

Project Number: KLB-0804

**"Ascorbic Acid Treatment of Fibroblast Tissue Rings to
Increase Collagen Production"**

A Major Qualifying Report

Submitted to the Faculty of

Worcester Polytechnic Institute

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Degree of Bachelor of Science

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Abstract

Mechanical conditioning is an important factor when designing tissue engineered blood vessels. In order for the tissue to undergo mechanical conditioning, the tissue itself must be able to withstand the strains and pressures applied to it. Collagen is naturally produced by cells and imparts strength to multicellular tissue, but treating the vascular tissue with ascorbic acid can further increase collagen production, thus making the tissue stronger. This work aims to examine ascorbic acid treatment to optimize collagen production in cell-derived engineered tissue rings, as well as to design a device to mechanically condition these ring constructs. This device will be used to dynamically culture tissue rings and optimize engineered tissue growth and maturation in culture.

Acknowledgements

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1.0 Introduction:

Coronary artery bypass surgery has become a widespread surgery in the United States, and since 2007 there have been more than 427,000 surgical procedures involving small-caliber blood vessels (American Heart Association, 2008). Coronary artery bypass surgery is necessary when a patient's artery becomes blocked and blood needs to be rerouted around the blockage to keep the blood flowing and tissue oxygenation (Schaffer, 2007). Currently, most of these surgeries are completed by taking autologous blood vessels from elsewhere in a patient's body, such as the internal mammary artery and saphenous vein, and using them to reroute blood around the blocked artery (Isenberg et al, 2006).

Due to complications and limitations of using autologous blood vessels for transplantation in coronary artery bypass surgery, tissue engineering is being explored as an alternative source of vascular grafts. The benefit of a tissue engineered method becomes apparent when recognizing the need for blood vessels that are small in diameter ($\leq 6\text{mm}$), similar to the ones they are replacing. Patients lacking in suitable small-caliber vessels for replacement in the coronary artery due to vascular disease, amputation, or previous harvest (Isenberg et al., 2006) would especially benefit from a tissue engineering approach to address the need for alternatives.

Many different techniques have been experimented with to produce a suitable vessel through tissue engineering techniques that can be used in coronary bypass surgeries. Although the ultimate goal is to generate a functional tissue replacement made entirely of living cells and their extracellular matrices, the process in which these vessels are constructed can vary. Five approaches that address the complicated architecture and unique mechanical properties of the vascular wall are endothelial cell (EC)-seeded synthetic grafts, cells suspended in fibrin or

collagen-gels, biodegradable synthetic polymer-based constructs (with or without seeding), cell sheet-based blood vessels, and decellularized tissue approaches to vascular graft engineering (Seliktar,2000). Though these novel procedures have made significant strides in creating fully functional engineered vasculature, significant limitations still prevent their clinical use. These include limitations in having the cells adhere to the synthetic polymers and ensuring the mechanical integrity of the constructs in vivo. Furthermore, the goal of developing a fully functional vascular graft will likely require knowledge gained from each one of these approaches.

The aim of this project is to assess the addition of varying concentrations of ascorbic acid to human dermal fibroblasts cultured as cell-derived tissue rings, to increase collagen production. An additional goal of this project was to create strong, collagen-rich tissue rings to mount and test in the Vessel Ring Bioreactor (KLB 0904, 2009), designed to mechanically condition the vascular rings as models for full-length, tubular blood vessels. Although it is beyond the scope of this project, of extreme relevance to the field of research would be to mechanically test and compare the viscoelastic properties of mechanically conditioned rings with those grown in a static culture.

2.0 Background

Coronary heart disease is the leading cause of death in men and women in the United States (Michaels, 2002). Consequently, coronary artery bypass graft (CABG) surgery is one of the most common operations performed throughout the world (Eagle, 2004). CABG surgeries are mainly conducted because plaque forms in the coronary arteries resulting in a process known as atherosclerosis, or hardening of the arterial walls (Parmet, 2008). This plaque blocks the arteries, decreasing blood flow and oxygen to the heart, causing angina and myocardial infarction (American Heart Association, 2008). CABG operations take blood vessels, usually from elsewhere in the patient's body, and use those vessels to bypass the blockage (Isenberg, 2006). Preferably, small caliber blood vessels like the saphenous vein, radial arteries and internal thoracic arteries should be used since they are close in size and function to the blocked vessel.

Figure 1 shows a cartoon of a CABG procedure.

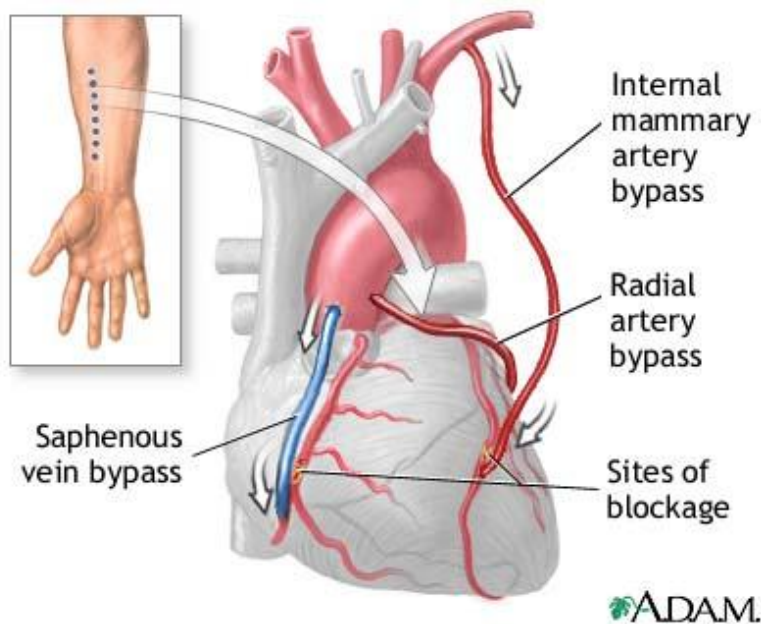


Figure 1 - Coronary Artery Bypass Graft (© 1997- 2008 A.D.A.M., Inc.)

Functional problems arise when small caliber (<6mm) are unavailable (Isenberg et. al., 2006). Although synthetic grafts have been explored as an alternative, the low blood flow environment in these arteries is associated with increased thrombogenicity and intimal hyperplasia, leading to synthetic graft failure (Shastri, 2004). Tissue engineering has emerged as a promising alternative that may limit the effects of thrombogenesis due to their biological origin. An ideal bioengineered graft would be mechanically strong and pliable, non-immunogenic, non-thrombogenic, easy to handle, and cheap to produce (Shastri, 2004).

One of the major differences between synthetic, tissue-engineered and autologous conduits is the presence in the latter of a living functional layer of endothelial cells resting on a metabolically active, smooth muscle cell media. Current synthetic grafts, lack these properties and are unable to 'heal' completely in humans, failing to achieve the necessary tissue ingrowth that would encourage coverage of the luminal surface by endothelial cells (Tiara et. al., 2001). These blood vessels, whether prosthetic or tissue-engineered must be able to withstand the pressures of the human body and they must possess the appropriate mechanical and biological properties needed to make them indistinguishable from the native cells and vessels (Isenberg et. al, 2006). It is widely held that 3 components are necessary for these criteria to be met: (1) a biocompatible component with high tensile strength to provide mechanical support (collagen fibers or their analogue); (2) a biocompatible elastic component to provide recoil and prevent aneurysm formation (elastin fibers and their analogue); and (3) a non-activated, confluent endothelium to prevent thrombosis (Isenberg and Tranquillo, 2003). The different techniques for engineering vasculature address these criteria, each with their own advantages and disadvantages.

