Assessment of Ascorbic Acid Effects on the Properties of Cell-Derived Tissue Rings

A thesis submitted to the faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Master of Science

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Abstract

We have developed a system to rapidly create three-dimensional tissue rings from aggregated cells. The ability to use cell-derived tissues to screen the effects of culture conditions on tissue mechanical function has not previously been reported. The first goal of this study was to evaluate the mechanical properties of cell-derived tissue rings in response to ascorbic acid, which has been shown to increase collagen content, resulting in increased mechanical strength. The second goal was to develop quantitative methods to evaluate the structure and composition of cell-derived tissue rings. Rat aortic smooth muscle cells ($1.33x10^6$ cells/ring) were seeded in agarose wells with 4 mm post diameters in DMEM supplemented with 10% FBS and ascorbic acid (0, 50, 150 μg/ml). After 7 days, the average thickness of the constructs reached 0.72 +/- 0.03 mm with no statistical differences between groups. Ultimate tensile strength values were higher in the ascorbic acid-treated groups compared to untreated controls. However, there was no significant difference between tissue rings treated with 50 and 150 μg/ml ascorbic acid. Biochemical analysis showed that ascorbic acid did not significantly affect total protein, collagen content or cell number. Image analysis of polarized light micrographs suggested that collagen fibril coverage increased in response to ascorbic acid treatment, although the differences between groups were insignificant. In addition to ascorbic acid treatment, we also subjected tissue rings to DTPA treatment to prolong ascorbic acid availability in culture medium, which resulted in weak and necrotic tissue rings. Reduced serum was also investigated in order to decrease cell proliferation, which resulted in decreased tissue thickness and increased mechanical strength. Overall, we successfully demonstrated that the mechanical properties of the tissue rings could be altered by ascorbic acid treatment, and developed a series of quantitative methods to measure tissue mechanics, composition and organization. The results of this study further support the

potential to use the tissue ring system as a high throughput screening method for studying the functional properties of three-dimensional engineered tissues.

Table of Contents

Table of Figures

Table of Tables

Chapter 1: Introduction

Over the past three decades, tissue engineering has emerged as a promising approach to creating blood vessel substitutes for clinical transplantation, as well as model systems to study vascular tissue function *in vitro*. Mechanical strength is particularly important for blood vessel substitutes to function properly *in vivo*. The majority of strategies for tissue engineered blood vessel synthesis have involved seeding cells within scaffolds made from synthetic[1-4] or natural polymers;[5-10] although, grafts composed entirely of cells and cell-derived extracellular matrix (ECM) have also been created successfully.[11] Such cell-derived tissue constructs have potential advantages over scaffold-based constructs including greater cell compatibility due to the lack of foreign materials and superior compliance.[12] In addition, "scaffoldless" engineered tissues allow direct assessment of the molecular mechanisms and functional consequences of cell-derived ECM synthesis and organization on tissue structure and material properties, independent of the chemical and mechanical properties of exogenous scaffold materials. Furthermore, three-dimensional (3-D) cell-derived ECM also mimics the cell-cell and cell-ECM interactions that occur in native tissues,[13] and decellularized cell-derived ECM has shown considerable promise as a material to support tissue engineering and repair.[14]

Despite some clinical success in creating cell-derived tissue-engineered blood vessels (TEBVs), the question still remains on how to achieve the required mechanical and physiological properties of transplantable tissues with less time. The ability to decrease the time window for cell-derived tissue synthesis may be achieved by optimizing culture conditions, and could therefore make cell-derived tissue-engineered blood vessels a more clinically attractive solution.

Numerous factors influence the properties of vascular grafts. Soluble factors such as TGF-β1, insulin, ascorbic acid and mechanical conditioning have been shown to improve mechanical

strength in TEBVs.[10, 15, 16] However, the complexity involved in creating vascular grafts that mimic native blood vessels requires extensive studies of the effects of cellular factors on the strength, composition, and function of tissue constructs. The solution to this problem is to develop a screening system that would allow multiple parameters to be studied simultaneously, and provide useful results within weeks.

The mechanical properties of blood vessels are often evaluated by performing uniaxial tensile testing on ringlets cut from blood vessels.[2, 10, 17, 18] The ringlet testing method inspired us to develop a high throughput screening system to assess the function of cell-derived vascular tissue constructs. In our previous studies, we created rounded bottom annular wells to allow cells to self-aggregate and form ring-shaped tissue constructs. This unique model system can be used to rapidly create three-dimensional tissue rings from rat smooth muscle cells (SMCs) within 8 days, and produces thick tissue rings from cells without any scaffold materials.[19] Uniaxial tensile testing has shown an average failure stress at 0.268 ± 0.136 MPa, average failure strain at 0.621 ± 0.117 mm/mm, and average stiffness at 0.626 ± 0.291 MPa. Fabricating tissue rings instead of blood vessels dramatically decreases the culture time required to systematically screen culture conditions to optimize tissue material properties (e.g., strength and stiffness).

The goal of the present study was to determine whether chemical augmentation of the medium during culture affects the mechanical and structural properties of cell-derived tissue rings. Collagen is one of the major contributors to the failure strength of the material,[20] and it is known to be influenced by ascorbic acid.[21-23] Therefore, we utilized the tissue ring system to assess the effect of ascorbic acid on tissue constructs by inspecting mechanical function, tissue morphology, biochemistry and histology, as well as micrograph image analysis (Figure 1). More specifically, the effect of ascorbic acid supplementation on tissue thickness, failure strength, cell

number, total protein, and collagen content were quantified. The results of this study support the premise of using tissue rings as a high throughput model system for studying the functional relationships between cell growth rate, ECM synthesis, cell phenotype, and mechanical properties of cell-derived tissue-engineered constructs.

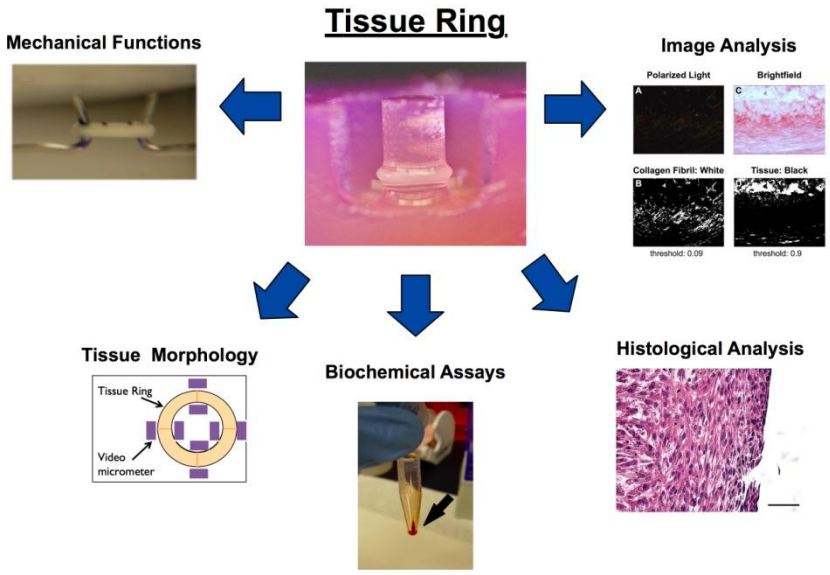

Figure 1. Schematic of the project approach with the effects of ascorbic acid on tissue rings are studied by quantitative assessment of functional endpoints.

Specific Aims

Specific Aim 1. **Evaluate the mechanical properties of cell-derived tissue rings in response to ascorbic acid treatment.** Mechanical properties and histology of tissue rings will be evaluated after culturing in media supplemented with various concentrations of ascorbic acid. The objective of this aim is to validate that the tissue ring system can be used as a model for studying the effects of culture conditions on 3-D tissue constructs. We hypothesize that the failure strength and stiffness of tissue rings will increase with addition of ascorbic acid to the medium during tissue ring seeding and culture.

Specific Aim 2**. Develop quantitative methods to evaluate the roles of collagen and cell number on tissue mechanical strength.** Collagen content and cell number will be assessed by biochemical assays and image analysis. The relationship between collagen quantity, cell number, and mechanical strength of the tissue constructs will be examined. The objective of this aim is to develop a series of methods that will provide quantitative measurements to evaluate the effects of collagen synthesis and cell proliferation on the functional properties of tissue rings.

Chapter 2: Background

The results of the present study strengthen the concept that the cell-derived tissue ring system is a useful tool for "high throughput" screening of soluble factors by showing that the mechanical properties of tissue rings can be altered with ascorbic acid treatment. This section provides a brief overview of the necessary background to explain the need and motivation for this project.

2.1 Clinical Need

Approximately 80,000,000 American adults have some form of cardiovascular disease. Among them, more than 500,000 patients required coronary artery bypass procedures in 2007.[24] This procedure involves bypassing the blocked artery with a graft that is typically taken from the internal thoracic arteries, saphenous vein or radial arteries of the patient.[25, 26] However, autologous grafts might not be the ideal option for some patients with unhealthy blood vessels, and other health complications.[27] Furthermore, harvesting an autologous vessel creates a second injury site, which lengthens the recovery time. An alternative source of blood vessels that is not burdened by the above complications and provides off-the-shelf availability is needed in order to provide better treatment to patients.

Large diameter blood vessels made from synthetic materials such as PTFE and Dacron are the primary options for clinical use when autologous vessels are not available. These synthetic materials are commercially available and easy to manufacture. However, compliance mismatch between the synthetic material and native vessel often lead to problems such as hyperplasia at the anastomoses.[28, 29] Furthermore, small diameter blood vessels made from

synthetic material are often linked to thromboses, infection and chronic inflammatory responses.[30-33] Therefore, alternative approaches are needed to address these limitations.

2.2 Tissue-Engineered Blood Vessels

The goal of TEBV development is to provide an answer to the inadequacy of vessels fabricated entirely from synthetic materials. The ideal design of a TEBV mimics the mechanical and biological properties of native blood vessels. Numerous approaches have been taken to create blood vessel substitutes. The major strategies include seeding cells within scaffolds made from synthetic[1-4] or natural polymers.[5-10] Blood vessels created from complete cell-derived approach have recently been used in clinical trials. L'Heureux *et al.* have created a completely cell-derived vessel without any scaffold by growing cells on tissue culture-treated plates and rolling them into a tubular structure.[34] Their blood vessel substitute achieved burst pressures of 2000 mmHg (compared to 1700 mmHg for human saphenous veins).[34]

One of the major advantages of the completely cell-derived approach is that ECM provides structural support and anchorage to cells, allows intracellular communication, and contributes to the mechanical properties of the tissue construct.[35-37] For example, collagen is one of the major ECM proteins that plays an important role in determining the failure strength of tissue.[38] Optimization of this ECM protein would help to create stronger vessel substitutes. Completely cell-derived materials, which encourage ECM protein deposition through cell-cell interactions, have been shown to create tissue constructs that are mechanically and biologically similar to native blood vessels.[27] Instead of remodeling the existing polymers in the scaffold material, cell-derived materials encourage cells to create their own matrix, which enhances the ECM-cell interactions.

Cell-derived tissues have weaker material properties initially, but strength builds up gradually as ECM is synthesized and organized by cells. Recent studies have demonstrated that TEBVs fabricated entirely from human cells without exogenous scaffolds show promising results *in vivo* after 3 months in culture.[27] This suggests cell-derived TEBVs may provide very good mechanical strength when the surgical need for a blood vessel substitute is not immediate. The challenge is to considerably decrease the TEBVs culture time to a clinically relevant time frame, while maintaining its strength.

2.3 Testing for Mechanical Properties

Since the mechanical properties of tissue engineered constructs are vital to their clinical implementation, mechanical testing of these materials provides valuable information about the properties of the material. Therefore it is imperative in the research and development process of creating vessel substitutes that mimic the native tissue.

Either one or both of the following test methods are often used to assess the mechanical properties of blood vessels: burst pressure testing of vessels, and uniaxial tensile testing of ring segments. During burst pressure testing, saline solution is infused into the vessel until the vessel bursts.[2, 17] On the other hand, tensile testing applies a uniaxial force to a ring segment of the tissue. The result can then be used to calculate the burst pressure of the vessel in a tubular form [2, 10, 17, 18] A number of common properties are measured in general: ultimate tensile strength (UTS), failure strain, and maximum tensile modulus (MTM)

$$\mathrm{UTS} = \frac{\text{force at failure}}{\text{cross sectional area}}\ ;\ \text{failure strain} = \frac{\Delta\,\text{length}}{\text{initial length}}\ ;\ \mathrm{MTM} = \text{max. ratio of}\ \left(\frac{\text{stress}}{\text{strain}}\right)$$

UTS is the measurement of tensile stress that is required to cause material failure. Failure strain is a ratio of material deformation at failure in one dimension to the original length of the material. MTM is the maximum ratio between the stress and strain values. It is determined by finding the steepest slope on the stress and strain curve. This parameter provides information about the stiffness of the tissue.

2.4 Collagen Synthesis and Organization

The organization, diameter and crosslinking of collagen fibrils dictate the material strength of the tissue construct.[20] Collagen fibrils are stretched, and deformed during loading until mechanical failure.[39] Studies of the mechanical characteristics of single collagen bundles have shown that the tangent modulus and failure stress are inversely proportional to the collagen fibril diameter. Smaller fibril diameters displayed higher failure stress and stiffness, since a large fiber is more likely to contain structural defects.[40] Furthermore, Martin *et al.* utilized collagen birefringence to show failure strength is higher in the longitudinal direction where collagen fibrils are aligned.[41]

Collagen molecules have a triple helical structure formed by joining three left-handed helices. Each chain is composed of a series of Gly-X-Y sequences, where Gly represents glycine and both X and Y could be any amino acid. Proline and hydroxyproline are the two molecules often found in the sequence. Proline at the Y position of the sequence can be enzymatically converted to hydroxyproline by prolylhydroxylase.[42] The increased presence of hydroxyproline in the sequence helps to stabilize the helical structure through stereoelectronic effects.[43]

2.5 Effects of Ascorbic Acid on Cell Culture

Ascorbic acid is essential to the crosslinking of collagen fibrils. Studies have found that ascorbic acid functions as a co-factor for synthesis of hydroxyproline and hydroxlysine.[21-23] Both hydroxyproline and hydroxlysine help to stabilize the triple helical structure of collagen, and play an important role in collagen crosslinking.

