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Effect of fatigue loading on structure and functional behaviour of fascicles from energy-storing tendons

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ABSTRACT

Tendons can broadly be categorized according to their function: those that act purely to position the limb and those that have an additional function as energy stores. Energy-storing tendons undergo many cycles of large deformations during locomotion, and so must be able to extend and recoil efficiently, rapidly and repeatedly. Our previous work has shown rotation in response to applied strain in fascicles from energy-storing tendons, indicating the presence of helical substructures which may provide greater elasticity and recovery. In the current study, we assessed how preconditioning and fatigue loading affect the ability of fascicles from the energy-storing equine superficial digital flexor tendon to extend and recoil. We hypothesized that preconditioned samples would exhibit changes in microstructural strain response, but would retain their ability to recover. We further hypothesized that fatigue loading would result in sample damage, causing further alterations in extension mechanisms and a significant reduction in sample recovery. The results broadly support these hypotheses: preconditioned samples showed some alterations in microstructural strain response, but were able to recover following the removal of load. However, fatigue loaded samples showed visual evidence of damage and exhibited further alterations in extension mechanisms, characterized by decreased rotation in response to applied strain. This was accompanied by increased hysteresis and decreased recovery. These results suggest that fatigue loading results in a compromised helix substructure, reducing the ability of energy-storing tendons to recoil. A decreased ability to recoil may lead to an impaired response to further loading, potentially increasing the likelihood of injury.

1. Introduction

Tendons provide the attachment from muscle to bone, facilitating movement of the limbs during locomotion. Specific tendons also act as energy stores, stretching and recoiling by up to 16% with each stride to decrease the energetic cost of locomotion [1,2]. To store and release sufficient energy in a useable form, these tendons need to be more elastic than tendons with a purely positional function [3,4]. Such differences in mechanical properties between tendon types must be conferred by differences in structural organization and composition. All tendons can be considered as hierarchical fibre-composite materials, in which type I collagen molecules are grouped together in a highly ordered fashion, forming subunits of increasing diameter [5], the largest of which is the fascicle. At the larger hierarchical levels, the collagenous units are interspersed with a predominantly non-collagenous matrix [6]. While the basic structure of all tendons is similar, numerous studies have documented structural and compositional differences between energy-storing and positional tendons [7–12].

We have previously observed rotation in response to applied strain within fascicles from energy-storing tendons, which suggests the presence of helical substructures [9]. We have previously proposed that this helical formation may provide a more elastic mechanism for extension and recoil than the viscous fibre sliding that governs extension in positional tendons [9]. Despite this specialization, energy-storing tendons such as the human Achilles and equine superficial digital flexor tendon (SDFT) are highly prone to injury [13–16]. Injury is thought to occur due to accumulation of microdamage over the course of many loading cycles, rather than as a sudden rupture [17]. In support of this, our recent work has

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demonstrated that cyclic fatigue loading results in alterations to the fascicle microstructure and response to applied strain [18]. We have shown that fatigued fascicles rotate less on extension, suggesting loss of the helix structure. However, if the helix structure is utilized by energy storing fascicles to provide better recoil than fibre sliding, fatigued fascicles may have reduced ability to recover following extension. In our previous work we assessed the effect of fatigue on fascicle extension mechanisms. In this study we now investigate how repetitive loading affects recoil mechanisms within tendon fascicles.

Furthermore, our previous studies compared the microstructural strain response of fatigue loaded fascicles with fascicles that had experienced no prior loading (controls) [9,19]. However, when assessing the loading response of soft tissues such as tendon, it is common practice to apply a few loading cycles (typically between 10 and 30 [4,20,21]) prior to testing to precondition the sample so it reaches a steady state [22]. Therefore previous studies could not distinguish between the effects of preconditioning and fatigue loading. Indeed, while preconditioning is universally accepted as a part of any mechanical testing protocol [23], the processes that occur within the tissue during preconditioning are not well understood. A few recent studies have demonstrated that during the first few cycles of loading there is a considerable degree of collagen fibre realignment and recruitment [24,25]. However, to the authors' knowledge, no previous studies have determined how both preconditioning and fatigue loading affect the microstructural strain response and recoil capacity of soft tissues.

The aim of this study was therefore to assess the effects of preconditioning and fatigue loading on extension and recoil mechanisms within fascicles from energy-storing tendons. This provides a greater understanding of the mechanisms occurring during preconditioning and allows a comparison of fatigue loaded samples to samples which have been preconditioned to reach a steady state. We have used equine tissue for our studies as the human Achilles and equine SDFT show remarkable similarities in terms of healthy function and injury risk [26,27].

