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Regional variation in the mechanical role of knee meniscus glycosaminoglycans

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Sanchez-Adams J, Willard VP, Athanasiou KA. Regional variation in the mechanical role of knee meniscus glycosaminoglycans. *J Appl Physiol* 111: 1590–1596, 2011. First published September 8, 2011; doi:10.1152/japplphysiol.00848.2011.—High compressive properties of cartilaginous tissues are commonly attributed to the sulfated glycosaminoglycan (GAG) fraction of the extracellular matrix (ECM), but this relationship has not been directly measured in the knee meniscus, which shows regional variation in GAG content. In this study, biopsies from each meniscus region (outer, middle, and inner) were either subjected to chondroitinase ABC (CABC) to remove all sulfated GAGs or not. Compressive testing revealed that GAG depletion in the inner and middle meniscus regions caused a significant decrease in modulus of relaxation (58% and 41% decreases, respectively, at 20% strain), and all regions exhibited a significant decrease in viscosity (outer: 29%; middle: 58%; inner: 62% decrease). Tensile properties following CABC treatment were unaffected for outer and middle meniscus specimens, but the inner meniscus displayed significant increases in Young's modulus (41% increase) and ultimate tensile stress (40% increase) following GAG depletion. These findings suggest that, in the outer meniscus, GAGs contribute to increasing tissue viscosity, whereas in the middle and inner meniscus, where GAGs are most abundant, these molecules also enhance the tissue's ability to withstand compressive loads. GAGs in the inner meniscus also contribute to reducing the circumferential tensile properties of the tissue, perhaps due to the pre-stress on the collagen network from increased hydration of the ECM. Understanding the mechanical role of GAGs in each region of the knee meniscus is important for understanding meniscus structure-function relationships and creating design criteria for functional meniscus tissue engineering efforts.

structure-function relationships; cartilage; chondroitinase abc; musculoskeletal

FUNCTIONING UNDER SHEAR, COMPRESSION, and tension, the knee meniscus relies on the complex organization of its biochemical constituents to distribute load and absorb shock in the joint. The meniscus bears between 45 and 75% of knee joint loads, which are estimated to be 2.7–4.9 times body weight while walking (37, 41). Unfortunately, the knee meniscus is prone to tears and degeneration, which are unable to heal effectively in the outer and middle portions of the tissue. These injuries are especially devastating in the inner, nonvascularized portion, where a healing response is absent (2, 5, 9, 16, 21, 32, 44). Tissue engineering efforts seek to address this problem by creating functional meniscus tissue for replacement. A complete understanding of the structure-function relationships that exist in the knee meniscus can help advance these tissue engineering efforts by identifying essential design criteria that can enhance the functionality of engineered tissues.

As a biphasic tissue, the knee meniscus relies on the interplay of solid and fluid components to achieve its viscoelastic characteristics (12, 33, 34, 38, 50). Composed of ~30% organic matter and 70% water, the meniscus is highly hydrated (9). The flow of the water fraction in and out of the tissue during loading plays a role in the tissue's viscoelastic behavior and allows for the exchange of nutrients between the synovial fluid and meniscus (14). The solid fraction, dominated by collagenous proteins and, to a lesser degree, sulfated glycosaminoglycans (GAGs), provides structure to the tissue. Collagen, the most abundant biochemical component in the meniscus, has been well characterized in terms of its distribution, organization, and mechanical contribution to meniscus mechanics. Microscopic and mechanical analyses of meniscal collagens have revealed that these proteins are organized mainly in the circumferential direction of the tissue, with some radially oriented fibers throughout (7, 15, 42). Tensile tests in the circumferential and radial directions indicate that tensile properties are 3- to 10-fold higher in the circumferential direction than in the radial direction (14, 42).

The mechanical contribution of sulfated GAGs in the meniscus, however, is not as well understood. Although collagen is abundant throughout the meniscus, sulfated GAG content is scarce in the outer region of the meniscus and increases in abundance moving radially inward. In tissues with high-sulfated GAG content, such as articular cartilage, GAGs mainly contribute to tissue compressive properties; the negatively charged aggrecan molecule, a proteoglycan with many GAG side chains, attracts water molecules and therefore helps to resist the flow of water out of the tissue while under compression (27, 34, 35, 43). In articular cartilage, however, sulfated glycosaminoglycans are, on average, eightfold more abundant than in the knee meniscus, which may indicate a difference in the mechanical role of GAGs in these two tissues (1, 20, 28, 29, 44).

