1:200, MyBioSource, San Diego, CA), matrix metalloproteinase-13 (MMP-13, rabbit anti-rat MMP-13, cross-reactivity with bovine MMP-13, 1:200, LSBio, Seattle, WA), and proliferating cell nuclear antigen (PCNA, rabbit anti-PCNA, 1:200, Abcam, Cambridge, MA) for 30 minutes at room temperature. Rabbit normal IgG (Vector Laboratory) was used as a negative control for polyclonal antibodies. Following incubation with primary antibody, the sections were rinsed three times in PBS and incubated with a second biotinylated antibody according to the manufacturer’s instructions (Universal Vectastain™ ABC kit). Color was developed with 3,38-diaminobenzidine (DAB kit, Vector Laboratory). Counterstaining was performed with Harris’s hematoxylin (Sigma-Aldrich, Saint Louis, MO) for KS and Col-2, and with Contrast RED (KPL, Laboratories, Gaithersburg, MD) for Col-1, MMP-13, and PCNA.

Data analysis of gene expression and biochemistry

The RQ of the expression of each gene was calculated according to the difference between the average of each condition (time of experiment in each group) and of the LOsm (control) at day 3, which was given a value of 1.0. RQ was analyzed using a one-way analysis of variance (ANOVA) followed by a Bonferroni test for comparison of all conditions, with p < 0.05 considered significantly different (SPSS, version 21; IBM, Armonk, NY). The experiments were conducted five times, using different cow tails each time. After outliers were eliminated with a Shapiro-Wilk test, statistical analyses of four or five samples were conducted. The concentrations of S-GAG and DNA at 3,
12, and 18 days were converted the amount per a pouch. The concentrations of S-GAG and DNA at 3, 12, and 18 days were converted to the amount per pouch. The amounts of S-GAG and DNA under HOsm and HOsm/HP at each time point, and in each group at 12 and 18 days, were compared to day-3 data using a Student’s t-test.

RESULTS
Cell viability
Viable cells with their characteristic sharp periphery were easily identified within aggregated clusters and individually in all experimental groups. Dead cells illuminated in red were rare, and small particles were often seen randomly within and around the aggregation (Fig. 3b). The viability of each group was more than 90%. There was no significant difference between groups (Fig. 3c). Although a few dead cells were seen in selected regions of interest under a microscope, retention of these dead cells was unavoidable, because cells/clusters formed a 3D mini-tissue-like structure within a semipermeable membrane pouch (molecular-weight cut-off of 500 kD).

Gene expression in NP cells in response to repetitive regimens of Osm and HP

Anabolic and regenerative turnover
We quantified the gene expression of anabolic molecules Acan, Csgalnact1, Has2, and Col2a1 in NP cells exposed to various regimens of combinations of OP and HP (Fig. 4a).

The bNP cells maintained steady levels of expression of Acan for 18 days in LOsm (control). On the other hand, in HOsm the expression of Acan was transiently upregulated at day 12 and declined at day 18. With HOsm/HP, bNP cells expressed significantly more Acan with time, i.e., 2.6, 4.2, and 5.2 times greater than that in LOsm at days 3, 12, and 18, respectively (p < 0.01). With cyHP and coHP, Acan expression increased significantly: 3 and 3.7 times compared to that in LOsm at day 12, respectively, and maintained roughly those levels until day 18. With H-LOsm/HP, bNP cells maintained Acan expression similar to that on day 3 for 18 days.

bNP cells maintained expression of Csgalnact1 at levels similar to that on day 3 for 18 days in LOsm control. On the other hand, Csgalnact1 increased significantly in HOsm: 1.7-, 2.6-, and 3.6-fold compared with LOsm control at days 3, 12, and 18 (p < 0.05), respectively. With HOsm/HP, bNP cells expressed significantly more Csgalnact1: 2.1-, 2.4-, and 1.5-fold more than with LOsm control at days 3, 12, and 18, respectively (p < 0.05). With cyHP and coHP, Csgalnact1 showed similar upregulation: 3.9 and 3.7 times that with LOsm control at day 12, respectively, and maintained those levels.
through day 18. With H-LOsm/HP, bNP cells significantly increased expression of Csgalnact1: 1.8 and 2 times that in LOsm at days 12 and 18, respectively (p < 0.05).

In the LOsm group control, expression of Has2 decreased with time. With HOsm/HP, however, Has2 was significantly higher (1.9 and 2.6 times) than that in LOsm control at days 12 and 18, respectively (p < 0.05). With other regimens, Has2 maintained levels similar to those seen on day 3 for 18 days.

In LOsm control, bNP cells decreased expression of Col2a1 with time. In HOsm, however, the expression of Col2a1 was maintained at day-3 levels for 18 days. With HOsm/HP, cyHP, and coHP, Col2a1 was increased 1.5, 2.0, and 2.9 times compared with LOsm levels at day 3, respectively (p < 0.05) and maintained similar levels until day 18. Col2a1 with H-LOsm/HP maintained levels similar to those seen on day 3 for 18 days.

Catabolic/degenerative turnover

We quantified gene expression of catabolic molecules Col1a1 and Mmp13 (Fig. 5a). bNP cells significantly increased expression of Col1a1 in LOsm control with time (p < 0.01). Col1a1 in HOsm and with HOsm/HP and H-LOsm/HP maintained levels similar to those on day 3 for 18 days. With cyHP and coHP Col1a1 showed a transient increase at day 12.

bNP cells significantly increased expression of Mmp13, which was 2.0 and 4.8 times greater on days 12 and 18 (p < 0.01), respectively than that in LOsm at day 3 (Fig. 5a). With other regimens Mmp13 was downregulated compared to LOsm at day 3 and maintained that level for 18 days.

