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The high-throughput phenotyping of the viscoelastic behavior of whole mouse intervertebral discs using a novel method of dynamic mechanical testing

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Abstract

Intervertebral disc (IVD) degeneration is highly correlated with lower back pain, and thus understanding the mechanisms of IVD degeneration is critical for the treatment of this disease. Utilizing mouse models to probe the mechanisms of degeneration is especially attractive due to the ease of manipulating mouse models and the availability of transgenics. Yet characterizing the mechanical behavior of mice IVDs remain challenging due to their minute size (approximately 540 μm in height and 1080 μm^2 in cross sectional area). We have thus developed a simple method to dynamically characterize the mechanical properties of intact mouse IVDs. The IVDs were dissected with the endplates intact, and dynamically compressed in the axial direction at 1% and 5% peak strains at 1 Hz. Utilizing this novel approach, we examined the effects of *in vitro* ribosylation and trypsin digestion for 24 or 72 hours on the viscoelastic behavior of the whole murine IVD. Trypsin treatment resulted in a decrease of proteoglycans and loss of disc height, while ribosylation had no effect on structure or proteoglycan composition. The 72 hour ribosylation group exhibited a stiffening of the disc, and both treatments significantly reduced viscous behavior of the IVDs, with the effects being more pronounced at 5% strain. Here we demonstrate a novel high-throughput method to mechanically characterize murine IVDs and detect strain-dependent differences in the elastic and the viscous behavior of the treated IVDs due to ribose and trypsin treatments.

Keywords

Murine model; intervertebral disc degeneration; trypsin; ribosylation; dynamic compression

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Introduction

Lower back pain is one of the most prevalent and expensive illnesses in today's society, affecting over 80% of people at some point in their lives (Hoy et al., 2012) and costing an estimated \$100 to \$200 billion dollars a year in the United States alone (Dagenais, Caro, & Haldeman, 2008). Additionally, intervertebral disc (IVD) degeneration is one of the strongest contributors to lower back pain (Cheung, 2010), and therefore understanding the mechanisms behind disc degeneration is critical in aiding the treatment of this disease.

The IVD is a fibrocartilaginous joint between two vertebral bodies. Each IVD has several rings of type I and type II collagen lamellae in the annulus fibrosus (Inoue & Takeda, 1975). The annulus surrounds an inner gelatinous nucleus pulposus, which is comprised of mainly type II collagen and proteoglycans, and is responsible for evenly distributing compressive loads between vertebral bodies (Adams, McNally, & Dolan, 1996). Two cartilaginous end plates lie above and below the IVD, connecting the IVD to the superior and inferior vertebral bodies (Grignon et al., 2000). The IVD, cartilaginous end plates, and adjacent vertebral bodies together make up a functional spinal unit (FSU).

Murine intervertebral discs are useful models to probe the mechanisms of degeneration due to the availability of transgenic strains. These knockout mouse models provide the opportunity to understand the regulatory and inflammatory processes that mediate IVD maintenance and degeneration (Hall & Cooke, 2011; Singh, Masuda, & An, 2005; Takao & Miyakawa, 2014). However, there is currently not a satisfactory system for characterizing the mechanical properties and functional integrity of isolated murine IVDs due to their minute size, which on average are only 540 μm in height and 1080 μm^2 in cross sectional area (O'Connell, Vresilovic, & Elliott, 2007). This is further confounded by the viscoelastic nature of the IVD, which would require either prolonged characterization by creep or dynamic mechanical characterization.

Bulk material test stands which are useful in characterizing tissues from larger mammals (Singh et al., 2005) often do not have a high enough resolution to detect subtle changes within the much smaller mouse IVDs. This problem has been alleviated by the use of custom built chambers or vices to anchor each specimen by gripping the vertebral bodies before mechanical testing (Bailey, Hargens, Cheng, & Lotz, 2014; Sarver & Elliott, 2004; Showalter et al., 2012). Although this allows the improved handling and manipulation of the sample, it is not possible to directly ascertain the mechanical behavior of IVD. The ability to directly quantify the mechanical behavior of the disc would be critical for understanding the mechanisms of disease progression, and the unique contributions of the disc to this process. It would also avoid confounding changes within the vertebral bodies with changes occurring in the intervertebral disc itself. To our knowledge, there has not been a published method that measures the mechanical properties of an isolated murine IVD without the vertebral bodies. Here we have developed a high-throughput method to measure the viscoelastic changes in the mechanical properties of isolated murine IVDs.

