Effect of elastase treatment on the structure and function of tendon

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Abstract

Elastin is an important extracellular matrix protein that is able to withstand large deformations and has been suggested to provide elastic recoil and resilience to numerous dynamic tissues. However, elastin's contribution to tendon mechanics is poorly understood. The objective of this study was to characterize the mechanical contribution of elastin to the macroscopic deformation of tendon. An elastase treatment was used to degrade elastin in excised tendon specimens and the elastic and viscous properties were characterized using quasi-static and hysteresis tensile tests. Multiphoton microscopy and biochemical assays were used to characterize the degradation profile of elastase on the treated tissue. The results indicated that the low stress-strain response and hysteresis of tendon were unaffected by the treatment, but the failure stress was significantly reduced. Moreover, elastase significantly degraded glycosaminoglycans and caused loosening of fibril crimp. These results suggest that elastin does not contribute to the low stress-strain response or elastic recoil of tendon as observed in other dynamic tissues. Further, elastase degrades non-elastin molecules in addition to elastin resulting in a complex alteration of the extracellular matrix and consequently tendon mechanics.

Introduction

Tendon is a complex hierarchical tissue that transmits large tensile forces from muscles to bones. Type I collagen is the main structural unit and is responsible for the high tensile strength of tendon. Noncollagenous structural molecules, including proteoglycans and glycoproteins, are also distributed throughout tendon (Riley, 2005). However, the extent to which these structural molecules affect the overall mechanical response of the tissue is not well understood.

Elastin has a low stiffness (E ~ 300-400 kPa) (Aaron and Gosline, 1981) compared to collagen (E ~ 4-11 GPa) (Wenger et al., 2007), but is highly resilient and able to withstand large deformations (~150% of initial length) (Gosline et al., 2002). Tendon is thought to consist of 2% elastin per dry weight (Lowry et al., 1941) deposited in the core of elastic fibres and surrounded by microfibrils (Mithieux and Weiss, 2005). Elastic fibres are oriented parallel to collagen fibrils, which run axially along tendon (Grant et al., 2013; Ippolito et al., 1980; Ritty et al., 2002; Smith et al., 2011).

The stress-strain response of tendon has a 'j-shape' and consists of a nonlinear and linear region (Rigby et al., 1959). The nonlinear response occurs at low strains (< 5%) and is thought to result from the straightening of crimped collagen fibrils (Butler et al., 1978). As the strain increases, fibril crimp disappears and the response becomes linear. Given the unique mechanical properties of elastin, researchers have hypothesized that elastin contributes to the low stress-strain response and elastic recoil of tendon (Greenlee et al., 1966; Millesi et al., 1995; Minns et al., 1973). However, there is a lack of experimental evidence to support this hypothesis.

Although the term 'elastic recoil' has been used throughout the literature it does not represent a mechanical property that can be easily measured. The term is commonly used to describe the ability of hollow organs, including lungs and arteries, to return to their unloaded

state following the removal of load (Martin and Sugihara, 1973). We have chosen to use mechanical hysteresis, which is a measure of the dissipated energy against internal friction during one loading cycle of the tissue to act as an appropriate indicator for a change in elastic recoil . The magnitude of hysteresis is dependent on the path of the unload portion of the curve, or return of the tissue to its original shape, therefore it can be used to measure a change in elastic recoil.

Previous studies have used targeted enzymatic treatments in combination with biomechanical testing to quantify the mechanical contribution of various structural molecules in tissue. Elastase degrades elastin and has been used to study the tissue mechanics of the esophagus (Fan et al., 2005), intervertebral disk (Jacobs et al., 2011), skin (Oxlund et al., 1988), and aortic valve (Vesely, 1997). Crude elastase has been shown to degrade collagenous proteins (Kafienah et al., 1998), so modern protocols use purified enzymes in the presence of protease inhibitors to reduce nonspecific degradation.

The aim of this study was to elucidate the role of elastin in the macroscopic mechanical behavior of tendon. We tested the hypothesis that elastic fibres contribute to the low stress-strain response and/or hysteresis of tendon by using an elastase treatment in combination with mechanical testing. Quasi-static and hysteresis mechanical tests were used to quantify the elastic and viscous properties of treated tendon specimens, which were compared using one way ANOVA. Moreover, the degradation profile of the elastase treatment was characterized through structural imaging and biochemical assays.

