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A Finalized Protocol for Testing Bovine Tendon to Study the Mechanical Effects of Elastin

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Abstract

Throughout this fall, I worked in Dr. Spencer Lake's Musculoskeletal Soft Tissue Laboratory to finish developing a protocol and design setup to enable us to study the effects of elastin on the mechanical properties of tendon. These mechanical properties can be determined using an enzyme called elastase, which degrades elastin to the point where it is non-functional. Once the elastin is degraded, the mechanical properties of the tendon without elastin will be tested using an Instron. More specifically, I worked to develop a repeatable and well-defined protocol for testing, surveyed the literature on elastase incubation times, and developed smaller-scale tests to find the ideal amount of an enzyme, elastase, that will be used in later experiments. Finally, I updated current guides to inform the rest of my laboratory of the protocols, and also developed a Matlab script for analyzing a region of interest in a tendon cross section. Through these various tasks, I was able to learn more about the mechanical properties of tendon from the preliminary experiments, which will inform research about elastase treated tendons in the Spring, 2019 semester.

Introduction/Background

Tendons are one of the most important parts of a human's musculoskeletal system. Even with their great importance, there is relatively little known about how the non-collagenous components of a tendon influence its mechanical properties. Dr. Lake's research lab has done extensive research on tendons, and more specifically, the mechanics of a protein in tendons called elastin (1, 3-4). Elastin exists predominantly in the interfascicular matrix (IFM), which is located between collagen fascicles and may link them together (1). It has been proposed that elastin has great mechanical importance in a tendon, for it may allow a tendon to recoil after being exposed to different stresses. Clinically, understanding elastin's effect 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 in genes related to elastic fibers (2). More in depth understanding would allow doctors to help patients with elastin deficiencies in a more informed and effective manner. Thus, future research on these syndromes can be streamlined towards developing a medication or cure.

However, there is little experimental evidence on how elastin affects the tensile mechanics of the tendon. Previous research in the Musculoskeletal Soft Tissue Lab using genetically modified mouse models has suggested that elastin does have 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). Therefore, comparing the contribution of elastin in tendons of different sizes will produce data explaining how elastin functions under a tensile load in tendon.

This comparison can be made by testing tendons, with and without enzymatic elastin degradation treatment, under the same loading conditions. Elastase is a promising candidate for this job, as it has been shown previously that elastase renders elastin non-functional in tendon (4, 5). However, the elastase cannot be utilized until a proper protocol has been created for testing control samples of bovine tendon in tension. Therefore, a repeatable tensile loading procedure was developed. The protocol uses elastase incubation to compare tendon with and without elastin. After the mechanical testing portion of the protocol was created, I then worked on developing the rest of the procedure. At this point, the complete testing procedure has been developed. In order to complete the entire protocol, guidelines were updated and smaller experiments were conducted to determine necessary incubation time in elastase. Thus, at the

beginning of next semester, our group will be ready to begin acquiring useful data from cow and human samples.

Methods

To begin testing on different species of tendon, we adapted the loading regimen from the mouse tendon experiments previously conducted on a biaxial loading machine in the Musculoskeletal Soft Tissue Lab (3), to the larger 5542 Instron Testing Machine shown in Fig. 1.

Figure 1: The machine used for all preliminary, current, and future testing. It is a 5542 Instron Testing Machine. Note these are not the clamps that were used during testing.

Because the bovine tendon samples are larger than the previously tested mice tendon samples, it was also necessary to design new clamps. The clamps were designed using Solidworks last spring and improved on this summer. Before machining the clamps from metal, plastic prototypes were produced using a 3D printer. This allowed us to test tendons while the

clamps were still in a prototype phase. Once the prototype showed consistency, the clamps were machined in a Washington University machine shop using aluminum. The final product of these clamps can be seen below in Fig. 2.

Figure 2: The final clamp design used for all preliminary, current, and future testing. On the left side of the clamps, there is a support bar in order to maintain a constant gauge length. The middle square pieces clamp the tendon.

