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Osmotic Loading and Growth Factor Supplementation Alters Tissue Growth of Intervertebral Disc Cells

Introduction: The intervertebral disc is a large fibrocartilaginous tissue, and its primary function to absorb and distribute large complex loads placed on the spine. A quarter of the disc's fluid is expelled and reimbibed during each diurnal cycle. The lower water content at the end of a diurnal cycle significantly alters the osmotic environment from 400 mOsm at maximum hydration to 550 mOsm. Recent studies demonstrated that osmolarity alters cell proliferation, gene expression, and matrix production of monolayer and micropellet cultures [1, 2]. However, these studies were short term and did not evaluate the mechanical integrity of matrix deposited *in vitro*. Tissue-engineering techniques can be utilized to culture cells under various osmotic conditions to evaluate extracellular matrix production and mechanical integrity [3].

The objective of this study was to determine the effect of long-term osmotic loading on matrix turnover and cell viability of disc cells. Typically, cells are given exogenic factors, such as TGFβ3 to stimulate matrix production; however, the effect of transient application of the growth factor can be species or age dependent. Therefore, the second objective was to determine the effect of transient supplementation of culture media with TGFβ3 on matrix production and cell proliferation. We hypothesize that cells will produce more native-like tissue at osmotic swelling conditions similar to a healthy disc, and that the effect of transient growth factor supplementation will be dependent on the donor's age.

Methods: Lumbar spine sections from juvenile cows (3-6 weeks) and caudal spine sections from adult cows (18 months) were acquired and intervertebral discs were removed aseptically with a scalpel. Discs were further dissected to separate the annulus fibrosus (AF) and the nucleus pulposus (NP). NP tissue was digested in serum media containing collagenase IV (Worthington, Lakewood, NJ). AF tissue was cultured in serum media and cells were allowed to crawl out of the tissue for two weeks. Cells were expanded for one passage (plating density = 45k cells/cm^2) in high-glucose media (DMEM) containing 10% FBS, 1% antibiotics, 0.5 ng/ml bFGF, 0.5 ng/ml PDGF, and 1 ng/ml of TGFβ1 [4].

Passaged cells were encapsulated in 2% w/v low-gelling agarose and samples were cored from the slab (4 mm diameter, 2.34 mm thickness; Young's modulus ~5 kPa). DMEM was diluted to make 300mOsm media by adding deionized, distilled water (starting osmolarity ~334 mOsm). Then, NaCl and KCl were added to the media to make 400 and 500 mOsm media. At day 14, constructs were divided into two groups for each osmotic loading condition. Half of the constructs continued to receive growth factor supplement and the other half was 'released' from the growth factor supplement. Constructs with NP cells were cultured for 42 days and constructs with AF cells were cultured for 28 days.

Mechanical and biochemical properties were measured (n = 5 per group). The equilibrium Young's modulus was determined at 10% strain under unconfined compression, and the dynamic modulus was measured from the response to a superposed sinusoidal input of ±1% strain at 0.5 Hz [2]. Glycoamionglycan (GAG) and collagen contents were measured and normalized by DNA to compare values between osmolarity groups. A one-way ANOVA (factor = osmolarity) was performed with a Bonferroni's post hoc test to determine differences in the biochemical and mechanical properties. A t-test was performed to transient and continuous growth factor supplementation. Significance was set at α=0.05.

