MANUFACTURING AND MECHANICAL TESTING OF BIOCHEMICALLY STIMULATED TISSUE-ENGINEERED BLOOD VESSEL CONSTRUCTS

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MANUFACTURING AND MECHANICAL TESTING OF BIOCHEMICALLY STIMULATED TISSUE-ENGINEERED BLOOD VESSEL CONSTRUCTS

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To my friends and family, especially my Mom and Dad.

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Rudolph Gleason, PhD, Assistant Professor, Department of Mechanical Engineering and Biomedical Engineering at Georgia Tech mentored this work. Julia Raykin, a graduate student in the Department of Biomedical Engineering, served as a co-mentor for this project.

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SUMMARY

Vitamin C (ascorbic acid), and transglutaminase have been shown to induce mechanical changes within collagen-based blood vessel constructs. Recent studies have found that the mechanical properties of tissue-engineered blood vessel constructs can be improved through chemical stimulation using these agents. In this study, we use vitamin C to induce extracellular matrix collagen synthesis, and transglutaminase to crosslink collagen. These chemical agents yield increased cell growth and enhance the mechanical strength and integrity of collagen based blood vessel constructs. However, the addition of each of these agents must be balanced, as high concentrations may be toxic to the cells. Finally, we also increase cell seeding density to modulate gel compaction and speed up construct development time. We aim to define the effects of vitamin C, transglutaminase, and initial cell seeding density through mechanical testing to confirm the findings. Preliminary results indicate that TG/AA have a significant impact on tensile strength of the constructs and that varying initial cell seeding density may have a positive trend. This research aims to provide the foundation necessary for a successful attack on the Holy Grail of cardiovascular tissue engineering: creating a small-diameter blood vessel substitute.

CHAPTER 1

INTRODUCTION

Atherosclerosis is an inflammatory disease that results in the narrowing of arteries and eventually impairs blood flow due to plaque buildup.[1] The narrowing of coronary arteries leads to decreased blood flow to the heart, ultimately leading to cardiac ischemia. Treatment for advanced stages of this disease involves coronary artery bypass graft surgery (CABG), the most commonly performed cardiac surgical procedure in the world.[2] Unfortunately, native blood vessels are not reliably available because they may be diseased or because such vessels were already used in previous surgery. Today, CABG surgery cannot be performed in patients who cannot provide native blood vessels. As a result, the replacement or repair of diseased vessels with natural or synthetic vascular grafts has become a routine treatment.[3] Efforts to create synthetic small diameter vascular grafts have been unsuccessful. Ultimately, an alternative to native blood vessels is needed, and thus, small-diameter blood vessel substitutes need to be further researched.[4] This project investigates whether certain biochemical stimulation and changes in initial cell composition can improve mechanical properties of tissue-engineered blood vessels.

CHAPTER 2

LITERATURE REVIEW

Vascular tissue engineering is dedicated to the creation of functional blood vessels *in vitro* primarily for treating cardiovascular disease through vascular grafts or studying vascular biology by creating improved vascular models. However, there are still many challenges and questions that must be answered before an adequate blood vessel alternative is available. Developing blood vessels with adequate mechanical strengths and hemocomatibility are two of the challenges we face.[5] The question that has to be answered for vascular tissue engineering to succeed is how to manipulate cell function in culture when the cells have been removed from their native environment. When placed in culture, vascular smooth muscle cells exhibit a synthetic phenotype with increased proliferation, increased matrix production, increased protease production, and altered cell contractility.[3] The main goal in vascular tissue engineering is to manipulate cell function and improve the properties of engineered tissues *in vitro* while still maintaining function similar to that of native vessels.

Chemical stimulation has been found to improve the mechanical properties of tissue-engineered blood vessel (TEBV) constructs in many studies. Specifically, chemical stimulation has been shown to induce mechanical changes leading to stronger TEBV constructs. Vitamin C (Ascorbic Acid, AA) has been shown to induce extracellular matrix (ECM) collagen. The resulting cell matrix is sufficient in strength to withstand pressures greater than those experienced *in vivo.* Specifically, AA is said to stimulate collagen synthesis, which yields cell growth.[3] However, the effects of AA on elasticity

are unknown. Some have reported that the AA-induced strength is accompanied by a significant increase in stiffness.

Transglutaminase (TG) has been established as a successful crosslinking agent of collagen to produce viable cell-seeded crosslinked gels with significantly enhanced mechanical strength.[6] It is also responsible for tissue stabilization because they form high molecular weight complexes through this chemical crosslinking. This is important because the endothelial cells that line the inner surface are involved in the functionality of vessel walls, which include the maintenance homeostasis within the blood, transport regulation, and cell signaling. Thus, it is paramount to have a synthetic vessel that is compatible with the arterial wall.

A fundamental problem with the current development process of collagen-based blood vessel constructs is the amount of time required to fully develop these constructs. With the ultimate goal of rapid development of constructs, it is important to minimize development time. Currently, the standard cell seeding density is 1×10^6 cells/ml.[4, 7-9] Previous studies have shown that using a cell seeding density over 1×10^6 cells/ml may have detrimental effects on the cells, resulting in cell apoptosis. However, it may be possible to increase the rate of gel compaction without sacrificing cell viability by optimizing initial cell seeding concentrations.

