Preconditioning is Correlated With Altered Collagen Fiber Alignment in Ligament

Kyle P. Quinn
Department of Bioengineering,
University of Pennsylvania,
Philadelphia, PA 19104

Beth A. Winkelstein
Department of Bioengineering,
University of Pennsylvania,
Philadelphia, PA 19104,
Department of Neurosurgery,
University of Pennsylvania,
Philadelphia, PA 19104
e-mail: winkelst@seas.upenn.edu

Although the mechanical phenomena associated with preconditioning are well-established, the underlying mechanisms responsible for this behavior are still not fully understood. Using quantitative polarized light imaging, this study assessed whether preconditioning alters the collagen fiber alignment of ligament tissue, and determined whether changes in fiber organization are associated with the reduced force and stiffness observed during loading. Collagen fiber alignment maps of facet capsular ligaments ($n=8$) were generated before and after 30 cycles of cyclic tensile loading, and alignment vectors were correlated between the maps to identify altered fiber organization. The change in peak force and tangent stiffness between the 1st and 30th cycle were determined from the force-displacement response, and the principal strain field of the capsular ligament after preconditioning was calculated from the fiber alignment images. The decreases in peak ligament force and tangent stiffness between the 1st and 30th cycles of preconditioning were significantly correlated ($R \geq 0.976$, $p < 0.0001$) with the change in correlation of fiber alignment vectors between maps. Furthermore, the decrease in ligament force was correlated with a rotation of the average fiber direction toward the direction of loading ($R = -0.730$; $p = 0.0396$). Decreases in peak force during loading and changes in fiber alignment after loading were correlated ($p \leq 0.0157$) with the average principal strain of the unloaded ligament after preconditioning. Through the use of a vector correlation algorithm, this study quantifies detectable changes to the internal microstructure of soft tissue produced by preconditioning and demonstrates that the reorganization of the capsular ligament's collagen fiber network, in addition to the viscoelasticity of its components, contribute to how the mechanical properties of the tissue change during its preconditioning. [DOI: 10.1115/1.4004205]

Keywords: preconditioning, vector correlation, facet capsular ligament, fiber realignment, cyclic loading

Introduction

Biological specimens exhibit a time dependence during mechanical testing that is generally characterized by a decrease in the load produced at a given displacement with each subsequent test [1]. The lack of a repeatable mechanical response during testing has led many researchers to precondition tissues prior to performing mechanical testing to establish constitutive models of mechanical behavior or define mechanical thresholds for tissue injury. Preconditioning protocols typically involve the application of cyclic subfailure loading in order to produce a repeatable force-displacement response [1–3]. The force associated with a given displacement typically decreases within the first 10 cycles of preconditioning, until a consistent force-displacement response is established between 10 and 30 cycles of loading [1,4,5]. Although the phenomenological force-displacement response of preconditioning is well-established [2,3,5–7], the specific microstructural mechanisms that drive this change in the mechanical properties still remain somewhat speculative. Experimental and theoretical studies have characterized the viscoelastic responses for a variety of biological tissues, but it has been established that standard viscoelastic models cannot explain the mechanical phenomenon associated with preconditioning [1,3,5,6]. Work by Viidik combining mechanical testing and light microscopy suggested that collagen fiber undulation was not fully recovered in highly aligned collagenous tissues after preconditioning [8]. As a result, it has been suggested that the internal microstructure of soft tissue is altered during preconditioning, leading to the change in the mechanical response that is not recoverable within minutes or hours after loading [1,8]. However, no study has directly quantified the reorganization of collagen fibers following preconditioning and assessed its correlation with the altered mechanical response of the tissue.

The microstructural organization of collagen fibers in soft tissue has been characterized through the use of polarized light in a variety of imaging studies [8–15]. Utilizing a quantitative polarized light imaging (QPLI) system, dynamic measurements of the collagen fiber alignment during mechanical preconditioning of collagen gels demonstrated that as the load is applied to the collagen matrix of the gel, the fibers rotate toward the direction of loading [14]. However, no detectable changes in fiber alignment were observed to persist when the gels were returned to zero displacement after preconditioning. More recently, a correlation calculation between fiber alignment vectors in sequential QPLI-derived alignment maps was used to identify and localize the occurrence of subfailure ligament damage in the facet capsular ligament [15,16]. That work demonstrated that vector correlation analysis can detect subtle changes in fiber organization, which makes it an ideal method to evaluate whether changes in collagen fiber organization are produced by preconditioning.

