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An Updated Protocol to Study the Effects of Elastase Treatment on the Mechanical Properties of Bovine Tendon

James Abraham
Washington University in St. Louis

Jeremy Eekhoff
Washington University in St. Louis

Spencer Lake
Washington University in St. Louis

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Abstract

Throughout this fall, I worked in Dr. Spencer Lake's Musculoskeletal Soft Tissue Laboratory testing bovine tendons to study the effects of elastin on the mechanical properties of tendon. To study the effects of elastin, the mechanical properties of tendon were compared when elastin was and was not functionally active. This process was completed by utilizing elastase, which enzymatically degrades elastin and renders it non-functional. Once the elastin was degraded, the mechanical properties of the tendon without elastin was tested using an Instron. Previously, 20 tendons were tested which included the bovine long digital extensor tendon (LDET) and bovine superficial digital flexor tendon (SDFT). However, after testing and data analysis, it was found that the test preparation system was flawed, and so the methods for testing were updated and validated. Overall, three bovine DDFT samples were used to validate the updated methods, and after mechanical struggles with the testing machine, the testing process was validated.

Introduction

Tendons act as one of the most important parts of a human's musculoskeletal system, as they are the connectors between bone and muscle. The compositional and organizational properties of tendons determine their ability to provide adequate mechanical function. While the collagen matrix is known to provide high tensile strength, relatively little is known about how the non-collagenous components of a tendon, and more specifically elastic fibers, influence its mechanical properties [1-3]. Elastic fibers are composed of elastin deposited onto a microfibrillar scaffold and exist predominantly in the interfascicular matrix (IFM), which is located between

collagen fascicles and may link them together [1, 4]. A schematic of the tendon structure can be seen in fig. 1 below.

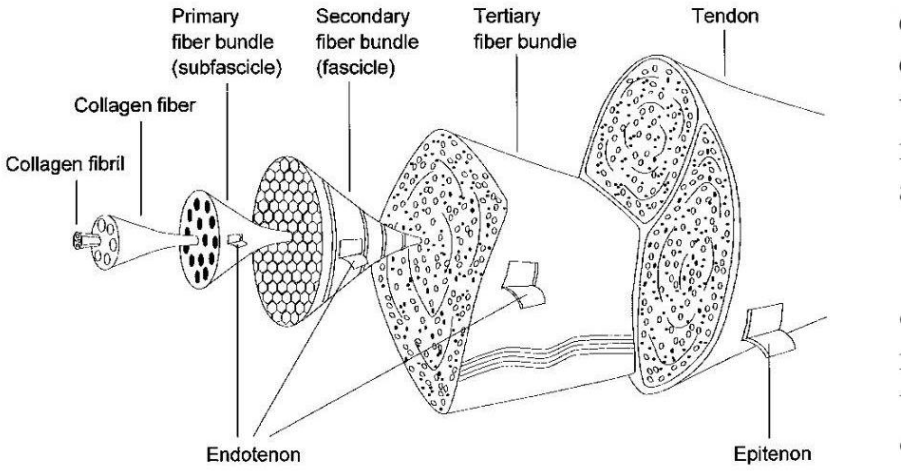

Figure 1: Composition of a tendon with more than one fascicle [5].

Elastic fibers have been suggested to provide important mechanical roles in tendon; for example, they may allow a tendon to recoil after being loaded. Clinically, understanding the effect of elastic fibers on the mechanical properties of tendon could facilitate future research on diseases such as Marfan Syndrome and Williams Syndrome, both of which stem from mutations to elastic fiber-related genes [5]. A more in-depth understanding would allow clinicians to help patients with elastic fiber deficiencies in a more informed and effective manner.

