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Evaluation of Tissue-engineered Tendon Enthesis Polymer Constructs

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Abstract

Both scientists and clinicians have proposed tissue engineering as the future of medicine. The possibilities for tissue engineering, that is, fabrication of tissues and organs in the laboratory and their translation to patients, appear to be endless, and many believe that this new approach in medicine will result in abolishing many common ailments, injuries, and congenital defects. Injuries to a tendon enthesis, the normal tissue connection between tendon and bone, are of particular concern to clinicians because of their frequency and failure to repair as a result of surgery. While these injuries may not be life threatening, they can certainly limit mobility and reduce the quality of life in those affected individuals. Fabrication of a tendon enthesis by tissue engineering would offer an alternative to the routine of surgery now performed and present potential for treatment and healing of the tissue now unavailable. In the current prospective study, polymer scaffolds created using polycaprolactone (PCL), poly-L-lactide (PLLA), or nano-polyglycolic acid (nPGA) were seeded with chondrocytes, tenocytes, and periosteum for the development of cartilage, tendon and bone, respectively, and then implanted into six athymic nude mice for a period of 10 weeks. One group of constructs (scaffolds and cells or tissue together) was tethered to the mice

in order to determine if mechanical forces improved or were required for tendon enthesis formation compared to a group of identical implanted constructs that were not tethered. Analysis by histology illustrated a noticeable increase in tissue formation around the area of anticipated enthesis in tethered constructs when compared to constructs that were not tethered. Based on these data, it is believed that mechanical tension (tethering) is required for the formation of a tendon enthesis.

Introduction

Orthopedic-related injuries in athletes and the elderly are a relatively common occurrence. In particular, injuries affecting tendons are of great concern because of the vulnerability of the tendon at its enthesis. For example, “Tennis Elbow” and “Jumpers Knee” are both enthesis-related injuries (Benjamin *et al.,* 2002). A tendon is composed of dense layers of connective tissue, principally collagen, and it serves to link separately to muscle and to bone (Nourissat *et al.,* 2010). Tendon also bridges between muscle and bone or between two bones. The enthesis is the tendon transitional structure that inserts into bone (Nourissat *et al.,* 2010). Its structure consists of an ordered tissue sequence of tendon, fibrocartilage and bone. The enthesis functions to aid movement, maintain joint stability, and dissipate forces of the skeletal system (Benjamin *et al.,* 2002; Nourissat *et al.,* 2010).

Injuries at the location of an enthesis are often detrimental because they can compound damage by destabilizing joints and altering the natural range of motion of the body (Nourissat *et al.,* 2010). Furthermore, the age of a patient and the type and extent

of injury can inhibit proper repair of the damage as, for example, in rotator cuff surgeries where the success rate is only 50% because the tendon enthesis fails to heal correctly (Nourissat *et al.,* 2010).

One prospective idea is to use tissue engineering in order to re-create an enthesis construct in the laboratory and subsequently implant it into a patient to augment or replace the injured tissue. It is thought that mechanical forces applied to an engineered enthesis will help speed its development. The goal of this project is to compare polymer constructs of entheses that are untethered (having no applied forces) or tethered (having applied forces) on their implantation and development in mice used to harbor the constructs. The project in this manner will evaluate whether applied forces are more suitable to assist the formation of a model enthesis. The study will help in understanding tendon enthesis tissue engineering in order to aid in the advancement of treatments for reducing recovery time and increasing healing following tendon enthesis injury.

Materials and Methods

Polymer constructs for the project were created using polymer scaffolds and human donor cells or tissues. The polymer scaffolds consisted of polycaprolactone (PCL), poly-L-lactide (PLLA), or nano-polyglycolic acid (nPGA) (Gunze Co., Kyoto, Japan). The construct consisted of two separate scaffold components, a PCL cube 1.0 x 2.0 x 0.5 cm in its dimensions, and a core sheet of PCL/PLLA that was sandwiched between two sheets of nPGA. The combined sheet measured 2 x 1 x 0.25 cm. Each piece of

the construct was connected with Vicryl sutures (See Figures 1 and 2) (Wade *et al.,* 2014).

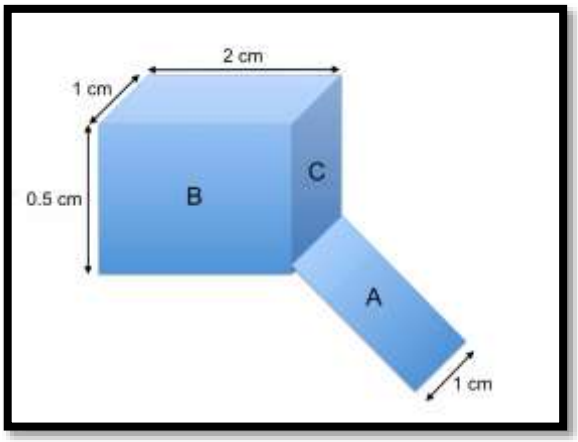

Figure 1. Experimental design of a composite tissue-engineered enthesis construct. Section A was seeded with tenocytes, section B was wrapped with periosteum, and the face of section B, which is identified as section C, was seeded with chondrocytes. The cell-seeded construct attempts to form an enthesis between Sections A and B, representing tendon and bone, respectively. (*Figure not drawn to scale).

