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Endothelialization Using Protein Substrates and Hydrogels for Aortic Valve Applications

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ENDOTHELIALIZATION USING PROTEIN SUBSTRATES AND HYDROGELS FOR
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Abstract

Aortic valve disease is currently the leading cause of cardiovascular morbidity in the United States.[1] In 2003, approximately 290,000 patients required heart valve replacement and that number is estimated to continue to rise as the average age of the population continues to increase.[2] However, the current valve replacement options have multiple limitations. Tissue engineering hopes to address these shortcomings by providing a viable valve that more closely mimics the native valve, structurally and functionally.[3] An ideal valve replacement should contain endothelial and interstitial cells, with the ability to remodel the extra-cellular matrix. While tissue-engineered heart valves are not uncommon in the field, retaining an adequate monolayer of endothelial cells on the scaffold surface remains an unresolved challenge. Without a functional endothelial layer, the implant can become thrombogenic, initiate an immune response, and become incapable of conducting necessary communications with the underlying interstitial cells. The focus of this series of studies is to create a seeding method that optimizes the adherence and uniformity of endothelial cells on an acellular heart valve scaffold.

This study has three approaches to maximize endothelial cell adhesion: the use of protein substrates, the application of hydrogels, and dynamic seeding. First, protein substrates – laminin and fibronectin – were applied to samples of decelled bovine pericardium and seeded with endothelial cells in static conditions. In the next study, hydrogels – PureCol, Q.Gel, and fibrin glue – were applied to the surfaces

of tissue samples followed by the aforementioned protein substrates and finally seeded statically with endothelial cells. A novel rotating device was then used to dynamically seed the decelled native valve. Finally the seeded valve was analyzed in vitro for cellular retention by exposing the valve to physiological shear stress in the heart valve bioreactor.

The use of substrates in combination with fibrin glue has shown to increase endothelial cell adhesion on bovine pericardium in proof-of-concept studies. A rotating device for valve seeding applications has shown positive results for uniform and confluent endothelial cell coverage. These studies will serve as a reference for future endothelialization studies as the field of cardiovascular tissue engineering strives to develop permanent solutions for patients with valve or vascular diseases.

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Part 1: Introduction and Background

Chapter 1: Anatomy and Physiology of the Heart

2.1 **Components of the cardiovascular system**

The heart is a part of the cardiovascular system and in charge of controlling the flow of blood throughout the body. Veins collect deoxygenated blood from the organs and tissues and carry it to the heart. The heart pumps the gathered deoxygenated blood to the lungs where the blood is oxygenated, and then pumps the oxygenated blood back to the organs and tissues. This hard-working system pumps about 7,000 liters of blood through the heart every day.[4]

2.1.1 **Tissue layers & the pericardium**

The wall of the heart itself is divided into 3 tissue layers: the epicardium (outermost layer), myocardium (middle layer), and the endocardium (innermost layer). The epicardium consists of connective tissue covered by endothelium, blood capillaries, and nerve fibers. It serves to reduce friction between the exterior of the heart wall and the outside covering described below. The myocardium controls the muscular contractions of the heart and is made of cardiac muscle tissue, connective tissue, blood capillaries, lymph capillaries, and nerve fibers. The endocardium lines all of the heart chambers and covers the heart valves described below. It is made of connective tissue, specialized muscle fibers, endothelium, and blood vessels.[4]

The entire heart is then wrapped in a complex covering called the pericardium. The pericardium is also comprised of three layers: a double layered serous

membrane and an outer fibrous sac. The innermost layer of the serous membrane directly touches the epicardium and is termed the visceral (meaning organ) pericardium. The middle layer, called the parietal pericardium, is formed from the folding of the visceral pericardium on top of itself. Finally, the outermost layer is a thick fibrous layer made of dense connective tissue called the fibrous pericardium.[4, 5]

2.1.2   Chambers and valves of the heart

The heart is divided into four chambers: the left atrium, the right atrium, the left ventricle, and the right ventricle. The atria are superior to the ventricles. The atria collect blood when delivered to the heart from the vena cava or the lungs and the ventricles contract to force blood out of the heart and into circulation. The chambers are shown in Figure 1 below.
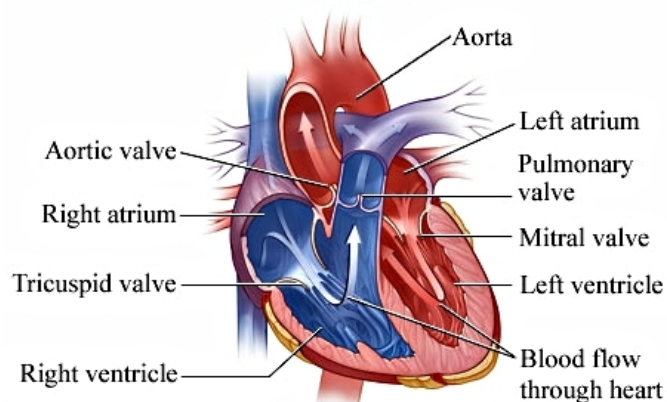
Figure 1: Coronal section of the heart showing the atria, ventricles, valves, and the flow of blood through the heart1

There are also four valves in the heart. The valve between the right ventricle and atrium is the called the tricuspid valve and the valve between the left ventricle and atrium is called the mitral valve (also known as the bicuspid valve). Generically

these valves are termed the atrioventricular valves and they control the flow of blood between the chambers. Additionally, there are valves that control the movement of blood out of the heart. The valve between the right atria and the pulmonary vessel is called the pulmonary valve and the valve between the left atria and the aorta is called the aortic valve.[4, 6] All four of the valves can be seen in the transverse cross section show in Figure 2 below.
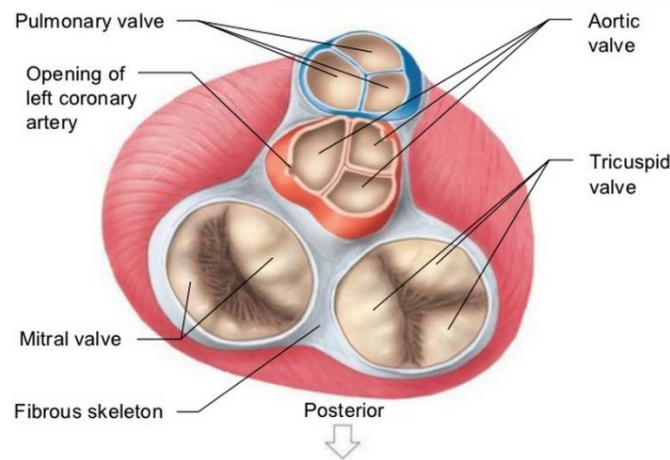
Figure 2: Superior view of the fibrous skeleton of the heart showing all four valves. The mitral and tricuspid valves are opened while the pulmonary and aortic valves are in the closed position.[4]

2.1.3 The route of blood flow

To fully understand the role of the chambers and valves described above, the route of blood flow should be traced. First, deoxygenated blood is carried through the veins and delivered to the right atrium. When the right atrium contracts (atrial systole and ventricular diastole), it forces blood out of the atrium, through the tricuspid valve, and into the right ventricle. From the right ventricle, the blood proceeds through the pulmonary valve and into the pulmonary arteries. The pulmonary arteries lead into the lungs where gas exchange occurs. The oxygenated

blood leaves the lungs through the pulmonary veins and is delivered to the left atria

of the heart. The blood flows from the left atria through the mitral valve into the left

ventricle. The left ventricle then contracts (ventricular systole and atrial diastole)

and blood is pushed out of the left ventricle and through the aorta in a large bolus.

The aorta branches into smaller arteries which carry the oxygenated blood to the

entire body.[4, 7] This process is best described in Figure 3 below.
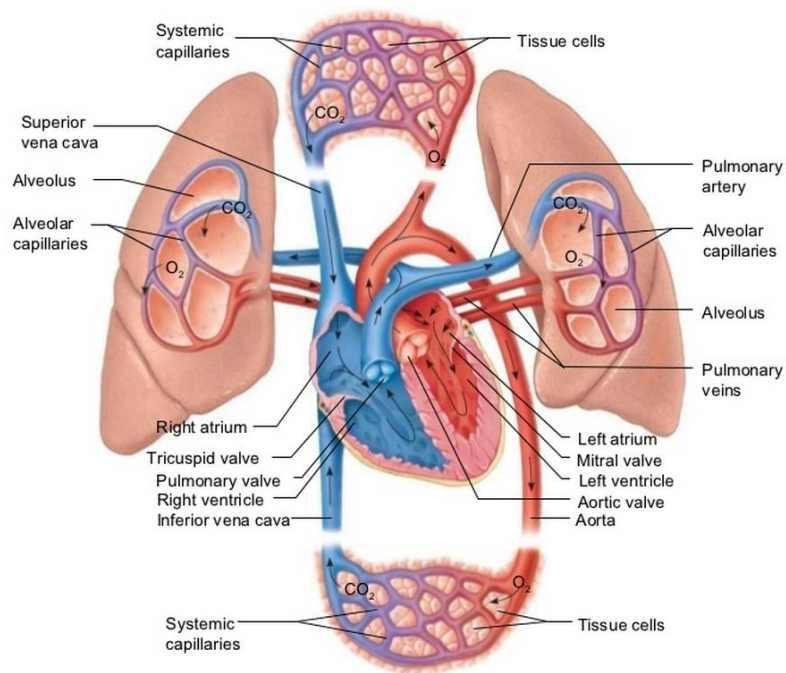
Figure 3: Detailing the route of blood flow in the heart and throughout the body. The right ventricle forces deoxygenated blood (in blue) into the lungs for gas exchange. The right ventricle pushes blood through the aorta and to the rest of the body.[4]

Chapter 2: The Anatomy and Physiology of the Aortic Valve

3.1 **Anatomy of the valve**

The aortic valve is made of three, equally sized, half-moon shaped cusps (or leaflets) a few hundred microns in thickness. Each cusp is attached to its respective sinus which is a physical expansion of the aortic root.[8] The aortic valve is located between the aorta, the largest artery in the body with a diameter of ~ 3 cm, and the left ventricle. It is positioned in the middle of the cardiac base, making it the centerpiece of the heart (shown in Figure 2 above). All four valves situated at the cardiac base are held in place by what is called the cardiac skeleton. The cardiac skeleton consists of rings of connective tissue that surround each of the valves and dense connective tissue that extends into the heart, between the ventricles.[4] Fibrous connective tissue surrounds the aortic valve, providing great support and making it the strongest portion of the cardiac skeleton.[9]

The cusp tissue is comprised of three layers: the fibrosa, the ventricularis, and the spongiosa. The fibrosa faces the aortic side of the passageway, the ventricularis faces the ventricular side, and the spongiosa is in between these two layers.[8] The fibrosa is the primary load-bearing surface and consists of densely packed type I collagen. The ventricularis is composed mostly of elastin to provide the ability to recoil during ventricular diastole. Lastly, the spongiosa provides overall cushioning and lubrication between the outer layers and is rich in glycosaminoglycans (GAGs).[1] There are two cell types that reside in this complex scaffold: endothelial cells and

interstitial cells. The endothelial cells cover both the outside surfaces, the fibrosa

and the ventricularis, and serve as a barrier to blood flow as well as control many

vital functions of the valve. The interstial cells are found in the center of the tissue

and are comparable to smooth muscle cells found in the vasculature intima.[10]