The process of proline hydroxylation requires the multienzyme complex prolyl 4-hydroxylase, which requires Fe^{2+} to function properly. During the catalytic cycle of hydroxylation, $Fe^{3+}$is produced as a by-product at the end of process. This ferric ion must be converted back to ferrous ion for the hydroxylation process to repeat.[44] Ascorbic acid functions as a cofactor to the reaction by reducing Fe^{3+} to Fe^{2+}(Figure 2).[45] Studies have shown that the absence of ascorbic acid prevents the hydroxylation process from continuing. Prolyl 4-hydroxylase activity is reactivated as ascorbic acid is added back to the medium.[46]

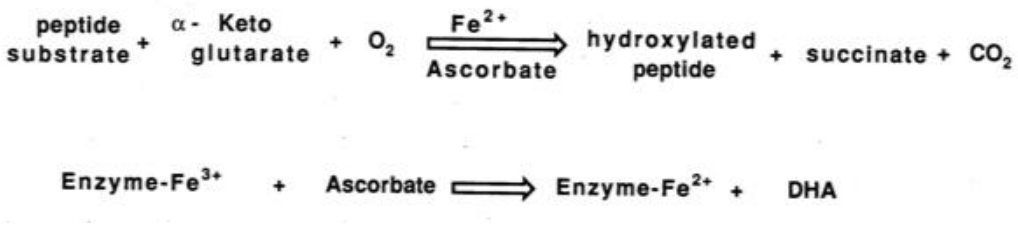

Figure 2. Schematic of the role of ascorbic acid during proline hydroxylation process in collagen crosslinking.[23]

The process of collagen synthesis and hydroxylation were mistaken as a single step at the study of collagen in the early studies.[47] These studies utilized hydroxyproline as a quantification assay to study collagen synthesis because hydroxyproline is often incorporated into collagen during post-translational modification. Later studies found that synthesis and crosslinking are two separate processes.[48, 49] The results of these early studies on collagen

synthesis using the hydroxyproline assay were later suspected to be inaccurate.[47] Collagen synthesis and hydroxylation were later found to be two separate steps.[50, 51]

Recent advancement in molecular biology allowed the collagen synthesis process to be studied from a different angle. The effects of ascorbic acid appeared to depend on the state of the targeted cells. Some studies have found that adding ascorbic acid to smooth muscle cells in culture stabilizes collagen mRNA levels,[52] and causes a 6-fold increase in procollagen production.[53] However, other studies on ascorbic acid have found ascorbic acid only increases collagen crosslinking, while synthesis and procollagen production are unaffected.[47, 54]

Further studies have shown the effects of ascorbic acid treatment are not limited to collagen. After the addition of ascorbic acid, smooth muscle cells changed from an elongated morphology to polygonal shape.[21-23, 55] Some studies have suggested that adding ascorbic acid increases cell proliferation,[55-57] while others found its effect was insignificant.[47, 52] Interestingly, ascorbic acid also affects elastin, another ECM structural protein. The level of elastin mRNA transcription decreases as much as 70% after ascorbic acid treatment.[52, 54] The degree of effect on elastin in these studies correlated to the concentration of ascorbic acid. While elastin production remains at a similar level after 5 days of treatment at 25 µg/ml, the production of this molecule drops significantly when cultures are exposed to 50 µg/ml of ascorbic acid for the same culture period.[52] These studies on elastin reinforce the need to optimize culture conditions when creating tissue-engineered blood vessels.

2.6 Stability of Ascorbic Acid in Culture Medium

Ascorbic acid has been shown to have a relatively short half-life in culture. Some studies have found this molecule completely disappeared from culture medium in 12 hours.[47] Feng *et*

al. have found the half-life of ascorbic acid decreased by 25% when O_2 levels were increased from 20% to 95% in a circulator.[58] Moreover, the temperature of storage could also affect ascorbic acid half-life. At 0°C, 83% of the original 300 µg/ml ascorbic acid still remained in medium after 4 days. However, 50% of the ascorbic acid disappeared within 1.5 hours when the medium is placed at 20°C due to oxidation reaction.[58]

2.7 Alternative Methods Used to Quantify Collagen

Collagen quantification is an important parameter in assessing tissue structure as a result of ascorbic acid treatment. The hydroxyproline assay has been used to measure collagen content. This assay measures the concentration of hydroxyproline, an important non-essential amino acid for collagen crosslinking.[59] Test samples for hydroxyproline assay are prepared by breaking down collagen protein into single amino acids through enzyme digestion. Therefore, this assay might be incompatible with procedures where protein structural integrity is required.

Sirius Red dye has been developed recently as another method to measure collagen content. The Sirius Red assay does not replace the hydroxyproline assay, but it provides measurement of a different parameter. Instead of measuring crosslinked collagen, Sirius Red dye only binds to newly synthesized, acid soluble collagen. The two main components of the dye are Direct Red 80 and picric acid. Sirius Red dye binds to collagen by allowing its sulphonated acid side-chain groups to interact with the basic amino acid side groups of collagen.[60] Upon binding of the molecule, Sirius Red dye lines up parallel to the orientation of collagen. Constantine *et al.* has documented the concentration of picric acid is not important to the proper function of the dye.[60] However, Sirius Red loses its collagen specificity when picric acid is

absent from the solution. This exact mechanism of picric acid-mediated collagen binding in the Sirius Red dye is still unknown.[61]

Recent studies have reported possible non-specific binding of Sirius Red dye to serum albumin in complex culture medium,[62] which potentially limits its use to tissue lysates (and not cultured cells). Despite this limitation, Sirius Red dye has been widely used in collagen research due to its simplicity.[63-65] Studies have shown that column ultrafiltration could be used to significantly decrease binding to serum albumin.[62] In contrast to the hydroxyproline assay, Sirius Red allows collagen content to be measured without subjecting the tissue to harsh chemicals that denature proteins. The Sirius Red method may therefore provide greater compatibility with multiple assays carried out from the same cell or tissue lysate.

2.8 Collagen Fibril Detection in Tissue with Polarized Light Microscopy

Polarized light microscope is a technique to visualize collagen fibrils in a tissue section. Unlike traditional light microscopy, the light that is arriving at the tissue sample is polarized to a single direction after passing through a polarizer. Optical anisotropy of collagen molecules causes birefringence, the decomposition of polarized light beam into two rays as it strikes the molecule. This results in two rays that travel at different velocities and vibrate in a plane 90° to each other. This allows collagen fibrils to be distinguished from other compounds in the tissue.[66] Birefringence of collagen fibrils can be observed without a specific stain. However, picrosirius red is often used to enhance the birefringence, which provides qualitative information about collagen fibril alignment (Figure 3). It can be difficult to use this technique to distinguish between crosslinked and uncrosslinked collagen.

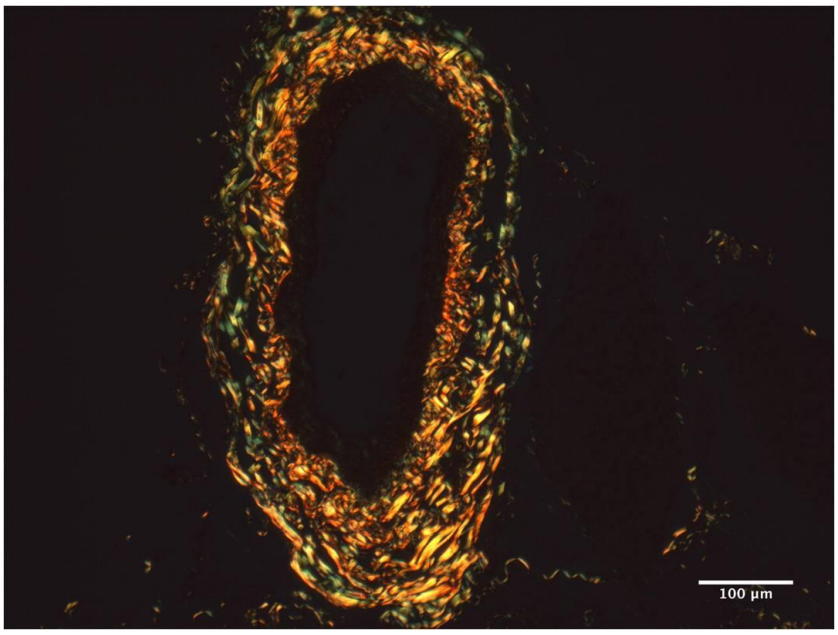

Figure 3. Rat artery stained with picrosirius red under polarized light. Scale bar: 100µm.

The goal of the study is to evaluate whether the structure, composition and function of the tissue rings can be changed by adding soluble factors to the medium. Herein, we have investigated the effect of ascorbic acid on tissue thickness and mechanical strength. We have also developed a series of quantitative methods to measure functional changes using different approaches and techniques.

Chapter 3: Methods

3.1 Custom Cell Culture Well Fabrication

Polycarbonate (Small Parts, Inc., Miramar, FL) was precision machined to create a mold to form annular wells with inner post diameters of 4 mm (4 mm ID). Polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, Midland, MI) was mixed at a 10:1 ratio (w/w) of base to curing agent, degassed for 1 hour, and poured onto the polycarbonate mold. After curing at 60°C for 4 hours, the PDMS was peeled from the mold and used as a template. Sterilized 2% agarose (w/v; Lonza, Rockland, ME) dissolved in Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Herndon, VA) was poured onto the PDMS template to form the cell-seeding wells. Individual agarose wells were cut away from the PDMS template and placed in 6-well plates. The agarose wells were equilibrated in DMEM supplemented with 10% fetal bovine serum (FBS; PAA, Ontario, Canada) and 1% penicillin/streptomycin (Mediatech, Herndon, VA) for 1 hour at 37°C and 5% CO_2 prior to the cell seeding. A schematic of the process for creating agarose wells and seeding cells is shown in Figure 4.

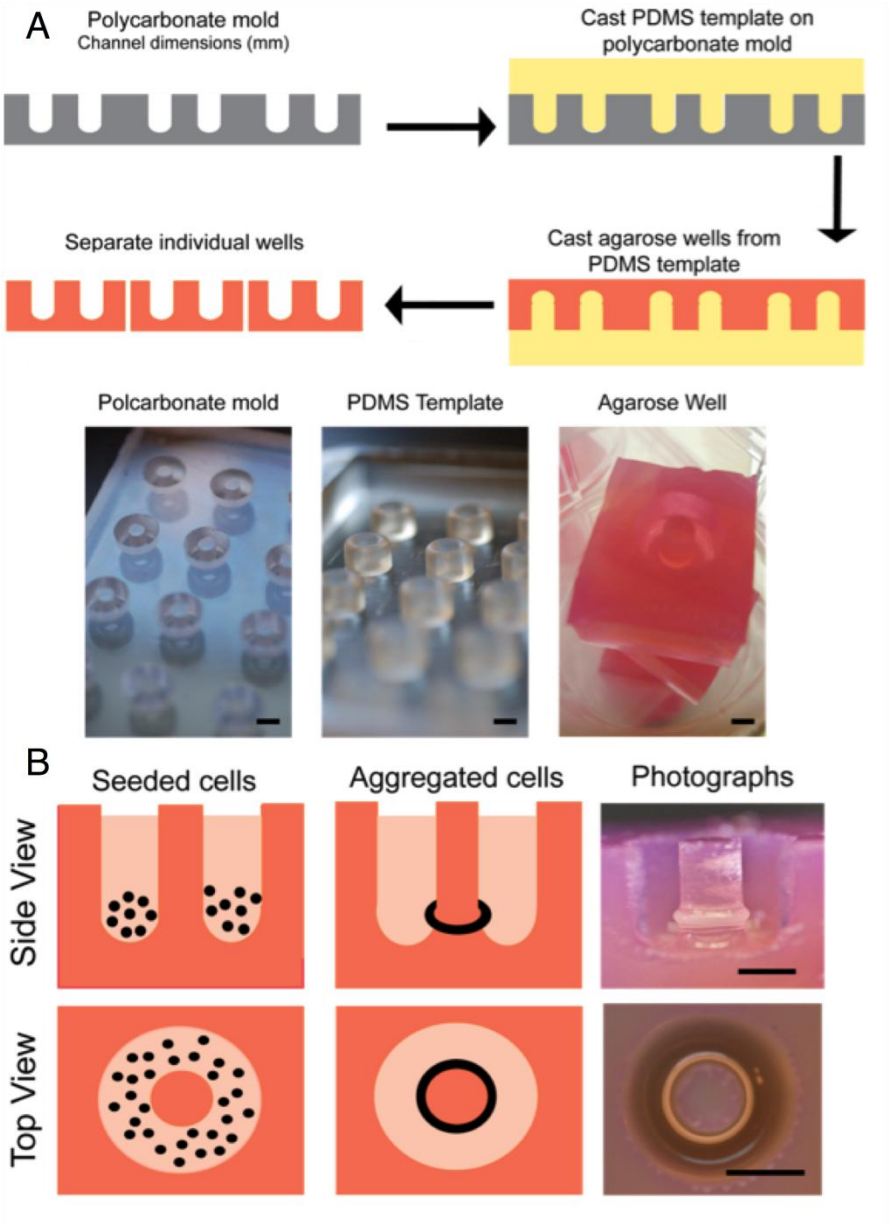

Figure 4. Schematic of tissue ring mold formation and photographs of components (A). Cell suspension pipetted into agarose wells. Within 24 hours, cells aggregated and contracted around posts to create tissue rings; Photographs of the side view and the top view of a 4 mm ID tissue ring after 7 days in culture (B). Scale bars: 4 mm.

3.2 Smooth Muscle Cell Culture And Seeding

Rat aortic smooth muscle cells (SMCs; cell line WKY 3M-22 derived from adult Wistar-Kyoto rats [67] provided by Dr. Thomas Wight) were cultured in DMEM (Mediatech, Manassas, VA) supplemented with 10% FBS (PAA, Ontario, Canada) and 1% penicillin/streptomycin (Mediatech, Manassas, VA). At 90% confluence, SMCs were trypsinized and re-suspended in culture medium containing the desired concentration of ascorbic acid (Sigma-Aldrich, St. Louis, MO). Each agarose well was seeded with 1.33×10^6 cells suspended in 410 µl culture medium (3.2×10^6 cells/ml). Plates were left undisturbed in the incubator for the first 48 hours after seeding, and culture media was changed every 48 hours for the culture duration of 7 days.

3.3 Ascorbic Acid Treatment

A stock solution of ascorbic acid in 100 mg/ml (Cat. 255564-100G, Sigma-Aldrich, St. Louis, MO) was prepared in DMEM, sterile filtered, and stored at 4°C. The stock ascorbic acid solution was diluted to its desired concentration in culture medium as needed. Culture medium supplemented with different concentrations of ascorbic acid was prepared at the beginning of each ring culture experiment, and stored at 4°C. Unused medium was discarded at the end of each 7 day experiment.

3.4 Tissue Ring Thickness Measurements

After 7 days in culture, the tissue rings were removed from the agarose wells and transferred to 60 mm Petri dishes filled with phosphate buffered saline (PBS) at room temperature. The rings were centered under a machine vision system (DVT Model 630, DVT Corporation, Atlanta, GA) and thickness measurements were acquired in four separate positions along the circumference of the ring using edge detection software (Framework 2.4.6, DVT;

Figure 5). The four measurements were averaged to yield the mean thickness for each sample. The measured resolution for the machine vision system was 44 µm. The cross-sectional area was assumed to be circular, and was calculated with the following equation:

$$\text{area} = 2 \bullet \left(\frac{\text{thickness}}{2} \right)^2 \bullet \pi$$

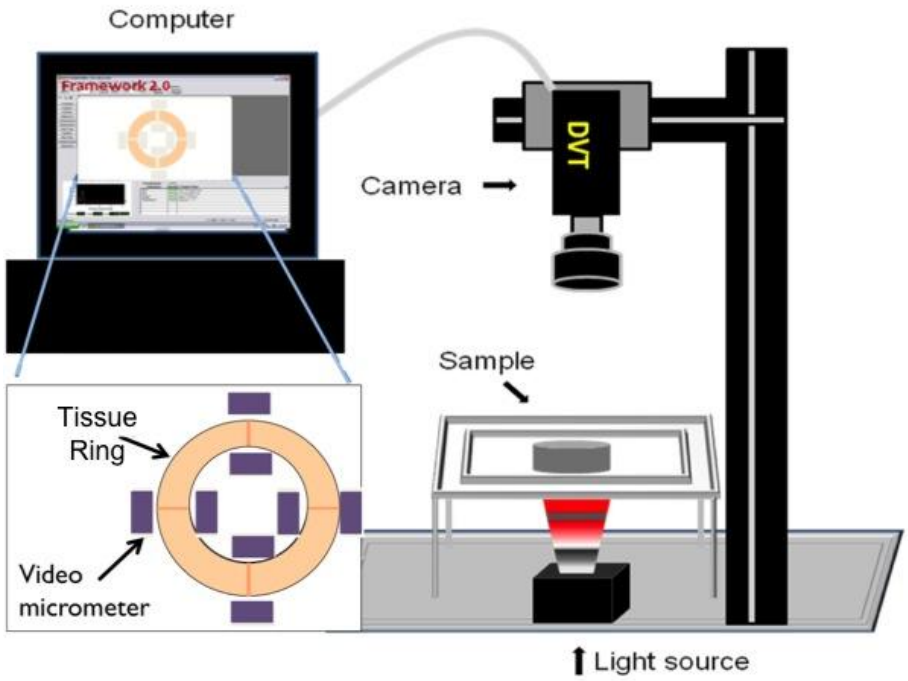

Figure 5. Schematic of digital vision system used to measure thickness of tissue rings using video micrometers at four locations.

