

A CONSTITUTIVE MODEL FOR THE HUMAN ANNULUS FIBROSUS THAT LINKS MATERIAL COEFFICIENTS WITH CROSSLINK DENSITY

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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 [1-3]. 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 methylglyoxal.

A particularly useful annular constitutive model would include coefficients that can be correlated with biochemical measures. 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 [4]. In the current study we modify this strain energy function by including an additional term, and validate the crosslinking terms by demonstrating that the coefficients associated with these terms can be correlated to the crosslink density.

METHODS

Specimens approximately 5mm wide, 10mm long, and 2mm thick were harvested from the anterior-lateral portion of 10 non-degenerate (Thompson Grade I and II) lumbar discs (age range 16 - 49), with the longest dimension coinciding with the axial direction. From four of the discs, a second test specimen was taken and was used as a control (age range 27 - 49), and both test and control specimens were subjected to mechanical testing. Tissue that was directly adjacent to the test and control specimens in the disc was dissected from the disc and returned separately to the freezer for later biochemical analysis. 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 tattoo 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 $\sigma = \frac{A}{B}(e^{Be} - 1)$ was fit to the stress-strain data.

Test specimens were incubated for one week at 37°C in a solution consisting of 200 mmol Tris/HCl, pH 8.8, containing methylglyoxal at a concentration of 100 mmol. Control specimens were incubated for one week at 37°C in a solution of 200 mmol Tris/HCl but without methylglyoxal. Samples were removed from the incubation buffers, 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 [5] and hydroxyproline (Woessner analysis) as a measure of collagen. Crosslink density was assumed to be proportional to pentosidine content normalized by collagen content.

We wanted to estimate the mechanical influence of the crosslinks only, without any adverse effects of the one-week incubation. To do so, we first calculated the percentage change in stress due to the sham protocol at a given strain. We then calculated this percent of the stress at the same strain from the mean biomechanical test data of the untreated test specimens, and added this amount on to the mean biomechanical test data of the glycated test specimens. This was repeated for multiple strains of the test protocol, and a new exponential curve was fit to this data.

We have previously proposed a strain energy function for the annulus consisting of a sum of four separate terms [4]. This was accomplished using the composite continuum theory for two fiber

families that was proposed by Spencer [6]. In the current study, we modified the strain energy function by adding a fifth term:

$$W = a_1(I_3 - 1/I_3)^2 + a_2(I_1 I_3^{-1/3} - 3)^2 + a_3(\exp(b_3(I_9 - 2)) - b_3 I_9) + a_4(\exp(b_4(I_{11} - I_9^2 + 2I_{10})) + a_5(\exp(b_5(J_9 - 2)) - b_5 J_9)$$

where I_1 , I_3 , I_9 , I_{10} , and I_{11} are invariant quantities that have been defined by Spencer [6] and J_9 is the sum of the squared stretches in directions perpendicular to the two fiber families. In the above strain energy function, the 1st and 2nd terms represent the response of the matrix, and the 3rd term models the collagen fibers. The quantity $I_{11} - I_9^2 + 2I_{10}$ is equivalent to the sum of the squared shear strains in the two fiber directions, therefore the 4th term represents the resistance of the crosslinks to shear deformation along the directions of the fibers. The 5th term represents the resistance of the crosslinks to stretching in the direction perpendicular to the fibers. Using previously described methods, we determined the values of the strain energy coefficients by conducting a non-linear regression to the mean elastic stress-strain response from wide range of experimental protocols, including both the axial tension datasets from the current study. Although the form of the mathematical expressions for these two experiments was identical, we employed different interaction coefficients for the two experiments. Specifically, we allowed a_4 and a_5 to take on different values for the two axial experiments but the matrix and the fiber coefficients were the same as in the other experiments. The equations were written such that a_4 and a_5 were the crosslinking coefficients for the equations that described the experimental results on the tissue before it had been incubated in the glycation solution, while $a_{4-cross}$ and $a_{5-cross}$ were the crosslinking coefficients for the equations that described the axial experiment on the tissue after it had been incubated in the glycation solution. Therefore, we determined best-fit values for the set of coefficients $\{a_1, a_2, a_3, b_3, a_4, b_4, a_5, b_5, a_{4-cross}, a_{5-cross}\}$.

RESULTS

The exponential equation represented the axial tension data well, with a correlation coefficient > 0.95 in all cases. The average mechanical behavior was characterized by $A=0.0942 \pm 0.0479$ and $B=11.1 \pm 4.74$ for the untreated specimens and by $A=0.132 \pm 0.0691$ and $B=10.1 \pm 3.06$ for the glycated specimens. This represents a statistically significant increase in the A coefficient ($p < 0.05$) and no statistically significant change in the B coefficient ($p > 0.1$) after incubation and leads to an average stress-strain curve that is slightly stiffer after incubation. The sham protocol, however, caused a significant decrease in the stiffness of the sham specimens, therefore we estimated that mechanical behavior after glycation without the adverse effect of the incubation was characterized by $A = 0.178$ and $B = 10.3$. These results show a more pronounced increase in stiffness with glycation and were used to model the annulus and to determine the material coefficients that appear in the strain energy function. The best-fit values for the strain energy coefficients were $\{0.0718, 0.00465, 0.000141, 33.1, 10.1, 0.00136, 0.000209, 5.22, 17.1, 0.00320\}$. These coefficients resulted in a good fit for all experimental deformations, including both axial tension datasets from the current study (Figure 1). The coefficient $a_{4-cross}$ was 69.1% greater than the coefficient a_4 while the coefficient $a_{5-cross}$ was 52.7% greater than a_5 . Our glycation protocol induced a pentosidine level of 27 mmol/mol collagen, while the pentosidine level in the discs of 20-40 year olds has been reported to be less than 10 mmol/mol collagen [7].

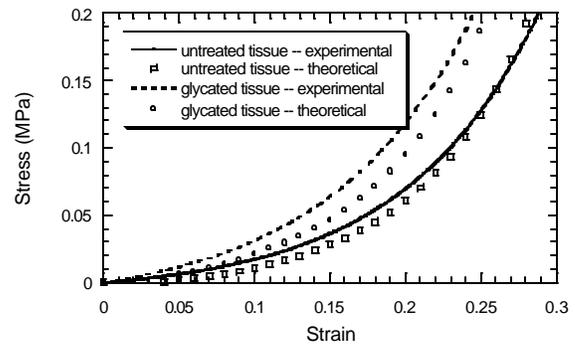


Figure 1: Elastic tensile behavior of the annulus in the axial direction both before and after incubation with methylglyoxal. Theoretical curves differ only by values of strain energy coefficients a_4 and a_5 .

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 [1-3].

By applying the experimental data to a strain energy function with separate terms to represent the effect of crosslinking, we have demonstrated that the mathematical expression captures the mechanical influence of the crosslinks. 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 terms 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 existing 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.

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