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MECHANOSTIMULATION OF A TENDON TISSUE ENGINEERED HUMAN UMBILICAL VEIN

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MECHANOSTIMULATION OF A TENDON TISSUE ENGINEERED HUMAN UMBILICAL VEIN

A THESIS APPROVED FOR THE
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BY

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For my parents, Holly and Ben Coffey, for all of their love, support, and great wine

Without whom none of my success would be possible

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Abstract

Tissue engineering has become a very popular method when combined with bioreactors for treating disorders of the musculoskeletal system. A scaffold that is biocompatible and bio-absorbable is seeded with the cells then applied a mechanical or chemical stimuli and growth factors that differentiate the cells on the construct into the accurate cell lineage. Tendon tissue engineering is the bridge between engineering, biology, and medicine that has the potential to create a biological substitute for tendon ruptures and restore them close to their full function and capabilities. The need for functional tendons for reconstructive surgeries is clear. Currently, autographs and allographs are a commonly used transplant. However, there are also issues with these solutions such as availability or donor site morbidity. Therefore, a tissue-engineered construct would be a welcome and more beneficial solution for severe tendon injuries. Our hypothesis is that due to the unique regenerative capacity of the human umbilical vein, functional tendons can be developed. The end-point of this project is the development of bio-functional tendons using this novel bio-matrix, to a point that animal studies can be conducted. The ultimate goal is the development of a prosthetic graft with success rates exceeding that of the current benchmark 'autologous tissue'.

This study investigated three main parameters of stimulating a human umbilical vein (HUV) construct seeded with mesenchymal stem cells (MSCs): frequency, duration, and force. The effects employed were varying the duration (0.5, 1, and 2 hours/day) and the frequency (0.5, 1, 2, cycles/min) of culture in the bioreactor for up to 7 and 14 days with a constant strain rate of 2%. It was found

that the highest proliferation rate was observed for the 14 day culture point with the extended (2 hours/day at 1 cycle/min) with a 117% increase compared to the 7 day extended group. Extracellular matrix fiber alignment and quality along with cellular penetration increased significantly with the extended duration and correlates with the upregulation of collagen type I (COL I). Although previous studies indicate that at earlier time points slower and shorter were best for construct development, at the 14 day time point longer durations were beneficial.

Introduction

Tissue engineering is a developing field that links biologics with engineering to promote tissue regeneration. Key components for successful tissue engineering is to have an appropriate cell or stem cell source typically derived from the patient via a cell biopsy. A scaffold that is biocompatible and bio-absorbable is seeded with the cells then applied a mechanical or chemical stimuli and growth factors that differentiate the cells on the construct into the accurate cell lineage. When implanted in a patient, the tissue construct is expected to influence extracellular matrix organization and construct degradation, limit any immune reactions, all while the native tissue remodels and regenerates. Tissue engineering has become a very popular method when combined with bioreactors for treating disorders of the musculoskeletal system.

Tendon Tissue Engineering

Tendons transition muscle to bone, a critical function of the musculoskeletal system [1]. Those affected by tendon injuries range from athletes who strain themselves to the elderly whose tendons degenerate. Mild aches and pains associate with less severe symptoms of tendon disorders. For more severe injuries, treatments range from electrical stimulation or orthopedic surgery in combination with tissue grafts to augment the injuries and promote tissue growth. Tendon injuries especially are difficult to treat surgically which is why research has grown considerably in the role of tissue engineering as an alternative repair method with increasingly innovative techniques. Tendon tissue engineering is the bridge between engineering, biology, and

medicine that has the potential to create a biological substitute for tendon ruptures and restore them close to their full function and capabilities.

Tendons provide the necessary mechanical properties to affectively transmit high loads and stress between the bone and muscle. They allow for a certain amount of elasticity, but also the ability to recover their original shapes during various stress conditions [1]. Tendons are mainly made up of collagen type I fibers with small amounts of collagen type III and elastin**.** These tissues consist almost entirely of collagen fibers, arranged in parallel bundles containing fibroblasts allowing for the tendon to withstand high uniaxial tensile loads as seen in Figure 1 [2]–[4].

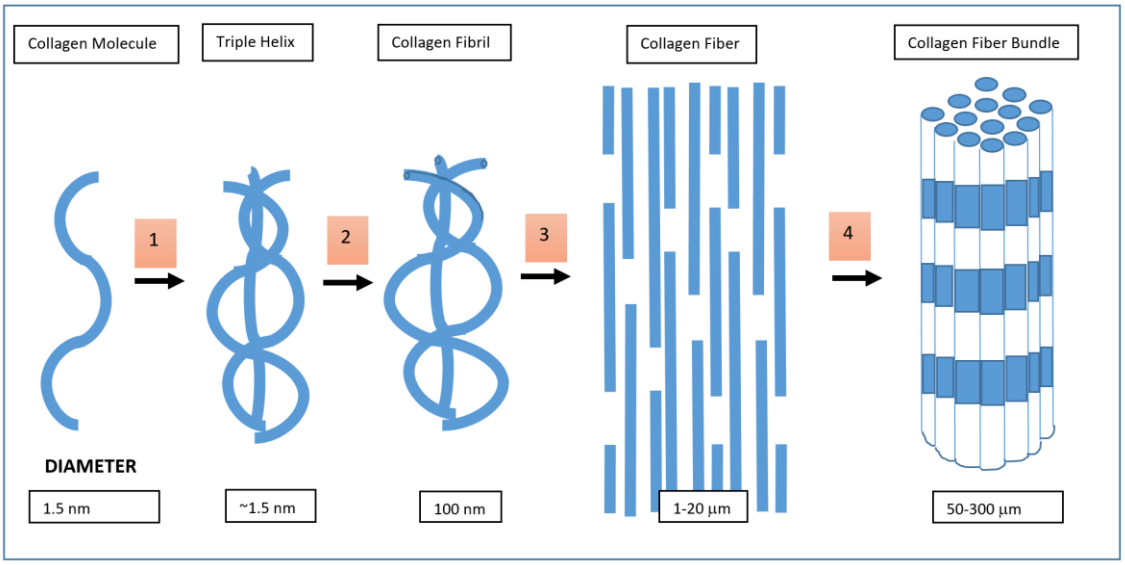

Figure 1: A collagen protein molecule is arranged (1) into a triple helix tropocollagen. The collagen fibril is made up of glycosylated propeptides (2) called procollagen in which the ends of the triple helix are trimmed (3) so that collagen fibrils can be stacked form a collagen fiber. Multiple collagen fibers (4) become a collagen fiber bundle that make up most of the tendon.

The collagen fiber bundles have a noticeable crimp pattern to them that tend to dampen transmitted forces going across them [5]. These fibroblasts, also known as tenocytes in the tendon, are the cells responsible for the production of the nonfibrous component of the matrix: the precursors of collagen, elastic fibers, and the extracellular matrix (ECM) [2]. Injuries in a tendon are influenced by the amount of force produced by the contraction of the muscle to which the tendon is attached and the cross-sectional area of the tendon in relation to that of its muscle [2]. Human tendons can undergo a strain of 0.10 and elastic stiffness of 700 N/mm^2 [3]. Tendons, depending on where they are in the body, are able to withstand tensile strengths ranging from 30-200 MPa, see Table 1 for more details. Research is focusing on creating tendon grafts as close to the native tendon in a specific anatomic location as possible with the condition that these grafts will be able to augment and reattach the ends of severed tendons. A variety of cell sources have been utilized in these research studies.

	Ultimate Tensile Strength (MPa)	**Young's Modulus (MPa)**	**Strain at Maximum Stress (%)**
Achilles Tendon	278	35	-15
Patellar Tendon	53.6 ± 10.0	504 ± 222	15 ± 6
Rotator Cuff Tendon (Anterior)	32.5 ± 5.3	421 ± 212	N/A
Rotator Cuff Tendon (Posterior)	4.1 ± 1.3	40 ± 15	N/A

Table 1: Typical Mechanical Properties of Tendons within the Human Body.[6]–[9]

Tendon Cell Types and Sources

Cells are the producers, modifiers, and recruiters that promote tendon tissue regeneration: responding to physical and chemical stimuli. There are several cell sources used in tendon tissue engineering including bone marrow mesenchymal stem cells (MSCs), adipose derived stem cells (ADSC), and adult tenocytes. Tenocytes and tenoblasts are the primary cell type found in tendon ECM. Tendon ECM is made up of several molecules such as collagens, elastin, proteoglycans, and glycoproteins. Studies increasingly stress the translation of physical stimuli to chemical stimuli, mechanotransduction, as a component to the differentiation of stem cells [10]. For example, tenocytes utilize gap junctions in conjunction with tendon fascicles to communicate responses between cells when undergoing tensile forces [11]. These gap junctions transport ions or other small molecules between tenocytes in response to these loads, coordinating the cell signaling cascade. The forces are potentially sensed by integrin receptors, focal adhesions, stretch activated ion channels, and the cytoskeleton [12]. Tenocytes express a variety of connexins such as connexins 26, 32, and 43 within the gap junctions [13]. The cell signaling cascade is activated in response to these components and turns on adaptor proteins such as FAK and paxillin [14]. Downstream proteins such as extracellular signal-regulated protein kinases 2 (ERK) and c-Jun N-terminal kinases (JNK) that activate and regulate signaling pathways to include mitogen-activated protein kinase [15], Rho-dependent kinase, NF-kB, PI3K, and protein kinase C pathways. These pathways lead the signal to modify the gene transcription, differentiation, cell survival, and gene expression. Growth hormones that are secreted to enhance collagen expression include

transforming growth factor beta (TGF-B), fibroblast growth factors (FGF), and growth differentiation factors (GDF). Transcription factors encode for collagen alpha-1[1] and collagen alpha-2[1] that are involved in collagen fibrillogenesis. Therefore, strain transmitted from a surface to a tenocyte via these pathways, provide for enhanced tendon generation.

Cell conditioning usually entails mechanical stimulation regimes such as oscillatory stretch cycles, vibrations, and multidimensional forces that are intermittently applied or one continuous motion. Force and duration are parameters that researchers often vary to try and optimize tendon regeneration. Tenocytes can dedifferentiate into immature fibroblasts in absence of stretching [16]. Type and magnitude of mechanical forces on tenocytes can have varying effects on gene expression [17]. Over-stimulation or under-stimulation regimes may lead tenocytes to induce collagen damage, increased metalloproteinase levels, and apoptosis [18]. Tenocytes, when properly stimulated, will exhibit markers involved in tenogenesis such as scleraxis (SCX) and tenascin-C (TNC), a glycoprotein that is a later term differentiation marker [19]. Unlike the differentiation of MScs towards bone osteoblastic or chrondroblastic lineage, tenocytic differentiation lacks a universally accepted set of markers that can be used to monitor their differentiation. SCX and TNC are the most commonly used markers by researchers when testing for tenocytic differentiation. Tenomodulin has been used as a marker for tenocytic differentiation and helps to increase tenocyte proliferation.

Aside from cell source, cell seeding also plays an influential role in achieving uniform cell seeding [20]. Tendon derived stem cells (TDSC) under cyclic tensile stain

of 0.5 Hz frequency and 4% amplitude showed a greater upregulation of TNC and SCX [21]. Other cell types such as ADSCs cultured on an electrospun fiber scaffold and undergoing cyclic tension showed an increased concentration of GDF-5 and have effective differential capabilities towards tenocytes for tendon repair [22]–[24]. A recent study was conducted that extracted dental pulp stem cells from a tooth to determine if they could act as a sufficient cell source for tendon tissue engineering [25]. Chen et al. statically cultured dental pulp stem cells (DPSC) onto PGA fiber scaffolds. The fibers were fixed to a custom-made spring that mechanically stretched it under tension. After three days the cell's gene expression was analyzed using real-time polymerase chain reaction (RT-PCR) and were found to express differentiation markers for collagen 1 and IV, SCX, and TNC, all common markers for tendon cells. This shows a novel use of DPSC-PGA constructs. Then the constructs were *in vivo* implanted into mouse models, also demonstrating that with mechanical loading DPSCs are a new potential stem cell source for tendon tissue engineering. It has yet to be identified which cell line is the best source of stem cells for tendon tissue engineering. It is an ongoing development using different stem cell types and stimulation parameters to determine which cell has the best accessibility while easily differentiated and proliferated into tenocytes.

Scaffolds for Tendon Tissue Engineering

A wide array of scaffolds have been investigated in tendon tissue engineering strategies aiming to improve the currently utilized clinical approaches. Clinical therapies include the use of grafts in severe tendon injuries utilizing autografts.

Although autografts have very low risk of graft rejection, they also suffer from donor site morbidity [26]. Allografts have been also employed using mostly tendons acquired from cadavers. However, they suffer from limited availability and excessive cost and potential disease transmission. Xenografts are becoming a popular alternative to regenerative medicine and in the case of tendons, they are mostly of bovine or porcine origin. Unfortunately, their low cost and relative abundance is countered by the need to perform decellularization prior to their use sense they would otherwise elicit a strong immune reaction. Obviously decellularized tendons lack many of the characteristics of healthy tendons and as such they have very limited reparative capacity.

Ideal biomaterials for tendon tissue engineering will assist in cell organization, provide a tendon like scaffold, and be amenable to mechanical stimulation required for neo-tendon tissue creation. Tendon biomaterials, which can be either natural or synthetic, have to be bio-absorbable, biocompatible, and biodegradable. They should not induce a cytotoxic response or an immune reaction in the body. The rate that the material degrades has to be proportional to the rate of neo-tendon formation, encouraging cell attachment *in vitro* and *in vivo*, tenocyte proliferation, and differentiation. Many tendon scaffolds, however, are limited by their ability for nutrient and growth factor mass transport to sustain a high cell number. Nutrient diffusion such as oxygen and glucose are important factors to consider for a tendon tissue scaffold and were investigated by Issa et al.

As previously mentioned, tendon scaffolds can either be derived from natural or synthetic biomaterials. Since the dry weight of a native tendon is more than 60% type

I collagen, a natural scaffold is most likely composed of a type I collagen. Other possible natural materials that can be used as tendon scaffolds include silk, fibronin, hyaluronic acid, and chitosan. Decellularized tissue can also be used as a scaffold and commercially produced decellularized scaffolds include porcine small intestine mucosa (SIS), and equine pericardium [26]. One major disadvantage to using natural scaffolds is their significantly lower mechanical tensile strength when compared to native tendon. However, xenografts and allografts are known for being able to retain most of their strength after decellularization [27]. A novel tissue engineered tendon scaffold created from decellularized human umbilical veins has also been seen to have significantly higher mechanical properties than other conventional natural scaffolds [28]. Although, the combination of seeding adult stem cells in decellularized HUVs and culturing them in a mechanical stimulator has shown great promise, the diffusion of oxygen and glucose from the luminal side exposed to circulating media to the cultured cells in the interior of the inverted HUV generated mass transport limitations that limited cell growth especially when high cellularity was achieved [29]. This indicated that 2 weeks was the longest viable culture time for a closed HUV construct with cells seeded in the interior [29]. Without the ability to effectively push nutrients and oxygen to outlying adult stem cells, it would be difficult to maintain cultures for longer time periods necessary to achieve higher cellularity and tensile properties matching the ones of tendons.

One advantage synthetic scaffolds have over their natural counterparts is their higher mechanical properties. Most are specifically tailored to have the precise mechanical properties necessary to promote tendon regeneration. However, synthetic

scaffolds suffer from poor cell attachment and require cytotoxicity and biocompatibility studies to confirm safety. Examples of polymer biomaterials found in the literature include polyglycolic acid (PGA), polylactic acid (PLA), PCL, and poly(lactic-co-glycolic) acid (PLGA). PLGA is more commonly used as a tendon scaffold due to its controllable mechanical strength and degradation rate during the fabrication process. In one study, electrospun nano fibers of PLGA were knitted into a biomimetic surface utilizing fibrin gel to allow for cell attachment [30]. The fibrin modified scaffold was able to demonstrate an increase in cell attachment and proliferation rates.

Bioreactor Types for Tendon Tissue Engineering

Bioreactors are becoming an essential component of tendon tissue engineering by providing the controllable environment necessary to influence stem cell differentiation through the implementation of mechanical stimulation and the mass transport of nutrients [31]. Most bioreactors will have a static base on one end of the tendon construct with an actuator on the other end that will stimulate the tissue with cyclic strain. Representative bioreactors are illustrated in Figure 2 below.

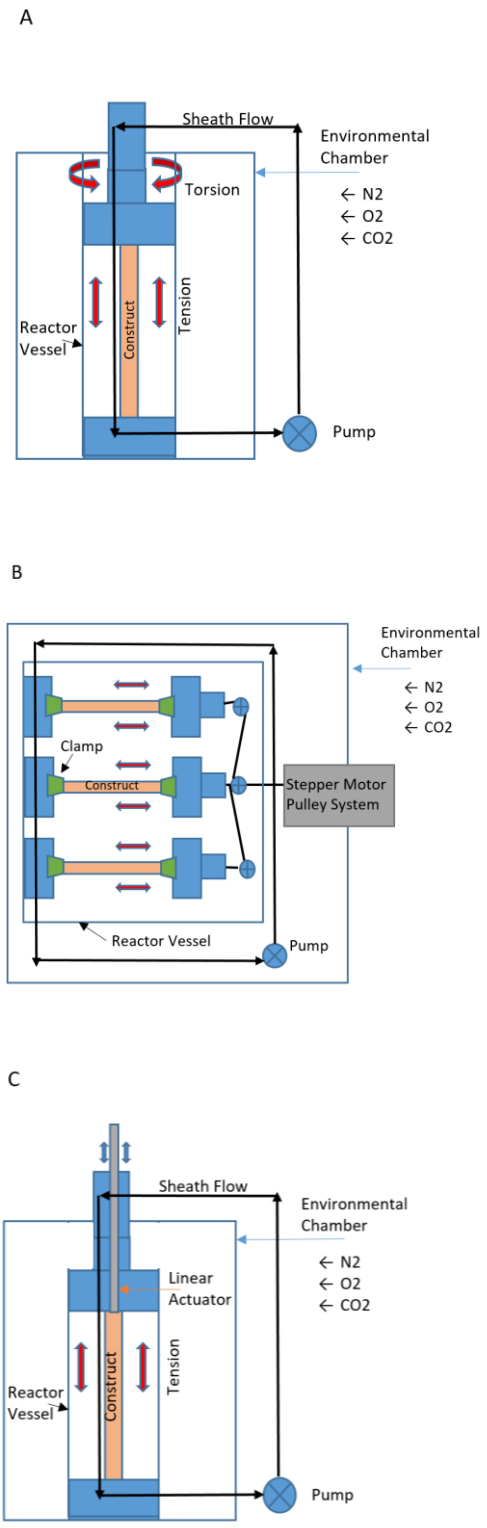

Figure 2: A) A schematic of a biaxial tensile bioreactor. B) A schematic of a uniaxial bioreactor stimulating multiple tendon scaffolds with a step motor utilizing a pulley system. C) A schematic of a uniaxial bioreactor using a linear step motor.