3.5 Mechanical Testing

The mechanical properties of the tissue rings were measured using a uniaxial testing machine (ElectroPuls E1000; Instron, Norwood, MA). The tissue rings were mounted between two small stainless steel pins (referred to as "grips") and submerged in PBS (shown

schematically in Figure 6). One grip was connected to an electromagnetic actuator and the other to a 1 N (±1 mN) load cell. Force, F, and displacement, were recorded continuously throughout the test at 10 Hz. A tare load of 5 mN was applied to the mounted ring and the gauge length (l_g) was recorded. The rings were then preconditioned for 8 cycles from the initial (tare) load to 50 kPa engineering stress (F/A) and then pulled to failure at a rate of 10 mm/min.

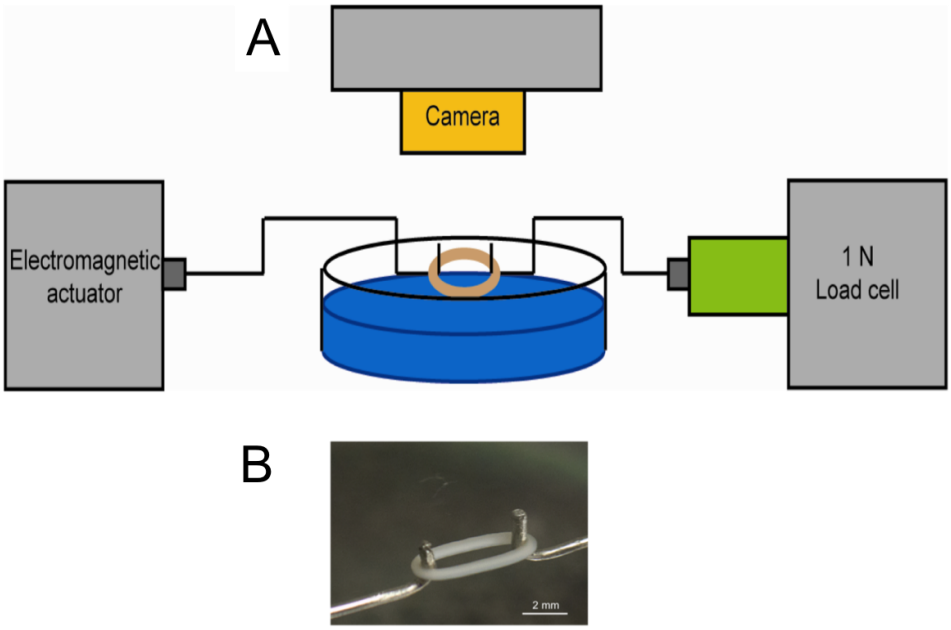

Figure 6. Schematic of uniaxial testing machine with a tissue ring mounted on the grips (A). One grip is attached to an electromagnetic actuator; the other end is attached to a 1N load cell. Photograph of a 4 mm tissue ring (cultured for 7 days) mounted for uniaxial testing (B); scale bar: 2mm.

The engineering stress and grip-to-grip strain ($\Delta l/l_g$) data were analyzed using MATLAB (The MathWorks, Inc., Natick, MA) to obtain the ultimate tensile strength (UTS), failure strain, and maximum tangent modulus (MTM, the maximum slope of the stress-strain curve) as illustrated in Figure 7.

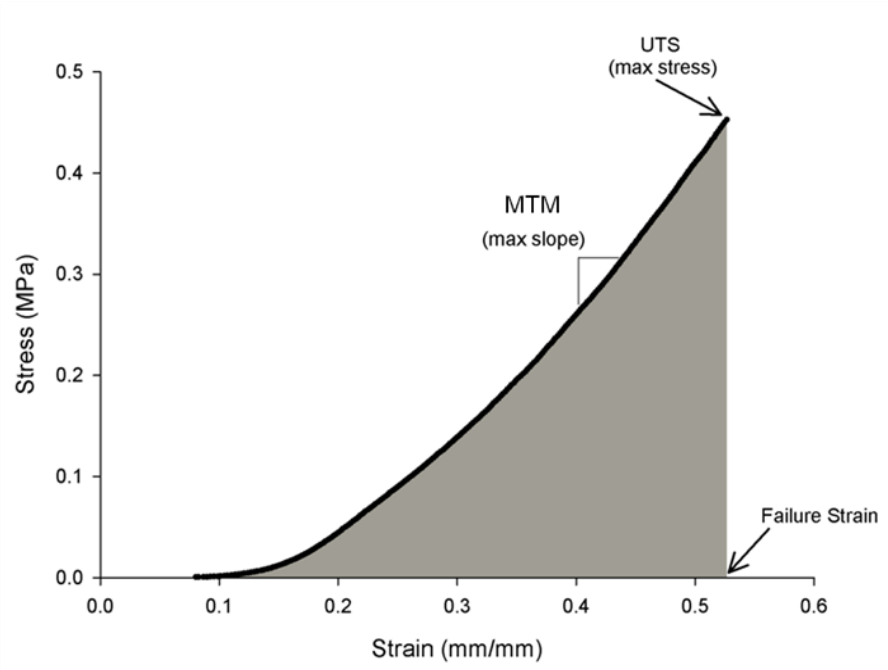

Figure 7. Example stress and strain plot obtained from a tissue ring sample.

3.6 Tissue Lysate Preparation

Each tested tissue ring was washed thoroughly in 1X phosphate buffered saline, dried with a Kim wipe, and transferred to a pre-labeled Eppendorf tube. The samples were then flash-frozen in liquid nitrogen, and stored at -80°C for downstream analysis.

A stock lysis solution was prepared by adding one tablet of proteinase inhibitor cocktail (Cat. 11836170001, Roche, Indianapolis, IN) to 10 ml of DNAse/RNAse-free water. An aliquot of 500 µl lysis solution was pipetted into the individual Eppendorf tubes that contained the tissue samples. The samples were broken down into solution with a tip sonicator (Misonix Micrson^{TM} XL-2000, Cole-Parmer) inserted into the tube for 10 seconds. The lysate was stored at -80°C until subsequent analysis was performed (Figure 8).

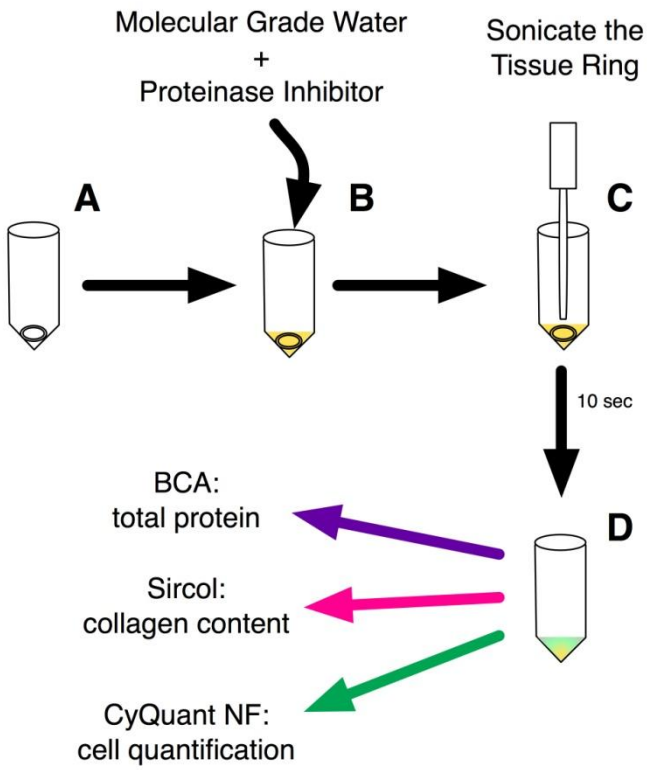

Figure 8. Schematic of the tissue ring lysate preparation process. The ring is placed in a tube (A), lysis solution is added (B), the sample is sonicated (C), and lysate is used for BCA, Sirius Red and CyQuant NF assays (D).

3.7 Total Protein Quantification

The total protein content of the tissue rings was quantified with a BCA™ Protein Assay Kit (Pierce, Rockford, IL). A fraction of the cell ring lysate (25 µl) was mixed with the 200 µl of BCA working reagent in a 96-well plate. The plate was incubated at 37°C for 30 minutes. Absorbance for each sample was measured at 562 nm using a microplate reader (SpectraMax 250, Molecular Devices, Sunnyvale, CA). A standard curve was prepared for each assay, using pre-diluted standards (bovine serum albumin, BSA; provided with the assay kit) of known protein concentrations (Figure 9).

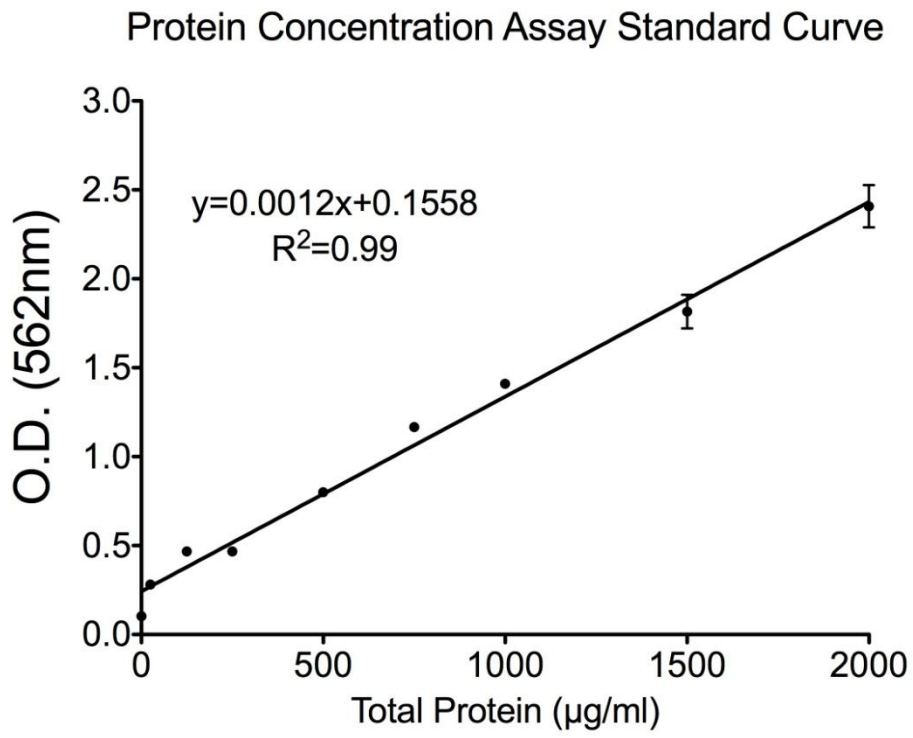

Figure 9. BCA assay standard curve. Optical density vs. known protein (BSA) concentration, reported as mean ±SD, n=3.

3.8 Collagen Quantification

Collagen was measured with a Sirius Red assay (reagents from Sigma; 0.1% each of Fast Green FCF and Direct Red 80 in Picric Acid)). A fraction of tissue lysate (100 µl) from each sample was mixed with 900 µl of dye on a rotator at room temperature for 30 minutes. The mixture was then centrifuged at 14,000 rpm for 10 minutes, after which the supernatant was poured out carefully without disturbing the pellet (Figure 10). The Eppendorf tubes were blotted on a paper towel to allow removal of unbound dye. The pellet was resuspended in 500 µl of 0.5N NaOH, and vortexed gently for 10 minutes. The sample (100 µl) absorbance was read at 550 nm on microplate reader (SpectraMax 250, Molecular Devices). A standard curve was prepared

from Type I Rat Tail Collagen (BD Bioscience, Bedford, MA), and was used to calculate the collagen content in each set of experiments (Figure 11).

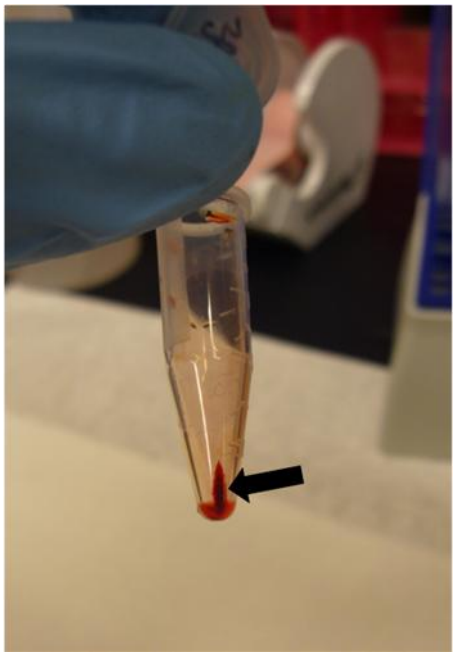

Figure 10. Photograph of an Eppendorf tube with Sirius Red dye bound to pelleted acid soluble collagen sample after the supernatant was removed. Arrow indicates Sirius Red dye-bound pellet.

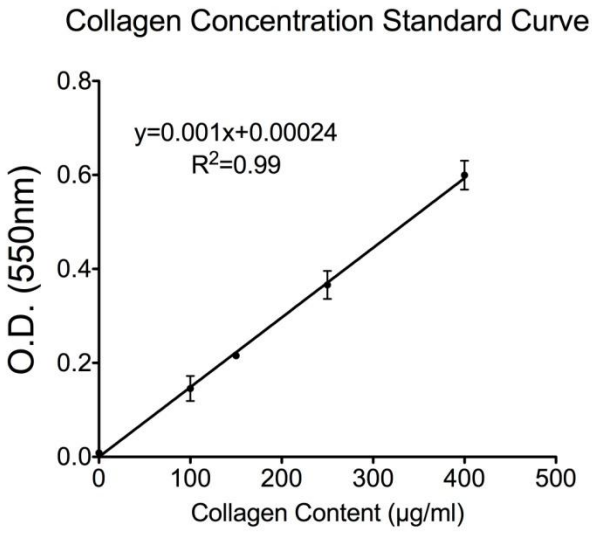

Figure 11. Sirius Red assay standard curve. Optical density vs. pre-diluted collagen samples of known concentrations, reported as mean ±SD, n=3.

3.9 Cell Number Quantification

The number of cells for each tissue ring was quantified using CyQuant NF (Invitrogen, Eugene, Oregon). A standard curve was prepared using known cell numbers for each experiment. The tissue lysate (1 µl) was mixed with 99 µl of DNAse/RNAse-free water in a well of a 96-well plate. CyQuant dye (100 µl) was then added to each well, mixed gently on a rotator for 30 seconds, and incubated at 37°C for 30 minutes protected from light. After the incubation period, the samples were read on a micro-plate reader (PerkinElmer Victor^3) with excitation set to 485 nm. The emission of the dye was detected at 530 nm. The cell number for individual samples was calculated by correlating the fluorescence reading to the standard curve that was prepared from pre-diluted cell lysates prepared from a known number of cells (Figure 12).

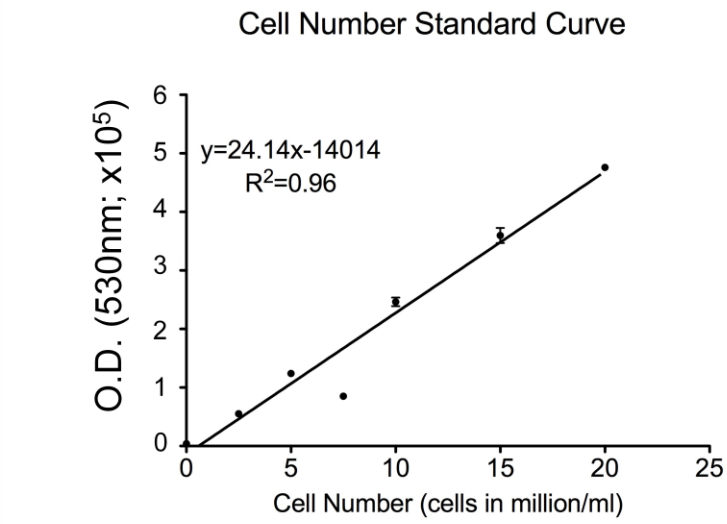

Figure 12. CyQuant NF standard curve. Optical density vs. known cell number, reported as mean ±SD, n=3.

