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Design and Validation of a Cyclic Strain Bioreactor to Condition Spatially-Selective Scaffolds in Dual Strain Regimes

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DESIGN AND VALIDATION OF A CYCLIC STRAIN BIOREACTOR TO CONDITION SPATIALLY-SELECTIVE SCAFFOLDS IN DUAL STRAIN REGIMES

by

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DEDICATION

I would like to dedicate my thesis to my wife, Mandy, and my parents, Greg and Lisa Goodhart, for your constant love and encouragement over the years. Thank you so much for continually supporting me and giving me the strength to persevere.

ABSTRACT

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The objective of this study was to design and validate a unique bioreactor design for applying spatially-selective, linear, cyclic strain to degradable polymeric fabric scaffolds. Image analysis of polyethylene terephthalate (PET) scaffolds subjected to 3% mechanical stretch showed that the stretched and unstretched portions of the scaffolds experienced 2.97±0.13% and 0.02±0.18% strain respectively. NIH-3T3 fibroblast cells were cultured on PET scaffolds and half of each scaffold was stretched 5% at 0.5 Hz for one hour per day for 14 days in the bioreactor. Scaffolds were assayed for cell viability and proliferation as well as glycosaminoglycan (GAG) and hydroxyproline concentration. Bioreactor scaffolds showed a 700% and 75% increase in DNA and hydroxyproline concentration respectively as well as a significant decrease in GAG concentration over the control scaffolds. Surprisingly, little differences were seen between stretched and unstretched portions of the scaffolds likely due to the conditioned media effect.

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CHAPTER 1

INTRODUCTION

In 2004, musculoskeletal injuries accounted for 60% of all traumatic injuries in the United States [1]. The tendon-bone interface represents one of the most vulnerable locations of the musculoskeletal system and accounts for 38% of all musculoskeletal injuries; almost twice as many as bone fractures [1].

Tendon injuries are graded based on severity – grade 1, 2, and 3 [2]. Grade 1 injuries consist of an overstretching of the tendon with some interstitial tearing; grade 2 injuries include partial tearing of the tendon; grade 3 injuries are severe tears to complete separation of the tendon. Typically grade 3 injuries are the only ones to ever require any surgical intervention. Healing times vary depending on the severity of the injury, but full recovery of serious musculoskeletal injuries is expected no sooner than 6 months after repair – even with the best recovery and physical therapy [3]. Healing takes many months for these injuries due to the very low vasculature found in the bone-tendon interface as a result of the calcified transition zones [4].

1.1. Tendon Organization/ Function

Tendon is the primary mechanism of force transduction from the muscles to the bones in the human body [5]. Every muscle has a tendon attached at every origin and insertion to allow for solid fixture onto the bone. The largest and most familiar tendon is the Achilles tendon that attaches the muscles in the calf to the calcaneus in the heel. Healthy tendon is comprised of dense regular connective tissue [5]. Fig. 1 shows a photomicrograph of a tendon magnified 500 times. A tendon consists of many collagen fibrils bundled together to form larger bundles that eventually result in a single tendon.

Fig. 2 schematically shows the multi-level components of a typical tendon. Due to the alignment of the collagen fibrils, tendon is primarily uniaxial and resists tensile stretching, as its prime function is direct transmission of force from muscle to bone. To transmit muscle forces to bone effectively, tendons are well anchored to bone. Because bone is a more rigid material, there is a gradual transition between the tendon and the bone. This transition is divided into four zones. Zone 1 is collagenous tendon; zone 2 is uncalcified fibrocartilage; zone 3 is calcified fibrocartilage; zone 4 is calcified bone (Fig. 2) [4].

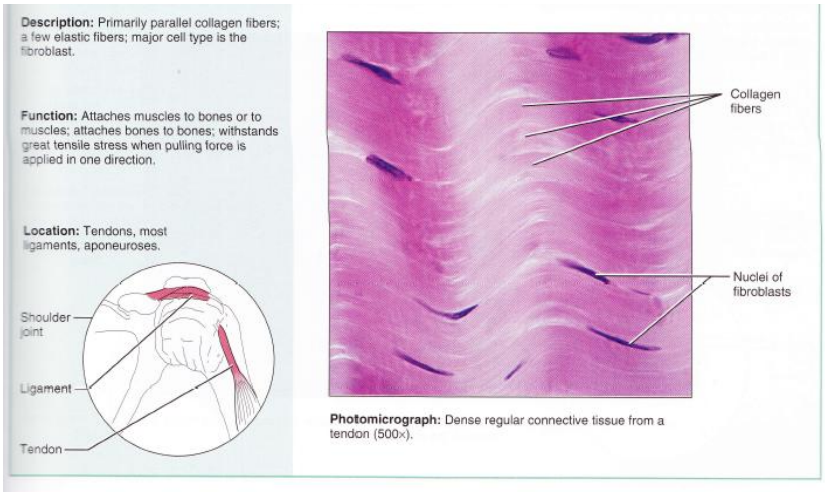

Figure 1: Photomicrograph of healthy tendon (500x). Depicts collagen fibers (translucent white) and fibroblasts (dark purple) [5]

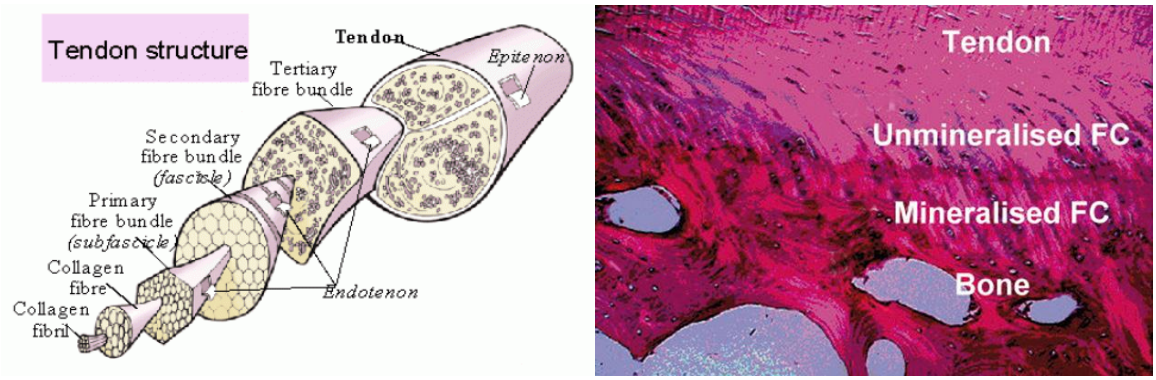

Figure 2: Microscopic structure of tendon (left) and the bone-tendon enthesis (right) [6,7]

Healthy tendon is 65-70% water; but, when dehydrated, 70% of its weight is type I collagen, 2% elastin, and 2-5% glycoproteins and proteoglycans. The remainder is tenocyte fibroblastic cells [7]. Glycosaminoglycans (GAGs) are a component of the proteoglycans found in tendon. Commonly found GAGs in tendon, such as decorin, Chondroitin-sulfate B, and Chondroitin-sulfate C, are responsible for the alignment and organization of the collagen fibrils [8]. During healing and regrowth, there are three stages: the first 72 hours are spent clearing the site of debris; days 5 through 28 are spent forming disorganized collagen; and after 28 days, the collagen is remodeled and realigned [9]. Therefore, it is expected that there will be a large increase in cell number during the stage 1 and the beginning of stage 2, a large increase in collagen during stage 2 plateauing towards the end of that stage, and an increase in GAGs beginning around days 14-18 [9].

A damaged tendon that is left unrepaired will lose most, if not all, of its ability to transduce force from the muscle to the bone. In a study performed by Mikolyzk et al, they investigated the histology of injured, steroid treated, and injured with steroid

treatment tendon in a rat model [10]. Fig. 3 shows the histological findings from their experiments. The differences between the injured tendon and the healthy (control) tendon are apparent. The tendon's elongated shape becomes much more compact as you see a much denser cluster of nuclei in the one week injury slide. After five weeks of healing, the collagen and fibroblast cells begin to have their elongated shape again. There tends to be a greater density of fibroblast nuclei at the injury site due to the body's natural reaction to strengthen the injury site.

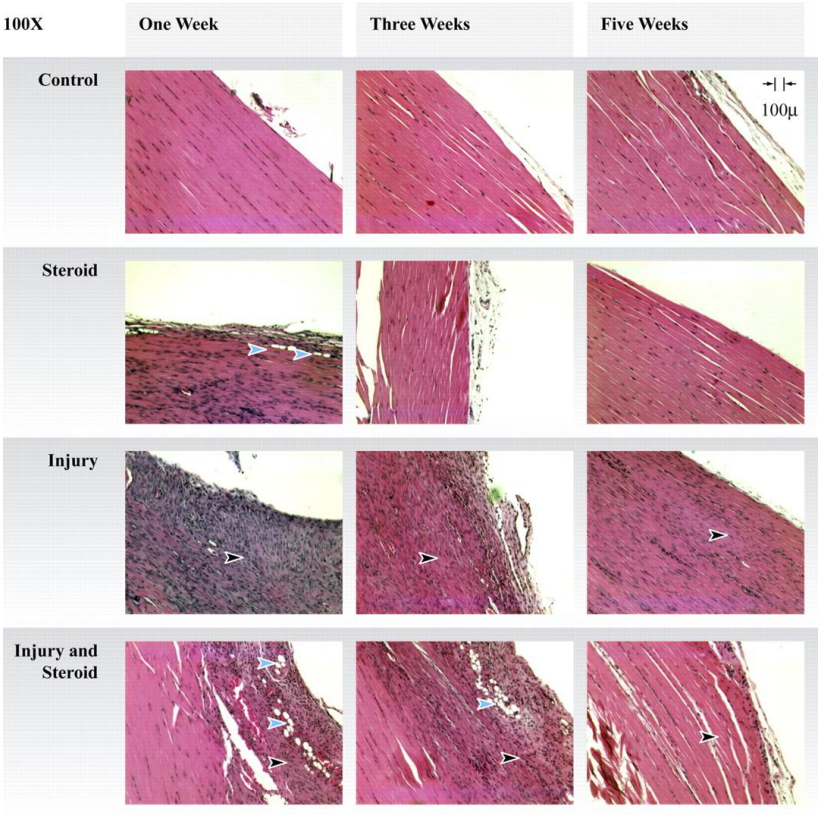

Figure 3: Histology of infraspinatus tendon in rat model performed by Mikolyzk et al. [10]

1.2. Current Treatment

When surgery is required, minor surgeries involve suturing the tendon together with 4-0 and 6-0 polypropylene monofilaments using a curved stainless steel needle and allowing it to heal [11, 12]. Sutures are satisfactory for minor tendon repair such as carpal flexor and extensor tendons, but are not satisfactory for major tendons such as the Achilles tendon or quadriceps tendon due to the large loads being transmitted by the larger muscle groups. Sutured tendons are able to withstand a tensile stress up to 0.6 MPa, which is far exceeded by the stresses experienced in the larger tendons [12]. Another greatly used repair technique involves using a bone tunnel to reattach damaged tendon [13-16]. Bone tunnels use bone plugs attached to tendon or tendon pulled through the tunnel and clamped to prevent pull-through [15]. Unfortunately, these methods are still far from perfect. Flexor tendon repair surgeries and Achilles tendon repair surgeries have been reported to have failure rates as high as 17% and 20% respectively [3,9]. As a result, major surgeries and recent experimental techniques now use tissue engineering to create scaffolds that aid in tendon repair [17-20]. Tissue engineering offers the potential for faster recovery and improved tendon repair over standard suturing. Results from tissue engineering studies look very promising, sparking a significant interest into the field of tissue engineering.

