Endothelialization of the Coronary Vasculature in Decellularized Heart Scaffolds

Jessica Lan Canavan
Clemson University
ENDOTHELIALIZATION OF THE CORONARY VASCULATURE IN DECELLULARIZED HEART 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
Jessica Lan Canavan
May 2016

Accepted by:
Dr. Dan Simionescu, Committee Chair
Dr. Agneta Simionescu
Dr. Ken Webb
ABSTRACT

In the United States, 900,000 people a year experience myocardial infarction (MI), making it the leading cause of death. Current treatment options such as coronary artery bypass procedure (CABG) and percutaneous coronary intervention (PCI) minimize damage to the myocardium, but do not guarantee restoration of blood flow and are associated with other complications such as renal failure. Heart transplants remain the sole existing therapy to restore cardiac function, but are limited by donor availability and chronic immune response. Many research groups have turned to tissue engineering to provide a strategy that restores function to the heart.

Our solution is to create an acellular myocardial scaffold seeded with the patient’s own cells, which will be implanted to replace ischemic myocardium and restore function to the heart. The goal of this study is to re-endothelialize the vasculature of the left coronary artery and its surrounding branches and capillaries. This will provide the vasculature needed to sustain a tissue engineered construct. First, rabbit hearts were prepared using an SDS based perfusion decellularization protocol. The produced scaffolds were then analyzed for preservation of the vasculature pathways beginning at the left coronary artery, and for removal of cellular components and retention of ECM components. Next, human aortic endothelial cells (ECs) and human adipose derived stem cells (ADSCs) were injected into the left coronary arteries of the hearts in order to test the ability of the cells to attach to the lumen of the acellular vessel walls.

The perfusion decellularization procedure resulted in complete preservation of the vasculature pathway beginning at the left coronary artery and ending in the corresponding vein. The scaffold was proven to be acellular and to have retained basic components of collagen and elastin. Hearts seeded with either ECs or ADSCs were found to have cells adhered to the walls of the vasculature, proving that both cell types are viable options for complete re-endothelialization of coronary vasculature in decellularized rabbit heart models.

This research will add to the effort of creating an alternative therapy to heart transplants using human donors for patients suffering from post-MI complications. It will also advance the field of cardiovascular
tissue engineering, by being the first study to both utilize rabbit heart models for xenogenic recellularized constructs, and utilize stem cells as a possible cell source for re-endothelialization of the coronary vasculature.
DEDICATION

For my family who has always taught me to follow my dreams, supported my decisions, and guided me to where I am today. For my brother Bryan as well, who has had the biggest influence on my life and whom I could not imagine life without. I also dedicate this thesis to my lifelong friends, with whom I can share anything and receive compassion and understanding.
ACKNOWLEDGEMENT

I would like to thank my advisor Dr. Dan Simionescu, for accepting me into his lab and guiding me through my graduate experience. Even prior to graduate school, he was a mentor for my research and became an inspiration to me for what I could possibly achieve in my academic career. With his wisdom and encouragement, I was able to fulfill my own goals for personal and professional growth in the laboratory setting. In addition, I acknowledge Dr. Agneta Simionescu for her role in my education in the classroom and in the laboratory.

I would also like to thank my committee members: Dr. Dan Simionescu, Dr. Agneta Simionescu, and Dr. Ken Webb. This accomplishment would not have been possible without you. I would like to recognize all of the Biocompatibility and Tissue Regeneration Lab members, with a special thanks to those who contributed directly to my experience by introducing me to the laboratory, teaching me necessary skills, and consulting with me on my research. This project developed from the prior work of Dr. Jason Schulte, and I am so grateful for the opportunity he has given me and the time he has taken to educate me about his work.

Lastly, I could not have begun my research without the help of Snow Creek Meat Processing Facility and the Godley Snell Animal Facility. All of my appreciation to the Clemson Department of Bioengineering as well, that has shaped my college experience and encouraged me to be involved in research.
# TABLE OF CONTENTS

Title Page...........................................................................................................................................i

Abstract .................................................................................................................................................. ii

Dedication ............................................................................................................................................... iv

Acknowledgement ...............................................................................................................................v

List of Tables .......................................................................................................................................... ix

List of Figures ......................................................................................................................................... x

Chapter One Introduction .........................................................................................................................1

1.1 Etiology & Pathology .......................................................................................................................1

1.2 Current Post-MI Therapies .............................................................................................................4
  1.2.1 Whole Organ Transplant ........................................................................................................ 4
  1.2.2 Percutaneous Coronary Intervention (PCI) .......................................................................... 5
  1.2.3 Coronary Artery Bypass Procedure (CABG) ....................................................................... 6
  1.2.4 Left Ventricular Assist Devices (LVADs) ........................................................................... 8

1.3 Introduction to Tissue Engineering ...............................................................................................9

1.4 Introduction to Cardiovascular Tissue Engineering ..................................................................11

1.5 Overview of Current Strategies in Cardiovascular Tissue Engineering ....................................12
  1.5.1 Cellular cardiomyoplasty ...................................................................................................... 12
  1.5.2 Injection of cells using a Biomaterial Delivery System ....................................................... 12
  1.5.3 Natural and Synthetic Scaffolds ........................................................................................... 13
  1.5.4 Cell Sheet Approach (Scaffold-Free) ................................................................................ 15
  1.5.5 Stem Cell Approach ............................................................................................................. 16
  1.5.6 Decellularization & Recellularization Approach ................................................................. 17

1.6 Current Strategies for Vascularization of Tissue Engineered Constructs .................................21
  1.6.1 Cell Based Approach .......................................................................................................... 21
  1.6.2 Scaffold Based Approach .................................................................................................... 22
  1.6.3 Bioreactor Approach .......................................................................................................... 23

1.7 Strategies for Endothelialization of Decellularized Scaffolds ...............................................25
  1.7.1 Kidneys .............................................................................................................................. 26
  1.7.2 Liver .................................................................................................................................... 26
Table of Contents (Continued)

1.7.3 Lung ........................................................................................................................... 27
1.7.4 Heart ......................................................................................................................... 29

1.8 Project Scope, Aims, and Significance ......................................................................... 33
1.8.1 Project Scope ............................................................................................................. 33
1.8.2 Project Aims ............................................................................................................... 35
1.8.3 Project Significance ................................................................................................... 36

Chapter Two Decellularization of Whole Hearts ................................................................ 37
2.1 Introduction ........................................................................................................... 37
2.2 Methods ................................................................................................................ 38
2.2.1 Whole Heart Preparation .......................................................................................... 38
2.2.2 Decellularization System Set Up ................................................................................ 39
2.2.3 Decellularization Procedure ...................................................................................... 39
2.2.4 Observation of Vascular Pathway Preservation ......................................................... 40
2.2.5 Histology .................................................................................................................... 40
2.3 Results ................................................................................................................... 41
2.4 Discussion ............................................................................................................. 45
2.4.1 Decellularization Cues by Mass Loss ......................................................................... 45
2.4.2 Decellularization Cues by Color Change .................................................................... 46
2.4.3 Preservation of the Vasculature Pathway ................................................................. 47
2.4.4 Removal of Cellular Content ...................................................................................... 48
2.4.5 Retention of ECM Components ................................................................................. 48
2.5 Conclusion ............................................................................................................ 50

Chapter Three Seeding of the Vasculature in a Decellularized Scaffold ............................... 52
3.1 Introduction ........................................................................................................... 52
3.2 Methods ................................................................................................................ 52
3.2.1 Overview of Seeding Experiment Evolution through Three Trials ............................ 52
3.2.2 Seeding Experiment Procedure ................................................................................. 54
3.3 Results ................................................................................................................... 58
3.4 Discussion ............................................................................................................. 60
3.4.1 Seeded Cell Types ...................................................................................................... 60
3.4.2 Attachment of Cells to Decellularized Coronary Vasculature System .......... 61
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4.3 Progression from Unsuccessful to Successful Seeding Experiments</td>
<td>61</td>
</tr>
<tr>
<td>3.5 Conclusion</td>
<td>63</td>
</tr>
<tr>
<td>Chapter Four Conclusions and Recommendations for the Future</td>
<td>65</td>
</tr>
<tr>
<td>4.1 Summary of Project Significance</td>
<td>65</td>
</tr>
<tr>
<td>4.2 Summary of Advancement toward Completion of Specific Aims</td>
<td>66</td>
</tr>
<tr>
<td>4.2.1 Aim 1</td>
<td>66</td>
</tr>
<tr>
<td>4.2.2 Aim 2</td>
<td>66</td>
</tr>
<tr>
<td>4.3 Recommendations for Future Studies</td>
<td>66</td>
</tr>
<tr>
<td>References</td>
<td>69</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1: Weight of Rabbit Hearts during Decellularization Process</td>
<td>41</td>
</tr>
<tr>
<td>Table 2: Mass Change of Rabbit Hearts during Decellularization Process</td>
<td>41</td>
</tr>
<tr>
<td>Table 3: Analysis of Collagen Content for Decellularized Tissue Samples</td>
<td>44</td>
</tr>
<tr>
<td>Table 4: Analysis of Collagen Content for Native Tissue Samples</td>
<td>45</td>
</tr>
<tr>
<td>Table 5: Comparison of Collagen Content of Decellularized and Native Tissue Samples</td>
<td>45</td>
</tr>
<tr>
<td>Table 6: Cell Population and Incubation Times for Three Cell Seeding Experiments</td>
<td>53</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1: Build-up of plaque (atherosclerosis) leads to a blockage of the left coronary artery, causing the death of myocardial tissue</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2: Diagram of PCI</td>
<td>6</td>
</tr>
<tr>
<td>Figure 3: Diagram of CABG procedure</td>
<td>7</td>
</tr>
<tr>
<td>Figure 4: Schematic of a patient with an implanted LVAD</td>
<td>9</td>
</tr>
<tr>
<td>Figure 5: Diagram of the concept of cell sheet stacking</td>
<td>15</td>
</tr>
<tr>
<td>Figure 6: Concept of decellularization of a heart followed by recellularization using a patient's own cells</td>
<td>20</td>
</tr>
<tr>
<td>Figure 7: Lung bioreactor designed by Petersen et al.</td>
<td>28</td>
</tr>
<tr>
<td>Figure 8: Native and decellularized porcine myocardium surrounding the left coronary artery</td>
<td>30</td>
</tr>
<tr>
<td>Figure 9: Schematic of a possible timeline for the use of the “living patch” tissue engineered product for post-MI treatment</td>
<td>35</td>
</tr>
<tr>
<td>Figure 10: Color change of whole rabbit hearts during decellularization process</td>
<td>42</td>
</tr>
<tr>
<td>Figure 11: Injection of red polymer into the left coronary artery (LCA) of a decellularized rabbit heart</td>
<td>43</td>
</tr>
<tr>
<td>Figure 12: Images of histological stains of native porcine myocardium and decellularized rabbit myocardium surrounding the left coronary artery at 100x (bar=100 µm)</td>
<td>44</td>
</tr>
<tr>
<td>Figure 13: Schematic of system configuration for seeding experiments of decellularized rabbit hearts</td>
<td>55</td>
</tr>
<tr>
<td>Figure 14: Schematic of decellularized rabbit heart location in decell chambers</td>
<td>56</td>
</tr>
<tr>
<td>Figure 15: Photograph of the injection of cells into the left coronary artery of a decellularized rabbit heart scaffold</td>
<td>57</td>
</tr>
<tr>
<td>Figure 16: DAPI and H&amp;E stain at 200x of Seeding Experiment #3 using human aortic endothelial cells (ECs)</td>
<td>58</td>
</tr>
<tr>
<td>Figure 17: DAPI and H&amp;E stain at 200x of Seeding Experiment #3 using human adipose derived stem cells (ADSCs)</td>
<td>59</td>
</tr>
</tbody>
</table>
Chapter One

INTRODUCTION

1.1 Etiology & Pathology

Cardiovascular disease has high mortality rates [1,2]. It is the leading cause of death worldwide, with an estimated 17.3 million deaths each year. This number is expected to increase to 23.6 million by 2030. In 2013, 1 in every 7 deaths was caused by heart disease, killing over 370,000 people annually in the United States [3]. Coronary artery disease, a sub-category of cardiovascular disease which includes myocardial infarction (MI), leaves 100 million people a year globally in need of some type of surgical intervention or other therapy [4]. The American College of Cardiology and the American Heart Association stated that 900,000 people each year in the United States alone experience MI, making it the leading cause of death in the United States [5,6]. In 2011, MI cost the healthcare industry $11.5 billion. By 2030, this number is expected to double. The cost of treatment of post-MI patients has a significant economic impact that will only increase with time [7]. When a patient experiences MI, a severe inflammatory response is induced, beginning with the invasion of macrophages into the affected tissues to phagocytize cells that have died and other debris. Over the next few weeks, granulation tissue is formed at the site of injury, which ultimately becomes collagenous scar tissue. This scar tissue is not vascularized and does not have the contractile function that healthy myocardium has. Its presence decreases the ability of the heart to function properly [8]. The damaged tissue and scar formation that accompany MI begin a degenerative process toward congestive heart failure (CHF). The prevalence of MI and CHF are a major public health concern [9]. The amount of people affected by MI demonstrates an immense need for an understanding of the disease and for development of treatments and therapies.

Atherosclerosis of the main coronary arteries has been discovered to be a major cause of cardiovascular disease and myocardial infarction. Atherosclerosis is a disease of the intima, or the inner layer of the arterial wall, where lipoprotein lipids including cholesterol enter the intima and begin to
aggregate to form a plaque, as shown in Figure 1. Inflammatory cells such as macrophages, t-lymphocytes, and mast cells are located in the plaque. Mast cells generally appear in the later stages of atherosclerotic plaque formation in humans, and are known to influence adjacent cells and extracellular matrix (ECM) by moderating the plaques stability. Mast cell proteases are released which activate matrix metalloproteases (MMPs) within the plaque that degrade various ECM components including collagen. This leads to erosion of the plaque’s superficial surface and ultimately to rupture of the plaque [6,10]. Plaque rupture accounts for 75% of all acute coronary thrombosis cases, with the remainder being caused by erosion of the surface of the plaque [10]. Once the plaque breaks free from the intima, it forms a thrombosis as it travels down the bloodstream. The thrombosis gets caught in smaller vessels and occludes blood flow, eliminating the supply of oxygen and nutrients to adjacent tissues. When the thrombus is located in the heart, most commonly in the left coronary artery, it causes myocardial infarction (MI) [6,10]. MI is marked by the death of cardiomyocytes, leading to reduced functionality of the heart [1,6]. Following myocardial infarction, inflammation occurs in the affected area, followed by cardiomyocytes apoptosis, the formation of a fibrous scar that cannot contract, and ultimately to congestive heart failure [1]. The affected tissue area becomes ischemic.

