Once Upon a Sheep:

A SEM Analysis of Ovine Patellar Enthesis

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Abstract

In tendon injury repair, the insertion of the tendon into the bone or enthesis often determines the quality of healing and poor enthesis often leads to treatment failure. However, natural enthesis is not very well understood and it is believed that the preparation methods of previous enthesis studies corrupted the observed mineral phases present. In order to address this controversy, this research prepared two sets of samples from where the patellar tendon joins the tibia in an ovine model. One set of samples was prepared for study via SEM using aqueous solvents whereas the other set was prepared using anhydrous solvents with the intention of comparing the mineral phases present between the two sets of samples. The fibrous material observed in the set of samples prepared using aqueous solvents was determined to be a membrane sheath and not tendon and thus the results could not be compared with the anhydrously prepared samples. The electron micrographs generated from the anhydrously prepared samples are ambiguous with some of them showing the physiologically incorrect scenario of the tendon connecting directly to the trabecular bone. Due to failure in experimental setup and ambiguous data no conclusions about the effect of sample preparation on mineralization phases could be drawn. Nevertheless, this work contributed to the field of research by producing electron micrographs of the patellar enthesis region in samples used prepared using anhydrous solvents

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1. Introduction and Background

This thesis aims to provide a thorough study of natural enthesis to aid in the development of clinical treatments for tendon injury. This thesis studies the natural enthesis region of the patellar tendon and the tibia via Scanning Electron Microscopy (SEM). Due to the interdisciplinary nature of this thesis, it is plausible that not all readers will be familiar with SEM analysis techniques. For convenience, a technical explanation of the operating principles of SEM microscopy and how those principles affect the results published in this work are included in the **Appendix**: **Scanning Electron Microscopy.**

Although standard methods of preparing biological specimens for study via SEM have been developed, the various procedures have generated a lot of controversy. For example, it is theorized by Landis, Paine, and Glimcher that the solid phase of Amorphous Calcium Phosphorus (ACP) is converted to poorly crystalline Hydroxyapatite when exposed to water. [1] In addition, it is hypothesized that the preparation of samples via aqueous solvents creates "changes in the size, shape, orientation, and location of the solid-phase calcium phosphorus constituents." [1] Conversely, ACP can be induced to mineralize even if not crystalline initially when prepared by anhydrous techniques [12] In order, to gain a complete picture of the enthesis region two sets of samples were generated. One set of samples were taken from a mature sheep. These samples were prepared for SEM analysis through the use of an aqueous fixation and infiltration procedure. The other set of samples was taken from a lamb and an anhydrous fixation and infiltration procedure was used. Both sets of samples were taken from where the patellar tendon joins the tibia. By studying the same region of the patellar tendon insertion sites with samples prepared with different experimental

procedures, it is expected that a better understanding of natural enthesis can be developed.

Tendon Injury

A tendon connects muscle to bone. Often, the tendon operates close to its physiological limits making injury common especially in high performance animals such as race horses.[2] A tendon often fails via partial tendon tears caused by chronic stress, although acute rupture of the tendon is certainly possible. [3]

As mentioned earlier, tendon injuries are most often caused by small tears created by repetitious and physiology stressful activities such as lifting or jumping. [3,4] Athletes, people over 40, as well as people with certain medical conditions such as tendonitis have a much greater probability of developing a tendon injury.[4] With severe enough injury, a tendon loses the ability to support mechanical load, which can result in an incapacitating injury.[4] For example, a patellar tendon tear can render patient's knee so instable that it will buckle whenever he or she puts weight on it. This injury would prohibit the patient from walking or carrying out other activities of daily living, drastically affecting the quality of life for the patient.

A rotator cuff injury, where the tendon detaches from the humerus bone in the shoulder, is a type of tendon injury.[5] This type of injury occurs with a frequency of ~ 4 people per 100,000 individuals.[5] Similarly, it's not just Achilles who has to worry about an arrow to the ankle, for injuries in the achilles tendon occur in 12-18 per 100,000 individuals.[5] Overall, injuries to the flexor and extensor tendon in the hand are the most common type of tendon injury reported by clinicians. [5]

In comparison to the tendon injuries described above, patellar tendon tears are relatively uncommon occurring in only ~.7 per 100,000 individuals.[5] The patellar tendon

connects to the patella bone (Kneecap) to the tibia.[4] It should be noted that some sources

refer to this region as the patellar ligament; however, in this work the term patellar tendon

is always used.[4] A patellar tendon tear occurs when the patellar tendon in the knee,

which detaches from either the patella or tibia which is shown below in **Figure 1**. [4]

Although of limited direct clinical application, patellar tendon tears have been widely

studied as a base model of natural healing. [5] In addition, a lot of the experimental

surgical techniques developed for tendon injury repair have been studied in the patellar

tendon because of its accessibility and the ease of using animal models to study the

tendon.[5] The patellar tendon was chosen to be studied in this work because of the

established academic practice of using the patellar tendon tear as a basic model for all

tendon injuries as well as the ease of obtaining an animal model, with similar in that

region to that of humans.

Figure 1: *Patellar Tendon Tear.* Figure reproduced from Source 3. Figure shows simplified
cutaway diagram of a human knee in order to display the relevant physiology and anatomy of the
human knee region.

For large tears, the tendon is surgically attached to the bone via sutures which as

shown in **Figure 2**. Typically, the surgeon shaves off the cortical bone surface of the

bone as most surgeons believe it helps the injury heal.[3] The underlying trabecular bone

is vascularized which may promote enthesis mineralization. The area of the injury is often kept immobilized for a time period after the injury. [4] Full healing can take as long as a year.[4] These surgeries tend to have a high failure rate; for example, rotator cuff injuries have failure rates ranging anywhere from 11% to 95% when measured two years following treatment.[6] Some treatments have been developed in order to decrease healing time, such as sutures that release calcium.
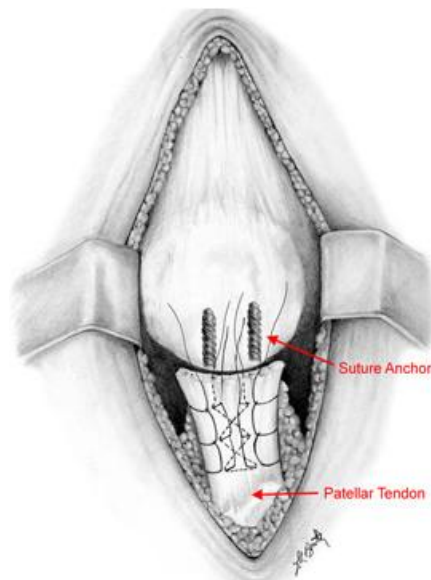
Figure 2: *Tendon Injury Repair.* Figure reproduced from Source 3. The diagram illustrates the suture anchor technique, which is of great clinical significance for severe tendon injuries. The tendon is permanently attached to the bone through the use of anchors. Over the course of approximately a year, the tendon undergoes enthesis, thereby healing the injury. High recovery times and failure rates make improved clinical techniques desirable. The figure demonstrates specifically a surgical technique for rejoining the patellar tendon to the patella bone in the knee.

Although tendon tears are relatively common, the tendon rarely fails at the tendon bone interface; however injured tendons must undergo enthesis.[7] Enthesis is the insertion of a tendon, ligament, capsule, or fascia into bone. [5] The formation and quality of the enthesis region after healing affects both recovery time, and the likelihood of treatment success in tendon repair injury; therefore, a thorough scientific understanding

of natural enthesis is needed in order to develop better clinical treatments for tendon repairs. [7]

The zone of enthesis is composed of four regions as shown in **Figure 3**. The four regions composing enthesis are: tendon, fibrocartilage, mineralized fibrocartilage, and finally bone.[5] For this study, it was important to understand not only the physiology of each region but also the materials composition and structure of each region. For reference, a thorough description of each of the regions is included below. After the systematic description of each of the individual zones of enthesis, the entire enthesis zone will be treated.
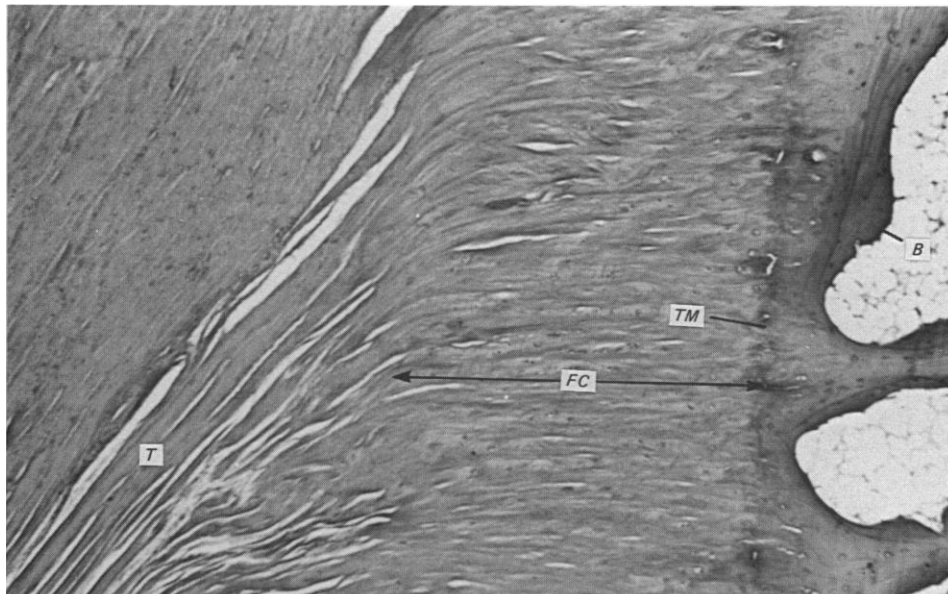
Figure 3: *Enthesis*. Figure reproduced from Source 7. Light microscope histology image of tendon and bone using a transmission light microscope, a green filter, and magnification of 60 times. The figure shows the four regions of enthesis: tendon, unmineralized fibrocartilage, mineralized fibrocartilage, and bone. T marks the tendon. The fibrous nature of tendon as well as tenocytes can be observed in the far left of the image. FC marks the unmineralized fibrocartilage region of enthesis. TM indicates the basophilic tidemark between the unmineralized fibrocartilage and the mineralized fibrocartilage that is indentified with some histology stains. The characteristic double d shape of Chrondocytes is observed in both the mineralized and the unmineralized sections but many more are observed after the tidemark. B marks bone.

Tendon

A tendon connects bone to muscle, and the contraction of muscles causes the tendons to pull on bones allowing for movement.[8] Tendons most often carry tensile forces from bone to muscle, although tendons can carry compressive forces when wrapped around the bone like a pulley.[8] Tendons are superstructure composites with the primary structure composed of fascicles.[8] The secondary structure of the tendon is the bunches of collagen fibrils that compose the fascicles. The tertiary structure of the tendon is the Type I collagen molecules that are stacked to form the collagen fibrils.[8] This arrangement of fibrils is shown in **Figure 4 (a)**.
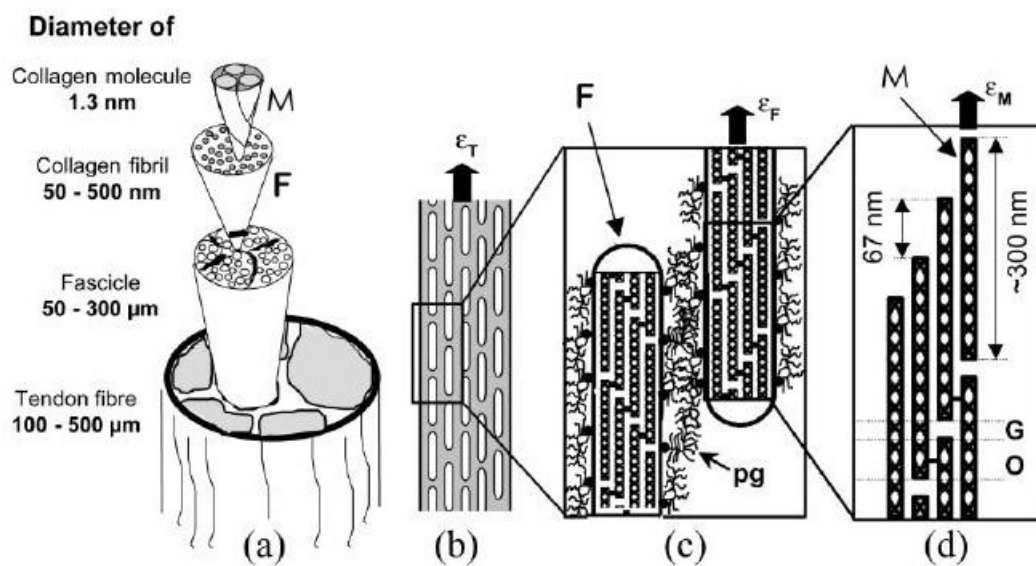
Figure 4: *Superstructure of Tendon.* Figure reproduced from Source 9. The figure caption is based on the caption from Figure 4 in Source 9. a). Shows the superstructure of tendon along with the relevant length scales. Tendon is a superstructure which is composed of bunches of fascicle. The fascicle itself is a composite of collagen fibrils and a proteoglycan (pg) matrix. The collagen fibrils are themselves composed of collagen molecules that are on order of 1.3 nm. Collagen has a triple helix structure. b). Shows a close up illustration of a tendon Fascicle. The tendon fascicle is composed of collagen fibers, which are represented in the figure as white rods. These collagen fibers are supported by a water rich proteoglycan matrix which is represented in the figure as the gray area. c). Demonstrates that the proteoglycan molecules attach to the outside of the collagen fibrils. The electrostatic interactions between the proteoglycan molecules on different collagen fibrils provide the mechanical connection that binds separate collagen fibrils together in order to form a fascicle. d). Demonstrates the 67 nm radial stacking of the collagen molecules. The average length of the collagen molecules is 300 nm.