The goal of this study was to use QPLI and vector correlation analysis to evaluate whether changes to the collagen fiber alignment of ligament tissue are related to the changes in its mechanical response typically observed during preconditioning. Isolated human cervical facet capsular ligaments were used in this study because QPLI has successfully been implemented in previous studies with this tissue. It was hypothesized that the change in ligament force and stiffness during preconditioning would be correlated with a change in collagen fiber alignment. Due to the possibility of sustained ligament deformation after preconditioning, the location of specific tissue regions was not assumed to be identical between the alignment maps acquired before and after preconditioning. Rather, a previously developed vector correlation tracking algorithm [17] was used to quantify both principal strain and altered fiber alignment after preconditioning. The relationships among the average fiber direction, the correlation of fiber alignment vectors, principal strain, and the ligament's force and stiffness during preconditioning were assessed through a multivariate analysis.

Methods

Human facet joints ($n=8$) were isolated from the C6/C7 spinal motion segments of fresh unembalmed cadavers (range: 47–79 years of age). All musculature and tendon insertions along the surface of the facet capsule were removed. Articular bone and cartilage were also removed along the medial-lateral axis to enable

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light transmission through the lateral aspect of the ligament. The two bony ends of the joint were cast with FlowStone (Whip Mix Corporation, Louisville, KY) in aluminum cups, which were then rigidly fixed to an Instron 5865 outfitted with a custom-built QPLI system [9]. The joint's resting position in the motion segment was replicated and used as the unloaded reference configuration for the mechanical testing. To verify that the integrity of bony fixation was maintained throughout testing, fiduciary markers drilled into each bone were monitored before and after preconditioning using a Phantom v9.1 camera (Vision Research; Wayne, NJ) with 18.52 pixels/mm resolution.

A series of three fiber alignment maps were acquired using the QPLI system as described previously [9,15,16]. The Phantom v9.1 camera used to image the specimens was outfitted with a circular analyzer and images were acquired as light was transmitted through a rotating linear polarizer and the ligament tissue. At each pixel, the optical axis and the degree of light retardation orthogonal to this axis were calculated and correspond to the average fiber direction and the strength of fiber alignment in that direction, respectively [9,14]. Following acquisition of the initial alignment maps, each specimen underwent a preconditioning protocol consisting of 30 cycles of tensile loading between 0 and 1 mm at 0.4 mm/s. Based on estimates of the human facet capsule length of each specimen (ranging from 5.16–8 mm), 1 mm of displacement during preconditioning was estimated to produce strains ranging from 0.125–0.194. Joint displacement of 1 mm does not produce altered fiber alignment or any other indication of damage to the capsular ligament during loading [16]. Further, failure of the human cervical facet capsular ligament occurs in tension at 3.62 ± 0.49 mm and transient decreases in ligament stiffness (i.e., ligament yield) are not observed until 2.01 ± 0.57 mm during loading [16]. Taking those displacement limits as the most liberal estimates of the mechanical tolerance for this ligament, together with the fact that the tensile load generated by a 1 mm displacement is approximately 5% of the tensile failure load for this ligament [18], suggests the preconditioning protocol was not capable of producing ligament damage. Force and displacement data were acquired at 1 kHz during cyclic loading using Instron's Bluehill software. Following the preconditioning protocol, alignment maps were again acquired of the ligament specimen in the reference configuration to identify changes in fiber alignment that may have been produced by preconditioning.

To characterize mechanical and microstructural changes produced by preconditioning, principal strain and altered fiber alignment were assessed between the alignment maps that were acquired before and after preconditioning. Tissue deformation that remained after preconditioning was defined using a grid of virtual markers with four-pixel spacing assigned to the first alignment map acquired prior to preconditioning [17]. These virtual markers were tracked through a sequence of five alignment maps: the first two maps acquired before preconditioning, two maps acquired after preconditioning, and a third map acquired *before* preconditioning. Marker displacements between each of those maps were calculated by maximizing the correlation of the local fiber alignment pattern between maps [17]. The final map in this sequence (acquired before preconditioning) was used to ensure that any differences in the maps before and after preconditioning could be separated from any potential error generated by the tracking of alignment through the five maps. If the location of a marker could not be tracked in the fifth map back to within 0.5 pixels of its original location in the first map, the marker was removed from further analysis [17]. As a result, fiber orientation and strain were only measured within the midsubstance region of the capsular ligament where light transmission was sufficient to allow for fiber alignment tracking between all maps. A mesh of three-node elements was generated from the virtual marker positions in the first alignment map, and the Lagrangian strain tensor was derived for each element in each alignment map using plane strain theory. First principal strain was calculated for each element from the maximum eigenvalue of the strain tensor.

