Improving Cell Seeding of Decellularized and Porated Porcine Arterial Scaffolds

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IMPROVING CELL SEEDING OF DECELLULARIZED AND PORATED PORCINE ARTERIAL SCAFFOLDS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Harrison Smallwood
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Accepted by:
Dr. Dan Simionescu, Committee Chair
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Dr. Christopher Wright
ABSTRACT

Coronary artery disease is one of the number one killers in the U.S. Current treatment of using CABG surgery has limitations from availability of autologous grafts, and low patency of artificial grafts. Tissue engineered grafts would ideally provide more availability, while also replicating the mechanical properties of the native grafts. Current issues in tissue engineering arteries lie in using an effective scaffold to get correct mechanical properties, and allowing for cell infiltration to assist in integrating with native tissue once implanted. This study shows the use of a decellularized and chemically and mechanically porated porcine carotid artery in mechanical characterization and cell seeding experiments. The mechanical properties all exhibit values like that of current autologous grafts. The cell seeding studies show data determining the viability of these porated scaffolds in cell infiltration. It also examines several seeding techniques and how they impact the location of cells. This study concludes that the decellularization and poration procedures result in a scaffold with suitable mechanical properties for CABG grafts. It also concludes that several different cells seeding methods can be effective in getting cells onto the scaffold, but some work remains on developing an ideal tissue.
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DEDICATION

I would like to dedicate this to my grandparents, Ronald, and Claire Taylor, for always pushing me to do more and always further my education.
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INTRODUCTION

Anatomy of the Heart

The heart is responsible for pumping blood to the lungs for oxygenation, then to the rest of the body to provide nutrients and oxygen to tissue. The heart has 4 chambers, left and right atrium, and left and right ventricle. Blood flows into the heart through the vena cava then into right atrium then right ventricle into pulmonary artery to lungs. Then it goes into pulmonary vein left atrium then left ventricle. The heart controls the blood flow into its chambers and surrounding vessels using 4 valves; pulmonary, mitral, aortic, and tricuspid. (National Heart, Lung, and Blood Institute, 2015)

Figure 1: A) location and orientation of heart in average person. B) Cross section of heart, identifying major features of the heart, and displaying direction of blood flow. (National Heart, Lung, and Blood Institute, 2015)
The left ventricle is thicker and pumps harder so it can deliver blood to the rest of the body. Immediately after the blood leaves the left ventricle it enters the aorta, which has 2 small branches immediately after the valve that go to the coronary arteries, which are responsible for vascularizing the outer layers of the heart.

Stats on CAD and Heart Disease

Heart disease is an issue of paramount importance in the US. It’s the then number one killer of both men and women, causing over half of male deaths in 2009. 610,000 Americans die every year from heart disease. (Centers for Disease Control, 2015) The costs of heart disease, both direct and indirect, is around $199.6 billion a year. (American Heart Association, 2017) High blood pressure, high LDL cholesterol and smoking are 3 of the biggest risk factors for heart disease, and almost half of Americans have at least one of these factors (Fryar, Chen, & Li, 2012). Heart disease mainly affects older patients, but as many as 4-10% of heart attacks occur before the age of 45. (Harvard Medical School, 2009)

What is CAD?

Coronary Artery Disease (CAD) is the most prevalent type of heart disease, responsible for the deaths of 370,000 people annually. (Centers for Disease Control, 2015) CAD is caused by buildup of plaque on the inner walls of the coronary artery, which provides blood flow and oxygen to the heart itself. This is known as atherosclerosis. The plaque buildup effectively narrows the artery restricting the blood
flow, leading to the patient suffering ischemia or heart attack without treatment. With
time CAD can weaken the muscles of the heart leading to heart failure or arrhythmias.
(National Heart, Lung, and Blood Institute, 2016)

