

1 **Effect of elastase treatment on the structure and function of tendon**

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1 **Abstract**

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

1 **Introduction**

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

8 Elastin has a low stiffness ($E \sim 300\text{-}400$ kPa) (Aaron and Gosline, 1981) compared to
9 collagen ($E \sim 4\text{-}11$ GPa) (Wenger et al., 2007), but is highly resilient and able to withstand
10 large deformations ($\sim 150\%$ of initial length) (Gosline et al., 2002). Tendon is thought to
11 consist of 2% elastin per dry weight (Lowry et al., 1941) deposited in the core of elastic
12 fibres and surrounded by microfibrils (Mithieux and Weiss, 2005). Elastic fibres are oriented
13 parallel to collagen fibrils, which run axially along tendon (Grant et al., 2013; Ippolito et al.,
14 1980; Ritty et al., 2002; Smith et al., 2011).

15 The stress-strain response of tendon has a 'j-shape' and consists of a nonlinear and linear
16 region (Rigby et al., 1959). The nonlinear response occurs at low strains ($< 5\%$) and is
17 thought to result from the straightening of crimped collagen fibrils (Butler et al., 1978). As
18 the strain increases, fibril crimp disappears and the response becomes linear. Given the
19 unique mechanical properties of elastin, researchers have hypothesized that elastin
20 contributes to the low stress-strain response and elastic recoil of tendon (Greenlee et al.,
21 1966; Millesi et al., 1995; Minns et al., 1973). However, there is a lack of experimental
22 evidence to support this hypothesis.

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

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

7 Previous studies have used targeted enzymatic treatments in combination with
8 biomechanical testing to quantify the mechanical contribution of various structural molecules
9 in tissue. Elastase degrades elastin and has been used to study the tissue mechanics of the
10 esophagus (Fan et al., 2005), intervertebral disk (Jacobs et al., 2011), skin (Oxlund et al.,
11 1988), and aortic valve (Vesely, 1997). Crude elastase has been shown to degrade
12 collagenous proteins (Kafienah et al., 1998), so modern protocols use purified enzymes in the
13 presence of protease inhibitors to reduce nonspecific degradation.

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

21 **Materials and Methods**

22 *Specimen Preparation*

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

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

4 *Enzyme Treatment*

5 A modified elastase protocol from Oxlund et al. (1988) was used to treat tendon
6 specimens. An elastase solution was created by combining 1 U/ml of elastase (Sigma E1250)
7 in buffered PBS solution (pH 8.6) with a trypsin inhibitor (0.1 mg/ml) (Sigma T6414).
8 Fascicles were transferred into a 36 well plate containing DMEM (n=30) buffered PBS
9 (n=30), or elastase solution (n=30). The fascicles were then placed in an incubator (37°C,
10 5% CO₂) for 36 h to promote digestion of elastin.

11 *Mechanical Testing*

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

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

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

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

3 The force and displacement were converted into engineering stress (F/A) and strain
4 ($\Delta L/L$) and plotted as stress-strain curves in Matlab (Mathworks, UK). The toe strain,
5 ultimate tensile strength (UTS), linear modulus, and failure strain were calculated for quasi-
6 static tests (Figure 1a). The toe strain was defined as the point where the slope of the curve
7 became constant as determined by the zero crossing of the numerical second differential. The
8 linear modulus was calculated using a linear least squares fit to all the data points between
9 5% and 85% of the UTS. The mechanical hysteresis was calculated by dividing the area
10 between the loading and unloading curves (energy dissipated) by the area under the loading
11 portion of the curve (energy input) (Figure 1b). One way ANOVA with a post hoc Tukey test
12 was used to compare the material constants in Prism (GraphPad, UK).

13 *Multiphoton Microscopy*

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

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

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

8 *Collagen-Elastin Scaffold*

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

16 The scaffolds were placed into a 36 well plate and treated with PBS or elastase then
17 incubated (37°C, 5% CO₂) for 36 h. Before and after incubation the scaffolds were imaged
18 using MPM to characterize the organization of collagen and elastin.

