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Multi-scale Structural and Tensile Mechanical Response of Annulus Fibrosus to Osmotic Loading

Woojin M. Han^{1,2}, Nandan L. Nerurkar¹, Lachlan J. Smith¹, Nathan T. Jacobs^{1,3}, Robert L. Mauck^{1,2}, and Dawn M. Elliott^{1,2,4}

¹Department of Orthopaedic Surgery, University of Pennsylvania, Philadelphia, PA 19104, USA

²Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104, USA

³Department of Mechanical Engineering and Applied Mechanics, University of Pennsylvania, Philadelphia, PA 19104, USA

⁴Biomedical Engineering, University of Delaware, Newark, DE 19716, USA

Abstract

This study investigates differential multi-scale structure and function relationships of the outer and inner annulus fibrosus (AF) to osmotic swelling in different buffer solutions by quantifying tensile mechanics, GAG content, water content and tissue swelling, and collagen fibril ultrastructure. In the outer AF, the tensile modulus decreased by over 70% with 0.15M PBS treatment but was unchanged with 2M PBS treatment. Moreover, the modulus loss following 0.15M PBS treatment was reversed when followed by 2M PBS treatment, potentially from increased interfibrillar and interlamellar shearing associated with fibril swelling. In contrast, the inner AF tensile modulus was unchanged by 0.15M PBS treatment and increased following 2M treatment. Transmission electron microscopy revealed that the mean collagen fibril diameters of the untreated outer and inner AF were 87.8 ± 27.9 and 71.0 ± 26.9 nm, respectively. In the outer AF, collagen fibril swelling was observed with both 0.15M and 2M PBS treatments, but inherently low GAG content remained unchanged. In the inner AF, 2M PBS treatment caused fibril swelling and GAG loss, suggesting that GAG plays a role in maintaining the structure of collagen fibrils leading to modulation of the native tissue mechanical properties. These results demonstrate important regional variations in structure and composition, and their influence on the heterogeneous mechanics of the AF. Moreover, because the composition and structure is altered as a consequence of progressive disc degeneration, quantification of these interactions is critical for study of the AF pathogenesis of degeneration and tissue engineering.

Keywords

tensile properties; collagen fibril diameter; extrafibrillar matrix; annulus fibrosus; ultrastructure; glycosaminoglycan

INTRODUCTION

The annulus fibrosus (AF) is a multi-lamellar fibrocartilagenous ring in the intervertebral disc. The AF composition and structure are critical to its physiological role, which is mechanical. The AF, comprised primarily of collagens and proteoglycans, possesses a

highly ordered structure: concentric lamellae are composed of collagen bundles arranged at alternating angles of approximately $\pm 30^\circ$ to the transverse axis, to form an angle-ply architecture.¹³ The collagen network of the AF consists mainly of types I and II. The relative ratio of type I to type II collagen gradually decreases from the outer to inner AF; the outer AF contains mostly type I collagen¹⁶ and innermost AF contains only type II collagen.¹⁷ Aggrecan, a high molecular weight aggregating proteoglycan, and hyaluronan, a non-sulfated glycosaminoglycan (GAG), contents gradually increase from the outer to inner AF.^{3,25,27,37,44} Furthermore, some members of small leucine rich proteoglycan such as decorin is found at a higher concentration in the outer AF compared to the inner AF.^{19,49} This variation biochemical content within the AF is in large part responsible for the heterogeneous AF mechanical properties.^{1,17,39} As disc degeneration progresses, many biochemical and structural changes occur, such as loss of proteoglycan, an increase in the collagen type I to II ratio, and eventually both structural and functional degradation.^{3,37,43,49} While AF degenerative changes are well-documented, how these manifest in the functional decline of AF mechanics is not fully understood. Therefore, it is crucial to investigate the relationships between structure, composition, and mechanical function.

