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BIOLOGICAL SCAFFOLDS FOR PERIPHERAL VASCULAR SURGERY

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BIOLOGICAL SCAFFOLDS FOR PERIPHERAL VASCULAR SURGERY

A Dissertation
Presented to
The Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by:
George Raymond Fercana Jr.
December 2013

Accepted by:
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Dr. Agneta Simionescu
Dr. Eugene M. Langan, III
Dr. Martine LaBerge
ABSTRACT

The gold standards for small diameter peripheral vascular graft replacement are autologous arteries or veins; however, one-third of patients lack such vessels due to previous vessel harvesting or advanced vascular disease. A promising approach for patients in this category is tissue engineering with off-the-shelf biological vascular grafts.

Three small diameter acellular scaffolds were developed and evaluated as vascular grafts. Porcine renal arteries (2-3 mm diameter, 20 mm length) were decellularized by immersion and stabilized with penta-galloyl glucose (PGG) with and without subsequent heparinization via carbodiimide chemistry. Bovine mammary (4-6 mm ID, 250 mm length) and femoral arteries (6-8 mm ID, 250 mm length) were decellularized in a purpose-designed, pressurized detergent perfusion system and stabilized with PGG. Decellularization completeness was confirmed by histology, DNA analysis and absence of xenoreactive galactose-(α1,3)-galactose antigen. Histology suggested good preservation of native collagen, elastin and basement membrane components collagen type IV, laminin, and fibronectin. Renal artery scaffolds stabilized with PGG showed increased resistance to elastase and heparinization increased resistance to collagenase. All scaffolds exhibited adequate values of burst pressure and diametrical compliance.

Acellular scaffolds were tested for biocompatibility, patency, thrombogenicity and host cell infiltration by intra-circulatory implantation in rats as abdominal aorta interposition grafts (renal artery scaffolds) and as femoral interposition grafts in minipigs (mammary artery scaffolds). Renal artery scaffolds exhibited 100% patency upon
explantation at 4 and 8 weeks and demonstrated revitalization with host cells staining positive for factor VIII and α-smooth muscle actin. Heparinized / PGG treated renal scaffolds exhibited the most promising short-term results in rats due to reduced thrombogenicity and intimal hyperplasia. Mammary artery scaffolds thrombosed within 1 week of implantation in the femoral position, but supported cellular infiltration. Preliminary studies also showed that cells could be seeded into specific tunics of the acellular scaffolds, thus generating revitalized grafts ready for implantation.

These results suggest that acellular scaffolds derived from arterial segments are good candidates for development of vascular grafts and that the combination of targeted matrix stabilization, heparinization and tunic-specific cell seeding might exhibit significant clinical potential for treatment of peripheral vascular diseases.
To my family, both immediate and extended, for all of their support during my education. Whether I was only able to see you during family reunions, eat dinner with you several times a year, make crazy desserts with you over the holidays, fry the Thanksgiving turkey with you, share your company at a Clemson football game, enjoy shared daily morning conversations with you, hang out together when we are both free, or help you work on your personal statements and résumés, these are powerful memories that I will never forget and I look forward to making more of them with you in the future.
ACKNOWLEDGEMENTS

One of my most powerful motivators is “opportunity” and the ability to capitalize on it to learn skills and advance my career. Although there are too many people to mention here, I want to acknowledge several individuals who have made significant contributions to my development over the years. My graduate education would not have been possible without Drs. Dan and Agneta Simionescu who generously took me into their laboratory and patiently supported me during my development as a scientist.

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My labmates were with me from the beginning. I have learned a great deal from all of you. I would not be where I am now without you working with me to design bioreactors, troubleshoot experiments, teach me complicated techniques, or discuss interesting literature topics. Jeremy Mercuri, Tom Chuang, Lee Sierad, Betsy Tedder, Richard Pascal, Jason Schulte, James Chow, Mike Jaeggli, Devon Bowser, Margarita Portilla, Allison Kennamer, Tasha Topoluk, Grace Dion and Katy Jaeggli. Thank you, Snow Creek Meat Processing for the generous donation of porcine tissues towards my research.

I want to also acknowledge Dr. Martine LaBerge for heartfelt advisement over the years as well as helping me find temporary work during my undergraduate education. Thank you Drs. Dean, Nagatomi, Mefford and Kletetschka for introducing me to research.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xvi</td>
</tr>
<tr>
<td>CHAPTER 1: REVIEW OF LITERATURE</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Vascular Replacements: Current Options and Clinical Need for Small Diameter Replacements</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Necessary Properties for the Ideal Vascular Graft</td>
<td>2</td>
</tr>
<tr>
<td>1.3 The Allure of Natural Biopolymers</td>
<td>6</td>
</tr>
<tr>
<td>1.3.1 Current Biopolymer Options: Collagen</td>
<td>7</td>
</tr>
<tr>
<td>1.3.2 Current Biopolymer Options: Elastin</td>
<td>8</td>
</tr>
<tr>
<td>1.3.3 Tissue Decellularization – The Alternative to Polymeric Fabrication</td>
<td>10</td>
</tr>
<tr>
<td>1.4 Scaffolds for Cardiovascular Tissue Engineering</td>
<td>13</td>
</tr>
<tr>
<td>1.5 Host Response to Biomaterials Implantation</td>
<td>15</td>
</tr>
<tr>
<td>1.6 Challenges in Cardiovascular Tissue Engineering</td>
<td>18</td>
</tr>
<tr>
<td>1.6.1 Existing Inflammation: The Bane of Tissue Engineered Vascular Grafts</td>
<td>19</td>
</tr>
</tbody>
</table>
3.2.1.6 Scaffold Characterization – Cytocompatibility ........................................ 43
3.2.2 Results ........................................................................................................ 44
3.2.2.1 Decellularization .................................................................................. 44
3.2.2.2 Scaffold Characterization – Histology ................................................. 45
3.2.2.3 Scaffold Characterization – Mechanical and Biochemical Properties .......................................................... 47
3.2.2.4 Scaffold Characterization – Cytocompatibility .................................. 50
3.2.3 Discussion .................................................................................................. 50
3.2.3.1 Scaffold Characterization – Histology and Cytocompatibility ............... 50
3.2.3.2 Scaffold Characterization – Mechanical and Biochemical Properties .......................................................... 54
3.2.4 Other Remarks .......................................................................................... 58
3.3 References ..................................................................................................... 59
3.4 Development of Porcine Renal Artery Scaffolds ......................................... 62
3.4.1 Introduction .................................................................................................. 62
3.4.2 Materials and Methods ............................................................................... 62
3.4.2.1 Scaffold Preparation – Dissection and Isolation .................................. 62
3.4.2.2 Scaffold Preparation – Decellularization .............................................. 64
3.4.2.3 Scaffold Preparation – Stabilization (PGG) .......................................... 65
3.4.2.4 Scaffold Preparation – Stabilization (Heparinization) ............................ 65
3.4.2.5 Scaffold Preparation – Sterilization ....................................................... 66
3.4.2.6 Scaffold Characterization – Histology ................................................. 66
3.4.2.7 Scaffold Characterization – Mechanical and Biochemical Properties .......................................................... 67

3.4.2.8 Scaffold Characterization – Heparin Quantification .......................................................... 67

3.4.2.9 Scaffold Characterization – Denaturation Temperature Determination .......................................................... 67

3.4.3 Results ........................................................................................................................................ 68

3.4.3.1 Scaffold Preparation – Decellularization ........................................................................ 68

3.4.3.2 Scaffold Characterization – Histology ........................................................................ 68

3.4.3.3 Scaffold Characterization – Mechanical and Biochemical Properties .......................................................... 68

3.4.3.4 Scaffold Characterization – Heparin Quantification .......................................................... 71

3.4.3.5 Scaffold Characterization – Denaturation Temperature Determination .......................................................... 72

3.4.4 Discussion ........................................................................................................................................ 73

3.4.4.1 Scaffold Preparation – Decellularization ........................................................................ 73

3.4.4.2 Scaffold Characterization – Histology, Mechanical and Biochemical Properties .......................................................... 74

3.4.4.3 Scaffold Characterization – Heparinization and Heparin Quantification .......................................................... 75

3.4.4.4 Scaffold Characterization – Denaturation Temperature Determination .......................................................... 76

3.5 References ........................................................................................................................................ 76

CHAPTER 4: SEEDING OF LONG VASCULAR SCAFFOLDS WITH TUNIC-SPECIFIC CELL TYPES .......................................................... 78

4.1 Introduction ........................................................................................................................................ 78
5.2.1.1 Heparinization for Implants .............................................. 97
5.2.1.2 Implant Groups ............................................................ 97
5.2.1.3 Graft Implantation ......................................................... 98
5.2.1.4 Graft Removal .............................................................. 99
5.2.1.5 Histology and Quantification .......................................... 99
5.2.1.6 Statistical Analysis ....................................................... 100
5.2.1.2 Bovine Mammary Artery Scaffolds ................................. 100
5.2.1.2.1 Preparation for Implantation ..................................... 100
5.2.1.2.2 Graft Implantation .................................................... 101
5.2.1.2.3 Graft Removal .......................................................... 101
5.2.1.2.4 Histology and Quantification ...................................... 102
5.2.1.2.5 SEM ........................................................................ 102
5.2.2 Results ............................................................................ 102
5.2.2.1 Porcine Renal Artery Scaffolds ...................................... 102
5.2.2.1.1 Implants and Surgical Handling ................................. 103
5.2.2.1.2 Histology and Evaluation of Implants ......................... 103
5.2.2.1.3 Histological Evaluation of Implants:
                   Calcification .......................................................... 105
5.2.2.2 Bovine Mammary Artery Scaffolds ................................. 107
5.2.2.2.1 Histological Evaluation of Implants .......................... 107
5.2.2.2.2 SEM ....................................................................... 109
5.2.3 Discussion ....................................................................... 110
5.2.3.1 Porcine Renal Artery Scaffolds .................................... 110
5.2.3.1.1 Histological Evaluation of Implants ........................................ 110

5.2.3.2 Bovine Mammary Artery Scaffolds ........................................ 112

5.2.3.2.1 Histological and SEM Evaluation of Implants .......................... 112

5.3 References ................................................................................ 113

CHAPTER 6: TRANSLATIONAL PERSPECTIVES ........................................ 114

6.1 Introduction .............................................................................. 114

6.2 Endovascular Management of a Complicated Type B Aortic Dissection in the Presence of an Aberrant Right Subclavian Artery Without Subclavian Artery Reconstruction – A Single Case Report ....................................................... 115

6.2.1 Abstract ................................................................................. 115

6.2.2 Introduction ........................................................................... 115

6.2.3 Case Report .......................................................................... 116

6.2.4 Discussion ............................................................................ 117

6.3 References ................................................................................ 120

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK ......................................................... 121

7.1 Conclusions .............................................................................. 121

7.1.1 Development of Bovine Mammary and Femoral Artery Scaffolds .............................................................. 121

7.1.2 Development of Porcine Renal Artery Scaffolds ............... 122

7.1.3 Seeding of Long Vascular Scaffolds with Tunic-Specific Cell Types .............................................................. 123

7.1.4 Preclinical Evaluation of Scaffold Biocompatibility .............. 123
7.4 References ......................................................................................................................... 134
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>3</td>
</tr>
<tr>
<td>1.3</td>
<td>15</td>
</tr>
</tbody>
</table>

1.1 Overview of Sizes and Preferential Choices for Vascular Grafts .......................... 2

1.2 Overview of Advantages and Disadvantages of Synthetic Vascular Grafts ................ 3

1.3 Overview of Advantages and Disadvantages of Biological Vascular Grafts ................. 15
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>5</td>
</tr>
<tr>
<td>1.2</td>
<td>9</td>
</tr>
<tr>
<td>1.3</td>
<td>18</td>
</tr>
<tr>
<td>3.1</td>
<td>45</td>
</tr>
<tr>
<td>3.2</td>
<td>46</td>
</tr>
<tr>
<td>3.3</td>
<td>48</td>
</tr>
<tr>
<td>3.4</td>
<td>49</td>
</tr>
<tr>
<td>3.5</td>
<td>51</td>
</tr>
<tr>
<td>3.6</td>
<td>52</td>
</tr>
<tr>
<td>3.7</td>
<td>52</td>
</tr>
<tr>
<td>3.8</td>
<td>58</td>
</tr>
<tr>
<td>3.9</td>
<td>63</td>
</tr>
<tr>
<td>3.10</td>
<td>65</td>
</tr>
<tr>
<td>3.11</td>
<td>69</td>
</tr>
<tr>
<td>3.12</td>
<td>70</td>
</tr>
<tr>
<td>3.13</td>
<td>70</td>
</tr>
</tbody>
</table>
3.14 Enzymatic Resistance of PGG-treated and Heparinized Renal Artery Scaffolds ................................................................. 71
3.15 Heparin Content of Renal Artery Scaffolds ................................................. 72
3.16 Denaturation Temperature of Heparinized Renal Artery Scaffolds ................................................................. 73
4.1 Tunic-specific Cell Seeding of Mammary Artery Scaffolds ................................................................. 86
4.2 External Stent Facilitated Endothelial Cell Seeding ............................................. 87
4.3 External Stent Facilitated Human ADSC Seeding Procedure ................................................................. 89
4.4 Fluorescent Imaging of Human ADSC Seeding ................................................ 89
5.1 Implanted Renal Artery Scaffolds ................................................................. 98
5.2 Histological Evaluation of Renal Artery Implants ............................................. 104
5.3 Characterization of Gross Histological Renal Artery Implant Properties ................................................................. 105
5.4 Quantification of Elastin Within Implanted Renal Artery Scaffolds ................................................................. 106
5.5 Implant Calcification Measurement ............................................................. 107
5.6 Overall Renal Artery Scaffold Biocompatibility ................................................ 108
5.7 Histological Overview of Bovine Mammary Artery Implants ................................................................. 109
5.8 SEM analysis of Explanted Mammary Artery Scaffolds ................................................................. 110
CHAPTER 1: REVIEW OF LITERATURE

(This work has been published in International Journal of Inflammation. 2011, Article ID 958247, 11 pages as well as Polymers for Vascular and Urogenital Applications. 2012:39-58)

1.1 Vascular Replacements: Current Options and Clinical Need for Small Diameter Replacements

Current treatment options for vascular diseases, regardless of location in the vasculature, include balloon angioplasty, stent placement, graft bypass surgery, and use of pharmacological agents \[1-5\]. The total need for vascular grafts has been estimated to be more than 1.4 million in the USA alone \[6\]. These can be divided into three categories, in order of decreasing diameter (Table 1). The large and medium caliber synthetic grafts are used in the thoracic and abdominal cavities with good long-term outcomes. Almost 1,200,000 small-caliber grafts (< 6mm) are used every year for vascular access, to relieve lower limb ischemia and for coronary bypass surgery. Autologous veins or arteries that come from the same patient needing the operation are the “gold standard” for replacement of small caliber arteries but in 30-40% of patients these are not available due to prior harvesting or pre-existing conditions\[7\]. In these latter cases synthetic grafts are used, but they provide poor outcomes, as 50% of these will occlude within 5 years \[8\], potentially leading to amputation.

In the search for small caliber vascular substitutes, a range of materials and methodologies have been evaluated\[9\] but most exhibit poor mid-term performance. These
include (i) synthetic grafts such as ePTFE, PET, polyurethanes, PGA and PLA\(^{[10]}\) (Table 2) and (ii) bioprosthetic grafts\(^{[2]}\) (autologous – from self, allogeneic – from same species, xenogeneic – from different species) (Table 3). Taken together, as multiple bypasses are sometimes required and availability of autologous arteries or veins is limited, there is clearly a significant unmet clinical need for small caliber vascular grafts.

<table>
<thead>
<tr>
<th>Vascular substitute choice</th>
<th>Vascular regions</th>
<th>Hemodialysis arterio-venous access</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Large-caliber arteries (≥ 8 mm)</td>
<td>Medium-caliber arteries (6-8 mm)</td>
</tr>
<tr>
<td>Aorta, such vessels, iliac and common femoral arteries</td>
<td>Carotid, subclavian, common femoral, visceral and saphenous arteries</td>
<td>Coronary, below-the-knee, tibial and peroneal arteries</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1st choice</th>
<th>2nd choice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prosthesis (Dacron, ePTFE)</td>
<td>Prosthesis or autograft (equal)</td>
</tr>
<tr>
<td>Arterial or venous autograft</td>
<td>Saphenous spiral vein graft, deep venous autograft</td>
</tr>
<tr>
<td>Native material</td>
<td>ePTFE, PU, xenografts, biografts, TEBV (clinical trial)</td>
</tr>
</tbody>
</table>

Table 1.1. Overview of sizes and preferential choices for vascular grafts. Taken from \(^{[2]}\)

1.2 Necessary Properties for the Ideal Vascular Graft

In the realm of biomedical engineering, a large breadth of materials have been investigated for vascular replacement applications in order to achieve necessary properties intrinsic to the ideal vascular replacement\(^{[11]}\). The properly functioning vascular graft needs to accommodate aforementioned mechanical properties in addition to mimicking the native compliance of the vessel to be anastomosed. In addition to the aforementioned qualities, vascular grafts will also require adequate performance regarding implant biocompatibility, such as resistance to calcification and thrombosis, which are typically modulated using
alterations to the material’s surface chemistry via known anti-coagulants such as variations of heparin\cite{12,13} or treating the material to remove cytotoxic molecules \cite{14-16}. The scaffold should also be resistant to degradation by enzymes naturally found in vivo, notably collagenases and elastases, in order to resist aneurysmal dilatation and rupture following implantation.

<table>
<thead>
<tr>
<th>Synthetic vascular grafts</th>
<th>PET (Dacron, Terylon)</th>
<th>ePTFE (Teflon, Gore-Tex)</th>
<th>Polyurethane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Woven Knitted</td>
<td>Low-porosity (&lt;50 μm IND)</td>
<td>High-porosity (&gt;45 μm IND)</td>
<td>Fibrillar Foamy</td>
</tr>
</tbody>
</table>

**Advantages**
- Better stability, lower permeability and less bleeding
- Greater porosity, tissue ingrowth and radial dilatation
- Biostability, no dilation over time
- Biostability, better cell ingrowth
- Compliance, good hemocompatibility, less thrombogenicity

**Disadvantages**
- Reduced compliance and tissue incorporation, low porosity, fraying at edges, infection risk
- Dilation over time, infection risk
- Stiff bleeding, limited incorporation, infection risk, perigraft sepsis formation
- Late neointimal desquamation in 90 μm IND, infection risk
- Biodegradation in first generation, infection risk, cannulogenic

**Healing**
- Intimal fibrous capsule, outer collagen capsule, scarring, intimal hyperplasia
- Fibrous intimal coverage, very sporadic endothelium, transmural intimal hyperplasia in animals
- Luminal fibrin and platelet carpet, connective tissue capsule with foreign body giant cells, no transmural tissue ingrowth
- Macrophages and polymorphonuclear invasion, capillary sprouting, fibroblast migration, certain engorgement, thicker neointima, endothelialization in animals
- Thin intimal fibrous layer, outside foreign body cells, limited ingrowth
- Better ingrowth with bigger pores

**First use**
- Ku et al. 1957
- Norton and Eiseman 1975
- Boreeox and Pierce 1967

**Review e.g.**
- Xue and Greisler 2003
- Nichols et al. 2004
- Tiwari et al. 2002

**Table 1.2.** Overview of advantages and disadvantages of synthetic vascular grafts. Taken from \cite{2}

Burst pressure is an intrinsic property of tubular materials and is tested by cannulating both ends of the graft and connecting a piezoelectric digital pressure transducer at one end and a peristaltic pump (or syringe pump) at the opposite end. The system is filled with saline, and pressure is slowly increased. Burst pressure is recorded when a sharp
drop in internal graft pressure occurs. Native arteries exhibit a burst pressure of 2,000 or more mmHg\textsuperscript{17}.

Physiological compliance is defined as the increase in graft diameter as pressure increases from 80 to 120 mmHg. Compliance measurement is performed as follows: Grafts are cannulated with one end connected to a piezoelectric digital pressure transducer and the other adapted to a saline reservoir. The pressure is raised until the transducer reads 80 mmHg hydrostatic pressure, and digital images are taken for external diameter measurement. Then, the pressure is increased to 120 mmHg, and the diameter is measured again. Calculated differences in diameter between these pressures are then used to calculate overall diametrical compliance\textsuperscript{18}. This is an important parameter to engineer because of known effects of compliance mismatch on graft performance\textsuperscript{19}.

Once satisfactory material properties have been established for the proposed vascular graft, the material must also prove to be non-cytotoxic, porous, and allow retention of shear resistant endothelial cells (EC) [intima], vascular smooth muscle cells (VSMC)[media], and fibroblasts (FB) [adventitia] into the three tunics native to blood vessels (\textbf{Figure 1.1}). Pending infiltration of these cell types, the material must permit degradation and matrix remodeling by the host’s own cells to produce a patient-tailored, autologous replacement according to the recent paradigm of regenerative medicine\textsuperscript{22}.