Materials and Methods

Specimen Preparation

Tails from Sprague Dawley rats (350 +/- 12 g, n = 12) were obtained from animals that were euthanized for an unrelated study. The skin was decontaminated with 5% ethanol and 10 fascicles (length ~ 20 cm) were excised from the distal region of each tail under a laminar

flow hood within one hour of sacrifice. The fascicles were placed in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS) and 1% penicillin–streptomycin to maintain tissue integrity.

Enzyme Treatment

A modified elastase protocol from Oxlund et al. (1988) was used to treat tendon specimens. An elastase solution was created by combining 1 U/ml of elastase (Sigma E1250) in buffered PBS solution (pH 8.6) with a trypsin inhibitor (0.1 mg/ml) (Sigma T6414). Fascicles were transferred into a 36 well plate containing DMEM (n=30) buffered PBS (n=30), or elastase solution (n=30). The fascicles were then placed in an incubator (37°C, 5% CO_2) for 36 h to promote digestion of elastin.

Mechanical Testing

A tensile testing machine (Bose, UK) was used to characterize the material properties of treated RTTFs. A PBS bath at room temperature (24°C) was used to hydrate the samples during mechanical testing and custom-built tensile testing clamps were used to grip the RTTFs, which minimized stress concentrations and prevented slippage.

The diameter of the RTTFs was measured at six locations along the testing region using a digital micrometer (Keyence, UK) following incubation. Between each measurement the specimens were hydrated with PBS to avoid measurement errors due to water loss. The average diameter was used to calculate the cross sectional area assuming the geometry of fascicles to be cylindrical.

A sinusoidal displacement corresponding to 5% strain was applied to the specimen for 30 cycles to precondition the tissue. Following preconditioning the gauge length was measured using a calibrated stereomicroscope (± 0.1 mm, Zeiss, UK). In quasi-static tests the specimens were subjected to ramped displacement to failure at a rate of 0.05 mm/s and the force and grip-to-grip displacement recorded at 20 points per second. Hysteresis tests were

conducted by applying a ramped displacement corresponding to 5% strain to the tendon specimen at a rate of 0.05 mm/s followed by a ramped displacement to the resting length.

The force and displacement were converted into engineering stress (F/A) and strain ($\Delta L/L$) and plotted as stress-strain curves in Matlab (Mathworks, UK). The toe strain, ultimate tensile strength (UTS), linear modulus, and failure strain were calculated for quasi-static tests (Figure 1a). The toe strain was defined as the point where the slope of the curve became constant as determined by the zero crossing of the numerical second differential. The linear modulus was calculated using a linear least squares fit to all the data points between 5% and 85% of the UTS. The mechanical hysteresis was calculated by dividing the area between the loading and unloading curves (energy dissipated) by the area under the loading portion of the curve (energy input) (Figure 1b). One way ANOVA with a post hoc Tukey test was used to compare the material constants in Prism (GraphPad, UK).

Multiphoton Microscopy

Multiphoton microscopy was used to visualize collagen and elastin structures in tendon specimens and scaffolds using the second harmonic generation (SHG) and two photon fluorescence (TPF) signals, respectively. Specimens were placed on slides and three drops of PBS were added to the sample to prevent dehydration. Samples were viewed under an inverted confocal laser scanning microscope (Zeiss LSM 710, USA) fitted with a tuneable pulsed femtosecond titanium-sapphire Chameleon laser (Coherent, USA) operating at 860 nm. A 20x 1.3 NA objective lens with 0.4 µm axial resolution was used to collect emission spectra at 430 nm and 520 nm corresponding to the SHG and TPF signals, respectively. Band pass filters (430/50 nm, 520/50 nm) were used to collect the signals in nondescanned mode at 0.9 µm incremental steps though the specimen creating a 3D image. The average power at the sample was 15 mW and the spectra acquisition time was 8 s. Images were

averaged over 5 frames to reduce background noise and there was no sign of photo-damage during imaging.

A 20 mm portion of each rat tail tendon fascicle (n=120) was used to quantify the crimp structure of the specimen after the corresponding treatment. A tile scan was used to collect four images along the tendon corresponding to a length of 3.4 mm. The image was imported into Image J (NIH, US) and the crimp length and angle (Figure 4) were measured using the angle and line segment tool.

