THE EFFECT OF BIOCHEMICAL STIMULATION ON MECHANICAL PROPERTIES OF COLLAGEN-BASED TISSUE ENGINEERED BLOOD VESSELS

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THE EFFECT OF BIOCHEMICAL STIMULATION ON MECHANICAL PROPERTIES OF COLLAGEN-BASED TISSUE ENGINEERED BLOOD VESSELS

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SUMMARY

The successful creation of a living blood vessel substitute for bypass surgery can overcome current problems associated with grafting the patient's own vascular tissue. A fully biological vessel comprised of smooth muscle cells (SMC's) in collagen is an attractive alternative due to its potential for long-term remodeling and vasoactivity, but these vessels are limited by inherent physical weaknesses. This study determines how the mechanical properties of tissue engineered blood vessels made with the collagen/SMC method can be improved by biochemical stimulation. Vessels were prepared by embedding rat aortic smooth muscle cells in a collagen gel and culturing the constructs for two weeks to allow matrix remodeling. Experimental vessels were treated with transglutaminase and cultured in media supplemented with ascorbic acid, TGF-beta, and insulin. Mechanical properties of these vessels were measured by using tensile testing of ring samples from the vessels and applying internal pressure loads over whole constructs. The treated vessels demonstrated statistically significant improvements in both ultimate tensile strength and burst pressure relative to control vessels. These results indicate that biochemical stimulation can facilitate improvements in mechanical properties of tissue engineered blood vessels.

INTRODUCTION

Motivation

Approximately half a million coronary artery bypass surgeries are performed in the United States each year.[1] This procedure is done when a patient's coronary arteries have narrowed to where blood flow is significantly reduced and a heart attack is either imminent or has already occurred. Typically, the narrowed artery is bypassed by using a graft of vascular tissue from elsewhere in the patient's body. The practice of using tissue grafts, however, is not always possible since the patient's other vessels might be unavailable due to previous surgery, or because they are also diseased. As a result, efforts are being made in the field of tissue engineering to successfully make implantable blood vessel substitutes that do not require native tissue from the patient.

There are several requirements for a successful tissue engineered blood vessel (TEBV). It must be immunologically safe, meaning the patient's immune system will not reject the implant. The interior surface of the vessel that contacts blood needs to be non-thrombogenic, which means that it should not cause unwanted blood clots. The vessel should also be mechanically strong enough to withstand blood pressures *in vivo*. Lastly, an ideal small-diameter substitute is able to demonstrate the vasoactivity of native blood vessels, meaning it can dilate or constrict when prompted.[1]

Overview of Current State

Several technologies for engineering a blood vessel substitute are being investigated. Approaches that involve synthetic grafts or scaffolds tend to yield vessels

that are more mechanically stable, but the non-biological components of these vessels place limitations on their ability to be successfully incorporated into a human's circulatory system. Engineered vessels that consist solely of biological components, such as cells and extracellular matrix (ECM) proteins, are better suited for demonstrating vasoactivity, but to date they have failed to be physically strong enough to withstand blood pressure in the body. This literature review will focus on a particular method of engineering fully biological blood vessels constituted of smooth muscle cells (SMC's) and collagen, as well as the efforts being made to improve the vessels' mechanical properties.

The Collagen-Based Approach

The collagen-based model for engineering blood vessel substitutes is comprised of vascular SMC's embedded in a collagen gel matrix. This model roughly mimics the structure of the media, or middle layer, of native blood vessels. The primary advantage of the collagen-based approach is that the biological nature of the collagen matrix allows the cells to remodel the construct during culture time and promote functional vasoactivity demonstrated by a normal blood vessel. In addition, the interior surface of these vessels can presumably be lined with endothelial cells, like native vessels, so that they will not be thrombogenic. The major issue that arises with this model, however, is that collagen gels have an inherent physical weakness that can limit their ability to withstand *in vivo* blood pressure. Although a synthetic reinforcement can be used to increase the engineered vessel's strength, this additional structural component places limits on the construct's ability to undergo biological adaptation. As a result, research focuses on finding ways to

improve the mechanical strength of TEBV's made with the collagen-based model without the use of a synthetic support.[1]

