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Indentation Properties and Glycosaminoglycan Content of Human Menisci in the Deep Zone

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Abstract

Menisci are two crescent shaped fibrocartilaginous structures that provide fundamental load distribution and support within the knee joint. Their unique shape transmits axial stresses (i.e. 'body force') into hoop or radial stresses. The menisci are primarily an inhomogeneous aggregate of glycosaminoglycans (GAGs) supporting bulk compression and type I collagen fibrils sustaining tension. It has been shown that the meniscal superficial layers are functionally homogeneous throughout the three distinct regions (anterior, central and posterior) using a 300 μm diameter spherical indenter tip, but the deep zone of the meniscus has yet to be mechanically characterized at this scale. Furthermore, the distribution and intensity of GAG throughout the human meniscal cross-section has not been examined. This study investigated the mechanical properties, via indentation, of the human deep zone meniscus among three regions of the lateral and medial menisci. The distribution of GAG's through the cross-section was also documented. Results for the deep zone of the meniscus showed the medial posterior region to have a significantly greater instantaneous elastic modulus than the central region. No significant differences were seen for equilibrium modulus when comparing regions or hemijoint. Histological results revealed that GAG's are not present until at least $\sim 600 \mu\text{m}$ from the meniscal surface. Understanding the role and distribution of GAG within the human meniscus in conjunction with the material properties of the meniscus will aid in the design of tissue engineered meniscal replacements.

Keywords

Knee; Menisci; Glycosaminoglycans; Material Properties

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1.0 Introduction

Human menisci are two crescent shaped fibrocartilaginous structures that serve numerous functions within the knee including load transmission, improving joint congruity, and reducing friction between the tibia and femur [1–3]. The menisci wrap around the contours of the femoral condyles and mitigate stresses otherwise transmitted to the tibial plateau, thereby safeguarding the underlying articular cartilage and preventing osteoarthritis (OA) [4–7]. Diffusing approximately 70% of the load in the knee as hoop/circumferential stresses the menisci are also subjected to compressive and shear forces due to sliding and screw-home mechanisms of the joint [1], [8], [9]. Under this complex loading environment structural integrity is preserved by an inhomogeneous aggregation of solid constituents and interstitial fluid [10], [11].

Meniscal structure and function has been shown to be both depth and circumferentially dependent, as there are multiple hierarchical levels of collagen fibril orientations within the menisci [12–15]. Within the deep zone of the meniscus, type I collagen fibers are thought to be predominately circumferentially aligned and bolster meniscal strength in tension. Additionally, proteoglycans and interstitial fluid form a surrounding matrix supporting compressive loads via water-affine sulfated glycosaminoglycans (GAGs), a proteoglycan side-chain [11], [13], [16–19]. However, less is known about the depth dependent distribution of GAGs within human menisci. Recently, it has been shown that the depletion of sulfated GAG has a significant effect on the biphasic mechanical behavior of the bovine meniscus, further clarifying the structure-function relationship of GAG in the meniscus [19].

Mechanical testing has identified regional inhomogeneity of the human menisci among the anterior, central, and posterior regions of both lateral and medial menisci when tested in tension, compression, and shear (Figure 1) [10], [12], [14], [20], [21]. Specifically, investigation of the deep zone of the medial meniscus has shown significant variation between regions, with the posterior region having a significantly smaller compressive modulus (at physiological loading rate) than the anterior region [12]. The meniscal surface has previously been studied utilizing creep-indentation modalities [14], [22]. The equilibrium elastic modulus determined using a 300 μm diameter spherical indenter tip [22] was an order of magnitude greater than that of a study employing a 1 mm flat ended porous indenter tip [14]. The smaller indentation tip may have better isolated the superficial layer of the meniscus, whereas the larger indenter tip may have engaged the superficial *and* deep zone within the meniscus; therefore, it is necessary to isolate the deep zone to investigate regional meniscal properties.