3.10 Histology

Tissue rings were fixed for 4 hours in 10% neutral-buffered formalin and embedded in paraffin. Five micrometer sections were cut and adhered to Superfrost Plus slides (VWR, West Chester, PA). The sections were stained with hematoxylin and eosin (H&E; reagents from Richard Allan Scientific, Kalamazoo, MI), and Fast Green/Picrosirius Red (reagents from Sigma; 0.1% each of Fast Green FCF and Direct Red 80 in Picric Acid) and images were acquired on an upright microscope (Leica DMLB2) equipped with a digital camera (Leica DFC 480).

Polarized light images of samples stained with Picrosirius Red alone were acquired using an inverted microscope (Olympus, IX81) with a digital camera (Olympus, Q-Color 5). A linear polarizer was placed between the light source and the specimen, while the analyzer was installed in the light path between the specimen and the camera. The analyzer was rotated until maximum light diminishment was obtained prior to image acquisition.

3.11 Analysis Of Area Fraction of Collagen Fibrils

Samples stained with Picrosirius Red were each positioned under an inverted microscope (Olympus, IX81), and polarized light images (exposure time: 360 ms) were taken with a 20X objective. Without disturbing the slide, a bright field image was acquired at the same location by switching the polarizer to the "off" position (exposure time: 12 ms). Four identical locations were sampled for each ring under the same lighting intensities, and aforementioned exposures. For incomplete tissue rings, two out of four locations were imaged (Figure 13).

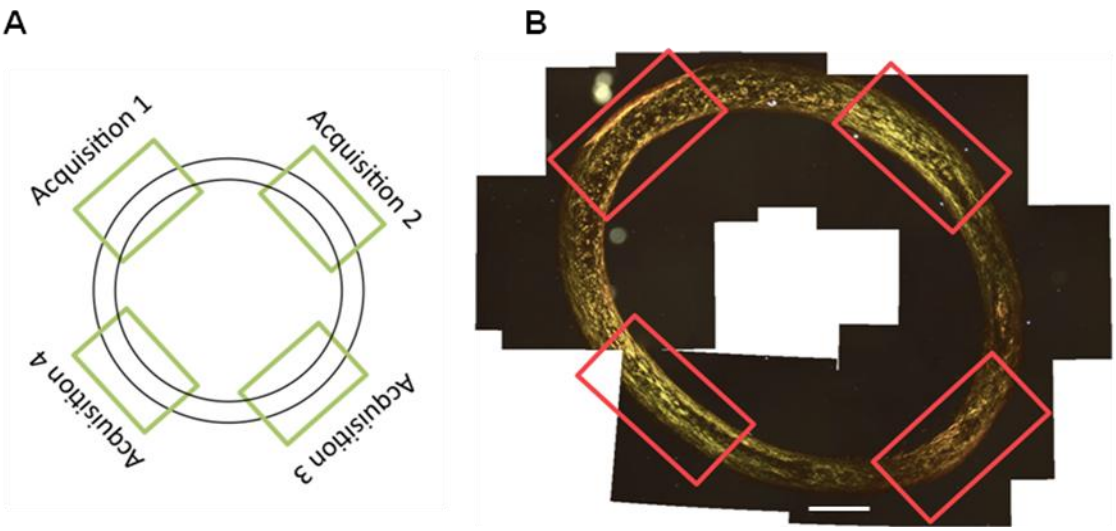

Figure 13. Schematic of image acquisition locations (A). Multiple images of a tissue ring under polarized light merged to a single image (B). The red boxes show the actual locations of image acquisition for area fraction analysis.

These images were analyzed with a custom developed image analysis program written in MATLAB (The MathWorks, Inc., Natick, MA). The program first converted the color images (Figure 14A) to black and white with a threshold set to 0.09. The collagen appeared in white (Figure 14B). The same procedure was applied to the bright field image (Figure 14C), however with the threshold was set to 0.90. The tissue area was thereby converted to black (Figure 14D). The area fraction of collagen fibrils for each sample was calculated using the following equation:

$$\text{fibril area fraction}(\%) = \frac{\text{fibril pixels}}{\text{tissue pixels}} \bullet 100$$

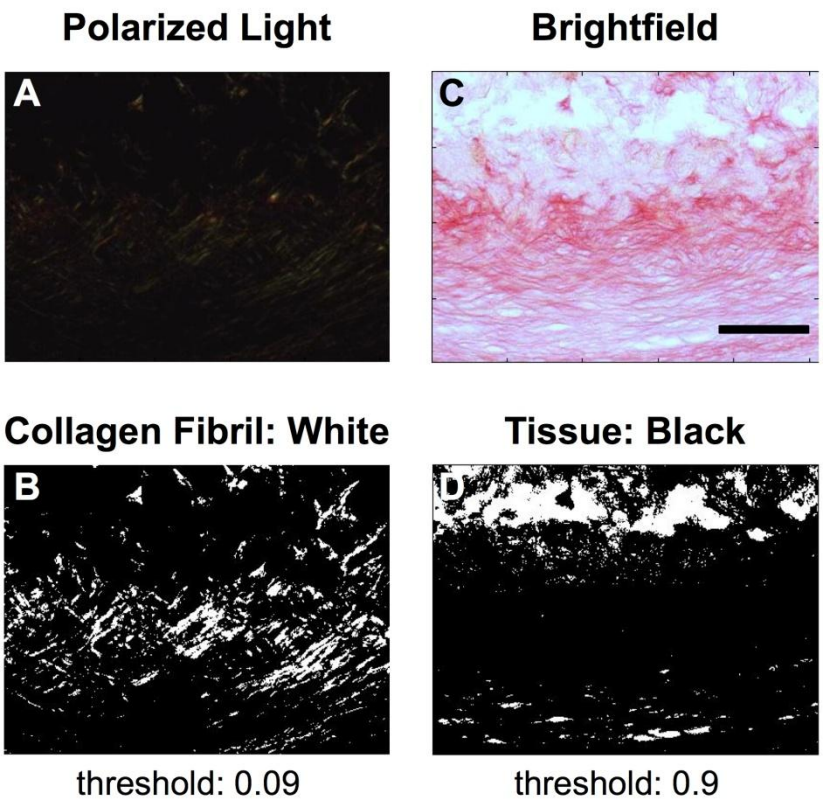

Figure 14. Output from MATLAB image analysis. Tissue ring under polarized light (A); Collagen fibers in thresholded image (white) (B); Bright field image of tissue ring stained with Picrosirius Red (C) (scale bar was superimposed post-image analysis, scalebar: 100 µm); Thresholded bright field image (black) (D); All images were taken under the same magnification.

3.12 Image Analysis of Cell Distribution

Histological staining such as hematoxylin and eosin (H&E) provided only qualitative information about cell distribution within the tissue rings. We thus developed a second image analysis program in MATLAB that provided a method for quantitative analysis of this parameter when combined with immunohistochemistry.

To visualize cell proliferation, deparaffinized, rehydrated histological sections were stained with the tissue ring with mouse monoclonal anti-human PCNA, which cross-reacted with

rat (0.2 µg/ml, Abcam, Cambridge, MA) for 1 hour, and labeled with anti-mouse immunoglobulin, conjugated to Alexa Fluor 488 (5 µg/ml, Invitrogen) for 45 minutes. The tissue sample was then counter stained with Hoechst 33342 dye (10µg/ml; Invitrogen, Eugene, OR) for 3 minutes, rinsed with PBS and coverslipped with aqueous mounting medium (Prolong Gold, Invitrogen). Images (in 1344 x1024 pixel) were then acquired in .tif format with an inverted microscope (Olympus, IX81), and were processed using the custom developed image analysis program. The program first converted the color image to black and white image by setting threshold 0.5. It then measured white pixel distribution across the image and plotted on a histogram.

3.13 Low Serum Treatment

Rat SMCs (1.33x106 cells) were seeded into each agarose mold in DMEM supplemented with 10% FBS. After 48 hours in culture, one group was switched to 2% FBS, another was switched to 2% FBS supplemented with 50 µg/ml of ascorbic acid. A third group remained in standard 10% FBS. Medium was changed every 48 hours. The tissue rings were harvested for thickness measurement, mechanical testing, and histological analysis after 7 days in culture.

3.14 Reducing Ascorbic Acid Degradation in Culture Medium with DTPA

Four conditions were tested in this experiment. DTPA (Cat. D6518, Sigma) stock solution was created by dissolving the chemical in DMEM, followed by sterile filtration. Medium with different supplements were prepared the day before seeding. Rat SMCs were seeded in medium contained 10% FBS. They were switched to 2% FBS 48 hours after seeding and supplemented with soluble factors as described in earlier section. Medium was changed

every 48 hours. Thickness measurements, mechanical testing, and histological analysis were conducted after 7 days in culture.

Table 1. Experimental setup for the DTPA study

	DTPA (5 µg/ml)	
Ascorbic Acid (50 µg/ml)	- ascorbic acid/- DTPA	- ascorbic acid/+ DTPA
	+ ascorbic acid /- DTPA	+ ascorbic acid/+ DTPA

3.15 Statistics

The data are reported as mean ± standard error (S.E.M.) for the tissue ring thickness values (4 measurements were obtained per ring sample) and collagen fibrils area fraction (4 measurements were obtained per ring sample). Mechanical and biochemical measurements are reported as mean ± standard deviation (S.D.).

A one-way ANOVA was used to analyze the effects of ascorbic acid treatment on tissue ring thickness, mechanical properties, biochemical assays, and image analysis. Tukey post hoc analysis was used to identify significant differences ($p < 0.05$) between parameter values. The correlation between data sets (e.g. cell number v. tissue ring thickness) was calculated using a Pearson product-moment correlation coefficient. In contrast to linear regression analysis, where cause and effect relationships are measured, Pearson correlations only calculate the strength of a linear association between two variables. The coefficient of correlation ranged from -1 to 1. Coefficient values close to 1 indicate a strong positive correlation. A coefficient close to 0 suggests the linear association is insignificant. Coefficient values close to -1 suggest an inverse

correlation between variables. All statistics in this report was prepared with Prism (Version 5.0b, GraphPad Software, Inc., La Jolla, CA).

Chapter 4: Results

4.1 Ascorbic Acid Treatment Has No Effect On Tissue Ring Thickness

The effects of ascorbic acid treatment on the mechanical strength and stiffness of the tissue rings were evaluated. The different groups were treated with 0, 50, and 150 µg/ml of ascorbic acid. These numbers were chosen based on reported concentrations applied in previous studies to improve collagen synthesis.[15, 34, 68] The cross sectional area for mechanical testing was calculated by measuring the thickness of the tissue using a digital vision system. The thickness values for untreated and ascorbic acid-treated tissue rings were not significantly different from group to group (0.714 ±0.047 mm, 0.726 ±0.030 mm and 0.707 ±0.012 mm for samples treated with 0, 50, and 150 µg/ml of ascorbic acid, respectively; Figure 15).

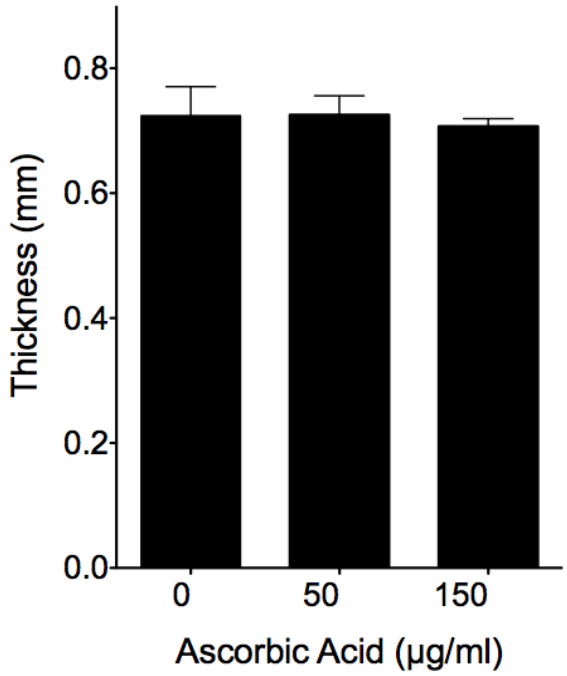

Figure 15. Tissue ring thickness after 7 days of treatment with different concentrations of ascorbic acid. Four thickness measurements were obtained for each ring sample. (mean ± S.E.M., n=5)

4.2 Ascorbic Acid Treatment Does Not Significantly Increase Cell Number

The insignificant difference in thickness led us to investigate whether cell number for each ring was affected by ascorbic acid treatment. A previous study suggested that cell number is correlated to the thickness of tissue.[69] Therefore, we decided to investigate whether a similar relationship existed in the tissue rings. The number of cells within individual tissue rings was quantified using CyQuant NF, a fluorescent dye that binds to DNA. On average, the cell number reached $4.9 \times 10^6 \pm 1.3 \times 10^6$ after 7 days in culture. Although statistical analysis indicated that the ascorbic acid treatment had no significant effect on the cell number in the tissue rings (Figure 16), the thickness of the tissue rings was found to correlate with the cell number across all samples (Figure 17).

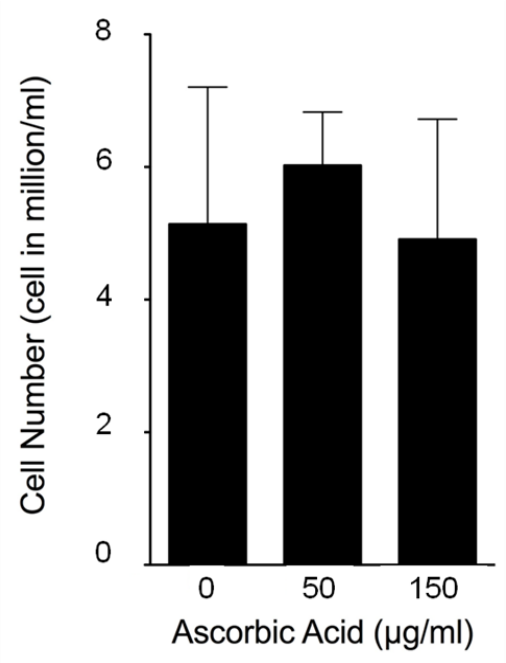

Figure 16. Effect of ascorbic acid treatment on cell number within tissue rings (values are expressed as mean ±S.D., n=5).

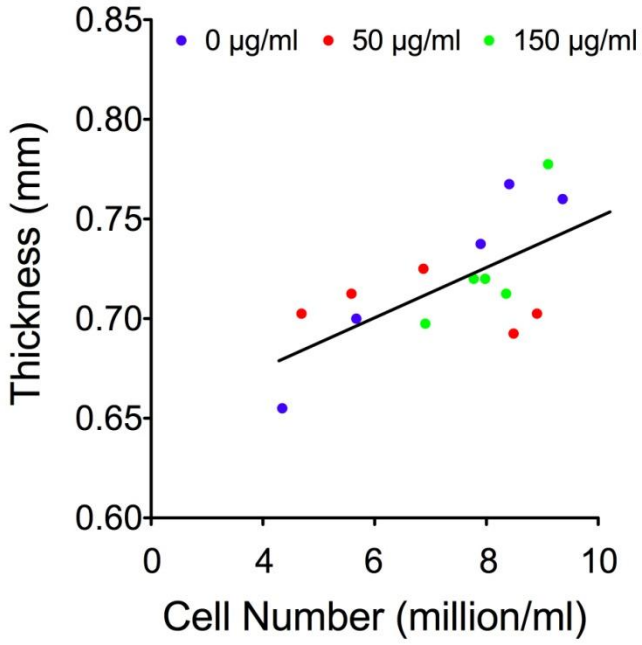

Figure 17. Pearson correlation between cell number and thickness. The line indicates a significant correlation ($p < 0.05$; Pearson r: 0.66; R^2: 0.44).