The fascicles that are bundled together to from tendon are a composite of collagen fibers and a proteoglycan-rich matrix which is shown in **Figure 4 (b)**. [8] By weight, water composes 60% to 70% of the tendon complex.[2] The proteoglycan proteins attach closely to the fibrils which is shown in **Figure 4 (c).**[9] Of the dry matter, Type I collagen fibrils compose as much as 86% of the tendon complex.[9] Type I collagen is the predominant type of collagen found in vertebrates.[8] Type I collagen is a triple helical structure with two strands of the form α1 and another strand of the form α2 which provides the collagen with elasticity.[2] The hierarchical nature of tendon and the electrostatic cross-linking of the proteoglycan-rich matrix are also theorized to contribute to the elastic nature of the tendon complex. The complex tendon superstructure is a major contributing factor to the high mechanical loads tendons can support.[2]

As described earlier, a tendon is a fibrous composite. Ligaments, which connect bone to bone, are structurally very similar to tendons, and most of the physiological description of tendon will apply to ligaments as well; however, ligaments are not directly discussed in this text. Tendon is often substituted for ligament in ligament replacement procedures anterior curiciate ligament (ACL). The highly orientated nature of the collagen bundles allows tendon to be easily differentiated from bone due the row of parallel fibers. A histological image of tendon taken using a light microscope is shown in **Figure 5**. The fibrous nature of tendon also provides information about the orientation and state of compression of the tendon samples.[10] In addition, uncompressed tendon has a wave-like nature, called crimp, which can be seen in **Figure 5**.[10] Previous histology studies of tendon provide reference points that allow us to identify the state of the tendon persevered by our sample preparation techniques.
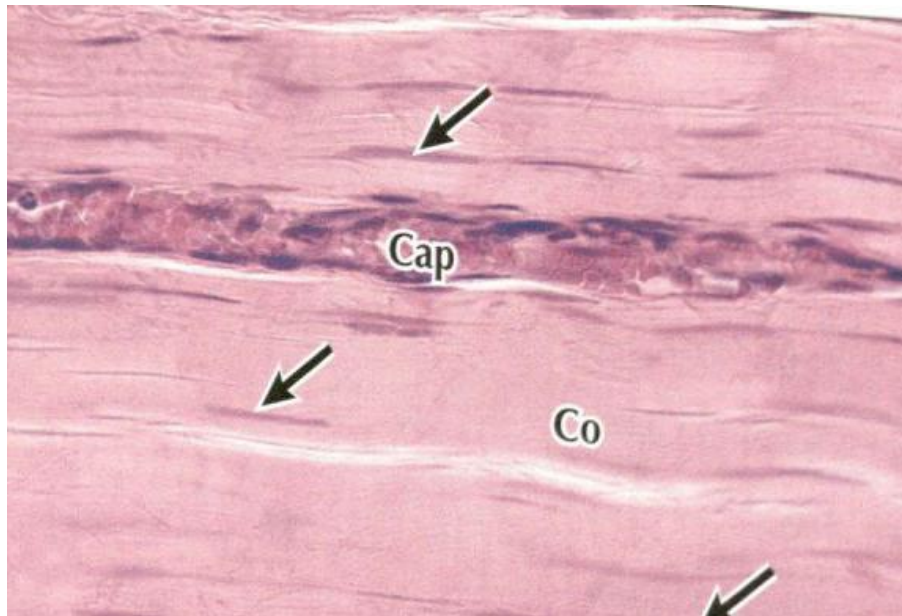
Figure 5: *Tendon Cross Section*. Figure reproduced from Source 10. Figure shows a longitudinal cross section of tendon taken via a light microscope stained using Hematoxylin and Eosin magnified 100 times.. The arrows point to inactive fibroblast. The section marked Cap is a capillary running through the tendon. The collagen fibrils are marked with CO. The wave like nature of the collagen is called crimp and it indicates that the tendon is uncompressed

The discussion above is a general description of the structure and composition of a tendon. Depending upon the location and function of the tendon under discussion, a tendon might have other structural components. For example, tendons under compressive forces such as those located in joints, will often have a significant minority component of Type II collagen. [7]

The specialized cells of the tendon, which are called tenocytes, are a type of elongated fibroblast cell that excerte collagen molecules and the extracellular matrix.[7] Fibroblasts are the most common cell in dense connective tissue such as tendon.[10] The unique flat and tapered shape of tenocytes is useful for histology studies because the distinctive shape provides a convenient physiological marker for identifying tendon.[7,10] The tenocytes are distributed sparingly in the tendon, typically occurring around the

collagen bundles. Tendon has a direct interface with unmineralized fibrocartilage which shares many structural and compositional similarities with tendon.

Fibrocartilage

Fibrocartilage is found when tendon joins bone or in the menisci of synovial joints and in intervertebral discs. [10] Fibrocartilage is an amalgamation hyaline cartilage and dense connective tissue such as ligament or tendon. [10] Fibrocartilage is distinct from other types of cartilage in its the high proportions of Type I collagen and the lack of perichondrial layer that is essential for hyaline and elastic cartilage growth. [10] The hybridization of dense regular connective tissue and cartilage produces notable mechanical properties.[10] The Type I collagen in the fibrocartilage extracellular matrix provides the material with great tensile strength, whereas the regions that are physiologically similar to hyaline cartilage provide the fibrocartilage with compressive strength.[10] For that reason, the fibrocartilage region of the enthesis reason is conjectured to be of great importance in the capacity of transferring shear loads from the bone.[1] Furthermore, it has been speculated that the regeneration of the fibrocartilage region can determine the quality of the tendon injury healing.[7]

The fibrous nature of fibrocartilage is one of the characteristic features of histological images of fibrocartilage, as shown in **Figure 6**.[10] Moreover, in histology studies conducted via a light microscope and stained with Eosin because the collagen fibers absorb the dye giving the fibers a distinctive pinkish appearance.[10] The cells that are found commonly in the fibrocartilage region fibroblasts and chondocyctes can also be used to identify the fibrocartilage in histology studies. [10]

Figure 6: *Fibrocartilage Cross Section.* Figure reproduced from Source 10. Figure shows a cross section of fibrocartilage stained with Hematoxylin and Eosin. Image was taken with a light microscope and at a magnification of 380 times. The arrows mark some of the collagen fibers, the M connotes the matrix, whereas the C marks the chondrocytes.

Chondocyctes, which are shown below in **Figure 7**, are a type of fibroblast that secrete and synthesize the components of the extracellular matrix.[10] Mature fibrocartilage is marked by a mixture of fibroblasts and chondrocyctes as fibroblasts differentiate into chondocyctes over time.[10] Chondrocyctes make useful markers in both light microscopy and electron microscopy because often have D shape and often occur in pairs because the cells split into half.[7] This arrangement is sometimes referred to as incestuous nest in the academic literature and is shown in **Figure 7**. Chondrocytes are approximately 10 µm in size.[7]
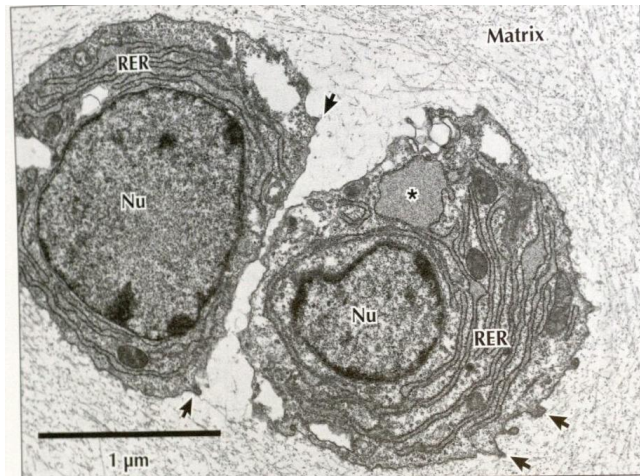
Figure 7: *Chondrocytes.* Figure reproduced from Source 10. Figure shows an electron micrograph of an incestuous nest of chondrocytes. Image was taken at 35,000 times magnification. Chondrocytes are a type of fibroblast that secretes collagen. They are a distinctive feature of fibrocartilage and often have a D shape and appear in pairs.

This tidemark is lost in this electron microscopy because the specific light absorbing stains were not used .[7] Reese observed a gradient in phosphorus and calcium in the Fibrocartilage extending 20 to 40 µM from the bone interface.[7] The entire fibrocartilage region observed by Reese was of the order 100µm indicating that there were two distinct layers in the fibrocartilage region.[7] In addition, Reese showed that there is not only a mineralization gradient in the fibrocartilage zone, but a gradient of cell differentiation as well.[7] Reese observed larger, more globular cells near the interface of the bone and smaller, more oblong cells that resemble tenocytes closer to the patellar tendon.[7] Further discussion of the cell differentiation observed by Reese appears in **Section 2.1**. The fibrocartilage region connects bone, which is described in detail in the next section, to tendon.

Bone

Bone, which is the organ that comprises our entire skeletal system, is a composite material composed of both an organic and an inorganic phase.[11] The organic phase is composed of a number of proteins with collagen fibrils being the most relevant to this work. The collagen fibrils that compose bone form a 3 dimensional network in space.[11] The mineral phase of bone is a calcium-phosphate compound that is most often cited as being a poorly crystalline Hydroxyapatite.[7] The exact nature of bone's ceramic phase practically in the case of newly mineralized bone is still a matter of scientific controversy and is covered in more detail in **Section 1.4.1**. The excellent mechanical properties of bone are due to its hierarchal superstructure and composite nature. [7] The approximately 20% of bone that is composed of organic proteins improves the compressive strength of bone and makes bone far less brittle than traditional ceramics. [7] The structure of bone is shown below in **Figure 8**.
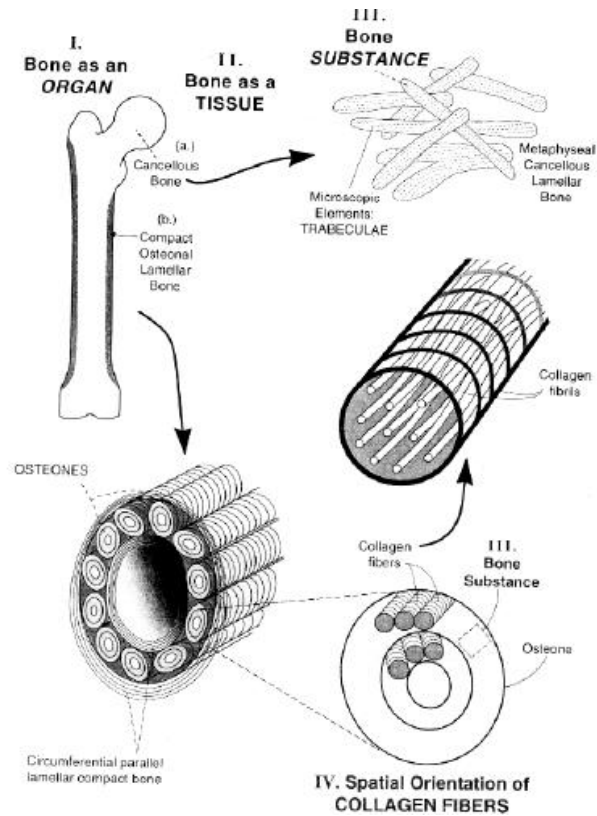
Figure 8: *Structure of Bone*. Figure reproduced from Source 11. Figure displays the structure of bone from the macrostructure component most people are familiar with down to the particular arrangement of the orientation of the cells and collagen 3D network.

On the macroscale there are two types of bone cortical bone and trabecular bone which are shown in **Figure 9**. The Cortical bone is dense and compact. This portion of the bone directly interfaces with the fibrocartilage region during enthesis. As mentioned previously this portion of the bone is shaved during tendon repair injury surgery in 90% of the surgeries.[12] Surgeons chose to shave these part of the bone because it is believed to decrease healing time.[12] This assumption is supported by findings that sutures that release calcium result in a faster healing time because the Calcium seems to be crucial in trigging the process of mineralization.[12] The second layer of the bone is called either Trabecular, Spongy or Cancelus bone.[13] Although it is not directly studied in this work,

the wavy nature of Trabecular bone shown in **Figure 9** makes for a convenient marker

for determining sample orientation.
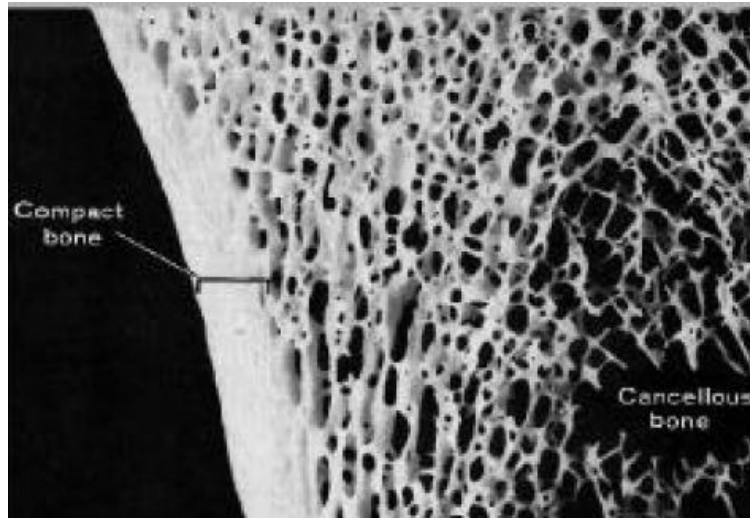
Figure 9: *Cortical and Trabecular Bone.* Figure reproduced from Source 13. Figure displays the compact section of the cortical bone that directly interfaces with fibrocartilage in regions where tendons or ligaments are undergoing enthesis. The section marked cancellous bone is also known as Trabecular bone or spongy bone. The wavy texture of Trabecular bone makes it a distinctive feature in electron microscopy studies.

