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Helical sub-structures in energy-storing tendons provide a possible mechanism for efficient energy storage and return

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ABSTRACT

The predominant function of tendons is to position the limb during locomotion. Specific tendons also act as energy stores. Energy-storing (ES) tendons are prone to injury, the incidence of which increases with age. This is likely related to their function; ES tendons are exposed to higher strains and require a greater ability to recoil than positional tendons. The specialized properties of ES tendons are thought to be achieved through structural and compositional differences. However, little is known about structure–function relationships in tendons. This study uses fascicles from the equine superficial digital flexor (SDFT) and common digital extensor (CDET) as examples of ES and positional tendons. We hypothesized that extension and recoil behaviour at the micro-level would differ between tendon types, and would alter with age in the injury-prone SDFT. Supporting this, the results show that extension in the CDET is dominated by fibre sliding. By contrast, greater rotation was observed in the SDFT, suggesting a helical component to fascicles in this tendon. This was accompanied by greater recovery and less hysteresis loss in SDFT samples. In samples from aged SDFTs, the amount of rotation and the ability to recover decreased, while hysteresis loss increased. These findings indicate that fascicles in the ES SDFT may have a helical structure, enabling the more efficient recoil observed. Further, the helix structure appears to alter with ageing; this coincides with a reduction in the ability of SDFT fascicles to recoil. This may affect tendon fatigue resistance and predispose aged tendons to injury.

1. Introduction

Tendons have multiple mechanical requirements which are specific to tendon type and relate to different functional roles. Precise mechanical properties are essential for efficient function and are conferred on the tendon by a complex hierarchical structure, in which highly aligned type I collagen is grouped together, forming sub-units of increasing diameter. Triple-helical collagen molecules are cross-linked together to form fibrils, which aggregate to form fibres. The fibres combine, forming fascicles, which are the largest sub-structural unit of tendons. Each hierarchical level is interspersed with a small amount of proteoglycan-rich matrix [1]. Structural and compositional alterations throughout this hierarchy are thought to result in the distinct mechanical properties required by functionally different tendons.

Tendons can be broadly categorized by their function; while all tendons have a positional role, some also operate as energy stores to decrease the energetic cost of locomotion [2]. Positional and energy-storing (ES) tendons have differing mechanical demands. To enable efficient force transfer, positional tendons need to be relatively stiff, whereas ES tendons require a degree of compliance to maximize energy storage [3,4], and they also need to recoil rapidly to return energy to the system [5]. Further, ES tendons need to be able to withstand large extensions; strains of up to 11% and 16% have been recorded in the human Achilles tendon and equine superficial digital flexor tendon (SDFT), respectively, during maximal exercise [6,7]. This extension capacity is not required in positional tendons such as the human anterior tibialis tendon and equine common digital extensor tendon (CDET), which experience strains in the region of 2–3% during normal use [8,9]. Correspondingly, previous work has shown that failure properties differ between tendon types, with the equine SDFT failing at higher strains than the CDET when tested in vitro [10,11].

Despite this ability to withstand higher strains, energy-storing tendons have a much greater predisposition to injury than

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positional tendons, likely due to the extremely high strains they experience in vivo. The initiation and progression of tendon injury is similar between humans and horses [12,13]; the human Achilles is the most commonly injured tendon in runners [14], and the equine SDFT is also highly susceptible to injury, specifically localized to the tendon core in the mid-metacarpal region (for a detailed review see Thorpe et al. [15] and references therein). Further, the risk of injury increases significantly with age in both human and equine ES tendons [14,16–18]. However, it has not been fully established how the structure of functionally distinct tendons is specialized for their specific roles, and if any age-related alterations occur to the structure of ES tendons which predisposes them to injury.

Previous studies have investigated micromechanics within isolated fascicles as it is possible to remove these from tendons without damaging their structure, and they provide a complete unit that is suitable in size for microstructural analysis. Studies using this approach have established that fascicle extension occurs predominantly as a result of sliding between adjacent fibres and fibrils, with only a small amount of extension occurring within the collagen units themselves [19–26]. Recoverability has also been assessed, with the results showing that both fibre sliding and fibre extension are reversible after the application of up to 5% strain [20,24]. The majority of studies have used rat tail tendon fascicles as this provides a simple model to develop an understanding of tendon micromechanics, and few studies have assessed how mechanics vary between tendon types. However, one recent study has reported that the microstructural stress relaxation response differs between the functionally distinct porcine SDFT and CDET, resulting in more rapid stress relaxation in fascicles from the positional CDET [27].