To date, mechanical testing of meniscal tissue has been primarily conducted in tension, and compression on samples 1–3 mm in size, or using indenter tips of 1 mm [10], [12], [14], [19], [20], [23]. Research studying articular cartilage has shown material properties with a spherical indenter tip (2.5 mm radius) is effective in detecting stiffness changes following elastase digestion of collagen [24]. Indentation with a smaller size tip can be utilized to better isolate zones within the menisci [25], [26]. A complete proteoglycan chain is suspected to be approximately 1.2 μm in length, while most Type I collagen fibril bundles have a diameter $> 10 \mu\text{m}$, and are composed of 35 nm diameter fibrils [11], [13], [15], [27]. Therefore, the objective of this study was to quantify and compare the viscoelastic mechanical properties of the human meniscus in the deep zone, and explore quantitative histological GAG measurements. To accomplish this, indentation of the anterior, central and posterior regions of the lateral and medial human meniscus was performed. This characterization will provide an improved understanding of the mechanical behavior at a biologically relevant scale and will lead to advancements in tissue engineering of meniscal replacements.

2.0 Materials and Methods

2.1 Specimen preparation

Eight human non-arthritic knees (ages 50–65, avg. age 58) (NDRI, Philadelphia, PA) were procured with institutional review board approval (May 20, 2011) and the lateral and medial menisci were harvested, placed in a 0.9% saline solution and frozen at -20°C until the time of testing. It has previously been proven that meniscus and cartilage do not have significantly different material properties following a freeze-thaw cycle, and histology has corroborated this by showing biochemical content did not significantly change following freeze-thaw [28–30]. Each meniscus was thawed, trisected into anterior, central, and posterior regional sections, and lastly subdivided into two adjacent 2–3 mm wide sections from each region, one for mechanical testing and the other for histological analysis (Figure 1). For the histological samples, India ink was applied to the proximal surface for identification during imaging. Prior to mechanical testing, the proximal (femoral contacting) 2mm and distal (tibia contacting) 2mm were removed from the sample cross-section to expose the deep zone of the meniscus (Figure 1). Samples were trimmed on the non-testing face, to ensure indentation was conducted perpendicular to the meniscal deep zone. Nitrocellulose was applied to the face opposite of indentation (either proximal or distal meniscal superficial layer) followed by double-stick tape to facilitate adhesion between the sample and the well puck where the sample was placed during testing. Saline solution was filled in around the well to keep the specimen fully hydrated throughout the test.

Meniscal cross-sections from each region and anatomical location were processed for paraffin embedding, to then undergo a histological analysis for GAG distribution [31]. Once samples were processed with paraffin, 6 μm thick sections were sliced using a microtome (Shandon AS325, Thermo Electron Corp., Waltham, MA) and stained to identify GAG content. Specimens were first stained in Weigert's iron hematoxylin working solution for 10 minutes, followed by a 10 minute water rinse. Samples were then stained using Fast Green FCF solution for 5 minutes with a 10 second rinse of 1.0% acetic acid. Lastly, each sample was stained with 0.1% Safranin-O for 10 minutes and dehydrated using 95% and 100% ethanol and cleared using Xylene [32], [33].

2.2 Mechanical Testing Procedure

Mechanical indentation was performed at room temperature on the deep zone of the meniscus, on both samples from each region, using a commercially available indenter (Nanoindenter, Agilent Technologies, Santa Clara, CA) with a 300 μm diameter spherical ruby tip (Agilent Nano Measurements, Indianapolis, IN). Ten spatially randomized indents were performed on each meniscal sample, ensuring each indent was normal to the surface (Figure 1). Indents were spaced at least 250 μm apart to avoid possible residual effects from prior indents [25]. Creep indentation was performed using a trapezoidal loading sequence with a 5 second rise time and 1 mN hold for 90 seconds. These testing parameters were chosen based on preliminary testing results.

Resultant displacement-time data was curve-fit, using the Matlab optimization toolbox (Mathworks, Natick, MA), to a viscoelastic model developed by Oyen et al. [34], [35]. Instantaneous and equilibrium elastic moduli were calculated for all three regions, on both deep-proximal and deep-distal meniscal surfaces using previously outlined methods with the assumption that Poisson's ratio of the human meniscus is 0.38 [36]. From the Prony series used to describe the material creep, two time constants were determined (τ_1 , τ_2). Furthermore, an elastic-fraction was calculated by taking the ratio between equilibrium and instantaneous elastic modulus. This term describes material elastic/viscous behavior, with a value equal to 1 signifying a perfectly elastic material and a value equal to 0 embodying a

perfectly viscous material [37]. Material property values from each indent were calculated and then averaged to gain an overall average material property value from each sample to encompass the meniscal surface.