4.3 Ascorbic Acid Treatment Increases Tissue Ring Strength And Stiffness

The overall mechanical strength values were higher in the ascorbic acid-treated rings. The average ultimate tensile strength (UTS) values were 0.242 ±0.034 MPa, 0.408 ±0.029 MPa and 0.409 ±0.076 MPa for tissues treated with 0, 50, and 150 µg/ml of ascorbic acid treatment, respectively. The addition of ascorbic acid (50µg/ml) to the medium increased the tissue failure strength by almost 70%. However, there was no statistically significant difference in strength between tissue rings treated with 50 µg/ml and 150 µg/ml ascorbic acid. Both MTM and failure strain results followed a similar trend as UTS. While ascorbic acid increased the stiffness of the tissue rings compared to untreated control rings, no significant difference was detected between the treated groups. The failure strain increased by 26% and 17% for 50 and 150 µg/ml ascorbic acid treatment compared to the untreated group. The stiffness increased 39% and 47% with 50 and 150 µg/ml ascorbic acid treatments (Figure 18).

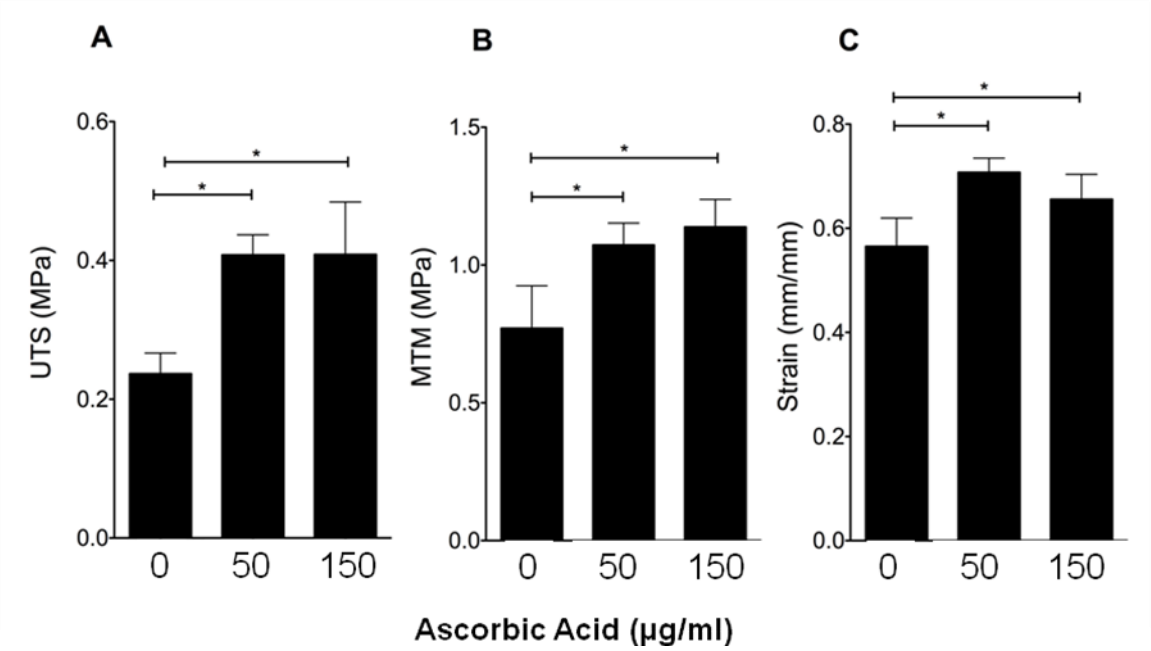

Figure 18. Mechanical properties of tissue rings. Uniaxial tensile testing was used to determine UTS (A), MTM (B) and failure strain (C), as a function of different levels of ascorbic acid treatment. All values are reported as mean ± S.D.; n=5. The asterisks indicate statistical differences between sample groups ($p<0.05$).

4.4 Acid Soluble Collagen Content Was Not Affected By Ascorbic Acid Treatment

The total protein content of each tissue ring was measured using the BCA assay. The average total protein content was 1,052 ± 204 µg/ml. Statistical analysis suggested ascorbic acid treatment had no significant effect on the total protein quantity (Figure 19). Collagen content for each ring was subsequently measured with the Sirius Red assay. The measurement was then normalized to cell number for comparison. The average collagen content per cell for groups treated with 0, 50 and 150 µg/ml of ascorbic acid were 81 ±32, 56 ±10 and 92 ±30 pg respectively (Figure 20). The level of collagen content was not affected by ascorbic acid treatment. Interestingly, the amount of collagen produced on a "per cell" basis appeared inversely proportional to the number of cells in that tissue ring sample (Figure 21).

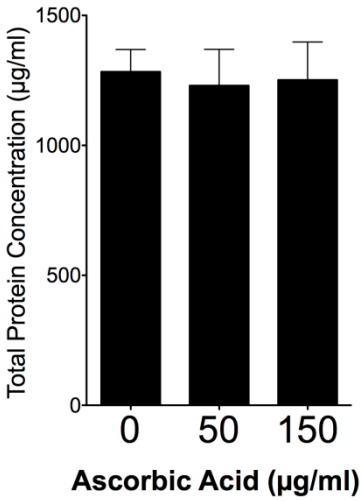

Figure 19. Effects of ascorbic acid treatment on total protein; values are expressed as mean ±S.D., n=5.

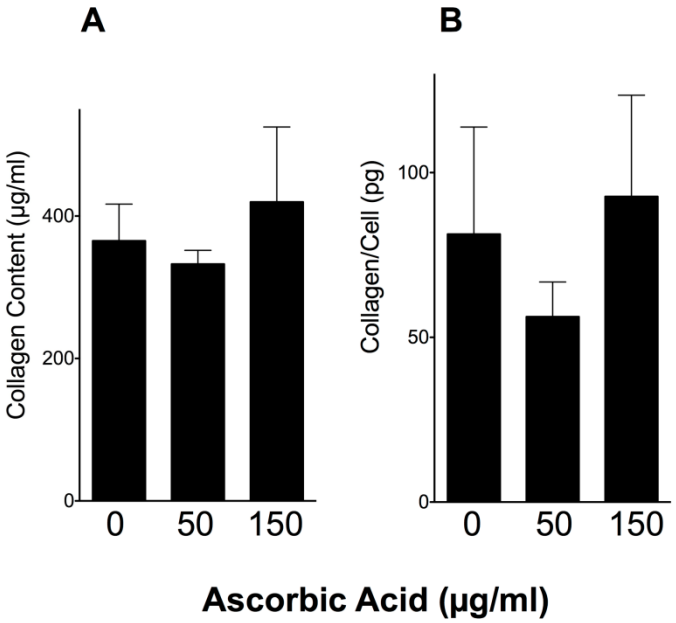

Figure 20. Effects of ascorbic acid treatment on collagen (A); Effects of ascorbic acid treatment on collagen content per cell (B); values are expressed as mean ±S.D., n=5.

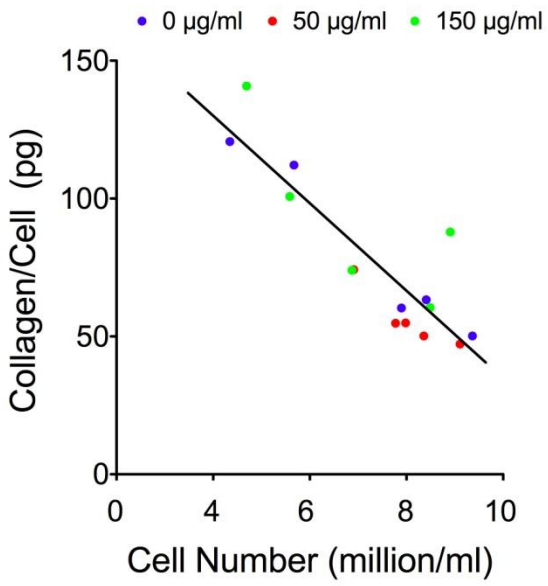

Figure 21. Pearson correlation between collagen content per cell and cell number. The line indicates significant correlation ($p < 0.05$; Pearson r: -0.88; R^2:0.78).

4.5 Histological Analysis

Histology was utilized to provide visual assessment of the structure and organization of the tissue constructs. Representative micrographs of tissue rings cultured with different concentrations of ascorbic acid are shown in Figure 22. In all tissue rings, cell density appeared highest along the outer edges of the rings, which is consistent with the results of our previous study.[19] Necrosis was observed at the center of some of the tissues (Figure 23). Fast Green/Picrosirius Red staining showed collagen deposition in all tissue rings after 7 days in culture. This observation was supported by polarized light microscopy.

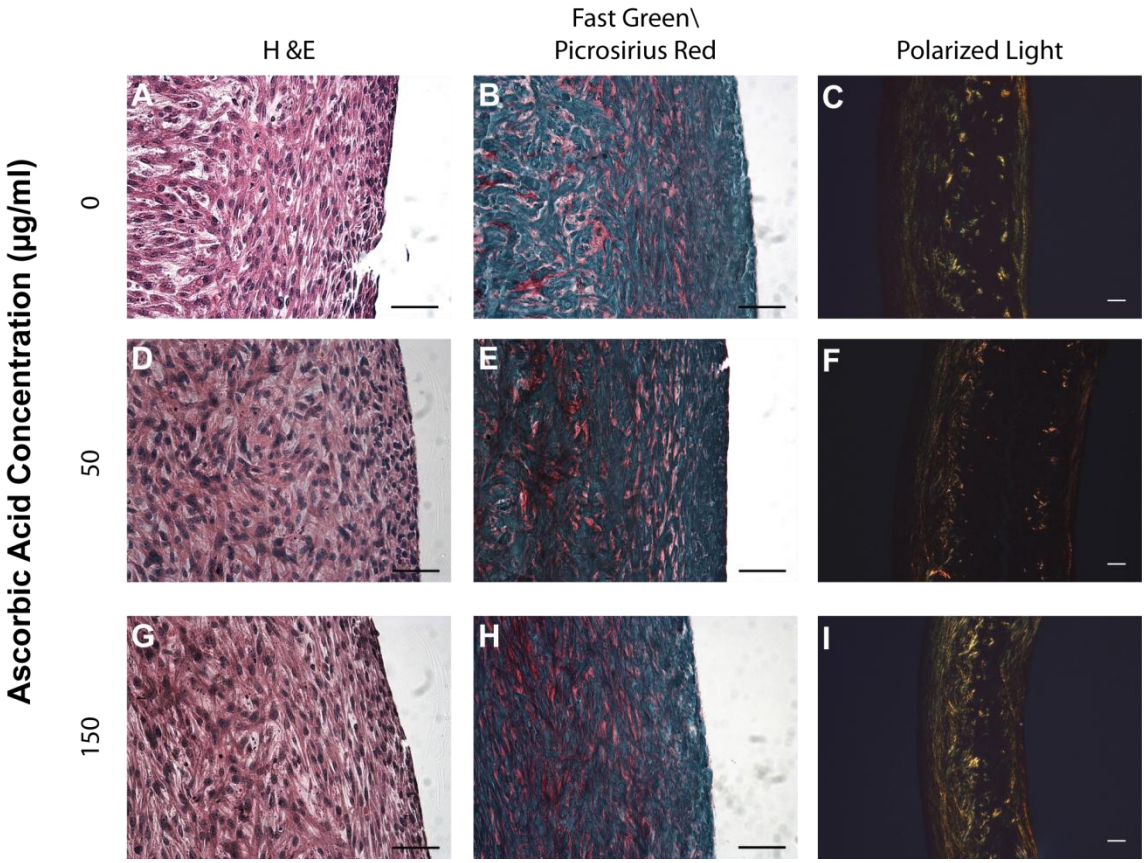

Figure 22. Tissue ring morphology. Representative photomicrographs of tissue rings cultured with 0, 50 and 150 µg/ml of ascorbic acid for 7 days. Tissue rings were stained with H&E (A, D, G), and Fast Green/Picrosirius Red (B, E, H). Images of tissue rings stained with Picrosirius Red alone were also obtained under polarized light (C, F, I). Scale bars: 50 µm.

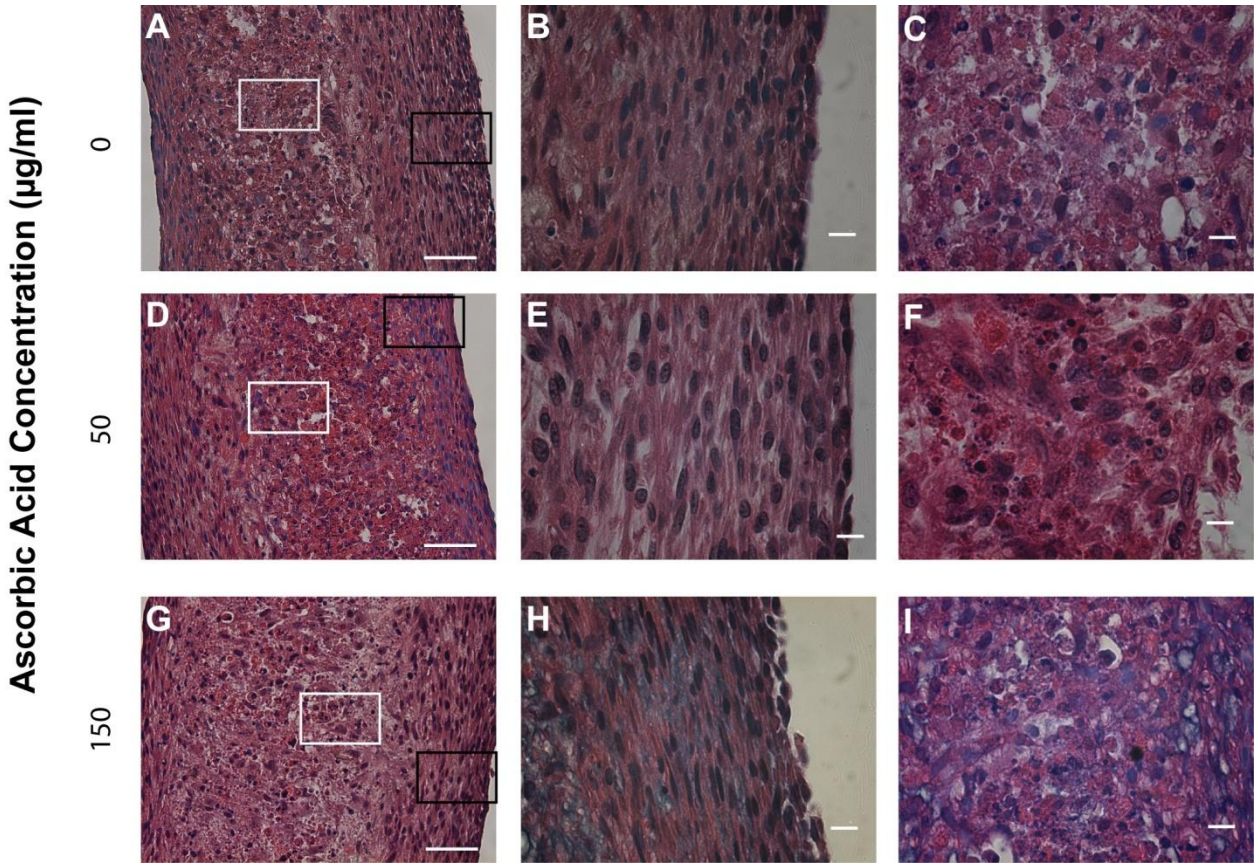

Figure 23. Representative photomicrographs of tissue rings cultured with 0, 50 and 150 µg/ml of ascorbic acid for 7 days. Low magnification view of the rings stained with H&E (A, D, G, scale bar: 50 µm) show the overall morphology of the rings. The boxes indicate the regions of interest magnified in panels B, E, H (black boxes) and C, F, I (white boxes). Scale bar: 10 µm for B, E, H, C, F, I.

