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Impacts of Maturation on the Micromechanics of the Meniscus Extracellular Matrix

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Abstract

To elucidate how maturation impacts the structure and mechanics of meniscus extracellular matrix (ECM) at the length scale of collagen fibrils and fibers, we tested the micromechanical properties of fetal and adult bovine menisci via atomic force microscopy (AFM)-nanoindentation. For circumferential fibers, we detected significant increase in the effective indentation modulus, E_{ind} , with age. Such impact is in agreement with the increase in the fibril diameter and collagen alignment during maturation, and is more pronounced in the outer zone, where collagen fibrils are more aligned and packed. Meanwhile, maturation also markedly increases the E_{ind} of radial tie fibers, but not those of intact surface or superficial layer. These results provide new insights into the effect of maturation on the assembly of meniscus ECM, and enable the design of new meniscus repair strategies by modulating the local ECM structure and mechanical behaviors.

Keywords

meniscus; maturation; collagen fibers; extracellular matrix; nanoindentation

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Conflict of interest statement

The authors of this study have no personal or financial conflicts of interest with this work. All authors were fully involved in the study and preparation of this manuscript and the material within has not been and will not be submitted for publication elsewhere.

1. Introduction

The meniscus plays essential biomechanical roles in the loading, stability and shock absorption of the knee (Makris et al., 2011). These specialized biomechanical properties are endowed by its type I collagen fiber-dominated, hierarchically structured extracellular matrix (ECM) (Petersen and Tillmann, 1998). Since meniscus tear or maceration is one common injury leading to the development of post-traumatic osteoarthritis (OA) in young adults (Badlani et al., 2013), repair and regeneration of native meniscus is imperative to the restoration of joint function. Unfortunately, the self-repair capabilities of mature meniscus ECM is very limited, especially in the proteoglycan-rich, avascular inner region (Mauck and Burdick, 2015). In contrast, at the fetal stage, meniscus has robust self-repair capability, which is progressively lost during maturation (Ionescu et al., 2011). In addition to the reduction in cell density (Ionescu et al., 2011), decrease in vascularity (King, 1990) and alteration in cell metabolism (Bland and Ashhurst, 1996), one salient change is the alteration of the ECM modulus and collagen fiber alignment (Qu et al., 2015). Maturation-associated ECM structural changes can increase the impedance of blood vessel infiltration and cell migration that is critical for self-healing (Di Giancamillo et al., 2017). Therefore, understanding the impact of maturation on the local structure and mechanical properties of the meniscus ECM will provide new insights into the biomechanical cues and self-repair mechanisms of native meniscus, and will assist the design of novel tissue engineering strategies (Qu et al., 2018).

This study aims to uncover how maturation affects the local nanostructural and micromechanical properties of the meniscus ECM in an orientation- and region-specific manner. Applying atomic force microscopy (AFM)-nanoindentation, we compared the effective indentation moduli between fetal and adult bovine menisci, which were interpreted together with our recently published work on the micromechanics of juvenile bovine meniscus (Li et al., 2017). To acknowledge the structural complexity of the meniscus ECM, each structural unit was tested and compared between the two ages, including the surface, superficial layer, radial tie fibers and circumferential fiber bundles (Fig. 1a,b). Since our recent study showed that the elastic modulus of juvenile meniscus ECM is highly sensitive to subtle changes in collagen fibril nanostructure, while the time-dependent, viscoelastic properties are not (Li et al., 2017), this study focused on age-dependent variations in the elastic indentation modulus. Furthermore, we interpreted variations of the moduli in the context of collagen fibril structure and ECM composition to elucidate the structure-mechanics relationship. We focused this study on the medial meniscus, which receives more attention than the lateral meniscus (McNulty and Guilak, 2015) due to its higher susceptibility to injury (Kilcoyne et al., 2012). We expect these results to yield new insights into the biomechanics and development of native meniscus ECM, which will set a basis for improving meniscus repair strategies by modulating local ECM micromechanical behaviors.