Cellular characteristic markers (cell proliferation, adhesion, phenotypes)

We quantified the gene expression of cellular characteristic molecules: Pcna, ItgaV, and Cdh2 (Fig. 6a). In LOsm (control), expression of Pcna was reduced considerably (50%) at days 12 and 18. With HOsm/HP, Pcna was significantly increased: 3.7 and 4.3 times greater than that in LOsm control at days 12 and 18, respectively (p < 0.05). With cyHP and H-LOsm/HP and in HOsm, Pcna maintained expression levels on day 3 for 18 days.

With HOsm/HP the expression of ItgaV was significantly upregulated at day 18; however, with other regimens it maintained day 3 expression levels for 18 days.

In LOsm (control) and with cyHP, the gene expression of Cdh2 decreased 20% and 50% at day 18 compared to that at day 3 in LOsm, respectively. In HOsm, Cdh2 was upregulated at day 3 compared to that in LOsm and maintained that level for 18 days. With HOsm/HP, Cdh2 increased significantly with time compared to that in LOsm control at day 3 (p < 0.05). With coHP Cdh2 doubled expression compared to day 3 at day 18. With H-LOsm/HP Cdh2 doubled expression compared to day 3 at day 12 and remained the same level at 18 days.

bNP cells showed a trend of increasing Ctgf in HOsm and H-LOsm/HP for 18 days.
(Fig. 6a). However, with other regimens the expression of Ctgf showed a trend towards
downregulation or maintained day-3 levels for 18 days.

**Immunohistology**

We characterized gene expression immunohistologically by evaluating the quality
of accumulated ECM, the localization of degenerative enzymes, and cell proliferation.

We used KS antibody staining to evaluate the quality of a component of aggrecan,
typical cartilaginous ECM, around NP cells (Fig. 4b). KS showed homogeneous
distribution associated with large gaps in the absence of HP. However, with HOsm/HP
NP cells/clusters showed denser accumulation of KS with time. The distinct difference
was that a dense area of ECM free of gaps was seen with HOsm/HP at day 18.

We stained with COL-2 antibody to evaluate the quality of typical cartilaginous
ECM in NP (Fig. 4b). In HOsm, COL-2 staining revealed low levels of accumulation
and the presence of gaps at day 18. With HOsm/HP, COL-2 showed considerably more
intense accumulation and homogeneity in NP clusters with time.

We stained with COL-1 antibody to identify fibrous ECM (Fig. 5b). In LOsm, COL-
1 increased in intensity with time. In HOsm COL-1 accumulated within NP clusters for
18 days. However, COL-1 intensity faded with HOsm/HP and H-LOsm/HP over 18
days.

We stained with MMP-13 antibody to assess the degeneration of ECM (Fig. 5b). In
the LOsm group, there was intense MMP-13 staining in ECM of NP cells/clusters at day
18. In HOsm, MMP-13 staining was slight around cells, and with other regimens, there
was no detectable MMP-13 within NP clusters.

We stained with PCNA antibody to identify proliferating cells and to semi-
quantitatively evaluate the number of these cells (Fig. 6b). PCNA-positive cells were
common within the NP cell clusters under all regimens and were more abundant with
HOsm, HOsm/HP, and H-LOsm/HP than with LOsm.

**Accumulation of S-GAG and DNA**

Accumulated S-GAG and DNA were measured (Fig. 7). Compared to day 3, the
amount of S-GAG produced by NP cells/clusters with HOsm/HP increased 2.1-fold
(statistically significant) by day 18 (P < 0.01). The amount of S-GAG with HOsm
increased between days 3 and 18, but statistical significance was not reached. The
amount of DNA in each group remained steady over 18 days of culture. There was
no statistical significance between the effects of HOsm and HOsm/HP. The S-
GAG/DNA ratio by NP cells/clusters with HOsm/HP increased significantly (1.9-
fold) by day 18 compared to day 3 (P < 0.01).

**DISCUSSION**
We intended to elicit anabolic turnover in primary and high viable bNP cells under alternating HP mode at HOsm medium *in vitro*. By mimicking diurnal spinal loading on NP cells, we hypothesized that altering cyHP followed by coHP in HOsm medium would stimulate anabolic turnover in bNP cells. To elucidate the effects of these stresses on anabolic turnover, we compared LOsm (control, medium osmolality 320 mOsm/kg H$_2$O), HOsm (no HP control, medium osmolality 450 mOsm/kg H$_2$O), HOsm/cyHP (continuous cyclic HP at 0.2 – 0.7 MPa, 0.5 Hz), HOsm/coHP (continuous constant HP at 0.3 MPa), repetitive HOsm/HP (alternating HP modes: cyclic HP for 2 days followed by constant HP for 1 day), and repetitive H-LOsm/HP (HOsm/cyclic HP – LOsm/constant HP) (Fig. 2). By systematically comparing the effects of these culture conditions, we intended to prove our hypothesis.

**Rationale of HOsm (estimated level in NP tissue) compared to LOsm (saline level at 320 mOsm/kg H$_2$O)**

In our recent study, bNP cells/clusters showed upregulated gene expression of ECM molecules in HOsm at day 7 (Mizuno et al., 2019). We postulated that the HOsm group mimicked conditions similar to those in normal NP tissue, where HOsm is higher than osmotic pressure (OP) in physiological fluid (320 mOsm/kg H$_2$O). However, because this OP in intra-NP tissue (defined as ECM-associated OP) was the estimated value converted from the fixed charged density, (Wuertz et al., 2007) it was unclear whether the osmolality in the culture medium was equivalent to that within the ECM around each cell (Neidlinger-Wilke et al., 2014). We used enzymatically isolated NP cells/clusters that were consistently exposed to HOsm in culture medium. We believe that HOsm interacted directly with bNP cells, because of the negligible accumulation of ECM at the start of culture. **However, newly synthesized ECM accumulated around cells with time.** At later time points, the effects of HOsm may be replaced by ECM-associated OP or synergistically increased with ECM. At LOsm *Col1a1* and *Mmp13* were significantly upregulated compared to HOsm with/without HP. HOsm and/or ECM-associated OP can inhibit synthesis of fibrotic ECM and enzymatic degradation. The comparison of HOsm with LOsm *in vitro* addresses the possibility that degenerated NP with AF injury (loss of intradiscal pressure) or loss of ECM increases the risk of progressive degeneration. To maintain anabolic turnover and support the regeneration of NP, a highly osmotic environment would be necessary.

