Effect of loading stress on regenerative characteristics in inner and outer bovine annulus fibrosus cells

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INTRODUCTION: Lumbar intervertebral disc herniation is a common spinal disorder that is often treated with discectomy surgery when conservative treatments fail. A few surgical devices have been designed to prevent recurrent herniation after discectomy. However, none of them was intended to induce the biological healing of annulus fibrosus (AF) wounds. The optimal treatment should incorporate some means for stimulating cellular and matrix integration by resident cells to obtain robust tissue repair. To explore such treatment strategies, we need to understand the cellular characteristics of AF cells under loading. It is known that AF is exposed to compressive and tensile stresses, and these complex stresses can be defined as combined hydrostatic pressure (HP) and deviatoric stress (Strain). We hypothesized that compressive loading recapitulated with combined HP and Strain reduces metabolic turnover in extracellular matrix (ECM) and cell proliferation. We evaluated the effect of HP and DS in the physiologically relevant range on ECM turnover and cell proliferation of inner and outer AF cells.

METHODS: AF tissues (margin: >2 mm from NP and >2 mm from outer ligament) were harvested from bovine caudal intervertebral discs (livestock 1-2 years old) (Fig. 1). Subsequently, the inner half of AF (inner AF) and outer half of AF (outer AF) were isolated and digested in 0.2% collagenase at 37˚C overnight on a rotator. The digests were collected by centrifugation and rinsed in PBS. Inner and outer AF cells were suspended in neutralized 0.3% collagen type-I solution, and 1x 10^5 cells/25 µl were injected into a semipermeable membrane tubing (1Ø x 25 mm long, hollow fiber, 500 kD molecular cut-off)[1]. Both ends of the tubing were closed with clips, forming a pouch, and incubated in Dulbecco minimum essential medium/Ham's F12 medium (1:1) with 10% fetal bovine serum and antibiotics. The pouches were assigned to four loading conditions as follows: (1) Unloaded control; (2) HP: cyclic HP at 0.2-0.7 MPa, 0.5Hz for 2 days followed by constant HP at 0.3 MPa for 1 day repeated twice; (3) Strain: continuous arc-bending strain at 0-0.4%, 0.5 Hz; (4) HP/Strain: combined HP and DS mimicking spinal movement (Fig. 1). The pouches were incubated using a pressure/perfusion culture system with/without arc-bending strain module[1,2]. AF cells were harvested at 3 and 6 days and evaluated for gene expression of metabolic molecules. Total RNA was extracted using a RNeasy kit (Qiagen) and amplified with a reverse transcription kit (ThermoFisher). Gene expression of anabolic (*Acan, Col-1, Col-2, Eln*), catabolic (*Mmp13*), and cellular molecules (*Timp2, Pcna*) were quantified with RT-qPCR using TaqMan probes (QuanStudio version 1.4, Applied Biosystems) and compared with the endogenous control (*Gapdh)* (n=5). Relative quantities (RQs) of the expression of each gene were analyzed by the $2^{-\Delta\Delta Ct}$ method, and the value of the unloaded control of inner AF cells at 3 days was set at 1.0. For histological evaluation, AF cells were fixed with 2% paraformaldehyde, embedded in paraffin, and cut into 7-µm sections. Immunostaining with antibodies corresponding to gene expressions was performed. Two-way ANOVA was performed between groups on the same day and between 3 and 6 days within each group. $p<0.05$ was considered statistically significant (GraphPad Prism 9, GraphPad Software).

RESULTS: Inner AF cells showed significantly increased levels of *Acan*, and *Timp2* by 6 days under HP ($p < 0.05$), with a trend toward increased *Mmp13* (Fig. 2). Outer AF cells demonstrated a significant decline in *Col-2* under Strain and HP/Strain ($p < 0.05$) and a trend toward suppression of *Col-1* and *Eln* expression compared to HP and unloaded control. On the other hand, *Pcna* increased significantly under Strain and HP/Strain in inner AF and declined under unloaded and HP in outer AF ($p < 0.05$). Immunohistology findings supported reductions in the expression of matrix genes, with lower intensity of keratan sulfate, collagen type-2, and collagen type-1 under HP/Strain compared to other loading conditions (Fig. 3).