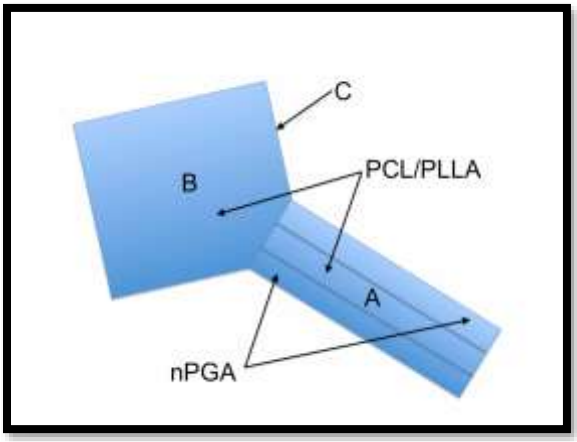

Figure 2. A cross-section of the tissue-engineered designed enthesis shows the sandwich-like structure of Section A composed of nPGA and PCL/PLLA. (Figure not drawn to scale).

Cells (cartilage cells or chondrocytes and tendon cells or tenocytes) and tissue (periosteum, composed of both chondrocytes and bone cells or osteoblasts) were obtained from the knee of a 51-year-old female donor using methods described in a previous study (Isogai *et al.,* 1999).

The enthesis was created by seeding (placing) or suturing tissue-cultured chondrocytes, tenocytes and periosteum isolated from the donor knee onto or about the various scaffolds that subsequently comprised the enthesis constructs. Cells were seeded at a density of 1×10^6 cells per scaffold and remained in culture for a period of one week in order to allow adequate time for them to attach to the scaffolds. Figure 3 shows a series of culture plates with cells and constructs being incubated in culture media.

Figure 3. A six-well plate contains cells and constructs suspended in cell media (pink solution) before being implanted in nude mice.

After a week, the constructs were implanted into 5-week-old athymic (nude) mice (Harlan-Sprague-Dawley Laboratories, Indianapolis, IN) for a period of 10 weeks. One group of constructs was sutured (tethered) with Vicryl thread to muscle or bone in the mice and mechanical forces were presumed to act on them as the mice walked, ran or went about normal activity in their cages. A second group of constructs was implanted but not sutured (untethered) in other mice. After the elapsed 10-week time frame of implantation, mice were sacrificed using CO_2 asphyxiation and the constructs were surgically retrieved. The animals used in this study were cared for and maintained in the Comparative Medicine Unit of the Northeast Ohio Medical University (NEOMED), Rootstown, OH. The work strictly followed policies described by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC) at NEOMED. A nude mouse and a retrieved tissue-engineered construct developed after 10 weeks of implantation are shown in Figure 4.

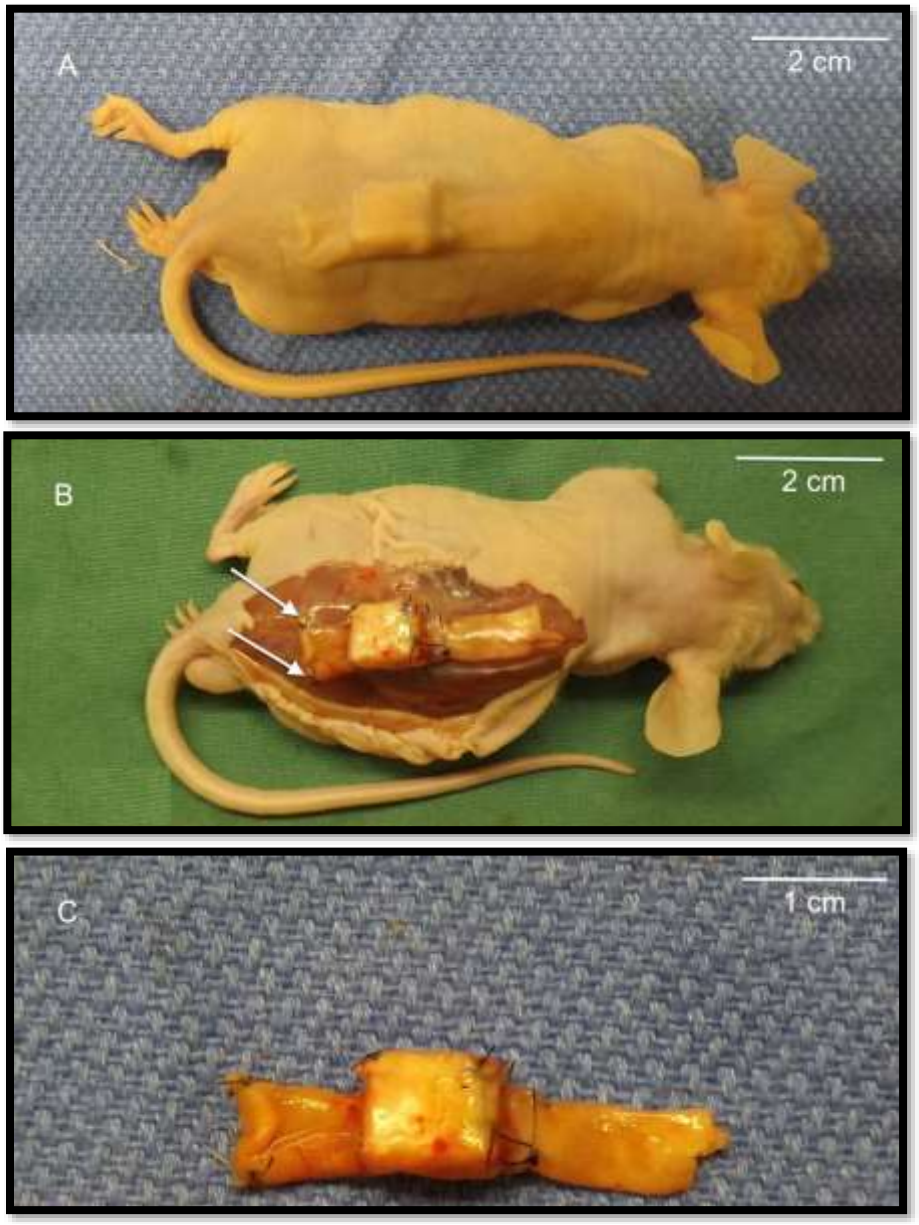

Figure 4. A sacrificed nude mouse showing (a) an enthesis construct implanted and thethered on the back of the animal and (b) the appearance of the construct as the skin is opened to retrieve the specimen. Sutures connecting the sections of the construct are visible (arrows). (c) There is notable tissue growth present on the construct sections after the construct has been removed from the mouse. Sections A (tenocytes), B (periodsteum), and C (chondrocytes) are labeled as such.