In load-bearing soft tissues, it is generally accepted that collagen resists tension and proteoglycan resists compression, however the relationships between structure, composition, and tensile mechanical function remain poorly understood. In tissues with high GAG content (>25% dry weight) such as articular cartilage, enzymatic removal of GAG increases tensile modulus.^{4,5,45} Similar findings have been reported in various engineered cartilage, fibrocartilage, and the inner AF.^{32–34} In contrast, in tissues with relatively low GAG content such as tendon and ligament (<1% dry weight), depletion of GAG has little or no effect on tensile modulus.^{29,46,54} Because GAG content increases radially from the outer AF inward,^{3,25,37} the relationship between AF mechanics, GAG content, and potential GAG loss by diffusion following prolonged periods in PBS remains unknown.

In vitro tissue-level studies require mechanical testing in aqueous buffers such as PBS to prevent tissue dehydration. It is therefore important to examine how PBS buffer affects tissue function. Moreover, the varying GAG content, and associated fixed charge density, from outer to inner AF suggest that the response to high and low PBS osmolarity may also be different with AF radial position. Previous studies in tendon and ligament have been conflicting: soaking tendon fascicles in PBS decreased tensile modulus,^{46,47} testing patellar tendon in PBS bath increased strength and modulus,²² and treating ligament in buffer had no effect on modulus.²⁹ The influence of aqueous *in vitro* testing environment is further confounded by the contrasting findings on the relationship of collagen fibril tensile modulus and environmental salt concentration.^{20,51} Soaking ligament in buffer solutions, as a control for enzymatic digestion, alters the mechanics and the tissue water content.^{14,24,52}

Collagen ultrastructure, specifically fibril diameter, has been suggested as a crucial determinant of tissue tensile modulus.^{2,12,40–42,48} In addition, collagen cross-links, fibril length, and concentration within matrix have been implied to have significant roles in force transmission in ligament, tendon, and engineered extracellular matrix.^{8,11,40–42} Differences in collagen fibril ultrastructure may exist across AF regions, and it is possible that tissue treatments prior to and during mechanical testing can alter this ultrastructure, affecting tissue mechanics in a region-specific manner.^{20,24,46} Therefore, tissue ultrastructure should be considered in the investigation of the composition, structure, and function relationships.

The objective of this study was to investigate multi-scale structure and function relationships of the outer and inner AF in response to osmotic loading in isotonic (0.15M) and hypertonic (2M) buffer solutions by quantifying tensile mechanics, GAG content, water content, tissue swelling, and collagen fibril ultrastructure. Because the mechanical properties, biochemical

composition, and structure vary noticeably between the inner and outer AF,^{3,16,17,25,37} it was hypothesized that the functional contributions of treatment would differ between the inner and outer AF. A secondary objective was to address the implication for the effect of the PBS baths when interpreting mechanical studies of many fibrous soft tissues. The multi-scale AF structure and function relationships quantified here are necessary to understand pathogenesis of degeneration and develop engineered and biological AF treatments.

MATERIALS AND METHODS

Sample Preparation and Treatment

Intervertebral discs were isolated from adult bovine caudal spines. Bovine caudal discs exhibit similar structure and mechanics to human discs, thus making them a reliable and accessible model for intervertebral disc research.^{9,26,35} Inner and outer AF tissue was excised from the harvested discs using a scalpel. Each sample was sectioned to 1.5 mm thickness on a freezing-stage microtome at -20°C . Samples were further cut to dimensions of $14 \times 3 \times 1.5$ mm (circumferential length $\times$ axial width $\times$ radial thickness) while frozen. Untreated control samples were wrapped in gauze and stored immediately at -20°C until mechanical testing.