Several groups have attempted to create small diameter blood vessels, but in all of these studies, there are still more questions that need to be answered. Many of these same groups determined that collagen has a significant positive impact on cell attachment and cell signaling, yet that it is intrinsically weak.[3-7] Stegemann and Nerem found that collagen type 1 matrices caused a shift in the smooth muscle cell phenotype towards a

more synthetic state, characterized in part by a loss of important contractile proteins.[5] They also determined that collagen decreased cellular proliferation due to cell-cell contact inhibition as the gels compact. Accordingly, Stegemann and Nerem verified that biochemical stimulation such as TGF-ß affects gel compaction. As a result, other biochemical stimulation treatments such as ascorbic acid and retinoic acid can be used to see their effects.

In another study, Ogle et al. (2002) performed uniaxial mechanical tests on their constructs.[4] Biaxial mechanical tests, however, may yield more accurate and meaningful results. Finally, Orban et al. (2004) found that enzymes such as TG could be used to crosslink cell-seeded collagen gels for improved mechanical strength; however, the addition of high concentrations of TG could be toxic to the cells.[6] Finding an ideal concentration of TG can solve this problem of cytotoxicity, which would allow for stiffer constructs and more desirable mechanical properties. Thus, all of these studies paved the way for this research to find ways to improve the mechanical strength and integrity of blood vessel constructs.

The specific goal of this research, in addition to determining an optimal cell seeding concentration, is to treat blood vessel constructs with AA and TG and to test the mechanical strength and integrity of the constructs after the introduction of these agents. The purpose of this research is to provide the foundation necessary for a successful attack on the Holy Grail of cardiovascular tissue engineering, creating a small-diameter blood vessel substitute.[10]

CHAPTER 3

MATERIALS AND METHODS

The first of the experiments was broken down into four components: (1) smooth muscle cells were cultured. (2) Collagen based blood vessel constructs were created with different concentrations of TG. Fixed cell concentrations were used in each construct. (3) The constructs were treated with ascorbic acid during incubation. (4) The constructs were subjected to mechanical testing on specific sections. The second experiment was similarly completed as the first, but with varying cell seeding concentrations. The data was then analyzed to verify the effects of each of the individual treatments. The research performed resulted in the determination of the preliminary effects of AA and TG on mechanical strength and integrity of blood vessel constructs as well as the determination of the effects of varying initial cell seeding densities.

3.1 Culturing of Rat Aortic Smooth Muscle Cells (RASMCs)

Adult rat aortic smooth muscle cells were cultured to create blood vessel constructs. The cultures were maintained in high-glucose Dulbecco's modified eagle medium (DMEM) supplemented with L-glutamine (Sigma), penicillin-streptomycin (Hyclone), and 10% fetal bovine serum (FBS) (Cellgro). Cells were stored in liquid nitrogen if not used immediately. Cells were subsequently thawed and cultured according to methods described by Seliktar et al.[4] They were grown below a passage of 15. Once the cells were ready, they were used in the fabrication of blood vessel constructs.

3.2 Fabrication of Blood Vessel Constructs

3.2.1 Initial Preparation

First, the collagen solution was prepared by the addition of 0.02 N acetic acid to lyophilized collagen to gain a final concentration of 4mg/ml. Next, 5X Dulbecco's modified eagle medium (DMEM) was prepared using powdered DMEM. The last solution prepared was 0.1 M NaOH through dilution. Mandrels were formed using a 3mm hollow glass rod. Two layers of rubber stoppers were placed on both ends of the mandrel and sized for fitting a glass test-tube. Finally, all instruments, test tubes, caps, and assembled mandrels were autoclaved. Once preparation was complete, the materials were used for creating the construct solution and used in construct assembly.

3.2.2 Construct Solution

Initially, 5X DMEM, collagen solution and NaOH were placed on ice. Cells were then be trypsinized and counted. The cell count was inputted into a construct spreadsheet to calculate the amount of cell suspension, NaOH, 5X DMEM and collagen needed for making the constructs to achieve the final concentrations: 5 ml of final solution per test tube/construct, 1 million cells/ml, 2 mg/ml collagen, 1 ml 5X DMEM to 4 ml bovine collagen solution, 1 ml NaOH to 6 ml bovine collagen solution, and used the remainder of the determined volume for the culture medium. Next, the calculated volume of cell suspension was centrifuged and placed on ice. The construct solution was made by gently resuspending the cells in the calculated volume of culture medium to which 5X DMEM was added to collagen and was mixed followed by the addition of NaOH to collagen. The cell suspension to collagen solution was quickly added and gently mixed and left on ice, making sure the solution was thoroughly mixed avoiding bubbles. In experiments with

varying cell seeding concentrations, concentrations of 2.5 million [2.5X] cells/ml and 5 million [5X] cells/ml were used.