**Rationale behind regimen of combined HOsm and HP**

In our recent study (Mizuno et al., 2019), we demonstrated that a seven-micrometer-thick section showed gaps (non-stained empty spaces) within the accumulated ECM synthesized by the bNP cells in HOsm. On the other hand, such gaps were absent from the LOsm control sample. If these gaps were created due to...
ECM degeneration, there would be less upregulation of anabolic gene expression and/or upregulation of catabolic gene expression. Another possible cause is weak entanglement of ECM due to excess absorption of fluid by ECM associated with high OP. Although these gaps may be unavoidable when forming amorphous NP tissue, we sought to identify a specific regimen of physicochemical stresses to prevent the formation of these gaps. In normal IVD, NP is compartmentalized within the IVD segment, which always maintains intradiscal pressure. Thus we mimicked this intradiscal pressure using coHP mode. More recently, we demonstrated that cyclic HP followed by constant HP prevented the formation of gaps with bNP cells over 6 days (Takeoka et al., 2020). To assess the consequences of coHP for metabolic turnover in NP cells, we compared the effects of a repetitive regimen of cyHP at 0.2 - 0.7 MPa, 0.5 Hz, and HOsm for 2 days followed by coHP at 0.3 MPa, and HOsm for 1 day over 18 days with other regimens systematically chosen. Since coHP loading prevented formation of ECM gaps, intradiscal pressure should be maintained for NP regeneration.

Rationale for the magnitude and mode of HP

A number of studies investigating the effects of pure HP or compressive stresses on metabolic turnover in NP cells have been conducted using monolayered NP cell culture, three-dimensional cell culture in gel (e.g., alginate, Neidlinger-Wilke et al., 2014), NP explants, organ culture (Gantenbein et al., 2006; Emanuel et al., 2015), and animal models (Alini et al., 2008). Through various positions and types of spinal motion, native NP tissues are under compressive stresses, which include pure HP and pure deviatoric (distortional or shear) stress (Carter and Beaupre, 2001). Since normal NP tissue is abundantly hydrated, we postulated that the stress was primarily pure HP in NP tissue (Urban and McMullin, 1988) with accompanying intradiscal pressure in IVD (Sato et al., 1999; Walter et al., 2011; Wilke et al., 1999). Thus, we intended to recapitulate the effects of pure HP in NP cells within an amorphous hydrated tissue compartment in this study.

We chose bNP cells isolated from bovine tails, because the physicochemical properties seen in human IVD are similar in several aspects to those in bovine tails. The range of motion of the human lumbar spine is similar to that of the bovine tail (Alini et al., 2008; Demers et al., 2004). Furthermore, the swelling pressure of NP in bovine tails is similar to that found in human lumbar IVD, estimated at 0.25 MPa, which was converted from the height of vertebrae (Oshima et al., 1993). Intradiscal pressure in human lumbar discs in prone and recumbent positions was estimated at 0.1 – 0.3 MPa, (Wilke et al., 1999) which can be defined as constant HP. In addition, dynamic spinal motion was critical to maintain metabolic turnover in avascular IVD (Neidlinger-Wilke et al., 2014). It has been reported that the magnitude and mode of HP vary depending on spinal position and physical activity (Gantenbein et al., 2006; Neidlinger-Wilke et al., 2014). Dynamic magnitudes have been estimated to range from 0.2 to over 3 MPa, and
the constant magnitude would be around 0.2 MPa regardless of posture or activity (Alini et al., 2008; Sato et al., 1999). Occasionally, the IVD is subject to a higher-magnitude HP (>3 MPa), which leads to degeneration in IVD cells, mediating increases in MMPs and decreasing ECM synthesis (Alini et al., 2008; Kasra et al., 2006). When NP cells were incubated under physiological HP ranging between 0.3 and 1 MPa, ECM proteoglycan synthesis was upregulated, while other conditions caused a downregulation in ECM and upregulation of catabolic signaling (Handa et al., 1997; Hutton et al., 1997; Hutton et al., 2001; Ishihara et al., 1996; Le Maitre et al., 2008; Mizuno et al., 2019). Based upon the above physiological magnitude and mode of HP, we established our in-vitro regimen of HP loading to recapitulate anabolic turnover. Although this regimen was designed for our in vitro study, it is very close to what occurs naturally in human spinal loading. This may be a considerable advantage for our dynamic translational model of the human IVD.

Rationale for duration of HP loading

The durations of one regimen of HP and total duration of repetitive regimens of HP were varied for research purposes (Gantenbein et al., 2006). For example, some researchers loaded HP for a few hours per day followed by no HP for the rest of the day, repeated each day for several days (Gantenbein et al., 2006; Kasra et al., 2006). In our previous in-vitro studies, we set HP loading for 3 days in order to determine possible differences in gene expression between the no-HP control and the HP-loading group (Mizuno et al., 2019; Ogawa and Mizuno, 2011, Ogura et al., 2018). We also included repetitive regimens to explore the effects of accumulated ECM on isolated NP cells/clusters over time. Thus, we created repetitive regimens that cycled six times over 18 days and evaluated quantitative gene expression, accumulation of ECMs, and immunohistology at three time points over 18 days. The gene expression profiles showed an increasing trend from 3 to 18 days, and a plateau either 3 - 18 days or 12 - 18 days. We speculate that the increased Acan under HOsm/HP was stimulated with newly accumulated ECM as positive feedback over 18 days. In addition, gene expression of Col1a1 under HOsm plateaued, whereas it was significantly upregulated under LOsm. Thus, Col1a1 expression was sensitive to osmolality. Furthermore, Mmp13 was suppressed under any regimen involving HP over 18 days. Overall, we believe that regimens repeated 6 times over 18 days recapitulated altering metabolic balances. By manipulating these regimens and duration, our NP cells/clusters model has the potential to reproduce homeostasis and regenerative process in NP tissue.