Specimens were removed from the mice and fixed in 10% neutral buffered formalin for a minimum of 24 hours. Samples were then washed three times for 15 minutes each in distilled H_2O (dH_2O), washed three times for 15 minutes each in 70% ethanol (EtOH), processed in a Leica model ASP300S tissue processor (Leica, Buffalo Grove, IL), and embedded in paraffin wax (Richard-Allan Scientific, Kalamazoo, MI) using a Leica model EG1150H tissue embedder.

The paraffin-embedded samples are normally kept at -20°C for long-term storage. However, the samples were placed at -80°C prior to sectioning for histology, to help harden the block and aid in sectioning. Embedded samples were sectioned at 5-6 µm thicknesses using a Leica model RM2255 microtome and a tungsten carbide blade. Sections were next floated on a Leica model HI1210 water bath for collection and mounting. The sections were mounted on Superfrost Excell slides (Thermo Scientific, Portsmouth, NH) and allowed to dry overnight in a vertical position for complete water removal.

Sections on slides were subsequently heated at 60ºC in an oven for one hour. The slides were then de-paraffinized in three washes of xylene for 5 minutes each and rinsed and rehydrated for 2 minutes each in EtOH of decreasing concentrations of 100%, 95%, and 70%. The slides were next stained with toludine blue (FisherBiotech, Logan, UT) for 1 minute and then rinsed with 2 washes of dH_2O to clear excess stain. The slides were then dehydrated for 2 minutes in EtOH at concentrations of 70%, 95%, and 100%, and then they were washed three times in xylene for 5 minutes each.

One or two drops of di-n-butyl phthalate in xylene (DPX) (Sigma Life Science, St. Louis, MO), were applied as a mounting agent, and the slides were cover-slipped. The

slides were allowed to dry in a hood for 24 to 36 hours in order to remove the xylene solvent.

Toluidine blue staining was used to observe and identify cell nuclei and general morphology of the tissues comprising the enthesis constructs. Picrosirius red staining (Pfaltz & Bauer, Inc., Waterbury, CT) was used for the observation of collagen fiber structure. Staining procedures were obtained from protocols adapted for use by the Landis Laboratory.

Cover-slipped slides were cleaned with a razorblade and 70% EtOH. They were then examined by light microscopy using an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan). Section images were captured using MicroSuite Basic Edition version 2.5 (Olympus, Tokyo, Japan) and subsequently compiled using Microsoft ICE version 1.4.4 (Microsoft Corporation, Redmond, WA). Montages of several individual images recorded under light microscopy were subsequently stitched together with Microsoft Image Composite Editor (ICE®).

Results

Tethered constructs were compared with constructs that were not tethered after retrieval from mice following 10 weeks of implantation. Untethered and tethered constructs were stained with toluidine blue and are shown in Figures 5 and 6, respectively.

Visual comparison with toluidine blue staining demonstrates overall morphology of representative tethered and untethered constructs. The area of anticipated enthesis formation is indicated by the box outline for each construct. Each image shown is a

montage of several individual images recorded under light microscopy and stitched together as noted in Materials and Methods above. The labels 785L and 786 refer to identification numbers for the individual untethered and tethered specimen constructs, respectively.

In Figure 5, an untethered construct, there is a noticeable lack of tissue formation at the area of anticipated enthesis. Additionally, it appears that the PCL/PLLA and nPGA sheet folded in on itself during the incubation period. This factor most likely resulted from the absence of tethering forces to hold it in place.