However, there is little experimental evidence on how elastic fibers affect the tensile mechanics of tendon. Previous research using genetically modified mouse models has suggested that elastin has an effect on a tendon's mechanical properties, but the effect was less than initially anticipated. Because of the vast difference in tendon size between mice and larger animals, the distribution of the elastin within the tendon and IFM may also be quite different [3]. This is mostly due to the fact that larger tendons are made up of many fascicles while mouse tendons are similar to a single fascicle. This means the larger tendons will have more elastin, and

therefore will have a larger potential for mechanical properties to change. Therefore, comparing the contribution of elastin in larger tendons with a prevalent IFM may better elucidate how elastin functions under tensile load in tendon.

In order to isolate the mechanical effect of elastic fibers, comparisons can be made by testing tendons before and after enzymatic elastin degradation treatment under the same loading conditions. Previous studies have used elastase to determine the effects of elastin digestion on the quasi-static mechanical properties of tendon and ligament; however, no studies to date have investigated changes to viscoelastic properties in tension with elastase treatment [3,7]. In this study, bovine long digital extensor tendon (LDET) and superficial digital flexor tendon (SDFT), which are functionally analogous to the human tibialis anterior tendon and the Achilles tendon respectively, were used as representative tendons for large species. A figure of their anatomy inside a cow hoof can be shown below in fig. 2 where the blue shows the LDET and the red shows the SDFT.

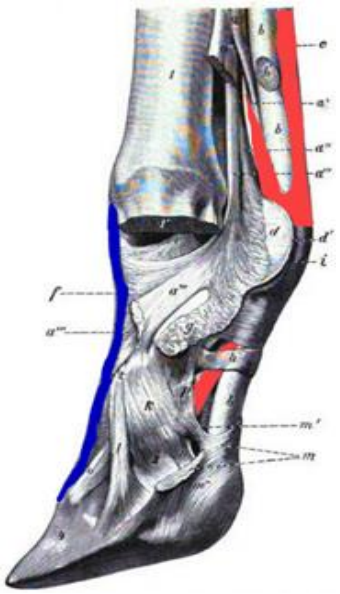

Figure 2: A picture of the anatomy of a bovine hoof with blue highlights showing the LDET and red showing the SDFT.

These two tendon types were chosen because of their function in the cow and the predicted role of elastin in functionally distinct tendons. The LDET is a positional tendon while the SDFT is an energy storing tendon, which means the SDFT would be subject to much higher loads and strains. Due to the function of elastin, it is predicted that SDFTs would utilize elastin more, and therefore show a larger change in mechanical properties when elastin is degraded. This would give us a more comprehensive analysis of the function of elastin in tendon. Overall, the purpose of this study was to utilize mechanical testing before and after elastase incubation to determine the role of elastin on the viscoelastic mechanical properties of tendon.

Previous Work:

In previous semesters, 20 bovine tendons were tested using a flawed protocol. It was found that for a significant amount of samples, the mechanical properties before and after a PBS incubation, which is our control experiment, would vary. This means that any changes during the elastase incubation would be unknown because the changes in the tendon's mechanical properties were not isolated. Upon analysis, our group was unsure if the changes in parameters such as linear modulus were due to the varying protocol or because the elastase incubation was successful. The parameters measured include transition stress and strain, linear modulus, equilibrium stress, peak stress, and percent relaxation. It was found that there were differences between the control and elastase groups, but the degree of change due to the elastase treatment is unknown because of the control results. For all of the analysis, the post treatment test was subtracted from the pre-treatment test, showing the change of each specified parameter. The previous results described can be found in fig. 3.