Collagen-Elastin Scaffold

Freeze-dried collagen-elastin scaffolds were prepared using type I collagen from bovine Achilles tendon (Sigma C9879) and bovine nuchal ligament (Sigma E1625) to characterize the degradation of elastase. A 1:1 collagen-elastin ratio suspension was mixed in 0.05 M acetic acid (pH 3.2) and cast into polytetrafluoroethylene cylindrical molds 50 mm in diameter. The suspension was cooled to -80°C and then placed in a freeze dryer (Christ I-5, UK) for 24 h in a vacuum at 0.05 mbar. The ice crystals were removed by sublimation, which left porous solid scaffolds.

The scaffolds were placed into a 36 well plate and treated with PBS or elastase then incubated (37°C, 5% CO_2) for 36 h. Before and after incubation the scaffolds were imaged using MPM to characterize the organization of collagen and elastin.

Biochemical Assays

PBS and elastase treated specimens were subjected to DMMB (Farndale et al., 1986) and hydroxyproline (Reddy and Enwemeka, 1996) assays to quantify the degradation of GAGs and type I collagen, respectively. Briefly, tendon specimens were rinsed in PBS, weighed, digested, subjected to a colorimetric agent, and quantified using a spectrophotometer (Spectramax Plus, UK). For the DMMB assay, papain was used to digest the specimens and chondroitin sulfate (C9819, Sigma Aldrich, UK) was used to create a standard curve.

Similarly, tendon specimens for the hydroxyproline assay were autoclaved and then digested with Chloromine-T reagent and hydroxyproline (H54409, Sigma Aldrich, UK) was used to create a standard curve.

Results

The mechanical response of tendon specimens followed the expected curve for quasi-static and hysteresis tensile tests (Figure 1). Preconditioning led to repeatable results and the mechanical properties of fresh tendons (modulus 327 ± 29 MPa) were comparable to those published in the literature (Screen et al., 2004). The majority (98%) of tendons failed at the midpoint of tendon through fibre pullout and sliding did not occur at the grips as observed with a stereomicroscope.

Quasi-static tensile tests indicated that there was a large decrease ($p<0.001$) in the UTS of elastase treated specimens compared to fresh tendon (Figure 2). Elastase treated tendon ruptured after sustaining a mean maximum stress of 11.8 MPa compared to 30.5 MPa in fresh specimens. There was a small, but significant ($p<0.05$) decrease in the linear modulus of elastase treated tendon compared to fresh samples. However, there was not an observable difference ($p>0.05$) in the toe strain and hysteresis of elastase treated specimens compared to fresh tendon. The mechanical properties of PBS incubated specimens varied from that of fresh tissue in quasi-static tests. The linear modulus decreased ($p<0.01$) indicating a reduction in the stiffness of the tissue. Moreover, the failure strain of PBS treated specimens was greater ($p<0.01$) than that of fresh tissue. Similar to elastase, PBS did not affect the toe strain or hysteresis of tendon specimens ($p>0.05$). Tendon incubated in DMEM had similar mechanical properties to fresh tendon ($p>0.05$).

The RTTFs emitted a strong SHG signal and the fibril crimp was easily distinguished. There was little variation in the crimp angle and wavelength of fresh tendon fascicles excised from animals used in this study. DMEM specimens had a similar fibril crimp structure to

fresh tendon ($p>0.05$), with a large crimp angle and small crimp length (Figure 3 and Figure 4). However, PBS treated specimens had a loose fibril crimp structure compared to fresh specimens and the crimp angle was significantly reduced while the length increased ($p<0.001$). Elastase treated specimens had a longer wavelength and a reduced crimp angle compared to fresh specimens ($p<0.001$). Specimens incubated in PBS sustained an increase in diameter ($p<0.01$), while other treatments did not vary from fresh specimens ($p>0.05$).

Collagen-elastin scaffolds had a random distribution of collagen and elastin as observed with the SHG and TPF signals, respectively (Figure 5). Collagen formed smaller fibres than elastin, but both had a fibrous structure. When treated with the elastase protocol, the collagen structure appeared unaffected by the treatment, but the elastin had been completely degraded. Moreover, elastic fibres were not visible in tendon treated with elastase as observed with the TPF signal. Colocalization imaging showed that the SHG and TPF signals originated from independent sources (data not shown).

Both the DMMB and hydroxyproline assays gave consistent results for the glycosaminoglycan and collagen content of fresh and treated specimens (Figure 6). The hydroxyproline content did not vary across treatments ($p>0.05$) and the GAG content was reduced in elastase treated tendon ($p<0.001$).