Samples subject to treatments were introduced to both isotonic (0.15M) and hypertonic (2M) PBS (pH 7.2) to investigate differential multi-scale structure and function relationships in response to osmotic loading by quantifying tensile mechanics, GAG content, water content, tissue swelling, and collagen fibril ultrastructure. Treating samples in a solution with high salt concentration (2M PBS) creates an osmotic gradient that decreases tissue water content and prevent swelling. Osmolarity of PBS was determined based on NaCl concentration (2M PBS: 2M NaCl, 118.26 mM Na_2HPO_4 , 39.42 mM KCl, 21.9 mM KH_2PO_4). For both outer and inner AF, samples were classified into 6 groups: samples treated in 1) 0.15M PBS for 6-hours (6h-0.15M), 2) 2M PBS for 6-hours (6h-2M), 3) 0.15M PBS for 12-hours (12h-0.15M), 4) 2M PBS for 12-hours (12h-2M), 5) 0.15M PBS for the first 6-hours, then subsequently treated in 2M PBS for additional 6-hours (0.15 $\rightarrow$ 2M), 6) and untreated control samples (untreated). All treatments were performed at 37°C under gentle agitation. After treatment, sample was gently blotted and wet weight was measured using an analytical balance. Cross-sectional area was measured with a custom non-contact laser device that recorded five width and thickness measurements to determine the mean cross-sectional area.^{29,34,38} For samples treated for 12 hours, cross-sectional area and wet weight were measured at 0, 6, and 12 hour time points to determine change in these parameters on a matched-sample basis. After these measurements the sample was wrapped in gauze and stored at -20°C for no longer than 3 days until mechanical testing.

Uniaxial Tensile Testing

6 samples per group and region were mechanically tested. Prior to mechanical testing, sample was thawed at room temperature for 30 minutes. Each sample was lightly speckle-coated with black enamel paint using an airbrush for optical surface strain analysis.^{15,34} Uniaxial tensile testing was performed using an Instron 5542 (Instron, Norwood, MA). Samples in groups 1 and 3 were tested in a 0.15M PBS, and samples in groups 2, 4 and 5 were tested in a 2M PBS bath. Samples were placed into custom-made serrated grips and loaded into the Instron. A 5 min preload of 0.1N was applied. Samples were then preconditioned for 15 cycles to 0.1% strain at a rate of 0.5%/sec to minimize grip effects. Samples were tested to failure at a strain rate of 0.1%/sec. Digital images of the sample mid-substance were captured every 5 sec (1 image per 0.5% strain increment).

Stress was calculated as load divided by the initial cross-sectional area. Lagrangian strain was calculated from acquired images using Vic-2D texture correlation software (Correlated Solutions Inc., Columbia, SC). Linear modulus was calculated using a custom-written MATLAB (The Mathworks Inc., Natick, MA) program that performed a regression to the linear region of stress-strain curve.

Glycosaminoglycan Content

After mechanical testing, each sample was dried overnight in a 60°C oven and dry weight was measured (n=6 per group). Percent water content was determined by subtracting sample dry weight from wet weight and dividing by wet weight. The dried sample was then digested with papain solution at 60°C overnight. Total GAG content was determined via the 1,9-dimethylmethylene blue dye binding (DMMB) assay and normalized by dry weight.^{28,34} Dry weights from 0, 6, and 12 hour time points were compared on a sample-by-sample basis to determine potential salt migrating into the tissue.

Electron Microscopy

The cross-sectional plane of the collagen fibrils in both inner and outer AF were imaged via transmission electron microscopy (TEM) to determine the fibril diameter distribution and percent extrafibrillar space of the untreated (n=1) and 6 hr treated groups (0.15M and 2M; n=1 each). Two adjacent caudal discs were dissected from an initially frozen bovine tail. Samples were prepared under a dissecting microscope to aid visualization of fiber alignment, and then trimmed to rectangular blocks (4 × 3 mm) with the long axis parallel to one of the fiber directions using a scalpel. Samples did not undergo any freeze/thaw cycles upon dissection.