Studies show that dynamic stretching is beneficial for tendon tissue engineering when compared to static samples. Static studies typically have poor cell yield resulting from the lack of media flow and mechanical stimuli. Although commercial bioreactors are available, they can also be custom built to mimic the native tendon environment, conform to the scaffold size, and apply uniaxial tension force using pneumatic actuators, step or linear motors. One end of the scaffold can remain static while the other has loads applied via an actuator or motor as previously mentioned. The parameters applied to a construct in a bioreactor can vary widely based off of the scaffold thickness and strength as well as the attributes of the cell line. Stronger forces might snap a weaker tissue scaffold or cause cell apoptosis.

Commercially Available Bioreactors

Commercially available bioreactors include the ElectroForce reactor and the Instron LigaGen reactor that are popular bioreactors of choice for many studies, however, they are also very expensive. The ElectroForce system, originally developed by Bose, uses electromagnetic motors and is able to provide tension, compression, and pulsatile forces. The Instron LigaGen system utilizes a linear motor to apply tension to samples. The Instron LigaGen bioreactor is used in several tendon tissue engineering studies. Saber et al. seeded rabbit tenocytes onto rabbit flexor tendons in order to create additional tendon material. The constructs underwent a uniaxial strain of 1.25 N over 5 days at 1 cycle/min in 1 hour periods. It was found that the constructs that underwent cyclic loading with the tenocytes greatly improved the elastic modulus of the tendons when compared to constructs without the cyclic loading with elastic moduli of 1091

$\pm$ 169 MPa and 632 $\pm$ 86 MPa respectively [32]. Woon et al. also utilized the LigaGen bioreactor to load human flexor tendons seeded with adult dermal fibroblasts to improve the mechanical properties of the allograft before implantation. Constructs with cells that were dynamically loaded at 1.25 N for 5 days had much higher ultimate tensile strengths and elastic moduli when compared to unloaded constructs and constructs loaded without cells with all samples expressing tenogenic markers [33], [34]. The LigaGen system was also the choice for Thorfinn et al. This study seeded tenocytes onto rabbit flexor tendons and cultured them dynamically for 5 days before being implanted in rabbits for 4 weeks. It was demonstrated that the loaded constructs had improved ultimate tensile strength and elastic modules than the unloaded construct [35].

Pneumatic Motors for Uniaxial Strain

Pneumatic motors are very easy to keep sterile and clean and can apply high force loads. However the air component can increase friction around the motor and decrease force output. The even distribution of force is crucial when applying dynamic loading to a tendon construct to influence the tenogenic differentiation of cells. Pneumatic bioreactors use air to stretch the dish the sample is on as seen in the study by Juncosa-Melvin et al. MSCs were taken and seeded onto collagen sponges and a strain protocol of 2.4% was applied to learn how the tensile stress stimulus affects the tenocytic gene expression of the construct [36]. A second study using a pneumatic bioreactor tested whether mechanical stimulation (5 min for 8 hours a day) applied at a peak strain of 2.4% for two weeks would increase the stiffness of mesenchymal stem

cell-collagen sponge tissue constructs [37]. The constructs that were stimulated showed about 3x greater collagen I and collagen III expression. They also had a greater linear stiffness and modulus.

Stepper Motors for Uniaxial Strain

Stepper motors are very precise and can utilize pulley systems and cranks to apply tensile forces to multiple samples [38]. Paxton et al. designed a custom uniaxial bioreactor to perform mechanical stretch using a stepper motor fixed to 11 tissue engineered constructs. Different stimulation conditions can be applied to the scaffolds based on stretch frequency, amplitude, and duration [39]. They found that intermittent stretch programs increased the collagen content of the grafts when compared to a continuous stretch program. One study tested xenografts seeded with bone marrow mesenchymal stem cells and subjected them to 0%, 3%, and 5% strain in a custom bioreactor that utilized four stepper motors in parallel to apply strain [40]. The cyclic strain increased the tenocytic gene expression of SCX when compared to its static counterpart and at 3% strain the tendon scaffold exhibited almost the same mechanical functions as the native tendon. Youngstrom et al. conducted another study with this type of bioreactor to determine which cell line underwent the greatest amount of tenogenesis. BMSCs, ADSCs, and TDSCs were seeded onto equine tendon and it was shown that TDSCs had the highest degree of tendon cell phenotypes when subjected to cyclic loading [41]. The MSCs and ADSCs, however, still exhibited tendon gene markers after cyclic stimulation just not to the degree of TDSCs. Unfortunately in a clinical setting, retrieving enough tendon stem cells for an adequate graft could prove

challenging. Wang et al. designed a programmable mechanical stimulation uniaxial bioreactor to apply different cyclic tensile strain on rabbit tendons. Tendons that were stimulated with 6% strain were shown to look more like the native tendon when compared to the tendons stimulated at 3% strain [42]. Although in the conclusion of this study an optimal range of test controls was identified, such optimal conditions can only be attributed to the specific scaffolds utilized in the study and cannot be generalized further.

Linear Motors for Uniaxial Strain

Linear motors are more common when testing individual samples. A modular bioreactor that consists of a linear motor, media circulation system, and culture chamber was used to apply cyclic tensile strain to P(LLA-CL)/Col scaffolds seeded with TDSCs in order to test the effect different frequencies and amplitudes [21]. Frequencies of 0.3 Hz, 0.5 Hz, and 1.0 Hz and amplitudes of 2%, 4%, 8% were used and it was found that at 0.5 Hz and 4% amplitude the TDSCs remained viable, expressing SCX, TNC, and collagen type I markers. These results further strengthen the conjecture that mechanical stimulation is a necessary component of tenogenic differentiation. One study compared a decellularized tendon scaffold seeded with allogeneic tenocytes loaded with cyclic strain against a tendon scaffold that was left static for 7 days. This study showed that in the absence of cyclic stimulation, the tendon scaffold degraded significantly with decreased tensile properties and elevated levels of matrix metalloproteinase-2 expression [43]. Wang et al. applied strain rates of 5-6% on rabbit Achilles tendons that were not decellularized to examine the

degeneration effects in the absence of mechanical stimulus. Tendons that underwent cyclic loading in the bioreactor were able to maintain tendon homeostasis for up to 12 days while the tendons that did not undergo cyclic loading had severely disrupted collagen fibers and lower tensile strength and stiffness.[44]

Mechanical stimulation is critical to the functionality of tendons. Deng et al. seeded ADSCs onto a tendon scaffold made of PGA/PLA fibers. A bioreactor stimulated the cell seeded scaffolds at a frequency of 3 times per minute and a stretch amplitude of $\frac{1}{10}$ the length of the constructs for 5 weeks. The constructs were implanted *in vivo* in rabbits with time points taken after 12, 21, and 45 weeks. Results showed that at the 45 week time point there was an increase in neo-tendon formation within the rabbit [45]. Constructs implanted without cells and without mechanical stimulation via the bioreactor had significantly decreased collagen fibril formation. It is interesting to note the range of frequencies and magnitude of forces used due to the variance of tendon scaffold architecture. A novel cyclic mechanical stimulation bioreactor was designed to control frequencies, intensities, and waveforms acting on MSC seeded Human Umbilical Vein (HUV) scaffolds. Abousleiman et al. had the bioreactor specifically designed for promoting tendon tissue engineering. The bioreactor can culture three samples at a time and stimulate them through tension from a linear actuator that is controlled by a signal converter [28]. Investigating the use of a HUV as a tendon scaffold resulted in significantly stiffer and tougher constructs when seeded with MSCs and stimulated with tension when compared to constructs without the mechanical stimuli by over 100%. Although the results of the ultimate tensile

strength were one magnitude lower than a native tendon, documenting further stimulation parameters could prove promising.

Bioreactors for Biaxial Strain

Since not all tendons undergo purely uniaxial strain, some are biaxial, bioreactors have been custom built that apply both tension and torsion forces on a tendon construct. Altman et al. seeded bone marrow stromal cells onto silk fiber matrices before culturing in a bioreactor for 21 days with 90 degrees rotational and 2 mm translational deformations at 0.0167 Hz [46]. Results of this study showed elongated BMSCs and an increased cross-sectional cell density. This bioreactor tries to closely mimic the physiological environment of tendons and ligaments as much as possible. Another bioreactor built by Lee et al. also applies tension and torsion. Porcine tibialis tendons were decellularized and stimulated in the bioreactor with 110% tension and 90 degrees of torsion for 7 days. The ultimate tensile strength of the decellularized tendon was found to be greater than the normal tendon [47].

In conclusion, mechanical stimulation via cyclic stretching has been shown to play a critical role in cell activity and tendon tissue health.

Chemical Stimulation

Aside from stimulating tissue engineered tendon constructs using mechanostimulation, it is also possible to implement chemical stimulation. The chemical stimuli must still drive stem cell differentiation into the appropriate lineage. Cytokines are biochemical cues that influence progenitor cells, however their exact

mechanisms are still unknown. Cytokines that have been identified as influencing tendon progenitor cells can include transforming growth factor-beta, insulin-like growth factor, and vascular endothelial growth factors.

When tendons are inflamed, the most active cytokine is transforming growth factor-beta [48]. TGF-beta helps tendon cells regulate cell differentiation and cell phenotype. TGF-beta also induces ECM protein expression [49]–[51]. Other growth differentiation factors, such as GDFs-5, -6, and -7 are part of the TGF-beta family and form a sub group, the bone morphogenetic protein family [52]. These growth factors are critical to the cellular proliferation and differentiation functions, but most importantly regulating tendon tissue repair and development [53]–[58].

Exploring the role of GDF-5, also known as BMP-14 [59], within biomaterials was investigated in its role for engineering tendon and ligaments [60]. GDF-5 is typically limited to affecting cellular proliferation and maintaining a significantly higher stiffness, whereas TGF-beta was able to improve the production of collagen type 1 [61]. A study by Hayashi et al. showed that combining bone marrow stem cells with GDF-5 accelerated tendon healing activity when compared to treating a collagen gel without the growth factor [52]. When human adipose stem cells were treated with GDF-5 and ascorbic acid within a copolymer filament scaffold, collagen production increased and tenogenic gene expression were upregulated [62]. Stem cells that were maintained on poly(DL-lactide-co-glycolide) fiber scaffolds with GDF-5 showed an increase in cell proliferation and scleraxis upregulation then when the cells were not treated with GDF-5 [22, p. 5]. Investigating the role of GDF-6 showed no

improvements and no differences in collagen formation in tendon models utilizing mesenchymal stem cells [63].

GDF-7, also known as BMP-12, has been investigated for its use in tendon tissue engineering. One study by Lee et al. treated collagen scaffolds with GDF-7 that were seeded with bone marrow stem cells. There were positive effects on the scaffolds such as an increase in cellular proliferation and upregulation of tenascin-C [64]. Kishore et al synthesized collagen threads that were electrochemically aligned that mimics the extracellular microenvironment of tendon tissues. Using these as scaffolds, GDF-7 was used to treat the seeded stem cells and indicated it potential of a tendon tissue source due to the upregulation of tendon gene expression markers [65].

Studies have shown that stem cells treated with basic fibroblast growth factor (bFGF) have indicated increase in cellular proliferation and collagen type III production [66]–[68]. Similar results were reported by Sahoo et al. when mesenchymal stem cells were treated with bFGF on silk/PLGA fiber constructs and there was an upregulation of collagen type I [30], [69], [70]. In the presence of bFGF, a study by Hankemeier et al. demonstrated an enhanced presence of collagen I and smooth muscle actin on BMSC's for tendon tissue development [71]. BMSC seeded silk fiber matrices that are RGD-modified were treated with bFGF. After 5 days of stimulation the study showed an increase in cell activity and tissue development [72]–[74]. Collagen membranes seeded with canine MSCs were treated with bFGF to see the benefits for tendon tissue regeneration. In all cases there was in increase in cell proliferation [75]. Another study looked at the effect of bFGF on the response of rat patellar tendon fibroblasts, and again confirmed enhanced cellular proliferation [76].

A critical growth factor involved in angiogenesis is vascular endothelial growth factor (VEGF), however it is usually only upregulated at the end of inflammation during tendon healing [77]. A study that supplemented BMSCs with VEGF on prepared constructs found that the cells had significant increase in cell growth when compared to non-VEGF supplemented cells constructs [78]. Insulin-like growth factor (IGF-I) is associated with an increase in collagen productions in various tendon tissue engineering models. IGF-I was shown to stimulate fibroblast proliferation and ECM synthesis [79]. Cell attachment and activity was affected when human MSCs were supplemented with IGF-I and there was an increase in collagen types I and III synthesis [80]–[82].

One of the main goals of tendon tissue engineering is to prime cells to create a functional tendon tissue construct. Oscillatory mechanical stimulation is still the gold standard for influencing tenocyte differentiation, although growth factors are clearly found to help complement many of these regimes to further the healing process. There is still a need for further research into tendon biology and how to effectively utilize the specific tendon cellular pathways to promote differentiation into the desired cell lineages.

Project Aim

Over 4 million patients suffer from tendon injuries each year in the United States alone. Approximately 250,000 patients will require some form of surgery due to rotator cuff tendon injuries [83]. In severe cases, a tendon replacement graft is often needed for recovery [84], [85]. These can be in the form of an autograft, allograft,

xenografts, or synthetic grafts. However, these grafts can lead to donor site morbidity, cause immune reactions, or result in insufficient healing [53], [86]. Tendon tissue engineering has emerged as a new approach generating grafts for the treatment of tendon injuries [87]. Common tendon tissue engineering approaches involve the combination of adult stem cells in bio-scaffolds together with a period of *in vitro* culture in the presence of mechanical stimulation aiming in the development of appropriate tendon graft substitutes [88], [89].

One of the key objectives for a tendon scaffold is to provide a strong 3 dimensional foundation for which cells can adhere and thrive [90]. Biological scaffolds that are naturally acellular are most desirable for tendon tissue engineering due to the fact that when properly decellularized, they tend to elude the immune response. Biological scaffolds typically include collagen, fibronin, hyaluronic acid, chitosan, and small intestinal submucosa [91], [92]. However, these constructs are often inferior to the strength required to match human tendons necessitating the use of alternative sources [93]–[96]. Human perinatal tissues, such as the human umbilical cord, have been known to have clinical success as allogeneic biomaterials [97], [98]. Human umbilical veins (HUV) are a valuable resource for vascular tissue engineering due to their low antigenicity and ability to support cell seeding and growth [99]. Although the HUV has been explored for use in various vascular applications, previous studies explored its use as a tendon construct due to its mechanical properties and extracellular matrix composition [100]. The use of the HUV in vascular tissue engineering involves endothelial cells seeded on the luminal side and smooth muscle cells on the abluminal side. In contrast, in tendon tissue engineering, the decellularized HUVs is often

inverted prior to seeding with cells usually seeded in the interior of the construct (abluminal side) that is known as wharton's jelly, while the luminal side now forms the exterior of the construct [101], [102].

Along with an appropriate tendon scaffold and cell source, mechanical stimulation is necessary to achieve efficient cell proliferation and proper differentiation. Typically, this is achieved by applying cyclic stretching to a cell-seeded tendon scaffold and culturing the constructs in a bioreactor. It is widely known that tenocytes positively respond to mechanical stimulation such as cyclic stretching [21], [103]. Various studies have shown that mechanical stimulation via cyclic loading is beneficial when compared to static controls and results in a significantly higher ultimate tensile strength and elastic modulus [28], [32]–[35], [39], [102], [104]. One positive outcome of seeding the cells at the interior of an inverted HUV was the observed migration towards the exterior of the construct that became significant beyond the first week of culture reaching a penetration of 75% [29], [101]. Mechanical stimulation must have played a significant role in this migration since in its absence the migratory capacity of the seeded cells was diminished.

The main goal of this study was to develop tendon grafts using decellularized HUVs seeded with adult stem cells and cultured under mechanical stimulation. It is hypothesized that by varying frequency and duration of the mechanical stimulation on the HUV/MSC construct will affect the construct's properties. At early culture time points, mechanical stimulation is hypothesized to be more beneficial as the cells migrate throughout the scaffold and adapt to the HUV environment. Previously, this was explored for 7 day culture time points. This study investigates the effects of slower

and faster frequency as compared to previous studies along with a shorter and longer duration of stimulation for up to 14 days of culture.

Materials and Methods

Cell Source

MSCs were harvested from femurs and tibias of 6-8 year old male Wistar rats (Harlan, Indianapolis, IN) weighing between 150 g and 200 g using prior established protocols [105]. The bones were extracted aseptically and the marrow was flushed from the bone with α-MEM. The α-MEM (Life Technologies, Grand Island, NY) was supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA) and 1% Antibiotic:Antimycotic (Gemini Bio-Products, West Sacramento, CA) as seen in the procedures listed in Appendix A. Bone aspirates were suspended in alpha minimum essential medium. The marrow was then broken up and cultured in T-75 culture plates (Fisher Scientific, Hampton, NH). After 3-4 days, the media was changed to remove any non-adherent cells. MSCs were then cultured until about 75% confluency and then passaged. Passage 2 cells were used for all experiments.

Human Umbilical Cord Preparation

Human umbilical cords (HUC) were obtained from the Women's Delivery Center at the Norman Regional Hospital (Norman, OK) from full term placentas (figure 3B). The HUCs were stored at 4^{o}C for no more than 7 days after delivery before being collected then cleaned (figure 3A) and prepared according to previous methods to extract the HUV and as seen in Appendix B and Appendix C [101].

Briefly, the HUC is cut into 7 cm long sections and then mounted onto a stainless steel mandrel and frozen at -80°C overnight (figure 3C).