These clamps have been used for all preliminary testing. As the new clamps expanded the size range of tendons that could be tested, a protocol with the ability to test tendons of sizes varying from mouse-range to bovine-range was necessary. The protocol that was used previously for mice (3), was changed and refined until it gave consistent and repeatable results for bovine superficial digital flexor tendons, deep digital flexor tendons, and long digital extensor tendons. A displacement versus time representation of our protocol can be seen in Fig. 3 below. From this testing, we are able to extract data about the elastic modulus, hysteresis, tensile strength, and

many other mechanical properties of the tendon. I then wrote out this protocol so the lab can repeat the same testing procedure at a later time, and this protocol was updated throughout the semester. A single test generally involves one loading regimen as shown in Fig. 3, a 24-hour incubation period, followed by the same loading regimen.

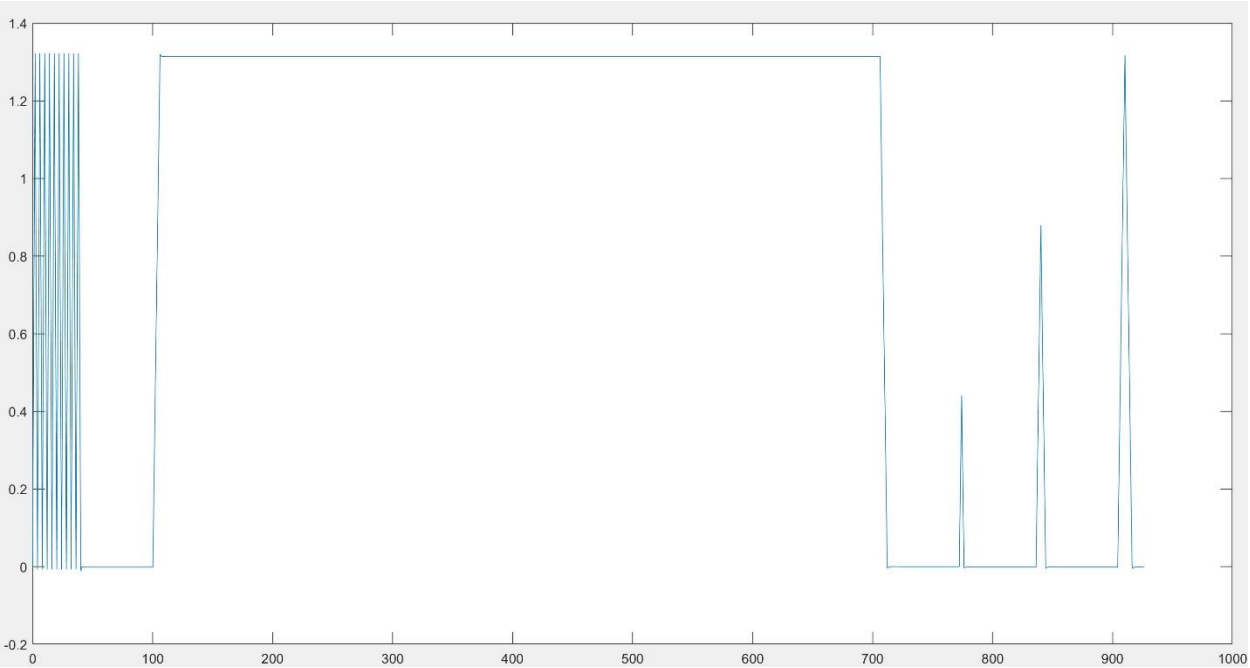

Figure 3: A displacement versus time graph of the Instron during testing. This will be an example of the protocol used in all future experiments. This protocol corresponds to the last row of the table in the appendix.

In addition to mechanical testing, we plan to compare structural differences between tendons from different species using two-photon microscopy. Our imaging technique uses second-harmonic generation, autofluorescence, and Hoechst 33342 to image collagen, elastin, and cell nuclei, respectively, in three separate channels to generate three-dimensional images. Both tendon fascicles and the IFM, which should be analyzed independently, can be present in a single image. Therefore, I created an ROI Matlab code that can extract a specific ROI that is

drawn by the user. This is used to better analyze the images for the content of elastin in different compartments of the tendon.