Methods

Thirteen C57/BL6 skeletally mature mice between 6 months and 8 months in age were euthanized and 3 intact lumbar vertebrae-disc-vertebrae FSUs (39 in total) were dissected from each animal as per Washington University in St. Louis Animal Studies Committee approval. The lumbar spine levels used were lumbar 1–2, lumbar 3–4, and lumbar 5–6, with posterior elements removed. The FSUs were rinsed in saline and cleaned of soft tissues using dissecting scissors prior to chemical treatment.

Chemical Treatment

We utilized two approaches to selectively modify the tissue-level mechanics of the discs: collagen crosslinking by ribose incubation (Jazini et al., 2012; Wagner, Reiser, & Lotz, 2006) and targeted proteoglycan cleavage by trypsin (Mwale et al., 2008; Périé et al., 2006). The segments were randomly assigned to five groups: ribose 24 hr (n = 8), ribose 72 hr (n = 8), trypsin 24 hr (n = 7), trypsin 72 hr (n = 7), and controls (n = 9). Increased nonenzymatic collagen crosslinking as well as a loss of GAGs have both been associated, among other changes, with disc degeneration (Pokharna and Phillips, 1998). The treatments were administered for either 24 hours or 72 hours to elicit a dose-dependent response. 0.6M ribose solution was prepared using D-Ribose (Sigma-Aldrich, St. Louis, MO) in PBS (pH = 7.2) with 5mM Benzamide (Sigma-Aldrich, St. Louis, MO). Trypsin solution 0.05% Trypsin-EDTA (Life Technologies, Carlsbad, CA) in PBS (pH = 7.2). The FSUs were incubated in 1 ml of treatment solution at 37°C for either 24 or 72 hours. After treatment, the discs were washed 3 times with PBS to remove any active reagent, wrapped in PBS soaked gauze, and stored at -20°C to prevent further degradation of the proteoglycan matrix.

Mechanical Testing

Just prior to mechanical testing, each sample was thawed and the intact intervertebral discs were isolated by carefully separating at the interface between the vertebrae and cartilage endplates. Cuts were made directly above the superior cartilage endplate and directly below the inferior cartilage endplate while carefully preserving the structural integrity of each endplate, utilizing a #11 blade scalpel aided by a dissection microscope (M400 Photomakroskop; Wild, Heerbrugg, Switzerland), (Fig 1). The endplates were kept intact to maintain the structure and ensure proper compressive behavior of the IVDs (MacLean, Owen, & Iatridis, 2007). The isolated IVDs were then attached to 1 cm × 1 cm × 0.3 cm aluminum platens and placed into a glass petri dish filled with PBS. Samples were kept in the PBS bath before, during, and between testing trials, and there was no visible signs of swelling or measurable damage.

The mechanical properties of the isolated intervertebral discs were determined using dynamic compression on a microindentation system (BioDent; Active Life Scientific, Santa Barbara, CA) with a 2.39 mm non-porous, flat probe. The probe's load cell resolution is 0.001 N, and the system's Piezo actuator resolution is 0.01 micron. In the PBS bath, each sample was moved into position under the probe tip by gripping the aluminum platen. The indenter tip was aligned over each sample so that the probe covered the entire diameter of the disc. Each disc was loaded sinusoidally at 2 different testing magnitudes of 1% strain

and 5% strain at 1 Hz for 20 cycles with a 0.03N preload (Fig 2A). Three technical replicates were done for each sample at each strain level with at least 30 min resting period between trials, resulting in a total of 6 measurements per sample. The loading slope value was obtained from the linear region of the force displacement curve and the loss tangent value was obtained by taking the tangent of the phase angle between the force and displacement curves. Our pilot studies indicated that there is no detectable mechanical damage to the samples at these testing parameters. These samples were maintained in physiological PBS solution (pH = 7.2) during and between trials to simulate the osmotic pressures found in the body and maintain hydration of the IVD (Costi, Hearn, & Fazzalari, 2002) (Fig 2B, 2C).