Progression

The coronary artery has 3 layers: the tunica intima composed of endothelial cells and (in contrast to many other mammals) a thin layer of smooth muscle cells. The tunica media is composed of smooth muscle cells in a complex extracellular matrix containing Elastin, the tunica adventitia is composed of fibroblasts and collagen and elastin (Nemeno-Guanzon, et al., 2012). Atherogenesis begins initially with leukocytes binding to the endothelial monolayer then migrating inward. The monocytes begin to mature into macrophages, and then through uptake of lipids, become foam cells. (Hansson,
Figure 2: progression of atherosclerosis over a patient's lifetime. (Della Rocca & Pepine, 2010)

Intimal migration of medial SMCs occurs, coupled with increased proliferation of medial and intimal SMCs inside the intimal layer. There is also more production of collagen, elastin, and proteoglycans. SMCs and macrophages can die in this environment, and often their contents end up trapped in what is known as the necrotic core. All of this leads to narrowing of the artery and eventual occlusion through thrombosis, when the fibrous cap becomes loose and is released into the lumen. (Libby, Ridker, & Hansson, 2011)
Current treatments

The two main treatments for CAD consist of percutaneous coronary intervention (PCI), and coronary artery bypass graft surgery (CABG). PCI is a noninvasive surgery that places a stent where the blockage in the artery is, opening the lumen of the coronary, and allowing free flow of blood. CABG is an invasive open-heart surgery where a using a graft (using an autologous artery/vein from elsewhere in body, or from a cadaver, or synthetic or tissue engineered vessels.) the blocked portion of the artery is completely bypassed and blood flow is diverted through the new vessels instead. (National Heart, Lung, and Blood Institute, 2012)

Figure 3: A) Shows the location of the heart B) Shows location of bypass grafts after CABG surgery (National Heart, Lung, and Blood Institute, 2012)
CABG has increased reduction in myocardial infarctions, and is considered a better option for multi-vessel CAD. For patients, unable to withstand the trauma of open-heart surgery, CABG may not be a viable option. Minimally invasive CABG surgery is possible and has been in use since late 90’s early 2000’s, but requires a complex system for the operations that many surgeons aren’t trained on, and requires a very long learning curve. (Diodato & Chedrawy, 2014) There is also an issue in suitable grafts for CABG surgery. The most commonly grafted blood vessels are the internal mammary artery and the saphenous vein, but who experience atherosclerosis in their coronary artery commonly have it developing in other blood vessels that would otherwise be suitable grafts. Patients who must undergo multiple bypass surgeries are at even greater disadvantage because they may have suitable autologous grafts for one or two grafts but not the rest. (Pashneh-Tala, Macneil, & Claeyssens, 2016) Synthetic scaffolds have proven to be viable alternatives for large and medium diameter arteries, but suffer from poor patency in small diameter arteries. (Pashneh-Tala, Macneil, & Claeyssens, 2016). Much of the poor patency may result from mismatched compliance of the graft and native tissue, lack of or damaged endothelial cells, trauma from surgery, and hemodynamic factors. (Pashneh-Tala, Macneil, & Claeyssens, 2016)
Tissue Engineering Paradigm

The tissue engineering paradigm consists of taking a patient’s cells, isolating the desired cell type(s), cell proliferation and then seeding on some type of scaffold then implantation into the body. Scaffolds can be artificial, allogeneic, or xenogeneic, but are usually chosen to be biodegradable, biocompatible, and having similar mechanical properties as the tissue that they replace. There is evidence of graft mechanical properties being modified sometime after implantation due to effects of vascular integration. (Pashneh-Tala, Macneil, & Claeyssens, 2016)

The methods by which grafts integrate into an animal or human host isn’t obvious. Neutrophils and monocytes from the host are suspected to be involved, but it’s not clear how, and to what degree a graft preseeded with cells would interact with
them and affect this process. There are many unanswered questions when it comes to tissue engineering, the best cell types, scaffold materials, scaffold structure, and cell integration methods are still being determined. Proper graft hemodynamics may have an effect on vascular integration and calcification rates, and can't be ignored. As tissue engineering continues to develop, more clinical use and improved understanding of what’s needed for an effective graft will lead to commercially viable grafts. (Pashneh-Tala, Macneil, & Claeysens, 2016)

