Histological Examination of Ruptured Tendons

by

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In collaboration with

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Abstract:

Ruptured tendons are becoming more of an issue that physicians are dealing with. This could be due to an increase in obesity rates, overuse, or the increase in participation in physical sports. Since ruptured tendons are not as mechanically strong, and the structural integrity is diminished, there must be a change in structure at the microscopic level. In this research, pig specimens came from Perdue Meat Packing Company in Sioux Center. Tendons were extracted from these specimens, grown in cell media for a few days, dehydrated, infiltrated, embedded, sectioned, stained, and analyzed. A rubric was designed to test the differences in fiber structure, fiber arrangement, rounding of the nuclei, regional variations in cellularity, decreased collagen stainability, and hyalinization. There were differences between a control and ruptured tendon in each of these variables except that of hyalinization. A better understanding of these structural differences might provide physicians with information to preventing ruptured tendons in humans.

Introduction:

Tendons play a crucial role in the musculoskeletal system, helping the body move, serving as connection points between various muscles and bones; without them, movement would be severely limited. Tendons are made of dense regular connective tissue consisting of collagen fibers running in the same direction, tenocytes with their corresponding nuclei, and fibroblasts. Tendons play a prominent role in the body, serving as attachment points from various muscles to bones. When muscles are contracted, different tendons pull on the bones, giving people the ability to mobilize. In comparison to vascularized muscles, tendons lack a direct blood supply. Although having a non-functional, ruptured tendon puts detrimental limitations on living organisms, the structural differences between a wild-type and ruptured tendon are not well

understood. Knowing the histology of tendons places significant value in determining these structural differences. Currently, a major obstacle in the field is that tendons, without the microscope and proper staining techniques, are indistinguishable between wild-type and ruptured. The Hematoxylin and Eosin (H&E) stain and the Alcian Blue/Periodic acid-Schiff (AB/PAS) stain pointed a clear distinction in arrangement of collagen and cells between wild-type and mutant samples. The H&E stain helped identify and differentiates the types of cells, tissues, and it provided important information about the pattern, shape, and structure of cells in these samples. The AB/PAS stain put more of an emphasis on the mucins and basement membranes and any sugars in the samples. The feasibility of this action is supported by the fact that tendons undergo constant stress during daily functions; a permanently altered tendon would change its composition. Thus, these stains allowed the collagen fiber arrangement, nuclei of the tenocytes, and fibroblasts to be observed in a ruptured/normal tendon.

Not knowing the specifics of the anatomical differences in a ruptured tendon poses many questions resulting in uncertainty in today's world. Due to overuse or excessive force, tendon injuries have become a common clinical problem. Damaged tendons heal slowly and rarely retain the structural integrity and mechanical strength of a healthy tendon, which often results in clinical challenges as well as patient burden (Wu et al., 2017). This research explains why damaged tendons do not retain their structural integrity and mechanical strength by broadening the knowledge of the greater scientific community. Tendinopathies are becoming more of an issue with the rise in higher participation in excessive physical activities. “In the general population, the incidence of Achilles tendon ruptures, a typical injury among 30- and 50-year-old men, is up to 1%. Leppilahti and Orava reported that 80% of these ruptures occurred during sporting activities…Achilles tendinopathies appear more frequently with a lifetime risk of 52%

in former elite male runners…24% among competitive athletes and 18% among athletes aged younger than 45 years" (Wu et al., 2017). This research gives an answer to why athletes are more prone to Achilles injuries as the realm of sports is becoming increasingly popular. It shows the effect excessive strain, the equivalent to playing sports, has on the histological structures of tendons.

Knowing the specific structures damaged from a tendon sample provides much information to the general field of medicine with what is taking place in tendinopathic tendons. Rather than resorting to surgery, new treatment interventions could possibly be established based on the findings of this study. Perhaps diet could play a prominent role in the healing process. For example, eating foods rich in vitamin C, zinc, copper, and amino acids can increase the levels of collagen in the body (Watson, 2017). All in all, the findings of the microscopic examination of histologically stained ruptured tendons provided great entail to the wider scientific community.

Methods:

Pig knees were obtained from Perdue Premium Meat Company. Upon arrival, the knee joints were stored in the cooler. In the same day, they were placed on a tray and various tendons around the knee joint were dissected with a scalpel and scissors. These tendons were cut into thin sections. The control tendons were kept as is. The ruptured tendons were ruptured by means of a couple of vice grips. The tendons were shaped in an hourglass shape to make this process feasible. (See Appendix 1)

Half of the tendon samples were plated in DMEM cell media containing 10% FBS with penicillin and streptomycin to promote a metabolic response and growth. The other half were plated in DMEM cell media containing 0.2% FBS with penicillin and streptomycin to represent

serum starvation, a limited metabolic cell response. All were then placed in a 37-degree Celsius incubator for 3 days. (See Appendix 1)

The 3 x 3mm samples were placed in 20mL of sterile 10% neutral-buffered formalin for 48 hours immediately after incubation.