Discussion

In this study mechanical testing, multiphoton microscopy, and biochemical assays were used to analyze the structural and functional changes of tendon treated with elastase to better understand the mechanical role of elastin in tendon. Mechanical tests indicated that elastase did not affect the low stress-strain response or hysteresis of tendon, but significantly reduced the failure stress. Moreover, elastase caused loosening of crimp of collagen fibrils and significantly degraded glycosaminoglycans.

The literature suggests that elastin contributes to the low stress-strain response and elastic recoil of tendon, but there remains a lack of evidence to support this theory, which has been primarily derived from cardiovascular studies. The composition, organization, and loading conditions of cardiovascular tissue are significantly different from those of tendon. For example, the human aorta consists of 30% elastin (Lowry et al., 1941) which forms lamellae and experiences circumferential stresses up to 16 kPa (Khanafer et al., 2011), while human Achilles tendon consists of 2% elastin (Lowry et al., 1941) which is distributed in fascicles and experiences tensile stresses of up to 70 MPa (Magnusson and Kjaer, 2003). Therefore, the applicability of this theory needs to be verified for tendon given the large variation in tissue composition, structure, and loading conditions. Results of the present study do not support the use of cardiovascular theories to describe tendon mechanics because the low stress-strain response and hysteresis were unaffected by elastase.

There is much interest in the structure of fibril crimp because diseased tendon experiences notable variation in crimp angle and periodicity (Tuite et al., 1997). Fibril crimp has been suggested to act as a shock absorber protecting tendon from impact loading (Frank, 2004; O'Brien, 1997) although the organization and origin of fibril crimp is a highly debated topic. Structural studies have speculated that elastin is responsible for maintaining fibril crimp (Caldini et al., 1990; Cooper et al., 1970) and the undulation of crimp following an elastase treatment has been used to support this theory (Oakes and Bialkower, 1977). We have confirmed that elastase significantly reduces fibril crimp in tendon, but attribute this result to both the removal of elastin as well as the nonspecific degradation of the extracellular matrix, including glycosaminoglycans. Fibril crimp is likely dependent on the structural integrity of the ground substance that surrounds collagen fibrils and not solely a function of elastin. Recent studies have shown that tenocytes are capable of inducing fibril crimp through a cytoskeletally mediated mechanism (Gardner, 2012; Herchenhan et al., 2011). Mechanical

results of the present study indicated that there is a significant difference in the crimp length and angle between tendons treated with DMEM and PBS. A live-dead stain (data not shown) indicated that the majority of cells survived incubation in DMEM for 36 hours and no cells survived when incubated in PBS. This observation may provide supporting evidence for cell-induced crimp although tissue swelling also influences the tissue structure.

In addition to structural observations, there are two major enzyme treatment studies that have been cited in support for the mechanical role of elastin in tendon. Minns et al. (1973) used alpha-amylase and EDTA to degrade the ground substance of the Achilles tendon and Millesi et al. (1995) used elastase to degrade elastin in the tendon associated with the palmaris longus muscle. Our results correlate well with the study conducted by Minns et al. (1973), who found the breaking strength of tendon to be significantly reduced following degradation of the ground substance. However, their focus was on the time-dependent properties of tendon and they did not mention changes in the toe region following treatment. Millesi et al. (1995) used elastase to degrade elastin and documented an increase in residual strain following treatment. Our results conflict with this result as the hysteresis loop of treated and untreated specimens did not significantly vary. Millesi et al. (1995) failed to mention the use of preconditioning in their mechanical testing protocol, which is key to achieving repeatable results. Yahia and Drouin (1990) showed that the hysteresis area of the first cycle of preconditioning in elastase treated specimens was greater than that of fresh tendon, but the difference disappeared after eleven loading cycles. We found a similar result, with the first cycle of preconditioning leading to an apparent residual strain due to an increase in the length of the tendon (data not shown).

