Queen Mary University of London
School of Engineering and Materials Science

EMS618U – Cell and Tissue Mechanics
Mechanical Testing of Tendons Lab Report

Name:	Student ID:
JATINDER DHALIWAL	220451019

Abstract

The aim of this report is to investigate the effect of storing tendon tissue in phosphate-buffered saline (PBS) prior to testing under tensile loads. Samples of rat tail tendons were taken and split into two groups – one group were dissected and incubated in PBS for one week and frozen up until the experiment, and the second group were dissected and frozen immediately until the experiment. Two mechanical tests were conducted – a stress relaxation and a stretch-to-failure test, to observe the behaviour of both sets of samples in each type of test. From the stress relaxation test, it was found that the fresh-frozen tendon relaxed at a lower rate than the incubated tendons, highlighting that changes to the internal structure of the tendons were caused by PBS. In the stretch-to-failure test, the results suggested that the ultimate tensile strength and toughness of the incubated tendons were significantly lower than that of the fresh-frozen tendons, further reinforcing the change of mechanical properties and viscoelastic behaviour of the tendons, depending on the storage method. This report is designed as follows – an Introduction section to display the background and theory of tendon structure and provide a hypothesis for this experiment, a Methods section to explain in detail the procedure for the experiment, a Results section showing data collected from the experiment, and a Discussion and Conclusions section which discuss the obtained results in further detail, linking back to literature.

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Introduction

Tendons act as “mechanical bridges” between muscle and bone, transmitting forces from muscle to bone tissue to facilitate movement [1]. They primarily consist of type I collagen fibres and proteoglycans, with other types of collagen also being present. The type I collagen fibres allow the tendon to resist tensile forces, while the high hydrophilicity of proteoglycans contributes to the viscous behaviour, where the stress or strain response is dependent on the rate at which the tendon is loaded [2,3]. Additionally, tendons have a hierarchical structure, starting from collagen molecules, which are the subunits of collagen fibrils at the nanoscale, the gathering of collagen fibrils to form collagen fibres at the microscale and the combining of fibres to form fascicles, which are enclosed by a connective tissue sheath known as the epitenon [4]. The tensile strength of a tendon is attributed to this hierarchy, as it allows forces to be distributed along the various components of the tendon, hence being able to withstand a relatively high load. Generally, the higher the tendon elasticity, the greater the muscle shortening. Therefore, tendons play a major role in physical performance by regulating muscle contraction force and inducing joint movement. However, tendon tissue inherently has a poor turnover and blood supply, highlighting that once tendon tissue is damaged, it can never fully regenerate. Although scar tissue is formed, the mechanical properties differ from that of normal tissue, meaning that the tissue function will not be restored [5].

The aim of this experiment is to explore the effect of incubating tendon tissue in phosphate buffered saline (PBS) using explants of rat tail tendons. Buffer solutions are typically used to maintain the hydration of a tissue before mechanical testing. However, PBS has been shown to increase the water content of a tendon, swelling the tissue, which in turn, decreases the tensile modulus, thus affecting the results obtained from mechanical tests. Furthermore, the swelling of the tendon more readily allows solutes from the buffer to diffuse into the tissue, potentially altering the structure and/or mechanics [6]. Based on this information, it can be expected that the incubated tendon sample will exhibit a greater stress relaxation than the fresh-frozen sample due to the reduced stiffness, allowing for more stress to be lost over time. The fresh-frozen sample can be expected to relax at a lower rate, due to its higher resistance to deformation. Also, the stretch-to-failure test may show higher peak stress for the fresh-frozen tendon, indicating a greater ultimate tensile strength (UTS). These factors highlight the probable mode of failure for both groups – the incubated sample would likely fail in a ductile manner, while the fresh-frozen sample may demonstrate brittle failure. The brittle nature of the fresh-frozen sample could indicate a lower strain at failure, due to reduced elongation, as opposed to the incubated sample, where the ductility and lower stiffness allow greater elongation at failure.