Mechanical and Biochemical Stimulation

Dynamic mechanical conditioning of the collagen/SMC vessels has shown promise as a technique for improving the physical strength of the vessels. In a study by Seliktar et al, the constructs were cultured around a silicon sleeve and subjected to circumferential strain that cycled between 0% and 10% at a frequency of 1 Hz. In other words, the interior of the sleeve was pressurized and depressurized such that the diameter of the construct would vary between its original value and 10% greater. The conditioned constructs exhibited a significant increase in yield stress and ultimate stress when uniaxial mechanics were tested by cutting the vessel into rings and stretching the rings until failure.[2] In a later study by Syedain et al., the applied periodic strain was increased in four steps from 5% to 15% over a 3 week period. This method of conditioning, called incremental cyclic distension (ICD), further improved the mechanical strength of the vessels relative to vessels that cycled between the static values for the entire 3 weeks.[3] Thus, applying mechanical stress to constructs as they are cultured helps to overcome the strength limitations that currently hinder the collagen/SMC model.

Biochemical stimulation can be used to favorably affect the cell-mediated remodeling that occurs when collagen constructs are cultured. Tissue transglutaminase (TG) is an enzyme that cross-links collagen gels by catalyzing the formation of an amide bond between glutamine and lysine residues in the polypeptide chains of collagen fibers.[4] This reaction, which is dependent on the presence of Ca^{+2} ions, has been shown

by Orban et al. to significantly increase the burst pressure of collagen constructs without being toxic to the smooth muscle cells.[5] The researchers of this study, however, acknowledged that burst pressure tests are only a simple comparison of strength that do not take into account dimensions and fail to give information on axial mechanics. Also, these constructs were only cultured for 7 days, while Girton et al. found that it takes about 14 days for them to fully remodel.[6] Thus, the data from this study may not accurately represent burst pressures of the engineered vessels in their mature form.

Ascorbic acid (AA) is known to stimulate SMC's to produce more extracellular matrix, and a correlated improvement in uniaxial ultimate tensile stress (UTS) occurred when the culture media for constructs was supplemented with AA by Ogle et al.[7] Insulin similarly causes increased collagen production in vascular SMC's,[8] so it could potentially be beneficial to improving the strength of collagen TEBV's. However, the effects of insulin in tissue engineering applications have yet to be thoroughly explored.

Transforming growth factor beta (TGF-beta) causes phenotypic changes in SMC's that are not yet fully understood but can lead to favorable extracellular matrix remodeling as the cells in constructs compact the collagen gel. Consequently, blood vessel constructs cultured in media containing TGF-beta experience a higher degree of gel compaction and exhibit a histologically more dense matrix.[9] This means TGF-beta may also be useful in improving the mechanics of the collagen/SMC engineered vessels.

Summary

It has been established that certain biochemical factors can cause improvement in the mechanical integrity of TEBV's made with the collagen gel approach. There is not,

however, significant knowledge on the effects of using these previously identified treatments (TG, AA, TGF-beta, and insulin) in combination with each other. It is unknown if all of the treatments together can exhibit mechanical improvements beyond what the individual treatments accomplish, or if similarities in the mechanisms of action result in negligible benefits of adding more biochemical factors. Evidence at least exists for a synergistic relationship between TGF-beta and insulin,[10] but for several treatments the effects are unknown.

Comprehensive quantification of the favorable changes in mechanical properties is also lacking. Employing multiple testing methods will give a more complete evaluation of mechanical properties. Ring tests can be used as a relatively simple method of testing tensile properties,[11] and particle tracking on these tests are useful to analyze true local strain.[2] Since blood pressure is a load applied to vessels *in vivo*, circumferential mechanics are important factors to consider and can be assessed using pressure vs. diameter tests intended to simulate conditions within the body.

Thus, the goal of this project on collagen-based TEBV's is to research a new experimental group of biochemical treatments, consisting of TG, TGF-beta, AA, and insulin, using a more diverse mechanical testing approach that includes both uni-directional tension and internal pressure loads. Blood vessel constructs were fabricated by embedding rat aortic smooth muscle cells (RASMC's) in a type I bovine collagen gel, then allowing the constructs to compact for two-weeks in culture media. For the experimental constructs, the gel contained TG and the culture media was supplemented with AA, insulin, and TGF-β. Unidirectional mechanics and tensile strength were evaluated using ring samples from the constructs, and internal pressures were applied to

whole constructs for burst pressure, creep, and fatigue experiments. The data for experimental and control constructs were compared to determine the changes caused by the biochemical treatments of interest.