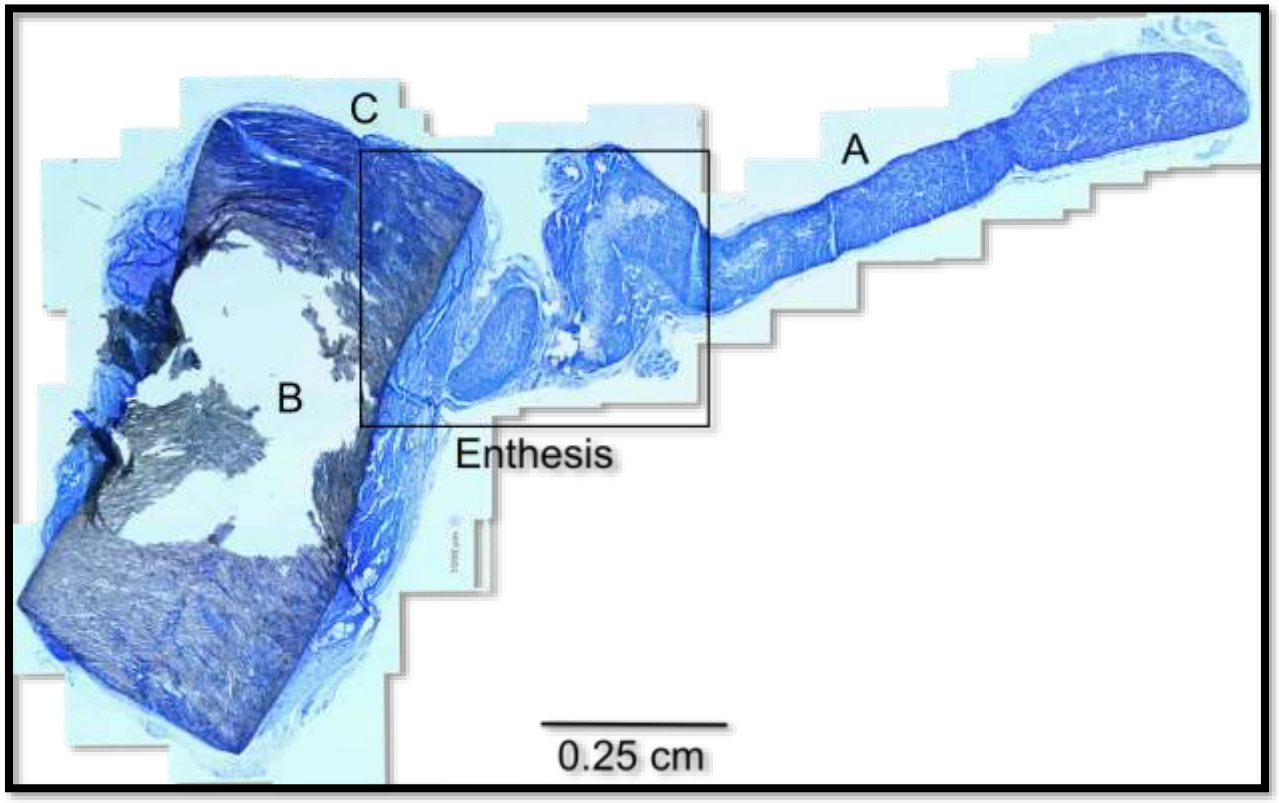

Figure 5. Specimen No. 785L — An untethered construct stained with toluidine blue. The areas A, B, and C refer to tenocyte, periosteum, and chondrocyte seeding, respectively, and as shown in Figure 1 above. The boxed area indicates the site of the anticipated enthesis. The area inside the box appears to have little tissue formation.

Additionally, the tenocyte-seeded sheet (A) appears to have folded in upon itself in the absence of any tethering.

In Figure 6, a tethered construct, there is a definite increase in tissue formation when compared to the untethered construct in Figure 5. There is no folding of the PCL/PLLA and nPGA sheet, and the outlined tissue in the enthesis area is much larger overall when compared to Figure 5.

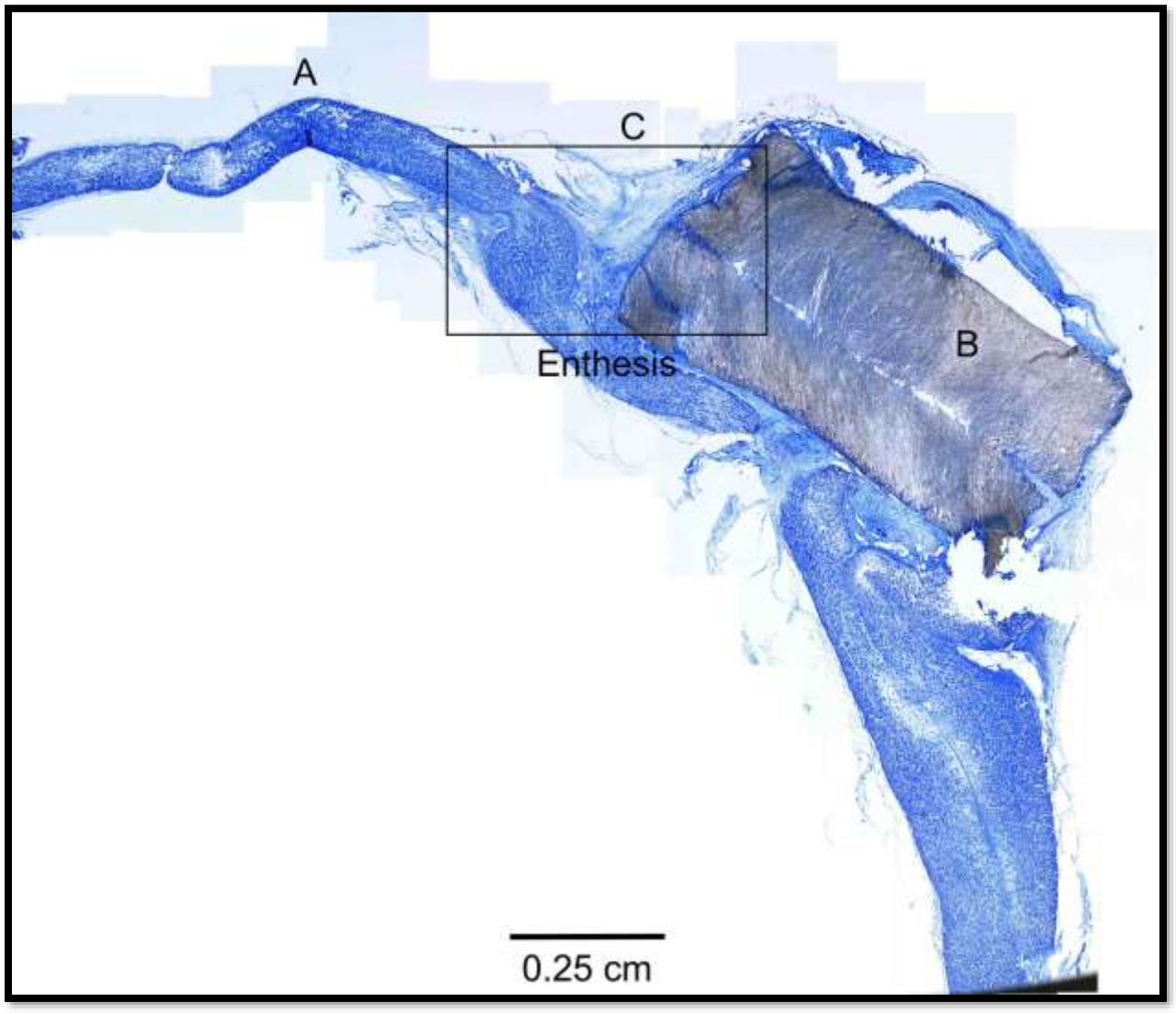

Figure 6. Specimen No. 786L — A tethered construct stained with toluidine blue that shows the area around the anticipated site of enthesis. The areas A, B, and C refer to

tenocyte, periosteum, and chondrocyte seeding, respectively. There is a noticeable increase in tissue formation inside the boxed area of anticipated enthesis. Additionally, there is no folding of the tenocyte-seeded sheet (A).