As indicated in the structural analysis and degradation profile, tendon is subjected to nonspecific degradation when exposed to elastase. Elastase is a powerful enzyme that can degrade a host of structural units in the ECM and has been shown to decrease GAG content

by 32-92%, while having a limited effect on type I collagen (Barbir et al., 2010; Jacobs et al., 2011; Smith et al., 2008). We found that elastase decreased GAG content by 36% as well as degraded elastin in tissue engineered scaffolds while having an insignificant effect on type I collagen. Therefore, the mechanical analysis following an elastase treatment should be interpreted as the degradation of a range of ECM structural molecules rather than elastin alone. The mechanical contribution of GAGs to tendon mechanics has received much interest, but their role is poorly understood. Our results indicated that degradation of the ground substance reduces the ultimate tensile strength of tendon. Although elastase does degrade GAGs, the tissue does not experience the same mechanical changes to tissue exposed to enzymes that specifically degrade GAGs. By comparing the degradation profile of elastase and GAG specific enzymes the mechanical role of various structural molecules may be characterized.

Our results indicated that incubating tendon specimens in PBS caused the tissue to swell. The diameter increased by 16% and the fibril crimp was more loosely organized than fresh or DMEM treated tendon. Previous studies have shown that the inter-fibril spacing of tendon increases after incubated in PBS and that the there is a significant increase in the water content (Han et al., 2000; Lujan et al., 2009; Screen et al., 2006; Screen et al., 2005). However, the mechanical properties of collagen fibrils were not altered following PBS incubation (Svensson et al., 2010). Our results indicated that PBS incubated specimens failed at a larger strain, which confirms previous findings (Chimich et al., 1992). An increased failure strain suggests that there is an increase in sliding following PBS treatment. Although tendon specimens treated with elastase were incubated in PBS, the effect of the enzyme exceeded the effects of the incubation media. However, the results of the enzyme treatment should be interpreted as a combination of the PBS treatment and elastase degradation. An increase in fibril diameter due to tissue swelling would affect the linear modulus and ultimate

tensile strength of tendon. Therefore, tissue swelling may be partly responsible for the decrease in UTS seen in PBS and elastase treated specimens. Tendon specimens treated with DMEM did not appear to experience tendon swelling and the mechanical properties were not significantly different from fresh tissue.

The current study was conducted to provide a detailed description of the effect of elastase on the structure and function of tendon. The enzyme treatment was based on a protocol used in several studies examining the structure-function relationship of dynamic tissues. Although the enzyme treatment, mechanical testing, and imaging were highly controlled, there are several limitations of the current work. First, we used rat tail tendon fascicles as tissue specimens because they minimize inter-animal variation. The fascicles offer several benefits over alternative tendons because they have a consistent diameter, repeatable mechanical properties, and do not require sectioning before imaging. However, by using RTTFs, we can only speculate about the effect of elastase on other types of tendon. It has been shown that areas of tendon that experience larger deformations contain a higher concentration of elastic fibres (Ritty et al., 2002) and the role of elastin may be significant in these regions. Moreover, by testing RTTFs we failed to test the mechanical contribution of the inter-fascicle space or endotenon sheath. It has been shown that elastic fibres are distributed between collagen fascicles in a loose mesh like organization (Grant et al., 2013; Korol et al., 2007). Future studies, conducted on whole tendon may be compared with the results of the current study to distinguish between the mechanical contribution of the sheath and tendon proper. Second, the focus of the current study was on the low stress-strain response and hysteresis of tendon. However, elastic fibres may contribute to additional time dependent properties including the stress-relaxation and creep response. Third, although the elastase treatment protocol was designed to limit nonspecific degradation, non-elastin proteins were degraded

by the enzyme. The enzyme used for the study was highly purified, but a more specific enzyme could yield more conclusive evidence.

In conclusion, we investigated the contribution of elastin to the mechanical properties of tendon to determine if it contributes to the low stress-strain response and elastic recoil of tendon as suggested in the literature. We found that elastase did not affect the toe region of the curve or the mechanical hysteresis, but significantly reduced the failure properties of tendon. Although elastase was very efficient at degrading elastin in collagen-elastin scaffolds, the degradation profile of tendon indicated that noncollagenous proteins were affected by the treatment. Therefore, the results should be interpreted as the effect of elastase on the structure and function of tendon and not necessarily the role of elastin in tendon. The theory that elastin contributes to the low stress-strain response and elastic recoil of tendon requires more evidence and future studies are required to determine its exact role, which may include non-mechanical functions.