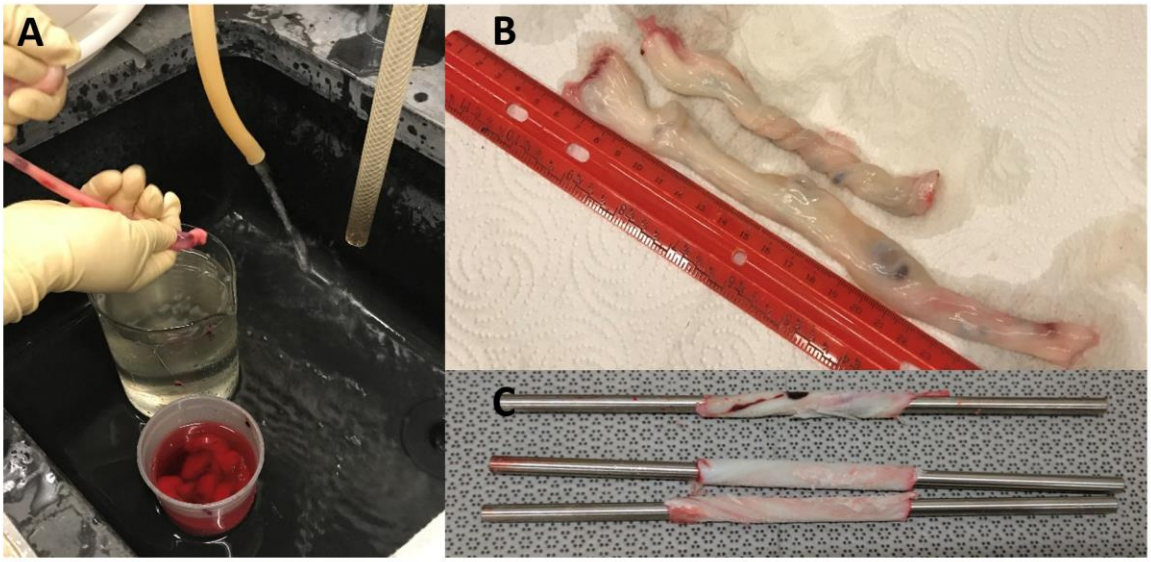

Figure 3: (A) Human Umbilical Cords are being thoroughly washed with distilled water. (B) A close up of the HUC before being cut to size and (C) inserted onto steel mandrels to be frozen overnight at -80ºC

The HUC was then removed from the vein using a computerized lathe (figure 4). The resulting HUV was dissected from the HUC and resulted in a 7cm long tubular construct that had a wall thickness of 0.75 mm and an outer diameter of 6.75 ± 0.25 mm. After extraction the HUV was inverted so that the abluminal side (Wharton's Jelly) would be on the interior of the scaffold as seen in figure 5.

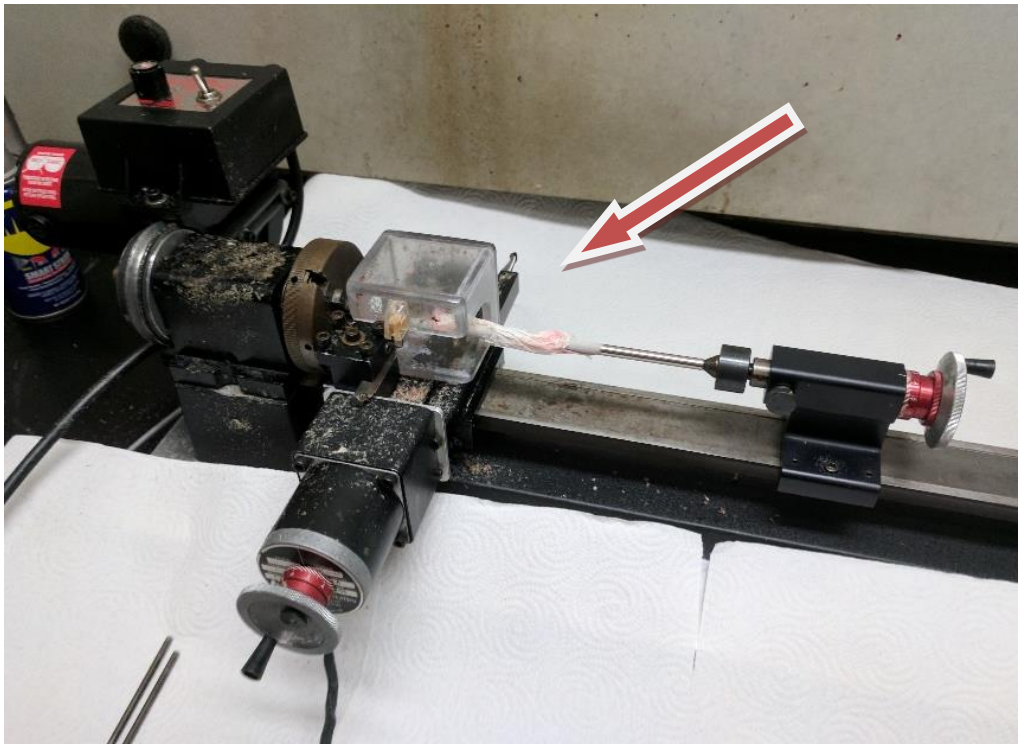

Figure 4: A HUC frozen onto a steel mandrel is prepped for HUV extraction and locked into place via a computerized lathe

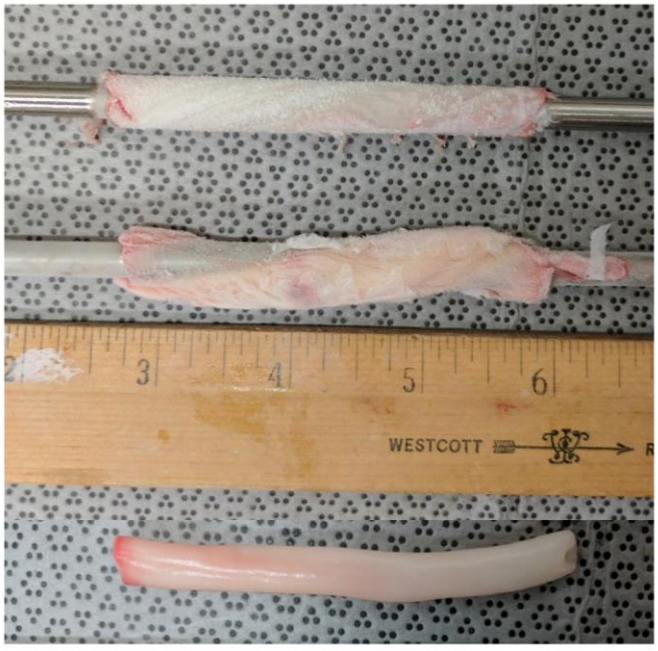

Figure 5: the HUC (middle cord) before being lathed, the resulting HUV (top cord) after extraction and the final inverted HUV (bottom)

The cords were then decellularized and washed in a 1% sodium dodecyl sulfate (JT Baker, Center Valley, PA), ethanol, and 0.2% peracetic acid washes (Sigma Aldrich, St. Louis, MO). The resulting scaffold was then pH adjusted in phosphate buffered saline to 7.2-7.4 with the resulting scaffold shown in figure 6. Scaffolds were kept for a maximum of 5 days at 4°C prior to use.

Figure 6: Inverted and decellularized HUV

Bioreactor Setup

Decellularized HUV sections were immersed in standard media at 37^{o}C prior to seeding with MSCs. The cells were washed with 5 ml of PBS then detached using 2 ml of 0.25% trypsin (Invitrogen, Carlsbad, CA) for 5 minutes. A cell pellet was formed using centrifugation and then mixed with 2 mg/ml of collagen type I (BD Biosciences, San Jose, CA) at a density of 1 million cells/mL following Appendix D. Special end adapters were attached to the HUV that are designed to hold the HUV in place during the seeding procedure (figure 7A-C). Using these adapters it is possible to use a 1 ml pipette to load 0.6 mL of the cell-collagen mixture into the interior of the HUV and sealed (figure 7D). The seeded constructs were then incubated at 37^{0}C to

allow the collagen hydrogel to polymerize for 2 hours. The constructs were then placed into the bioreactor for varying duration and frequency of 14 days as seen in figure 8.

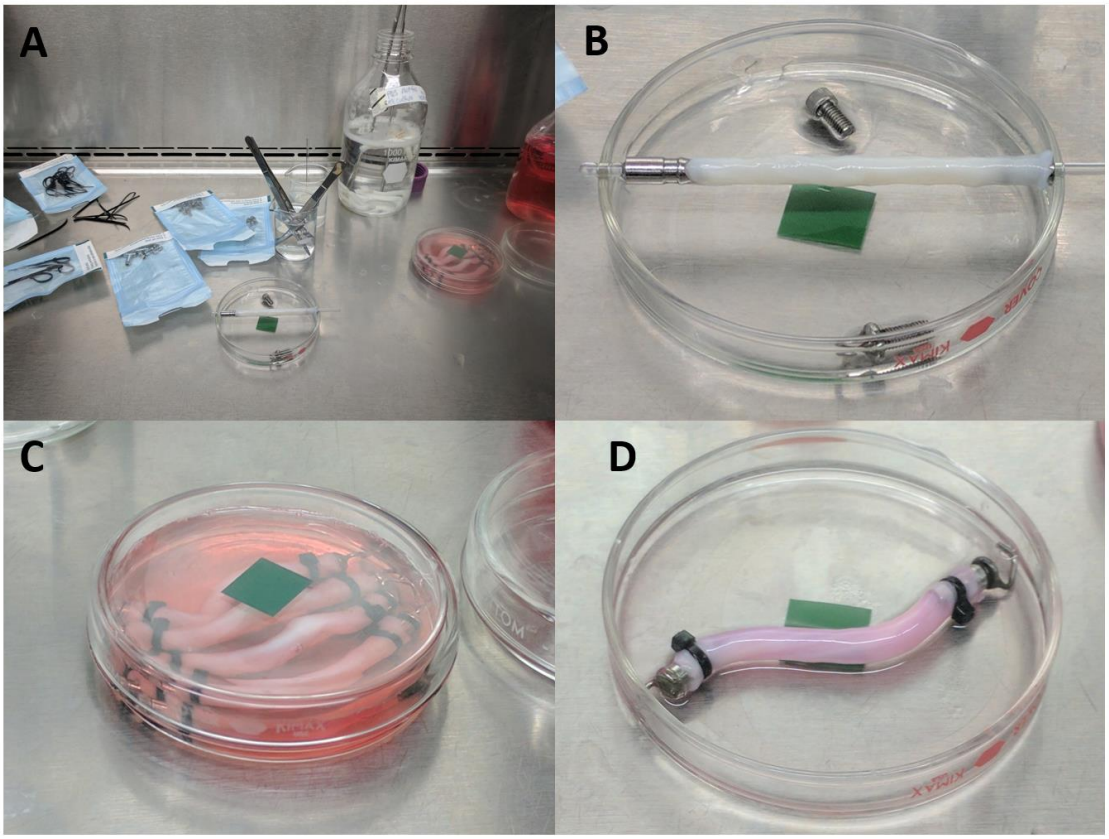

Figure 7: (A) set up to attaching the special adapters to each end of the HUV (B) Close up of an unseeded decellularized HUV with the adapters set in place (C) Incubating the HUV in media in preparation for seeding (D) An MSC seeded HUV sealed with the adapters in place via zip ties

At this point, mechanical stimulation was applied daily to the constructs according to the specific experimental groups described in Table 2. A control group was included in which constructs were prepared as described above but without the use of mechanical stimulation. Regular stimulation was performed at 2% strain for 1 hour/day at 1 cycle/minute meaning the construct was strained and then relaxed 1 time per minute. Using this as a starting point, the stimulation frequency was varied under

two different conditions: 1 cycle/2 minutes (slow) or 2 cycles/minute (fast). In addition, the duration of the stimulation was also changed; constructs were subjected to either 0.5 hour/day (brief) or 2 hours/day (extended) durations at 1 cycle/minute. The regular stimulation was based on previous studies which were shown to be beneficial to the HUV/MSC construct. The variations were chosen to create a difference in mechanical stimulation without exposing the construct to too much stimulation, which has been shown to be detrimental to the tendon tissue engineer constructs [29], [100], [101]. The whole bioreactor system is kept in a humidified incubator at 37°C, 5% CO_2, and 95% air. The load is transferred from the actuator to the samples via a piston that connects to the triangular plate which in turn is hooked to the three constructs. The media in the bioreactor system is replaced every three days with alpha-MEM supplemented with FBS following the procedure depicted in Appendix E. The two reservoir system allows the changing of media without having to move the bioreactor out of the incubator, minimizing contamination events. After culturing, constructs were removed from the bioreactors and prepared according to the specific analysis being performed.

	Strain	**Frequency**	**Duration**
Static	None	None	None
Regular	2%	1 cycle/minute	1 hour/day
Slow	2%	1 cycle/2 minutes	1 hour/day
Fast	2%	2 cycles/minute	1 hour/day
Brief	2%	1 cycle/minute	0.5 hour/day
Extended	2%	1 cycle/minute	2 hours/day

Table 2: Description of stimulation parameters for the various experimental groups

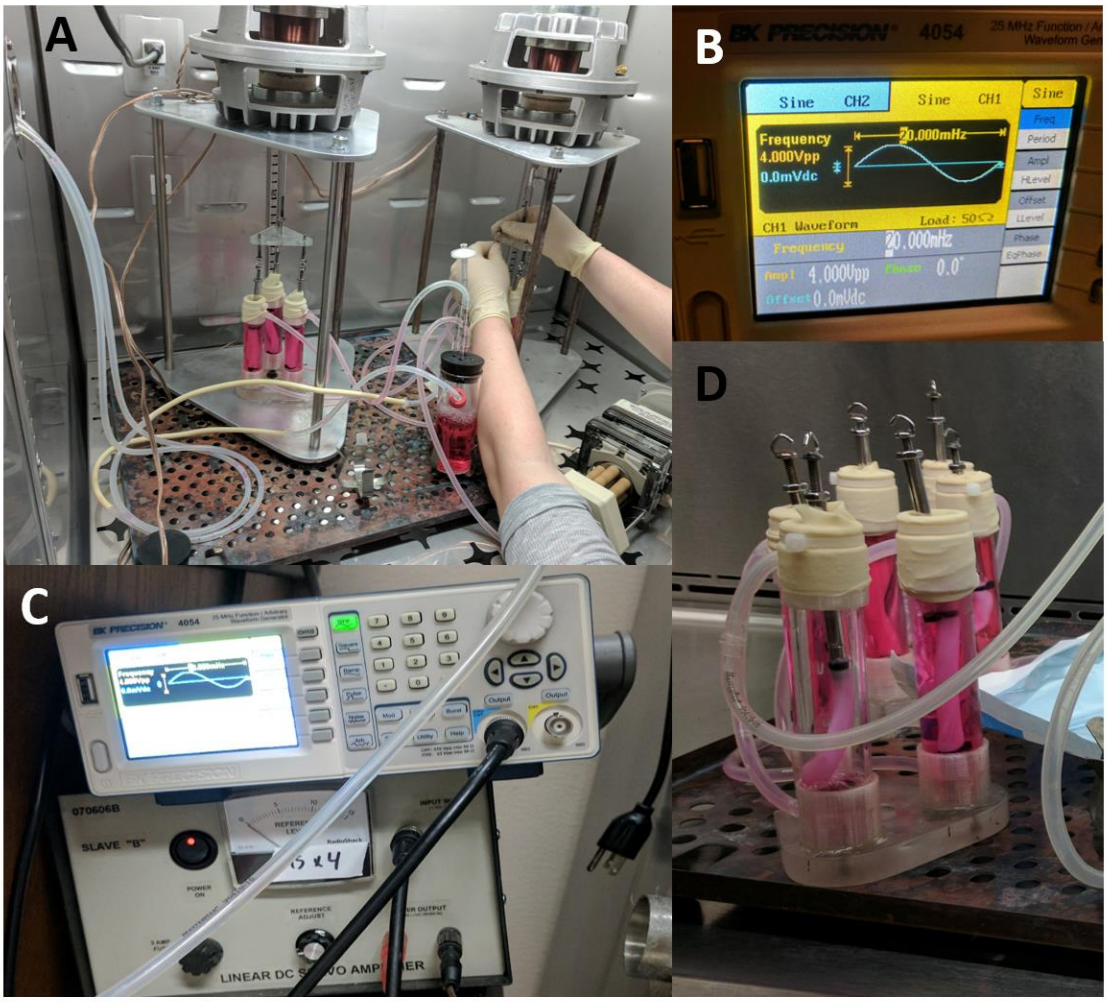

Figure 8: Depiction of the bioreactor. (A) Design of the bioreactor including reservoirs for continuous media circulation and linear motor (B) Close up of a B&K Precision 4054 Signal Generator (C) Signal Generator and Amplifier (D) A close up of the HUV constructs in the reactor vessels

Cellularity Analysis

To determine the amount of cells on the construct, DNA quantification was performed on at least three constructs per culture duration, with triplicate sections tested from each construct. After culture, ringlets of about 5 mm long were taken from the top, middle, and bottom of the construct for representative sampling. The tissue are then digested in 1 mL of water with 200 U/mL of collagenase type I overnight at 37°C as per Appendix F. Following collagen digestion, samples were sonicated for 5 seconds and then subjected to three cycles of freeze/thaw to ensure complete lysis of the cells and release of the contained DNA. The solution was then measured for DNA content using a Quant-iT PicoGreen dsDNA Assay kit (Life Technologies, Grand Island, NY). Fluorescence of the solution was measured at 480 nm/528 nm excitation/emission wavelengths. DNA content was correlated to cell number using a known DNA concentration of cells (7 pg/cell) that was previously determined via in-house studies [20], [28], [100]. The picogreen DNA assay gives an indirect indication of cell viability by showing an increase in DNA concentration, and thus an increase in cell number per construct, with time.