Finally, I completed several smaller tasks throughout the semester. Through literature search, I found that most researches use an elastase incubation between 6-48 hours. To learn more about the penetration of elastase, small cuts of tendon were placed in food dye and observed. Based on other's research, we incubated tendon in food dye for 6,12,24, and 48 hours. Food dye was used instead of elastase to mitigate cost. While food dye is not a perfect model for elastase, our group was able to gain a rough, visual estimation of what to expect from elastase penetration. Additionally, I developed plastic tubs to hold the elastase solution for when full-scale testing begins. I updated various guides and protocols so that others can utilize the new procedure, and I planned out the testing that will be conducted next semester.

Results

Below, are three Figures showing the hysteresis curves with a 2, 4, and 6% extension. In all the plots the red curve shows the hysteresis curves before a 24-hour PBS incubation. The green curves show post incubation hysteresis curves. There are several other samples that were tested besides these three samples, but these samples represent results from our final protocol. Through out the semester I gathered data to re-verify that this is an adequate protocol. These re-verification results of our final protocol show that the slope of the curves is consistent before and after incubation, meaning no mechanical properties were changed as a result of our loading protocol. Therefore, when the sample is incubated in elastase rather than PBS, any change in mechanical properties will be due to elastase incubation as opposed to failures of the protocol.

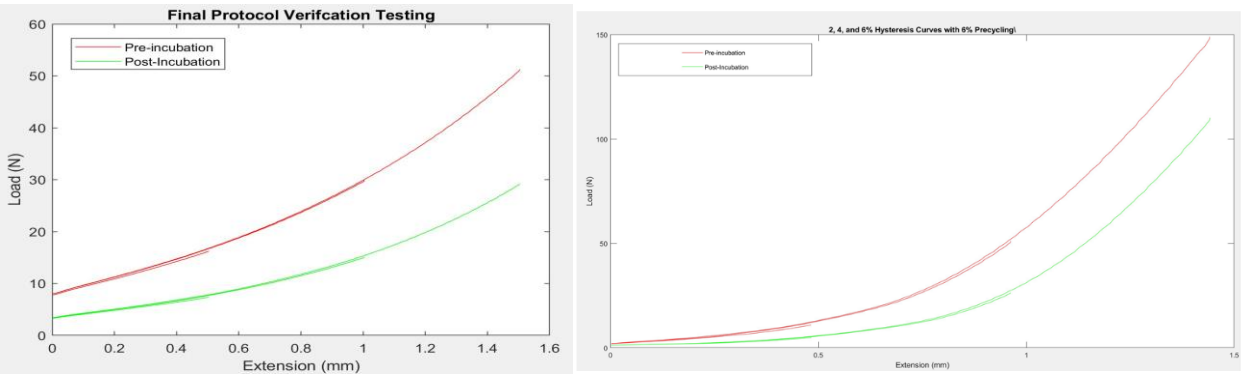

Figure 4 (a-b): 2%, 4% and 6% curves using our final protocol as described in the methods section

Final Protocol Description

The final protocol begins with the tendon clamped in the newly designed clamps, as shown above in Fig. 2, and then incubated overnight in PBS to allow the tendon to swell. The following day, the tendon will be put into cyclic tension, in which the sample will be cycled between 0 - 6% strain for 10 cycles. This is to establish a consistent strain history in the sample, which can have an effect on further testing in a viscoelastic material such as tendon. Next, the tendon will be held in tension at 6% strain for 10 minutes. This is to measure the viscous properties of the tendon as the stress relaxes during the test. Finally, the tendon will undergo three increasing ramps of tension. The first goes to 2% strain, followed by 4% strain, and finally 6% strain. Then the clamps will be taken out of the Instron, incubated once more in PBS or elastase for 24 hours. The above loading protocol will then be repeated. Control tendons will be incubated in PBS, while the experimental group will be incubated in elastase for 24 hours at a specific concentration between tests. A sample of the loading regimen, which graphs displacement of the clamps versus time can be seen in Fig. 3 above.