MATERIALS AND METHODS

Cell Culture

Rat aortic smooth muscle cells (RASMC) were cultured in a monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin, and 10% fetal bovine serum (FBS) at standard cell culture conditions (37° C, 5% CO_2). Detachment and seeding of the cells was accomplished using phosphate-buffered saline (PBS) and 0.05% trypsin.

Collagen Construct Preparation

Bovine collagen type I was dissolved to a concentration of 4 mg/ml in 0.02 N acetic acid. The construct mixture was prepared by first mixing 5X DMEM and 0.1 M NaOH such that the complete mixture would had a 1:4 volume ratio of 5X DMEM: collagen solution, and 1:6 ratio of NaOH: collagen solution. The collagen solution was then added to the DMEM-NaOH mixture so that the final concentration of collagen would be 2 mg/ml. Lastly, a cell suspension in 1X DMEM was added to make the cell concentration 10^6 cells/ml in the complete mixture.

For constructs treated with TG (experimental constructs), a 5000:1 weight ratio of collagen:TG was used in the complete construct mixture, since this has been found to be the optimal concentration.[5] Also in accordance with the Orban study, the construct mixture contained 2.5 mM $CaCl_2$ and 1 mM dithiothreitol (DTT).

The complete construct mixture was pipetted into test tubes at 5 to 7 ml per tube, then a glass mandrel was inserted into each tube. Gels were allowed to solidify about the

central mandrel in the cell culture incubator for approximately 45 minutes, after which they were removed and placed in the DMEM solution previously described (see Figure 1). The culture media for the experimental constructs contained 2 ug/ml of insulin, 1 ng/ml of TGF-beta, and 50 ug/ml of Ascorbic Acid in accordance with the studies listed in the background section. The gels were cut away from the rubber ends of the central mandrel so that they could compact freely. Vessels were cultured in the conditions previously described with media being changed weekly and the experimental media being re-supplemented three times weekly to account for biochemical degradation (see Figure 2).

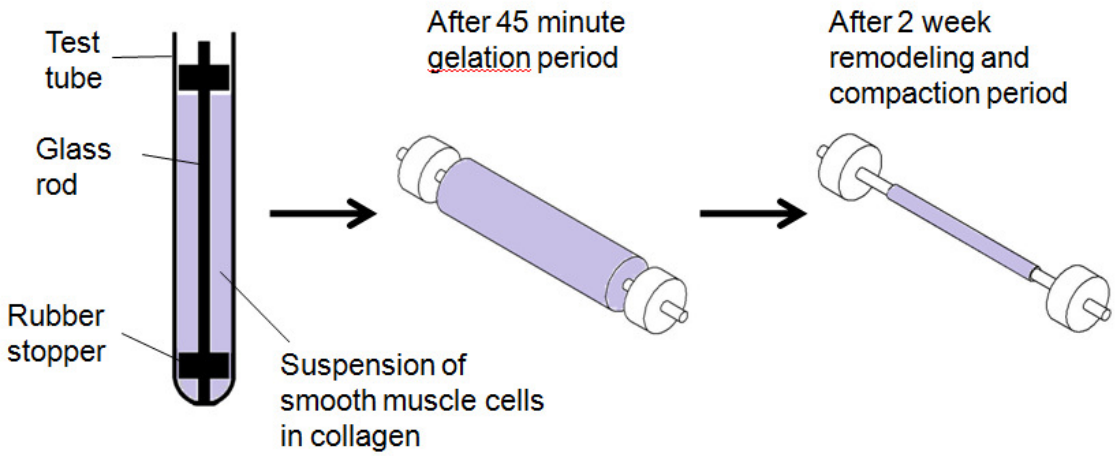
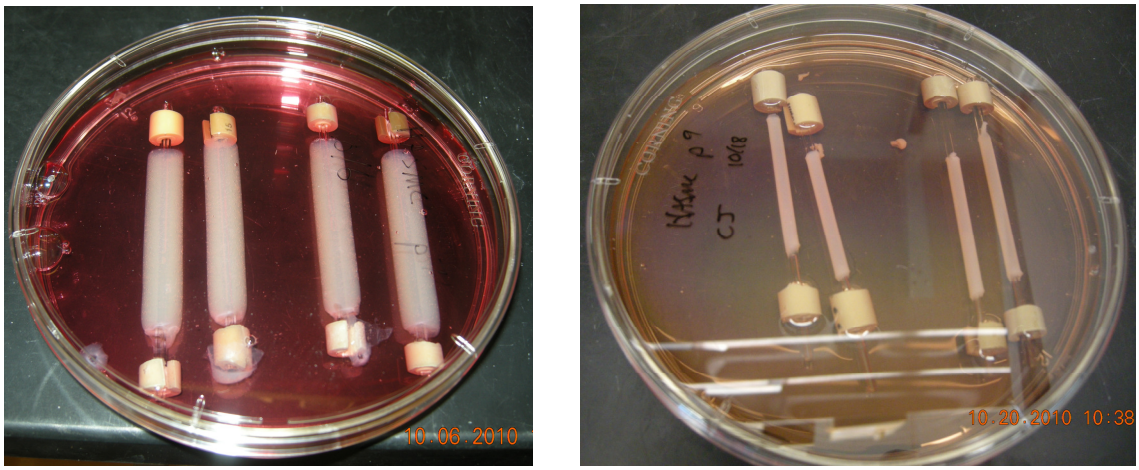