Methods

Storing Tendons

As previously mentioned, explants were taken from rat tail tendon, and separated into two groups. The first group were dissected and incubated in phosphate buffered saline (PBS) for one week, and frozen at -22ºC up until the experiment. This group was referred to as the “incubated” group. The second group were dissected and immediately frozen at -22ºC, which assumes that the mechanical properties of the tendon remained unchanged until the experiment was carried out. This group was labelled the “fresh-frozen” group. All samples were placed in

storage boxes and covered with tissue paper, to maintain the hydration of the tendons incubated in PBS.

Sample Preparation

The Instron testing machine was used for the mechanical testing in this experiment. The first test specimen was removed from the storage box and both ends of the sample were placed in each of the Instron machine's loading platens. Once clamped into place, a small preload was applied to the tendon to ensure that the tendon was taut before beginning the experiment. Using Vernier callipers, the length and diameter of the sample were measured and recorded. The load was zeroed on the software after the length of the taut tendon was measured. Finally, a small amount of water was sprayed onto the sample, ensuring that it remained hydrated, concluding the sample preparation stage of the experiment. This preparation method was applied to both groups of samples for both types of tests.

Mechanical Testing

One of the mechanical tests carried out was a stress relaxation test. Firstly, an incubated tendon was subjected to a strain of 5%, with a displacement rate of 0.8mm/s. The sample was held at this tension for three minutes, and the software connected to the Instron machine recorded the force at a frequency of 25 Hz. After three minutes, the data was saved and the incubated tendon sample was removed from the platens. A tendon from the fresh-frozen group was then prepared using the method mentioned previously, and the steps for the stress relaxation test were repeated for this tendon sample. Once both datasets were obtained, a graph of tensile stress against time was plotted, and, assuming exponential decay of stress, the following method was used to determine the relaxation time:

$$\sigma = \sigma_0 e^{-\frac{t}{\tau}} + \sigma_1 \tag{1}$$

Taking natural logarithms on both sides and creating a linear equation in the form $y = mx + c$:

$$\ln(\sigma - \sigma_1) = -\frac{t}{\tau} + \ln(\sigma_0) \tag{2}$$

where σ is the stress at time t in MPa, σ_0 is the initial stress in MPa, σ_1 is the residual stress in MPa and τ is the relaxation time constant, with units in seconds. Using this linear equation, a graph of $\ln(\sigma - \sigma_1)$ against time was plotted, from which the relaxation time constant could then be calculated through the equation:

$$\tau = -\frac{1}{m} \tag{3}$$

where m is the slope of the logarithmic graph.

Finally, the percentage drop in stress for each sample was calculated as a measure of how much each tendon had relaxed:

$$\%\ \text{Reduction} = \frac{\sigma_0 - \sigma_1}{\sigma_0} \times 100 \tag{4}$$

The second mechanical test was a stretch-to-failure test. An incubated tendon was prepared using the aforementioned method and subjected to 100% strain at a displacement rate of 0.5mm/s, as the large strain value ensured that the tendon would reach failure. Similar to the stress relaxation test, the force was recorded at a rate of 25 Hz. Once the tendon had reached

the failure point, the data was saved and the incubated tendon sample was removed. A sample of the fresh-frozen tendons was prepared and the stretch-to-failure test procedure was carried out in the same manner. A graph of tensile stress against tensile strain was then plotted and the tangent modulus was calculated using the following equation:

$$E_t = \frac{\Delta\sigma}{\Delta\varepsilon} \tag{5}$$

where E_t is the tangent modulus in MPa, $\Delta\sigma$ is the change in stress in MPa and $\Delta\varepsilon$ is the change in strain. The UTS for both samples were also assessed by comparing the peak stresses on both stress-strain curves. The toughness of each tendon, which is the amount of energy absorbed up to failure, was found by calculating the area under both curves using the trapezium rule:

$$U = \sum_{i=1}^{n-1} \frac{1}{2}(\varepsilon_{i+1} - \varepsilon_i)(\sigma_{i+1} + \sigma_i) \tag{6}$$

where U is the toughness in MJ/m^3, ε is the strain and σ is the stress in MPa.