Although regenerative medicine is still in its infancy with regards to vascular applications, the concept of tailoring a material to become a patient’s own tissue inspires researchers to push the limits of conventional research towards producing translational replacements. Such replacements would combine scientific expertise, xenogenic tissues,
human cells, and \textit{in vitro} bioreactors capable of mimicking the physiological environment seen by vascular grafts to produce commercially available grafts capable of being stored in a hospital setting and utilized on an “off-the-shelf” basis. This concept of bridging the gap between laboratory bench and the patient’s bedside elicits the phrase “bench to bedside” and encompasses the goals of translational research. The following section will discuss material choices for TEVGs and a brief explanation of research leading to these material suggestions.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Representation of the three tunics native to a blood vessel. Intima – ECs, media – VSMCs, adventitia – FBs. (B) Structural differences between muscular arteries (left) and elastic arteries (left); note differences in elastin content dependent on the artery type. Taken from \cite{20}.}
\end{figure}
1.3 The Allure of Natural Biopolymers

The two main categories of available scaffolds are biological and synthetic (Table 3). Biologic scaffolds are naturally occurring materials, often derived from an animal, to which cells readily bind onto\textsuperscript{[21]} and are able to remodel\textsuperscript{[17]}; however, biological scaffolds can have the tendency to degenerate rapidly after implantation when not appropriately stabilized\textsuperscript{[22]}. These properties can be better controlled using chemical or physical methods of cross-linking or stabilization which reduce biodegradability and also enhance tensile strength. Synthetic scaffolds share the major advantage that material properties can be tailored to the target tissue or organ, are easy to process and are very consistent between batches. However, some polymers exhibit poor cellular interactions and may also leach residual monomers, catalysts and degradation byproducts which may be toxic or elicit inflammation after implantation.

When deciding the optimum material for a vascular graft, one should keep in mind the previously discussed ‘ideal’ properties for such a scaffold. What often leads researchers to utilizing biopolymers such as collagen or elastin-based proteins is the unique properties of said biopolymers which often provide the ideal characteristics for the necessary properties due to their natural origins. For example, elastin has been recently shown to not only function as a completely non-thrombogenic surface, but to also provide elasticity to tissues in order to achieve necessary compliance and cyclic fatigue strength\textsuperscript{[23, 24]}. Collagen-based scaffolds, conversely, provide rigidity that will impart mechanical strength, potential for rapid degradation and remodeling, but also contribute to thrombus formation, due to the material’s high thrombogenicity. With the combination of necessary
mechanical properties and coveted *in vivo* properties, biopolymers are often the “natural” choice for researchers. Interestingly, researchers are observing that neither collagen- nor elastin-based vascular replacements retain sufficient mechanical properties when used individually for functional vascular replacements in arterial positions\[^{25}\].

### 1.3.1 Current Biopolymer Options: Collagen

The ECM protein collagen exists in the body in numerous forms and is widely accepted to be deposited rapidly into *in vivo* implants either in the form of a fibrous capsule of collagen matrix, indicating a host response leading to implant rejection, or neocollagen formation in the implant. Such neocollagen formation often indicates a positive implant remodeling which can be verified using immunohistochemistry on histological tissue sections. As the most abundant protein in the body\[^{26}\], collagen is readily available and has been experimented with thoroughly in the literature for the purpose of engineering vascular grafts; an example of such is below.

With experimentation into hydrogel-based substrates generally producing inadequate mechanical properties for the vascular constructs as discussed by Patel et al\[^{25}\], researchers began to investigate mechanical conditioning on hydrogels in hopes of elucidating the key increased mechanical strength for hydrogel-based vascular constructs. It was later suggested by L’Heureux et al\[^{27}\] that mechanical forces should be applied to collagen gels in order to induce alignment of collagen fibrils and seeded smooth muscle cells in circumferential fashion, as seen in natural vessels\[^{28, 29}\]. When this methodology
was first applied in attempts at producing a hollow, cylindrical artery using a mandrel-based approach by Hirai and Matsuda[30], mechanical strength was not sufficient for implantation into arterial positions. Follow-up experimentation utilized glycation mechanisms in addition to lysyl oxidase-based cross-linking of fibers, however, again, mechanical strength of the collagen-based gels proved to be insufficient[31, 32]. These findings as well as reviews in the field suggest the necessary introduction of another material in addition to collagen in order to achieve adequate mechanical properties. Some researchers suggest the supplementation of vascular construct materials currently in the literature with elastin to improve resilience and impart the necessary mechanical properties for engineering vascular grafts[25].

1.3.2 Current Biopolymer Options: Elastin

One of the least synthesized extracellular matrix fibers in the human body, elastin, provides properties tantamount to a functional tissue engineered vessel, but is widely known for the resilience it imparts to biological tissues[23]. An example of a current methodology for utilizing this ECM protein includes electrospinning, in which the fibers are spun with varying voltages, distances between the plate and needle, and solvents to dissolve the fiber for the process. In order for a TEVG to be remodeled by the host with acceptable mechanical properties, said construct should induce elastin biosynthesis during remodeling in vivo. However, currently there have been no large successes in elastin biosynthesis for TE constructs.
Figure 1.2. Example decellularization protocols for (A) thin laminates such as pericardium, (B) thicker laminates such as dermis, (C) fatty, amorphous tissues such as adipose, (D) composite tissues or whole simple organs such as trachea, and (E) whole vital organs such as liver. Arrow lengths represent relative exposure times for each processing step. Rinse steps for agent removal and sterilization methods are not shown to simplify comparison. (F) Representative images of the gross appearance of intact rat liver subjected to decellularization: (left to right) before, during, and after decellularization; decellularized liver perfused with blue dye. (G) Representative photomicrographs showing no nuclear staining after whole organ decellularization: (left to right) native rat liver H&E; decellularized liver-ECM H&E; native rat liver DAPI; liver-ECM DAPI. Scale bars are 50 mm. Taken from[^33].

An example of several previous efforts conducted in the field in order to incite elastin biosynthesis utilized combinations of fibrin and collagen. Other growth factors and conditions involving smooth muscle cells known to produce the coveted elastin were
utilized in these experiments, but no elastin was produced by their efforts\cite{34-37}. Research into differences between fibrin- and collagen-based hydrogel scaffolds conducted by Long and Tranquillo did, however, elucidate that when murine neonatal smooth muscle cells are seeded into both collagen and fibrin gels, the fibrin gels produce significantly more elastin than those made of collagen, indicating that perhaps fibrin is a much more favorable substrate for elastin biosynthesis\cite{37}. Investigating the effects of another hydrogel substrate on elastin biosynthesis, work done by Ramamurthi’s group compared performance of neonatal rat smooth muscle cells in both hyaluronan gels and monolayer conditions on tissue culture polystyrene. The results showed that elastin was indeed produced, and in the fenestrated form native to the internal elastic lamina and concentric lamellae inside the media of blood vessels\cite{38}. The research from these groups suggest that elastin biosynthesis is upregulated when used in specific three-dimensional substrates, but the key ingredients necessary to repeatedly and dependably produce elastin are not fully understood.

1.3.3 Tissue Decellularization – The Alternative to Polymeric Fabrication

The act of gathering specific biologically inspired polymers and integrating them methodically and precisely into a mechanically stable structure via hydrogels, mandrels, or other commonly utilized substrates is a technique favored by many research groups. This particular methodology can easily be included into the category of a ‘bottom-up’ fabrication approach in which a biologically viable scaffold is essentially ‘created from scratch’. This process retains advantages due to the seemingly unlimited variability
afforded by such a technique in which the researcher specifically selects the desired components and implements them into a scaffold.

However, investigators in the field are also adopting a ‘top-down’ approach for scaffold fabrication. For this process, donor tissue, of either xenogenic or human origin is meticulously treated to remove the undesired antigens, soluble proteins and cellular remnants (Figure 1.2). This step, referred to “decellularization” is essential because of the possibility of immune rejection – upon implantation. A second prerequisite is to ensure that the extracellular matrix composition and 3D integrity are fully retained after decellularization. This in turn will determine mechanical properties of the scaffolds. This process is also similarly advantageous in that researchers can fine-tune their approach to retain specific ECM compositions and porosities while further augmenting their process by choosing a different donor tissue as deemed necessary. An example of such would be the choice of either a muscular or elastic artery for decellularization and scaffold treatment: each artery type will provide different mechanical properties and porosity.

With the advent of decellularization for production of tissue engineered scaffolds came numerous questions regarding the extent of decellularization necessary in order to retain necessary mechanical properties[39]. However, the question of extent of decellularization with respect to leftover nucleic acids and the consequences of such presence in a scaffold have been explored thoroughly since then[33]. With this newfound understanding, researchers often are combining several known decellularization methods, such as detergents, acids / bases, alcohols, and also biologic agents such as nucleases and
proteases. Crapo et al clarify that the choice of the optimum decellularization medium is
often determined by four components, (i) cell density (ii) total cell content, (iii) lipid
content and (iv) thickness\cite{33}; readers are directed to this excellent review for further details
regarding current clinical options and mechanisms utilized to deliver the necessary
treatment agents to the tissue as well as a general overview of possible sterilization options
for biological scaffolds, and their consequences of use, before implantation and use.

Following successful decellularization of a scaffold, the researcher must then
stabilize the ECM of the scaffold in order to render the scaffold resistant to matrix
metalloproteinases that are capable of degrading natural ECM-based scaffolds in vivo\cite{22}.
Following stabilization, scaffolds should be characterized with respect to mechanical
properties, biochemical properties and biocompatibility as described previously (see
Necessary Properties for the Ideal Vascular Graft)\cite{40} and discussed in greater depth later
in this chapter. Pending in vitro characterization, the scaffold is then ready for the initial in
vivo biocompatibility test for scaffolds, subcutaneous implantation in a small animal
model, to assess potential for inflammation and calcification upon functional implantation.
A functional implantation utilizes a large animal model and necessitates the implantation
of the fully prepared scaffold in the position it was originally intended and evaluated for
mechanical and biocompatible properties. For example, a tissue engineered aortic valve
would be implanted in the position of the aortic valve within a large animal. Pending
successful large animal studies, the potential for clinical trials arise in which the scaffold
is implanted in human patients to assess safety, toxicity, and efficacy before finally
bringing the device to market after many years of testing.
1.4 Scaffolds for Cardiovascular Tissue Engineering

Tissue engineering (TE), aided by emerging stem cell technology, holds immense potential for the treatment of cardiovascular (CV) diseases, as progress has been made in engineering the various components of the CV system, including blood vessels, heart valves, and cardiac muscle [41]. The goal of CVTE is to create or regenerate a functional structure populated with cells capable of continuously remodeling the extracellular matrix (ECM). Optimal replacements for failed CV components would be biocompatible tissues that have the potential to rapidly restore the lost function and slowly regenerate by remodeling while implanted. This is a bioengineer’s dream come true and increasingly more reports show that we are slowly getting closer to viable solutions [42].

The specific concepts of TE include creation of a suitably shaped scaffold, repopulation with the appropriate cells (endothelium, smooth muscle cells, fibroblasts), ensuring that cells are placed in their appropriate tissue “niche” (e.g. lumen, media, adventitia – see Figure 1), promotion of neovascularization (where needed), and dynamic mechanical conditioning [43] to slowly adapt cells to loads and prepare constructs for implantation. Scaffolds for CVTE must be non-cytotoxic, biocompatible, biodegradable with safe by-products, and highly porous yet mechanically stable for the appropriate functions [44]. Bioscaffolds derived from xenogeneic ECM as well as synthetic polymers have been used in numerous TE applications (Table 3). ECM is the natural scaffold for tissue and organ morphogenesis, maintenance and reconstruction following injury, and is associated with constructive tissue remodeling. The 3D organization of its components clearly distinguishes ECM from synthetic scaffolds [45].
The processing of ECM biomaterials for medical use involves decellularization of mammalian tissues in order to remove all epitopes associated with cells, especially the terminal galactose alpha 1,3 galactose (alpha-Gal) [46], expressed on the cell surface of all mammals except those of humans and old world primates [47]. It is known from earlier studies that pure collagen scaffolds degrade slowly and do not calcify in subdermal implantation models [37–39]. Aldehyde crosslinked ECM calcifies after implantation [40], thus, limiting the use of glutaraldehyde-treated valve bioprostheses. Notably, increasing the extent of matrix cross-linking by adding amine bridges [41] significantly reduced calcification when tested in a variety of animal models [42–45].

At early stages, implanted scaffolds are infiltrated by macrophages which degrade ECM slowly by means of secreted matrix metalloproteinases (MMPs); at later stages scaffolds are infiltrated by fibroblasts which initiate repair and remodeling processes [48]. Although degrading collagen scaffolds do not accumulate calcium deposits in vivo, degrading elastin scaffolds have a natural tendency to calcify unless stabilized [49]. While the mechanisms of this process are still under investigation, it is evident that matrikines such as elastin peptides can induce osteogenic responses in smooth muscle cells and fibroblasts and thus mediate calcification [50]. Thus it is apparent that there is a dire need for a method to stabilize elastin inside tissue engineered cardiovascular replacements prior to implantation.
### Table 1.3. Overview of advantages and disadvantages of biological vascular grafts. Taken from [2].

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Autografts</th>
<th>Allografts (homografts)</th>
<th>Xenografts (heterografts)</th>
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<tr>
<td>Adjetant</td>
<td>Arterial</td>
<td>Venous</td>
<td>Arterial</td>
</tr>
<tr>
<td>Closest approximation, less</td>
<td></td>
<td>Durable and versatile,</td>
<td>Off-the-shelf availability,</td>
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<tr>
<td>diameter mismatch, internal</td>
<td></td>
<td>good resistance, relative</td>
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<tr>
<td>vascular anatomy, anatomically</td>
<td></td>
<td>availability</td>
<td>resistance to infection,</td>
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<tr>
<td>nearby, excellent function</td>
<td></td>
<td></td>
<td>transplant-reipient patients</td>
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<tr>
<td>Disadvantages</td>
<td></td>
<td></td>
<td>Antigenicity, graft</td>
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<tr>
<td>Availability, vasospasm (radial</td>
<td></td>
<td>Overall injury,</td>
<td>deterioration, early</td>
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<td>vein graft disease</td>
<td>occlusion, chronic</td>
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<td></td>
<td></td>
<td>rejection, intake of drugs,</td>
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<td></td>
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<td>infection risk</td>
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<tr>
<td>Healing</td>
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<td>Endothelial desquamation,</td>
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<td>Intra-gl thickening, myocardial</td>
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<td>vein dilatation, wall</td>
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<td>hypertension (radial artery)</td>
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<td>thickening, stenosis,</td>
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<td></td>
<td>neointimal hyperplasia</td>
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<td>First use</td>
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<td>Jaboulay and Brian 1896</td>
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<td>Gross <em>et al.</em> 1948</td>
<td>Linton 1955</td>
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<td>Review e.g.</td>
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<td></td>
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<td>Durdi <em>et al.</em> 2002</td>
<td>Schmidt and Dries 2004</td>
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</table>

**1.5 Host Response to Biomaterial Implantation**

Following all necessary preparation and treatment, newly created scaffolds are ready to replace an inflamed, thrombotic, atherosclerotic, calcified or infected cardiovascular tissue. Tissue engineering and regenerative medicine are on the verge of clinical translation and hold great therapeutic potential. However, progress in the field is critically hampered by uncontrolled, chronic implant-host interactions and more specifically by chronic inflammation.

When challenged by an implanted biomaterial, the body selects two or more of its three defense mechanisms existent in the “armamentarium”: hemostasis/coagulation, immune reactions and inflammation. While we have the ability to control the first two
mechanisms relatively well using drugs, chronic cardiovascular inflammation is more difficult to manage. Moreover, the clinical consequences of chronic inflammation including uncontrolled cell proliferation, fibrosis, calcification and sclerosis are almost impossible to treat pharmaceutically \[53, 54\].

After implantation, cardiovascular devices typically undergo a process similar to wound healing \[53\]. Following an initial blood-material interaction where fibrin is deposited on the luminal surface, inflammatory processes occur around the implanted construct. In initial stages, neutrophils and monocytes migrate to the interface between the implant surface and the injured tissue. During the granulation phase, phagocytes remove debris due to trauma and then provide signals for fibroblasts and smooth muscle cells to start remodeling. This phase lasts 2-3 weeks in humans and ideally will culminate with complete healing. However, the inflammatory response may continue for months or years and thus may lead to chronic inflammation. The consequences of this deleterious process include intimal thickening, tissue overgrowth (pannus formation), foreign body reactions, granulation, fibrosis and ectopic calcification. The mechanisms of these pathological phenomena are not fully understood, but it is known that monocytes / macrophages are observed within implanted constructs until they fully degrade it or, if the implant is not degradable, for the life of the implant\[54\]. Mineralization of synthetic or biologic scaffolds is end-stage pathology, generally irreversible and untreatable. It is also commonly accepted that calcification may not occur if scaffolds degrade slowly and the matrix is capable of remodeling \[55\].
In most studies on chronic inflammation reported to date, a “diffuse mononuclear infiltrate” was described together with “frustrated macrophages” a.k.a., foreign body giant cells \(^{56}\). The latter secrete large amounts of proteases and reactive oxygen species which in turn promote implant degradation, loosening and eventual failure. Recent evidence suggests that infiltrated monocytes / macrophages produce important cytokines, growth factors, and matrix-remodeling proteases (specifically metalloproteinases). Monocytes / macrophages express markers typical of proinflammatory macrophages (type M1) or remodeling macrophages (type M2) depending on whether the implanted scaffolds are degradable or not\(^{57}\). Additionally, recruited monocytes release vascular endothelial growth factors and thus may be involved in undesired neo-vascularization of CVTE implants\(^{58}\).

Clearly, a better understanding of the underlying pathways appears crucial for controlling the fate of implants and modulating inflammatory reactions in such a way as to induce implant healing and remodeling as compared to fibrosis and/or degeneration. One way of approaching this issue is developing imaging modalities and discovering new biomarkers of inflammation which would help further understanding of inflammatory diseases and discerning events related to inflammation in CVTE implants\(^{59}\). Examples include detection of vascular cell adhesion molecules, markers for proteases, integrin labeling and uptake of oxidized low density lipoprotein in atherosclerotic lesions\(^{60-63}\). Circulating C-reactive protein has become particularly favored as an inflammatory marker due to its long half-life and chemical stability\(^{64-66}\) in addition to its ability to predict cardiovascular events\(^{67}\) and evaluate the effectiveness of clinical anti-inflammatory
treatment options\cite{68,69}. One example of recent advances in molecular imaging modalities demonstrates ability to image complicated inflammatory mechanisms such as atherosclerosis (Figure 1.3)\cite{70}.

![Figure 1.3](image)

**Figure 1.3.** Targeted microbubble assisted imaging of inflammatory aortic plaque in a mouse model using molecular contrast ultrasound modality in (a) asymptomatic, (b) intermediate, and (c) severe inflammatory conditions. Taken from\cite{70}.

### 1.6 Challenges in Cardiovascular Tissue Engineering

Little is known about the effect of the pathological status of a tissue, organ or patient on the fate of CVTE devices and constructs. However it is reasonable to believe that the pre-existing pathology or existing risk factors would influence long-term outcomes of device implantation. For example, implantation of a vascular graft in an atherosclerosis-prone patient results in decreased patency\cite{71}.

The surgical procedure may also affect host reactivity. In the first several weeks, surgery-related tissue trauma induces an expected inflammatory response coupled to a wound healing reaction. The response follows the known pathways of inflammation followed by repair and healing. This portion of the implant-host interaction timeline is
necessary to heal the excised tissues and establish a “working interface” between the implant and host. Since the extent of the host response depends on the degree of surgical injury, more and more CVTE approaches envision devices that could be delivered and implanted using less traumatic, minimally invasive (percutaneous, laparoscopic, endovascular) approaches\(^\text{[72]}\).

The nature of the implant also has a great impact on its clinical outcome. Ideally the CVTE implant should not be immunogenic, nor should it induce thromboembolic complications, or excessive and prolonged inflammation. Unfortunately, few biomaterials exist which can be considered completely inert. For example, ePTFE and Dacron vascular grafts function well in large diameter graft applications without endothelial cell coverage but when used in peripheral applications (small diameter), one half of them occlude within the first five years of implantation\(^\text{[73]}\).

1.6.1 Existing Inflammation: The Bane of Tissue Engineered Vascular Grafts

One of the most complicated aspects of designing a replacement for diseased tissue is incorporating measures which prevent the device from succumbing to the same fate as the diseased tissue it is replacing. The clinical experience with transplantation of vascularized organs has uncovered several aspects useful for TEVGs. In heart transplants, it has been reported that as the duration of time post-implant increases, patient mortality also increases\(^\text{[74]}\). The culprit for this problem has been denoted as graft arterial disease (GAD)\(^\text{[75]}\), characterized by intimal hyperplasia, denudation of the medial layers, fibrosis of adventitial layers, and vasoconstriction\(^\text{[76]}\).
GAD differs from atherosclerosis by not being related to fatty streak deposition and by developing circumferentially and not focally\cite{77}. Shimizu and Mitchell\cite{75} also reported that symptoms of ischemia are often masked in transplanted tissues due to the lack of innervation, only furthering the convolution of the disease. Therefore, even following successful organ transplantation and the necessary immunosuppression medication that ensues, GAD can arise and threaten the health of both the transplanted organ and the patient. Placing TEVGs into a compromised site would likely decrease the life of the vascular construct, especially due to the expression of chemokines that recruit inflammatory cells which are key to allograft rejection\cite{78, 79}. GAD has been reported to incite host in-growth of endothelial cells and intimal smooth muscle-like cells, which are reported to be different from medial-smooth muscle cells,\cite{80} into the allograft vasculature\cite{81-83}. On the upside, such cellular ingrowth properties could potentially be harnessed to further TE strategies for vascular constructs.