![Figure 1: Build-up of plaque (atherosclerosis) leads to a blockage of the left coronary artery, causing the death of myocardial tissue](image taken from medicalassessmentonline.com)
Ischemia occurs when blood supply to a tissue is restricted, lowering the levels of glucose and oxygen delivered to that tissue. Without adequate amounts of oxygen and glucose for an extended period of time, cells cannot perform vital metabolic functions and will undergo apoptosis. Depending on the extent of the damage, tissues can either become dysfunctional or undergo necrosis [4]. Damaged cardiac tissue does not have the natural ability to completely repair itself and restore function like other tissue types such as epithelium do. This is due to the inability of cardiomyocytes to replicate and replace damaged cells. There are cardiac stem cells within the myocardium, but they are limited in number and are not prevalent enough to repair the amount of ischemic tissue caused by MI. Instead, fibrous scar tissue develops. This scar tissue keeps the organ intact, but does not aid the heart in restoring function [1,11]. The goal of therapy following MI is to either avoid scar tissue formation or replace the scar tissue with healthy functional tissue [11]. The goal of cardiovascular tissue engineering is to create a functional tissue scaffold to replace damaged cardiac tissue by and restore full functionality to the heart [1,2,8]. Functional tissue needs a vascular network to sustain itself, and is a one of the major challenges in tissue engineering [12].

It is vital that a complete and functional vascular network is present in any strategy for restoring function to damaged areas of the heart following myocardial infarction. Some strategies focus on repairing or rebuilding the existing network of blood vessels, such as bypass surgery and angioplasty, while others strive to create new vasculature such as stem cell therapy and cardiovascular tissue engineering strategies [4]. Tissue engineering therapies rely on a few key components. The first is a cell source, which will be determined by the individual patient the product is intended for, as well as the type of tissue being repaired. A scaffold to mimic ECM properties and to seed the cells on is also imperative. This can be made of natural or synthetic materials, or a combination of the two. Lastly, many tissue engineering solutions include growth factors to help promote angiogenesis in order to form a blood vessel network to sustain the living construct [1,2,4,8,13].
1.2 Current Post-MI Therapies

1.2.1 Whole Organ Transplant

One solution for patients who have experienced MI is to receive a heart transplant. This option surgically removes the entire damaged heart and replaces it with a healthy heart from a human donor. The Registry of the International Society for Heart and Lung Transplantation has released information that between the years of 2004-2007, 41% of all adult heart transplants in the world were initially caused by coronary artery disease. Non-coronary cardiomyopathy accounts for 45% of adult heart transplants. Of the patients receiving a heart transplant, 27% of them were using a left ventricular assist device (LVAD) prior to the surgery, which is a significant increase from the previous ten years. To provide insight into the success rate of this type of therapy, the transplant half-life, or time at which half of all of those patients who received a heart transplant remained alive, was 10 years in 2007. Data has also demonstrated that the survival rate within the first 6 months following the operation falls drastically with increasing time. After the first six months, the survival rate is linear [14]. This makes the first half year following surgery a critical time to monitor and provide the patient with the appropriate drugs and treatments.

The incidence of issues the recipient may have following heart transplantation, such as graft failure, multiple-organ failure, and acute rejection is dependent based off of recipient and donor variables. Some recipient factors that increase risk of problems and ultimately morbidity include recipient age, creatine levels, weight, and pre-transplant panel-reactive antibodies, while some donor factors that have been found to have a significant influence on the recipient patient outcome include donor age and weight [14]. An even more significant factor in the success of receiving a heart transplant is the number of available donors. There is a severe shortage of donors compared to the number of patients needing a new heart. An additional limitation of heart transplants is that patients must take immunosuppressant drugs for the remainder of their lives, which arguably decreases patient quality of life and can lead to further complications such as patient compliance in taking the drug [1]. These factors demonstrate major limitations in heart transplant as a therapy for those who have suffered MI. Factors attributed to the recipient’s health and existing conditions, as well as the health of the donor, will always be a variable in the
clinical outcome for heart transplants as well. An optimal treatment option would include a product or procedure that has verification and validation standards in place. This would minimize the variability in the treatment and help improve clinical outcomes. Other existing therapies that do not involve a donor, such as LVADs and coronary artery bypass surgeries (CABG), do involve testable products that can be verified to achieve a set standard of safety and efficacy in the lab. These therapies can improve mortality rates following MI, but are limited greatly by not being able to restore function to the afflicted myocardial tissue. Heart transplants do restore function to the heart, and are the sole existing treatment to do so. However, heart transplants have many limitations, and only allow patients to survive 10 years on average following the procedure [1,14]. It is no surprise that a number of technologies have been developed to reduce the need of heart transplants, and are continuing to be researched and improved.

1.2.2 Percutaneous Coronary Intervention (PCI)

Another mechanical treatment option for patients who have experienced MI is percutaneous coronary intervention (PCI), a reperfusion therapy, formally known as angioplasty with a stent. PCI can be performed without a stent as well, but yields inferior results [6]. Application of PCI with a stent involves reaching the area of blood vessel that has been partially or completely occluded by buildup of atherosclerotic plaque using a thin flexible tube. This tube deploys a stent which expands to open the lumen of the blood vessel, and to depress the plaque. This increases the diameter for blood flow, as shown in Figure 2. When performed in the coronary artery, PCI successfully increases blood flow through the affected area in 90-95% of patients who experienced MI [5,6,15]. PCI is a widely accepted as a very effective post-MI treatment, and has been shown to minimize damage done to the myocardium [15]. A large number of reports and sources including the American College of Cardiology recommend having a patient undergo PCI within 90 minutes of initially entering a hospital following MI, providing that the necessary experienced personnel to perform the procedure are present [16,17]. This time limit is intended to minimize the damage done to the myocardium due to tissue ischemia. Focusing on reducing ischemic time is the key to achieve greater preservation of the myocardium. However, only about one third of patients who received a PCI end up with adequate reperfusion or restoration of blood flow to the coronary artery.
Any amount of ischemic tissue will result in an infarct scar that is not functional, and this area will increase if PCI does not provide enough blood flow to the affected tissue area. Although PCI has been shown to improve patient clinical outcomes following MI in some cases, it still has significant limitations. Other treatment options must be explored to achieve better prognosis by reducing tissue ischemia and therefore infarct scar size for patients who have suffered MI.

**Figure 2:** Diagram of PCI, where a stent is inserted into the blocked left coronary artery and deployed to open the lumen and restore blood flow

### 1.2.3 Coronary Artery Bypass Procedure (CABG)

An additional well known and commonly performed mechanical surgery technique for patients who have MI is coronary artery bypass grafting (CABG) procedure. This procedure involves harvesting a healthy vein or artery from a different location in the patient’s body and grafting it onto the blocked coronary artery. The graft bypasses the blockage and creates a new pathway for blood to flow, keeping the surrounding tissue vascularized, as shown in **Figure 3**. The use of this procedure is so prominent that it consumes more resources worldwide than any other single procedure for cardiovascular diseases [18].

Increased risk for mortality for patients undergoing a CABG procedure has been linked to several main factors, including age, sex, record of prior cardiovascular surgeries, and the amount of stenosis in the left coronary artery. For example, women are at a higher risk of morbidity following a CABG procedure due to

---

2 Image taken from myhealth.alberta.ca.
smaller diameter of the lumens of the affected arteries. Other complications that have been reported after a CABG procedure include renal dysfunction, development of neurological abnormalities, and post-operative stroke [18]. One study found that the quality of life five years after a CABG procedure was heavily dependent on the patient’s quality of life prior to the procedure. The average quality of was lower if the patient was a female and or had a history of diabetes mellitus as well. However, it was also found that 75% of patients did not experience cardiovascular tissue ischemia for five years following CABG procedure [19]. CABG procedure for post-MI patients can decrease damage to the myocardium, but is not without limitations and complications. Research has been conducted to compare the clinical outcomes of patients who underwent CABG versus PCI. Studies have shown that rates of variables such as one year mortality, MI, and major cardiovascular and cerebrovascular complications are about 10% higher for patients who underwent PCI with stents compared to patients who underwent CABG procedure [20–22]. CABG has also been shown to more successful for patients with diabetes [20]. While CABG is a widely used and accepted as a successful treatment for MI, it is not optimal and can lead to other health issues.

**Figure 3:** Diagram of CABG procedure. A healthy vessel from the patient's body is harvested and then grafted to connect the area of the left coronary artery that has restricted blood flow to the aorta. This creates an alternative pathway for blood flow.\(^3\)

\(^3\) Image taken from drbillsukala.com.au.
1.2.4 Left Ventricular Assist Devices (LVADs)

A final method of treatment for patients with ischemic tissue due to MI is the use of left ventricular assist devices (LVADs). LVADs are used for patients with end-stage heart failure as a permanent alternative to heart transplant. The device’s popularity grew due to the insufficient available donors for heart transplant. The LVAD assists proper function of the damaged host heart, as opposed to replacing the whole heart. The LVAD is implanted next to the left ventricle and is attached to the left ventricle and to the aorta. Blood flow from the lungs enters the left ventricle and subsequently the device, which moves the oxygen rich blood to the aorta to enter systemic circulation. The LVAD has an external component called the driveline, which is a power cable that exits the skin and plugs into a power source worn on the patient’s body, as shown in Figure 4. One study that included 208 patients that were implanted with an LVAD found that the 1 year mortality rate was 56%. The greatest factor in determining the success of the LVAD was the timing of the implantation. The chances of survival were higher if major complications had not developed prior to implantation [23]. In spite of the high mortality rate in patients who received an LVAD, it must be recognized that these patients had or were close to end stage heart failure, and had no other treatment option besides a heart transplant, which was often unavailable. This made LVADs a revolutionary invention. However, besides mortality rates, the use of LVADs presents other limitations. The external component protruding through the skin causes concerns about infection, while the need for electrical energy and device maintenance interferes with a patient’s quality of life. Overall, the LVAD is not an optimal solution for patients with damaged myocardium around the left ventricle.
In summary, the only current post-MI therapy that restores function to cardiac tissue is a heart transplant. Heart transplants are severely limited by donor availability, complications associated with major surgery, and a lifetime of immunosuppressant treatments [24]. Alternative therapies such as PCI and CABG are associated with their own side effects and complications. Implantation of a LVAD can improve heart functionality, but is also accompanied by downsides such as risk of infection, device maintenance, and a decreased quality of life. Limitations of existing therapies in all fields of medicine have given rise to a new era of research, tissue engineering, with a goal of finding a superior treatment for diseases that cannot currently be cured. For the millions of patients worldwide who experience MI, cardiovascular tissue engineering offers new hope for therapies that aim to restore function to the heart, eliminating the need for a human donor.

1.3 Introduction to Tissue Engineering

Tissue engineering is considered by many to be the future of medicine, and the ultimate solution for disease. Beginning around the 1980’s when human cells were first utilized to create a therapeutic

---

4 Image taken from mylvad.com
scaffold, this growing field implies that treatments can be developed that cure diseases and conditions that were previously too complex and difficult to even treat [8,25]. The goal of tissue engineering is to provide a method of treatment that regenerates a patient’s own cells to restore functionality to tissues and organs. This is done without biocompatibility issues or mounting an immune response, which are current limitations to almost all existing technologies and therapies for diseases of the body.

There are three fundamental pieces to tissue engineering: cells, scaffolds, and growth factors [8,25,26]. The first, cells, must come from an appropriate and viable source. Cells can be harvested from autologous sources (from the patient to which the final treatment will be delivered to), allogenic sources (from the same species), or xenogenic sources (from a different species). It is well known that allogenic sources cause an immune response, and xenogenic sources cause an even more severe immune response. Xenogenic sources are the easiest to obtain in great quantities, but are the worst option for immunogenic response. Autologous cells are the optimal choice in terms of biocompatibility. However, they are also the most difficult cells to obtain in adequate numbers [25]. They must be expanded in cell culture, which takes more time, resources, and can introduce more variables and complications. Stem cells, or undifferentiated cells with the capacity to differentiate into a number of different cell types, are often thought of when tissue engineering comes to mind. Some common stem cell types being used in therapies include adipose-derived adult stem cells (ASCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and various bone marrow-derived adult stem cells (BMDSCs). Induced pluripotent stem cells (iPSCs), or differentiated cells that have been exposed to specific transcription factors causing them to revert back to an undifferentiated state, constitute a major area of research as well [1,8,24]. These cells are used in tissue engineered constructs with the intention that they will take cues from their environment and differentiate into an appropriate cell type for the intended application, as well as provide a self-regenerating source of new cells for the construct.

Scaffolds, which support and house the cells, can be made from natural or synthetic materials, or a combination thereof. Examples of common synthetic materials used in tissue engineering include poly-lactic acid (PLA) and poly-glycolic acid (PGA), and examples of natural materials include collagen and fibrin [27]. The function of the scaffold in a tissue engineered construct is to assist in cell proliferation and
differentiation, and provide mechanical strength and support [25]. A scaffold is not limited by the requirement of possessing all of these qualities. They can have just one or multiple of these qualities, depending on the application.

Lastly, growth factors are commonly added to tissue engineered constructs in order to promote cell growth, proliferation, differentiation, adhesion to the scaffold, and proper function. Some of the most commonly used growth factors are bone morphogenic proteins (BMPs), and pro-angiogenic factors such as vascular epithelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) [25,28]. The most prominent challenge with growth factors is the method and efficacy of delivery. It has been shown that bolus injections to the target site are not effective because the injected molecules quickly leave the target area. Other methods of delivery such as using DNA plasmids containing the gene for creating the desired growth factor are being researched [25].

The three factors of cells, scaffolds, and growth factors are combined in various ways and analyzed to create possible therapies in all fields of tissue engineering. Cardiovascular tissue engineering, or tissue engineering of the heart and its associated tissues and components, is no exception in utilizing these key building blocks.

1.4 Introduction to Cardiovascular Tissue Engineering

The goal of cardiovascular tissue engineering is to restore the integrity and function of damaged tissues in the heart [1,8]. In addition to the general elements of cells, scaffold, and growth factors that make up a tissue engineered construct, cardiovascular engineering of the myocardium is unique in that it requires electromechanical coupling, the ability to endure and maintain stable contractile function, and the need to be adequately vascularized [8]. This makes bioengineering the heart a unique and complex venture. Various sections of the heart, such as heart valves and the myocardium, have different structures and residing cells. The myocardium in particular houses cardiomyocytes which are responsible for coordinated contraction of the heart, along with pacemaker cells which are specialized cardiomyocytes. Cardiac fibroblasts and cells found in vasculature such as endothelial cells and smooth muscle cells are present in native ventricular wall as well, all residing in a collagen based ECM [29,30]. The following overview of
existing cardiovascular tissue engineering therapies for patients who have suffered MI will focus on myocardial tissue regeneration. The research presented afterward will concentrate on the myocardial tissue surrounding the left coronary artery and its adjacent branches and capillaries. This specific area is targeted for therapeutic restoration due to its high prevalence of severe damage following myocardial infarction in patients.