The mechanical role of sulfated GAGs in musculoskeletal tissues has been investigated through selective digestion of sulfated GAGs and subsequent mechanical testing. A common method of depleting GAGs from native tissues is to use the catabolic enzyme chondroitinase ABC (CABC), which depolymerizes chondroitin sulfate, dermatan sulfate, and, to a lesser degree, hyaluronan (17). CABC has been used previously to investigate the contribution of GAGs to the mechanical properties of various musculoskeletal tissues. GAG-depletion of articular cartilage has been shown to reduce the tissue's compressive modulus and increase tissue permeability (23). In the tendon, a tissue similar to the knee meniscus in terms of GAG content, results suggest that sulfated GAGs impart higher

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tissue viscosity in the transverse direction (perpendicular to collagen alignment) (19). A study investigating the human medial collateral ligament, however, showed that dynamic viscoelastic properties of the tissue were largely unchanged by sulfated GAG removal (26). These results show that GAGs in different tissues play varying roles in the tissue's mechanical properties, indicating that they may affect regional meniscus mechanics as well.

This study, therefore, investigates the regional contribution of sulfated GAGs to meniscus compressive and tensile mechanics. CABC is used to deplete GAGs from each meniscus region, and compressive and tensile material properties are compared between depleted and control specimens. It is hypothesized that GAG depletion will have varying effects on material properties regionally in the meniscus. The results from this study should inform further tissue engineering efforts to recapitulate the meniscus and allow for a better understanding of regional variations in meniscus mechanics.

MATERIALS AND METHODS

Medial menisci from 2-wk-old bovine knees (Research 87, Boston, MA) were surgically removed and frozen in saline solution with protease inhibitors until treatment and mechanical testing were performed. In *phase 1* of the study, the minimum treatment time required to remove all of the sulfated GAGs (sGAG) from each region was determined. Samples from each region (inner, middle, and outer meniscus) were dissected from the tissue and either treated with chondroitinase ABC (CABC) in an activation solution or placed in the activation solution without CABC (untreated control). Treated samples were placed in a 1 U/ml chondroitinase ABC (CABC) (Sigma-Aldrich, St. Louis, MO) solution containing 50 mM Tris, 60 mM sodium acetate, and 0.02% bovine serum albumin and incubated with gentle shaking at 37°C. Following treatment or incubation in buffer, samples were placed in an inactivation solution (1 mM Zn^{2+} with 50 mM Tris) for 15 min with gentle shaking at 37°C. Three samples from each region were treated for either 1, 3, 6, 12, and 24 h. Biochemical analysis was performed on each sample to determine sGAG and collagen content per dry weight of tissue. sGAG content vs. time data were fit with an exponential decay model, and the half-life for GAG depletion for each region was determined. A one-way ANOVA was performed on the data for each region with a significance level of $P < 0.05$.

The appropriate treatment time determined in *phase 1* was then carried forward to *phase 2*, where compressive and tensile mechanical testing was performed on each GAG-depleted region and untreated control. For compressive samples, 3-mm dermal punches were used to obtain inner, middle, and outer meniscus samples, which were cut to ~1 mm thickness with razorblades. Unconfined compression stress-relaxation testing was performed in the axial direction on each tissue sample. The diameter and sample thickness was determined just before testing. Samples were preconditioned with 15 cycles of 0–5% compressive strain, and then stress-relaxation tests were carried out at 10 and 20% strain. As described previously, a Kelvin solid viscoelastic model was fit to the data to yield the following compressive material properties at each strain level: instantaneous modulus (E_i), modulus of relaxation (E_r), and viscosity (μ) (4). For tensile samples, circumferential strips of the inner, middle, and outer portions of the meniscus were cut into dog bone shapes with an average thickness of 1 mm. Sample thickness and gauge length was determined just before mechanical testing. Tensile strain-to-failure testing was carried out in the circumferential direction of the tissue, with preconditioning of 15 cycles of 0–2% strain, followed by tensile testing at 1% strain/s. The linear portion of each stress-strain curve was used to determine the Young's modulus (E_y) of each sample, and the ultimate tensile stress (UTS) was determined from each curve. An integration of the stress-strain curve was used to determine the toughness, or energy to failure, of each sample. GAG depletion was verified for each region histologically using Safranin-O staining as well as biochemically. Collagen content of the regional samples was also determined biochemically.