In this study, we tested the hypotheses that: (1) preconditioning will alter the microstructural extension and recoil mechanisms in fascicles, but fascicles will retain their ability to recover after the removal of load; (2) cyclic fatigue loading will result in fascicle damage, causing further alterations in microstructural extension and recoil mechanisms and reduced ability to recover.

2. Materials and methods

2.1. Sample collection and preparation

Forelimbs distal to the carpus were collected from half- to full-bred, skeletally mature, thoroughbred horses (aged 3 to 6 years, $n = 10$), euthanized at a commercial equine abattoir. We have previously shown an intact helical fascicle structure in tendons from this young age group [9]. Only tendons which had no macroscopic evidence of previous tendon injury at post-mortem examination were included in the study (approximately 1 in 20 SDFTs harvested show evidence of injury). The SDFT was dissected free from the limbs from the level of the carpus to the metacarpophalangeal joint, and wrapped in tissue paper dampened with phosphate buffered saline, and then in aluminium foil. Samples were stored frozen at −20 °C in sealed bags within 24 h of animal death for up to 6 months. It has previously been shown that one freeze–thaw cycle does not affect tendon mechanical properties [28]. On the day of testing, the tendons were allowed to thaw at room temperature and fascicles (8–12 fascicles per tendon, ~25 mm in length, diameter of 0.2–0.4 mm) were isolated from the mid-metacarpal region of the tendon by cutting with a scalpel longitudinally through the tendon (Fig. S1). Fascicle hydration was maintained by storing the fascicles on tissue paper dampened with Dulbecco's modified Eagles medium (DMEM). Fascicle diameter was measured continuously along a 10 mm region in the mid-portion of the fascicle using a laser micrometer, scanning perpendicular to the fascicle [10]. The smallest diameter recorded was used to estimate fascicle cross-sectional area, assuming a circular cross-section. While previous studies have demonstrated that fascicle cross-section within the equine SDFT may be irregular [29], we have previously demonstrated that assuming a circular cross-section to calculate cross-sectional area results in an overestimation of 4% [10]. All experiments were performed at room temperature. Fascicles were observed carefully during each experiment to ensure that only one fascicle was being tested.

2.2. Mechanical testing protocols

Fascicles were stained with the collagen stain 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein hydrochloride at a concentration of 2 mg ml^{-1} in 0.1 M sodium bicarbonate buffer, pH 9 for 20 min. Following staining, the fascicles were washed in two changes of DMEM for 20 min. Fascicles from each tendon were then randomly assigned to three groups: control ($n = 3$ per tendon), preconditioned (PC; $n = 3$–4 per tendon) and fatigue loaded (FL; $n = 3$–4 per tendon). Control samples remained unloaded, while PC and FL samples were secured in custom-made chambers at a resting grip-to-grip distance of 10 mm [30]. Each chamber was placed in a materials testing machine (Electropuls E1000, Instron) and a preload of 0.2 N was applied to remove any slack from the sample and determine the resting length. We have previously shown that fascicle failure strain is more consistent between samples than failure stress [10], and so to determine the appropriate load to apply for the subsequent cyclic tests, one loading cycle to a displacement of 1 mm (10% strain, equivalent to 50% of predicted fascicle failure strain) was applied, and the maximum load reached at this displacement was recorded. A cyclic creep test was then performed for either 30 cycles for PC samples or 1800 cycles for FL samples at 1 Hz, using the load recorded at 10% strain as the maximum load for each cycle, and 0.2 N as the minimum load. During each test, force and displacement data were recorded at a frequency of 100 Hz. The displacement at 0.2 N in the last cycle was used to calculate the increase in sample length in both the PC and FL groups. We chose to apply 30 loading cycles to samples in the PC group, as we have previously shown that this is within the primary phase of the creep curve, but provides a relatively stable curve compared to the first few cycles of loading [18]. 1800 loading cycles was applied to the FL group as this has previously been shown to be sufficient to induce mild damage within SDFT fascicles, but is well below the average number of cycles to failure, which we have shown to be in excess of 16,000 cycles in this tendon type [18], and so would be within the secondary portion of the creep curve.

2.3. Calculation of hysteresis

To determine the extent of damage with FL, the percentage increase in hysteresis from cycle 30 (end of PC) to cycle 1800 (end of FL) was calculated from the mechanical testing data (GraphPad Prism).

2.4. Determination of extension and recoil mechanisms

The microstructural strain response of the control samples was assessed within 1 h of staining. The strain response of samples in the PC and FL groups was assessed immediately after loading. Each fascicle was fixed into the tensile straining rig at a resting grip-to-grip length of 10 mm. Each fascicle was viewed under the laser