Histology

0.5 cm Sections of the tissue were fixed in 10% formalin (Azer Scientific, Morgantown, PA) and then dehydrated in order to be embedded in paraffin (VWR, Radnor, PA) following Appendix G. The embedded samples were then sectioned according to Appendix H and placed on Fisherbrand frosted slides (Fisher Scientific, Hampton, NH) and stained with hematoxylin and eosin by the protocol depicted in

Appendix H. Haematoxylin is a dark violet stain that is basic/positive. It binds to DNA/RNA in the nucleus of cells. Eosin is a pink/red stain that is acidic/negative and binds to substances such as collagen. The shade of the Eosin is also affected by the corresponding acid wash to remove the stain but the collagen is still noticeably different than the much darker stained cells making them easier to distinguish. Images were obtained using a Nikon Eclipse E800 microscope. Differences in color in histological slides may also be due to differences in light balances when using the microscope. Lateral sections of the construct were analyzed with ImageJ software and the FibrilTool plug-in to determine the fiber alignment [106]. This was reported in terms of anisotropy of the fibrils where 0 indicates no directional dependence of fibrils while 1 indicates a complete alignment in one direction. Cross sections were examined to analyze cellular penetration using ImageJ software.

Mechanical Analysis

Entire constructs were tested for their mechanical properties using a uniaxial tensile testing frame (Untied Testing Systems, model SSTM-2K, Flint, MI) following the protocol in Appendix J. The force vs displacement data was measured and recorded. Samples were then tested until failure at 1%/second. Samples were preconditioned for 5 cycles to minimize hysteresis before testing to failure.

RT-PCR Gene Expression

Gene expression was measured utilizing quantitative RT-PCR. Tissue samples were placed into RNAlater (Life Technologies, Grand Island, NY) at -80°C until analysis.

The tissue was then homogenized utilizing Trizol (Life Technologies, Grand Island, NY) and a tissue grinder as referred to in Appendix K for RNA isolation. The Trizol was then recovered and treated to isolate the RNA according to manufacturer procedures. The resultant mRNA was confirmed to be phenol-free using the nano-drop one (thermos scientific, Waltham, MA) then reverse transcribed using a RNA-to-cDNA kit (Sigma Aldrich, St. Louis, MO) and qTower^{3}G thermal cycler (analytic-jena, Strasse, Germany). The primers (TaqMan Gene Expression Assays, Foster City, CA) utilized for RT-PCR are given in table 3 [107]–[109], [110, p. 2]. The PCR analysis was done utilizing the TaqMan Universal PCR Master Mix (ThermoFisher, Waltham, MA) and the qTower^{3}G. Analysis was performed utilizing the $2^{-\Delta\Delta Ct}$ method with a static control at day 14. Genes in the experimental groups were normalized utilizing the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and comparison between controls and the experimental group were done utilizing the $2^{-\Delta\Delta Ct}$ method of comparing experimental gene targets to the GAPDH housekeeping gene and then comparing that change to a control.

	Forward Primer (5' to 3')	**Reverse Primer (5' to 3')**
GAPDH	CCATTCTTCCACCTTTGATGCT	TGTTGCTGTAGCCATATTCATTGT
Collagen Type I	ATCAGCCCAAACCCCAAGGAGA	CGCAGGAAGGTCAGCTGGATAG
Collagen Type II	CCACGCTCAAGTCCCTCAAC	AGTCACCGCTCTTCCACTCG
RUNX-2	CCTGAACTCTGCACCAAGTCCT	TCATCTGGCTCAGATAGGAGGG

Table 3: Primers utilized in RT-PCR reactions

Statistical Analysis

All analysis was performed utilizing ANOVA and Bonferroni Post tests for significance between individual groups. A $p < 0.05$ was used for significance with a confidence level >95% and was determined using SigmaPlot software. Sample sizes of 3 or more was used for each analysis. 6 samples or more were utilized for mechanical testing. All results were expressed as mean values ± standard deviation.

Results

Cellularity

Sections from three different locations on each of three seeded HUVs were tested for cellularity. The cellularity of the non-decellularized HUV was also compared to each wash step of the decellularization process to confirm low cellularity before the HUVs are seeded from 0.5 cm samples that were analyzed in triplicate as seen in figure 9. A statistically significant decrease in cell number was measured in the samples stored in PBS for two days as compared to the non-decellularized HUV samples. Confirming the prior research in the lab that decellularizing removes a significant amount of the existing cells, therefore no additional contribution in cellularity is from the HUV itself [100], [101]. Seven day data in this section was acquired from a previous study [102].

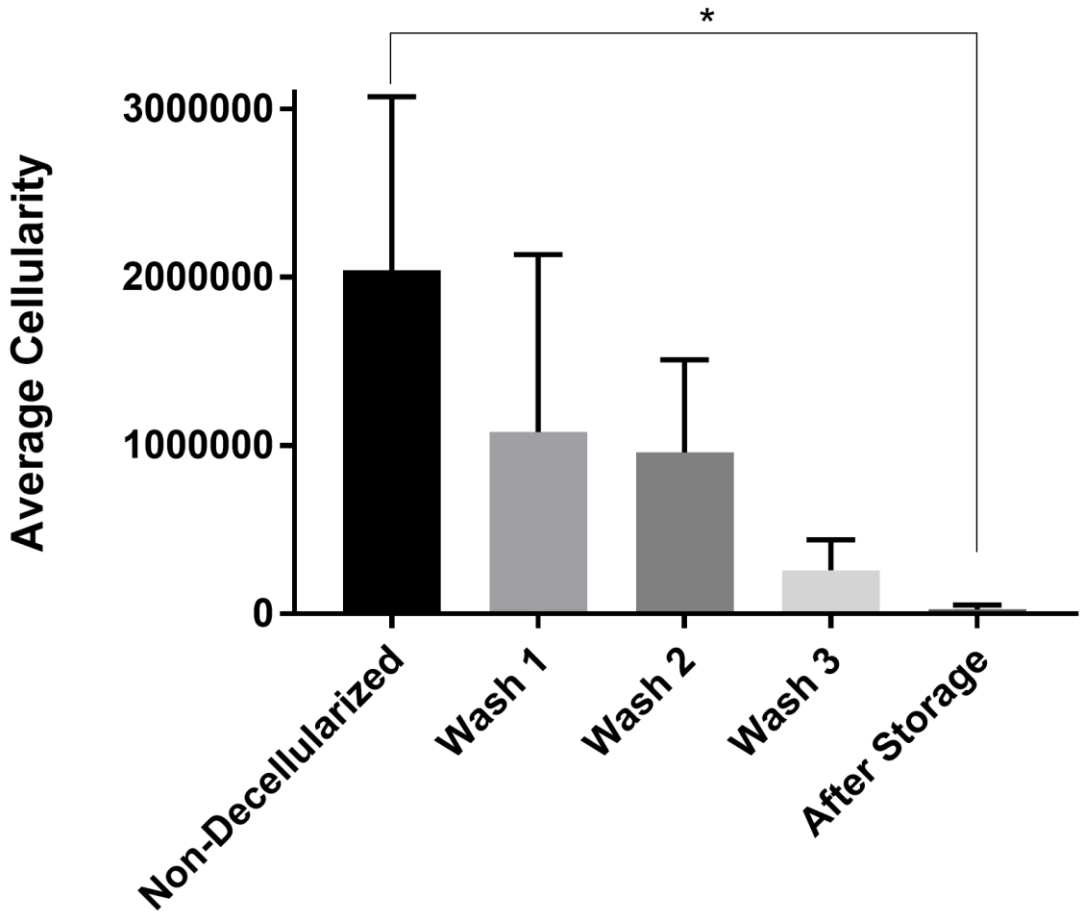

Figure 9: Average cellularity of the non-decellularized HUV and after each wash in the decellularization process. After the final wash with water, samples were stored for two days in sterile PB and a final sample was taken from this "After Storage" step. * indicates $p < 0.05$ compared between samples. Data represented as mean ± SEM. A sample size of n = 6 was used.

Figures 10 and 11 show the cellularity of the MSC/HUV constructs after 7 and 14 days of culturing from the different stimulation and duration changes. The dashed line indicates the initial cell seeding density of 600,000 cells per construct.

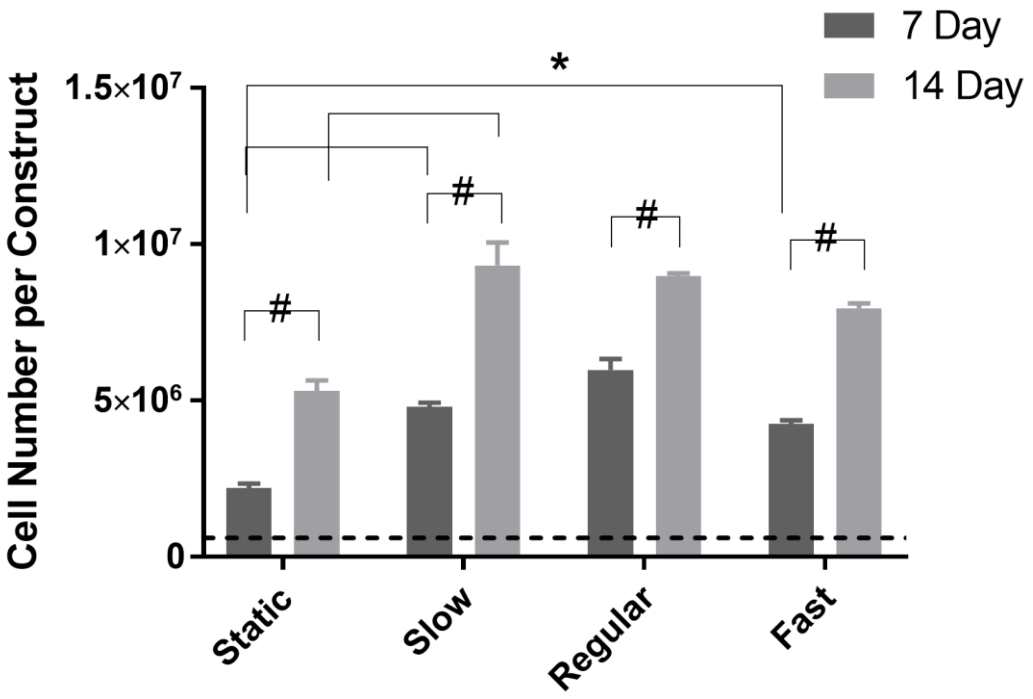

Figure 10: Cellularity of the HUV/MSC construct as a function of the frequency of the stimulation at 7 days and 14 days. * indicates $p < 0.05$ compared between the 7 day and 14 day samples. # indicates $p < 0.05$ comparing the 14 day to the static control. Data represented as mean ± SEM. A sample size of n = 6 was used. Dashed line indicates initial seeding density of 600,000 cells/construct

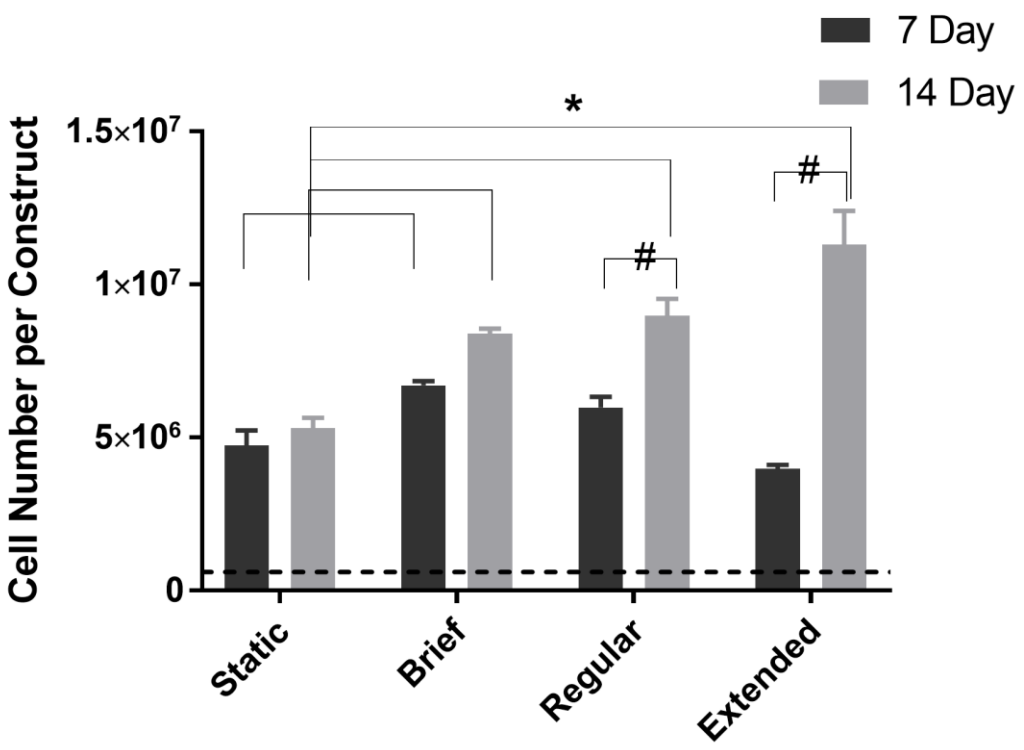

Figure 11: Cellularity of the HUV/MSC construct as a function of the duration of the stimulation at 7 days and 14 days. * indicates p < 0.05 compared between the 7 day and 14 day samples. # indicates p < 0.05 comparing the 14 day to the static control. Data represented as mean ± SEM. A sample size of n = 6 was used. Dashed line indicates initial seeding density of 600,000 cells/construct.

In most cases for the MSC/HUV construct, there was a significant increase in cell number between the 7 day and 14 day samples with the exception of the brief duration (8.39 ± 1.66 million cells/construct). There was also statistical significance between the 14 day extended duration, and the 14 day regular and slow frequencies (figure 10) when compared to the 14 day static control. The extended duration (figure 11) of stimulation was shown to have the greatest increase in the number of cells with 11.3 ± 3.3 million cells/construct, compared to the static culture, which only had 5.31 ± 1.01 million cells/construct, a 112% increase compared to static. The extended duration (figure 11) also was the best group investigated in terms of increase in cellularity when compared to its 7 day counterpart, with a 180% increase.

Mechanical Analysis

Testing the MSC/HUV constructs longitudinally until failure resulted in a typical viscoelastic behavior due to the rearrangement of fibers upon stretching. Thus the constructs were preconditioned for 5 cycles prior to loading till failure as seen in Figure 12.

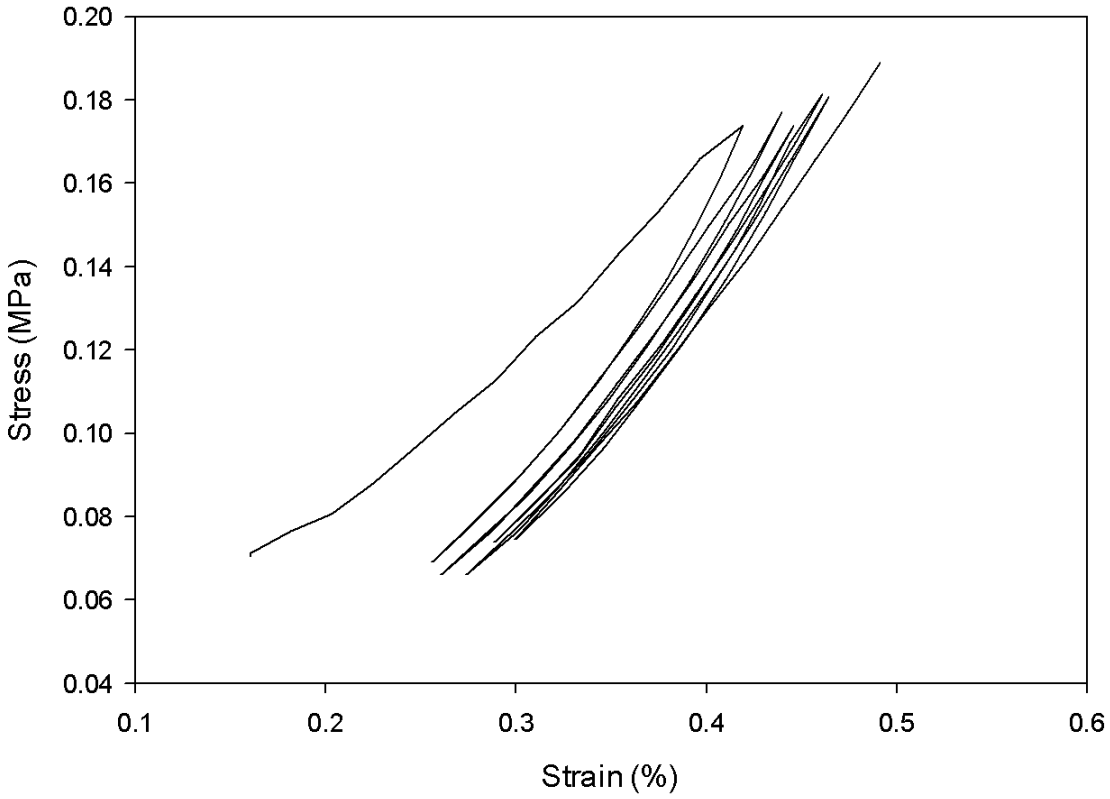

Figure 12: Toe region of stress-strain curves of HUVs preconditioned for 5 cycles. After 5 preconditioning cycles the load and unload paths are less likely to undergo hysteresis

Figure 13 shows a typical stress stain plot for HUVs. Figure 14 is a comparison of the ultimate tensile strength of the non-decellularized HUV compared to its

decellularized counterpart after two days of storage in PBS. Decellularization has slightly increased the ultimate tensile strength but there is not statistically significant difference.