Scaffolds

Scaffolds can be made from a variety of materials (PLA, PGA, collagen, silk, chitosan, among many others) in a variety of manners (electrospinning, 3d printing, gels, decellularized xenogeneic tissue) (Nemeno-Guanzon, et al., 2012). Scaffolds ideally should have similar compliance and burst pressure as a coronary artery, or saphenous vein or internal mammary artery (the most common autologous grafts). There has been incredible variety in the materials proposed for scaffolds, the cells used to seed them, and the ways that the tests were quantified making. Some have favored an approach using no scaffolds, instead relying on the extracellular matrix the cells are capable of naturally producing. (Nemeno-Guanzon, et al., 2012) The architecture of the scaffold should have a porated structure to allow for cell infiltration, and for adequate nutrient diffusion to the cells that migrate inward. Scaffolds must also be biocompatible, as any material that would cause rejection is unacceptable. For scaffolds to be commercially
viable and have the most impact in the treatment of medicine, they need to be able to be scaled up to mass production. (O'Brien, 2011)

Stem Cells

One of stem cells most popular uses are in the fields of regenerative medicine and tissue engineering. Stem cells are unspecialized cells have the unique ability to develop and change into a wide variety of cell types. There are 3 existing types of stem cells, Adult Stem Cells (ASC), embryonic Stem cells (ESC), and induced pluripotent stem cells (iPSC). ASCs are present throughout the body in very small numbers, but are most easily obtained through bone marrow or adipose tissue. The cells can differentiate into the cell types of the tissue they reside in. (i.e. hematopoietic stem cells won’t develop into neurons, but can differentiate into the various types of blood cells). iPSCs are genetically modified stem cells, created by altering the gene expression of normal cells, reverting them back to a stem cell, capable of differentiation into cell types of all 3 germ layers. All types have some pros and cons associated with them: ESCs have numerous ethical issues associated with them, and aren’t guaranteed to be a perfect immune match with the patient, requiring the patient to take immunosuppressants. ASCs can be more difficult to both retrieve and proliferate to number large enough for seeding applications. iPSCs still have little clinical data, and may potentially be teratocarcinogenic, causing tumors. (Riha, Lin, Lumsden, Yao, & Chen, 2005)
3 Phases of Graft Failure.

There are 3 distinct phases of graft failure; Early stage failure occurs within 30 days of surgery and is usually related to surgical complications, acute thrombosis, or flow disturbances. Midterm failure is 3 months to 2 years, caused by neointimal hyperplasia, obstructing the lumen, and late term failure is more than 2 years and caused by atherosclerotic degradation. (Catto, Fare, Freddi, & Tanzi, 2014)
AIM 1: MECHANICAL PROPERTIES OF PORATED ARTERIES

Methods

Tissue Preparation

Arteries are obtained from Animal Tech (Texas), and shipped on ice. The arteries are porcine carotid arteries that were obtained from pigs slaughtered the previous day. The arteries are placed in saline solution on ice. Using hands and scissors, the arteries had all extraneous fat or branches trimmed off, taking care not to inadvertently puncture or damage the artery. Arteries had all blood or blood clots removed, until tissue no longer appeared red. Samples were set aside and frozen at -80 °C for DNA extraction with other samples being placed in 10% formalin for histology. Arteries were mounted with barbed Luer adaptors on each side, using zip ties to secure. Using a syringe with a Luer connection push saline through the artery to clean and locate any branches or holes are. All identified openings were sutured shut, and the arteries were re-infused with saline to verify that they were all shut. Arteries were put into 30 mM EDTA, 0.02% Sodium Azide with pH of 7 (overnight solution) overnight in refrigerator.
Decellularization

The decellularization circuit was assembled as described in (Fercana, et al., 2014) creating two parallel circuits with a volume of ~1.4 L. Appropriately sized Luer barbed adaptors were used to equip each circuit with 6 arteries. The system was then filled
with overnight solution. And run with pressure (~50 mmHg) then rinsed using ddi water procedure (Rinse using ~5 L of ddi water without pressure, then 2.5 L with pressure).

