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Effect of orientation and targeted extracellular matrix degradation on annulus fibrosus shear mechanical properties

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Abstract

The intervertebral disc experiences combinations of compression, torsion, and bending that subject the disc substructures, particularly the annulus fibrosus (AF), to multidirectional loads and deformations. Combined tensile and shear loading is a particularly important loading paradigm, as compressive loads place the AF in circumferential hoop tension, and spine torsion or bending induces AF shear. Yet the anisotropy of AF mechanical properties in shear, as well as important structure-function mechanisms governing this response, are not well understood. The objective of this study, therefore, was to investigate the effects of tissue orientation and enzymatic degradation of glycosaminoglycan (GAG) and elastin on AF shear mechanical properties. Significant anisotropy was found: the circumferential shear modulus, $G_{\theta z}$, was an order of magnitude greater than the radial shear modulus, $G_{r\theta}$. In the circumferential direction, prestrain significantly increased the shear modulus, suggesting an important role for collagen fiber stretch in shear properties for this orientation. While not significant and highly variable, ChABC treatment increased the circumferential shear modulus compared to PBS control ($p=0.15$). Together with the established literature for tensile loading of fiber-reinforced GAG-rich tissues, the trends for changes in shear modulus with ChABC treatment reflect complex, structure-function relationships between GAG and collagen that potentially occur over several hierarchical scales. Elastase treatment caused no difference in shear modulus with respect to PBS control for either radial or circumferential orientation. Elastase digestion did not significantly affect shear modulus; further contributing to the notion that circumferential shear modulus is dominated by collagen fiber stretch. The results of this study highlight the complexity of the structure-function relationships that govern the mechanical response of the AF in radial and circumferential shear, and provide new and more accurate data for the validation of material models and tissue-engineered disc replacements

Keywords

biomechanics; glycosaminoglycan; elastin; disc degeneration; intervertebral disc; anisotropy

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1. INTRODUCTION

The function of the intervertebral disc is primarily mechanical, providing spinal stability while simultaneously permitting motion and energy dissipation. The disc experiences combinations of compression, torsion, and bending that subject its substructures, particularly the annulus fibrosus (AF), to multidirectional loads and deformations. The AF is a highly organized fibrocartilagenous ring of concentric lamellae reinforced by collagen fibers oriented at oblique angles to the disc's circumferential plane and whose orientation alternates with each consecutive lamella. AF composition and structure renders it capable of withstanding the loading environment experienced in vivo. Combined AF tensile and shear loading is a particularly important loading paradigm, as compressive disc loads place the AF in circumferential hoop tension, and torsion or bending induces AF shear. The anisotropy of AF mechanical properties in shear, however, as well as important structure-function relationships governing this response, are not well understood.

Torsion testing of intact vertebra-disc-vertebra segments, in combination with simple material models [1,2], suggests that the AF shear modulus is 8-20 MPa [3,4]. Fiber-reinforced constitutive models similarly predict the AF shear modulus to be on the order of 10 MPa and also indicate significant shear anisotropy, with the circumferential shear modulus (in the plane of the collagen fibers, $G_{\theta z}$, Figure 1) predicted to be 100 times greater than the radial modulus (transverse to the lamella, $G_{r\theta}$, Figure 1) [5]. In contrast, tissue-level shear studies using cubic and cylindrical samples have reported lower modulus and less anisotropy, with a circumferential shear modulus of 0.06 MPa [6,7] and a radial modulus 0.03 MPa [6], only a 2 fold difference. Shear tests of planar AF strips with a 10% prestrain have a shear modulus of 0.4 MPa [6], higher than cubic and cylindrical samples, but still lower than the expected ~10 MPa. The higher planar AF shear modulus may be due to greater engagement of collagen fibers, which was not enforced by the boundary conditions applied in cubic and cylindrical tests. Radial shear modulus has not been measured using this same configuration, and structure-function relationships have not been investigated.

Elucidation of structure-function relationships at the tissue level is important to understand the function of normal tissue, the impact of the degenerative process, and for the design of engineered constructs. Structure-function relationships in the AF are dependent not only on collagen fibers, but also on other matrix constituents, such as proteoglycans and elastin [8,9]. The glycosaminoglycan (GAG) side chains associated with the proteoglycans carry a fixed negative charge and are considered to contribute to the compressive properties of musculoskeletal tissues by generating an osmotic swelling pressure [8,9]. The nature of the contribution of GAG to the tensile and shear properties of fiber-reinforced tissues, however, is unclear. A tensile load-transfer mechanism has been proposed in which forces between adjacent collagen fibrils are transferred via interactions of GAG attached to the collagen fibrils [10-12]; however, multiple studies in both tendon and ligament have failed to demonstrate a significant mechanical contribution by GAG in tension [13-17] or shear [17]. GAG constitutes just 1% of dry weight for tendon and ligament, while in the AF it constitutes up to 20%, indicating it may play a more significant tensile role in this tissue, similar to other GAG-rich tissues such as cartilage [18,19]. Indeed, enzymatic digestion studies of both native AF tissue and engineered AF constructs suggest GAGs play a role in the AF tensile mechanics, but that it may be more pronounced for the inner AF than the outer AF [20,21]. To date, the contribution of GAG to AF shear mechanical properties has not been investigated.

Elastic fibers are found in abundance across multiple tissues that undergo large deformations. The extensibility and recoverability of heart and lung tissues, for example, are

dependent on elastic fibers, and their enzymatic removal results in impaired mechanical properties [22-25]. Elastic fibers are found throughout the AF [26-29]. Elastic fiber density is highest in regions of the AF that experience large tensile deformations, such as the outer and posterolateral AF, and between concentric layers of lamellae, running perpendicular to the lamellar plane [30]. Although the quantity of elastin within the AF is small in comparison to other tissues (such as heart and lung), the highly organized architecture of the elastic fiber network is suggestive of an important functional role [26]. Indeed, elimination of elastic fibers via elastase digestion decreased the tensile modulus of human AF in the radial direction [31]. Additionally, elastase treatment of radially oriented samples has been observed to increase intralamellar shear strain, but in circumferentially oriented samples there was no apparent effect of elastase treatment on shear strain [32]. The potential roles of elastic fibers in contributing to the load response of the AF in shear are unknown.

The objective of this study was to investigate the effects of tissue orientation and enzymatic degradation of GAG and elastin on AF shear mechanical properties. It was hypothesized that the circumferential shear modulus would be an order of magnitude greater than the radial shear modulus, and that increasing tensile prestrain in the circumferential direction would increase the shear modulus. Further, we hypothesized that GAG and elastin degradation would significantly reduce the shear modulus for both orientations.

2. METHODS

The AF was removed from bovine caudal discs via sharp dissection and planar rectangular samples $3 \times 15 \times 1.5$ mm (width $\times$ length $\times$ thickness) were prepared using a freezing stage microtome. Samples were prepared in two orientations: circumferential and radial (Figure 1). Circumferential samples were aligned in the plane of the collagen fibers, with length oriented along the circumferential (θ) axis, width along the axial (z) axis, and thickness along the radial (r) axis. Radial samples had length along the radial axis, width along the circumferential axis, and thickness along the axial axis. For each orientation, samples were assigned to one of 4 groups ($n=6$ per group): untreated, 0.15M phosphate buffered saline (PBS) control, chondroitinase-ABC treatment (ChABC), and elastase treatment (elastase). The PBS control was included to account for effects of the long buffer soak times associated with enzyme treatments and consisted of 1 ml 0.15M (1X) PBS with the following protease inhibitors: 10 mM N-ethylmaleimide (NEM), 5 mM benzamidine hydrochloride (Benz-HCl), 1 mM phenylmethylsulfonyl fluoride (PMSF). Pilot studies indicated that PBS had a similar effect on modulus in uniaxial tension as ChABC buffer (no enzyme), and we therefore chose PBS to be a simple control of both enzyme treatment groups. ChABC treatment consisted of 1 ml of 0.05 M Tris-HCl, 0.06 M sodium acetate buffer (pH 8.0) containing 1U ChABC and the same protease inhibitors as for PBS. Elastase treatment consisted of 1 ml of 0.2 M Tris-HCl buffer (pH 8.6) with 3U elastase, and protease inhibitors (NEM, Benz-HCl, 3 mg soybean trypsin inhibitor). Soybean trypsin inhibitor was included as it has been shown to inhibit degradation of collagen associated with elastase treatment [33]. All reagents were obtained from Sigma Aldrich (St Louis, USA). Treatment followed established protocols [31]: the sample was submersed in the appropriate treatment solution at 37° C for 36 hr under gentle agitation. Following treatment the sample was washed in PBS for 30 min at 4° C then frozen at -20° C until testing. Untreated samples were not placed in solution and were kept frozen until testing.

Prior to testing, each sample was equilibrated for 30 min in PBS at 4° C and cross-sectional area measured with a laser-based system [34]. The sample was speckle coated with black enamel paint using an airbrush to create texture patterns needed for optical surface strain measurements. Mechanical testing was performed using a custom-designed apparatus that enabled the application of combined tensile and shear strains (Figure 2A and 2B) [35]. The

sample was placed in custom serrated grips installed in a PBS bath that was mounted on an x-y translation table (M426A, Newport, Irving, CA). Tensile prestrain was applied using Vernier micrometers (resolution 1.0 μm , Newport, Irving, CA) attached to the translation table and the tensile load was measured using a submersible 9.8 N load cell (model 31 submersible, Honeywell, Minneapolis, MN). The device was integrated with a mechanical testing system (Model 5848, Instron, Norwood, MA) which applied the shear displacement and measured shear force.

Following placement in the testing system, shear testing protocol for the radial orientation included a 15 min unloaded equilibration, followed by 2% tensile prestrain to eliminate slack. Prestrain was chosen over a prestress as very little stress is generated for radially oriented AF samples until a large strain is induced, because these samples have no collagen fiber reinforcements in the radial direction. While preconditioning is used in fiber-reinforced tissues to create a uniform loading history, preconditioning was not used for radial samples. Preliminary studies showed that radial samples undergo irreversible deformation during preconditioning, similar to that observed in transverse tendon tensile testing [36]. Thus, a shear deformation ramp to $+10^\circ$ shear strain at $3^\circ/\text{min}$ was immediately applied following prestrain. During this ramp, force and deformation were recorded for shear modulus calculation.

The shear testing protocol for the circumferential orientation included a 15 min 2% tensile prestrain, followed by 20 shear preconditioning cycles to $\pm 10^\circ$ at 0.05 Hz, followed by a quasi-static $3^\circ/\text{min}$ triangular ramp to $\pm 10^\circ$ shear strain, during which force and deformation were recorded for shear modulus calculation. The entire protocol was repeated at a 10% tensile prestrain. Thus, circumferential samples were tested with the collagen fibers initially in the toe-region (2% prestrain), and with the fibers engaged in the linear region of the stress-strain response (10% prestrain). For both radial and circumferential tests, images were acquired with a digital camera (Model A102f; Basler, Exton, PA) at 5 sec intervals throughout the shear ramp test for calculation of two-dimensional Lagrangian tissue strain.

Shear stress (τ) was calculated during the shear deformation by dividing the shear load (from the Instron's vertical axis) by the sample cross sectional area. Images acquired during the shear ramp were analyzed with VIC 2D (Correlated Solutions, Columbia, SC), using the extensometer feature. Ten tissue sub-regions spanning the central two thirds of each sample were tracked throughout the shear ramp and the x-y location at each 5 sec interval was exported to Matlab (MathWorks, Inc, Natick, MA), for calculation of Lagrangian strain (E) using a custom written program [37,38]. Briefly, the deformation gradient:

$$F = \frac{\partial x}{\partial X},$$

where X represents the undeformed and x the deformed state, was calculated. The Lagrangian finite strain tensor was calculated from the deformation gradient as:

$$E = \frac{1}{2} (F^T F - I).$$

The shear modulus (circumferential: $G_{\theta z}$, radial: $G_{r\theta}$) was calculated from the shear stress-strain response using linear regression. For the circumferential orientation, shear modulus was calculated for both the positive and negative portions of the triangular shear ramp. The effect of tensile prestrain on circumferential modulus was determined using a two-tailed student's t-test for each treatment separately. The effect of orientation (circumferential vs.

radial) was determined using a t-test to compare $G_{\theta z}$ vs. $G_{r\theta}$ for each test group separately. To evaluate the effect of enzymatic degradation on shear modulus, a one-way analysis of variance (levels: PBS control, ChABC, and elastase) with a Tukey post-hoc test was performed separately for the circumferential and radial orientation. Significance was set at $p < 0.05$.

To evaluate the effectiveness of each treatment, tissue GAG content was measured using the dimethylmethylene blue assay [39] following mechanical testing. GAG was normalized by tissue dry weight. Separate samples underwent the treatments described above and were evaluated histologically for the presence or absence of elastic fibers using Miller's stain [30] and for GAG using alcian blue.

3. RESULTS

The AF shear response exhibited nonlinearity and anisotropy. The stress-strain response for the AF in radial shear was linear, while in the circumferential direction, a range of stress-strain responses, from linear to nonlinear, was observed (Figure 3). The variability between linear and nonlinear samples was similar to the variability within each group (linear or nonlinear) in part due to the high inter-sample variability observed in AF shear. Therefore, a mean was taken across all samples. The standard deviation for this study is consistent with previous studies on annulus shear [6,7]. For untreated samples, the circumferential shear modulus, $G_{\theta z}$, with 2% prestrain was 8 times higher than the radial shear modulus $G_{r\theta}$, and 44 times higher with 10% prestrain (Figure 4A). The effect of prestrain on circumferential modulus was significant: $G_{\theta z}$ for untreated samples at 10% prestrain was 4 times greater than the 2% prestrain modulus (Figure 4A).

The consequence of buffer soak was next determined through comparison of untreated and PBS treatment groups, followed by evaluation of the effect of selective matrix removal, which were compared to PBS treatment. Radial shear modulus, $G_{r\theta}$, significantly decreased from 15 kPa to 3 kPa with PBS treatment, five times lower than untreated ($p < 0.05$). The radial shear moduli for ChABC and elastase treatments were not significantly different than for PBS treatment (Figure 4B). The shear circumferential modulus, $G_{\theta z}$, was not significantly affected by PBS treatment (Figure 4C). The shear circumferential modulus was not significantly altered by ChABC and elastase treatments compared to PBS, however, some interesting trends were observed (Figure 4D). The circumferential shear modulus with ChABC treatment was 1.34 MPa, four times greater than PBS control ($G_{\theta z}$ 0.33), this effect was marginally insignificant ($p = 0.15$). Further, the increase in shear modulus observed with higher tensile prestrain remained for the PBS and enzyme treatment groups (Figure 4D) as did the relationships between treatment groups at 2% and 10% prestrain.

To confirm enzymatic removal of tissue components, the total amount of GAG in the tissue was measured. Pooling samples from the two orientations, the PBS treatment group experienced a 51% decrease in GAG content compared to untreated ($p < 0.05$). The ChABC treatment had 36% reduced GAG ($p < 0.05$) and elastase treatment had 70% reduced GAG ($p < 0.05$) compared to PBS treatment (Figure 5). Elastase treatment removed two times more GAG than PBS treatment compared to untreated. These values were consistent with histological evaluation: alcian blue staining indicated greater GAG content for untreated and a decrease in GAG levels from untreated to PBS, ChABC, and finally elastase treatment (Figure 6 A-D). GAG removal appeared to be uniform across the length and width of the samples. Miller's stain confirmed removal of elastic fibers with elastase treatment and preservation of elastic fibers for untreated (Figure 6E and F), and other treatment groups (not shown).

4. DISCUSSION

This study used planar shear testing to quantify the effects of testing orientation and selective matrix degradation on AF shear modulus. Consistent with the study hypothesis, shear modulus was found to be highly anisotropic: the circumferential shear modulus, $G_{\theta z}$, was an order of magnitude greater than the radial shear modulus, $G_{r\theta}$. In the radial direction, shear loads are supported by interactions between the extrafibrillar matrix and collagen fibers [5], interconnections between adjacent collagen fibers [40], and interconnections between lamellae [41]. In the circumferential direction, prestrain significantly increased the shear modulus, suggesting an important role for collagen fiber stretch in shear properties for this orientation. The circumferential shear modulus with a 10% tensile preload ($G_{\theta z}=0.66$ MPa) was an order of magnitude higher than previous tissue experiments with cubic or cylindrical samples (0.06-0.4 MPa) [6,7], was similar to planar shear with preload [6] (Figure 4C), but was much lower than expected based on disc torsion experiments and constitutive models (~ 10 MPa) [2,5]. Axial torsion tests of bone-disc-bone segments quantify shear modulus in the $z\theta$ direction [2]. However the current study measured the shear modulus in the θz direction due to disc height limitations that prevented testing in the $z\theta$ direction (Figure 2B, C). For an orthotropic material, $G_{z\theta} = G_{\theta z}$, due to symmetry, however the discontinuities caused by the AF multi-lamellar structure and tissue nonlinearities may have affected that assumption. Non-symmetric shear moduli and artifacts due to sample aspect ratio have also been observed in shear mechanics of ligament and tissue engineering scaffolds, and have been confirmed using finite element models [35,42-44]. Aspect ratio for the current study was chosen to limit sample size related artifacts, based upon finite element studies of human ligament mechanics [44], and our own finite element studies of annulus and tissue-engineered constructs [42].

To further investigate the disparity between AF tissue and expected shear modulus from disc torsion and modeling studies, we evaluated the tissue strain and the amount of fiber stretch during the shear experiment. While the applied shear strain at the grips for 10° shear deformation applied in this experiment was $E_{\theta z}=0.088$, the measured tissue surface strain in the central two-thirds of the sample was approximately half the applied amount, $E_{\theta z}=0.043$. Assuming affine deformations and a 45° fiber angle (fiber angle for bovine annulus), the collagen fiber stretch during 0 to 10° shear deformation was estimated to be 1.03. After a 10% tensile prestrain that theoretically places the collagen fibers in the linear-region, additional fiber stretch ($\lambda = 1.03$) during shearing should have resulted in much higher forces than measured. Non-affine behavior has been observed in AF tensile loading in the axial (z) direction [45] and in supraspinatus tendon loading in the transverse direction [46,47]. In these studies the fibers likely rotated within the deforming matrix rather than straining identically as required for the affine assumption [5,45-47]. This phenomenon would have reduced the measured circumferential shear modulus from its true value. In situ the AF fibers are constrained by the vertebral bone and affine deformations are more likely to occur. These experimental limitations may explain the large variability observed in the stress-strain response and the lower than expected measured shear moduli.

The second objective was to use targeted enzymatic degradation to evaluate the contribution of GAG and elastic fibers to AF shear modulus. Contrary to our hypothesis, there was no significant difference between PBS and enzyme treatments (ChABC or elastase) for either orientation (Figure 4B, 4D). This negative finding is likely related to the study limitation where a decrease in shear modulus was observed for PBS treatment compared to untreated tissue (only significant for radial shear). This may be in part due to diffusive GAG loss (Figure 5, 6). Reduced shear modulus and GAG loss following extended PBS immersion is consistent with previously observed reduced tension and compression modulus across a spectrum of GAG-rich fibrous tissues [15, 48, 49], although such findings are not universal

[16, 17]. Additionally, it is difficult to account for the effects of swelling and transmission of charged ions during the enzymatic digestion. The PBS and untreated samples were observed to have similar cross-sectional area (data now shown), indicating that similar volumetric swelling occurred for untreated samples during the 30 min equilibrium soak and PBS samples during the 36 hr treatment. The cause for the disparity between untreated and PBS samples is unknown, and may be related to the treatment process, including being heated to 37 deg and placed on a rocker for 36 hr, and to internal fibril changes as previously shown in uniaxial tensile tests [21]. Freezing the samples does not have an effect on disc subfailure mechanics [50] and as all samples, even the untreated group, underwent at least one freeze-thaw cycle, any minor effect would be encountered by all samples. However, to account for the effects of the treatment as observed by the difference between the untreated and PBS groups, all digestion groups were compared to the PBS group, which underwent the same treatment procedure. A full understanding of each of these effects warrants additional study.

Chondroitinase ABC treatment was used to investigate the contribution of GAG to AF shear modulus. While not significant and highly variable, ChABC treatment tended to increase the circumferential shear modulus compared to PBS treatment ($p=0.15$) (Fig 4D), but showed no such trend in the radial direction. While ChABC removed more GAG than PBS, degradation was not complete, likely due in part to the inability of ChABC to degrade keratin sulfate. Were ChABC able to more fully degrade annulus GAG, the increase in modulus with respect to PBS treatment may have been greater. These findings suggest a potential role for interactions between GAG and collagen that, contrary to expectations, *increases* overall stiffness when the GAG is selectively digested. While these shear modulus observations are limited by high variability, they are supported by enzymatic degradation studies in uniaxial tension for AF tissue-engineered constructs [20] and for both inner and outer AF in uniaxial tension [20,21], where ChABC treatment increased the tensile modulus compared to PBS treatment. These effects were highly dependent on tissue harvest location (outer AF was not significant, inner AF was significant, Figure 7A). This further supports the notion that the tensile role of GAG is, in part, a function of the quantity of GAG in the tissue, yet the complexity of this relationship exceeds composition alone, and depends also on tissue type, location, and maturity [14,18]. In contrast, the effect of ChABC induced GAG depletion in reducing compressive modulus is widely documented and is explained by the associated loss of osmotic pressure [51-55]. Overall there is a growing evidence for structural interactions between GAG and collagen that affects tissue function, likely at different hierarchical levels, but as the results of this and other studies demonstrate, this is not a simple mechanism and warrants further investigation.

Elastase treatment was used to investigate the contribution of elastic fibers to the AF shear modulus. Elastase treatment caused no difference in shear modulus with respect to PBS treatment for either radial or circumferential orientation. The radial modulus of elastase treated samples, however, was significantly lower than the untreated group, consistent with studies of human AF in radial tension, where elastase treatment reduced the uniaxial modulus compared to both untreated and ChABC groups [31]. Although we hypothesized that circumferential shear modulus would be reduced by elastase treatment due to expected disruption of collagen-elastic fiber interconnections, no difference was observed for $G_{\theta z}$ between the PBS and elastase groups. Microstructural studies have shown that elastase treatment has no effect on normalized stretch and rotation of collagen fibers for bovine AF in circumferential shear [32]. Thus, in circumferential shear, collagen fibers in both PBS and elastase treated samples are likely to contribute equally, explaining the unchanged circumferential modulus. Elastase treatment removed more GAG than either PBS or ChABC treatment. Despite this, and in contrast to the trend for ChABC to increase shear modulus, elastase samples were unchanged. This may indicate that elastic fibers provide a subtle but

noticeable mechanical support to AF in shear, however, the predominate mechanism appears to be collagen fiber stretch.

5. CONCLUSIONS

This study demonstrated that AF shear modulus is highly anisotropic and that fiber stretch contributes significantly to the circumferential shear modulus. The circumferential shear modulus was larger than for previous studies with cubic and cylindrical samples, confirming a role for fiber stretch, but still lower than expected compared with torsion testing and constitutive modeling, suggesting non-affine deformations may be occurring for these boundary conditions. Together with the established literature for tensile loading of fiber-reinforced GAG-rich tissues, the trends for changes in shear modulus with ChABC treatment reflect complex structure-function relationships between GAG and collagen that potentially occur over several hierarchical scales. Elastase digestion did not significantly affect circumferential shear modulus; supporting the notion that circumferential shear modulus is dominated by collagen fiber stretch. The results of this study highlight the complexity of the structure-function relationships that govern the mechanical response of the AF in radial and circumferential shear, and provide new and more accurate data for the validation of material models and tissue-engineered disc replacements.

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REFERENCES

1. Elliott DM, Sarver JJ. Young investigator award winner: validation of the mouse and rat disc as mechanical models of the human lumbar disc. *Spine (Phila Pa 1976)*. 2004; 29(7):713–22. [PubMed: 15087791]
2. Costi JJ, et al. Frequency-dependent behavior of the intervertebral disc in response to each of six degree of freedom dynamic loading: solid phase and fluid phase contributions. *Spine (Phila Pa 1976)*. 2008; 33(16):1731–8. [PubMed: 18628705]
3. Spilker RL, Jakobs DM, Schultz AB. Material constants for a finite element model of the intervertebral disk with a fiber composite annulus. *J Biomech Eng*. 1986; 108(1):1–11. [PubMed: 3959546]
4. Yin L, Elliott DM. A homogenization model of the annulus fibrosus. *J Biomech*. 2005; 38(8):1674–84. [PubMed: 15958225]
5. Guerin HL, Elliott DM. Quantifying the contributions of structure to annulus fibrosus mechanical function using a nonlinear, anisotropic, hyperelastic model. *J Orthop Res*. 2007; 25(4):508–16. [PubMed: 17149747]
6. Fujita Y, et al. Anisotropic shear behavior of the annulus fibrosus: effect of harvest site and tissue prestrain. *Med Eng Phys*. 2000; 22(5):349–57. [PubMed: 11121768]
7. Iatridis JC, et al. Shear mechanical properties of human lumbar annulus fibrosus. *J Orthop Res*. 1999; 17(5):732–7. [PubMed: 10569484]
8. Urban JP, et al. Swelling pressures of proteoglycans at the concentrations found in cartilaginous tissues. *Biorheology*. 1979; 16(6):447–64. [PubMed: 534768]
9. Urban JP, McMullin JF. Swelling pressure of the intervertebral disc: influence of proteoglycan and collagen contents. *Biorheology*. 1985; 22(2):145–57. [PubMed: 3986322]
10. Cribb AM, Scott JE. Tendon response to tensile stress: an ultrastructural investigation of collagen:proteoglycan interactions in stressed tendon. *J Anat*. 1995; 187(Pt 2):423–8. [PubMed: 7592005]

11. Liao J, Vesely I. Skewness angle of interfibrillar proteoglycans increases with applied load on mitral valve chordae tendineae. *J Biomech.* 2007; 40(2):390–8. [PubMed: 16483580]
12. Redaelli A, et al. Possible role of decorin glycosaminoglycans in fibril to fibril force transfer in relative mature tendons—a computational study from molecular to microstructural level. *J Biomech.* 2003; 36(10):1555–69. [PubMed: 14499303]
13. Millesi H, et al. Biomechanical properties of normal tendons, normal palmar aponeuroses, and tissues from patients with Dupuytren's disease subjected to elastase and chondroitinase treatment. *Clin Biomech (Bristol, Avon).* 1995; 10(1):29–35.
14. Rigozzi S, Muller R, Snedeker JG. Local strain measurement reveals a varied regional dependence of tensile tendon mechanics on glycosaminoglycan content. *J Biomech.* 2009; 42(10):1547–52. [PubMed: 19394024]
15. Screen HR, et al. The influence of swelling and matrix degradation on the microstructural integrity of tendon. *Acta Biomater.* 2006; 2(5):505–13. [PubMed: 16839828]
16. Lujan TJ, et al. Effect of dermatan sulfate glycosaminoglycans on the quasi-static material properties of the human medial collateral ligament. *J Orthop Res.* 2007; 25(7):894–903. [PubMed: 17343278]
17. Lujan TJ, et al. Contribution of glycosaminoglycans to viscoelastic tensile behavior of human ligament. *J Appl Physiol.* 2009; 106(2):423–31. [PubMed: 19074575]
18. Asanbaeva A, et al. Articular cartilage tensile integrity: modulation by matrix depletion is maturation-dependent. *Arch Biochem Biophys.* 2008; 474(1):175–82. [PubMed: 18394422]
19. Schmidt MB, et al. Effects of proteoglycan extraction on the tensile behavior of articular cartilage. *J Orthop Res.* 1990; 8(3):353–63. [PubMed: 2324854]
20. Nerurkar NL, et al. Homologous structure-function relationships between native fibrocartilage and tissue engineered from MSC-seeded nanofibrous scaffolds. *Biomaterials.*
21. Han WJ, T N, Nerurkar NL, Smith LJ, Mauck RL, Elliott DM. Differential Tensile Mechanical Behavior of the Inner and Outer Annulus Fibrosus Following Treatment with Chondroitinase ABC and Buffer Solutions. *Transactions of the Orthopaedic Research Society.* 2011; 625
22. Lee TC, et al. The effect of elastin damage on the mechanics of the aortic valve. *J Biomech.* 2001; 34(2):203–10. [PubMed: 11165284]
23. Ujiie Y, et al. Degradation of noncollagenous components by neutrophil elastase reduces the mechanical strength of rat periodontal ligament. *J Periodontal Res.* 2008; 43(1):22–31. [PubMed: 18230103]
24. Vesely I. The role of elastin in aortic valve mechanics. *J Biomech.* 1998; 31(2):115–23. [PubMed: 9593204]
25. Yuan H, et al. Effects of collagenase and elastase on the mechanical properties of lung tissue strips. *J Appl Physiol.* 2000; 89(1):3–14. [PubMed: 10904029]
26. Smith LJ, Fazzalari NL. The elastic fibre network of the human lumbar anulus fibrosus: architecture, mechanical function and potential role in the progression of intervertebral disc degeneration. *Eur Spine J.* 2009
27. Cloyd JM, Elliott DM. Elastin content correlates with human disc degeneration in the anulus fibrosus and nucleus pulposus. *Spine.* 2007; 32(17):1826–31. [PubMed: 17762289]
28. Yu J, et al. The elastic fiber network of the anulus fibrosus of the normal and scoliotic human intervertebral disc. *Spine.* 2005; 30(16):1815–20. [PubMed: 16103849]
29. Yu J, et al. The elastic fiber network of the anulus fibrosus of the normal and scoliotic human intervertebral disc. *Spine (Phila Pa 1976).* 2005; 30(16):1815–20. [PubMed: 16103849]
30. Smith LJ, Fazzalari NL. Regional variations in the density and arrangement of elastic fibres in the anulus fibrosus of the human lumbar disc. *J Anat.* 2006; 209(3):359–67. [PubMed: 16928204]
31. Smith LJ, et al. Elastic fibers enhance the mechanical integrity of the human lumbar anulus fibrosus in the radial direction. *Ann Biomed Eng.* 2008; 36(2):214–23. [PubMed: 18066662]
32. Michalek AJ, et al. Measurement of local strains in intervertebral disc anulus fibrosus tissue under dynamic shear: contributions of matrix fiber orientation and elastin content. *J Biomech.* 2009; 42(14):2279–85. [PubMed: 19664773]

33. Missirlis YF. Use of enzymolysis techniques in studying the mechanical properties of connective tissue components. *J Bioeng.* 1977; 1(3):215–22. [PubMed: 28317]
34. Peltz CD, et al. Mechanical properties of the long-head of the biceps tendon are altered in the presence of rotator cuff tears in a rat model. *J Orthop Res.* 2009; 27(3):416–20. [PubMed: 18924143]
35. Weiss JA, Gardiner JC, Bonifasi-Lista C. Ligament material behavior is nonlinear, viscoelastic and rate-independent under shear loading. *J Biomech.* 2002; 35(7):943–50. [PubMed: 12052396]
36. Lynch HA, et al. Effect of fiber orientation and strain rate on the nonlinear uniaxial tensile material properties of tendon. *J Biomech Eng.* 2003; 125(5):726–31. [PubMed: 14618932]
37. Thomopoulos S, et al. Collagen fiber alignment does not explain mechanical anisotropy in fibroblast populated collagen gels. *J Biomech Eng.* 2007; 129(5):642–50. [PubMed: 17887889]
38. Lake SP, et al. Effect of fiber distribution and realignment on the nonlinear and inhomogeneous mechanical properties of human supraspinatus tendon under longitudinal tensile loading. *J Orthop Res.* 2009; 27(12):1596–602. [PubMed: 19544524]
39. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta.* 1986; 883(2):173–7. [PubMed: 3091074]
40. Pezowicz CA, Robertson PA, Broom ND. Intralamellar relationships within the collagenous architecture of the annulus fibrosus imaged in its fully hydrated state. *J Anat.* 2005; 207(4):299–312. [PubMed: 16191160]
41. Pezowicz CA, Robertson PA, Broom ND. The structural basis of interlamellar cohesion in the intervertebral disc wall. *J Anat.* 2006; 208(3):317–30. [PubMed: 16533315]
42. Driscoll TP, et al. Fiber Angle and Aspect Ratio Influence the Shear Mechanics of Oriented Electrospun Nanofibrous Scaffolds. *Journal of the Mechanical Behavior of Biomedical Materials.* In Review.
43. Bonifasi-Lista C, et al. Viscoelastic properties of the human medial collateral ligament under longitudinal, transverse and shear loading. *J Orthop Res.* 2005; 23(1):67–76. [PubMed: 15607877]
44. Gardiner JC, Weiss JA. Simple shear testing of parallel-fibered planar soft tissues. *J Biomech Eng.* 2001; 123(2):170–5. [PubMed: 11340878]
45. O'Connell GD, Guerin HL, Elliott DM. Theoretical and uniaxial experimental evaluation of human annulus fibrosus degeneration. *J Biomech Eng.* 2009; 131(11):111007. [PubMed: 20353258]
46. Lake SP, et al. Tensile properties and fiber alignment of human supraspinatus tendon in the transverse direction demonstrate inhomogeneity, nonlinearity, and regional isotropy. *J Biomech.* 43(4):727–32. [PubMed: 19900677]
47. Cortes DH, et al. Characterizing the mechanical contribution of fiber angular distribution in connective tissue: comparison of two modeling approaches. *Biomech Model Mechanobiol.* 9(5): 651–8. [PubMed: 20148345]
48. Perie DS, et al. Correlating material properties with tissue composition in enzymatically digested bovine annulus fibrosus and nucleus pulposus tissue. *Ann Biomed Eng.* 2006; 34(5):769–77. [PubMed: 16598654]
49. Fessel G, Snedeker JG. Evidence against proteoglycan mediated collagen fibril load transmission and dynamic viscoelasticity in tendon. *Matrix Biol.* 2009; 28(8):503–10. [PubMed: 19698786]
50. Dhillon N, Bass EC, Lotz JC. Effect of frozen storage on the creep behavior of human intervertebral discs. *Spine (Phila Pa 1976).* 2001; 26(8):883–8. [PubMed: 11317110]
51. Henninger HB, et al. Effect of sulfated glycosaminoglycan digestion on the transverse permeability of medial collateral ligament. *J Biomech.* 43(13):2567–73. [PubMed: 20627251]
52. Basalo IM, et al. Cartilage interstitial fluid load support in unconfined compression following enzymatic digestion. *J Biomech Eng.* 2004; 126(6):779–86. [PubMed: 15796336]
53. Katta J, et al. The effect of glycosaminoglycan depletion on the friction and deformation of articular cartilage. *Proc Inst Mech Eng H.* 2008; 222(1):1–11. [PubMed: 18335713]
54. Korhonen RK, et al. Fibril reinforced poroelastic model predicts specifically mechanical behavior of normal, proteoglycan depleted and collagen degraded articular cartilage. *J Biomech.* 2003; 36(9):1373–9. [PubMed: 12893046]

55. Maroudas A, Schneiderman R. “Free” and “exchangeable” or “trapped” and “non-exchangeable” water in cartilage. *J Orthop Res.* 1987; 5(1):133–8. [PubMed: 3819905]

Figure 1.

Schematic of the annulus fibrosus showing radial (r) and circumferential (circ, θ) sample orientations, with applied boundary conditions (tensile prestrain and shear strain directions) indicated by arrows. $G_{r\theta}$ = radial shear modulus and $G_{\theta z}$ = circumferential shear modulus, z = spine axial direction.

Figure 2.

A. Shear testing device, showing directions of applied strains B. Schematic illustration of boundary conditions, including shear angle γ and shear stress τ_{ij} . By convention, the i -direction represents the long axis of the sample, while the j -direction represents the direction at which shear is applied. C. Schematic illustration of the shear deformation, $\gamma_{z\theta}$, experienced by the AF when the intervertebral disc undergoes torsion.

Figure 3.

Representative stress-strain plots for circumferential samples illustrating typical response the varied degree of nonlinearity in the responses. UT-L and UT-NL indicate untreated response for linear and nonlinear response patterns.

Figure 4.

Shear modulus mean and standard deviation. A) Untreated radial and circumferential (circ), *significantly different from radial, +significantly different from circ 2% B) Radial following treatment, *significantly different to untreated C) Circumferential untreated vs PBS treated, D) Circumferential following treatments. *significantly different to same group at 2% prestrain

Figure 5. GAG content with radial and circumferential samples pooled. *significantly different to untreated, and **significantly different to PBS treated

Figure 6. Histology results. Representative alcian blue staining for A. untreated, B. PBS treated C. ChABC treated, and D. elastase treated samples showing. Scale bars for A-D = 100 μm E-F) Millers stain of E. untreated and F. elastase treated samples (arrows = examples of elastic fibres). Scale bar for E-D = 20 μm

Figure 7.

Previously reported results for uniaxial tension [20,21] A. Tensile modulus. B. GAG content of outer and inner AF for PBS and ChABC treated samples. Mean $\pm$ SD