Atherosclerosis has similarities to GAD, but ultimately differs in the involvement of lipoproteins and foam cells entrapped within the intima, which form the characteristic localized fatty streaks\cite{64}. After several decades of research, we now know that atherosclerosis is a consequence of chronic inflammation\cite{84} with elements of immune system activation\cite{85}. The main question that bioengineers have to address now is: how to develop a TEVG that would be protected from atherosclerosis after implantation in an atherosclerotic-prone patient? Regardless of the mechanisms perpetuating disease progression, studies into pathogenesis of atherosclerosis and other inflammatory diseases point to the activation of endothelial cells as the critical starting point for the disease\cite{64, 86}. 
Thus, knowledge of mechanisms of endothelium activation and its role in promoting inflammation would need to be incorporated into future TEVGs\textsuperscript{[64]}.

1.6.2 Improving Outcomes of Implanted Vascular Replacements

Technical challenges in CVTE are plentiful, but the “final frontier” is the healthy, quiescent endothelium\textsuperscript{[87]}. This monolayer of cells that naturally covers all blood-contacting tissues acts as a dynamic and selective barrier by maintaining a non-thrombogenic surface\textsuperscript{[88, 89]}, controls the transfer of molecules across the vascular wall\textsuperscript{[88, 89]}, modulates blood flow and vascular resistance\textsuperscript{[88, 89]}, regulates immune and inflammatory reactions\textsuperscript{[88, 89]} and also interacts with underlying cells to regulate their growth and proliferation\textsuperscript{[88, 89]}. Activation of the endothelium by cytokines, bacterial products, hemodynamic forces, lipids, and other agents, induces expression of a new and radically different cell phenotype. Activated endothelium expresses new adhesion molecules on its surface and secretes chemokines, growth factors, vasoactive mediators and coagulation proteins\textsuperscript{[88, 89]}. Dysfunctional endothelium becomes adhesive to inflammatory cells, exposes thrombogenic surfaces and thus promotes inflammation, atherosclerosis and thromboembolism\textsuperscript{[90]}. Activation of other cardiovascular cells such as vascular smooth muscle cells and cardiac fibroblasts also contribute significantly to cardiovascular pathology by initiating intimal hyperplasia\textsuperscript{[91]} and cardiac fibrosis\textsuperscript{[92]}, respectively.

Overall, the presence, integrity and state of activation of an endothelial surface at the implant-host interface can “make or break” a tissue engineered cardiovascular device.
It is thus clear that the secret to successful CVTE is gaining control over inflammation by modulating the endothelium, the “ultimate interface”. In addition to biological grafts, the presence of an intact endothelium can also drastically alter the outcomes of even synthetic prostheses; this was demonstrated by *in vitro* endothelialization of ePTFE grafts, a procedure pioneered by Prof. Peter Zilla in South Africa more than 20 years ago[^93]. In this process the patients’ own endothelial cells were harvested and grown on synthetic ePTFE grafts prior to implantation. This resulted in an improvement in graft patency similar to values obtainable with autologous vessels[^94].

### 1.7 Final Remarks

The field of vascular tissue engineering necessitates advances in cell seeding, cell ingrowth, elastin biosynthesis, and nutrient perfusion before any large clinical breakthroughs are to be expected. Scaffolds need to be altered so that they are less inflammatory and also that they perform well in pro-inflammatory environments. For these objectives, testing TEVGs in healthy and pathological animal models would be useful. The most immediate area in need of improvement is the development of endothelialized scaffolds that are resistant to inflammation.

CVTE holds great potential to solve some of the biggest current health issues. The prospects of using scaffolds, cells, and chemical or mechanical stimuli to create functional tissues are hugely exciting as the field attains skills that were previously unimaginable. After an initial period of hype and hope, we are now closer to clinical application of CVTE; however, we need to increase our control over inflammation and its clinical consequences.
One approach could involve making scaffolds from carefully screened, intact molecules (synthetic or biologic), and repopulating the scaffolds with interstitial cells and endothelial cells \textit{in vitro}. This should be followed by conditioning the constructs in bioreactors until cells “gain control” over the remodeling process and the constructs show clear signs of regeneration. Only then would tissue engineered products be ready for implantation in the targeted dynamic cardiovascular sites.

1.8 References


CHAPTER 2: RESEARCH MOTIVATION, SPECIFIC AIMS, AND PROJECT SIGNIFICANCE

2.1 Introduction

There is no small diameter vascular graft that solves the lack of long-term patency problem following implantation. Examples abound of attempts to vitalize synthetic\cite{95} grafts or extracellular matrix-based (ECM)\cite{96} scaffolds with endothelial cells in order to improve patency and decrease thrombogenicity. However, synthetic grafts often do not heal at the anastomosis due to persistent inflammation\cite{94}, and the majority of products do not taper along their length as do natural vessels\cite{97}. Additionally, non-degradable synthetic materials often remodel very little over implant duration\cite{98,99} while biological scaffolds hold the potential to be remodeled\cite{98,99} and grow with the patient over time\cite{100}. Since products on the market and currently in research fall short of the requisites for vascular grafts, the need for a small diameter graft capable of discouraging aneurysmal dilatation and chronic inflammation while sustaining resistance to thrombus formation and long term patency throughout the duration of implantation is perpetuated.

Certain ECM stabilizing agents used with scaffolds may incite calcification\cite{101}. Contrarily, our group showed that scaffolds stabilized with pentagalloyl glucose (PGG) resist calcification\cite{102} and ECM degrading enzymes\cite{103}. PGG treatment can also inhibit inflammatory cytokines\cite{104}. Additionally, scaffolds can retain a bioactive “niche” that encourages cellular differentiation\cite{105,106}. Preliminary studies suggest that proposed scaffolds are cell-free and complete with a preserved basal lamina for naturally enhanced
cell-ECM adhesion, a primarily collagen and elastin-based ECM, and burst pressures near native arterial values.

The **proposed goal** is to produce bioactive, tapering vascular constructs resistant to dilatation and thrombosis in **preclinical** circulatory models. An **approach** to meet this goal includes the use of acellular scaffolds stabilized with PGG and seeded with autologous endothelial cells to produce **autologous constructs**.

### 2.2 Specific Aims

**Aim 1 (Chapter 3): Characterization of ECM-stabilized vascular scaffolds**

**Hypothesis:** Xenogeneic vessels can be decellularized and stabilized with PGG to produce cytocompatible scaffolds with appropriate ECM integrity and enzymatic resistance.

**Proposed Approach:** a) Develop a vascular graft bioreactor, designed for vascular scaffold preparation, cell seeding, and graft conditioning; b) Decellularize bovine femoral and internal mammary as well as porcine renal arteries, stabilize them chemically, and evaluate their biochemical and mechanical properties, as well as their biodegradation.

**Expected Outcomes:** Stabilized scaffolds will be acellular, cytocompatible, exhibit retention of collagen and elastin, mechanical properties similar to native tissue and resistance to enzymes.

**Aim 2 (Chapter 4): Seeding long vascular scaffolds with tunic-specific cell types**

**Hypothesis:** Cytocompatible PGG-treated scaffolds promote cell viability in the short term.
**Proposed Approach:** a) Commercially available mature endothelial cells will be seeded onto the scaffold lumen, smooth muscle cells into media and fibroblasts onto the adventitia. To improve luminal coverage, repeated seeding and rotations of the scaffold will be used. Seeded scaffolds will be exposed to dynamic culture or static culture as controls and cells stained for viability. b) Scaffolds seeded intra-luminally with adipose-derived stem cells will be cultured for up to 10 days and stained for viability and endothelial markers.

**Expected Outcomes:** Seeded cells will retain viability following static and dynamic conditioning.

**Aim 3 (Chapter 5): Preclinical evaluation of scaffold biocompatibility**

**Hypothesis:** Biological scaffolds are appropriate conduits for vascular surgery.

**Proposed Approach:** a) Implant 2 mm diameter x 1 cm long scaffolds as infrarenal vascular grafts in the abdominal aorta of rats for 4 and 8 weeks and evaluate biocompatibility, thrombosis, patency and intimal hyperplasia (collaboration with vascular surgeons in South Africa). b) Implant 4mm diameter x 4cm long cell-free scaffolds for 1 week in a pilot study on 2 mini-pigs and evaluate biocompatibility, thrombosis, patency and intimal hyperplasia (collaboration with Dr. Langan at GHS).

**Expected Outcomes:** Cytocompatible scaffolds will discourage inflammation, promote cellular infiltration and exhibit properties of a biocompatible material in circulatory animal models.
2.3 Significance of Proposed Project

A great deal of biomedical research conducted in the current time is well-motivated with the goal of improving overall patient health in some capacity, however, the potential for immediate application of these products in a clinical setting is sometimes underdeveloped due to lack of strong working relationships with active clinicians and surgeons. Since the inception of the work described herein, all development of this project has benefitted greatly from the collaboration of the author and local vascular and cardiothoracic surgeons who have clarified the major clinical shortcomings with regards to vascular bypass grafting. Essentially, this project was developed from the ground-up with strong surgical collaboration in order to develop grafts that could potentially save the life, limb, or improve the overall quality of life for current patients living with vascular disease. Although the scope of the present work is limited by academic requirements, follow-up studies using the scaffolds and techniques discussed in this document may potentially further this work to achieve the overall long-term goal of developing bioactive scaffolds for peripheral vascular surgery.

2.4 References


CHAPTER 3: CHARACTERIZATION OF ECM-STABILIZED VASCULAR GRAFTS

3.1 Introduction

Vascular replacement comes in three sizes: large caliber (> 8 mm inner diameter [ID]), medium caliber (6-8 mm ID), and small caliber (< 6 mm ID). The gold standard for bypass grafting is the autologous saphenous vein, however, approximately one-third of all patients lack viable autologous vessels for transplantation due to previous vessel harvesting or advanced vascular disease\(^1\). Patients in this category must use synthetic grafts due to lack of other options. Synthetic grafts for large caliber vascular replacements are currently being used with acceptable long term patency\(^2\). However, synthetic grafts exhibit unacceptable long-term patency when implanted in small diameter coronary and peripheral arteries due to progressive thrombosis\(^3\). Furthermore, the Centers for Disease Control report that for 2007, more than 800,000 individuals died of cardiovascular disease in the US alone, mainly due to small diameter vascular disease\(^4\). Clearly, a great need exists for vascular replacements that remain patent and resist thrombosis through the implant duration.

Currently marketed synthetic conduits include polytetrafluoroethylene (PTFE, W.L. Gore and Associates, Flagstaff, AZ) and exhibit four-year below-knee patency of 12% (requiring revision surgery) versus 49% for autologous saphenous vein (ASV)\(^5\). Heparinized ePTFE (Gore) was found to have four-year patency of approximately 59% vs 70% for ASV\(^6\), which is an upgrade from the previous technology (PTFE), but still requires improvement. Cryopreserved allograft vein (Cryolife, Kennesaw, GA), an
alternative to synthetic vessels, claims infection resistance\cite{7}, but also has poor patency (18% at two years)\cite{8}. These results solidify ASV as the gold standard. A promising approach for patients in need of a small caliber bypass vessel is tissue engineering, with the ultimate goal of creating patient-specific scaffolds seeded with autologous cells and capable of \textit{in situ} remodeling over time.

\subsection*{3.1.1 Current Tissue Engineering Paradigms:}

Tissue engineering strategies typically employ the use of a substrate for cell adhesion, proliferation, and integration – commonly referred to as a scaffold – seeded with an appropriate cell population, depending on the application. The field of tissue engineering showcases various different approaches for scaffold production which are produced either in top-down fashion (a starting material is processed and broken down to achieve a refined product) or bottom-up fashion (building blocks are carefully assembled to produce a final product). Scaffolds produced in bottom-up fashion provide a large degree of specificity in design, but are currently hindered by difficulties in scaling up these processes\cite{9}. Conversely, top-down processing of natural tissues, through a process known as decellularization, can be tailored to remove unwanted components and cells and leave behind an acellular ECM scaffold which can retain many desired native components, including existing vascular networks\cite{10} and bioactive cell niches\cite{11, 12}, which are difficult to reproduce in bottom-up fashion\cite{13, 14}.

ECM scaffolds of biological origin can be naturally degraded using cellular enzymes to break down the fibers comprising these scaffolds\cite{15}. These enzymes, known as
matrix metalloproteinases (MMPs) are endowed to certain cell types to breakdown these fibers. This mechanism of scaffold-fiber breakdown is accelerated in vivo\textsuperscript{[16-17]} and can lead to graft dilatation and aneurysm formation\textsuperscript{[18-19]}. These scaffolds are often treated with a cross-linking agent in order to stabilize the fibers comprising the scaffold and render the scaffold resistant to enzyme-mediated breakdown, but not all products are stabilized\textsuperscript{[18-19]}.

Numerous stabilizing agents are used in research; however, the majority of these agents impart an irreversible stabilization, which often leads to implant failure through inability to be broken down over time, heightened disease progression, and cytotoxicity\textsuperscript{[20-21]}. This irreversible stabilization also hinders natural remodeling of tissue fibers, in which existing fibers are degraded and new fibers are replaced. Maintaining a scaffold capable of remodeling may enhance natural cellular infiltration by allowing scaffold fibers to be degraded and remodeled by contacting cells\textsuperscript{[21, 22]}. Scaffolds stabilized with PGG resist calcification\textsuperscript{[23]} and ECM degrading enzymes\textsuperscript{[17]} and potentially support natural scaffold remodeling\textsuperscript{[24]}.

3.1.2 Strategies for Small Diameter Vascular Tissue Engineering

The majority of tissue engineered grafts maintain a constant diameter along their length\textsuperscript{[25]}, which could be viewed as not ideal. For peripheral bypass, conduits are generally connected to a larger diameter inflow source, such as the femoral artery, and a smaller diameter outflow source, such as the distal popliteal artery or other femorocrural arteries\textsuperscript{[26]}. Because the implanted conduit is breaching a gap between medium- and small-diameter vasculature, a tapered blood vessel of decreasing diameter along its length is favored, in
order to achieve a more appropriate blood flow regime for the endothelial cells lining the conduit\[27\]. Fortunately, most mammalian vessels naturally taper, which is an advantage for the vascular tissue engineer.

Xenogeneic tissue sourcing is a favorable option for researchers for various reasons. Porcine vasculature, for example, has been noted for its similarity to human vasculature\[28\]; however, we found porcine options did not retain the required diameter (6 mm ID) and length (≥ 25 cm) for human vascular replacements. Therefore, this tissue source was used for the development of smaller grafts for use in smaller animal models. The next most readily available, and previously cited option was a bovine host\[29\]. Due to the increased animal size, bovine vessels were substantially larger than those of a porcine host. The bovine internal mammary artery, which was observed to have a proximal ID of approximately 6 mm and a distal ID of approximately 3 mm when collected, was chosen for use because of the vessel’s intrinsic radical taper over its length (~25 cm). The observed high degree of tapering would allow for a more anatomically correct transition from larger inflow to smaller outflow circulation or the ability to be appropriately cut to size for testing in animal models or other scenarios where only a small segment of graft is needed. Prior to cell seeding experimentation, the vessel will be rendered acellular to create an ECM-based scaffold. Biological scaffolds can potentially retain an endogenous, bioactive “niche” that encourages cellular differentiation\[11, 12\]. For these reasons, PGG stabilized biological scaffolds were chosen for further characterization for potential as peripheral bypass grafts.
3.2 Development of Bovine Mammary and Femoral Artery Scaffolds

3.2.1 Materials and Methods

3.2.1.1 Scaffold Preparation – Decellularization

Previous efforts to decellularize long vessels (>25 cm) through chemical immersion yielded incomplete native cell removal (data not shown). A bioprocessor was constructed (Figures 3.1, 3.2D) capable of decellularizing native vessels and also serving as a bioreactor for culturing seeded scaffolds. Prior to decellularization, native bovine mammary arteries (taper from 6 mm to 3 mm ID along their length (Figure 3.2B)) and femoral arteries (taper from an 8 mm to 6 mm inner diameter (ID) along their length (Figure 3.2A)) (Animal Technologies, Tyler, TX) were cleaned of adherent tissue and their branches were cut and sutured shut (4-0 Ethibond braided suture with RB-1 needle, Ethicon Inc., Somerville, NJ) (Figure 3.2A, 3.2B, 3.2C, 3.2E). Once arteries were cleaned of adherent tissue, they were placed in an “overnight solution” consisting of 30 mM Ethylenediaminetetraacetic acid (EDTA, Fisher Scientific) and 0.02% sodium azide (NaN₃, Fisher Scientific) dissolved in distilled, deionized water (ddH₂O) and stored shaking at 4°C until all artery branches were appropriately sutured shut.

Following incubation in overnight solution, sutured arteries were then mounted in the bioprocessor. Before decellularization, mounted arteries were rinsed with 5 L of unpressurized ddH₂O and 2.5 L of pressurized ddH₂O (this process will be referred to the “ddH₂O protocol” throughout the rest of this publication) to flush all remnants of overnight solution from the arteries and the system. Rinsed arteries were treated with sodium dodecyl sulfate (SDS, Fisher Scientific, Pittsburgh, PA) for 12 days (changing SDS at 6 days),
exposed to the ddH$_2$O protocol, then exposed to 3 separate 1-hour incubations with 70% ethanol (Pharmco-Aaper, Belmont, NC), demonstrating transmural diffusion along the arteries’ length (Figure 3.2F). Following another round of the ddH$_2$O protocol, arteries were incubated in 0.1 M sodium hydroxide (NaOH, Fisher Scientific) for 2 hours and then exposed to the ddH$_2$O protocol again. Following ddH$_2$O rinsing, arteries were incubated overnight in 1x phosphate buffered saline (PBS, Corning-Cellgro, Manassas, VA). Arteries were then exposed for 96 hours at room temperature to a solution of 720 munits/mL deoxyribonuclease (DNase, Worthington Biochemical Corp., Lakewood, NJ), 720 munits/mL ribonuclease (RNase, Fisher Scientific) and 5 mM MgCl dissolved in 1x PBS and the pH adjusted to 7.5. Following 96 hours, the system was flushed with 1x PBS for 1 hour. All incubations were exposed to 80 mmHg pressure, unless otherwise specified, to achieve decellularization. Scaffold decellularization efficacy was qualitatively assessed using histological 4',6-diamidino-2-phenylindole (DAPI, Vector Labs, Burlingame, CA) staining for DNA and quantitatively with a fluorescence-based PicoGreen assay for total double-stranded DNA content (Invitrogen, Grand Island, NY) using a Gemini XPS Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA); data was normalized to sample dry weight.

### 3.2.1.2 Scaffold Preparation - Stabilization

Following decellularization, scaffolds were stabilized with sterile-filtered 0.15 % pentagalloyl glucose (PGG, N.V. Ajinomoto OmniChem S.A., Wetteren, Belgium) in 50 mM dibasic sodium phosphate buffer with 20% isopropanol in saline, at pH 5.5 and room
temperature, without perfusion or pressure, for 24 hours. After stabilization with PGG, scaffolds were rinsed twice with 1x PBS, incubated in 70% ethanol for 10 minutes, exposed to the ddH₂O protocol, then rinsed 6 separate times with 1x PBS for 24 hours. All incubations exposed to 80 mmHg pressure, unless otherwise specified.

3.2.1.3 Scaffold Preparation - Sterilization

Stabilized scaffolds were sterilized with 0.1% peracetic acid dissolved in 1x PBS and adjusted to pH 7.0 (Fisher Scientific) for 2 hours. Following sterilization, scaffolds were exposed to the ddH₂O protocol, then rinsed twice with 1x PBS, with the last rinse incubating for 20 minutes. Sterilized and rinsed scaffolds were then stored in a solution of 1x PBS and 1% penicillin-streptomycin-amphotericin (PSA, Corning-Cellgro). All solutions were sterile-filtered prior to use.

3.2.1.4 Scaffold Characterization – Histology

Completely acellular scaffolds were analyzed histologically with hematoxylin and eosin (H&E, Fisher Scientific) for cell nuclei and ECM morphology, Gomori’s trichrome (Poly Scientific R&D Corp., Bay Shore, NY) for collagen and muscle protein, and Verhoeff’s van Gieson stain for elastic tissue (Poly Scientific) to qualitatively assess total ECM integrity of the acellular scaffolds (Figures 2, 3). In preparation for in vitro cell seeding studies, scaffolds were then analyzed using immunohistochemical methods for the presence of three basal lamina components, laminin (Abcam, Cambridge, MA), collagen type IV (Abcam) and fibronectin (EMD Millipore, Darmstadt, Germany). The basal lamina
is a matrix of cell-secreted biomolecules and proteins found in native tissue which connects cells to their surrounding ECM and greatly facilitates natural cellular adhesion to scaffolds without application of exogenous ECM substrates \cite{30} (Figures 2, 3).

