



GAG depletion increases the stress-relaxation response of tendon fascicles, but does not influence recovery



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ABSTRACT

Cyclic and static loading regimes are commonly used to study tenocyte metabolism in vitro and to improve our understanding of exercise-associated tendon pathologies. The aims of our study were to investigate if cyclic and static stress relaxation affected the mechanical properties of tendon fascicles differently, if this effect was reversible after a recovery period, and if the removal of glycosaminoglycans (GAGs) affected sample recovery. Tendon fascicles were dissected from bovine-foot extensors and subjected to 14% cyclic (1 Hz) or static tensile strain for 30 min. Additional fascicles were incubated overnight in buffer with 0.5 U chondroitinase ABC or in buffer alone prior to the static stress-relaxation regime. To assess the effect of different stress-relaxation regimes, a quasi-static test to failure was carried out, either directly post loading or after a 2 h recovery period, and compared with unloaded control fascicles. Both stress-relaxation regimes led to a significant reduction in fascicle failure stress and strain, but this was more pronounced in the cyclically loaded specimens. Removal of GAGs led to more stress relaxation and greater reductions in failure stress after static loading compared to controls. The reduction in mechanical properties was partially reversible in all samples, given a recovery period of 2 h. This has implications for mechanical testing protocols, as a time delay between fatiguing specimens and characterization of mechanical properties will affect the results. GAGs appear to protect tendon fascicles from fatigue effects, possibly by enabling sample hydration.

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1. Introduction

Exercise or mechanical loading is an important stimulus, sensed by tenocytes, which subsequently initiate tendon matrix remodeling. A certain amount of loading is required to keep the tendon in a healthy and functional state, while higher loads can lead to tendon adaptation to exercise and an increase in performance [1]. However, overloading has been associated with the initiation of tendinopathies [2]. It has yet to be established how much loading can be tolerated by the tendon, optimizing adaptation without resulting in the development of tendinopathies. Animal studies aiming to experimentally induce Achilles tendinopathy with intense loading generally fail to initiate pathological changes, pointing towards a high loading tolerance in the Achilles tendon [3–5]. Characterizing the effects of specific loading conditions on the mechanical properties of tendon will help us to understand how the tissue responds to loading.

Cyclic and static stress-relaxation loading regimes are commonly used to study tenocyte metabolism and to investigate mechanotransduction behaviour in vitro [6–9]. As such, it is important to characterize tendon under these loading conditions, to establish how the tissue may respond to, and recover from, both static and cyclic stress relaxation tests. To improve understanding of the tendon tissue response to stress relaxation, two experiments were conducted. The aim of the first experiment was to investigate if cyclic and static stress relaxation protocols affect the mechanical properties of tendon fascicles differently and if any of the mechanical changes were reversible with a recovery period. We hypothesized that cyclic loading would be more damaging, as represented by a reduction in failure properties, and that recovery would be limited in both loading environments, but particularly with cyclic loading.

The concept of a mechanostat set point suggests that tendon cells sense and generate internal forces to maintain tissue homeostasis, such as constant cytoskeletal tension in response to changing loads [10]. However, passive processes (non-cell-mediated) might also contribute to the retensioning of the tendon after stress relaxation. To investigate passive recovery processes, in the absence of any living cells, the experiment was conducted with freeze-thawed tendon tissue.

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Passive viscoelastic processes are likely to incorporate water movement. Indeed, tissue hydration has been shown to influence the mechanical properties of tendon fascicles [11], whilst tendons lose water when loaded under tension [12]. Glycosaminoglycans (GAGs), which are hydrophilic in nature, seemed a likely candidate to influence this process, encouraging sample rehydration and thereby influencing the mechanical properties of the tissue. The precise role of GAGs in tendon tissue and their contribution to the mechanical behaviour of tendons is still debated. It has been suggested that GAGs are involved in lateral force transmission between neighbouring fibrils, forming cross-links between collagen molecules, and contributing to the mechanical strength of the tissue [13]. However, recent studies could not confirm this hypothetical mechanism; enzymatic removal of GAGs had no effect on the failure properties or viscoelasticity of either human patellar tendon [14] or medial collateral ligament fascicles [15], neither did it influence the elastic behaviour (modulus) of rat tail tendon fascicles [16]. However, one study has shown an elevated GAG content upon static loading of cultured rat tail tendon fascicles, which is also associated with an increase in elastic modulus [17]. This indicates that GAG concentration may influence the mechanical properties of tendon tissue. To the best of our knowledge, the influence of GAG content on the tissue response to stress relaxation and recovery has not yet been investigated.

The aim of the second experiment was therefore to subject GAG-depleted tendon tissue to static stress relaxation, hypothesizing that removal of GAGs would increase the stress-relaxation effect and reduce or completely abolish the recovery effect.