4.6 Assessment of Area Fraction of Collagen Fibrils by Image Analysis

To quantify the collagen fibril coverage in each tissue, histological sections were stained with Picrosirius Red (without Fast Green counter stain) and images were acquired under polarized light. The area fraction of collagen fibrils was calculated based on the pixel density using a custom MATLAB program. An upward trend in area fraction was observed with increasing ascorbic acid concentration. However, statistical analysis suggested that the difference in area fraction of collagen fibrils between groups was not significant (Figure 24).

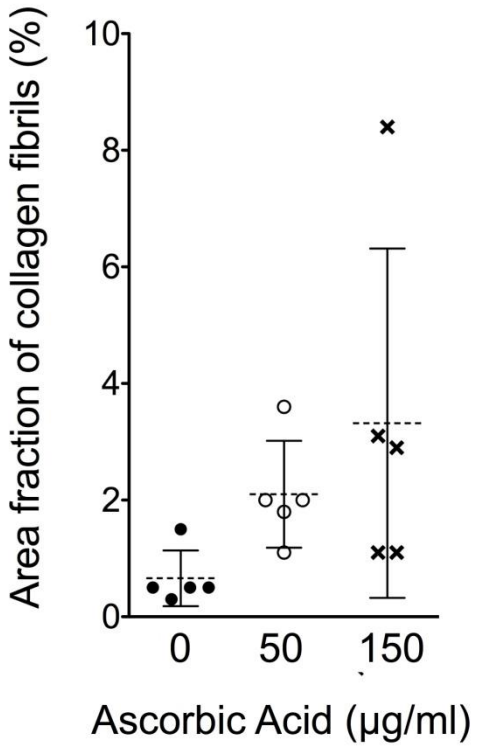

Figure 24. Effects of ascorbic acid treatment on collagen fibril area fraction; Symbols indicate the mean value for each ring, dotted lines denoted the mean value for the group (error bars are expressed as mean ± S.E.M., n=5).

4.7 Image Analysis for Cell Distribution

All tissue ring samples appeared to share a similar pattern of cell distribution. To follow up on this observation, tissue sections were stained with Hoechst for nuclei, and PCNA for cell proliferation. Image analysis based on Hoechst staining indicated that most of the RASMCs were distributed at the two edges of the tissue. A pixel histogram of PCNA-stained samples also suggested most of the cell proliferation occurred at the edges of the tissue. Interestingly, very little proliferation was found at the center of the tissue (Figure 25).

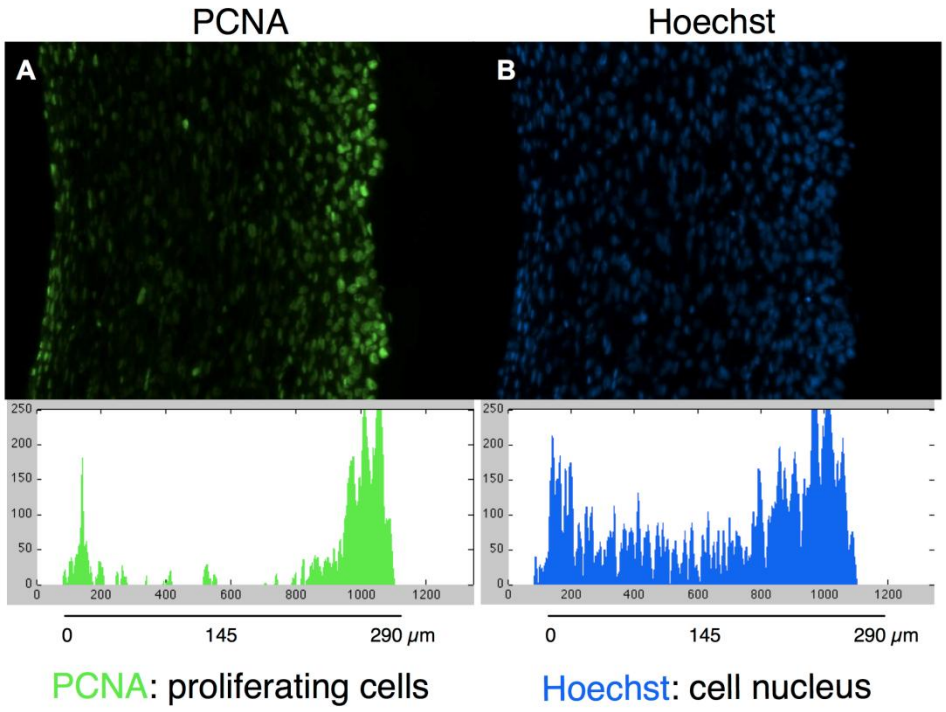

Figure 25. Representative results from cell distribution image analysis in tissue rings. Tissue ring section stained with PCNA (green) and proliferating cells distribution is represented on a histogram (A); The same tissue counterstained with Hoechst (Blue) and the cell nucleus distribution is represented on histogram (B); Scale bar: 50 µm.

4.8 Ascorbic Acid Treatment at 0, 5, 50 µg/ml

The previous experiment showed ascorbic acid treatment at concentrations of 50 and 150 µg/ml had no significant effect on tissue ring thickness, but significantly increased the mechanical properties compared to the untreated group. Therefore, we decided to determine whether lower concentrations of ascorbic acid would also lead to changes in mechanical properties. The average thickness of these constructs reached 0.705 ±0.03 mm at day 7, which is comparable to rings treated with 0, 50, and 150 µg/ml of ascorbic acid (0.716±0.03 mm).

Ascorbic acid treatment at 5 µg/ml did not increase the thickness of the tissue significantly compared to untreated controls (Figure 26).

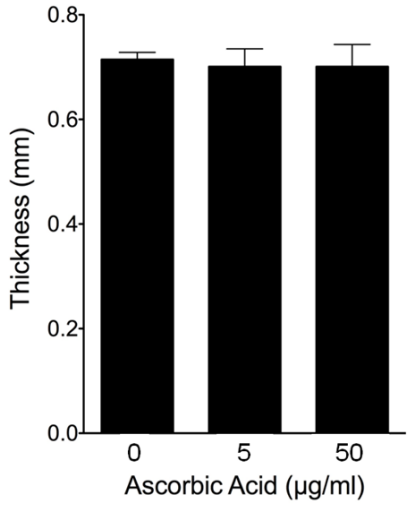

Figure 26. Thickness measurements of ascorbic acid treated rings (0, 5 and 50 µg/ml).

The average ultimate tensile strength values were 0.245 ±0.011, 0.247 ±0.026 and 0.352 ±0.056 MPa. The failure strength of tissue rings treated with 50 µg/ml of ascorbic acid was significantly higher than the control (0 µg/ml) and the group that was treated with 5 µg/ml. Stiffness of these tissues followed a similar trend. No significant difference was found between the control and 5 µg/ml treatment group, while tissue rings treated with 50 µg/ml of ascorbic acid were significantly higher than the other two groups (Figure 27). Interestingly, the UTS, MTM, and failure strain values were lower than the previous experiment (0, 50, 150 µg/ml).

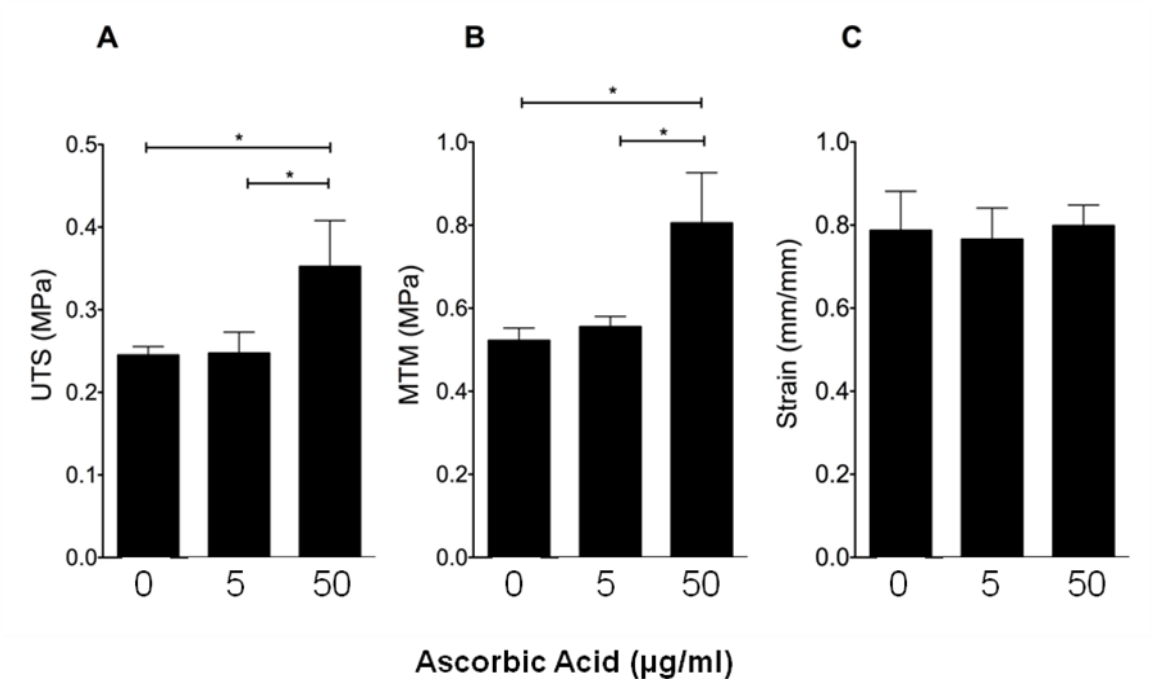

Figure 27. Mechanical properties of cell-derived vascular tissue rings. Uniaxial tensile testing was used to determine UTS (A), MTM (B) and failure strain (C), as a function of different levels of ascorbic acid treatment. The asterisks indicate statistical differences (All values are reported as mean ± S.D.; n=5; $p<0.05$).

Representative micrographs of tissue rings cultured in 0, 5, and 50 µg/ml of ascorbic acid of are shown in Figure 28. In all tissue rings, cell density appeared highest along the outer edges of the rings (in direct contact with cell culture medium), whereas the number of cells per area appeared to decrease at the center of the rings. Cells appeared to be aligned circumferentially at both edges of the rings. Fast green/picrosirius red staining showed collagen deposition in all the tissue rings after 7 days in culture. Under polarized light, the majority of collagen fibers appeared yellow in micrographs.

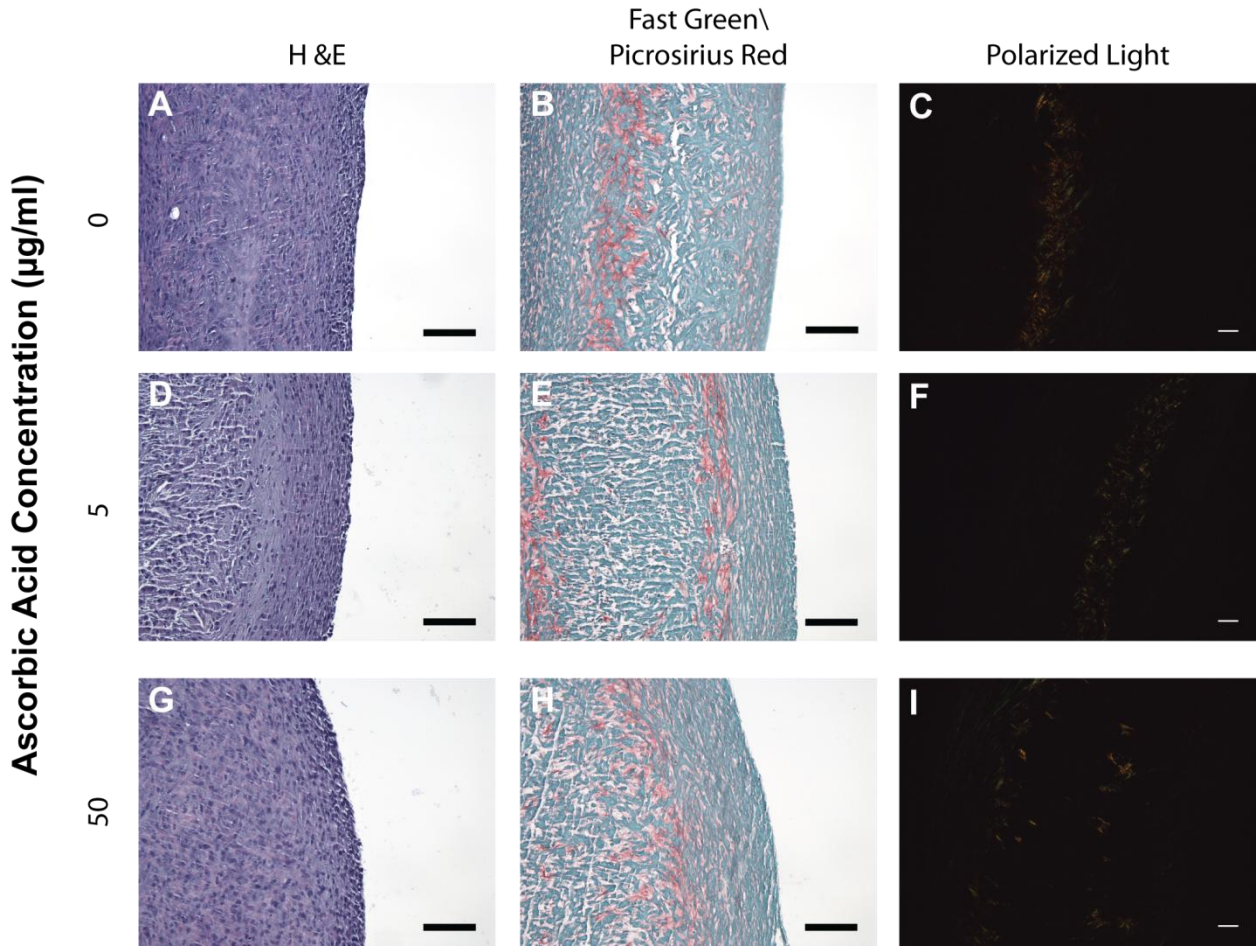

Figure 28. Tissue ring morphology. Representative photomicrographs of 4 mm inner diameter tissue rings cultured with 0, 5 and 50 µg/ml of ascorbic acid for 7 days. Images of tissue rings stained with H&E (A, D, G), and Fast Green\ Picrosirius Red were taken in bright field (B, E, H). Tissue rings stained with Picrosirius Red alone imaged under polarized light (C, F, I). Scale bars: 50 µm.

4.9 Low Serum Treatment

We hypothesized that low serum conditions would decrease the thickness of tissue rings, while increasing the mechanical strength. Thickness measurement showed that lowering the serum concentration in the culture medium significantly decreased the tissue ring thickness by 15%, while there were no statistical differences between tissue rings cultured in 2% FBS supplemented with 50 µg/ml of ascorbic acid and tissue rings cultured in (10% FBS) serum. The

thicknesses were 0.65 ±0.02mm, 0.56 ±0.04mm and 0.61 ±0.02mm for 10% serum treatment, 2% serum, and 2% serum with 50 µg/ml of ascorbic acid respectively.