3.2.1.5 Scaffold Characterization – Mechanical and Biochemical Properties

Scaffolds were analyzed quantitatively for burst pressure using a peristaltic pump to progressively fill sections with 1x PBS and a pressure transducer (Cole Parmer, Vernon Hills, IL) to record the scaffold’s ultimate pressure before rupture (Figure 3.5A, 3.5B and 3.6A). Diametrical compliance measurements made use of the same peristaltic pump and pressure transducer in addition to a Lumix digital camera (Panasonic Corp., Secaucus, NJ). Briefly, scaffold sections were exposed to 80 mmHg and 120 mmHg, capturing an image at each pressure setting, then the diametrical distension was measured by importing images into SolidWorks computer-aided design software (Dassault Systems, Vélizy-Villacoublay, France) and differences in distension were noted for each scaffold between 80 and 120 mmHg at 6 total positions perpendicular to the scaffold sides (Figure 3.5K). Diametrical compliance was then calculated by inputting these measured differences into equations discussed by Hamilton’s group \cite{31} (Figure 3.5F, 3.6B).

Suture retention strength was calculated by cutting scaffolds into 10x20 mm sections, clamping one end to an MTS test frame (MTS Systems Corp., Eden Prarie, MN), and placing a single 4-0 braided suture 2 mm from the free edge and tied to the test frame. Secured sections were preloaded to 0.005 N and extended to failure at 5 mm/min using a 10N test frame (MTS Systems Corp.) (Figure 3.5G, 3.6C).
Ring opening angle measurements, a metric of circumferential tension within vascular tissues first discussed by Fung’s group\cite{Fung1974}, made use of 2 mm thick transversely cut scaffold sections transferred to petri dishes and filled with 1x PBS to the tissue surface prior to splaying the sections open with a scalpel blade (Figure 3.5C, 3.5D). Once scaffolds were splayed open, the tissues were allowed to equilibrate for 15 minutes in 1X PBS while rotating at 30 RPM on an orbital shaker at room temperature. Upon completion of incubation, a top-down image of the section was captured and the ring opening angle was calculated by importing the image into SolidWorks and measuring the cut tissue’s angle similar to the methods employed for diametrical compliance (Figure 3.5E, 3.5H; femoral scaffold ring opening data not shown).

Total tissue collagen and elastin content was calculated by measuring, with the help of Dr. Barry Starcher (University of Texas Health Science Center at Tyler, Tyler, TX), total tissue hydroxyproline (a component of natural tissue collagen) and desmosine (a component of natural tissue elastin) and normalized to dry tissue weight. Measured values were then applied to known tissue ratios\cite{Starcher1991, Starcher1993} to approximate total tissue collagen and elastin per sample (Figure 3.5I and 3.6D).

Scaffold resistance to enzymatic degradation by collagenase and elastase made use of chromatographically purified type VII collagenase (Sigma-Aldrich Corp., St. Louis, MO) and high purity elastase (Elastin Products Company, Inc., Owensville, MO). Samples were cut into 3x3 mm squares, lyophilized and their masses recorded on an analytical balance (Ohaus Corp., Parsippany, NJ) to obtain dry tissue mass. The enzyme digestion buffer utilized for both enzymes consisted of 1 mM CaCl$_2$ (Calcium Chloride, Fisher
Scientific) and 0.02% NaN₃ (Sodium azide, Fisher Scientific) in 100 mM Tris (hydroxymethyl) aminomethane (Tris, EMD Millipore), at pH 7.8. Digestion buffers were prepared as separate stock solutions at a final concentration of 20 units/mL ultrapure type VII collagenase (Sigma-Aldrich) or 6.25 units/mL elastase (Elastin Products Company) respectively. Scaffold samples were placed in DNase/RNase-free microcentrifuge tubes (Fisher Scientific) and filled with 1 mL of the desired enzyme digestion solution. Samples were incubated at 37°C using an Excella E24 climate-controlled orbital shaker (Eppendorf, Hamburg, Germany) for an duration of 24 hours. After incubation, samples were centrifuged at 12,000 RPM, the supernatant discarded, rinsed with 1.5 mL of ddH₂O and centrifuged at 12,000 RPM. Three total rinsing cycles were utilized prior to discarding the supernatant, lyophilizing the digested samples, recording the difference between the original and digested tissue sample masses and calculating the total percent tissue loss during digestion (Figure 3.5J and 3.6E).

3.2.1.6 Scaffold Characterization – Cytocompatibility

Before large-scale cell seeding studies can be conducted, it must be established that the stabilized scaffolds are not toxic to contacting cells (cytocompatible). To assess this metric, small 2 mm ring sections were prepared by transversely sectioning a PGG-stabilized scaffold. These sections were then sterilized using 0.1% peracetic acid, as described previously (Scaffold Preparation – Sterilization). Sterilized scaffolds were then placed in a sterile 24-well plate (Greiner Bio-One, Monroe, NC) carefully wetted with
culture medium (10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1% antibiotic solution (Corning-Cellgro), and 89% Dulbecco’s modification of eagle’s medium (Corning-Cellgro)), taking note to only fill the wells with enough medium to almost cover the surface of the section. 10,000 rat fibroblasts (Passage 10 from Sprague-Dawley rats, Harlan Laboratories, Indianapolis, IN) were then seeded in dropwise fashion on to the scaffolds’ surface and allowed to incubate for 4 hours before gently filling each well with enough medium to cover the surface of the seeded scaffolds. These seeded scaffolds were cultured for a total of 3 days with no shaking, stirring, or media changing for the duration of culture, before imaging for cell viability using Calcein AM staining at 25x total magnification (Life Technologies, Grand Island, NY; Figure 3.7).

3.2.2 Results

3.2.2.1 Decellularization

Complete scaffold decellularization was confirmed qualitatively by noting removal of cell nuclei with DAPI staining for DNA (Figure 3.2G, 3.2H, 3.2J, 3.2K) as well as a quantitative PicoGreen assay for total double-stranded DNA content (Figure 3.2I, 3.2L). DAPI micrographs were overlayed with natural elastin autofluorescence micrographs in order to improve visualization of scaffold media and emphasize the lack of visible DNA within it. PicoGreen assays showed an approximate 90% and 94% reduction in DNA content for the mammary and femoral scaffolds, respectively.
Figure 3.1 – Overview of bioprocessor design and assembly. Computer-aided design image depicts tissue enclosures are tilted slightly by a base which screws into both tissue enclosures (right). This tilt allows air bubbles to naturally leave the system at the top of the enclosures, indicated by the black lines (right). Support rods (right) hold the pressure heads (left) in place, which allow the retention of hydrostatic pressure within the system. A peristaltic pump perfuses the system using manifolds to direct fluid flow (left).

3.2.2.2 Scaffold Characterization – Histology

Qualitative histology suggests preservation of overall arterial matrix morphology (Figure 3.3A, 3.3D, 3.4A, 3.4D), native collagen (Figure 3.3B, 3.3E, 3.4B, 3.4E) and elastin (Figure 3.3C, 3.3F, 3.4C, 3.4F) after decellularization. Additionally, immunohistochemical staining shows native basal lamina laminin, (Figure 3.3G, 3.3H, 3.3K, 3.3L, 3.4G, 3.4K) collagen type IV, (Figure 3.3I, 3.3J, 3.3M, 3.3N, 3.4H, 3.4L) and fibronectin (Figure 3.3O, 3.3P, 3.3S, 3.3T, 3.4I, 3.4M) are preserved following decellularization. The absence of the powerful xenoreactive galactose (α1,3) glucose (α-gal) antigen was also confirmed with immunohistochemistry in mammary (Figure 3.3Q, 3.3R, 3.3U, 3.3V) and femoral scaffolds (Figure 3.4J, 3.4N).
Figure 3.2 – Bovine femoral (A) and mammary (B) arteries are cleaned of adherent tissue, the branches sutured shut (C), then the arteries are mounted (E) in a bioprocessor (D) and perfused with various chemicals and solvents to achieve transmural diffusion-mediated decellularization of scaffolds (F). DNA removal is confirmed with qualitative DAPI stain (G, H, J, K) and quantitative PicoGreen for double-stranded DNA (I, L). Image H enlarged to show absence of DNA.
3.2.2.3 Scaffold Characterization – Mechanical and Biochemical Properties

In mechanical properties, PGG-treated mammary scaffolds were found to be not statistically different from fresh tissue in both burst pressure (Figure 3.5A) and suture retention strength (Figure 3.5G). However, decellularized (decell group) and PGG-treated mammary scaffolds were statistically different from fresh tissue in diametrical compliance (Figure 3.5F) and ring opening angle (Figure 3.5H). Contrarily, PGG-treated femoral scaffolds were statistically different in burst pressure (Figure 3.6A), diametrical compliance (Figure 3.6B), suture retention strength (Figure 3.6C), and ring opening angle (data not shown). When testing femoral scaffold ring opening angles, both decellularized and PGG-treated femoral scaffolds collapsed upon themselves once cut open during testing, creating negative angles. However, fresh femoral arteries were found to open similarly to fresh mammary arteries as well as decellularized and PGG-treated mammary scaffolds.

In biochemical properties, decellularized mammary arteries were found to be statistically different from fresh tissue in percent collagen and elastin content (Figure 3.5I). When testing enzyme resistance in vitro, PGG-treated mammary scaffolds were found to be not statistically different from fresh tissue for collagenase resistance at all tested timepoints (5, 7, and 11 months of storage following PGG-treatment). However, PGG-treated mammary scaffolds stored for 7 and 11 months following PGG-treatment were statistically different from fresh arteries for elastase resistance (Figure 3.5J). Decellularized femoral arteries, in the context of percent collagen and elastin content, were not statistically different from fresh arteries for percent collagen content; however,
Figure 3.3 – Mammary artery scaffolds stained with H&E (A, D), Trichrome (B, E), and VVG (C, F) to verify cell removal and retention of endogenous collagen (B, E) and elastin (C, F). Immunostaining for basal lamina laminin (G, H, K, L), collagen type IV (I, J, M, N) and fibronectin (O, P, S, T) suggest retention of the basal lamina. Xenoreactive epitope α-gal absent from scaffolds (Q, R, U, V). Scale bars = 50 µm.
Figure 3.4 – Femoral artery scaffolds stained with H&E (A, D), Trichrome (B, E), and VVG (C, F) to verify cell removal and retention of endogenous collagen (B, E) and elastin (C, F). Immunostaining for basal lamina laminin (G, K), collagen type IV (H, L) and fibronectin (I, M) suggest retention of the basal lamina. Xenoreactive epitope α-gal notably absent from scaffolds (J, N). All scale bars = 50 µm.
decellularized femoral arteries were found to be statistically different in terms of percent elastin content (Figure 3.6D). Enzyme resistance testing for femoral scaffolds suggests that PGG-treated femoral scaffolds are statistically different from fresh arteries, for both collagenase and elastase (Figure 3.6E). All statistical analyses made use of 1-factor ANOVA with \( \alpha=0.05 \).

3.2.2.4 Scaffold Characterization – Cytocompatibility

Following tissue culture of seeded scaffolds for 3 days, imaging with Calcein AM suggested that cells naturally aligned circumferentially on the media and exhibited an elongated, spindle-shaped morphology. Additionally, cells that attached to the luminal surface of the scaffold seemed to align longitudinally on the scaffold (Figure 3.7).

3.2.3 Discussion

3.2.3.1 Scaffold Characterization – Histology and Cytocompatibility

Arguably the largest road blocks to widespread use of xenogeneic tissue for biological scaffolds are the presence of residual DNA\(^{35}\), cellular proteins such as smooth muscle\(^{36}\), and xenoreactive epitopes such as alpha-gal\(^{37}\) within the resultant scaffold matrix. Although researchers have a reasonable understanding of what approximate levels may lead to an immune response when implanted, Badylak’s group has recently stated that threshold levels of these and many more metrics for decellularized scaffolds have yet to be established and agreed upon by the community\(^{36}\). We are presenting two vascular scaffolds which have over 90\% reduction in DNA content per dry tissue mass, no visible nuclei in H&E or DAPI stains, no visible smooth muscle in trichrome stains,
Figure 3.5 – Mammary artery scaffolds mechanically and biochemically characterized. Burst pressure (A, B – n=9 Fresh, n=8 Decell, and n=8 PGG), diametrical compliance (F, K – n=8 for all groups), suture retention strength (G – n=8 for all groups), ring opening angle (H, C, D, E – n=16 Fresh, n=24 Decell, and n=8 PGG), total tissue hydroxyproline and desmosine (I – n=8 for all groups), and enzyme resistance (J – n=10 for all groups) were all compared between fresh, Decell, and PGG treatment groups. * denotes statistical difference from Fresh, # denotes statistical difference from Decell, ^ denotes statistical difference from PGG - 7mo group.
Figure 3.6 – Femoral artery scaffolds mechanically and biochemically characterized. Burst pressure (A – n=8 Fresh, n=11 Decell, and n=6 PGG). Diametrical compliance (B – n=9 Fresh, n=9 Decell, and n=6 PGG). Suture retention strength (C – n=8 for all groups). Total tissue hydroxyproline and desmosine (D – n=8 for all groups). Enzyme resistance (E – n=10 for all groups) were all compared between fresh, Decell, and PGG treatment groups. * denotes statistical difference from Fresh, # denotes statistical difference from Decell.

Figure 3.7 – Cytocompatibility of PGG-stabilized scaffolds. Viable seeded cells following 3 day incubation are stained bright green. Natural tissue autofluorescence is a much lighter green.
and no alpha-gal epitope visible following histochemical analysis. We therefore consider these scaffolds decellularized.

Another tough obstacle for tissue engineered scaffolds to overcome \textit{in vivo} is the necessity of a capillary network within scaffolds to ensure seeded cell and/or ingrowing cell survival in the long term. Our group has recently shown the retention of capillary networks within perfusion-decellularized myocardium\cite{38}, which adds credibility to a decellularization-based approach for scaffold production. Although we do not demonstrate capillary network retention using the same metrics, microvessels supplying the adventitia of our scaffold can be seen in the images Figure 3.3H, 3.3L, 3.3N, and 3.3T suggesting at least the retention of adventitial microvessels within our scaffolds which could potentially improve \textit{in vivo} performance in the long term.

Probably one of the biggest assets to a decellularization-based top-down approach to scaffold production is the potential to retain a bioactive matrix capable of not only facilitated cell adhesion through the retention of basal lamina components, but also the mounting evidence that endogenous ECM-based “niches” serve as queues that are able to induce cellular differentiation into desired cell types\cite{11, 12}. Future studies will need to be done to support this niche hypothesis for our scaffolds, but we do present retention of basal lamina fibronectin, laminin, and collagen type IV on both scaffolds. It should be noted that the presence of these components is often times isolated to specific areas of the scaffolds such as the lumen of microvessels (Figures 3.3L, 3.3T and Figure 3.4K), the lining of the intima (Figure 3.3S and 3.4G), and observed presence in the media but not in the adventitia (Figures 3.3L, 3.3T, 3.4K) which imply a unique distribution of these basal lamina
components within our scaffolds. Furthermore, seeded cells initially studied for cytotoxicity of scaffolds displayed a natural tendency to align according to the natural orientation of native smooth muscle cells in the media of fresh vessels (circumferentially) which further supports the feasibility of a preserved bioactive niche within our scaffolds. Future stem cell differentiation studies will be necessary to support the hypothesis of a preserved niche within the presented scaffolds.

3.2.3.2 Scaffold Characterization – Mechanical and Biochemical Properties

In addition to scaffold histological properties, the mechanical properties of PGG-treated scaffolds with respect to burst pressure, diametrical compliance, suture retention strength and ring-opening angle must be investigated in order to completely characterize scaffolds with clinically-relevant metrics. Mechanical properties of vascular scaffolds can potentially be compared to either native tissue before treatment in the case of decellularization-based approaches\textsuperscript{[39]}, diseased human peripheral vessels in need of replacement\textsuperscript{[31]}, healthy human peripheral vessels\textsuperscript{[40]}, or in some cases, combinations of these options\textsuperscript{[31]}. Although debate exists as to which route of comparison is the most clinically relevant, it cannot be denied that the most frequently replaced vascular tissue is that of the much stiffer, diseased origin. Regardless, the goal of replacing diseased vasculature is to bring the surrounding vasculature closer to a disease-free condition. We compare our PGG-treated scaffolds to their fresh and decellularized counterparts for each mechanical test in order to show the change in mechanical properties observed following decellularization, and only additionally compare to human arteries at desired peripheral
implantation sites in the case of diametrical compliance in order to make the data more meaningful and convey the clinical applications of that specific set of data. Our PGG-treated scaffolds exhibit burst pressures well above physiological conditions, so a human comparison is not necessary and suture retention strength is difficult to compare between groups due to the inherent variation in the conditions used by groups\[^{41-43}\]. Therefore, scaffold suture retention strength is only compared to fresh and decellularized tissue.

Both presented scaffolds exhibit similar trends across all three treatment groups in terms of burst pressure and diametrical compliance. When comparing the compliance of our scaffolds to native human arteries, Hamilton’s group has reported that healthy patients of 67 years of age, on average, have diametrical compliance values of their proximal superficial femoral arteries, distal superficial femoral arteries, and popliteal arteries of 6.1, 3.8 and 4.7 percent per 100 mmHg [(%/mmHg)x10\(^{-2}\)]\[^{31}\], respectively. These arteries were chosen to compare against because most frequently peripheral bypasses are initiated at various locations of the femoral artery (inflow site more proximal depending on extent of vascular disease) and anastomosed to below-knee popliteal arteries in what is commonly referred to as femoropopliteal bypass. Our mammary and femoral scaffolds display compliance values of 6.18% (± 0.72) and 5.79% (± 0.61) percent per 100 mmHg, respectively, which fall in between the values for the proximal superficial femoral arteries and popliteal arteries reported by Hamilton’s group\[^{31}\].

Both PGG-treated scaffolds show increased suture retention strength when compared to fresh tissue, although this difference was not statistically significant for the mammary scaffold. L'Heureux’s group recently reported fresh human internal mammary
artery suture retention strength at 120 mm/min strain rate as retaining 138 (±50) gmf before tearing\[^{40}\]. However, our results are likely not comparable because our group used a strain rate of 5mm/min, as was also reported by McFetridge’s group\[^{41}\].

Although the femoral scaffold ring opening data is not shown here, the mammary scaffold ring opening data is included in order to present another metric that affirms the similarity between decellularized and PGG-treated scaffolds. Arterial ring opening angle, as first discussed by Fung’s group\[^{32}\], is a metric of residual circumferential stress within arteries which was tested to investigate the effect of PGG stabilization on the presented scaffolds. Seeing as there is no statistical difference between the mammary decellularized and PGG-stabilized scaffold ring opening angle in addition to both the femoral decellularized and PGG-stabilized scaffolds behaving in similar fashion when tested, it can be suggested that PGG-stabilization does not affect ring opening angle for either presented scaffolds.

With burst pressures and suture retention strength well above those for fresh tissue, and diametrical compliance values approximately matching those of human femoral and popliteal arteries, the proposed PGG-treated scaffolds should be regarded as retaining optimal mechanical properties for implantation.

Quantitative assessment for total tissue collagen and elastin content per dry tissue mass is rarely done on biological scaffolds. We include these tests for the discussed scaffolds because the sum of the percentages of decellularized collagen and elastin content per scaffold dry weight add to approximately 100%, which suggests that the majority of cellular proteins are removed by decellularization. Therefore, it is assumed that the
scaffolds are comprised almost entirely of collagen and elastin. The data also suggests about 10% higher elastin content in the femoral scaffolds than the mammary scaffolds, which may provide some explanation for the observed higher suture retention strength and burst pressure as well as lower diametrical compliance in the femoral scaffolds than the mammary scaffolds.

Very little data exists on enzymatic resistance for PGG-treated scaffolds. Previous work from our group explored elastase resistance of PGG-treated pure bovine neck ligament elastin\cite{44}, elastase resistance of vascular scaffolds stabilized with increasing concentrations of PGG\cite{24}, and an approximately 1 week timecourse study of collagenase degradation for heart valve scaffolds stabilized with PGG\cite{21}. To the best of our knowledge, this is the first publication to discuss the effects of long term storage of PGG-treated scaffolds on both collagenase and elastase resistance. Isenburg et al. previously suggested that storing PGG-stabilized scaffolds at pH 5.5 may lead to increased longevity of PGG stabilization and scaffold resistance to collagenase and elastase, however, supporting data was not shown\cite{44}. The data presented in this publication seems to support this hypothesis. All tissues used for the studies discussed in this publication were stored at a pH of 7.0. Future studies may investigate scaffold storage in a lower pH solution in order to preserve scaffold enzymatic resistance following treatment with PGG.
3.2.4 Other Remarks

With scaffold lengths of approximately 25 cm, these grafts are long enough to be considered for clinical use. The intrinsic differences in geometry between the two scaffolds also point to unique potential applications for each scaffold. For example, the observed large degree of taper in the mammary scaffold (approximately 6 mm to 3 mm along its length) may make this scaffold an excellent candidate for coronary bypass, where 25 cm of graft should prove to be more than enough in most cases. The femoral scaffold, because it tapers very little along its length (approximately 8 mm to 6 mm), could serve as a potential graft for cases when portions of the iliac or femoral arteries may need to be replaced. Most importantly, the needs of each patient are different and numerous potential combinations exist for each separate scaffold as a bypass graft.

