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Investigating mechanisms of tendon damage by measuring multi-scale recovery following tensile loading

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ABSTRACT

Tendon pathology is associated with damage. While tendon damage is likely initiated by mechanical loading, little is known about the specific etiology. Damage is defined as an irreversible change in the microstructure that alters the macroscopic mechanical parameters. In tendon, the link between mechanical loading and microstructural damage, resulting in macroscopic changes, is not fully elucidated. In addition, tendon damage at the macroscale has been proposed to initiate when tendon is loaded beyond a strain threshold, yet the metrics to define the damage threshold are not determined. We conducted multi-scale mechanical testing to investigate the mechanism of tendon damage by simultaneously quantifying macroscale mechanical and microstructural changes. At the microscale, we observe full recovery of the fibril strain and only partial recovery of the interfibrillar sliding, indicating that the damage initiates at the interfibrillar structures. We show that non-recoverable sliding is a mechanism for tendon damage and is responsible for the macroscale decreased linear modulus and elongated toe-region observed at the fascicle-level, and these macroscale properties are appropriate metrics that reflect tendon damage. We concluded that the inflection point of the stress-strain curve represents the damage threshold and, therefore, may be a useful parameter for future studies. Establishing the mechanism of damage at multiple length scales can improve prevention and rehabilitation strategies for tendon pathology.

Statement of Significance

Tendon pathology is associated with mechanically induced damage. Damage, as defined in engineering, is an irreversible change in microstructure that alters the macroscopic mechanical properties. Although microstructural damage and changes to macroscale mechanics are likely, this link to microstructural change was not yet established. We conducted multiscale mechanical testing to investigate the mechanism of tendon damage by simultaneously quantifying macroscale mechanical and microstructural changes. We showed that non-recoverable sliding between collagen fibrils is a mechanism for tendon damage. Establishing the mechanism of damage at multiple length scales can improve prevention and rehabilitation strategies for tendon pathology.

1. Introduction

Tendon pathology, including pain and dysfunction, is common in both sports and occupational settings, and there is a need to understand the source of pathology to improve prevention and rehabilitation strategies [1,2]. Many studies associate tendon pathology with microstructural changes and damage initiated by mechanical loading [3–9], yet the specific etiology of tendon damage remains unknown. Although many studies have addressed damage in tendon, quantification of tendon damage remains challenging, in part because a precise definition with appropriate engineering context, has generally not been used for tendon.

In this study, we define damage, consistent with the definition established in engineering and applied to other materials, as an irreversible change in the microstructure that alters the macroscopic mechanical parameters [10]. Examples of microstructural changes include microcracks in metals [10–13], interlamellar debonding in polymers [14,15], matrix microcracking in

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composites [16,17], and voids in ceramics [18]; all of these irreversible rearrangements in microstructure produce impaired macroscopic mechanical properties, such as lowered modulus, are initiated by microscale deformation. In tendon, histological [19] and microscopic [20,21] studies have shown microstructural changes that appear to represent damage, however, these were not directly linked to mechanics. Similarly, changes to macroscale mechanical behavior such as reduced modulus and decreased failure stress and strain are well-known when tendon is loaded to high stresses and/or for multiple cycles [1,6,22–24]. While these are likely in response to microstructural damage, the link to microstructural change is not fully elucidated. Thus, evaluation of tendon at multiple length scales to identify the microstructural source is a critical aspect in the study of tendon damage.

Another key feature in the definition of damage, in addition to microstructural changes described above, is that it must be irreversible. Tendon, due to its viscoelastic properties, can partially recover its macroscale mechanical properties after loading [21,25]. However, many studies attempted to quantify the macroscale mechanical response immediately after loading, without decoupling the effects of time-dependent recovery from permanent change [26,27]. Quantification of damage must distinguish between the irreversible (non-recoverable, permanent) and recoverable changes in mechanical parameters (strictly related to mechanical effects, separate from cell contributions to healing).

The microstructural location for tendon damage is unclear. Some studies have identified structural changes of fibrils in the form of collagen kinking and discontinuities [20,22] or increase in D-period [28], while other studies suggest damage is localized to the interfibrillar structure, which connects adjacent collagen fibrils together [26,29]. It is possible that both occur or that damage in one location may precede the other. Mathematical shear lag modeling of tendon has suggested that plastic deformation of the interfibrillar structure replicates the macroscale mechanical behavior of tendon fascicles (matching the equilibrium stresses and predicting fibril strain); however, elastic deformation of fibrils cannot replicate this macroscale mechanical behavior [29,30]. Thus, shear lag modeling suggests that microstructural damage is localized to the interfibrillar structure and produces the observed changes in mechanical response at the fascicle-level. In this study we aim to experimentally identify the microstructural source of damage by investigating tendon structure and deformation at the fibril-level. Throughout this study, as in previous work [28–35], we define the microscale structures observed using confocal microscopy as "fibril-level" because they are at the hierarchical level below the tendon fascicle and there is no evidence of fibers in the tissue [32,36].