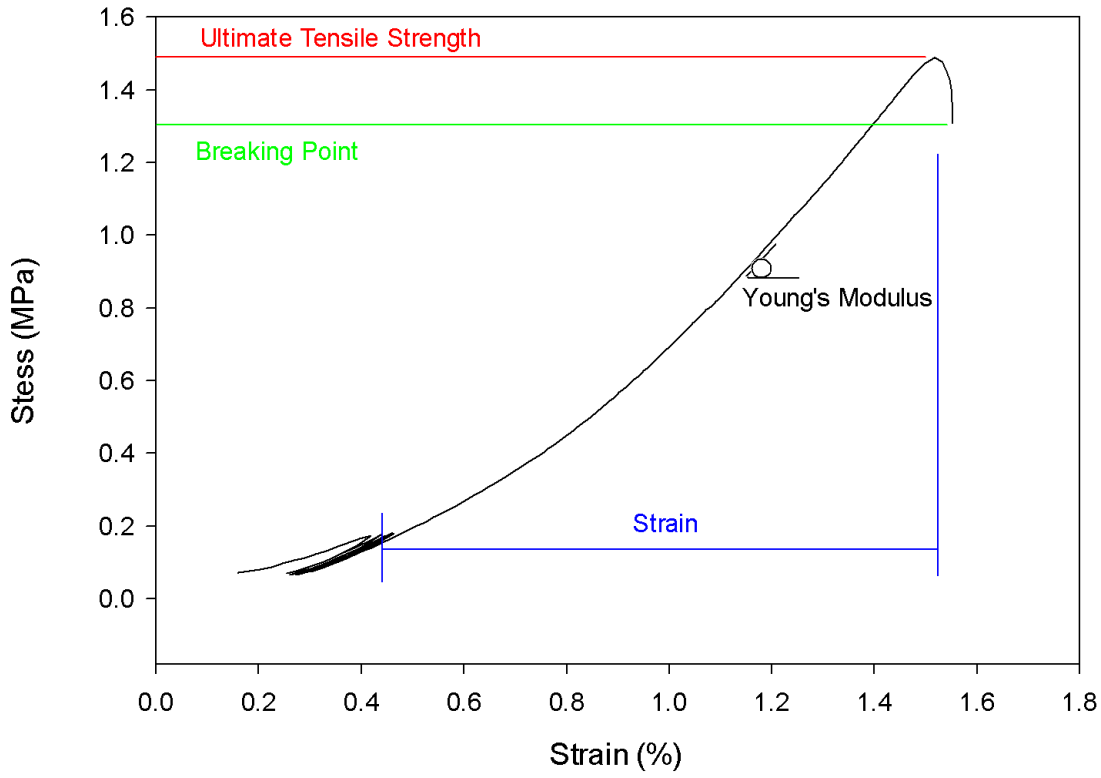

Figure 13: Typical stress strain curve of HUV. The curve has a bell shape with a "toe region" consisting of the preconditioning cycles, a linear region located at the young's modulus, a maxima at ultimate stress seen at the tip of the curve, and then a final decrease in strength at the failure point.

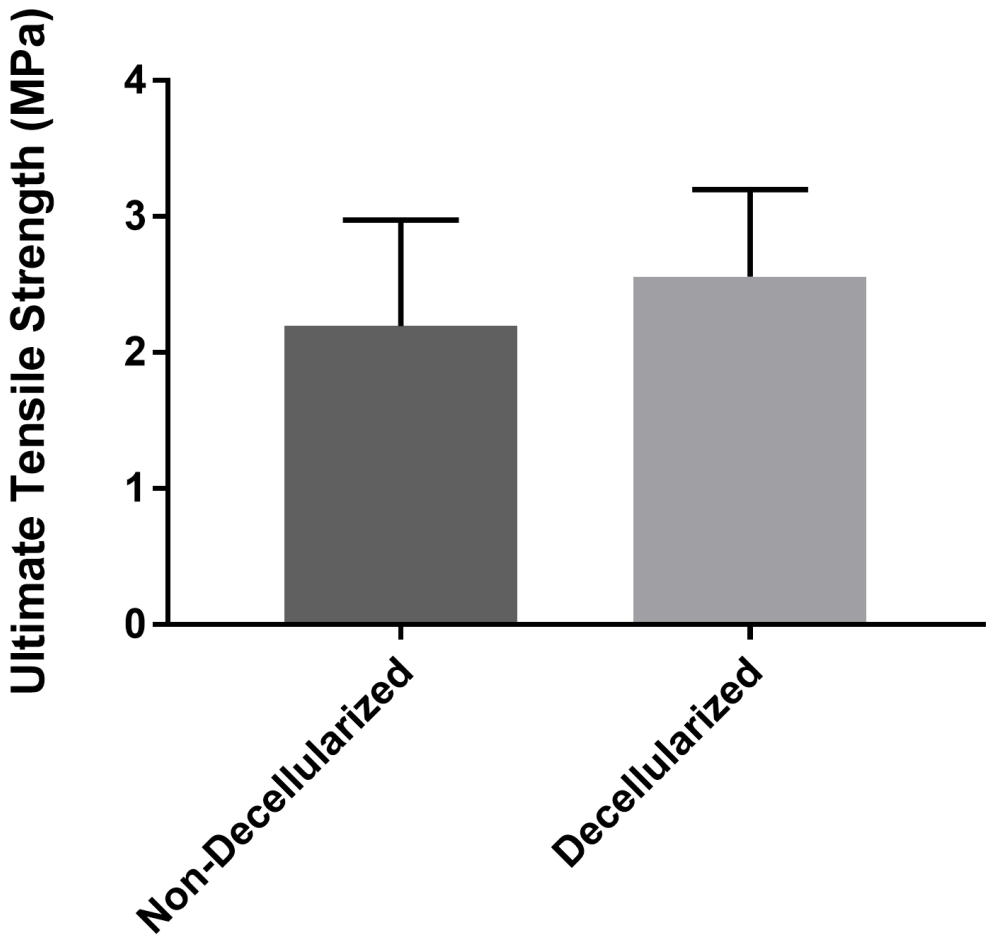

Figure 14: Ultimate Tensile Strength for a non-decellularized HUV and a decellularized HUV construct. Data represented as mean ± SEM. A sample size of n = 3 was used.

Prior studies showed that mechanical testing at 7 days revealed that the various stimulation groups or the type of stimulation did not result in significant changes compared to the static group. Values for the 7 day ranged from 1.06 ± 0.34 MPa for the fast group to 1.58 ± 0.35 MPa for the slow group [102]. Figure 15 shows how the 14 day results differed from the 7 day data. Only the extended stimulation duration resulted in a significant increase between the 7 day and 14 day data, as well as a statistically significant increase when compared to the 14 day static control. The

extended group had an ultimate tensile strength of 3.28 ± 0.63 MPa whereas the 14 day static control was only 1.74 ± 0.64 MPa.

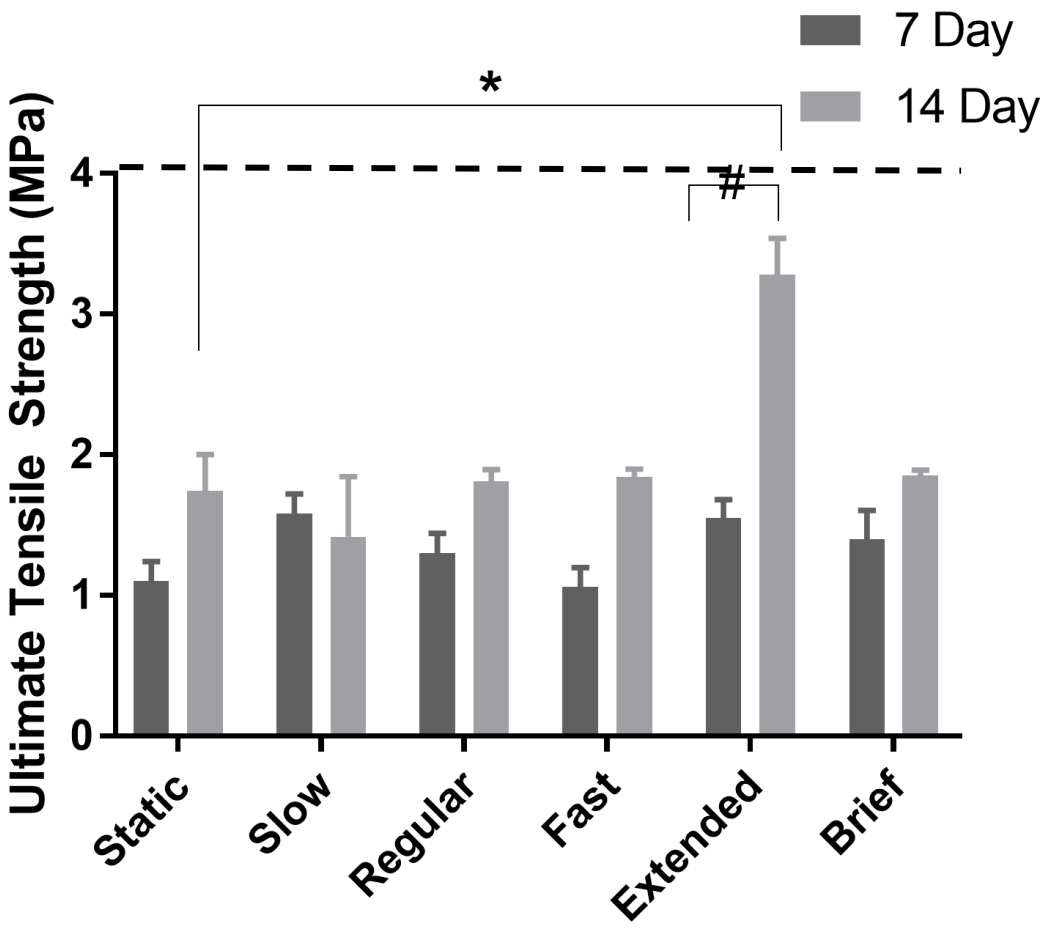

Figure 15: Ultimate tensile strengths for the various experimental groups at 7 and 14 days of culture. * indicates $p < 0.05$ compared between the 7 day and 14 day samples. # indicates $p < 0.05$ comparing the 14 day to the static control. Data is represented as mean ± SEM. A sample size of n = 6 was used. Dashed line indicates the beginning Ultimate Tensile Strength value of a natural human tendon.

Although the extended group seems to almost double compared to the other groups, it is actually very similar to preliminary in house studies [28], [100], [102], [111]. At one point, one in house study showed a value significantly greater than the extended group for much longer culture durations [111].

Histology

Decellularization was effective in removing cellular components. This is clearly demonstrated in the H&E stained cross sections of the HUV scaffold (figure 16) before and after decellularization. Cell bodies are hardly visible in figure 16B and the fibrils are easier to distinguish compared to figure 16A.

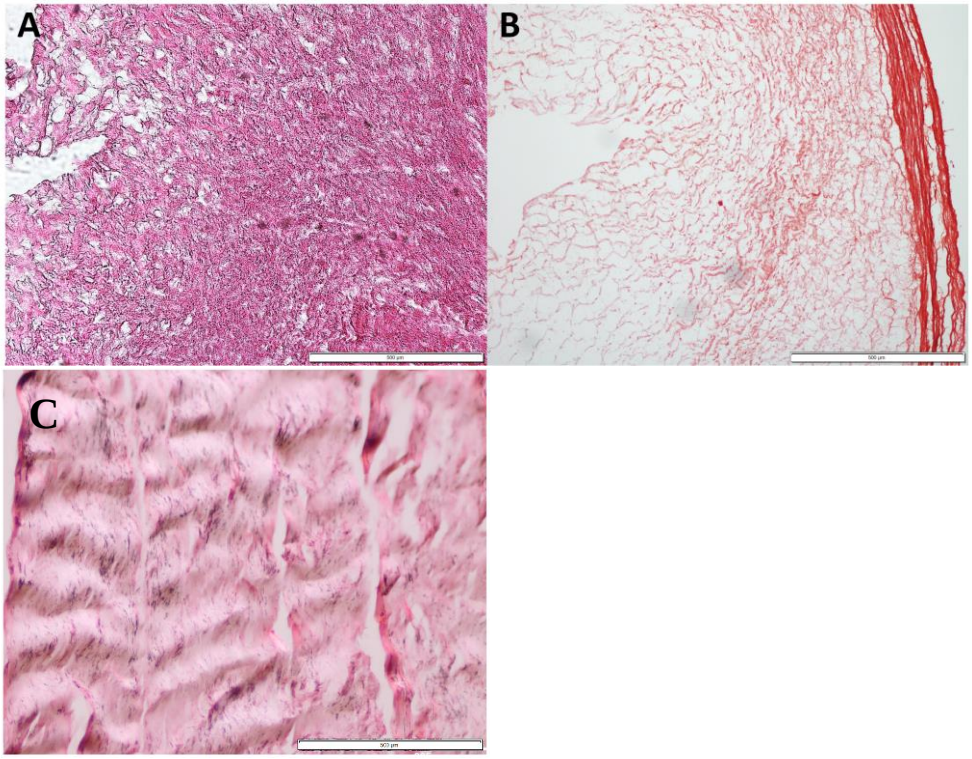

Figure 16: (A) Non-Decellularized HUV (B) decellularized HUV (C) Native Tendon (porcine) where you can see a noticeable crimp pattern in the collagen fibers characteristic of a tendon. There is a noticeable thickness and lack of disruption to the native tendon. Scale bar = 500 µm

Cross sections of the luminal (vein) side of the HUV were compared to the abluminal (Wharton's Jelly) side to confirm the need to invert the HUV prior to cell seeding (figure 17) due to dense nature of the luminal side. In figure 17, the luminal side appears to be a deeper red than the Wharton's Jelly side, but this is just due to the

light balance on the microscope being affected by the different densities. The luminal side is dense because its purpose was to prevent the back flow of blood and contains collagen and elastic fibers (also stains pink/red). The abluminal side (figure 17B) consists of a network of collagen fibers that used to host stem cells, ground substance, hyaluronic acid, and macrophages. After decellularization what is left is the network of collagen fibers.

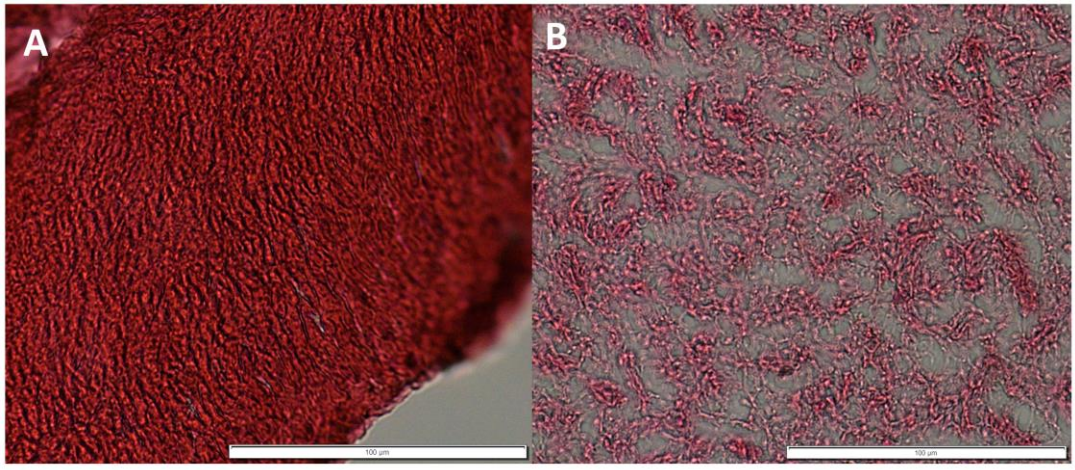

Figure 17: (A) luminal side of the human umbilical vein (B) Wharton's Jelly (abluminal) side of the HUV. Scale bar = 100 µm. Light balances from the microscope at these magnifications create "dark spots" or "shadows" due to different depths between fibers. If these were violet or dark violet it would indicated a stained cell, however these dark spots are only increasingly darker shades of red.

Figure 18, seen below, indicates a representative image of a histological image of a tendon cross section. You can see the darker stain of the cell in the magnified box because it is also elongated along the fibrils, a known characteristic of fibroblasts previously mentioned. Figure 19 shows the difference in the histology between a non-decellularized HUV and a decellularized HUV. Figure 20 shows the cellular penetration via cross sections of the constructs. Cells in all cases are primarily located

in the interior of wall of the MSC/HUV construct, with quite a bit of migration into the walls, reaching a maximum distance of about 89% ± 3% of the wall thickness (Table 4).

	14 Day Average	Std. Dev	7 Day Average	Std. Dev
Static	58%	8%	24%	5%
Regular	85% *	2%	16%	4%
Slow	71%	2%	13%	4%
Fast	63%	9%	29%	10%
Brief	80% *	7%	29%	8%
Extended	89% *	3%	29%	14%

Table 4: Maximum cell penetration depth as determined by histological cross sections of the frequency and duration group between 7 and 14 days. * indicates $p < 0.05$ for the experimental group compared to the static control. Data represented as a mean ± standard deviation.

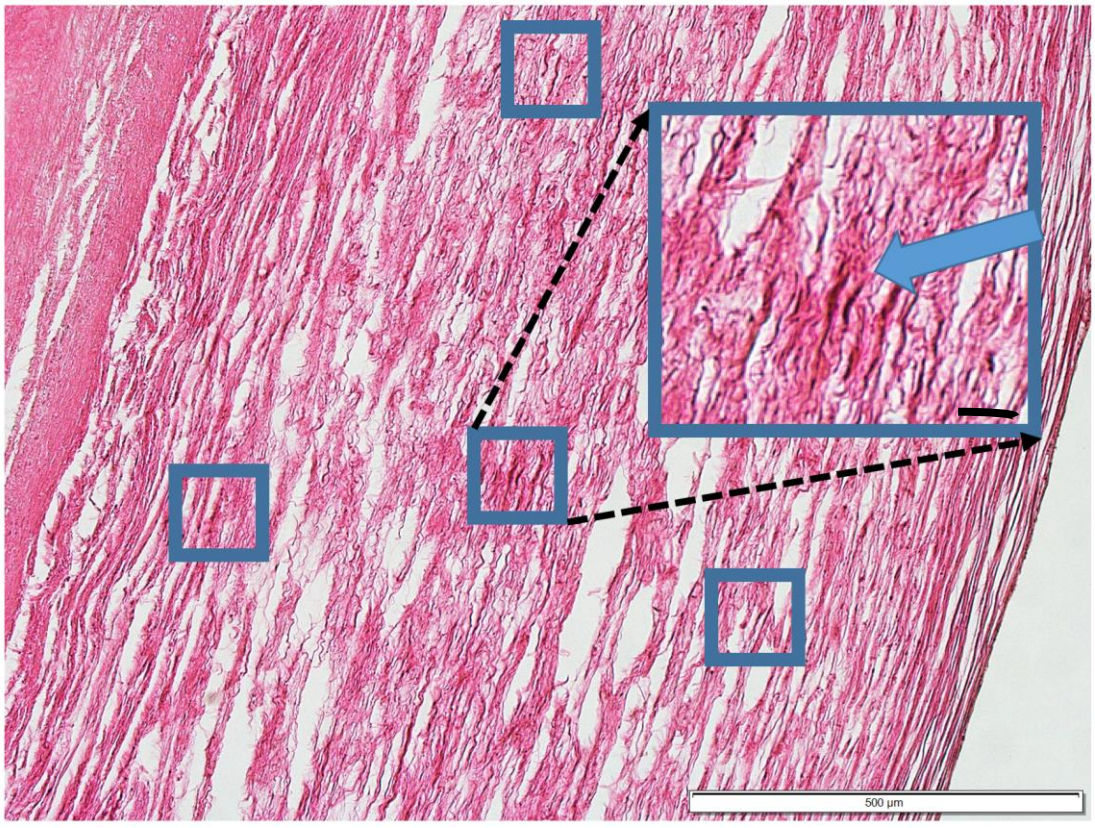

Figure 18: A representative image of a histological cross section with Scale bar = 500 µm with a zoomed in image of a representative cell with scale bar = 100 µm. Other representative cell locations are enclosed in boxes.