Together these layers serve to create a very durable and unique composite material.
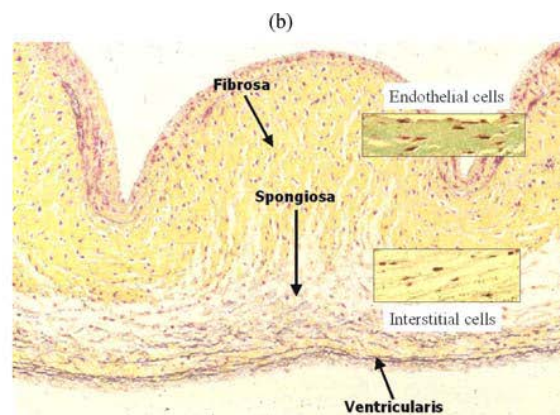
Figure 4: The three layers of a native cusp - Fibrosa, spongiosa, and ventricularis. Endothelial cells can be seen on the fibrosa and ventriucularis while interstitial cells are found in the spongiosa.[3]

3.2   Biomechanics of the valve

The sinuses function to prevent the valve leaflet from completely touching the

aortic wall and getting stuck when opened and to help to facilitate valve closure by

creating vortices inside the sinuses when the leaflets are opened. The actual opening

and closing of the valve is controlled by the creation of a pressure gradient when

blood flows into the sinuses. The opening of the valve is triggered when the

pressure gradient changes when the valve is closed and flow slows down. Pressure

on the inflow side becomes higher than the pressure on outflow side allowing the

leaflets to naturally fall open. When the valve is opened (ventricular systole), the leaflets push against the sinuses and allow blood to flow out of the ventricle and through the aorta.[11] As the myocardium relaxes, the pressure in the ventricle (inflow side) drops below the pressure in the aorta (outflow side) causing the valve to snap shut (ventricular diastole). When the valve is closed, the leaflets come together and meet along a fibrous region a small distance from the free edge of the leaflet, called the commissure.[9] Additionally, the sinuses function to relieve abnormal stresses on the leaflets by facilitating a smooth closure. The sinuses are able to close smoothly by creating eddy currents that cause the leaflets to begin closing before systole is complete, allowing minimal fluid flow to be required for the valves to completely close. These flow patterns are detailed in Figure 7.
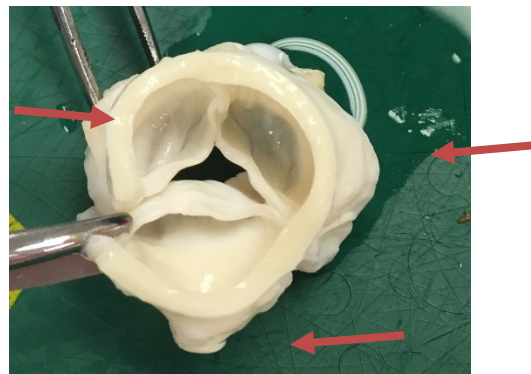
Figure 5: Decellularized explanted porcine aortic valve oriented with the outflow side up. The arrows are demonstrating the location of the sinuses, the cavity behind each of the leaflets that extrudes from the aortic root.
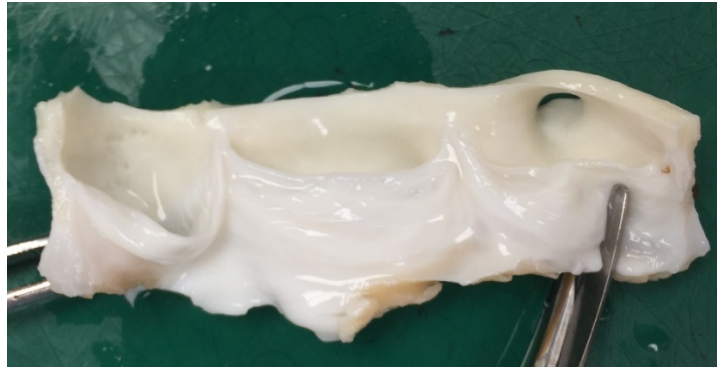
Figure 6: Decellularized explanted porcine aortic valve that has been cut along the aortic root and opened up to view the 3 cusps, or leaflets. The sinuses are the regions behind each of the cusps.

Substantial back pressure is created on the valve when it is closed creating a highly demanding environment for the valve to reside.[1] Additionally, the aorta and the aortic valve are exposed to extreme mechanical forces which each cardiac cycle when the blood is pushed through at a high velocity to ensure it reaches all tissues. To give an idea, the peak velocity of the blood through the aortic valve is 1.35 ± 0.35 m/s.[8] The aorta's viscoelastic properties allow for the expansion and recoil at the reception of this high blood stroke volume before the blood is passed into the arteries. The aortic valve undergoes similar cyclic stretch in order to maintain proper homeostatic flow dynamics in these challenging conditions. The endothelial cells help conduct these cyclic stretch and relax mechanisms as described in detail in section 4.1.
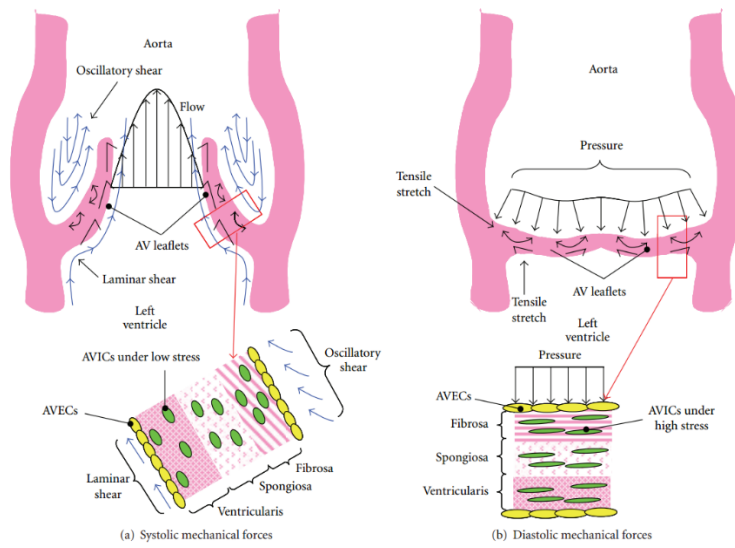
Figure 7: Eddy currents are created in the sinuses when blood flows through the valve which aid in the closing of the valve. When the valve is closed, pressure increases on the inflow side of the valve which pushes the valve back open.[11]

Chapter 3: Aortic Valve Pathology

With heart disease as the primary cause of disability and death in the Western world, it accounts for 40% of all postnatal deaths (about 750,000 people) in the United States annually.[12] Specifically valvular heart disease, a subset of heart disease, is when proper functioning of a valve is compromised. Valvular heart disease is generally characterized by regurgitation, stenosis, or sometimes both of a heart valve. Stenosis is the failure of a valve to open completely so blood does not flow freely (shown in Figure 8). If a valve is reguritant it does not close properly and allows retrograde flow, or backflow, of blood back into the left ventricle instead of being properly expelled through the aorta. Valve regurgitation is shown in Figure 9. The severity and rate of development of valvular disease can range from physiologically unimportant to rapidly fatal. The severity, duration, cause, and rate of onset can affect secondary changes in the heart, vessels, and other organs.[11, 12]

Valvular disease can be a congenital defect, acquired later in life as a result from cardiovascular disease, or a consequence of rheumatic fever. Aortic regurgitation can result from disease of the valve cusps or damage to the supporting structures without primary changes in the actual valve itself. It can be acute but is usually chronic caused by a dilation in the ascending aorta, related to hypertension and aging. Stenosis, however, is almost always chronic and due to calcification.[12]
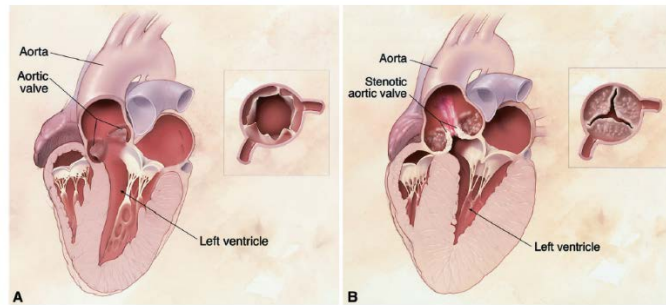
Figure 8: Stenotic aortic heart valve during systole is shown in figure B and compared to a healthy valve in figure A.[13]
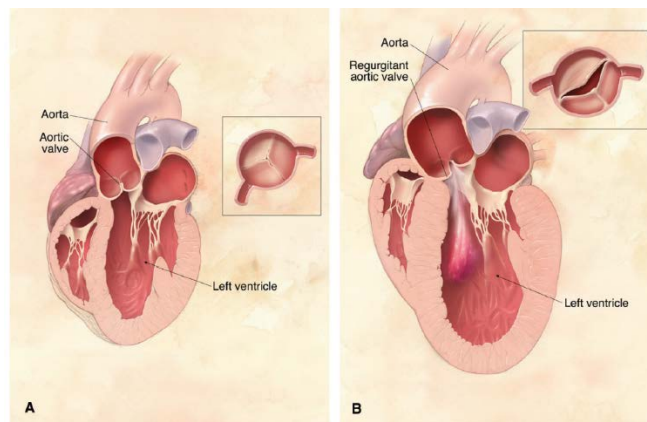
Figure 9: Regurgitant aortic valve during diastole shown in figure B compared with a health valve during diastole shown in figure A.[13]

4.1   Calcification

As discussed earlier, the aortic valve resides in a mechanically challenging environment due to the extensive number of cardiac cycles, substantial tissue fatigue deformations incurred with each cycle, and high pressure gradients created across the closed valve. Calcification can be induced by either mechanical wear and tear from the environment, from aging, or a congenitally developed bicuspid aortic valve. Morphologically, a calcified heart valve contains heaps of calcified masses within the cusps that eventually protrude through the outflow surface of the valve

and into the sinuses. As calcium deposits accumulate, the valve opening becomes impaired.[12]

Valvular oxidative stress is increased in patients with end stage aortic valve stenosis and has been tied to the pathogenesis of aortic valve calcification. Oxidative stress induces calcification by leading to increased reactive oxygen species, amplified osteogenic signaling, and reduced blood lipids.[14, 15]