Picrosirius red staining is used to identify the presence of collagen fibers in histological sections. When viewed under light microscopy, collagen fibers appear bright red over a yellow background. When viewed under polarized light, the presence of thicker collagen fibers appears red-orange, while thinner fibers appear green in color because of differences in birefringence as a function of fiber diameter. Figures 7-10 indicate overall morphology of untethered and tethered constructs when viewed under non-polarized and polarized light. The boxed area indicates the site of anticipated enthesis.

In Figure 7, an unpolarized untethered construct, there is little to no tissue formation present around the area of anticipated enthesis. Based on the amount of Picrosirius red saturation present, it is possible that there is a high percentage of collagen in the construct. However, it is probable that certain polymers have high affinities for stains in a similar way that proteins and tissues react with chemical staining. Thus, an estimate of the collagen content alone is difficult to make.

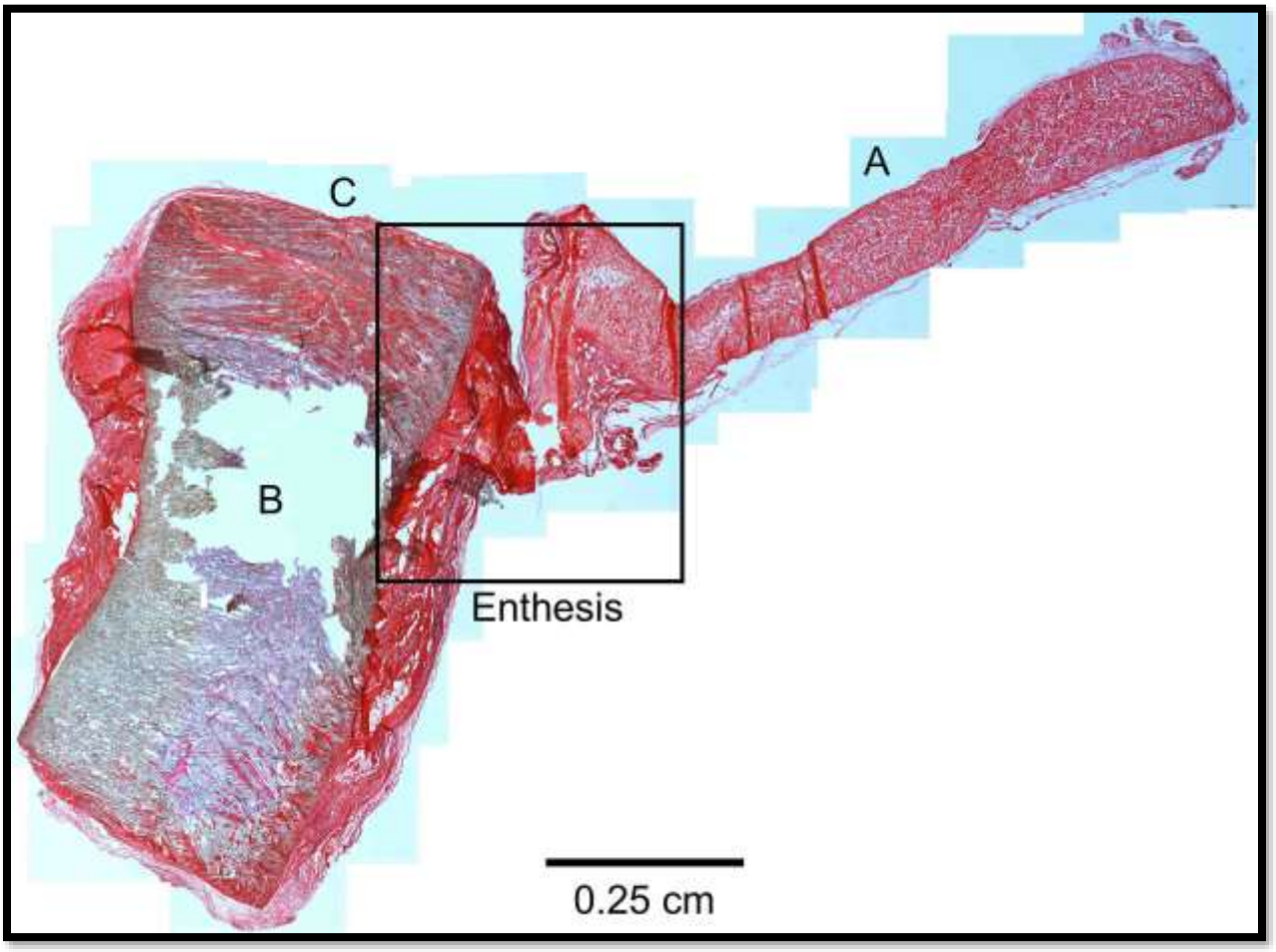

Figure 7. Specimen No. 785L — An untethered construct stained with Safranin-O red as viewed under non-polarized light conditions. The areas A, B, and C refer to tenocyte, periosteum, and chondrocyte wrapping or seeding, respectively. There is very little tissue formation present in the boxed area of anticipated enthesis.