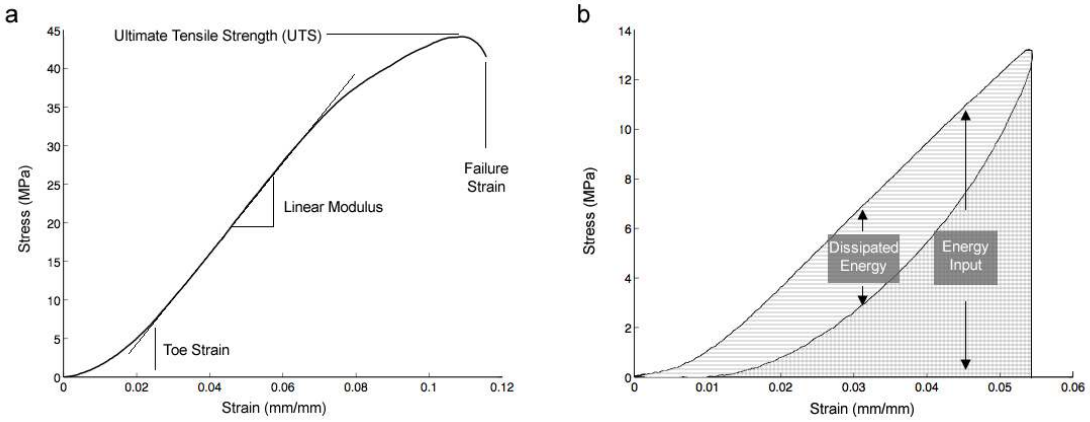

Figure 1 – Representative stress-strain plots for (a) quasi-static test-to-failure and (b) mechanical hysteresis tensile tests. The toe strain, linear modulus, ultimate tensile strength, and failure strain were used to compare quasi-static tests and the mechanical hysteresis (dissipated energy / energy input) was used to compare hysteresis tests

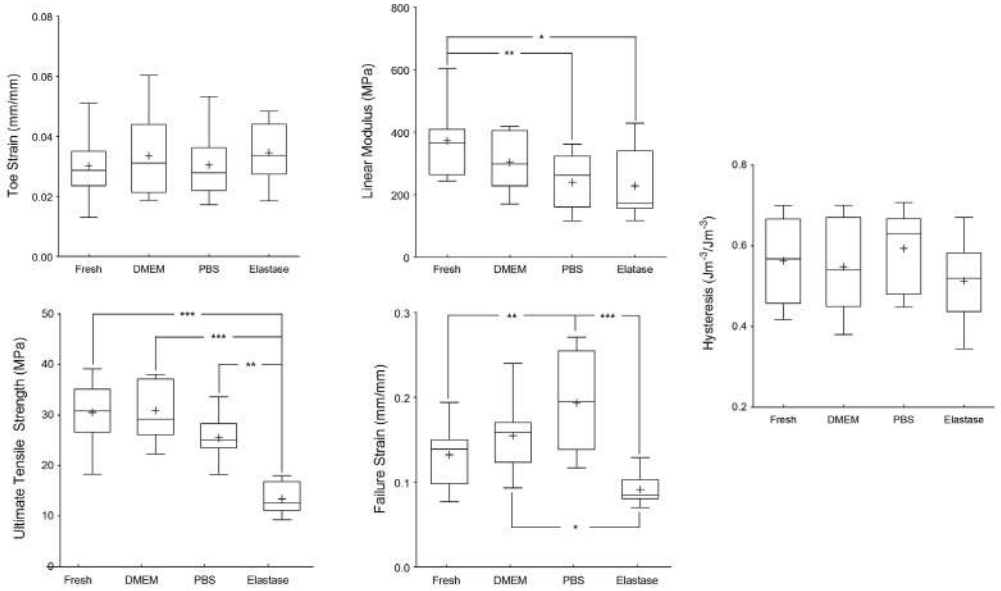

Figure 2 – Tensile test statistical summary for Fresh, DMEM, PBS, and elastase treated tendon (one way ANOVA, post hoc Tukey, * P <0.05, **P<0.01, ***P<0.001). The largest significant differences were observed in the ultimate tensile strength of elastase treated specimens. Treatments did not have a significant effect on the toe strain and hysteresis response.