Four to six samples were used for each region and treatment group, and Student's *t*-tests were performed between treated and untreated groups. Statistical significance was set at $P < 0.05$.

RESULTS

Results from *phase 1* of the study are shown in Fig. 1. Inner, middle, and outer meniscus samples were treated with CABC for 0, 1, 3, 6, 12, or 24 h. In the untreated state, the inner meniscus contained the most sulfated GAG per dry weight (3.88 ± 1.5%) compared with the outer (0.91 ± 0.33%) and middle (1.2 ± 0.42%) regions. When treated with CABC, it was found that the outer and middle meniscus displayed similar GAG depletion profiles, with half-lives of 0.325 and 0.456 h, respectively. In contrast, the inner meniscus GAG depletion profile displayed the longest time to full depletion, with a half-life of 0.899 h. Collagen content for each region was unaffected by CABC treatment, and it was found that the inner meniscus had statistically less collagen than the outer and

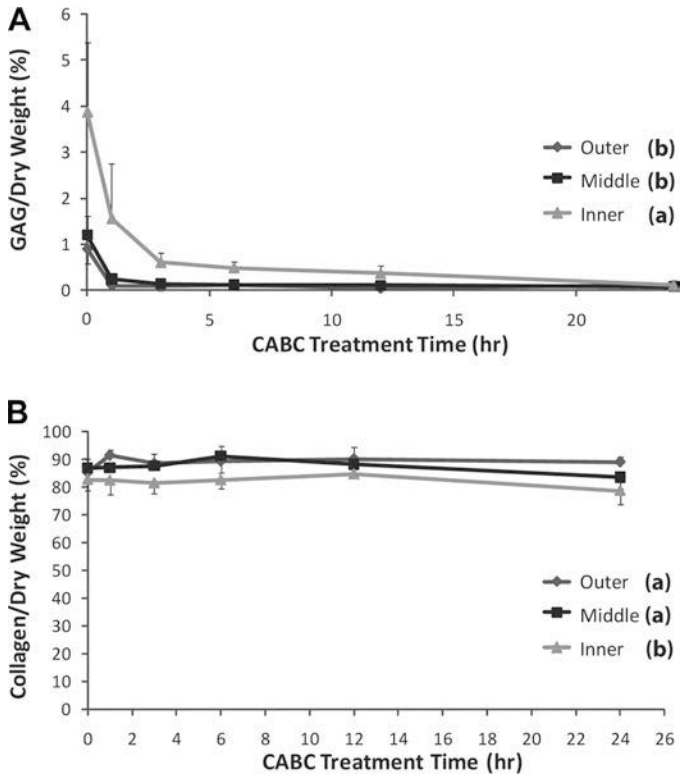

Fig. 1. Temporal effects of chondroitinase ABC (CABC) treatment in different meniscus regions. Percent sulfated glycosaminoglycan (GAG) content (*A*) and total collagen content (*B*) per dry weight was measured for outer, middle, and inner meniscus specimens treated with CABC for 0, 1, 3, 6, 12, and 24 h. Inner meniscus specimens contained more sulfated GAG per dry weight than the outer and middle specimens before CABC treatment and required longer treatment time to reach full GAG depletion. Collagen content remained unchanged in all groups in response to CABC treatment, and the inner meniscus was found to contain statistically less collagen than the outer and middle meniscus. Each data point represents the average measurement and standard deviation. Significant results from the one-way ANOVA performed on the data are shown in the legend, where groups not connected by the same letter are statistically different from each other.

middle meniscus. The outer and middle meniscus contained 89.01 ± 4.80% and 87.07 ± 4.62% total collagen, respectively, whereas the inner meniscus contained 82.04 ± 3.75%. Based on these results, it was determined that the middle and outer meniscus specimens would be treated with CABC for 3 h, and the inner meniscus specimens would be treated for 24 h to ensure full GAG depletion in *phase 2*.