1.5 Overview of Current Strategies in Cardiovascular Tissue Engineering

1.5.1 Cellular cardiomyoplasty

Cardiomyocytes are limited in their ability to naturally regenerate and restore function to damage infarct myocardial tissue [30,31]. The cellular cardiomyoplasty strategy involves either cardiomyocytes, stem cells, or growth factors being delivered directly into the damaged myocardium to regenerate the cardiomyocyte population in the target area. There are no scaffold or growth factors being introduced to the heart with this strategy, making it simpler with less room for error, as well as cheaper due to requirement of minimal resources when compared to more complex tissue engineering strategies. However, current issues with this simpler method include biocompatibility with the use of autologous cells, and cell retention in the target area [1,31]. If stem cells are used as the cellular component, unwanted differentiation is also a concern. Blood supply to the injected cells presents a complication when the target injection area is MI scar tissue. The scar tissue does not contain any vasculature to sustain the injected cells [1]. There is also concern if the injected cells do not integrate properly into the host tissue and disrupt the electrical conduction pathways within the heart, affecting cardiomyocyte contraction [31].

1.5.2 Injection of cells using a Biomaterial Delivery System

Cells can also be delivered to myocardium using a biomaterial as a delivery vessel, such as a hydrogel. For example, Christman et al. found that combining fibrin glue with myoblast cells and injecting them into ischemic rat myocardium increased cell survivability when compared to injecting myoblasts without a vehicle. Results also indicated that using a biomaterial as a delivery system increased retention of
the injected cells in the target area, led to a reduction in scar size, and exhibited the beginning of
vascularization within the ischemic tissue [32]. A study by Ye et al. using a fibrin gel seeded with human
aortic myofibroblasts discussed the possibility of including growth factors or bioactive molecules to create
an environment for the cells that more accurately mimics their native environment, as well as adding
proteinase inhibitors to control the degradation rate of the fibrin gel [33]. An injectable alginate hydrogel
made by Landa et al. found that the gel provided structural support to the fibrous scar tissue in infarct rat
models. Although this model did not include cells, it highlights the benefits of using a biomaterial for
injection into ischemic myocardial tissue [34]. However, approaches similar to these have a major
limitation in that they do not address making structural and biomechanical changes to the damaged
myocardium. Successfully restoring function to damaged myocardium necessitates the delivery of
appropriate cell types as well as restoration of ECM properties. Many biomaterials used in these injection
approaches do not mimic the mechanical properties of the natural myocardial ECM, and are limited by the
same properties that allow them to flow through a needle or injection apparatus [1]. Their simplicity also
lacks domains for cell attachment as well as signals to promote cell differentiation, migration, and
proliferation. To remedy these limitations, biomaterials can be made into scaffolds with various geometries
and properties that are implanted into the host tissue.

1.5.3 Natural and Synthetic Scaffolds

Scaffolds for tissue engineering can be made of natural or synthetic materials, or a combination of
both. Synthetic scaffolds for cardiovascular tissue engineering are often made of materials such as poly-
lactic acid (PLA) and poly-glycolic acid (PGA). As with any manufactured synthetic product, synthetic
scaffolds can be made in large scale production, eliminating the issue of availability [35]. An additional
attractive advantage of synthetically made scaffolds is the amount of control over physical, geometrical,
and chemical properties the researcher has [8,26,36]. For example, electrospinning is a technique capable
of creating scaffolds with a defined high porosity and surface area, which can be optimized to aid in cell
invasion and migration, drug delivery, as well as exchange of nutrients, oxygen, and waste [37]. Laser
sintering and salt or particulate leaching can be used to create specific porosities as well [1,38]. Synthetic
scaffolds can also be made of biodegradable materials, which is advantageous for applications in which it is optimal that the scaffold not permanently reside in the host. The rate of degradation for biodegradable scaffolds can be controlled to a degree as well. This allows the scaffold to be tailored for specific applications. Even though they are considered biocompatible, most synthetic polymers used for cardiovascular tissue engineering cause some degree of immune response upon degradation. For example, PLA and PGA and their copolymers (PLGA) lower the pH of adjacent tissue as they degrade due to their acidic degradation products, causing an immune response. In addition, no currently existing biomaterial or combination thereof is as detailed and complex as the extracellular matrix (ECM), the natural scaffold found in tissues, and they do not possess the same molecular geometries and mechanical properties. For example, natural collagen fibers can recover from larger deformations than PLA/PGA/PLGA can [39]. Using natural materials for scaffolds can provide properties more similar to native ECM.

Natural scaffolds are made from materials found in nature. Studies have utilized materials from human tissue, such as collagen, gelatin, fibrin, and elastin [32,33,37,40–42]. Materials can also be taken from other species, such as silk from spiders and chitosan from insects, or alginate from plants [34,43,44]. Using natural materials that have inherent biological properties provides several advantages that synthetic materials lack, such as superior biocompatibility. Another important aspect of using natural scaffolds is the native geometries they possess [35]. For example, many natural scaffolds have binding sites for proteins and cells, and can be influenced by cell signaling molecules as well as have an influence on cellular function [8,45]. However, a limitation of using natural materials for scaffolds is that their mechanical and chemical properties are not easily manipulated [26]. It is also a challenge to purify natural materials from their sources, and there are concerns about immunogenicity and transmission of disease [45]. The availability of these scaffolds is also limited to the availability of the organisms required [35]. An enhanced category of natural scaffolds are those made through a decellularization process of healthy tissue. Discussed later in the review, decellularized natural scaffolds are able to overcome the limitations of the previously mentioned natural materials.

In short, synthetic scaffolds are reproducible and made with standardized protocols, which gives them a great advantage over natural scaffolds, which cannot be reproduced or tailored in any way [12]. On
the other hand, natural scaffolds provide inherent biological properties needed for cellular function. The choice between synthetic or natural scaffolds is dependent on the desired application.

1.5.4 Cell Sheet Approach (Scaffold-Free)

This approach consists of creating monolayer cell sheets and stacking them to build a thin tissue construct, as shown in Figure 5. The technology and procedure utilized protects cell-cell connections during detachment from the sheets, allowing the cells to establish electrical connections with one another. Cell surface structures are also preserved during the detachment process as well. This technology is valuable due to its ability to make a construct with sufficient contractile properties, as well as the ability to combine cells without extra matrix material [27,46]. A study done by Miyahara et al. used mesenchymal stem cells (MSCs) to create cell sheets that were implanted on the surface of an infarct scar in a rat model. They found that the addition of these contracting cells reduced stress on the left ventricular wall, which improved overall cardiac function [46]. However, with the lack of matrix comes fragility of the construct. In addition, the attainable thickness of the sheet is limited by the lack of vascularization. Vascularization itself remains a key challenge in cell sheet approaches as well as in tissue engineering in general [8,27].

Figure 5: Diagram of the concept of cell sheet stacking. Removal process of cell monolayers from sheets not shown

---

5 Image taken from rsif.royalsocietypublishing.org.
1.5.5 Stem Cell Approach

When using a stem cell based approach, selecting an appropriate cell source is a challenge. The cell source must be easily accessible, widely available, and be expandable in culture. There are ethical concerns surrounding the use of embryonic stem cells, encouraging the use adult stem cells to avoid such obstacles [24]. Common non-embryonic stem cells used in cardiovascular approaches include skeletal myoblasts, adipose derived stem cells, resident cardiac stem cells, bone-marrow derived stem cells, mesenchymal stem cells, induced pluripotent stem cells, multi-potent adult progenitor cells, and endothelial progenitor cells [26]. In clinical application, a study by Chen et al. demonstrated that injecting bone marrow mesenchymal stem cells into the left coronary artery of post-MI patients significantly improved the function of the left ventricle [47]. Many other studies have investigated the therapeutic effects of bone marrow stem cell injections to MI patients, including the TOPCARE-AMI study, which showed reduced infarct scar size and reduced left ventricular volume [48]. Another study by Lunde et al. injected autologous bone marrow derived mononuclear cells into the coronary arteries of patients 6 days after MI. However, they did not find any therapeutic effects on left ventricular function [49]. The MYSTAR study also used bone marrow derived mononuclear cells, but delivered them by injection into two different routes, the myocardium and intracoronary infusion. Patients also received treatment at one of two different time points, the first being around one month after MI and the second 3-4 months after MI. Looking at variables such as left ventricular volume and ventricular wall motion, no significant differences were found between the early and late time point groups. However, both time point groups showed a trend toward decreased systolic volume, and a significant reduction in infarct scar size [50].

Stem cells can be selected as the only cells in a given tissue engineered therapy, or they can be combined with mature differentiated cells. A study by Caspi et al. showed that multi-culture poly-lactic/poly-glycolic acid scaffolds containing endothelial cells (ECs), human embryonic stem cell-derived cardiomyocytes (hESC-CM), human umbilical vein ECs (HUVEC), and embryonic fibroblasts cells (EmF) resulted in a significantly higher number and density of blood vessels, and a higher EC density compared to cultures without EmF. Results also showed a higher proliferation rate of cardiomyocytes in scaffolds co-
cultured along with ECs, compared to single cultures of cardiomyocytes [51]. Another study by Lesman et al. found similar results with a tri-culture tissue construct [38]. These studies show great potential in combining various differentiated and undifferentiated cell types to maximize the efficacy of a tissue engineered construct.

1.5.6 Decellularization & Recellularization Approach

One of the most recent tissue engineering strategies to emerge is decellularization of native tissues and organs. This method involves using allogenic or xenogenic tissues and completely removing the cellular components and DNA content, while simultaneously preserving as much of the structure and components of the ECM as possible. ECM components that are found in the majority of human tissues are collagen type I, glycosaminoglycans (GAGs), fibronectin, and laminin [52]. The advantages and possible clinical benefits of using decellularized scaffolds are very clear and alluring. Using cadaveric or xenogenic tissue sources could eliminate the need for donor organs, which is the main limitation of heart transplants, the only existing therapy to restore function to the heart [1]. Using native ECM also avoids the numerous limitations associated with synthetic materials, including the challenge of mimicking the complex and not completely understood ECM, and the adverse effects associated with a synthetic material’s degradation products. ECM scaffolds already possess optimal structure for specific cell populations and tissue types, which thus far is too complex to recreate in the laboratory from non-biologic materials [26]. The use of decellularized scaffolds provides seeded cells with the most accommodating and nurturing environment, with built in cues for differentiation, proliferation, migration, and cellular function [53,54]. For example, a scaffold’s elastic modulus directs mesenchymal stem cell (MSC) differentiation pathways [55,56]. In addition, it has been proven that the ECM’s mechanical properties play an important role in cardiac muscle contraction [53]. The ECM also creates a complex and dynamic environment for resident cells, and alters its structure and composition in response to changes in cellular metabolic activity [52]. The exact mechanisms and pathways that allow ECM to influence cell behavior is not fully understood, but it is clear that the ECM does possess this quality. Using a decellularized myocardium scaffold for a cardiovascular tissue engineered construct will preserve the natural mechanical properties and dynamic remodeling
properties, and subsequently retain the ability to differentiate seeded or invading stem cells into cell types appropriate for myocardial tissue.

There are three main obstacles for developing decellularization techniques. The first is selecting an appropriate native tissue or organ for a specific application. If only a portion of tissue is needed, this is not a cumbersome problem. However, for whole organ tissue engineering, the selected native tissue must be sized to fit the patient. Clearly, allogenic sources would provide the best shape and size for humans. However, live donors are limited, and cadaveric donors are limited as well. There is also concern that the tissues may have begun to change structure or degrade after the death of the donor [57]. The most widely available option are xenogenic sources, which are more difficult to appropriately size. In respect to whole heart engineering, porcine models are closest in size to human hearts and are widely available from slaughterhouses [26]. The second challenge is the decellularization of the tissue, or removal of cellular components and DNA. Especially important for xenogenic tissues, this step is critical in ensuring that the host does not reject the implant, and does not have to take immunosuppressant drugs for the rest of their life. Choosing the optimal decellularization solutions for a given application is a challenge as well. The most effect solutions for a given tissue will depend on the residing cell types, tissue density, lipid concentration, and thickness of the tissue. Common agents used in decellularization processes include but are not limited to acids and bases, hypotonic and hypertonic solutions, detergents, alcohols, and enzymatic solutions. It is also important to know that each decellularization solution will cause some extent of damage to the ECM, which is the last main barrier for decellularization techniques [54]. A procedure must be optimized to retain the maximum amount of ECM structure and components possible. A perfect decellularized construct would be identical to the native construct, without the cells. This has not yet been achieved for any tissue or organ. However, it is possible that a decellularized scaffold with only a percentage of ECM components left will be feasible for the intended application. Finding an appropriate solution for an application that effectively removes cellular content and minimizes damage to ECM components of interest is a key consideration in experimental design for decellularization.

It is logical that each tissue type and organ has a different ideal decellularization method. Many decellularization trials have utilized combinations of mechanical and physical separation methods [26].
Enzymatic and chemical solutions have also been employed, and one study has even investigated the use of microwave radiation to aid in the decellularization process [58]. It is well known that detergents and enzymatic solutions that solubilize cell membranes can damage the ECM and decrease its mechanical and structural properties. Specifically, sodium dodecyl sulfate (SDS), which is often used in combination with other agents in decellularization processes, is known to degrade collagen [26,57]. Therefore, it is essential to perform research to find the best decellularization methods for cardiovascular tissues that effectively remove cells while minimizing destruction of the ECM. It is also important to note that synthetic or natural biomaterials can be used in conjunction with decellularized matrices, as this combination may yield superior results than an acellular scaffold alone [8].

Perfusion decellularization, or decellularization by flowing solutions through natural channels in the organ, has shown promising results for cardiovascular tissue engineering, as well as whole organ decellularization of kidneys and lungs [57]. Ott et al. were the first to use a decellularized heart matrix and recellularize it with stem cells in 2008. They developed a perfusion decellularization method using retrograde flow through the aorta in cadaveric rat hearts. They found that using SDS in solutions resulted in a greater removal of DNA than the use of polyethylene glycol (PEG), Triton-X 100, or enzyme based solutions. The nuclei were successfully removed, and ECM components including collagen types I and II, laminin, and fibronectin were preserved. Mechanical testing revealed the decellularized rat hearts to be anisotropic for stress-strain testing [59]. Another study by Guyette et al. using perfusion decellularization through the aorta investigated combinations of SDS, PEG, and Triton-X for rat, porcine, and human whole heart models. The decellularized matrices were analyzed for quantification of remaining collagen, elastin, and glycosaminoglycans. Between 10-30% of each component in comparison to native heart tissue by weight was retained in the scaffolds. The results showed that decellularization of whole hearts of varying sizes is possible, and that human hearts can be completely decellularized in about a week. Knowledge of a timeframe will become important in the future once decellularization strategies are made available for use in clinical applications. However, one limitation to this study is that it is unknown if and to what extent age or disease of the donor has affected the ECM, which may render the source inadequate for clinical use. This study also did not investigate recellularization techniques, which is the next step following the
decellularization of whole organ constructs [57]. For cell seeding, or recellularization of the scaffold, the scaffold is combined in some manner with cells harvested from the intended patient [27]. These cells can be stem cells, mature differentiated cells, or a combination of both [59]. The concept of decellularization of a whole organ followed by subsequent recellularization is demonstrated in Figure 6. The goal is to obtain adequate population numbers of all relevant cell types in a decellularized scaffold so that it mimics the native organ as close as possible.