Additionally, low shear stresses and disturbed flow on the valve surface have also been hypothesized to lead to a myriad of pro-calcific effects including: endothelial cell apoptosis and increased permeability, reduced release of paracrine signaling molecules like nitric oxide, increased inflammatory cell recruitment, and the diffusion of inflammatory molecules into the tissue. When endothelial permeability is compromised, macrophages can infiltrate into the tissue (shown in Figure 10), become activated, and express proteinases. These proteinases, such as metalloproteinases (MMP2 and MMP4), may lead to osteogenic differentiation of myofibroblasts or smooth muscle cells.[16] Nitric oxide paracrine signaling of valvular endothelial cells to interstitial cells is thought to be the mechanism by which valvular endothelial cells activate the valvular interstitial cells.[17] Activated interstitial cells can then differentiate into myofibroblasts or osteoblasts which lead to the production of collagen and calcification.[14]
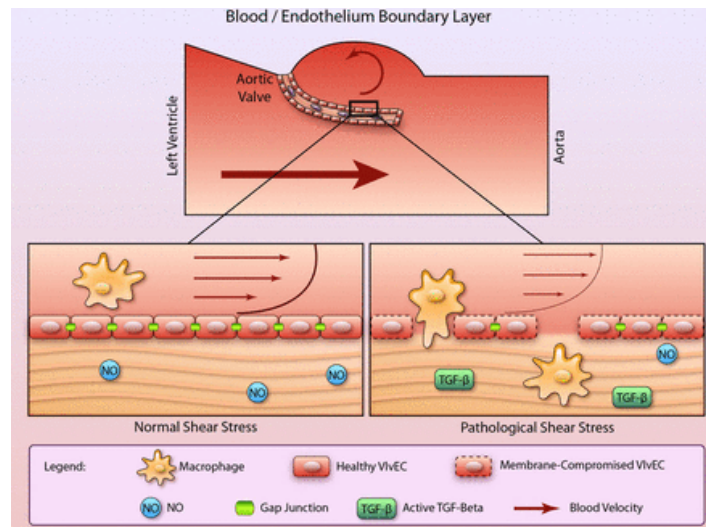
Figure 10: Increased nonspecific permeability of the endothelial cells from abnormal shear stress, allowing macrophages to invade the interior of the leaflet tissue.[17]

4.2 Current treatment options

If diagnosed with valvular heart disease today, there are two treatment options: a mechanical or a bioprosthetic heart valve replacement. The mechanical valve replacements are made of artificial materials such as carbon, metal, or polymers. These valves offer decent hemodynamic functionality and long-term durability; however they are known to induce thrombosis and systemic thromboembolism which require the patient to take anti-coagulants, such are warfarin, for the rest of his or her life.[18] Thrombosis is induced from the disruption of flow and damage to blood cells, especially around the hinges of the prosthesis.[19] Anti-coagulation therapies are generally associated with an increased risk in hemorrhage thus is unsuitable for many active lifestyles or expecting mothers.

Bioprosthetic valves are fully or partially created from animal or human tissue. These valves are less thrombogenic than the mechanical options due to their more natural anatomy to the native valve, thus they usually do not require anti-coagulation drugs. Current bioprosthetic valves on the market are nonviable and fixed with gluteraldehyde to prevent an immune response. However, since there are no living cells to repair the tissue from normal wear and tear the valve is subject to total structural dysfunction.[19] This deterioration can stem from intrinsic or extrinsic mineralization (calcification) caused from the host's immune system, the implant design, or the induced shear stresses on the valve. Abnormal value motion is another factor that could contribute to the deterioration of the valve.[20] Therefore, the tissue valves have a shorter lifetime and must be replaced every 10-15 years. In a study by Hammermeister, it was found that 15% of the patients given an aortic bioprosthetic valve replacements and 36% of the patients given a mitral bioprosthetic valve replacement saw structural failure at 11 years post implantation.[18]

Of the 285,000 annual valve replacement surgeries, about 40% of these cases chose the bioprosthetic valve.[20] Both valves face major complications such as systemic embolism, significant bleeding, regurgitation, thrombosis, endocarditis, reoperation, and even death. The bioprosthetic valves are popular among the older population who likely will not face an additional operation in their lifetime. Due to the aging population however, there has been an increase in use of the tissue valves.[20]

Chapter 4: Valvular Endothelial Cells & their Interactions with ECM

5.1 <u>**The roles of valvular endothelium**</u>

The study of endothelial cells, especially valvular endothelial cells, is a fairly new field of study and much information remains unknown about these cells and their functioning. However, it is known that endothelial cells (EC) play crucial roles in the maintenance of blood and tissue homeostasis. This includes coagulation, transduction of mechanical and biochemical signals, and tissue permeability.[21] Figure 11 below shows the topology of a healthy native endothelial layer. The endothelial cells appear to have a "cobblestone" morphology because they are spread out and cover every surface of the tissue's surface. The following sections will describe the specific roles of the endothelium.
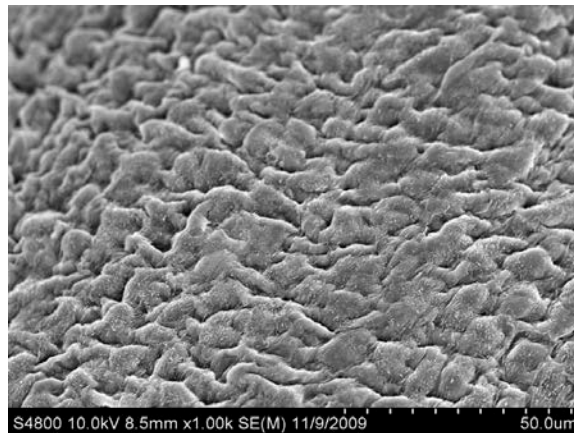
Figure 11: Scanning electron microscope image of a native endothelium of porcine aortic valve cusp. Courtesy of Lee Sierad.

5.1.1 Coagulation

   The coagulation cascade is initiated when the endothelium becomes injured. The endothelial cells mediate coagulation by secreting tissue factor, which leads to the formation of fibrin, when stimulated by inflammatory cytokines and shear stresses. Von Willebrand Factor (vWF), expressed by the endothelium, aids in the adhesion of platelets and fibrin to the activated surface. Since fibrin formation is increased at higher shear rates, it is important the valvular endothelium remains non-activated to prevent the formation of thrombus.[22] However, it is interesting to note that it has been observed that shear stress induced valvular endothelial cells rarely result in thrombosis compared to vascular endothelial cells.[17]

5.1.2 Maintenance of mechanical properties

   Endothelial cells aid in the maintenance of vasomotor tone through the secretion of vasoactive agents such as nitric oxide (NO).[21] NO is a vasodilator that functions with the tissue's underlying smooth muscle cells. Valvular endothelial cells release NO when exposed to shear stress in order to control the mechanical properties of the tissue. For example, it has been shown that these agents increase the elastic modulus of the tissue by a significant amount.[23] The specific mechanism lies in the current of calcium. The release of vasoactive agents controls the smooth muscle cells and myofibroblasts which in turn regulate the flow of calcium into the tissue. Additionally, in vitro studies have shown that valve tissue can contract and dilate in response to vasoactive agents just like vascular endothelial cells, thus changing the

actual dimensions of the leaflets.[22] The change in dimensions is thought to aid in the

alignment of leaflets along their lines of commissure and complete closing.

5.1.3   Regulation of immune response

Endothelial cells play an important role in regulating immunogenicity and

inflammation of the underlying tissue. A healthy endothelium provides a non-

immunogenic surface by protecting against leukocyte rolling and diapedesis, the

primary inflammatory response. The endothelium also controls the recruitment of

circulating inflammatory markers such as cytokines and the release of inflammatory

markers such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion

molecule-1 (VCAM-1), von Willebrand factor, plasminogen activator inhibitor-1, and

thrombomodulin.[24] Thus, endothelial cells are one of the most immunostimulatory

cell types in the body.[25]

5.1.4   Tissue permeability

It is also the endothelium's responsibility to determine how the underlying tissue

will respond to certain substances such as coagulation factors, lipids, nutrients, and

molecular stimulants that circulate in the blood [22]. Intact, healthy endothelium is

tightly connected by intercellular junctions, including connexins and vascular

endothelial cadherin, and thus do not allow many macromolecules such as

lipoproteins and albumin to enter the tissue.[26] However, it is worth noting that

valvular endothelial cells have been shown to have an increased permeability

compared to vascular endothelial cells with the exception of lipoprotein uptake. It is

thought the valve is less permeable to LDL (low density lipoproteins) because it is already saturated with LDL compared to vasculature.[22]

5.2   <u>**The extracellular matrix**</u>

Endothelial cells line the blood vessels and all blood-contacting surfaces, acting as a barrier between circulation and the underlying tissue. First, it is important to note that valvular endothelial cells have a slightly different morphology and functional role than vascular endothelial cells due to their varying mechanical environments. In vivo, valvular endothelial cells are elongated, resembling ellipsoids, with the long axis of the cell parallel to underlying tissue's collagenous circumferential pattern and perpendicular to the direction of flow. Alternatively, vascular endothelial cells align parallel to the direction of flow. The alignment of both types of endothelial cells relies on changes within cytoskeletal actin filaments and the formation of stress fibers along the long axis of the cell when exposed to shear stress.[22]

The extracellular matrix (ECM) is an interconnected network of macromolecules that provides critical support for the endothelium. It is nature's biologic scaffold material for cells because it is created and excreted by the residing cells and is thus in homeostasis with the surrounding microenvironment. The ECM consists of structural proteins, specialized proteins, and glycosaminoglycans but its exact composition can vary depending on its location.[27] Proteoglycans form a gel-like "ground substance" where fibrous proteins are embedded. Polysaccharide gels help

resist compressive forces and allow for the permeability of necessary molecules. Collagen gives the ECM strength and organization and elastin provides resilience.

Fibronectin and laminin are adhesive glycoproteins part of the ECM and serve to attach cells to the ECM by binding the ECM components (such as collagen) to cell surface integrins. Fibronectin exists on the cell surface or in the extracellular space and consists of the sequence arginine-glycine-aspartic acid (RGD) that is known to serve as the specific binding site for cell surface integrins. Fibronectin is also known to have roles in cell growth, migration and differentiation.[28] Laminin is a major component in basement membranes (BM), aids in BM organization, and has been shown to enhance cell attachment and spreading.[29]
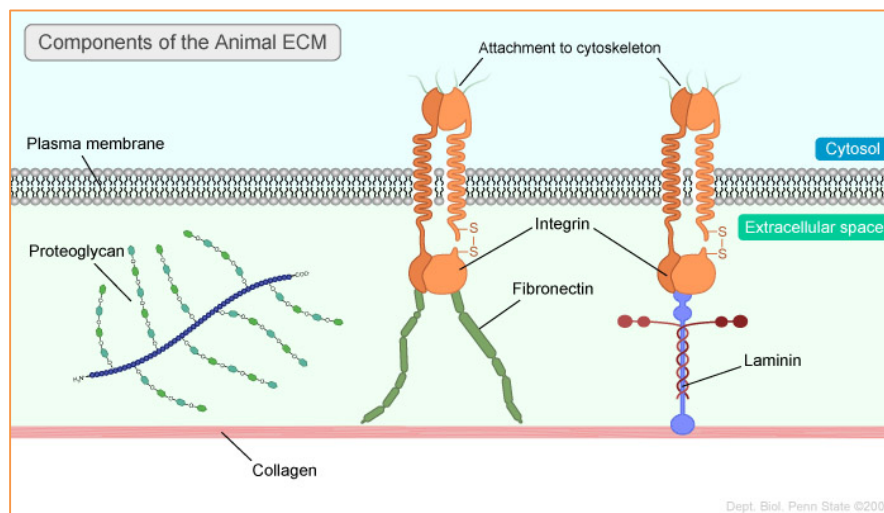
Figure 12: Adhesive proteins fibronectin and laminin create bridges between the ECM and neighboring cells by attaching the collagen in the ECM to the cell surface integrins.