3.2.3 Construct Assembly

Test tubes were put into test tube holders and 5 ml of construct solution was added into each test tube. Using mandrel grabbers, a mandrel was inserted into each test tube. The test tubes were then capped and placed in an incubator for 30-45 minutes to allow for gelation and polymerization. Once the constructs were determined to have gelled, the caps were removed and a glass pipette was used to aspirate a very small amount of fluid and gel from inside the glass rods to prevent hydraulic lock. Mandrel grabbers were then used again to remove the constructs from the test tubes, which were placed in culture dishes in 120 ml of medium. The constructs were released from the stoppers with scissors and mandrel grabbers for support, and any excess material was discarded. Finally, the medium was replaced as necessary due to metabolic activity during incubation (See **Figure 1**).

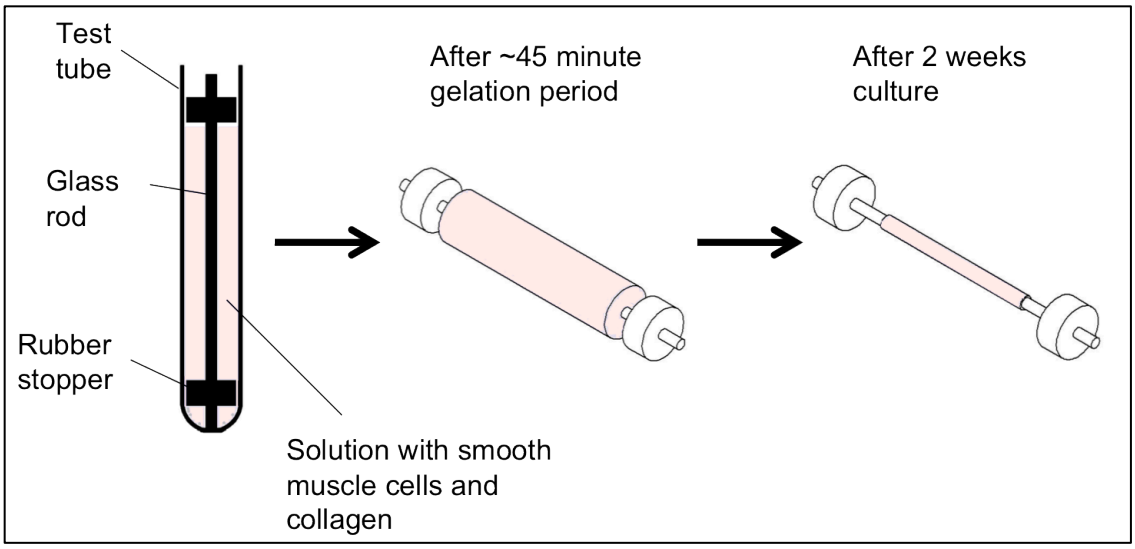

Figure 1: Summary of Construct Assembly. The construct solution is first prepared and added to a cell solution. Once mixed, the resulting solution is transferred into individual test tubes and mandrels are placed into each test tube. The test tubes were then placed in an incubator for 30-45 minutes or until polymerization. Following polymerization, the constructs were removed from the test tubes and placed in culture dishes with media and incubated for the duration of the experiment. Once compacted, the constructs look like the final image on the right.

3.3 Optimization of Transglutaminase Crosslinking

The manufactured constructs were treated with 2 µg/ml (1:1000 C:E), 0.4 µg/ml (1,:5,000 C:E) and 0.2 µg/ml (1:10,000 C:E) of TG to find the optimal concentration for successful crosslinking during the construct solution fabrication. A 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay was used to analyze end groups to find the extent of reaction through transamidation. The number of free amine groups was quantified by TNBS absorption. After gelation, a solution containing 4% $NaHCO_3$ and 0.5% TNBS was added to each well, and the plates were incubated in the dark at 37°C for 2 hours. 6 M HCl was added and the temperature was maintained at 37°C until

solubilization of the gel. The resulting solutions were aliquotted into a 96-well plate. Finally, the absorbance was measured at 345nm using a microplate spectrophotometer.[6]

3.4 Treatment with Ascorbic Acid

To demonstrate the effects of AA on the biomechanical properties of the tissue-engineered blood vessels, 0.3 mM AA was added to the media. Media was changed approximately 3 times per week. The effects were measured at 14 days and compared to the controls.[5]

3.5 Mechanical Testing

3.5.1 Ring Tests

Mechanical testing was performed 14 days after construct formulation. The tensile properties of the constructs were measured using 5 mm wide rings cut from the tubular constructs. Carbon markers were placed on the rings to allow for the measurement of the true local strain of the material during testing upon particle tracking of images taken during testing. The rings were placed between two hooks on an Instron mechanical testing device in phosphate buffered solution (PBS). After being preconditioned by 5 stretches to 1 mm greater the unstretched length, the construct rings were stretched to failure at constant displacement rates of 0.1 mm/s, 1 mm/s, and 10 mm/s as the load/extension data was recorded. A force transducer and video imaging system was used to ensure the accuracy of the measurements and for further analysis.[4] These tests were performed on constructs treated with TG/AA and controls. See **Figure 2** for a visual representation of setup.