Elastase Penetration Verification

In the past, our group was using a 6 hour incubation period. However, this amount of time is not sufficient for the elastase to penetrate the tendon. Therefore, an appropriate amount of time for the incubation period was determined. To do this, we used food dye to model elastase penetration. Although the two molecules have different molecular weights, we felt as though food dye could be a rough guide for studying elastase penetration patterns. It was found that waiting 24 hours has a significant increase on food coloring penetration compared to 6 hours. Below in Fig. 5 are pictures comparing tendons incubated in food dye for 6 hours and 24 hours.

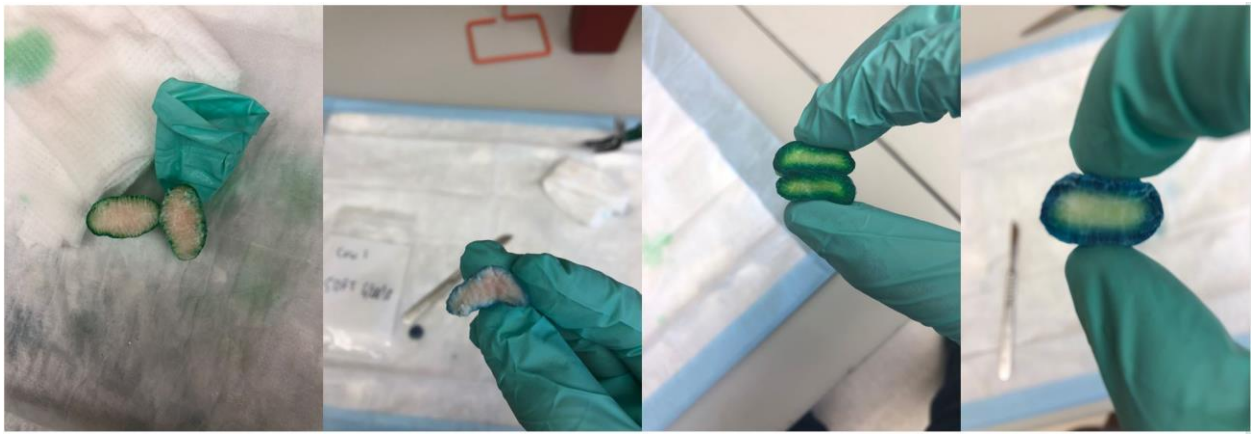

Figure 5 (a-d): 5(a-b) are shown in the left two images. They show penetration of food dye after 6 hours of incubation. 5(c-d) are shown in the right two images. They show penetration of food dye into the tendon after 24 hours.

As seen in Fig. 5 above, it is clear that the 24 hour incubation in food dye penetrated significantly deeper than just a 6 hour penetration. This indicated that elastase likely would not significantly penetrate the tendon with a 6 hour incubation, which means it would not have a significant effect on the tendon. However the 24 hour incubation allowed food dye to penetrate significantly deeper, which means elastase is more likely to have a uniform effect on the elastin in the tendon. After several tests done by other members in the Musculoskeletal Soft Tissue Laboratory, it was confirmed that a 24 hour incubation time was necessary for a significant

penetration into the tendon. This longer incubation time did not affect the results found in a control protocol as shown above in Fig. 4.

Testing Details for Spring 2019

In order to be ready to test next semester, a well-defined plan must be created. This involves deciding what species of tendon will be tested, how many tests will be conducted, and the timing of the tests. Our group decided on using three different species of tendon and two types of tendon from each species. We will also be using a sample size of five samples for our control and elastase incubated tests. This leads to 12 total groups, leading to 60 different tendons, and 120 total tests through out next semester. A table outlining the testing specimens can be seen in Fig. 6 below.