The basement membrane is a specialized type of ECM that provides an attachment site for cells to adjacent tissues. The basement membrane consists mostly of type IV collagen, laminin, nidogen/entactin, and perlecan and plays an important role in cell growth and tissue remodeling. The type IV collagen and laminin form individual networks crucial for BM stability, and the nidogen/entactin and perlecan bridge the laminin and type IV collagen networks to create the basement membrane.[30, 31]

Specifically, valvular endothelial cell ECM is composed of an elastin-myofibroblast network on the ventricularis and circumferentially aligned collagenous bundles on the fibrosa.[22] One way the ECM provides structural and organizational stability for endothelial cells by allowing the EC binding to it. EC adhesion to the ECM is essential for EC proliferation, migration, morphogenesis, and viability. Additionally, this relationship helps to mediate cell-to-cell communications that could control cell apoptosis, proliferation, and cell morphology.[32]

5.3   Dysfunctional Endothelium

A healthy endothelium is important for vital functioning of the underlying tissue because of the many aforementioned duties of the endothelium. However, if the endothelium becomes dysfunctional it has the ability to induce a host of detrimental responses. An activated, or dysfunctional, endothelium can become thrombogenic, pro-inflammatory, alter proper interstitial cell functioning, and incapable of tissue remodeling.[15] Thus, endothelial dysfunction is considered a principal initiator in

valvular pathology. Increased oxidative stress is one of the major players in activating endothelial cells. Dysfunctional endothelium has increased nonspecific permeability and allows the uptake of coagulation factors that stimulate platelet adhesion and fibrin synthesis. Activated valvular endothelial cells also have an increased expression of adhesion receptors (VCAM-1, ICAM-1, and E-selectin) which recruits and allows invasion of monocytes and leukocytes. Invaded monocytes and leukocytes 1) differentiate into macrophages and attempt to engulf lipids and 2) secrete cytokines that stimulate smooth muscle cell differentiation.[33]

Chapter 5: Heart Valve Tissue-Engineering Approach

6.1   The Need for a Long-Term Solution

As aforementioned, current heart valve replacement options come with severe limitations. Mechanical heart valve replacements induce thrombosis and require patients to take a lifetime of anti-coagulation therapy while bioprosethic replacements suffer from mechanical degradation and must be replaced after 10 – 15 years. The ideal heart valve replacement would have the ability to self-repair, remodel, and allow for somatic growth. In order for a valve to have these features, it must be viable, or contain living cells. Creating a viable heart valve comes with an extensive list of objectives. In order to support cell growth, the scaffold must also not be cytotoxic or induce a chronic immune response. It should have the ability to fight off inflammatory cells to prevent chronic inflammation that can lead to valve degradation and calcification. However, if these challenges can be overcome, a viable heart valve replacement offering could provide a lifetime solution, increased patient life time, decrease cardiovascular related morbidity, and cut healthcare cost drastically.

6.2   The Tissue Engineering Process

Tissue-engineering is the process of producing therapeutic or diagnostic tools that use living cells or attracts endogenous cells to aid tissue formation or regeneration. Tissue-engineering (TE) is believed to be the future of organ and

tissue repair due the ability to tailor each aspect of the product's development to fit the needs of the patient. According to the tissue-engineering paradigm, a synthetic polymer or biologic material scaffold is chosen and then treated to improve the mechanical and/or biocompatibility properties. Either differentiated cells or undifferentiated stem cells are then added to the scaffold to create a viable product. The construct is placed in vitro environment that is metabolically and mechanically supportive to allow cell proliferation and extracellular matrix production. In vitro environments are often created by using bioreactors to stimulate the physiological conditions where the final product would be implemented. In vitro testing also includes using animal models to evaluate mechanical stability, immune response, and cellular interactions of the construct with the native environment. Next, the TE product is implanted in vivo in hopes it will recapitulate the normal functioning and of the native organ or tissue. [34] The diagram below show the tissue-engineering paradigm.
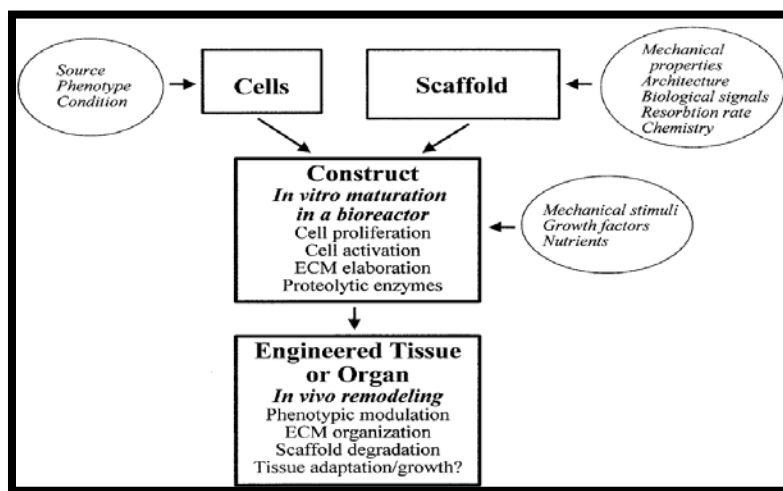
Figure 13: Tissue-engineering paradigm[34]

As aforementioned, a healthy endothelium is an imperative part of native tissues; therefore, a viable, TE solution would also require a functional monolayer of endothelial cells on the scaffold's surface to prevent immune rejection, mechanical degradation, and thrombus formation, as well as to ensure proper overall functionality of the tissue. However achieving a well-attached and complete monolayer of endothelial cells has been proven to be challenging in the field. An effective method to re-endothelialize acellular tissues has not yet been discovered or cited in literature. Without an adequate method for endothelialization, tissue-engineered constructs will not be successful in vivo. The following section reviews endothelialization methods currently being researched found in literature and their shortcomings.

6.3   Review of Current Research in Endothelialization Methods

6.3.1   Decellularization

Much research is being conducted to develop a tissue-engineered aortic valve by using decellularized xenogeneic scaffolds. Often these scaffolds consist of porcine or ovine valves or are made from bovine pericardium. Depending on the scaffold of choice and the endothelialization method, a method is chosen to remove all native cells and agents that could illicit an immune response after implantation. One decellularization method is to conduct a series of Trypsin/EDTA washes. In a study conducted by Cebotari et al, human aortic valves were taken from cadavers and decellularized with this method. The study reported removal of all cells and 98% of

the host DNA from the valve tissue while maintaining the fibrillar structure and collagen organization.[35] However, when the xenogeneic valve was tested in pigs in vivo, aneurysmal dilatation and elastin degradation of the matrix occurred which then elicited an immune response in the extracellular matrix. Another detergent decellularization method has been utilized in several studies, where a series of detergents, such as Trion and/or sodium deoxycholate, is used and followed by washes in enzymatic washes DNase and RNase to remove any remaining nuclear components. One study reported a cell-free structure across the thickness of the leaflet with native wavelike collagen formations intact. However, the tissue was loosened with widened interfibrillar spaces creating pores in the scaffold.[36] It is also reported that this method completely removes all cells and cellular remnants. [37]
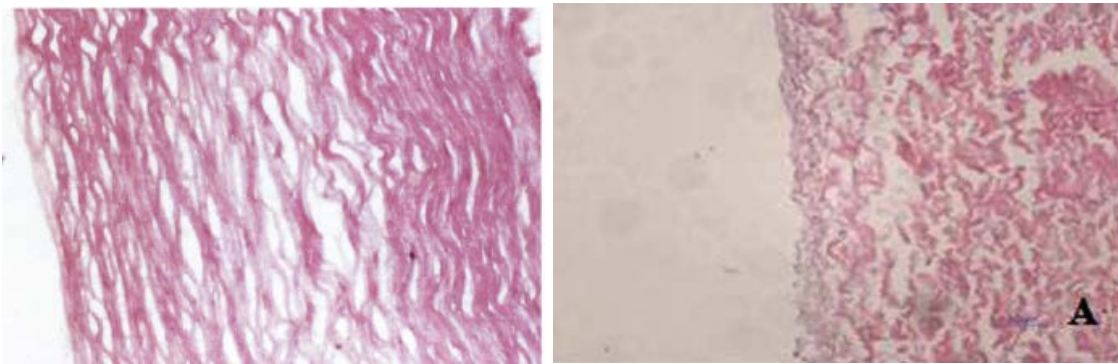
Figure 14: Left hand image shows a valve cusp decellularized with trypsin and has intact collagen organization but still shows some cells[35] while the right handed imaged was decelled with detergents and shows no cells but has a porous structure[37]

6.3.2 Scaffold Stabilization

Enzymes, detergents, spit freezing, and radiation used in decellularization techniques can impact the tissue preservation and the valve efficiency.[38] If the scaffold is deteriorated during the decell process, the scaffold is sometimes

stabilized with an agent that cross-links the tissue to enhance the mechanical properties after it has been decelled. Non-cross linked scaffolds may degrade too quickly and further threaten the functionality of the valve.[38] Cross-linking agents found it literature can consist of small concentrations of formaldehyde, dialdehyde starch, epoxy compounds, gluteraldehyde, or polyphenolic tannins.[39, 40] While aldehydes are resistant to collagenase degradation they are also cytotoxic and are resistant to cell infiltration so this method is not very common if the intent is to seed cells.[38, 40]

6.3.3   Cell Seeding

Cells are then seeded on the valve. Often know differentiated endothelial cells are used in endothelialization studies.[41] In the re-cellularization study by Yang et al, a saphenous vein vascular cell suspension was used and allowed to differentiate directionally to show the identities of endothelial cells, smooth muscle cells, and fibroblasts in vivo.[37] Some studies use stem cells that are more readily available but then must be differentiated into endothelial cells.[42] The density of cells used is can vary from study to study. Cebotari et al optimized their concentration to 2×10^5 per square centimeter of tissue[35] and Bader et al used 1×10^5 per square centimeter.[36] While Lichtenberg et al suggested a "suitable number of cells" was 1.2×10^7 cells per square centimeter.[41] Sometimes researchers utilize additional biologic materials found in the extracellular matrix as cell carriers such as collagen, elastin, hyaluronic acid, fibronectin, and fibrin to promote cell adhesion on the scaffold surface since the decellularized scaffold is often lacking a basement membrane.[38] After the

endothelial cells have been added to the valve in suspension, the valve is usually put in some type of rotational device or is manually rotated to allow the cells to cover all surfaces of the valves.[35, 41]

6.3.4 Fluid Flow Devices (Bioreactors)

The valves are generally rotated for 12 - 24 hours before placing the seeded valve in dynamic conditions. Generally a pulsatile flow loop is created by flowing culture medium through the valve at slow rates and then increased over time.[35, 41] By putting the cells in an environment that mimics the mechanical loading of the tissues in vivo, the cells are able to respond how they would in native conditions. The objective of these devices is to produce flow through the valve, apply pressure and shear stress, and to bend the valve. Two types of dynamic systems are used for in vitro tests – simple systems that induce stretching and bending of samples not in the shape of a valve and complex systems that induce physiological loading to a valve. Dynamic cultivation of cells has beneficial effects on vascular and valvular cells in forming a continuous layer of cells, differentiate cells, and fully mature the underlying tissues for improved mechanical properties.[38, 43]

6.3.5 Analysis of Endothelial Cells Attachment

When the endothelial cells are stimulated with the proper biochemical and mechanical signals, they spread out in the aforementioned "cobblestone" morphology, lay down a basement membrane, bind to the basement membrane via cell surface integrins, and bind to neighboring endothelial cells via cadherins. To prove the cells are expressing endothelial cell markers and functioning, the scaffolds

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are often tagged with anti-bodies for von Willebrand Factor and vascular

endothelial cadherins using immunohistochemistry or immunofluorescence.