Figure 6: Concept of decellularization of a heart followed by recellularization using a patient's own cells. A functional heart is created for surgical implantation to replace a damaged heart.

In summary, three main goals must be accomplished to successfully create a therapeutic cardiovascular tissue engineered construct. The key components include a density of specific cell populations equivalent to that of the native myocardial tissue, the ability to contract in synchrony using

---

6 Image taken from revespecardiol.org.
electrical signals, and adequate vasculature [8]. More specifically, an implantable construct must successfully integrate into the host tissue without an inflammatory response and with successful electrical coupling with the host’s heart contractions [24]. One of the most significant obstacles in tissue engineering is vascularization of scaffolds. In tissues that rely on the circulatory system for sustenance, cells are no more than 100-200 microns away from a capillary. This distance is optimal for exchange of nutrients, oxygen, and wastes between the cell and the blood vessel network [12,24,28]. Various strategies have been developed to attempt to adequately vascularize tissue engineered constructs.

1.6 Current Strategies for Vascularization of Tissue Engineered Constructs

1.6.1 Cell Based Approach

Cell based strategies revolve around inducing angiogenesis, or the formation of new blood vessels, within tissue engineered constructs. The first cell based strategy utilizes an in vitro prevascularization of scaffolds that occurs before implantation into a host. One method of accomplishing this involves culturing ECs in vitro in order to induce angiogenesis, possibly along with other supporting cell types such as fibroblasts. The aim is to create a partial vessel network, and then implant the network into the ischemic tissue where the construct will integrate with surrounding host vasculature. An advantage of this method is that invasion of host blood vessels into the scaffold is not required, saving time. The vessels in the scaffold are anastomosed with the adjacent existing vessels. However, microscopic vessels are not able to be surgically connected between the two areas. This can limit the amount of cells that receive adequate blood supply [12].

Another method is an in vivo prevascularization approach, where a cell-free scaffold is created and then implanted into a healthy tissue within the patient. The implant is left for a period of time sufficient to allow for the invasion of blood vessels that will eventually create an adequate vasculature system. The implant is then removed from the first surgical site, and implanted into the site of injury in order to deliver a vascular network to the avascular ischemic tissue. Various studies have proven that host cells in healthy tissue areas are able to invade certain synthetic scaffolds and create a network of blood vessels [60–62].
However, this method has not yet been tested on *in vivo* infarct scar tissue. Advantages of this method include an autologous and complete vascular network within the scaffold that is capable of sustaining all cells in the construct. Limitations of this method include the risks and resources involved with performing three surgeries, as well as the time needed for the scaffold to become vascularized [12].

Various studies have been able to modify the cell sheet (scaffold-free) technique to include a vascular network as well [63]. One study by Sekine et al. co-cultured rat cardiomyocytes and endothelial cells in petri dishes. The ECs were able to form vascular networks within the dishes. The networks were preserved when the sheets were removed from the dishes and layered on top of each other. These constructs were then transplanted onto infarct myocardial tissue. It was found that constructs containing ECs had more capillary formation compared to sheets lacking ECs, and were able to connect with host vasculature. The amount of infarct scar tissue was reduced and cardiac function was improved [64]. A similar cell sheet experiment showed an increase in the number of capillaries in the sheet containing smooth muscle cells compared to a fibroblast sheet control. There was also migration of smooth muscle cells from the implanted construct into host ischemic tissues, leading to angiogenesis [65].

Another strategy is to introduce growth factors and cytokines to the area lacking vasculature in order to promote migration and proliferation of cells involved in angiogenesis. Angiogenic growth factors activate endothelial progenitor cells to migrate toward to the target site, and drive vessel formation and endothelial cell maturation [12,28]. Some drug delivery techniques that have been utilized for inducing new blood vessel formation include polymeric carriers, hydrogel networks, and peptide carrier systems. The common angiogenesis promoting growth factors used in these techniques include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), TAT-HSP27, transforming growth factor beta (TGFbeta), and hepatocyte growth factor (HGF). All of these methods have been proven to increase angiogenesis in ischemic tissues [12,28,66–70].

### 1.6.2 Scaffold Based Approach

Physically assembled scaffolds can be designed to promote vascularization as well. These scaffolds can be made of natural or synthetic materials, or a combination of both, as mentioned previously.
In order to promote angiogenesis, properties such as elastic modulus, surface roughness, biodegradability, and specific geometries must be controlled to a high degree [12]. One study using polyethylene glycol (PEG) as the material for a scaffold found that introduction of matrix metalloproteases helps define the degradation rate of the scaffold. Control over the degradation rate has been associated with the ability to obtain a higher degree of blood vessel formation [71]. Porosity of the scaffold is another physical variable that is important in setting up a structural environment capable of supporting vessel formation. A study by Keskar et al. found that superporous hydrogel scaffolds made of polyethylene glycol diacrylate polymer were vascularized by host cells when implanted in mice [72]. In addition to controlling specific scaffold properties, using certain methods of creating the scaffolds can also promote vascularization. One scaffold fabrication method that is aimed at creating blood vessel network channels down to the capillary level is microfluidic systems [28,73]. This method combines a scaffold-based approach and a cell-based approach. Artificial capillary systems are formed from molds using biocompatible materials, and are then endothelialized by fluid flow through the channels. The ability to vary flow rate may be useful in this system because it has been proven that shear flow influences the traction force and migration of endothelial cells in blood vessels [74]. However, the microfluidic method is limited by the inability to influence the surface characteristics of the lumen of the channels [75]. Scaffold based approaches aimed at creating easily vascularized environments are limited by the same obstacles that synthetic and natural scaffolds face in general, such as inability to mimic the complex native ECM and difficulty in tailoring properties, respectively.

1.6.3 Bioreactor Approach

Bioreactors are *in vitro* chamber systems used to deliver flowing culture medium through seeded scaffolds. This increases cell viability and homogeneity throughout the scaffold, while simultaneously allowing greater exchange of nutrients, oxygen, and wastes between the cells and culture medium. Bioreactors can also provide electro-mechanical stimuli to influence and support tissue constructs, and aid in their formation [26,28,76]. Perfusion bioreactor systems are one category of bioreactors that were developed to more specifically address cell viability throughout the entire scaffold by utilizing tissue
engineered blood vessel constructs. These scaffolds can be made of a combination of synthetic or natural biomaterials, which are then seeded with cells found in native vasculature such as smooth muscle cells or endothelial cells. The scaffold is connected to the bioreactor and then culture medium is flowed through the system at a controlled rate. Shear forces along the lumen of the vessel can be adjusted to be similar to forces found in native arteries, arterioles, or capillaries [28]. A novel perfusion bioreactor study by Kofidis et al. seeded cardiomyocytes into a fibrin-glue based 3D matrix. Compared to constructs in unperfused bioreactors, those subjected to perfusion had greater cell viability and metabolic activity [77]. Another study by Dvir et al. used a fluid-distributing mesh to maximize exposure of cells to the flowing medium and oxygen, as well as nets to support the cells. They found that the constructs in the perfusion bioreactor resulted in higher cell viability compared to static control groups, due to increased exposure to oxygen and increased removal of cellular waste [78]. Taking perfusion bioreactors one step further, Barash et al. combined an electrical stimulator to a perfusion bioreactor, in order to deliver and control an electric stimulus to the seeded cardiomyocyte cells. They found that cell constructs in this system expressed greater elongation and number of striations compared to a static control group, as well as increased expression of the gap junction protein, which allows for cell coupling in myocardial tissues [79].

Many attempts at vascularizing ischemic tissues using cells involved in angiogenesis, angiogenesis promoting growth factors, and carefully designed scaffold structures have been made. Bioreactors have been utilized to provide stimuli to seeded scaffolds in order to increase the degree of vascularization in the constructs. Thus far, no method has successfully been able to create a vascularized tissue engineered product that is ready to move to the clinical trial phase and eventually to market. However, decellularized constructs seem to be a promising option. Due to their unique property of possessing natural structure and components of the ECM, decellularized scaffolds have been increasing explored as a viable option for tissue engineered constructs. Their inherent ability to support cell viability and influence cell behavior has spurred an incredible amount of research directed toward the vascularization of decellularized organs. The next section of this literature review will provide an overview
of re-endothelialization methods for decellularized scaffolds, with an emphasis on myocardial
decellularized tissues.

1.7 Strategies for Endothelialization of Decellularized Scaffolds

Due to a severe shortage of organ donors and a lack of existing therapies that restore function to
damaged tissues and organs, decellularization and subsequent recellularization of organs has spurred great
interest in the field of tissue engineering, especially within the past decade. Organs are taken from widely
available and accessible xenogenic sources, or from allogenic human cadavers. The concept is that cells are
removed from the organ in order to prevent an immune response in the host, and then the patient’s own
cells are seeded into the acellular ECM. This should provide a fully functional tissue construct or whole
organ to surgically replace damaged tissue in a patient. Despite many research groups working on this
strategy, there are still critical obstacles to be overcome before this type of therapy is successful for human
organs. One key challenge is adequate vascularization of the acellular ECM. Functional blood vessels have
a lumen that is lined with endothelial cells, a specialized type of epithelium. Endothelial cells are in
constant contact with blood in the body, and provide a non-thrombogenic surface for the circulatory
system. When a vessel is wounded and the endothelial monolayer is disrupted, whole blood components
come into contact with a cell type other than endothelial cells, which initiates the coagulation cascade.
Therefore, in a tissue engineered construct, the vascular network must provide pathways that are
completely lined with a monolayer of endothelial cells in order to sustain blood flow and exchange of
oxygen, nutrients, and waste. A key characteristic of vasculature’s ECM is the presence of a basal lamina,
or basement membrane, on which endothelial cells reside. The basal lamina is made of proteins, such as
collagen type IV, laminin, fibronectin, and nidogen, as well as proteoglycans such as perlecan. This
structure is especially important to preserve during a decellularization process, as it provides an attachment
point for endothelial cells and acts as a guide for regeneration and migration. One of the main reasons that
the whole organ tissue engineered constructs that require an extensive vascular network have not yet been
successfully created is the challenge of oxygen delivery [80,81]. Poor oxygen delivery indicates a deficient
vasculature network that is unable to reach all seeded cells of decellularized whole organs. In this section of
the literature review, various attempts at decellularizing and re-endothelializing organs including kidney, liver, lung, and heart which provide proof of concept will be reviewed.

1.7.1 Kidneys

In the United States, about 100,000 people annually are in need of a kidney transplant, and 13 of these patients die each year on the waiting list [82]. Kidney transplantation is the only existing therapy for severely damaged kidneys, so the notion that xenologous kidneys can be made into viable kidneys for transplant is exciting and has attracted researchers to the area [83]. It has been shown that rat, porcine, and cadaveric human kidneys can be effectively decellularized with retention of ECM components, including fibronectin, collagen types I and IV, and laminin [57]. One study by Nakayama et al. showed that rhesus monkey acellular kidneys were able to support migrating host cells [83]. In addition, another study by Sullivan et al. found that decellularized porcine kidneys retained ECM microstructure as well as vascular network pathways. These acellular scaffolds were also non-cytotoxic to human renal cells [84]. This study brings the engineered kidney one step closer to vascularization. More research is necessary to find methods to re-endothelialize the vascular pathways in acellular kidneys.

1.7.2 Liver

Similar to the state of available therapies for kidneys, severely damaged and failing livers have no existing treatment options besides organ transplantation, which is limited by a shortage of donors [81]. Uygun et al. used a decellularization process to create an acellular rat liver scaffold with intact microvasculature. Adult hepatocytes were seeded via perfusion through the portal vein. Histological staining revealed that injected cells remained located in and around the blood vessel pathways. Whole blood was then perfused through the vasculature, and further analysis revealed that the injected cells remained viable and showed signs of metabolic activity during the blood perfusion [81,85]. Another study by Baptista et al. used ferret livers for decellularization. The acellular scaffolds retained the native ECM structure and a majority of the vascular network. However, some small holes in the vascular pathways were detected during a leak test. When seeded with endothelial cells, it was found that the cells adhered to the
lumen of the larger vessels and capillaries. In some samples, endothelial cells were seeded via the portal vein, and were mainly deposited in the downstream periportal area. In other samples, endothelial cells were seeded into the vena cava, and were found in the pericentral area of the liver. A portion of the cells were found to be aligned in the direction of medium flow through the scaffold as well [86]. These studies provide evidence that decellularization of small whole livers is obtainable, and that vascularization of acellular liver scaffolds is possible. There are also implications that the point of delivery of endothelial cells may have an effect on the efficacy of re-endothelialization. Further studies must be completed to scale up the size of the tissue constructs to be relevant to human livers, and to overcome existing obstacles such as non-continuous vessel walls that are unable to be fully covered by a monolayer of endothelial cells. With further investigation, decellularized and recellularized livers may be a future alternative treatment to liver transplants.

1.7.3 Lung

Around 50 million people worldwide are living with end-stage lung disease [87]. Lungs do not have the capability at the cellular level to naturally regenerate injured tissues, forcing patients to seek some form of treatment. Lung transplants are currently the only available option for severely damaged lungs, creating a need and market for potential tissue engineered lungs [88]. Tissue engineered lungs require a few key components, including presence of appropriate cell types, retention of mechanical abilities to withstand inhalation and exhalation, a blood-oxygen barrier, and an adequate vasculature system [88]. Ott et al. were able to decellularize rat lungs while preserving the vasculature and airway network pathways. Using a bioreactor to provide physiological stimuli, the vasculature in the scaffolds were seeded with endothelial cells, while the airways were seeded with epithelial cells via the trachea. It was shown that the engineered lungs performed adequate gas exchange comparable to native lungs during in vitro and in vivo testing [87]. A study by Petersen et al. using a similar set up for rat lungs showed similar results. A diagram of their experimental set up is shown in Figure 7. However, the blood-oxygen barrier was not complete in these studies, as there was leakage of blood into the airways. The lack of adequate surfactant led to non-physiologic alveolar surface tension and subsequently a decreased inhalation volume as well. Although
many cells were found in the engineered lung that are present in native tissue, the populations were less than those found in native tissue. The engineered lungs did lack a small number of cell types seen in native lung tissue as well [87,88]. An additional study showed the feasibility of seeding acellular lung scaffolds with alveolar cells derived from iPSCs, which successfully adhered on to the lung matrix. This provides additional information on the possible cell sources that can be used for lung scaffold repopulation [89].