In Figure 8, a polarized untethered construct, there is very little tissue formation present around the area of anticipated enthesis. The red-orange fibers would seem to indicate the presence of collagen at the site of the anticipated enthesis. Other areas of the construct are darker in appearance and may indicate regions with little collagen that

showed an affinity for the stain and did not react under polarized light in this plane of view.

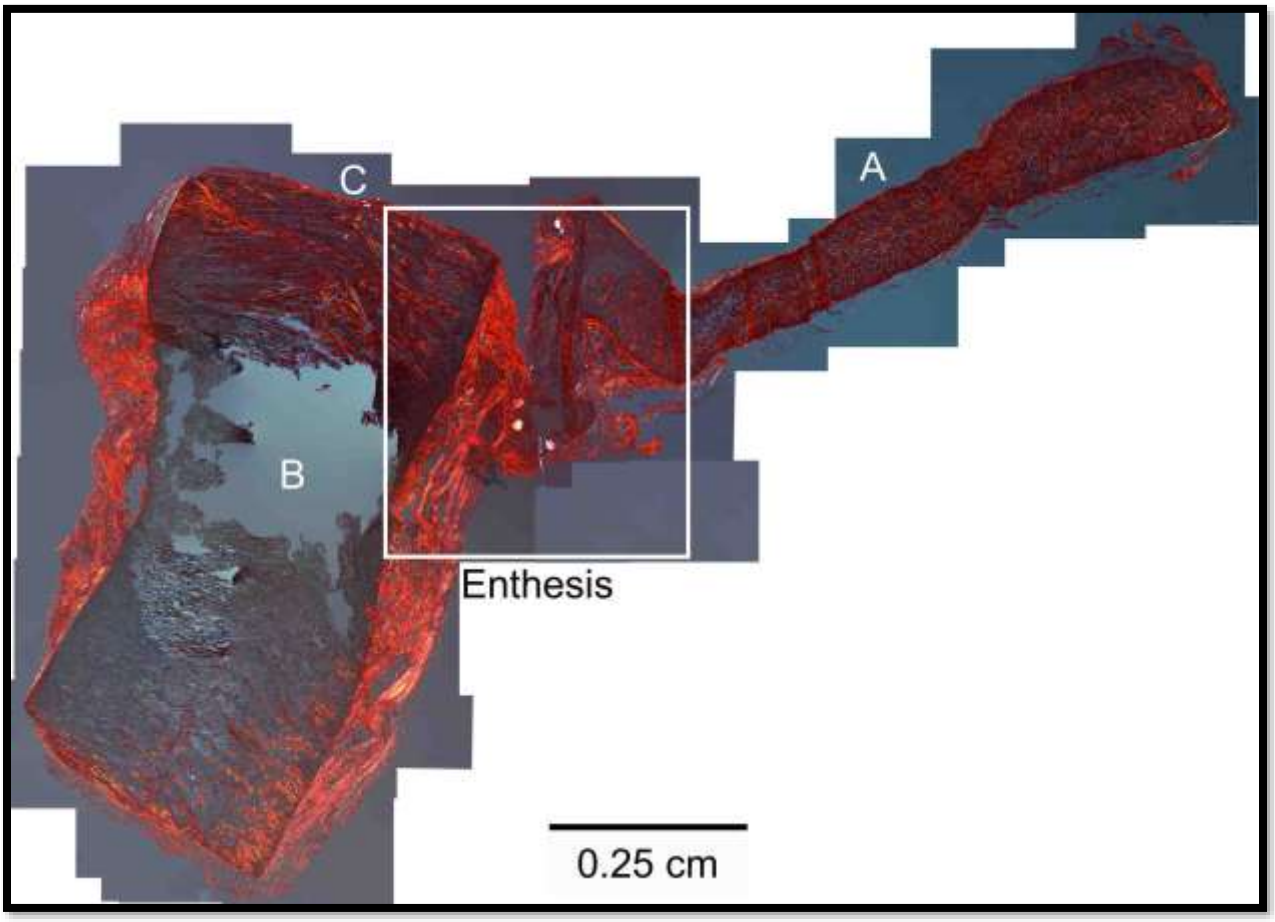

Figure 8. Specimen No. 785L — An untethered construct stained with Safranin-O red as viewed under polarized light conditions. The areas A, B, and C refer to tenocyte, periosteum, and chondrocyte wrapping or seeding, respectively. There is very little visible tissue formation present at the site of anticipated enthesis. The red-orange fibers indicate the presence of collagen fibers. Dark areas may indicate regions that acquired the stain, but did not contain collagen, or regions that are not reacting in this plane of view.

In Figure 9, an unpolarized tethered construct, there is very little tissue formation present at the site of enthesis when compared to Figure 6. It is possible that the tissue present was lost during the processing steps or continued sectioning on the microtome reached a depth on the construct where the tissue had not developed.