Hematoxylin and eosin are often used to stain cross sections of the scaffold to

evaluate if the cells have formed a monolayer on the surface of the scaffold.

Additionally, scanning electron microscopy images are often taken to view the cell

morphology. After it has been determined the endothelial cells are present, well

attached, and functioning on the scaffold's surface the valve undergoes in vivo

testing.

However, successfully achieving a complete monolayer of endothelial cells is very

difficult. Below are results common in literature which attempts to endothelialize

tissues.   Figure 15 is from a study where the collagen type IV was used to coat a

decellularized porcine aortic valve and then it was seeded with endothelial cells.

The figure shows the presence of collagen type IV and some endothelial cells.

However the study reports that "endothelial cells were detected on the luminal

surface as an incomplete monolayer."[41]
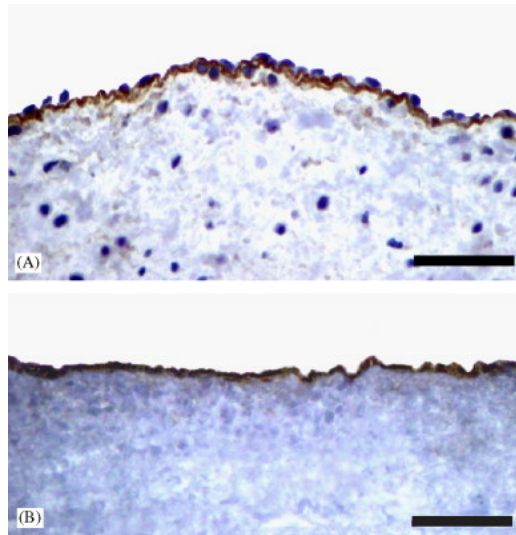
Figure 15: Immunohistochemistry for collagen type IV on native cusp (A) and re-endothelialized decellularized porcine valve cusp(B)[41]

Figure 16 is taken from a study that decellularized porcine aortic heart valves, treated the scaffolds with fibronectin and reseeded with marrow stromal cells. The figure shows the development of the 3 tissue layers shown in native cusp tissue after a 6 week incubation. However, the authors mention "the upper side of the leaflet was cellular and compact, whereas the lower ventricular side consisted of only loosened acellular matrix without cellular reconstitution."[43]
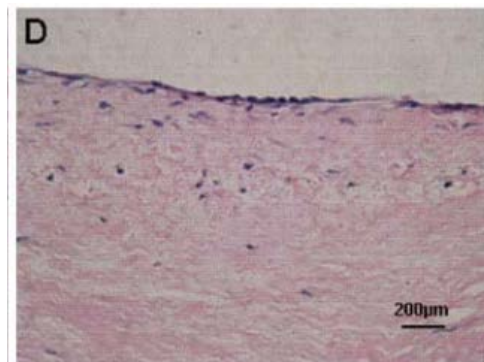
Figure 16: Hematoxylin and eosin stain showing the development of tissue layers and endothelial cells after 6 weeks of culture[43]

Another study showed scanning electron microscopy images of their bovine

pericardium valve after 30 days of in vivo implantation in mice. The cells are not

spread out in the "cobblestone" morphology meaning the endothelial cells are not

attached to the surface.
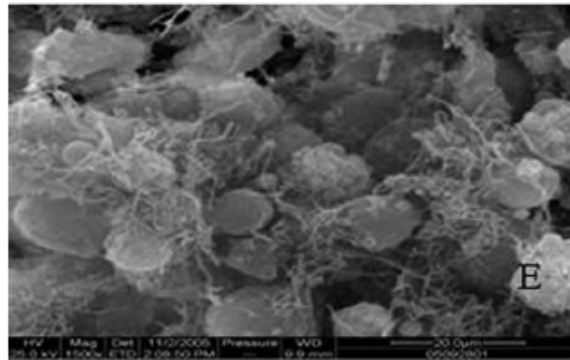
Figure 17: Scanning electron microscopy image of decellularized bovine pericardium reseeded with endothelial cells are implanted in vivo. Collagen fibers are intertwined with the cells and the morphology show spherical cells and not cell spreading.[37]

Chapter 6: Project Rationale and Aims

Current heart valve replacements can degrade or induce bleeding complications; therefore, there is a *need* for a permanent and effective solution for patients requiring heart valve replacements. As demonstrated in the chapter above, lots of tissue-engineering research has been done to create a viable heart valve replacement. There are combinations of methods used to decellulararize scaffolds, stabilize scaffolds, seed cells, and conduct in vitro testing. However, the ideal heart valve solution has not been created yet. The formation of an adequate endothelial layer still poses one of the largest problems in tissue-engineered solutions. The motivation behind my project is to achieve a layer of healthy endothelial cells for heart valve and other vasculature tissue-engineering applications where an endothelium is needed.

Specific aims of my project include:

- To achieve a confluent and uniform monolayer of endothelial cells on the scaffold's surface
- To optimize cellular attachment by the use of protein substrates and hydrogels
- To optimize cellular conditioning with dynamic seeding

Part 2: Research Methods, Results,

Discussion and Conclusion

Chapter 1: Materials and Methods

Summary Table of Studies

Study Name	Study Description and Purpose	Endpoints
Study 1 (EAF01-008) *Page 34*	Static seeding of TE valve with HADSCS and incremental rotation for analysis of cellular adhesion	H&E DAPI *(page 68)*
Study 2 (EAF01-008-2) *Page 41*	Static seeding of TE valve with HADSCS and incremental rotation followed by exposure to shear stress for analysis of cellular retention	DAPI *(page 70)*
Study 3 (EAF01-019) *Page 44*	Static seeding of pericardium samples with HCAECS and treated with adhesive protein substrates for increased cellular adhesion	H&E Live/Dead IHC *(page 71)*
Study 4 (EAF01-033) *Page 50*	Static seeding of tissue samples with HCAECS and treated with protein substrates and hydrogels for increased cellular adhesion	H&E Live/Dead *(page 75)*
Study 5 (EAF01-044) *Page 54*	Dynamic seeding of native valve with HCAECS and treated with protein substrates and fibrin glue for cellular adhesion analysis	Live/Dead IHC SEM DAPI *(page 80)*
Study 6 (EAF01-056) *Page 65*	Dynamic seeding of native valve with HAECS and treated with laminin and fibrin glue followed by in vitro testing for cellular retention analysis	DAPI *(page 87)*

1.1   Study 1: Static seeding of TE valve with incremental rotation in seeder for analysis of cellular adhesion

This study was designed and completed under the direction of Mike Jaeggli, a Ph.D candidate in the lab. The purpose of this study was to determine if a uniform monolayer of cells could be seeded on the surface of a tissue-engineered (TE) valve made of decelled and PGG fixed bovine pericardium.

1.1.1   Pericardium Decellularization

Fresh tissues were received, put on ice, and cleaned of excess fat and loose connective tissue. Tissues were placed in double-distilled water (ddH$_2$O) for 24 hours at 4°C for cell lysis. Tissues were then rinsed with fresh ddH$_2$O and transferred to a sterile bottle. All further steps were done with sterile solutions and using aseptic techniques. To remove cellular remnants, tissues were treated with one liter of a detergent solution consisting of 50 mM tris, 0.15% volume to volume ratio of triton x-100, 0.25% deoxycholic acid-sodium salt (DOC), 0.1% EDTA and 0.02% sodium azide for 3 days at room temperature on a shaker plate. After the 3 days, the detergent solution was replaced with fresh solution and the tissues were treated for an additional 3 days. Tissues were then washed in ddH$_2$O twice for 30 minutes and 70% ethanol twice for 30 minutes to remove the detergent solution. Tissues were then washed twice more with double-distilled water. Removal of residual nucleic acids was achieved by treatment for 24 hours at 37°C with 360 mUnits per mL of both deoxyribonuclease (DNase) and ribonuclease (RNase) dissolved in 1x Dulbecco's phosphate buffered saline (1xDPBS) containing 5 mM MgCl at a pH of 7.5. Tissues were washed twice with double-distilled water, 70% ethanol, and double-distilled water again. Tissues were stored at 4°C in 1XDPBS with 0.02% sodium azide until use.

1.1.2   Pericardium PGG Fixation & Neutralization

Pentagalloyl glucose (PGG) is an elastin cross-linker that is used to lightly fix

tissues in order to stabilize the tissue. 0.15% (PGG) solution was made by mixing

0.75g PGG dissolved in 100 mL of 100% isopropyl alcohol (IPA) and adding

dropwise to 400 mL of 50 mM Na_2hPO_4 pH balanced to 5.5. The solution was then

sterile filtered through a 0.2um filter and the pericardium was placed in the jar with

the solution and left to incubate on a shake plate at room temperature overnight.

The PGG solution was kept protected from light the entire time by covering each of

the solutions in aluminum foil. The next day the PGG solution was aspirated and the

pericardium was washed three times in 1XDPBS for 30 minutes each wash. The

tissue was then stored in 0.02% sodium azide until ready to use in order to prevent

bacterial growth. The day before using the fixed tissue, it was washed 3 times for 10

minutes in sterile 1x 1XDPBS on the shaker at room temperature and then

neutralized in 50:50 fetal bovine serum in Dulbecco's modified Eagle Medium plus

1% PSA antibiotics (Penicillin-Streptomycin-Amphotericin) overnight at room

temperature.

1.1.3   Mounting the Tissue into a Valve

The sterile, PGG fixed, and neutralized pericardium was cut into three leaflets

using a mold designed previously in the lab (Figure 13). The leaflets were then

sutured into one side of a novel 3D printed mount designed by another member in

the lab, Mike Jaeggli, and then crushed into the other side of the mount to mimic the

shape and dimensions of a modern bioprosthetic aortic valve (Figure 14). The

engineered valve were stored in a sterile jar of 1x 1XDPBS at 4°C.