Decellularization began 1% SDS run with pressure for 6 days, then drained and replaced with fresh 1% SDS solution for another 6 days. Another ddi water rinse protocol was conducted, followed by 3 separate 1 L infusions of 70% ethanol into each separate circuit. Each was spaced an hour apart. Then one more ddi water rinse performed, while using ddi water as a 30 minute with pressure. Ddi water was drained, and 0.1M NaOH solution was introduced and run for 2 Hours, then followed by another ddi water rinse cycle. Following the rinse cycle was introduction of 1x PBS which was run with pressure overnight. Next system was drained and then filled with DNAse/RNase solution (720 mUnits/mL DNAse, 720 mUnits/mL RNAse, 5mM MgCl, 1% antibiotic/antimycotic in 1x PBS adjusted to pH 7.5) Was run with DNAse/RNase solution for 96 hours. Next 1x PBS was introduced and run with pressure for 1 hour. To conclude decellularization process, conduct 3 separate ddi water rinse protocols with system running for ~20 minutes in between rinse steps. If any decellularized scaffolds were needed for histology/DNA analysis they were removed here, and replaced with tubing.
DNA Extraction

When artery samples were taken, small sections were placed in microcentrifuge tubes and stored at -80 °C until electrophoresis is performed. Electrophoresis was performed using fresh and decellularized (not acetic acid treated) samples. The samples were weighed to ensure they were less than 25 mg, and the values were recorded for quantification during the nanodrop. The <25 mg sample was placed into 1.5 mL microcentrifuge tube, and filled with 180 µL Buffer ATL and 20 µL of proteinase K. Incubate at 56 °C until tissue is completely lysed. Once lysed vortex for 15 seconds, then add 200 µL Buffer AL and mix by vortexing. Next 200 µL of ethanol was added then mixed by vortexing. The mixture was pipetted into a DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at 8000 rpm for 1 min. flow through was discarded along with collection tubes. Spin column placed into new 2 mL collection tube, and 500 µL buffer AW1. The tube was then centrifuged at 1 min at 8000 rpm. Flow through and collection tubes was discarded. It was then placed in a new collection tube with 500 µL of Buffer AW2 and centrifuged for 4 minutes at 13.2 rpm. It was then transferred to a new collection tube. DNA was eluted using 200 µL Buffer AE incubate for 1 min at room temperature. Then centrifuge for 1 minutes at 8000 rpm. Repeat step with buffer AE for increased DNA yield.

Nanodrop

Using the extracted DNA, a quantitative analysis was performed using Nanodrop 2000 Spectrophotometer (Thermo Scientific). First it was cleaned with DNA/RNA free
water, then buffer AE was ran as a control, followed by each sample of DNA. The stand was cleaned with DNA/RNA free water in between each measurement. Using the data, the amount of DNA was calculated and used with the weight of the tissue to determine DNA concentration in ng DNA/mg tissue.

Decellularization Results

**DNA Concentration of Decellularized and Fresh Tissue**

![Graph showing DNA concentration comparison between Decell and Fresh tissues](image)

*Figure 7: Concentration of DNA in tissue as determined by spectrophotometer*
Figure 8: Image of gel electrophoresis showing the size of DNA present in fresh and decellularized samples
Gel Electrophoresis

10x TBE buffer was prepared with 21.8 g TRIS, 11.1 g boric acid, 0.93 g EDTA, pH of 8.3 and brought to 200 mL with ddi water. Electrode buffer was prepared with 75 mL of 10x TBE buffer and 1425 mL of distilled deionized (ddi) water. Agarose gel was prepared using 100 mL 0.5x TBE and 1 g of agarose.

Agarose gel was placed in microwave for 1.5 minutes stopping every 30 second to stir. Allow gel to cool to 60 °C then add 5 µL of ethidium bromide, then pour gel into mold and place comb.
Prepare samples and standards by adding 8 µL of fluorescent dye into 32 µL of each sample. For the DNA ladder, use five parts DNA ladder and one-part dye. Load 20 µL of each sample into wells, then run at 100 V for 1 hour. Then image the gel using ChemiDoc XRS+ molecular imager with image lab software (Bio-Rad, Berkeley, Ca)

Embedding

Arteries were stored in 10% buffered formalin for 24+ hours. Sections of arteries were run in a tissue processor overnight, and embedded in paraffin wax. Then embed the tissue section in a block of wax in such a position that each section drawn from the block shows a cross section of the artery. Sections are then cut at 10 microns using a microtome, and placed onto the slides and then into an oven. Afterwards slides are stored until histology.