2.1 Definition of Mechanical Conditioning

An efficient arterial replacement graft needs to not only function biologically like a natural vessel but also must function mechanically like a natural vessel. Mechanically, a vessel needs to be able to withstand shear stress, pressure and stretching due to blood pressure, and longitudinal tension along the vessel (Isenberg et al, 2006). There have been different methods established to replicate natural vessel properties, such as varying cell culture conditions and cell types, but a promising, recently developed method is mechanical conditioning, or dynamic stimulation (Isenberg et al, 2006, Sung In Jeong et al., 2005, Cummings et al., 2004, Isenberg and Tranquillo, 2003). Several studies have shown that *in vitro*-cultured arterial cells that are exposed to mechanical signals, (like those experienced by natural vessels) develop many attributes of efficient vascular grafts for *in vivo* usage. Such attributes include enhanced mechanical strength, collagen production, cell alignment, and regulated phenotype of vascular smooth muscle cells (Isenberg et al, 2006, Sung In Jeong et al., 2005, Cummings et al., 2004, Isenberg and Tranquillo, 2003). It has been shown that mechanical stretching promotes the expression of type I and III collagens, fibronectin, and tenascin-C in cultured ligament fibroblasts (Sahoo et. al., 2007). Another study indicated that human bone marrow mesenchymal stem cells (hBMMSC) - even in the absence of biochemical regulators - could be induced to differentiate into ligament-like fibroblast by the application of physiologically relevant cyclic strains (Vunjak-Novakovic et. al., 2004). Therefore, mechanical conditioning has become a primary strategy for inducing tissue growth and maturation *in vitro* (Freed et. al., 2000). An assortment of bioreactors have been manufactured to expose cell structures to mechanical stimulation (Isenberg et al, 2006). However, these bioreactors have never been used to test the effects of different culture conditions, a goal that the team hopes the design accomplishes. These experimental parameters can be growth factors, such as cell and media type, that contribute to changes in the architecture of the extacellular matrix. As seen in Figure 2, the team plans to use

this bioreactor to combine both methods of cyclic distention and growth factors to obtain experimental results. The problem is illustrated in the schematic below.

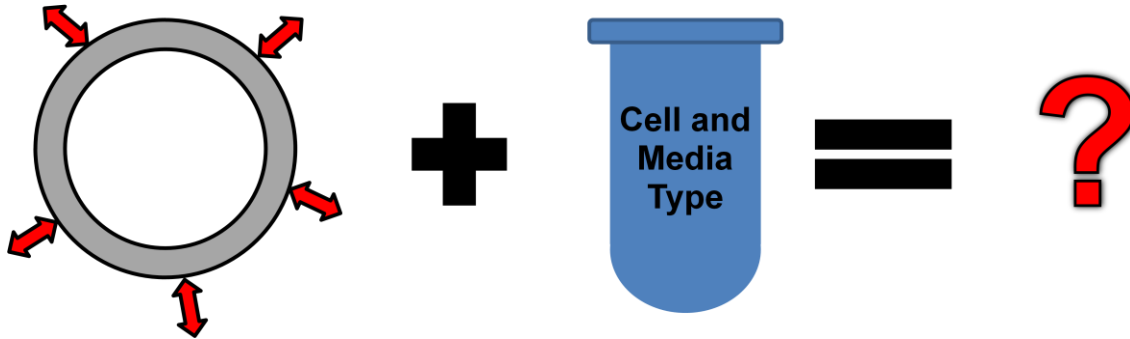


Figure 2 - Optimizing Effects of Dynamic Conditioning and Growth Factors

2.2 Tissue Engineered Blood Vessels

There are many different ways that blood vessels can be constructed using tissue-engineered techniques. The ultimate goal is to create a blood vessel made entirely of cells and their extracellular matrices. Many of these methods use scaffolds for the cells to aggregate around (Isenberg et al., 2006). One method starts with biodegradable scaffolds on which cells are seeded and allowed to produce their extracellular matrix (Isenberg et. al., 2006). Once visible tubes are formed the cells are washed away and only the matrices are left, leaving the tubes acellular (Schaffer, 2007). In theory these acellular tubes are then to be implanted into the patient and repopulated by the body's own cells (Schaffer, 2007). Biodegradable polymer scaffolds and biopolymer gels are also methods used to generate tubular vessels (Syedain et. al., 2008). The cells are seeded onto a multitude of different biodegradable synthetic polymer scaffolds or gels and once the cells have formed into vascular tubes the polymer dissolves leaving behind a hollow vascular tube (Isenberg et. al., 2006, Syedain et. al., 2008).

Another method to produce blood vessels is growing the cells into two dimensional sheets and then rolling them into the shape of a tube (L'Heureux et. al., 2007). The sheets are made completely from cells and no synthetic or exogenous materials are used (L'Heureux et. al., 2007). The tubes are created from multiple layers of cells that fuse together to create a tube that will withstand the pressures of the human body (Isenberg et. al., 2006). The cell sheets are grown on culture plate and then wrapped around a porous tubular mandrel and an outer layer of cells is wrapped around that to construct the adventitia, which is the outermost connective tissue of the vessel (L'Heureux et. al., 2007).

While all of the above methods ultimately end in the production of a tissue-engineered blood vessel they all have major problems that need to be addressed. All of the methods involving scaffolds as well as those vessels made from sheets have a lack of compliance that keeps the vessels from performing to their full potential (Isenberg et. al., 2006). The vessels made from decellularized tissues have experienced reduced tensile strength and a lack of compliance (Isenberg et. al., 2006). A lack of compliance implies high stiffness. The vessels made on biodegradable scaffolds have experienced premature weakening of the tissue (Isenberg et. al., 2006). The lack of compliance prevents the engineered vessels from maintaining their structural integrity, as such vessels undergo unrecoverable plastic deformation. Better methods for constructing tissue-engineered blood vessels have greatly improved the mechanical properties of these constructs.

Rather than generate an entire blood vessel and then take sections of it to mechanically test, some researchers are growing cellular rings that have the same properties as an entire blood vessel and are testing them as model representation of small diameter vasculature (Isenberg and Tranquillo, 2003). This representation is shown in the schematic below.

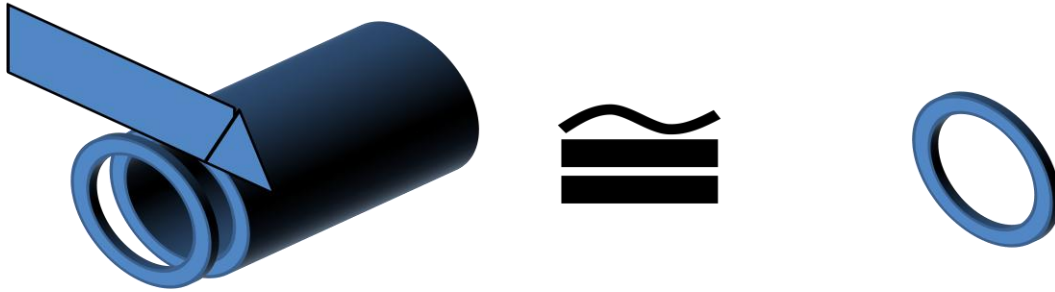


Figure 3 - Rings as Tube Models

This technique is both easy to replicate and time efficient. Since investigators have not yet been able to develop a fully functional bioartificial artery (BAA) that meets the requirements for mechanical strength and biological functionality (Isenberg and Tranquillo, 2003), the idea of creating rings that mimic BAAs is an approach that will allow researchers to have a biological model through which to study and improve the functionality of tissue-engineered vasculature.

2.3 Cell Derived Matrix

Patients that have undergone myocardial injury have scar tissue from local remodeling that inhibits the vessels from functioning properly and they must have vascular grafts implanted to continue smooth functioning of the heart and vessels (Bunda et al, 2007). In previous studies vascular rings, which are models for tissue engineered blood vessels, have been made from cells other than cardiac myocytes, so therefore the cellular matrix must be adjusted to withstand the pressures of the heart and blood vessels (Bunda et al, 2007).