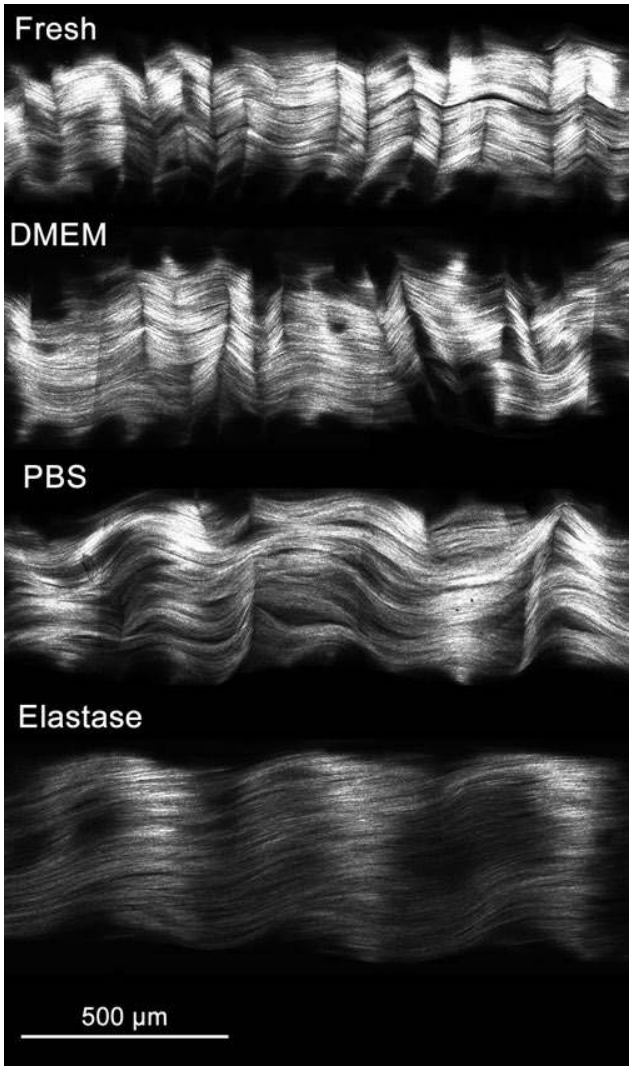

Figure 3 – Representative images acquired by the second harmonic generation signal of tendon depicting fibril crimp organization of fresh and treated (DMEM, PBS, and elastase) tendon specimens. Collagen fibril crimp is tightly organized in fresh and DMEM specimens with the organization becoming more loosely packed in PBS and elastase treated specimens. There is an observable increase in diameter of PBS and elastase treated specimens as compared to DMEM and fresh tendon.

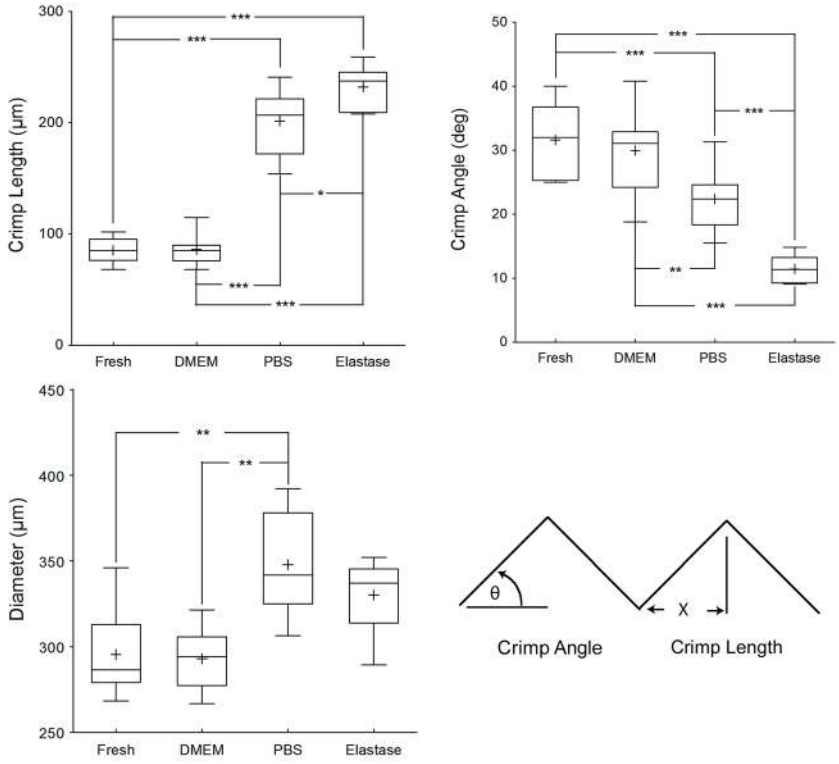

Figure 4 – Structural analysis statistical summary for Fresh, DMEM, PBS, and elastase treated tendon (one way ANOVA, post hoc Tukey, * P <0.05, **P<0.01, ***P<0.001). There was a significant increase in the crimp length and a significant decrease in crimp angle of PBS and elastase treated specimens depicting a loss of fibril crimp. The diameter was significantly larger in PBS specimens indicative of tissue swelling.