Important strides have been taken in the creation and vascularization of acellular lung scaffolds. Endothelial cells are able to be successfully adhered to the lumen of large and capillary sized blood vessels of acellular lung tissue, and have the ability to proliferate and participate in gas exchange. Studies have also indicated that exposing the seeded scaffold to physiologic conditions, such as ventilation of air, may help increase cell viability and functionality. Larger lung models must be investigated next, and further experiments designed to overcome limitations shown in current literature must be performed.

**Figure 7:** Lung bioreactor designed by Petersen et al. A decellularized rat lung is placed into the bioreactor, where it is subjected to vascular perfusion via the pulmonary artery and to a "breathing loop" via the trachea. Exposure to mimicked physiological stimuli aids in the formation of a recellularized lung scaffold, which is then implanted into rats for analysis [88]
1.7.4 Heart

22 million people are living with some stage of heart failure worldwide, limiting their treatment options to LVAD implantation or heart transplant [59]. Mechanical LVAD devices are associated with complications such as device maintenance and biocompatibility, while heart transplants are limited by the severe shortage of donors. The decellularization and recellularization approach to engineered whole hearts could become the golden standard of heart disease therapy if current obstacles are analyzed and overcome. One of the largest barriers is a need for a vasculature system. This section will divulge the details of studies that have been performed to re-endothelialize decellularized heart scaffolds, since this information is the most relevant to the study presented in this paper.

The first study of its kind, Ott et al. proved that their decellularization process for whole rat hearts was both effective in removing cellular components and retaining important ECM components including collagen type I, collagen type III, laminin, and fibronectin. The retention of these components led to a visibly intact basement membrane and relevant composition of vasculature ECM. Large blood vessels down to small capillary branches were shown to be present in the acellular scaffold, proving that the vasculature system pathways were intact. 20 million rat aortic endothelial cells were then seeded into the construct via the aorta, and sat statically for 45 minutes to allow for cell attachment to the matrix. The scaffold was then placed in a bioreactor for one week. At this time point, endothelial cells had successfully attached to the lumen of larger and smaller coronary vessels, and adhered to the lumen of vasculature in the ventricular cavities. Within the vasculature, the average endothelial cell density was found to be 264.8 ± 49.2 cells/mm² [59].

Another study done by Schulte et al. used a perfusion technique via a cannulated aorta to successfully decellularize a portion of porcine myocardium surrounding the left coronary artery, shown in Figure 8. ECM components were retained following this procedure as well. The vasculature pathways were shown to be completely intact, down to the level of the capillary. In addition, arterial burst pressure was measured to quantify the mechanical properties of the blood vessel pathways. No significant difference was found between native arterial burst pressures and burst pressures of the acellular scaffold vasculature.
pathways. Retaining mechanical strength of the blood vessels may have an important influence on the ability of cells to attach to the lumen for re-endothelialization. Preliminary cell seeding studies showed that the acellular scaffold was non-cytotoxic toward relevant cardiac cells, including rat dermal fibroblasts and neonatal rat cardiomyocytes. Further studies are needed to evaluate the efficacy of this intact vasculature pathway to be seeded with endothelial cells or stem cells [90].

Figure 8: Native and decellularized porcine myocardium surrounding the left coronary artery. The decellularized myocardium patch is cannulated via the left coronary artery and vein in order to allow for the perfusion of solutions. The red arrow indicates native direction of flow of oxygenated blood, while the blue arrow indicates flow of deoxygenated blood [90]

Finally, a novel study by Robertson et al. was the first study to re-endothelialize decellularized whole rat hearts through an arterial and a venous delivery site. After successful decellularization and preservation of ECM components, an acellular heart was transplanted into a rat to test the efficacy of the vascular pathway in vivo. As expected, the acellular blood vessels experienced blood clots, even when treated with anti-coagulants. This evidence further substantiates the need for re-endothelialization of decellularized vascular networks. Rat aortic endothelial cells were then injected into the heart scaffolds.
either through the aorta, the brachiocephalic artery, or a simultaneous combination of the inferior vena cava and brachiocephalic artery. The seeded scaffolds were allowed to sit statically for one hour to allow for adherence of the cells before being recannulated and exposed to flowing medium for one week. Analysis of the scaffolds indicated that the endothelial cells injected via the brachiocephalic artery or the combination route were distributed throughout the entire heart, and expressed endothelial nitric oxide synthase as well as von Willebrand factor. Interestingly, endothelial cells delivered through the aorta were not uniformly distributed throughout the entire tissue construct. Therefore, the aortic delivery group was not used in further testing. The combination route showed a greater number of cells present in the scaffold and an increased number of large and small vessels lined with endothelial cells compared to the other singular routes. Thrombogenicity in vivo was also reduced compared to non-seeded acellular scaffolds, which is a key finding because one of the main functions of blood vessels is to provide a non-thrombogenic surface to allow for blood circulation. In vitro, the hearts were transplanted into the abdomen of rats, and then explanted after one week. There was less clotting in the re-endothelialized scaffolds compared to the acellular scaffolds. It was also found that re-endothelialization of the acellular scaffolds before recellularization of the left ventricular wall with neonatal cardiac cells led to increased contractility compared to recellularized left ventricular walls that were not re-endothelialized [91].

Similar to what has been found for tissue engineered lungs, there is strong evidence that exposing a re-seeded whole heart scaffold to physiological conditions may increase the survivability, proliferation, migration, and functionality of cells. Although these studies show promising results dealing with the re-endothelialization of acellular whole heart scaffolds, they are not clinically relevant at the current scale. These types of experiments must be repeated in a larger more relevant heart, such as porcine or human hearts. Further studies should aim to use a bioreactor to recreate physiologic conditions in order to achieve a complete re-endothelialization of the vasculature lumen. Eventually, studies will include complete recellularization of each tissue and cell type in the whole heart in order to ultimately generate a fully functional whole heart product.
Myocardial infarction is just one subset of cardiovascular disease, which currently affects 17.3 million people worldwide [3]. Current treatment options are associated with complications and significant mortality rates, with heart transplants being the sole therapy to actually restore function to the damaged myocardial tissue. Heart transplants are limited by a shortage of donors, and have limitations such as the need for a lifetime of immunosuppressant drugs. The decellularization and recellularization approach to tissue engineering is applicable to whole organ constructs, and may be the key to creating widely available, functional, and biocompatible organs for transplantation. Incredible progress has been made in the development of successful decellularization methods for whole organs in the past decade, which simultaneously preserve ECM components and the native architecture of the organ. Various studies have been performed that were aimed at the recellularization of organs and re-endothelialization of their vascular networks, including the lungs, liver, and heart. Placing an emphasis on heart models, a few decellularization methods have been developed that allow for the retention of the vascular pathways and ECM components of blood vessels such as the basal lamina. However, the recellularization and re-endothelialization of whole hearts must still be improved, and scaled up in size to be clinically relevant to humans. So far, research has only been able to provide proof of concept for re-endothelialization of decellularized heart scaffolds. The current most successful re-endothelialized whole heart constructs have been limited to rat models. Obtaining a complete monolayer of endothelial cells lining the entire lumen of the vascular network has been elusive as well. Further testing is imperative to optimize a re-endothelialization procedure for whole heart constructs, which must then be applied to larger heart models.

In summary, there is a need for a treatment option for damaged myocardial tissue that is widely available and restores function to the patient’s tissue. Using decellularized and recellularized whole hearts could be a viable option, but more research must be done to further explore the efficacy of this strategy. One main obstacle for this approach is adequate vascularization of acellular whole heart scaffolds. Only a handful studies have focused on the re-endothelialization of acellular myocardial tissues, and have found encouraging results. Re-endothelialization must still be optimized to create a construct equivalent to native blood vessels, which have a fully non-thrombogenic blood vessel lumen and are capable of nutrient and waste transport, among other important roles.
1.8 Project Scope, Aims, and Significance

1.8.1 Project Scope

Myocardial infarction (MI) affects 900,000 people each year in the United States alone, making it the leading cause of death \[5,6\]. Current treatment options for MI patients, including coronary artery bypass procedure, percutaneous coronary intervention, and LVADS, do not address the ischemic myocardial tissue in the left ventricle associated with MI. This tissue eventually forms fibrous scar tissue, which is non-vascularized and does not possess contractile abilities necessary for cardiac function \[8\]. Due to the scar tissue hindering heart function, patients left with this scar tissue undergo a degenerative process leading to congestive heart failure (CHF) \[9\]. In 2010, it was reported that 2.0% of Americans had CHF \[92\]. In addition, in 2011, heart failure cost the United States $32 billion annually, a number which grows each year \[93\]. It is clear that CHF and MI present great public health and economic concern. In order to reduce the number of patients living with post-MI complications and CHF, new therapies must be developed to restore function to damaged myocardial tissue.

The ultimate goal of this research is to create an acellular myocardial scaffold from xenologous tissue that is seeded with a patient’s own cells. This scaffold would then surgically replace damaged and infarct tissue, restoring function to the heart. One key challenge in creating tissue engineered products using a decellularized scaffold is acquiring sufficient vasculature. The research discussed in this paper is focused on the re-endothelialization of an acellular heart scaffold, in order to achieve adequate vascularization of the construct. Further research will include recellularization of the acellular scaffold by the seeding of other cell types that are known to reside in native myocardium. One possible immediate application of this construct is a “living patch” of myocardium, encompassing the area surrounding the left coronary artery and vein. This section of tissue would be surgically implanted to replace scar tissue in patients with ischemic tissue in the left ventricle. The product would be biocompatible since it would contain only cells from the host, and would integrate successfully and restore function to that area of the heart. Figure 9 shows an estimated timeline for the creation and implantation of such a living patch, starting at the time the MI patient is admitted into a hospital. The timeframes and proposed steps are based
off of recommendations and or findings from various groups listed under the references for Figure 9. This figure is only an estimate and hypothesis of how the living patch product may be used, it is not based on research. With regards to the decision of how long to wait after the patient is admitted to the hospital to perform surgery, several factors must be taken into consideration. If the construct is implanted before scar tissue forms, it may be possible to reduce the ischemic area, reduce the amount of scar tissue formed, and increase the functionality of the damaged area. However, it is possible that introducing the construct at this stage in wound healing will expose it to a hostile environment as the host tissue undergoes the inflammatory stages of wound healing. This may disrupt or destroy portions of the implanted scaffold and decrease the viability of the scaffold’s cells. Due to these factors, it is proposed that the living patch be implanted after the scar tissue has fully formed in the host, when there is no inflammatory response in the surrounding areas [94]. In addition to a section of the myocardium, it could be possible to use this approach to recellularize a complete whole heart scaffold that could be used in place of a donor heart in a heart transplant procedure as well.
Figure 9: Schematic of a possible timeline for the use of the “living patch” tissue engineered product for post-MI treatment. The MI patient arrives at the hospital and measurements are taken of the patient’s heart. An animal heart of the correct size is harvested and the decellularization process begins. The following day, all relevant cells types are harvested from the patient and expansion in culture flasks begins. Endothelial cells in particular are harvested from the patient’s microvasculature or umbilical cord blood [95]. Over the following six weeks, the patient is allowed to stabilize prior to surgical intervention [15]. The decellularization process of the heart scaffold is completed in this timeframe [90]. After six weeks, the acellular scaffold is seeded with the cells previously harvested from the patient and allowed to incubate statically for 1 hour to allow for cell attachment. The construct is then hooked up to a bioreactor for 1 week to aid in cell proliferation and functioning [59,91]. After 1 week, the living patch flap is cut from the heart scaffold and surgery is performed to remove the damaged myocardial tissue from the patient’s heart and then suture in the engineered patch product.

1.8.2 Project Aims

Aim 1: To develop and characterize a decellularized whole rabbit heart scaffold for use in cardiovascular tissue engineering.

Hypothesis: Our perfusion decellularization protocol for whole rabbit hearts will produce a decellularized heart scaffold with an intact vasculature pathway that lacks cellular content and preserves extracellular (ECM) components.
**Aim 2:** To re-endothelialize the acellular vasculature pathways surrounding the left coronary artery with clinically relevant cell types.

**Hypothesis:** *Acellular left ventricular rabbit myocardium will contain a network of vascular channels capable of supporting attachment of relevant endothelium cell types.*

### 1.8.3 Project Significance

This research will bring our laboratory one step closer to creating a “living patch” product as a post-MI treatment. It will also contribute to the scientific community’s knowledge of cardiovascular tissue engineering and specifically re-endothelialization of acellular scaffolds. This information will hopefully provide insight into cellular interactions and pathways that allow for attachment to decellularized lumens of vascular networks. Overall, the project will aid in the advancement of the field of cardiovascular tissue engineering as well as contribute to the effort of providing new tools to surgeons and clinicians in order to ultimately treat patients suffering from coronary heart disease.
Chapter Two

DECELLULARIZATION OF WHOLE HEARTS

2.1 Introduction

Decellularization of whole organs, or removal of cells in xenogenic tissues that would otherwise cause a host immune response, is one strategy to create a scaffold on which a tissue engineering construct can be built. A group led by Ott et al. successfully decellularized whole rat hearts in 2008 [59]. However, the size of the rat heart renders it far from clinically relevant to the human body. Approaching an appropriate size, Schulte et al. decellularized whole porcine hearts [90]. Guyette et al. successfully decellularized whole human cadaveric hearts as well [57]. At the present time, no literature can be found on the decellularization of whole rabbit hearts. Although rabbit hearts are still not a clinically relevant size for human therapies, they are scaled up from rat hearts. In terms of size, cadaveric human hearts are the best choice. However, there are concerns that these hearts may have an altered structure or degraded tissue due to their post-mortem status [57]. Rabbit hearts are certainly candidates for a decellularized scaffold source, due to the fact that various studies have supported the general acceptance of similarity between rabbit and human hearts. The physiological alterations that occur in failing myocardial tissue following MI are similar, as well as the composition of sarcomeric proteins, whereas rats have a significantly different composition [96,97]. A rabbits heart beat in beats per minute is more similar to humans compared to rats [98]. In addition, the smaller size of the rabbit heart compared to a porcine heart or cadaveric human heart requires a lesser number of cells and resources for experiments. This research team believes using rabbit hearts to create decellularized whole xenogenic heart scaffolds is a clinically relevant and valuable step toward the ultimate goal of creating fully functional hearts for human transplantation.