The majority of these studies have used fluorescent dyes to visualize the cell nuclei under a confocal microscope and measured the displacement between nuclei in the same or adjacent rows to infer fibre extension and sliding. However, the relationship between the cells and their surrounding matrix will influence the observed response [28]. An alternative approach is to stain the collagen and then photobleach a grid into the dye. The deformation of the grid can then be quantified, allowing direct assessment of the matrix response to applied strain [28]. This approach has been used previously to investigate micromechanics in rat tail tendon fascicles and other collagen-rich tissues including the intervertebral disc [28,29]. These studies have ascertained that the microstructural response to applied strain is complex and heterogeneous, with fibre sliding dominating extension. However, mechanisms governing fascicle recovery after strain has been applied have not been investigated using this approach, and it has not been established if extension or recovery behaviour varies between functionally distinct tendons. Further, no studies have investigated the effect of increasing age on the microstructural strain response within tendon fascicles.

In this study, we investigated the extension and recovery mechanisms in fascicles from the high strain ES SDFT and low strain positional CDET from young and old horses. We hypothesized that the extension and recoil mechanisms would differ in fascicles from the equine SDFT and CDET, resulting in more efficient recoil in SDFT samples. We further hypothesized that ageing would result in altered extension mechanisms and less efficiency of recoil in SDFT fascicles, whereas the response of CDET fascicles would be unaltered with ageing.

2. Materials and methods

2.1. Sample collection and preparation

Forelimbs distal to the carpus were collected from half- to full-thoroughbred horses aged 3–6 years ($n = 12$, young group) and 17–20 years ($n = 12$, old group), euthanized at a commercial equine abattoir. Only tendons which had no evidence of previous tendon injury at post-mortem examination were included in the study. The SDFT and CDET were dissected free from the limbs from the level of the carpus to the metacarpophalangeal joint, wrapped in tissue paper dampened with phosphate buffered saline (PBS) and stored frozen at −20 °C wrapped in aluminium foil. It has previously been shown that one freeze–thaw cycle does not affect tendon mechanical properties [30]. On the day of testing, the tendons were allowed to thaw at room temperature and fascicles (~25 mm in length) were isolated by cutting with a scalpel longitudinally though the tendon using previously established protocols [31,32]. Fascicles were dissected from the core ($n = 3$ or 4 from each tendon) and periphery ($n = 3$ or 4 from each tendon) of the mid-metacarpal region of the SDFT and CDET. We have established that, while isolated fascicles show alterations in mechanical properties after freezing, there is no difference in the failure properties of fascicles dissected from fresh or frozen tendons (unpublished data). Fascicle hydration was maintained by storing the fascicles on tissue paper dampened with PBS. Fascicles were tested within a few hours of isolation to ensure that their structural integrity was maintained.

2.2. Mechanical testing protocols

2.2.1. Assessment of fibre level response to incrementally applied strain

Fascicles from the core and periphery of paired SDFTs and CDETs (6–8 fascicles) from a subset of four horses in the young age group were stained with the collagen stain 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein hydrochloride (5-DTAF) 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 PBS for 20 min, and secured in a custom-made tensile testing rig [28], at a resting length of 10 mm. Fascicles were maintained in PBS for the duration of the experiment. Initial experiments were performed to assess the effect of 5-DTAF on fascicle material properties (see Supplementary information).

Each fascicle was viewed under the laser scanning confocal microscope (TCS SP2, Leica Microsystems GmbH, Wetzlar, Germany) using a ×20 objective (HC PL Fluotar, Nikon, Kingston-Upon-Thames, UK). Fascicle alignment and orientation were checked under bright-field settings along the entire fascicle length to confirm that only single fascicles were tested. Any samples that showed evidence of gross torsion or damage were not tested. A tare load of ~0.1 N (range: 0.05–0.15 N) was applied. A grid was then photobleached onto the fascicle, using a 488 nm krypton–argon laser, encompassing a series of 2 μm thick lines, bleached in the central region of the fascicle, to create a grid of four squares, each 50 μm × 50 μm (Fig. 1a). The laser intensity was then reduced to the imaging range, and the sample imaged with the same objective lens at a resolution of 2048 × 2048 pixels, with each pixel measuring 0.18 × 0.18 μm. An image of the photobleached grid was taken in a focal plane ~20–25 μm from the sample surface. The fascicle was then strained to 2% at a rate of 1% s^{-1} and the grid was refocused, before imaging again (Fig. 1b and c). Incremental strains of 2% were applied up to a maximum of 10%, and the grid was imaged at each strain increment. There was a hold period of ~1 min before imaging at each increment, whilst the focal plane was located. Initial experiments were performed to ensure that overall applied strain was representative of mid-portion strains (see Supplementary information).

2.2.2. Determination of recoil capacity at fibre level

To assess the ability of the fascicles to recoil, a further 6–8 fascicles per tendon were taken from a subset ($n = 4$ paired SDFTs and CDETs) of the young tendon group. The fascicles were stained with