1.3. Tissue Engineering

One of the overall goals of tissue engineering is to use cells, materials, and biological substances to improve and repair biological function. This broad field spans from reconstructing heart valves to culturing new skin for burns to growing entire organs [21-22]. A specific, ongoing research focus in tissue engineering is repair and re-

attachment of the tendon at the bone-tendon enthesis [23-24]. To accomplish this re-attachment of the tendon, modified biomaterials are needed. Neo-tissue requires cells from which to grow, a scaffold or substrate to adhere to, and a medium to feed it [19]. Tendon is primarily comprised of tenocytes derived from fibroblasts and an extracellular matrix that is predominantly collagen [5].

Early attempts at implantable scaffold devices were similar to what was used by Joshi et al [23]. They were successful in growing fibroblasts on a Tecoflex® (medical-grade aliphatic polyether polyurethane) scaffold and improving the elastic modulus of the resulting hybrid. Improving the material properties of the implanted substrates is critical to alleviate stress on the repairing tendon so it is not reinjured. One must be careful though to avoid stress shielding that can actually hinder the effectiveness of the scaffold [23].

Other considerations taken were making scaffolds more biomimetic to improve tissue ingrowth and bond strength. Garvin et al. created bioartificial tendon that was from cultured tenocytes in a collagenous matrix to improve the growth environment on the flexible silicone rubber membrane [24]. The resulting tendon, while lacking in mechanical strength as compared to healthy tendon, improved the morphology and histology as evidenced by the genetic expression of collagen I, III, and XII, decorin, beta-actin, and tenascin. Collagen I, II, and XII are common collagen types found in tendon; decorin is a proteoglycan necessary for ECM organization; beta-actin is a type of actin useful for cell stability and motility; tenascin is a common glycoprotein found in connective tissue – especially tendon [24]. The presence of these matrix components,

such as collagen types I and II as well as decorin, is promising, as this experimental method has produced genetic expression for vital components to proper tendon function.

Because a second surgery to remove non-permanent fixtures would not be needed, engineers have been attempting to grow cells and tissues on degradable and non-degradable fabric scaffolds such as polyethylene terephthalate (PET), polypropylene, polyglycolic acid (PGA), and polylactic acid (PLA) [24-25]. As a result, recently, Deng et al. has experimented with growing human neo-tendon on a PGA scaffold [26]. Using a device to impart constant strain, they were able to culture human neo-tendon using human dermal fibroblasts. The resulting tissue was indistinguishable from the human tenocytes cultured from the same source. This achievement has allowed other researchers to use fibroblasts for tendon culture as opposed to tenocytes – fibroblasts being less differentiated and therefore more versatile [26].

1.3.1. Mechanical Stimulation

As evident from the study by Deng et al., mechanical stimulation was essential for creating healthy neo-tendon [26]. Early studies did not subject cells to mechanical forces, which may have limited organization/function of the engineered constructs. To simulate the mechanical factors in tendon development and organization, investigators have begun applying mechanical strain to engineered tendon constructs [27-28].

One of the most widely used bioreactors is the Flexcell Strain Unit that imposes a vacuum on a thin silicone elastomer membrane resulting in a strain on the cells adhered to the membrane [27]. Unfortunately, it is a very basic system that does not allow for segregated culture. Another very popular bioreactor used is the Bose Electroforce 3200 used by Butler et al., which, like the Flexcell system, does not allow for segregated

culture [29]. Because the bone-tendon interface contains many cell types, such as fibroblasts, osteoblasts and chondroblasts, and a variety of stress and strain magnitudes, many researchers have designed their own bioreactors to fit the desired stimuli for their engineered tissues [23, 27-28, 30-32]. For example, Titze et al. designed a system to vibrate fibroblasts approximately 1mm between 20 and 200 Hz, which is the type of stimulation experienced in laryngeal tissue during speech [30]. This system produced tissue containing elevated amounts of fibronectin, MMP-1, HA Synthase 2, CD 44, fibromodulin, and decorin; all of which are essential to vital laryngeal tissue. In contrast, Riboh et al. used entirely different mechanical conditions (4–8% strain at 0.1–1.0 Hz) to engineer flexor tendon-like tissue [27]. By applying intermittent cyclic strain, they saw increased cell proliferation over no strain and constant strain. Intermittent strain also increased total collagen I production, collagen I being the most prevalent collagen type in tendon [27]. Similar to Riboh et al., Saber et al. cultured flexor tendon using a bioreactor system with great success [33]. They applied a 1.25N load at 1 cycle/min for one hour alternating load and rest. Results of their study also confirmed better cell and collagen alignment. While these groups demonstrated great advances in neo-tendon construct engineering by growing organized tendon tissue *in vitro*, the bone-tendon interface is not addressed. In order for the engineered tissue to function properly, the tendon will need quality integration into bone.

1.3.2. Co-culture

In the case of injuries to the attachment site of a tendon to bone, such as tearing the tendon from the bone, two tissues are primarily involved – tendon and bone (as well as their associate cellular and matrix components). As a result, researchers have been looking into co-culture models using fibroblasts, osteoblasts, and non-living biologics. Wang et al. studied their recreation of the fibrocartilagenous region between the ACL and bone using fibroblasts and osteoblasts [34]. The two cell types were cultured on one substrate with an agarose barrier and after a week, were allowed to migrate into co-culture. The group studied the effects of this interaction and what effect various media additions, specifically ascorbic acid and beta-glycerophosphate (β-GP) would have on the cells. The co-culture yielded an increase in bone related alkaline phosphatase (ALP) activity and promisingly, an up-regulation of cartilage oligomeric matrix protein (COMP). Increased ALP activity signifies osteoblast activity and since the tendon-bone interface is comprised of zones of fibrocartilage, an up-regulation of COMP shows signs that a more effective attachment could be achieved. However, this study used an unsegregated co-culture with uncontrolled dispersion of cell types. Because the bone-tendon interface is comprised of 4 distinguishable zones, better segregation is desired [4].

1.3.3. Pre-conditioning

Pre-conditioning tissues on scaffolds significantly improves the performance of the engineered scaffolds [21,27,35]. Riboh et al. conditioned flexor tendons using constant and intermittent cyclic strain [26]. They were able to control collagen type 1 production and cell proliferation [27]. This phenomenon is also true for other tissues as well. Moon et al. demonstrated this pre-conditioning effect using muscle cells that, like

tendon, are very directionally dependent [35]. By cyclically straining the scaffolds during culture, the actin and myosin elongated and aligned in a parallel fashion in the direction of the strain as opposed to being balled and scattered. This idea of preconditioning was also used by Syedain et al. to engineer heart valves [21]. Due to conditioning their tissue engineered heart valves possessed an elastic modulus very similar to that of native cardiac tissue.

1.3.4. Dynamic Media

In traditional cell culture, cells are grown in flasks with a static medium. The medium is allowed to sit until the nutrients are used, it is discarded, and new medium replaced. Bioreactors, specifically perfusion bioreactors, allow the user to continuously perfuse fresh medium through the system. It has been shown that static culture significantly limits cell viability and matrix production in long-term cell studies and cultures [36]. Using a perfusion system bioreactor may alleviate the problems associated with this poor diffusion of medium. It has also been shown that increased fluid flow over certain cell types, such as osteoblasts and chondroblasts, increases extracellular matrix production through fluid shear [36-37]. Extracellular matrix is largely a strengthening agent of the tissue, so this results in a stronger scaffold [36-37]. To this point, several groups have studied the effects of mechanical stimulation, single culture models, co-culture models, static medium, and dynamic medium on cells. However, there are no studies on extensive combinations of these parameters such as co-culture in dynamic medium with mechanical stimulation. To be able to create a healthier, viable bone-tendon engineered construct, these conditions and improvements must be combined to create improved, novel designs.

1.4. Bioreactor Design

Our study goal is to combine these bioreactor/scaffold improvements and modifications into one bioreactor design. From our literature review, no current bioreactor design allows the user to cyclically strain a portion of the scaffold while maintaining no strain on another portion. The bioreactor design of this study aims to co-culture two different cell types with spatially selective strain. To do this, design ideas were reviewed from other published bioreactors designed specifically for muscle and tendon tissue and those using linear strain applicators [29,35,38]. Initially, this bioreactor will aid in preconditioning multiple spatially selective, co-cultured, degradable scaffolds. Each scaffold is engineered to imitate the two anchoring areas of the bone-tendon interface – bone and tendon. For the tendon-anchoring end, fibroblasts may be cultured on one half of each scaffold and exposed to cyclic strain while on the other half of the scaffold, i.e. the bone anchoring end, osteoblasts may be cultured in the absence of strain. This strategy is expected to enable the collagen and other matrix proteins in the fibroblasts/tendon end to align and grow on the scaffold due to the applied strain while the osteoblasts are shielded from the strain [27]. It has been shown that osteoblasts exposed to relatively large cyclic strain tend display fibrocartilagenous tendencies and create type II collagen especially in the presence of fibroblasts [34]. This design will also include a perfusion system to provide continuously flowing medium to the bioreactor chamber.

In addition, this bioreactor design will be able to accommodate a large number of testing parameters: strain, strain frequency, type of scaffold, length of scaffold, ratio of scaffold strained, and flow rate of perfused medium via computer control of the linear

mechanical actuator, clamp and pump system. These variables may be easily changeable at the start of any study by inputting changes in the computer program, removing selected clamps within bioreactor and/or adjusting pump controls. As was encountered by Syedain et al, very few bioreactor systems are able to control or record the strain transmitted to the scaffolds; but due to our ability to use position feedback, our design will be able to specify the stretch and record it.