Figure 1: Overview of vessel preparation



A **B**

Figure 2: Images of constructs before (A) and after (B) the two week remodeling period

Ring Tests

Constructs were tested 14 days after being made since research has found that no significant gel compaction or remodeling occurs after this time.[6] Rings with a length of approximately 4 mm were cut from the construct and given a light dusting of black carbon powder so that particle tracking could eventually be done on pictures taken during the tests.

The ring tests were conducted in PBS using the testing apparatus in Dr. Nerem's lab. The testing device recorded the load and displacement of the two hooks on which the rings are pulled (see Figures 3 and 4). 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 either 0.1 mm/s or 1 mm/s. Two cameras record images during the tests: one from the front of the ring and another from the side. Nominal stress and strain measurements for the construct rings were calculated using the load and extension

measurements taken by the testing equipment. The geometric dimensions of the rings in the unloaded state were found by referencing the length and thickness of the rings in the camera images with the testing hooks (which have a known diameter). Average ultimate tensile strength (UTS), which is the maximum stress sustained before tearing, was calculated for each treatment group and displacement rate.

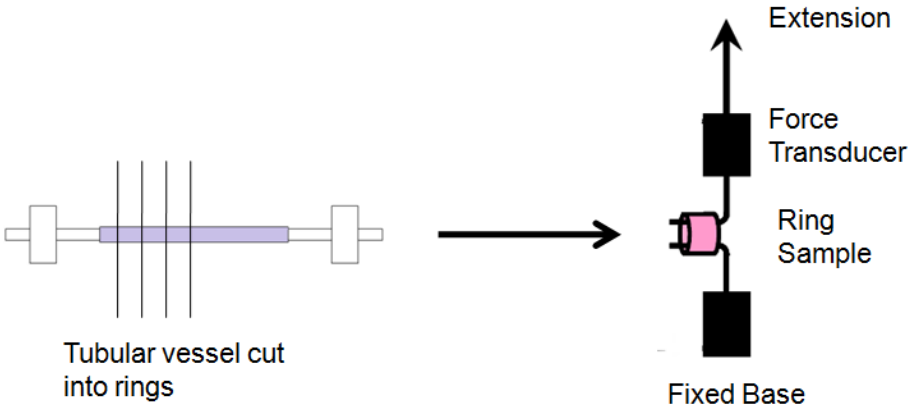


Figure 3: Schematic of ring tests

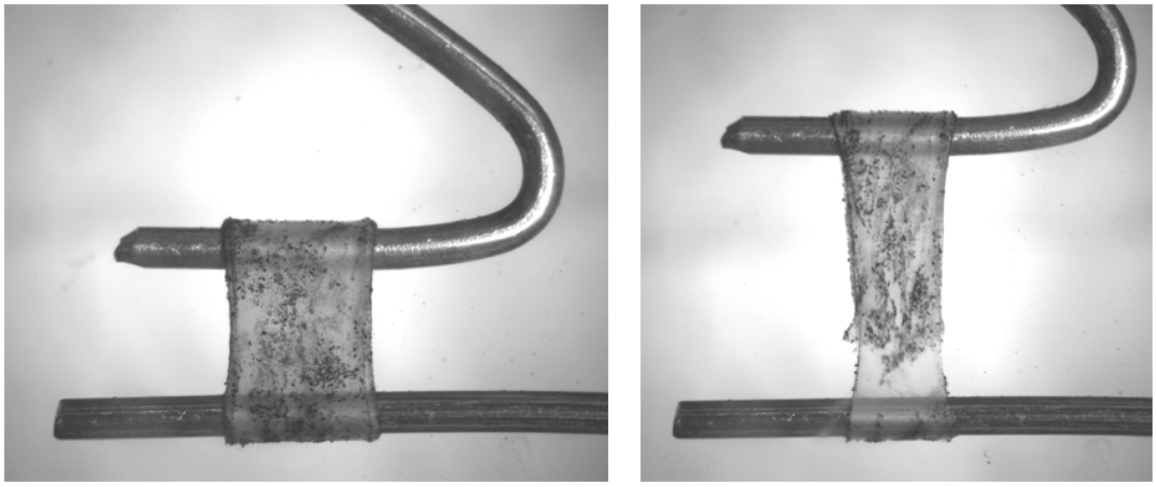


Figure 4: Picture of ring sample being tested

Pressure-Diameter Tests

For pressure testing, each end of the whole construct was slid onto a metal tube and secured using suture thread (see Figure 5). The testing set-up was a fluid "circuit" of PBS consisting of the following items in a loop: (1) a partially filled, stopper-sealed Erlenmeyer flask with several leads for tube connections, (2) a pressure transducer, (3) the construct in a bath of PBS, and (4) a second pressure transducer. The fluid pressure in the system was controlled by clamping shut one tube leading to the flask, then modifying the pressure in the air space above the fluid in the flask. The average of the two pressure readings was taken to be the pressure inside the construct. Burst pressure was measured by increasing the internal pressure at a constant rate, then recording the maximum pressure sustained before rupture.

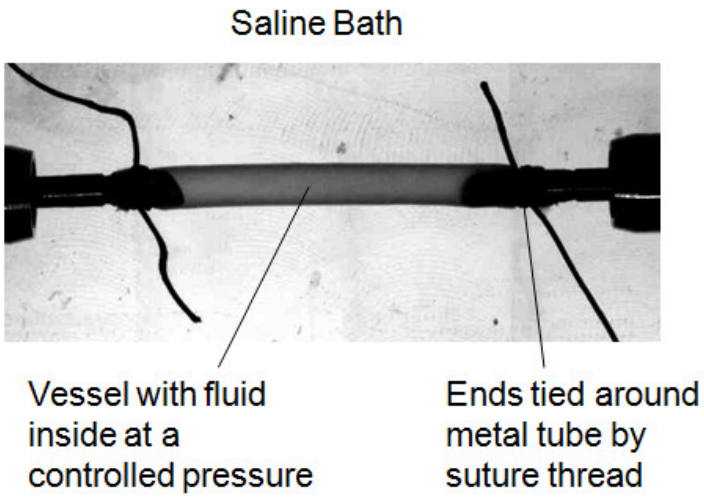


Figure 5: Image of vessel undergoing a burst pressure experiment

RESULTS

Ring tests and burst pressure tests were used to quantify the mechanical properties of the biochemically treated vessels as compared to the control vessels.