Histology

Sections were deparaffinized with xylene and ethanol then stained with Hematoxylin and Eosin (H&E) then dehydrated using ethanol and xylene, then equipped with a coverslip and imaged at 10x and 2.5x magnification. The same was done using Masson’s Trichrome stain to identify collagen, elastin, and glycosaminoglycans. 4',6-diamidino-2-phenylindole (DAPI) was used as a fluorescent stain to identify nuclei. Imaging was done with Axiovert 40 CFL microscope equipped with Axiocam mRC and Axiovision image software (Carl Zeiss)
Poration Methods

To begin the chemical poration, 0.2 M acetic acid was added to system and run with pressure for 1 hour. Then system was drained and arteries were removed and placed into 50 mL conical tubes which were then placed into -80 °C. The 50 mL conical tubes were then placed into lyophilization flask lyophilized for 72 hours. Following lyophilization scaffolds were removed and placed into 5% CO2 incubator for 6 hours for partial rehydration. Scaffolds were then set onto cutting mat and rolled along the main axis 10x on each side using 1 mm derma-roller. Scaffolds were then set into container with ddi water and allowed to rehydrate overnight.

Samples were then set into sterile 0.1% v/v Peracetic acid solution in 1x PBS. Containers were covered in foil and set onto shaker plate for 4 hours. Following sterilization, arteries were placed into sterile ddi water containing 1% antibiotic/antimycotic and stored until used in refrigerator at 4 °C.
Poration results

Figure 10: Average pore size of fresh, 1 hour, and 3-hour acetic acid treated. Dry is tissue dried through lyophilization, and wet refers to tissue rehydrated after lyophilization.
Connect the arteries were equipped with Luers and zip ties to secure them and connected to pump and reservoir to achieve pressures of 80 mmHg and 120 mmHg. Arteries were set into Pyrex container and on countertop system was adjusted to 80 mmHg, the Pyrex dish was lowered to achieve a pressure of 120 mmHg. Pressure was measured using PDAQview software and 50 psi transducer. A ruler was placed on the Pyrex dish, a picture was taken containing both ruler and artery at 80 mmHg and 120 mmHg. After testing a small section of the artery was cut, and a picture was taken with it and the ruler. Using ImageJ and the ruler as a reference, Outer diameter at 80 and 120 mmHg were calculated, along with wall thickness and resting Internal diameter, these values were then used to calculate the compliance for each arterial sample.
Burst Pressure Methods

The same experimental set up was used as when compliance was done, just using a 100 PSI transducer instead of 50 PSI. The arteries were equipped with burst pressure Luer adaptors and zip tied and ligated using 3.0 sutures. The system was pressurized to the highest rpm the peristaltic pump could obtain. Pump was run for 1 minute then pressure recording was stopped. Burst pressure was obtained by finding the max pressure in the samples.

Result

Figure 12: burst pressure values for saphenous vein, internal mammary artery, fresh porcine carotid, decellularized, and 1 and 3-hour acetic acid treated. (Pashneh-Tala, Macneil, & Claeysens, 2016) (Roy, Silacci, & Stergiopulos, 2005)
Discussion

As shown in Figure 8, you can see the substantial number of cells present in fresh tissue, and the complete lack of those cells in the decellularized images. The amount of DNA present, as determined by spectrophotometer, in the scaffolds is around 50 ng DNA/mg tissue which is considered sufficiently decellularized in literature (Crapo, Gilbert, & Badylak, 2011). The gel electrophoresis clearly depicts that the fresh samples had large DNA fragments, which is reasonable considering nothing had been done to the fresh samples to damage or breakdown the DNA. The decellularized lanes displayed no indicators of any DNA presence. The H&E and the DAPI stains on the...
decellularized scaffolds showed no cells present anywhere in the scaffold. This leads to the conclusion that the decellularization procedure is effective in removing all the cells from the scaffold.