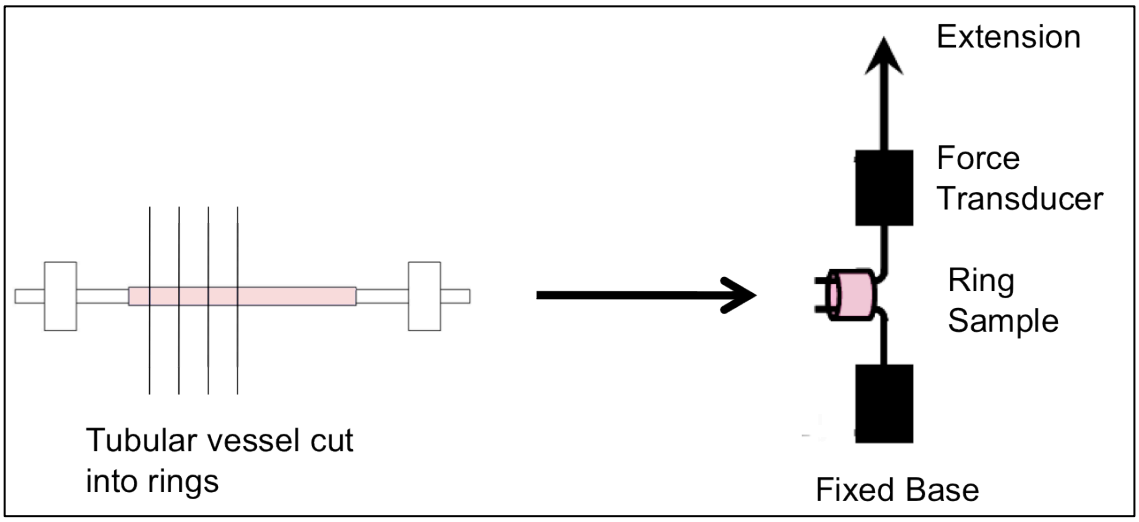

Figure 2: Ring Test Setup. 5 mm wide rings were cut from each tubular construct. The rings were placed between two hooks on an Instron mechanical testing device. The fixed base and ring samples were in a PBS bath.

3.5.2 Burst Pressure Tests

Pressure-diameter tests were performed 14 days after construct assembly. Each end of the construct was slid onto a metal cannula and secured using suture thread and placement grooves (See **Figure 3**). A mechanical testing apparatus composed of flow control, pressure control, axial motion control, and geometry-monitoring subsystems. A mechanical fluid circuit loop was created using PBS: (1) a reservoir of PBS was created using a stopper-sealed Erlenmeyer flask with leads for tube connections, (2) a pressure transducer, (3) a PBS bath for the construct, and (4) a second pressure transducer. The fluid pressure was controlled by blocking flow in the tube leading to the flask and then modifying the pressure in the air space above the fluid in the flask. The pressure inside the construct was calculated by taking the average of the two pressures at the pressure transducers. Increasing the internal pressure at a constant rate and recording the maximum pressure inside the construct before rupture measured burst pressure. The

diameter of the construct was constantly measured using webcam-based geometry-monitoring subsystem. All burst pressure and diameter data was collected using a custom coded National Instruments LabVIEW virtual instrument. These tests were completed on constructs with varying cell concentrations at the control (1X), 2.5X, and 5X.

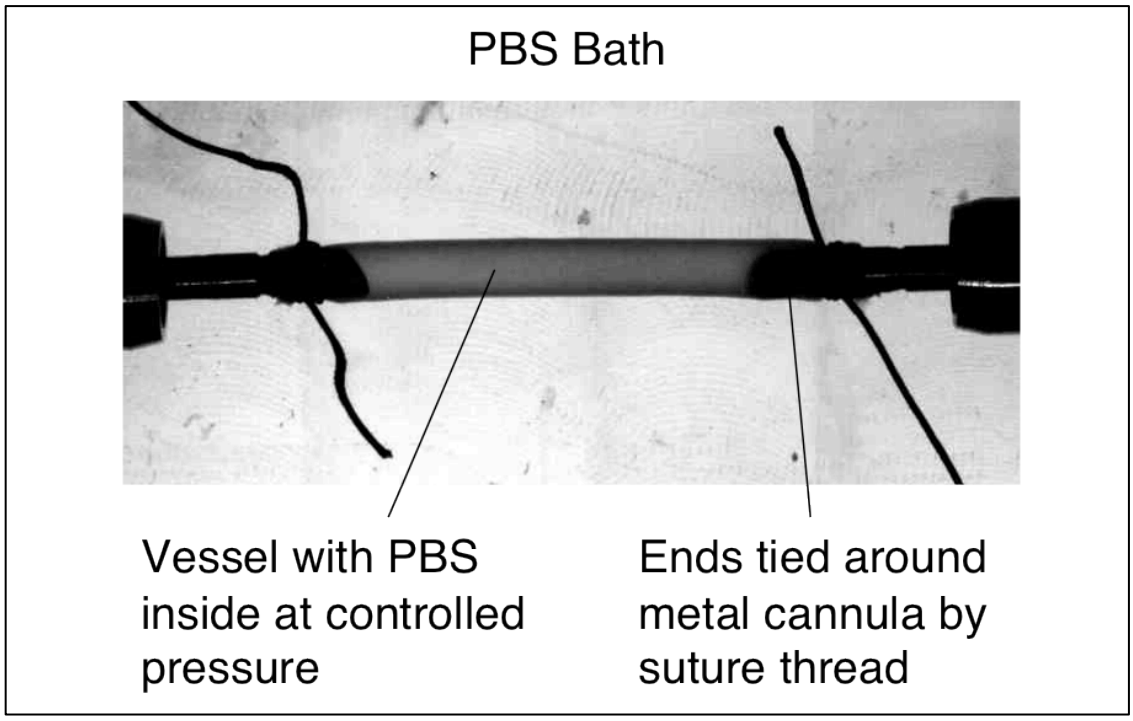

Figure 3: Burst Pressure Test Setup. Each end of the construct was placed on a metal cannula, which allowed for pressure modulation using PBS.