When creating decellularized scaffolds for use in tissue engineered constructs, there are two main goals. The first is to remove all cells and DNA content from the scaffold. The quantification of successful cell content removal has been defined in many different ways. For example, Guyette et al. used the color of
the decellularized heart as visual confirmation of complete decellularization, followed by quantitative validation of the decellularization process. This included tests such as detection of bioburden in the scaffolds [57]. Other tests used to determine decellularization efficacy include staining, such as DAPI stain for nuclei, and scanning electron microscopy (SEM) to detect presence of cells such as myofibers [59]. The second goal of a decellularized scaffold is to preserve as much of the extracellular matrix (ECM) components as possible. The ECM has been shown to contain environmental cues for cellular differentiation, proliferation, migration, and function [53,54]. Using decellularized scaffolds with native ECM structure and components may provide a huge advantage during the recellularization process. Characterization of decellularized scaffolds must be performed in order to analyze which ECM components were removed, damaged, or preserved during the given decellularization process. Which ECM components must be retained and in what acceptable quantity depends on the final application of the tissue engineered construct.

2.2 Methods

2.2.1 Whole Heart Preparation

10 rabbit hearts were obtained over a period of 4 days from Godley Snell Animal Facility in Clemson, SC. Each was gently pressed and massaged by hand immediately after harvesting to remove large blood clots and excess blood. The hearts were then rinsed in saline and excess fat was cut off. The hearts were stored in saline for 30 minutes during transportation to the lab. 1mL of warm 50mM EDTA in phosphate buffered saline (PBS) was perfused through the aorta 6 times using a 3mL syringe in order to wash out blood and prevent further clotting. All hearts were placed in a -20°C freezer while more hearts were obtained, and hearts from the last harvesting day were placed in the freezer overnight. This was done to keep the treatment consistent for all heart samples. Due to the small size of hearts, all valves, including the aortic valve, were kept intact. A “branching” set up of 1/8 inch inner diameter tubes was used to connect the 10 hearts inside the same decellularization (decell) chamber, hereby referred to as the rabbit
heart tree. Each heart was cannulated via the aorta, which was secured to the end of a 5/32 inch y-connector using 2.0 Vicryl Plus suture thread.

2.2.2 Decellularization System Set Up

The decell system was created by modifying the system used by Dr. Schulte in his porcine heart decellularization protocol [90]. The decell system included a decell chamber, two fluid reservoirs, and a multi-channel peristaltic pump (Masterflex, Cole-Parmer). The rabbit heart tree was connected inside of the decell chamber. The rest of the system was connected using ¼ inch inner diameter tubes. The first fluid reservoir was located 90cm above the cannulated aortas of the rabbit hearts. This elevation generated a hydrostatic pressure of about 80mmHg into the decell chamber. Assuming negligible flow losses through the system and that all assumptions for Bernoulli’s equation are met, the hydrostatic pressure reaching each heart was about 69mmHg. The flow rate was about 200mL/min. However, the actual pressure and flow rate through each rabbit heart was dependent on the individual unique resistance created by the heart tissue. The second fluid reservoir was located at an arbitrary distance beneath the decell chamber, which provided fluid back to the pump. 2L of fluid was used in each step of the decell process.

2.2.3 Decellularization Procedure

A solution of 30mM EDTA with 0.02% sodium azide (to help prevent contamination and bacterial growth in the heart tissue and system) in PBS without Ca/Mg (pH = 7.5) was perfused through the system for 12 hours in order to remove any residual clotted blood from the heart vasculature. Next, a 1% sodium dodecyl sulfate (SDS) solution with 0.02% sodium azide was perfused through the system. This solution was replaced with fresh 1% SDS with sodium azide solution every 4 days for 16 days, for total of four 1% SDS washes. The weights of the hearts were taken after each SDS decell solution change. Next, distilled deionized (ddi) water was pumped through the system for 4 hours. A solution of ddi water with 0.02% sodium azide was then perfused through the system for 12 hours, followed by another 4 hour wash of pure ddi water. Next, 70% ethanol was perfused through the system for 4 hours. Fresh 70% ethanol was run
through the system for 12 hours, followed by another 4 hour wash. Next, the ddW water washes of 4 hours, 12 hours with sodium azide, and another 4 hours was repeated. Then PBS without Ca/Mg was perfused for 4 hours, followed by 12 hours with 0.02% sodium azide, and then 4 hours without sodium azide. Next, 720 munits of both RNase/DNase in PBS with 5mM MgCl₂(H₂O)₆ and 0.02% sodium azide (pH = 7.4) was perfused through the system for 48 hours to ensure all cell and DNA content were removed. Finally, the PBS wash step of 4 hours, 12 hours with sodium azide, and another 4 hours was repeated. The decellularized rabbit hearts were then stored in PBS with 0.02% sodium azide and 0.001% protease inhibitor at 4 °C to help prevent contamination and growth of bacteria.

2.2.4 Observation of Vascular Pathway Preservation

A Batson’s Corrosion kit from Polysciences, Inc. was used to observe the degree of preservation of the vasculature pathway encompassing the left coronary artery, left coronary vein, and surrounding areas. The polymer was mixed with red dye to aid in visualization of the pathway. The polymer was slowly injected using a 3mL syringe into the left coronary artery of a decellularized rabbit heart. The injection process was halted immediately once the polymer appeared to stop its progression through the vasculature and instead began to burst through small capillaries near the surface of the heart, creating red beads as shown in Figure 11. The polymer was allowed to cure according to kit instructions and then the tissue macerated, or dissolved, to reveal a hardened polymer cast of the vasculature pathway.

2.2.5 Histology

Native porcine cardiac tissue was used as a control. Samples of the left coronary artery and the surrounding areas were taken from the control tissue and decellularized rabbit heart tissue for ECM analysis. Histological staining, including Hematoxylin and Eosin (H&E) and DAPI stain, were used to evaluate the presence of cells. A Luna stain was used to evaluate elastin content. The Luna stain protocol was taken from IHC World’s “Luna Staining Method and Protocol for Elastic Fibers and Mast Cells”. The only adjustment made was a substitution of the paraldehyde solution for acetaldehyde. A ready-made
methyl orange solution from Polyscientific Inc. was used, and the components for the Weigert’s Iron Hematoxylin Working Solution were obtained from Polyscientific as well. In addition, Masson’s Trichrome stain using a kit from Polyscientific Inc. was performed to evaluate present collagen. Quantification of collagen content from the trichrome stain images for the control group (n=5) and decellularized group (n=5) was calculated using the “Color Adjustment”, “Binary”, and “Analyze Particle” functions in the ImageJ software (NIH). The percent of collagen (stained blue) within each imaged tissue section was calculated.

2.3 Results

Table 1: Weight of Rabbit Hearts during Decellularization Process

<table>
<thead>
<tr>
<th>Hearts</th>
<th>Weight [g]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After tied onto y-connectors</td>
</tr>
<tr>
<td>1&amp;2</td>
<td>17.75</td>
</tr>
<tr>
<td>3&amp;4</td>
<td>16.67</td>
</tr>
<tr>
<td>5&amp;6</td>
<td>15.88</td>
</tr>
<tr>
<td>7&amp;8</td>
<td>16.64</td>
</tr>
<tr>
<td>9&amp;10</td>
<td>15.60</td>
</tr>
</tbody>
</table>

Table 2: Mass Change of Rabbit Hearts during Decellularization Process

<table>
<thead>
<tr>
<th>Hearts (n=5)</th>
<th>Mass Change [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After EDTA wash step</td>
</tr>
<tr>
<td>1&amp;2</td>
<td>57.75</td>
</tr>
<tr>
<td>3&amp;4</td>
<td>31.97</td>
</tr>
<tr>
<td>5&amp;6</td>
<td>57.43</td>
</tr>
<tr>
<td>7&amp;8</td>
<td>62.26</td>
</tr>
<tr>
<td>9&amp;10</td>
<td>66.67</td>
</tr>
<tr>
<td>Mean</td>
<td>55.22</td>
</tr>
<tr>
<td>Std Dev</td>
<td>13.53</td>
</tr>
</tbody>
</table>
Figure 10: Color change of whole rabbit hearts during decellularization process. A: After EDTA wash step, B: After SDS wash #1, C: After SDS wash #2, D: After SDS wash #3, E: After SDS wash #4. The hearts are in numerical order from left to right, starting with Heart #1 on the left.
Figure 11: Injection of red polymer into the left coronary artery (LCA) of a decellularized rabbit heart. A: The polymer traveled through the smaller vessels and capillaries branching from the LCA (balloon 1), B: The injected polymer eventually converged into the left coronary vein (balloon 2), proving that the vasculature pathway from the LCA to the left coronary vein is fully intact. Balloon 3 shows bursting of polymer through tissue wall
**Figure 12:** Images of histological stains of native porcine myocardium and decellularized rabbit myocardium surrounding the left coronary artery at 100x (bar=100 µm). Column A: H&E stain showing the presence of cell nuclei (blue), cytoplasmic proteins (dark pink), and collagen (pale pink) in the native tissue and absence of cell nuclei in the decellularized tissue. Column B: DAPI stain showing the presence of cell nuclei (blue) in the native tissue and absence of cell nuclei in the decellularized tissue. Column C: Luna Stain showing presence of elastin (purple) in the native tissue and decellularized tissue. Column D: Masson’s Trichrome stain showing the presence of muscle fibers (red), cytoplasm (light red), and collagen (blue) in both the native tissue and decellularized tissue.

**Table 3:** Analysis of Collagen Content for Decellularized Tissue Samples

<table>
<thead>
<tr>
<th>Image #</th>
<th>Collagen Area µm²</th>
<th>Total Area µm²</th>
<th>% Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3762.966</td>
<td>40716.499</td>
<td>9.242</td>
</tr>
<tr>
<td>2</td>
<td>18207.117</td>
<td>74658.776</td>
<td>24.387</td>
</tr>
<tr>
<td>3</td>
<td>20557.535</td>
<td>87109.968</td>
<td>23.6</td>
</tr>
<tr>
<td>4</td>
<td>20266.381</td>
<td>89920.202</td>
<td>22.538</td>
</tr>
<tr>
<td>5</td>
<td>16244.413</td>
<td>64693.983</td>
<td>25.11</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>15807.682</strong></td>
<td><strong>71419.886</strong></td>
<td><strong>20.975</strong></td>
</tr>
</tbody>
</table>
Table 4: Analysis of Collagen Content for Native Tissue Samples

<table>
<thead>
<tr>
<th>Image #</th>
<th>Collagen Area µm²</th>
<th>Total Area µm²</th>
<th>% Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14344.851</td>
<td>126856.449</td>
<td>11.308</td>
</tr>
<tr>
<td>2</td>
<td>17025.109</td>
<td>131310.298</td>
<td>12.966</td>
</tr>
<tr>
<td>3</td>
<td>14663.834</td>
<td>100549.947</td>
<td>14.584</td>
</tr>
<tr>
<td>4</td>
<td>34087.536</td>
<td>107244.584</td>
<td>31.785</td>
</tr>
<tr>
<td>5</td>
<td>53009.046</td>
<td>107971.835</td>
<td>49.095</td>
</tr>
<tr>
<td>Average</td>
<td>26626.075</td>
<td>114786.623</td>
<td>23.948</td>
</tr>
</tbody>
</table>

Table 5: Comparison of Collagen Content of Decellularized and Native Tissue Samples

<table>
<thead>
<tr>
<th>Tissue Sample</th>
<th>Average % Collagen</th>
<th>% Collagen Retention following Decellularization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decell RB</td>
<td>20.975</td>
<td>87.586</td>
</tr>
<tr>
<td>Native P</td>
<td>23.948</td>
<td></td>
</tr>
</tbody>
</table>

2.4 Discussion

2.4.1 Decellularization Cues by Mass Loss

The weights of the rabbit hearts, displayed in Table 1, were taken after the hearts were tied to the y-connectors for ease of measurement during the decellularization process. This weight includes the weight of the y-connector along with two hearts. The purpose of these measurements was to get an idea of the mass loss of the tissues as the decellularization progressed, not to obtain exact weights of each heart.

Releasing each heart from the y-connector and then re-cannulating after each measurement step would have
weakened the aorta of the hearts and eventually render then unable to be re-cannulated. The percent of mass change is shown in Table 2. Before each measurement, each heart was massaged to remove as much of the fluid inside the heart as possible to get a more accurate and precise reading of the weight. However, this is a variable in weight fluxuations that should be noted. Each weight measurement is an estimate. A positive % change correlates with an increased weight, as seen after the EDTA wash step. This is due to the retention of decellularization solutions within the heart tissue. Beyond this step, there is only one positive percent increase, for Hearts #3 & #4 after the first SDS wash. This could be due to increased fluid retention in the hearts. The remainder of the mass changes are negative, meaning mass was lost, which is expected as cells are removed from the tissues. It is also evident that the last SDS wash, SDS wash #4, was not as effective in removing mass from the hearts as the previous three SDS washes. Therefore, it could be concluded that for further experiments using similarly sized rabbit hearts, decellularization should be stopped after SDS wash #3 in order to shorten the process time, lessen tissue exposure to decellularization agents that could potentially damage the ECM, and preserve resources.

2.4.2 Decellularization Cues by Color Change

A photo was taken of the rabbit hearts after the EDTA step in the decell process and after each SDS wash to observe the changes in color, as displayed in Figure 10. A significant color change is noted after the first SDS wash step in all of the hearts. The loss of color is due to the removal of cellular components. Hearts #2 & #9 appear to have a residual blood clot inside one of the ventricles, which is evidently removed during the second SDS wash as they are not present in image C. All hearts appear to lose color after SDS wash #2. However, there is not visual evidence of further decellularization after SDS wash #3 & #4. Only using visual cues, it is unclear whether or not SDS washes #3 & #4 were necessary. Using the information of mass loss provided in Table 2, it could be concluded that SDS wash #3 did aid in decellularization, but SDS wash #4 may not have been effective in removal of additional cellular components. Previous studies have based perfusion decellularization times solely off of color change, or the appearance of a white and translucent organ, and have shown results of successful removal of cells and
preservation of ECM components using this criteria [57,59]. One study by Schulte et al. also monitored changes in mass to determine decellularization efficacy [90]. Further experiments with rabbit hearts would need to be performed with varied numbers of SDS wash steps in order to find the minimal number of SDS washes needed for complete decellularization. Less exposure to SDS will minimize the damage done to the ECM, as well as preserve resources and time.