Effects of repetitive regimen of HOsm/HP on metabolic turnover and cellular characteristics in NP cells
We categorized the effects of physicochemical stresses into regenerative/anabolic turnover, degenerative/catabolic turnover, and cellular characteristics (Fig. 4-6, Table 1).

**Regenerative/anabolic turnover**
Under repetitive HOsm/HP, several important constituents of aggrecan were upregulated with time: *Acan*, an aggrecan core protein that binds chondroitin sulfate and keratan sulfate chains to form large proteoglycans (Hascall and Heinegard, 1974); *Csgalnact1*, an enzyme that transfers chondroitin sulfate N-acetylglucosamine, to elongate chondroitin sulfate chains (Ishimaru et al., 2014; Sakai et al., 2007; Sato et al., 2011); and *Has2*, an essential enzyme to synthesize hyaluronan, which binds to the aggrecan core protein, producing stable accumulation of aggrecan around NP cells (Roughley et al., 2011; Vigetti et al., 2012) (Fig. 4, Table 1). Compared to HOsm/HP, Acan and Csgalnact1 also increased under continuous HOsm/cyHP and continuous HOsm/coHP; however, Has2 was not upregulated. If hyaluronan is produced insufficiently, secreted aggrecan does not stably accumulate around each cell due to the lack of binding sites (Hascall and Heinegard, 1974). Immunohistologic staining with KS antibody, revealed accumulation of KS around each NP cell and the absence of gaps in the ECM under repetitive HOsm/HP. Thus, repetition and switching between cyclic and constant HP played an anabolic role in forming NP ECM.

*Col2a1* was chosen as a typical phenotypic and anabolic marker of NP cells (Hayes et al., 2001). The upregulation of *Col2a1* significantly increased with time under repetitive HOsm/HP, continuous HOsm/cyHP, and continuous HOsm/coHP compared to HOsm and LOsm. Without HP, *Col2a1* expression remained steady in HOsm and declined in LOsm over 18 days. Thus, we think that osmolality in culture medium is a key condition to alter *Col2a1* expression. We speculate that this specificity of *Col2a1* upregulation was created synergistically by changing the cell volume due to HOsm and changing membrane channels *e.g.*, transient receptor potential cation channel subfamily V member 4 (TRPV4) (Johnson et al., 2014) because HP increased intracellular calcium concentration in chondrocytes (Mizuno, 2006). The effects of these physicochemical conditions, HOsm and HP on *Col2a1* will be clarified during further study using potential inhibitors.

**Catabolic/Degenerative turnover**
*Mmp13* expression was chosen as an enzymatic marker of the catabolism of collagen in NP cells (Le Maitre et al., 2004). Application of HP in any mode significantly diminished *Mmp13* expression, whereas it is significantly upregulated in LOsm. Although we have not examined the effects of deviatoric stress (Chan et al., 2013) or interactions with other tissues in this study, *Mmp13* was shown to be capable of destroying ECM (Naqvi and Buckley, 2015; Yuan et al., 2018) and stimulating...
angiogenesis. Therefore, HOsm and HP may have the potential to synergistically inhibit degeneration of NP tissue including angiogenic invasion.

Collagen type-I is an essential molecule in ECM; however, excess collagen type-1 increases the risk of forming fibrotic tissue, causing dehydration of NP tissue (Naqvi and Buckley, 2015; Yuan et al., 2018). Thus, we chose Colla1 as a marker of NP cell degeneration. Over 18-day culture, Col1a1 expression was significantly upregulated in LOsm compared to other regimens, whereas HOsm (regardless of HP) was capable of preventing fibrosis in NP and maintained hydration. However, HOsm in body fluid is not practical for regenerative therapy. To establish a HOsm-equivalent tissue environment, replenishing sulfated-ECM to NP will be a practical alternative for restoring high ECM-associated osmotic pressure.

Cellular characteristics

The upregulation of Pcna, a marker of proliferating NP cells (Dietrich et al., 1993); and Itgav, an adhesion molecule mediating cell proliferation and stabilizing cell adhesion within ECM (Gilchrist et al., 2007; Le Maitre et al., 2009; Nettles et al., 2014), were significantly higher with HOsm/HP compared to other HP regimens by day 18. This suggests that proliferating cells produced the adhesion molecule integrin V, which binds to fibronectin in ECM. NP tissue is composed of abundant chondroitin sulfate, which prevent cell adhesion. For regenerative therapy, the presence of collagen and fibronectin would be necessary to promote cell proliferation.

To evaluate changes in NP phenotypes, we measured gene expression of Cdh2 (Hwang et al., 2016; Risbud et al., 2014); under repetitive HOsm/HP its expression was similar to that of other characteristic cellular molecules (Pcna, Itgav). Since Cdh2 is a cell-cell contact molecule, proliferation of NP cells may efficiently occur within cell clusters, increasing to establish cell-cell contact. Although early studies showed that Cdh2 gene expression increases under cyclic HP and decreases under constant HP (Wang et al., 2017; Xu et al., 2018), our results indicate that repetitive HOsm/HP elicited a significantly larger increase in Cdh2 compared to cyHP and coHP loading. The effects of repetitively altering cyHP and coHP on Cdh2 expression will be clarified in further studies.