The samples were placed in a series of alcohol solutions to dehydrate. After being placed in alcohol, they were placed in xylene substitute to further the dehydration process. They were then placed in cassettes and infiltrated in molten paraffin for 2-3 hours. (See Appendix 1)

After infiltration, the samples were placed in warm metal molds filled with some paraffin wax and oriented properly. Once set in place and hardened, the remainder of the mold was filled with molten paraffin. This was left on the bench to harden. (See Appendix 1)

Once completely hardened, the blocks were faced, so the samples were near the surface of the wax. After facing, the blocks were chilled on ice with a small amount of water for about 15-20 minutes to rehydrate the tissue. After this time elapsed, sectioning was performed by carefully cutting the block and utilizing a paintbrush to move the paraffin film to the warm water bath. After sitting in the water for a couple of minutes, a super frost plus slide was used to remove it from the water bath. The slides dried overnight before staining. (See Appendix 1)

The slides were then stained with both Hematoxylin and Eosin as well as Alcian Blue / Periodic Acid Schiff. (See Appendix 1)

Results:

This research gave insight to identifying the structural differences between a control and ruptured tendon. All the results analyzed stemmed from the ruptured tendon slides. Control tendons were quantified within the ruptured tendon slides, since there were portions of

unruptured samples where rupturing did not occur. This was done because the tendons serving as the control samples were not placed in cell media as an oversight, so they could not be compared to ruptured tendons. It is important to note that the worst grading scale was done for both the ruptured and control tendons. For example, a sample could have looked normal across its entirety, but it may have had one abnormal part. Given this, the abnormal part was the result quantified, not the normal part. The results attained by me were compared to that of a former, well-versed, histology student who knew how to look at the variables tested. The variables tested were fiber structure (FS), fiber arrangement (FA), rounding of the nuclei (N), regional variations in cellularity (RVC), decreased collagen stainability (DCS), and hyalinization (H). These variables were replicated from the research article from Tallon, et al., 2001, which are found along the x-axis in figures 1-5. Both H&E and AB/PAS stains were used to assess these variables, using a four-point scoring system, where 0 indicates a normal appearance, 1 is slightly abnormal, 2 is moderately abnormal, and 3 is markedly abnormal. The AB/PAS stain did not stain any mucins, indicating no presence of proteoglycans, specifically glycosaminoglycan (GAG). If any proteoglycans were stained, they would have appeared a blue color in the AB/PAS stain, which there was no indication of throughout any of the analyses. Each sample stained a pink color, indicating no presence.

The distribution of the tendon pathologic scores is shown in figure 3. Regardless of whether the tendon was a control or ruptured, the 0 score is represented by a bright green color, 1 a darker green, 2 a darker red, and 3 a bright red. There is a clear difference between control (leftmost columns) and ruptured (rightmost columns) tendon samples. The graph represents an average quantitative analysis from the other student and me. This indicates there are differences regarding fiber structure, fiber arrangement, rounding of the nuclei, regional variations in

cellularity, and decreased collagen stainability. There was no difference in hyalinization between the control and ruptured tendons. The graph is on a percentage-based scale, because there were 8 control tendons, and 14 ruptured tendons.

To prove the comparative analysis was valid, figures 1 and 2 show a side-by-side comparison of the control and ruptured tendon pathologic scores. My analyses are represented on the leftmost columns whereas the other students are represented on the rightmost columns.

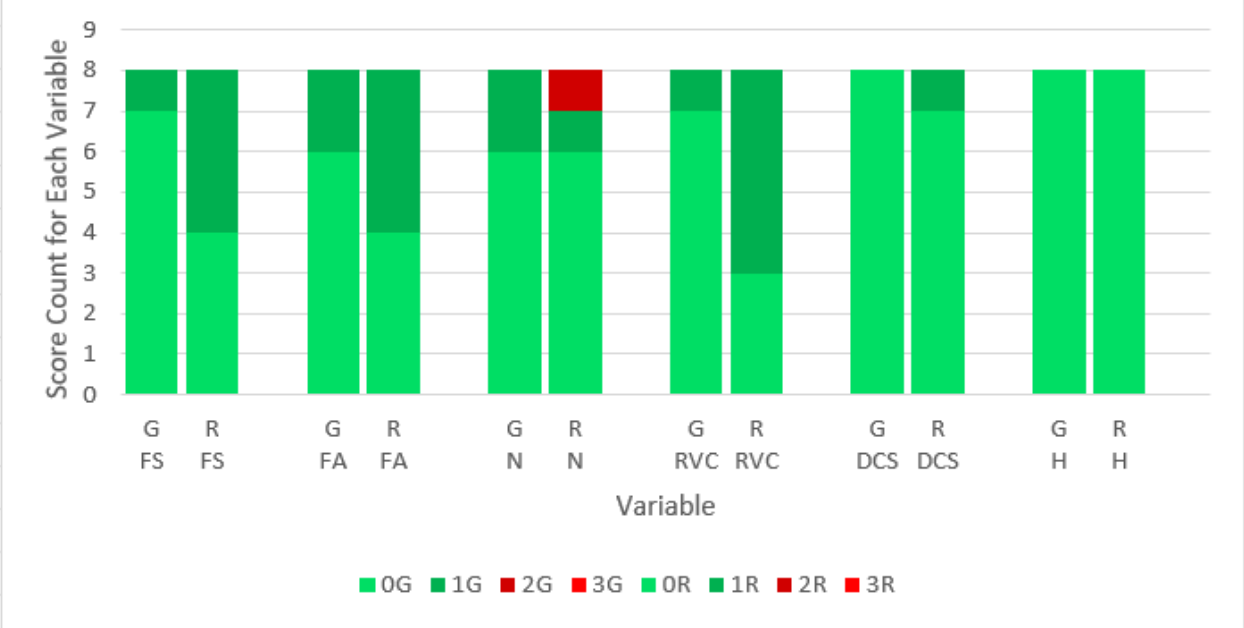

Figure 1: Comparative Analysis of the Control Tendon Pathologic Scores Distribution Between Grant and the Other Student. *Just the control tendons are shown here. The columns represent the variables mentioned earlier with a G indicating my analysis and an R indicating the other student's analysis. The color scheme is split up into my scoring system (0G-3G) and the other student's scoring system (0R-3R). The control tendon analysis was strikingly similar between both sets of people. The same principle is followed in figure 2, but it looks at strictly ruptured tendons.*