Lateral Sections of the HUV after 14 days seen in Figure 21, demonstrates the alignment or lack thereof, of the ECM fibers with the direction of stretching. Arrows that were input via ImageJ software indicate the direction of stretching. The stimulation groups demonstrated fiber alignment, or anisotropy, in the direction of stretching as indicated in table 5. Only the extended duration group at 14 days showed a statistically significant increase in fiber alignment as compared to the 14 day static control.

	14 Days Mean	Std. Dev.	7 Days Mean	Std. Dev.
Static	0.23	0.02	0.20	0.01
Brief	0.34	0.08	0.44	0.02
Slow	0.32	0.08	0.39	0.04
Regular	0.30	0.06	0.44	0.01
Fast	0.24	0.04	0.19	0.01
Extended	0.43 *	0.02	0.46	0.07

Table 5: Anisotropy values as determined by histological lateral sections of the frequency and duration group between 7 and 14 days. * indicates $p < 0.05$ for the experimental group compared to the static control for 14 day data set. Data represented as a mean ± standard deviation.

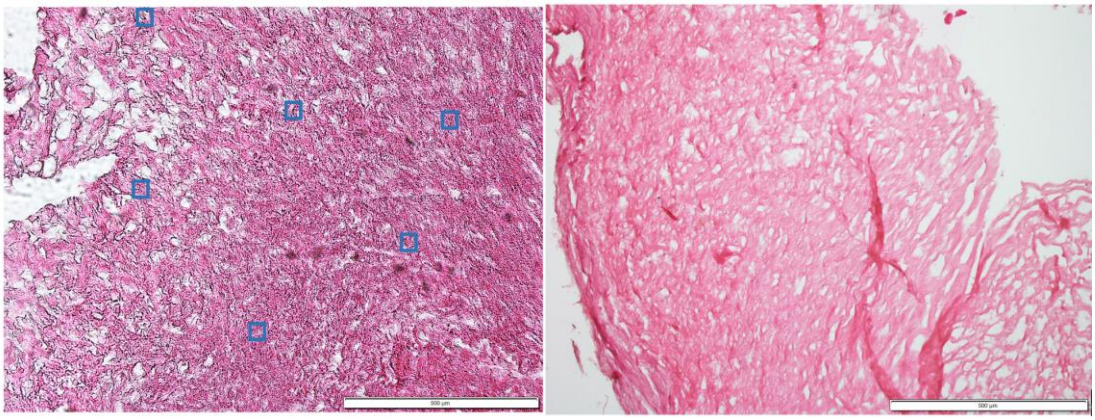

Figure 19: (Left) Non-decellularized HUV with boxes indicating where there are cells located (Right) Decellularized HUV. Scale bar = 500 µm.

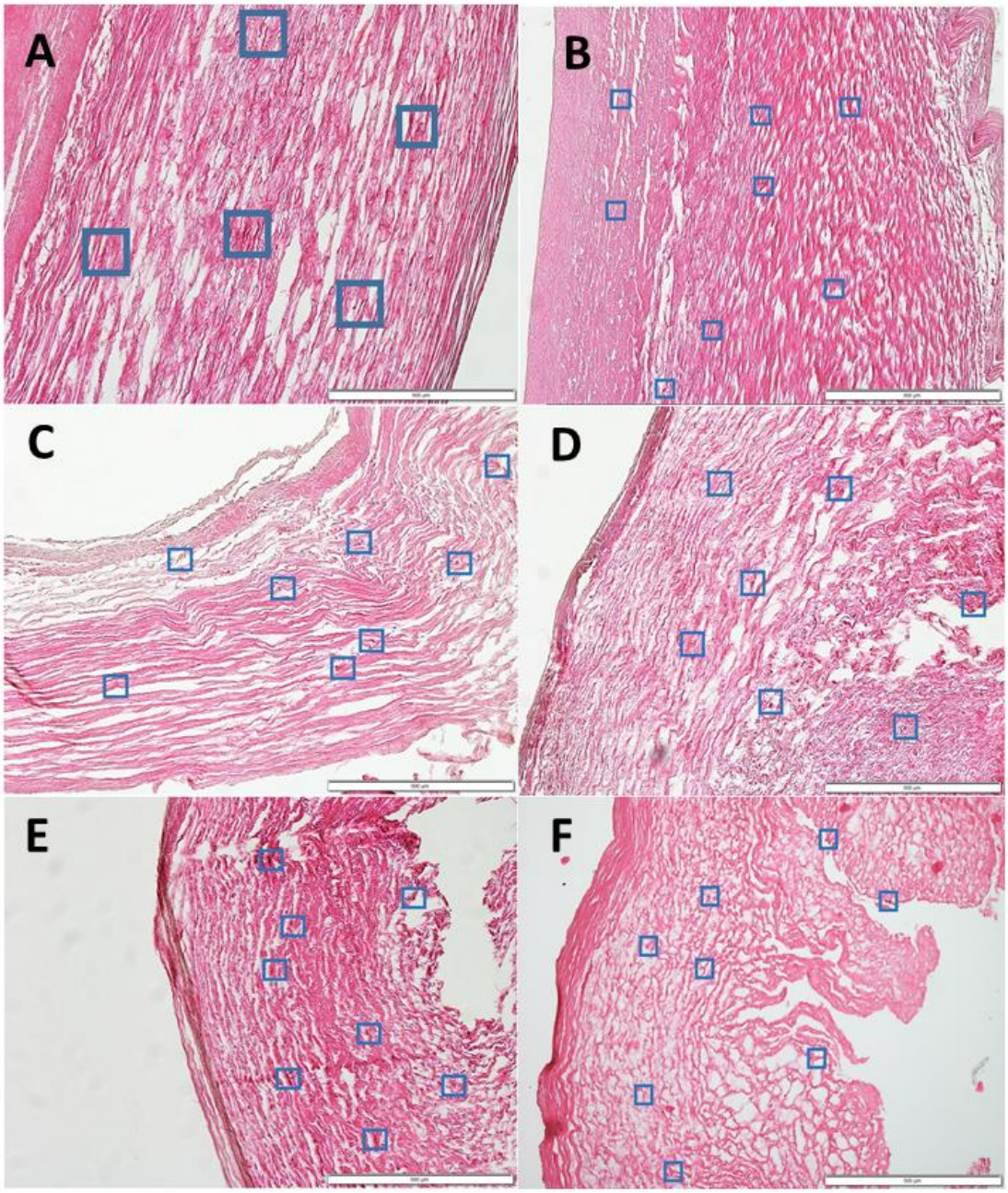

Figure 20: Histological Cross sections for the 14 day cross sections of the (A) Brief Stimulation (B) Extended Duration (C) Fast Duration (D) Regular Stimulation and Duration (E) Slow Stimulation (F) Static Control. Scale bar = 500 µm. Cell locations are indicated by the boxes as referenced in Figure 18.

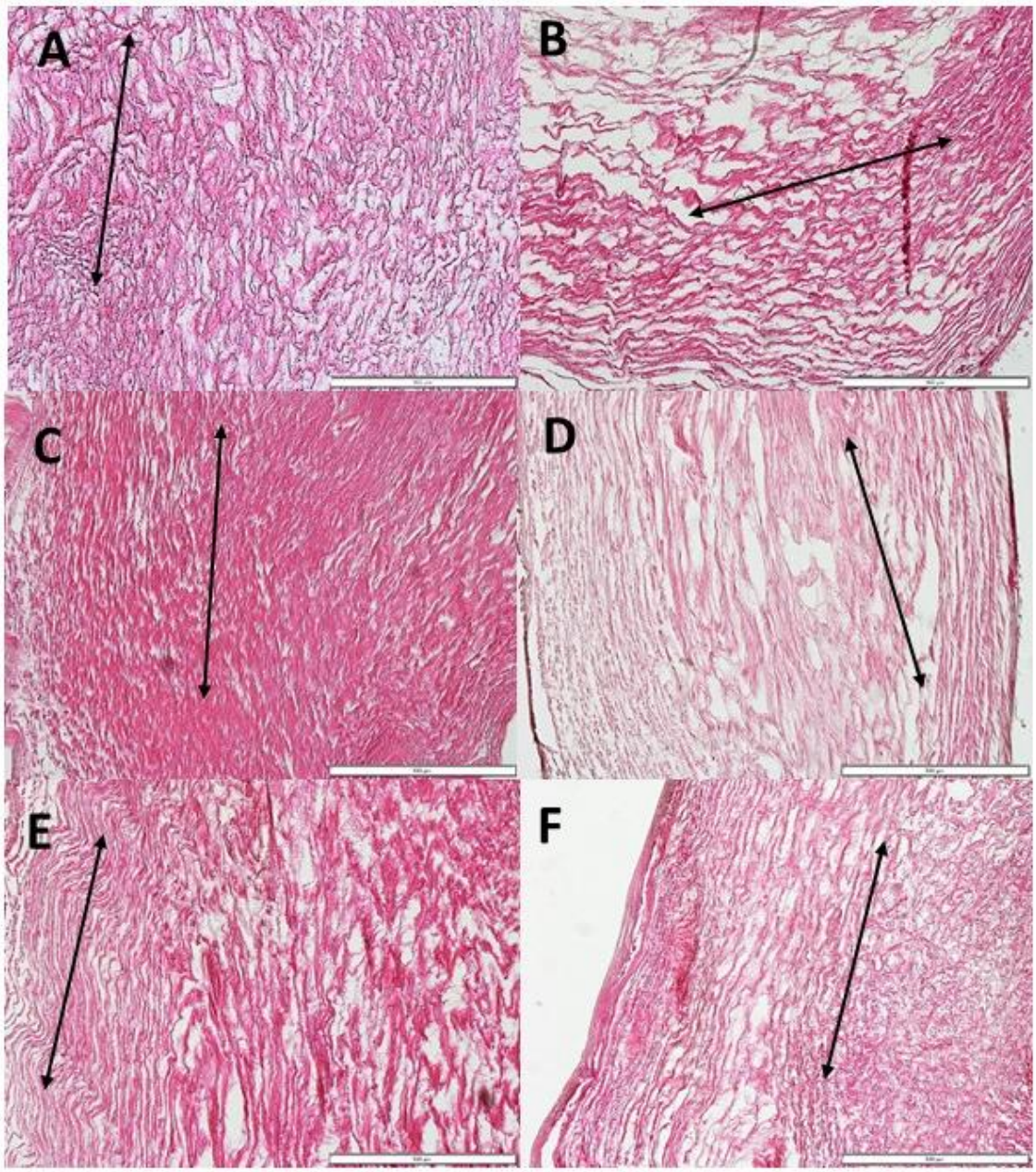

Figure 21: Histological longitudinal sections of the construct after 14 days for (A) Static control (B) Brief duration (C) Extended Duration (D) Fast Frequency (E) Slow Frequency and (F) Regular Stimulation. Arrows indicate direction of mechanical stretching and were averaged using the FibrilTool in ImageJ. Scale bar = 500 µm.

Gene Expression

Figure 22 is an example of the chart for the linear ct (threshold cycle) output given by the program qPCRsoft (Analytik Jena, Strasse, Germany). When converted into the logarithmic form, you can see the cycle number at which the fluorescence signal crosses the threshold (figure 23).

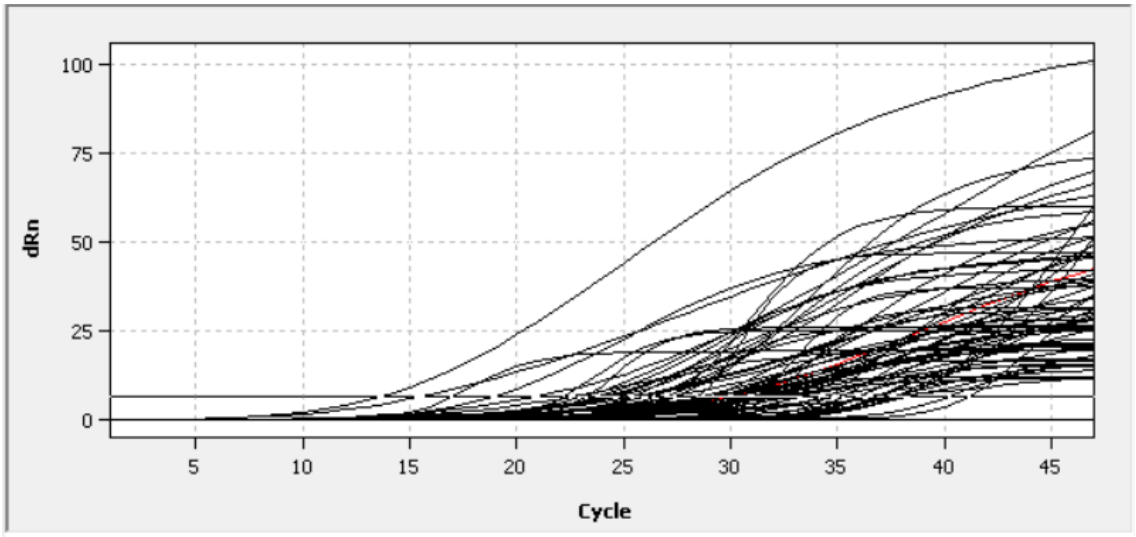

Figure 22: Linear Ct output from the qPCRsoft software used to analyze the RT-PCR data

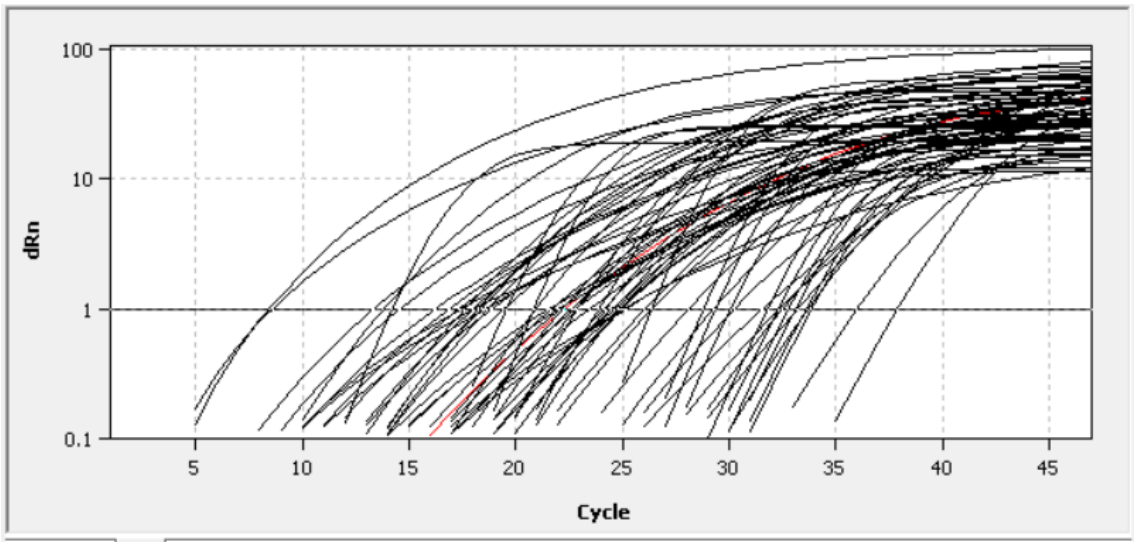

Figure 23: The exponential phase of the Ct (cycle threshold) from the qPCRsoft software used to analyze the RT-PCR data. The log Ct values are used in the $2^{-\Delta\Delta Ct}$ method. Noise is due to empty wells on the plate reader.

Figure 24 shows the gene expression relative to a 14 day static control utilizing the $2^{-\Delta\Delta Ct}$ method. The star indicates that there is a statistical significance between the experimental groups and the static control. Collagen type I (COL I) was examined as a tendon-related ECM gene marker to marker sure the mechanical properties were aligning in the preferred direction. All groups except the slow group showed an upregulation of COL I. Collagen type II is a non-tendon ECM related gene marker and was significantly downregulated for the Slow and Fast groups only. Only the Brief group showed a significantly downregulated instance for RUNX-2, a bone related gene marker.