The cells that these vessels are made of become an important decision because they have to be compliant with the rest of the system as well as be able to function like cells in blood

vessel. Smooth muscle cells and dermal fibroblasts are two types of cells that are very common among previous studies (Bunda et al, 2007).

The cell matrix is also a very important part of the vascular tissue. Different types of cells lay down different matrix components and it is crucial to have the correct ratio of cellular matrix components to create an ideal vascular tissue to be used in such grafts.

2.3.1 Collagen Production

Collagen fibers are made of tropocollagen which are triple helix and are composed of three extended protein chains that wrap around one another (Alberts et al, 2004). Collagen molecules are cross-linked in the ECM to form collagen fibrils which form collagen fibers which can be seen in Figure 4 (Berwal and Novakofski 1999). Collagen is a glycoprotein composed of galactose and glucose which have intermolecular cross-linkages and because of these, have high tensile strength (Berwal and Novakofski 1999). Collagen is more rigid than elastin and rarely branches like elastin usually does (Berwal and Novakofski 1999). Collagen is needed in the ECM because it keeps the tissue strong and rigid. Collagen is as strong as steel which will help the cells to withstand the pressures of the heart and vessels.

Hydroxyproline is a modified amino acid exclusive to collagen and ascorbate has been shown to contribute to the metabolic processes which produce hydroxyproline, leading to collagen production (Davidson et al, 1997). The amount of ascorbate added to the cells in culture has a direct affect on collagen production; the more ascorbate that is added, the more collagen is produced (Davidson et al, 1997).

Ascorbate is a cofactor in the enzyme activity of hydroxylase which stimulates collagen production and is needed for collagen to attain its triple helix formation under physiological

temperatures (Davidson et al, 2007). Based off of the data collected by Davidson et al, ascorbate added to human dermal-fibroblasts at varying concentrations (0, 0.5, 5, and 25 $\mu\text{g}/\text{mL}$) produced different amounts of collagen in the cell's ECM.

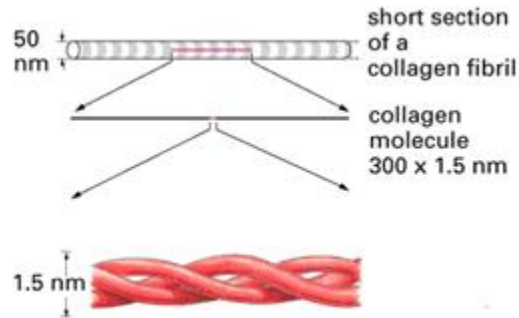


Figure 4 - Collagen Fiber (Alberts et al, 2004)

2.4 Hypothesis:

The working hypothesis for this project is that ascorbic acid treatment will increase collagen production and would thereby increase the tensile strength of the tissue. To test this hypothesis vascular rings were cultured and treated with varying concentrations of ascorbic acid and were analyzed for collagen production using histochemical methods.

3.0 Methods and Materials

3.1 Cell Culture

Human dermal fibroblasts were grown in DMEM (Mediatech, Herndon, VA) supplemented with: 10% fetal bovine serum (FBS), 1% non-essential amino acids, 1% pen/strep, 1% pyruvate, and 1% glutamine. This media will later be referred to as growth medium. The cells were grown in T75 flasks until they were 90% confluent in the incubator at 37°C and 5% CO₂. If the cells were from a frozen stock they were thawed and plated with 10mL of the growth medium. If the cells were passaged, the supernatant was aspirated out from the T75 flask, and the cells were washed with 5mL of phosphate buffer saline (PBS). The cells were then trypsinized with 5mL of trypsin (Media Tech), and were left to sit until the cells detached from the bottom of the flask. The cells were then mixed with 5mL of the growth medium to deactivate the trypsin and were transferred to a 15mL conical tube. The tube was then centrifuged for 5 minutes at 1,000 rpm. If the cells were being counted 100µL was added to 100µL of trypan blue, and 10 µL of the solution was added to each side of the hemocytometer and counted using a microscope with 100x magnification (10x objective). Figure 5 shows one of the grids of a hemocytometer. The four corners were counted on each side of the hemocytometer to calculate the total amount of cells. If the cell fell on the line, the cell was counted “in” if the line was on the top or right side of the square. If the cell fell on the bottom or left side of the square the cell was not counted. Once the number of cells in the four corners on both sides of the hemocytometer were counted, this number was divided by eight to get the number of cells per square and then multiplied by 10,000 to convert to the number of cells per mL. This number was then multiplied by 10 to get the total number of cells per T75 flask.

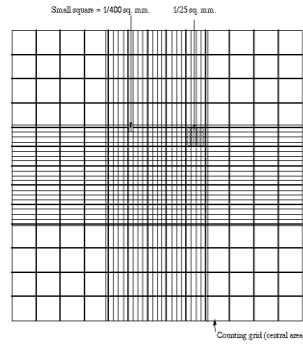


Figure 5 - Hemocytometer Chamber (Caprette, David, 2000)

3.2 Preparation of Cellular Rings

Once the human dermal fibroblasts were ready to be cultured into the rings the following protocols were used. The protocols found below follow the schematic found in Figure

6.

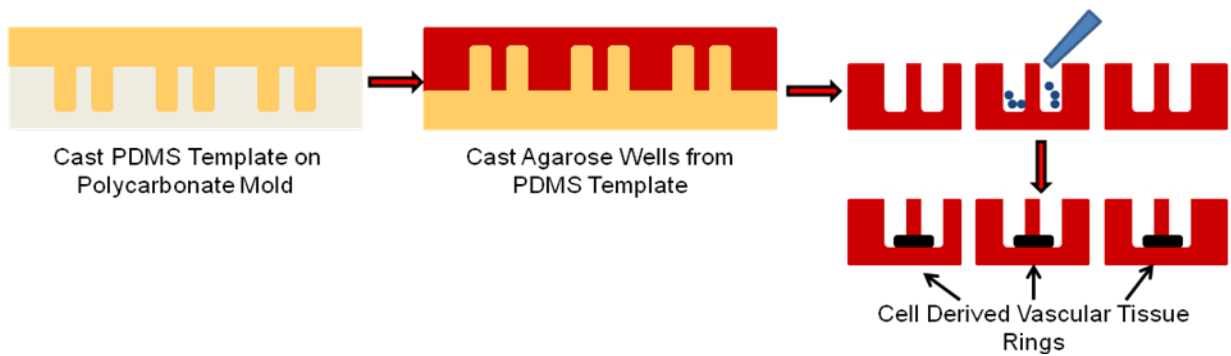


Figure 6 - Schematic of Ring Protocols

3.2.1 Making a PDMS Template



Figure 7 - Polycarbonate Mold

A polycarbonate mold was machine containing 15 annular wells with inner posts of 2 mm (Figure 7). The wells were machined with round bottoms so that the cells could aggregate around the posts to form rings. The polycarbonate mold was washed with warm soapy water to rid it of dirt and bacteria and tapped dry before each use. The polycarbonate mold was then placed in a plastic container and set aside. A mixture of 10:1 silicone elastomer base and silicone elastomer cure agent were used to create the PDMS (Polydimethylsiloxane, Sylgard 184 silicone elastomer kit, Dow Corning, Midland, MI) template; 110g of base and 11g of curer were mixed well and placed in a vacuum chamber to remove the bubbles for 20 minutes. The plastic container with the polycarbonate template was the filled with the PDMS and allowed to sit in the vacuum for 40 minutes to remove any additional bubbles. This container was then placed in the oven at 60°C overnight. The PDMS template and polycarbonate mold were cut out of the plastic container using a scalpel and the PDMS template was peeled off of the polycarbonate mold (Figure 8). Before the PDMS template could be used for any experiments it was placed in an autoclavable bag and autoclaved first. The PDMS template must be autoclaved before each experiment.