19 *Biochemical Assays*

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

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

4 **Results**

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

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

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

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


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

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

18 **Discussion**

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

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

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

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

6 In addition to structural observations, there are two major enzyme treatment studies that
7 have been cited in support for the mechanical role of elastin in tendon. Minns et al. (1973)
8 used alpha-amylase and EDTA to degrade the ground substance of the Achilles tendon and
9 Millesi et al. (1995) used elastase to degrade elastin in the tendon associated with the
10 palmaris longus muscle. Our results correlate well with the study conducted by Minns et al.
11 (1973), who found the breaking strength of tendon to be significantly reduced following
12 degradation of the ground substance. However, their focus was on the time-dependent
13 properties of tendon and they did not mention changes in the toe region following treatment.
14 Millesi et al. (1995) used elastase to degrade elastin and documented an increase in residual
15 strain following treatment. Our results conflict with this result as the hysteresis loop of
16 treated and untreated specimens did not significantly vary. Millesi et al. (1995) failed to
17 mention the use of preconditioning in their mechanical testing protocol, which is key to
18 achieving repeatable results. Yahia and Drouin (1990) showed that the hysteresis area of the
19 first cycle of preconditioning in elastase treated specimens was greater than that of fresh
20 tendon, but the difference disappeared after eleven loading cycles. We found a similar result,
21 with the first cycle of preconditioning leading to an apparent residual strain due to an increase
22 in the length of the tendon (data not shown).

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

1 by 32-92%, while having a limited effect on type I collagen (Barbir et al., 2010; Jacobs et al.,
2 2011; Smith et al., 2008). We found that elastase decreased GAG content by 36% as well as
3 degraded elastin in tissue engineered scaffolds while having an insignificant effect on type I
4 collagen. Therefore, the mechanical analysis following an elastase treatment should be
5 interpreted as the degradation of a range of ECM structural molecules rather than elastin
6 alone. The mechanical contribution of GAGs to tendon mechanics has received much
7 interest, but their role is poorly understood. Our results indicated that degradation of the
8 ground substance reduces the ultimate tensile strength of tendon. Although elastase does
9 degrade GAGs, the tissue does not experience the same mechanical changes to tissue exposed
10 to enzymes that specifically degrade GAGs. By comparing the degradation profile of elastase
11 and GAG specific enzymes the mechanical role of various structural molecules may be
12 characterized.

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

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

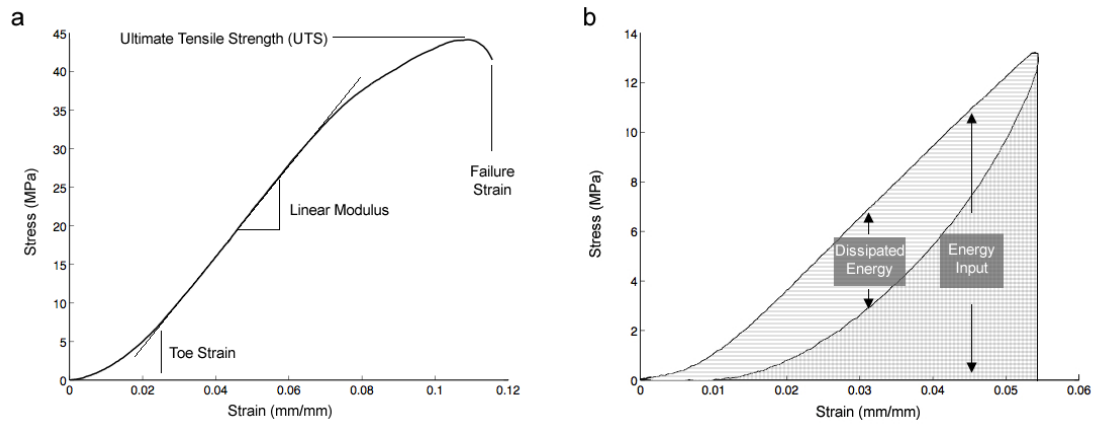
5 The current study was conducted to provide a detailed description of the effect of elastase
6 on the structure and function of tendon. The enzyme treatment was based on a protocol used
7 in several studies examining the structure-function relationship of dynamic tissues. Although
8 the enzyme treatment,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