2.3 Histological Analysis

Histological slides of meniscal cross-sections were analyzed to measure three relative quantitative properties: the average distance from the meniscal surface to the initiation of staining for GAG content (Figure 2A), the ratio of the GAG cross-sectional area to the meniscal cross-sectional area (Figure 2B) and the binding intensity fraction of GAG within the meniscal cross-section. Prior to the first two analyses, images had a color threshold applied using commercially available software (Image J) [38] to signify where GAG content was present. Average thickness of the meniscal surface – to – GAG was performed using commercial software (BIOQUANT Image Analysis Corporation, Nashville, TN) with sectioned lines drawn along the meniscus articulating surface and where GAG was visually present (Figure 2A). Measurements were made every 20 microns.

The ratio of GAG to meniscal cross-section area was performed using commercially available software (Image J) [38]. A region of interest (ROI) was first drawn around the section to encompass the area from meniscal proximal surface to distal surface and the area was calculated. A second ROI only surrounding the GAG content was then measured (Figure 2B). The ratio of meniscal GAG content area to meniscal cross-sectional area was then determined.

The binding intensity of the GAG stain was measured by selecting a ROI around a portion of the inner meniscal GAG content. The intensity values of red (R), green (G) and blue (B), with values from 0 to 255 assigned was measured and the proportion of red color (GAG stain) was calculated using the equation $r = R / (R^2 + G^2 + B^2)^{1/2}$. The intensity fraction of red (r) with respect to the other primary colors was quantified [31], [39].

2.4 Statistical Analysis

One way Analysis of Variance (ANOVA) was performed to determine differences among meniscal regions for quantitative values from indentation and histological analyses. When significant results were identified by ANOVA, a post-hoc Student's two-tailed *t*-test was conducted to compare individual regional values amongst one another. Additionally, material properties from indentation, GAG fraction intensity and GAG/meniscal area ratio values were all analyzed for significant differences using a Student's two tailed *t*-test to determine significant differences between same regions in the lateral and medial menisci. Quantitative histological values measuring the distance from meniscal surface to GAG content were statistically analyzed with the Student's two tailed *t*-test to compare proximal and distal surface values for respective regions and anatomical locations. $p < 0.05$ was considered significant for all tests.

3.0 Results

Individual indents resulted in a displacement-time curve with a typical linear region during the ramp loading phase, followed by a horizontally asymptotic curve during the held load, indicative of equilibrium (Figure 3). Equilibrium was defined when a change in displacement was $< 0.01\%$. The average time to equilibrium was approximately 67.5 seconds.

No significant difference was seen between deep zone indentation results from proximal or distal cut samples, so values from the two samples were averaged together. As numerous indents were conducted on each individual sample, the variation among the indents was

calculated to assess the homogeneity of each sample. The variation among indents on each sample was calculated to be less than 4% of the respective material property value, indicating homogeneity within a sample. The average indenter contact radius was determined using Hertzian contact as $r = \sqrt{Rd}$, where R is the radius of the indenter and d is the displacement into the surface. As the displacement into the meniscal surface was approximately 11 μm for each indent, the contact radius calculated was $41 \pm 2 \mu\text{m}$. The average contact area of indentation was approximately 5000 μm^2 .

Regional examination showed no significant differences for the lateral meniscus for any of the material properties; however the medial-central meniscal region was significantly different than posterior for both the instantaneous modulus and elastic- fraction properties (Figure 4A & C). There were no significant differences between respective lateral or medial regions for equilibrium elastic modulus (Figure 4B). The posterior region of the medial menisci had the largest instantaneous elastic modulus and a small equilibrium elastic modulus (Figure 4A & B), resulting in a low elastic- fraction (Figure 4C). There were no significant spatial differences for the two time constants (Table 1).

Visible inspection showed that GAG stain was weak along the proximal and distal meniscal surfaces (Figure 5). The central region for both the lateral and medial menisci had the largest GAG fraction intensity values when compared to the anterior and posterior regions (Table 2). No significant differences among all regions and anatomical locations were seen for the ratio of GAG area –to- meniscal area (Table 3).

