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Short communication

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 h 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 h 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.

1. 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–$200 billion dollars a year in the United States alone (Dagenais et al., 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 and 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 et al., 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 and Cooke, 2011; Singh et al., 2005; Takao and Miyakawa, 2014). However, there is currently no 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 et al.,

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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 that 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 et al., 2014; Sarver and 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.

2. 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.

2.1. 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 et al., 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 h ($n=8$), ribose 72 h ($n=8$), trypsin 24 h ($n=7$), trypsin 72 h ($n=7$), 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 h or 72 h to elicit a dose-dependent response. 0.6 M ribose solution was prepared using D-Ribose (Sigma-Aldrich, St. Louis, MO) in PBS (pH=7.2) with 5 mM 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 h. 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.

2.2. 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 Photomakroscop; Wild, Heerbrugg, Switzerland), (Fig. 1). The endplates were kept intact to maintain the structure and ensure proper compressive behavior of the IVDs (MacLean et al., 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 were 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 μm. 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 0.03 N 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 et al., 2002) (Fig. 2B and C).

2.3. 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).

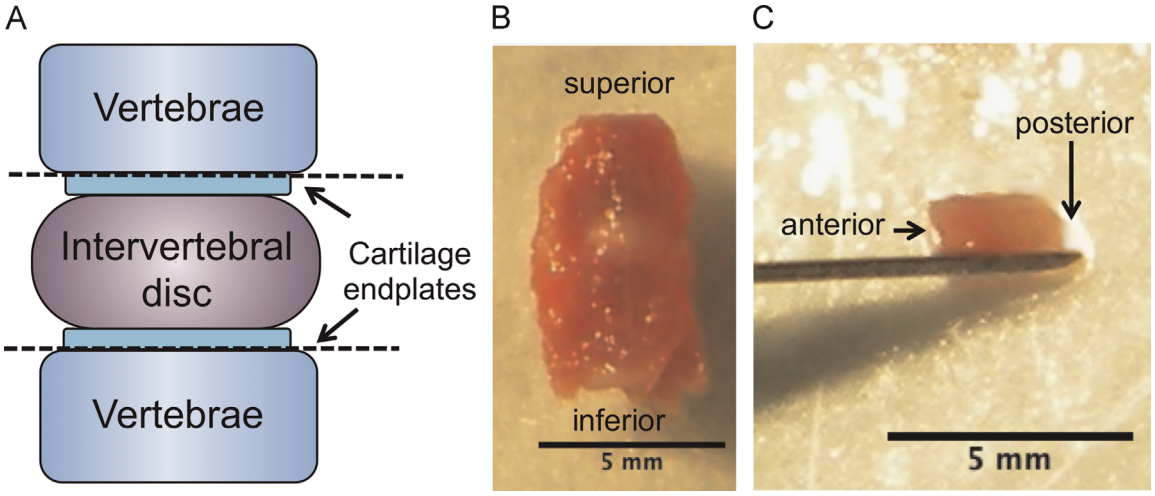

Fig. 1. Dissection schematic showing that cuts were made directly above the superior endplate and directly below the inferior endplate. (A) Intact murine intervertebral discs were isolated by carefully separating at the interface between the vertebrae and cartilage endplates of each functional spinal unit. (B) The vertebral bodies were removed but both the superior and inferior cartilaginous endplates were maintained (C).