The alignment vector correlation values between the positions of each virtual maker in each alignment map were recorded and used to assess the overall changes in the local fiber alignment. Similar to previous work [16], a change in alignment vector correlation of a virtual marker between two maps was used to determine the extent to which the fiber alignment surrounding that marker had changed. For each virtual marker, a baseline correlation measurement was calculated by averaging the vector correlation between the two maps acquired *before* preconditioning and the correlation between the two maps *after* preconditioning. Changes in fiber alignment produced by preconditioning were assessed by calculating the correlation between these two sets of maps before and after preconditioning. For each specimen, altered fiber alignment was measured by the average change in vector correlation between maps before and after preconditioning relative to the baseline correlation measurements. In addition, the average fiber direction, directional variance among pixels, and average light retardation (strength of alignment within a pixel) were computed for each specimen before and after preconditioning. To quantify any changes in the mechanical response produced during preconditioning, the force at 1 mm and the tangent stiffness at 0.5 mm were determined from the 1st and 30th cycle. Tangent stiffness was computed using a centered finite difference approximation as described in previous work [16].

A paired *t*-test determined whether the force and stiffness changed between the 1st and 30th cycle in the preconditioning protocol. A multivariate analysis was then performed to determine whether changes in the mechanical function could be explained by changes in the collagen fiber alignment. For each specimen, the difference before and after preconditioning was calculated for the following measurements: the average alignment vector correlation, the average fiber direction relative to the direction of loading, the directional variance, and the average retardation. The linear correlations among these changes in microstructural measurements, the average principal strain, and the differences in force and stiffness between the 1st and 30th cycle were calculated. Significant correlations were defined by $\alpha = 0.05$.

Results

The average peak force during the 1st cycle of preconditioning was 4.47 ± 5.57 N which significantly decreased ($p = 0.0184$) to 3.74 ± 4.74 N by the 30th cycle (Fig. 1). Similarly, the tangent stiffness at 0.5mm was 3.67 ± 4.06 N/mm during the 1st cycle, and decreased ($p = 0.0206$) to 2.68 ± 2.85 N/mm by the 30th cycle (Fig. 1). A total of 2399 virtual markers were tracked

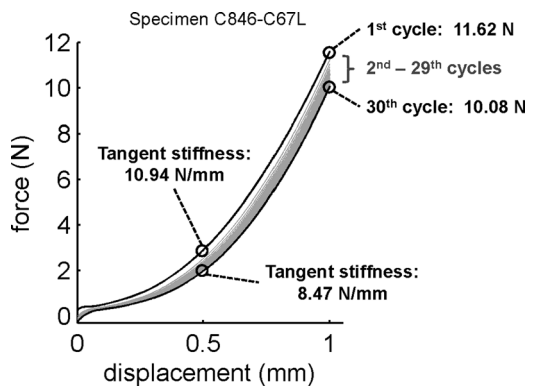

Fig. 1 The change in force and stiffness in a representative ligament specimen during preconditioning. The peak force decreased by 1.54 N and the tangent stiffness at 0.5 mm decreased by 2.47 N/mm between the 1st and 30th cycles. The force-displacement curves from only zero-to-peak displacement are shown for each cycle here.