Ring Tests

The results from ring tests are displayed in Figure 6. The control group consisted of engineered vessels made according to the standard protocol, while the treated group consisted of constructs that were treated with transglutaminase and cultured in media containing ascorbic acid, TGF-beta, and insulin. The number at the top of the graph is the rate at which the rings were stretched until failure, either 0.1 mm/s or 1 mm/s. Each of the four bars represents a sample size of N=6, and results are plotted as mean ± standard error. At an extension rate of 0.1 mm/s, treated constructs had an average ultimate tensile strength (UTS) of 107 ± 4 kPa while control constructs had an average UTS of 60 ± 3 kPa. At an extension rate of 1 mm/s, treated constructs had an average UTS of 133 ± 7 kPa while control constructs had an average UTS of 70 ± 4 kPa. For both extension rates, the difference between treated and control constructs was statistically significant ($p = 1.84e-6$ for 0.1 mm/s and $p = 3.94e-5$ for 1 mm/s).

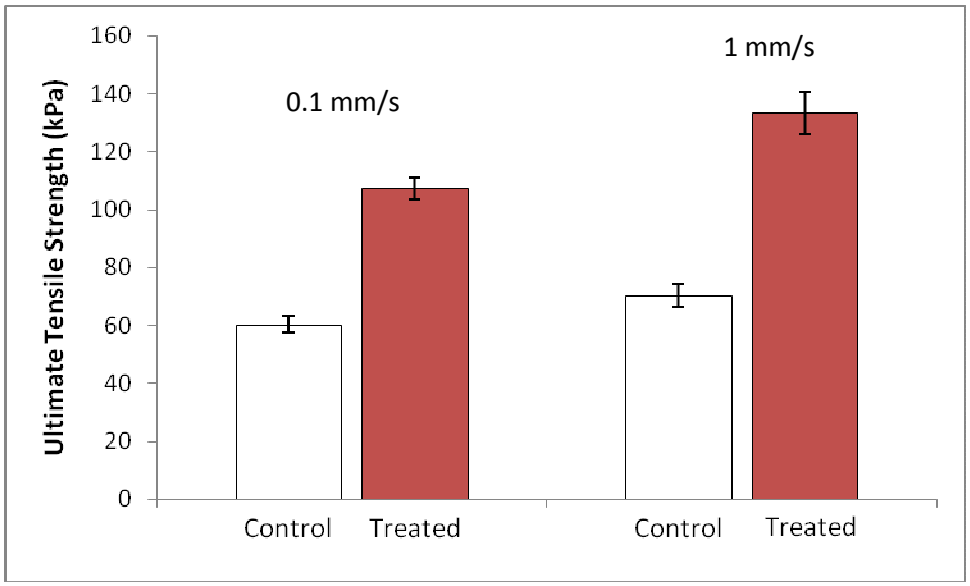


Figure 6: Ultimate Tensile Strength of Ring Samples of Control vs. Treated Constructs

Burst Pressure Tests

The results from the burst pressure tests are displayed in Figure 7. The control and treated groups had the same conditions as described in the previous section. The sample size for the control group was N = 8, while the sample size for the treated group was N = 5. Treated constructs had an average burst pressure of 211 ± 10 mmHg while control constructs had an average burst pressure of 144 ± 6 mmHg. This difference was statistically significant ($p = 4.46e\text{-}4$).