CTGF plays roles in both anabolic and catabolic turnover in NP cells. Ctgf is a paracrine marker promoting fibrotic ECM production and leading to degeneration of NP tissue (Ali et al., 2008; James et al., 2019; Tran et al., 2013). In HOsm and H-LOsm/HP, Ctgf showed a trend toward increased upregulation compared to other regimens over 18 days. These unique trends, however, have large standard deviations of RQs. Even though further studies clarifying the effects of CTGF will be needed, other physicochemical stresses e.g., deviatoric stress may affect CTGF due to more active fluid movement (Neidlinger-Wilke et al., 2005).
Effects of repetitive regimen of H-LOsm/HP to recapitulate abnormal NP under diurnal spinal motion

Normal NP tissue contains the negative fixed charged density of S-GAG, which creates ECM-associated high osmotic pressure. Axial compression and relaxation in NP tissue with the circadian rhythms of spinal motion results in movement of intra-NP fluid as well as changes in the volume of NP tissue within the IVD segment (Mavrogonatou and Kletsas, 2010). We mimicked these changes in OP and HP using our NP cells/clusters model. We examined the effects of repetitive regimens of HOs/m/HP (compressive NP with diurnal stress) followed by LOsm/coHP (bulging NP with no external stress but intradiscal HP) on metabolic turnover and cellular characteristics.

Comparing HOs/m/HP and H-LOsm/HP, the latter diminished the significant increase in gene expressions of most molecules or maintained lower levels over 18 days, except for Ctgf. More specifically LOsm diminished the repetitive and cumulative effects of HP. To clarify the roles of HOs/m and LOsm, we need to examine the effects of newly accumulated endogenous ECM, which has the potential to create ECM-associated OP. We should examine the LOsm after accumulation of ECM by NP cells instead of LOsm with no accumulation. (Hoffmann et al., 2009; Lodish et al., 2000).

Though H-LOsm/HP maintained most cellular characteristics, this regimen did not increase Col2a1 and showed a trend toward increasing Ctgf, indicating progressive degeneration. We hypothesize that once the IVD compartment is destroyed, it is very unlikely that metabolic turnover in NP cells can support regeneration on its own, clearing the way for biological therapeutic intervention.

Accumulation of S-GAG and proliferation and viability of NP cells

S-GAG and DNA of NP cells/clusters under HOsm and HOs/m/HP were compared using pooled NP cells/clusters isolated from 5 tails in one experiment (5 pouches at each time point) to avoid risk of batch to batch variation of NP cell isolation. Accumulation of S-GAG under HOsm and HOs/m/HP showed increasing trends and significant increase (P < 0.01) over 18 days, respectively. These increases should be consistent with upregulation of Acan and Csgalnact1 (Fig. 4, 7). There was, however, no significant difference between HOsm and HOs/m/HP. We believe a cumulative parameter of S-GAG and a short-term parameter of gene expression should include correlations among multiple molecules. Amount of DNA under HOsm and HOs/m/HP showed steady levels over 18 days of culture, whereas Pcna showed a slight increase under HOsm/HP compared to HOsm. Since Col-1a1 showed low levels under both conditions over 18 days, NP cells had limited binding ECM sites which caused less DNA amounts. For promoting regeneration, cell adhesion on ECM and cell proliferation should be stimulated with other stress factors or augmented ECM materials.
Conclusions

We demonstrated that a repetitive regimen of HOsm/HP increased gene expression and accumulation of ECM molecules constituting NP and increased or maintained proliferation capability and phenotypes of NP cells, while suppressing degenerative/catabolic molecules over 18 days. Although bNP cells showed greater anabolic turnover of Acan and Col2a1 under HOsm/HP, distinctive differences were found with Csgalnact1 and Has2 compared to continuous cyHP and continuous coHP. Thus, repetitive alternating cyHP and coHP can promote production of whole aggregan components (core protein, chondroitin sulfate) and binding site (hyaluronan). The repetitive regimen also increased cell proliferation (Pcna), and cell-cell (Cdh2) and cell-ECM (ItgV) adhesion molecules compared to other HP modes and no HP. Since cell proliferation is another important characteristic promoting regeneration in NP cells, repetitive regimens are necessary. Furthermore, HP with any mode and/or HOsm inhibited catabolic Mmp-13 and minimized expression of its counterpart, fibril collagen type-I (Col1a1) compared to LOsm. We expect to find that balanced production and degradation of molecules proceeds under repetitive regimens of HP.

Though amorphous NP tissue is under compressive stress, which consists of pure HP and pure deviatoric (distortional or shear) stress, our study demonstrated only the effects of pure HP on production of ECM and the cellular behavior of NP cells. In future studies we shall examine the effects of compressive stresses including deviatoric stress. With additional stresses we may reproduce upregulation of degenerative/catabolic turnover and cellular behavior. With a set of these stresses, we may model homeostasis or balance of anabolic and catabolic turnover in NP cells by manipulating HP and deviatoric stress.

This study tries to identify the effects of certain hydrostatic and osmotic pressures on the biology of the NP cells. With a better understanding of how the cells respond to physicochemical stresses, we will be much better armed with biologically treating the degenerating disc with either cell or gene-based therapies and other potential matrix enhancing therapies. Efforts to apply these tissue engineering and regenerative medicine strategies will need to consider these important physicochemical stresses that may have major impact on the survivability of such treatments.