Figure 18: 3D printed cusp mold shown on the left and the pericardium cusp after it had been cut out of the crush mount

Figure 19: Tissue engineered aortic valve from decellularized and PGG fixed bovine pericardium and sutured into novel mount

1.1.4   Endothelial Seeding of the Engineered Valve

The valve was pre-incubated in culture medium for 24 hours at 37°C. Using a

specially printed acrylic chamber, the valve was placed in the chamber aortic side

up and culture media (DMEM: HAM F12, 10% FBS, 1% PSA, 12.5 mL/L of media of

L-Glutamine) was poured into the chamber until it reached the top of the chamber. 3

mL of media were removed from the top of the valve. 3 million human adipose

derived stem cells (HADSCs) suspended in 1 mL of media were added to each of the

three cusps of the closed valve. The chamber was closed and carefully placed in the

incubator (aortic side up) at 37^{0}C for 6 hours. 3 million more HADSCs were pipetted

through the lumen of the valve suspended in 3 mL of media and introduced to the

ventricular sides of the valve and allowed to sit in a horizontal position so that cusp 1 was on the bottom side of the chamber in the incubator (position A). After 6 hours of incubation, 3 million HADSCs/3 mL were again added through the lumen of the valve and the valve was rotated so that the second cusp was allowed to sit on the bottom of the chamber (position B). After 6 hours of incubation in position B, 3 million HADSCs/3mL were again inserted through the lumen and the chamber was rotated so the final cusp was allowed to sit on the bottom side of the chamber and incubator for 6 hours (position C). A diagram of this procedure is in Figure 15 below.
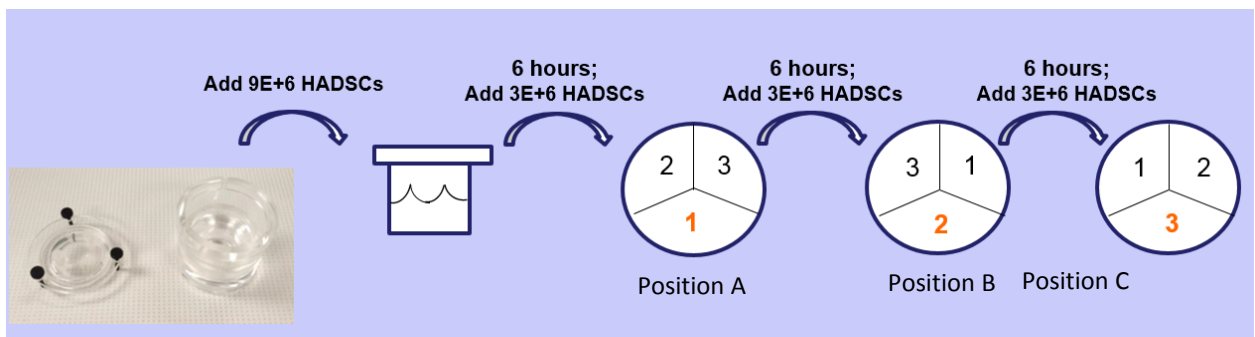
Figure 20: Valve endothelialization method in chamber and incrementally rotating chamber position

1.1.5 Staining and Imaging with DAPI

DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain used to image nuclei. Immediately after removing the valve from culture, the cusps were cut out of the mount and washed twice in prewarmed 1XDPBS. Two cusps were fixed in 10% phosphate buffered formalin and one was fixed in 4% paraformaldehyde (PFA). The cusp fixed in PFA was allowed to sit for 10 minutes at room temperature in the

fixative and then washed twice with 1XDPBS. In the meantime 100 ul of 1mg/mL

DAPI aliquot was added to 19.9 mL of 1XDPBS, vortexed, and covered in foil to

protect from light. The samples were then allowed to incubate in the DAPI solution

for 5 minutes at room temperature in the dark. The samples were rinsed twice with

1XDPBS and left in the 1XDPBS for imaging. The blue fluorescent filter was used to

image the section.

1.1.6 Staining and Imaging with Hematoxylin and Eosin

Histology allows observation of sample cross sections. The cross sections of

tissue baked on glass slides can then be stained with a variety of dies to label

cellular component of interest. A common histology stain is a combination of

hematoxylin and eosin. Hematoxylin stains nuclei dark purple and eosin stains

cytoplasm pink.

After being in the phosphate buffered formalin for 5 days, the cusps were taken

out of the fixative, first cut in symmetrical halves, and then cut vertically dividing

the cups half into thirds. Each section was placed in a processing cassette and run in

an automatic tissue processor to infiltrate the tissue with paraffin wax (Leica

Medium, T_m 56 - 58°C) according to the following preset protocol:

- 10% buffered formalin (1 min) at 37°C
- 10% buffered formalin (1 min) at 37°C
- 70% ethanol (15 min) at 37°C
- 80% ethanol (15 min) at 37°C
- 95% ethanol (15 min) at 37°C
- 95% ethanol (15 min) at 37°C
- 100% ethanol (15 min) at 37°C
- 100% ethanol (15 min) at 37°C
- Xylene (5 min) at 37°C
- Xylene (10 min) at 37°C
- Paraffin (15 min) at 60°C
- Paraffin (15 min) at 60°C
- Paraffin (15 min) at 60°C
- Paraffin (15 min) at 60°C

The cassettes were then removed from the processor and stored in a warming drawer with a thin layer of melted paraffin to prevent the samples from drying out. One week later the sections were removed from the drawer, embedded in blocks of hot paraffin, allowed to set on a cold plate set at -7°C and then placed in a small bucket of ice water. The cold blocks were sectioned at 5 um using an automatic microtome. The thin wax embedded sections were transferred into a shallow Pyrex dish of 44°C tap water carefully to prevent the sample from bunching and distorting the sample. Glass slides were inserted under the floating sections to retrieve the samples. The slides were put in a 60°C oven vertically and allowed to incubate overnight in order to melt the excess wax away from the section and leave only the tissue sample on the slide.

The next day the slides were removed from the oven, placed in a slide rack, and stained with fresh hematoxylin and eosin solutions (H&E). An established protocol

by the Clemson University Department of Bioengineering was used to deparaffinize in xylene, dehydrate in a series of ethanol dilutions, rehydrate in water, stain with hematoxylin and eosin, and re-dehydrate in ethanol dilutions and xylenes. The sections were taken out of the final xylene incubation and immediately coverslips were applied with paramount medium, allowed to dry, and then imaged using a light microscope.

1.2 <u>Study 2: Static seeding of TE valve with incremental rotation and exposure to shear stress for analysis of cellular retention</u>

This study was also designed and conducted under the guidance of Ph.D candidate, Mike Jaeggli as a continuation of previous experiment. Since an adequate layer of cells was effectively seeded on the valve's surface in study 1, the goal of this study was to determine if the cells would be retained when applied to shear stress.

1.2.1 Making of the Tissue-Engineered Valve

Bovine pericardium was decelled and fixed with half of the previously used concentration of PGG (0.075%) according to study 1 sections 1.1.1 and 1.1.2. The tissue was then sutured in the mount and crushed into the shape of a valve as described in section 1.1.3.

1.2.2 Cell Seeding

A slightly higher concentration of cells was used compared to study 1 but the exact same incremental rotating of the seeding chamber was used as described in

section 1.1.4. Additionally a different chamber was used to allow for better alignment of the seeding ports over the cusps. The valve fits ventricular side down in the new chamber just like in the previous chamber design; however, the new chamber has three ports on the top – one over the aortic side of each cusp – and one port on the bottom – for ventricular side seeding. These slight seeding changes are shown in the Figure 16. After the last seeding rotation in position C, the chamber was turned upside down (ventricular side up) and allowed to incubate for 24 hours before putting in the bioreactor.
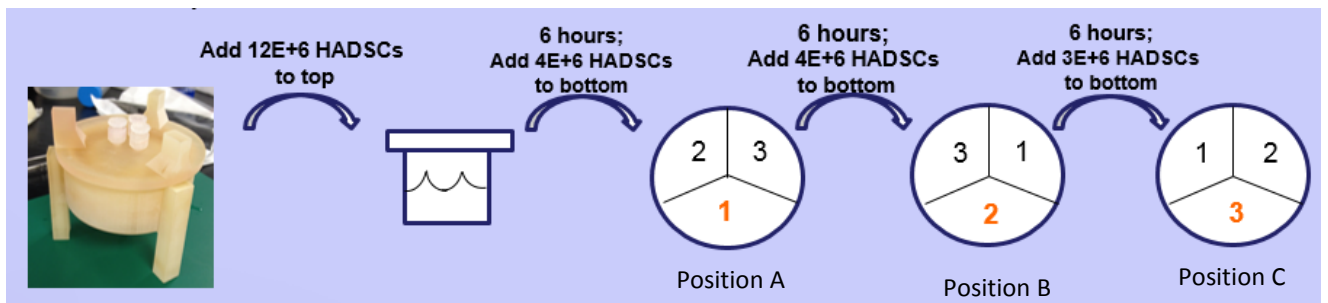
Figure 21: Study 2 endothelial cell seeding diagram

1.2.3   In Vitro Testing in the Heart Valve Bioreactor

After the valve was removed from the seeding chamber, it was placed in the heart valve bioreactor designed by Dr. Lee Sierad, a Ph.D. graduate and post-doctorate of the BTRL, and filled with culture media. The bioreactor is used to create physiological flow to study scaffolds in vitro by using bursts of air controlled by LabVIEW to force a silicone membrane (diaphragm) up and push media through the valve. Figure 18 and Figure 19 demonstrate the bioreactor how the membrane is used to open and close the valve. Volumetric flow rate, systolic pressure, beats per

minute, and diastolic pressure can be measured and adjusted and were done so according to the table below. After 24 hours in the bioreactor, it was noticed that the membrane was pierced and thus not functioning correctly and the valve was removed from the bioreactor and placed in 4% PFA and imaged with DAPI according to the protocol in study 1 (1.1.5).