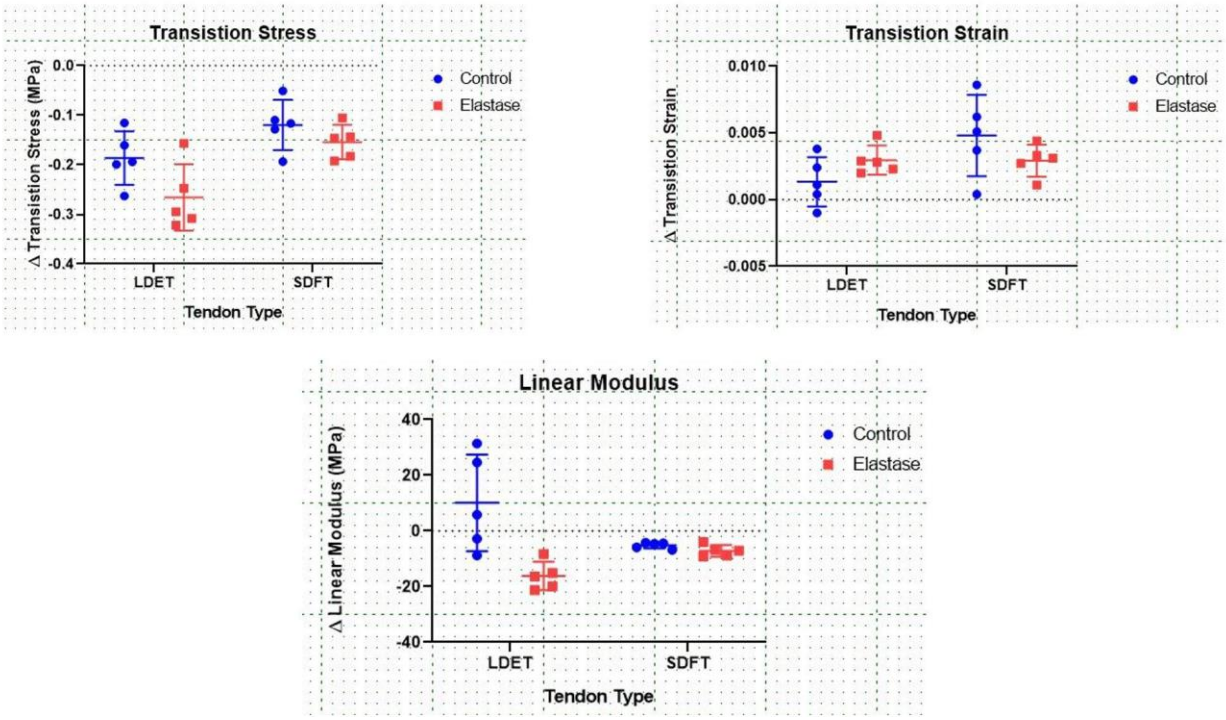

Figure 3: (top left) The change of transition stress before and after incubation for each tendon type, (top right) The change of transition strain before and after incubation for each tendon type, (bottom) The change of linear modulus before and after incubation for each tendon type

It is clear in from fig. 3 that the control and elastase groups differed from each other significantly. However, it is also important to note that the control groups rarely result in a difference that is near zero, which would indicate a successful test. Furthermore, there was also significant variability in some control groups, which was also an unexpected result. This is further verified in the rest of our measured parameters, as seen in fig. 4.