In *phase 2*, outer, middle, and inner meniscus explants were treated with CABC for the duration determined in *phase 1* and tested under compression and tension and compared with untreated controls. Histological and biochemical assessment of untreated and treated explants verified that GAG depletion was achieved for all three regions (Fig. 2). Additionally, biochemical analysis of collagen content for treated and untreated samples confirmed that no change in collagen content was observed in any of the regions (Fig. 2).

Compressive testing results are shown in Fig. 3. Unconfined compression stress-relaxation testing on CABC treated samples showed that GAG depletion reduced the coefficient of viscosity for all regions compared with untreated controls. For the inner and middle regions, GAG depletion also significantly reduced the tissue's modulus of relaxation and caused a trend lower in the tissue's instantaneous modulus. These statistical differences were seen both at the 10 and 20% strain levels. All fits to the experimental data produced R^2 values of >90%, indicating that the viscoelastic model used was a good approximation of the tissue's behavior.

Tensile material properties of control and GAG-depleted meniscus specimens are shown in Fig. 4. The tensile properties of the middle and outer meniscus were not significantly affected by CABC treatment either for ultimate tensile stress or Young's modulus. However, CABC treatment did significantly increase inner meniscus Young's modulus (40.75% over untreated control) and ultimate tensile stress (40.55% over untreated control). Additionally, inner energy to failure increased significantly with CABC treatment compared with the untreated control.

DISCUSSION

This study investigated the effects of sulfated GAG-depletion on the material properties of the inner, middle, and outer meniscus to elucidate structure-function relationships in the knee meniscus. Viscoelastic compressive testing showed that GAG depletion causes decreased tissue viscosity in all regions of the meniscus, as well as decreased modulus of relaxation in the inner and middle regions. Statistically significant decreases in compressive properties for all regions were observed at both 10 and 20% strain levels, indicating that GAGs are mechanically important at even low tissue strains. Tensile properties of the inner region were also found to be increased following GAG depletion, suggesting that sulfated GAGs play a role in the meniscus tensile characteristics. Understanding these contributions of sulfated GAGs to the mechanical properties of the meniscus can help explain the complex structure-function relationships that exist in the tissue. As the meniscus undergoes both static and dynamic compression, tension, and shear under normal loading conditions, it is vital to elucidate the major