![Possible combinatory use of mammary and femoral artery scaffolds](image)

**Figure 3.8**—Possible combinatory use of mammary and femoral artery scaffolds. Given the high suture retention strengths of both the femoral (A) and mammary (B) artery scaffolds, these scaffolds could potentially be sutured together in end-on-end fashion (C) in order to accomplish much longer or more complicated bypasses than separate scaffolds may allow. This is an important option given that most commercially available vascular grafts come in 25, 50, and 75 cm lengths and biological grafts are often limited in size.

Furthermore, these scaffolds could potentially be combined in end-to-end fashion to achieve longer or more complicated bypasses normally impossible for shorter, non-
tapering grafts (Figure 3.8). These characteristics, as well as the robust mechanical properties, approximate total composition of collagen and elastin, retention of endogenous bioactive basal lamina proteins, and the absence of the xenoreactive epitope alpha-gal from the tissue point to the potential for these scaffolds to function immediately upon implantation and eventually be remodeled and integrated into the patient. Future animal studies will be needed to test these claims and further elucidate the potential of these scaffolds to serve as bypass grafts in the context of regenerative medicine.

3.3 References


3.4 Development of Porcine Renal Artery Scaffolds

3.4.1 Introduction

Previous work in the laboratory laid the initial groundwork for a sodium hydroxide (NaOH)-based decellularization protocol using timed immersion and agitation of porcine carotid arteries\textsuperscript{17}. After discussing the clinical potential of these grafts with surgical collaborators, it was deemed necessary to develop much longer grafts in order to meet the unmet clinical need for long (≥ 25 cm) small diameter (≤ 6 mm) vascular grafts (see section 3.2 – Development of Bovine Mammary and Femoral Artery Scaffolds). In order to gain funding for large animal biocompatibility studies to assess the \textit{in vivo} biocompatibility of these larger scaffolds, previous \textit{in vivo} data would be necessary in order to support feasibility of these tissue engineered scaffolds to function as appropriate vascular grafts capable of discouraging thrombus formation and inflammation through the implant duration. To meet this requirement, renal artery scaffolds (1.5 cm long; 1-2 mm diameter) were prepared and characterized prior to \textit{in vivo} studies of these smaller porcine renal artery scaffolds.

3.4.2 Materials and Methods

3.4.2.1 Scaffold Preparation – Dissection and Isolation

In order to isolate renal arteries from porcine kidneys, a stepwise dissection protocol was established. Kidneys are collected from a local abattoir and stored on ice while in transit back to the laboratory. To remove the fascia from the kidney, an incision is made into the thickness of the tissue and the fascia is pulled away from the kidney before being
removed along with extraneous fat (Figure 3.9A). The renal artery “trunk” must now be isolated by dissecting away all extraneous fat and preserving the renal artery trunk as well as its downstream branches (Figure 3.9B).

**Figure 3.9** – Dissection and isolation of renal arteries. Porcine kidneys are cut to remove exterior fascia (A), the renal artery trunk isolated (B), then gross dissection is initiated by tearing the top and bottom (C) of the kidneys prior to gently prying the kidney walls from the renal artery trunk and its branches (D). Extraneous tissue is cut from the kidneys (E, F) prior to finalizing the dissection by isolating the entire arterial tree (G) and grouping branches for decellularization.

To begin dissecting the arterial tree from the kidney, slide a thumb through the top and bottom “edges” of the kidney (Figure 3.9C). Next, gently “pry” the wall of the kidney from the renal artery trunk and its branches (Figure 3.9D) – care must be taken to not damage the small arteries by using too much force. Once these steps are complete, the
kidneys are then cut through the “bottom” (inferior) region of the kidney into the ductwork of the urinary tract. Vascular supply stops just above this “wall”, which can be used as a landmark for how much extraneous kidney tissue can be removed. Now knowing how far to cut into the kidney, remove all extraneous tissue from the kidney (Figure 3.9E, 3.9F). Once unnecessary tissue is removed, gently and carefully dissect the entire arterial tree from the kidney (Figure 3.9G), leaving behind a hierarchy of branches ranging from ≥ 3 mm to < 1mm. Select the branches ranging from 1-2 mm diameter, cut them so that each segment is 10-20 mm in length, and group them with 4-5 branch segments per group (paying attention to maintain approximately the same amount of mass between each group) prior to decellularization.

3.4.2.2 Scaffold Preparation – Decellularization

Following dissection and isolation, each group of renal arteries are placed in separate 50 mL conical tubes and filled with 45 mL of 0.1M NaOH. Sealed tubes are then placed in a 37°C shaking water bath for 3 hours. Following 3 hours of NaOH treatment, the arteries are removed with aseptic technique and sterile forceps and placed into new 100 mL sterile specimen cups and filled with sterile ddH₂O. The arteries (still separately grouped as before) are now rinsed in ddH₂O for 5 total minutes using an orbital shaker set to 3.5 rpm. Following a total of 10 sterile ddH₂O rinses, the ddH₂O is decanted and replaced with sterile 1x phosphate buffered saline (PBS) in order to neutralize any residual alkalinity within the tissues. Follow-up with one additional PBS rinse. Scaffold decellularization efficacy was qualitatively assessed using histological DAPI staining (Vector Labs) and
agarose gel electrophoresis with ethidium bromide (Fisher Scientific) to show residual DNA within scaffold sections (Figure 3.11). The agarose gel was loaded with DNA standard, fresh renal artery “trunks” and fresh renal arteries (controls) as well as decellularized renal artery trunks and decellularized renal arteries.

3.4.2.3 Scaffold Preparation – Stabilization (PGG)

PGG solutions were prepared as described previously (3.2.1.2 Scaffold Preparation – Stabilization). Decellularized scaffolds were kept as previously grouped and filled with 40 mL of sterile-filtered PGG solution. Scaffolds were treated with PGG for 24 hours, shielded from light, with no additional shaking or agitation. Following treatment, scaffolds were rinsed for 10 minutes with sterile 1x PBS, 10 minutes in sterile 70% ethanol, and then rinsed for 24 hours in sterile 1x PBS. Scaffolds were rinsed for a total of six 24-hour periods before sterilization.

3.4.2.4 Scaffold Preparation – Stabilization (Heparinization)

To optimize the heparinization protocol, PGG and non-PGG treated scaffold samples were rinsed in phosphate buffered saline (PBS), reacted with Jeffamine (amine terminated polypropylene glycol, Huntsman, Figure 3.10 – Mechanism of tissue heparinization. Courtesy of Tim Pennel.
240mM in 0.25M MES buffer, pH=5) for 2 hours at room temperature using a combination of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccimimide (EDC/NHS, 300/10mM) as activator. After another PBS rinse, nitrous acid degraded (NAD) heparin (2mg/ml; in 0.15M NaCl; pH=3.9, solution containing 1mg/ml NaCNBH3) was coupled by reductive amination to the aminated and non-aminated control tissue samples by a 16 hour reaction at room temperature. The treatments were performed either with or without pre-soaking in Heparin and samples were subsequently rinsed and stored in sterile PBS.

3.4.2.5 Scaffold Preparation – Sterilization

Peracetic acid solutions were prepared as described previously (3.2.1.3 Scaffold Preparation – Stabilization). PGG-stabilized scaffolds were kept as previously grouped and filled with 45 mL of sterile-filtered peracetic acid solution. Scaffolds were shaken on an orbital shaker for a period of 6 hours to ensure sterilization. Following treatment, scaffolds were rinsed with sterile ddH2O for 1 hour, changing the sterile ddH2O every 15 minutes. Following ddH2O rinsing, two 10 minute 1x PBS rinses were conducted prior to storage of sterilized, stabilized scaffolds.

3.4.2.6 Scaffold Characterization – Histology

Completely acellular scaffolds were analyzed histologically with H&E (Fisher Scientific) for cell nuclei and ECM morphology, Masson’s trichrome (Poly Scientific R&D Corp.) for collagen and muscle protein and VVG stain for elastic tissue (Poly Scientific) to qualitatively assess total ECM integrity of the acellular scaffolds (Figure 3.12).
3.4.2.7 Scaffold Characterization – Mechanical and Biochemical Properties

PGG-treated renal artery scaffold burst pressure, diametrical compliance and resistance to enzymatic degradation by collagenase and elastase were measured as described previously (3.2.1.5 Scaffold Characterization – Mechanical and Biochemical Properties). All statistical analyses made use of 1-factor ANOVA with α=0.05.

3.4.2.8 Scaffold Characterization – Heparin Quantification

The heparin content of the samples (n=3) was quantitatively determined by 3-methyl-2-benzothiazinone hydrazone (MBTH) assay adapted from Riesenfeld and Roden[139] and similar to work previously described by our colleagues[140]. Briefly, after heparinized and non-heparinized control samples (in 250µl of deionized water; DI) were reacted with nitrous acid (250µl, 30 min, 0.025M HNO2, 0.9M HCl), the reaction was stopped using 250µL of 1 M ammonium sulfamate solution. The combined supernate and subsequent rinse (500µl 1M NaCl) were reacted with MBTH (11mM, 500µL, 15min, RT), complexed by addition of FeCl₃ (500µl, 31mM, 20min 50ºC) and the absorbance of cooled, resultant solutions determined by UV spectroscopy (660 nm, Shimadzu UV-1601PC). Heparin content (mg/g tissue) was calculated from the standard curves obtained in a similar manner but with omission of the sample and replacement of the initial 250 uL DI with the same volume of the standard solutions (0–600mg/250mL).

3.4.2.9 Scaffold Characterization – Denaturation Temperature Determination

Thermograms of tissue samples (5-10mg, n=3) in sealed aluminum sample pans were obtained at a heating rate of 10ºC/min (DSC 7; Perkin Elmer) and the onset
temperature of the denaturation endotherm recorded as the denaturation temperature.

3.4.3 Results

3.4.3.1 Scaffold Preparation – Decellularization

Complete scaffold decellularization was confirmed qualitatively by noting removal of cell nuclei with DAPI staining (Figure 3.11A, 3.11B) as well as agarose gel electrophoresis (Figure 3.11C). No residual DNA was visible in either test metric.

3.4.3.2 Scaffold Characterization – Histology

Qualitative histology suggests preservation of overall arterial matrix morphology (Figure 3.12A, 3.12E), native collagen (Figure 3.12B, 3.12F) and elastin (Figure 3.12C, 3.12G) after decellularization. Absence of the powerful xenoreactive galactose (α1,3) glucose (α-gal) antigen was also confirmed with immunohistochemistry (Figure 3.12D, 3.12H).

3.4.3.3 Scaffold Characterization – Mechanical and Biochemical Properties

Decellularized renal arteries were found to be statistically different from fresh arteries in terms of burst pressure (Figure 3.13 left), but not statistically different from fresh arteries in terms of diametrical compliance (Figure 3.13 right). For enzyme resistance, PGG-treated scaffolds showed an increased resistance to elastase that was statistically different from fresh (Figure 3.14) while heparinized scaffolds exhibited an increased resistance to collagenase that was also statistically different from fresh (Figure 3.14). All statistical analyses made use of 1-factor ANOVA with α=0.05.
Figure 3.11 – Characterization of scaffold decellularization. Renal artery scaffolds show no residual DNA following DAPI staining (B) when compared to fresh tissue (A). Agarose gel electrophoresis confirms no residual DNA within decellularized “trunks” and smaller renal arteries compared to fresh controls (C).
Figure 3.12 – Histological and immunohistochemical characterization of renal artery scaffolds. Scaffolds are stained with H&E (A, E), Trichrome (B, F) and VVG (C, G) to verify complete decellularization and retention of endogenous collagen (B, F) and elastin (C, F). Immunostain for α-gal (D, H) shows the powerful xenoreactive epitope is notably absent from scaffolds. All scale bars = 50 µm.

Figure 3.13 – Renal artery scaffolds mechanically characterized. Decellularized scaffolds exhibited a burst pressure that was statistically different from fresh (left – n = 6 for all groups) while no statistical difference was noted for diametrical compliance (right – n = 6 for all groups). * denotes statistical difference from Fresh.
3.4.3.4 Scaffold Characterization – Heparin Quantification

Control samples showed the appropriate low baseline levels of heparin, irrespective of PGG treatment presence. Low heparin values were also found when direct heparinization without the intermediate diamine coupling was attempted (for both PGG and non-PGG treated samples; no differences seen between the tissue samples which were pre-soaked or not). (Figure 3.15)

Much higher values were observed for samples that were aminated with Jeffamine prior to the reductive amination with heparin. The +PGG+JEFF+HEP group had slightly less heparin than the –PGG+JEFF+HEP group (no soaking, p<0.05), and pre-soaking did not significantly affect the heparin content of either the –PGG or +PGG tissue types.

![Renal Artery Mass Loss vs Tissue Treatment](image)

Figure 3.14 – Enzymatic resistance of PGG-treated and heparinized renal artery scaffolds (n=5 for all groups). * denotes statistical difference from Fresh, # denotes statistical difference from +PGG, +Hep, ^ denotes statistical difference from No PGG, +Hep, ~ denotes statistical difference from +PGG group.
3.4.3.5 *Scaffold Characterization – Denaturation Temperature Determination*

Jeffamine treatment resulted in significant increases in denaturation temperature (DT) (63 and 55%; p<0.001) in the DT of the –PGG and +PGG tissues respectively (*Figure 3.15*). There were no statistically significant differences between –PGG and +PGG in either of the three treatment groups (Control, Jeff or Jeff+Hep). Further heparinization of Jeffamine treated samples resulted in a small decrease in DT from 85 to 81 ºC, but the latter was still much increased over the control average of 53ºC.

*Figure 3.15* - Heparin content of EDVG (-PGG and +PGG) samples after heparinization with and without pretreatment with Jeffamine (with and without pre-soaking). Jeffamine treatment was shown to significantly increase heparin retention, both with and without pre-soaking. Courtesy of Tim Pennel.
Figure 3.16 – Denaturation temperature of heparinized scaffolds. Jeffamine treatment had a significant effect on scaffold DT while additional heparinization did not statistically alter DT. Courtesy of Tim Pennel.

3.4.4 Discussion

3.4.4.1 Scaffold Preparation – Decellularization

It has been previously suggested by Badylak’s group that decellularized scaffolds should retain 50 ng or less dsDNA (double-stranded DNA) per mg dry ECM weight in order to be considered decellularized, although it was also mentioned that this metric has yet to be agreed upon by the tissue engineering community.\textsuperscript{[130]} We utilize histological DAPI staining as a primary screening metric to confirm the lack of intact nuclei within the scaffold following decellularization. As a follow-up to confirm lack of residual DNA within decellularized scaffolds, it was decided to conduct agarose gel electrophoresis on these scaffolds.
This method makes use of purified DNA from digested biological tissue that is conjugated with ethidium bromide in order to convey a stronger ultraviolet fluorescence to the naturally fluorescent DNA. The agarose gel is a hydrogel which acts as a medium for the purified DNA to pass through once an electric current is applied to the system. In this process, larger, intact sections of DNA are able to move less through the gel while smaller, fragmented sections of DNA are more mobile and move farther through the gel. Therefore, if the scaffolds were incompletely decellularized, there would be either intact bands or “smears” observed in the gel (see Fresh tissue controls, Figure 3.11C), where DNA was present in the sample and migrated through the gel. It observed that the decellularized scaffolds show undetectable amounts of DNA following agarose gel electrophoresis. This observation coupled with the lack of nuclei observed following DAPI staining leads us to the conclusion that the scaffolds are fully decellularized and ready for further histological, mechanical and biochemical characterization.

3.4.4.2 Scaffold Characterization – Histology, Mechanical and Biochemical Properties

Although these scaffolds have not been characterized with as much rigor as the bovine mammary and femoral scaffolds, the decellularized scaffolds have been shown to not retain residual DNA while retaining endogenous collagen and elastin as well as physiologically-relevant burst pressure and diametrical compliance following decellularization. Additionally, PGG-treatment of these scaffolds resulted in an increased resistance to elastase, with heparinization providing additional resistance to collagenase. These properties make these scaffolds suitable for in vivo studies in that the most notable
requirements for a biocompatible scaffold have been met (see section 1.2 Necessary Properties for the Ideal Vascular Graft). Additionally, the size of these scaffolds makes them an ideal candidate for implantation into the abdominal aorta of rats, which requires a diameter of 1-2 mm and a length of 15 mm, both of which have been met by the scaffolds.

3.4.4.3 Scaffold Characterization – Heparinization and Heparin Quantification

The renal artery scaffolds described in this chapter are proposed to be implanted as acellular grafts in rat abdominal aortas (see Chapter 5 Preclinical Evaluation of Scaffold Biocompatibility). The acellular nature of these grafts, coupled with the high collagen content of these scaffolds was expected to be highly thrombogenic in vivo\textsuperscript{[141, 142]}. An approach to amend this presumed thrombogenicity involved grafting heparin to the scaffold surface using additional stabilization of renal artery scaffolds via EDC/NHS chemistry and treatment with Jeffamine in order to increase heparin retention on the scaffold surface (Figure 3.10). Heparin conjugation was chosen because of the molecule’s known ability to decrease thrombogenicity in vivo\textsuperscript{[141, 142]}. When heparin quantification was conducted, Jeffamine treatment was justified by noting significantly higher heparin content than scaffolds not treated with Jeffamine.

Another significant finding was that the process of heparinizing the scaffolds also brought about inherent resistance to collagenase in vitro (Figure 3.14). This unexpected resistance to collagenase, when coupled with the observed resistance to elastase that PGG treatment endows to scaffolds (Figure 3.14), results in a dual-fold, collagen-specific (heparin treatment) and elastin-specific (PGG treatment) stabilization process for
presumably overall improved enzymatic resistance in vivo. The heparin treatment process is suggested to be collagen-specific because PGG treatment did not confer statistically significant resistance to collagenase in vitro; additionally, PGG treatment did not confer statistically significant resistance to elastase in vitro (Figure 3.14). The unique properties of this dual-fold, collagen- and elastin-specific stabilization needs to be characterized further in future studies in order to elucidate further therapeutic potential.

3.4.4.4 Scaffold Characterization – Denaturation Temperature Determination

The observed increase in DT resulting from scaffold treatment with Jeffamine, independent of heparin treatment (Figure 3.16), is a significant finding which clarifies that the Jeffamine treatment process does in fact stabilize the renal artery scaffolds. The specificity of stabilization was further characterized by exposing heparinized grafts (these grafts were treated with Jeffamine prior to heparinization) to both collagenase and elastase resistance testing in order to determine the potential in vivo significance of this treatment (Figure 3.14). Future studies investigating the DT of PGG-treated vascular grafts in addition to the treatment groups described herein may be beneficial.

3.5 References


CHAPTER 4: SEEDING OF LONG VASCULAR SCAFFOLDS WITH TUNIC-SPECIFIC CELL TYPES

4.1 Introduction

The majority of life-threatening vascular disease occurs in small caliber blood vessels due to progressive vascular occlusion, resulting in decreased blood flow. Because of the challenge presented by producing an endothelialized small caliber replacement, these constructs are often times implanted in more forgiving sites closer to the heart to achieve decreased thrombogenicity, presumably due to the increased blood flow at these sites\cite{2, 143}. Seemingly, in order to achieve favorable patency in a flow-limited model of human peripheral circulation, complete luminal coverage of endothelium is required\cite{95}. Recently the question was raised by Badylak and colleagues as to whether or not 3D acellular scaffolds would be able to function \textit{in vivo} without \textit{ex vivo} cell seeding and conditioning in bioreactors. They pose the question as to if the natural cell infiltration observed \textit{in vivo} could replace the need for \textit{ex vivo} cell seeding and conditioning in bioreactors\cite{144}. Indeed, it is challenging to revitalize complex tissues with 100\% of the total amount of cells naturally observed \textit{in vivo} using \textit{ex vivo} cell seeding procedures.

Native blood vessels are vitalized by three main cell types, each specific to one tunic of the blood vessel. Endothelial cells are found lining the luminal surface of blood vessels\cite{122}. This “lining” is referred to as endothelium, the natural hemocompatible blood contacting surface\cite{122}. Smooth muscle cells reside within the thickness of the blood vessel, known as the media, and impart contractility to native vessels in order to control blood flow\cite{122}. These cells are commonly observed to be morphologically stretched in the
direction of contraction, which is typically in circumferential fashion within the media.\textsuperscript{122} The last primary cell type for blood vessels, known as fibroblasts, resides on the outermost surface of the vessel, known as the adventitia.\textsuperscript{122} These fibroblasts are known for their ability to synthesize and remodel native ECM, among other functions.\textsuperscript{122} Although the three vessel tunics are inhabited in a small capacity by other cell types, these represent the primary cell types which vitalize and embody native vessels. When attempting to tissue engineer a vascular graft, the question of what is the proper cell source arises and is based off of the potential application.

Creating a patient-specific vascular graft requires the combined use of an acellular scaffold and patient-derived cells. Several cell sources are currently accepted in the literature;\textsuperscript{143} however, only a select few sources can feasibly be used clinically to produce patient-specific cells. Numerous clinical trials have been conducted which made use of undifferentiated stem cells directly injected into desired sites,\textsuperscript{145} but the use of undifferentiated stem cells for disease treatments has had questionable efficacy and their overall safety of use is still in question.\textsuperscript{146} These and other findings have shifted some researchers towards the use of differentiated stem or progenitor cells as opposed to undifferentiated stem cells.

