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Short communication

Changes in the interfacial shear resistance of disc annulus fibrosus from genipin crosslinking

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ABSTRACT

Crosslinking soft tissue has become more common in tissue engineering applications, and recent studies have demonstrated that soft tissue mechanical behavior can be directly altered through crosslinking. Using a recently reported test method that shears adjacent disc lamella, the effect of genipin crosslinking on interlamellar shear resistance was studied using in vitro bovine disc annulus.

Specimens of adjacent lamella were dissected from four discs taken from three fresh frozen bovine tails. These specimens were paired and soaked in either 50 mM EPPS Phosphate (ph9) with 20 mM genipin at 37 °C for 4 h or in 50 mM EPPS Phosphate (ph9) of which twelve specimens (6 per treatment) were successfully tested and analyzed.

Crosslinked specimens were noted to have significantly higher yield force per width (59%), peak force per width (70%), and resilience (69%) compared to sham treated controls, supporting the hypothesis that genipin crosslinking increases the resistance to interlamellar shear of the annulus interface. Additionally, a possible dependency may exist between the interlamellar shear strength and neighboring lamella because of the bridging fiber network previously described by other investigators.

1. Introduction

The gross anatomy of the annulus fibrosus consists of concentric lamella composed of parallel collagen fibers with orientation that alters from adjacent lamella (Coventry et al., 1945). Tear like defects in the annulus are classified usually by location and direction of the tear, as in rim lesion (RL), concentric tear (CT), or radiating tear (RT) (Vernon-Roberts et al., 2007). Such tears are common in adult human discs and strongly correlated to age. CT tears, comprised primarily of delamination of adjacent lamellae, are the most common and the first to appear (Osti et al., 1990; Vernon-Roberts et al., 2007).

Mechanically, annular tears decrease motion segment stiffness in bending, flexion, and torsion (Fazzalari et al., 2001; Thompson et al., 2000) and CT tears may affect other types of tears due to increased stress near the structural defect (Goel et al., 1995; Osti et al., 1990) but a direct connection between annular tears and pain has only been weakly established. In Thompson's study all cadaver donors were described as having "no history of back pain" and Fazzalari's study utilized animals. Osti et al. (1990) postulated that pain provocation during discography was "closely related to the presence of tears extending to the outer lamellae of the annulus fibrosus". Freemont et al. (1997) demonstrated that painful discs had more sensory innervation and deeper innervation than pain free discs and aged and pain free discs.

Recently Weiler et al. (2002) discussed a connection between annulus tearing/degradation and the presence of matrix metalloproteinases and cytokines that may affect pain. While the presence of sensory innervation near a lesion where shearing stresses are increased (Goel et al., 1995) seems a reasonable mechanism for pain generation, it has yet to be proven true. Nevertheless, tears in the annulus is evidence of structural overload, and presents a structural failure meeting Adams and Roughley (2006) proposed definition for a degenerated disc.

In the intervertebral disc, the use of genipin crosslinking has been shown to alter several mechanical parameters which may be advantageous in treating degenerative discs and back pain. For example, studies have demonstrated increased circumferential stiffness (Chuang et al., 2007), yield stress, and resilience (Slusarewicz et al., 2011) of annular tissue. Such effects also extend to the intervertebral joint behavior including changes in axial neutral zone parameters (Barbir et al., 2010; Yerramalli et al., 2007), flexion – extension neutral zone parameters (Hedman et al., 2006; Kirking et al., 2013), and maximum disc bulge during axial loading (Slusarewicz et al., 2011). It was hypothesized that changes in the disc mechanical behavior with cross linking would also

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extend to an increased resistance against shearing of the interlamellar interface. If true, then exogenous crosslinking of the annulus may have potential therapeutic benefits for the treatment or prevention of disc tears; and along with increasing the circumferential tensile strength of the annulus, decreasing disc bulge, and stabilizing the intervertebral joint, cross linking may be useful in the treatment and prevention of lower back pain.

Recently, Gregory et al. (2011) described a method of testing the interface strength between disc lamellae, observing that the shear stresses that lead to debonding are concentrated at the edge of the bonded interface. The described test presents a useful tool for understanding interlamellar tears and their propagation. Using this method, the primary objective of the present study was to test the hypothesis that genipin cross linking would increase the force necessary to debond the interlamellar interface when subjected to shear loading.

2. Methods

Adjacent musculature was removed from three fresh frozen bovine tails and the spines stored wrapped in plastic. While frozen, the caudal disc locations were identified, cut along the mid sagittal plane, and excised from the endplate. The nucleus was removed and the annulus samples placed in 0.15 M PBS to soak overnight at 4 °C. The free swelling enlarged the lamella making identification and dissection of the lamella interface easier. After swelling, specimens were refrozen and then while still frozen hand-dissected to isolate a single interlamella interface (Fig. 1) from the outer half of the annulus. Additional adjacent lamellae were maintained on both sides of the interlamellar interface of the specimen. Samples were treated for 4 h at 37 °C in 50 mM EPPS Phosphate buffer (ph9) (sham) or 50 mM EPPS Phosphate (ph9) with 20 mM genipin at 37 °C (crosslink treated) and then stored overnight at 4 °C in their respective solutions.