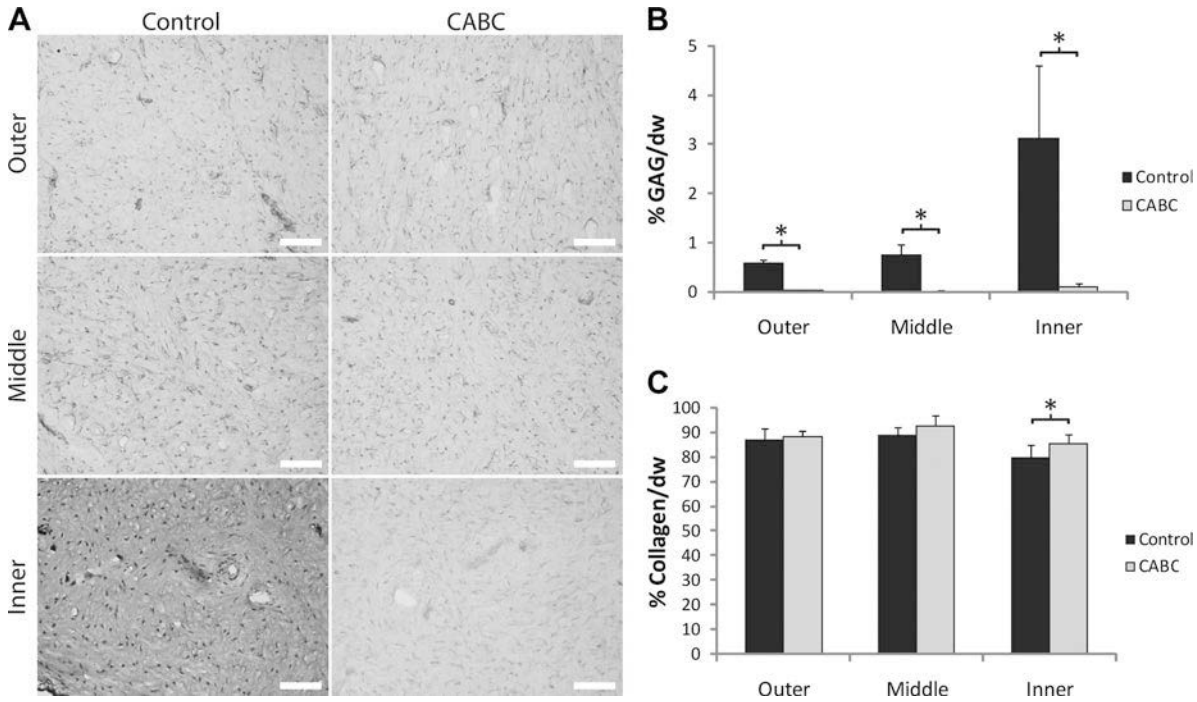

Fig. 2. Histology and biochemistry of control and CABC treated specimens. Safranin-O staining (*A*), and biochemical analyses for sulfated GAG content (*B*) and total collagen content (*C*) were performed on control and CABC-treated specimens from the outer, middle, and inner regions of the meniscus. Outer and middle specimens were treated with CABC for 3 h, and inner specimens were treated for 24 h, as determined in *phase 1*. Positive staining for sulfated GAGs could be detected readily in control specimens from the inner region and to a lesser degree in the middle and outer regions; all regions were negative for sulfated GAGs following CABC treatment (*A*). Biochemical analysis confirmed histological results, with all regions showing >95% decreases in GAG content following CABC treatment (*B*). Percent collagen content per dry weight in the outer and middle regions remained unchanged following GAG-depletion, and increased in the inner region (*C*). Scale bar: 100 μm. Student's *t*-tests were performed in *B* and *C*, with significance set at $P < 0.05$ and indicated by an asterisk.