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

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

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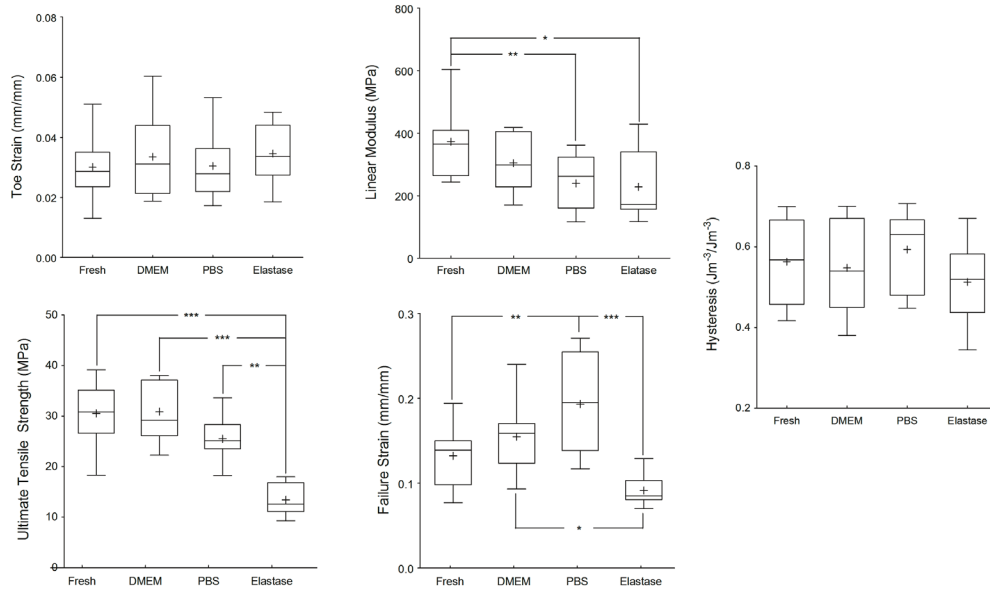
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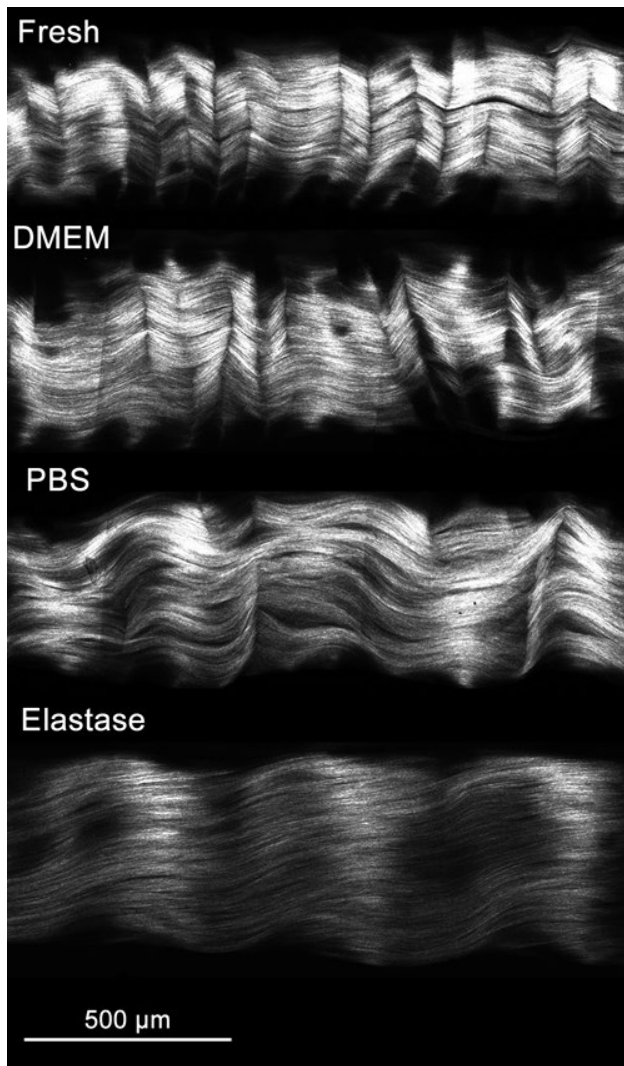
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Figure 1 – Representative stress-strain plots for (a) quasi-static test-to-failure and (b) mechanical hysteresis tensile tests. The toe strain, linear modulus, ultimate tensile strength, and failure strain were used to compare quasi-static tests and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