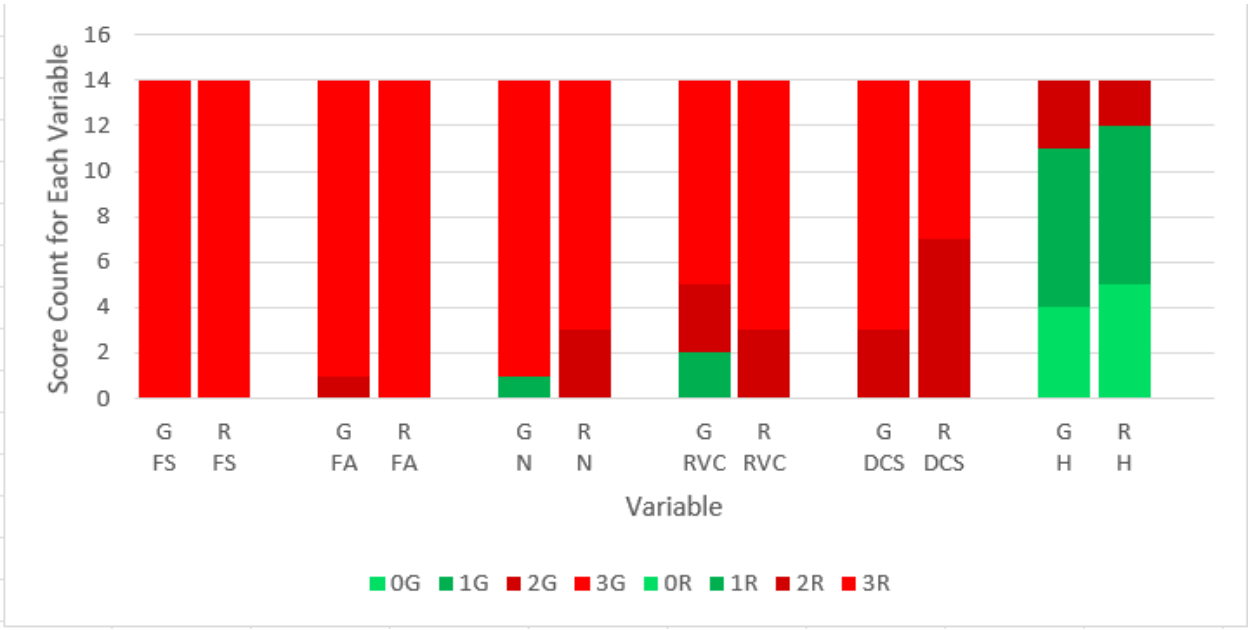

Figure 2: Comparative Analysis of the Ruptured Tendon Pathologic Scores Distribution Between Grant and the Other Student.

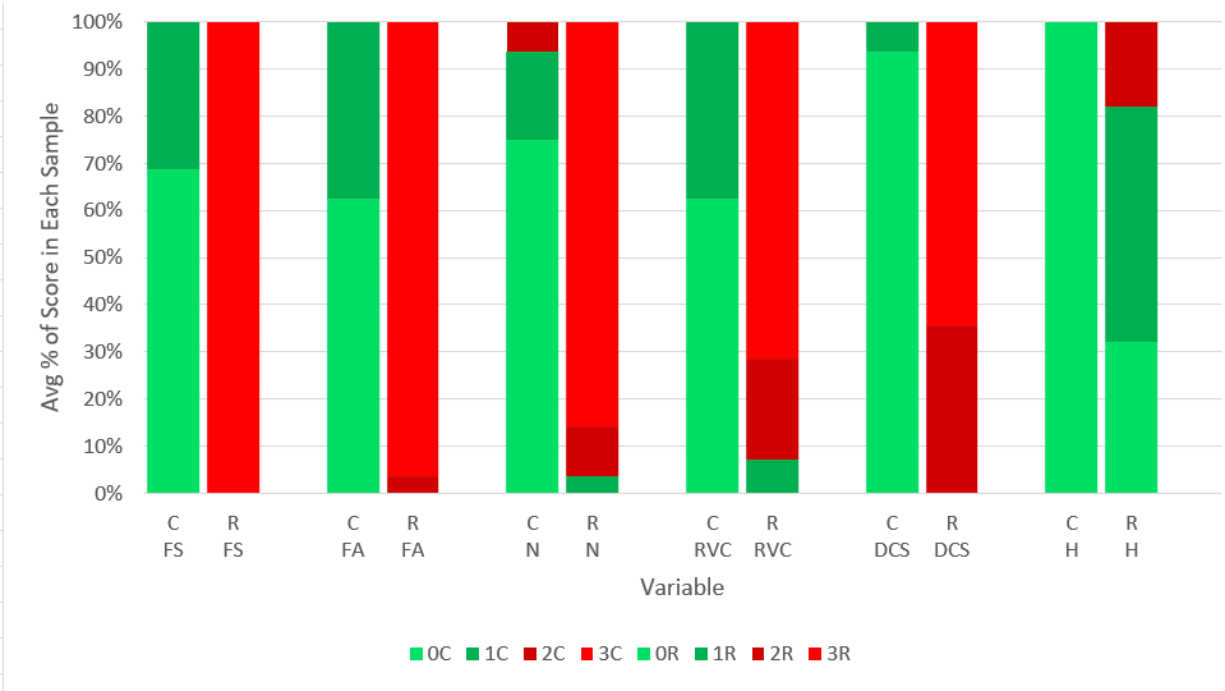

Figure 3: Average Distribution of Tendon Pathologic Scores Between Grant and the Other Student. *The other student and I each categorically had results in a table (table 1 in Appendix 1).*

These two tables were compiled into one table by taking the averages of each category in each variable. A graph was then generated, as shown here. It is through the combination of each of these analyses that differences are markedly observed apart from hyalinization.