Group Number	Species	Tendon	Number of Samples	Incubation Solution	Total Tests
1	Cow	Superficial Digital Flexor Tendon	5	PBS	10
2	Cow	Superficial Digital Flexor Tendon	5	Elastase	10
3	Cow	Long Digital Extensor Tendon	5	PBS	10
4	Cow	Long Digital Extensor Tendon	5	Elastase	10
5	Human	Achilles Tendon	5	PBS	10
6	Human	Achilles Tendon	5	Elastase	10
7	Human	Tibialis Anterior Tendon	5	PBS	10
8	Human	Tibialis Anterior Tendon	5	Elastase	10
9	Mouse	Achilles Tendon	5	PBS	10
10	Mouse	Achilles Tendon	5	Elastase	10
11	Mouse	Achilles Tendon	5	PBS	10
12	Mouse	Achilles Tendon	5	Elastase	10

Figure 6: A table of the different species, tendon types, sample size, and incubation solution that will be used.

Now that the number of tests is determined, it is important to plan times in which testing can occur. Over a week I timed myself timing a tendon from start to finish, and I found that the average time to finish one test, test a new sample, and add time to check activity of the elastase totaled to roughly 1 hour and 15 minutes. This included an added 10 minutes to account for any potential mishaps that might occur during testing. This ensured that testing could occur each day as needed. It was decided that each group, which is shown in one row of Fig. 6 above, should be tested over the course of one week, so testing will occur Monday through Saturday This will be repeated weekly for 12 weeks throughout the semester. This will allow us to compile all necessary data from this project in one semester, while also ensuring the groups were tested in as consistent of a manner as possible. Past these organizational projects, smaller tasks were completed such as creating plastic tubs for incubation which will minimize necessary elastase volume. These tasks were necessary to complete before testing begins next spring.

Analyzing a region of interest from a tendon

To produce more useful and relevant data, a Matlab code was created to create a code to analyze and extract a ROI from a tendon image. This code can be used in conjunction with existing analysis scripts, such as a script for finding the percent volume in an elastin image. In terms of its function, the user picks a stack of images that they want to create a ROI from and draws a region using their mouse over the image. The output is a stack of images and its respective image matrix of just the ROI. This new image stack with just the specified ROI can then be analyzed using other existing codes. In Fig. 6 below, a progression of the code can be seen for a single slice of the tendon image. The only difference between Fig. 6 and the actual code is that the code iterates the ROI through a stack of images rather than just one image.

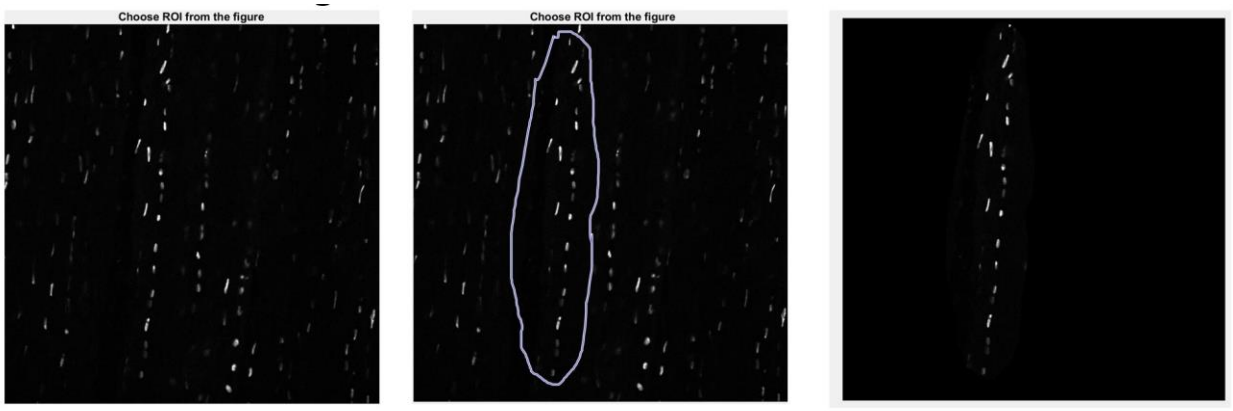

Figure 7(a-c): Figure 7a is the image on the left and it shows the original uploaded image. Figure 7b is the middle image and it shows a theoretical ROI that a user would draw over the picture. Figure 7c is the image on the right and it shows the output of the code, which is the ROI with the rest of the image erased.