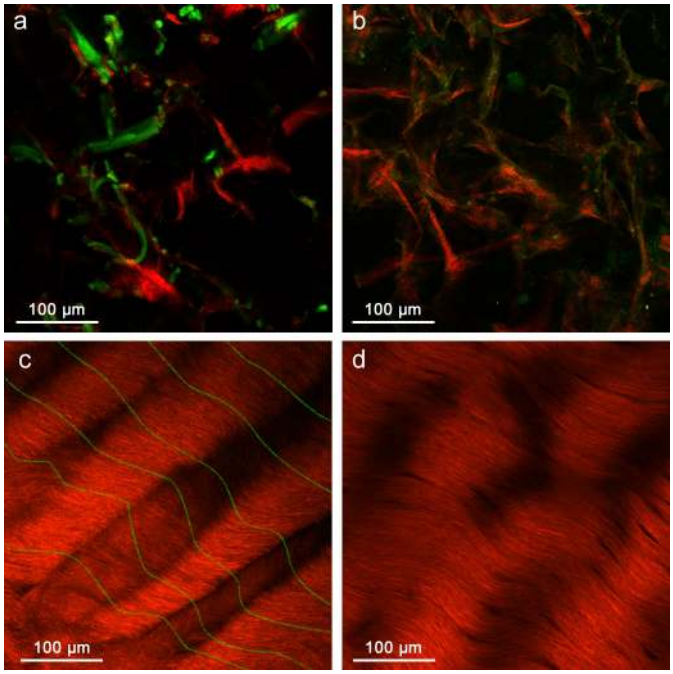

Figure 5 – Multiphoton microscopy analysis of elastin-collagen scaffolds and rat tail tendon fascicles treated with elastase. The second harmonic generation signal (red) was used to identify collagen fibres and the two photon fluorescence signal (green) for elastin fibres. Collagen and elastin fibres were visible in (a) PBS treated scaffolds and (c) fresh rat tail tendon fascicles. Elastin fibres were largely degraded following elastase treatment of (b) collagen-elastin scaffolds and (d) rat tail tendon fascicle.

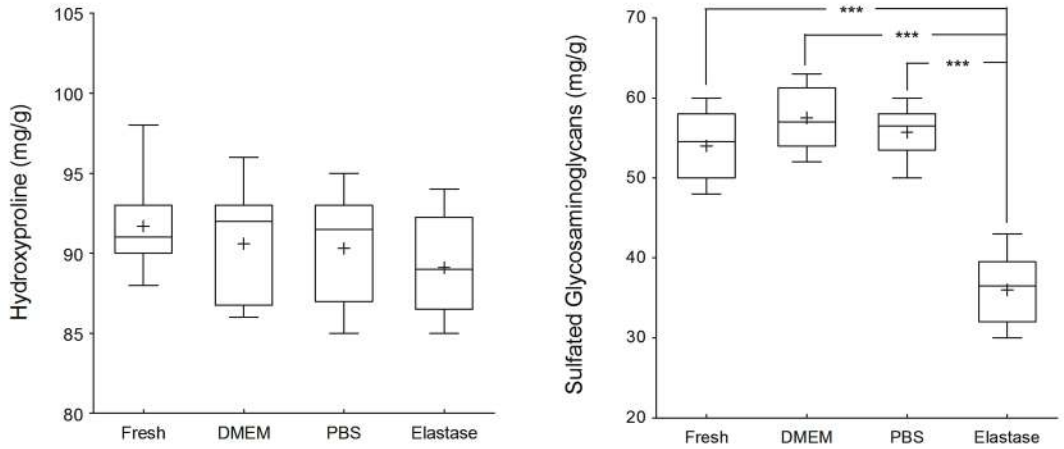

Figure 6 – Results of biochemical assays measuring the (a) collagen content and (b) glycosaminoglycan content of fresh and treated (DMEM, PBS, elastase) tendon (one way ANOVA, post hoc Tukey, * P <0.05, **P<0.01, ***P<0.001). Collagen content did not significantly vary between treatments, while the glycosaminoglycan content was significantly reduced in elastase treated tendon.

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