Figure 8 - PDMS Template

3.2.2 Media Concentrations of Ascorbic Acid

Cells were treated with varying concentrations of ascorbic acid: 0, 10 μ g/mL, and 100 μ g/mL ascorbic acid. To create these concentrations 0.1 g of Ascorbic Acid (Sigma) was dissolved in 10mL of serum free DMEM. This solution was then sterilized by passing through a 0.2 μ m sterile filter and into a 15mL conical tube. This tube was labeled 100 μ g/mL and became the ascorbic acid stock solution (high concentration stock; 0.01 g/mL). Then 1mL of the 100 μ g/mL ascorbic acid solution was placed in a new 15mL conical tube and added to this was 9mL of serum free media. This solution was 0.001g/mL ascorbic acid and became the low concentration of ascorbic acid stock solution. The control for the experiment was 0 μ g/mL ascorbic acid. This was created by placing 10mL of serum free media in a separate tube and labeled 0 μ g/mL.

Once the stock solutions were made 0.5 mL of each stock solution was added to 49.5mL of growth medium. This media was then used for the following procedures. The working concentrations of ascorbic acid applied to the fibroblasts were 0 μ g/mL, 10 μ g/mL, and 100 μ g/mL. Figure 9 shows a schematic of the serial dilutions used to create the stock solutions and working media.

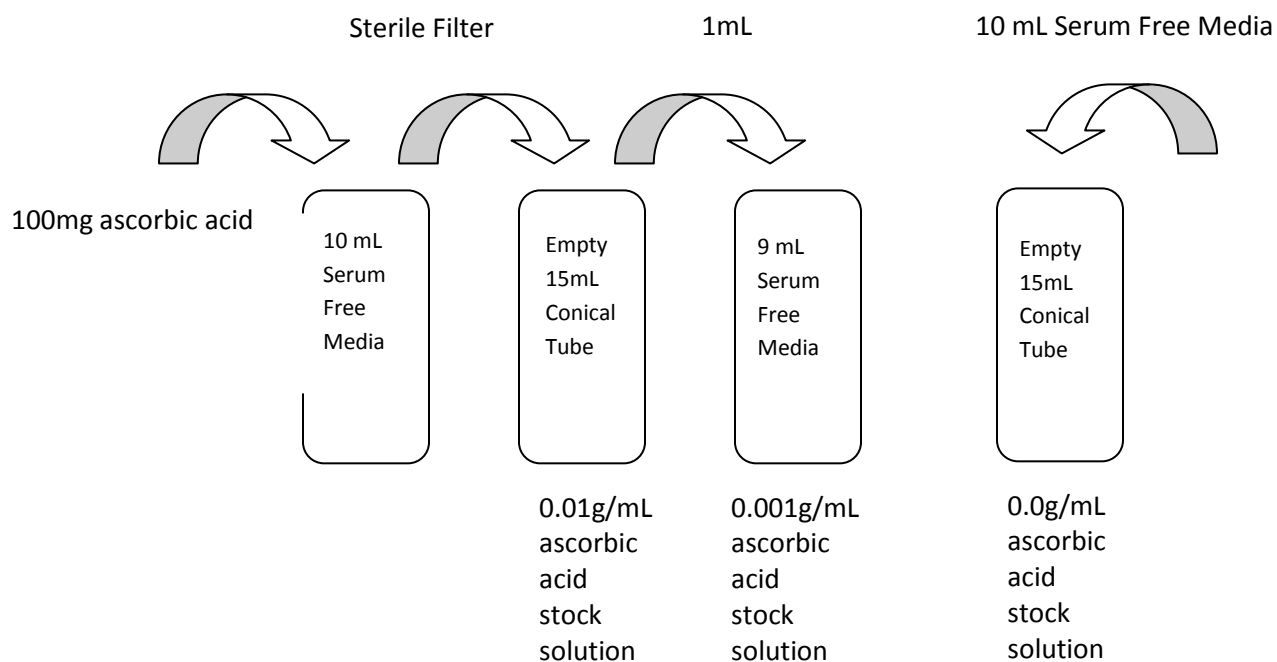


Figure 9 - Preparation of Ascorbic Acid Stock Solution

3.2.3 Making Agarose

Each PDMS template required 100mL of 2% agarose to fill the template. The 2% agarose mixture was made using 2g of agarose mixed with 100mL of serum free DMEM. This mixture was then autoclaved at 121°C. For each replication of the entire experiment 3 PDMS molds were used so a total of 6g of agarose and 300mL of serum free DMEM were autoclaved at the same time.

3.2.4 Making Agarose Wells

Once the agarose was autoclaved and still hot, it was transferred into the PDMS templates in the cell culture hood. Each inner ring of the molds was filled carefully using an automatic pipetter set at 100µL. Once each of the inner rings was full and no air bubbles were seen, the rest of the mold was filled using a 25mL pipette. This same process occurred for all

three PDMS templates. These molds were then left to solidify in the cell culture hood for 1 hour.

Once the agarose molds had solidified, each well was individually cut out of the mold, and placed into a 6 well plate. Before this part of the experiment began, two spatulas and a scalpel must have been autoclaved in an autoclavable bag. Six, 6 well sterile plates must have also been obtained before beginning. Each well was then carefully cut out of the mold using the scalpel and placed into a well of the 6 well plates. For each experiment, 32 rings were made so a total of six, 6 well plates were used. Once all the wells were cut and placed into the appropriate dishes, media was added to allow them to equilibrate. Each well was filled with 6mL of media, and each media type (0 μ g/mL, 10 μ g/mL, and 100 μ g/mL) was used to fill three plates. Overall there were twelve wells for each type of media. These wells were left to equilibrate in media, in the incubator for 90 minutes prior to cell seeding.

3.2.5 Seeding Rings

Once the wells were equilibrated they were seeded with cells. Each ring started with 1 million cells and each six well plated required approximately one T75 flask of HDF. The cells were trypsinized from the flask using the protocol found in (3.1 Cell Culture) but before the cells were placed in a 15mL conical tube they were counted using the protocol found in 3.1 Cell Culture. Once the total number of cells was known, a customized program, developed by Jason Hu in the Rolle Lab, gave the exact amount of media that needed to be centrifuged to make six rings. This amount of cell suspension was centrifuged at 1,000 rpm for 5 minutes. The supernatant was then removed, and added to it was 816 μ L of the corresponding media. If the 6 well plates were equilibrated with 0 μ g/mL media, then 0 μ g/mL media was added to the cells to create rings. 136 μ L of media was used to seed each ring. The rings were seeded around in the

inner well of the mold in a circular motion. Once the rings were seeded they were left undisturbed for 48 hours in the incubator and were fed every two days after that and harvested at 7 and 14 days. Figure 10 shows one ring around the agarose post in the well. Each time the cells were fed they were fed with the appropriate media.