Samples were fixed overnight at 4°C in 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.4), then washed several times in buffer and post-fixed for 1 hour in 2% osmium tetroxide. After brief washes with deionized water, samples were stained *en bloc* in 2% aqueous uranyl acetate for 30 minutes, rinsed again in deionized water prior to dehydration in a graded ethanol series, and finally infiltrated and embedded in EMBED-812 (Electron Microscopy Sciences, Hatfield, PA). Sections were stained with uranyl acetate and lead citrate prior to examination in a JEOL 1010 electron microscope (JEOL Ltd., Peabody, MA) fitted with a Hamamatsu digital camera (Hamamatsu Corp., Japan) and AMT advantage image capture software (Advanced Microscopy Techniques Corp., Woburn, MA).

Ten images at 60,000X magnification were chosen randomly from different locations within the sample. ImageJ (NIH, Bethesda, MD) was used to measure collagen fibril diameters (n=300). Fibril diameter distribution was binned in 20 nm intervals and normalized to the total number of measured fibrils. Ten images at 20,000X magnification were used to determine percent extrafibrillar space (n=10), calculated using a custom-written MATLAB program that converted each gray scale TEM image to a binary image and calculated the ratio of the white pixel area (no fibrils) to the total pixel area.

Statistical Analysis

For all statistics, significance was set at the 95 percent confidence level ($p < 0.05$). For GAG content and tensile modulus, a one-way ANOVA with Tukey's post-hoc analysis was used to test for significant difference between groups (factor: treatment, levels: untreated, 0.15M, 2M for the 6-hour groups, levels: untreated, 0.15M, 0.15→2M, 2M for the 12-hour groups). Statistics were performed separately for the outer and inner AF. The untreated inner and outer AF groups were compared with two-tailed Student's t-test. For the change in cross-sectional area and water content, a one-factor repeated measure ANOVA (factor: time,

levels: 0, 6, 12 hours) with Tukey's post-hoc was applied to test for significant difference within each treatment group.

To compare fibril diameter distributions between groups in each of the outer and inner AF, statistical analyses were performed as previously described.¹⁸ For each fibril diameter distribution, sample median, median-to-first quartile distance, and median-to-third quartile distance were calculated (n=10). These values assess the center, the spread, and the degree of skewness of the distributions.¹⁸ Distributions of the sample medians and median-to-quartile distances were compared to the corresponding untreated group via the Kolmogorov-Smirnov test. For percent extrafibrillar space, a one-way ANOVA with Tukey's post-hoc analysis was performed. The untreated inner and outer AF groups were compared with two-tailed Student's t-test.

RESULTS

Mechanical Properties

Linear modulus was significantly greater for the outer AF than the inner (17.22 ± 7.69 and 2.65 ± 1.03 MPa respectively, $p < 0.05$, Fig. 1). In the outer AF, treatment in 0.15M PBS for both 6 and 12 hours decreased the tensile modulus by 89 and 74%, respectively, compared to untreated ($p < 0.05$, Fig. 1). However, 2M PBS treatment for both 6 and 12 hours maintained the modulus at the untreated levels (Fig. 1). The reduction in modulus observed with 0.15M PBS treatment was reversed if followed by 2M PBS (0.15→2M), where no significant difference was observed relative to either untreated or 2M PBS groups (Fig. 1).

In contrast to the outer AF, tensile modulus of the inner AF samples treated in 0.15M PBS for 6 and 12 hours remained unchanged compared to untreated (Fig. 1). However, 2M PBS treatment for 6 and 12 hours increased the modulus 250 and 130%, respectively, compared to untreated ($p < 0.05$, Fig. 1).

GAG Content, Cross-Sectional Area and Water Content

The mean dry weights of untreated and 0.15M, 2M PBS treated groups were not statistically different, ensuring insignificant effect of salt solution confounding water and GAG content measurements (data not shown). The GAG content in the untreated outer AF was $6.65 \pm 3.48\%$ dry weight and the inner AF was $19.75 \pm 4.85\%$ dry weight ($p < 0.05$, Fig. 2), similar to previously reported findings.²⁵ In the outer AF, no significant change in GAG content was observed following any PBS treatment (Fig. 2). In the inner AF, treatment with 2M PBS resulted in a 43–60% loss of GAG compared to the untreated group ($p < 0.05$, Fig. 2). No significant changes were observed following 0.15M and 0.15→2M PBS treatments.