The distance from meniscal surface to the initiation of GAG staining ranged from approximately 500 to 1500 μm , with the medial meniscus having greater distance values than the lateral region (Figure 6). Only the lateral posterior region displayed a significant difference between the two meniscal surfaces ($607 \pm 38 \mu\text{m}$ on the distal surface compared to $826 \pm 83 \mu\text{m}$ on the proximal surface). Distal surface posterior values were significantly different between lateral and medial menisci, while proximal surface values were significantly different between the anterior and posterior regions for the lateral menisci (Figure 6). Additionally, it can be seen that the medial menisci displayed a much higher variability throughout all regions when compared to the lateral menisci for both proximal and distal values (Figure 6).

4.0 Discussion

This is the first study to isolate and examine the deep zone of the human meniscus with a small indenter tip. Indentation was a viable test modality to gather local mechanical properties from the deep zone of human menisci and results were repetitive for each proximal-side and distal-side meniscal deep zone sample. Equilibrium moduli results from this study are approximately two orders of magnitude larger than another compressive based study, also conducted at room temperature ($\sim 1.5 \text{ MPa}$ vs. $\sim 0.03 \text{ MPa}$) [12]. Interestingly, properties measured in this study are on the same order of magnitude as previous indentation results for both articular cartilage and meniscus, with similar testing parameters and environments [22], [29], [40], [41].

In the current study, the medial-posterior region had the lowest elastic-fraction value (Figure 3C), agreeing with prior indentation-based investigation showing the medial posterior region to have a lower aggregate modulus compared to the anterior region [14]. In contrast to these studies, an unconfined compression study on cubic shaped human meniscal samples by Chia et al., 2008 showed the medial anterior region to have a statistically greater instantaneous elastic modulus when compared to the posterior region [12]. Clinically, the medial posterior region is torn most frequently [42–48]. The relationship between indentation modulus and

increased tear frequency is interesting and may provide insight into meniscal injury mechanics.

It is interesting to note that while articular cartilage is known to have roughly 8 times as much proteoglycan content as the meniscus, the indentation moduli do not appear to reflect this [16], [49–51]. The current study reports equilibrium elastic modulus for meniscus (superficial and deep) to be 1.5 ± 0.04 MPa, while previous indentation studies on articular cartilage reported equilibrium elastic modulus values of approximately 2.9 ± 0.4 MPa, only a 2 fold increase, not 8 fold as seen with proteoglycan content [22], [40], [41], [52]. Permeability coefficients for meniscus and cartilage appear to be very similar [14], [53] and may help explain these results.

Only one other study has reported regional sulfated GAG values, finding no regional variations; however, the study did not isolate GAG from the deep zone, but rather the entire cross-section [10]. Thus, our study is unique in that GAG intensity was measured for deep zones of each region of the meniscus main body (anterior, central and posterior). This is the first study to our knowledge that quantifies the thickness of the superficial zone that is essentially devoid of GAG. Our data shows a significant difference in thickness of the GAG superficial zone between the anterior and posterior regions of the lateral meniscus. It can be seen that GAG is not present until approximately 600 μm from the meniscal surface (Figure 5) and only encompasses approximately 50% of the meniscus area (Table 2). This discovery suggests that GAG within the meniscus does not have the same depth dependency as collagen fibrils. The meniscal superficial layer, a taut network of collagen fibrils oriented parallel to meniscal surface is suspected to be approximately 100–200 μm throughout each meniscal region [13], [54].

The current study shows that the bulk of GAG is concentrated in the deep zone of the meniscal cross-section. Taken together with the GAG-less superficial zone, this may explain why indentation testing required a longer time to achieve equilibrium condition as compared to the GAG-less meniscal surface; 67 seconds compared to 57 seconds [22]. The GAG-less meniscal surface will likely not retain as much interstitial fluid as the GAG-rich deep zone, resulting in less resistance to compression and time to reach equilibrium. These times to reach equilibrium (67–57 seconds) are similar to previous literature in which time to equilibrium (~50 seconds) was reported for the medial and lateral articular cartilage hemijoints[55]. Others have chemically depleted GAG from articular cartilage and shown that with 55% deletion of GAG there is no change in the time constants[56]. It is important to note that time constants have been shown to be influenced by the ramp loading rate in polymers[57]. For slower loading rates, creep also occurs during the loading time. A study using atomic force microscopy nanoindentation studied material properties of normal-healthy and proteoglycan depleted articular cartilage [58]. In this study, depletion of proteoglycans significantly decreased indentation modulus. Further, The material properties obtained from the deep zone in the current study are similar to previous results on the superficial layer despite differences between these zones in collagen fiber orientation and GAG content [22]. For both the superficial and deep zones, equilibrium moduli are approximately 1.5 ± 0.04 MPa and instantaneous moduli are 3.5 ± 0.1 MPa [22]. This surprising result warrants further investigation as to the structures that are engaged during indentation with a 300 μm diameter spherical tip.