One disc per tail was dissected into four specimens producing 12 specimens from 3 tails. A second disc was taken from one tail yielding three additional samples resulting in 15 total samples. To reduce inter-specimen variability, samples were paired with two sham and two genipin treated samples from each disc, and each sham/genipin pair further grouped by size. Specimen interface width, thickness and length were measured using calipers after treatment and immediately before testing.

Mechanical testing was conducted using a Test Resources R-1000 frame and 100 N load cell. Custom rake fixtures were used to clamp the specimens (Fig. 2). The location of the interlamellar lap joint boundaries were marked on the lamina plane and joint midpoint was similarly indicated on the side of the specimen. Two digital cameras monitored the specimens and from the images, stretch of the joint was calculated using the boundary marks. The force – displacement output of the R-1000 was synchronized with the stretch by tracking the clamp positions. Three preconditioning cycles were performed at 1%/s to 10% strain and testing was carried out at 2%/s (Gregory et al., 2011). No attempt was made to test the effects of genipin at differing strain rates.

Using Octave, stiffness was calculated by fitting a line (Fig. 3) through the linear region of the force – displacement curve (normalized by lap joint width to compare directly with Gregory et al. (2011)). Yield force was taken where the measured data deviated from the linear fit by 2%. Peak force was taken as the largest force measured. Work was calculated as the area under the force – displacement curve up to yield and to peak. Statistical significance was tested using the Wilcoxon Rank Sum test on the paired differences with Stata R11.

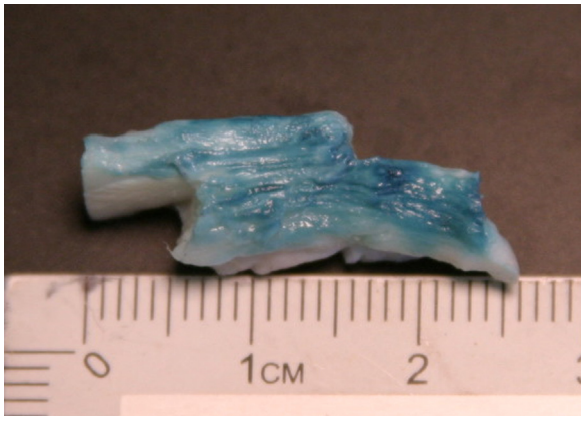

Fig. 1. Bovine annulus specimens after dissection to isolate a single interlamellar interface. Note presence of multiple lamella on either side of interface. Surface was colored with blue dye to enhance lamella boundaries.

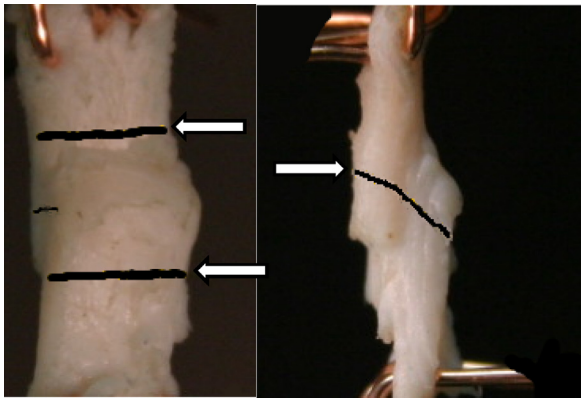

Fig. 2. Lamellar interface plane view (left) showing interface boundaries tracked during the experiment (arrows) and side view (right) showing marking indicating midpoint of interface.

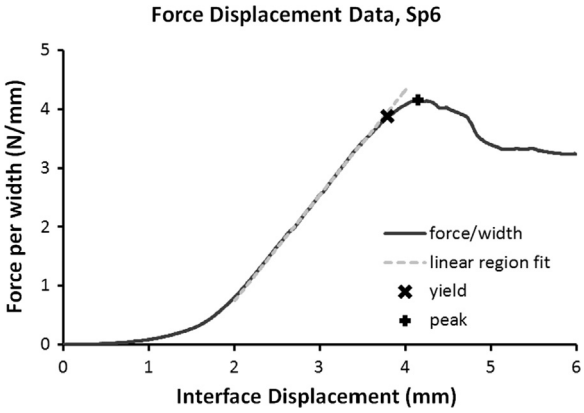

Fig. 3. Example of the force displacement data with the yield point, peak point, and linear region stiffness.

3. Results

Of the fifteen specimens six sham and six genipin treated specimens were successfully tested. These specimens all failed by debonding of the interlamellar interface. As the crosshead displaced, specimens would stretch as observed by continuity of the indicator mark across the midpoint of the interface. At peak force, the lamella adjacent to the interface would slip but not completely debond. As the displacement continued, the slipping would increase and the force would decrease.

The mean yield force per width was 2.43 N/mm for sham specimens (Table 1), 3.87 N/mm for genipin specimens (59% greater than sham specimens), and the paired difference between the groups was 1.81 N/mm (Fig. 4) which was statistically significant ($p < .018$). The mean peak force per width was 2.65 N/mm for sham specimens, 4.50 N/mm for genipin specimens (70% greater than sham specimens), and the paired difference between the groups was 2.23 N/mm which was statistically significant ($p < .018$). The mean work to yield was 2.16 J/mm for sham specimens, 3.65 J/mm for genipin specimens (69% greater), and the paired difference between the groups was 2.38 J/mm (Fig. 5) which was statistically significant ($p < .018$).