2. Methods

2.1 Sample preparation

Medial menisci were harvested from fetal (later 2nd to 3rd trimester, $n = 3$) and adult (18–30 months old, $n = 4$) cows. The outer one-third (outer zone) and inner one-third (inner zone)

tissues of each meniscus were isolated and embedded in Optimal Cutting Temperature (OCT) media. The middle zone was not included since its properties are similar to those of the outer zone (Li et al., 2017). Tissue from each zone was cryo-sectioned transversely and radially onto glass slides to produce $\sim 20 \mu\text{m}$ thick horizontal and vertical sections, respectively (Fig. 1c). Samples were stored at -20°C for less than one week until AFM nanoindentation. In addition to fetal and adult menisci, we also included the results of juvenile bovine menisci (< 3 months old) obtained by our recent study (Li et al., 2017) to provide a more comprehensive analysis on the age-dependence of meniscus ECM micromechanics.

2.2 AFM-nanoindentation

On each section, AFM-nanoindentation was performed with a microspherical tip ($R \approx 5 \mu\text{m}$, $k \approx 0.6 \text{ N/m}$, NanoAndMore, Watsonville, CA) and a Dimension Icon AFM (BrukerNano, Santa Barbara, CA) at $\approx 10 \mu\text{m/s}$ indentation rate up to a maximum load of $\approx 80 \text{ nN}$ (Fig. 1d). For each vertical section, testing locations include the cross-section of circumferential fiber bundles, the longitudinal sections of radial tie fibers and superficial layer (Fig. 1a). In addition, to elucidate the anisotropy of circumferential fibers, we tested the longitudinal section of these fibers on horizontal sections (Fig. 1b). For each microstructural region on each section (Fig. 1b,c), indentation was performed on ~ 10 – 15 different locations. At each location, effective indentation modulus, E_{ind} , was calculated via the finite thickness-corrected Hertz model (Dimitriadis et al., 2002). During all indentation measurements, tissues were immersed in PBS (0.15 M , $\text{pH} = 7.4$) with protease inhibitors (Pierce). After nanoindentation, each section was fixed with Karnovsky's fixative and dehydrated in ethanol + hexamethyldisilazane (HMDS) to preserve the collagen fibril architecture (Bray et al., 1993).

2.3 Collagen nanostructure analysis

To reveal the microstructure of collagen fibers, outer one-third (outer zone) and inner one-third (inner zone) menisci were isolated, fixed with 4% paraformaldehyde, embedded in paraffin, and microtomed to produce $6\text{-}\mu\text{m}$ -thick vertical and horizontal sections. Sections were stained with Alcian Blue for proteoglycans, PicroSirius Red for collagens, and visualized under polarized light microscopy (PLM, Olympus BX51). To reveal the nanostructure of collagen fibrils, following our established procedure (Li et al., 2017), immediately after AFM tests, each section was fixed, dehydrated, HMDS-dried, and imaged via scanning electron microscopy (SEM, Zeiss Supra 50VP) or tapping mode AFM (Bruker). The collagen fibril diameter, d_{coh} , and fibril alignment orientation angle, θ , of the circumferential fibers, were measured from each image via ImageJ (≈ 100 fibrils per image). After offsetting the values of θ with the average value, we fitted the distribution of θ with the von Mises probability density function, and the output concentration parameter, κ , was used as the indicator of the degree of fibril alignment.

2.4 Statistical analysis

We applied the Kruskal-Wallis test followed by the Tukey-Kramer multiple comparison to examine the differences of E_{ind} among three age groups in both the inner and outer zones. Data were pooled from different animals, as we did not find significant differences between

individual animals. For structural data, one-way ANOVA followed by the Tukey-Kramer multiple comparison was used to compare different structural units in each of the inner and outer zone. For fibril orientation data, θ , the Mardia and Jupp test of concentration equality (Mardia and Jupp, 2000) with the Bonferroni correction, was applied to compare the von Mises concentration parameter κ among the three age groups. A p -value of less than 0.05 was taken as statistically significant.