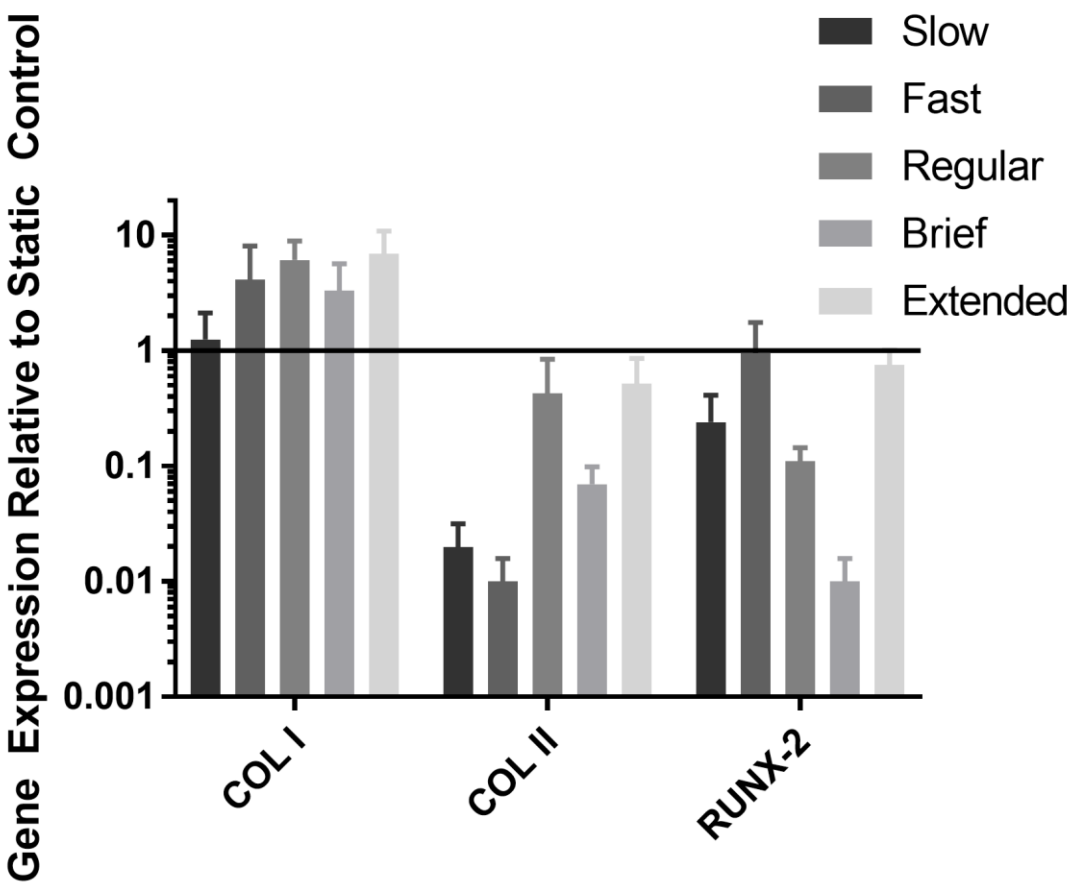

Figure 24: RT-PCR results for tendon-related ECM component (COL I), non-tendon related ECM marker (COL II) and non-tendon (bone) related differentiation marker (RUNX-2) for 14 day culture time points compared to a static MSC/HUV construct control. Data represented as a mean ± SEM. A sample size of n = 3 was used.

Discussion

The main goal of this study was to develop tendon grafts using decellularized HUVs seeded with adult stem cells and cultured under mechanical stimulation that permit the creation of tendon-like grafts with mechanical properties approaching the ones of natural tendons. Previous studies have used the inverted decellularized HUV and seeded MSCs in the hollow space in the cylindrical specimen. Such constructs cultured under mechanical stimulation demonstrated significant cell growth up to two weeks and major rearrangements on the ECM (evident by fiber alignment and improved mechanical properties). It is hypothesized that by varying frequency and duration of the mechanical stimulation on the HUV/MSC construct will affect the construct's properties. At early culture time points, mechanical stimulation is hypothesized to be more beneficial as the cells migrate throughout the scaffold and adapt to the HUV environment. Previously, this was explored for 7 day culture time points. This study investigates the effects of slower and faster frequency as compared to previous studies along with a shorter and longer duration of stimulation for up to 14 days of culture. Analyzing the cellular proliferation, extracellular matrix organization, mechanical properties, and gene expression of the cells present will help elicit the best stimulation parameter from the study.

The average cellularity was shown to significantly decrease between the non-decellularized HUV and after the final step of the decellularization protocol and storage in sterile PBS for two days. This confirms the documented results via figure 16 that the decellularization of the HUV had only minor disruptions to the ECM. Examining the differences between the luminal and the abluminal side of the HUV

(figure 17) confirms that the dense nature of the luminal side deters cells from penetrating. The Wharton's Jelly side however, has a lot more adequate spacing of collagen fibers to allow cellular movements showing the necessity of inverting the HUV contrast before seeding with MSCs.

Uniformity and distribution of cellularity throughout the MSC/HUV construct was accounted for by evaluating DNA quantification tests in three different sections taken from the top, middle, and bottom of the constructs and averaging the results. Previous results for 7 day culture stimulation groups showed significant increases in cellular proliferation against static controls for the slow and regular frequency stimulations. 7 day culture of the brief duration also showed significant increase in cellularity. For the 14 day culture groups, all but the brief showed a significant increase in cellular proliferation as compared to the 7 day culture groups. The slow group resulted in a 76% cellular increase as compared to the 7 days and the extended group increased by about 80%. Unlike the 7 day group, the 14 day brief stimulation did not have statistical significance when compared to the static control. This can be explained with other studies showing that too little stimulation can limit the beneficial effects of oscillatory motion on MSCs [112]. The fast group also did not increase much compared to the static control, this may be because the frequency of stimulation was beyond what the cells needed as they tried to migrate through the HUV construct, causing cell apoptosis. The slow frequency was significantly increased compared to the static control for the 14 day culture period.

A significant increase in cellular proliferation for 14 days of culture is also reflected in the histological analysis. Although similar to previous studies where full

penetration was eluded, mainly due to the luminal side of the HUV, the 14 day groups all were able to penetrate a majority of the scaffold (Table 4). Seven days of stimulation seem to not be enough time for significant improvements in mechanical properties to occur, whereas at the 14 day culture groups, variations among the stimulation changes is becoming more apparent. Mechanical stimulation clearly enhances the migratory capacity of HUVs through the Wharton's Jelly. This also begs the question whether it is the fibril thickness or the overall fiber alignment that is attributing to the strength of the tendon construct. Although would it really matter of the goal of the construct is to reach an appropriate strength to match a given tendon and augment the injury.

Fiber alignment and ECM deposition (Figure 21) is also affected by mechanical stimulation on the MSC/HUV construct. The only statistically significant increase in anisotropy for the 14 day culture period was the extended group with a 186% increase compared to the 14 day static control. The static control (figure 21-A) fibers had a random orientation and a less dense alignment as compared to the extended duration (figure 21-C) which produced much thicker and aligned fibers. This can be attributed to the significant increase in cellularity and upregulation of collagen type I (figure 24). The extended duration is also giving the cells more access to migrating through the collagen fibers of the Wharton's Jelly assuming that the pull on the construct is causing cells to pull from one fiber to the other and increase collagen secretion. The other 14 day stimulation groups had fibers that were not as dense as the extended and were not statistically different than the static control in terms of anisotropy. All stimulation groups showed a significant upregulation in COL I

expression excluding the slow group. This can be due to some noise in the slow sample as previous 7 day results showed a significant upregulation of COL I and the slow group significantly downregulated the non-tendon ECM gene marker (COL II). A bone gene (RUNX-2) was analyzed as a non-tendon type gene marker showing that all the stimulation groups were downregulated compared to the static control. The fast group barely downregulated the RUNX-2 gene indicating that the increased stimulation frequency has the potential to cause unwarranted effects of shear on the cells. In contrast, the brief group significantly downregulated the RUNX-2 gene showing that the short duration did not cause the cells much shear stress.

The increase in cellularity, Extracellular matrix reorganization, and cell migration through the MSC/HUV construct were accompanied by an increase in the mechanical properties of the constructs. As previously reported, there are no significant increases in ultimate tensile strength after 7 days of stimulation for any of the stimulation groups. Most likely this is due to cells adjusting to the new environment. However, at 14 days, the only significant increase in ultimate tensile strength as compared to the static control was the extended duration. This can be attribute to its increased cellularity and being the only group to have a statistically significant increase in fiber alignment. The combination of stretching and having cells present in the HUV show a significant increase in mechanical properties. After 14 days of stimulation, the extended duration in this study had an ultimate tensile strength value (3.28 ± 0.63 MPa) nearing the range of the posterior supraspinatus (rotator cuff) tendon (Avg 4.1 ± 1.3 MPa).

Although the tendon construct appears stronger, due to the increased cellularity and fibril thickness, it is able to withstand a greater fore than the other groups. This is important due to the need to reach an ultimate tensile strength as near to native tendons as possible, with the goal being to create a graph that can augment an injury. Augmenting a tendon injury with a scaffold close in mechanical properties will help the healing process and allow the native tendon to retain a greater amount of its original strength. With this in mind, *in vivo* studies will be necessary to examine the long term potential of this tendon construct. While significant results were seen up to 14 days by varying stimulation parameters, it is still necessary to continue culture successfully for up to 28 days before moving into *in vivo* studies. With continued optimization of culture parameters, the results are promising for a tissue engineered tendon replacement.

Conclusion

The results clearly show the ability to change the mechanical properties of the MSC/HUV construct by varying the frequency and duration of stimulation. After 14 days of culture for various stimulation groups, an increase in cell number compared to the 7 day groups was measured for all groups excluding the 14 day brief duration. Mechanically stimulating constructs for an extended duration increased the proliferation rate of MSCs and increased the fiber alignment and fiber thickness, giving the construct a tendon like appearance. Cell growth tends to directly contribute to the extracellular matrix quality reflecting the upregulation of COL I, a tendon-like ECM gene marker. Moreover, mechanostimulation resulted in the extended group having a significantly stronger and stiffer construct when compared to its 7 day and 14 day static counterparts. Overall, longer durations such as extended and the regular stimulation allowed for increased cellular proliferation while maintaining the mechanical properties necessary to promote tenocytic differentiation past 14 day culture periods.

Future Works

The goal of this study was to optimize the stimulation parameters for up to 14 days of culture. While it was found that the 14 day extended duration significantly increased the mechanical properties of the construct, the 7 day data showed that the regular stimulation had the best increase in cellularity. With the goal of increasing the mechanical properties further at the 14 day time point, it would be worth investigating combining the parameters for the 7 day and 14 day groups by stimulating the MSC/HUV construct at the regular stimulation for the initial 7 days, then switching it to the extended for the final week and analyzing the results. The 14 day slower frequency also had increased cellular and mechanical properties despite not achieving as high results as the extended. This could be investigated by stimulating a construct by combining the slow frequency and at the extended duration. It is also necessary to include a negative control (constructs stimulated without cells) to further show that it is the cells influencing the overall fibril realignment. Further improvements could include the use of an Alamar Blue stain that will show a closer approximation

If either of those stimulation groups pans out, it would also be worth exploring the effects of adding growth factors to the culture, such as TGF-beta, on the tenocytic differentiation capabilities past the 14 day culture period and how it may impact construct development.

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Appendix A: Extracting Stem Cells from Rats

Supplies:

- 2x 2 blue towels packs
- 2x gauze (3 stacks) packs
- 2 basins (2 packs)
- 1 plastic container – scissors, skin tweezers, normal tweezers, 2 scissor clamps, small scissors, small tweezers
- 5x 5 mL syringes w/ α-MEM
- MSC Extraction – 250 mL beaker, scissor clamp, small scissor, bone cutter, medium tweezers
- Bone media – α-MEM with antibiotic

Prepare α-MEM

1. Filter Medium BEFORE adding antibiotic and FBS
2. Store the extra 100 ml medium without antibiotic. Will be used to store bones.
3. Add FBS and antibiotic to filtered medium

Preparing the site for Dissection

1. Clean table with soap and water
2. Wipe with alcohol
3. Cover a small area (for surgery) in the middle of the table with aluminum foil
4. Place two blue absorbent pads on top of aluminum foil
5. Use one more blue pad to cover the rat
    a. Cut a circle in the middle of the pad big enough to see dissection part (leg) of the rat
6. Place one blue pad to right of dissection site. This is used to place dissection tools on
7. Fill up glass beaker with alcohol and put dissecting tools in it
8. Fill on white bowl with alcohol
9. Fill the other white bowl with iodine

Rat prep:

1. Retrieve rat
    a. Let rat rest for at least one day after delivery before performing this procedure. 3 or more days is best.
2. CO_2 asphyxiation

    a. Plug tube in gas chamber
    b. Close the CO2 supply
    c. Place rat in chamber (one at a time and do not let the other rats see)
    d. After about 6 minutes in chamber, feel for heart beat
3. Shave hind quarters
4. Soak in ethanol – about 5 minutes while prepping other stuff
5. On bench – Paper towels, then foil, then blue sheet, then rat, the blue sheet
6. 1 basin is triodine, 1 basin is EtOH
    a. Container is 70% EtOH
    b. Spray packs as though area is sterile
7. Iodine entire area around leg, wash with EtOH – 1 pass per gauze with EtOH wash
8. Cut about a thumb width in corner of top of blue towel

Surgery:

9. Use hand to stretch skin, start at the top of the hip, use constant pressure, keep blade straight and go to ankle/foot
10. Make sure to handle all instruments aseptically
11. Peel skin away (filet it)
12. Cut down on white line from hip to knee (femur)
13. Cut meat away from bone
14. Cut ligament at hip to open it up then cut around to free it
15. White line is tibia, cut around pop it up
16. Break at joint next to heel
17. Use back of blade or gauze to peel flesh
18. Once free, pop knee off
19. Little meat on top is fine, going to cut below spur
20. Put in bone media
21. Repeat steps 8-18 with new blade on other leg
22. Engulf the rest of the rat with gloves and place in biohazard freezer

Extracting Bone Marrow from Bones:

NOTES

- Usually you flush cells (drill through bone using syringe) from the part of the bone that forms the knee. You cut the other end so that bone marrow is flushed out of the end that is away from the knee
- Flush all bone marrow from all rats into one falcon tube then aliquot in several T-75 flasks. This way you will get an equal number of cells from all rats.
- Each rat makes 4 to 5 T75 flasks

Materials

1. Blue stand for falcon tubes
2. Falcon tube with bones (2 tibia and 2 femur per rat)
3. One empty falcon tube per rat
4. Needles filled up with media
5. α-MEM
6. Pipettes
7. Four T75 flasks per rat
8. Sterilize
    a. Two blue absorbent towels, two tweezers to hold bones, two bone cutters

PROTOCOL

1. Work above absorbent towels
2. Use scissor clamp to hold
3. Get rid of remaining flesh
4. Open the falcon tubes
5. Hold the femur with tweezers and cut the large part filled with tissue
6. Stick the needle on the other side and drill until the bone marrow become visible
7. Flush media in bone until marrow falls into falcon tube
8. For tibia, cut the small end and flush through the large end
9. Break up marrow with plastic pipette and remove bone fragments
10. 4-5 flasks for all marrow
11. Change media after 4 days – be very gentle, do not use PBS on first feeding because the cells are still loosely attached
12. For second change – use PBS to wash hematopoietic cells away
13. When ready to be split – about 1 week or so, do 1:5 split

Appendix B: Preparing Veins and Lathing

Supplies

- Steel Mandrels
- Glass Rod
- Paper Towels
- Styrofoam Container
- Decontamination supplies (bleach, detergent, water, ethanol)
- PPE – Lab coat, face mask, goggles, double gloves
- WD-40

Procedure

1. Put on PPE prior to working with veins.
2. Cut vein about 1 inch away from placenta and also cut off about 1 inch from the other end.
3. Measure and cut to length, use tubing guides in vial as an estimate, about 8.5-9 cm.
4. Insert glass rod into vein and feed it through.
5. Use the vein/rod to slip the vein onto the stainless steel mandrel
6. Straighten and stretch veins as much as possible.
7. Roll veins individually in paper towels.
8. Group 4-5 veins in a paper towel.
9. Place veins in a Styrofoam container and place into -80^{o}C freezer in D214.
10. Allow to freeze overnight prior to lathing.
11. Clean working area that was near blood with bleach, detergent, water, and ethanol. This will include the sink and most likely biohood. Discard any biological waste in specialized waste bags.
12. When ready to lathe, cover surrounding lathe area with paper towels to contain mess.
13. Open up the software and load appropriate program.
    a. Go one directory above cnc (Turn Master Pro directory) and open Brandon folder
    b. Program will be in there (0.75 cm thickness)
14. Set up lathe and place blade next to an empty mandrel (basically touching) manually using knob in front to set z direction initially. Make sure computer control is off!
15. Turn on computer control and use the software to jog the blade to the left, near the chuck.
    a. Control menu → Jog Tool
16. Set origin to the bottom and left in machine parameters.
    a. Options menu → Machine Parameters

17. Initialize this as the zero position.
    a. Control menu → Init As Start Position
18. Manually move the blade out 5 cm in by typing in +5 in the z direction box of moving blade.
    a. Control menu → Move to point
19. Initialize as zero position again.
    a. Control menu → Init As Start Position
20. Run the program once to ensure proper operation. Use the menu or F5 key shortcut
21. Take a pack of 4-5 veins out from the freezer.
22. Remove the paper towel from a vein as best as possible, don't worry if some is stuck and place into the lathe, tighten chuck and cone to secure the mandrel.
23. Place the splatter guard and turn on rotation.
24. Start the program and allow the lathe to complete the program, ensuring that the motor, blade, and process operate properly.
25. Take out vein and repeat for as many as needed.
26. Once finished, clean lathe. Place any waste that touched tissue or has blood in biological waste bag.
27. Wash the blade, blade holder, guard, cone, and any tools with soap and water.
28. Wipe out any tissue residue from the lathe with paper towels.
29. Once finished, clean lathe with soap and water. Dry thoroughly.
30. Spray a towel with ethanol and wipe lathe.
31. Lubricate with WD-40 or a grease as needed.
32. Place waste bag in refrigerator in D211.

Appendix C: Processing Veins and Decellularizing

Supplies

- DI Water – Autoclaved, 4 bottles
- Sodium Dodecyl Sulfate (SDS)
- Peracetic Acid – Light sensitive keep out of light as much as possible.
- Ethanol (95%)
- 10x Phosphate Buffer – Autoclaved, 1 bottle

Procedure

- Prior to decellularization process cords as needed.
    - If keeping as a cylinder, use special long, wire grippers to invert vein so that the Wharton's jelly side is inside (rougher) and vein is outside (smooth).
        - Cut to about 6.5 cm in length
    - If using flat veins, cut open lengthwise and trim to desired length, about 5 cm.
- If you have more than 5 pieces of tissue, use 1 L bottles (750 mL of liquid), otherwise use 500 mL bottles (375 mL of liquid).

Washing Steps

1. SDS for 24 hours
    a. 750 mL: 7.5 g of SDS in 750 mL of DI water
    b. 375 mL: 3.75 g of SDS in 750 mL of DI water
2. DI water
    a. Wash for 10 minutes, 20 minutes, 30 minutes, and then 24 hours
3. Ethanol for 24 hours
    a. 750 mL: 600 mL of 95% ethanol and 150 mL of DI Water
    b. 375 mL: 300 mL of 95% ethanol and 75 mL of DI Water
4. DI water
    a. Wash for 10 minutes, 20 minutes, 30 minutes
5. Peracetic Acid for 2 hours
    a. 750 mL: 720 mL of DI water, 30 mL of 95% ethanol, and 1.5 mL of peracetic acid
    b. 375 mL: 360 mL of DI water, 15 mL of 95% ethanol, and 0.75 mL of peracetic acid
    c. Once cords are in bottle and shaking, cover to protect from light.
    d. Afterwards, cords are now sterile and following washing steps must be done under the bio-hood with sterile instruments (long tweezers). Cords will look white.
6. DI Water

    a. Wash for 10 minutes, 20 minutes, 30 minutes, and then 24 hours
    b. After 24 hours and transfer to next step, check pH of water.
7. 10x Phosphate Buffer
    a. See phosphate buffer protocol for recipe
    b. After 24 hours, check pH to make sure between 7.2-7.4. If not, wash for another 24 hours in new 10x phosphate buffer.
8. Store cords in autoclaved 10x PBS in refrigerator. Use within one week.