In the outer AF, treatment in 0.15M PBS for 6 and 12 hours resulted in a significant 40% increase in cross-sectional area from the 0-hour initial state (Fig. 3, $p < 0.05$). After 12 hours in 2M PBS, a significant decrease in cross-sectional area and water was observed relative to the 6-hour state (Fig. 3 and 4). Inner AF water content decreased after 6 and 12 hours of treatment in 0.15M PBS from the initial state ($p < 0.05$, Fig. 4), however area was unchanged. After 6 and 12 hours of treatment in 2M PBS, a significant decrease in cross-sectional area from the initial state and in water content from the 6-hour state was observed (Fig. 3 and 4). The changes observed with 0.15M PBS treatment were generally reversed if followed by 2M PBS (0.15→2M), where the final values were consistent with 2M PBS groups (Fig. 3 and 4).

Electron Microscopy

Electron microscopy was performed on the 6 hr treatment groups. The mean collagen fibril diameter of the untreated outer AF (87.8 ± 27.9 nm, Fig. 5A, Table 1) was larger than the collagen fibril diameter of the inner AF (71.0 ± 26.9 nm, Fig. 5D, Table 1). The median of the outer AF fibril diameter was significantly greater than the fibril diameter of the inner AF, whereas both median-to-first and median-to-third quartile distances were similar ($p > 0.05$), suggesting that the outer AF has comparable fibril distribution than the inner AF, but larger fibril diameter (Table 1).

High magnification images of the outer AF revealed apparent swollen fibrils in the 0.15M and 2M PBS samples compared to the untreated (Fig. 6A–C). The inner AF showed no evident morphological differences between the untreated and 0.15M PBS samples (Fig. 6D–E). However, the fibril organization of the 2M PBS sample appeared denser than the other groups (Fig. 6F). Similar observations were made in low magnification images of the outer and inner AF (Fig. 7A–F). In both outer and inner AF, percent extrafibrillar space of the 2M sample was significantly reduced from the untreated (Fig. 8). Percent extrafibrillar spaces of the 0.15M PBS group was unaffected compared to untreated.

For the outer AF, the distributions of both treatment groups (0.15M and 2M) had larger fibril diameters, as indicated by significant increases in medians, compared to the untreated (Fig. 5A–C, Table 1). The mean fibril diameters of the samples treated in 0.15M and 2M PBS increased by 10.1 and 35.5 nm, respectively (Table 1). The median-to-first quartile distances of the 2M PBS group significantly increased from the untreated, and the 0.15M group had significantly decreased median-to-third quartile distance compared to the untreated, collectively supporting the overall shift in distribution toward larger fibril diameter with both treatments (Table 1).

For the inner AF, the collagen fibril distribution of 0.15M was unchanged compared to the untreated (Fig. 5E, Table 1). On the other hand, the mean fibril diameter increased by 34.5 nm for 2M PBS group from the untreated (Fig. 5F, Table 1). Significantly increased median values of the 2M PBS group support increased fibril diameter with treatments (Table 1). The distribution was generally unaffected by treatments (Table 1).

DISCUSSION

This study demonstrated different multi-scale structure and function response of the outer and inner AF to osmotic treatments in isotonic (0.15M) and hypertonic (2M) buffer solutions, including the tensile modulus, GAG content, water content and tissue swelling, and collagen fibril ultrastructure. For the first time, to the best of authors' knowledge, collagen fibril diameters of inner and outer AF were measured. The mechanical function results in the study can be interpreted as due to the effects of three measured factors: GAG content, tissue swelling, and collagen ultrastructure, as discussed below for the inner and outer AF.