Osteoblasts are the cells that create bone. [7] They serve to secrete the extra

cellular matrix and serve to calcify the sections of the unmineralized matrix.[7] Osteocytes

are Osteoblasts that have become enclosed on all sides by the matrix.[7] The distinction

between the two types of cells exists because their cell morphology and function are

different.[11] Osteocytes are distinguished by the ability to reabsorb the matrix.[7] Bone

manages to adapt to its environment with complex signaling pathways that are not yet

fully understood.[7] Tendon and fibrocartilage also vary their structure and to a lesser

extent their composition depending upon many environmental factors [1] Determining the

molecular pathways for these physiological changes and how injury affects them, is an interesting avenue of future research

Bone Mineralization

Although bone mineralization has been the subject of a great deal of scientific research, the extact nature of the materials components and the order of the minerialization phase is a matter of great scientific contraversy.[1] Bone mineralization, when the extracelluar matrix and a ceramic calcium-phosphate phase merge to from a ceramic composite, transfigures the mechanical properties of both materials. [11] An image displaying both mineralized and unmineralized sections of bone is shown in **Figure 10**.
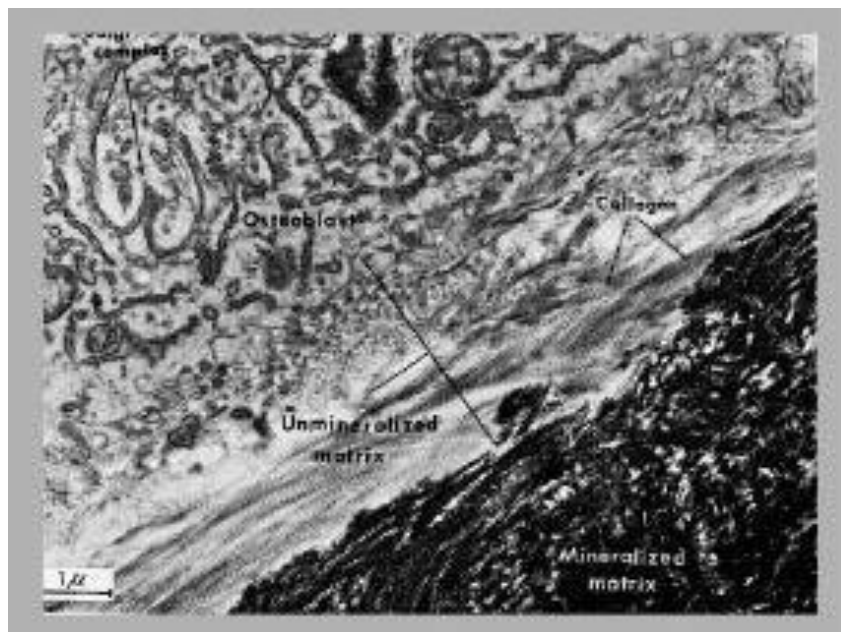
Figure 10: *Unmineralized and Mineralized Sections of Bone.* Figure reproduced from Source 13. The Electron Micrograph connotes the difference between mineralized and unmineralized sections of bone.

One of the biggest controversies in this field of study is whether the mineral phase of newly mineralized bone or fibrocartilage is poorly crystalline hydroxyapatite or Amorphous Calcium Phosphate (ACP).[1,12,7,14] Traditionally, it has been stated that bone is a poorly crystalline phase of hydroxyappatie based on X-ray diffraction patterns.[14] The X-Ray diffraction patterns of bone were did not perfectly align with the patterns for hydroxapaptite found in nature but this was presumed to be the differences in crystalline structure that were induced by the complex biological environment.[14] The research of Hobbs et all of which my thesis is a continuation, concluded that the phase was ACP in nanometer sized clusters that originated from the mitochondria.[12]

Enthesis

As described above enthesis is the insertion of a tendon or ligament into the bone.[7] The region has four general areas: tendon, unmineralized fibrocartilage, mineralized fibrocartilage, and bone.[7] Benjamin et all did an extensive study of enthesis by light microscope histology.[7] One of their key findings was the observation of the collagen fibers of the tendon extending past the tendon to the collagen and chondrocyte matrix of unmineralized fibrocartilage. [7]

The region is very important in the transference of sheer forces between bone and tendon.[7] It is also suspected that the quality of the enthesis region after injury is a major factor in determining the risk of repeated injury.[7]

2. Literature Review

It is the intent describe in this section is to describe previous studies of relevance to this thesis. This literature review is not intended to be a complete and through overview of all work on the subject of enthesis. The works covered in this section are papers relevent in explaining the experimental results of this thesis.

Thesis of Willie Mae Reese

The thesis of Willie Mae Reese, which is cited in the references section as Source 7, is the direct academic primogenitor of this thesis. Reese studied the enthesis region of where the patellar tendon attaches to tibia in an Ovine model using SEM images and Energy X-Ray dispersive analysis comparable to the work done in this thesis.[7] Aqueous solvents such as a 10% neutral buffered formalin solution were used to prepare the samples studied by Reese.[7] Continuing where the research of Reese stopped, this thesis compares samples prepared using aqueous solvents to those prepared mostly anhydrously in order to gain a more complete picture of natural enthesis. Some studies have shown that the use of aqueous solvents leads to the corruption of calcium-phosphorus mineral phases present in the sample as explained in detail in **Section 2.3;** therefore, it was deemed relevant to prepare and study another set of samples.[1,7] A summary of Reese's findings follows immediately below.

In her work Reese, observed a cartilage layer that varied in thickness from 100 to 300 μM. [7] This Cartilage layer is shown in **Figure 11**; however, the cartilage layer does not display a characteristic fibrous nature that is observed oftentimes in histology studies of enthesis.[7,10] Reese noticed a remarkable amount of interdigitation where chondrocytes were surrounded on three sides by bone.[7] This interdigitation is shown in **Figure 12**.

Reese speculated that the reported strength of the interface between the tendon and the bone is due to the significant degree of interdigitation.[7] As mentioned previously, there is typically a tide mark in light microscope cross sections separating the mineralized and the unmineralized fibrocartilage regions.[7] As expected Reese did not observe the tidemark in the fibrocartilage zone because the samples used in this work were not stained. Nonetheless, she observed a gradient of phosphorus and calcium extending 20 to 40 µM into the fibrocartilage region indicating that there was a mineralized region.[7] This gradient of Phosphorus-calcium is shown in **Figure 13**.
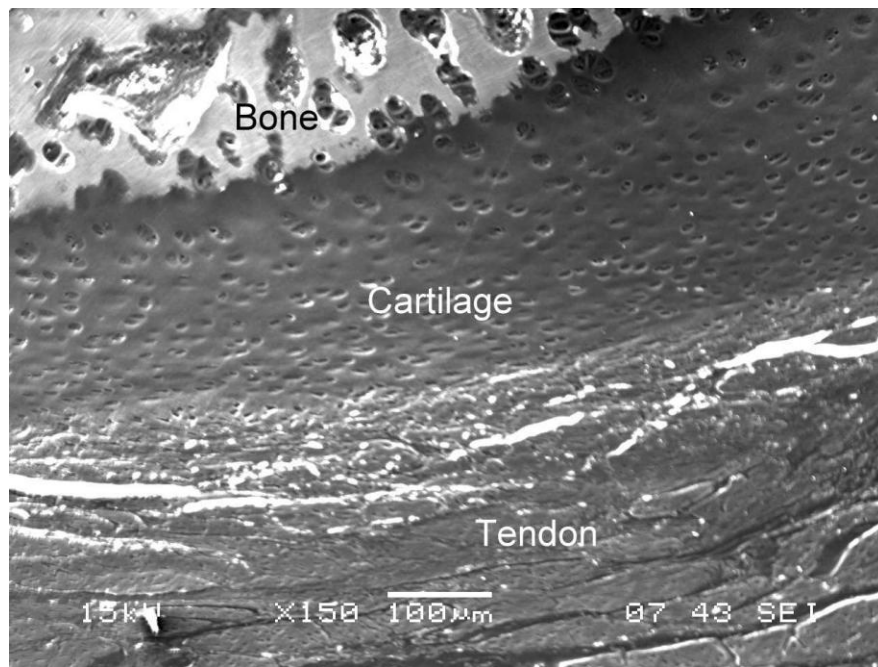
Figure 11 *Cartilage layer Observed by Reese.* Figure reproduced from Source 7. Figure shows Electron Micrograph of Ovine Patellar Tendon Enthesis.
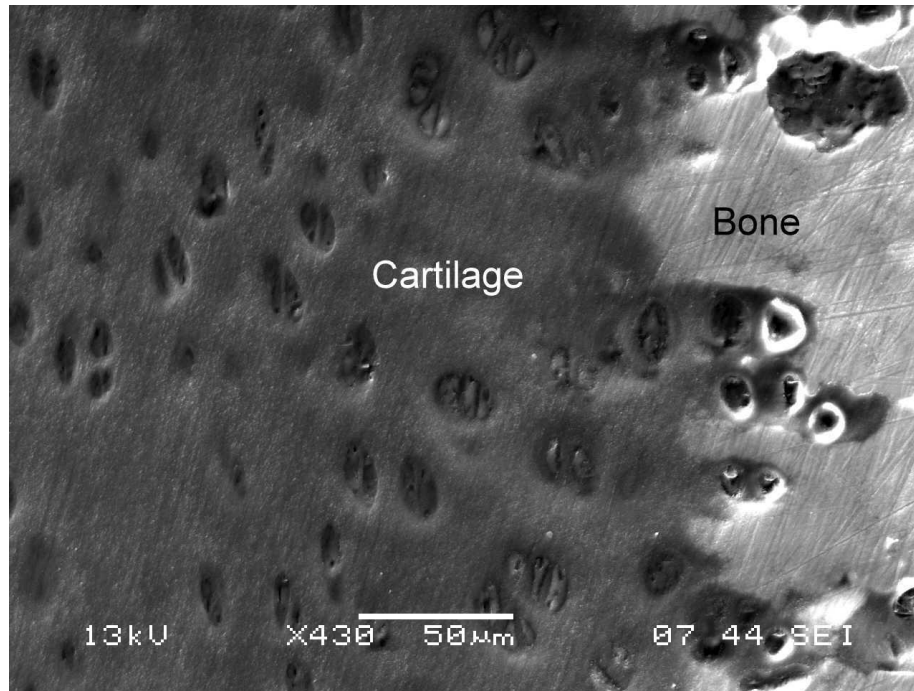
Figure 12: *Interdigitation Observed by Reese. Figure* reproduced from Source 7. The figure displays a highly magnified image of where the fibrocartilage region meets bone. The characteristic chondrocyctes of the fibrocartilage region are enclosed on three sides by bone. This interdigitation between the interfaces is characteristic of the region and leads to very strong mechanical properties.
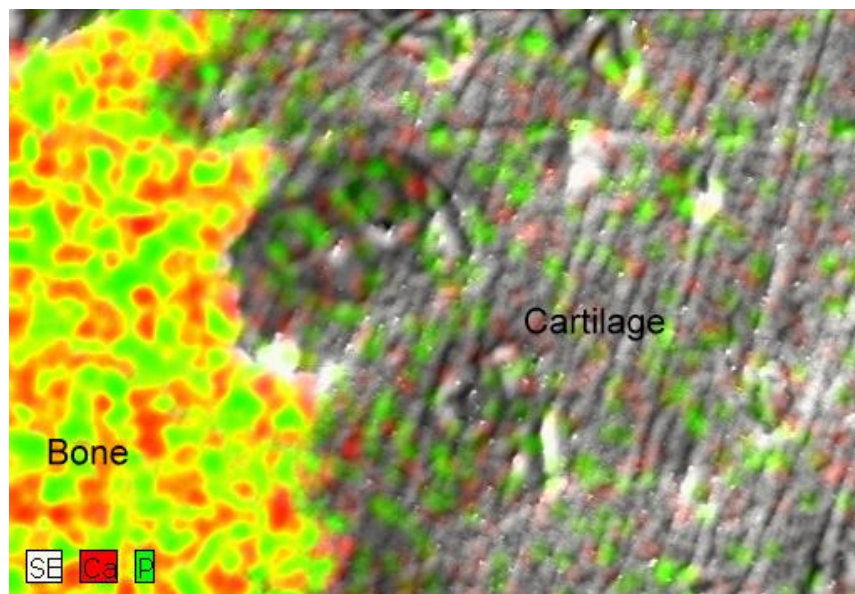
Figure 13: *Calcium-Phosphorus Mapping Observed by Reese.* Figure reproduced from Source 7. Figure Overlay of the image and X-Ray dispersive energy atomic mapping of patellar tendon ovine enthesis. Calcium is highlighted in Red and phosphorus is highlighted in Green. Signal is the most intense along the bone, but a gradient extending into the cartilage zone. Signals

extending beyond 20μM – 40 μM of the fibrocartilage zone are either due to proteins that contain phosphorus and calcium or an artifact of polishing.

Although the fibrocartilage region was identified by the chondrocytes cells, Reese observed cell differentiation in the fibrocartilage region.[7] This cell differentiation is shown in **Figure 15**. A cellular gradient is not entirely unexpected because osteocytes, chondrocytes, and tenocytes all derive from the same mesenchymal stem cells; however, it seems to indicate that the chemical markers that influence cell differentiation are based upon physical location which opens up many other avenues for research.[7,10]
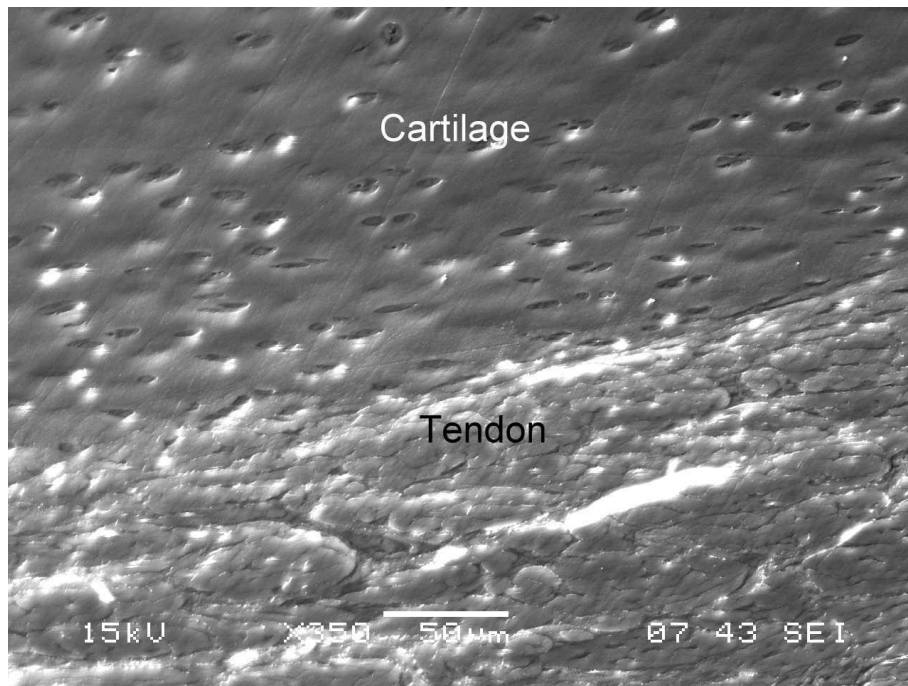
Figure 14: *Cell Differentiation Observed by Reese.* Figure reproduced from Source 7. Figure is an electron micrograph of Ovine patellar tendon enthesis at high magnification. Cells in the fibrocartilage zone become flatter and less globular near the interface with the tendon. The cells differentiate throughout the fibrocartilage layer resembling chondrocytes closer to the bone and resembling tenocytes closer to the tendon.

