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The influence of swelling and matrix degradation on the microstructural integrity of tendon

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Abstract

Tendon is multi-level fibre composite material, responsible for the transmission of forces from muscles to the skeleton. It is composed of a hierarchical arrangement of collagenous units surrounded by a proteoglycan-rich matrix, arranged to support strain transfer, and thus contribute to the mechanical behaviour of tendon. This study examines the effect of swelling and enzymatic degradation on structural integrity at different levels of the tendon hierarchy. Biochemical and microstructural analysis are used to examine the effects of incubation on the composition and swelling of the matrix, prior to a mechanical characterisation of sample integrity. Results indicated significant swelling of tendon fibrils and interfibrillar matrix after incubation in phosphate buffered saline, leading to a reduction in ultimate tensile load, with failure initiated between fibrils and sub-fibrils. In contrast, incubation with the enzyme chondroitinase ABC resulted in a total removal of glycosaminoglycan from the samples, and a subsequent reduction in the extent of swelling. These fascicles also demonstrated an increase in failure loads, with failure predominating between fibres. The findings from this work confirm the importance of the non-collagenous matrix components in controlling strain transfer within tendon structures. It also highlights the necessity to maintain samples within a suitable and controlled environment prior to testing.
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1. Introduction

Tendon is a fibrous material, responsible for transmitting the forces generated by muscles to the skeletal system, thus facilitating locomotion [1]. It is composed predominantly of crimped type I collagen fibres, aligned in the direction of loading, and arranged in a number of hierarchical levels. These collagen units impart both high tensile strength and stiffness, whilst still enabling flexibility and improving hydration of the tendon [2]. Structural integrity is maintained by a combination of collagen crosslinks, and a proteoglycan-rich matrix binding together the collagen components [3,4]. Individual collagen molecules, and the sub-fibril collagen units are thought to be discontinuous components, tightly bound by minimally extensible collagen crosslinks [5]. However, the structural arrangement of the fibril, fibre and fascicle levels of the tendon hierarchy is less clear. Experimental assessment of their length remains inconclusive, although studies generally indicate that fibrils and fibres are also discontinuous units [6,7]. The compositional analysis of the surrounding matrix indicates the presence of proteoglycans, predominantly decorin, as well as fibronectin and other glycoproteins in small quantities [8].

The structural arrangement of the tendon matrix has important consequences for its mechanical characteristics.

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Tendon exhibits a strain response characterised by a non-linear toe region, followed by a quasi-linear region prior to gross failure. During the initial toe region, the crimped collagen fibres straighten, and once fully aligned, they are increasingly recruited to the direction of loading [9]. Beyond this, further tendon extension requires the structure to respond as a fibre composite material, such that gross extension involves either the extension of the individual collagen components, or relative sliding between them [10]. Continuous fibres are not necessary for structural integrity, provided the shear forces acting along the surface of a fibre are equal to the tensile forces along its length. This relationship is thus dependent on the diameter and length of the fibres, as well as the mechanical characteristics of both the collagen and its associated linkages.

The minimum fibre length over which structural integrity of tendon can be maintained has been estimated to be approximately 30–100 μm [11]. This so-called critical length lies well within the experimentally-derived fibril length of between 1.0 and 5.9 mm [7,12]. Synchrotron X-ray diffraction studies of the tendon matrix have demonstrated that only 40% of gross tendon extension can be accounted for at the molecular level, implying that approximately 60% of tendon extension occurs due to sliding between one or more of the structural levels of collagen [13–15]. Accordingly, tendon extension mechanisms are dominated by the ability of the non-collagenous matrix to resist shear forces at each hierarchical level. Previous studies by the authors have demonstrated a significant fibre sliding behaviour contributing to tendon extension, during both linear extension and within the failure region of the tendon strain response [16].

The strain transfer behaviour of the non-collagenous matrix has been examined using various techniques. For example, electron microscopy has demonstrated the presence of decorin between collagen fibrils, attached to the D-period of the collagen molecule by its protein core [17]. This has led to several hypotheses describing proteoglycan–collagen linkages, and the mechanisms by which they may facilitate load transfer. Decorin has a horseshoe shaped core protein, with an internal diameter of approximately 2.5 nm, enabling it to bind around the surface of the circular collagen fibrils [18]. Within tendon, each decorin molecule has a single dermatan sulphate glycosaminoglycan (GAG) chain, covalently bound to one edge of the core protein, imparting mobility to align orthogonally to the fibrils and couple with other GAG chains [19]. It has been suggested that interactions between adjacent GAG chains contribute to the interfibrillar linkages by the development of a 'shape molecule', comprising collagen–decorin core protein–dermatan sulphate GAG chain–decorin core protein–collagen [20]. Alternative studies have suggested different mechanisms for providing inter-fibrillar linkages, involving the adhesion molecule fibronectin binding between decorin molecules [21]. This hypothesis was examined with the use of the pentapeptide NKISK, which inhibits fibronectin–decorin binding. *In vivo* studies demonstrated tendon lengthening, suggesting a reduction in the interfibrillar binding integrity in the absence of fibronectin–decorin bonds [22]. In addition, computational models based on molecular interaction forces between collagen and decorin are used increasingly to predict the mechanical properties of tendon [23].

Proteoglycans are highly hydrophilic in nature, which is important for both lubrication and water retention within tendon [24]. As such, the state of tendon hydration may strongly influence its mechanical behaviour. In a previous study, the authors examined the effects of incubating fascicles in different solutions on fibre sliding behaviour and gross mechanical characteristics [25]. Data demonstrated that after incubation in phosphate buffered saline (PBS), the contribution of fibril sliding to fascicle extension was significantly greater than that found in non-incubated samples. The present *in vitro* study investigates the effects of incubation in PBS and chondroitinase ABC on both the swelling and composition of the non-collagenous matrix, hypothesising that swelling will alter the ultrastructure of fascicles, and subsequently reduce their microstructural integrity.

2. Materials and methods

2.1. Fascicle isolation and incubation

Collagen fascicles, approximately 60 mm in length, were teased from the tails of male Wistar rats aged between 4 and 6 months, within 2 h of death. Fascicles were either examined immediately after analysis, or incubated overnight at 37 °C in a solution designed to disrupt or swell the non-collagenous matrix. The solutions tested were (1) 1 ml PBS (Sigma, Poole, UK) to swell the matrix, and (2) 1 ml PBS containing 0.5 U of an essentially proteinase-free preparation of the enzyme chondroitinase ABC (Sigma, Poole, UK), which selectively cleaves GAG chains from their proteoglycan core protein [26]. After incubation, fascicles were rinsed briefly in 1 ml PBS prior to analysis by one of the following techniques: biochemical analysis ($n = 30$), microstructural analysis ($n = 3$), or mechanical testing ($n = 25$), where n numbers denote the number of fascicles in each treatment group.

2.2. Biochemical analysis

Fascicle weight was recorded before and after incubation. The specimens were lyophilised and weighed again, in order to determine the water content and percentage increase in wet weight. Fascicles were assayed to determine GAG and collagen content. Samples for GAG analysis were digested overnight at 60 °C in 0.4 U ml^{-1} papain (suspension from *papaya latex*, Sigma–Aldrich, Poole, UK) in 55 mM sodium citrate, 150 mM sodium chloride, 5 mM ethylenediamine-tetraacetic acid (EDTA) (all reagents from BDH Laboratory Supplies, Poole, UK) and 5 mM cysteine hydrochloride (Sigma–Aldrich, Poole, UK) in

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