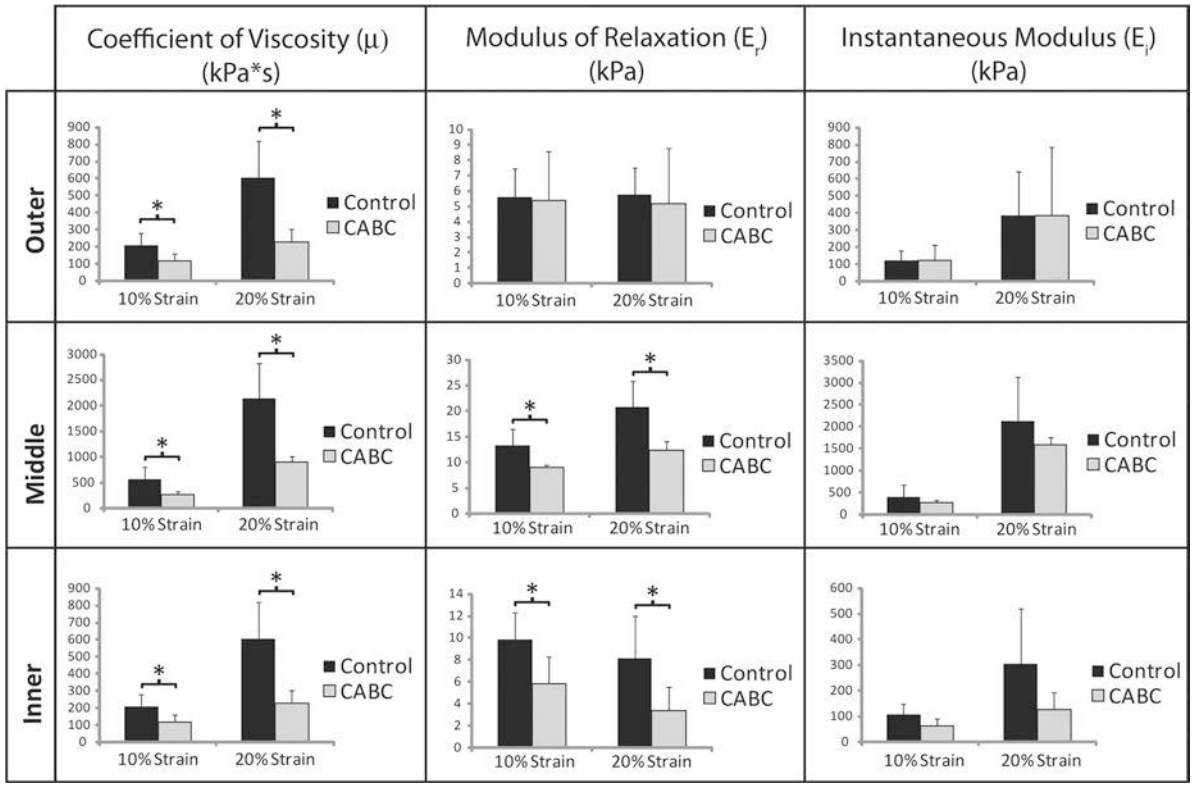

Fig. 3. Compressive properties of control and GAG-depleted regional meniscus specimens. Unconfined compression stress-relaxation tests at 10 and 20% strain levels were performed on outer, middle, and inner meniscus specimens either treated with CABC or not (control). Similar results were observed at both 10 and 20% strain levels. All regions displayed a decrease in tissue viscosity in response to GAG depletion. The modulus of relaxation remained unchanged in outer meniscus specimens following CABC treatment but decreased in middle and inner regions. No region showed differences in instantaneous modulus following GAG depletion. Student's *t*-tests were performed at each strain level with significance set at $P < 0.05$ (indicated by an asterisk).

contributors to this mechanically important tissue. Many investigations have shown that the presence of the meniscus in the knee protects the articulating cartilage from the progression of osteoarthritis (8, 11, 24). Unfortunately, the meniscus is prone to injury, has little self-regenerative capacity, and repair techniques are often insufficient to restore full functionality. Tissue engineering efforts aim to create functional replacement tissues and depend on detailed design criteria to achieve this goal. Although many studies exist bearing out the types, organization, and mechanics of collagens in the meniscus, much less is known about the mechanical contribution of GAGs regionally in the tissue. To our knowledge, this study is the first to assess the regional contributions of sulfated GAGs to viscoelastic meniscus mechanics.

Meniscus degeneration is often associated with inflammation of the knee joint and manifests through a variety of detrimental changes to the tissue including GAG catabolism (13, 18, 49). Although to our knowledge no studies have specifically addressed GAG release regionally in the degenerating meniscus, there is abundant evidence that meniscus GAGs are susceptible to degradation when the tissue is in an inflammatory environment (13, 25, 30, 31, 45, 48, 49). Proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) have both been shown to promote GAG degradation by enhancing the production of matrix metalloproteinases and aggrecanases (25, 45). Therefore, the observed differences in the mechanical properties of intact and GAG-depleted tissues seen in this study may also be applicable to pathological changes in the meniscus when exposed to inflammation.

The success of this study depended on an efficient GAG-removal technique, and CABC proved to be an effective mode of depleting sulfated GAGs from the bovine meniscus (see Fig. 2). In the present study, all regions of the meniscus subjected to CABC treatment showed >95% decreases in sulfated GAG content. Since CABC is known to act mainly on chondroitin and dermatan sulfate molecules (17), it follows that these comprise the vast majority of GAGs in the bovine meniscus. This is in agreement with previous characterization of the human meniscus, in which the GAG distribution was found to be 40% chondroitin-6-sulfate, 10–20% chondroitin-4-sulfate, and 20–30% dermatan sulfate (20). These similarities between bovine and human menisci could indicate that the structure-function relationships borne out in this study may have broader clinical relevance.