Study 2 valve conditions created by the bioreactor

Beats per minute	21.4
Stroke volume (mL)	1.43
Systolic Pressure (mmHg)	12
Diastolic Pressure (mmHg)	4
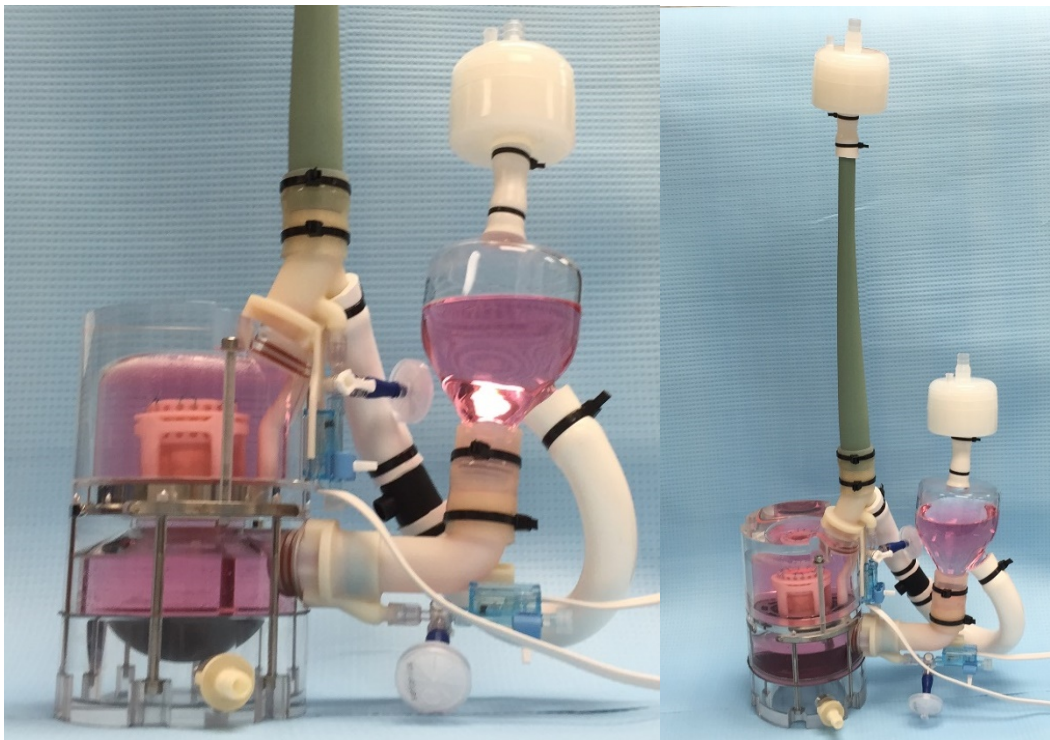
Figure 22: Heart valve bioreactor filled with culture media and focused on the valve mounted in the aortic chamber on the left and showing the entire system on the right.
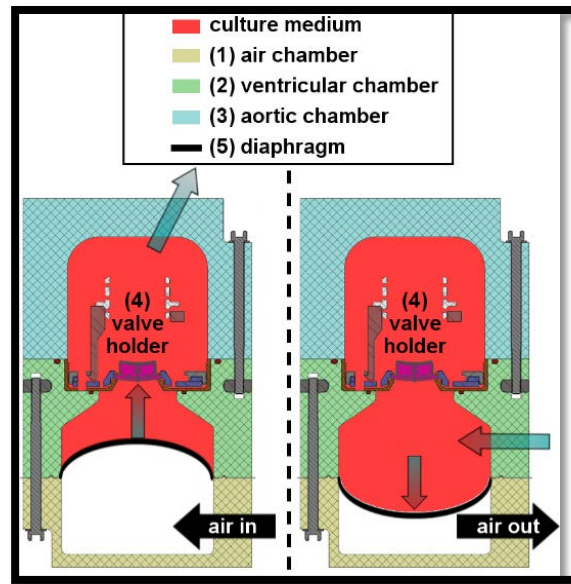
Figure 23: Heart valve bioreactor can create physiological flow by creating bursts of air that force a membrane up which in turn pushed culture media through the valve
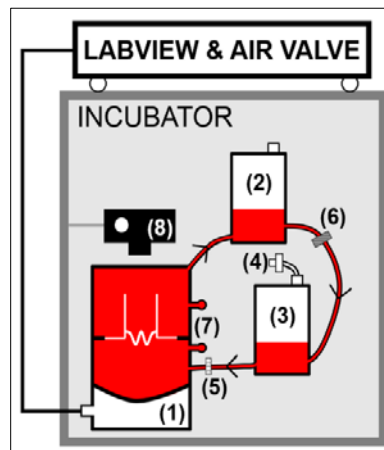
Figure 24: Heart valve bioreactor system composed of air chamber (1), media reservoirs (2,3) air filter (4), one-way check valves (5,6), pressure transducers (7) and a webcam (8).

1.3   Study 3: Static seeding of bovine pericardium tissue samples with endothelial cells and with added adhesive protein substrates for increased cellular adhesion

Since inadequate cellular retention was observed after the tissue-engineered

valve was statically seeded and put in the bioreactor in study 2, the objective of this

study was to determine if adhesive protein substrates could be used to increase the strength of cellular attachment on the scaffold's surface. Since endothelial cells have unique structural and behavioral characteristics in vivo, this study and the subsequent studies were completed with known endothelial cells to effectively analyze endothelial cell attachment and functioning.

1.3.1 Pericardium Sample Preparation

Bovine pericardium was decellularized, PGG fixed in 0.075% PGG, and neutralized according to the protocols in sections 1.1.1 and 1.1.2. The pericardium was then cut into 2.5 cm x 2.5 cm square samples and divided in a 6 well plate.

1.3.2 Coating with Fibronectin and Laminin Solutions

11 ug/mL of fibronectin (1 mg/mL from bovine plasma) in 1XDPBS was sterile filtered through a 0.2um filter. 5 ug/mL of laminin (1mg/mL from Engelbreth-Holm-Swarm mouse sarcolemma) in 1XDPBS was also filtered through a 0.2 um filter. All steps from this point forward were conducted under sterile conditions using aseptic technique and sterile solutions and tools. 1 mL of the laminin solution, 1 mL of the fibronectin solution, and 1 mL of warmed fetal bovine serum (FBS) for control was pipetted on the respectively labeled tissue samples. The well plate was sealed with Parafilm and the samples incubated in the solutions at room temperature overnight. The next day the solutions were aspirated and the samples were washed 3 times with sterile 1XDPBS.
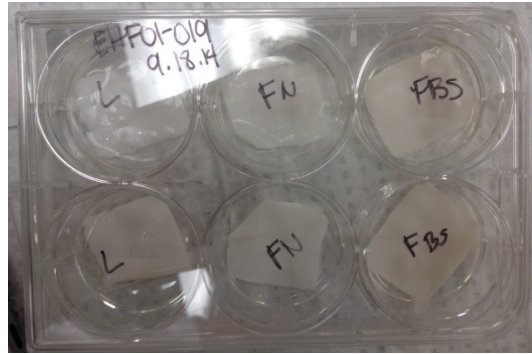
Figure 25: Six-well-plate layout of study where "L" marks the laminin samples, "FN" marks the fibronectin samples, and "FBS" marks the control samples

1.3.3 Block Nonspecific Binding on the Tissue Surface

0.02% bovine serum albumin (BSA) in 1XDPBS was prepared and sterile filtered into a conical tube. A beaker of ddH$_2$O was warmed to 45°C on a hot plate and the conical was placed in the hot water for 10 minutes. The 1XDPBS was aspirated from the wells and 1 mL of the heat-treated BSA was pipetted on the each of the samples and allowed to incubate for 2 hours at 37°C. The BSA solution was aspirated and tissues were rinsed with 1XDPBS 3 times.

1.3.4 Drop Seeding Tissue Samples with Endothelial Cells

Passage 5 human coronary artery endothelial cells (HCAECs) was trypsinized with 10 mL of trypsin-EDTA solution in a 5x dilution with prewarmed, sterile 1XDPBS for 5 minutes at 37°C (followed cell product protocol). The reaction was quenched with 10 mL of endothelial cell growth culture media from ATCC kit (Ascorbic acid, FBS, rh EGF, Heparin Sulfate, L-Glutamine, rh VEGF, rh FGF-b, rh IGF-1, Hydrocortisone in basal media). The flasks were rinsed with sterile 1XDPBS twice to remove the residual cells. The cell solution was spun down in a centrifuge at 200xg for 3 minutes. The supernatant was removed and the pellet was resuspended

in 3 mL of new prewarmed culture media. A scepter was used to count the cells and 2.94×10^3 cells were added to the center of each sample in a small volume of media (200uL) to minimize cell runoff. The well plate was placed in the 37^oC incubator and allowed to incubate for 8 hours. More media was then added to the wells until it covered the tissue by a 3-4 millimeters and the samples were allowed to incubate for another 24 hours in the incubator.

One laminin, one fibronectin, and one control sample was put in a new six well plate, rinsed twice with 1XDPBS, and fixed with 4% PFA for immunohistochemistry (IHC) and H&E staining. The other samples were analyzed with Live/Dead described in section 1.3.5 below.

1.3.5   Live/Dead Assay

Live/Dead assay is used to evaluate the viability or toxicity of cells. Calcein AM stains viable cells by fluorescing when uptaken and multiplied by esterase activity in the cytosol. EthD-1 fluoresces in dead cells because it is only able to penetrate the membranes in cells where the membrane is compromised and then stains the nucleic acids. 4mM Calcein AM and 2mM Ethidium homodimer-1 (EthD-1) from Molecular Probes were used. 6 ul of the EthD-1 was added to 3 mL 1XDPBS in a conical and vortexed. 1.5 ul of Calcein AM was added the conical and the solution was vortexed again.

The media was removed from the wells and the samples were rinsed with prewarmed 1XDPBS. One laminin, one fibronectin, and one control sample was placed in a new well plate with 1 mL of the prepared Live/Dead solution on each

sample. The well plate was then covered in foil to protect from light and put in the 37°C incubator for 20 minutes. The samples were placed on a glass slide (cell seeded side down) and viewed on a fluorescent microscope with a red filter to see any dead cells and a green filter to show the live cells.

1.3.6 Immunohistochemistry for Analysis of Laminin Presence

This immunohistochemistry (IHC) protocol was taken from a graduate from the lab, James Chow. Samples were sectioned onto glass slides on as described in the H&E histology in section 1.1.6. The slides were then deparaffinized in xylene and ethanol solutions and then rehydrated to water. For antigen retrieval, the Slides were put in 10 mM citric acid monohydrate pH balanced to 6.0 in a microwavable slide rack and coplin jar. The slides were microwaved on half power for 5 minutes and then the temperature was checked to see if it had reached 90-100°C and more citric acid solution was added if any had boiled over. The samples were microwaved for an additional 20 minutes, checking the temperature and refilling the coplin jar with citric acid solution if needed. The slides were then allowed to cool at room temperature for 1 – 2 hours. The samples were then rinsed in tris buffer saline (TBS) pH balanced to 7.5 twice for 5 minutes each rinse. A Kim wipe was then used to wipe excess buffer from the slides and a wax pen (Vector Immedge Pen) was used to individually circle the tissue sections on the slides. The wax was allowed to dry for 2-3 minutes before putting back in the TBS. For tissue permeabilization, the slides were rinsed twice in 0.025% triton for 5 minutes each rinse and then rinsed once in TBS for 5 minutes. To block non-specific binding, the sections were

incubated in 1.5% normal blocking serum (horse normal serum in TBS from
Vectastain Kit) for 45 minutes. The normal blocking serum was wicked well from
the slides and then the primary antibody [anti-laminin from Abcam, ab 11575, at a
concentration of 4ug/mL in TNB buffer (1mL blocking reagent solution from
Vector's Western Blot kit in 19 mL TBS)] was added to the positive sections and TBS
was applied to the negative control sections. The primary antibody was incubated
on the samples overnight at 4^oC in a self-made humidity chamber (slides were
placed over wet paper towels in a glass Pyrex dish and covered with cling wrap.
Following incubation, the slides were rinsed twice in TBS for 5 minutes each rinse.
To block the endogenous peroxidase, the slides were incubated in a solution of 0.3%
H_2O_2 and 0.3% normal horse serum in TBS for 30 minutes. The slides were then
rinsed in TBS for 5 minutes prior to applying the secondary biotinylated antibody
(30 ul normal horse blocking serum and 10 ul anti-rabbit in 2 mL TBS) for 30
minutes at room temperature. The slides were once again rinsed with TBS for 5
minutes and then incubated in ready to use Avidin-Biotin Complex (ABC) for 30
minutes at room temperature followed by rinsing twice in TBS for 5 minutes. The
antibody was carefully observed while developed with DAB substrate solution (5
mL water, 2 drops Buffer Stock Solution, 4 drops DAB solution from Vector, and 2
drops H_2O_2). The samples were carefully observed under a light microscope for a
brownish color to appear signifying the antibody has been developed. Once the
brown tint is observed, the reaction was immediately quenched with distilled water
(the anti-laminin took a full 5 minutes to develop). All slides were rinsed in tap

water 5 minutes and counter stained in diluted hematoxylin (50% hematoxylin and 50% water) for 45 seconds, rinsed in water, dehydrated in xylene and ethanol solutions, cleared with clearing agent, and cover-slipped with mounting medium. The slides were imaged under a light microscope.