Tendons were placed in DMEM cell media containing 10% FBS with antibiotics to act as serum. The goal of this was to provide the tendons with an environment where they could respond and make any regeneration actions to compensate for the forceful rupture. The serum provided growth supplements to ensure this was possible. The tendons that were placed in DMEM cell media containing 0.2% FBS with antibiotics acted as serum-starvation. The aim of this was just the opposite, the cells did not have a chance to regenerate and proliferate due to the absence of growth supplements. Figures 4 and 5 show there was no difference in the comparison of the DMEM cell medias containing serum vs. serum-starvation.

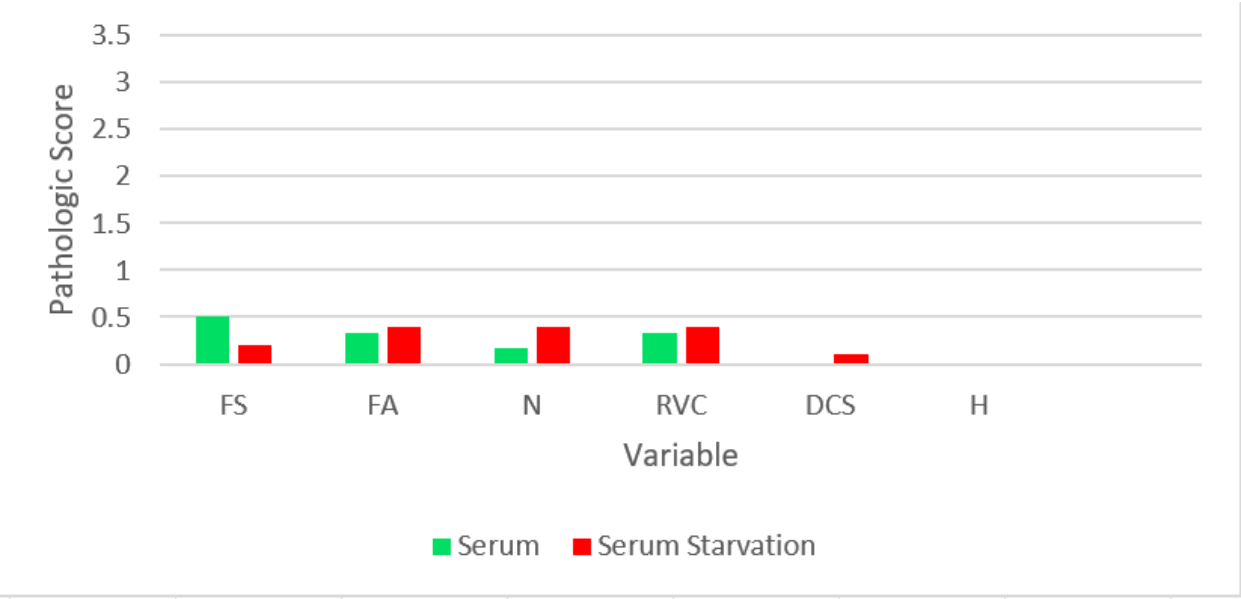

Figure 4: Average Comparative Analysis Serum vs. Serum Starvation in Control Tendons. *Of a sample size of 8 and a maximum score of 3, the differences between serum and serum starvation*

in control tendons was minimal. Graphed here is the average of the compiled data between both the other student and me. The hyalinization variable in control tendons was a 0, which is why there is no graphical column here. The same principle can be applied to figure 5, which looks at strictly ruptured tendons.

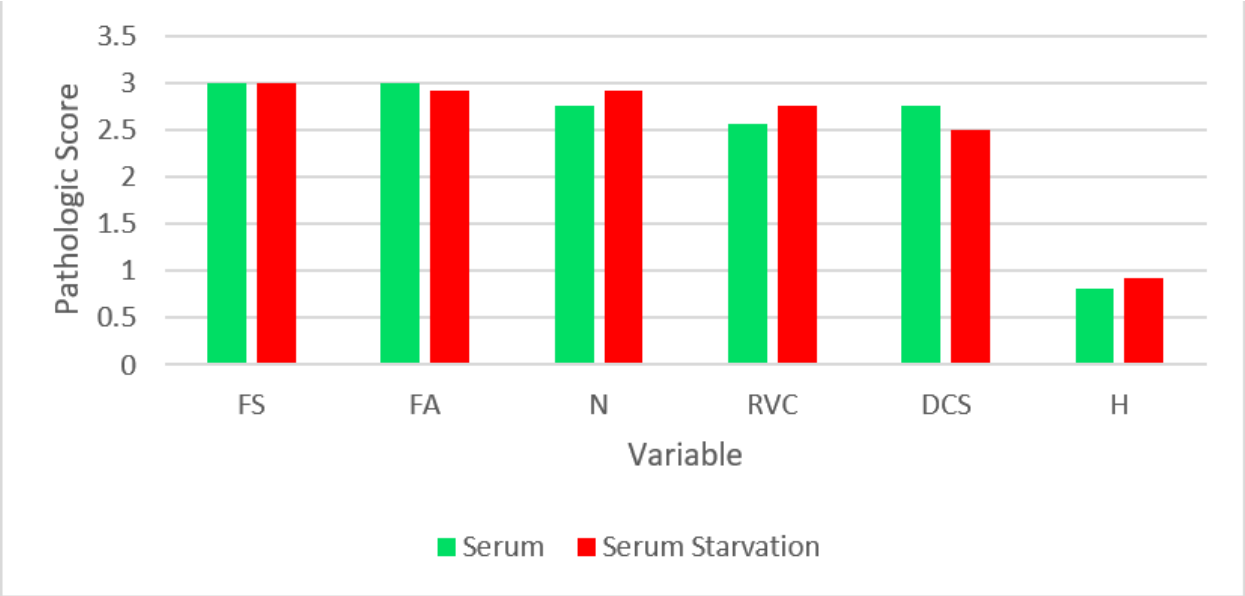

Figure 5: Average Comparative Analysis Serum vs. Serum Starvation in Ruptured Tendons

To get a visual representation of what was analyzed, pictures were taken from a microscope camera. In the control specimens, the collagen fibers were arranged close and parallel to each other (Figure 6).