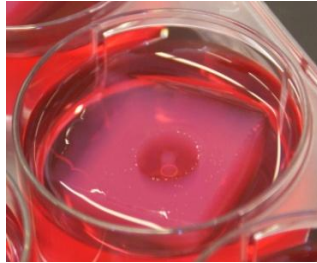


Figure 10 - HDF Ring in Well after 7 Days

3.3 Experimental Design

The following experimental design was used for the seeding the rings:

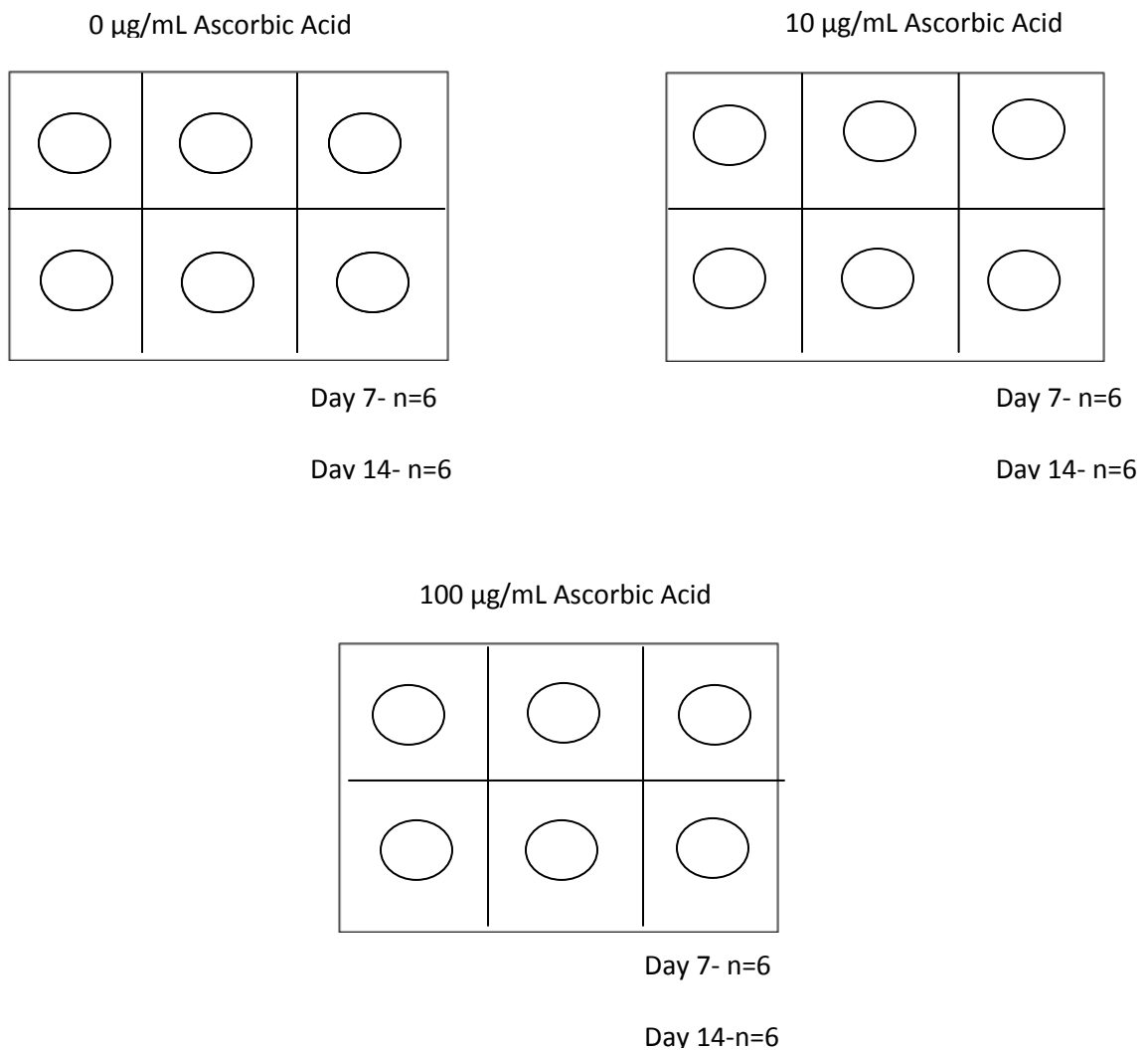


Figure 11 - Ascorbic Acid Experimental Design

For each experiment, two 6 well plated were seeded per ascorbic acid concentration. Each time frame had one 6 well plate and the entire experiment was repeated three times. Three rings from each plate (n=3) were harvested for histochemical staining and the other three were frozen for Sircol (Sigma) and CyQuant (Invitrogen) assays.

3.4 Harvesting Vascular Rings

3.4.1 Harvesting 7 and 14 Day Rings

When the rings had been cultured for 7 or 14 days, each set of rings was harvested. Using a pair of bent needle nose forceps, the rings were slid off of their posts one by one and placed in PBS which can be seen in Figure 12. They were then dabbed dry on Lens Paper and placed in a 2 in X 2 in lens paper square to retain the ring within the cassette during processing. The lens paper was then folded and placed in a cassette which was placed in neutral buffer formalin. This was done for each concentration at each time stamp. The samples were then processed and embedded in paraffin. The cassettes were then cut in 5 μ m sections and placed onto glass slides.

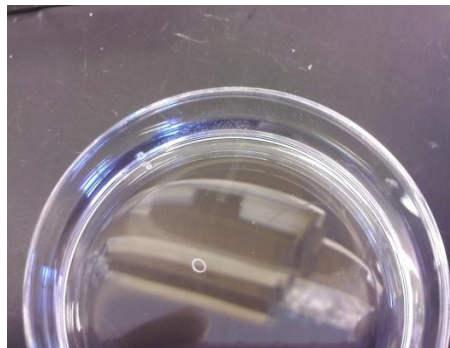


Figure 12 - HDF ring washing in PBS

3.4.2 Histochemical Staining

For each ring, one of the slides prepared was used for a Masson trichrome stain and another was used for a Movat's pentachrome staining. The protocols for these procedures can be found in Appendix A.

The Masson trichrome stains three different colors with each color corresponding different cellular and matrix components. Cytoplasm and keratin stained red. Nuclei stained black, and collagen stained blue.

The modified Movat's pentachrome stains five different colors with each color corresponding to a different part of the tissue. Nuclei and elastic fibers stained black, glycosaminoglycans stained blue, cells and cytoplasm stained red, and collagen stained yellow.

4.0 Results

The entire experiment was repeated three times for each concentration and each time point for a total of 36 rings for each experiment. Due to contamination and non-salvageable rings, not all of the rings for each concentration were harvested and analyzed. Table 1 shows the total amount of rings harvested and analyzed for trichrome and pentachrome staining. There were also 8 rings frozen for sircol and cyquant assays which were not completed due to time restraints.

Table 1 - Total Number of HDF Rings Analyzed

	Day 7			Day 14		
Experiment	0µg/mL	10µg/mL	100µg/mL	0µg/mL	10µg/mL	100µg/mL
1*	0	0	0	0	0	0
2	1	3	3	2	0	0
3	2	0	1	0	0	2
Total:	3	3	4	2	0	2

*Experiment 1 produced zero rings to improper seeding methods.

Vascular rings were also cultured for a mechanical conditioning device that is discussed in Chapter 6. These rings were cultured in growth medium without the addition of ascorbic acid. For this experiment six rings were cultured at a time and the entire experiment was repeated four times. At this time none of the rings have successfully been mechanically conditioned due to contamination or breakage of the ring.

4.1 7 Day Treatments

Each salvageable ring from the 7day Ascorbic Acid treatments was harvested and stained using the trichrome and pentachrome staining. A representative sample from all of the experiments can be seen in Figure 13.