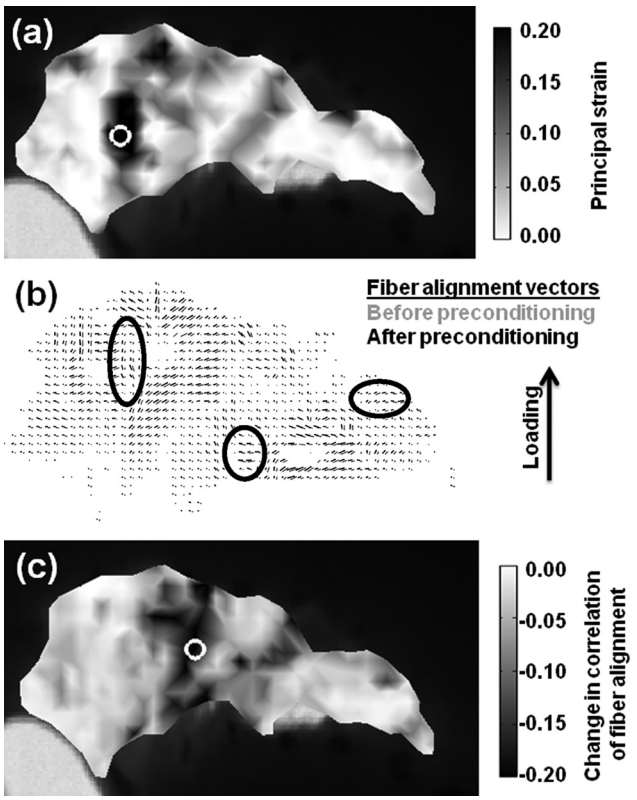

Fig. 2 Changes in principal strain and collagen fiber alignment after preconditioning in the specimen shown in Fig. 1. (*a*) First principal strain remained in certain regions of the tissue after unloading. (*b*) A slight change in the direction and magnitude of fiber alignment vectors toward the direction of loading was detectable in some regions of the capsular ligament (circled) after preconditioning. Those regions typically corresponded to areas of the ligament where larger principal strains were also measured, as shown in (*a*). (*c*) Decreases in the correlation of fiber alignment were detected in all specimens following preconditioning. The locations with the largest principal strain and change in correlation are indicated by circles in (*a*) and (*c*).

between the alignment maps acquired before and after preconditioning, with an average of 300 ± 142 markers assigned to each specimen. An average principal strain of 0.030 ± 0.011 remained in the ligament when each sample returned to zero displacement after the preconditioning protocol was completed (Fig. 2(*a*)). This establishment of principal strain in the ligament after preconditioning was significantly correlated with the decrease in both force ($R = -0.806$; $p = 0.0157$) and stiffness ($R = -0.807$; $p = 0.0155$) (Table 1).

The initial average fiber direction was oriented $47.8° \pm 29.9°$ from the direction of loading. Following preconditioning the average fiber direction was found to have rotated $11.4° \pm 22.7°$ toward the direction of loading (Fig. 2(*b*)). The average directional variance among pixel-wise alignment vectors was 0.714 ± 0.135 prior to preconditioning, and a small increase of 0.032 ± 0.073 was produced after preconditioning. Similarly, the average light retardation at each pixel was $10.6° \pm 2.2°$ prior to preconditioning, and only a small increase of $0.03° \pm 0.30°$ was observed after preconditioning. The average change in the correlation of fiber alignment when tracking virtual markers between maps acquired before and after preconditioning was -0.041 ± 0.027 (Fig. 2(*c*)).

The change in force between the 1st and 30th cycles of preconditioning was significantly correlated with the change in correlation of fiber alignment ($R = 0.979$, $p < 0.0001$) and the rotation of the average fiber direction toward the direction of loading ($R = -0.730$; $p = 0.0396$) (Fig. 3(*a*), Table 1). The change in the correlation of fiber alignment between maps acquired before and after preconditioning was also significantly correlated with the change in tangent stiffness between the 1st and 30th cycles ($R = 0.976$; $p < 0.0001$) and the principal strain produced by preconditioning ($R = -0.825$; $p < 0.0118$) (Fig. 3(*b*), Table 1). No additional correlations with statistical significance were identified among any of the measurements of force, strain, and fiber alignment.