Anhydrous Fixation of Bone Tissue

Whether the mineralization of the fibrocartilage matches the mineralization of bone, is a matter of rigorous scientific debate.[12] It is still a matter of controversy whether this mineralization is an Amorphous Calcium- Phosphorus (ACP) crystal complex or a

poorly crystalline hydroxyapatite.[12] In addition, it has been speculated that the mineral complexes observed by some researchers have been due to artifacts introduced during sample preparation. [1]

For example, Landis et all propose that the observed mineral phases are due to the means of sample preparation introducing unwanted artifacts.[1] For example, Landis et all contest the results of previous studies because in studies both in vitro and of actual bone material ACP has been shown to convert to poorly crystalline Hydroxyapatite when exposed to water. [1] With conversion between different mineral phases already established, it is not unreasonable to assume that transformations in mineral size, shape, and orientation also occur. [1] Further concerns on the validity of calcium-phosphorus crystal structures obtained from samples fabricated using aquesously prepared solvents arise from the observed dissolution on calcium-phosphorus structures when exposed to water or commonly used electron microscopic stains. [1]

Landis et all prepared samples of the periostal cuff and surface of the bone from embryonic and postnatal mice without the introduction of aqueous solvents.[1] This work by Landis et all formed the basis of the fixation procedure for anhydrous samples that is detailed in **Section 3.2**.

Limitations of an Ovine Patellar Tendon Enthesis Model

One of the advantages of using a sheep is that a sheep's knee is loaded throughout the entire gait cycle similar to the strains imposed upon the human knee. [6] In addition, the stress-strain curves of the forces applied to the knee during walking have a similar shape.[SL] Although the species are very different, it appears the morphology and

physiology such as attachment site location, bone geometry, and soft tissue mechanics are similar enough to produce analogous stress strain curves between ovine and human models.[6] Conversely, pig based models undergo a tendon repair process that better matches the natural healing process of humans. [6] Nonetheless considering that natural enthesis is the focus of this thesis, the ovine patellar tendon is a very good model for the human knee.

3. Methods and Materials

They were two sets of ovine enthesis samples studied in this thesis. One set was originally prepared in 2010 as part of the experimental portion of the thesis of Willie Mae Reese which is cited in the references section as **Source 7**. These samples were prepared using aqueous solvents. After a period of approximately two years, these samples were polished again and coated with a layer of carbon 15 nm thick. The second set of samples discussed in this work were prepared by the author of this thesis using anhydrous methods of preparation. Both sets of samples were analyzed by SEM and X-Ray energy dispersive analysis (XEDS).

SEM Enthesis Samples Prepared via Aqueous Fixation

The following section is based on the account in Reese's thesis. A section of the tibia with the tendon attached was collected from a 2 year old male sheep which had been euthanized following an unrelated surgical procedure. The samples were supplied by Smith & Nephew LLC, Endoscopy Division, Mansfield Massachusetts.

The ovine samples were fixed in 10% neutral buffered formalin kept at $4^{°}$C and gently shaken for 24 hours. Samples were rinsed with Phosphate Buffered Solution (PBS) three times. Samples were soaked in PBS at $4^{°}$C and gently shaken to remove formalin. The samples were then dehydrated by soaking in the following series of ethanol baths: 50%, 75%,85%,95%,100%, and finally 100% undiluted ethyl alcohol . Samples were soaked in each series of the ethanol baths for 30 minutes.

The samples were infiltrated with a ratio of 1:2 volume ratio of fresh LR White Resin^{TM} to ethanol under vacuum at 4°C. Followed by a second infiltration with 1:1 ratio

of resin to alcohol under the same conditions. Finally, the samples were embedded in pure LR White Resin™ for 24 to 48 hours in a closed container at 60° C in order to fully cross-link the resin. The samples were polished using 400, 800, and 1200 girt silicon carbide abrasive. Some of the samples were sputter coated with a thin layer of gold although the gold layer is not recommended because it interfered with the EDS mapping of calcium and phosphorous.

After a period of two years, the samples were polished again to remove the gold coating and remove cracking in the resin near the surface. First the samples were polished for 30 seconds using 4000 grit silicon carbide paper, using pure ethanol as the lubricating solvent. The samples were placed in an ultrasonic bath for 1 minute while immersed in ethanol in order to remove silicon carbide particles that might have become embedded in the plastic. It was speculated that loose silicon carbide particles embedded in the cracks of the specimen could have resulted in large gashes. Next, the samples were polished with a 0.3μm-alumina particles suspended in ethanol. The samples were then placed in an ultrasonic bath for 5 minutes with the samples placed in a beaker containing ethanol in order to remove alumina from the surface of the sample. The presence of Alumina would have corrupted the results shown in **Section 4** as the ceramic phase of the alumina appears have brighter than bone which distorts the contrast of the SEM images. Additionally, the high atomic number of Aluminum can also decrease the accuracy and resolution of EDS mappings. Lastly, the samples were evaporation coated with 15 nm of amorphous carbon deposited in a vacuum chamber.

SEM Enthesis Samples Prepared via Anhydrous Fixation

A lamb's knee was obtained from The Meat House butcher shop, Brookline, MA. The knee came as part of a vacuum-packed leg of lamb. Although the age of the animal is not precisely known, lambs in the United States must be slaughtered before 14 months. A portion of the femur and the tibia along with the knee joint was separated from the rest of the lamb with butchering tools. Most of the muscle was removed by the butcher. The knee was wrapped in plastic, then in butcher paper. The knee was then stored in s freezer double bagged in Ziploc plastic bags. Before dissection, the knee was thawed in the refrigerator. Further flesh was removed using a Bard-Parker BD Surgical Blade No.11 made out of stainless steel. Instruments were cleaned with isopropyl alcohol before and after. The sheep sample was further dissected to leave only the patellar tendon attached to the patella and the tibia.

The samples were frozen for approximately one month. The samples were then fixed with an anhydrous fixation procedure based upon the one described by Landis, Paine, and Glimcher in **Source 1**. Each of the samples was placed in a beaker filled with 100 mL of anhydrous ethylene glycol. The beakers were placed in a vacuum desiccator which was evacuated by a rotary pump for one hour. While maintaining vacuum, the desiccator was placed in an ice bath in order to keep the samples at 4° C. The ice bath + desiccator were placed on a mechanical shaker, where the samples were moderately agitated for 36 hours. The ice was replaced periodically throughout the 36 hours to insure that the temperature was maintained at 4° C. Although the samples were intended to have been under vacuum for the entire fixation process, a leak was discovered after the

samples were fixed. The samples were unintentionally exposed to an atmospheric environment for the entirety of the fixation process.

After 40 hours, the ethylene glycol was replaced with the monomethyl ether of ethylene glycol (also known as cellosolve). The cellosolve was supplied by Electron Microscopy Sciences Inc,. The samples were maintained at 4° C and at atmospheric pressure for 12 hours while continuing to be moderately agitated. After 12 hours, the cellosolve was changed, although the temperature, pressure and agitation were maintained. The cellosolve was removed after 16 hours.

Next, some of the samples were trimmed using a Dremel^{TM} hand tool. A reinforced steel cutting wheel attachment was used. The tools were disinfected and cleaned with ethanol. The Dremel tool resulted in the loss of some of the material of the bone as well as a burning of the bone. The hand tool is recommended only for trimming regions of bone where the tendon does not intersect the bone. For trimming sections of the sample that included tendon, the samples were cut by using a razor blade as a chisel by placing the edge of the razor blade along the sample and striking it with a hammer.

Next, the samples were infiltrated and embedded in resin. The embedding procedure followed by Landis et al could not be used because the resin used by them was discontinued in 1978. Instead, it was decided to follow the infiltration and embedding procedure outlined in the thesis of Reese in **Source 7**. The resin used in that work was LR White Medium Grade which is widely used as an embedding medium for biological samples prepared for electron microscopy. The LR White Medium Grade resin was

supplied by Electron Microscopy Sciences Inc, already catalyzed and kept refrigerated until used.

The samples were dehydrated in a series of ethanol baths of 50%, 75%, 95%, and 100% for a time of 30 minutes each. Then the samples were infiltrated with a 1:2 volume ratio of LR White Resin to 100% ethanol. The samples were maintained at 4° C for the majority of 14 hours. A failure in experimental method led to the samples reaching room temperature for a period of approximately 2 hours. The samples were intended to be infiltrated under vacuum; however, due to in the failure in experimental arrangement the samples with the 1:2 volume ratio were unintentionally infiltrated at atmospheric pressure. Next, the samples were infiltrated in loosely capped scintillation bottles with a 1:1 volume ration of LR White Resin to 200 Proof Ethanol for 16 hours. The samples were kept under vacuum and at 4° C. Due to another failure in experimental arrangement, the samples lost vacuum and may have been exposed to a slight amount of water. Finally the samples were infiltrated with pure LR White Resin for 24 hours.

The samples were embedded in pure LR White Resin by pouring resin into 1.25 inch diameter silicon molds and placing the samples in the molds. The molds were then placed in a closed glass container in order to avoid the introduction of Oxygen which is known to interfere with the curing process. The samples were heated in a furnace at 58°C for 24 hours. It should be noted that the first time the samples were embedded they were done under vacuum which caused the plastic that the samples were embedded in to become brittle and introduced many voids. As much plastic as possible was removed by either cutting the plastic or breaking the plastic away although the original biological

sample was still covered on all sides by plastic. The samples were then embedded again following the procedure above.

The samples were cut using a diamond saw with Isopropyl alcohol used as the lubricating solvent. The samples were polished with the same polishing procedure outlined in **Section 3.1**. The samples were sputter coated with 30 nm of Carbon. The conductive layer was increased to 30 nm of Carbon because electron beam drift interfered with the EDS mappings in the samples prepared in aqueous solvents.

4. Results

Samples Prepared Using Aqueous Solvents

Figure 15, which is shown below, displays an electron micrograph taken of the patellar tendon enthesis region of a sample prepared using a aqueous fixation system and infiltration system. The image was taken with SEM backscatter mode. The textured light gray region of the micrograph represents a fibrous material. It was determined that the material was fibrous because the region is composed of mostly parallel strands that are typical of fibrous materials. The parts of the image that are a darker gray and look smooth is the plastic that the samples were embedded in. The sample is plagued with a severe amount of cracking. Although the majority of the cracks affect the plastic embedding matrix which that is of zero interest experimentally, parts of the fibrous material under study are corrupted by the cracking. Small flashes of white in the image, which represents the presence of high atomic number compounds, are visible mainly around the cracking and selected parts of the fibrous material.
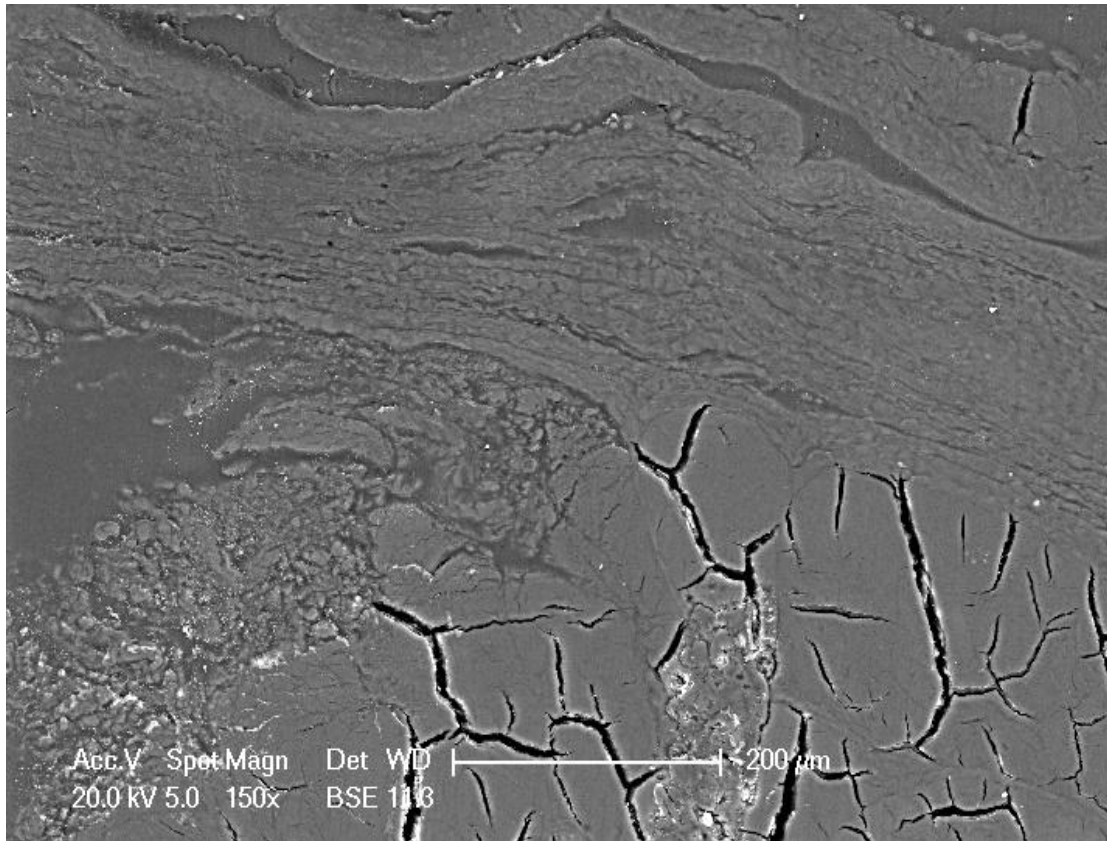
Figure 15: *Close-up of Fibrous Material.* Figure shows an electron micrograph of the approximate region where the patellar tendon meets the bone at the relatively low magnification of 150. Region was taken from an Ovine Model and specimen samples were prepared using aqueous solvents. Image was taken using the Back Scatter Mode of the SEM; therefore, the brighter regions correspond to regions that contain higher atomic number element. The textured, light gray region is a fibrous material. The region that is a smooth, dark gray is the plastic embedding matrix of the sample. The long narrow black regions are cracks in the sample.