Appendix D: Setting Up Tendon Bioreactor

Notes

- See pictures for bioreactor, scaffolds with clips, double hook screws joined together, the bioreactor top (hook screw joined with glove finger)

Supplies

- Bioreactor
- Inlet and Outlet Tubing
- Media change tubing with stopper for incubator.
- Latex gloves (large)
- Zip Ties
- Zip Tie gun
- Stainless steel clips (I've had good luck with badge clips from Hobby Lobby, they didn't rust recently, however, prior ones had, so test this out before you use them)
    - May want to see if we can buy or make some custom reusable ones to avoid this and possibly not even have to use paper clips
- Non-rusting paper clips (Plastic coated ones work well)
- Hook screws
- Cells
- Media
- PBS
- Trypsin
- Petrie Dish – autoclaved, 1 petri dish per 3 cords, and one extra for working
- 2 x 100 mL Beakers – autoclaved
- 2 x round nose tweezers - autoclaved
- 0.2 µm filter
- Force meter
- 2 x media bottle with two arms.
- Peristaltic Pump
- Ethylene Oxide Ampule

Sterilizing Equipment

33. In one large autoclave bag place bioreactor. Cover the tops and tubing ends with aluminum foil. Be careful as these are fragile.
34. In one bag place media reservoir and cover with foil. Also place breathing tube (short tube on one arm of reservoir) in bag to sterilize.

35. In another bag put the inlet and outlet tubing. Cover pipette tips and tubing ends with foil. On the syringe end of the stopper, wrap a paper towel around it to prevent puncture of the bag.
36. Put the media feed tube in another large bag. Include y-fittings and tubing if doing two bioreactors. Cover with foil.
37. Take two hook screws and a nut and use the nut to join the two screws together. This will attach the top of the scaffold to the bioreactor.
38. Cut off the fingers of a latex glove cut a slit in the tips. Slip one of these over the combined hook screws. Place a second glove finger over the first and zip tie below the first to ensure a seal. Need 3 of these per bioreactor.
39. You need two clips per scaffold. Place a non-rusting paper clip (to attach to hook) on one clip for each scaffold. Leave the other clip not attached (this will attach to the top).
40. Place the other adapters and screws into a small autoclave bag. Include enough zip ties for both ends.
41. Place some gloves into autoclave bags to help when putting clips on scaffolds in a sterile environment.
42. Put equipment in the chemical sterilizer and sterilize for 24 hours.

Calibrating Stimulation

1. Hook up force meter to bioreactor.
2. At rest should be at about 0.5 pounds force.
3. One cycle should be 1 lbf. This equates to 5% strain initially. With plastic deformation due to constant stretch at rest, this is 2% strain.
    a. At highest force stretch, meter should be at 1 lbf.
    b. At lowest force, meter should be at 0 lbf.
    c. Adjust these on Amplitude variable knob on Wavetek.
        i. Should be on -40 dB and use small knob to fine tune. Can adjust to -20 or other If needed.
4. Calibrate frequency by counting how many cycles/min (0.5, 1, 2, etc.)
    a. Adjust these using large start freq knob on the top left of the Wavetek. This should be enough, if not, can adjust using Freq (Hz) Vernier knob further.

Preparing Cords

1. Slip a vein onto the small glass rod, using the petri dish to stabilize.
2. Slip on an adapter on one end and zip tie into place. Do the other size.
3. Remove from the rod, and on one side, using both tweezers, screw a hook into an adapter.
4. Place the cord in a petri dish and cover with media. Repeat with the other two.
5. Allow to sit while preparing cells.

Preparing Cells

1. Lift cells normally, with enough cells as needed. Typical amounts are 1 or 3 million cells/mL collagen solution.
2. Once you have a cell pellet, dissolve in 2 mL of collagen solution.
    a. For 2 mL of solution:
        i. 0.8 mL collagen
        ii. 0.2 mL 10x PBS
        iii. 0.02 mL NaOH
        iv. 0.98 mL sterile water

Seeding Veins

1. Using a 1 mL pipette, inject 0.6 mL of collagen/cell solution into the vein. It may not all fit, go slowly and coax it in.
2. Seal the open end with another hook adapter and place back into petri dish.
3. Repeat this with the other cords.
4. Place in incubator and allow to incubate for 1-2 hours.

Assembling Bioreactor

1. Set up bioreactor on tray in biohood in D214 next to tall incubator without motor.
2. Attach tubing to bioreactor and reservoirs. Wait for media feeding tube.
3. Fill reservoirs with 270 mL of media and place breather tube on reservoir with filter attached to allow for extra oxygenation.
4. Using sterile gloves and tweezers, attach a clip to each side of the scaffold.
5. Place into bioreactor and cover with glove fingers. Repeat with other two.
6. Pump media into bioreactor and until the circuit is full of media. Do not go more than 1 mL/minute as cells are exposed to the flow now.
7. Stop the pump and clamp hoses to prevent draining.
8. Attach media feeding tube to reservoirs, leave other end covered with foil.
9. Move bioreactor to incubator, quickly place the other media feeding tube ends back into biohood and into media bottles.
10. Place motor on tray and screw bioreactor into the base.
11. Hook up circulation tubing to pump and circulate media at speed 1.
12. Hook cords onto triangular plate and attach to motor.
13. Close incubator, turn on CO_2, and allow to equilibrate, usually an hour or so.
14. Run desired stimulation.

Appendix E: Changing Media for Tendon Bioreactor

Supplies

- α-MEM
- 2x 250 mL media bottle with spouts
    - One bottle for emptying and one bottle for filling

Procedure

1. (Optional) If running 2+ bioreactors, make sure that the split media change tubing is clamped for one of the reactors
2. Fill one bottle with 180 mL of fresh media.
3. Turn media change pump on reverse to remove media, set to max flow
4. Clamp the tubing between the two reactors to isolate feed and return.
5. Remove all media from outflow bottle or until media is low on the fill bottle of the reactor
6. Increase the flow rate of the bioreactor to speed 3
    a. Do not do this if running bioreactor with “flat” tendons exposed to media flow
7. Pump the 180 mL of fresh media into the reactor
8. Shut of media filling pump
9. Remove clip between bioreactor reservoirs
10. Reduce flow back to speed 1 on reactor pump.
11. Repeat for other bioreactor if needed.

Appendix F: Preparing Tissue for DNA Assay

Notes:

- Make sure to filter Collagenase

Materials:

- 24 well plate
- Sharp end Scissors

Protocol:

1. Cut 0.5mm of tissue and place in 24 well plate
2. Chop into little pieces using scissors
3. Add 2ml of collagenase (200 units/ml = 0.21 mg/ml) in each well
4. Incubate overnight at 37^{o}C
5. Sonicate for 5 seconds
6. Add 200 ul of Trypsin 10X (0.5%) to each well
7. Incubate at 37^{o}C for 1 hour
8. Sonicate for 10 seconds
9. Apply 2 freeze thaw cycles
10. Test for DNA

Appendix G: Histology

NOTES:

- Make sure new 100% that has not started collecting water/diluting
- Extra changes of 100% is to make sure sample is completely dehydrated and no excess water
- Extra changes of Paraffin is to remove all of the excess Xylene
- If a step is done repeatedly, use new solution each time
- Glass vials work well to hold solutions
- If orientation is important, place in cassettes and use petri dishes instead of vials
- Use 95% ethanol to make diluted ethanol solutions
- Make sure 100% ethanol is sealed well to maintain purity
- All steps at room temperature unless noted

Supplies

- Glass vials
- 95% Ethanol
- 100% Ethanol
- Formalin
- Clear Rite 3
- Paraffin
- Molds
- Cassettes

Protocol

1. Place tissue in 10% Formalin for 1 hour
    a. If not processing immediately, leave tissue in formalin over night and store in refrigerator in 70% ethanol for long term storage
2. Place tissue in 85% ethanol for 1 hour
3. Place tissue in 95% ethanol for 1 hour
4. Place tissue in 95% ethanol for 1 hour
5. Place tissue in 100% ethanol for 45 minutes
6. Place tissue in 100% ethanol for 45 minutes
7. Place tissue in 100% ethanol for 45 minutes
8. Place tissue in Clear Rite 3 for 1 hour at 45ºC
9. Place tissue in Clear Rite 3 for 1 hour at 45ºC
10. If not already in a cassette, place tissue in cassette
11. Place tissue in Paraffin for 2 hours at 60ºC in heated vacuum chamber in D211

    a. Stir every half hour to ensure mixing and thorough penetration of paraffin
12. To prepare for embedding, place a small layer of melted paraffin in mold and allow to stiffen, but not completely harden
13. Take tissue out of paraffin bath and cassette. Place tissue in desired orientation in the mold, using the stiff paraffin to hold it.
14. Fill mold with paraffin
15. Place cassette base in mold securely
16. Let sample cool on counter or freezing table in D211
17. Remove from mold when completely hardened. Prior to sectioning, place in freezer to cool.

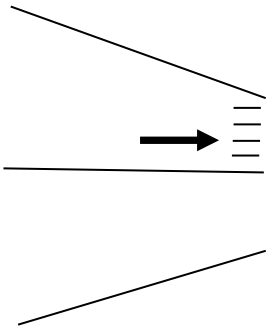
Appendix H: Sectioning Embedded Samples with Microtome

NOTES

- Cut section to a 7 µm thickness
- Use one half/side of the blade to cut to conserve blades
- If rips are occurring, replace blade
- Water bath should be at 37-40 ^{o}C
- Use pencil to write on frosted slides
    - Sharpie will be removed by solvents used in histology
- If paraffin is too warm, it will curl and not cut well
- Use Kimwipes to clean water bath if needed of extra sections

SUPPLIES

- Histology Blades (Replace if needed)
- Kim Wipes
- Large Paint Brush
- Small Paint Brush
- Tweezers
- Water Bath – Tap water
- Drying Oven
- Frosted Slides
    - Regular slides work too if frosted not available



Blade Angle Diagram

(Recommended)

Protocol

18. Put blade/blade holder in microtome and adjust to desired angle.
19. Flip engagement lever to the right to adjust stage manually
20. Move stage back completely
21. Put sample in the holder
22. Adjust thickness to appropriate levels
23. Move sample down to blade level
24. Move blade to position right before sample
25. Move sample to the top
26. Lock stage in with engagement lever

27. Rotate handle using smooth, fast motions
28. Once cutting, don't go too fast, and continue to use smooth operation
    a. If too fast or sample catches, it can break the paraffin block off the holder
29. Use small paintbrush to hold sample ribbon as it is cutting
30. Once into sample, take a section of ribbon and put into water bath
    a. Use small paint brush to end of ribbon
    b. At the sample, use tweezers to grab and move
31. Once in water bath, allow for smoothing of the ribbon if crimped from cutting
32. Use the edge of a slide to break ribbon into appropriate size sections
33. To mount on to slide, dip slide underneath the section of ribbon and fish it onto the slide
34. Lay slide on paper towel and allow to air dry for half hour
35. Remove any excess water with Kimwipes
    a. Excess water may bubble when oven drying
36. Label slides, place into metal holder, and place into oven set at 45^{o}C
37. Leave overnight and remove for staining.

Appendix I: Hematoxylin and Eosin Staining

NOTES

- Histology jars in D211 hold between 200 and 250 mL of solution
- If only performing 1 or 2 samples, coplin jars are available.
- When transitioning, dip slides a couple time before letting rest in solution to coat slides better, especially important when going to different types of solutions
- If mounting medium is too thick, cut with clear-rite to dilute, but don't dilute too much
- If hematoxylin has particulate on top, skim with kimwipe to remove
- If eosin isn't staining well, add a drop or two of glacial acetic acid to improve
- Process can be optimized to individual samples if need be
- Anything after color is tap water
- Acid alcohol is cleaning agent
- Ammonia water is a bluing agent for hematoxyline
- Filter your hematoxylin routinely
- Rotate solutions periodically and change one at a time
- Clean out and replace all solutions every 6 weeks
- Eosin lasts about a week

MATERIALS:

1. metal slide container
2. oven at 62-63˚C and another one at 45˚C
3. Acid Alcohol→3960ml of 70% alcohol and 40ml of HCl
4. Ammonia Water→10 ml ammonia hydroxide and 3984ml DI water
5. DI and tap water
6. 5 containers with Xylene, 3 with 100%, 2 with 50%Xylene/50%Alcohol, 3 with 95%, 1 with 70%, 1 with 80%, 1 with Hematoxyline, 1 with Eosin, 4 with DI water, 1 with tap water
7. Timer of some sort

PROTOCOL

1. Mount tissue onto slides from 38˚C water bath
2. Put slides into metal slide container and place into oven
3. Bake slides 25 minutes at around 62-63˚C (a few points above melting point of paraffin which is 55-57˚C)
4. Wait a little while after the oven for the slides to cool down
5. Xylene 3x3min
6. 50% Xylene/50% Alcohol 1 min
7. 100% alcohol 1 min
8. 95% 1 min

9. 80% 1 min
10. 70% 1 min
11. DI Rinse
12. Hematoxyline 5 min
13. Tap water Rinse
14. Acid Alcohol 1 min
15. DI Rinse
16. Ammonia water 1 min
17. DI Rinse
18. Eosin 1 min
19. DI Rinse
20. 95% 2x1 min
21. 100% 2x1 min
22. 50% Xylene/50% Alcohol 1 min
23. Xylene 2x1min
24. Bake overnight at 45 ˚C

MOUNTING

- Put a few drops of Acrymount (mounting medium)on slide and gently place cover slip on top so there are no air bubbles
- Wipe of extra mounting medium with gauze
- Leave at room temperature overnight
- You can add some xylene to mounting medium to thin it but not too much otherwise may lose staining or it may not mount

Appendix J: Tensile Testing Protocol

NOTES:

- The Tensile Machine is located in Room C214
- Only use during evening hours to not disturb the Grady Lab

MATERIALS:

1. Gloves
2. Your samples
3. Extra clamps if you need them
4. Calipers or a ruler to take measurements

PROTOCOL:

1. Power on Computer and Monitor
    a. Click on admin on computer, no password
    b. Computer is a mess, everyone uses it (bring new mouse)
2. The clamps are on an airline, the Foot pedal- down closes, button in back you kick to open
3. Power on Machine
    a. Togs moves clamps
4. Go to DATUM Program
    1. Q exits, foot petal operates clamps
    2. Window pops up, go to specimen prep
    3. Go to specimen prep
        a. Open, find
    4. Brandon Tensile on HUV, double click, should open a new screen
        a. Before specimen testing clicked, load tissue samples into machine
            i. Use calipers to know the distance between clamps/ a ruler to know the distance being stretched
            ii. Wear gloves
            iii. As much of tissue out as possible
            iv. Just enough in clamp to hold in place
            v. Hold onto samples when you hit foot pedal, it does not always lock
        b. Ok
    5. Before “To Specimen Testing’ load cords into machine
        a. Measure widths and thickness of samples (length)
    6. No go to specimen testing
    7. Hit “E”- to select a channel, “3”-this is the channel that we are using, then “Q” resets everything

8. “T” clears what was there, then “T” again starts the process. There should be a pre-stretch cycle. Won’t work well if not clamped correctly or is non-homogenous material
    a. Pre stretch flexes the material
    b. Takes out inelastic deformation to begin with so you have your actual starting and go from there
9. “ enter button“ resets- open clamps, to return, comes back to where it was
    a. Creating a crv file, which saves data then will make an excel sheet
10. “O” once, puts some markers on it, hit “O” again, now done
11. “Q” kills it and takes you back to specimen prep
12. Data just in software still
13. Click Graph, export curve button will now appear, choose graph, then export curve
    a. hit graph, graph will pop up
14. now can put in new specimen and then re-hit specimen prep
15. Desktop, shortcut to explot, top file
16. Close out of everything when done
17. Click shortcut to explots, default sticks everything
    a. Make a folder to save files, copy paste it in

Appendix K: RNA Isolation

SUPPLIES

- Tissue Grinder
- DNase/RNase Free Water
- Trizol
- Chloroform
- 100% Ethanol
- 100% Isopropyl Alcohol
- DNase/RNase Free PCR Micro-centrifuge Tubes and Pipette Tips

PROTOCOL

1. Autoclave any glassware/equipment needed. This may include beakers, tweezers, tissue grinder, scissors, etc.
2. Add 1ml per 50-100mg tissue of trizol. If using grinder, put this into the tissue grinder and homogenize the tissue
3. Allow to incubate for 5 minutes at room temperature to degrade tissue further
4. Split solution into 1ml aliquots in centrifuge tubes. Add 200 ul of chloroform to each tube, cap, and mix by rigorous inversion for ~15 seconds
5. Incubate for 2 minutes at room temperature
6. Centrifuge at 12,000 RPM's for 15 minutes at 4 degrees Celsius
7. Pipette the clear, aqueous phase into a new centrifuge tube
8. Add 500 ul of isopropyl alcohol to the centrifuge tube
9. Incubate at room temp for 10 min
10. Centrifuge at 12,000 RPM's for 10 minutes at 4 degrees Celsius
11. Remove supernatant leaving pellet at the bottom. This pellet may be small and/or clear- sometimes hard to see
12. Wash the pellet with 1ml 75% ethanol made with DNase/RNase free water and 100% ethanol.
13. Vortex sample and centrifuge at 8,000 RPM's for 5 minutes at 4 degrees Celsius. Pellet may now be a more whitish color after this step
14. Remove supernatant and allow pellet to air dry for 5-15 minutes. Do not use vacuum.
15. Add DNase/RNase Free Water to desired concentration
16. Dissolve pellet by incubating at 55-60 degrees Celsius for 10 minutes
17. Mix solution by vortexing
18. Pellet is not ready for reverse transcription or stored at -20 degrees Celsius