Introduction: The physical properties of the annulus fibrosus are critical to the intervertebral disc’s biomechanical function: alterations with degeneration and aging can contribute directly to joint dysfunction and pain. Historically, the material properties of fibrocartilaginous tissue have been attributed to both collagenous and non-collagenous features of the tissue architecture. For example, increases in tissue stiffness have been correlated with increases in advance glycation end product (AGE) crosslinks in many tissues including skin, tendon, and articular cartilage. These crosslinks may be responsible for age-related intervertebral disc stiffening. To study the effects of AGE crosslinks on the material properties of the annulus, we subjected tissue specimens to mechanical tests both before and after they had been incubated with methyl glyoxal.

A mathematical model that links tissue architecture to annular material properties is critical for understanding disc disease etiology and progression. We have previously reported an annular strain energy function with separate terms representing specific tissue features, including a term that represents the mechanical contribution of collagen crosslinks\(^6\). In the current study we validate this crosslinking term by demonstrating that it accounts for the mechanical changes induced by an increased level of annular AGE crosslinks.

Methods: Specimens approximately 5mm wide, 10mm long, and 2mm thick were harvested from the anterior-lateral portion of 5 non-degenerate (Thompson Grade I and II) lumbar discs (age range 27-38), with the longest dimension coinciding with the axial direction. At the ends of each specimen, 4 loops consisting of continuous suture were sewn and were used to grip the tissue. In the middle of the specimen, visual targets for strain measurement were created by applying dots of black ink to the tissue in a 3x3 grid with the tip of a pin. The specimens were submerged in 0.15 M saline and protease inhibitor solution during testing. Using custom testing apparatus and software that allows for real-time and non-contact strain measurement, the specimens were first preconditioned with 10 cycles to 0.04 MPa at a strain rate of approximately 0.005 sec\(^{-1}\). The specimens were returned to a zero-stress state and then tested to 0.2 MPa at a strain rate of 0.0001sec\(^{-1}\). At the end of testing, specimens were removed from the testing apparatus and allowed to equilibrate for at least one hour. As a general description of the data, an exponential curve of the form \(\sigma = \gamma_0 (e^{b_1 I_1} - 1)\) was fit to the stress-strain data, and the stiffness modulus at 0.2 MPa was determined.

Test specimens were incubated for one week at 37°C in a solution consisting of 200 mmol Tris/HCl, pH 8.8, containing methyl glyoxal at a concentration of 100 mmol. Samples were removed from the incubation buffer, rinsed with PBS, and the above axial tension test protocol was repeated. A preliminary study determined the AGE crosslink density induced by this in vitro glycation protocol by assaying for pentosidine (RP-HPLC) as a reliable marker of AGE crosslinking\(^3\) and hydroxyproline (Woessner analysis) as a measure of collagen.

We have previously proposed a strain energy function for the annulus and hydroxyproline (Woessner analysis) as a measure of collagen. Specimens were submerged in 0.15 M saline and protease inhibitor solution during testing. Using custom testing apparatus and software that allows for real-time and non-contact strain measurement, the specimens were first preconditioned with 10 cycles to 0.04 MPa at a strain rate of approximately 0.005 sec\(^{-1}\). The specimens were returned to a zero-stress state and then tested to 0.2 MPa at a strain rate of 0.0001sec\(^{-1}\). At the end of testing, specimens were removed from the testing apparatus and allowed to equilibrate for at least one hour. As a general description of the data, an exponential curve of the form \(\sigma = \gamma_0 (e^{b_1 I_1} - 1)\) was fit to the stress-strain data, and the stiffness modulus at 0.2 MPa was determined.

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We have previously proposed a strain energy function for the annulus consisting of a sum of separate terms: \(W = a_1(I_1-1)/I_1 \cdot a_2(I_1^{-1/3} - 1) + a_3(\exp(b_1 I_1) - b_1 I_1) + a_4(\exp(b_2 I_2) - b_2 I_2)\) where the \(I_1\) and \(I_2\) terms represent the response of the matrix, the \(3^{rd}\) term models the collagen fibers, and the \(4^{th}\) term represents the contribution of the crosslinks\(^6\). Using previously described methods, we determined the values of the strain energy coefficients \(a_1, a_2, a_3, b_1, a_4, b_2\) by conducting a non-linear regression to the mean elastic stress-strain response from wide range of experimental protocols, including the axial tension dataset from the untreated tissue in the current study. We then conducted another non-linear regression to just the mean axial stress-strain data from the glycated tissue, allowing only the coefficients \(a_1\) and \(b_1\) to vary and setting the rest of the coefficients equal to the previously determined values.

Results: The exponential equation represented the axial tension data well, with a correlation coefficient > 0.98 in all cases. The average mechanical behavior was characterized by \(A=0.111\) ± 0.0391 and \(B=10.5\) ± 1.99 for the untreated specimens and by \(A=0.138\) ± 0.0899 and \(B=12.0\) ± 2.65 for the glycated specimens. Glycation resulted in a 15% increase in stiffness at 0.2 MPa (2.54 ± 0.425 vs 2.21 ± 0.3, p < 0.05). Our glycation protocol induced a pentosidine level of 27mmol/mol collagen, while the pentosidine level in the discs of 20-40 year olds has been reported to be less than 10mmol/mol collagen\(^1\). The best fit values for the six strain energy coefficients were \(0.00399, 0.106, 0.000166, 29.9, 0.00549, 3.40\). These coefficients resulted in stress-strain curves that lie within one standard deviation for all experimental deformations, including the axial tension data from the untreated tissue in the current study (Figure 1). Additionally, we found an excellent fit to the tension data from the glycated tissue with values of \(a_1 = 0.00658\) and \(b_1 = 4.84\) and all other coefficients as listed above (Figure 1).

Discussion: Using an in vitro glycation protocol, the mechanical effects of AGE crosslinks (of one of the many biochemical processes that occurs with aging in vivo) can be isolated and studied. Our experimental results show that an increased level of AGE crosslinking correlates with an increased stiffness in the annulus in the axial direction. These results are consistent with previously reported findings in many tissues such as skin, tendon and cartilage\(^2,4,6\).

By applying the experimental data to a strain energy function with a separate term to represent the effect of crosslinking, we have demonstrated that the mathematical expression captures the mechanical influence of the crosslinking. Specifically, we have determined that the changes in axial tensile behavior due only to increased levels of AGE crosslinks can be modeled by increasing the coefficients in the crosslinking term of the strain energy function. This is a first step towards developing a mechanistic constitutive relationship that correlates specific features of tissue architecture to material properties. If successful, this mechanistic approach will be a significant advance beyond exiting phenomenological constitutive models, and may be used in the future to elucidate the structure-function relationships of the annulus and the pathomechanics of aging and degeneration in the intervertebral disc.

References:
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