The histology in figure 11 the increase in opens pores for cells to potentially migrate into is apparent. The chart in figure 10 displays a quantitative visual analysis showing the substantial increase in porosity from the acetic acid treatment.

The burst pressure of the decellularized and acetic acid treated scaffolds is much lower than the burst pressure of native arteries. This doesn’t preclude them from being suitable grafts however, because their burst pressures are still many times higher than the systolic pressure of even a hypertensive patient (>160 mmHg) (American Heart Association, 2016).
AIM 2: CELL SEEDING STUDIES

Methods

Cells

Rat adipose derived stem cells were thawed and began culture in T75 cell culture flask, at passage 20, cells were fed media consisting of Dulbecco’s Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS), and 1% antibiotic/antimycotic and expanded to a total of 12 million cells.

1st Cell Seeding

Luer barbed adaptors, forceps, were sterilized the day before. Using the sterilized arteries from in 1x PBS from earlier, Arteries were placed in 0.2 M acetic acid again for 2 hours, then lyophilize the arteries again sterile. Scaffolds were equipped with a Luer adaptor on each end, and 6 arteries were injected with 1 mL of 2.02 million cells per ml. Arteries were placed in T175 flasks filled halfway with media, and left into incubator for 2 days.

DAPI & L/D

Two arteries used for DAPI and Live Dead staining. The DAPI was composed of 3 mL DPBS and 3 µL of DAPI stain. The Live Dead stain was composed of 3 mL DPBS, 1.5 µL calcein AM, and 6 µL ethidium homodimer-1. Sections of arteries were immersed in the stains for 30 minutes, and were insulated from light using aluminum foil. Images taken
using Axiovert 40 CFL microscope equipped with Axiocam mRC and Axiovision image software (Carl Zeiss)

Results

Figure 14: H&E and DAPI images of fresh decellularized and cell seeded tissue

2nd Cell Seeding

Arteries were decellularized and porated and sterilized as described earlier.

Again, rat ADSCs were cultured and expanded. Arteries were set into sterile acetic acid

Figure 15: Diagram depicting the difference in the groups for cell rolled versus noncell rolled samples.
for 1 hour, then placed into 50 ml conical tubes and put in -80 °C freezer overnight. They were then lyophilized steriley for 48 hours. Arteries were placed in a petri dish containing ~50 million cells suspended in 30 ml of media (DMEM, 10% FBS, 1% ab/am). They were then placed in incubator for 2 hours for the cells to settle. Arteries were then removed from incubator and rolled 10x on each side with the derma-roller. Arteries then placed into T175 flasks, filled approximately halfway, and set into incubator for 7 days. Arteries then removed and used for Live/Dead, DAPI, and histology.

2nd Seeding Results

*Figure 16: Live and Dead images at 10x and 2.5x of cell rolled and noncell rolled scaffolds. Bright green indicates live cells, and bright red indicates dead cells.*
Figure 17: DAPI images at 10x and 2.5x of cell rolled and noncell rolled scaffolds. Bright blue indicates cells.
Figure 18: Masson’s Trichrome stain of cell rolled and noncell rolled scaffolds. Red indicates elastin, blue indicates collagen, and yellow indicates GAG’s.

Figure 19: H&E stain of cell rolled and noncell rolled. Dark purple indicates nuclei of cells.
3rd Cell Seeding

Arteries were again decellularized in the same manner as described above, then treated with acetic acid and left in -80 °C overnight. Arteries then lyophilized for ~2 days. Seeding used 3 cell lines, human aortic endothelial cells (hAECs) starting from passage 1 and using endothelial growth media (EGM) (Lonza), human aortic adventitial fibroblasts (hAoAF) at passage 6 fed with media made of 1% antibiotic/antimycotic, 10% FBS, and 89% DMEM. The third cell line was human aortic smooth muscle cells (hASMC’s) started at passage 4 grown in smooth muscle growth media (SGM) (Lonza).
Arteries were set in incubator for 6 hours, then derma-rolled 10x on each side with 1 mm derma-roller, and then set into ddH2O water overnight. Arteries were then lyophilized again for 2 days.

