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Research Paper

Early stage fatigue damage occurs in bovine tendon fascicles in the absence of changes in mechanics at either the gross or micro-structural level

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ABSTRACT

Many tendon injuries are believed to result from repetitive motion or overuse, leading to the accumulation of micro-damage over time. *In vitro* fatigue loading can be used to characterise damage during repeated use and investigate how this may relate to the aetiology of tendinopathy.

This study considered the effect of fatigue loading on fascicles from two functionally distinct bovine tendons: the digital extensor and deep digital flexor. Micro-scale extension mechanisms were investigated in fascicles before or after a period of cyclic creep loading, comparing two different measurement techniques – the displacement of a photo-bleached grid and the use of nuclei as fiducial markers.

Whilst visual damage was clearly identified after only 300 cycles of creep loading, these visual changes did not affect either gross fascicle mechanics or fascicle microstructural extension mechanisms over the 900 fatigue cycles investigated. However, significantly greater fibre sliding was measured when observing grid deformation rather than the analysis of nuclei movement. Measurement of microstructural extension with both techniques was localised and this may explain the absence of change in microstructural deformation in response to fatigue loading. Alternatively, the data may demonstrate that fascicles can withstand a degree of matrix disruption with no impact on mechanics. Whilst use of a photo-bleached grid to directly measure the collagen is the best indicator of matrix deformation, nuclei tracking may provide a better measure of the strain perceived directly by the cells.

1. Introduction

Many tendon injuries (tendinopathies) are believed to result from repetitive motion, or overuse, which creates 'micro-trauma' that accumulates over time and can initiate catabolic cell behaviour (Lin et al, 2004; Riley 2004, 2005). To understand the processes behind tendinopathy, a range of model systems have been developed to simulate tendon overuse, characterise

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the development of fatigue damage, and investigate how this may relate to the aetiology of tendinopathy (Shepherd and Screen, 2013b). *In vitro* models provide very controlled loading conditions, in which to investigate the mechanics of fatigue damage and the nature of tendon failure. Data from these studies have shown strain to be the primary mechanical parameter governing tendon damage accumulation and injury (Schechtman and Bader, 1997; Wren et al., 2003). They have also highlighted that changes in matrix structure proceed non-linearly, accelerating before rupture (Parent et al., 2011) and that the onset of visual matrix damage precedes statistically significant mechanical weakening of the tendon (Fung et al., 2009; Shepherd et al., 2014). The damage hypothesis introduced by Wang is based upon the understanding that damaged material no longer contributes to stiffness or strength whereas intact material makes a full contribution to both (Wang and Ker, 1995).

Whilst *in vitro* tendon fatigue analysis has traditionally considered whole tendon mechanics, a recent body of work has focused on isolated fascicle fatigue (Legerlotz et al., 2013; Maeda et al., 2007; Screen, 2003; Screen et al., 2003, 2005a; Thorpe et al., 2013a,b). The fascicle size scale is of benefit, as the testing of viable tendon sections is simpler, enabling investigation into factors such as cellular mechanotransduction responses (Banes et al., 1999a,b) and the role of inflammation (Devkota et al., 2007; Flick et al., 2006). Fascicles can be removed from bulk tendon with relative ease, providing a complete unit with a comparatively consistent cross sectional area for analysis (Shepherd and Screen, 2013b; Thorpe et al., 2013a), in which the considerable issues associated with gripping whole tendon samples can be overcomed. Fascicle testing also allows for far more straightforward imaging of matrix damage generation (Shepherd et al., 2014), and analysis of fatigue effects on tissue micro-mechanics and cellular morphology (Cheng and Screen, 2007, 2004a, 2003; Thorpe et al., 2013a). Considering the extent of variability in biological tissues (Ker, 2007), investigating fascicle characteristics can also ensure inter-animal variation is taken into account.

Previous studies of fascicle micro-mechanics have shown crimp straightening and fibre extension to be the dominant extension mechanisms at low applied strains, with fibre sliding dominating beyond the toe region (Cheng and Screen, 2007; Goulam Houssen et al., 2011; Gupta et al., 2010; Screen et al., 2004a; Thorpe et al., 2013a). In studies across a range of tendon types, including rat tail tendon fascicles, (Cheng and Screen, 2007; Screen, 2008; Screen et al., 2004a, 2003, 2004b) more highly loaded bovine tendons (Screen et al., 2013), and also energy storing and positional equine tendons (Thorpe et al., 2013a; Thorpe et al., 2014a,b), local strains along fibres have consistently been reported to be smaller than applied strains, as a result of the composite structure of tendon and reliance on fibre sliding for tendon extension.

However, despite this growing body of data concerning tendon micromechanics, there are relatively few studies directly comparing micromechanics in functionally distinct tendons (Thorpe et al., 2013a, 2014a,b), with none in the bovine model, and few studies investigating the effects of fatigue damage on the micromechanics of tendon at the fascicle and fibre levels (Thorpe et al., 2014a,b). Such comparisons are important, in light of the growing body of evidence outlining structural and mechanical differences between tendons with different mechanical functions (Smith et al., 2002; Stanley et al., 2006; Thorpe et al., 2013a, 2012). Whilst data indicates that energy storing tendons are more fatigue resistant, there is still evidence that tendinopathy may arise from mechanical fatigue damage, and there is a need to understand how fatigue damage initiates and propagates in different tendon types, to establish why some tendons are more prone to injury.

In previous micromechanical studies, tenocyte nuclei have been stained with a fluorescent dye and their movement tracked during straining under a confocal microscope. The tenocytes align along the tendon fibres and an assumption is made that the cell movement is the same as that of the fibres. The cell nuclei no doubt provide convenient, regular shaped markers for the analysis of local strains, but the strains recorded will be highly dependent upon the association between the cells and the surrounding matrix (Screen et al., 2004a). Recent studies have considered an alternative approach to characterising tissue micro-strains, in which the collagen matrix is stained, a grid photo-bleached onto the collagen, and the deformation of the grid in response to applied strain monitored (Cheng and Screen, 2007; Thorpe et al., 2013a, 2014a). However, it currently remains unclear how comparable measures taken with these two techniques may be. This work therefore had three key aims:

1. To investigate micro-scale extension mechanisms in fascicles from two functionally distinct tendons of the bovine hoof – the deep digital flexor and the digital extensor.
2. To investigate the effects of fatigue loading on fascicle micro-mechanics, hypothesising that the relative importance of fibre extension and fibre sliding within a fascicle will change as a result of fatigue damage. It is also hypothesised that fatigue damage, and thus changes in micro-mechanics, will be less significant in the more energy storing flexor tendon.
3. To compare two techniques for characterising micromechanics: bleaching a grid on the matrix to directly measure collagen deformation, and using the cells as fiducial markers of fibre movement.

2. Materials and methods

2.1. *Tendon source and fascicle dissection*

The feet of healthy bovines (male steers between 18 and 36 months of age) with no observed tendon injury were sourced from a local abattoir. The tensional regions of the three digital extensor tendons and the deep digital flexor tendon were removed within 24 h of slaughter. Tendons were either frozen immediately upon dissection (−20 °C; max duration of 30 days) or used within 12 h of removal.

Upon defrosting and during fascicle dissection, hydration was maintained with Dulbecco's Modified Eagles Medium (DMEM). Fascicles of length at least 20 mm were carefully dissected and maintained under DMEM hydration until use.