Structural and Compositional Data

The wet weight and height of each isolated disc was taken prior to mechanical testing utilizing an analytical balance (A-200DS; Denver Instrument Company, Bohemia, NY) and a laser micrometer (Keyence, Itasca, IL) respectively. The height was calculated from the average of three measurements taken along the diameter of the disc, one height from the mid-diameter and two heights from halfway between the midline and edges of the disc. Proteoglycan content was quantified using the colorimetric dimethyl-methylene blue assay with chondroitin sulfate from bovine cartilage (Sigma-Aldrich, St. Louis, MO) standards, and was normalized to wet weight of the IVD. Functional and compositional data were compared using a one-way ANOVA test with Tukey post-hoc multiple comparisons. A p-value of less than 0.05 is considered significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA).

Results

A total of 234 mechanical tests were taken during three testing sessions, with a total testing period of about 7 hours including the time needed to isolate the IVD and mount the discs. This results in a throughput rate of approximately 33 tests per hour. The variance of loading slope and tan delta of the control group were 2.44% and 5.82%, respectively.

Wet weight measurements of the control discs and the treatment discs revealed a significant reduction in wet weight due to 72 hours of trypsin treatment (-26.5%; $p = 0.0055$; Fig 3A). Correspondingly, the 72 hours of trypsin treatment also resulted in a significant reduction of disc height (-52.9%; $p = 0.0025$; Fig 3B) and proteoglycan content (-54.4%; $p = 0.0030$; Fig 3C).

Mechanical testing of the intact mouse IVDs revealed strain-dependent differences in the resulting viscoelastic behavior from the ribosylation and trypsin treatments. At 1% peak strain levels, the dynamic testing revealed a dose-dependent decline of tan delta in the trypsin-treated groups, with a 33.6% reduction ($p = 0.0428$; Fig 4A) in the 24 hour trypsinized group, and a 46.2% reduction ($p = 0.0025$; Fig 4B) in the 72 hour trypsinized group. Additionally there was a 43.2% reduction of tan delta in the 72 hour ribosylated group ($p = 0.0026$; Fig 4A). At the 5% peak strain levels, both of the 72 hour treated groups revealed significant losses in tan delta with a 52.6% reduction of tan delta in the 72 hour ribosylated group ($p = 0.0003$; Fig 4B), and a 45.9% reduction of tan delta in the 72 hour

trypsinized group ($p = 0.0034$; Fig 4B). The 72 hour ribose group was also 32.4% stiffer than the control ($p = 0.0342$; Fig 4D).

Discussion

Here we have described a novel technique to characterize the mechanical behavior of isolated murine IVDs. Because of the relative ease of the dissection (less than 2 minutes per sample) and the short testing duration (30 seconds per test), this method is suitable for high-throughput phenotyping of mouse IVDs. Our results detected significant changes in the mechanical behavior of chemically treated murine IVDs.

Both trypsin and ribose treatments altered the viscoelastic behavior of the IVDs from the control group, resulting in decreased tan delta, yet the mechanism for the loss of viscous behavior appears to be unique due to each treatment. Trypsin is a serine protease that catalyzes the hydrolysis of peptide bond of proteoglycans, which are a major constituent of the nucleus pulposus of the IVD, and trypsin incubation resulted in the time-dependent deterioration of the proteoglycan content as measured by DMMB (Fig 3C). The loss of proteoglycan decreases the disc's ability at maintaining hydration under load (Costi et al., 2002), and we accordingly observed the loss of tan delta in the trypsin-treated groups at both strains (Fig 4A,4B). Furthermore, the overall content of proteoglycans was correlated with the tan delta of the control, trypsin 24 hr, and trypsin 72 hr groups in both 1% ($r = 0.4843$; $p = 0.0165$; Fig 5A) and the 5% strain amplitude tests ($r = 0.5947$; $p = 0.0022$; Fig 5B).