**hASMC Seeding**

43 million cells were split into 4 petri dishes containing 3 mL of SGM for 10.7 million cells in each dish, and an average cell density of 0.357 million cells per mL media. Eleven sterilized arteries were removed and placed into petri dishes with 2 containing 3 arteries and 2 containing 2 arteries. There was a noticeable color change when the arteries were placed in the media, indicating a pH change. They were then set into incubator for 1 hour at 37 °C at 5% CO2. Arteries were placed into petri dish and derma-rolled 10x on each side with 1 mm derma-roller. Afterwards, arteries placed into T175 flask and filled with 50 mL SGM. Arteries were placed on an orbital shaker inside of an incubator for 5 days with SGM changes every other day.

**hAEC and hAoAF Seeding**

The T175 flasks were removed from incubator, and then arteries removed using sterile forceps. Both ends of the artery were equipped with Luer barbed adaptors, with one end of each being also equipped with a Luer cap. Using a 1 mL Luer lock syringe, 1 mL of media containing 10.5 million hAECs, was injected into the lumen of the artery. The syringe was removed, and each artery was carefully placed back into a new T175 flask, with care taken not to allow cell suspension to leak out into flask. Then T175s
were put back into incubator (without shaker) for 2 hours to give the HAECs time to attach. Next the T175s were removed once more to be seeded with hAoAFs. Using a 5 mL serological pipette, 1 mL of media containing 0.815 million hAoAFs was slowly dropped onto the adventitial layer of each artery. After each artery has been seeded, the flasks were put back into incubator (no shaker) overnight. The next morning 25 mL of media (1% antibiotic/antimycotic, 10% FBS, 89% DMEM) was added and shaker was turned on at a low speed. They were left in incubator for 1 week then removed and imaged with Live/Dead, DAPI, and immunofluorescence.

Immunofluorescence

Arteries were fixed in 4% paraformaldehyde or 10% neutral buffered formalin. For ~24 hours. Processed with a tissue processor, and then embedded in paraffin blocks. Blocks were sectioned and placed onto slide, with 2 sections per slide, and baked in oven overnight. Slides were deparaffinized, and then immersed in 10 mM citric acid at 90 °C for 30 minutes, then cooled to room temperature for 30 minutes, and immersed into distilled water. Sections on the slides were circumscribed with a wax pen, the wet with 1x PBS. PBS was removed and then 0.3% Triton in 1x PBS was placed for 10 minutes at room temperature. Sections were then covered in blocking solution (1 % BSA, 2% FBS, 0.05% Triton in 1x PBS, filtered) for 2 hours at room temperature. The blocking solution was removed and the primary antibody (for αSMA 0.5 µg/mL ab7817, for vimentin 5µL/mL ab92547, for CD31 5µL/mL Abbiotec 250589, all in 1:1 ratio of blocking solution and 1x PBS) was placed onto the sections and left for 2 hours. The primary antibody was
removed, slides rinsed 3 times with PBS, and the secondary antibody (Alexa Fluor 488 and 594, anti-rabbit/mouse accordingly, 4 µL/mL, in 1:1 ratio of blocking solution and 1x PBS) was placed for 2 hours in the darkness. Then the antibody was removed and the slides were cover slipped using Vectashield DAPI mounting media. Images taken using Axiovert 40 CFL microscope equipped with Axiocam mRC and Axiovision image software (Carl Zeiss).

Results

Figure 21: Immunofluorescence stain using anti αSMA as primary and red Alexa Fluor 588 as the secondary, overlaid with DAPI image
Figure 22: Immunofluorescence stain using anti vimentin as primary and green Alexa Fluor 588 as the secondary, overlaid with DAPI image
Discussion

From the first seeding, the scaffolds have a propensity for reseeding. In only two days, noticeable cell attachment has occurred. Across several parts of the artery. In all the images taken, it was expected to find some that would be indicative of damage done by the derma-roller to the artery. None of the images obtained displayed any sort of damage, which led to the idea that the rehydration was allowing the elastin to be indented, and then “bounce back” without any sort of opening generated to allow for the infiltration of cells.