Table 1 Correlations among fiber and mechanical metrics

Comparison	Correlation	*p*-value
Change in correlation of fiber alignment Change in force	0.979	< 0.0001
Change in correlation of fiber alignment Change in stiffness	0.976	< 0.0001
Change in correlation of fiber alignment Principal strain	−0.825	0.0118
Principal strain Change in stiffness	−0.807	0.0155
Principal strain Change in force	−0.806	0.0157
Fiber rotation toward loading direction Change in force	−0.730	0.0396
Fiber rotation toward loading direction Change in stiffness	−0.620	0.1011
Change in retardation Change in force	−0.074	0.8617
Change in directional variance Change in stiffness	0.064	0.8803
Fiber rotation toward loading direction Change in stiffness	−0.030	0.9438
Change in directional variance Change in force	0.024	0.9550

Discussion

This study demonstrates a strong correlation between changes in collagen fiber alignment and changes in the mechanical response of ligament tissue during a cyclic tensile preconditioning protocol (Table 1). Only small modifications in average fiber direction ($11.4° \pm 22.7°$ rotation toward loading direction) and strength of alignment ($0.03° \pm 0.30°$ change in retardation) were

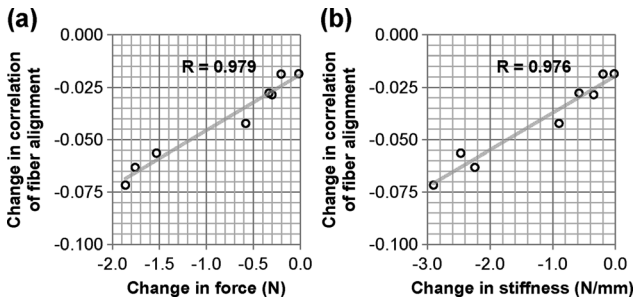

Fig. 3 Altered fiber alignment correlates with a change in mechanical response during preconditioning. The reduced correlation of fiber alignment vectors between maps acquired before and after preconditioning was strongly correlated ($p < 0.0001$) with (*a*) the decrease in ligament force between the 1st and 30th cycles and (*b*) the decrease in tangent stiffness from the 1st to the 30th cycles of preconditioning.

detected after preconditioning (Fig. 2(*b*)). Previous work has demonstrated that collagen fiber alignment varies throughout the mechanical preconditioning process [8,14]. However, quantifying any microstructural changes that remain in soft tissue after preconditioning has presented an experimental challenge. By utilizing a vector correlation tracking algorithm in this study, small asynchronous fiber rotations could be detected through a decrease in the vector correlation between fiber alignment maps acquired before and after preconditioning (Fig. 2(*c*)). The strong correlation between the reduced force response during preconditioning (Fig. 1) and the changes in fiber alignment after preconditioning (Fig. 3(*a*)) suggest both viscoelasticity and microstructural reorganization contribute to the time-history dependence of the mechanical properties of soft tissues. Although a previous theoretical model of preconditioning has suggested that both a viscoelastic component and a fiber-lengthening mechanism are responsible for the preconditioning mechanical response of tendon [5], the inclusion of fiber realignment through a multiscale fiber network model of collagenous soft tissue [19] may help to further elucidate the mechanisms that drive the preconditioning mechanical phenomena.

Fiber alignment was inferred in this study through changes in the overall birefringence of the ligament tissue. Therefore, the organization of individual fibers could not be visualized. As a result, it remains unknown whether the altered fiber alignment inferred here is produced by the microstructural failure or plastic deformation of some extracellular matrix component(s) or simply a different fiber network organization that achieves static equilibrium when the tissue is unloaded. In addition, the composition and microstructural organization of the facet capsular ligament has not been fully explored. Previous QPLI studies of the facet capsule demonstrate far greater spatial variability in the collagen fiber direction compared to more organized, cablelike ligaments [15,16] (Fig. 2(*b*)). Imaging approaches with enhanced resolution, such as nonlinear optical microscopy [20,21] or electron microscopy [22,23], could help to define the structural organization of the tissue at smaller scales and also contextualize the changes in alignment vector correlation that were detected in this study. Because a reduced force response was correlated with a rotation of the fiber alignment toward the direction of loading (Table I), the preconditioned state may be altered with any deformation in a direction orthogonal to the loading direction applied during preconditioning. Accordingly, this work may indicate that even if preconditioning is initially performed on a tissue, its microstructure may change between the multiple biaxial test protocols frequently used to form constitutive models of tissue [24]. Additional efforts to understand the role of fiber network reorganization under multiple loading scenarios may provide insight into how sensitive the collagen fiber alignment established by preconditioning is to subsequent loading. Nonetheless, the current study provides the first evidence of the microstructural reorganization assumed to occur in soft tissues during a standard cyclic preconditioning protocol.

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