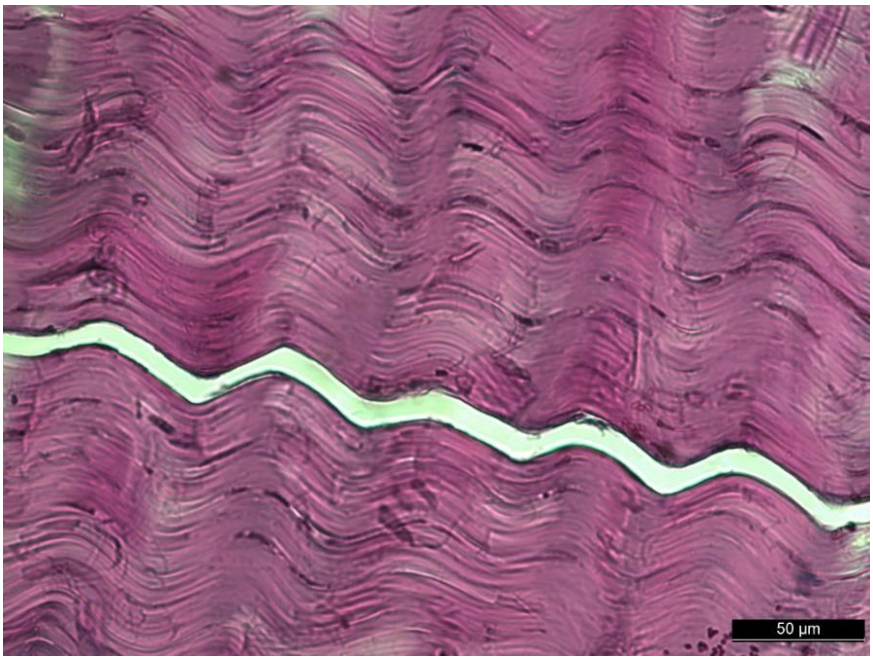

Figure 6: AB/PAS 20x Control Tendon. *Closely arranged, and intact collagen fibers. The nuclei are flat, there is no hypercellularity, there is no hyalinization, and this is stained darkly.*

Ruptured tendons, on the other hand, had collagen fibers that were disarranged, the nuclei were round, the cells were hypercellular, and the overall sample was stained lighter in color.

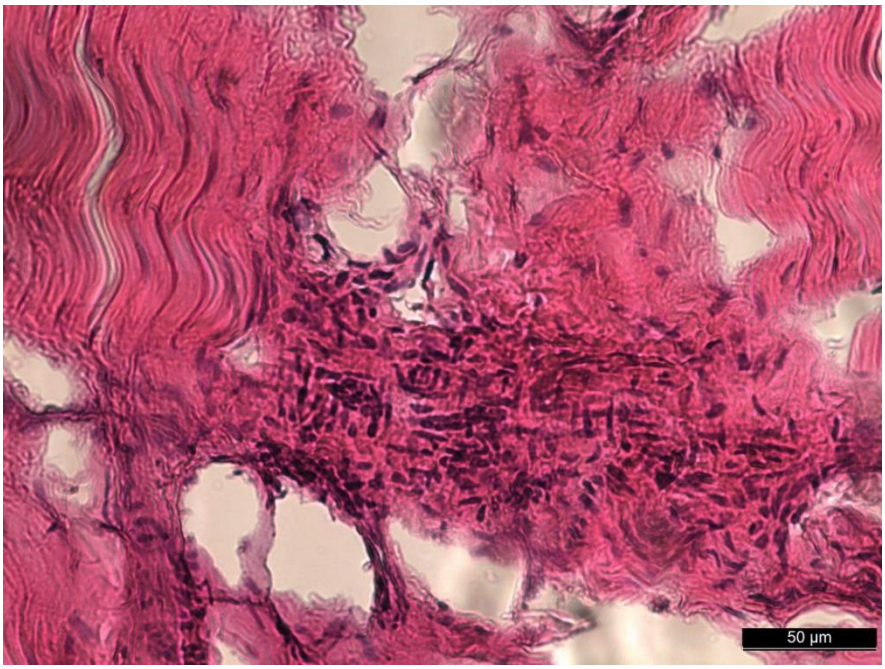

Figure 7: H&E 20x Hypercellular, Round Nuclei, and Disarranged Collagen Fibers in a Ruptured Tendon. *In this sample, there is a control tendon on the far left where the collagen fibers are arranged parallel to one another, with good structure, and flat nuclei. However, the middle of this image shows a large region of hypercellularity with disarranged collagen fibers to the top.*