CHAPTER 2

AIMS

The goal of this project is to design and validate a cyclic strain bioreactor system capable of stretching multiple scaffolds while isolating a portion of each scaffold from the stretching. This bioreactor system will be important in the field of tissue engineering by providing a resource to investigate tendon and ligament tissue regeneration and wound healing leading to improved function and faster healing times for major musculoskeletal injuries. As the design lends itself to co-culture, it may also provide a platform for exploring the material-mechanical effects on tendon and bone cell differentiation, proliferation, and extracellular matrix production and organization to better understand the healing and repair/regeneration process at the bone-tendon enthesis. The aims of this project are to:

1. Design and construct a cyclic strain bioreactor system capable of stretching multiple scaffolds simultaneously while isolating a specified portion of each scaffold to receive no stretch.
2. Measure the mechanical strain experienced on the scaffolds.
3. Evaluate the cellular response to mechanical stimulation via the bioreactor system using NIH-3T3 fibroblasts.
4. Characterize the matrix response to mechanical stimulation via the bioreactor system using NIH-3T3 fibroblasts.

CHAPTER 3

MANUSCTRIPT

Design and Validation of a Cyclic Strain Bioreactor to Condition Spatially-Selective Scaffolds in Dual Strain Regimes

Annals of Biomedical Engineering

ABSTRACT

The objective of this study was to design and validate a unique bioreactor design for applying spatially selective, linear, cyclic strain to degradable and non-degradable polymeric fabric scaffolds. This system uses a novel three-clamp design to apply cyclic strain via a computer controlled linear actuator to a specified zone of a scaffold while isolating the remainder of the scaffold from strain. Image analysis of polyethylene terephthalate (PET) woven scaffolds subjected to a 3% mechanical stretch demonstrated that the stretched portion of the scaffold experienced 2.97±0.13% strain (mean ± standard deviation) while the unstretched portion experienced 0.02±0.18% strain. NIH-3T3 fibroblast cells were cultured on the PET scaffolds and half of each scaffold was stretched 5% at 0.5 Hz for one hour per day for 14 days in the bioreactor. Cells were checked for viability and proliferation at the end of the 14 day period and levels of glycosaminoglycan (GAG) and collagen (hydroxyproline) were measured as indicators of extracellular matrix production. Scaffolds in the bioreactor showed a seven-fold increase in cell number over scaffolds cultured statically in tissue culture plastic petri dishes (control). Bioreactor scaffolds showed a lower concentration of GAG deposition per cell as compared to the control scaffolds largely due to the great increase in cell number. A

75% increase in hydroxyproline concentration per cell was seen in the bioreactor stretched scaffolds as compared to the control scaffolds. Surprisingly, little differences were experienced between the stretched and unstretched portions of the scaffolds for this study. This is largely attributed to the conditioned media effect. Results indicate that the bioreactor system is capable of applying spatially-selective, linear, cyclic strain to cells growing on polymeric fabric scaffolds and evaluating the cellular and matrix responses to the applied strains.

Keywords – Bioreactor, Cyclic strain, Fibroblast, Tendon, Cell-culture

INTRODUCTION

Tissue engineering strategies have shown promise in aiding the repair of damaged tendons.[4-5,16,22] Studies have shown that the addition of cells on scaffold-based constructs has greatly improved the constructs' elastic moduli, tensile strength, and biological response of native tissue as compared to synthetic/non-cell materials.[6,8,10]

Because tendons are mechanically responsive, studies have examined the effects of mechanical stretch on engineered tendon tissues by a variety of mechanisms such as linear actuators, perfusion chambers, electric motors, vacuum systems, and audio speakers.[3,6,14,23] In general, cell-scaffold constructs subjected to mechanical strain showed increased stiffness and tensile strength over statically cultured constructs.[3,14] The addition of cyclic strain showed increased collagen type I production as well as cytoskeletal rearrangement.[3,14] Intermittent cyclic strain caused increased cell proliferation while constant cyclic strain led to cell-mediated apoptosis.[14]

Because of the complexity of the bone-tendon enthesis, one challenge that still remains is the integration of engineered tendon into bone because these constructs lack the normal transition zones in the bone-tendon enthesis.[3,6,14,23] To address this problem, researchers have co-cultured tenocytes or fibroblasts, and osteoblasts.[1,11,20-21] Wang et al. showed the ability to create an unstrained construct divided into three zones: primarily fibroblastic, primarily osteoblastic, and a third zone that was a mix of fibroblasts and osteoblasts.[21]

While bone is also a mechanically responsive tissue, if subjected to the same large strains as tendon, it tends to form fibrocartilagenous tissue instead of bone.[21] In order to engineer tendon that mimics the bone-tendon enthesis, it would be ideal to be able to use large mechanical stretch to stimulate tendon formation on one end of the scaffold while minimizing tensile stretch on the other end to favor bone formation. Therefore, the study aim was to design bioreactor able to support the engineering of scaffolds with differentiated osseous and fibroblastic attachment zones.

This design was initially validated using fibroblast culture to investigate the difference in cellular and matrix response to the different strain zones on the scaffold. Responses were characterized by cell proliferation, glycosaminoglycan (GAG) deposition, and collagen deposition onto the scaffold. The presence of these elements of tendon has been shown to indicate healthy tendon tissue.[21]

MATERIALS AND METHODS

Bioreactor System

The bioreactor system design was modified from a commercially available bioreactor (BOSE Electroforce 3200) to provide differential stretch to portions of the scaffolds. The overall system is composed of two major sections: a baseplate (composed of 6061 aluminum alloy) and a removable polycarbonate chamber (Fig. 1). The chamber is removable from the base and replaced with another chamber so concurrent tests may be performed. This system uses a linear actuator to apply linear, uniaxial strain to a group of parallel scaffolds fixed inside the chamber. The overall dimensions of the system are 7in. x 17.5in. which allows it to fit inside of a standard cell incubator.

The bioreactor chamber is constructed of ¼" plates of polycarbonate fitted together using 316L stainless steel socket head cap screws and then sealed with silicone (100% Clear Aquarium Silicone, Marineland) along both the outside and inside edges to make the chamber watertight. The clamps, screws, and actuator inside the chamber are also made of 316L stainless steel.

The novel 3-clamp design involves three clamps: an actuating, fixed, and adjustable clamp (Fig. 2). This design allows scaffolds of different materials and sizes, as well as portions of scaffold subjected to mechanical perturbation. The actuating clamp is connected to the linear actuator and is used to mechanically stretch the scaffold by pulling on one end of the scaffold. The fixed clamp is used to hold the opposite end of the scaffold in a fixed position. The adjustable clamp may be used to create two distinct zones of mechanical stretch (a non-stretched zone (A) and a stretched zone (B)) by creating a fixed section in the central region of the scaffold. The adjustable clamp may

be moved to other positions designated by the extra holes visible in Fig. 2. This adjustability allows for varying scaffold lengths and larger/smaller percentages of the scaffold to be stretched. In addition, different cell types may be seeded in zones A and B allowing for spatially segregated co-cultures as well as intermixed co-cultures. After stretching begins, the fixed clamp and actuating clamp are intended to remain in place; but, the adjustable clamp may be removed for non-segregated co-culture when two cell types are used.

Medium is pumped with a Masterflex pump through the bioreactor chamber at 60-70 mL/min through an inlet to the side of the scaffolds and an outlet at the opposite corner. Gas permeable tubing (Cole-Parmer, Vernon Hills, IL, USA) allows for CO_2 and O_2 gas exchange as the medium circulates. The bioreactor chamber and tubing are sterilized using steam autoclave at 121°C and 15 psi for 20 minutes.

A LabVIEW program (v. 8.0 National Instruments, Austin, TX) was created to control the 2" stroke 150lb force linear actuator, with potentiometer feedback (FA-PO-150-12-2, Firgelli Automations, Surrey, BC, Canada), that is used to mechanically stretch the scaffolds. The program is used to control frequency, magnitude, and duration of mechanical stretch to meet experimental conditions.

Mechanical Characterization

To characterize the mechanical performance of the bioreactor, 4 Polyethylene Terephthalate (PET) (Biomedical Structures, Warwick, RI, USA) fabric samples (10mm x 80mm) were clamped in the bioreactor and marked with small graphite dots (Fig. 3). The LabVIEW program was set up to apply a 3% stretch chosen as an expected applied

stretch to be used with this bioreactor. 3% is also large enough to measure a noticeable strain but also small enough that the accuracy/repeatability of the system can be tested. The LabVIEW program was run through 24 strain cycles and pictures taken at each step in the cycle. Observed strain was recorded from the measurements taken.

Cellular Characterization

Six large PET scaffolds (8mm x 87.5mm) and seven small PET scaffolds (8mm x 25mm) were cut from a large spool and their cut ends lightly melted by touching them to a heated steel bar to prevent fraying. Scaffolds were sterilized by sonicating in each of the following solutions for 30 minutes: 1% Ivory dish soap solution, deionized (DI) water, and a second time in DI water. Samples were placed in 70% ethanol for 30 minutes and finally sterilized by exposure to UV light for at least 30 minutes per side.

After sterilization, but before seeding, scaffolds were rinsed with sterile 1x PBS and then pre-exposed to culture medium, Dulbecco's Modified Eagle Medium High Glucose (HyQ® DMEM, HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, Cat. No. 30-2020, ATCC) and 1% antibiotic/antimycotic (AB/AM, 100 U/mL penicillin, 100µg/mL streptomycin, 0.25 µg/mL amphotericin B) by soaking the scaffolds in sterile 50mL centrifuge tubes for at least 30 minutes to allow the medium to penetrate the scaffold and proteins to start attaching.

NIH-3T3 mouse fibroblast cells were cultured in DMEM-High Glucose + 10%FBS + 1% AB/AM at 37°C with 5% CO_2. Cells were pipetted at a density of $1x10^5$ cells/cm^2 in four spots along the length of six large (8mm x 87.5mm) and seven small

(8mm x 25mm) scaffolds, and kept in three 3.5in. diameter TCP petri dishes and seven individual 1.5in. TCP petri dishes respectively overnight to allow attachment.

Two groups were tested in this experiment: a control group (static culture) and an experimental group (bioreactor). The experimental group was divided into two subgroups. Subgroup 1 of the experimental group was cultured in the bioreactor, but experienced no stretch. Subgroup 2 of the experimental group was also cultured in the bioreactor, but was subjected to 5% mechanical stretch at 0.5Hz for one hour per day for 14 consecutive days. Table 1 lists the characteristics for each experimental group.