This code proved to be quite effective, as it was successful with drawing several different ROIs and on several different images. The user has the ability to redraw and move the ROI after it is made, which is extremely useful when there is user error. This ROI code works by creating a mask over the image, so everything outside the specified ROI would be given an intensity value of zero, making it black. This code will be used in the future in several ways, but in the immediate future it will be used for validation of our elastase incubation. Images of various depths of tendon will be analyzed to show whether or not the percent of elastin in the tendon has decreased after elastase incubation, and this ROI code will be used to specify more specifically where elastin would be located, which will give our group more accurate verification results.

Discussion

As stated above, it is preferable to have the 2, 4 and 6% hysteresis curves coinciding as closely as possible and for the curves to be parallel in order to confirm uniform stiffness. This allows us to compare elastase treated tendons with PBS treated tendons. In order to determine the best protocol for testing, we compared the hysteresis loops before and after a 24 hour incubation in PBS, and tried to see which slopes matched. Much of the changes to the protocol were

concentrated on the preconditioning and stress relaxation portion of the loading protocol. This led to a finalized protocol, which can be seen in terms of displacement in Fig. 3 above, and the final hysteresis curves from the test can be seen in Fig. 4. Although the hysteresis curves in Fig. 4 do not coincide perfectly, the slopes are both parallel, which means the stiffness pre and post incubation are the same. This means that we can make comparisons in the future between the stiffness of elastase treated tendons and the control tendons, which are just incubated in PBS.

Even though we decided the protocol used from Fig. 4 is best, it still has its limitations. Although the hysteresis slopes are the same, and therefore the stiffnesses are roughly the same, the tensile force was decreased for the post-incubation test. Based on the different loading protocols performed, this was deemed to be necessary due to a trade-off between similar forces and similar stiffnesses. Because consistent stiffness was deemed more important than consistent force, this limitation is acceptable for our purposes. Overall, we are content with our final protocol and are confident it will produce consistent results when elastase incubation is utilized.

Along the same lines, we are also satisfied with the sample size and specimens used. All the tendons used will be energy storing tendons, which means elastin is utilized more often. This suggests that the change of mechanical properties due to elastase incubation will be more noticeable. Furthermore, cow tendon is extremely similar to human tendon in terms of size and number of fascicles. The only significant difference between the two tendons is the concentration of elastin. This will give useful results, as we will be able to better understand the effects of elastin in a human tendon. We were slightly concerned that our sample size of five per tendon group might be too small; however, due to the consistency of preliminary tests and the work of other researchers who have conducted similar experiments, we believe a sample size of five is adequate for our project. Finally, we believe the scheduling of our testing is optimized, as it is

important to ensure that the groups are tested as consistently as possible. If the groups were tested during different weeks, it is more likely that errors or changes could occur during testing.

Overall, this semester's work has mostly been based around finishing our methods for this upcoming test. Much of the work of previous semesters has been on the protocol described above, while this semester was more focused on loose ends such as incubation time, reverification of the protocol, updating guidelines for the protocol, finding proper specimens and groups for testing, scheduling testing, and finally validating our elastase incubation. All of these tasks were completed, which means our group is primed to begin acquiring data in the future. So, although the work this semester was not as robust or data driven as previous semesters, it was still very necessary to complete the work in order to acquire consistent and true data for the future.

Conclusion

Overall, the completion and validation of methods for the semester means that our group can finally start to understand the relationship between the mechanical properties of tendon and elastin. These results are exciting and important, as we can apply what we learn from these larger tendons to a human model. This allows us to know more about diseases such as Marfan Syndrome, which is caused by a mutation in elastic fiber-related genes. Therefore we can learn if the problems associated with Marfan Syndrome are due to the change of mechanical properties in tendon because of the deficiency of elastin. In conclusion, this semester's work has gotten us closer to discovering more about the relationship between elastin and tendon mechanics.

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