Figure 16, which is displayed below, shows a low magnification electron micrograph of the fibrous material meeting bone. The image was taken in the back scatter mode of the SEM. The bone can easily be identified because the large proportions of phosphorus and calcium in the bone cause the bone to appear a white or silver color in SEM back scatter mode. The fibrous material was identified by the parallel strands composing it.
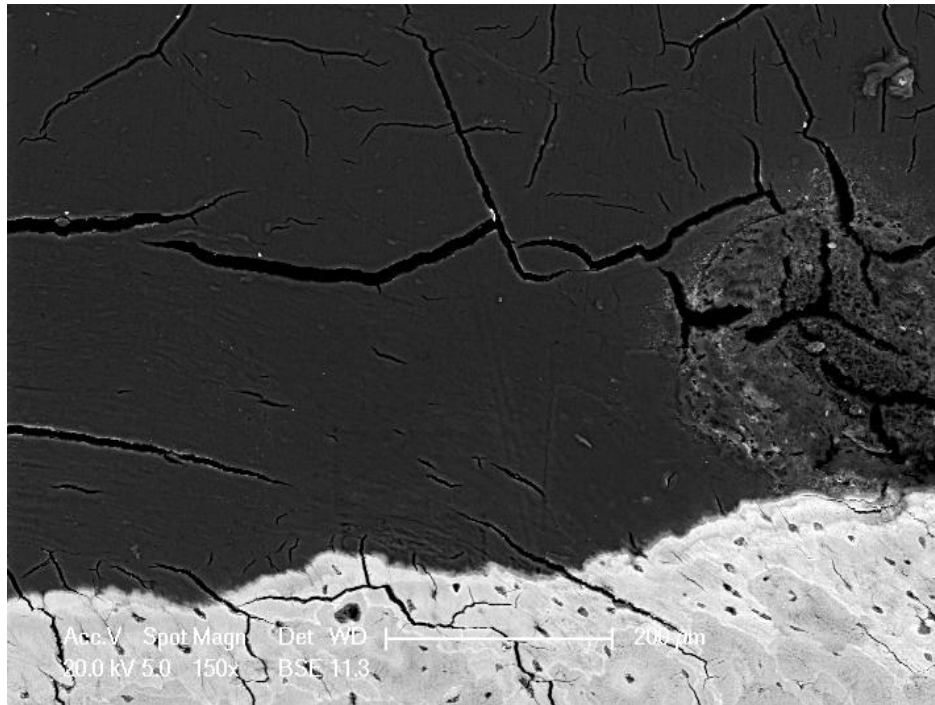
Figure 16: *Fibrous Material Interface with the Bone.* Image displays an electron micrograph at a magnification of 150. Image was taken in Back Scatter Mode. Image display the interface between a fibrous material and bone from the area at or near where the patellar tendon attaches to the tibia. Images were taken from an Ovine model and the samples were prepared using aqueous solvents.

Figure 17 displays a high magnification of the fibrous material meeting the interface with the bone. The parallel strands of the fibrous material are clearly shown. At the edge of the bone, there appears to be either a gradient in the small bone. In a region on the order of 1 μM, the coloring along the edge of the bone is less intensely bright than the surrounding bone. This image was taken in the SEM back scatter SEM which indicates that there is a gradient of phosphorous and calcium at the edge of the bone.

Figure 18 displays a high magnification electron micrograph the porous region in the right portion of **Figure 17**. This region is porous and some of the pores have the shape and size of typical fibroblast and chondrocyte cells. However, the cracking in the matrix obscures the section and overall the image is too ambiguous to determine whether or not

fibrocartilage is present.
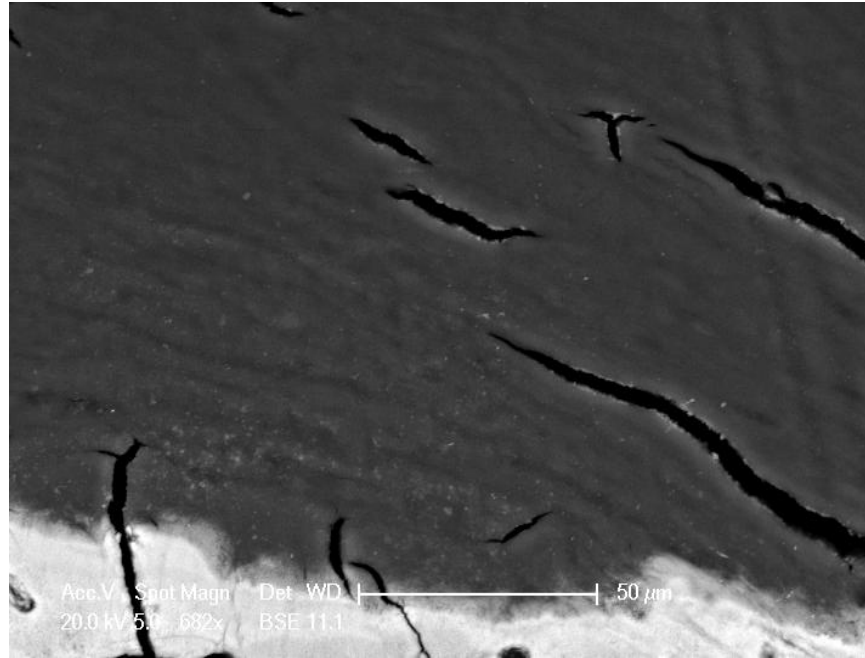
Figure 17:*Close up of an Interface.* Image was taken in Back Scatter Mode in the SEM. Highly magnified image (682 x) of where the fibrous material connects with the bone.
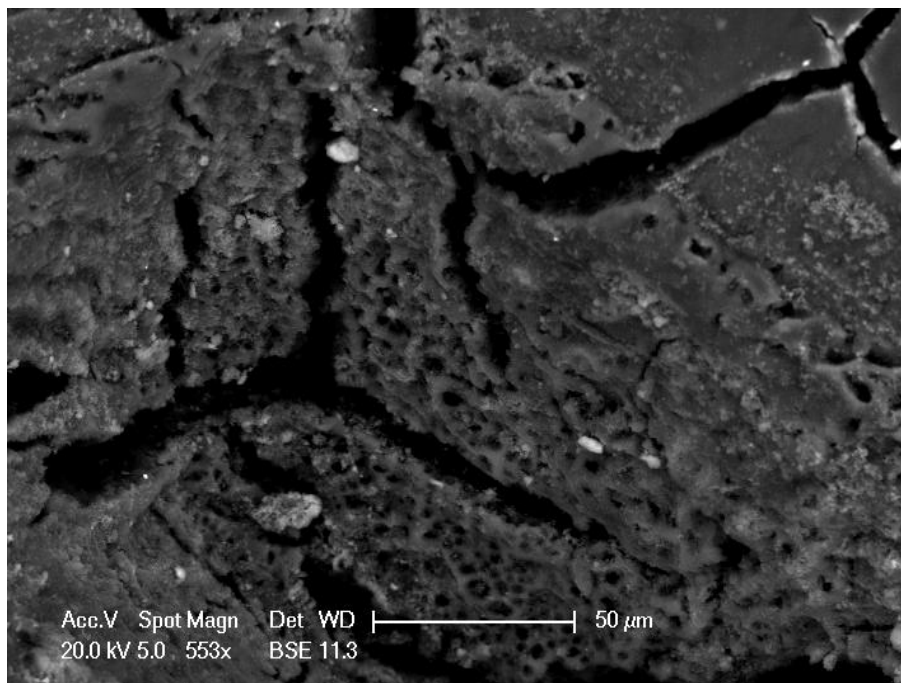
Figure 18: *Possible Fibrocartilage Region.* Image is highly magnified (553 X) and the SEM is in backscatter mode. Region is approximately where the patellar tendon intersects with the tibia. Image displays things that could be possible cellular activity.

Figures 19 and **20** display electron micrographs as well as EDS mappings of the samples described above. The black area in the images is the organic phase. A brighter intensity corresponds to a greater concentration of the mapped element. Maps of calcium and phosphorous mapped separately shown in **Figure 19** and a map of both elements superimposed on the same image is shown in **Figure 20**. A slight decrease in the intensity of the mapping is visible along the edge of the bone.
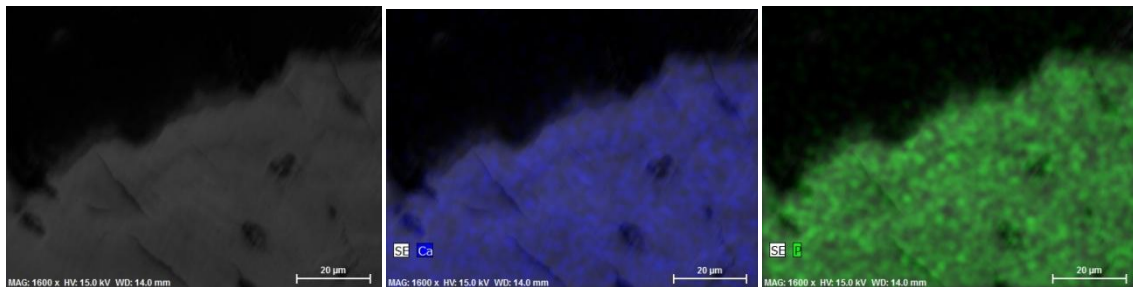
Figure 19: *Interface image with Phosphorus and Calcium Overlay.* Electron micrograph is where the fibrous material joins the bone and is highly magnified is the left image. EDS mapping of calcium is the middle image. EDS mapping of Phosphorus is shown on the right.
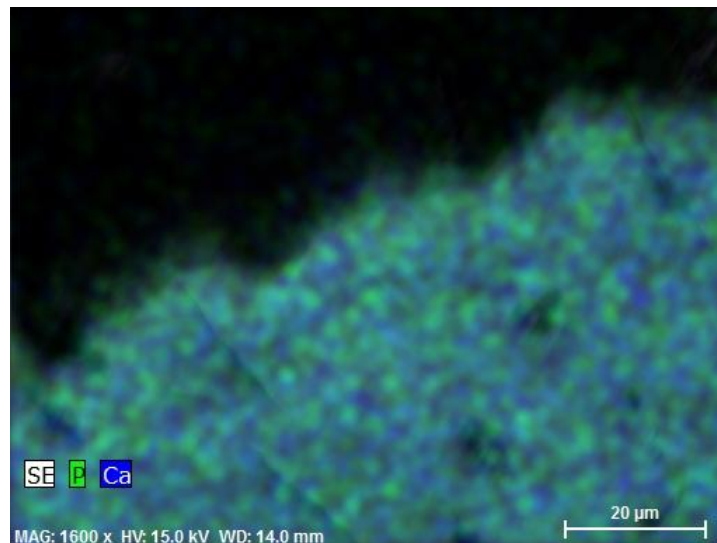
Figure 20: *EDS Mapping of Phosphorous and Calcium.* Figure displays an electron Micrograph and EDS mapping of the approximate region where the patellar region joins the tibia *in an ovine model. The samples were prepared using aqueous preparation methods.*

Samples Prepared using Anhydrous Solvents

Intersection between the Tibia and what is believed to be the Patellar Tendon is shown in **Figure 21** at low magnification, in **Figure 22** at moderate magnification, and in **Figure 23** at high magnification. **Figure 24** also shows an electron micrograph of the interface between the tendon and the tibia. **Figures 25** and **Figures 26** show XEDS mapping of calcium and phosphorus of the region shown in **Figure 24**. All of these SEM electron micrographs were generated in the back-scattered mode in an environmental SEM at low vacuum with the samples not coated with a conductive carbon coating. The samples were prepared from an ovine specimen and anhydrous solvents were used in their manufacture.

Figure 21 displays an electron micrograph of natural Patellar enthesis at a magnification of 150 times. The region of the left of the electron micrograph has a white appearance which indicates that it is composed of heavier atomic elements. It is known from sample preparation and sample set up that this region is bone. To the right of the bone there is a region that is steel grey and textured. Parallel strands can be observed in this region. The darker color indicates that this region is composed of light atomic elements such as hydrogen, oxygen, carbon. The region that is a very dark grey and smooth is the plastic resin that the sample is embedded in.

Acc.V Spot Magn   Det WD ├─────────────┤ 200 µm
20.0 kV 3.0 150x   BSE 11.0 0.8 Torr
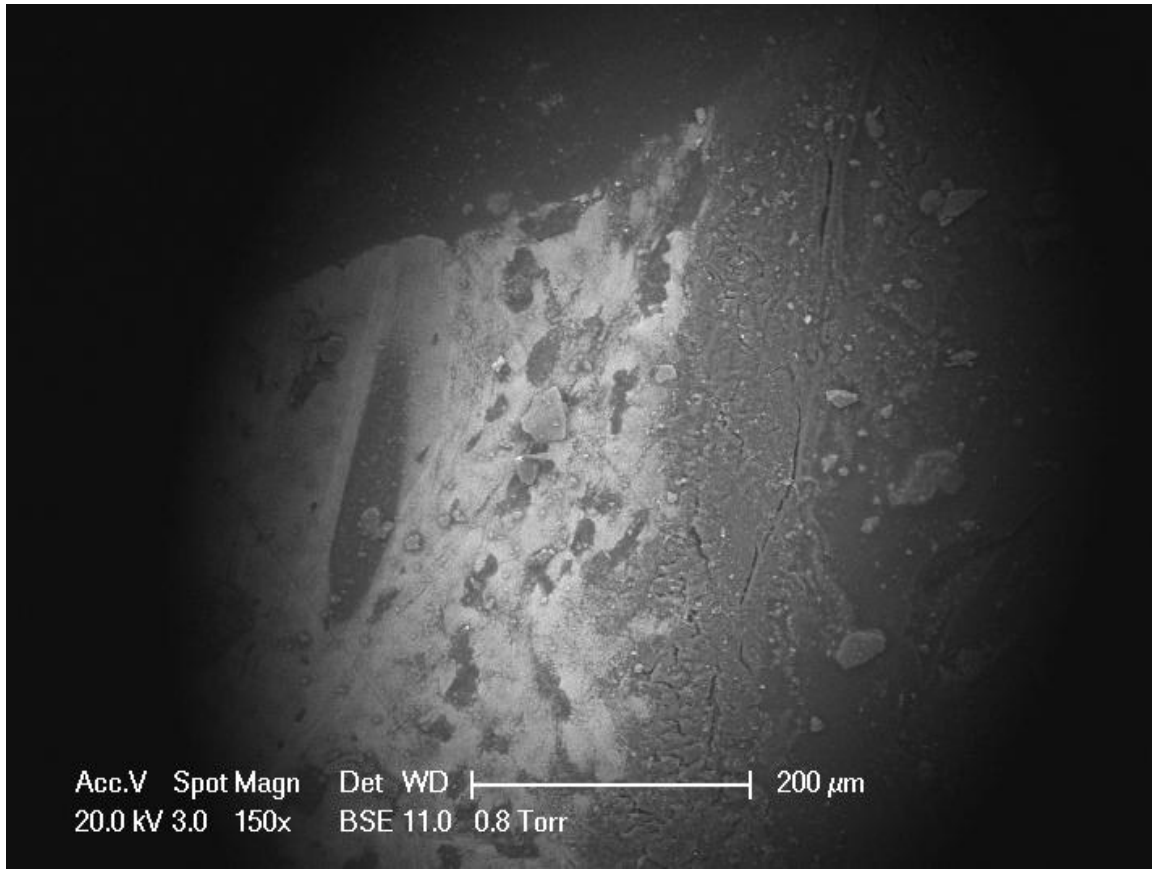
Figure 21: *Anhydrously Prepared Intersection Between Tendon and Bone.* Figure displays in low magnification, the intersection between the Patellar Tendon and the Tibia in an ovine model prepared mostly anhydrously. Taken in a low vacuum mode in an Environmental SEM. The sample was not coated with carbon. The white region located mostly in the left portion of the image is the Tibia The dark gray fibrous region in the right of the image is believed to be the Patellar tendon.