The Inner AF

Role of GAG and Tissue Swelling—The inner AF experienced both an increased modulus and GAG loss with 2M PBS treatment (Fig. 1 and 2), which is consistent with the observed effect of GAG loss due to chondroitinase ABC treatment in articular cartilage,^{4,5,45} GAG-rich engineered constructs,^{32–34} and inner AF.³⁴ High native GAG content (~25% dry weight) is a similarity across these tissues. It is theorized that the inner AF becomes stiffer with GAG depletion because the rotation and recruitment of collagen fibers in the direction of loading becomes less constricted.^{6,34}

The inner AF tissue did not swell during treatments, rather 0.15M PBS treatment resulted in a lower water content and 12-hour 2M PBS treatment resulted in both lower cross-sectional area and water content (Fig. 3 and 4). One possible explanation for the absence of swelling with 0.15M PBS treatment is that the inner AF is highly hydrated *in situ*, as it lies in direct apposition to the nucleus pulposus, which has a very high GAG and water content with osmolality of approximately 0.45 to 0.55 Osm.⁵³ The tissue expelled water during 2M PBS treatment to maintain osmotic equilibrium *ex vivo*.

Ultrastructure—Fibril diameter and extrafibrillar space were consistent with observations of the modulus and cross-sectional area. 2M PBS treatment increased the mean fibril diameter by 50% and reduced extrafibrillar space by 50% (Fig. 8, Table 1). As the tissue compacts in 2M hypertonic solution, it is likely that some water leaves the tissue (water content decreased) while some water enters the fibrils (diameter increased and extrafibrillar space decreased). As 6-hour 2M PBS treatment also lost GAG, it is possible that GAG is an extrafibrillar matrix molecule that contributes to preventing collagen fibril swelling. For instance, it has been shown that decorin-deficiency during tendon development causes distorted collagen fibrils with increase fibril diameter.⁵⁶ In addition a structural investigation on SLRP revealed that collagen II and aggrecan are joined to collagen VI via SLRP, providing interconnected mesh-like networks within the ECM.⁵⁵

The Outer AF

Role of GAG and Tissue Swelling—The outer AF exhibited significantly different mechanical responses to treatments than the inner AF. In the outer AF, modulus decreased 80–90% with 0.15M PBS compared to the untreated (Fig. 1). Treatment in 2M PBS did not affect tensile modulus (Fig. 1). Past studies on tendon soaked in PBS recorded similar observations, where the tensile modulus of tendon fascicles in 0.15M PBS decreased significantly.^{46,47} Electron microscopy suggested that swelling increased the distance between collagen fibrils, potentially affecting the hypothesized mechanical load transfer interactions between collagen and GAG.^{46,47} A similar phenomenon was observed in the outer AF in shear, where PBS treatment decreased the shear modulus.²⁸ Because the GAG content is relatively low in the outer AF (~7% DW) and no significant change in GAG content was observed, the role of GAG in the outer AF is difficult to interpret.

Maintenance of the outer AF modulus with 2M PBS treatment suggests that 2M PBS reduces or prevents tissue swelling, which is supported by decreased cross-sectional area and water content (Fig. 3 and 4). Analogously, samples soaked in 0.15M PBS increased cross-sectional area from the original state, suggesting bulk tissue swelling (Fig. 3). Samples that have been swollen and subsequently un-swollen (0.15→2M) also were able to recover their tensile modulus, suggesting that the dramatic loss in tensile modulus that occurs with PBS is in fact reversible when the sample is subjected to hypertonic conditions. Osmolarity of buffer solution influences the water content of the tissue, which subsequently has an effect on mechanical properties.^{20,24,52} For instance, increased dilatational stress generated from tissue hydration is positively related to creep, stress and cyclic relaxation responses, giving dry tissues stiffer properties compared to hydrated tissues.^{7,14,23,24,30,31,36,52}