3.6 Statistical Analysis

Ring test data were analyzed by calculating the mean ultimate tensile load for each group (Control, TG/AA) of constructs at different extension rates (0.1 mm/s, 1 mm/s, 10 mm/s) after 14 days of treatment. These values were compared within each extension rate using the two-sample t-test function, ttest2, in Mathworks Matlab r2010a.

The hypothesis for this test is that the mean ultimate tensile loads are not equal. The null hypothesis is that the means are equal. Burst pressure test data were analyzed by taking the mean pressure peak attained until specimen failure and comparing each test group (Control – [1X], [2.5X], [5X]) to another using a two-sample t-test function in Mathworks Matlab. The hypothesis for this test is that the mean bust pressures are not equal. The null hypothesis is that the means are equal. No statistical analysis was performed on the TNBS assay data because the data obtained did not contain analyzable information.

CHAPTER 4

RESULTS

4.1 Transglutaminase Crosslinking

Amine group quantification was used to attempt to measure the extent of crosslinking. As the ratio of collagen:enzyme was varied between 10,000:1, 5000:1, 1000:1 (w/w), including a non-crosslinked control, the TNBS absorbance should have decreased thereby signifying a decrease in amine groups present with increasing enzyme concentration. Analyses should have indicated a significant difference in number of amine groups present between the non-crosslinked and all of the crosslinked disks. However, the assay failed to yield any results. No tubular constructs were made with different TG concentrations, so burst pressure tests were not performed. Representative results are indicated in **Figure 4** and **Figure 5**.

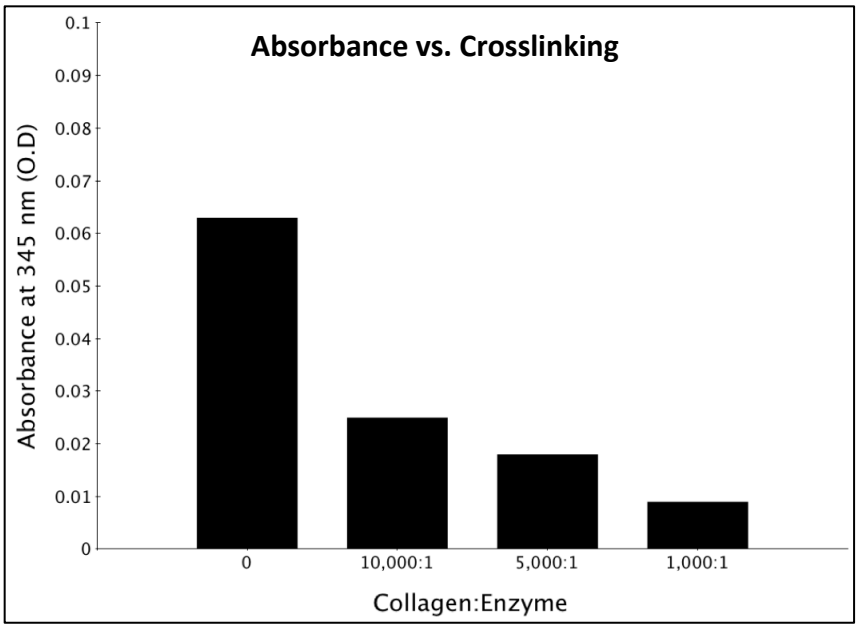

Figure 4: Representative Amine Group Content of Crosslinked Collagen Gels. Average TNBS absorption (345 nm) is plotted against TG concentration. The value at 0 collagen:enzyme represents the non-crosslinked control. This is to verify that collagen crosslinking has occurred.