After seeding and incubation overnight, scaffolds were divided into their respective groups. On day 1, the six scaffolds were clamped in the bioreactor and 250mL of DMEM-High Glucose medium further supplemented with 1mM beta-glycerophosphate (β-GP) was added. The six smaller scaffolds, sized such to represent the same surface area as the other groups, remained in their petri dishes and 2mL of medium was added to each. The remaining small scaffold selected for determining initial cell attachment was removed, rinsed with sterile 1x PBS, and then stored at -20°C. Stretching on day 1 began 2 hours after clamping to allow any cells that may have become dislodged or perturbed to reattach to the scaffold.

Over the 14-day period, the medium was supplemented with 10μg/mL of ascorbic acid every 3 days as ascorbic acid has been proven to improve type I collagen deposition in fibroblast cells.[9] The medium of the control scaffolds was changed every 3 days. However, due to the much greater volume in the medium reservoir of the bioreactor, the medium of the experimental group scaffolds was changed every 6 days.

Experimental parameters were chosen based on their use in the literature. The stretching protocol is similar to that used by other groups such as Garvin et al. and Riboh et al.[8,14] Daily intermittent straining was chosen as it has been shown that intermittent straining increases collagen I production and cell proliferation.[14] 14 days is also a common time for tendon tissue engineering bioreactors.[14,23] Concentrations of β-GP and ascorbic acid were chosen as the having the most positive effect on cell proliferation and collagen I production in tests performed by Wang et al.[21] Mechanical stretching of the scaffolds took place in a cell incubator (37°C, 5% CO_2, 95% humidity).

Scaffolds were removed from the bioreactor and petri dishes 2 hours following the final stretch of day 14. All scaffolds were cut into three approximately equal sized sections (8mm x 8mm) and their surface area recorded (Fig. 4). By sectioning the scaffolds, we are able to determine if the matrix on the scaffolds is uniform over the whole scaffold as well as across all of the scaffolds. If any non-uniformity is noted, it may be possible to determine if effects are caused by proximity of the clamps (section 1, 2, or 3) or by position of the scaffold (scaffold A-F).

To remove the cells for analysis, each scaffold section was rinsed with sterile 1x PBS and placed into labeled Erlenmeyer tubes with 1mL of 50μg/mL Proteinase K (Pro-K, Promega Corporation, Madison, WI) solution (50μg/mL Pro-K, 50mM Tris-HCL, 50mM $CaCl_2$ buffer, pH 7.5) and placed overnight in an oven at 60°C. The following day, 50μL of proteinase inhibitor (phenylmethylsulfonyl fluoride (PMSF), MP Biomedicals, Solon, OH, USA) was added and a sonic dismembraner (Fisher Scientific Sonic Dismembraner 550, Fisher Scientific, Pittsburg, PA, USA, Setting 2) was used to disrupt any remaining cells and matrix. This cell lysate was used to estimate cell number.

One scaffold was not digested in the Pro-K solution and used instead to view cells on scaffold using a Live/Dead® assay (Invitrogen, Grand Island, NY, USA). From the assay protocol, viable cells will fluoresce green and dead cells will fluoresce red.

The PicoGreen assay (Invitrogen, Grand Island, NY, USA) was to measure DNA as an indicator of the number of cells on scaffolds. For the assay, the 100 µL of PicoGreen reagent was added to 20µL of the cell/matrix lysate in 80 µL of tris-HCL-EDTA buffer in a 96 well opaque microplate. The plates were read at 485 nm excitation and 528 nm emission using a spectrophotometer. A linear standard curve (2 µg/mL serial dilution) was prepared from a DNA standard (100µg/mL). DNA was normalized to surface area, ngDNA/mm^2, for each scaffold section.

Glycosaminoglycans are polysaccharides that are a major component of tendon extracellular matrix.[13] To quantify the concentration of GAG, the Alcian blue assay was used. 250µL of each prepared sample (cell lysate with Alcian blue) and 100µL of each prepared chondroitin sulfate standard (400 µg/mL serial dilution) was transferred to a 96-well plate and the absorbance read at 620nm. Sample concentrations of GAG were then normalized by DNA to ngGAG/ngDNA.

Elaboration of collagen by cells is an indicator of cellular matrix production. The hydroxyproline assay was used to measure collagen. According to Stegemann and Salter, hydroxyproline can be linearly translated to collagen by multiplying by a factor of 7.46, i.e. 1 µg/mL hydroxyproline is equal to 7.46 µg/mL collagen.[19] First, 25 µL of prepared hydroxyproline (HP) standard (Sigma-Aldrich, St. Louis, MO, USA) (100 µg/mL serial dilution) and 25µL of 16x concentrated prepared sample were added to each well of a 96-well plate. Prepared chloramine-T solution (112.5µL) was added and incubated at room

temp for 20 min. Immediately following, 125µL of prepared Ehrlich's reagent were added to each well, incubated for 20 minutes at 65°C in an oven, and the absorbance read at 550 nm. Sample concentrations of collagen were then normalized by DNA to ngHP/ngDNA.

Statistical Analysis

Means among section position and scaffold location were calculated and a two-factor ANOVAs performed; one factor was the individual scaffolds (A-E) and the other factor was scaffold section (1-3) to one another. This was done for each of the three groups. Differences were considered significant at $p<0.05$. Differences were determined using Tukey's Post-hoc testing.

When no significant differences were determined, results were pooled and one-way ANOVA was performed to test differences between test groups (n=5) for the GAG and hydroxyproline data. Differences were determined using Tukey's Post hoc testing and were considered significant at $p<0.05$.

RESULTS

Mechanical Characterization

Fig. 5 shows the LabVIEW waveform for the actuator to apply mechanical stretch to test scaffolds in the bioreactor. The large spikes at the end of each plateau are a phenomenon caused by the sampling and recording and are not representative of stretched applied to the scaffolds.

Results of the video analysis of the reference dots on the 4 PET scaffolds subjected to the programmed 3% stretch at 0.1 Hz for 24 cycles is shown in Fig. 6. The figure shows the mechanical stretch of the stretched portion and the non-stretched portion of the scaffolds. The average strain experienced by the stretched scaffolds was 2.97±0.13%. The average strain experienced by the unstretched scaffolds was 0.02±0.18%.

From the camera resolution limitations, visual representation of 0% strain was very difficult to obtain. However, the resolution of the pictures taken was 1 pixel~0.5% strain, so values less than 0.5% were assumed to be 0%. Because the LabVIEW program was designed as an open loop control system, errors from one stretch cycle do not translate to the following stretch cycle as evidenced by the positive and negative differences of cycles 10-15.

Cellular Characterization

Images of the experimental and control scaffolds stained with the Live/Dead reagent at the end of the 14-day culture period are shown in Fig. 7. Images were taken of each of the three assayed sections in each group at magnifications of 10x and 20x. The images showed that there were many live/viable cells on all scaffold groups and scaffold sections. The cells uniformly covered the majority of the surface area of the scaffolds and cells were observed growing on fabric fibers within the scaffold.

Using measured cell DNA data, two-way ANOVA was performed on test samples to determine if there were any effects based on proximity of cells on scaffolds to the clamps or on position of scaffolds in the bioreactor. No statistical differences were found

between zones or proximity of cells to clamps with in each experimental group ($p>0.18$) nor were differences detected for position of scaffolds in bioreactor for each experimental group. Since there were no effects of position on the scaffold or position within the bioreactor based on DNA data, scaffolds were then treated as a single test unit in subsequent analyses.

Fig. 8 shows the results of total DNA on scaffolds in each experimental group after 14 days. One-way ANOVA revealed that the DNA concentration in all groups was not equal ($p<1x10^{-6}$). A large increase was found in the DNA concentration of the bioreactor unstretched group as compared to the control group ($p<1x10^{-6}$) as well as the bioreactor stretched group as compared to the control group ($p<1x10^{-6}$). However, no difference was found between the DNA concentration of the bioreactor unstretched group and bioreactor stretched group ($p>0.90$).

Matrix Characterization

To investigate the effect of mechanical stretch on levels of extracellular matrix, GAG was measured via the Alcian Blue assay and then normalized to DNA. One-way ANOVA with post-hoc Tukey tests showed that the amount of GAG per DNA was statistically lower in the bioreactor as compared to the static culture ($p<0.001$), but there was no difference between strained and unstrained sections of scaffolds in the bioreactor (Fig. 9) ($p>0.99$).

Hydroxyproline (HYP), like GAG, was normalized to DNA and showed a difference between experimental groups (Fig. 10) ($p<0.01$). The bioreactor unstretched group showed an increase in hydroxyproline per DNA as compared to the control group

($p<0.05$) as did the bioreactor stretched group ($p<0.01$). The bioreactor unstretched and stretched groups still showed no significant differences in hydroxyproline deposition when normalized to DNA ($p>0.25$).

DISCUSSION

This study presented an enhanced bioreactor design that is able to cyclically strain preferential zones of multiple, fabric, cell-seeded scaffolds in a controlled *in vitro* setting. This system has potential to develop degradable and non-degradable fabric scaffolds with improved matrix deposition over basic statically cultured scaffolds. Analysis with photographic video demonstrated that the system is able to deliver accurate and repeatable cyclic stretch to a designated portion of each scaffold while isolating the remainder of the scaffold from stretch. In addition, the system may accommodate numerous variables including, scaffold length, width, and material as well as stretch zone, magnitude, frequency, and schedule. Furthermore, each bioreactor chamber could interchange with a second identical bioreactor chamber for concurrent studies using similar or differing experimental conditions.

Minor variations of less than 10% of the desired strain magnitude were noticed between stretch cycles, which were attributed mostly to the process of the marking for the video analysis. The method used to mark the scaffolds for analysis was sufficient for initial validation purposes, but lacked fine resolution. Many methods were attempted to mark the scaffolds to measure the distance between marks – graphite providing the most accurate method. The camera used also had a limited resolution. For more exact values, a camera or microscope with higher resolution would give decreased error to the measurements taken. However, the system did prove that it could consistently stretch as

low as 1% and at least as high as 10% for a 1-inch scaffold (data not shown). Unfortunately, over a 14 day period, there was some laxity noticed in the scaffolds likely due to viscoelasticity.