2.4.3 Preservation of the Vasculature Pathway

Perfusing decellularization solutions through the aorta was expected to result in preservation of the vasculature pathways within the rabbit heart scaffolds. The perfusion decellularization technique for whole organs has been shown to preserve the major vascular pathways and microvasculature in organs including kidneys, lungs, livers, and hearts [59,81,84–86,88,90]. In order to determine if our decellularization protocol yielded similar results to previous literature studies, a corrosion cascade kit was used. As shown in Figure 11, injection of a polymer into the left coronary artery revealed that the vessels branching off of the left coronary artery are still intact, as well as the capillaries. These capillary pathways are connected to branches of the left coronary vein, and eventually converge into the left coronary vein. This proves that the vasculature pathway beginning at the left coronary artery and ending at the left coronary vein was preserved. This finding is in agreement with current literature in the field. A greater volume of injected polymer that highlighted more capillaries and completely filled the left coronary vein would have been optimal. However, this was not able to be accomplished because polymer began to burst from the capillaries onto the surface of the tissue, as shown in Balloon 3 of Figure 11. Usually, with corrosion cascade kits, the tissue is macerated and a solid cast of the target pathway remains. Due to the small size of the rabbit heart vessels, the polymer folded onto itself during the maceration of the tissue. The outcome is not shown in this paper, as it is unable to provide additional information about the vasculature of the rabbit heart.
2.4.4 Removal of Cellular Content

In general, tissue decellularization involves removal of cellular content as well as preservation of ECM components. The first can be measured in a variety of ways, including quantification of the amount of bioburden in the flowing solutions over time, histological and immunofluorescent staining, scanning electron microscopy imaging, and biochemical quantification of DNA content [57,59]. In this study, an H&E stain was performed on the native and decellularized myocardium tissue, as shown in Column A of Figure 12. The cell nuclei are stained blue. There is clearly a complete absence of cells in the decellularized tissue. To further assess cell removal, a DAPI stain which fluorescently binds to A-T regions of DNA within the cell nuclei was used, as shown in Column B of Figure 12. This stain confirmed that no cell nuclei are present in the decellularized tissue, which was expected with the decellularization protocol utilized. Many studies using perfusion decellularization have cited SDS as the most effective detergent for decellularization, and have shown results of sufficient removal of cellular content using histological data and quantitative data [57,59,91]. Our findings of cellular content removal by an SDS based decellularization protocol concur with the conclusions of these studies.

2.4.5 Retention of ECM Components

The second main goal of tissue decellularization is to preserve as much of the native ECM structure and components as possible, in order to create a scaffold that closely mimics native tissue. Histological stains and other forms of analysis have been employed in previous studies to qualitatively and quantitatively analyze target ECM components. The components deemed the most crucial to retain is dependent on the intended final application of the scaffold. In this study, the aim is to re-endothelialize the vascular pathway of rabbit hearts. Therefore, retention of ECM components related to blood vessels and endothelialization mechanisms are the most important, such as those found in the basal lamina. The basal lamina is the thin ECM sheet that endothelial cells lining blood vessels adhere to. It consists of collagen type IV, elastin, laminin, fibronectin, and nidogen, as well as proteoglycans and glycosaminoglycans. Due to constraints of time, collagen and elastin content was examined in this study. A Luna Stain for elastin was
performed on native and decellularized tissue. In Column C of Figure 12, elastin fibers are stained purple. It is evident that elastin was preserved during the decellularization process. Column D of Figure 12 shows a Masson’s Trichrome stain of the native and decellularized myocardium. Muscle fibers are dyed red, cytoplasm light red, and collagen blue. It is evident that collagen was retained in the decellularized tissue. For quantitative analysis, ImageJ software was used to analyze the percent of collagen seen on histological sections imaged at 200x stained with Masson’s Trichrome stain. Table 3 shows the raw data for the decellularized rabbit heart sections, while Table 4 shows the raw data for the native porcine tissue sections. As highlighted in Table 5, there was about 87.5% retention of collagen content in the decellularized samples compared to the native tissue samples. However, due to the structural changes that the decellularized rabbit heart tissue underwent during the decellularization and tissue sectioning process, it was difficult to find arteries and veins in the decellularized histology sections taken for this analysis. If these structures could have been identified, then collagen content of the left coronary artery along with only myocardium and capillaries could have been more accurately compared between the two groups. If this study is repeated, more attention will be paid to get cleaner decellularized tissue sections in order to get more accurate comparison of collagen content between native and decell tissues. With the current data, it is still very clear that the decellularization process resulted in excellent collagen retention. A previous study performed by Schulte et al. using a decellularization procedure that was modified for our rabbit heart study visually confirmed the presence of collagen and elastin in the acellular myocardium using Masson’s Trichrome stain and Verhoeff’s Van Gieson stain, respectively. Quantification of collagen and elastin content from the experimental group that underwent a decellularization procedure most similar to the one in this study was obtained using a total hydroxyproline and desmosine analysis. The mean collagen weight percentage and elastin weight percentage was significantly different compared to the native myocardium tissue. The ratio of collagen to elastin weight percentage for the SDS treatment group was found to be around 30.5%. Further analysis also revealed that ECM components including collagen IV, laminin, and fibronectin, were well preserved [90]. Due to the similarity of decellularization process used, we expect our decellularized rabbit myocardium to have similar ratios of collagen and elastin, as well as suspect that collagen IV, laminin, ad fibronectin were preserved. In addition to preservation of ECM components,
retention of the mechanical and structural properties are of interest in order to create an optimal
decellularized scaffold. In the study by Schulte et al., uniaxial tensile testing of decellularized and native
myocardium revealed that the decellularized myocardium possessed a significantly higher elastic modulus
than the native tissue. To analyze the structure of the scaffold, histological analysis was used to identify and
measure cylindrical voids present in the acellular scaffold, presumably left behind by removal of various
cell types. The pores created by removal of cardiomyocytes were about 20 x 40 µm in size [90]. Future
studies aimed at analyzing the decellularized rabbit heart myocardium should include quantification of
elastin content, DNA content, analysis of structural and mechanical properties such as pore size and elastic
modulus, as well as characterization of other ECM components found in native myocardium.

2.5 Conclusion

Based off of previous literature where groups have decellularized whole organs, it is sufficient to use
observed changes in color and mass to estimate when the removal of cellular components is complete.
However, based on the results presented in this section, four SDS washes performed as described in the
methods section may not be optimal. Further testing is needed to determine the minimum number of SDS
washes for rabbit hearts needed to effectively remove cellular components. Washes beyond the minimal
requirement will not impede the decellularization process, but may lead to increased destruction of the
ECM. The vasculature pathway beginning at the left coronary artery and ending at the left coronary vein
was found to be intact, which was expected due to previous accounts of perfusion decellularization being
used to preserve blood vessel pathways. The efficacy of the decellularization process in terms of cell
removal and ECM component retention was qualitatively analyzed using histology, along with a
quantitative analysis of collagen using ImageJ software. The perfusion decellularization protocol using 1%
SDS was successful in removing cellular content from rabbit hearts while preserving various ECM
components, including cytoplasm, muscle fibers, elastin, and collagen. It is assumed that additional ECM
components associated with the basal lamina were retained, including laminin and fibronectin, based off of
the findings of the work of Schulte et al. The preservation of ECM components and the vasculature
network structure is critical for the next aim of the study, which is to re-endothelialize the acellular blood vessel pathways of decellularized rabbit hearts. This is the first study of its kind to decellularize whole rabbit hearts and utilize them as a scaffold for a tissue engineered construct. Currently, only whole rat hearts have been decellularized. They are also the only whole heart source to have undergone attempts at recellularization. The rabbit heart is larger than the rat heart and accepted as similar to human hearts, and is therefore closer to a clinically relevant whole heart model for eventual human use.
Chapter Three

SEEDING OF THE VASCULATURE IN A DECELLULARIZED SCAFFOLD

3.1 Introduction

One of the key challenges in creating tissue engineered constructs is providing adequate vasculature. In tissues that require vascularization for sustenance, each cell must be no more than 100-200 µm away from a capillary [12,24,28]. Vascularization also provides a non-thrombogenic surface for blood to flow, and has an important role in immune responses and wound healing. This means that successful recellularization of a whole heart must contain, along with other appropriate cell types, a full monolayer of endothelial cells lining the lumens within the vasculature tree. Several attempts have previously been made to re-endothelialize decellularized scaffolds derived from whole livers, lungs, and hearts [59,81,85–88,91]. All were able to confirm attachment of endothelial cells to the lumen of the vasculature in large vessels and in capillaries. However, none have been successful in creating a monolayer of endothelial cells. During analysis, leaks occurred in airways for lungs and clotting occurred in hearts, indicating an incomplete layer of cells [88,91]. More research is necessary for successful re-endothelialization of the vascular tree in decellularized whole organs.

3.2 Methods

3.2.1 Overview of Seeding Experiment Evolution through Three Trials

Three seeding experiments using the decellularized rabbit hearts were performed. The first and second seeding experiments each used one heart, while the third used four hearts. In all of the experiments, the hearts remained cannulated via the aorta to the same Y-connector as they were during the decellularization procedure. In the first two seeding experiments, a stopper was placed on the opposing end of the Y-connector to direct fluid flow into the single hearts. These system configurations required only one
decell chamber, and the height of the fluid reservoir was 10cm above the cannulated aortas. For the third experiment, two decell chambers were used, and the height from the fluid reservoir to the aortas was 21cm. The tissue fixation step was altered between experiments as well. In experiment #1, 3mL of 10% buffered formalin solution was injected using a 3mL syringe directly into the left coronary artery 3 times. The heart was then placed back into the pump system and 300mL of formalin was perfused through the heart at 50rpm for 24 hours. For experiment #2, 3mL of buffered formalin was injected using a 3mL syringe into the left coronary artery 3 times, and then the heart was placed in a p-cup with formalin for static fixation for 12 hours. Experiment #3 used only the static fixation technique. In addition, the differences between the three seeding experiments included altered cell population numbers and incubation times, as highlighted in Table 6. The procedure for seeding experiment #3 is explained in detail below.

**Table 6: Cell Population and Incubation Times for Three Cell Seeding Experiments**

<table>
<thead>
<tr>
<th>Seeding Experiment</th>
<th># of Hearts</th>
<th>Cell Type</th>
<th>Cell Population</th>
<th>Incubation Time in Scaffold</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>1</td>
<td>Human Aortic Endothelial Cells</td>
<td>6.5 million cells</td>
<td>18 hours</td>
</tr>
<tr>
<td>#2</td>
<td>1</td>
<td>Human Aortic Endothelial Cells</td>
<td>2 million cells</td>
<td>6 hours</td>
</tr>
<tr>
<td>#3</td>
<td>2</td>
<td>Human Aortic Endothelial Cells</td>
<td>2.8 million cells</td>
<td>6 hours &amp; 18 hours</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Human Adipose Derived Stem Cells</td>
<td>380,000 cells</td>
<td>6 hours &amp; 18 hours</td>
</tr>
</tbody>
</table>
3.2.2 Seeding Experiment Procedure

All system components were sterilized with EtO prior to use. The system configuration, shown in Figure 13, has main five components, including one fluid reservoir, two decell chambers, a sterile air filter, and a pump (323, Watson Marlow). Each step of the seeding protocol involving sterile tissue was performed inside a sterile cell culture hood. Four decellularized rabbit hearts were connected inside the decell chambers as shown in Figure 14. 600mL of a 0.1% peracetic acid solution was added into the system via the fluid reservoir to sterilize the tissue. The system was removed from the sterile hood and placed on the lab bench where it was connected to the pump. The peracetic acid was perfused through the system for 4 hours at 50rpm. The peracetic acid solution was then removed from the system, and 400mL of 1X PBS without Ca/Mg was perfused through the system for 1 hour to rinse out the peracetic acid. The PBS solution was then exchanged for fresh PBS and pumped through the system for 2 hours. A final PBS rinse was then performed for 15 minutes. The PBS was then removed from the system, and 400mL of 50% fetal bovine serum (FBS) and 50% DMEM solution was perfused through the system for 12 hours in order to coat the tissue with fibronectin to aid in cell binding. At this time, human aortic endothelial cells (ECs) and human adipose derives stem cells (ADSCs) were recovered from flasks and suspended in EGM-2 (Lonza Clonetics®) medium and 1X DMEM (Dulbecco’s Modification of Eagle’s Medium) (Corning cellgro®) with 9% FBS and 1% anti-biotic medium, respectively. During the recovery process, each cell solution was centrifuged and resuspended twice to remove any residual trypsin.
Figure 13: Schematic of system configuration for seeding experiments of decellularized rabbit hearts. Red arrows indicate direction of fluid flow
1mL of suspended ECs was placed in a 3mL syringe and injected directly into the left coronary artery of Heart #1 located in Decell Chamber A, as depicted in Figure 15. Another 1mL of suspended ECs was injected in the same fashion into the left coronary artery of Heart #3 in Decell Chamber B. Two drops of suspended ECs was placed in well A1 of a 96 well plate. Next, 1mL of suspended ADSCs was injected into the left coronary artery of Heart #2 in Decell Chamber A, and another 1mL into Heart #4 in Decell Chamber B. Two drops of this solution was added to well B1 of the 96 well plate. The system and well plate were placed in a cell incubator for 6 hours. After 6 hours, Hearts #1 and #2 were removed from the system and placed in separate p-cups containing 10% buffered formalin solution for static fixation. The well plates containing cells were observed for cell attachment and viability using a light microscope. After 12 additional hours, for an incubation time of 18 hours total, Hearts #2 and #4 were removed from the incubator and placed in p-cups with formalin for static fixation. The well plate was again observed for cell viability. All tissues were left in formalin overnight. The tissues were then sectioned and stained using H&E and DAPI stain to detect presence of cells.
Figure 15: Photograph of the injection of cells into the left coronary artery of a decellularized rabbit heart scaffold
3.3 Results

**Figure 16**: DAPI and H&E stain at 200x of Seeding Experiment #3 using human aortic endothelial cells (ECs). The arrows point toward cells in the images. Panels A1-A3 show that ECs are present in the decellularized rabbit heart scaffold that underwent an incubation time of 6 hours. Panels B1-B3 show that ECs are present in the scaffolds that underwent an incubation time of 18 hours as well. The DAPI Overlay images shows that the ECs have attached to the lumen of a decellularized vessel pathway for both incubation time treatments.
**Figure 17:** DAPI and H&E stain at 200x of Seeding Experiment #3 using human adipose derived stem cells (ADSCs). The arrows point toward cells in the images. Panels C1-C3 show that ADSCs are present in the decellularized rabbit heart scaffold that underwent an incubation time of 6 hours. Panels D1-D3 show that ADSCs are present in the scaffolds that underwent an incubation time of 18 hours as well. The DAPI Overlay images shows that the ADSCs have attached to the lumen of a decellularized vessel pathway for both incubation time treatments.
3.4 Discussion

3.4.1 Seeded Cell Types

The preliminary first and second seeding experiments used one rabbit heart each in order to conserve resources while working through and correcting issues to make the protocol successful in reseeding the vasculature of the acellular left coronary artery. DAPI and H&E results from these two experiments are not shown due to the lack of cells attached to the vasculature walls. The third experiment used four hearts in order to increase the number of variables tested that could possibly contribute to the absence of cells seen in the first two experiments. The third seeding experiment included two cell types, human aortic endothelial cells (ECs) and human adipose derived stem cells (ADSCs). ECs were used as a control group and to test the efficacy of the seeding procedure, since native heart vasculature is lined with a monolayer of ECs. However, in clinical application, harvesting ECs from the patient in a quantity sufficient to seed a human sized decellularized heart scaffold may take an impractical amount of time and resources. One study found than in healthy subjects, 1-3 cells per 1 mL of circulating blood is an endothelial cell [99]. Stem cells, or a combination of stem cells and endothelial cells, may be the optimal choice for cell types injected into decellularized coronary vasculature. So far, current literature shows that stem cells have not been used in attempts to re-endothelialize decellularized coronary vasculature [59,91]. In our study, ADSCs were used to assess the efficacy of using stem cells to aid in re-endothelialization. ADSCs are easily harvested from patients in large quantities. Endothelial progenitor cells (EPCs) were also considered for use in this study, but were not utilized due to time constraints and difficulties of harvesting large quantities. Several publications have shown that it is possible to direct these two autologous stem cell types toward differentiation into ECs, which are needed to create a non-thrombogenic surface in the vasculature pathway. It is also possible to differentiate ADSCs and EPCs into cardiomyocytes. Cardiomyocytes are a key cell type in myocardium and must be integrated into the final tissue engineered construct, making ADSCs and EPCs an even more attractive option for cell sourcing. Methods for inducing cell proliferation and expansion in vitro have also been published for these cell types [100–102]. The optimal cell type or
combination of cell types for re-endothelialization of a decellularized coronary vasculature system has not yet been explored, but it is clear that there are several options of cell sources to consider.