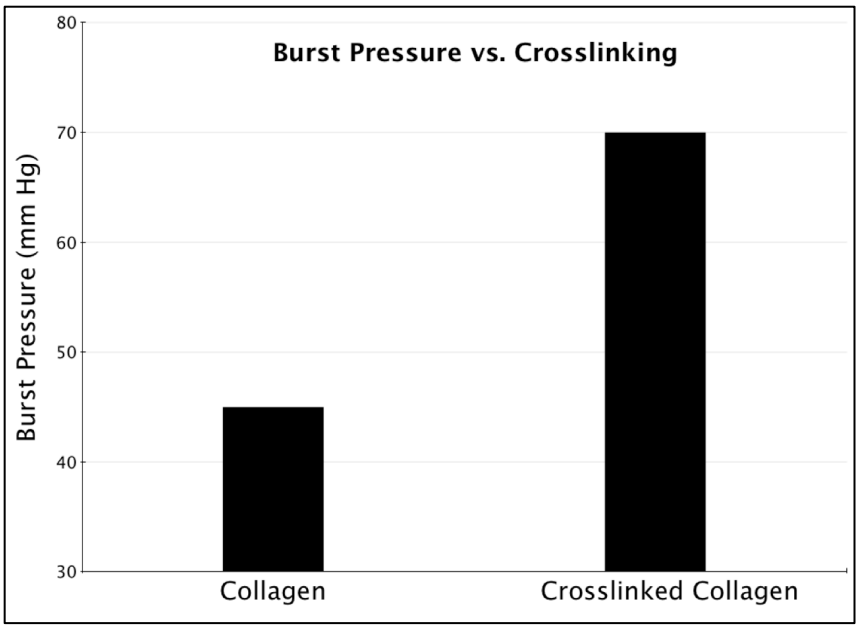

Figure 5: Representative Mean Burst Pressure of Constructs. Burst pressure was measured by peak pressure (mm Hg) attained prior to specimen failure. Control versus 5,000:1 Collagen:TG shown. Crosslinking collagen improves burst pressure resistance, allowing the constructs to still function at high pressures.

4.2 Transglutaminase/Ascorbic Acid Treatment

Ring tests were performed on TG/AA treated and control tubular constructs to compare ultimate tensile load at uniaxial extension of 0.1 mm/s, 1 mm/s and 10 mm/s. A two-sample t-test with $\alpha = 0.05$ was used to confirm that the addition of TG/AA had a significant effect on ultimate tensile load. TG/AA treated TEBVs had significantly more strength at 0.1 mm/s and 10 mm/s uniaxial extension than the controls. Uniaxial loading at 1 mm/s did not yield any statistically significant results due to high standard deviation. 10 mm/s uniaxial loading resulted in higher ultimate tensile load recordings for TG/AA treated TEBVs than any other group at any extension rate. The p-values at 0.1 mm/s, 1 mm/s, and 10 mm/s uniaxial extension were 0.0071, 0.0743, and 0.0408. Ultimate tensile load of each of the groups is shown in **Figure 6.** A representative load vs. extension plot for a control at 10 mm/s uniaxial extension is shown in **Figure 7**.

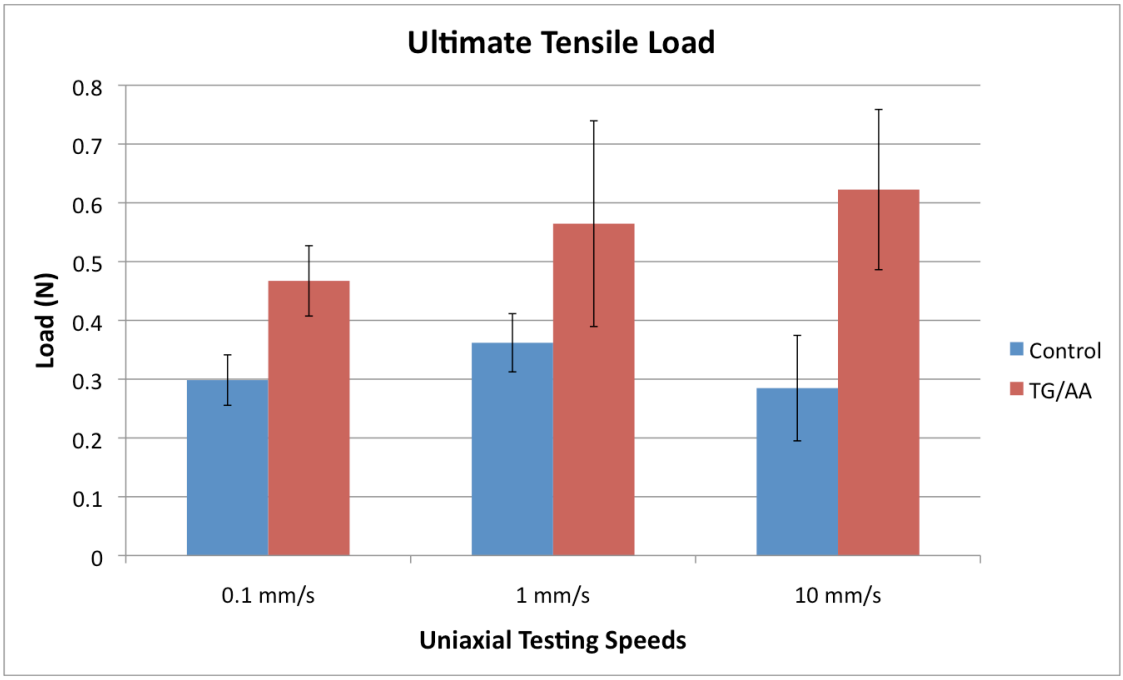

Figure 6: Ultimate Tensile Load of Tubular Construct Controls and TG/AA Treated Constructs during Uniaxial Testing. TG/AA treated constructs tested at 0.1 mm/s and 10 mm/s were significantly stronger than controls. 5,000:1 Collagen:TG, 0.3 mM AA shown. Error bars, means ± SD; Control: n=4, TG/AA: n =3,2,2, α=0.05.