Ultrastructure—Both osmolality treatments resulted in greater mean fibril diameter relative to the untreated for the outer AF. Unlike the shift in fibril diameter distribution with swelling in 2M PBS treated inner AF (Fig. 5F), the distributions of treated outer AF groups did not shift to the right in a uniform manner. It is postulated that smaller fibrils in the outer AF swelled more extensively compared to the larger fibrils. The samples following 0.15M PBS treatment resulted in a reduction in the modulus, suggesting that larger fibril diameter is related to lower tensile modulus. A previous finding that compared the ultrastructure of

individual collagen fibrils with mechanical properties of the nucleus pulposus demonstrated that the compressive modulus linearly increases with the mean fibril diameter.² Similarly, a three-dimensional matrix prepared from type I collagen with smaller fibril diameters exhibited increased tensile linear modulus and failure stress compared to the matrix made up of fibrils with larger diameter, further highlighting the importance of fibril diameter on tissue mechanical function.⁴¹ Multi-scale models of collagen fibrils have suggested that tensile stiffness of collagen fibrils is inversely related to the number of tropocollagen molecules in a fibril's cross-section, supporting that tensile stiffness decreases as fibril diameter increases.¹² The mechanical behavior of collagen fibrils is dependent on collagen fibril geometry (diameter) as well as structural features (spatial variation) within the fibrils, which may exhibit different fibril crimping characteristics and shear behavior.⁴⁸

Despite overall increased fibril diameters in the 2M PBS group compared to the untreated (Fig. 5C), the modulus was maintained compared to the untreated (Fig. 1). Possibly, more compact fibril organization induced with hypertonic solution, along with decreased water content, may have prevented additional reduction of the modulus from increased interfibrillar and interlamellar shearing (Fig. 8).^{21,28}

Additional Factors and Limitations—A limitation in the current study was that only one tissue sample per group underwent TEM imaging, although 10 individual regions of the sample were analyzed and 300 fibril diameters were measured. In addition, the TEM samples were prepared from fresh-frozen tissue. While this may alter the ultrastructure, no effects of freezing were apparent. Moreover, the data analysis was comparative with respect to osmotic treatment and AF region, so any effect of freezing should carry across all analyses.

Collagen cross-links may also be involved in tissue swelling and tensile properties, although they were not quantified in this study. Single fibrils from an adult human patellar tendon with a high content of mature cross-links had no relationship between tensile properties and environment salts,⁵⁰ while a fibril from a reconstituted bovine Achilles tendon with little or no mature cross-links had a twofold increase in tensile modulus with increased salt concentration in a phosphate buffer.²⁰ The importance of mature cross-links for mechanical stability also has been established in rat aorta, where a 49% decrease in pyridinoline resulted in a significantly reduced maximum load and stiffness.¹⁰

The outer and inner AF are clearly distinguished by their collagen organization as well by collagen type. The AF collagen network becomes more organized from the nucleus pulposus radially outward and the ratio of type I to type II increases.^{16,17} The collagen fibrils of the inner AF (20–140nm) were smaller than the collagen fibrils of the outer AF (20–160nm; Fig. 5). It is possible that varying fibril diameters in the outer and inner AF arise from different types of collagen. It is likely that different types of collagen with varying geometry have distinct mechanical roles.^{2,12,48}

Conclusion—The inner and outer AF mechanical behaviors are distinct and their responses to osmotic loading in isotonic and hypertonic buffers vary significantly due to regional variations in disc ultrastructure and composition. The present work demonstrates inhomogeneous multi-scale relationships between AF composition, structure and tensile mechanical function, and how aqueous environment can significantly alter the tissue properties. These observations show the importance of considering the effect of PBS baths when interpreting fibrous soft tissue mechanical studies. Moreover, because the composition and structure of the AF is altered as a consequence of progressive disc degeneration, quantification of these interactions are critical for study of pathogenesis of degeneration and development of engineered and biological treatments for the AF.

Acknowledgments

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Figure 1.