3.4.2 Attachment of Cells to Decellularized Coronary Vasculature System

Figure 16 shows the results for Hearts #1 and #3 from Seeding Experiment #3 that were injected with ECs and incubated for 6 and 18 hours, respectively. Figure 17 shows results for Hearts #2 and #4 that were injected with ADSCs and incubated for 6 and 18 hours. All image panels of DAPI and H&E show the presence of cells in the scaffolds. The DAPI Overlay images show that the location of these cells are along the wall of the vasculature lumen, where endothelium is found in native tissues. More cells are seen in the hearts injected with ECs compared to ADSCs, presumably because there was a much greater population of ECs available for injection compared to ADSCs. There is no evidence supporting or refuting that ECs have a greater or lesser ability to attach to the decellularized matrix than ADSCs. Further studies would be necessary to investigate this idea.

The success of all four of the experimental groups in Seeding Experiment #3 provides additional information on the characterization the decellularized scaffold as well. It is evident that the decellularization procedure allowed a sufficient amount and number of ECM components and structural properties to be retained in order to allow for support of attachment of ECs and ADSCs.

3.4.3 Progression from Unsuccessful to Successful Seeding Experiments

Possible variables that were considered to possibly affect the outcomes of the experiments included extended incubation times that could have deprived the cells of oxygen and nutrition due to absence of flow of media during incubation, inflicted mechanical damage leading to cell death from being injected through a small syringe needle tip, the number of injected cells, and the fixation strategy that may cause detachment of cells from the vessel walls.

Due to the success of Seeding Experiment #3 and failure of the previous experiments, several conclusions can be made. First, an incubation time of 6 hours is long enough to allow for attachment of
cells to the vasculature lumen, and an incubation time of 18 hours is short enough to retain cell viability within the incubator without flowing or exchanging media during incubation for the left coronary artery pathway in rabbit hearts. Second, using a syringe to inject the cells by hand at a rate of .5mL/min was sufficient to allow for cell attachment. Also, ejection through the syringe tip did not cause damage leading to the death of cells. This was further confirmed by analysis of the 96 well plate containing ECs and ADSCs ejected through the syringe and incubated for 6 hours and 18 hours. Cells remained viable and attached to the well plate throughout the duration of the study, proving that ejection through the syringe did not cause cell death.

In addition, the number of cells necessary for cell attachment to the lumen of the decellularized vasculature injected was also a concern. Less ECs were used in Seeding Experiment #3 compared to Seeding Experiment #1, suggesting that the reason for the success of the first and failure of the latter was not due to cell population. In a study done by Robertson et al., 2.0 x 10^7 rat aortic endothelial cells were perfused into the aorta of decellularized rat hearts, which are significantly smaller than rabbit hearts [91]. A study by Ott et al. also used 20 million rat aortic endothelial cells seeded through the aorta in their procedure [59]. The implication would be that re-endothelializing a rabbit heart would take many more cells than this in order to successfully promote cell attachment. However, these studies did not test the lower limit of cells needed for successful attachment of ECs to vasculature walls. Due to the success of Seeding Experiment #3, which used 2.8 million EC cells for Hearts #1 and #3 and only 380,000 ADSCs for Hearts #2 and #4, this study concludes that the mentioned number of cells are enough to successfully attach ECs and ADSCs, respectively, to the lumen of a decellularized vasculature scaffold. Our study shows that less cells than have been previously reported in published experiments are required for attachment of cells to a decellularized vascular wall. However, of course, more cells are needed to create a complete monolayer of cells along the lumen.

Finally, it is probable that the reason for the failure of Seeding Experiments #1 and #2 was the fixation procedure. The fixation strategy originally consisted of injection of formalin directly into the coronary artery at .5mL/min by hand, followed by perfusion of formalin using the entire pump system through the heart. The perfusion step was not performed in the second experiment. In the third, both the
injection step and perfusion step were not performed, and the hearts were only statically fixed in formalin. Injecting formalin by hand, as well as perfusing formalin through the pump system, may have detached any injected cells that had previously attached themselves to the vessel walls. Seeding Experiment #3 only used static fixation, and cells were found in the scaffold. However, it several literature studies, cells were seeded in decellularized rat whole organs and then placed in a bioreactor where media constantly flowed through the construct [59,88,91]. The cells remained attached to the vasculature lumen. It is possible that injection of formalin by hand in this study was too fast or too inconsistent, causing cells to detach. It is also possible that perfusion of media, which has a different density and fluid properties than formalin, does not cause detachment of cells. It is known that physiological shear stress induced by blood flow aids in endothelial cell migration and proliferation. Controlled flow in the bioreactor studies probably aided in EC attachment and proliferation. In addition, a lesser number of injected cells would be required if they were able to proliferate and migrate once attached to the ECM in order to create a complete monolayer. It is suggested that subsequent studies utilize a bioreactor which is able to perfuse media and cells through the coronary vasculature at a physiological rate and pressure, as well as deliver an electrical pulse stimulus similar to that received by the native heart. A bioreactor that closely mimics all stimuli experienced by native hearts in vivo should provide the optimal environment for cells to build a tissue engineered heart.

3.5 Conclusion

The last seeding experiment performed was successful, as decellularized rabbit hearts injected with either ECs or ADSCs were found to have cells attached to the acellular lumen walls of the coronary vasculature pathway. The results prove that the composition and structure of the decellularized matrix is capable of supporting EC and ADSC attachment. This is the first study directed toward re-endothelialization of decellularized coronary vasculature using a type of stem cell, and it proved that ADSCs are capable of adhering to the lumen walls. Therefore, the decellularization process presented creates an effective model for tissue engineered scaffolds. It is apparent that for manual injection of cells by hand, a static fixation step rather than injection or perfusion of formalin is necessary to retain attachment of
This study also shows that 2.8 million ECs and 380,000 ADSCs are sufficient numbers to allow for attachment of cells to the scaffold. The number of ECs sufficient to allow for attachment of cells to the acellular lumens is much lower than has been previously shown in literature [59,91]. This is the first step toward complete re-endothelialization of the vasculature in decellularized scaffolds. A greater number of cells is needed to create a complete monolayer covering the walls of the coronary vessels, which currently remains an elusive accomplishment in the field. It is suggested that an enhanced bioreactor is built that delivers several stimuli at physiological levels to the tissue engineered construct, in order to mimic a native heart as closely as possible. This should aid in cell attachment, migration, and proliferation along the vessel lumens of the coronary vasculature, as well as allow the injected cells to receive fresh flowing media. The scaffold can then be incubated for an extended period of time while maintaining cell viability, which will allow time for cell proliferation and migration. This could lessen the amount of harvested cells needed as well as aid in the complete monolayer coverage of the vessel walls. It is also recommended to continue the use of rabbit hearts, as they provide a larger model than rat hearts which are currently the only animal model used for decellularization and recellularization of hearts, besides preliminary re-seeding studies of a porcine heart performed by Schulte et al [90]. Rabbit hearts are more clinically relevant, as they are closer in size to human hearts compared to rat hearts, and have proven similarities to human hearts such as sarcomeric protein composition [97]. Porcine hearts remain the most similar in size to human hearts, and therefore potentially the most clinically relevant model. However, for the stage of research that the field is currently in, it is recommended to use rabbit hearts over porcine hearts because they require less resources and cells. Once the rabbit heart is able to be fully reseeded with all clinically relevant cell types, then a larger model could be pursued.
4.1 **Summary of Project Significance**

Around every 42 seconds, an American will experience myocardial infarction (MI), or blockage of the coronary artery, which leads to varying degrees of myocardium necrosis and complications. The prevalence of coronary artery disease, including MI, in American adults at least 20 years of age is 6.2% according to the American Heart Association. Coronary heart disease cost the healthcare industry $10.4 billion and MI cost $11.5 billion in 2011. By 2030, the cost of coronary heart disease is expected to double \[7\]. The large population affected by coronary artery disease and MI makes it a major public health concern, and presents a large economic burden for the United States. It is clear that there is a critical need for development of post-MI treatments for these patients.

Current therapies and standards of care for treating post-MI patients with myocardial ischemia do not restore function to the cardiac tissue, with the exception of heart transplants which are limited by a shortage of donors and the need for immunosuppressive drugs. There is clearly room for innovation to develop a novel treatment to restore function to damaged areas of patients’ hearts. We chose to base our approach on tissue engineering using a decellularized heart scaffold, due to the many advantages of using a natural scaffold such as having a pre-made environment for injection of autologous cells that supports cell functionality. Our work is focused on replacing myocardium around the left coronary artery and the surrounding vessel network, as this is the most common site of damage for MI patients. In order to create a fully functional myocardial patch for implantation, the construct must be adequately vascularized. This study is centered on the re-endothelialization of the left coronary artery vasculature pathway in a decellularized rabbit heart scaffold.
4.2 Summary of Advancement toward Completion of Specific Aims

4.2.1 Aim 1: To develop and characterize a decellularized whole rabbit heart scaffold for use in cardiovascular tissue engineering

Whole rabbit hearts were successfully decellularized using a perfusion decellularization system. This procedure preserved the vascular pathways of the left coronary artery, left coronary vein, and associated vessel branches and capillaries, as shown by the use of a corrosion cascade kit. The efficacy of the decellularization of the ECM was determined by the complete removal of cellular components and retention of ECM components of elastin, collagen, and cytoplasm, as shown by histological staining.

4.2.2 Aim 2: To re-endothelialize the acellular vasculature pathways surrounding the left coronary artery with clinically relevant cell types

Decellularized rabbit hearts were sterilized and then coated with fibronectin. ECs and ADSCs were then injected by hand using a syringe into the left coronary artery of the hearts. DAPI and H&E histological staining showed attachment of the two cell types to the lumen of the vasculature pathways in all treatment groups of varied incubation times of 6 hours or 18 hours. Prior seeding experiment trials revealed that perfusion fixation and injection of formalin into the left coronary artery caused cell detachment from the vessel walls. A static fixation technique is necessary to allow cells to remain attached to the ECM. The success of the seeding experiment also proves that the decellularized scaffold contains sufficient structural properties and components to support the attachment of ECs and ADSCs.

4.3 Recommendations for Future Studies

The research presented is aimed toward the development of a post-MI treatment for patients with damaged myocardium. The next step in this study is to increase the injected cell population in order to create a complete monolayer of cells along the lumen of the decellularized vasculature pathways. It is
recommended that a bioreactor capable of providing physiologically relevant stimuli such as flow rate, pressure, and electrical pulses to the tissue construct is built. Using a model that provides a similar environment to native \textit{in vivo} hearts should aid in cell attachment to the ECM, as well as cell proliferation, migration, and functionality. A system that constantly perfuses fresh medium through the tissue construct will also allow for the cells to be incubated and allowed to migrate and proliferate for an extended period of time, increasing the probability of creating a complete monolayer along the vessel walls. The resulting scaffold will then be tested for non-thrombogenicity. Once a fully functional and non-thrombogenic vasculature system of the left coronary artery is established, other relevant cell types can be seeded into the myocardium to create a fully re-cellularized myocardium patch. This can also be extended to the entire vasculature system and to the entire heart. The bioreactor system capable of delivering physiological stimuli may aid in the seeding of the other cell types into the myocardium as well. Following \textit{in vivo} studies to prove efficacy, the study can then be scaled up to porcine hearts, which are more similar in size to human hearts.

In terms of contributing to the field of cardiovascular engineering, our study has proven the efficacy of using decellularized rabbit hearts as a tissue engineering scaffold, which are more similar to human hearts compared to rat hearts. This study has also shown that a lesser number of ECs are needed for cell adherence to an acellular vasculature wall than previously reported in literature. This is the first study to use a type of stem cell, specifically ADSCs, in attempted re-endothelialization of acellular coronary vasculature. We have shown that ADSCs are capable of adhering to coronary vessel walls. This suggests that stem cells may be a viable cell source to aid in the creation of a complete monolayer of endothelial cells in acellular vasculature pathways. A combination of stem cells and ECs may be more effective at facilitating cell proliferation and migration to create this monolayer compared to one cell type as well. Further studies can also be performed on the analysis of the decellularized scaffold and seeded cellular interactions. The decellularized scaffold can be tested for quantitative analysis of every known component of native ECM, including but not limited to laminin, proteoglycans, fibronectin, and nidogen. The scaffold can also be tested for mechanical properties such as elastic modulus and porosity, and compared to values taken from native
myocardium. In addition, the specific mechanisms that allow for EC attachment to the decellularized ECM could be studied, as well as those allowing for attachment of various stem cells including ADSCs and EPCs. This fundamental knowledge would increase our understanding of cardiovascular tissue engineering, and lead to the development of more effective tissue engineering therapies.
REFERENCES


damage and the potential for left ventricular repair after primary percutaneous coronary intervention,” Am. Heart J., 160(6 SUPPL).


Lunde, K., Solheim, S., Aakhus, S., Arnesen, H., Abdelnoor, M., Egeland, T., Endresen, K., Ilebekk,