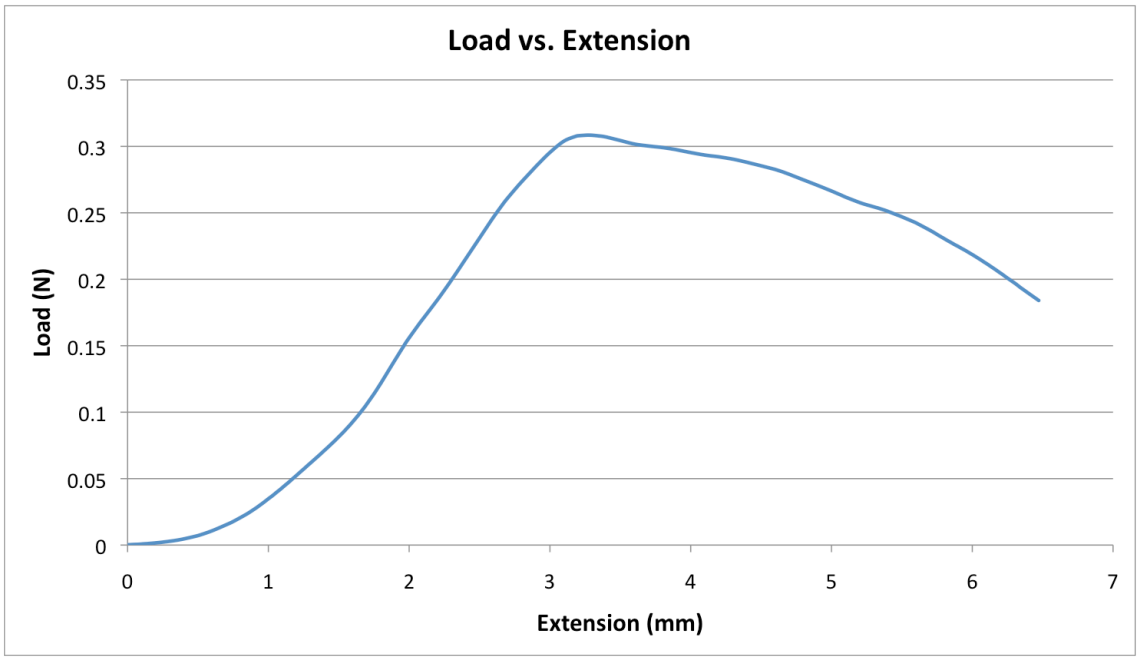

Figure 7: Representative Load vs. Extension Plot for TEBV. Uniaxial extension at 10 mm/s was performed on ring sample from control group following 14 days of incubation.

4.3 Initial Cell Seeding Density

A two-sample t-test with $\alpha = 0.05$ was used to determine that the burst pressures recorded for each group (Control – [1X], [2.5X], [5X]) were not significantly different. The burst pressure tests were performed on tubular constructs with varying initial seeding concentrations. Although the results were not statistically significant, there was an increasing trend in burst pressure, with the [5X] having the highest pressure as seen in **Figure 8**. Comparing the [2.5X] to the control, [2.5X] to the [5X], and [5X] to the control yielded p-values of 0.4949, 0.5076, and 0.3262, respectively.