One favorable patient-specific cell source is adipose tissue, from which adipose-derived stem cells (ADSCs) can be isolated.\textsuperscript{147} With this process in mind, an adipose-derived cell population can be isolated from a patient, expanded in culture, and differentiated or sorted into the desired cell type(s) for the scaffold. One of the three main vascular cell types, endothelial cells (ECs), lining the vessel lumen, have proven
challenging to seed onto vascular scaffolds in a confluent monolayer. In theory, stabilized scaffolds combined with autologous cells can be implanted into the original patient to achieve gradual remodeling and host integration over time. This is the ultimate goal of tissue engineering: creating “translational” products that can bring laboratory science directly to the patient, breaching the gap between laboratory bench and patient bedside. In this chapter, we present our experience with revitalizing vascular scaffolds with tunic-specific cells, improving luminal endothelial cell coverage of vascular scaffolds, and experimentation with seeding of human ADSCs onto vascular scaffolds.

4.2 Cell Seeding of Bovine Mammary Artery Scaffolds

4.2.1 Materials and Methods

4.2.1.1 Tunic-specific Cell Seeding

Following confirmation of scaffold decellularization and characterization, efforts were taken to revitalize each distinct vascular tunic (intima, media, adventitia) of a stabilized and sterilized scaffold with the appropriate cell type. Human aortic smooth muscle cells (HASMCs – passage 11, Invitrogen, Grand Island, NY) were injected into the media with a syringe and repeating dispenser (Hamilton Company, Reno, Nevada) at 10 μL of cell suspension (5 x 10⁶ cells in 500 μL total syringe volume) per injection (Figure 4.1A), and cultured in static conditions for 4 days in Dulbeccos modification of Eagle medium (DMEM, Corning-Cellgro) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, Georgia) and 1% antibiotic solution (Corning-Cellgro); this medium was used for all cell types. Human umbilical vein endothelial cells (HUVECs –
passage 8, Fisher Scientific) were then perfused into the lumen of the scaffold using Luer-lock barbed fittings and a Luer-Lock syringe at a density of $2 \times 10^5$ cells (Figure 4.1B), rotated gently for 6 hours (not shown), then cultured under static conditions for 1 day. Human aortic fibroblasts (HAFBs – passage 8, Lonza, Walkersville, MD) were then seeded dropwise onto the adventitia using a Luer-lock 22-gauge needle and Luer-Lock syringe at a density of $5.5 \times 10^6$ cells (Figure 4.1C). After HAFB seeding, the scaffold was seeded with all three cell types.

The scaffold seeded in all three vessel tunics was cultured for an additional 8 days under static conditions. Following static incubation, the scaffold was cut in half, with half immediately processed and imaged (static, control) and the other half mounted aseptically in a bioreactor (Figure 4.1D) then perfused in the bioreactor for 5 days (dynamic) at 37°C (Figure 4.1E). Approximate volumetric flow rates for dynamic conditioning were as follows: day 1 = 56.6 mL/min, day 2 = 62.3 mL/min, day 3 = 67.9 mL/min, day 4 = 73.6 mL/min and day 5 = 84.9 mL/min.

Following *in vitro* tissue culture, scaffolds from the control and dynamic groups were analyzed for cellular viability on the luminal and adventitial surfaces as well as within the media using Calcein AM and ethidium homodimer-1 staining (Life Technologies, Grand Island, NY) for viable and dead cells, respectively (25x total magnification) (Figure 4.1F, 4.1I, 4.1H, 4.1K). In order to better visualize the cells inhabiting the media, scaffolds from both groups were analyzed histologically with DAPI staining (100x total magnification) (Figure 4.1G, 4.1J).
4.2.1.2 Endothelialization

As a follow-up to the tunic-specific cell seeding study, an external stent facilitated endothelial cell seeding study was conducted. An external stent was designed using SolidWorks software (Figure 4.2A) and constructed using a rapid prototyping machine (3D Systems, Rock Hill, SC). Using the external stent as a lattice framework support structure, a sterile mammary artery scaffold stabilized with PGG was inserted into the external stent with suture wrapped around the vessel and secured between stent struts in order to freely suspend the scaffold within the stent (Figure 4.2B-D). HUVECs – passage 20 were then seeded at a density of $6 \times 10^6$ cells into the scaffold lumen (Figure 4.2E), the scaffold ends were closed with Luer-lock plugs (Cole Parmer) (Figure 4.2F), then placed within a tissue culture flask filled with M199 medium (Corning-Cellgro) supplemented with 10% FBS (Atlanta Biologicals) and 1% antibiotic solution (Corning-Cellgro), and then incubated for 1 hour at $37^\circ$C (Figure 4.2H). The construct was then rotated 90° clockwise and placed back in the incubator for a period of 1 hour (Figure 4.2I). This rotation process was repeated for a total duration of 4 hours, at which point the construct was unsealed on the right side (Figure 4.2F) and left incubating at $37^\circ$C overnight. The following day, this entire cell seeding process was repeated again with a density of $5 \times 10^6$ cells (Figure 4.2J). Following the second day of cell seeding, the construct was incubated at $37^\circ$C, with the right side of the construct unsealed, for a total of 10 days of static tissue culture, with media being gently pipetted into the right section of the scaffold (Figure 4.2F) twice daily in an attempt to improve nutrient/waste exchange for seeded cells. The seeded
scaffold was bathed in approximately 80 mL of media, which was changed twice during the experiment.

Following the static tissue culture incubation, the scaffold was sectioned into three large pieces and the lumen was visualized for HUVEC retention using Calcein AM (Figure 4.2K-M) as described in the tunic-specific seeding study.

4.2.1.3 Seeding and Differentiation of Human ADSCs

Once it was demonstrated that stent-facilitated endothelial cell seeding is able to seed an appreciable amount of cells along the entire length of a mammary artery scaffold, experimentation was conducted to reproduce these results with human ADSCs. Another goal of this study was to demonstrate the preserved endothelial niche within the lumen/intima of the decellularized scaffolds by observing if the human ADSCs seeded on the scaffolds would show signs of differentiation into cells of endothelial lineage following 10 days of static culture. The cells used for this study were purified from human adipose tissue using the following procedure. An approach to meet this goal includes immunofluorescence staining of control human ADSCs and human ADSCs seeded onto the decellularized scaffold for CD34 (a marker indicating “stemness” or ability to differentiate into different cell types\cite{148}, sheep anti-human antibody, R&D Systems, Minneapolis, MN) and for CD31 (an endothelial marker\cite{148}, rabbit anti-human antibody Abcam). Both Alexa-fluor conjugated antibodies (donkey anti-sheep and goat anti-rabbit) were purchased from Invitrogen. In theory, cells of endothelial lineage would stain positive for CD34 and CD31 while control human ADSCs would only stain positive for CD34.
Human adipose tissue was collected according to the procedures within an approved IRB protocol (Institutional Review Board) and placed into MesenPRO RS Medium (Life Technologies) with 1% antibiotic solution (Corning-Cellgro). The fat was then minced using two scalpel blades and placed into a solution of collagenase type I (1 mg/mL, Sigma) in 1x PBS and shaken at 37°C for 1 hour. Following digestion, the solution was filtered through 100 µm sieves and centrifuged at 1000 rpm for 5 minutes. Once the supernatant was decanted, the cell pellet was resuspended in a solution of 155 mM ammonium chloride (NH₄Cl, Fisher Scientific) and 0.1 mM EDTA (Fisher Scientific) in 1x PBS for 5 minutes at room temperature to lyse remaining erythrocytes. The solution was then centrifuged at 1000 rpm for 10 minutes, the supernatant decanted, and the pellet resuspended in MesenPRO RS Medium, the cells counted, and plated in T-175 tissue culture-treated flasks (Fisher Scientific) at a density of 5000 cells/cm². After 4 hours of incubation, the floating cells were removed by washing in fresh MesenPRO RS Medium. Adherent cells were maintained at sub-confluent levels and replated at 5000 cells/cm² for the duration of culture.

The design of the study included 2 control groups of human ADSCs grown on tissue culture plates and 1 experimental group of human ADSCs seeded onto the lumen of a sterile mammary artery scaffold. Control 1 included cells grown to confluence in a 12-well plate (Greiner Bio-One), control 2 included cells grown to 70% confluence in a 12-well plate, and the experimental group involved external stent facilitated cell seeding using the scaffold mounting procedures described previously (4.2.1.2 Endothelialization).
Once the scaffold was appropriately mounted in the external stent, human ADSCs were seeded over a 4 day period with the orientation of the scaffold being held constant each night following seeding and the scaffold being rotated 90° before subsequent cell seeding (Figure 4.3); all cell seeding steps made use of passage 8 human ADSCs. Aside from the different cell type used and daily cell seeding used herein, all procedures related to cell seeding were identical to those mentioned previously (4.2.1.2 Endothelialization). For cell seeding: on day one, approximately 5.5 million cells were seeded into the lumen of the scaffold; on day 2, approximately 7 million cells were seeded; on day 3, approximately 5.5 million cells were seeded; on day 4, approximately 5.8 million cells were seeded. When imaging seeded cells for all conditions, three separate images (DAPI – blue, CD34 – green, CD31 – red) were taken for each specific image and overlayed to produce 1 representative image of all three channels.

4.2.2 Results

4.2.2.1 Tunic-specific Cell Seeding

Static-conditioned scaffolds stained with Calcein AM (Figure 4.1F, 4.1H) display a higher number of cells on the luminal and adventitial surfaces compared to dynamic-conditioned scaffolds when stained with Calcein AM (Figure 4.1I, 4.1K). Conversely, transverse vessel sections histologically stained with DAPI suggest higher cell retention in the media for the dynamic group (Figure 4.1J), with cells notably absent from the media of the control group (Figure 4.1G).
Figure 4.1 – Tunic-specific cell seeding of mammary scaffolds. HASMCs were injected into the scaffold media (A), HUVECs perfused into the lumen (B), and HAFBs seeded in dropwise fashion onto the adventitia (C) before either static culture or dynamic culture in a bioreactor (D, E). Following conditioning, scaffolds were assessed with either Calcein AM (F, H, I, K) or DAPI (G, J) to show viability and relative cell distribution within each tunic.
Figure 4.2 – An external stent was designed in SolidWorks (A), rapid prototyped, and sterilized. A sterile mammary artery scaffold was placed inside the stent (B), secured by wrapping suture around the artery and through the stent wall (C, D), seeded with HUVECs (E), sealed (F), and placed into a tissue culture flask (G) with the orientation maintained by sterile tubes within the flask (H). The seeded scaffold was rotated every hour (I), then one cap removed and allowed to incubate overnight. A similar process was utilized for day 2 (J). Following 10 days of static culture, large proximal, medial, and distal sections were imaged with Calcein AM to detect cell viability and approximate cell confluence (K, L, M).
4.2.2.2 Endothelialization

Following the 2 day HUVEC seeding procedure and 10 day static culture, representative fluorescent Calcein AM micrographs of the three large sections of seeded scaffold suggest slightly different degrees of confluence along the scaffold length. The left most section (the distal section from the inlet used for changing media) was found to have an estimated over 80% confluence (Figure 4.2K), the middle section retained approximately 70% confluence in most areas (Figure 4.2L), and the right most section (the section used as the inlet for changing media) was found to have an estimated 50% confluence (Figure 4.2M).

4.2.2.3 Seeding and Differentiation of Human ADSCs

Human ADSCs grown to 70% confluence on tissue culture plastic (control 1) stained positive for cell nuclei (DAPI) while expressing minimal staining for CD34 and undetectable staining for CD31 (Figure 4.4A, 4.4D). Human ADSCs grown to 100% confluence on tissue culture plastic (control 2) also stained positive for cell nuclei (DAPI) while expressing minimal staining for CD34 and undetectable staining for CD31 (Figure 4.4B, 4.4E). Human ADSCs seeded onto a mammary artery scaffold and grown for 10 days in static culture stained positive for cell nuclei (DAPI), but expressed a different (smaller) nuclear morphology than the cells grown on tissue culture plastic. Additionally, these cells expressed minimal staining for CD34 and undetectable staining for CD31 (Figure 4.4C, 4.4F).
Figure 4.3 – External stent facilitated human ADSC seeding. Following 4 days of cell seeding, the cell-seeded scaffold was incubated for 10 days of static culture with twice daily media changes.

Figure 4.4 – Fluorescent imaging of human ADSC seeding. Human ADSCs were seeded onto either tissue culture plastic (A, B, D, E) or a mammary artery scaffold and stained using DAPI (blue) or immunofluorescence for CD34 (green) and CD31 (red). * denotes that vascular tissues express natural elastin autofluorescence (green), not to be confused as positive CD34 staining.

4.2.3 Discussion

4.2.3.1 Tunic-specific Cell Seeding

After analyzing the results of this study, several areas of improvement for future studies were established. It became apparent that the HUVEC seeding density of $2 \times 10^5$
cells/scaffold was too low of a seeding density in order to achieve total seeded cell confluence along the entire length of the scaffold. Because of the low seeded cell retention on the intima and adventitia, future dynamic seeded cell conditioning studies could make use of the following changes in order to increase overall cell seeding efficacy: make use of a lower initial flow rate (McFetridge’s group recently published work using an initial flow rate of 1.65 mL/min\textsuperscript{[149]}), a lower end fluid flow rate (volumetric flow rate in the popliteal artery was measured to be 72 ± 5 mL/min\textsuperscript{[150]}), a smaller incremental increase in flow rate (perhaps approximately 5.6 mL/min daily increases overwhelmed seeded cells), more time to reach the end flow rate and possibly improvements in cell seeding technique or the use of a higher number of seeded cells.

When assessing the HASMC retention within the media, a lack of seeded cells in the media of static-conditioned scaffolds as opposed to the relative abundance of seeded cells in the media of dynamic-conditioned scaffolds was observed. Because the dynamic scaffolds were statically conditioned for the same amount of time as the static scaffolds (13 days), no differences in seeded cell retention would arise from culture period alone. Therefore, the cells present in the dynamic scaffold media remained in place through both static and dynamic culture. Under those pretenses, the most likely explanation for the difference observed between the seeded scaffold media from the static and dynamic groups would be the inherent variability in distribution of seeded cells. The 5 µm histological section from the static group chosen for imaging may have not had cells in it while cells may have existed within 10 µm or more from the imaged site. Future studies will have to
improve upon HASMC seeding techniques in order to achieve more uniform seeded cell distribution within scaffolds.

The results from this study suggest that cells can be placed into all three tunics of PGG-stabilized scaffolds and retain viability following 23 days of total tissue culture (13 days for the static group), including 5 days of perfusion-based tissue culture. Although DAPI staining is not an indicator of cell viability, the media from the dynamic-conditioned scaffold shows tight spherical nuclei which imply cellular viability, as apoptotic cells would display blebbed nuclei and not intact, spherical nuclei (Figure 4.2). Future studies will be needed to explore improved cell seeding techniques for the media as well as other avenues to convey seeded cell viability in the media.

4.2.3.2 Endothelialization

Previous cell seeding studies have shed light on the need for high seeding densities for scaffolds in order to reach confluence (Figure 4.1). Furthermore, scaffold orientation and rotation during seeding have also been shown to be paramount to attain confluence (Figure 4.2). During these earlier studies (Figure 4.1), scaffolds were continuously rotated during one round of cell seeding in anticipation of achieving a dense, uniform cell layer following seeded scaffold conditioning. After analyzing the results from the external stent-based endothelialization study (Figure 4.2), we believe that further modifications in cell seeding procedures will be necessary for attaining over 90% confluent cell populations along the entire length of the scaffold. One other potential modification to improve seeded cell confluence may include changing media on both ends of the seeded scaffold as
opposed to using only one side, as discussed in the methods section, in order to expose both ends of the scaffold to some fluid flow and possibly enhance cellular preparation for dynamic conditioning.

4.2.3.3 Seeding and Differentiation of Human ADSCs

The design of this study was chosen based on recent results showing that stem cells can be differentiated along particular lineages simply by culturing seeded stem cells on scaffolds with a preserved ECM niche\textsuperscript{105, 106}. Mammary artery scaffolds were chosen as a candidate to test this hypothesis because of the previously discussed extensive retention of a vascular basal lamina within the ECM of the decellularized mammary and femoral artery scaffolds (see section 3.2.2.2 Scaffold Characterization – Histology and corresponding figures). It was therefore hypothesized that properly cultured human ADSCs seeded onto the lumen of a mammary artery scaffold would differentiate into cells of an endothelial lineage because of the preserved vascular ECM niche and recent literature which suggests the feasibility of this approach\textsuperscript{105, 106}.

Two controls were used for this study in order to confirm the differentiation potential of the human ADSCs prior to cell seeding onto the scaffold. In terms of cell seeding/plating density, researchers have mentioned the need for low density culture initiation in order to assess progenitor potential for ADSCs isolated from lipoaspirates\textsuperscript{148, 151}. From this assertion it is insinuated that when stem cells are grown to confluence, they exhibit a decreased potential to differentiate into different cell types/lineages; this potential to differentiate is also known as “stemness”. For this reason, two controls were used for
Control 1 was utilized to establish that when the human ADSCs are cultured appropriately, they should be able to retain their stemness (stain positive for CD34). On the other hand, control 2 was used to demonstrate that when the same human ADSCs are cultured past the appropriate density and allowed to reach confluence, these human ADSCs would no longer express CD34 and would therefore be exhibiting a decrease in stemness. By demonstrating the ability to retain or decrease the stemness of the human ADSCs utilized for this study, these controls would support the feasibility of the human ADSCs to differentiate into an endothelial lineage when seeded and cultured on a vascular scaffold with an intact niche.

Although the results from this study did not suggest that human ADSCs seeded onto a sterile mammary scaffold were able to differentiate into cells of endothelial lineage following static culture, it should be noted that the cells from both control groups did not express the human ADSC marker CD34, as was expected. One of the implications of these results is that the cells utilized for this study were perhaps not appropriately purified as human ADSCs and that the cell population was heterogeneous in nature. This implication is supported by the previously discussed characteristic heterogeneity of the cell pellet formed by centrifuging the resultant solution of adipose tissue digested by collagenase\textsuperscript{148}; this was the procedure utilized for the isolation of human ADSCs for this study. Evidence of a heterogeneous cell population can be seen experimentally by the noted differences in nuclear morphology observed in all stained images (Figure 4.4).

Furthermore, although the cellular isolation protocol utilized for this study has been published and noted a yield of human ADSCs\textsuperscript{147}, it is also noted that without the use of
fluorescence-activated cell sorting employing both positive and negative controls, isolation of a pure human ADSC population is difficult to attain\[148\]. If this study was reproduced with human ADSCs purified by fluorescence-activated cell sorting, the results would likely be different and may confirm the original hypothesis of this study, that a scaffold containing a preserved ECM niche can support differentiation of seeded stem cells.

4.3 References


CHAPTER 5: PRECLINICAL EVALUATION OF SCAFFOLD BIOCOMPATIBILITY

5.1 Introduction

Individuals familiar with medical devices understand that the use of class III medical devices will most likely require extensive animal testing prior to clinical trials. One often complicated side of the requirement for animal testing is the frequently realized discrepancies between animal models and the humans the devices were originally designed for. These discrepancies can include differences in size, metabolism, cellular response and many more categories. The challenge for biomedical engineers is to not only implant their device in an animal model and show that it works, but this same device must work in its present form without manipulating the core facets of the device when needing to scale up for human testing.

In the field of tissue engineering, biocompatibility has been evaluated with a wide range of animal models, and within those models, different permutations of implant sites also exist\(^{[143]}\). For example, surely a graft that is subdermally implanted in the back of animal will elicit a different response from the host than if it was implanted as a vascular conduit that is constantly pulsing and in contact with blood. Furthermore, most medical devices are designed to be implanted for numerous years before the device fails, but most animal studies can only investigate host response and implant function for short periods often under one year in duration. Therefore, the very nature of animal studies involves trying to design a device for humans, but having to modify the device to work in a smaller
animal with (hopefully) comparable anatomy and then extrapolate on the short term data produced by the study in order to gain insights on potential long term performance.

There are indeed many challenges to be overcome when designing a medical device. In this chapter, the implantation of two different vascular graft scaffolds will be discussed in the context of two animal models. The biocompatibility of these scaffolds will be analyzed and insights gained from these studies will be used to improve these scaffolds and/or promote future animal studies to further characterize the host response to the discussed vascular scaffolds.

5.2 Analysis of Vascular Scaffold Biocompatibility in Circulatory Models

5.2.1 Materials and Methods

5.2.1.1 Porcine Renal Artery Scaffolds

5.2.1.1.1 Heparinization for Implants

Porcine renal artery scaffolds were prepared and heparinized for implantation prepared as discussed previously (section 3.3 Development of Porcine Renal Artery Scaffolds) via Jeffamine functionalization (without pre-soaking) and heparin attachment using 0.2µm-filtered solutions under sterile conditions in a laminar flow hood. The following implant groups were thus generated. Groups 1: no PGG, no Heparin (-PGG-HEP); Group 2: PGG, no Heparin (+PGG-HEP); Group 3: no PGG, Heparin (-PGG+HEP); and Group 4: PGG followed by Heparin (+PGG+HEP).