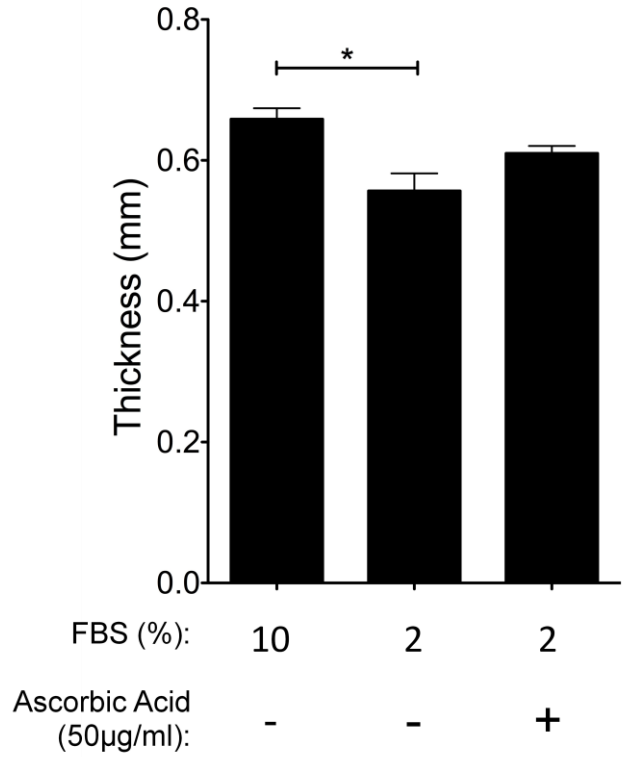

Figure 29. Effect of serum level on tissue ring thickness; Asterisks indicate statistical differences (All values are reported as mean ± S.E.M.; n=3. $p<0.05$).

Tissue rings cultured in low serum also had higher mechanical strength compared to those grown in 10% serum. The average UTS for these tissue rings was about two times higher than those cultured in 10% serum. Interestingly, the addition of ascorbic acid to cultures treated with 2% serum did not increase the mechanical strength significantly (Figure 30).

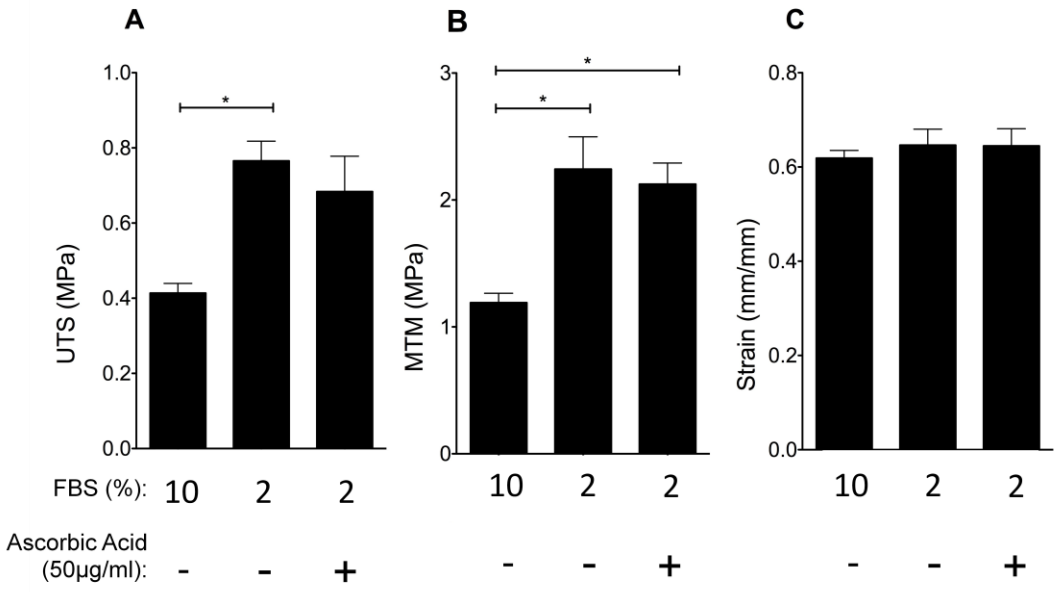

Figure 30. Mechanical properties of cell-derived vascular tissue rings. Uniaxial tensile testing was used to determine (A) ultimate tensile strength (B), stiffness and (C) failure strain as a function of different levels of ascorbic acid treatment. All values are reported as mean ± S.D.; n=3. The asterisks indicate statistical differences between sample groups ($p<0.05$).

Histological analysis showed higher cell density along the edges of the rings. The number of cells per area appeared to decrease towards the centers of the rings. However, necrosis was not found at the center of the tissue.

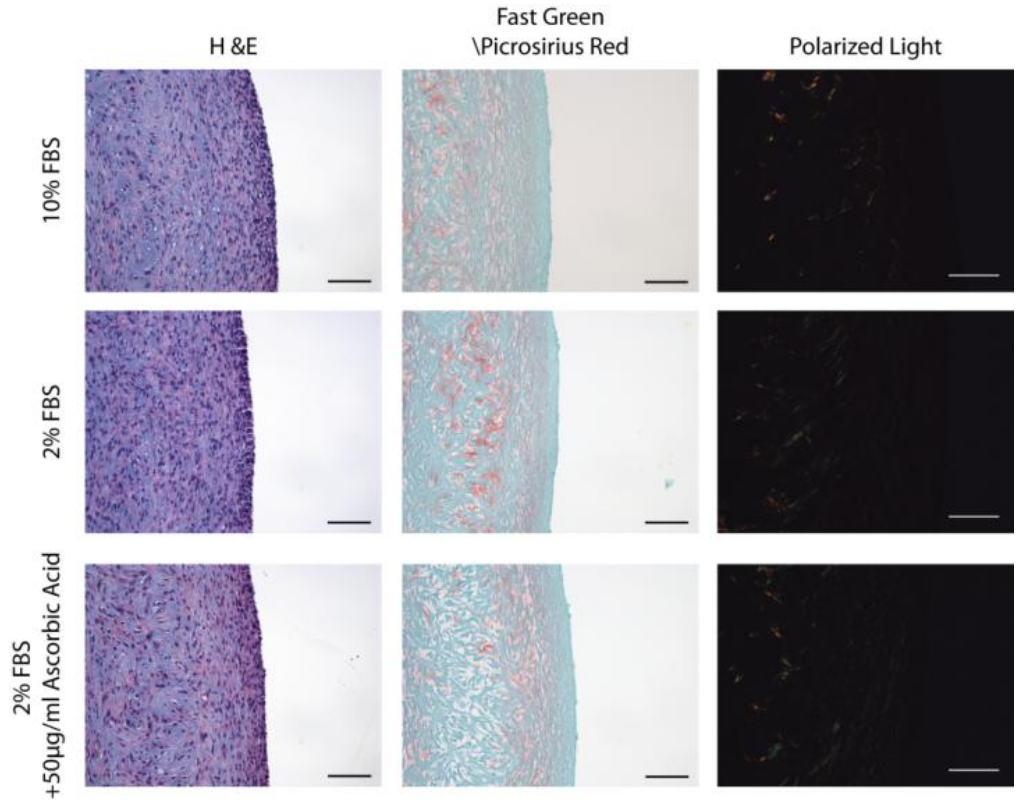

Figure 31. Representative photomicrographs of tissue rings stained with H&E, Fast Green/Picrosirius Red, and Picrosirius Red under polarized light. (scale bar: 100 µm)

4.10 Reducing Ascorbic Acid Degradation Rate in Culture Medium with DTPA

Diethylenetriaminepentaacetic acid (DTPA) is a polyaminocarboxlic acid that sequesters metal ions that otherwise decomposes ascorbic acid.[70] Previous studies have shown the degradation rate of ascorbic acid in culture medium varies depending on the culture environment.[47, 48] The ability to increase the presence of ascorbic acid in culture medium is expected to enhance the effectiveness of this molecule. A study conducted by Saxena *et al.* on the effect ascorbic acid on crystallins, a protein in the lens of the eye, showed that DTPA prevents oxidation of ascorbic acid molecules.[70] However, the use of DTPA has not been documented in the field of tissue engineering. Previous experiments we conducted with 2% serum showed addition of 50 µg/ml of ascorbic acid did not increase mechanical strength

significantly. This led us to believe that the low serum environment increased the degradation of ascorbic acid. In this experiment, we attempted to determine the effect of DTPA in tissue rings.

Measurement analysis showed that adding DTPA to the culture medium decreased the overall thickness of tissue rings. The thicknesses were 0.74±0.03, 0.70±0.03, 0.62±0.02 and 0.65±0.03 mm for rings in the -DTPA /-ascorbic acid, -DTPA /+ascorbic acid, +DTPA /-ascorbic acid and +DTPA /+ascorbic acid treatment groups, respectively (Figure 32).

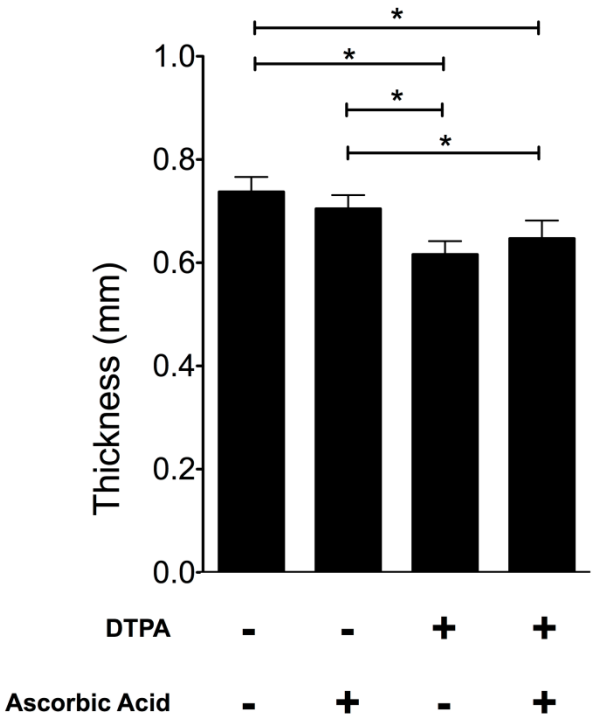

Figure 32. Effect of DTPA on thickness of tissue rings; The asterisks indicate statistical differences (All values are reported as mean ± S.E.M.; n=3. $p<0.05$).

The addition of DTPA to the culture medium also decreased the failure strength of the tissue rings by as much as 100%. The failure strength were 0.20±0.01, 0.21±0.05, 0.09±0.02 and 0.07±0.01 MPa for -DTPA /-ascorbic acid, -DTPA /+ascorbic acid, +DTPA /-ascorbic acid and

+DTPA /+ascorbic acid respectively. Failure strain of the tissue followed a similar trend. However, there were no significant differences in MTM between groups (Figure 33).

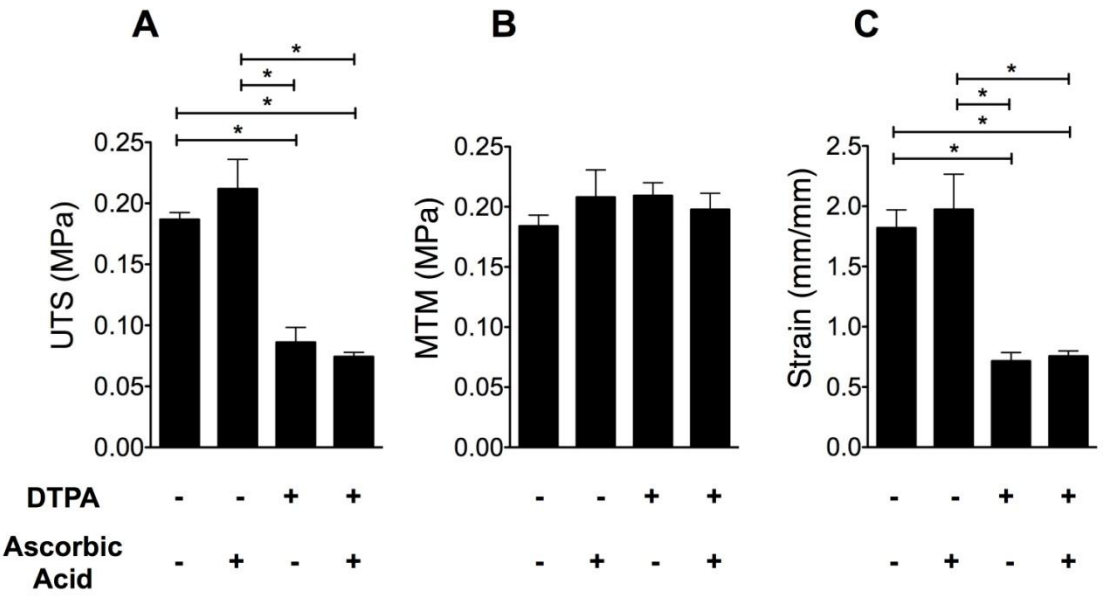

Figure 33. Effect of DTPA on the mechanical properties of tissue rings. Uniaxial tensile testing was used to determine UTS (A), stiffness (B) and failure strain (C) as a function of different levels of ascorbic acid treatment. The asterisks indicate statistical differences between sample groups (all values are reported as mean ± S.D., n=3, $p<0.05$).

Histological analysis revealed that DTPA-treated groups had large areas of necrosis throughout the tissue. In contrast to the treatment groups, the untreated groups had limited amounts of necrosis at the center of the tissue rings (Figure 34).

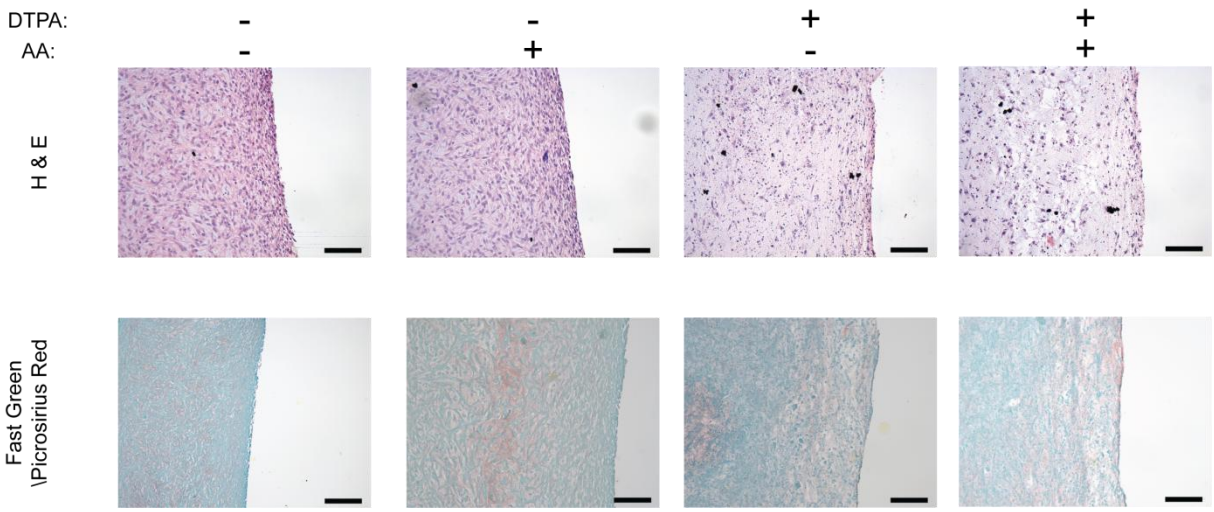

Figure 34. Tissue ring morphology under different DTPA treatments. Scale bar: 50 µm.

Chapter 5: Discussion and Conclusions

In the present study, we have examined the feasibility of manipulating the structural and mechanical properties of cell-derived tissue rings. The first objective of this project was to provide proof-of-concept of our ability to alter the mechanical properties of tissue rings by treatment with ascorbic acid. The second objective was to develop methods that would provide quantitative measurements of the function of tissue rings. The results of this study have further strengthened the original concept of using this novel approach to generate cell-derived tissue constructs for "high throughput" screening of factors such as media supplementation and cell source for their effects on the structural and functional properties of engineered tissues.

