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Proteoglycans contribute locally to swelling, but globally to compressive mechanics, in intact cervine medial meniscus

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ABSTRACT

Loss of charged proteoglycans in the knee meniscus, which aid in the support of compressive loads by entraining water, is an effect of degeneration and is often associated with osteoarthritis. In healthy menisci, proteoglycan content is highest in the inner white zone and decreases towards the peripheral red zone. We hypothesized that loss of proteoglycans would reduce both osmotic swelling and compressive stiffness, spatially localized to the avascular white zone of the meniscus. This hypothesis was tested by targeted enzymatic digestion of proteoglycans using hyaluronidase in intact cervine medial menisci. Mechanics were quantified by creep indentation on the femoral surface. Osmotic swelling changes were assessed by measuring collagen fiber crimp period in the radial-axial plane in the lamellar layer along both the tibial and femoral contacting surfaces. All measurements were made in the inner, middle, and outer zones of the anterior, central, and posterior regions. Mechanical measurements showed variation in creep behavior with anatomical location, along with spatially uniform decreases in viscosity (average of 21%) and creep stiffness (average of 15%) with hyaluronidase treatment. Lamellar collagen crimp period was significantly decreased (average of 27%) by hyaluronidase, indicating a decrease in osmotic swelling, with the largest decreases seen in locations with the highest proteoglycan content. Taken together, these results suggest that while proteoglycans have localized effects on meniscus swelling, the resulting effect on compressive properties is distributed throughout the tissue.

1. Introduction

The menisci are a pair of fibrocartilaginous structures in the intraarticular space of the knee which aid in the distribution of compressive stresses in the adjacent cartilage surfaces during loading of the joint (Andrews et al., 2017). Each meniscus (medial and lateral) is crescent shaped and anchored via ligamentous attachments at its anterior and posterior ends. The bulk of the meniscus consists of large bundles of Type I collagen (Herwig et al., 1984), which run along the circumferential axis, transitioning to ligamentous attachment horns at the anterior and posterior ends. These bundles are strengthened by a network of radial and vertical tie fibers (Andrews et al., 2014), as well as a thin lamellar layer which covers the femoral and tibial contact surfaces (Petersen and Tillmann, 1998) Collagen fibers are able to be subjected to large tensile strains due, in part, to their propensity to form a regular crimped pattern when relaxed (Diamant et al., 1972; Aspden et al., 1985) Collagen fiber crimp period is often used as a measurement of both applied (Miller et al., 2012) and residual (Duclos and Michalek, 2017) strain in soft tissues.

The remainder of the dry mass of the meniscus is made up of proteoglycans, which are large protein molecules with highly charged polysaccharide side chains (glycosaminoglycans). Proteoglycans, by way of fixed electrostatic charges, allow tissues to imbibe water and support compressive loads. Though proteoglycans make up an average of only 2.5% of dry weight in the meniscus (Herwig et al., 1984) (compared to 15% in articular cartilage (Maroudas et al., 1980) and 40% in intervertebral disc nucleus pulposus (Iatridis et al., 2007)) their high degree of localization in areas between circumferential collagen fiber bundles suggests an important function (Ling et al., 2016). The local concentration of proteoglycans varies in both the circumferential (from anterior to posterior) (Danso et al., 2017) and radial (from 8% of dry weight in the inner white zone to 2% of dry weight outer red zone) (Nakano et al., 1997; Sanchez-Adams and Willard, 2011) directions. Meniscus degeneration, which is often associated with knee osteoarthritis, is characterized by decreases in cell viability and proliferation along with proteoglycan loss (Melrose et al., 2008; Lopez-Franco et al., 2016). As proteoglycan content varies with

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anatomical locations, alterations in osmotic swelling and compressive stiffness subsequent to proteoglycan loss will result in an uneven distribution of contact stresses in the adjacent cartilage surfaces, potentially accelerating osteoarthritis (Maher et al., 2017).

Previous testing of bovine meniscus explants showed a complex role of proteoglycans, with removal causing a decrease in viscosity in all zones and a decrease in relaxation modulus in the white zone only, consistent with the factor of four difference in proteoglycan content relative to the red zone (Sanchez-Adams and Willard, 2011). While previous studies have shown a decrease in bulk compressive modulus of meniscus tissue explants (Yasura et al., 2007) with proteoglycan loss, the effect on the intact meniscus, with more physiological boundary conditions surrounding the test location, is unknown. Proteoglycan loss is expected to have two effects on meniscal contact mechanics: decreasing the effective compressive modulus of deeper layers of the tissue (via decreased osmotic pressure) and decreasing the tensile stiffness of the lamellar layer (via slackening of collagen fibers). Both of these effects are expected to be sensitive to specimen boundary conditions, with boundary permeability affecting fluid flow and fiber continuity affecting lamellar layer tension.