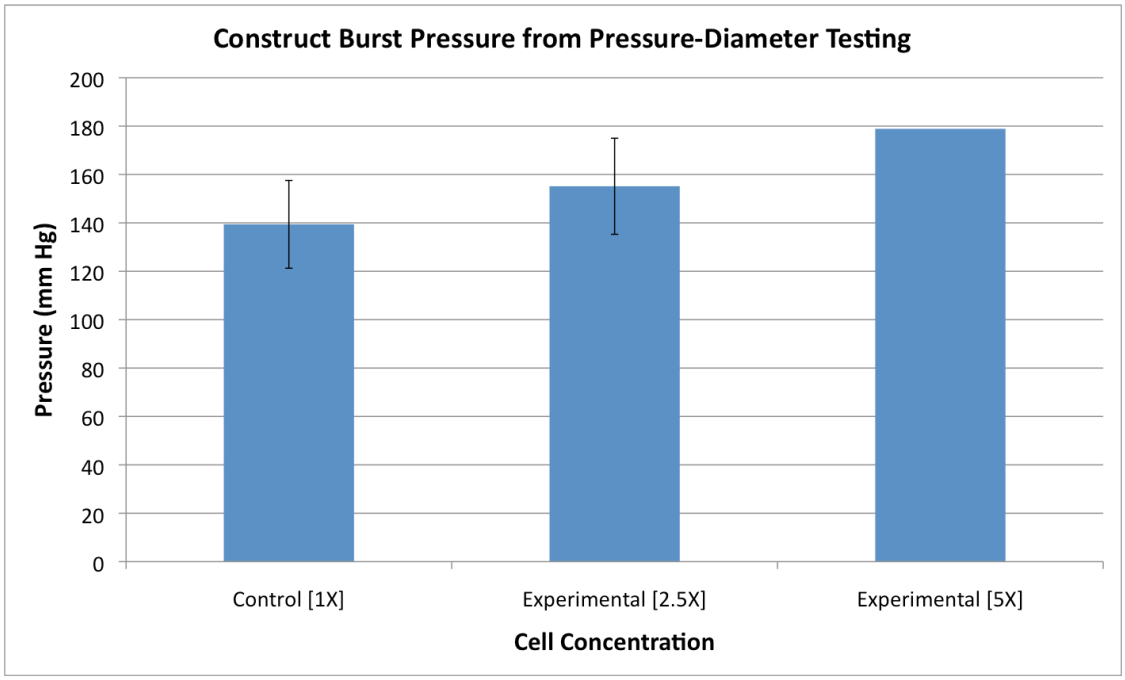

Figure 8: Mean Burst Pressure of Tubular Constructs with Varying Initial Cell Seeding Concentrations. Burst pressure was defined as the peak pressure (mm Hg) attained prior to specimen failure. [2.5X] and [5X] constructs did have higher burst pressures, but the increase was not significant. Error bars, means ± SD; [1X]: n=2, [2.5X]: n=2, [5X]: n=1, α=0.05.

CHAPTER 5

DISCUSSION

The importance of finding a means to improve the mechanical strength and integrity of collagen-based tissue engineered blood vessels is widely recognized and is one of the most pressing issues in the vascular tissue engineering community. Collagen-based blood vessel constructs have been intrinsically weak since conception. This research was conducted to determine whether crosslinking collagen, adding exogenous biochemical stimuli, and varying initial cell seeding densities would positively influence the mechanical properties of collagen based TEBVs. It was hypothesized that these stimuli and changes in initial properties would affect the mechanical properties of TEBVs.

It was determined that the addition of TG/AA to the TEBVs exhibited increased tensile strength from the data obtained from ring tests. Although the 1 mm/s extension did not yield significant improvement, this issue can most likely be handled through testing a larger sample size because the standard deviations for this particular set was almost three times more than the data obtained from 0.1 mm/s extension. The results obtained, however, support the hypothesis that the addition of TG/AA improves the mechanical properties of TEBVs.

Another facet of the development process of TEBVs that was examined was the initial cell seeding density when manufacturing the cells. Results from burst pressure tests showed that the increased concentration of cells increased the vessel's ability to withstand pressure. However, the increase was not statistically significant. This is mainly due to the fact that very few samples yielded results. The original sample size per group

was n = 3, but some vessels were found to have defects such as shorter lengths and tears. Leaks also occurred to ruin some vessels. Thus, the sample size fell to n = 2 and even n = 1 for the [5X] samples. Performing this portion with a greater sample size again may yield statistical significance.

The third specific aim of this study was to determine the optimal TG concentration for collagen crosslinking. The protocol for making disks to verify collagen crosslinking was performed correctly, however, when completing the experiment with the TNBS assay, the absorbance plate reader did not give sensible data because 0.0000 was outputted for all the wells in the plate. It is unknown why this happened; however, it may be due to the concentrations being inaccurate. The concentrations, nonetheless, were verified to be correct.

Crosslinking collagen in the constructs through the addition of TG has previously been proven to facilitate structurally robust constructs by increasing their mechanical strength and integrity.[6] However, that previous research was done with bone muscle cells, and not aortic smooth muscle cells. Ascorbic acid has been widely recognized in increasing the collagen content within the extracellular matrix of cells.[3] Thus, it was externally added to the media after the constructs were fabricated. Mechanical testing was performed in the form of ring tests and burst pressure tests. The results obtained conformed to expectations, but their impact was less noticeable than anticipated. A greater sample size would in general solve the issue because of previous results that lacked statistical significance.

Further limitations of this experiment were present, which compromised some results that were as a result unusable. There are many possible situations than could have

adversely affected the results of this research. Some of these may include equipment failure, compromised sterility of the constructs, inadequate mixing of construct solutions, lack of CO_2 due to tank depletion, incomplete data acquisition, or improper plate readings for assays.

Future studies should include variations of TG concentrations along with AA proceeded with ring tests. Vitamin A (Retinoic Acid, RA) has been demonstrated to induce elastin synthesis in isolated vascular smooth muscle cells (VSMCs), which is important for the mechanical properties of the constructs.[3] Thus, this should be looked into for further manipulating the mechanical properties of TEBVs. Beyond looking at just tensile strength, modulus and other material properties should be analyzed as well with these given treatments. TGF-ß and insulin have also been shown to improve the mechanical properties of TEBVs.[11] Biaxial testing should also be performed on future vessels; this was not attempted because of lack of scope and time.

CHAPTER 6

CONCLUSIONS

The hypothesis that TG/AA have an impact on the mechanical properties of TEBVs was supported by the findings of this research. Biochemical stimulation has been identified as a means for improving the mechanical properties, such as tensile stress, load, yield, and modulus. Furthermore, it was determined that increasing initial cell seeding density may have an impact on the development time of these constructs and without degrading any mechanical properties. Biochemical stimulation is a critical technique that must be further explored to improve the strength and integrity of these vessels. Eventually, the mechanical integrity of the constructs will be improved upon using these techniques and may one day lead to synthetic small diameter blood vessels that can be used in humans.

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