GAG depletion affected biochemical content differently in the inner region compared with the outer and middle regions. Following GAG-depletion of the inner region, an increase in percent collagen per dry weight was observed (see Fig. 2). This is likely because the inner region contains ~4% GAG per dry weight, and when this fraction is removed, there is a concomitant increase in collagen fraction. The same result is not observed in the outer and middle regions. This is likely because GAGs in these regions do not comprise such a large fraction of the dry weight, and therefore minute increases in collagen content between control and GAG-depleted groups could not

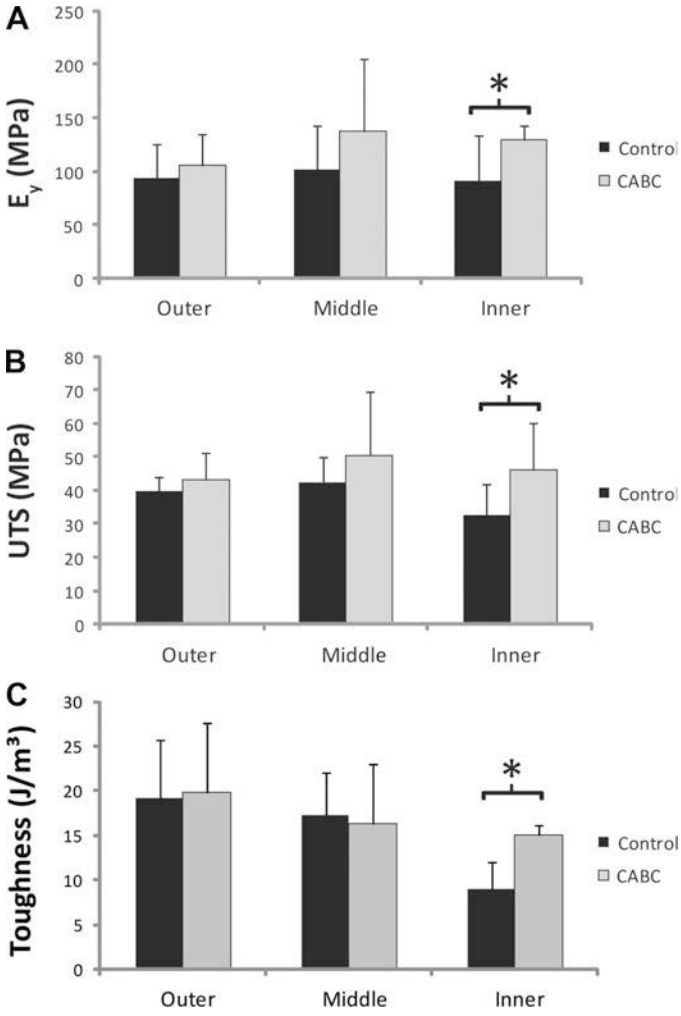

Fig. 4. Tensile material properties of GAG-depleted meniscus regions. Young's modulus (*A*), ultimate tensile stress (*B*), and toughness (*C*) were measured for outer, middle, and inner meniscus regions subjected to GAG depletion (CABC) or not (control). Outer and middle meniscus regions did not show any significant difference in either Young's modulus or UTS following GAG depletion. Inner meniscus specimens, however, showed a statistically significant increase in both parameters after CABC treatment. Student's *t*-tests were performed on these data with significance set at $P < 0.05$ (indicated by an asterisk).

be detected. These differences in the effect of GAG depletion on biochemical content, however, were not predictive of the effect of GAG depletion on compressive properties.

As hypothesized, the viscoelastic compressive properties of the meniscus were affected by sulfated GAG depletion, and these effects varied regionally. The present data establish that, in compression, all regions of the tissue rely on sulfated GAG to impart viscosity, even the outer region where these proteins are most scarce. In the middle and inner regions, where sulfated GAGs are more abundant, both the viscosity and modulus of relaxation of the tissue are reliant on sulfated GAG, indicating that the molecule is also important in supporting compressive loads. This overall change in viscosity is in agreement with the literature on compressive mechanical properties of GAG-depleted ligaments and cartilages (19, 23). GAG removal in porcine medial collateral ligament (MCL) causes overall increases in tissue permeability. Since ligament tissue is similar in biochemical content and organization to the outer meniscus in the circumferential direction, the observed changes in viscosity in the present study for the outer meniscus were found to closely match the change in permeability for GAG-depleted ligaments. Thus it appears that the coefficient of permeability reported in these studies is inversely proportional to viscosity. Increased permeability has also been observed in GAG-depleted articular cartilage explants, along with a decrease in lubrication of the tissue (23). Although not measured in the present study, it is possible that GAG depletion of the meniscus also has an effect on the lubrication properties of the tissue. Therefore, these results show that changes in compressive properties resulting from GAG depletion are commensurate with the native GAG content in each region.