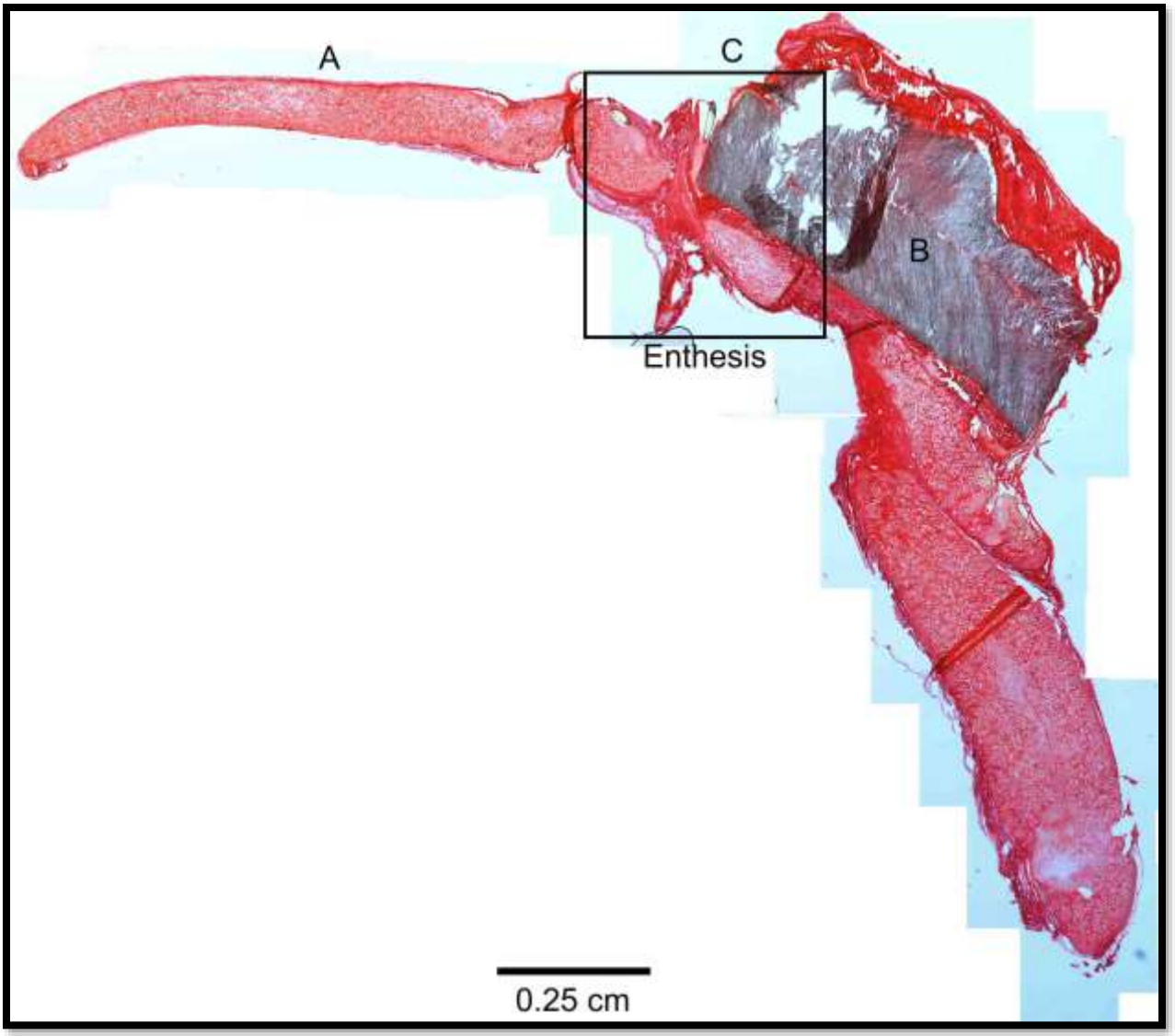

Figure 9. Specimen No. 786 — A tethered construct stained with Safranin-O red as viewed under non-polarized light conditions. The areas A, B, and C refer to tenocyte, periosteum, and chondrocyte wrapping or seeding, respectively. There is very little tissue present in the area of the anticipated enthesis.

In Figure 10, a polarized tethered construct, a much clearer view of the construct is visible. There are very few polarized fibers present around the indicated area of enthesis and additionally along the tenocyte-seeded sheet (A). The bright yellow orange region appears to contain a high level of collagen formation compared to other areas on the construct. This region comprises the surface of the construct that would be parallel to the skin of the mouse. Additionally, when viewed under polarized light, it appears the right sheet (A) has been folded over. It is possible that the construct was not sutured with enough tension during the tethering process. This aspect is only clearly visible when stained with Picrosirus Red and viewed under polarized light conditions.