Acknowledgements

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FIGURE LEGENDS

Fig. 1  Nucleus pulposus cells/clusters culture using a semipermeable membrane pouch module and hydrostatic pressure/perfusion culture system

a) Histological validation of a harvested nucleus pulposus.  b) A semipermeable membrane pouch for enclosing nucleus pulposus cells/clusters.  The pouch was made of polyvinylidene difluoride (1.0 mm in diameter, 1.2 mm outer diameter, 30 mm in length, 500 KD MWCO).  c) A diagram of the rationale of a semipermeable membrane pouch.  Larger molecules e.g., ECM is retained within the pouch, whereas smaller molecules (< 500 kD) in- and out-fluxes through the membrane.  d) A diagram of hydrostatic pressure/perfusion culture system.  The culture system has three components: 1) a pump unit, 2) a pressure culture chamber unit, and 3) a backpressure control unit.  The culture chamber has a flexible plastic film (tetrafluoroethylene perfluoroalkyl vinyl ether) that separates the culture chamber from 4) the adjacent water compression chamber.  Water in the compression chamber was compressed with 8) an actuator driven piston, and hydrostatic pressure is transduced to the medium through the flexible plastic film.  Hydrostatic pressure in the medium and in the water chamber is equivalent so that the pressure within the water compression chamber is monitored with a pressure sensor.  Each unit is connected with pressure-proof unions.  Outlet of a backpressure control unit and inlet of a medium perfusion pump unit were connected to a medium bag by silicon tubing allowing gas exchange but completely closed to air.  5) Culture medium is kept in a clinically available blood donor bag, hung in an incubator.  6) Culture medium is replenished with a piston pump.  7,8,9) Each unit is attached to an actuator in a control system.  The maximum magnitude of backpressure is regulated with a spring-attached actuator for constant and cyclic hydrostatic pressure.  These culture units are completely closed and isolated from the ambient environment.  e) A photograph of hydrostatic pressure/perfusion culture module.  f) Pouches within a pressure/perfusion culture chamber.  g) Hydrostatic pressure/perfusion culture system.

Fig. 2.  Systematic culture conditions to compare interactions of changes in hydrostatic pressure and osmotic pressure.  HP: cyclic hydrostatic pressure at 0.2 - 0.7 MPa, 0.5 Hz for 2 days followed by constant hydrostatic pressure at 0.3 MPa for 1 day.  L\text{O}_{\text{osm}}: low osmolality at 320 mOsm/kg H\text{$_2$}O.  H\text{O}_{\text{osm}}: high osmolality at 450 mOsm/kg H\text{$_2$}O.  H-L\text{O}_{\text{osm}}: High osmolality for 2 days followed by low osmolality for 1 day.  no HP: atmospheric pressure.

Fig. 3.  Cell viability.  a) Sample preparation from 3D NP cells/clusters ejected from a pouch, minced, and stained with fluorescent indicators.  b) NP cells stained with
fluorescent indicators. Live cells in green, dead cells in red, and nuclei in blue. c) Cell viability (n=3).

Fig. 4. Gene expression profiles and immunohistology of anabolic molecules by NP cells/clusters in response to repetitive changes in cyclic followed by constant hydrostatic pressure in high or low osmolality. a) Relative quantity (RQ) of the expression of aggrecan core protein (Acan), chondroitin sulfate N-acetylgalactosaminyltransferase 1 (Csglnact1), hyaluronan synthase 2 (Has2) and collagen type-2 (Col2a1). Bars indicate Mean ± SD (n=5). Two-way ANOVA was conducted between regimens at the same day (**,*: p < 0.01, 0.05) and between 12 or 18 days and control at 3 days of each regimen (##,*: p < 0.01, 0.05). b) The accumulation of sub-molecule of aggrecan core protein, keratan sulfate (KS) and collagen type-II (Col-2) at 18 days. Each molecule accumulated is stained in brown and counterstained with hematoxylin in blue. Arrows indicate intense accumulation of KS and Col-2. Neg Cont: Negative control. LOsm: low osmolality at 320 mOsm/kg H₂O. HOsm: high osmolality at 450 mOsm/kg H₂O. HP: cyclic hydrostatic pressure at 0.2 - 0.7 MPa, 0.5 Hz for 2 days followed by constant hydrostatic pressure at 0.3 MPa for 1 day. H-LOsm: High osmolality for 2 days followed by low osmolality for 1 day. no HP: atmospheric pressure. Each section is 7 µm thick and scale bar indicates 50 µm.

Fig. 5. Gene expression profiles and immunohistology of catabolic molecules by NP cells/clusters in response to repetitive changes in cyclic followed by constant hydrostatic pressure in high or low osmolality. a) Relative quantity (RQ) of the expression of matrix metalloprotein-13 (Mmp13), and collagen type-I (Col1a1). Bars indicate Mean ± SD (n=5). Two-way ANOVA was conducted between regimens at the same day (**,*: p < 0.01, 0.05) and between 12 or 18 days and control at 3 days of each regimen (##,*: p < 0.01, 0.05). b) The accumulation of collagen type-I (Col-1) and matrix metalloprotein-13 (MMP-13) at 18 days. Each molecule is stained in black and counterstained with Contrast Red in red. Arrows indicate intense accumulation of each molecule. Neg Cont: Negative control. LOsm: low osmolality at 320 mOsm/kg H₂O. HOsm: high osmolality at 450 mOsm/kg H₂O. HP: cyclic hydrostatic pressure at 0.2 - 0.7 MPa, 0.5 Hz for 2 days followed by constant hydrostatic pressure at 0.3 MPa for 1 day. H-LOsm: High osmolality for 2 days followed by low osmolality for 1 day. no HP: atmospheric pressure. Each section is 7 µm thick and scale bar indicates 50 µm.