3. Results

To elucidate the impact of maturation on meniscus ECM nanostructure and micromechanics, we compared the results obtained from fetal and adult bovine menisci from this study, and results from juvenile bovine menisci, which were adapted from our recently published work (Li et al., 2017). Specifically, AFM-nanoindentation detected significant differences in E_{ind} among the three age cohorts (Fig. 2). On the horizontal section, where indentation was performed normal to the fibril axis, in both inner and outer zones, fetal meniscus had significantly lower modulus compared to juvenile and adult tissues, while the latter two had similar moduli (Fig. 2a). On the vertical section, where indentation was performed parallel to the fibril axis, the same trend was observed in the inner zone. In the outer zone, however, all three age cohorts had different moduli (Fig. 2b). This trend was in agreement with variations in the collagen structure (Fig. 3). At the microscale, or fiber-level, in both inner and outer zones, PLM images showed thicker, more aligned collagen fibers in adult tissues (Fig. 3a). This trend was similar at the nanoscale, or fibril-level, as shown by SEM (Fig. 3b). Structural analysis suggested significantly larger diameters from fetal to juvenile to adult tissues in both inner and outer zones (Fig. 3c). Meanwhile, in the inner zone, fibrils in the fetal tissue were less aligned than juvenile and adult tissues (smaller κ), while the latter two had similar κ . In the outer zone, values of κ increased from fetal to juvenile to adult tissues (Fig. 3d).

Significant impact of maturation was also observed for other structural units, albeit in a differentiated manner (Fig. 4). For these units, indentation was performed normal to the fibril axis. For the intact surface, there was no significant age-dependence in the inner zone, but a marginal increase in E_{ind} from fetal to adult tissues in the outer zone (Fig. 4a). In addition, E_{ind} did not show significant age-dependence for the superficial layer (Fig. 4b), but exhibited significant increases with age for radial tie fibers (Fig. 4c).

4. Discussion

This study elucidates how maturation affects the nanostructure and micromechanical properties of the meniscus ECM. Under microspherical tips ($R \approx 5 \mu\text{m}$), the moduli represent local ECM deformation behaviors at the fibril and fiber levels, which complements current understanding of meniscus biomechanics at the tissue level (Mow et al., 2003). For the circumferential fibers, the age-dependent increase in modulus (Fig. 2) of the outer zone can be mainly attributed to the increase in collagen fibril diameter, packing and alignment at the nanoscale (Fig. 3). As shown by our previous study (Li et al., 2017), anisotropy of E_{ind} is originated from differences in the deformation modes of collagen fibrils: indentation parallel to the fibril axis (on the vertical section) results in laterally constrained fibril compression,

while indentation normal to the fibril axis (on the horizontal section) results in local fibril uncrimping and sliding, which are more compliant (Li et al., 2017). During maturation, collagen fibrils form more organized, densely packed bundles (Fig. 3), which provide higher resistance in both deformation modes, yielding higher modulus. Our results suggest that such structural changes have more pronounced impacts on the lateral constraints between adjacent fibrils, leading to a greater degree of stiffening when indentation is parallel to fibrils. Therefore, adult meniscus exhibits increased anisotropy compared to juvenile and fetal tissues.

In the inner zone, where the fibrils are less organized and there is higher concentration of “amorphous” proteoglycans (Sanchez-Adams et al., 2011; Sanchez-Adams et al., 2013; Vanderploeg et al., 2012), maturation has a lesser impact on E_{ind} . It appears that maturation-governed stiffening is primarily manifested through altering the organization of collagen fibrils. Meanwhile, during maturation, aggrecan molecules become less glycosylated with shorter GAG chains and more fragmented, leading to reduced molecular stiffness, as shown for hyaline cartilage (Ng et al., 2003). This phenomenon can partially offset the increase in collagen fibril organization, contributing to the reduced stiffening in the inner zone (Fig. 2).

In adult meniscus, self-healing is mostly absent in the inner zone (Kawamura et al., 2003). During post-natal growth, local fibril stiffening, together with increased fibril packing, impede the infiltration of blood vessels through the circumferential fiber bundles, thus inhibiting angiogenesis (Di Giancamillo et al., 2017). This effect could contribute to the reduced vascularity in the inner zone, and thus, its inability of self-healing. Meanwhile, upon injury, the stiffer matrix impedes the migration of cells from the vascular outer zone to the avascular inner zone. Modulation of local micromechanical environment could provide a valuable means to improve meniscus repair by facilitating angiogenesis and cell migration.