Finally, for the stretch-to-failure test, the strain at failure is another parameter that was compared. In terms of qualitative data, the post-yield behaviour was briefly compared to explore how stress drops in both tendons after reaching the yield stress.

It should be noted that each test was only carried out once for each of the sample groups, totalling four tests, due to the limited number of samples available. Therefore, the focus of this experiment was not to obtain a reliable set of repeats.

Results

Stress Relaxation

To obtain the stress relaxation curve, a graph of tensile stress against time for each sample was plotted using the data recorded in the stress relaxation experiment, shown in Figure 1.

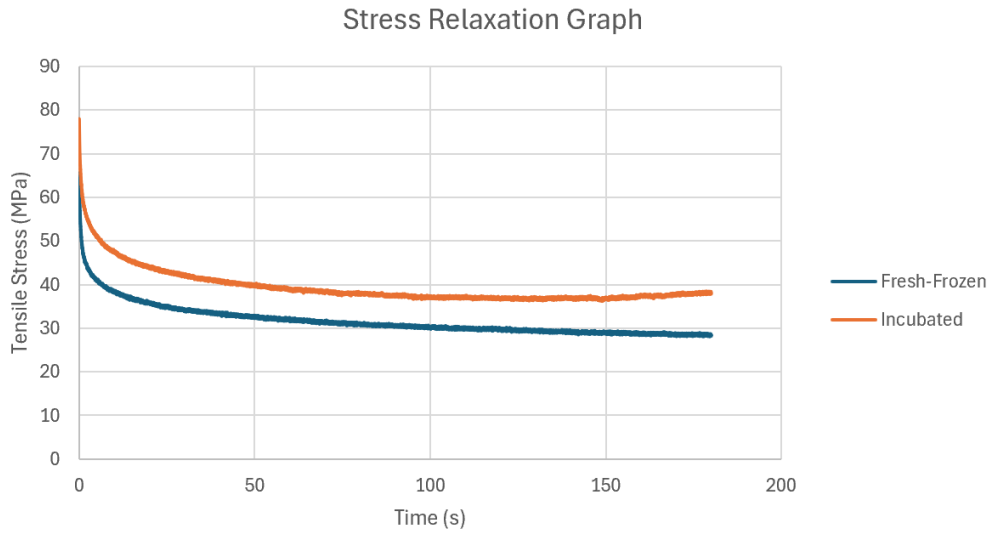

Figure 1 – Graph of Tensile Stress against Time for Stress Relaxation for PBS-incubated and fresh-frozen samples. This plot shows the stress relaxation curve after a strain of 5% was achieved. The data points during loading were not included in the graph.

From initial inspection, the curve for the PBS-incubated sample curved upwards slightly from approximately 150 to 180 seconds. This was not an expected result, as the stress should theoretically continue to decrease until it eventually reaches zero, meaning that the upward curve could have been caused either by experimental error or the mechanical changes the PBS potentially caused on the tendon. The incubated sample displayed a higher initial stress value which can be seen when examining the starting data points for each tendon, at 78.0 MPa (3 S.F.), compared to 65.3 MPa (3 S.F.) after the loading phase. This trend carried on throughout the whole relaxation phase, where the tensile stress for the incubated sample remained higher than the fresh-frozen samples, implying that the PBS potentially induced structural changes enabling the tendon to retain a higher stress throughout testing. Both samples exhibited relatively high residual stress, indicating that there may have been biological factors that influenced the long-term relaxation behaviour of both tendon types. The incubated sample had a greater value of residual stress, at 37.8 MPa (3 S.F.), compared to 28.2 MPa (3 S.F.) for the fresh-frozen tendon, possibly highlighting the effect of PBS on the behaviour of the tendon sample during stress relaxation.

The relaxation time constant was calculated using a logarithmic graph, as mentioned in the Methods section, shown in Figures 2 and 3.