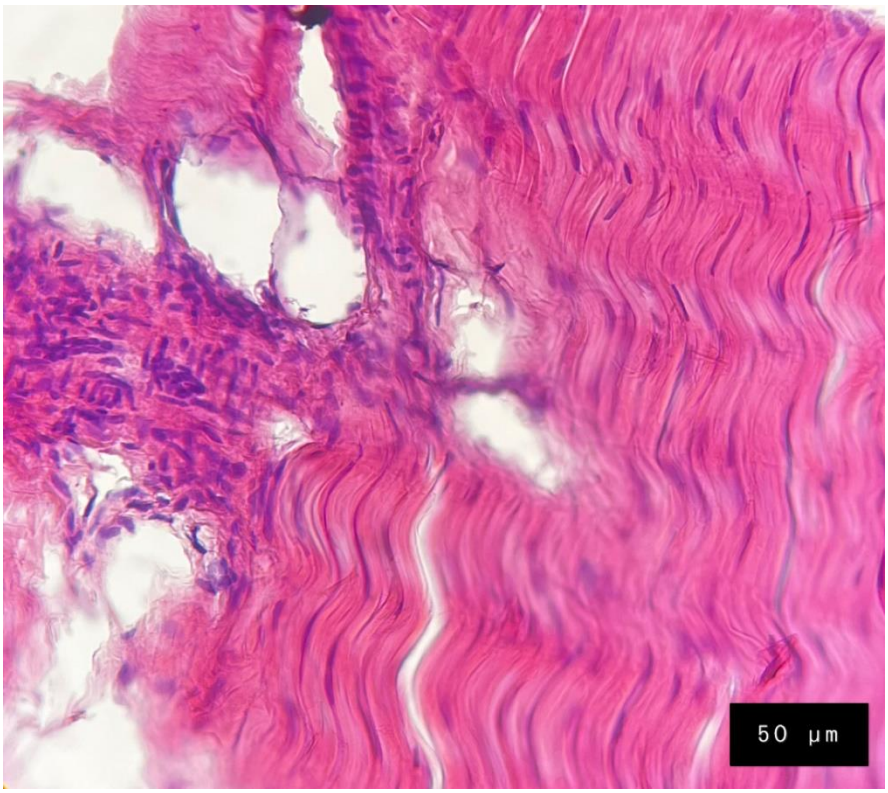

Figure 8: H&E 20x Ruptured Tendon Consisting of Regional Variations in Cellularity.

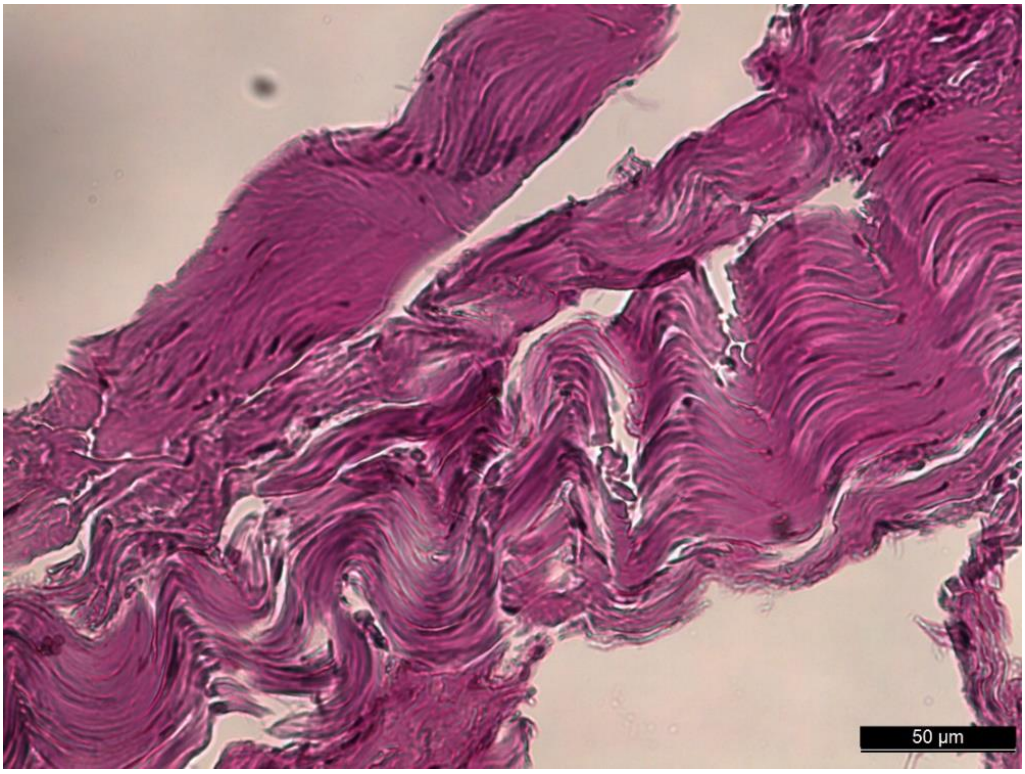

Figure 9: AB/PAS 20x Ruptured Tendon Consisting of Wavy Collagen Fibers.

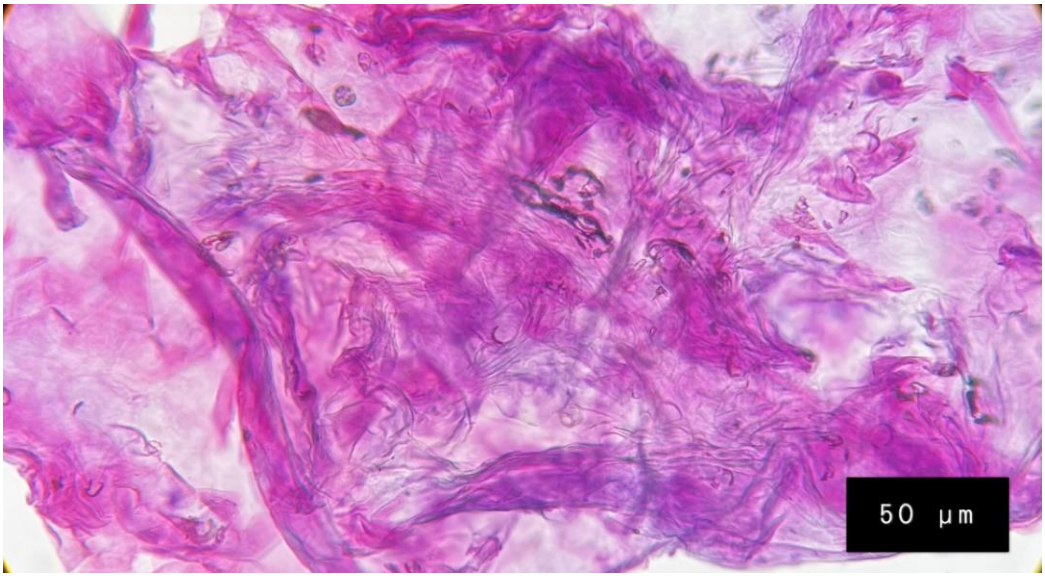

Figure 10: AB/PAS 20x Ruptured Tendon Consisting of Decreased Collagen Stainability.