Results: **NP cells:** Compressive mechanical properties of juvenile NP seeded constructs were greatest at 400 mOsm (p < 0.001; Fig. 1A). Long-term viability of cells from juvenile and adult donors decreased with increasing osmolarity (p < 0.001; Fig. 2A). Transient supplementation of TGFβ3 improved mechanical and biochemical (i.e. GAG and collagen) properties of constructs with juvenile NP cells (p < 0.05; Fig. 1A & 3A). Adult NP cells cultured at 300 mOsm had the highest mechanical properties (p < 0.02; Fig. 1C). GAG and collagen contents were not affected by osmotic loading (p > 0.05; Fig 3C), which was comparable to previous studies using monolayer cultures [1]. The effect of TGFβ3 on adult NP seeded constructs was not clear, because the effect of TGFβ3 was dependent on the osmotic condition. That is, at 300 mOsm continuous supplementation of TGFβ3 resulted in higher GAG production; however, at 400 mOsm, GAG deposition was greater with transient growth factor supplement (p = 0.01; Fig. 3C). **AF cells:** Mechanical and biochemical properties were greatest at 300 mOsm for juvenile AF cells (p < 0.03; Fig. 1B & 3B). Similar to NP cells, cell viability of AF cells from juvenile donors decreased with increasing osmolarity (p = 0.01). The Young's modulus of constructs seeded with adult AF cells was dependent on the osmotic condition, where constructs cultured in 300 mOsm had the highest mechanical properties. Transient supplementation of TGFβ3 only altered mechanical properties at 300 mOsm (Fig. 1B & D). Interestingly, the GAG content, which is usually correlated with compressive mechanical properties, was not altered with transient TGFβ3 supplement (Fig. 3B & D). GAG production and cell viability of adult AF cell seeded constructs were not dependent on osmolarity (p > 0.15; Fig. 3D).

Discussion: This study investigated the effect of osmotic conditions and transient TGFβ3 supplement on juvenile and adult disc cells. Constructs seeded with juvenile NP cells were the most productive, resulting in the highest GAG content and stiffest constructs. In contrast, adult NP constructs had the lowest matrix production and mechanical properties. The effect of TGFβ3 supplementation observed in juvenile and adult cells is comparable to previously reported observations of chondrocytes, where juvenile cells deposit more extracellular matrix with transient supplementation [5].

Although there are significant differences between AF and NP cells, the close proximity may provide a reasonable cell source for developing a biological repair strategy for the disc by 'reprogramming' AF cells. Matrix production and mechanical properties achieved by AF cells were similar regardless of donor age. These results demonstrate that when AF cells are cultured in a similar environment to NP cells, the AF cells are capable of developing tissue that is mechanically and biochemically similar to the engineered tissue developed by the NP cells. Moreover, adult NP and AF cells had similar responses to TGFβ3 and osmotic loading, suggesting that these cells may be sufficient for developing a biological repair strategy for either tissue.

As future treatments look towards biological repair strategies for the intervertebral disc, it's possible that a combination of osmotic loading and chemical stimuli will be used, depending on the desired goal (i.e. cell viability vs. matrix production). The results shown here demonstrate the age-dependent response of TGFβ3 supplement. Recently, growth factor injections have been used to treat low back pain. Future work will evaluate the effect of TGFβ3 supplement on human disc cells. In conclusion, this study demonstrates that matrix production and cell viability are greatly affected by long term osmotic loading and TGFβ3 supplementation.

Significance: Biochemical and mechanical properties of engineered tissues developed with juvenile NP cells were higher with transient TGFβ3 supplementation. Osmotic loading had a greater effect on NP cells than AF cells, which may represent differences in osmotic loading experienced *in situ,* where the NP is likely to see larger changes in osmotic loading during a diurnal cycle.

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References: [1] Neidlinger-Wilke, C$^+$ JOR 2012; [2] O'Connell, GD$^+$ ORS, 2012; [3] Smith, LJ$^+$ ORS, 2011; [4] O'Connell, GD$^+$ JKS 2012; [5] Ng, KW$^+$ Ann Biomed Eng, 2011.