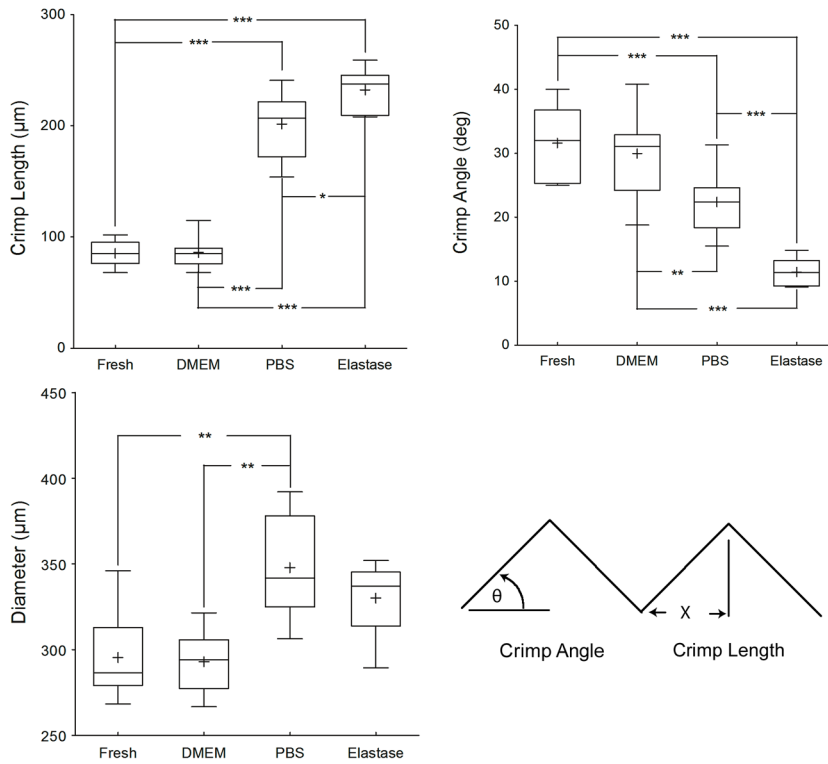


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 2 Figure 2 – Tensile test statistical summary for Fresh, DMEM, PBS, and elastase treated
 3 tendon (one way ANOVA, post hoc Tukey, * P <0.05, **P<0.01, ***P<0.001). The largest
 4 significant differences were observed in the ultimate tensile strength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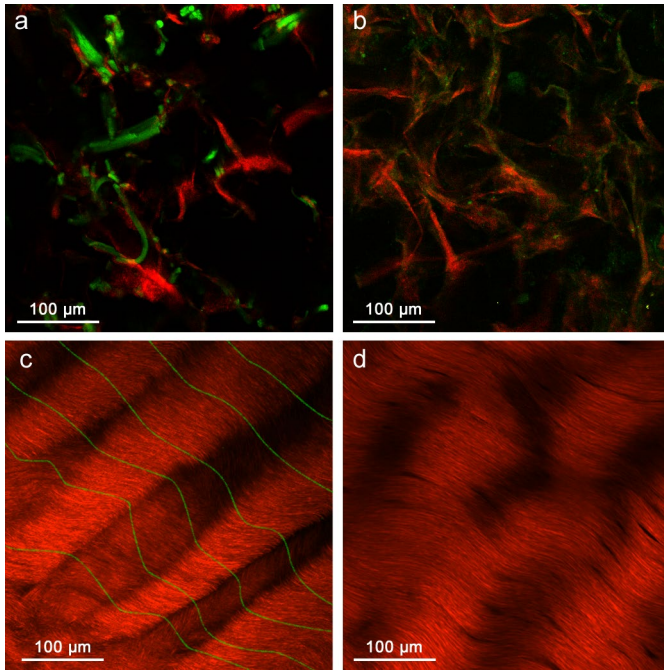


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Figure 3 – Representative images acquired by the second harmonic generation signal of tendon depicting fibril crimp organization of fresh and treated (DMEM, PBS, and elastase) tendon specimens. Collagen fibril crimp is tightly organized in fresh and 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.

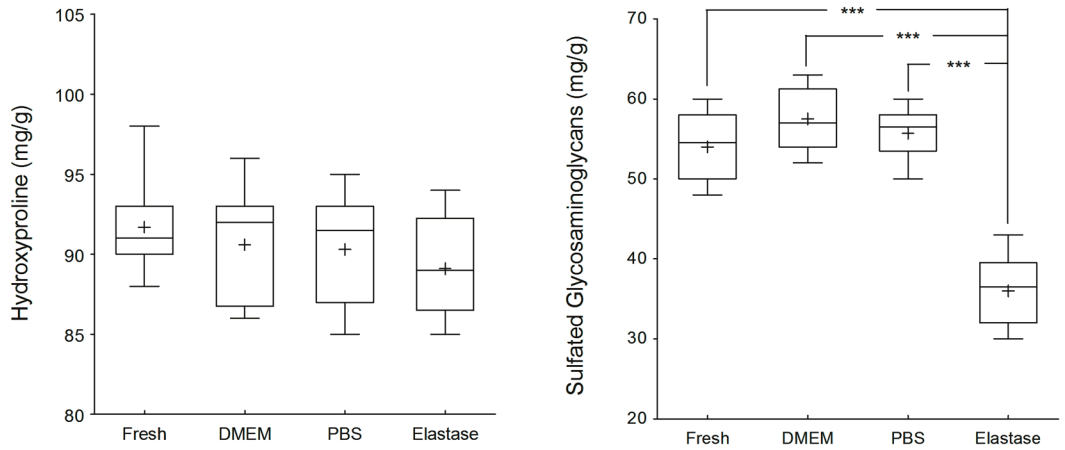


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 2 Figure 4 – Structural analysis statistical summary for Fresh, DMEM, PBS, and elastase
 3 treated tendon (one way ANOVA, post hoc Tukey, * P <0.05, **P<0.01, ***P<0.001).
 4 There was a significant increase in the crimp length and a significant decrease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



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



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