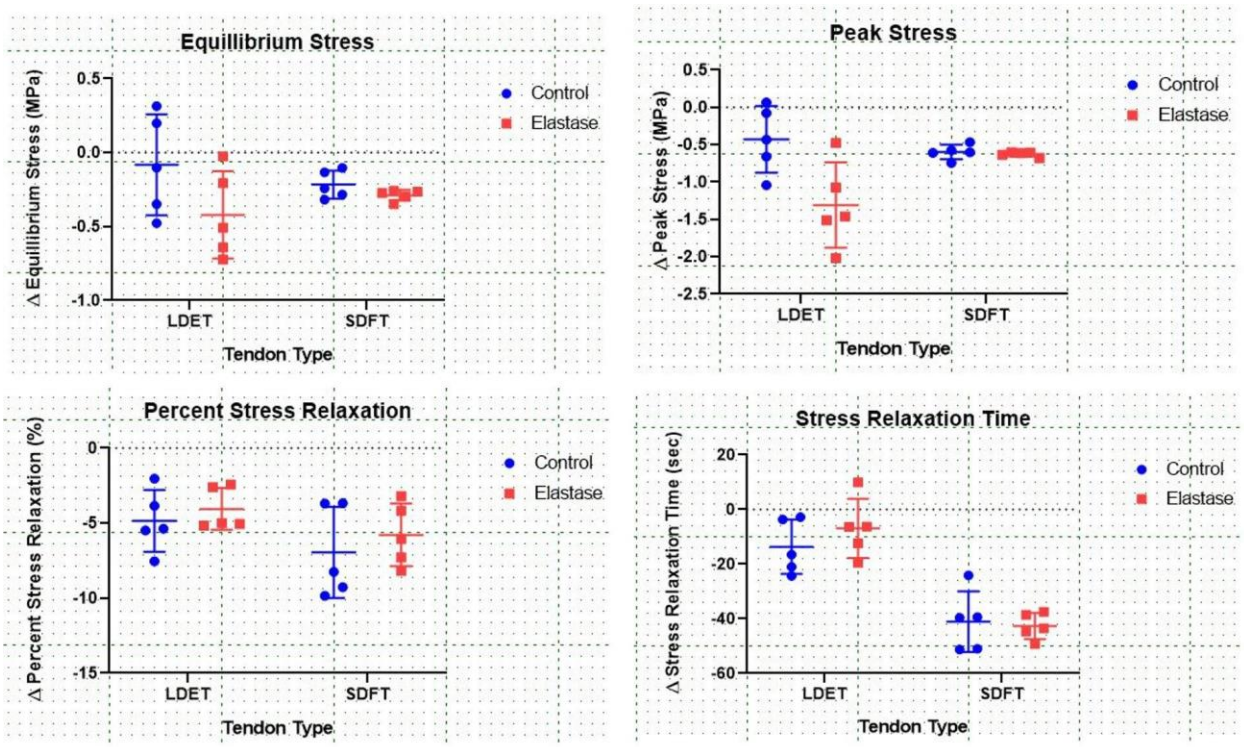

Figure 4: (top left) The change in equilibrium stress before and after incubation for each tendon type, (top right) The change in peak stress before and after incubation for each tendon type, (bottom left) The change in percent relaxation before and after incubation for each tendon type, (bottom right) The change in stress relaxation time before and after incubation for each tendon type.

So, with the analysis from these tests, our group concluded that there is a potential for elastase incubation to affect the mechanics of tendon, but a new set of tests must be done with an updated protocol. This is because the degree of effect of elastase penetration must be verified. So, this semester's work was focused on finalizing an updated protocol and verifying that the control test's mechanics remain unchanged. After researching other group's testing procedures, we realized the mechanics have likely changed due to the hydration of the tissue. During the test, we clamped the tendon with force to ensure there was no slipping during the test, but in doing this, liquid was also exuded from the tendon ends. Then, as the tendon was incubated, liquid was reabsorbed into the tendon, which means the tissue will loosen in the clamps. Therefore, on the post-incubation test, the clamp strain will remain constant, but the tissue strain will change,

resulting in varying mechanics. So, the testing procedure was updated so that the tissue strain remained constant pre and post incubation. To do this, four major updates on the testing procedure were made including thinning the tendon, utilizing visual strain tracking, drying the ends of the tendon, and tightening the clamps again after the post incubation test.

Methods

As stated previously, the goal of this semester was to improve our testing protocol so that there are negligible differences in our control experiment results. Some changes include drying the tendon, tightening the clamps before each test, using visual strain tracking, and thinning the tendon. Again, these changes were done to minimize the effect of liquid exudation after clamping and tightening. Each of these steps procedures are outlined below.

Dissection:

Bovine LDETs and SDFTs were acquired from a local abattoir and were dissected fresh from the lower portion of the hoof. The superficial digital flexor tendon (SDFT), deep digital flexor tendon (DDFT), and long digital extensor tendon (LDET) were dissected and cut to a length of roughly 100 mm. Any extra muscle, fat, or other tissue was carefully removed using a scalpel. The dissected tendons were also visually inspected to ensure there were no physical defects. The specimen were then wrapped in PBS soaked gauze and frozen at -20°C until use. Once a sample was ready for use, the sample was thawed in room temperature PBS for roughly 30 minutes.