DISCUSSION: To address therapeutic strategies for AF wounds, we compared the characteristics of inner and outer AF cells under HP or Strain or HP/Strain, because inner and outer AF are exposed to different loading stimuli in daily spinal loading. Since we intend to promote tissue repair, gene expression and the corresponding molecules in the ECM turnover and cell proliferation were evaluated. Our results show HP/Strain inhibits ECM remodeling while promoting cell proliferation. To promote regeneration, continuous compressive loading should be avoided, as it switches the metabolism to a state where tissue repair and remodeling are limited. Since both ECM remodeling and cell proliferation are essential for tissue repair, switching HP/Strain on and off may be optimal for tissue repair. This finding may be translated as the cycle of spinal movement and resting promotes AF wound healing in clinical situations. Further study with another loading regimen will elucidate the effect of on- and off- loading cycles on AF tissue repair.

SIGNIFICANCE: Continuous compressive loading (combined HP and Strain) appears to inhibit metabolic turnover while increasing cell proliferation in both inner and outer AF. Continuous compressive loading should be avoided for the regeneration of AF.

REFERENCES: (1) Mizuno S +. J Orthop Res. 2019. (2) Ogura T +. J Tissue Eng Regen Med. 2019

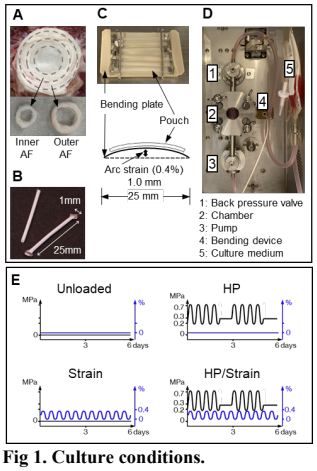

Fig 1. Culture conditions.
A) Isolation of inner and outer AF tissues.
B) A semipermeable membrane pouch.
C) Device and schema of arc bending strain.
D) Pressure/perfusion culture system
E) Culture conditions.

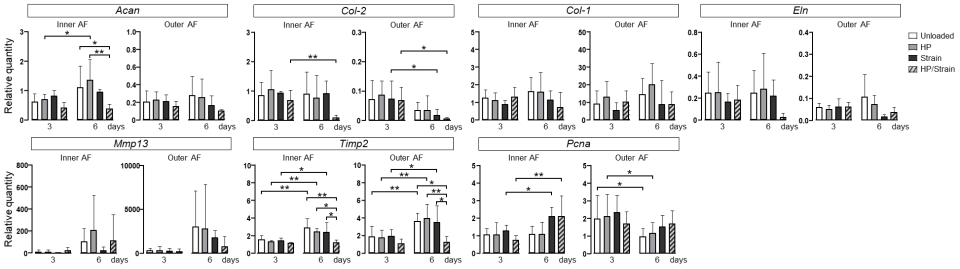

Fig 2. Gene expression in inner and outer AF cells.

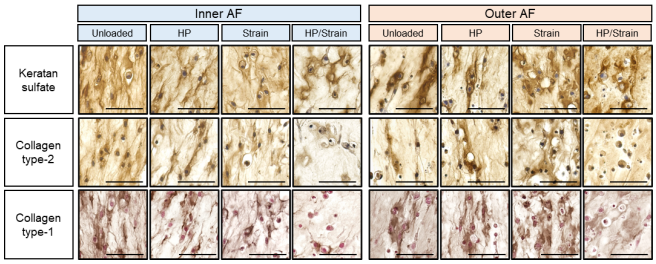

Fig 3. The accumulation of extracellular matrix molecules.