This is what led to the second experiment. With the hypothesis that as cells were in suspension, and partially attached during the derma-rolling procedure, it would help provide an
avenue for the cells to infiltrate. As seen in the noncell rolled trichrome (Figure 18) and the cell rolled H&E (Figure 19), there appears to be a straight-line puncture through all three layers of the tissue. In the H&E picture, the cells are present along the crack edge. This supports the idea the previous method of rehydration may have been insufficient. The trichrome is less effective in displaying the presence of cells, but does show that the puncturing does not significantly alter the make-up of the tissue. Both cell rolled and noncell rolled proved to be successful in cell acquisition. In the future, this study could be repeated with just cell rolling and no injection to try and quantify how many cells are coming from each seeding method, how much the method is impacting the location cells attach to, and observe for a longer period. The graph in Figure 20 shows the mean and standard deviation of the cells counted from DAPI and live images. There isn’t any significant difference between the two, and both sample sets had large variance. Using a 2-tailed t-test, and n=12, the p-value was 0.899.

The third seeding incorporated hAECs, hASMCs, and hAoAFs to replicate the cells that dominate the intimal, medial, and adventitial layers respectively. By using the cell rolled method in a similar manner as was used in the previous experiment, muscle cells were expected to migrate more into the medial layers of the scaffold. This can be seen in Figure 21 which depicts the location of alpha Smooth Muscle Actin (αSMA), a common muscle cell marker. The presence of red throughout the scaffold indicates that muscle cells had the ability to migrate throughout all the layers. The vimentin stain has the fluorescence concentrated towards the adventitial layer of the artery. This is largely because that’s the location of the seeding, and the hAoAFs had less time to infiltrate into the scaffold. The CD31 stain was unsuccessful, and the imaging needs to be reattempted. Those images could be very useful since the hAECs had the highest seeding
density of any of the cell types. The live image depicts a near monolayer along the luminal surface, which would be very promising. The results of this experiment merit repeating due to the complications of the pH change during the hASMC seeding. The change is believed to be from incomplete lyophilization which would have left some residual peracetic acid present in the scaffold leftover from sterilization.

Future experiments based off these results could have a repeat of the 3rd seeding with 3 cell types, but taking one sample out for histology at various time points to observe the migration of the cells. Future studies could also employ a bioreactor to observe the effect of the pressure gradient on the recellularization, and any effects on the location of the different cell types. Another study could attempt to differentiate endothelial, smooth muscle, and fibroblast cells from adipose derived stem cells and use those in seeding the scaffold. This is more replicative of how a tissue engineered scaffold would function practically. Eventually an animal study could be done using pigs or rats to see if the seeded scaffolds can compare to just decellularized scaffolds, or other graft materials.
CONCLUSIONS

1) Decellularization and mechanical characterization
   a. The compliance is within ranges of currently acceptable grafts, and the burst pressure of the artery while lower than autologous vessels, is still many times above standard blood pressures even in a hypertensive patient
   b. The decellularization shows ~50 ng DNA/mg tissue, the gel electrophoresis displays no bands in the decellularized samples, and all histology of decellularized scaffolds lacks any evidence of cells, ensuring that the current decellularization procedure is effective at removing all cells from the artery.

2) Cell seeding #1
   a. The cells have shown that they will attach to the decellularized scaffold, but there was little evidence to show actual infiltration.

3) Cell Seeding #2
   a. There was evidence of the derma-rolling piercing through the scaffold, and that cells were present throughout the crevice created.
   b. There was no significant difference between the number of cells counted in the Cell rolled and noncell rolled scaffolds.
   c. The derma-rolling process did not significantly alter the overall structure of the artery
4) Cell Seeding #3

a. αSMA was present in all layers of the artery, including the medial layers suggesting that the hASMCs could migrate into the medial layer.

b. The vimentin stain suggested the concentration of the hAoAFs in the adventitial layer of the scaffold where they were seeded.

c. The CD31 stain was inconclusive and not displayed, another stain for the hAECs needs to be done.

d. The live images showed evidence of a monolayer along the luminal surface.
REFERENCES