5.2.1.1.2 Implant Groups
A total of 51 rats were implanted with vascular grafts (Figure 5.1) for n=12 per group (3 rats were replaced due to premature graft rupture). The following groups were formed. Group 1: untreated (-Hep - PGG); n=14 rats. Group 2: PGG treatment only (-Hep +PGG); n=13 rats. Group 3: Heparin/EDC only (+Hep - PGG); n=12 rats and Group 4: PGG followed by Heparin (+PGG +Hep); n=12 rats. Each of these groups was divided in half, with half of the group being implanted for a duration of 4 weeks and the other half implanted for a duration of 8 weeks.

![Figure 5.1 - Implanted renal artery scaffolds. Arrows indicate anastomoses. Courtesy of Tim Pennel.](image)

5.2.1.1.3 Graft Implantation

All animal experiments were approved by the Animal Research and Ethics Committee of the University of Cape Town and were in compliance with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, South Africa.

Male Wistar rats weighing 350-510 grams were induced with isoflurane 5% anaesthesia and were maintained with 2% isoflurane spontaneously breathing via a conical mask. Sterility was maintained throughout the procedure and a warming pad was used to
regulate temperature. Following a mid-line laparotomy the aorta was dissected free of the inferior vena cava and surrounding tissues and all perforating arteries between the left renal artery and the iliac bifurcation were ligated. In all cases the inferior mesenteric artery was preserved. A single dose of intravenous heparin (1mg/kg) was administered and grafts of 10mm in length were implanted into the infrarenal aorta by end-to-end anastomoses using 9-0 Nylon interrupted sutures. The abdomen was closed in layers and Buprenorphine (0.1mg/kg) was administered subcutaneously twice daily for three days. No anticoagulation medication was administered after surgery.

5.2.1.4 Graft Removal

At time of explant, the abdomen was opened and the graft inspected for patency by observation of pulsation in the distal aorta, under general anaesthesia, per the implant procedure. Animals were euthanized by exsanguination following 1mg/kg heparin administration via the inferior vena cava. The aorta was flushed with PBS via the apex of the left ventricle until clear of blood (±150ml), following which the aorta was perfusion fixed with formalin. The graft was then excised, cross-sectioned for macro-photography and processed for histology.

5.2.1.5 Histology and Quantification

Tissue samples were dehydrated, embedded in paraffin, sectioned and processed for histological examination. Hematoxylin-eosin and Masson’s trichrome were used for basic light histological analysis (Figure 5.2). Immunohistochemical identification of
endothelial cells was performed with vWF/FVIII (Figure 5.6). Elastin and calcification was identified with orcein (Figure 5.4) and alizarin red stain (Figure 5.5) respectively, and glycosaminoglycan were stained with Alcian blue (Figure 5.5). Quantification of elastin and measurement of wall thickness, intimal hyperplasia and clot area were performed on trichrome stained sections with Visiopharm xyz and Adobe Photoshop CS6 on mid-graft cross-sections. Graft dilatation was determined as a surrogate of internal elastic lamina diameter (IEL diam.), the luminal distance excluding neointima and thrombus (Figure 5.2).

5.2.1.1.6 Statistical Analysis

Statistical tests were performed with STATA (StataCorp. 2011. *Stata Statistical Software: Release 11*. College Station, TX: StataCorp LP) Results were expressed as mean±SD for continuous variables. All continuous data was confirmed as nonparametric (Shapiro-Wilkinson) interrogated with Mann Whitney test for statistical significance. Kruskal-Wallis equality-of-populations rank test was used for multiple group analysis. Categorical data was tested with the Fishers exact, and the level of significance was set at P< .05. Burst pressures, diametrical compliance and enzyme resistance were compared using ANOVA, and effect of heparinization and denaturation temperatures with student’s t-test.

5.2.1.2 Bovine Mammary Artery Scaffolds

5.2.1.2.1 Preparation for Implantation

Bovine mammary artery scaffolds were prepared for implantation as discussed previously (see section 3.2 Development of Bovine Mammary and Femoral Artery
Scaffolds Artery Scaffolds). This study was structured as a pilot study to evaluate the *in vivo* performance and feasibility of the bovine mammary artery scaffold as a conduit in the femoral artery position of a mini-pig in the short term. One total scaffold was implanted in one Yucatan mini-pig for a period of one week.

5.2.1.2.2 Graft Implantation

The animal experiment was approved by the Animal Research Committee of the Godley-Snell Research Center at Clemson University. This facility is AAALAC (Association for Assessment and Accredidation of Laboratory Animal Care) accredited and regulated by the USDA (United States Department of Agriculture).

Male mini-pigs weighing 40 kilograms were induced with isoflurane 5% anesthesia and were maintained with 2% isoflurane spontaneously breathing via intubation. Sterility was maintained throughout the procedure. Following an inguinal incision, the femoral artery was dissected free of surrounding tissues. A single dose of intravenous heparin (1400 units) was administered and grafts of 3cm in length were implanted into the femoral artery by end-to-end anastomoses using 5-0 prolene running sutures. To improve patency and decrease vasospasm following the operation, Papaverine, a vasodilator, was administered dropwise onto the anastomoses. The incision was closed in layers and no anticoagulation medication was administered after surgery.

5.2.1.2.3 Graft Removal

At time of explant, the original inguinal incision was reopened and the graft inspected for patency by observation of pulsation in the distal vasculature, under general
anaesthesia, per the implant procedure. Following a single dose of 1400 units of heparin administered through the ear vein port, the proximal femoral artery was clamped, flushed with PBS until clear of blood, the graft and anastomosed native tissue was excised, and the remaining arterial segments were sealed with electrocautery. The excised graft was then processed for histology and scanning electron microscopy (SEM). The animal was euthanized by a lethal dose of pentobarbital.

5.2.1.2.4 Histology and Quantification

Tissue samples were fixed in 10% neutral buffered formalin (Fisher Scientific) dehydrated, embedded in paraffin, sectioned and processed for histological examination. Hematoxylin-eosin and Masson’s trichrome were used for light histological analysis (Figure 5.7).

5.2.1.2.5 SEM

Tissue samples were fixed using Karnovsky’s fixative [2% paraformaldehyde (Fisher Scientific), 2.5% glutaraldehyde (Fisher Scientific) and 0.1M PBS (Corning-Cellgro) in distilled water, pH 7.2] and dehydrated in Hexamethyldisilazane (HMDS, Electron Microscopy Sciences, Hatfield, PA) before critical point drying using graded ethanol and imaged using SEM (Figure 5.8).

5.2.2 Results

5.2.2.1 Porcine Renal Artery Scaffolds
Porcine renal artery scaffolds were characterized and results discussed previously (see section 3.4.3 Results).

**5.2.2.1 Implants and Surgical Handling**

Three grafts ruptured prior to explant (2x -Hep -PGG, 1x -Hep +PGG) all of which occurred after six weeks of implant. Rupture was confirmed at autopsy with abdominal blood and macroscopic evidence of wall defect. These grafts were subsequently replaced and all of the animals that survived to explant were patent (48/48, 100%). Although the surgeon was not blinded at the time of graft implant, surgical handling was subjectively superior in the heparinized set.

**5.2.2.1.2 Histological Evaluation of Implants**

All grafts demonstrated prominent perigraft tissue infiltration from the adventitia towards to the lumen but only in some cases penetrating the media. This cellular infiltration was mainly composed of lymphocytes with few spindle shaped fibroblast, multinucleated foreign body giant cells and macrophages (Figure 5.6). Very few, to no granulocytes were noted in the graft wall. Capillary ingrowth penetrated the graft wall up to, but not beyond the media. Endothelial cells covering the lumen of the graft were noted with cross sectional coverage of 36±28% across all groups. There was no statistically significant difference in the remodelling, morphological cell type, vascularity or endothelial coverage between the four groups (Figure 5.2).
The luminal area was statistically larger in the unheparinized than the heparinized set (5.81±10.04 mm² vs. 1.26 mm², p=0.01), despite the trend towards more thrombus in these samples (0.33±0.72 mm² vs. 1.44±0.02 mm², not significant). The IEL diameter was also much greater in the unheparinized set compared to heparin treatment (3.51±1.64mm vs. 2.26±0.68, p=0.003). This difference was not seen in the pre-implant analysis of the same samples (2.11±0.45mm vs. 2.39±0.71mm, not significant). The graft wall was also significantly thinner in the unheparinized set (0.47±0.26mm² vs. 0.78±0.27mm², not significant).

**Figure 5.2** – Histological evaluation of renal artery implants. Each implant group is histologically processed, stained with Masson’s trichrome, imaged using light microscopy, then digitized in order to calculate the observed cross-sectional area of the graft wall, clot, intimal hyperplasia, and lumen. Courtesy of Tim Pennel.
p<0001). PGG treatment showed no influence on wall thickness (PGG; 0.43±0.15mm² vs. Untreated; 0.50±0.33mm², NS) (Figure 5.3).

Figure 5.3 – Characterization of gross histological implant properties. Trends were observed to be similar between the cross sectional wall area and wall thickness of implants. Similarly, the IEL diameter and the luminal area of implants were also observed to have similar trends. Courtesy of Tim Pennel.

5.2.2.1.3 Histological Evaluation of Implants: Calcification

Three samples (2x -PGG+Hep, 1x +PGG+Hep) stained positive for glycosaminoglycans with a histological appearance of chondroid metaplasia in the neointima. All three of these samples also stained positively for calcium. Two unheparinized samples (1x-PGG-Hep, 1x +PGG-Hep) stained positive for calcium in the neointima without chondroid metaplasia. No grafts showed evidence of calcium in the graft wall. Intimal hyperplasia increased across all four groups from four weeks to eight
weeks (1.2±0.76 mm$^2$, 1.66±0.65 mm$^2$, p=0.04) as a result of peri-anastomotic pannus ingrowth (Figure 5.5).

Figure 5.4 – Quantification of elastin within implanted renal artery scaffolds. These data suggest that combined PGG- and heparin-treatment of scaffolds yields the highest elastin retention in vivo. Courtesy of Tim Pennel.
Figure 5.5 – Implant calcification measurement. Histologically processed scaffolds were stained for alcian blue (left) for glycosaminoglycan (blue) and cartilage (red) content in addition to alizarin red staining (right) for calcium formation. Observed calcification was then quantified (top). Courtesy of Tim Pennel.

5.2.2.2 Bovine Mammary Artery Scaffolds

Bovine mammary artery scaffolds were characterized and results discussed previously (see section 3.2.2 Results).

5.2.2.1 Histological Evaluation of Implants

Excised graft was noted to be thrombosed upon explant (not shown), but demonstrated prominent perigraft tissue infiltration from the adventitia towards to the lumen but not completely penetrating the media (Figure 5.7B, 5.7C). Masson’s trichrome image of explanted scaffold suggests some smooth muscle cell infiltration, indicated by the
red staining observed in the media and around the nuclei of infiltrating cells. This red stain is notably absent from the control graft (Figure 5.7H) which was implanted for 1 minute then excised. Both time zero micrographs confirm the scaffolds were acellular upon implantation (Figure 5.7E, 5.7H). Therefore, all observed cells in explanted grafts are infiltrating host cells (Figure 5.7 – F, I). Intimal hyperplasia was not observed in excised grafts.

Figure 5.6 – Overall porcine renal artery scaffold biocompatibility. Grafts implanted into rat abdominal aorta show a large degree of cellular infiltration. Cells staining positive for α-smooth muscle actin (A, D, G), von Willebrand Factor (B) and factor VIII (E), macrophage ED-1 (C) and ED-2 (F) are presented, along with masson’s trichrome (I) to display general explant morphology. Courtesy of Tim Pennel.
5.2.2.2.2 SEM

SEM imaging confirmed that the excised scaffold was not endothelialized *in vivo* during the implant period (Figure 5.8E); SEM images of fresh tissue confirmed this finding (Figure 5.8B). This finding was further confirmed by analyzing the anastomosis of the native and scaffold tissue, where the scaffold surface is notably absent of cells (Figure 5.8A, 5.8D, 5.8F). A side-view of a control graft distinctly portrays the dense vascular media (left half) and fibrous vascular adventitia (Figure 5.8C).

![Figure 5.7](image)

*Figure 5.7* – Histological overview of *in vivo* bovine mammary artery scaffold performance. Following scaffold implantation into a mini-pig femoral artery (A), the graft was left in place for 1 week before explant. H&E staining of the anastomosis (B, C) showed greater cellular infiltration than mid-graft sections visualized with H&E and Masson’s trichrome (F, I) when compared to fresh tissue (D, G) and control grafts excised following 1 minute of implantation (E, H).
5.2.3 Discussion

5.2.3.1 Porcine Renal Artery Scaffolds

5.2.3.1.1 Histological Evaluation of Implants

The immunofluorescence and light micrographs of the excised grafts provide an overall insight into the host response observed following 8 week explantation. Although several grafts suggested nearly complete recellularization of the media with cells staining positive for α-smooth muscle actin (Figure 5.6A, 5.6D, 5.6G), this was not observed in all grafts across the 4 week and 8 week timepoint. Similarly, a large percentage of grafts were shown to retain an intact endothelial layer, visualized with von Willebrand Factor (Figure 5.6B) and factor VIII staining (Figure 5.6E). In the outermost adventitia, cells were commonly shown to also stain for the macrophage markers ED-1 (Figure 5.6C) and ED-2 (Figure 5.6F). Additionally, cells found in and immediately deep to the intima were shown to commonly stain positive for vimentin (Figure 5.6H). These findings, coupled with the few instances of graft calcification (Figure 5.5 – bottom right) and cartilage formation (Figure 5.5 – bottom left) observed contribute to the general conclusion that these grafts are indeed biocompatible.

![Figure 5.8 – SEM analysis of explanted mammary artery scaffolds.](image)
Although cartilage formation is not ideal, this observation still points to the overall conclusion that the grafts are indeed biocompatible, given the cells’ ability to remodel the matrix. Also, the large degree of α-smooth muscle actin positive staining cells infiltrating and aligning circumferentially in the media provide further evidence strengthening the conclusion of biocompatibility for these grafts. Additional staining for prolyl-4-hydroxylase to highlight neo-collagen formation may provide additional insights into the extent of vascular graft remodeling.

The quantitative analysis of the excised vascular grafts provided results which may require additional experimentation in order to investigate further. The initial hypothesis was that heparinization would improve hemocompatibility of the vascular grafts, but there was no significant difference in patency between unheparinized and heparinized treatment groups. Furthermore, the luminal area and IEL diameter was statistically larger in the unheparinized groups than the heparinized groups. When these results are coupled with the observed thinner graft walls in the unheparinized groups, these findings suggest that unheparinized groups may be dilatating over implant duration.

Although this possibility is not ideal, it is plausible, especially given the increased enzymatic resistance of heparinized grafts observed in vitro (see section 3.3.3.3 Scaffold Characterization – Mechanical and Biochemical Properties and corresponding enzymatic resistance figure). Perhaps future experimentation with grafts that receive a lesser degree of heparinization may increase the grafts susceptibility to be degraded by in vivo proteases and therefore also increase the observed graft lumen at the same 8 week timepoint.
The observed increase in graft wall thickness in the heparinized groups may possibly indicate a host response that is attempting to encapsulate the grafts as opposed to remodeling the grafts. Such results imply that the current degree of heparinization may impart too much graft fixation and negatively affect graft biocompatibility. If this is truly what is happening *in vivo*, then the observed decrease in wall thickness observed in the unheparinized groups may not be an actual *decrease* in wall thickness in the unheparinized groups, but an *increase* in wall thickness observed in the heparinized groups. As mentioned earlier in this section, the results gathered from this study can be analyzed from various angles and future studies are needed to gain more concrete conclusions as to the positive and detrimental attributes of heparin and PGG-treatment.

5.2.3.2 Bovine Mammary Artery Scaffolds

5.2.3.2.1 Histological and SEM Evaluation of Implants

This study was much smaller in scale than the renal artery study with only one treatment group (decellularized, PGG-treated graft) and only a short time period of evaluation (1 week). The cellular infiltration that was observed in the anastomosis (*Figure 5.7B*) was similar to those from the mid-graft sections (*Figure 5.7C, 5.7F, 5.7I*) in that the cellular infiltrate seemed to be deep to the granulation tissue observed on the outside of the graft wall. Immunohistochemical analysis will need to be done to determine the extent of infiltration for particular cell types. The staining employed for the porcine renal artery scaffolds was quite thorough and the bovine mammary artery scaffold explant analysis would benefit greatly from this breadth of staining. The cells observed in the media of the
excised graft are staining red in the Masson’s trichrome stain (Figure 5.71), but more extensive analysis will be necessary to infer if these cells are indeed smooth muscle cells.

The excised graft was observed to be thrombosed upon removal, but this result should not be unexpected, as it is widely accepted in the literature that unendothelialized grafts often thrombose when implanted in more challenging peripheral positions\[^2\]. The results of this study do suggest that the grafts are able to be infiltrated by host cells even in the short term period of 1 week; therefore, a high degree of cellular infiltration should be expected if this study was able to be performed for 4 and 8 weeks, as done for the renal artery scaffolds. With our described ability to endothelialize long bovine mammary arteries \textit{in vitro} (see section 4.2.2.2 Endothelialization), future studies using endothelialized bovine mammary arteries implanted for 4 and 8 weeks may actually be able to discourage thrombosis during the implant duration.

Although the media and adventitia of these scaffolds would be acellular upon implantation, the implanted bovine mammary artery scaffold has recruited a relatively large influx of cells for only 1 week of implantation and when coupled with a hemocompatible autologous endothelial layer, these cell-seeded scaffolds could provide promising results.

5.3 References

CHAPTER 6: TRANSLATIONAL PERSPECTIVES

6.1 Introduction

As mentioned briefly earlier in this document (see section 2.3 Significance of Proposed Project), a large portion of the work described herein, specifically, the development of the bovine mammary and femoral arteries, was largely inspired by rich collaborations with local vascular and cardiothoracic surgeons. During the many shared discussions both inside the operating room and the laboratory, they clarified shortcomings of current vascular replacements and inspired several developments, the most profound of which are,  

1) the research into long (> 25cm) vascular grafts, which spawned the inception of the device used for the decellularization of the xenogeneic arteries and culturing of cell-seeded constructs and  

2) the need for biological grafts of 8 mm to 6 mm diameter taper, which catalyzed the development of the bovine femoral artery in addition to the bovine mammary artery which tapers from, on average, 6 mm to 3 mm along its length; all of which are discussed earlier in this document (see Chapter 3: Characterization of Cytocompatible ECM-Stabilized Vascular Grafts) .

In addition to the many fruitful discussions regarding potential research directions and analysis of the produced results, a project was undertaken wherein a surgical case report was prepared for publication based on a unique case handled by local vascular surgeons. The information from this case report was gathered and written-up in accordance with all patient information guidelines in addition to the institutional review board documents from the related hospital. Below is the manuscript describing endovascular intervention for type B aortic dissection in the presence of an aberrant right subclavian artery (ARSA).
6.2 Endovascular Management of a Complicated Type B Aortic Dissection In the Presence of An Aberrant Right Subclavian Artery Without Subclavian Artery Reconstruction - A Single Case Report

6.2.1 ABSTRACT

Aortic dissection is the most common aortic catastrophe encountered by practitioners. For patients with Type B dissection who develop complications, endovascular stent grafting has become the most frequently employed therapeutic modality. Unfortunately, aberrant aortic anatomy can complicate the planning and implementation of this modality. Endovascular management for patients with aortic dissection in the presence of an aberrant right subclavian artery (ARSA) has been reported in several cases; however, in the majority of these cases the decision was made to reconstruct the right subclavian artery prior to deploying the aortic graft[152, 153]. We report a case of a complicated type B aortic dissection and associated ARSA treated with endovascular stent grafting without subclavian artery reconstruction.

6.2.2 INTRODUCTION

The optimal treatment of type B aortic dissections has been a matter of debate amongst physicians[154, 155]. Patients with this condition are initially treated pharmacologically and monitored for the development of complications[156, 157]. Thoracic endovascular aneurysm repair (TEVAR) has emerged as an increasingly accepted alternative to open interventions in patients who develop complications; however, the long
term outcomes following this treatment have yet to be established\textsuperscript{[158]}. The presence of aberrant aortic arch anatomy makes treatment of these patients particularly complex. While there are sporadic reports of type B aortic dissection treatment with TEVAR, most include reconstruction of the right subclavian artery in conjunction with TEVAR\textsuperscript{[152, 153]}. The purpose of this report is to discuss endovascular treatment of a complicated type B aortic dissection in a patient with an ARSA who was treated with a single stent graft covering the origin of the ARSA without subclavian artery reconstruction.

**6.2.3 CASE REPORT**

A 44-year-old African American female presented to the emergency room with the chief complaint of the acute onset of sharp chest pain radiating to her back coupled with shortness of breath. Past medical history included chronic obstructive pulmonary disease, morbid obesity, congestive heart failure and hypertension. Patient was noted to be hypertensive with initial blood pressure of 199/112, pulse of 102 and oxygen saturations of 94\% on 4 liters of oxygen. Due to the patient’s morbid obesity, vascular access was difficult to initiate. A V/Q scan was obtained to rule out pulmonary embolism as a cause for her hypoxia. The results were indeterminant for pulmonary embolism. A PICC line was placed to establish vascular access. This revealed the presence of an acute type B aortic dissection with a proximal descending thoracic aortic pseudoaneurysm and associated hemothorax. The patient was also noted to have an ARSA. The presence of the pseudoaneurysm and hemothorax prompted the decision to treat the patient via endovascular stent grafting.
Given the urgency of the situation combined with the patient’s intact vertebral artery system, the decision was made not to reconstruct the right subclavian artery prior to placement of the stent graft. The patient was approached via right femoral artery cutdown and a left brachial artery puncture. A thoracic aortic stent graft (W.L. Gore TAG, Flagstaff, AZ) was selected for use with size being determined both by CT angiography and intravascular ultrasound (IVUS). The graft was placed proximal to the ARSA origin and allowed to impinge on the origin of the left subclavian artery. The patient stabilized but remained critically ill. After a complicated hospitalization requiring tracheostomy placement, the patient was transferred to a long-term acute care (LTAC) facility for ventilator weaning. Follow-up CT angiogram demonstrated good positioning of stent graft with no evidence of endoleak.