As naturally occurring meniscus degeneration is a multifactorial condition, characterized by tearing (Englund et al., 2009) and collagen fiber derangement (Fahmy et al., 1983) in addition to proteoglycan loss, determining the effects of proteoglycan loss alone is difficult using human cadaveric tissue. A more precise picture of the role of proteoglycans in meniscus structure and function may be obtained by targeted enzymatic digestion of healthy tissue using either chondroitinase ABC (Sanchez-Adams and Willard, 2011) or hyaluronidase (Yasura et al., 2007). As healthy human menisci are highly variable and difficult to obtain, various large animal models including sheep (Chevrier et al., 2009), pigs (Aspden et al., 1985), and cows (Andrews et al., 2014) have been used as tissue sources. Our lab has recently validated the North American white tailed deer (odicoileus virginianus) as an ex-vivo model of the human knee (Zaino et al., 2017). Of quadruped species, deer have knee range of motion, bony morphometry, and ligament strength closest to those of humans. Deer menisci are also of comparable size and structure to those of humans.

We hypothesized that loss of proteoglycans would reduce osmotic swelling and alter viscoelastic compressive properties, with a most pronounced effect in the avascular white zone of the meniscus. This hypothesis was tested by enzymatic digestion of proteoglycans from healthy intact cervine medial menisci and assessing compressive mechanics via creep indentation and swelling via lamellar layer crimp period.

2. Methods

2.1. Specimens

Eight left legs from wild harvested white tailed deer (5 male, 3 female, all approximately 3 years old) were obtained from a local meat processor. Medial menisci were removed via transection of the anterior and posterior horn attachments, wrapped in saline soaked tissues and frozen at −80 °C.

2.2. Enzyme treatment

Menisci were randomly divided into two experimental groups (n = 4 per group), hyaluronidase treated (HD) and PBS control. Enzyme treated specimens were thawed and placed in a 50 mL centrifuge tube with 35 mL of phosphate buffered saline (pH 6.5) and 44 mg of hyaluronidase (Type I-S from bovine testes, 400–1000 U/mg, Sigma-Aldrich, Sigma-Aldrich, St. Louis, MO). PBS control specimens were placed in saline only. All specimens were incubated at 37 °C for 24 h, then rinsed in PBS, wrapped tightly in aluminum foil and frozen at −20 °C until testing. Enzyme penetration into the tissue was verified by imaging specimens treated with fluorescently labeled hyaluronidase at various time points (see supplemental materials). The enzyme treatment was determined by preliminary calculations to be well in excess of that required to remove all proteoglycans from the tissue.

2.3. Indentation

Localized compressive mechanical properties were assessed using a creep indentation protocol. The indenter consisted of a 1.6 mm diameter probe with spherical tip attached to a steel rod in a linear ball bearing slide. Parallel to the steel rod was a linear potentiometer (Bourns Inc, Riverside, CA) connected to a data acquisition card (National Instruments, Austin, TX) and recorded using LabVIEW (National Instruments, Austin, TX). The height-voltage relationship of the indenter was calibrated using a dial caliper verifying linearity within the range of heights used for testing and a measurement precision of ±17 μm.

Specimens were thawed to room temperature immediately prior to testing and pressed into a dish of non-hardening clay (Sargent Art, Hazleton, PA) to support the tibial contact surface. Nine test points were located on the femoral contact surface of the specimen; inner, middle, and outer zones of the anterior, central, and posterior regions (Fig. 1). The specimen holder was mounted to an articulating ball head (Dolica, Rancho Cucamonga, CA) attached to an x-y-z translation table on a custom built indenter instrument. The stage and ball head were adjusted prior to each indentation test such that the surface of the specimen at the point being tested was perpendicular to the probe tip.

At each test point, the indenter was manually lowered into contact with the specimen and allowed to equilibrate under its own weight (0.36 N) for 60 s. A 2.17 N weight was then placed on top of the indenter, which was allowed to creep for 150 s. Pilot studies showed average creep time constants of approximately 50 s, and 150 s was chosen to provide sufficient data for curve fitting while minimizing total test time. Time and displacement data were collected at 20 Hz throughout each test. As the ball head mechanism used to position the specimen precluded testing in a fluid bath, the surface of the specimen was periodically moistened with PBS between indentation tests to prevent dehydration. The pattern of indentations at the nine test points was offset from specimen to specimen to ensure that they were not tested in the same order for any two specimens.

Creep displacement (D(t)) under static load (F_0) for each test was fit using a Kelvin-Voigt viscoelastic solid model (Eq. (1)) using MATLAB (Mathworks, Natick, MA) (Fig. 2). Goodness of fit suggested that for our measured displacements, this linear model was appropriate. Equilibrium (R_1) and creep (R_2) stiffnesses along with viscosity (η) were recorded for each test.

$$D(t) = \left(\frac{F_0}{R_1} - \frac{F_0}{R_2}\right) + \frac{F_0}{R_2}\left(1 - e^{\frac{-R_2 t}{\eta}}\right) \quad (1)$$

2.4. Histology

Following testing, menisci were fixed in approximately 30 mL of 10% neutral buffered formalin (Electron Microscopy Sciences, Hatfield, PA) at 4 °C for a minimum of 24 h. Specimens were then frozen at −20 °C and cut into anterior, central, and posterior blocks. The blocks were embedded in OCT compound (Sakura Finetek, Torrance. CA) and two sets of transverse cryosections (40 μm)

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