Damage in tendon at the fascicle-level has been proposed to initiate when tendon is loaded beyond a strain threshold [37–40], but metrics to define the damage threshold are not well established. While some studies use a non-recoverable length change (i.e., laxity) to identify the threshold [41–43], others use the beginning of strain-softening behavior [22,44]. A recent study hypothesized that the inflection point in the stress-strain curve, which marks the point where the response shifts from strain-stiffening to strain-softening, may mark the damage threshold in soft tissue [45]. Thus, we will investigate if the inflection point in the stress-strain curve represents the initiation of damage.

In summary, the objective of this study was to elucidate the tendon damage mechanisms. The key definition of damage, an irreversible change in the microstructure that alters the macroscopic mechanical parameters, was applied. Microstructural changes were quantified at the fibril-level and macroscale mechanical changes were simultaneously quantified at the fascicle-level. Experiments were designed to ensure damage was measured only as a non-recoverable change in mechanical behavior by allowing the tissue to recover after applying potentially damaging loading, and the threshold for damage initiation was determined. Understanding the mechanism of damage can improve prevention and rehabilitation strategies for tendon pathology.

2. Methods

2.1. Sample preparation

Rat tail tendon fascicles were harvested from eleven 6–8 month old Sprague-Dawley male rats that were sacrificed for a separate IACUC-approved study. These tails were previously frozen at −20 C, where the maximum number of freeze thaw cycle was limited to three [46,47]. Our pilot study showed that there was no effect of freezing on tissue for any of the mechanical testing parameters [47]. On the day of the experiment, each fascicle was dissected from a tail. We stained each sample with 10 μg/ml 5-DTAF (5-(4,6-Dichlorotriazinyl) aminofluorescein, Life Technologies) to minimize the effect of DTAF on mechanics [31]. We tested on a custom-made uniaxial testing device with PBS bath mounted on an inverted confocal microscope (LSM 5 LIVE, objective Plan-Apochromat 10x/0.45) as previously described [29]. Each sample was soaked in PBS to stabilize pH at room temperature for at least 3 h before testing to allow the sample to reach equilibrium, which was determined by monitoring cross-sectional area in our pilot study. In addition, all the samples were kept at room temperature from the beginning of DTAF staining to the end of mechanical testing.

2.2. Mechanical testing protocol

Mechanical testing on a total of 8 groups with n = 7 fascicles per group was performed. Each fascicle was randomly assigned to a strain level 2, 4, 6, or 8%, while also randomizing the number of freeze-thaw cycles for each strain group (on average 2 freeze-thaw cycles were used per group). In addition, within each strain level, each fascicle was randomly assigned to either "No Rest" or "Rest" group. We preloaded each fascicle to 0.5 g (~5 mN) to define the reference length and preconditioned with 5 cycles of 4% grip strain (i.e., grip-to-grip displacement). After ramping to its target strain level, we held the strain level constant for 15 min to reach equilibrium, followed by unloading to the reference length. The "No Rest" or "Rest" group was used to compare the effect of 60-min unloaded rest on fascicle-level parameters and separate the recoverable from non-recoverable deformation (Fig. 1). The No Rest group was immediately loaded to failure after unloading, whereas the Rest group was held at its reference length for 60 min before loading to failure.

The fascicle-level parameters calculated from the initial ramp were defined as "Baseline" and the parameters calculated from the ramp-to-failure were defined as "Diagnostic" (Fig. 1). Note that the parameters quantified at the Baseline for all four strain levels and both No Rest and Rest groups are identical. The strain rate for all loading and unloading was 1%/s, and only the fascicles that failed in the mid-substance were included in the following analysis. We excluded samples that failed at the grip (n = 2) or slipped during preconditioning (n = 3) from this study and additional fascicles were added to achieve desired sample size. Thus, all the samples failed at mid-substance.

2.3. Data acquisition

To measure the tissue strain, we applied two ink markers directly on the tendon using a permanent marker, and imaged with CCD camera to track displacement of markers using digital image