Both histological and mechanical results from this work will prove imperative to better develop a structure function relationship and effectively tissue engineer meniscal replacements [59]. Documentation of the regional mechanical properties of the deep zone of the menisci will help understand the circumferential and radial inhomogeneity of the human menisci and lead to advancements in meniscal replacement design.

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Figure 1.

Schematic of human menisci showing where three regions are located and what testing slices resembled. Enlarged meniscal cross-section is shown. Two 2–3 mm slices were taken from each region for mechanical testing and histology analysis. Both analyses were conducted in the middle meniscus (inner, middle and outer meniscus labeled) section and indentation was normal to the deep zone meniscus cut surface. Dashed lines represent specimen cutting.

Figure 2.

Representative histology images that have had a color threshold applied to show location of GAG content with A) showing outline of meniscal surface region without GAG content and B) showing the outline of meniscal cross-sectional area ROI and GAG cross-sectional area ROI. Figure 2A was used to calculate the average distance from meniscal surface to the presence of GAG and Figure 2B was used to calculate the ratio of meniscal GAG to cross-sectional area.

Figure 3. Representative time-displacement curve showing best fit of the creep function to the displacement data.

Figure 4.

A. Average instantaneous elastic modulus- B. Average equilibrium elastic modulus- C. Average Elastic-Fraction values from indentation conducted on the deep zone of human meniscal samples * represents a significant difference between the connecting lines for regions ($p < 0.05$). Error bars represent standard error.

Figure 5.
Image showing a stained medial anterior specimen. Red stain = GAG

Figure 6.

Average distance from meniscal surface to GAG presence values for both proximal and distal surfaces of the anterior, central and posterior regions from lateral and medial menisci. ▼ represents a significant difference between proximal and distal surface values; # represents a significant difference between the connecting lines for regions within respective anatomical locations and * represents a significant difference between the connecting lines between anatomical locations within respective regions ($p < 0.05$). Error bars represent standard error.

Table 1

Time constants from viscoelastic indentation (mean $\pm$ standard deviation).

	τ_1, τ_2 (seconds)		
	Anterior	Central	Posterior
Lateral	4.7 $\pm$ 1.1,	4.8 $\pm$ 1.1,	4.6 $\pm$ 0.5,
	41.3 $\pm$ 9.1	42.4 $\pm$ 4.0	41.1 $\pm$ 4.8
Medial	4.7 $\pm$ 0.9,	4.4 $\pm$ 0.7,	4.8 $\pm$ 1.0,
	45.6 $\pm$ 6.4	41.2 $\pm$ 13.3	53.1 $\pm$ 16.1

Table 2

GAG fraction intensity measured from histological images of regional meniscal cross-sections (mean $\pm$ standard error).

	GAG fraction Intensity		
	Anterior	Central	Posterior
Lateral	0.64 $\pm$ 0.01 *	0.65 $\pm$ 0.03	0.60 $\pm$ 0.02
Medial	0.61 $\pm$ 0.02	0.63 $\pm$ 0.03	0.60 $\pm$ 0.02

* represents a significant difference from the lateral – posterior region

Table 3

Ratio of GAG - to - Meniscal Area measured from threshold histological images of regional meniscal cross-sections (mean $\pm$ standard error).

	Ratio of GAG - to - Meniscal Area		
	Anterior	Central	Posterior
Lateral	0.60 $\pm$ 0.04	0.58 $\pm$ 0.09	0.50 $\pm$ 0.05
Medial	0.50 $\pm$ 0.06	0.46 $\pm$ 0.09	0.45 $\pm$ 0.09