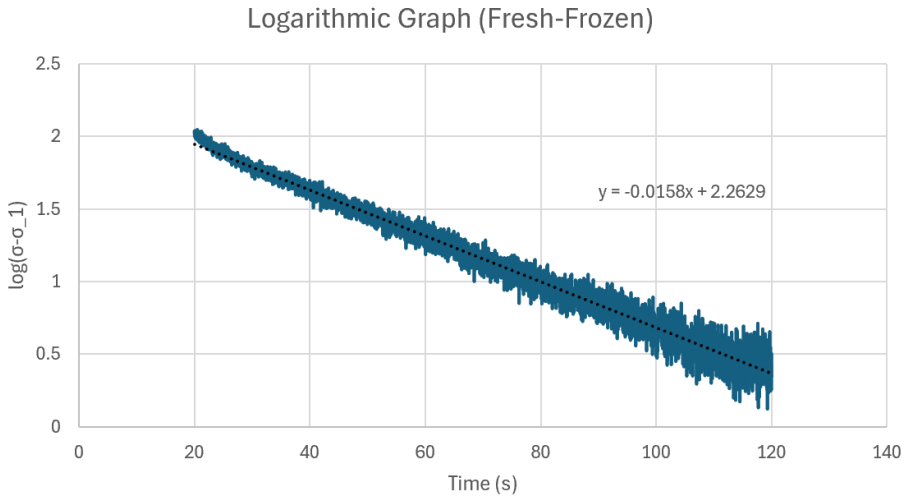

Figure 2 – Logarithmic graph for the fresh-frozen tendon.

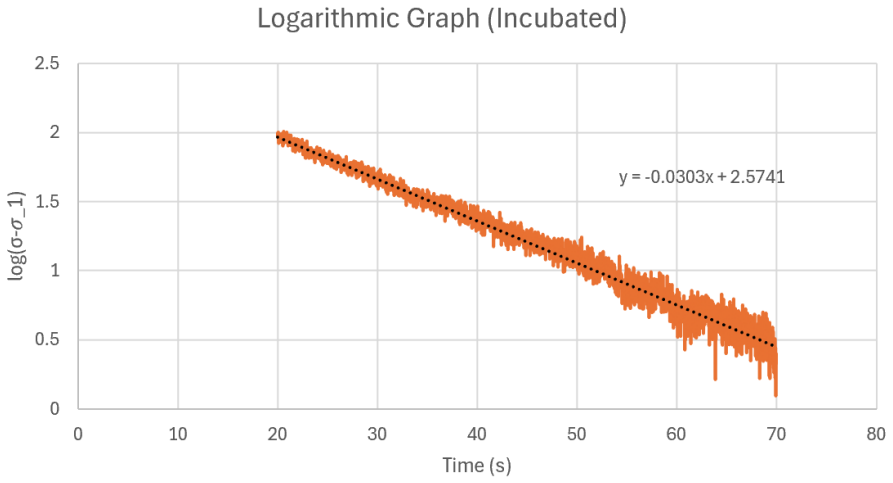

Figure 3 – Logarithmic graph for the incubated tendon.

The stress values from 20 seconds to 120 seconds were plotted on the fresh-frozen tendon graph and 20 to 70 seconds for the incubated tendon graph, thus eliminating any deviations from linearity and negative stresses, which would be considered invalid in the case of logarithms.

The slope for the fresh-frozen sample was determined to be -0.0158. Therefore, using Equation (3) the relaxation time constant for the fresh-frozen tendon was calculated as follows:

$$\tau = -\frac{1}{(-0.0158)} = 63.3 \text{ seconds (3 S.F.)}$$

For the PBS-incubated tendon, the slope was -0.0303, and the resulting relaxation time constant:

$$\tau = -\frac{1}{(-0.0303)} = 33.0 \text{ seconds (3 S.F.)}$$

These results are consistent with the expected outcome, as the higher time constant suggests that the fresh-frozen tendon relaxes slower than the incubated sample. Alongside this, using Equation (4), the percentage reduction of stress for the incubated sample was 51.5% (3 S.F.) and for the fresh-frozen tendon, it was 56.8% (3 S.F.).