Linear-region tensile modulus (MPa) of the *untreated* and treatment groups of both outer and inner AF. Statistical analysis performed on groups within each time point plus the respective *untreated* group. *0.15* → *2M* = 6h 0.15M PBS + 6h 2M PBS. * = $p < 0.05$ compared to *untreated*. ★ = $p < 0.05$ compared to *0.15M*. # = $p < 0.05$ compared to the inner AF *untreated*.

Figure 2.

GAG content (% DW) of both outer and inner AF. Statistical analysis performed on groups within each time point plus the respective *untreated* group. DW = dry weight. *0.15→2M* = 6h 0.15M PBS + 6h 2M PBS. * = $p < 0.05$ compared to *untreated*. # = $p < 0.05$ compared to the inner AF *untreated*.

Figure 3.

Percent change in cross-sectional area normalized to 0-hour time point. $0.15 \rightarrow 2M = 6h$ $0.15M$ PBS + $6h$ $2M$ PBS. * = $p < 0.05$ compared to respective 0-hour measurements. # = $p < 0.05$ compared to respective 6-hour measurements.

Figure 4.

Percent change in water content normalized to 0-hour time point. $0.15 \rightarrow 2M$ = 6h 0.15M PBS + 6h 2M PBS. * = $p < 0.05$ compared to respective 0-hour measurements. # = $p < 0.05$ compared to respective 6-hour measurements.

Figure 5. Distribution of collagen fibril diameter (nm) in the AF. **A:** Outer AF *untreated*, **B:** Outer AF *0.15M*, **C:** Outer AF *2M*, **D:** Inner AF *untreated*, **E:** Inner AF *0.15M*, **F:** Inner *2M* AF. Black lines represent means of respective *untreated* groups.

Figure 6.

Representative TEM images of collagen fibrils in the outer and inner AF taken at 60000X magnification. **A:** Outer AF *untreated*, **B:** Outer AF *0.15M*, **C:** Outer AF *2M*, **D:** Inner AF *untreated*, **E:** Inner AF *0.15M*, **F:** Inner AF *2M*. Bars = 500 nm.

Figure 7. Representative TEM images of collagen fibrils in the outer and inner AF taken at 20000X magnification. **A:** Outer AF *untreated*, **B:** Outer AF *0.15M*, **C:** Outer AF *2M*, **D:** Inner AF *untreated*, **E:** Inner AF *0.15M*, **F:** Inner AF *2M*. Bars = 500 nm.

Figure 8. Percent extrafibrillar space of the *untreated*, *0.15M*, and *2M* outer/inner AF groups. * = $p < 0.05$ compared to *untreated*.

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Table 1

Summary of mean fibril diameter, median, median-to-first quartile distance (Median-Q1), and median-to-third quartile distance (Median-Q3) of collagen fibril diameter distributions of different groups (nm).

	Mean Diameter		Median		Median-Q1		Median-Q3	
	Mean ± SD		Mean ± SD		Mean ± SD	p	Mean ± SD	p
Outer AF								
Untreated [#]	87.8 ± 27.9		85.1 ± 7.0	0.03	17.7 ± 4.9	n.s	25.2 ± 7.0	n.s
6h 0.15M	97.9 ± 25.8		100.2 ± 6.8	0.001	19.4 ± 7.2	n.s	16.9 ± 5.0	0.03
6h 2M	123.3 ± 39.6		130.3 ± 14.1	<0.001	33.6 ± 17.0	0.007	24.1 ± 10.3	n.s
Inner AF								
Untreated	71.0 ± 26.9		71.2 ± 8.4	-	20.5 ± 7.1	-	18.9 ± 6.1	-
6h 0.15M	80.6 ± 27.2		82.2 ± 9.1	n.s	22.7 ± 7.2	n.s	20.0 ± 7.4	n.s
6h 2M	105.5 ± 26.0		104.5 ± 7.1	<0.001	17.0 ± 6.5	n.s	20.3 ± 9.6	n.s

p values are obtained from two-sample Kolmogorov-Smirnov tests against the corresponding native values.

[#] = compared to the *untreated* inner AF.