Discussion:

The variables of fiber structure, fiber arrangement, rounding of the nuclei, regional variations in cellularity, and decreased collagen stainability differ in control tendons compared to that of ruptured tendons, but not hyalinization. Within the control and ruptured tendons, half of the samples were placed in cell media containing serum, and the other half were placed in serum starvation cell media; this variable did not affect any of the validated results, which were all looked over and quantified from a former histology student to take away my biases.

Although this research was representative of the literature used, there were some key differences. In the literature, control tendons were extrapolated from "patients undergoing amputation for peripheral vascular disease admitted to the department of vascular surgery of Aberdeen Royal Infirmary, and patients who died of cardiovascular accidents while inpatients at Aberdeen Royal Infirmary" (Tallon, et al., 2001). Ruptured tendons were extrapolated from "patients who sustained a unilateral subcutaneous tear of the Achilles tendon repaired in the trauma theater at Aberdeen Royal Infirmary. During surgical repair of the ruptured tendon, performed within 48 hours of the rupture, samples were removed" (Tallon, et al., 2001). The literature also explored *tendinopathic* Achilles tendons, which this research did not. Given this, these ruptured tendons occurred in the interworks of the body where there was a bodily response such as vascularization, and the presence of GAG to be secreted at tenocyte sites.

This research extracted all tendons from freshly killed pigs at Perdue Premium Packing Company located in Sioux Center, Iowa. The pigs were slaughtered the same day the tendons were extracted. However, rupturing these tendons occurred when the pig was already dead, and outside of their body's natural healing process, so there were no regenerative capabilities compared to that of the literature. Given this, the research introduced placing half of the ruptured

tendons in aliquoted cell media containing serum and the other half of the ruptured tendons in cell media lacking serum. Serum, specifically fetal bovine serum (FBS), provides an environment as if the tendon was in the body, having cellular growth and metabolically responding to abrupt changes (rupturing). They were placed in a 37-degree Celsius incubator for 3 days.

Cell culturing was not used in the literature. Given this, the forcible ruptures of the tendons in this research were not tendinopathic prior to rupture, contrary to the literature, yet similar results were attained. This research, however, was not able to point out blood vessels to compare any vascularization because there is not a wide array of blood supply in tendons, and the tendons were not in the inner workings of the body, so it had no chance to incorporate that into its healing process in the serum. Similarly, the AB/PAS stain was not able to pick up the presence of GAG simply because the tendons lacked a source from where proteoglycans could be excreted from. Although the tendons in this research were not tendinopathic prior to the rupture, this research says tendons do not have to necessarily be tendinopathic prior to ruptures to see notable differences.

If one were to continue this research, some next steps could be to culture the tendons for about six weeks without introducing any infections to the tendons. Six weeks is about how long it takes for proteoglycans to make their presence known in a ruptured tendon. A comparison analysis of this could be followed. Another way someone could extend this research is to see how diet in pigs affects how their tendons are ruptured. For example, if a pig consumed a diet supplementing collagen synthesis, that could potentially reduce histological structural deficiencies.

Some limitations throughout this experiment are that the tendons were not ruptured prior to the extraction, so there was less availability of vascularity and the secretion of proteoglycans to

promote the healing process. This made the comparison between the control and ruptured tendons less comprehensive. During the embedding and sectioning process for the ruptured tendons, it was difficult to do this properly as they were frayed and disarranged, making sectioning difficult. During analysis of the ruptured tendons, they were much smaller fragments due to its general disarrangement, making it more difficult to properly quantify. I did have more bias and had deductive reasoning when analyzing my results, however a former histology student served as a second set of eyes to quantify my results blindly, which took away my biases. The results could have been analyzed by a greater number of people to ensure accuracy. Lastly, increasing the number of samples of both control and ruptured tendons would have only made my results more reliable.

Acknowledgements:

Perdue Meat Packing Company
For supplying the pig specimens to which was the foundation of this research.

Steve Bogaard
For making sure there were enough supplies to conduct this research. He also retreived the pig specimens so that extraction could begin.

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For looking over the submitted work and making any revisions that were necessary. He also made sure the research was coming along at a good rate.

Dr. Robbin Eppinga
For aliquoting the serum and serum-starvation cell mediums so that cell culture could be done.

Riley Doenhoefer
The former histology student, serving as a second set of eyes to analyze my data.

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Appendix 1

Extraction

Pig knees arrived from Perdue Premium Meat Company. This company provides a humane way to process pork. The plant provides packaged pork products that are meat case-ready for retailers. "The pigs processed here are raised organically and antibiotic-free as well as raised in more traditional means" (Hytrek, 2020). Upon arrival, the knee joints were stored in the cooler. In the same day, they were placed on a tray and various tendons around the knee joint were dissected with a scalpel and scissors. These tendons were cut into approximately 3 x 3mm sections, corresponding to how they would look during the embedding process. The control tendons were kept as is. The ruptured tendons were ruptured by means of a couple of vice grips. The tendons were shaped in an hourglass shape to make this process feasible.

Cell Culture

To simulate the natural body's process of healing and having the cells metabolically change to such an extreme rupturing stimulus, cell culture was utilized. Professor Eppinga aliquoted DMEM cell media containing 10% FBS with penicillin and streptomycin to promote a metabolic response and growth, to which half of the tendon samples were placed in. The other half were plated in aliquoted DMEM cell media containing 0.2% FBS with penicillin and streptomycin to represent serum starvation, a limited metabolic cell response. Each of these DMEM cell media solutions occupied three wells of a six well plate. Prior to their plating in the wells, the tendon samples were rinsed in 70% ethanol to clean them. They were then placed in a 37-degree Celsius incubator for 3 days. Proper aseptic technique was conducted to ensure sterilization underneath the laminar flow hood.