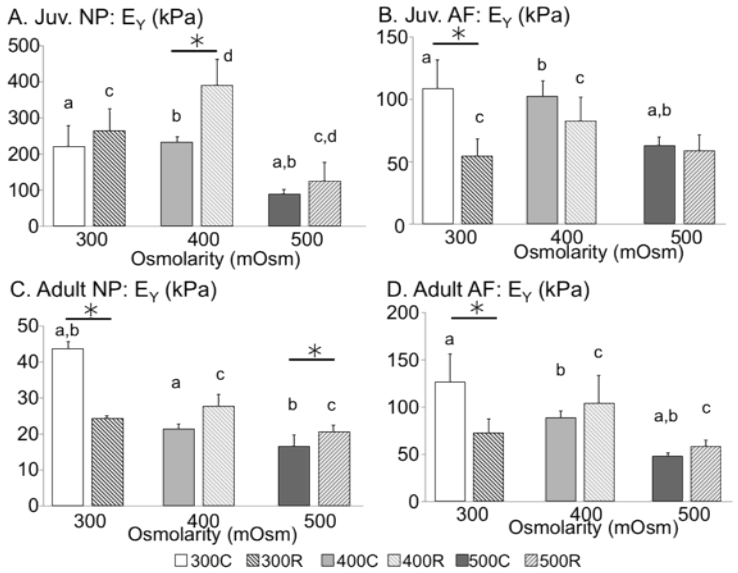

Fig 1. Compressive Young's modulus for constructs seeded with (A & C) NP and (B & D) AF cells. Cells were obtained from juvenile (A-B) and adult (C-D) cows. Groups with the same letter represent differences between osmotic conditions. * represents significant differences with TGFβ3 supplementation.

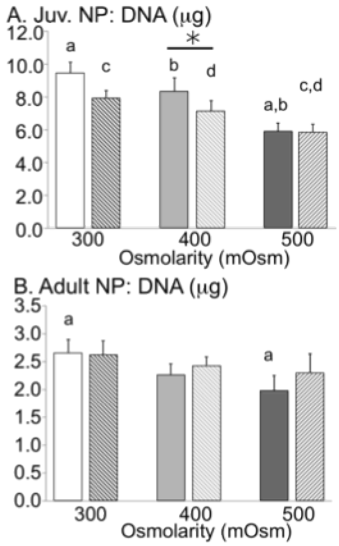

Fig 2. DNA content for (A) juvenile and (B) adult NP constructs. Groups with the same letter represent differences with osmotic conditions. * represents significant differences with TGFβ3. See Fig 1 for legend.

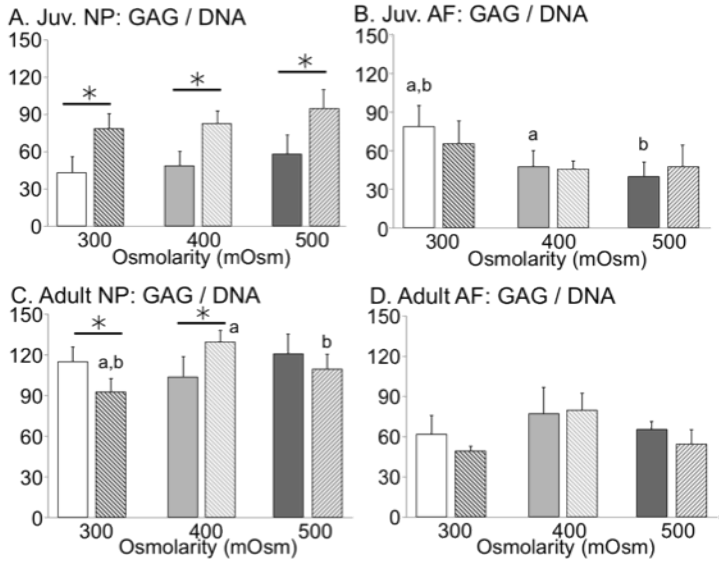

Fig 3. GAG content normalized by DNA for constructs seeded with (A & C) NP and (B & D) AF cells. Cells were obtained from juvenile (A-B) and adult (C-D) cows. Groups with the same letter represent differences with osmotic conditions. * represents significant differences with TGFβ3. See Fig 1 for legend.