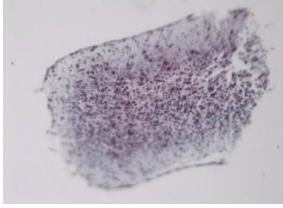
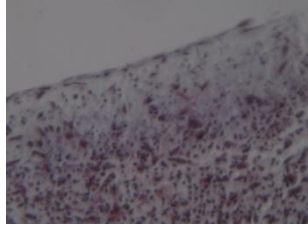
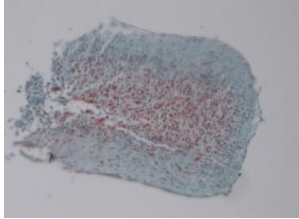
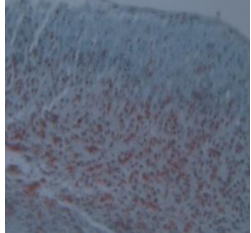
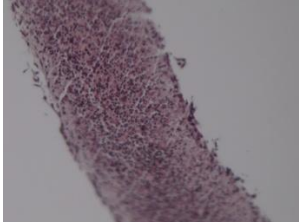
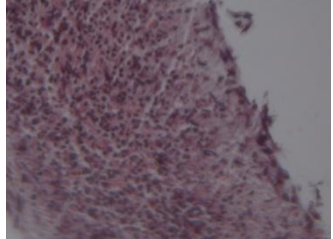
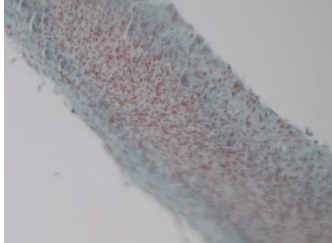
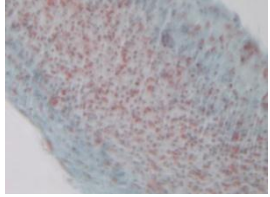
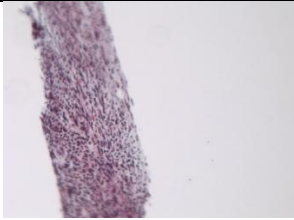
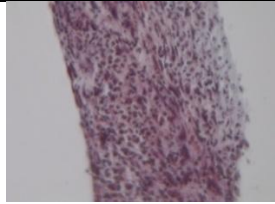
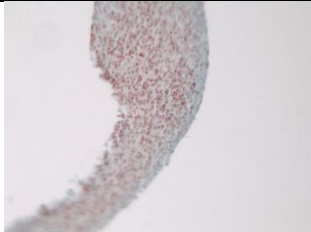
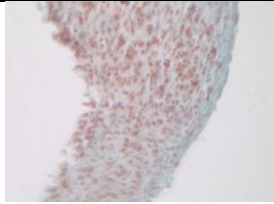
Treatment	Trichrome 20x	Trichrome 40x	Pentachrome 20x	Pentachrome 40x
0µg/mL				
10µg/mL				
100µg/mL				

Figure 13 - 7 Day Treatments

4.2 14 Day Treatments

Due to contamination and non-salvageable rings there were not enough rings from the 14 day treatments to have accurate results. The images below were parts of rings or clumps of cells that were grown at the given concentrations and stained.

4.2.1 0µg/mL Ascorbic Acid Treatments at 14 Days

Figure 14 shows the rings harvested at 14 day with 0µg/mL Ascorbic Acid.

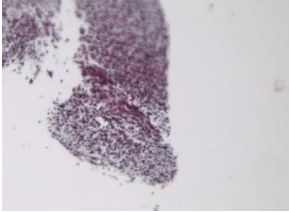
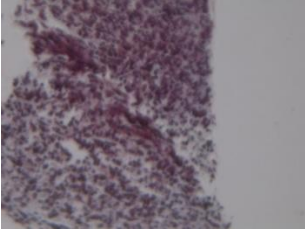
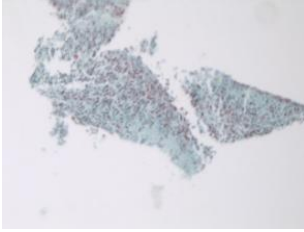
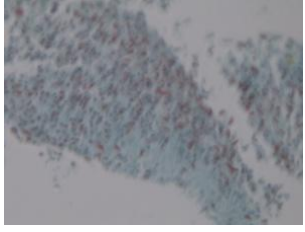
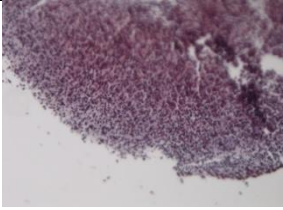
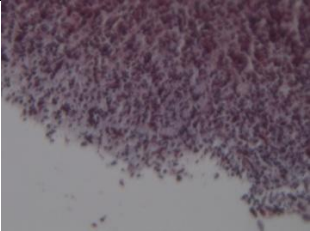
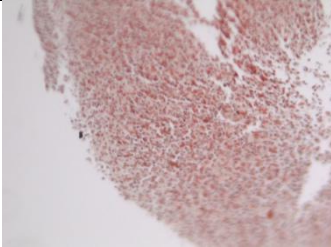

Trial #	Ring #	Trichrome 20x	Trichrome 40x	Pentachrome 20x	Pentachrome 40x
1	2				
1	3**				

Figure 14 - 14 day 0 μ g/mL Ascorbic Acid Trichrome and Pentachrome Stains

**Trial 1, Ring 3 was not actually a ring. The cells were in a ball and the 'clump' of cells was tested.

4.2.2 10 μ g/mL Ascorbic Acid Treatments at 14 Days

There were no salvageable rings for the 14 day 10x Ascorbic Acid treatments for either trials due to contamination and/or poor harvesting methods.

4.2.3 100µg/mL Ascorbic Acid Treatments at 14 Days

Figure 15 shows the rings harvested at 14 day with 100µg/mL Ascorbic Acid.

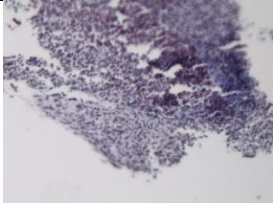
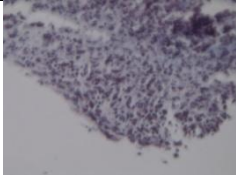

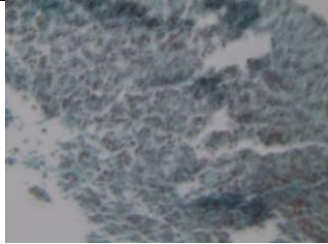
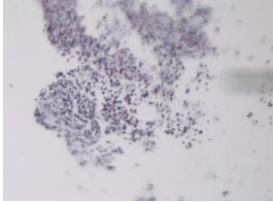
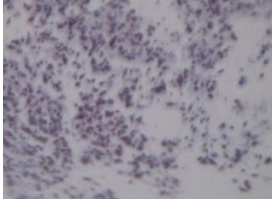
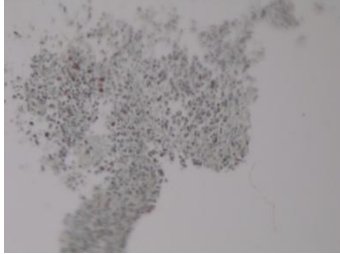
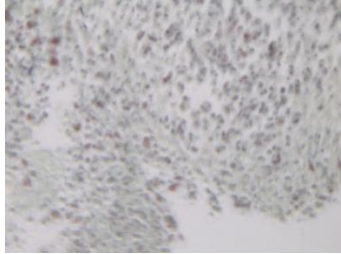
Trial #	Ring #	Trichrome 20x	Trichrome 40x	Pentachrome 20x	Pentachrome 40x
2	1				
2	2				

Figure 15 - 14 day 100µg/mL Ascorbic Acid Trichrome and Pentachrome Stains

5.0 Discussion

Over the past five months three full experiments were completed and a total of 22 rings were harvested. A total of 14 rings were analyzed using trichrome and pentachrome stains while 8 rings were frozen for sircol and cyquant assays which were not completed due to time constraints. With increasing amounts of ascorbic acid, it was shown that there was a visible change in the ECM of this human dermal fibroblast tissue. This can be seen in Figure 10 by the increase in 'red' color and the decrease in 'blue' seen in the pentachrome stain. This observation suggests that there was a decrease in glycosaminoglycans and an increase in cells and cytoplasm within the tissue. Although collagen was not easily seen in the tissue, a decrease in glycosaminoglycans and an increase in cells and cytoplasm could potentially make the tissue stronger which would accomplish the overall goal of the project; to increase the initial strength of the tissue. There were no noticeable changes in the 7day treatments when it came to the trichrome stains which could be because the black and dark blue stains are so similar that it was hard to distinguish the two.