Stretch-to-Failure

Using the results from the stretch-to-failure experiment, a graph of tensile stress against tensile strain was plotted, which is displayed in Figure 4.

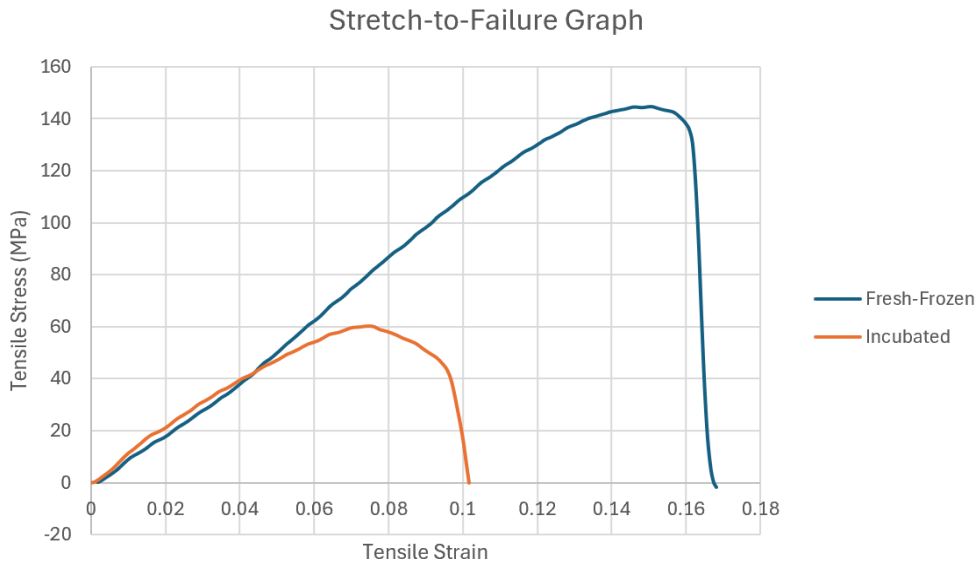

Figure 4 – Graph of Tensile Stress against Tensile Strain for the fresh-frozen and PBS-incubated samples to study behaviour when stretched to failure. The data points after stress reached below zero for both samples were excluded from the graph.

When comparing the strain at failure for both tendons, it can be seen that the fresh-frozen sample began to fail after reaching approximately 15% strain, while the PBS-incubated sample failed at a much lower strain, approximately 7.5% strain. This implies that the fresh-frozen tendon exhibited higher ductility than the PBS-incubated tendon, which reinforces the fact that PBS causes structural changes in the tendon. The post-yield behaviour shows that the fresh-frozen tendon had a sharp reduction in stress. Conversely, the behaviour of the PBS-incubated sample shows a gradual reduction in stress, followed by a rapid drop to zero from 9.5% strain.

Using Equation (5), the tangent modulus for both samples was calculated using two intervals higher and lower than 3% strain. This was done to account for any noise in the data, by selecting points that follow the overall trend. 3% strain was chosen because it was one of the points at which both materials were still behaving elastically, giving a more accurate measure of the modulus of each material.

For the fresh-frozen sample, the tangent modulus at 3% strain was determined to be:

$$E_t\ (3\%) = \frac{29.26264 - 26.42798}{0.03175 - 0.02846} = 862 \text{ MPa (3 S.F.)}$$

Using the same method for the PBS-incubated sample:

$$E_t\ (3\%) = \frac{32.23828 - 29.49778}{0.03169 - 0.02837} = 825 \text{ MPa (3 S.F.)}$$

Based on these results, the fresh-frozen tendon possesses a higher stiffness at 3% strain than the PBS-incubated sample, which aligns with the original hypothesis that the hydration and swelling the PBS provides to the tendon tissue may decrease its stiffness.