Figure 22 shows a region of the interface between the bone and the tendon at

1,000 times the magnification. The region is the same in **Figure 21** but was taken at a

greater magnification. The light gray region is shown to the left of the electron

micrograph is composed of heavier elements such as phosphorus and calcium. It is

known from sample preparation and sample orientation that the bone is in the left region

of the micrograph. A series of parallel lines that follow the orientation of the light grey

regions is observable. Dark grey lines located in the steel grey region of the electron

micrograph are cracks in the embedding plastic. Little pockets of brightness can be

observed in the steel grey region. The steel grey section of the electron micrograph

contains light elements hydrogen, carbon, and oxygen. The little pockets are composed of

heavier atomic elements. The parallel strands that were observable in the steel grey

region at the lower magnification are no longer visible.
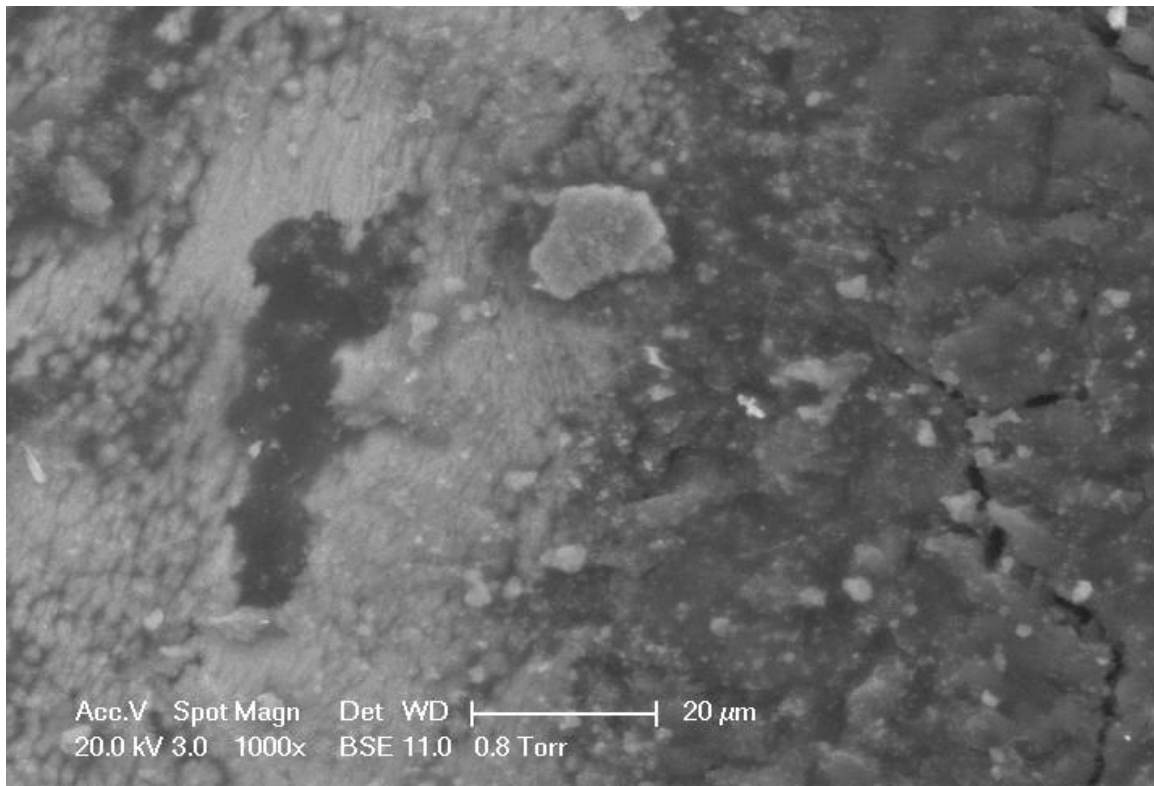
Figure 22: *Moderately Magnified Tendon-Bone Interface.* Figure displays a higher magnification of the region shown in Figure 21. The figure shows the intersection between the Patellar Tendon and the Tibia in an ovine model prepared mostly anhydrously. Image was taken while using low vacuum mode in an Environmental SEM. The sample was not coated with carbon. The white region located mostly in the left portion of the image is the Tibia The dark gray fibrous region in the right of the image is believed to be the Patellar tendon.

Figure 23 displays an electron micrograph of the tendon-bone interface taken at a

magnification of 3000. The parallel lines in the light grey region of are more noticeable in

this figure than in **Figure 22**. In addition, the regions are in the white, bright region are in

the steel grey region of the micrograph.
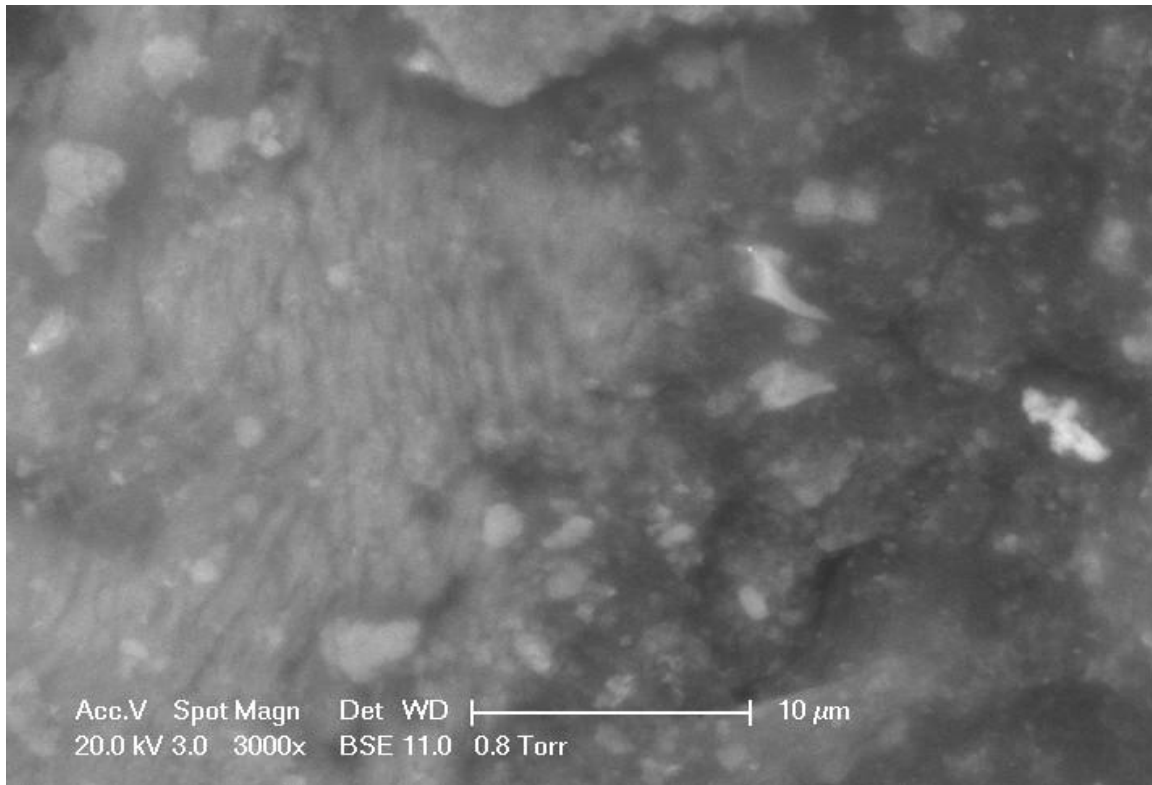
Figure 23: *Highly Magnified Tendon Bone Interface.* Image displays a section of the region shown in Figure 21 at very high magnification. Image was taken at the very edge of the tendon-bone junction. In comparison to the white of the bone in Figure 21, this figure has a medium gray in the left hand image. A series of parallel lines in the mild grey region indicates that this region might be fibrous indicating that they region might by of mineralized fibrocartilage. The figure shows the intersection between the Patellar Tendon and the Tibia in an ovine model prepared mostly anhydrously.

Figure 24 displays an electron micrograph of the tendon-bone junction at a magnification of 500 times. It is known from sample preparation and orientation the bone will be on the left side of the electron micrograph and the fibrous material on the right as seen in **Figure 21**. The middle section is a very bright white. Since the intensities of backscattered images are a function of the atomic number of the elements that compose them, it appears as though the middle section of the micrograph contains heavier elements than the left of the micrograph which is different than **Figures 21-23**.
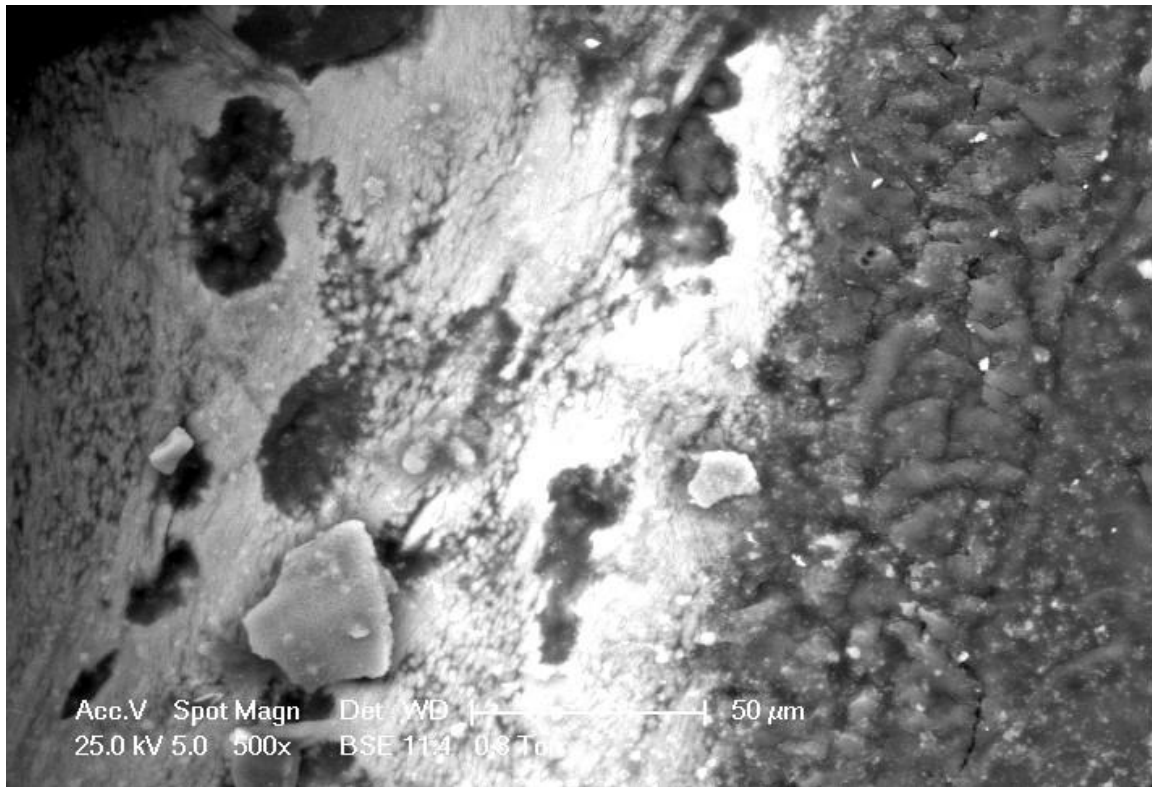
Figure 24: *Region Studied via XEDS Mapping.* The figure shows the intersection between the Patellar Tendon and the Tibia in an ovine model prepared mostly anhydrously. Image was taken while using low vacuum mode in an Environmental SEM. The sample was not coated with carbon. The white region located mostly in the left portion of the image is the Tibia The dark gray fibrous region in the right of the image is believed to be the Patellar tendon