There were no useful results from the 14day treatments due to contamination in the lab and rings that were not salvageable. Some of the rings were ruined as they were being taken off of the agarose posts. These rings fell apart and could not be analyzed for collagen content.

In an ideal situation, each experiment would have produced 32 viable rings, but overall contamination was an ongoing problem for both 7 and 14 day treatments leaving only a total of 14 rings that could be analyzed for collagen.

Davidson published a paper in 1996 using varying concentrations of ascorbic acid cultured with pig skin fibroblasts (Davidson et al, 1996). They stained the tissue sections with

hematoxylin and eosin, which was not done during this project, but they did see an increase in collagen production with an increase in concentration of ascorbic acid.

Overall there was not a noticeable increase in collagen production within the human dermal fibroblast rings, but there was a significant change in the ECM of the 7day treatments. This shows that the ascorbic acid could be increasing the strength of the tissue just not in the manner that was first expected.

5.1 Future Work

This project leaves many opportunities for future work. The rings originally frozen for sircol and cyquant assays are still in the freezer so that these tests can be done at a later date. Both of these assays analyze for DNA content which will be able to be correlated with cell count. Once the cell count is known for the final rings, it can be compared to the original 1 million cells that were seeded.

The rings can also be treated with different growth factors to increase different proteins within the cellular tissue that might help to increase strength and flexibility so that the rings can better withstand mechanical testing.

Once the optimal culture conditions have been developed, the rings can be mechanically conditioned to further increase tissue strength. Both of these methods seem very promising for increasing tissue strength and reaching the ultimate goal of creating a tissue that can be used to make vessels for coronary artery bypass surgeries. The future work with these rings has many different directions it can take and all of them seem very promising.

6.0 Mechanical Conditioning Device (Ali et al, 2009)

This chapter is a small part in a much larger Major Qualifying Project that was completed with three mechanical engineer students and me. Chapter six of this paper shows the biological aspect of the vessel ring bioreactor as well as the finished design.

6.1 The final mechanical conditioning device

This device was designed to mechanically condition vascular tissue. This device will be used to dynamically condition rings of vascular tissue created by cells in culture without the use of a scaffold. This device creates a high-throughput and accurate yet inexpensive, dual-regulated pressure source as well as an electronic valve controller to cyclically inflate up to 32 ring segments cultured on a series of silicone tubes encased in standard 50mL sterile conical tubes. This device will be used to determine the dynamic culture conditions that optimize engineered vessel growth and maturation in culture (Figure 16).

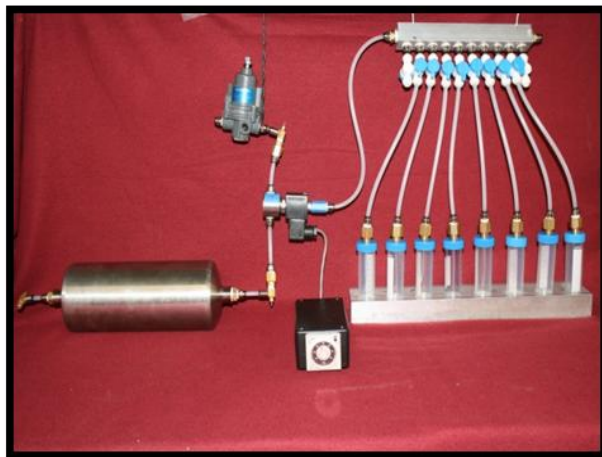


Figure 16 - Final Design

6.2 Individual Media Chambers

Tissue engineered vascular rings will be placed on flexible, inflatable tubing, with up to four rings on each tube, as shown in Figure 17. To ensure cell compatibility, and the appropriate mechanical strength for dynamic loading, silicone was recognized and verified as a suitable option. The outside diameter of the silicone tubing will be slightly smaller than the vascular rings. The flexible silicone tubing is connected to a threaded barb, by a heat-sensitive sleeve. The threaded barb screws onto the cap of a media chamber. A hole was drilled on to the cap to ensure that the threaded barb fits through the cap. An air pressure fitting screws on to the top of the barb on the opposite side of the cap. On the underside of the media chamber cap, between the cap and the barb connector's hex head, an o-ring ensures that there is no leaking between the hole made in the cap and the outside environment. The barb and the silicone tube are housed inside the media chamber, with the appropriate media. A sterile air filter was attached to the cap to allow the media to obtain oxygen. Eight such units are attached to the manifold (Figure 16). The numerous separate chambers allow for the high throughput required of the design. The 50mL conical tube bases can be changed easily when fresh media is required for feedings since they are readily available in large quantities.

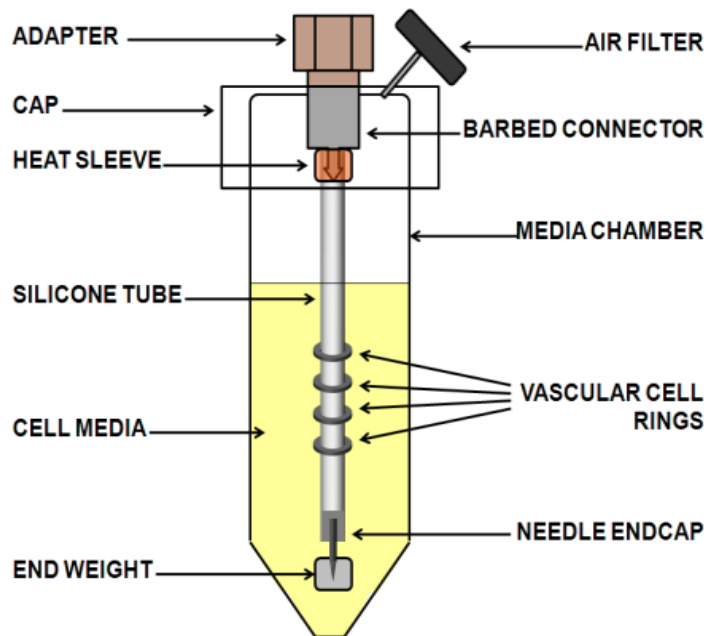


Figure 17 - Individual Media Chamber

6.2.1 Ring Method for Media Chambers

The same method for seeding rings for the media chambers was described in chapter 3.2. The only difference between these two methods is that the rings cultured for the mechanical conditioning device were cultured in growth medium instead of media with ascorbic acid.

6.3 Loading the Vascular Rings

In order for the device to mechanically condition the vascular rings in culture one must be able to successfully load the rings onto the silicone tubes without damaging them. To test the silicone tubing idea rings were cultured and loading of these rings was attempted. The attempted loading occurred on the bench top so the rings were not viable after testing. When this machine is used the ring loading process must occur within the cell culture hood to maintain sterility. After many attempts, the design seen in Figure 18 was established. A

hypodermic needle was glued into the bottom on the silicone tubing which allows the user to pierce the center of the agarose well to help hold the tube in place. Once the silicone tube is in the center of the agarose well the user can take bent needle nose forceps and 'roll' the ring up the tube to its desired location (Figure 19). The tube with the ring on it will then be placed in a 50mL conical tube with the desired media.