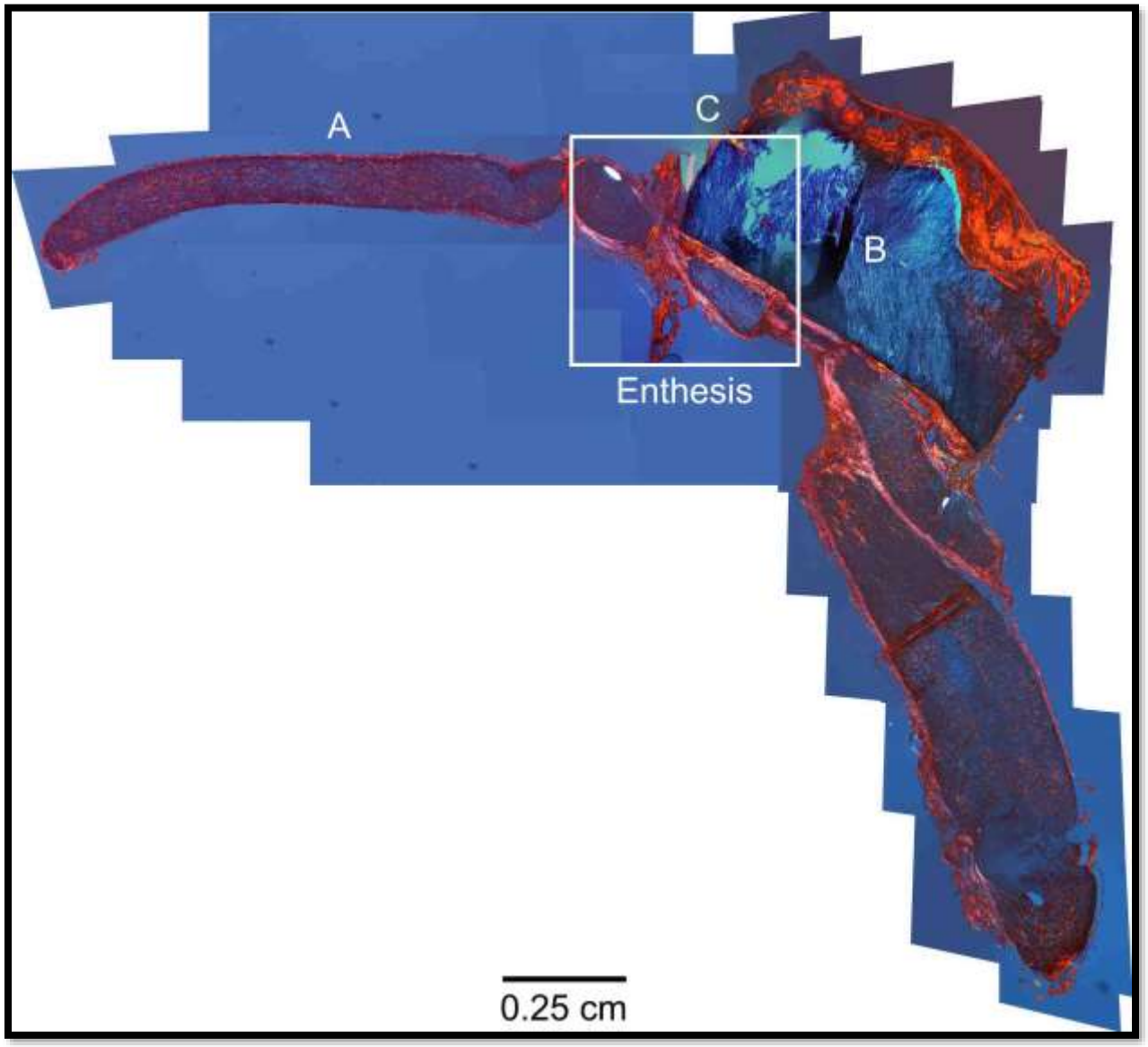

Figure 10. Specimen No. 786 — A tethered construct stained with Safranin-O red as viewed under polarized light conditions. The areas A, B, and C refer to tenocyte, periosteum, and chondrocyte wrapping or seeding, respectively. There are very few polarized collagen fibers located in the area of enthesis. Most of the visible polarization is occurring in the yellow-orange region.

Discussion

These tissue-engineered constructs are relatively difficult to section intact. Of the samples sectioned in the course of this study, the best examples have been provided in the Figures. Based on direct comparisons, there is a definitive indication that tissue formation increased in the tethered compared untethered regions of the respective enthesis. As previously mentioned, there is certain literature evidence to suggest that the application of mechanical force (tension) is required for the proper development of a tendon enthesis. The study conducted here is inadequate at this time to contribute information in this context. Greater numbers of these implanted specimens and more detailed analyses are needed to provide conclusive data.

Further studies will require the use of immunohistochemical staining in order to confirm the presence of possible proteins and other molecules that have developed with time in the tissue-engineered constructs. These would include but are not limited to types I, II, and III collagen, decorin, and aggrecan, for example. Type I collagen is the most common form of fibrous connective tissue that forms the tendon connection between muscle and bone. However, during the transition from tendon to bone, type II collagen is predominantly found (Thomopoulos *et al.,* 2007). Type III collagen may be complementary to types I and II collagen. Decorin is a proteoglycan that aids in extra-cellular matrix organization and assembly (Reed and Iozzo, 2002). Aggrecan is a proteoglycan component of the extracellular matrix that provides resistance to compression forces (Hardingham *et al.,* 1994; Kiani *et al.,* 2002).

Additionally, it would be beneficial to examine the expression of genes that might characterize the developing tissue-engineered enthesis. Such an examination would

involve the use of reverse transcription-polymerase chain reaction (RT-PCR) analysis. Separate specimens implanted and retrieved from nude mice would be required in this case as they would be placed in a buffer preservative for RT-PCR studies and not in NBF, which is useful for histology and immunohistochemistry.

Finally, it is possible that increasing the time of implantation *in vivo* in nude mice will produce results of viable constructs that are more mature in their development. For example, Thomopoulos *et al.* found that enthesis samples harvested at later periods produced more conclusive results (Thomopoulos *et al.,* 2007). It is believed that the implantation time period for this study could be increased from 10 to 15 or 20 weeks in order to allow more time for greater enthesis tissue formation.

Conclusions

The samples in this study are too few in number to draw a concrete conclusion about mechanical forces and enthesis formation. These are only 10 week samples and therefore not enough time to form a well developed enthesis. There are very few literature studies that are related to the design of a tissue-engineered enthesis as described for this project. One investigation published by Thomopoulos *et al.* presented results in which a tendon enthesis was developed in a mouse model (Thomopoulos *et al.,* 2007). Tension was either applied or absent in the study so that its effects on enthesis formation could be compared. Decreased tension was reported to prevent bone mineralization of the tendon enthesis in this model. When the model with applied tension was examined histologically, the enthesis was determined to have robust columnar fibrochondrocytes that were perpendicular in relation to the bone in the model.

The model lacking tension (untethered) had abnormally hypertrophic chondrocytes and poor cellular alignment. The study here has not examined cell structural features of the tissue-engineered enthesis constructs so no details are comparable to the data of Thomopoulos *et al.* (2007). Additional work in which other constructs will be retrieved and documented following implantation in nude mice should provide supplemental information that can be used to gain further insight into such models and which can be compared to published studies.

Acknowledgments

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