Sample Thinning:

For this test, all samples were thinned to 4 mm by removing tissue on both sides for consistency. A microtome (Make, Model, City, NJ, USA) was utilized for the thinning process. The microtome stage was set to -20°C, and OTC was used to help freeze the tendon. Roughly 250 microns were shaved off of one side before the sample was flipped and shaved down to 4 mm. a 3D printed 4mm block was used to measure the thickness of the tendon on the microtome, and calipers were used to verify the thickness of the tendon.

Drying:

Once the sample was thinned, 3 layers of 2-ply PBS soaked gauze were wrapped around the midsection of the tendon. The midsection length is about 40 mm long. Then, the tendon with the soaked gauze was placed in a hot room maintained at 50 °C for 6 hours, which dries the ends of the sample. Next, a cross sectional area measurement of the midsection using a laser scanner (Keyence, LJ-V7080, Osaka, Japan) was taken, and the height of the tendon was verified to be roughly 4mm. A custom MATLAB code was used to then calculate the average cross-sectional area (CSA).

Biomechanical Testing:

Currently, the only samples that have been tested using the updated protocol include several DDFTs and 3 SDFTs. However, in the future, bovine SDFT (n=10) and LDET (n=10) tendons will be used for testing. The samples were secured using custom aluminum clamps with 10x10mm pieces of sandpaper on each side of the clamped end. Once secured, plumber's putty was used to fill in any gaps between the two clamp faces along all of the perimeter. This is done to ensure the dried clamped ends remain dry. Next, four small plastic beads dyed with Verhoeff's

stain were placed on the midsection of the tendon using animal tissue superglue. This is done in order to conduct an optical strain analysis. An image of the clamped tendon with beads can be seen in fig. 5 below.

Next, a camera was placed (Nikon, DSLR, Tokyo, Japan) on a tripod in front of the testing machine. Next, the clamps were connected to the actuators of a uniaxial mechanical testing machine (Instron, 5542, Norwood, MA, USA), and a 0.2 MPa preload was applied to the tendon. The clamp to clamp distance was measured and then we set the gauge length in Bluehill Universal before starting the test. The tests consists of preconditioning, which consists of 10 cycles to 6% strain, stress relaxation, which consists of a ramp to 6% strain held for 10 minutes, and finally a hysteresis test, which consists of a 2, 4, and 6% strain ramp. All tests were conducted at a rate of 1mm/s, and there was a 1 minute pause between each portion of the test.

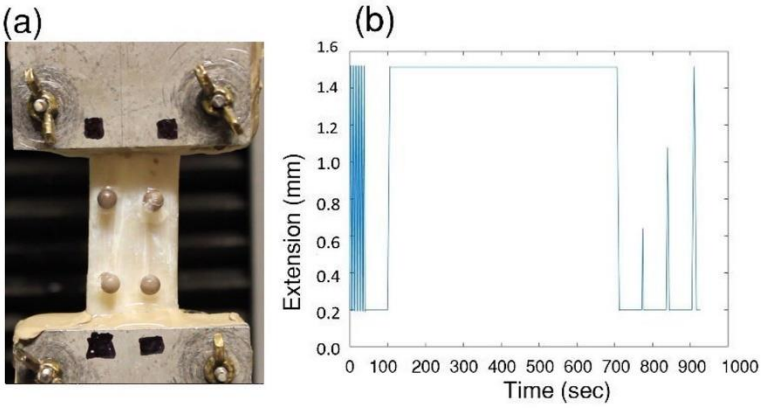

Figure 5: (a) A SDFT sample loaded in the custom-made clamps. Samples were loaded in tension in the Instron 5542. (b) A time versus displacement graph for the testing protocol. The protocol for the pre and post incubation tensile testing protocol included 10 cycles of preconditioning at 6% strain, followed by a 10-minute stress relaxation at 6% strain and hysteresis curves to 2, 4 and 6% strain.