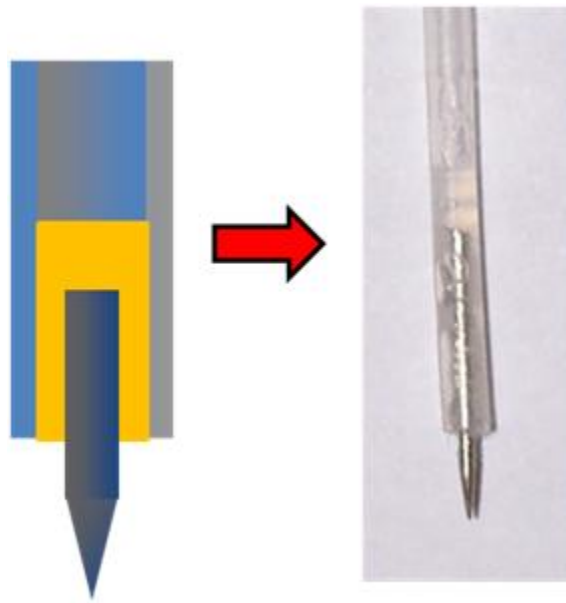


Figure 18 - Silicone end tubing design

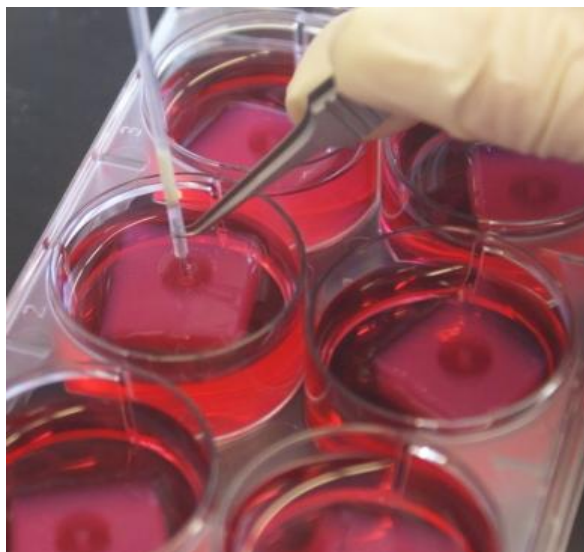


Figure 19 - Loading the Rings

During this project the rings were not successfully loaded onto the silicone tubing because the tubing used was 2mm and the post the rings were grown in was 2mm. In order for the rings to successfully be loaded onto the tubing these two must line up perfectly without any room for the rings to roll up inside of the silicone tubing. To fix this problem the rings will be grown around 2.25mm posts during future work.

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8.0 Appendix

Masson Trichrome Protocol

The following reagents were used for the trichrome staining:

Bouin's Solution (Sigma HT10-1-128)

Weigert Iron Hematoxylin:

-Solution A (Sigma HT107-500mL)

-Solution B (Sigma HT109-500mL)

-Working Solution: Mix equal parts of solutions A and B

Biebrich Scarlet-Acid Fuchsin Solution (HT151-250mL)

Phosphomolybdic/Phosphotungstic Acid Solution

-Working Solution

-1 part: Phosphomolybdic Acid Solution (HT153-250mL)

-1 Part: Phosphotungstic Acid Solution (HT152-250mL)

-2Part: Distilled Water

Aniline Blue Solution 1%

Acetic Acid Solution 1%

-1 mL: Glacial acetic acid

-99 mL: Distilled Water

Procedure of Trichrome Staining: (Used for slides set in Paraffin)

1. Dehydrated slides in Xylene I for 3 minutes
2. Dehydrated slides in Xylene II for 3 minutes
3. Dehydrated slides in Xylene III for 3 minutes
4. Dehydrated slides in 100% ETOH for 3 minutes

5. Dehydrated slides in 100% ETOH for 3 minutes
6. Dehydrated in 95% ETOH for 1 minute
7. Dehydrated in 70% ETOH for 1 minute
8. Rinsed slides well with distilled water
9. Mordant the sections in Bouin solution for 1 hour at 56°C
10. Removed slides from oven, allowed to cool, washed in running water until the yellow color disappeared
11. Stained sections in Weigert hematoxylin for 10 minutes
12. Washed in warm running tap water for 10 minutes
13. Stained sections in Beirich scarlet-acid fuchsin solution for 3 minutes
14. Rinsed in distilled water
15. Places slides in Phosphomoybdic/Phosphotungstic acid solution for 15 minutes. Solution was discarded after use.
16. Stained sections were placed in aniline blue solution for 5 minutes
17. Slides were rinsed in distilled water
18. Slides were placed in 1% acetic acid solution for 5 minutes. Solution was discarded after use.
19. Rinsed in several changes of tap water
20. Slides were dehydrated through several graded alcohols, clear in two changes of Xylene and Mounted with cytooseal.

Modified Movat's Pentachrome Staining

The following reagents were used for the Pentachrome stain:

1% Alcian Blue (good for 6 months):

-Alcian Blue (8GX) -1 g

-Distilled water- 100 mL

Alkaline Alcohol (good for 3 months):

-95% ethanol -45 mL

-Ammonium hydroxide, conc- 5 mL

Weigert's Stain Stock Solutions:

Alcoholic Hematoxylin 2% (good for 3 months)

-Hematoxylin (Sigma H3136) – 10g

-95% ethanol- 500 mL

Ferric Chloride

-Ferric Chloride Hexahydrate- 12.4 g

-Distilled Water- 500 mL

-HCl, conc- 5 mL

Iodine Solution (good for 6 months)**

-Iodine- 2 g

-Potassium Iodide(KI) – 4 g

-Distilled water- 100 mL

**Dissolved 4g of potassium Iodide in 20mL of distilled water. Added 2g of Iodide and completely dissolved then added the remaining 80mL of distilled water.

Working Solution***:

-Hematoxylin 2% - 30mL

-Ferric Chloride- 20mL

-Iodine Solution – 10mL

***Mixed in the order given, discarded after use.

Crocein Scarlet-Acid Fuchsin

1% Crocein Scarlet (good for 6 months)

-Crocein Scarlet M00 3B- 1g

-Distilled water- 99.5 mL

-Acetic Acid, conc- 0.5 mL

0.01% Acid Fuchin (good for 6 months)

-Acid Fuchsin- 0.1 g

-Distilled water- 99.5 mL

-Acetic Acid, conc- 0.5 mL

Working Solution:

4 parts 1% Crocein Scarlet

1 part 0.01% Acid Fuchin

Acetic Acid 1%

Acetic acid, conc Glacial- 1mL

Distilled water- 99mL

Phosphotungstic Acid 5%

Phosphotungstic Acid- 5g

Distilled Water- 100 mL

*Alcoholic Saffron**** (good for 1 year)*

Saffron (Sigma S8381)- 6g

Absolute EtOH- 100 mL

****Placed in an air tight bottle in 60°C oven for 48 hours to extract saffron

Procedure for Pentachrome staining (Used for slides set in Paraffin)

1. Deparaffinized and hydrated to dH₂O
2. Preheated alkaline alcohol in 60°C oven or water bath
3. Mordant in Bouin's in 80-90°C water bath for 10 minutes. (contained formaldehyde and picric acid-seal bucket with parafilm to minimize fumes; Sigma HT10-1-32)
4. Cooled for 10 minutes in room temperature water
5. Rinsed under running water for 10 minutes
6. Placed in 1% Alcian Blue for 20 minutes (At this time the Weigert's and Crocein Scarlet Acid Fuchsin working solutions were prepared)
7. Rinsed under running water for 10 minutes
8. Placed in alkaline alcohol at 60°C for 10 minutes
9. Rinsed under running water for 5 minutes
10. Placed in Weigert's for 60 minutes
11. Washed for 5 minutes in running water, then rinsed in double distilled water for 10 minutes
12. Placed in Crocein Scarlet Acid Fuchsin for 45 seconds
13. Rinsed twice (2X) for 5 minutes in water
14. Placed in 5% Phosphotungstic Acid for 5 minutes 9the for 10 and 20 minutes for progressively less elastic fibers)
15. Transferred directly to 1% acetic acid for 5 minutes
16. Rinsed twice (2x) for 5 minutes in water
17. Dehydrated in short series 1 change for 1 minute each in 95% EtOH, Twice in 100% EtOH
18. Placed in alcoholic saffron for 60 minutes
19. Rinsed in two changes of Absolute EtOH, 1 minute for each
20. Cleared in xylene and mounted