In this study, a viscoelastic model was used to approximate the compressive characteristics of control and GAG-depleted meniscus specimens and was found to closely match the observed mechanical behavior of both groups. Although other studies have used the biphasic model to describe native meniscus mechanics (12, 38), this model requires that sufficient interstitial pressure be generated in the tissue on initial loading. Because the present study depleted GAGs from the meniscus, reducing the viscosity of the tissue in the experimental groups but also the negative charge in the tissue, this effect may have made such an assumption invalid. Therefore, the Kelvin solid model was used and was found to produce fits with R^2 values of >90% in both control and experimental groups. This is in agreement with previous work in which viscoelastic models have been used to describe the mechanics of other fibrocartilaginous tissues with characteristics similar to the knee meniscus such as the temporomandibular joint disc (3, 4, 22). Therefore, the data presented here and previous work on fibrocartilaginous tissues indicate that both the biphasic and viscoelastic models may be appropriate for approximating meniscus mechanics.

Tensile testing on GAG-depleted meniscus regions showed a significant increase in inner region properties but had no statistically significant effect in the outer and middle regions. Since the inner region contains the most sulfated GAG of all the meniscus regions, it is possible that the GAG content imparts a pre-stress on the resident collagen molecules, a phenomenon that has been modeled in GAG-rich tissues such as articular cartilage (40). If the same principles are applied to the inner meniscus, in its normal state sulfated GAGs attract water molecules, which increases hydrostatic pressure and imparts a pre-stress to the collagen network. Once the GAG is depleted and the pre-stress is removed, the apparent tensile properties of the tissue increase. This increase could be a result of minute decreases in tissue volume as the GAGs are removed. As tissue volume decreases, the same force would be applied to a smaller cross-sectional area, resulting in a higher calculated stress and higher tensile properties. Although not directly measured in this study, decreased tissue volume and increased tensile properties have been noted in CABC-treated articular cartilage, as well as tissue engineered articular cartilage constructs, which are both rich in sulfated GAG (6, 36). In the other two regions, where GAGs are not as abundant, the pre-stress and volume changes may not be significant enough to make a difference in the tensile properties measured. These results show, therefore, that sulfated GAGs play a role in inner meniscus tensile properties.

Finally, it is pertinent to note that, although GAGs were not found to affect many mechanical properties of the outer me-

niscus, they do play a role in meniscus development and extracellular matrix organization throughout the tissue. Both chondroitin sulfates and dermatan sulfates are usually found associated with protein cores, forming large or small proteoglycans. Although aggrecan is perhaps the most commonly discussed proteoglycan in the meniscus due to its large size and highly negative charge, there are also other small proteoglycans in the tissue such as decorin, biglycan, and fibromodulin (29, 39). Since these proteoglycans are smaller, their main functions involve collagen fibrillogenesis and organization, but they may also be contributors to tissue viscosity as seen in the present study. Decorin, for example, is most abundant in the outer portion of the meniscus and is known to regulate collagen fibril size during development (10, 39, 46). Biglycan and fibromodulin are found mostly in the inner portion of the meniscus, are known to be more abundant during development, and are theorized to be important in tissues that undergo compressive loading (39, 47). Therefore, the role of proteoglycans and GAGs in the knee meniscus is likely varied and dependent on both the region and developmental state of the tissue.

In conclusion, this study showed that GAGs in the knee meniscus contribute significantly to the viscoelastic properties of the meniscus, especially in the inner and middle regions where GAGs are most abundant. These regional variations in the contribution of GAGs to meniscus mechanics illustrate the importance of these molecules to the overall function of this tissue. Therefore, when engineering the meniscus, constructs should use, produce, or mimic the regional distribution of GAGs to attain native viscoelastic mechanics.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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