Fig. 6. Gene expression profiles and immunohistology of cellular characteristic molecules (proliferation and adhesion) by NP cells/clusters in response to repetitive changes in cyclic followed by constant hydrostatic pressure in high or low osmolality. a) Relative quantity (RQ) of the expression of proliferating cell nuclear antigen (Pena), N-
cadherin (Cdh2), integrin-V (ItgaV), and connective tissue growth factor (Ctgf). Bars indicate Mean ± SD (n=5). Two-way ANOVA was conducted between regimens at the same day (**,*: p < 0.01, 0.05) and between 12 or 18 days and control at 3 days of each regimen (##,#: p < 0.01, 0.05). b) The proliferating cells exhibited with proliferating nuclear antibody (PCNA) is stained in black and counterstained with Contrast Red in red. Arrows indicate examples of PCNA positive cells. Neg Cont: Negative control.

LOsm: low osmolality at 320 mOsm/kg H$_2$O. HOsm: high osmolality at 450 mOsm/kg H$_2$O. HP: cyclic hydrostatic pressure at 0.2 - 0.7 MPa, 0.5 Hz for 2 days followed by constant hydrostatic pressure at 0.3 MPa for 1 day. H-LOsm: High osmolality for 2 days followed by low osmolality for 1 day. no HP: atmospheric pressure. Each section is 7 µm thick and scale bar indicates 50 µm.

**Fig. 7.** Amounts of accumulated S-GAG and DNA produced by bNP cells/clusters under HOsm and HOsm/HP over 18 days. a) The amount of S-GAG. produced by NP cells/clusters. b) The amount of DNA. c) The ratio of S-GAG/DNA. Bars indicate Mean ± SD (n=5).

**Table. 1.** Effects of Changes in Physicochemical Stresses on Metabolic Turnover in Bovine Normal Nucleus Pulposus Tissue models (cells/clusters) over 18 days (five repetitions)

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Fig. 1 Nucleus pulposus cells/clusters culture using a semipermeable membrane pouch module and hydrostatic pressure/perfusion culture system

- a) Histological validation of a harvested nucleus pulposus.
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- e) Culture medium is kept in a clinically available blood donor bag, hung in an incubator.
- f) Culture medium is replenished with a piston pump.
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172x60mm (600 x 600 DPI)
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182x127mm (300 x 300 DPI)
Fig. 6. Gene expression profiles and immunohistology of cellular characteristic molecules (proliferation and adhesion) by NP cells/clusters in response to repetitive changes in cyclic followed by constant hydrostatic pressure in high or low osmolality. a) Relative quantity (RQ) of the expression of proliferating cell nuclear antigen (Pcna), N-cadherin (Cdh2), integrin-V (ItgaV), and connective tissue growth factor (Ctgf). Bars indicate Mean ± SD (n=5). Two-way ANOVA was conducted between regimens at the same day (**, *: p < 0.01, 0.05) and between 12 or 18 days and control at 3 days of each regimen (##, #: p < 0.01, 0.05). b) The proliferating cells exhibited with proliferating nuclear antibody (PCNA) is stained in black and counterstained with Contrast Red in red. Arrows indicate examples of PCNA positive cells. Neg Cont: Negative control. LOsm: low osmolality at 320 mOsm/kg H2O. HOsm: high osmolality at 450 mOsm/kg H2O. HP: cyclic hydrostatic pressure at 0.2 - 0.7 MPa, 0.5 Hz for 2 days followed by constant hydrostatic pressure at 0.3 MPa for 1 day. H-LOsm: High osmolality for 2 days followed by low osmolality for 1 day. no HP: atmospheric pressure. Each section is 7 µm thick and scale bar indicates 50 µm.
Fig. 7. Amounts of accumulated S-GAG and DNA produced by bNP cells/clusters under HOsm and HOsm/HP over 18 days. a) The amount of S-GAG produced by NP cells/clusters. b) The amount of DNA. c) The ratio of S-GAG/DNA. Bars indicate Mean ± SD (n=5).

166x63mm (300 x 300 DPI)
Table 1. Effects of Changes in Physicochemical Stresses on Metabolic Turnover in Bovine Normal Nucleus Pulposus Tissue models (cells/clusters) over 18 days (five repetitions)

<table>
<thead>
<tr>
<th>Gene Condition</th>
<th>Regenerative/Anabolic</th>
<th>Degenerative/Catabolic</th>
<th>Phenotype/Behavior</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Acan</td>
<td>Csgalact1</td>
<td>Has2</td>
</tr>
<tr>
<td>LOsm</td>
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<tr>
<td>HOSm</td>
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<td>HOSm/HP</td>
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<td>HOSm/coHP</td>
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<td>HOSm/cyHP</td>
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<tr>
<td>H-LOsm/HP</td>
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</tbody>
</table>

- Nearly constant
- Slight decrease
- Moderate/great decrease
- Small increase
- Moderate increase
- Great increase
Scientific Editor Comments:
The authors addressed all reviewers’ comments in an appropriate manner; the quality of the manuscript is significantly improved compared to the previous version. There are minor corrections to undertake.

Authors: We corrected wrong descriptions and Figures and improved data analysis (ratio). We also added our response to Reviewer 2’s question.

Reviewer(s)’ Comments to Author:

Reviewer: 1
Recommendation: 1. Accept as is
Comments: (There are no comments.)
Additional Questions:
Original findings: YES
Aim: Clear and relevant

Introduction: Much improved and well read.

Materials & Methods
Described well enough to allow repetition of the work: YES
Materials and methods comments: Far more detailed than in previous submission. Now acceptable.

Results - Errors/comments: All prior concerns addressed

Discussion - Errors/comments: All prior concerns addressed

Conclusion - Errors/comments: All prior concerns addressed

References - Errors/comments: All prior concerns addressed

Illustrations- Quality: All prior concerns addressed

General Errors/comments not listed in the sections above:

If the paper is acceptable (1 or 2 - See Recommendations below), please assess the significance/character of this paper.: Original research of interest to specialists.
In addition to reviewing the paper as critically as you would for any other respected journal, we desire relevant questions such as may arise at a conference where this paper is presented as written. Questions which bring out additional information or which challenge the authors’ approach, findings, or conclusions, are particularly welcome. While some of these questions may be attended to by appropriate text changes, MOST questions and authors’ replies will be published with the paper as "Discussion with Reviewers" (with the reviewers named, as thanks for reviewing, unless requested specifically not to be named.