After the completion of the test, the clamps were secured and placed in a solution bath for 24 hours. This bath was held in an acrylic plastic container, and it contained PBS for the control

samples (n=5) and elastase solution (n=5) for the elastin deficient samples. The bath was placed on a stir plate with a stir bar and covered with plastic to minimize volume loss from evaporation. The elastase solution contained pure porcine elastase (Elastin Products Company, Owensville, MO) and soybean trypsin inhibitor in PBS. The solution was kept at 37 degrees Celsius for all the tests, and the activity of the elastase was kept around 4 units/mL. This value was found by measuring the activity of the elastase solution on N-Suc-$(Ala)_3$-pNitroanilide, a substrate which creates a measurable colorimetric change at 410nm when cleaved by elastase. When the activity of the elastase solution drops below 3.5 units/mL, it was replenished with more elastase and the activity was verified again. After 24 hours in the bath solution, the clamps were removed and the same mechanical test was completed. It is important to note that the elastase samples have not been testing yet, but this will be the protocol used for future testing.

Analysis:

The linear actuator position and force were measured at a 10 Hz sampling rate. All parameters were calculated by finding the difference between the pre and post incubation tests. The parameters that were calculated include the stress differences, the peak stress difference, the equilibrium stress difference, the percent relaxation difference, the toe modulus difference, and linear modulus difference. Many of these parameters were determined using a transition point, which was found using bilinear curve fitting. An example transition point on a loading curve can be seen below. Note that in fig. 6, the stress is analogous to the stress and the extension is the same as the strain.

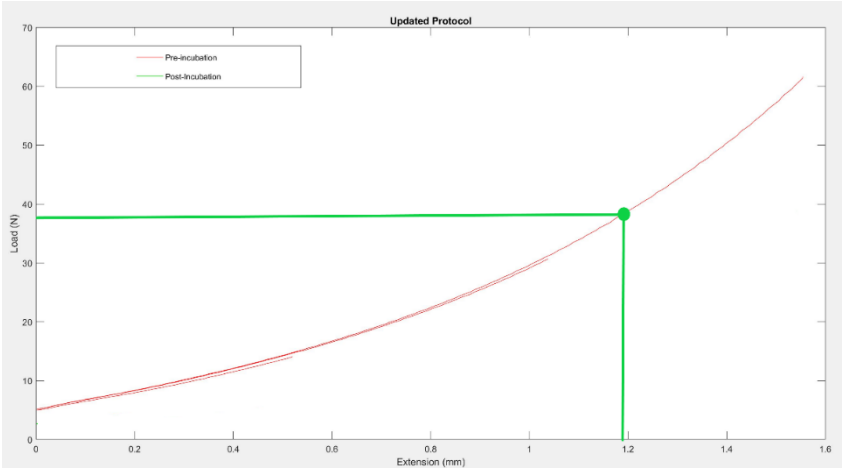

Figure 6: The green dot shows a sample transition point on a load-extension curve and the green lines show the corresponding load and extension

Results

As noted previously, there were several parameters changed for the protocol, so a sample was tested utilizing each of the changes, before the entire test was verified with several samples. To start, the tendon thinning was first verified to ensure there were no major mechanical property changes. It was expected that there would be some changes in the stress before and after thinning the tendon, but ideally, they would be minimal. As shown in fig. 7, it was found that thinning the tendon had a small change on the stress of the tendon, but not enough to rule it out from the testing protocol. Therefore, the thinning portion of the protocol was validated to be used in the experiment.