In order to show that the function of the tissue rings can be altered by adding a soluble factor, we have decided to supplement ascorbic acid to our culture medium. This decision was made because the practice of supplementing ascorbic acid to culture medium has been widely used in the field of tissue engineering. Ascorbic acid is known to play an important role in crosslink of collagen fibrils.[42, 71, 72] Studies conducted on cells grown on tissue culture plastic have also suggested that ascorbic acid has an effect on cell morphology.[21-23] In this study, the tissue rings were treated with varying concentrations of ascorbic acid for 7 days. The effects of ascorbic acid on the tissue were examined by measuring tissue thickness, mechanical properties, cell number, protein and acid soluble collagen content.

The effect of ascorbic acid on tissue ring thickness was measured after 7 days in culture. We found that ascorbic acid did not have a significant effect on the tissue thickness. The average thickness for the tissue rings was 0.64±0.07 mm, which was comparable to the thickness measurements we obtained from our previous studies on 8 day-old rings.[19] A study published

by Livoti *et al.* suggested that the thickness of cell-derived tissue constructs directly correlated with cell number.[69] Since the effect of ascorbic acid on thickness was insignificant, this led us to believe that treatment had no effect on cell number. Therefore, we further quantified this parameter by using the CyQuant NF assay to quantify total DNA content as a measure of cell number in tissue rings. The results of this assay agreed with the Livoti *et al.* finding. The average cell number for tissue rings was $5.1 \pm 1.5 \times 10^6$ cells/ml, and statistical analysis of the data showed that the difference in cell number between treatment groups was not significant.

The insignificance in cell number differences suggested that ascorbic acid had little influence on cell proliferation. Results from previous studies on the effect of ascorbic acid on cell proliferation in 2-D are cell stage specific.[47, 52, 55-57] Moreover, most of the studies on this topic have been conducted on tissue culture-treated plastic, which is different from the unique environment cells faced in the 3-D tissue rings. It is known that SMC grown in 3-D collagen matrix proliferate slower than those grown on 2-D surfaces due to cell-ECM interaction.[73, 74] Therefore the insignificant difference in cell number between treatment groups could be a characteristic of the 3-D culture environment.

The availability of ascorbic acid might have also limited its effect on cell proliferation. It has been shown that ascorbic acid disappeared from culture medium within 24 hr after its addition.[47] We have attempted to prevent ascorbic acid's oxidation by adding DTPA, a metal chelator, to the culture medium. However, the addition of this chelator led to extensive necrosis throughout the tissue, and significant decrease in UTS and failure strain. Since the oxidization of molecules is part of many cellular pathways, it is possible that the addition of DTPA to the culture medium have interrupted essential pathways in the cells.

The second functional end point we tested was the effect of ascorbic acid on mechanical properties. Overall, the cell-derived tissue rings were strong compared to reported values for ring segments from engineered vascular tissue equivalents cultured for similar time periods. The average UTS for tissue rings treated with 0 and 50 μg/ml of ascorbic were 0.24 ±0.03 MPa and 0.41 ±0.03 MPa respectively. Both of these measurements exceed those reported for rings of engineered tissues made with smooth muscle cells cultured statically within collagen (0.016 MPa at 8 days)[10] and collagen/fibrin mixtures (0.028 MPa at 7 days)[75] for approximately the same culture duration. With only 50 μg/ml of ascorbic acid treatment, the strength of our rings at 7 days approached that for SMC-populated fibrin gels cultured for 3 weeks with TGF-β1 and insulin (0.476 MPa).[5] In a similar study using a completely cell-derived tissue engineering approach, three weeks culture time was required for constructs created from fibroblasts and treated with 150 μg/ml of ascorbic acid to reach 0.313 MPa.[15]

A previous study on tissue culture-treated plates showed that ascorbic acid concentrations between 0 and 5 μg/ml were sufficient to increase collagen production in tissue culture.[54] However, it was not possible to assess the effects of these low ascorbic acid concentrations on mechanical function. Therefore, we decided to test the effect of 5 μg/ml ascorbic acid on the mechanical properties of tissue rings. After 7 days in culture, tissue rings treated with 50 μg/ml of ascorbic acid significantly increased the UTS of the tissue ring, while the UTS of 5 μg/ml treatment group was not significantly different from the untreated controls. It is known that the mechanical strength of tissue constructs is proportional to its degree of collagen fiber crosslinking.[76] Therefore, it was possible that collagen production did increase in culture as suggested by de Clerck *et al.*,[54] while the degree of crosslinking was not sufficient to increase failure strength for 5 μg/ml treatment groups. Additional studies will need to be performed to

fully characterize collagen content in tissue rings treated with lower concentrations of ascorbic acid. Due to the lack of increase in mechanical strength, biochemical analysis was not pursued.

Interestingly, the mechanical properties between 50 and 150 µg/ml of ascorbic acid treatment groups were not significantly different. Dosage studies with ascorbic acid have shown that its effect on producing insoluble collagen became less significant at concentrations greater than 50 µg/ml.[54] Moreover, the effects of ascorbic acid are not limited to effects on collagen; studies have shown that high concentrations of ascorbic acid impact the deposition of other ECM molecules such as elastin and glycosaminoglycans.[52, 54, 55, 77, 78] This effect might have impacted the overall structure of the tissue rings, and resulted in insignificant differences in mechanical properties between the two treatment groups.

In order to examine the effect of ascorbic acid on the molecular composition of tissue rings, we used Sirius Red assay to evaluate the collagen content in tissue rings. In contrast to the hydroxyproline assay, Sirius Red measures collagen content without breaking the protein down into amino acids. This allowed other protein-based assays, such as the BCA assay, to be performed on the same lysate without additional preparation. Results of the assay indicated the amount of acid soluble collagen content in tissue rings was not affected by the addition of ascorbic acid treatment. However, instead of measuring the entire collagen content, Sirius Red dye only binds to newly synthesized, acid soluble collagen. It was possible that ascorbic acid contributed mainly to collagen crosslinking,[76, 79] while its effect on synthesizing collagen was insignificant. Although the degree of crosslinking was not measured in this study, we speculated the increase in UTS for 50 µg/ml and 150 µg/ml treatment groups are the result of increased collagen crosslinking.[80]

Inspection of tissue rings stained with Hoechst and PCNA allowed us to evaluate cell

distribution and proliferation in the tissue. Image analysis indicated that most of the proliferation occurred at the inner and outer edges of the tissue rings, where the tissue was in direct contact with the medium, resulting in higher cell density at the edges of the rings (Figure 25). This observation suggested the possibility that SMC subpopulations, along with structural and phenotypic inhomogeneity may exist within the tissue.

Histological evaluation of the tissue rings provided insight in addition to the biochemical and mechanical assessments. Collagen fibers were observed with the assistance of polarized light microscopy, and the alignment of the fibers was assessed visually. Collagen fiber studies have suggested that a higher degree of alignment could lead to an increase in UTS.[20] This could be another factor in addition to crosslinking that contributed to the higher strength we found in 50 and 150 μg/ml treatment group. However, further inspection of the images acquired in this study does not suggest that the fibers are preferentially oriented in any specific way (data not shown).

Apparent inconsistency in the distribution of fibrils may be the result of uneven cutting of the tissue specimen during processing. It was likely that the tissue rings were not laid down completely flat in the mold during embedding. We sometimes found one side of the untested ring tissue sections appeared thinner than the other side, suggesting that they may have been embedded or sectioned at an oblique angle. No major differences in the thicknesses around the circumference of the ring were found with DVT measurements.

Image analysis of the area fraction of collagen fibrils showed an upward trend in response to ascorbic acid treatment, but the difference was not significant. The large variance in coverage for individual treatments could be the result of low statistical power. Post experimental analysis with one-way ANOVA showed the statistical power was 0.2, which is far below the desirable power value of 0.8.[81] Histological sections were 5 μm in thickness, while the tissue ring was

700 μm thick. Only one section per sample was used in the calculation, and it was difficult to calculate the fibril fraction coverage for the entire tissue from a single plane. The second possible explanation was the location of the section. It was possible that these sections were cut from different depths in the tissue from ring sample to ring sample. Although histology and image analysis only provided a snapshot of a complex tissue at one plane, it still gave valuable information about the morphology of the tissue. The image analysis method could potentially become more accurate by measuring multiple sections from this same tissue, thus increasing the sample size and the power of the statistical analysis.

Tissue rings stained with H&E allowed visualization of the general morphology and organization of cells within the tissue rings. Possible tissue necrosis was observed at the centers of the rings, indicated by fragmented nuclei (Figure 22). Although diffusion was not studied, the thickness of the rings may have impeded diffusion and contributed to this finding. *In-vivo* studies of tissue engineering scaffolds suggested that cells located 0.2 mm from a vascular network were either necrotic or inactive.[82] Tissue ring wall thickness values were substantially thicker (up to 0.726 mm) than other reported cell-derived tissue constructs, which may be partially explained by the high density of cell seeding to form rings and high proliferation rate of rat SMCs.

In addition to increase in tissue thickness, highly proliferative cells also discourage ECM synthesis.[83] Pilot data presented in Chapter 4 showed that increased UTS could be obtained in the absence of ascorbic acid treatment by switching tissue rings from 10% FBS to 2% FBS 48 hours after seeding, while the thickness of the rings decreased significantly. Although cell number was not quantified in this particular pilot study, the increase in UTS and decreased cell number suggests that cells may have successfully switched from the proliferative stage to a more quiescent state. This can be further verified with α-smooth muscle actin stain.

While the effect of ascorbic acid treatment on mechanical properties was consistent throughout the study, batch-to-batch variation (such as failure strength values) of the control groups was found between experiments. Similar variation was also found in the previous studies we conducted.[19] This appeared to be intrinsic to biological tissue, and not limited to tissue rings.[27] For example, TEBVs made by cell-sheet technology using cells from the same patients have shown high variability from patient-to-patient.[27] Regardless of intrinsic biologic variability, batch-to-batch variation should be minimized to strengthen the power of analysis using the tissue ring model system. Tighter control of medium supplements, tracking of passage number, and cell source could be implemented to decrease the intra-batch variability.

In conclusion, we successfully demonstrated that the mechanical properties of the tissue rings could be altered by adding ascorbic acid. We also developed a series of methods to evaluate the structure and function of cell-derived tissue constructs. We have laid down the foundation for using the tissue ring system as a screening method to conduct functional studies, and could ultimately optimize the culture conditions for cell-derived tissue-engineered blood vessel fabrication.

Chapter 6: Future Work and Implications

In the present study, we have successfully demonstrated proof-of-concept in using the tissue ring system to study the effect of soluble factors on extracellular matrix synthesis and mechanical properties by treatment with ascorbic acid. Future studies should focus on further characterizing the tissue ring system, and further optimizing the culture conditions to manipulate ECM synthesis and organization.

6.1 Optimize Ascorbic Acid Treatment

Future studies should focus on optimizing the ascorbic acid treatment conditions for tissue rings. Histology from the present study suggested nuclear fragmentation at the center of the tissue rings. Furthermore, image analysis has shown higher cell proliferation at the two edges of the tissue, where it was in direct contact with the culture medium. This led us to believe that decreasing the thickness of the tissue rings would improve ascorbic acid and other nutrient diffusion. We have conducted a pilot study in which cell culture medium was supplemented with 2% FBS (compared to 10% FBS), and found this condition significantly decreased tissue thickness. Moreover, the mechanical strength of the tissue rings cultured in 2% FBS in the absence of ascorbic acid increased ~100% in contrast to the control group. This indicated that culturing tissue rings in low or no serum may be beneficial and should be explored in the future.

The availability of ascorbic acid in culture medium under the current feeding protocol might be a limiting factor for using this molecule to optimize mechanical strength in the future. Ascorbic acid has been shown to degrade within 12 hours in culture medium through an oxidation reaction.[47, 58] Since culture medium along with ascorbic acid were changed every 48 hr during the present study, it was likely that the tissue rings were cultured in the absence of

ascorbic acid for extended period of time. Future work on optimizing tissue strength with ascorbic acid should consider replenishing this soluble factor on a more regular basis. It is also recommended to switch to magnesium L-ascorbate or sodium L-ascorbate for better stability.

6.2 Inhibit Collagen Crosslink

The present study showed that ascorbic acid had no significant effect on the content of newly synthesized soluble collagen, but published studies suggest that increase in failure strength is due to collagen crosslink.[76] Future studies should examine the effect of crosslink on mechanical properties, and its relationship to ascorbic acid. Beta-aminopropionitrile (BAPN) could be used for inhibition of collagen crosslink. Mechanical properties could then be evaluated at day 7. The degree of crosslink could be quantified with ninhydrin assay. [84]

Collagen content could also be evaluated from the tissue and culture medium at different time points. Recent studies have suggested that Sirius Red dye also binds to serum albumin in medium. Despite the method for separating albumin from collagen proposed in the same study,[62] hydroxyproline assay should be used in addition to the Sirius Red assay.

6.3 Improve Accuracy Of Image Analysis Of Collagen Fibril Area Fraction

Images were taken from a single histological section for each tissue ring sample under linear polarized light, and analyzed with the custom developed image analysis program. Although treatment groups were not statistically different from each other, an upward trend in collagen fibril area fraction was found. We believed the lack in statistical significance was due to low sampling power. This could be improved by taking multiple sections throughout the tissue in a pre-defined increment. Another improvement that could be made is to replace the linear polarizer with a circular polarizer. Linear polarizers create “blind” spots on the image, while

circular polarizers do not have this problem.[66, 85, 86] We have avoided this problem by only taking four images at specific locations where all fibrils were visible. Using a circular polarizer would allow more images to be taken for each sample, thus leading to a higher sample number.

6.4 Optimize Collagen Fiber Orientation To Improve Mechanical Properties

Collagen fibers were found in all the tissue samples. However, the lack of orientation for these fibers was not comparable to the native blood vessel. It is known that higher mechanical strength could be obtained through circumferential collagen fiber alignment along the vessel wall.[87, 88] However, histological analysis indicated that this alignment is still lacking in the system at present. This was not surprising since the tissue rings were cultured in a static environment, while native blood vessels are under constant cyclical stress. Studies have found dynamic mechanical conditioning improved the orientation of collagen fibers, therefore leading to better mechanical strength.[10, 89-91] A similar approach could be taken in the future to improve the strength of the tissue rings.

Although the current techniques we have described in the earlier chapter are tailored for the tissue ring system, new methods are needed to quantify fiber alignment. Existing optical techniques could serve as a guide to develop these methods for quantifying collagen fiber orientation, and its correlation to mechanical strength could be compared.[89, 92, 93]

6.5 Optimize Culture For Elastin Expression

While the orientation of the collagen fibers can be improved through dynamic mechanical conditioning, development of other parameters still requires the optimization of soluble factors. We have found that elastin, the protein that contributes to the contractile function of blood vessels, is absent from the tissue rings. Since the expression of this protein is vital to mimic

natural blood vessel structure and function, future focus should be put on studying this molecule in tissue rings.

A method pioneered by Wight *et al.* increased elastin formation in tissue by overexpressing versican V3.[94] Similar approaches can be taken to increase elastin production in tissue rings by using retroviral vectors to induce expression of V3 in cells used to generate rings, as described in the previous study.[95] Other potential approaches may include utilizing cytokines and growth factors. Studies have found that transforming growth factor-β1 (TGF-β 1) and insulin-like growth gactor-I (IGF-I) have the ability to modulate the expression of elastin.[96, 97]

6.6 Measure Compliance of Tissue Rings

The importance of compliance is often overlooked in the development of engineered blood vessel substitutes, but it is believed to be critical for proper pulsatile blood flow patterns through the vessel.[98] Compliance is defined as the relative change in vessel diameter for a given change in pressure within the physiologic range. Compliance of tissue-engineered blood vessels is mainly determined by the elastin content.[10] In the case of the tissue rings, additional effort has to be put into increasing elastin protein expression. Compliance testing would serve as an additional valuable parameter to measure toward assessing the functional contributions of elastin in engineered tissue constructs.

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