The other structural units exhibit differentiated age-dependence. The radial tie fibers and superficial layer share similar structure of radially oriented fibers, and play similar functions of integrating circumferential fiber bundles (Bansal et al., 2017; Skaggs et al., 1994). However, at the fetal stage, radial tie fibers appear to be less developed than superficial layer, as evidenced by the much lower modulus. As a result, radial tie fibers yield pronounced age-dependence (Fig. 4c), which is absent in the superficial layer (Fig. 4b). From fetal to mature ages, the circumferential fibers not only become increasingly aligned and packed, but are also interdigitated by stiffer, more organized radial tie fibers. These trends together contribute to the increased impedance to cell migration in the adult tissue. Interestingly, in murine meniscus, neither did our study (Li et al., 2015) nor previous work (Gamer et al., 2016) report well-defined radial tie fibers, which may be attributed to the fact that the relatively fast growth of murine meniscus does not provide the timeframe necessary for developing this complex structure.

In summary, this study highlights the impacts of maturation on the nanostructure and micromechanical properties of meniscus ECM. Notably, maturation-associated stiffening of the circumferential fiber bundles and radial tie fibers in the outer zone could be a major factor contributing to the low vascularity of the inner zone and the high impedance to cell migration in adult tissues (Han et al., 2016; Mauck and Burdick, 2015). This new

biomechanical understanding of native meniscus will provide a benchmark for improving meniscus repair through tuning local ECM micromechanical properties.

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Fig. 1.

Experimental set-up of atomic force microscopy (AFM)-nanoindentation on bovine meniscus. (a) Schematic of the meniscus extracellular matrix (ECM) highlighting the four major ECM structural units: surface, superficial layer, radial tie fibers and circumferential fibers, as well as the zonal variation. (b) Atomic force microscopy (AFM) tapping mode image showing collagen fibril bundle and waviness from the outer zone circumferential fiber of fetal bovine meniscus. (c) Schematics of AFM-nanoindentation test on the horizontal and vertical sections, where the fiber axis orientation within each interior structural unit is shown. (d) Representative indentation force versus depth ($F-D$) curves measured on the vertical section of adult and fetal circumferential fibers in outer zone meniscus and corresponding Hertz model fit (solid line, tip radius $R \approx 5 \mu\text{m}$, spring constant $k \approx 0.6 \text{ N/m}$, $R^2 > 0.99$). Panels a) and c) are adapted from (Li et al., 2017) with permission.

Fig. 2.

Comparison of the effective indentation modulus, E_{ind} , of the ECM circumferential fibers among fetal (F), juvenile (J) and adult (A) bovine menisci, measured on a) horizontal section, b) vertical section. The corresponding scanning electron microscopy (SEM) images are shown for adult meniscus to illustrate the collagen fibril structure on each section type (mean $\pm$ 95% CI from > 30 locations pooled from $n = 3$ animals (fetal, juvenile) and $n = 4$ (adult) animals; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ from Kruskal-Wallis test followed by Tukey-Kramer multiple comparison; data of juvenile meniscus are adapted from (Li et al., 2017)).

Fig. 3.

Fiber-level microstructure and fibril-level nanostructure of fetal and adult meniscus ECMs. (a) PicroSirius Red and Alcian blue staining under polarized light microscopy images of each region and direction highlight the differences between fetal and adult tissues. (b) Scanning electron microscopy (SEM) images show the dimension and alignment of collagen fibrils in the circumferential fibers. (c) Box-and-whisker plot of collagen fibril diameter, d_{col} distribution for circumferential fibers among fetal (F), juvenile (J) and adult (A) meniscus (one-way ANOVA followed by Tukey-Kramer multiple comparison). (d) von Mises concentration parameter, κ , among the three age cohorts (mean $\pm$ 95% CI, Mardia and

Jupp test of equal concentration followed by Bonferroni correction). Panels c and d are obtained from 300 fibrils pooled from $n = 3$ animals (fetal, juvenile) and $n = 4$ (adult) animals; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$; data of juvenile meniscus are adapted from (Li et al., 2017).

Fig. 4.

Comparison of the effective indentation modulus, E_{ind} , of a) intact surface, b) superficial layer vertical section and c) radial tie fibers vertical section among fetal (F), juvenile (J) and adult (A) bovine menisci for both outer and inner zones. All the tests were performed where the indentation axis is normal to the fibril axis (mean $\pm$ 95% CI from > 30 locations pooled from $n = 3$ animals (fetal, juvenile) and $n = 4$ (adult) animals; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ from Kruskal-Wallis test followed by Tukey-Kramer multiple comparison; data of juvenile meniscus are adapted from (Li et al., 2017)).