Figure 25 and **Figure 26** display the XEDS mappings of calcium and phosphorus respectively of the region shown in **Figure 24**. Unlike most XEDS mappings, **Figure 25** and **Figure 26** not are overlaid with the original image. The bone is to the left of the electron micrograph. In both figures, the left is very brightly colored with the element that the XEDS is mapping with the right of the electron micrograph being black with a few isolated marks of color. At the edge between the brightly colored region which is bone and the dark region which is the organic material there appears to be a slight gradient in decrease from color to black.
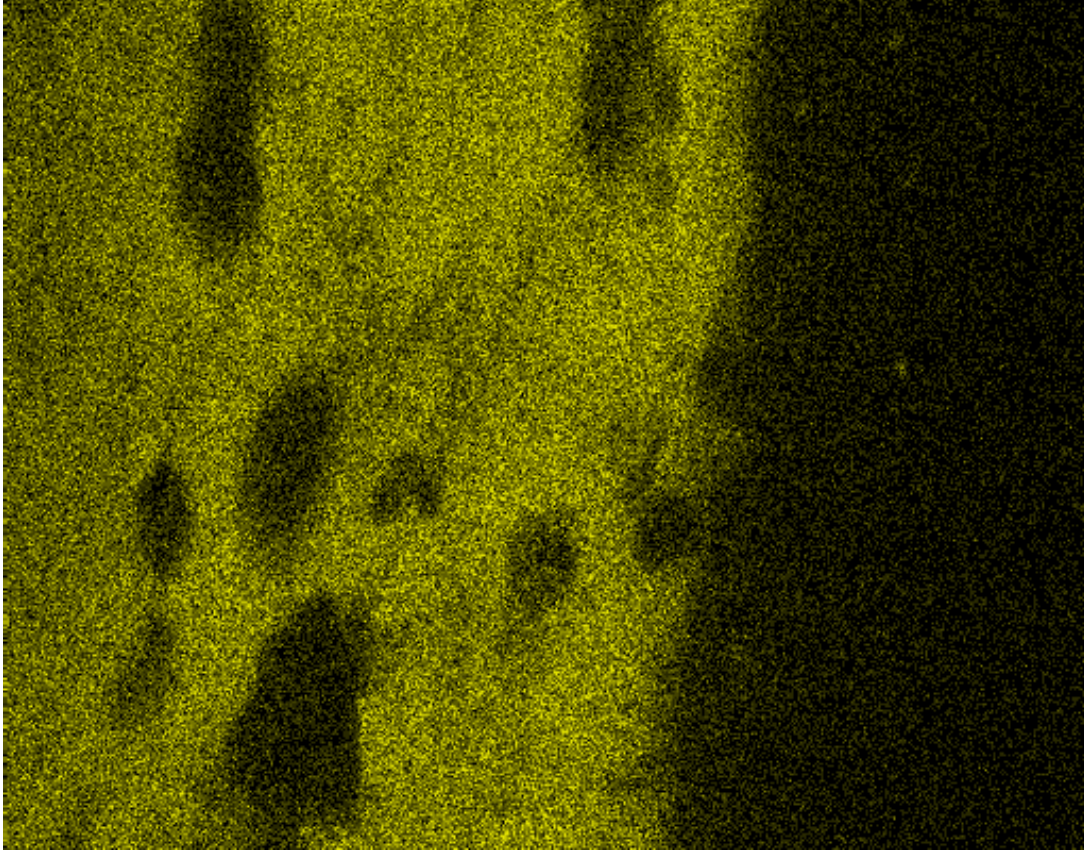
Figure 25: *Calcium XEDS Mapping.* XEDS mapping of the element Calcium. Map was generated from the image shown in Figure 24. Image was taken while using low vacuum mode in an Environmental SEM. The sample was not coated with carbon. Region is of the intersection of the Patellar tendon and the Tibia in an Ovine model.
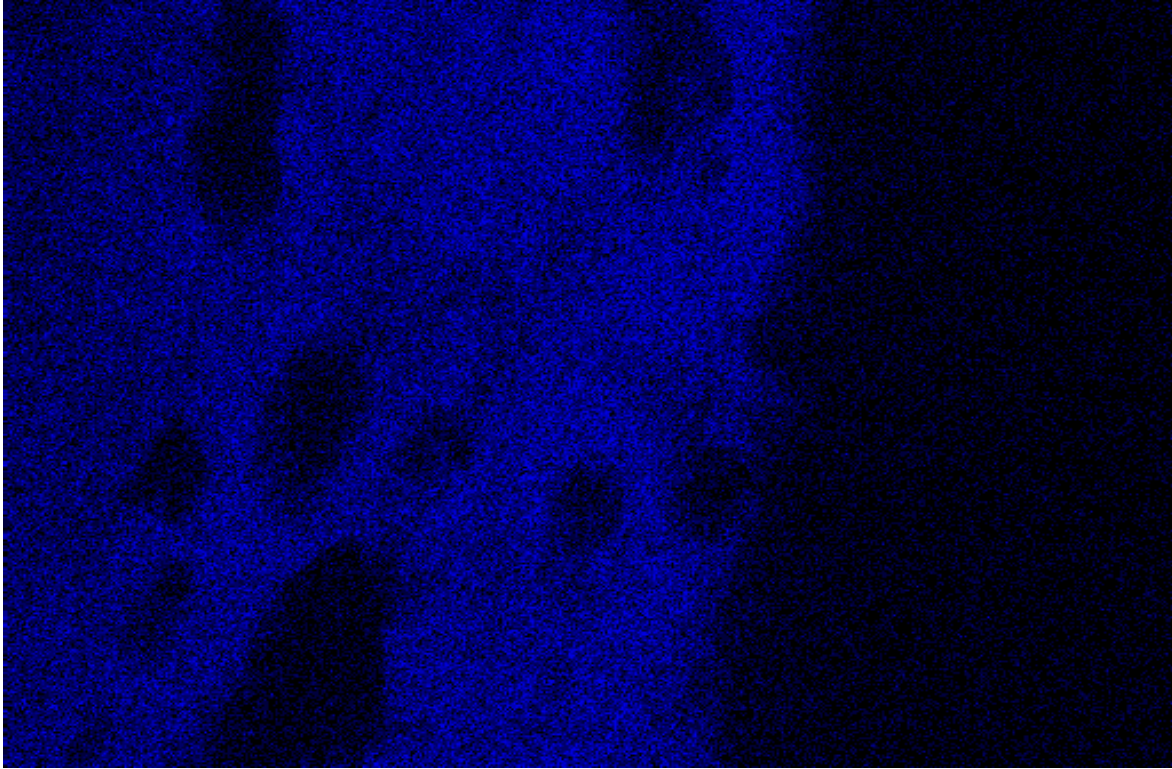
Figure 26: *Phosphorus XEDS Mapping.* Figure displays XEDS mapping of the element Calcium. Map was generated from the image shown in Figure 24. Image was taken while using low vacuum mode in an Environmental SEM. The sample was not coated with carbon. Region is of the intersection of the Patellar tendon and the Tibia in an Ovine model.

5. Discussion

The Fibrous Material in Samples Prepared Using Aqueous Solvents

  The nature of the fibrous material of the samples prepared via aqueous means was ambiguous. This fibrous material is shown in great detail in the **Figures 15**, **16**, and **17** located above in **Section 4.1**. The observed parallel strands of the material shown in **Figure 15** show that the material is definitely fibrous. The texture of the material is distinguishable from the surrounding embedding matrix which as shown in **Figure 15** is a dark gray and smoother than the fibrous region. This region contains a higher portion of cracks than the fibrous material. However, if the material was tendon, tenocytes should be observed. As can be seen in **Figures 15-17**, nothing resembling cells can be observed in the fibrous material. The fibrous material extended quite a long distance before intersecting with the bone. Although images of the entire region are not included, the electron micrographs of **Figures 15-17** are representative.

  Besides, if the fibrous material was tendon there should be an intermediate layer of fibrocartilage between then tendon and the bone.[7] **Figure 18** has a region whose visual appearance and texture seems to match the electron micrographs of fibrocartilage shown in other studies.[7] Equally important, cells appear to be present.

  Nevertheless, this apparent fibrocartilage region does not seem to have a direct interface with both the fibrous material and bone. In addition, the electron micrograph is seriously obscured by cracking in the embedding matrix. Some areas that appear to be the partial mineralization expected in fibrocartilage between tendon and bone could in fact be Alumina particles trapped in cracks in the matrix. Upon consultation with Dr. Hao

Wang, it was decided that this fibrous material was a membrane on the surface of the bone and not actually tendon.

These aqueously prepared samples were embedded in a plastic matrix and then sectioned for study in the SEM. The interface between tendon and bone is three dimensional. Hence, it is plausible that not all of the sections retained the interface between tendon and bone which resulted in the specimen samples analyzed above containing a membrane sheath over the bone but not a region of enthesis.

The Fibrous Material in the Anhydrous Prepared Solvents

It is known from the methods of sample preparation that the samples prepared using anhydrous solvents that were studied in this work did contain the intersection between the patellar tendon and the tibia. However, it is ambiguous that the region is shown in **Figures 21-26** is the region of the interface between the patellar tendon and the tibia.

The steel grey region on the right side of the electron micrograph was determined to be a fibrous material based on its textured appearance and the parallel strands that compose it. **Figure 21** seems to show a porous region in the bone that is mostly likely trabecular bone. Trabecular bone is underneath the cortical bone and does not directly connect to the tendon. Therefore, if the right of the electron micrograph was in fact the tendon then it seems to indicate that the tendon is connecting to the trabecular bone which shouldn't be the case. Perhaps the layer of cortical bone is too small to be resolved in **Figure 21**. The regions are too large to be cells. **Figure 24** displays a region of brighter intensity between the bone and the fibrous material which could be the cortical bone.

Figure 23, which displays a highly magnified region between the tendon-bone junction, has a mild grey region on the left side of the electron micrograph that is marked with straight parallel lines. The parallel line are much straighter than is typically found in fibrous materials, but the fact that the follow the orientation of the mineral phase would seem to indicate that they are not an artifact left by polishing scratches. These parallel lines could indicate that this mild grey region might be fibrous. The location between tendon and bone, the fibrous nature and the mild grey color which indicates some presence of phosphorus and calcium but not as much as found in the bone indicates that this region is mineralized fibrocartilage.

The Gradient of Phosphorus and Calcium

The overlay of the image as well as a Mapping of both Phosphorous and Calcium is shown above in **Figure 20**. At the very edge of the bone interface there appears to be a slight gradient of phosphorus but this is most likely an artifact of the electron drift experienced during this data collection which is described in more detail in **Section 5.3.** As mentioned previously in **Section 2.1,** the observed mineralized gradient region was on the order of 20-40 μM. No gradient this large is observed in either **Figure 19** or **Figure 20**. This lack of a gradient provides further evidence that the material shown in **Figures 15**- **17** is not the actual insertion site of the tendon into bone.

Figures 25 and **26** seem to display a slight decrease in intensity going from the region on the left that marks the bone and the black region on the right of the electron micrograph in the junction between the two regions. This gradient could be a gradient of phosphorus and calcium that is characteristic of the unmineralized fibrocartilage zone.

However, this decrease in intensity seems to be on the order of a few micrometers which is an order of magnitude smaller than the expected fibrocartilage zone.

It is interesting to note that **Figure 24** seems to indicate a very bright region in the middle of the electron micrograph. This increase in the intensity is reflected in **Figure 26** which seems to indicate that the middle of the electron micrograph has a greater degree of mineralization that the left. The bone is to the left and it is interesting that this area of greater mineralization seems to be in-between the bone and the tendon. It is possible that this region of greater mineralization represents the denser cortical bone or a possible enthesis site.

Sources of Error

The EDS mappings shown in **Figures 19** and **20** were corrupted by electron drift. Although various spot size, and accelerating voltage combinations were tried a slight electron drift could not be avoided. It is believed that the electron drift was due to the material charging up even though the samples were coated with 15nm of Carbon.

The samples prepared via aqueous means were severely cracked. It is believed that the cracks initially developed during sample creation due to differences in their thermal coefficient of expansion between bone and plastic. These cracks were aggravated by atmospheric attack over time, as well as polishing the samples with water used as the lubricating medium. Although polishing the samples using ethanol as the lubricating medium eliminated a lot of the surface cracks and the cracks were concentrated in the experimentally irrelevant sections of the sample, the quality of the results was seriously hindered by this cracking.

Although it was intended that the samples prepared using anhydrous solvents were to be prepared in an environment that was entirely anhydrous, due to a failure in experimental set up the samples might have been exposed to a minute amount of water. Therefore, it must be assumed that if phases of poorly crystalline hydroxyapatite were found in the sample that it could be corrupted ACP.

In addition, during the sample fixation and infiltration process the samples were not under vacuum. Infiltrating the samples under a vacuum, removes the oxygen that might be found in the pores of the sample that could to pockets of trapped and air and improper curing of the samples. The embedding process did not work the first time and then the plastic embedding resin was chipped away and the samples were re-embeded. Overall, a lot of things in sample preparation happened in less than optimal conditions. These samples prepared using anhydrous solvents should probably not be used to study the mineral phases of natural enthesis because it has been established that the mineral phases of enthesis are very delicate.

6. Conclusion

Summary

In tendon injury repair, the insertion of the tendon into the bone or enthesis often determines the quality of healing and poor enthesis often leads to treatment failure. However, natural enthesis is not very well understood which makes developing additional clinical treatments for tendon injury repair difficult. In addition, previous studies about natural enthesis especially those that concluded that the calcium-phosphate mineral phases were those of poorly crystalline hydroxyapatite have been divisive. One of the reasons for the controversy, is that many of the natural enthesis samples were prepared using aqueous solvents and it has been established elsewhere in academia that amorphous calcium-phosphate converts to poorly crystalline hydroxyapatite upon exposure to water.

The intention of this thesis was to ascertain the effect that sample preparation had on the natural enthesis. For this means, two sets of samples were prepared from where the patellar tendon intersects the tibia in an ovine model. One set of samples was taken from a mature sheep and prepared using aqueous solvents. The other set of samples were prepared from a lamb and prepared using anhydrous solvents although a failure in experimental setup did lead to some exposure of water during the process. Both sets of samples were embedded in LR White Resin and studied using SEM. The SEM was used in back-scattered mode. In addition, XEDS mapping were generated for select regions.

It was determined upon consultation with Dr. Hao Wang that the samples that were prepared using aqueous solvents did not in fact display the junction of the patellar

tendon and the tibia. Although a fibrous material was clearly present and is in visible in

Figures 16-18. The material was determined to be fibrous because the SEM electron

micrograph showed that the region was composed of parallel strands. However, no

fibrocartilage zone was observed between the fibrous material and the bone. The lack of a

fibrocartilage zone and observable tenocytes were the reasons it was decided that the

fibrous material was not the patellar tendon but a membrane covering the bone. It was

speculated that do to the three dimensional nature of the tendon-bone interface that not all

of the two dimensional cross sections generated from the original sample captured the

actual interface with the tendon. In addition, the samples prepared using aqueous solvents

were plagued with sever cracking that obscured the quality of results that could be

obtained.

The results prepared using anhydrous solvents were ambiguous. Although it is

known from the sample preparation that the samples studied certainly had the region

where the patellar tendon directly attaches to the tibia, it is unclear that this intersection is

captured in **Figures 21-26. Figure 21** seems to show the fibrous material directly

attaching to the trabecular portions of bone which is physiologically incorrect. However,

Figure 24 and **Figure 26** seem to show the trabecular bone, followed by the cortical

bone, and then the tendon. Perhaps the layer of cortical bone is too small to be resolved in

Figure 21. **Figure 23**, seems to show the mineralized fibrocartilage zone. No cellular

activity was observed in the samples prepared anhydrously.