However, the proteoglycan-tan delta relationship was disrupted in the ribose treatment groups (Fig 5C, D). Ribose is a highly reactive sugar that undergoes non-enzymatic glycation of proteins which produces advanced glycation end-products (AGEs) that can occur as collagen crosslinks between type I and type II collagen fibrils in the annulus fibrosis of the IVD (Jazini et al., 2012; Wagner et al., 2006). In addition to collagen, proteoglycans can also be cross-linked (Sivan et al., 2006) and previous work has shown that the increase of AGEs can adversely affect IVD's ability to retain water independently of its proteoglycan content (Jazini et al., 2012). Therefore, the decoupling of the proteoglycan-tan delta behavior may be a hallmark of non-enzymatic glycation. Non-enzymatic glycation has been associated with aging and degeneration (Pokharna & Phillips, 1998; Sivan et al., 2006); and diabetics are particularly susceptible to the accumulation of AGEs (Monnier, Kohn, & Cerami, 1984) and elevated disc injuries (Jhawar, Fuchs, Colditz, & Stampfer, 2006). Taken together, the loss of energy dissipation in the ribosylated tissue and the disruption of the proteoglycan-viscous relationship may be the mechanical mechanism for the increased susceptibility to damage and injury in AGEs-rich discs.

There are several limitations that should be noted with this technique. The testing of the samples is conducted with parallel surfaces that constrain the samples in unconfined compression and there may be uneven strain distribution of the endplate-bound intervertebral discs. We have addressed this by randomizing our experiments such that biological replicates of the same level segments were evenly distributed across treatment groups, but we suggest that a pivoting platen be implemented for samples with large lumbar curvature. Based our pilot tests, we found that at 1–5% strains the tests were highly

repeatable and no notable mechanical changes occurred in the samples with repeated testing. At strain magnitudes higher than 5%, we often observed mechanical damage in the form of stiffness loss in the samples, which is consistent with observations by other authors (O'Connell, Vresilovic, & Elliott, 2007; Showalter et al., 2012; Walter et al., 2011). We would thus continue to recommend these testing conditions to evaluation the mechanical function of the intervertebral disc.

It is also worth noting that the phenotyping approach here represent a first order analysis, and more detailed materials testing, such as by biphasic methods (Iatridis et al., 1998) would be recommended to fully capture the material level changes occurring in the disc. Nevertheless, we believe that the method we have described here would be useful screening phenotypes in mouse tissues, particularly in transgenics targeting the IVD (e.g., Abe et al., 2012).

In conclusion, we describe here a novel method to characterize intact murine intervertebral discs in a high throughput manner. The ability to test the isolated intervertebral discs provides the precise mechanical behavior and may reveal insights in the IVD's contributions towards the structural function of the spine.

Acknowledgments

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Fig. 1.

Dissection schematic showing that cuts were made directly above the superior endplate and directly below the inferior endplate. (A) Intact murine intervertebral discs were isolated by carefully separating at the interface between the vertebrae and cartilage endplates of each functional spinal unit. (B) The vertebral bodies were removed but both the superior and inferior cartilaginous endplates were maintained. (C)

Fig. 2.

Testing configuration for isolated intact murine intervertebral discs. (A,B) The samples are kept in a PBS bath throughout the testing and between trials to prevent dehydration. Testing waveform for 1% and 5% strain. (C)

Fig. 3.

Structural and compositional properties of each isolated intervertebral disc was measured, including wet weight (A), disc height (B), and proteoglycan concentration (C). Treatment by ribosylation did not affect wet weight, disc height, or proteoglycan concentration. Treatment by trypsin after 72 hours resulted in significant losses in wet weight ($p < 0.01$) and disc height ($p < 0.01$), concomitant with a significant reduction of proteoglycan concentration ($p < 0.01$).

Fig. 4.

Viscoelastic differences as measured by tan delta were observed in both the ribosylated and trypsinized groups. At a testing magnitude of 1% strain (A), the trypsin treated groups showed a dose dependent response with tan delta (ANOVA, $p < 0.05$), while only the ribose 72 hour group showed a reduction in tan delta (ANOVA, $p < 0.01$). At a testing magnitude 5% strain (B), both treatments after 72 hours resulted in a decreased response in tan delta (ANOVA, $p < 0.01$). Elastic differences as measured by loading slope were not consistently observed across testing magnitudes or treatment groups. At a testing magnitude of 1% strain (C), no differences in the elastic properties of the discs were measured between any of the treatment groups and the controls. At a testing magnitude of 5% strain (D), only the ribose 72 hour group showed an increased loading slope (ANOVA, $p < 0.05$).

Fig. 5.

In non-ribosylated discs (A,B), which comprised of the trypsin treated the discs and the controls, the proteoglycan concentration is positively correlated with the viscoelastic response of the IVDs. ($p < 0.01$). Ribosylation (C,D) disrupts the correlation between proteoglycan concentration and viscoelasticity.