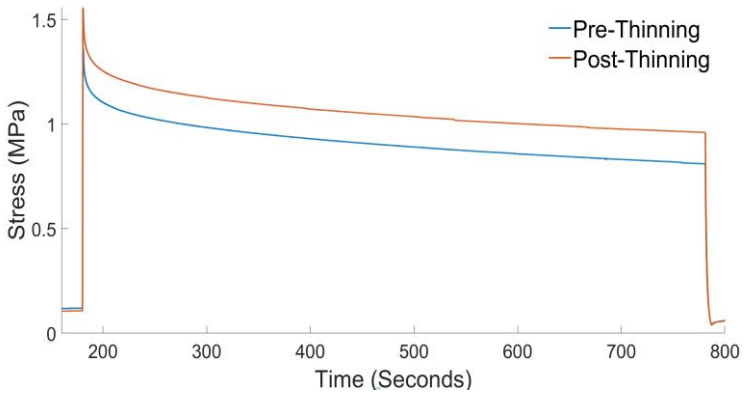

Figure 7: Force vs. time graph shows a comparison of mechanics before and after thinning. It is clear thinning the tendon does not majorly affect mechanics and therefore thinning the tendon will not create any inconsistent results.

Next the clamp tightening and drying were validated, and it was found that drying the ends of the tendon that were clamped, utilizing plumbers putty in the cracks of the clamp to maintain a watertight area around the dried tendon, and tightening the clamps after each test creates much more consistent results, as shown in fig. 8 below.

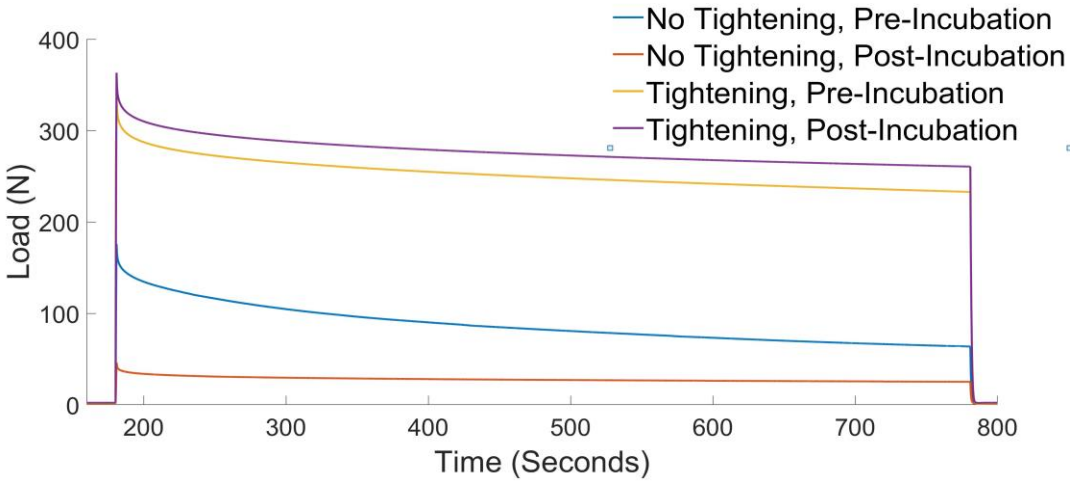

Figure 8: Validation of Drying the Clamped Tendon Ends and Tightening the Clamps After Incubation, Which Created More Consistent Results

Since all of the changes to the protocol created more consistent results, a complete test was done on SDFT tendons. The results of these tests can be shown, with the blue curve representing pre incubation results and red representing post incubation results. As seen in fig. 9, the load differences between pre and post incubation tests are less than 7% on average, which is negligible for validation results. These results are also in the process of being verified with the visual strain tracking code. This means that our new protocol was successful, and our group can move to retesting bovine LDET and SDFT tendons.