Due to failure in experimental setup as well as the ambiguousness of the data

generated no conclusions can be drawn regarding the effect of sample preparation has on

the mineral phases present during natural enthesis. Nevertheless, this work contributed to

the field of research by producing electron micrographs of the patellar enthesis region in samples used prepared using anhydrous solvents.

Limitations

The samples prepared with aqueous solvents were over two years old and were subject to atmospheric attack that led cracking in the matrix that obscured the regions of enthesis that could be observed. Some of this cracking was able to be eliminated by re-polishing the samples using 4000 grit Silicon Carbide paper and Ethanol. Additionally, it was ambiguous whether or not the region studied in the samples prepared with aqueous solvents was actually of the enthesis region. The samples prepared anhydrously were not kept under vacuum during certain steps of the fixation and infiltration procedure. The samples might have been exposed to slight quantities of water which may have corrupted the mineral phases observed. In consideration of the less than optimal sample quality, it is recommended that the experiment be repeated.

Future Work

The non proteoglycan proteins of the tendon which composes approximately 20% of the dry matter of the tendon are receiving an increasingly research focus because of their role in tendon matrix remolding and their speculated potential as such as molecular red flags that could alert to the presence of tendon injury. [2] For example, a high level of Cartilage Oligomeric Matrix Protein, which composes 3 % of the tendon matrix, has been found to correspond to high mechanical strength in the tendon. [SO] However the histology via electron microscopy experiments in this work, cannot determine the presence of

individual proteins. Further research on natural enthesis, should combine the physiology studies provided by histology with more advanced studies of protein [2]

In addition, the properties and composition of tendon and bone both change with patterns of exercise, diet and age.[7,10,1] Although this work, compared the enthesis region of a fully mature sheep to that of a lamb it was hard to attribute differences that were due to age due to the differences in preparation and condition of the samples. An age spectrum of enthesis samples should be studied in order to determine if a physical change in the enthesis region could be partially responsible for the large increase in tendon injuries with age. In particular, it would be interesting to see if the interdigitation observed by Reese decreases in volume fraction of the enthesis region as a function of age. [7]

Several different SEM samples were created from where the patellar tendon joined the tibia. In addition, one SEM sample was prepared from where the patellar tendon connected to the Patella. However, the sampling size is very small because all of these samples came from one knee joint of one lamb. Electron Microscopy studies require large amounts of skilled labor and expensive equipment time that make sample sets of 10 to 100 specimens' non practical. In addition, it is desired that as few animals as possible be sacrificed. In view of these external factors that limit sample size, it would be of great academic benefit if a quantitative metric could be devised that would convert the unique features of each histological image into a common language which would facilitate the comparison of work between different researchers. In addition, a quantative metric of the enthesis region would be useful in determining how the enthesis region after injury compares with natural enthesis.

7. References

[1] Landis, William, Mary Paine, and Melvin Glimcher. "Electron Microscopic Observation of Bone TIssue Prepared Anhydrously in Oranganic Solvents."*Jornal Of Ultrastructure Research*. 59.1 (1977):

[2] Smith, R.K. . "Physiology of Tendon and Ligament." Ithaca, New York: International Veterinary Information Service, 2005. 1903.1205. Web. 1 Apr. 2012. <http://www.igloo.lv/horses/harm/physiology_of_tendon.html>.

[3] "Patellar Tendon Tear." *OrthoInfo*. American Academy of Orthopedic Surgeons, 2009. Web. 25 Mar 2012. <http://orthoinfo.aaos.org/topic.cfm?topic=a00512>.

[4] "Rotator Cuff Injury ." *Health Information-Mayo Clinic*. Mayo Clinic, Aug. 21, 2010. Web. 25 Mar 2012. <http://www.mayoclinic.com/health/rotator-cuff-injury/DS00192>.

[5] Doschank, M.R., and R.F. Zernicke. "Structure, Function, and Adaptation of Bone-Tendon and Bone-Ligament Complexes." *journal of Musculoskeletal Neuronal interactions*. 5.1 (2005): 35-40. Print.

[6] **SL** Shearn, J.T, K.R.C Kinneberg, N.A. Dyment, M.T. Galloway, K. Kenter, C. Wylie, and D.I Butler. "Tendon Tissue Engineering: Progress, Challenges and translation to the clinic." *Journal of Musculoskeletal Neuronal interactions*. 11.2 (2011): 163-173. Web. 26 Mar. 2012. <http://www.igert.org/system/content_item_assets/files/1053/10SHEARN.pdf?1321629403>.

[7] Reese, Willie Mae. "The Natural Interface between Bone and Tendon: SEM Observations in an Ovine Model." Thesis. Massachusetts Institute of Technology, 2010. Print.

[8] "Structure and Function of Ligaments and Tendons."*BME/ME 456 Biomechanics*. University of Michigan , n.d. Web. 31 Mar 2012. <http://www.engin.umich.edu/class/bme456/ligten/ligten.htm>.

[9] Fratzl, Peter. "Cellulose and Collagen: From Fibres to Tissue ."*Current Opinion in Colloid and Interface Science*. 8. (2003): 32-39. Print.

[10] Ovalle, William, and Patrick Nahirney. *Netter's Essential Histology*. Philadelphia: Eleseveir, 2008. 51-71. Print.

[11] Glimcher, Melvin. "Bone: Nature of the Calcium Phosphate Crystals and Cellular, Structural, and Physical Chemical Mechanisms in Their Formation." *Reviews in Mineralogy and Geochemsitry*. 64. (2006): 223-282. Print. <http://rimg.geoscienceworld.org/content/64/1/223.full.pdf html>.

[12] Hobbs, Linn, Hao Wang, Alexandra Porter, Mark Walton, and Nicholas Cotton. "Microsture and Ultrastructure of Healing Tendon-Bone Interface." Presentation Slides

[13] . "Introduction: Bone Structure and Composition." . N.p., n.d. Web. 5 May 2012. <http://www.eng.tau.ac.il/~gefen/BB-Lec1.PDF>

[14] Landis, William, and Melvin Glimcher. "Electron Diffraction and Electron Probe Microanalysis of the Mineral Phase of Bone Tissue Prepared by Anhydrous Techniques ." *Journal of Ultrastructure Research*. 63.2 (1978): 188-223. Web. 20 May. 2012. <http://www.sciencedirect.com/science/article/pii/S0022532078800744>.

[15] Werstenfeld, Louis, Thomas Wronski, Jeffery Hollinger, and Thomas Einhorn. "Application of Histomorphometric Methods to the Study of Bone Repair." *Journal of Bone and Mineral Research*.

[X16] N.p., n.d. Web. 2 Mar 2012. <ece480.cankaya.edu.tr/uploads/files/7_SEM.pdf>.

[17] Sarig, Sara. "Aspartic Acid nucleates the apatite crystallites of bone: a hypothesis." *Bone*. 35. (2004): 108-113. Print.

[18] Galler, David. "Adventures in Scaning Electron Microsocpy." *Slides from SEM Training on 1/21/12*. Print

[19] Goodge, John. "Back Scattered Electron Detector."*Integrating Research and Euducation*. Carleton College n.d. Web. 2 Mar

[20] Henry, Darrell. "Generation of Characteristic X-Rays."*Integrating Research and Education*. Carleton College, n.d. Web. 8 Mar 2012. <http://serc.carleton.edu/research_education/geochemsheets/xrays.html>.

[21] T.Boonrungisman, Elgentleman, R.Carzoiniga, N.DEvans, D.W. McComb, A. Porter, and S. Stevens. "The Role of Intracellular Calcium Phosphate in Osteoblast-Mediated Bone Apatite Formation" Submitted to Proceedings of the National Academy of Sciences. (2012).

Appendix

Scanning Electron Microscopy

. The results published in **Section 4: Results**, come in either the form of images taken by the SEM or in X-Ray dispersive maps that measure atomic distribution of those images. Consequently, a fundamental understanding of how the SEM generates these images and what the images represent is essential to the comprehension of this work. Therefore, this section serves to provide an overview of the fundamental operating principles of Scanning Electron Microscopy for those unfamiliar or unaccustomed to the technique. Naturally, this section is not intended to be a complete theoretical overview or operation guide to SEM histology; however, this section does describe the aspects of SEM histology that are relevant to **Section 4: Results.**

The process of bone mineralization is frequently characterized through samples that were stained with specific organic or inorganic compounds that impart color or contrast to certain types of tissue. These histological stains facilitate the study of biological materials through the transmission light microscopy of thin cross sections.[15] However, scanning electron microscopy (SEM) offer several advantages over traditional histology studies. SEM can resolve features that are a fraction of a micrometer and can magnify images tens to thousands of times, a resolution enhancement that is key to the study of the early stages of bone mineralization. [16] Transmission electron microscopy (TEM), which is the direct analogue of the histological studies described above, can provide further resolution enhancements. The operating principles of TEM are not described below because TEM studies were not carried out in this work. SEM and Tem can provide information about crystalline structure. Energy Dispersive X-Ray Analysis

(XEDS)can be used in conjunction with either SEM or TEM and provides information about atomic composition. The combination of the two techniques described above allows   hydroxyapatite, which is the major inorganic component of bone mineralization, can be indentified based on its calculated atomic ratio between calcium and phosphorus.
[12,14, 15]

Whereas light microscopes use light to generate images, an SEM uses an electron beam. The surface of the sample must be slightly conductive in order to prevent the electron beam from allowing charge to build up in the sample. In naturally non-conductive samples, such as the bone- tendon interfaces studied in this work, a conductive coating is applied to an SEM sample. In this work, the SEM samples were coated with carbon instead of the more typical gold-platinum combination because the high atomic number of the gold would obscure and dilute the back-scatter electrons used to image the bone-tendon interface being studied in this work.[18,17] The electron beam generates secondary electrons, back-scatter electrons, and characteristic X-rays along with other sources of energy irrelevant to this work.[18]

Most SEM images are produced in secondary electron mode, so named because the SEM detectors are configured to measure the low energy secondary electrons generated through inelastic collisions between the beam electrons and the electrons of the sample. The roughness of the sample surface will affect the number of secondary electrons that reach the SEM secondary detector at any given point; therefore, the contrast of the final SEM image in secondary mode represents surface morphology.[18] For this work, SEM secondary mode is of rather limited use because the topology of the samples reflects sample preparation and not of the underlying biological mechanisms. In

the present case the sample surfaces are deliberately polished as part of sample preparation.

In the operating mode typically referred to as back-scatter mode, the SEM detectors are configured to measure measures back-scattered electrons, which are scattered from the sample surface along the original electron trajectory. These back scattered electrons are created by elastic collisions between the electrons and the atoms of the sample surface.[19] By definition, these elastic collisions mostly preserve the original kinetic energy of the electron, although the direction of travel changes. [19]The probability of an elastic collision occurring increases greatly with increasing atomic mass due to the atom's greater cross sectional area for scattering. [18] In the final gray-scale SEM image, regions containing lighter atomic elements appear darker than the regions containing heavier atomic elements. The SEM back-scatter mode does not determine atomic composition as brightness intensities are relative and dependent upon experimental setup. However, SEM back scatter mode is extremely useful in showing the spatial segregation of different phases of the material. In the case of bone mineralization, the inorganic phase of bone ,which contains mostly calcium and phosphorus ,will appear white whereas the tendon and the fibrocartilage region are composed mostly of carbon, hydrogen, and oxygen will appear a dark gray. The visual differentiation between tendon and bone allows the very early stages of enthesis after tendon injury to be detected.[18]

While the SEM in back scatter mode allows for the study of the progression of the bone and tendon interface during enthesis, XEDS allows for the atomic composition and composition ratio of mineralized bone to be calculated. XEDS measures the characteristic energy generated when the collisions between the SEM electron beam and atomic

electrons in the sample excite atomic inner shell electrons to an outer shell orbital or to the continuum[20]

Atomic electrons are distributed in many shell and sub-shell orbitals. Each of these shells and sub-shell orbitals are at a different energy level. However, there is a limit to how many electrons each orbital can hold. Electrons seek to be at the lowest energy state possible; therefore, when the SEM excites the inner shell electrons out of their orbitals creating inner shell holes in for example the K shell, the electrons in the higher shell orbitals suck as the L shell or M shell orbitals are accelerated into the empty states and in the process the electrons give off an X-ray corresponding to the energy difference between the shells.[20] The energy and the wavelength of the X-ray produced are therefore characteristic of the atom that produced it. The energies unique to each atom have previously been tabulated; therefore, the atoms that compose the near-surface of the sample can easily be identified.[20]

Most XEDS detectors cannot distinguish between the energies produced with elements with a lower atomic number than 11. [18] This lower resolution bound means that many of the elements found in tendon such as nitrogen, carbon, oxygen and hydrogen are difficult or impossible to detect.[18] In addition, atoms might have multiple characteristic energies due to a large number of orbitals and the emission peak resolution may be insufficient to distinguish particular atoms from others.; therefore, it is likely that energy spectra of different atoms will overlap.[18] The investigator must use his or her experience and judgment in order to match the detected peaks to the known energy peaks of the elements. This overlap can lead the investigator preclude certain elements based upon the unlikely-hood of their presence in the sample. Such analyses depend upon

careful sample preparation in order to insure that the sample has not been contaminated for example by processing agents. Once the elements have been identified through XEDS, the atomic ratios of certain elements can be calculated.

Calculation of the atomic ratios of calcium and phosphorus in indentified tissue mineral phases is useful in straightening out suspected processes of the tendon-bone junction mineralization . [12] The atomic ratio of crystals is a function of both chemistry and crystal structure, and for a particular crystal mineral structure the atomic ratio is fixed. For example, hydroxyapatite ($Ca_{10}(PO_4)_{6(}OH)_2$) has a molar ratio of calcium-phosphorus of 1.62.[12] In vivo measurements of a poorly crystalline hydroxyapatite have found an atomic molar ratio between 1.45 and 1.62.[14,21] Hobbs et all determined that the nano-crystalline mineral phases in fibrocartilage were Amorphous Calcium-Phosphorus (ACP) not poorly crystalline Hydroxyapatite because the calcium phosphorus ratio did not match the reported ratio for Hydroxyapatite.[12] Enthesis stages of tissue mineralization may involve other calcium-phosphate mineral crystalline phases such as brushite ($CaHPO_4 * 2H_2O$) which has a calcium-phosphorus molar ratio of 1.2 or more poorly defined with widely ranging ratios or even completely amorphous phases before reaching a calcium-phosphorus molar ratio of 1.62.