The patient progressed at the LTAC facility to the point of requiring only continuous positive airway pressure (CPAP) with supplemental oxygen and was receiving physical therapy. Unfortunately, she developed acute respiratory decompensation followed by cardiovascular collapse while transferring from her bed and expired. Post mortem analysis revealed an intact stent graft in good position with no evidence of leakage or other complication. The cause of death was determined to be a saddle pulmonary embolus extending from the pulmonic valve to the deep bilateral pulmonary arteries.

6.2.4 DISCUSSION

The ideal treatment for type B aortic dissection has yet to be determined. While type B aortic dissection is very common, trials are ongoing to assess various treatment
modalities. Type B aortic dissection in the presence of an ARSA is uncommon, but has been reported in the literature\textsuperscript{[154]}. However, the field suffers from a low incidence of treatment for this population with a TEVAR approach\textsuperscript{[152]}. An even lower incidence of endovascular management for complicated aortic dissections exists without the use of open reconstruction in conjunction with stent graft placement. The need to assess long term TEVAR treatment safety and efficacy spawned the INSTEAD (Investigation of STEnt grafts in Aortic Dissection) trial, which assessed two current treatment options, medical treatment alone or endovascular repair coupled with medical treatment\textsuperscript{[158]}.

The study suggested that although “favorable remodeling” of the aorta was observed, patient survivability was not affected in the mid-term of two year follow-up\textsuperscript{[158]}. Following the 2 year INSTEAD trial, the patients were assessed between years 2 and 5 after initial treatment as part of the INSTEAD-XL trial and the findings were recently reported\textsuperscript{[159]}. Among the findings of the study were that TEVAR, when used in patients with uncomplicated type B aortic dissection, improves late patient outcomes. Furthermore, the use of TEVAR in addition to medical treatment improves overall 5 year patient survival. It should be noted that although this trial provided significant insights into the initial safety of TEVAR for type B aortic dissection, this trial did not provide insights regarding TEVAR cases involving aberrant aortic anatomy. Given the low incidence of patients presenting with aberrant anatomy and an aortic dissection it is unlikely that we will ever have evidence-based treatment algorithms. Current case reports of patients such
as ours who require TEVAR in the face of aortic dissection and an ARSA have typically utilized subclavian artery reconstruction in combination with TEVAR\textsuperscript{152}.

Our case is the second case to report TEVAR with right subclavian exclusion using a single stent graft without reconstruction\textsuperscript{160}. Another similar case was also reported, however, the deployed stent graft did not cover the ARSA\textsuperscript{161}, which is different from our approach. While current recommendations are to reconstruct the left subclavian artery when it requires coverage with TEVAR, there are no recommendataions for patients with aberrant anatomy such as an ARSA. In theory, the risk should be similar for left subclavian artery coverage. In an ideal elective setting we would likely reconstruct prior to coverage. In this case, however, given the patient’s clinical instability and morbid obesity, we elected not to reconstruct the artery. While the ultimate patient outcome was far from ideal, her demise was not secondary to either hemorrhagic complications related to ineffective aortic treatment nor were they related to cerebrovascular complications as a result of lack of subclavian artery reconstruction.

In conclusion, TEVAR shows promise as a means of treating complicated type B aortic dissection in the presence of ARSA. Our case suggests that subclavian artery reconstruction may not be required in all patients receiving TEVAR for type B aortic dissection.
6.3 References


CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

7.1 Conclusions

A significant, unmet clinical need still exists for long, small diameter (< 6 mm) vascular bypass grafts that can be utilized in various peripheral positions such as the lower limbs and coronary arteries while resisting thrombosis during the implant duration. More importantly, small diameter bypass grafts currently in development must not only meet the requirements for such a graft, but also outperform the current gold standard for bypass grafting, the autologous saphenous vein. The tough metric of outperforming autologous vasculature is important because although implanting developed grafts would fix the problems associated with donor site morbidity after harvesting autologous vessels, if the grafts do not beat the current gold standard in performance, these products will likely not be adopted for use.

We believe that a promising approach to meet this unmet clinical need would involve a tissue engineering approach making use of acellular scaffolds of both small and medium (8-6 mm) caliber in order to potentially provide grafts large enough to connect to the larger inflow vessels such as the femoral artery while also retaining diameters small enough to reach peripheral and coronary vessels. After exploring this approach in the previous sections of this document, the following conclusions were drawn:

7.1.1 Development of Bovine Mammary and Femoral Artery Scaffolds
1. A system was constructed capable of serving as both a decellularization device and an aseptic perfusion bioreactor for culture of up to six simultaneous native vessels or cell-seeded scaffolds.

2. Native bovine mammary and femoral arteries could be fully decellularized, resulting in an acellular matrix free of residual cellular nuclei and the powerful xeno-reactive epitope α-gal. The resulting acellular ECM scaffold retained endogenous collagen and elastin in all three native vascular tunics as well as an intact basal lamina “niche” containing laminin, collagen type IV and fibronectin.

3. The mechanical properties of the described scaffolds meet or exceed the mechanical criteria necessary for functional arteries and often times match the mechanical properties mentioned in the literature for in situ human peripheral arteries.

4. Cell seeded scaffolds were cytocompatible in the short term period of 3 days.

7.1.2 Development of Porcine Renal Artery Scaffolds

1. A protocol was established to efficiently dissect and isolate native renal arteries from porcine kidneys.

2. Native porcine renal arteries were fully decellularized, resulting in an acellular matrix free of residual cellular nuclei and the powerful xeno-reactive epitope α-gal.

3. The mechanical properties of the described scaffolds are similar to those of native controls.
4. PGG-treated renal artery scaffolds showed a significant resistance to elastase *in vitro*. Heparinization treatment endowed scaffolds with a significant resistance to collagenase *in vitro*.

7.1.3 Seeding of Long Vascular Scaffolds with Tunic-Specific Cell Types

1. Bovine mammary artery scaffolds were cytocompatible and were capable of retaining viability of seeded cells up to 15 days total culture time.

2. Bovine mammary artery scaffolds can be endothelialized along the entire scaffold length.

3. Future studies are needed in order to establish the stem cell differentiation potential of the preserved vascular “niche” in the described scaffolds.

7.1.4 Preclinical Evaluation of Scaffold Biocompatibility

1. Acellular porcine renal artery scaffolds retained 100% patency following 4 and 8 week implantation in the rat abodimal aorta position.

2. Explanted and histologically processed porcine renal artery graft sections displayed instances of revitalization of the tunica media and tunica intima with cells expressing markers pertinent for each specific tunic (α-smooth muscle actin, factor VIII, von Willebrand factor, respectively) which suggest the scaffolds are not only biocompatible, but potentially recellularize *in vivo* with tunic-appropriate cell types.
3. Bovine mammary arteries implanted into the femoral artery of Yucatan mini-pigs for 1 week were patent upon implantation, but occluded in vivo. Although significant cellular infiltration was observed, future studies will be needed to improve graft patency and conclude on graft biocompatibility.

7.2 Potential Clinical Uses for Bovine Mammary and Femoral Arteries

With scaffold lengths of approximately 25 cm, these grafts are long enough to be considered for clinical use. The intrinsic differences in geometry between the two scaffolds also point to unique potential applications for each scaffold. For example, the observed large degree of taper in the mammary scaffold (approximately 6 mm to 3 mm along its length) may make this scaffold an excellent candidate for coronary bypass, where 25 cm of graft should prove to be more than enough in most cases. The femoral scaffold, because it tapers very little along its length (approximately 8 mm to 6 mm), could serve as a potential graft for cases when portions of the iliac or femoral arteries may need to be replaced. Most importantly, the needs of each patient are different and numerous potential combinations exist for each separate scaffold as a bypass graft. Furthermore, these scaffolds could potentially be combined in end-to-end fashion to achieve longer or more complicated bypasses normally impossible for shorter, non-tapering grafts (see section 3.2.4 Other Remarks).

These characteristics, as well as the robust mechanical properties, approximate total composition of collagen and elastin, retention of endogenous bioactive basal lamina proteins, cytocompatibility, and absence of the xenoreactive epitope alpha-gal point to the
potential for these grafts to function immediately upon implantation and eventually be remodeled and integrated into the patient. Future animal studies using cell-seeded scaffolds will be needed to test these claims and further elucidate the potential of these scaffolds to serve as bypass grafts in the context of regenerative medicine.

7.3 Recommendations for Future Work

Our working premise is that autologously endothelialized long vascular scaffolds stabilized with PGG provide the ideal replacement peripheral vascular graft. Autologous cell sourcing resolves the problem of graft versus host disease and implant rejection that commonly accompanies allogeneic or xenogeneic cell sourcing. Stabilization of scaffolds with PGG prior to combining with cells will presumably ameliorate the dilatation and aneurysm formation that commonly plagues non-stabilized biological grafts by imparting to our scaffolds resistance to ECM degrading enzymes\(^{16, 22}\). In addition to enzymatic resistance, PGG treatment of biological tissues has been shown to reduce calcification \textit{in vivo} as well\(^{16, 22}\). Based on our previously demonstrated cytocompatibility of multiple scaffolds produced in our laboratories with several different cell types, we believe the bovine internal mammary and femoral artery scaffolds will serve as excellent vascular replacements that will retain patency throughout the duration of implantation. A potential experimental plan to further assess the \textit{in vivo} performance of these arteries is proposed according to two aims based on the goal of autologous cell sourcing to produce fully autologous vascular grafts to be evaluated in a preclinical canine model.
7.3.1 Proposed Specific Aims

Aim 1: Endothelial Cell Seeding and Progressive Adaptation to Physiologic Shear

Hypothesis: Acellular scaffolds seeded with endothelial cells differentiated from ADSCs will embody autologous constructs capable of forming shear-adapted endothelium.

Proposed Approach: Canine adipose tissue will be isolated from which ADSCs will be purified, expanded in culture, differentiated into endothelial cells and seeded onto vascular scaffolds. Seeded constructs will be mounted into a vascular bioreactor and progressively adapted to physiological shear stress to establish confluent cell seeding and adaptation procedures for future in vivo experimentation.

Expected Outcomes: Cytocompatible scaffolds will exhibit native cellular removal, retention of collagen and elastin, mechanical properties similar to native tissue and resistance to ECM degrading enzymes.

Aim 2. Preclinical evaluation of shear-adapted autologous constructs

Hypothesis: Our tapering bioactive constructs with autologous, shear-adapted endothelium will achieve greater biocompatibility than currently marketed acellular synthetic conduits.

Proposed Approach: A canine model will be employed to compare the in vivo biocompatibility of our proposed endothelialized constructs to expanded polytetrafluoroethylene (ePTFE) conduits. Autologous adipose tissue will be isolated, then ADSCs will be purified and differentiated into cells of endothelial lineage. Differentiated
cells will be seeded onto scaffolds and adapted to physiological shear. Autologous constructs will be implanted as a conduit in peripheral arterial circulation for up to 4 weeks.

**Expected Outcomes:** Vascular constructs with confluent endothelium will sustain patency while resisting thrombus formation and dilatation throughout the implant duration.

### 7.3.2 Proposed Research Design and Methods

#### 7.3.2.1 Methods for Aim 1

**Aim 1: Endothelial Cell Seeding and Progressive Adaptation to Physiologic Shear**

#### 7.3.2.1.1 Optimize Isolation of ADSCs:

Stem cell isolation from adipose tissue and protocols for differentiating these ADSCs into various different cell types has been heavily discussed in the literature.[147] These protocols which have been tailored for isolation of human cell types will be adapted for use with a canine host. Canine hosts were chosen because they represent the smallest species with the most anatomically comparable sizes for vasculature (not shown).

Adipose tissue will be collected from a local abattoir, stored in DMEM supplemented with 10% FBS and 2% antibiotic solution, and transported back to the laboratory. Tissue will then be minced using scalpels, and digested using collagenase solutions buffered in PBS at 37°C. The digested solution will then be filtered through cell-safe sieves, with the cells collected into an erythrocyte lysis solution buffered with PBS and incubated for 10 minutes at room temperature. Following incubation, cells will be spun down using a centrifuge and the supernatant decanted. Pelleted cells will then be resuspended in the aforementioned DMEM solution and plated in a culture flask. Cell
adhesion will be monitored for three days, then the media changed and cells cultured to confluence prior to differentiation into endothelial cells (ECs).

Before any ADSC-based cell seeding can be conducted, the cells must be shown to present endothelial markers as previously discussed\textsuperscript{[162]}. Cells will be placed into well plates, and using immunohistochemical methods will be stained for von Willebrand factor as well as EN4 to confirm the cells are of an endothelial lineage. ADSC-derived ECs will be used for construct endothelialization and shear adaptation.

**7.3.2.1.2 Confluent ADSC-derived EC Seeding on Scaffolds:**

Previous cell seeding studies have shed light on the need for high seeding densities for scaffolds in order to reach confluence. Furthermore, scaffold orientation and rotation during seeding have also been shown to be paramount (see section \textbf{4.2.2.2 Endothelialization} and related figure). During these earlier studies, scaffolds were continuously rotated during cell seeding in anticipation of achieving a dense, uniform cell layer. Although the initial results are encouraging, we believe that repetitive cell seeding coupled with overnight static incubation and rotation are key for attaining confluent cell populations along the entire length of the scaffold (see section \textbf{4.2.2.3 Seeding and Differentiation of Human ADSCs}).

**7.3.2.1.3 Condition and Adapt Confluent ADSC-derived ECs to Physiological Shear:**

Literature in the field has shown successful flow conditioning of short, flat sections of seeded constructs using parallel plate-based flow chambers\textsuperscript{[163]}. We believe that by using
gradual increases in fluid flow rate and overnight flow conditioning periods as previously described,[149] we can successfully replicate these results using our perfusion bioreactor system. We propose a 7 day increasing flow ramp-up period wherein after adjusting fluid flow for each day (except days 1 and 2), flow will be maintained constant until the next day, when it will be gradually increased again. Days 1 and 2 would utilize enough fluid flow to prevent fluid stagnation, and day 7 would utilize a flow rate of at least 1.5 L/min. Final flow rates will be selected depending on the implant site ([human] common femoral artery – 284 mL/min, superficial femoral artery – 152 mL/min, popliteal artery – 72 mL/min).[150]

7.3.2.2 Milestones for Aim 1

7.3.2.2.1 Optimize Isolation of ADSCs:

ADSC isolation “optimization” will be defined by immunohistochemical staining showing positive markers for EN4 and von Willebrand Factor, known endothelial markers, using immunofluorescence-based methods as discussed previously[162]. Plated cells are expected to stain positive for both antigens to confirm that the isolated cells are committed to an endothelial-like lineage.

7.3.2.2.2 Confluent ADSC-derived EC seeding on Scaffolds:

“Confluent” cell seeding will be defined by achieving at least 70% confluence of seeded constructs within the proximal third, medial third, and distal third of seeded constructs in at least n = 3 separate areas within each section after staining with Calcein
AM. Confluence will be calculated by measuring the surface area of seeded cells divided by the total visible area in captured images using ImageJ software (National Institutes of Health, Bethesda, MD). A metric of 70% seeded cell confluence is proposed because seeded scaffolds will be undergoing additional perfusion culture immediately following cell seeding and tissue culture, which is expected to incite additional cell proliferation, cell-cell attachment, and shear-induced flattening.

7.3.2.2.3 Condition and Adapt Confluent ADSC-derived ECs to Physiological Shear:

Given that seeded scaffolds have now undergone two rounds of tissue culture (one static during seeding and one dynamic to align to flow), “confluence” is now defined by achieving at least 80% confluence within all three sections with n = 3 as described above.

7.3.2.3 Challenges and Alternative Approaches for Aim 1:

We are anticipating difficulties in placing enough cells onto scaffolds in order to achieve the stated confluence milestones. However, we plan to overcome these difficulties by simply seeding more cells per seeding cycle than the previously mentioned 5+ million cells. Pure gravity-based cell seeding is a refined enough process that we strongly believe that when enough cells are seeded per seeding cycle, the stated milestones can be met (see section 4.2.2.2 Endothelialization and associated figure). Previous experimentation in our group has already ruled out chemotaxis-, magnetic-, and pressure-based methods as efficient cell seeding techniques (not shown).
With respect to flow conditioning of seeded constructs, we are expecting a great deal of experimentation will be necessary to establish flow rates that will align cells appropriately while not forcibly shearing them off the construct. Amending these difficulties can take several different possibilities in terms of experimental design, but will ultimately have to be resolved by increasing static culture periods, dynamic culture periods, seeding more cells per cycle, or combinations of these options.

Regardless of the highly publicized nature of primary cell isolation from any tissue source, challenges will likely be incurred when attempting to isolate and expand large numbers of the ADSC populations. We propose to increase primary tissue explant size during preliminary experimentation until the procedures for cell isolation can be refined enough to only require the small animal tissue explant sizes that are enforced by IACUC and IRB animal use and care protocols. Circulating endothelial progenitor cells\cite{164}, endothelial cells sorted from the lipoaspirate stromal vascular fraction\cite{162}, and EC-differentiated dermal fibroblasts following epithelial-mesenchymal transition\cite{165} can also be utilized for experimentation.

In terms of confirming endothelial lineage for cells differentiated from ADSCs, there are several accepted methods and immunohistochemical stains that can be conducted. If difficulties are incurred with either the von Willebrand factor or EN4 stains, the factor VIII EC marker may be used. If additional confirmation of endothelial lineage is required, gene analysis can be conducted using polymerase chain reaction and protein analysis can be conducted using Western blot analysis to show that the cells are expressing genes and synthesizing proteins typical for endothelial cells.
7.3.2.4 Methods for Aim 2

Aim 2. Preclinical evaluation of shear-adapted autologous constructs

7.3.2.4.1 Four Week Implantation of Endothelialized Autologous Canine Constructs

Autologous EC populations will be isolated from 6 separate canine hosts, with shear stress adapted constructs being prepared as discussed previously (Aim 1). Following progressive shear stress adaptation of constructs, grafts will be implanted into the peripheral circulation of each respective host, using ePTFE contralateral controls (W.L. Gore, Flagstaff, AZ), for up to 4 weeks prior to explantation. Explant biocompatibility analysis will involve immunohistochemical staining for endothelial cells, macrophages, smooth muscle cells, fibroblasts, neutrophils, and lymphocytes using semi-quantitative histomorphometric analysis. Overall ECM integrity will be assessed with hematoxylin and eosin, Gomori’s trichrome, and Verhoeff’s van Gieson stains in addition to analysis of calcification with alizarin red staining. Additionally, scaffold hemocompatibility will be analyzed by observing explant luminal surfaces using scanning electron microscopy for platelet activation. Scaffold dilatation will be assessed by measuring and comparing in situ diameter at implant and explant.

7.3.2.5 Milestones for Aim 2

8.3.2.5.1 Four Week Implantation of Endothelialized Autologous Canine Constructs

Explanted constructs should display a retained shear-adapted endothelial monolayer, which is not expected to be observed from explants in the 1 week scaffold implantation study. Preliminary in vivo data corresponding to stabilized scaffold
implantation (Figure 5.6) has shown host smooth muscle-like cell infiltration following 8 week implantation in rat abdominal aorta. Therefore, we anticipate similar cell infiltration will occur within our seeded constructs over the implant duration. Because the scaffold material is of biological origin, we anticipate infiltrating smooth muscle-like cells to take on a contractile, ellipsoid morphology (Figure 5.6) as opposed to the characteristically spherical synthetic morphology which we expect to observe in the ePTFE grafts based on previous studies[166]. Furthermore, because of the advantage of retaining an autologous endothelial cell layer, we also expect to observe host fibroblast repopulation of the adventitia. Minimal lymphocyte, neutrophil, and macrophage infiltration is also expected, leaving the media and intima largely free of these cells. Graft dilatation is not expected and graft diameter should be within ± 0.5 mm of initial implant.

7.3.2.6 Challenges and Alternative Approaches for Aim 2

The peripheral circulation is a challenging implantation site for both humans and animals. Although the constructs utilized in this study will be endothelialized prior to implantation, the constructs may experience vasospasm and an overall decrease in inner diameter at the anastomoses. In attempts to ameliorate this, grafts will be implanted and consistently monitored for signs of occlusion secondary to vasospasm. If palpable pulse is noted to decrease distal to the implanted construct, vasodilating pharmaceuticals will be applied topically to the anastomoses in hopes of breaking the vasospasm. If the animal is showing signs of peripheral ischemia, the dosage of heparin will be increased and low-dose
vasodilating pharmaceuticals will be administered in the short term to combat the initial inflammation following implantation, pending veterinarian approval.

7.4 References