Investigating the effect of location within the bioreactor and the proximity to a clamp revealed that the scaffolds were statistically equivalent and could be treated as uniform. Live/Dead staining demonstrated that cells remained viable on the scaffolds in both the bioreactor and in control cultures over the 14-day experimental time frame. Non-viable cells were also evident on the scaffolds. The viable cells on the scaffolds in the bioreactor are still at a much greater density than the viable cells on the control scaffolds, despite the increase in non-viable cells. Also, the cells in the bioreactor, especially the stretched cells, began to show alignment along the scaffold fibers. Riboh et al. also noticed this phenomenon with cytoskeletal and nuclear rearrangement due to uniaxial cyclic stress.[14]

Quantitative analysis of the DNA concentration on the scaffolds supported our qualitative findings. Intermittent cyclic strain increased DNA concentration seven times on the scaffolds in the bioreactor as compared to the control scaffolds. This agreed with other studies investigating the effects of varying schedules of intermittent cyclic strain over static culture.[2,14] Application of mechanical strain to these cells activates the mitogen-activated protein kinase pathway as well as mechanosensitive calcium channels initiating the proliferative response.[14] However, our findings did not agree with Screen et al. who did not see any change in cell proliferation on a cyclically strained tendon.[17] However, their studies were only performed for 24 hours and used a grafted tendon as their scaffold material.[17]

Initially, it was surprising that there were no differences detected in DNA and elaboration of extracellular matrix components between strained and non-strained scaffold sections since cyclic strain has been shown to have such a large effect over the static control petri dish scaffolds. It was expected that the proliferation increase of the strained scaffolds over the unstrained scaffolds would be much greater than the unstrained scaffolds over the control scaffolds. We attribute this to a concept known as conditioned media since the media was recycled by the pump system. Multiple groups have shown that instead of directly or mechanically stimulating cells, replacing the culture medium with a conditioned culture medium can induce a response from the cells based on chemical signals present in the medium.[7,12,18] For example, by using medium that was used to stretch osteoblast cells, Soma et al. was able to induce osteoclast proliferation by using the discarded medium as a conditioned medium. It contained all of the cellular signals telling osteoblasts to proliferate, which in turn caused the osteoclast cells to initiate the homeostatic tendencies by proliferating.[18] This same phenomenon may be occurring inside the bioreactor as the unstretched scaffolds shared medium with the strained scaffolds. The cells on the unstrained scaffolds are receiving the same cellular signals as the cells on the strained scaffolds, but not the mechanical stimulation.

The tendon reconstruction schedule, proposed by Rust et al., shows that cell proliferation peaks around day 5, followed by a large increase in unorganized collagen deposition (days 5 – 28).[15] The collagen deposition overlaps slightly with an increase in GAG deposition beginning around day 21 and increasing through day 28 and beyond.[15]

Our analysis occurred in the middle of the collagen growth peak based on Rust et al., which would ideally show us if cyclic strain increased the concentration of collagen

as well as the rate of production. We found a 44% increase in hydroxyproline concentration on the unstrained scaffolds in the bioreactor as well as a 75% increase in hydroxyproline concentration on the strained scaffolds in the bioreactor as compared to the control scaffolds. A similar trend has been previously reported by Riboh et al. as intermittent cyclic strain was shown to increase type I collagen production using fibroblastic cells.[14] The strained scaffolds cultured by Screen et al showed no significant increase in hydroxyproline concentration over the unstrained control scaffolds.[17] Interestingly though, there was a large increase in hydroxyproline concentration present in the medium of the strained scaffolds over the unstrained scaffolds.[17]

Analysis of GAG concentration on the scaffolds in the bioreactor shows an interesting trend. Overall, there was a greater mass of GAG on the bioreactor scaffolds than on the control scaffolds, but the static control petri dish scaffolds deposited four times more GAG per cell on the scaffolds as the bioreactor scaffolds. It would appear that cyclic strain had a negative effect on GAG deposition; but the trend is misleading because of the drastically large increase in DNA concentration on the bioreactor scaffolds. At day 14, when these scaffolds were assayed, the large increase in GAG deposition proposed by Rust et al. would not have begun; therefore, it is not surprising to have a lower concentration of GAG per cell on the bioreactor scaffolds due to the large increase in bioreactor cell proliferation. Sampling around day 28 may provide a better investigative time point to analyze the bioreactor's effect on the elaboration of GAG. While these results seem surprising, our findings do agree with Screen et al. where they saw a statistically significant decrease in GAG concentration on their strained scaffolds as compared to their unstrained scaffolds.[17]

A unique bioreactor system was designed, developed, and shown to apply a known, user-specified, cyclic strain to cells growing on polymeric fabric scaffolds. The strain environment experienced by the cells was quantified and reproducible. Strain was able to be isolated from a specified portion of each scaffold while stretching the remainder. Differences in cellular proliferation, GAG concentration, and hydroxyproline concentration were measured based on static and dynamic culture conditions experienced by the cells. The bioreactor system may be used to evaluate the effects of mechanical stretch on degradable and non-degradable fabric scaffolds.

ACKNOWLEDGEMENTS

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TABLES:

Table 1: Experimental group characteristics

Group	Location	Stretch Schedule
Control	TCP Petri Dish	No stretch
Unstretched	Bioreactor	No stretch
Stretched	Bioreactor	5%, 0.5Hz, 1hr daily, 14 days

FIGURES:

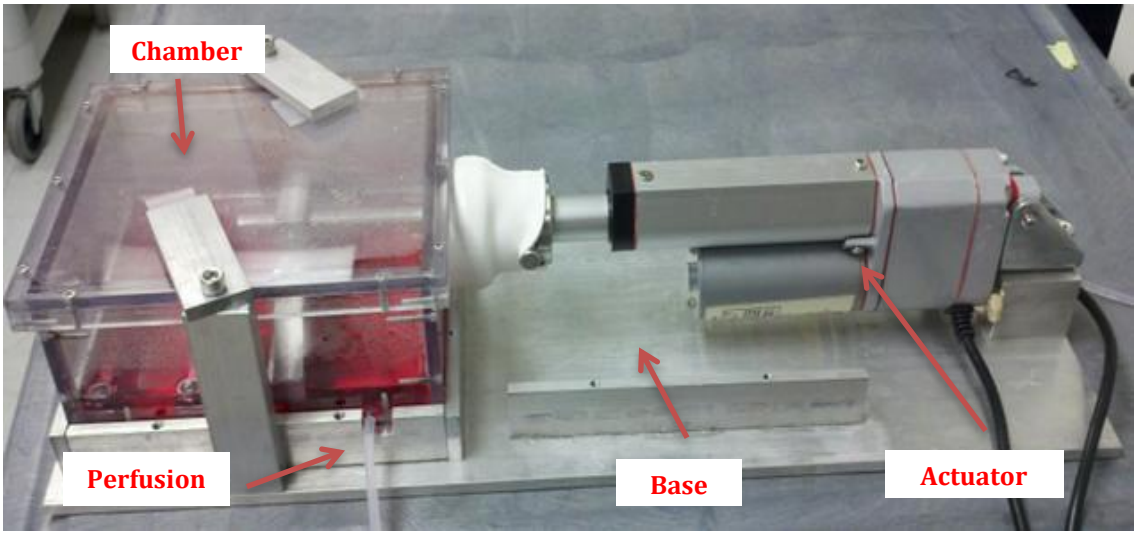

Figure 1: Bioreactor chamber in stretching base

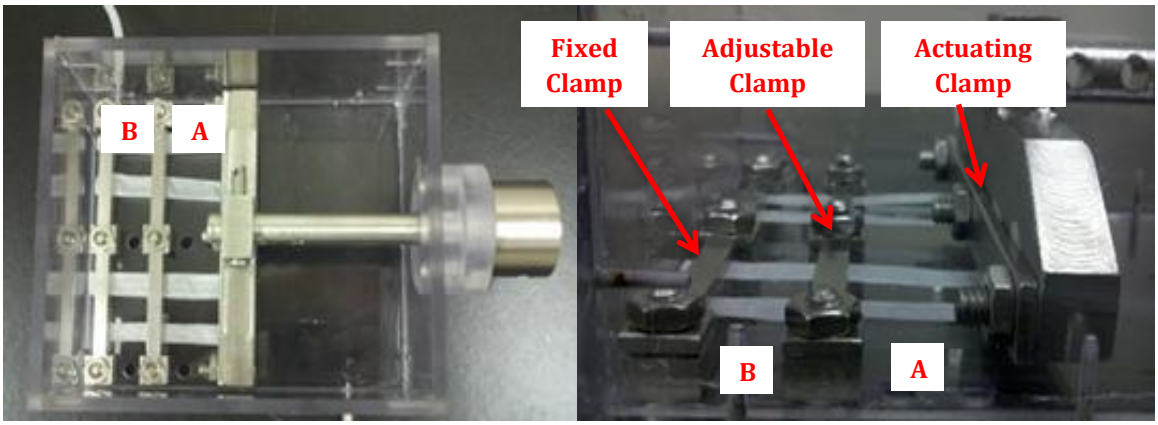

Figure 2: Bioreactor chamber three-clamp design. (A) Portion of the scaffold experiencing strain. (B) Portion of the scaffold that remains under 0% strain conditions.

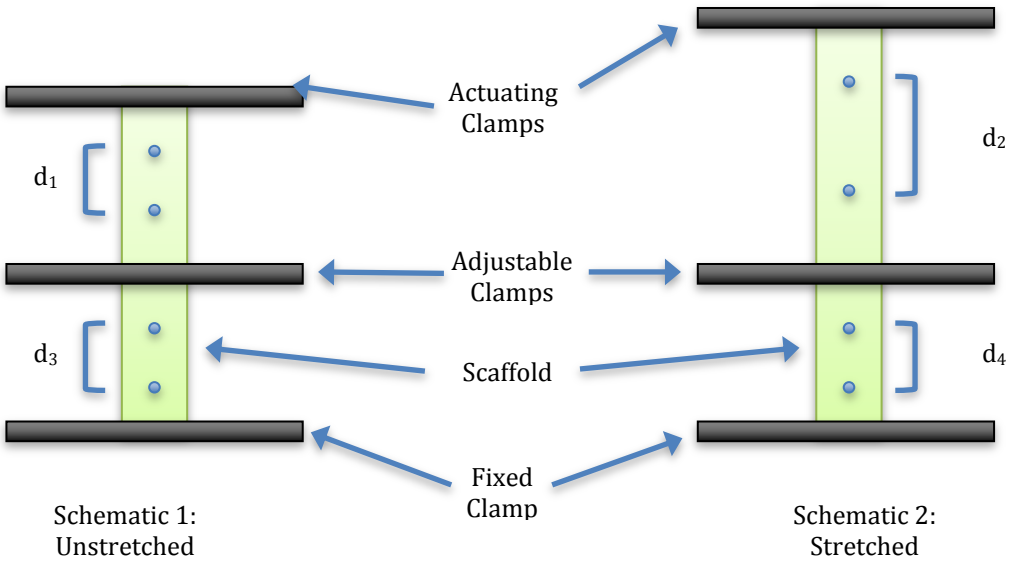

Figure 3: Two schematics of the strain characterization. Schematic 1 represents the photo of an unstretched scaffold. Schematic 2 represents a picture of the same scaffold under a specified stretch. Comparing to Fig. 2, d_1 and d_2 are markers inside of zone A while d_3 and d_4 are markers inside of zone B.