Examining the peak stresses gave an insight into the ultimate tensile strength of both samples. The fresh-frozen sample had a much higher peak stress value, at 144 MPa (3 S.F.). In comparison, the incubated sample had a peak stress of 60.2 MPa (3 S.F.), indicating that the fresh-frozen sample has a much greater ultimate tensile strength, and therefore a greater load-bearing capacity.

Using Equation (6), the toughness of the fresh-frozen and incubated samples was determined to be 12.0 MJ/m^3 (3 S.F.) and 2.70 MJ/m^3 (3 S.F.), respectively. This clearly shows that the fresh-frozen tendon can absorb a significantly higher amount of energy before failing, resulting in a higher resistance to fracture.

Discussion and Conclusions

Throughout this experiment, managing elements of the setup proved to be quite challenging, particularly maintaining the hydration of each tendon sample, clamping the samples onto the Instron machine's platens and measuring the diameter using Vernier callipers. The method that was used to maintain hydration was to periodically spray the samples with water. The main issue with this is that the spray may have introduced an additional load to the sample during testing, affecting the results obtained. An alternative approach to hydrating the sample would be to place a drop of water onto two pieces of plastic and hold them on either side of the tendon without touching it. Due to the surface adhesion of water, the water droplets will attach and hydrate the tendon while minimising error. Clamping the samples to the platens was a difficult task, as it required careful precision in not over-tightening the plates, as well as keeping the tendon upright and presenting a greater portion for testing. A more effective method for clamping the tendons would be to glue the ends of the tendons onto a piece of cardboard with an ovular cutout in the centre. The cardboard could then be clamped onto the Instron machine, allowing for easier placement and almost the entire tendon can be subjected to the load applied in each test. Finally, an error may have arisen from measuring the diameter of the tendon samples. The callipers had a high probability of squeezing the sample, changing its diameter and providing an inaccurate value. A simple solution for this would be to instead use a pressure-

sensitive calliper, which would stop contracting as soon as contact was made with the sample, hence eliminating errors in measurement.

An unexpected occurrence in this experiment was the upward curvature at the end of the stress relaxation curve for the PBS-incubated sample. This may have been caused by an experimental error, such as a change in strain during the relaxation phase, which could have increased the stress. A discrepancy of this nature is unlikely to be related to a fault in the Instron machine, but rather an unintended oversight during testing. Another, and more probable, cause for this outcome may have been a change in the structure of the tendon during relaxation. A paper which investigated stress relaxation for healthy right ventricular wall tissue stated that an upward curvature may be a result of the straightening and increased recruitment of collagen fibres at high strains, contributing to the load-bearing properties of the tissue, meaning the tissue can withstand higher stress in a specific direction [7]. A similar occurrence may appear in tendon tissue due to the abundance of collagen fibres, which explains the unconventional behaviour of the curve displayed in Figure 1.

The PBS-incubated sample maintained a higher stress than the fresh-frozen sample throughout the relaxation phase. This may have resulted from the incubated tendon maintaining a greater degree of hydration throughout testing, which would facilitate the alignment of collagen fibres in the tendon, allowing for higher stress to be retained during relaxation. On the other hand, the fresh-frozen tendon may not have been adequately hydrated, thus reducing the structural changes in adaptation to the load applied and not retaining as high stress throughout.

Both samples exhibited a relatively high residual stress at 180 seconds. The reason for this may be that due to the hierarchical structure of tendons, the load, as mentioned previously, can be distributed across the various components that compose the tendon at different scales, hence being able to retain higher stress in the structure. Alongside this, the collagen fibres present in tendons are able to slide past each other when under a unidirectional load, thus allowing for greater stress retention. However, this experiment may not have allowed sufficient time for the tendons to relax to a lower residual stress. Consequently, the residual stress after stress relaxation in the tendon samples may have been different provided more time was given to test the tendons.