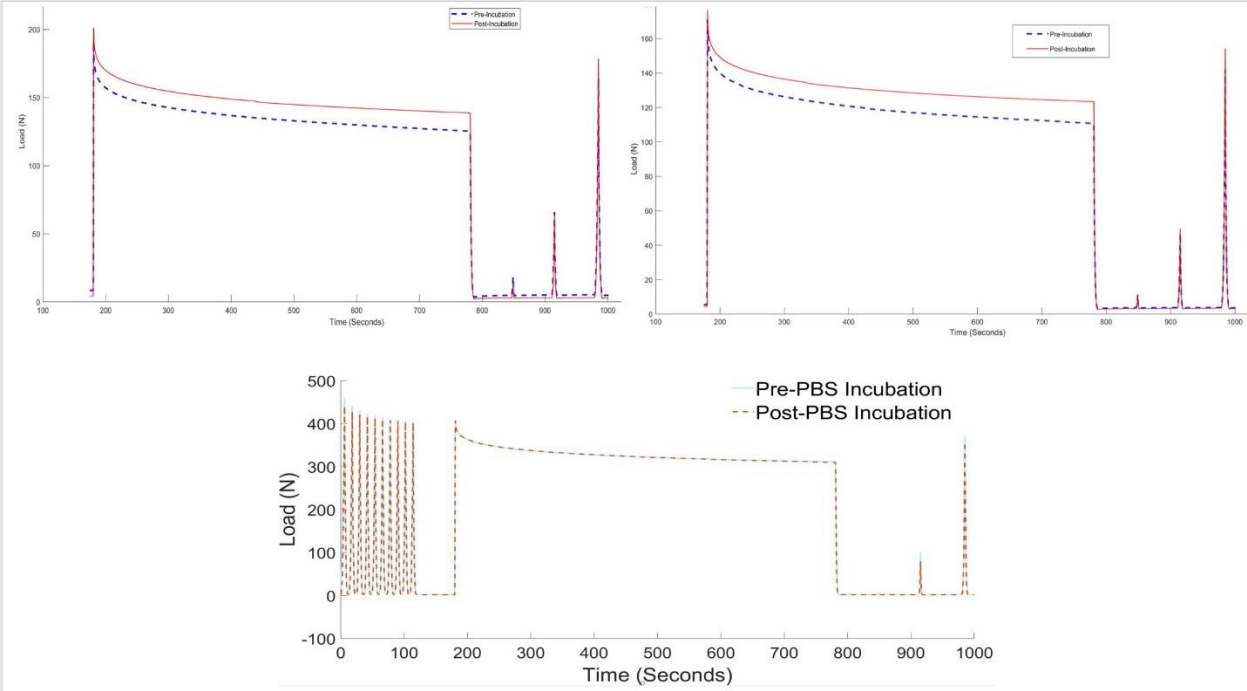

Figure 9: Load Vs. Time Graphs of Three SDFTs Used for Testing Validation

Discussion

After several validation tests, it was found that the new protocol was successful. The pre and post tests for control samples do not have significant differences, and changes in the

mechanical properties between tests has been minimized. Obviously, the changes will never be perfect due to the nature of tissue mechanics testing, but overall, the protocol is consistent enough to be used. It is believed the changes are small enough that they will not overshadow any mechanical property changes due to elastin degradation. Unfortunately, through out this past semester, the Instron testing machine has failed several times, which has halted testing. Because of this, only validation tests have been completed, while true testing will begin next semester. With that being said, we are confident with the protocol and its validation, which means the semester was a success.

Conclusion and Future Work

As stated previously, it was found that previous testing on LDET and SDFT samples were invalid due to testing protocol problems. This is due to clamp loosening, and low tissue strain relative to the clamp strain. Through out this past summer and semester, a new protocol was created which involves keeping the ends of the tendon dry, tightening the clamps, using visual strain tracking, and thinning the tendon to improve elastase penetration. Upon validation, it was found that the mechanics no longer varied significantly, unlike previous rounds of testing. This suggests the protocol was successful and in the future, five bovine SDFT, five bovine LDET, five human Achilles tendons and five human tibialis anterior tendons will be tested using PBS and elastase incubation. This will provide information about the effects of elastin on the mechanical properties of energy storing and positional tendons.

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