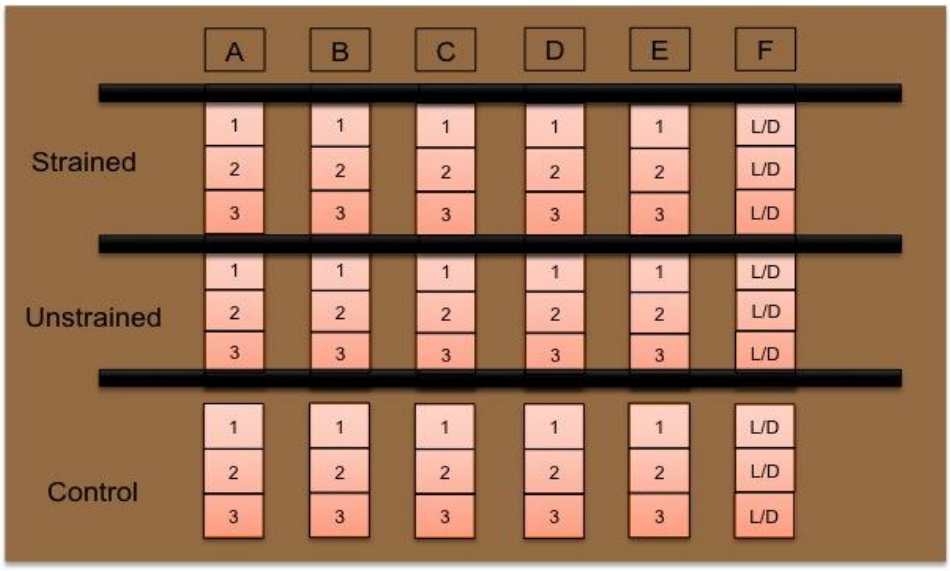

Figure 4: Relative position of each section of scaffold for assays. Position 1, 2, and 3 refer to position on the scaffold and proximity to clamps. L/D refers to sections of the scaffolds designated for Live/Dead imaging.

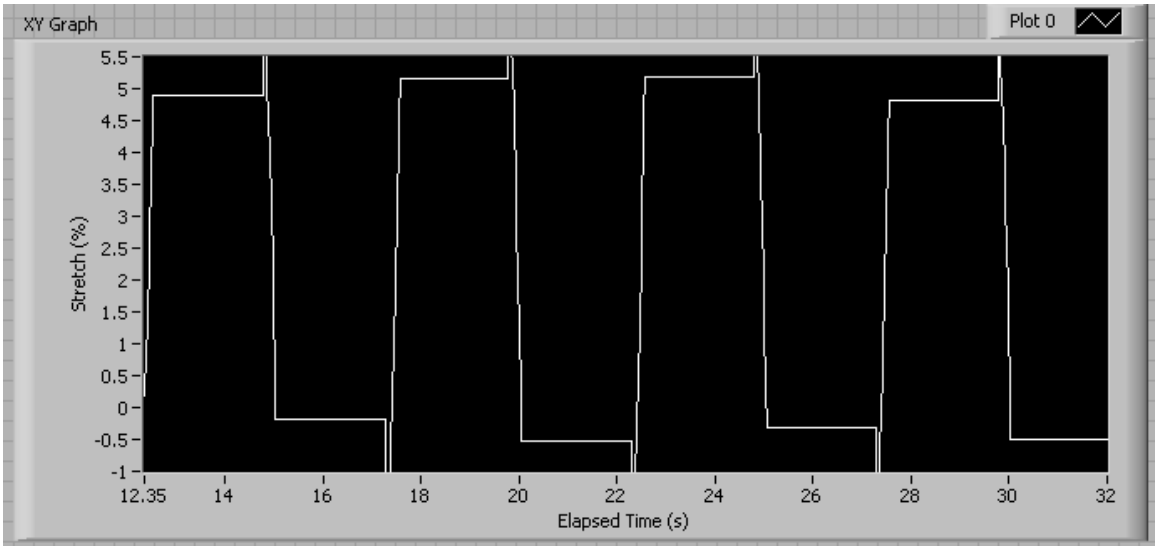

Figure 5: Sample waveform for controlling actuator to apply mechanical stretch to scaffolds in bioreactor.

Parameters set were 5% stretch at 0.2 Hz.

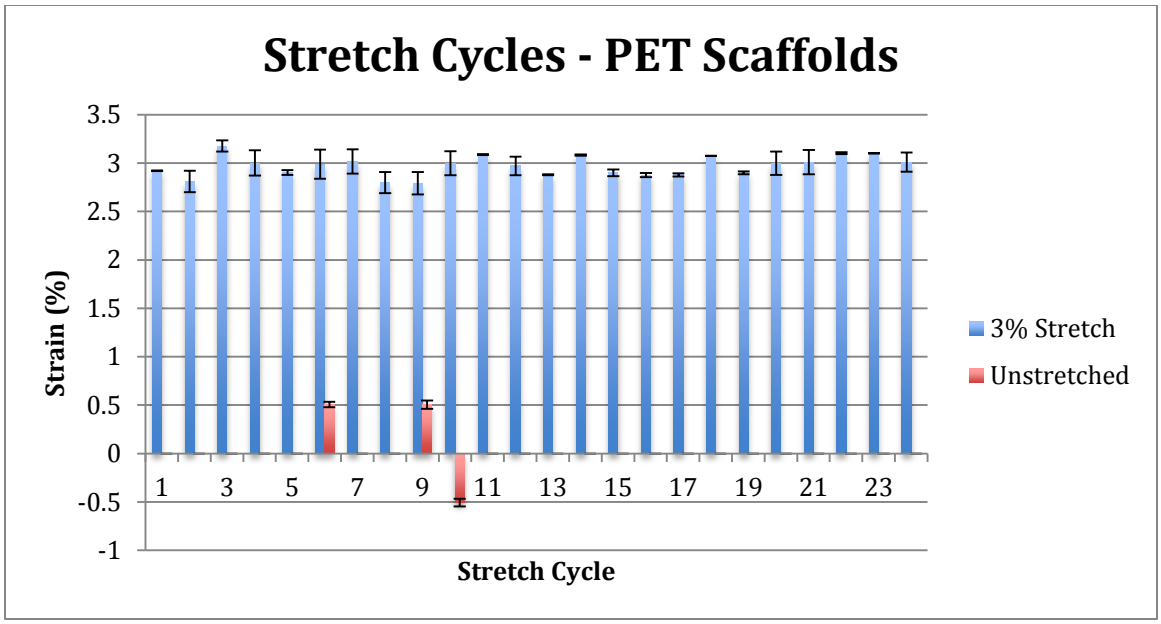

Figure 6: Stretch cycles of the PET scaffolds in the bioreactor. Each bar represents the average strain across all four scaffolds for that stretch cycle. Error bars represent one standard deviation. n=4.

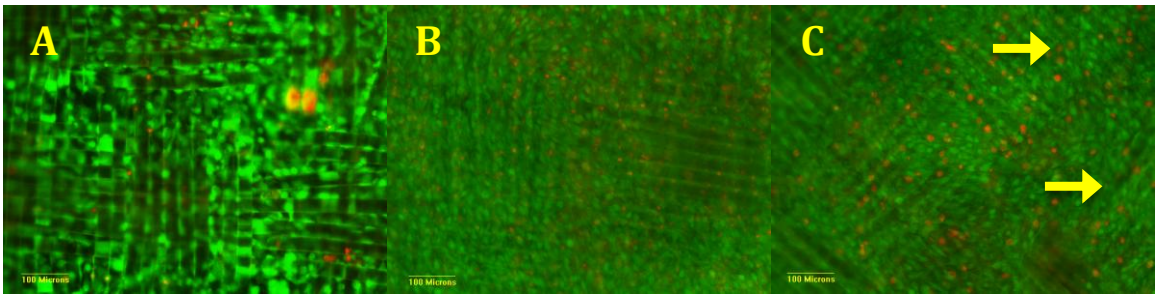

Figure 7: Live/Dead images of section 2 of each scaffold magnified at 10x. Green represents those cells that are alive/viable and red represents those cells that are dead/non-viable. A) Static Petri Dish Control; B) Unstretched; C) Stretched. Arrows signify cells aligning along scaffold fibers.

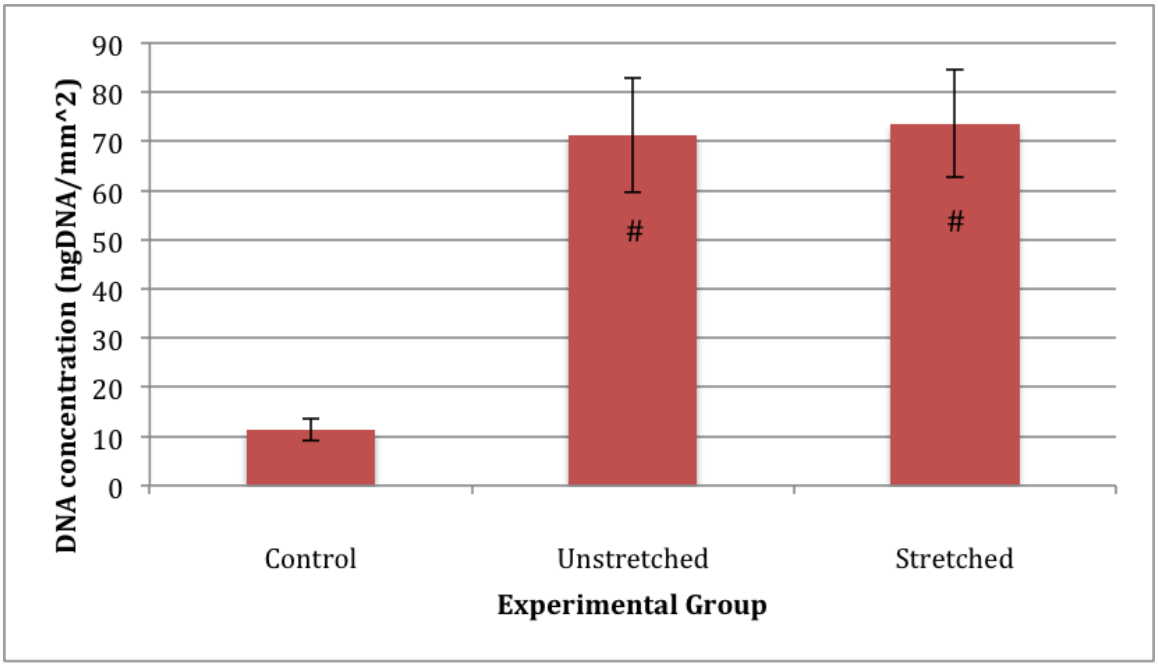

Figure 8: DNA concentration of each experimental group. Data from all three sections of each scaffold were combined to compare the effect had over the whole scaffold. n=5. Error bars represent one standard deviation. #Statistically different than control ($p<1x10^{-6}$).