2. Materials and methods

Tendon fascicles were dissected from the bovine foot medial, lateral or common digital extensor tendon ($n = 8$). These three tendons are localized next to each other on the anterior face of the front foot, perform the same function and have the same mechanical properties [18]. The lateral digital extensor tendon extends the lateral digit (digit IV), the medial digital extensor tendon extends the medial digit (digit III), and the common digital extensor tendon splits in two ends and extends both digits. Previous studies have indicated no differences in their mechanical properties [18]. All tendons were obtained from eight young, healthy animals (male steers between 18 and 36 months of age) from a local abattoir and frozen at -20°C until used. For each fascicle, the diameter was determined along a 1 cm region in the middle of the fascicle, using a laser micrometer (LSM-501, Mitituyo, Japan). The smallest diameter recorded was used to calculate the cross-sectional area (CSA), assuming a circular shape.

Two experiments were conducted: the first experiment compared cyclic and static stress-relaxation protocols and the effect of 2 h recovery, while the effect of GAG depletion on static stress relaxation and recovery was investigated in the second experiment. Both experiments were performed with tendons from the same animals. For the control groups (Con; Con_B; Con_Ch) four fascicles per animal were used, while two fascicles per animal per condition were used in the stress-relaxation groups (SR; SRC; SR_2 h; SRC_2 h; SR_Ch; SR_Ch_2 h). See Table 1 for a schematic representation of the groups. For statistical analysis the mean value per animal per condition was used.

2.1. First experiment: Cyclic and static stress-relaxation comparison

Fascicles were secured in individual custom-made stainless steel loading chambers with a grip to grip distance of 10 mm as described previously [8]. Each chamber was filled with Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 U ml $^{-1}$

penicillin and 0.05 mg ml $^{-1}$ streptomycin. The fascicles were either directly tested to failure (Con) or first subjected to stress-relaxation tests. For the stress-relaxation test, the fascicles were strained for 30 min, either statically (SR), or cyclically at a frequency of 1 Hz (SRC) to 14% strain superimposed on a 2% strain off-set. For the static stress-relaxation test the ramp speed was 0.5 m ms $^{-1}$, resulting in a rise-time of 3.2 s. 14% strain was chosen, as previous experiments had shown that this leads to a significant reduction in the failure properties of fascicles after 30 min of cyclic stress relaxation [8]. 14% strain corresponded to 60% of the strain at failure in these 10 mm long samples. In short specimens the failure strain is affected by gripping effects, so this would be equivalent to 6% strain when gripping effects are removed [19]. Fascicles were loaded to failure directly after the stress-relaxation test. Another two groups were subjected to identical stress-relaxation protocols, but were then given a recovery period of 2 h prior to quasi-static testing to failure (SR_2 h and SRC_2 h).

During the stress-relaxation test, peak stress was monitored at 1 Hz using a BOSE loading frame with a 225 N load cell (BOSE Corporation, Eden Prairie, MN, USA). For the failure tests the fascicles remained in the loading chambers and each chamber was individually secured in a materials testing machine (Bionix100, MTS, 50 N load cell). Fascicles were then loaded to failure at a rate of 1 m ms $^{-1}$ at room temperature. Force and deformation were both continuously recorded at 50 Hz and engineering stress and strain calculated using the initial CSA and length of the sample. From the resulting data, the point at which a 0.1 N load was detected was located, and defined as the test start point. The original sample length was corrected accordingly.

2.2. Second experiment: effect of GAG depletion on stress relaxation and recovery

Fascicles were subjected to overnight incubation (15–16 h) in buffer alone (Con_B) or buffer with 0.5 U chondroitinase ABC (catalog no 2905, Sigma-Aldrich, St Louis, MO, USA) (Con_Ch; SR_Ch; SR_Ch_2 h) at 37 °C. The buffer solution was 50 mM Tris, 60 mM sodium acetate, in 0.02% bovine serum albumin at pH 8, while chondroitinase ABC enzymatically cleaves GAG chains from their proteoglycan core protein [20]. The fascicles were then secured in loading chambers filled with DMEM, and either tested to failure directly (Con_B and Con_Ch), or tested after being subjected to 30 min static stress relaxation with (SR_Ch_2 h) or without (SR_Ch) 2 h of recovery (Table 1). Subsequent to the failure test, fascicles were harvested and the GAG content determined by a spectrophotometric SAG assay.

2.3. GAG assay

The GAG assay was performed as described previously [21]. Briefly, fascicles were lyophilized overnight and fascicle dry weight was recorded. Samples were digested overnight in 0.4 U ml $^{-1}$ papain (Sigma-Aldrich, Poole, UK) at 60 °C. The digest supernatant was mixed with 1,9-dimethylmethylene blue (DMB) and the GAG content determined by spectrophotometry at a wavelength of 595 nm, using a standard curve of chondroitin-4-sulfate derived from bovine cartilage.

2.4. Statistical analyses

We used SPSS 16.0 for Windows including a Bonferroni-type correction to account for multiple comparisons. A one-way ANOVA was used to compare failure properties of control fascicles with those subjected to stress relaxation or buffer (Con to SR, SRC, SR_2 h and SRC_2 h; Con_Ch to SR_Ch and SR_Ch_2 h; Con_B to Con_Ch). Two-way ANOVAs were used to analyse the effect of

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