When comparing the time constants for each sample, an important factor taken into account was that different time ranges were used for both samples, to give a more accurate representation of the linear behaviour during stress relaxation. It could be argued that the time constant for the fresh-frozen sample was more accurate, due to including a wider range of time, 20 to 120 seconds, as opposed to the incubated sample, which was calculated from 20 to 70 seconds. However, the time constant for the fresh-frozen sample was significantly larger than for the incubated sample, meaning that although the relaxation behaviour for the incubated sample may not have been interpreted to a high degree of accuracy, it can clearly be seen that the fresh-frozen sample relaxed at a lower rate than the incubated sample. This is reinforced when considering the structure of each sample. The fresh-frozen sample was likely able to hold internal stress for an extended period due to a collagen network with increased integrity, thus increasing the time it takes to relax. Yet for the incubated sample, the PBS may have induced structural changes, reducing the internal tension throughout the tendon, hence relaxing at a quicker rate.

During the stretch-to-failure experiment, the incubated samples failed at approximately half of the strain that the fresh-frozen samples failed at. This may be explained by the decrease in stiffness of the tendon that the PBS causes, as suggested by the literature. A study performed on rat tail tendons investigated the effect of incubating samples in PBS, and it found that swelling of the tissue as well as microstructural changes occur as a result. This decreases the modulus and consequently, the tendon becomes less resistant to deformation, which provides evidence for the result shown in Figure 4.

When comparing the post-yield behaviour of both samples, the fresh-frozen tendon displayed a very steep decrease in stress, which may have been attributed to the tearing of collagen fibres, thus rapidly losing its load-bearing capacity. This is supported by a study that explored the effect of freeze-thawing human posterior tibial tendons. The results from this experiment showed splitting and fragmentation of collagen fibres, which rapidly reduces the ability of the tendon to bear the tensile load [8]. In contrast, for the incubated sample, the PBS may explain the initial slow decrease, as the sample may have been able to bear some of the load past failure due to adequate hydration of the tissue, reducing the likelihood of immediate tears forming post-failure. However, at approximately 9.5% strain, the tendon was likely torn significantly, hence the quicker drop in stress.

The tangent modulus for the fresh-frozen sample was calculated to be higher than the incubated sample, which was the expected result, as PBS swells the tendon tissue and reduces the tensile modulus, as outlined in the literature. Comparing the ultimate tensile strength of each tendon showed that the fresh-frozen tendon could withstand a higher amount of stress than the incubated sample. Similar to the reasoning behind why the fresh-frozen tendon relaxed at a lower rate during stress relaxation, the robustness of the collagen fibres may have resulted in greater peak stress. For the incubated samples, the reduction in stiffness may have impacted the UTS, hence failing at a significantly lower stress. Finally, the toughness of the incubated samples was calculated to be lower than that of the fresh-frozen tendon. With regards to the stress-strain curve, a reduced stiffness correlates with lower toughness, as stress would increase slower with strain, decreasing the area under the curve, which explains this result for toughness.

To summarise, the aims of this experiment were to investigate the mechanical properties of rat tail tendon samples during tensile loading, along with observing the effects of PBS incubation on the behaviour during stress relaxation and stretch-to-failure tests. These aims have been achieved, as from the results of each test, both sets of samples, PBS-incubated and fresh-frozen, showed a large difference in behaviour. Therefore, overall, this experiment was successful in analysing the effect of storing tendons in PBS prior to testing compared to freezing them directly after dissection. However, the reliability of the data can be argued, as there were not enough samples tested to provide statistical repeats. The use of repeats in experiments can eliminate any anomalous data, which could potentially change the outcomes of the tests. Although this is the case, when comparing results to the literature, it can be seen that most of the explanations concluded in other articles reinforced the data obtained from this experiment.

In future experiments involving tensile testing of tendons, more caution could be applied to minimise sources of error during sample preparation, specifically the hydration, clamping and measurement methods. In addition, other factors such as temperature and alternative buffer solutions could be analysed, increasing the possibilities for optimal tendon storage for the preservation of mechanical properties.

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