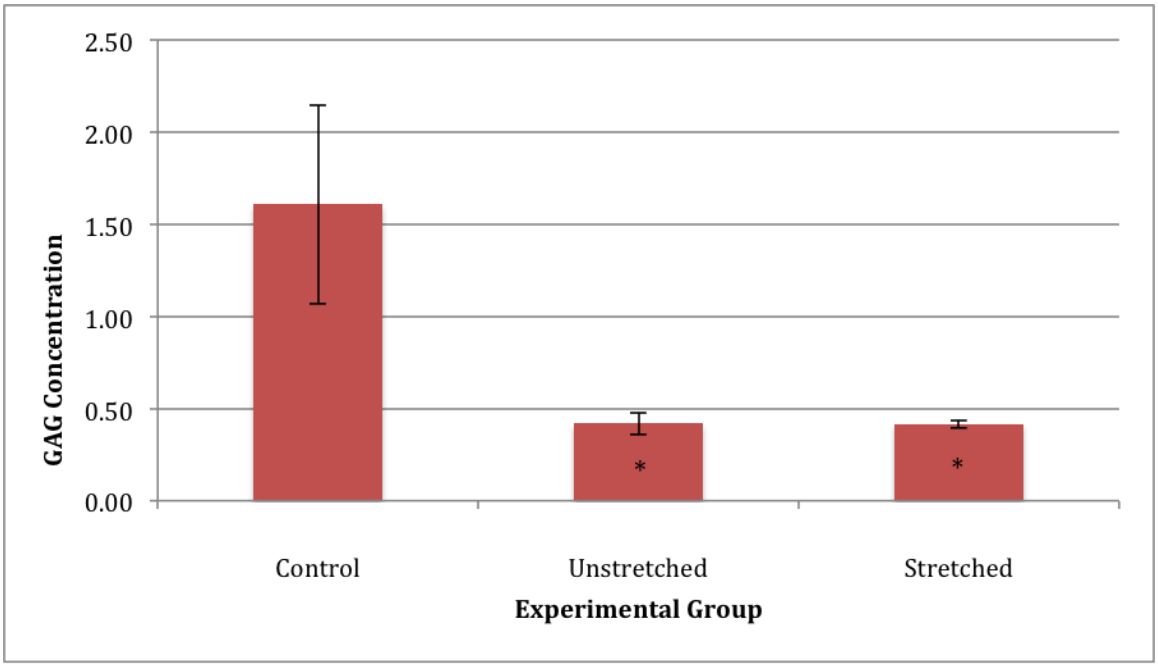

Figure 9: GAG deposition normalized to DNA (ngGAG/ngDNA). n=5. *Statistically different than control ($p<0.001$).

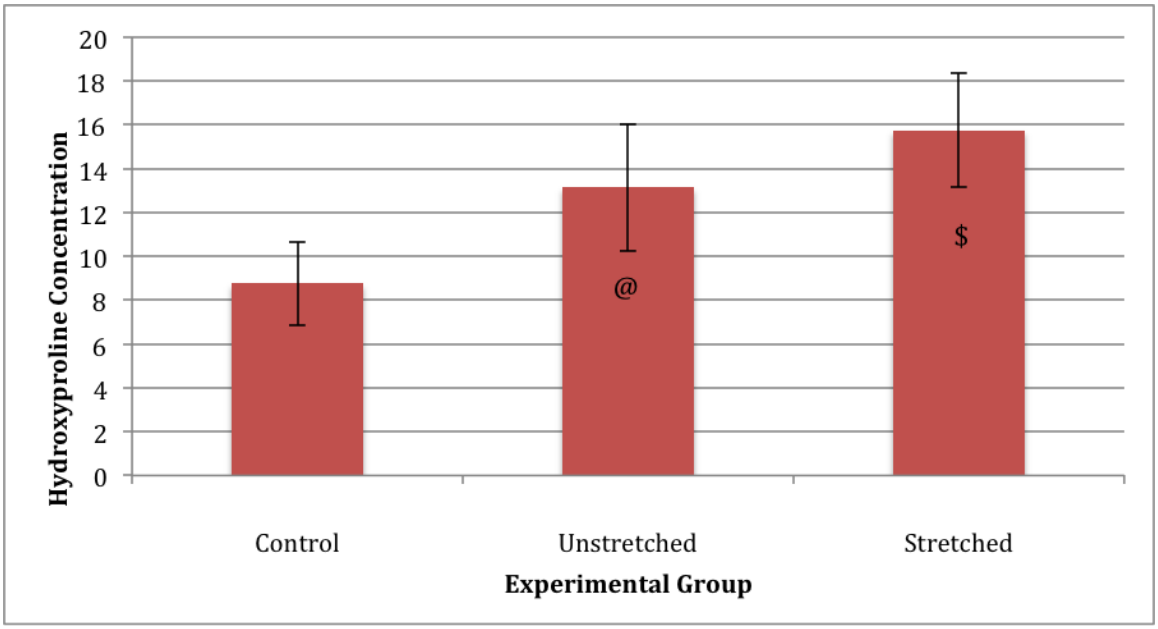

Figure 10: Hydroxyproline deposition normalized to DNA (ngHYP/ngDNA). n=5. @Statistically different than control ($p<0.05$). $Statistically different than control ($p<0.01$).

CHAPTER 4

FUTURE STUDIES

In future studies with this bioreactor system, it is suggested that multiple cell types be used in co-culture under varying mechanical load conditions. This study used only fibroblast cells; but, by using different cells such as NIH-3T3 fibroblasts and MC-3T3 osteoblasts, it may be possible to investigate how this system affects co-culture. In addition, it would be interesting to investigate the effects that the bioreactor has on cells that are not fully differentiated. Aside from altering the cell types used, other scaffold substrates would be beneficial to test such as a degradable polymer like poly-lactic acid (PLA) or poly-glycolic acid (PGA) as they are commonly used in tendon reconstruction surgeries.

For this study, we investigated only one loading scenario – 5% at 5 Hz for one hour daily. Future studies could be conducted to characterize the effects of varying the strain magnitude, frequency, and schedule. Also, this study was ended after 14 days. It would be beneficial to test the bioreactor at other important time points in the tendon reconstruction schedule such as 5 days for cell proliferation, 21 days for collagen and GAG, or 28 days for GAG.

A more in depth study of specific extracellular matrix elements would be very beneficial as well. Our assays tested for the presence of any GAGs as well as all hydroxyproline. Specific GAGs such as decorin and chondroitin sulfate are very important in healthy tendon, but serve very different purposes. Assaying for specific elements would better inform us where improvements have been made in tendon reconstruction. The same is true for the hydroxyproline – it does not test for specific

collagen types. Testing for specific collagen types, such as types I and III, would indicate growth of tendon and bone verses type II which would indicate growth of a more fibrocartilagenous tissue. This knowledge would allow us to better map our imitation of the bone-tendon enthesis transition zone. Performing gel permeation chromatography (GPC) would also allow us to see other matrix elements, such as growth factors, that have been genetically upregulated, but are otherwise difficult to test.

CHAPTER 5

CONCLUSIONS

A unique bioreactor system was designed, developed, and shown to apply a known, user-specified, cyclic strain to cells growing on polymeric fabric scaffolds. The strain environment experienced by the cells was quantified and reproducible. Strain was able to be isolated from a specified portion of each scaffold while stretching the remainder. Differences in cellular proliferation, GAG concentration, and hydroxyproline concentration were measured based on static and dynamic culture conditions experienced by the cells. The bioreactor system may be used to evaluate the effects of mechanical stretch on degradable and non-degradable fabric scaffolds.

CHAPTER 6

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CHAPTER 7

APPENDIX

The mechanical characterization was performed using two different materials: PET, as shown previously, and polylactic acid (PLA). The data for the PLA scaffolds was not included since that scaffold material was not used in the cellular and matrix characterizations. Figure A1 shows the mechanical strain data for the PLA scaffolds undergoing 5% stretch.

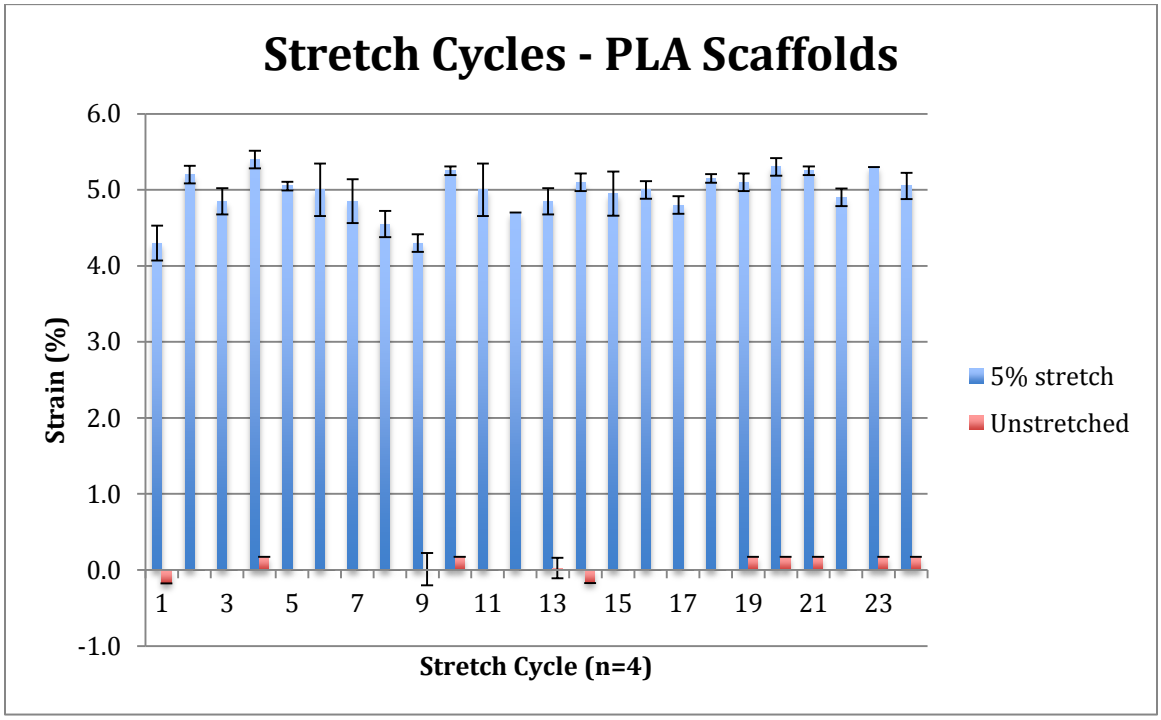

Figure A1: Stretch cycles of the PET scaffolds in the bioreactor. Each bar represents the average strain across all four scaffolds for that stretch cycle. Error bars represent one standard deviation. n=4.