Please add your questions here. <i>(Please insert N/A into the text box below if you do not answer 1 or 2)</i>: N/A

Reviewers are named in the published paper by default. Please confirm you agree, or request anonymity.: I request my review remains anonymous.

Have the authors provided all the information as indicated in the ARRIVE checklist document?: Not applicable

ARRIVE Checklist Document comment:

**********************************************************
Reviewer: 2
Recommendation: 2. Recommend acceptance with listed textual revision.
Comments: (There are no comments.)
Additional Questions:
Original findings: YES

Aim: The aim of this study is to test the effect of repetitive changes of hydrostatic pressure on bovine nucleus pulposus cells under high osmolality conditions. It was hypothesized that such loading would lead to anabolic responses of the cells.

Introduction: Fine

Materials & Methods
Described well enough to allow repetition of the work: YES

Materials and methods comments: Page 6, line 37: Please replace "TaqMan probes" by "TaqMan gene expression assays"; since these assays contain the primers and the fluorescent probes.

Authors: Corrected. (Page 6, line 37)
Results - Errors/comments: Page 9, line 7: Please correct "significantly different" to "significant difference"
Authors: Corrected. (Page 9, line 13)

Fig. 4a, 5a, 6a. Please change figure legends in graphs to correct abbreviations (LOsm; HOsm).
Authors: Corrected.

Fig. 4-6, captions. Change "osmotic pressure" to "osmolality".
Authors: Corrected.

Fig. 7. Please add standard deviations.
Authors: We added the standard deviation to Figure 7c.

After the standard deviation was calculated, the change in the ratio of S-GAG/DNA under HOsm/HP between days 3 and 18 was statistically significant (P < 0.01). We added this to the results. (Methods: Page 9, line 2-4; Results: Page 11 line 29-37)

We initially thought that including the ratio of S-GAG/DNA was unnecessary because it was a secondary analysis. In addition, sometimes the S-GAG/DNA ratio runs the risk of emphasizing a false negative. For example, cartilaginous cells simultaneously accumulate abundant S-GAG within a defined compartment or a cell scaffold and elute it into the culture medium. If we only measured the S-GAG and DNA within the compartment or the scaffold, the eluted S-GAG in the medium is excluded. As this reviewer reminded us, however, a semipermeable membrane pouch (MWCO: < 500 kD) has the ability to confine the S-GAG and DNA. Thus, we agree that the ratio of S-GAG and DNA is a valuable statistic to compare the effects of HOsm/HP over time. We will continue to evaluate the newly synthesized ECM in detail in future studies.

Discussion - Errors/comments: Fine.

Conclusion - Errors/comments: Fine.

References - Errors/comments: Fine.

Illustrations- Quality: ok.

General Errors/comments not listed in the sections above:
If the paper is acceptable (1 or 2 - See Recommendations below), please assess the
significance/character of this paper: Original research of interest to specialists
In addition to reviewing the paper as critically as you would for any other respected journal, we
decline relevant questions such as may arise at a conference where this paper is presented as
written. Questions which bring out additional information or which challenge the authors’
approach, findings, or conclusions, are particularly welcome. While some of these questions
may be attended to by appropriate text changes, MOST questions and authors' replies will be
published with the paper as 'Discussion with Reviewers' (with the reviewers named, as thanks
for reviewing, unless requested specifically not to be named.

Please add your questions here. <i>(Please insert N/A into the text box below if you do not
answer 1 or 2)</i>:

Hydrostatic pressure has also been shown to have beneficial effects on the phenotype of
articular chondrocytes. Are there any differences in the optimal loading regimes between
intervertebral disc and cartilage cells?

Authors: We previously demonstrated that bovine articular chondrocytes stimulated the
upregulation of cartilaginous matrix genes with cyclic hydrostatic pressure 0 – 0.5 MPa, 0.5 Hz
(Mizuno S and Ogawa R. Using changes in hydrostatic and osmotic pressure manipulate
metabolic function in chondrocytes. Am J Physiol Cell Physiol 2011; 300: C1234-45). Using the
same culture system, bovine nucleus pulposus cells/clusters also stimulated the upregulation of
cartilaginous matrix genes with HP at cyclic 0 – 0.5 MPa, 0.5 Hz (Mizuno et al., 2019). However,
extracellular matrix accumulated by NP cells showed gaps (spatial voids) when 7-µm thick
sections were stained with keratan sulfate antibody, whereas articular chondrocytes did not
form gaps. Based on this histological difference, we hypothesized that constant HP prevents
formation of gaps because the NP is under intradiscal pressure. We demonstrated that
HOsm/cyclic HP at 0.2 - 0.7 MPa for 2 days followed by HOsm/constant HP at 0.3 MPa for 1 day
upregulated cartilaginous matrix molecules and reduced gap formation (Takeoka, 2020). In the
current study, we repeated this regimen of HOsm/HP over 18 days and showed synthesis and
accumulation of cartilaginous molecules using gene expression, immunohistologic, and
biochemical markers. When we design any experiments to determine differences between more
than two treatment groups, we are careful to choose advantageous markers and treatments.
Thus, though our findings should be interpreted with the normal limitations of any in vitro
experiments, the data should be suitable for extrapolation of the effects of HP in vivo. As we
gather more information on varied culture conditions, we will establish an intervertebral disc
model for developing regenerative therapeutic strategies.

Reviewers are named in the published paper by default. Please confirm you agree, or request
anonymity: I confirm my name will be made public with the paper
Have the authors provided all the information as indicated in the ARRIVE checklist document?
Not applicable    ARRIVE Checklist Document comment: