Assessment of Microscale Mechanical Properties of Murine Intervertebral Discs with Aging

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INTRODUCTION: Intervertebral disc (IVD) degeneration afflicts a significant portion of the population, contributing to the high incidence of low back pain[1]. While injury and pathological loading are drivers of morphological and microstructural changes associated with human disc degeneration[2,3], age-related changes in the nucleus pulposus (NP) and annulus fibrosis (AF) may also play a role in spontaneous disc degeneration not attributable to injury, altering the distribution of biomechanical force within the disc and spine as a whole[3-8]. Due to the complexity of studying aging in human subjects, murine models have served as an attractive alternative to study aging effects in the musculoskeletal system. Prior studies in murine models demonstrate evidence of reduced disc height[9], reduced glycosaminoglycan (GAG) and water content[8], increased fibrosis[10], tissue stiffening[11], and disc tears and herniation primarily in the dorsolateral region of the AF[12] with age. However, changes in the micromechanical properties of the unique tissues within the IVD with age have not been investigated, with a considerable lack of research involving the use of atomic force microscopy (AFM), a particularly effective method for interrogating microscale mechanical properties. Therefore, we aim to determine age-related alterations in tissue micromechanical properties, composition, and morphology for the NP, inner annulus fibrosus (iAF), and outer annulus fibrosis (oAF) for murine IVD. We hypothesize that aged mice will exhibit morphological hallmarks of aging (increased fibrosis of NP and reduced distinction between NP and AF), accompanied by tissue stiffening in the NP and AF.

METHODS: Intact lumbar (L4/5) IVDs were harvested from C57BL6 mice at 6-7 or 20+ months (M) of age, with N=3 mice per age group. Samples were snap frozen, and later embedded in OCT and stored at -80ºC. Embedded samples were cryosectioned into 20µm thick transverse sections midplane to visualize both the NP and AF regions. Section thickness was previously optimized by performing AFM on 20, 35, and 50µm thick sections, and consistent force-indentation curves (within tissue type) were observed using 20µm sections. GAG content was evaluated using Safranin-O and Fast Green Staining. Contact mode AFM was performed on serial frozen sections (Asylum MFP-3D, 6.1µm diameter polystyrene colloidal indenter) on each hydrated section per sample at 3-4 locations within the iAF and oAF, and 3 locations within the NP (**Figure 1B**). Force-indentation curves were analyzed using the Hertz model to extract Young's modulus (E_Y). Overall comparisons were determined by conducting an ANOVA on a general linear model, with Tukey post-hoc tests (correcting for multiple comparisons) to specifically interrogate modulus differences in the following comparison: 1) between tissue types within age, 2) within tissue type across age, 3) between AF regions across age, and 4) across AF regions within age. Additionally, t-tests were conducted to compare NP modulus across age due to heteroscedasticity relative to the AF that may confound post-hoc tests. Statistical analyses were conducted in R (version 4.1.2)[13,14].

RESULTS: Visual evaluation of IVD histological staining revealed characteristic morphological changes associated with age, including reduced cell count and increased fibrosis within the NP, increased circularity of cells and widened lamellae within the AF, and lamellar penetration of the NP (**Figure 1A**). Additionally, we reproducibly observe a statistically significant increase in E_Y in the AF relative to the NP, and a trend towards a higher modulus in the oAF relative to the iAF (**Figure 1C**). With age, we observed statistically significant increases in E_Y in the oAF and NP, but not in the iAF (**Figure 1C**). Regional differences in the AF are observed with the ventral aspect of the AF having higher E_Y than both the dorsal and lateral aspects in aged mice (**Figure 1D**). However, the lateral aspect of the AF had a higher modulus in younger mice. Additionally, ventral AF modulus increases with age, with lateral AF modulus decreasing. Tissue E_Y varied considerably between donors controlling for both tissue and age, indicating variability between donors.

DISCUSSION: We demonstrate higher microscale compressive stiffness in the AF relative to the NP reflective of tissue-specific microstructure and composition. Increased stiffness of the oAF and NP with age is consistent with morphological changes including increased fibrotic content and a redistribution of mechanical loading in aged IVDs from NP to AF regions that accompanies reduced water content of the NP[1,3,4]. Significant changes in iAF stiffness across age were not observed, potentially due to limited sample size and high variability between donors. Regional differences in the AF of older mice show increased stiffness in the ventral aspect of the disc, consistent with previous studies demonstrating a decrease in AF modulus from ventral to dorsolateral[15]. However, this is not shown in the younger mice, potentially reflective of the limited visible region available for testing in some samples and low sample size. Overall, we demonstrate both morphological and mechanical differences between the NP, iAF, and oAF that are reflective of both morphological differences and age-associated changes.

SIGNIFICANCE/CLINICAL RELEVANCE: Identifying variability in both microstructural and micromechanical properties of the tissues within the IVD and the corresponding changes associated with age is reflective of age-related degeneration and may improve the characterization of properties that influence the tissue and region-specific vulnerability towards injury and degeneration.

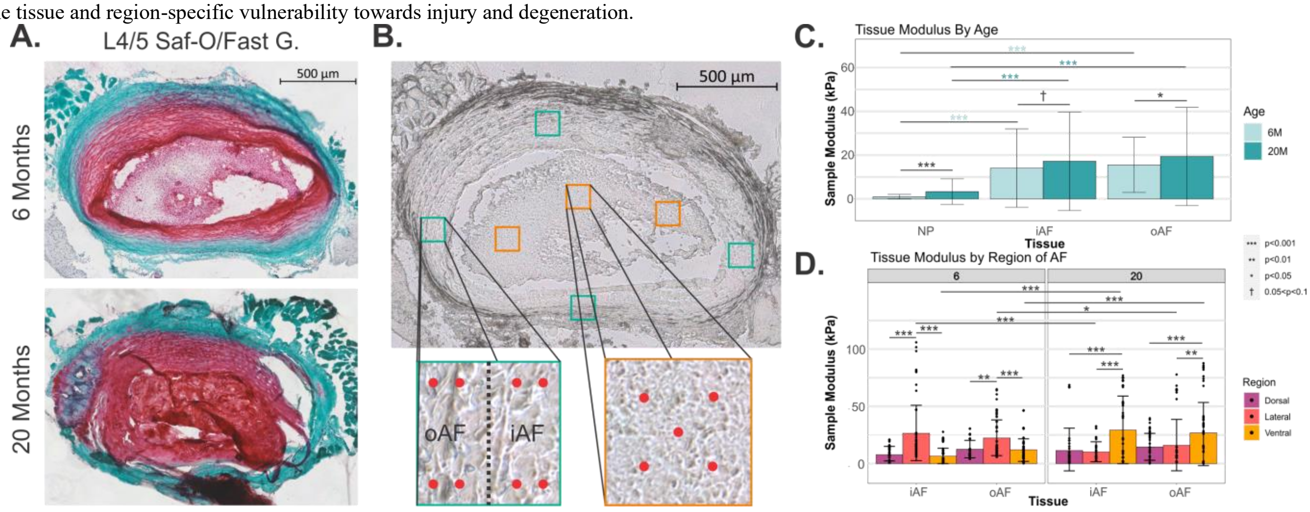

Figure 1. A) GAG staining for evaluation of age-related histological changes. **B)** AFM testing locations to derive NP, iAF, and oAF mechanical properties, as well as regional differences in AF. **C)** Tissue E_Y for 6M and 20M. **D)** Regional AF E_Y for 6M and 20M.

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