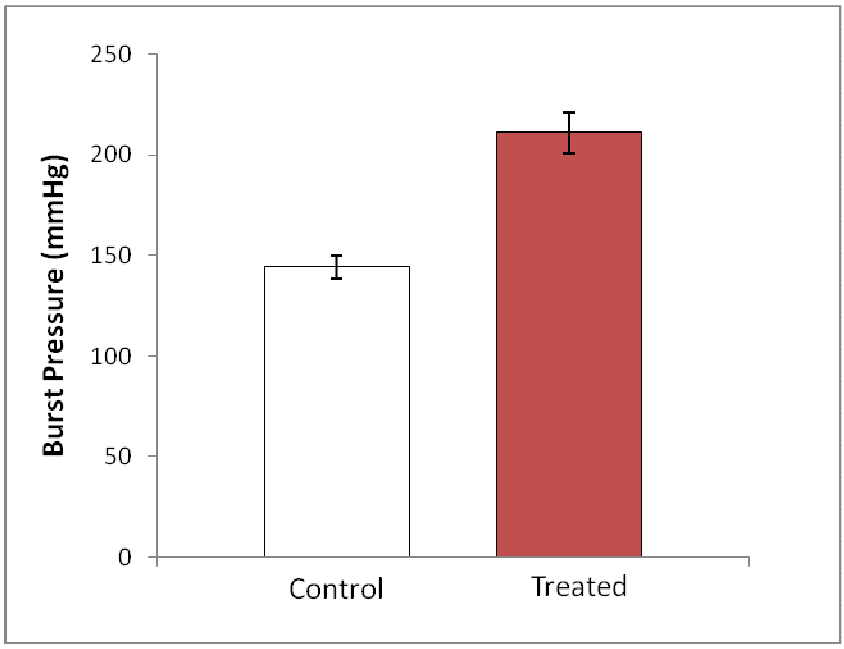


Figure 7: Average Burst Pressure of Control vs. Treated Constructs

DISCUSSION

The results indicate that treatment with TG, AA, insulin, and TGF-beta significantly improves the mechanical properties of engineered blood vessel constructs consisting of SMC's in collagen. Compared to control vessels, the treated vessels were able to withstand greater applied loads before rupturing during both tensile and pressure testing.

Compared to constructs in other studies treated only with TG and cultured for 7 days, the vessels in this experiment were substantially stronger based on the results of burst pressure experiments. Orban et al. reported that constructs treated with TG had an average burst pressure of 71 ± 4 mmHg after 7 days.[5] In comparison, vessels in this experiment that were treated with TG in addition to AA, TGF-beta, and insulin had an average burst pressure of 211 ± 10 mmHg after 14 days. Thus, the additional culture time combined with the three additional biochemical treatments results in further improvements in mechanical strength.

The primary take-away from this project is that biochemical stimulation of collagen-based blood vessel constructs seeded with smooth muscle cells produces favorable changes in the mechanical properties of the tissue. The strength of vessels that are treated with the four factors of interest in this study, however, remains insufficient for these vessels to be used as tissue substitutes for applications such as bypass surgery. The human saphenous vein, which is typically used as the substitute vessel during bypass surgery, has a burst pressure that is roughly one order of magnitude greater, in the range of 1,680 to 2,273 mmHg.[12] Additional strengthening techniques such as mechanical

conditioning are required if the collagen/SMC engineered vessels will ever be feasible as replacement grafts, but biochemical stimulation is a technique that can help meet the requirements for mechanical strength. Once these vessels can be engineered to have the necessary mechanical integrity, researchers will be one step closer to manufacturing blood vessel substitutes that eliminate the need for grafting native tissue during bypass surgery.

FUTURE RECOMMENDATIONS

Characterization of biological and/or structural changes caused by these factors will lead to a better understanding of the mechanisms by which the strength has been improved. Collagen content of the vessels may be increased due to ascorbic acid and insulin stimulating the SMC's to produce more ECM. The extent of crosslinking should also be greater in constructs that have been treated with TG. TGF-beta has previously been shown to lead to greater compaction of the vessels, so the structure of the treated vessels will presumably be denser than that of untreated vessels. These hypotheses should be tested with the appropriate biological assays to confirm that the chemicals are inducing the expected changes.

Increasing the number of experimental groups will reveal more information about which of the biochemical treatments are the most beneficial to mechanical strength. This experiment only used a control group with none of the treatments and an experimental group with all four of the treatments, under the expectation that future work will test mechanical properties when only one, two, or three of the treatments are used. At the very least, repeating these methods on a group with just TG and a group with just the soluble factors in the culture media will give insight to their relative contributions. Ideally, there would be 16 (2^4) experimental groups so that all possible combinations of treatments are used. Data can then be analyzed with ANOVA to see the effects of each treatment so that researchers will know which factors are the most promising.

A wider variety of pressure tests should also be conducted to more comprehensively analyze vessel mechanics. The burst pressure experiments in this study,

which increased pressure until vessel failure, were a relatively simple way to assess strength. Vessels *in vivo*, however, experience pressures that oscillate between systolic and diastolic blood pressures. Thus, a more appropriate mechanical test would cycle the internal pressure between an upper and lower value and analyze the deformation of the engineered vessels compared to native vessels.

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