The Live/Dead assay was performed for all scaffold sections under 10x and 20x magnification. Only section 2 under 10x magnification was shows previously. Figures A2, A3, and A4 show the Live/Dead images of the control, unstretched, and stretched scaffolds respectively. Images on the left (A, C, and E) show 10x magnification and the images on the right (B, D, and F) show 20x magnification.

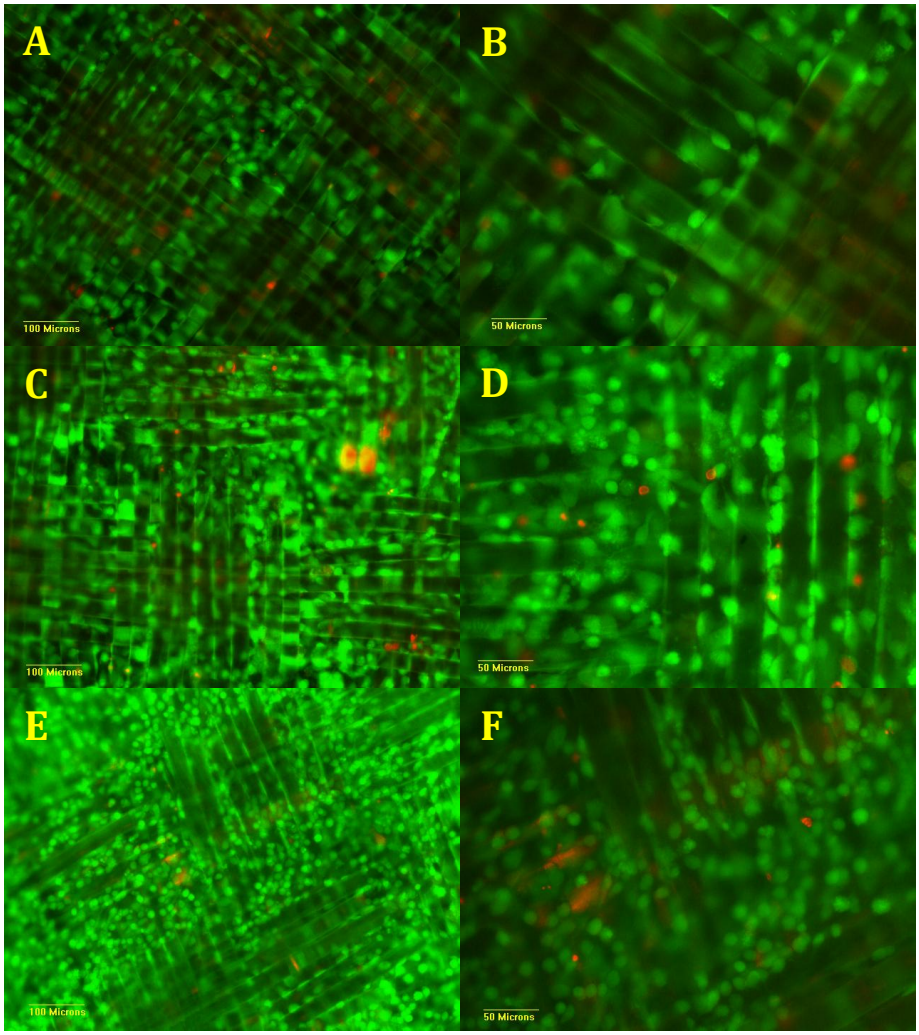

Figure A2: Live/Dead images of the control scaffolds. Green represents those cells that are alive and red represents those cells that are dead. A) Section 1, 10x. B) Section 1, 20x. C) Section 2, 10x. D) Section 2, 20x. E) Section 3, 10x. F) Section 3, 20x.

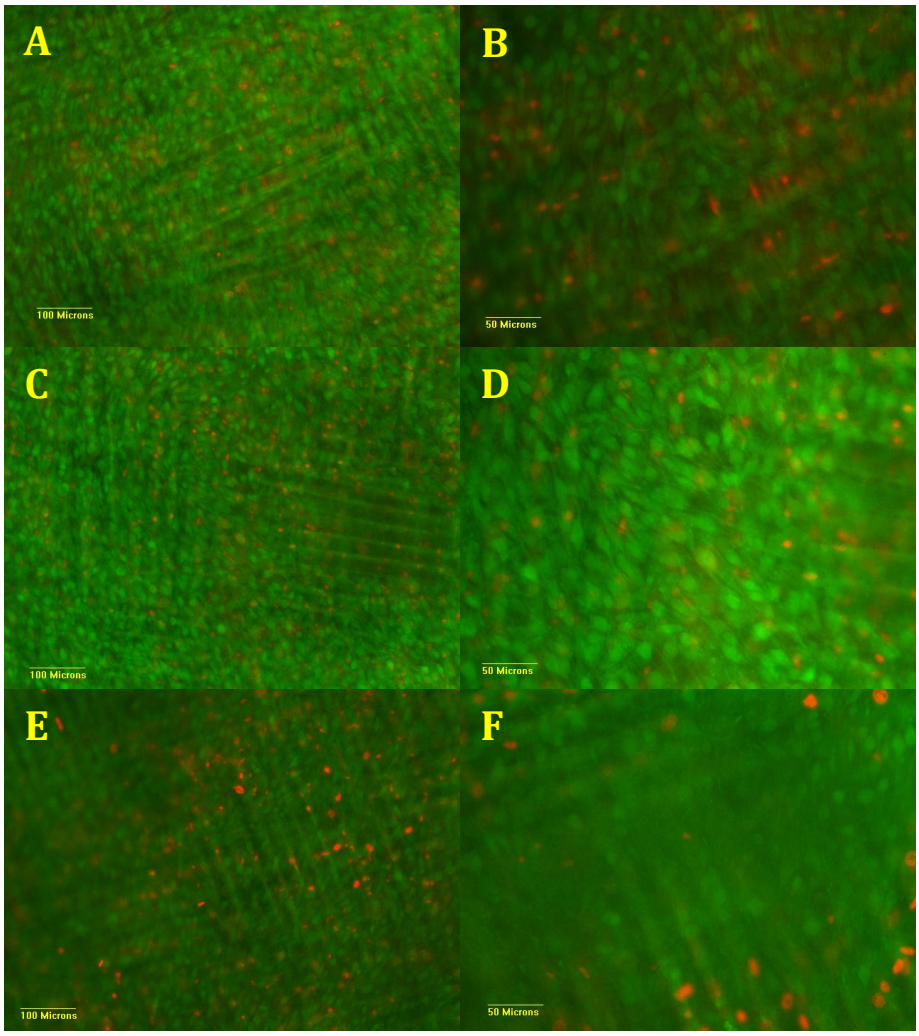

Figure A3: Live/Dead images of the bioreactor unstretched scaffolds. Green represents those cells that are alive and red represents those cells that are dead. A) Section 1, 10x. B) Section 1, 20x. C) Section 2, 10x. D) Section 2, 20x. E) Section 3, 10x. F) Section 3, 20x.

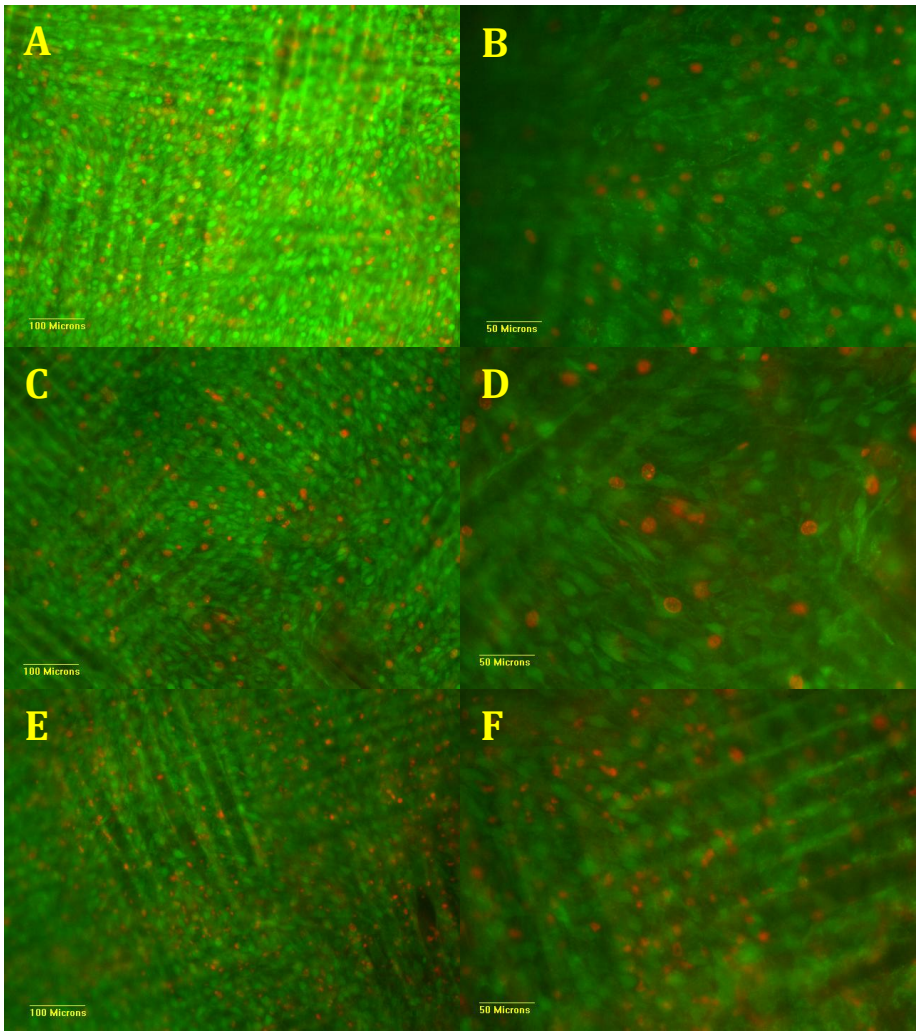

Figure A4: Live/Dead images of the bioreactor stretched scaffolds. Green represents those cells that are alive and red represents those cells that are dead. A) Section 1, 10x. B) Section 1, 20x. C) Section 2, 10x. D) Section 2, 20x. E) Section 3, 10x. F) Section 3, 20x.