Fixation

The 3 x 3mm samples were placed in 20mL sterile 10% neutral-buffered formalin for 48 hours immediately after incubation.

Tissue Dehydration, Clearing, and Infiltration

The samples were placed in the following solutions to dehydrate:

- 70% alcohol → 1-2 hours
- 95% alcohol → 1-2 hours
- 95% alcohol / 5% glycerin → 0.5-1hour x 2 (another beaker with solution was prepared)
- Xylene substitute → 1.5 hours x 3 (another 2 beakers with solution were prepared)

They were then placed in a labelled cassette-holder and transferred to molten paraffin for infiltration for 2-3 hours.

Embedding

The tissues were warm so that the paraffin that infiltrated them melted. They were placed on a metal hot plate, so they could be warmed up. The metal molds and forceps were also on this hot plate to ensure efficiency. When the tissues were ready to embed, a metal mold of the appropriate size was taken from the hot place and filled with paraffin. The tissue was removed from the cassette with warm forceps and oriented properly from the plate to the metal mold. The mold was transferred to the working bench and the tissue sample was pressed firmly on the mold as the paraffin was starting to harden (turning white). The entire mold was not full of paraffin. When the bottom turned white/hardened, a cassette was placed on top, and more paraffin was added. This was then placed on the workbench to completely harden overnight.

Sectioning

All the blocks made were faced. There was some paraffin between the tissue and the surface of the block, so it was “faced” until the tissue was on the surface of the block so that it was included in the sections. When facing, 5-10μm was removed at a time. The block was removed frequently and held to the light to see if the tissue was at the surface yet. After facing, the blocks were chilled on ice with a small amount of water for about 15-20 minutes to rehydrate the tissue. While the sections were on ice, the water bath temperature was set between 42-47°C.

After this time elapsed, the microtome was set for 3μm. The block was moved carefully to the blade, and the sectioning was performed by carefully utilizing a paintbrush and blowing lightly underneath the sections as they are cut. Once cut, the microtome was locked, and a paint brush was used to carefully lift the last section of the ribbon from the blade. The section was slowly moved over to the water bath and carefully placed into it by rolling the paint brush so that it was no longer under the last section. After sitting in the water for a couple of minutes, a super frost plus slide was used to remove it from the water bath by sticking it on it. The slides dried overnight before staining.

H&E Staining Protocol

Step	Reagent	Times	Exact
1	Xylene	3:00	
2	Xylene	3:00	
3	Xylene	3:00	
4	100% Alcohol	1:00	
5	100% Alcohol	1:00	
6	100% Alcohol	1:00	
7	95% Alcohol	1:00	
8	Tap Water	1:30	
9	D I Water	1:00	
10	Hematoxylin	5:00	Yes
11	Tap Water	0:05	
12	Circulating Tap Water	3:00	
13	95% Alcohol	1:00	
14	Eosin	1:00	Yes
15	100% Alcohol	1:00	
16	100% Alcohol	1:00	
17	100% Alcohol	1:00	
18	Xylene	1:00	
19	Xylene	1:00	
20	Xylene	1:00	

The circulating tap was on a stir plate with the stirrer on when stained. The slides were transferred here for this step. The reagents were changed when necessary. The stained slides were dried overnight before adding mounting medium to them to avoid air bubbles.

AB/PAS Staining Protocol

#	**Action**	**With**	**Time** **Mins**	**Details**
1	Deparaffinize	Xylene Substitute, 2 changes	5	5 mins each change
2	Rinse	Absolute Alcohol, 3 changes	1	1 min each change
3	Rinse	Running DI Water	1	
4	Immerse	Alcian Blue Stain pH 2.5	30	Once complete, rinse in running DI water for 1 min
5	Immerse	0.5% Periodic Acid	5	Once complete, rinse in running DI water for 1 min
6	Immerse	Optimized Schiff's Solution	15	
7	Rinse	Running Tap Water	5	
8	Immerse	Modified Mayer's Hematoxylin	1-5	Once complete, rinse in running tap water for 1 min
9	Dehydrate	Absolute Alcohol, 3 changes	1	1 min each change
10	Clear	Xylene Substitute, 3 changes	1	1 min each change
11	Coverslip	Permanent Mounting Media	---	

Table 1: Grant and the Other Student's Distribution of Tendon Pathologic Scores Analysis

Distribution of Tendon Pathologic Scores - Grant Analysis								
	Control Tendon (N=8)				Ruptured Tendon (N=14)			
Variable	0	1	2	3	0	1	2	3
Fiber Structure	7	1	0	0	0	0	0	14
Fiber Arrangement	6	2	0	0	0	0	1	13
Rounding of the Nuclei	6	2	0	0	0	1	0	13
Regional Variations in Cellularity	7	1	0	0	0	2	3	9
Decreased Collagen Stainability	8	0	0	0	0	0	3	11
Hyalinization	8	0	0	0	4	7	3	0
Distribution of Tendon Pathologic Scores - Riley Analysis								
	Control Tendon (N=8)				Ruptured Tendon (N=14)			
Variable	0	1	2	3	0	1	2	3
Fiber Structure	4	4	0	0	0	0	0	14
Fiber Arrangement	4	4	0	0	0	0	0	14
Rounding of the Nuclei	6	1	1	0	0	0	3	11
Regional Variations in Cellularity	3	5	0	0	0	0	3	11
Decreased Collagen Stainability	7	1	0	0	0	0	7	7
Hyalinization	8	0	0	0	5	7	2	0

The bar graphs in figures 1-5 were made using the average of these two data sets. The top data set is representative of my analysis of the data, and the bottom data set is representative of the other student's analysis of the data.