1.4 **Study 4: Static seeding of bovine pericardium tissue samples with endothelial cells treated with fibronectin and laminin protein substrates and PureCol, Q.Gel, and Fibrin Glue hydrogels for increased cellular adhesion**

Since endothelial cell-to-cell communication and spreading out is an imperative part of the cells' functioning, study 4 aimed to increase cell attachment by providing an even surface that more closely mimics the in vivo extra-cellular matrix and basement membrane where native endothelial cells reside.

1.4.1 Sample Preparation and Experimental Design

Decelled, PGG fixed (0.075% PGG) bovine pericardium was prepared as described in previous methods. 3 different hydrogels – Q.Gel, PureCol, and fibrin glue – were prepared and applied to 12 mm pericardium punches in a 24 well plate as detailed in the following sections. Each combination of gel and substrate was repeated for analysis at 2 time points: 3 day and 7 day.
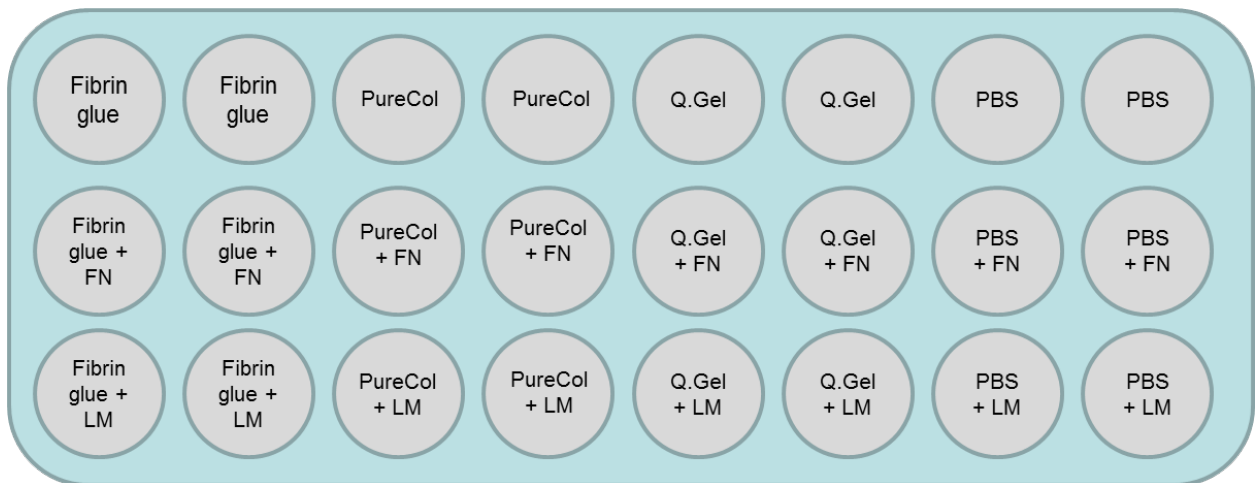
Figure 26: Experimental layout of combination of the 3 gels, 2 substrate solutions, and controls in a 24 well plate.

1.4.2 Scaffold Modification with Fibrin Glue

This protocol was adapted from an article by the Hoerstrup lab published in Biomaterials.[44] When fibrinogen (human from Sigma) and thrombin (65% clottable protein from human plasma from Sigma) are mixed at 37°C an enzymatic reaction occurs and an irreversible gel forms. 20 mg/mL fibrinogen was prepared by dissolving 40 mg fibrinogen in 1 mL 1XDPBS and sterile filtering the solution into a sterile conical and putting the solution on ice. 12.5 units/mL of thrombin was made by dissolving 250 ul of sterile filtered Dulbecco's Modified Eagle Media (DMEM) pH balanced to 6.3 in 25 unit thrombin (comes sterile in glass jar with rubber lid). The thrombin was then diluted to 12.5 units/mL by adding 750 ul of sterile filtered 2mM CaCl solution [222 mg CaCl in 100 mL HEPES buffered saline (HBS)] and put in ice. *Immediately* before putting the gel on the tissue, 150 ul of the thrombin solution was thoroughly mixed in 150 ul of the fibrinogen solution and quickly pipetted 50 ul

directly onto the respective pericardium samples. The samples were also incubated at 37°C for 1.5 hours. Excess gel, not on the tissue, was aspirated and the samples were rinsed twice with sterile 1XDPBS.

1.4.3   Scaffold Modification with PureCol

PureCol is 0.5% pure bovine type 1 collagen in DMEM/F-12 media and forms a gel when warmed to 37°C. Prepared PureCol solution (3.1mg/mL bovine type 1 collagen from Advanced Biomatrix) was diluted to 50 ug/mL in 1 mL of sterile filtered 0.01 M HCl solution in ddH$_2$O. 100 ul of the gel solution was added to the respective 12 mm punches of pericardium in the 24 well plate and allowed to incubate at 37°C for 1.5 hours. After incubation, any gel that had run off of the tissue was aspirated and the samples were rinsed twice in sterile 1XDPBS.

1.4.4   Scaffold Modification with Q.Gel

Q.Gel is a synthetic extracellular matrix that forms a gel when warmed to 37°C. 500 ul of gel was made by adding 400 ul of Buffer A (3D Matrix #2101) to the vial Q.Gel powder (3D Matrix #1001) prewarmed to room temperature and vortexing for 2 seconds. The buffer and the power come in sterile one time use containers, so there was no sterile filtering of this solution. 50 ul of the prepared gel was then pipetted on the respective tissue samples and incubated at 37°C for 1.5 hours. Any remaining gel on the bottom of the well was aspirated and the samples were rinsed twice with sterile 1XDPBS.
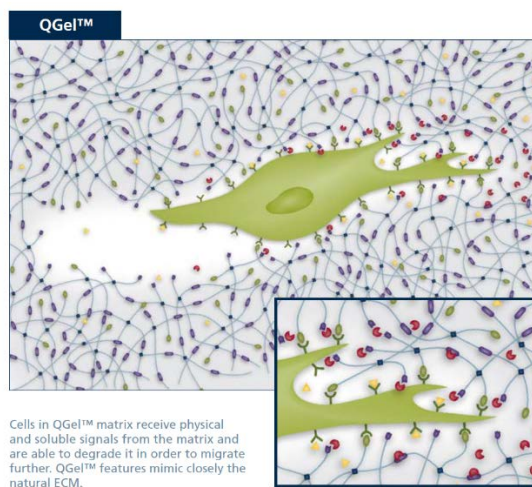
Figure 27: Q.Gel provides an environment that closely mimics the native environment for cells by providing physical and soluble signals.[45]

1.4.5 Scaffold Modification with Substrates and Cell Seeding

The respective samples were then incubated in 500 ul of 5 ug/mL laminin, 5 ug/mL fibronectin, or 1XDPBS for control for 24 hours as detailed in section 1.3.2. $1x10^5$ hCAECs suspended in 200 ul of endothelial culture media was drop seeded on the samples in each well as detailed in section 1.3.4. 500 ul of culture media was added to completely cover the samples 4 hours later.

1.4.6 Cellular Adhesion, Coverage, and Viability Analysis

After 3 days of incubation, one of each sample (12 samples total) was rinsed with 1XDPBS and cut in half. The media was changed on the remaining 12 samples reserved for analysis on day 7 of incubation. Of the samples cut in half, half was fixed in 10% phosphate buffered formalin for H&E and the other half was put in prepared Live/Dead solution. Live/Dead assay was followed as described in section 1.3.5. The fixed samples were put in processing cassettes after two weeks in 10% formalin in 4°C. Since the scaffolds were coated in hydrogels, they are subject to

deterioration in the automatic processor due to the harsh vacuuming paraffin

infiltration steps. Therefore the automatic processor was specially programmed and

run according to a protocol found in literature for processing and paraffin

embedding hydrogels opposed to the aforementioned processing protocol.[46]

Tissue scaffold with hydrogel surface modification processing protocol:

(Note: this protocol is only beneficial for the samples with hydrogels on their surface and may overly dehydrate unmodified samples.)
- 10% buffered formalin (1 min) at 37^{oC}
- 10% buffered formalin (1 min) at 37^{oC}
- 70% ethanol (60 min) at 37^{oC}
- 80% ethanol (60 min) at 37^{oC}
- 95% ethanol (60 min) at 37^{oC}
- 100% ethanol (60 min) at 37^{oC}
- 100% ethanol (60 min) at 37^{oC}
- 100% ethanol (60 min) at 37^{oC}
- Xylene (60 min) at 37^{oC}
- Xylene (60 min) at 37^{oC}
- Paraffin (15 min) at 58^{oC}
- Paraffin (15 min) at 58^{oC}
- Paraffin (15 min) at 58^{oC}

Paraffin embedding, sectioning, staining, and imaging was following as described in

section 1.1.6. The Live/Dead and histology steps were repeated for the 7 day time

point samples as well.

1.5 Study 5: Application of fibrin glue hydrogel and protein substrate surface modifications on decelled and harvested porcine native valves, dynamic seeding, and cellular adhesion and uniform coverage analysis

After finding positive results in study 4 for endothelial cell attachment and

spreading on the fibrin glue and protein substrate combination samples compared

to the Q.Gel, the PureCol, or the controls, this study attempted to apply this surface modification method to a valve. Another aim of this study was to determine if there is a conclusive preference for laminin or fibronectin.

1.5.1   Aortic Valve Harvesting and Decellularization by Immersion

Porcine hearts were donated from Snow Creek Meat Processing Facility in Seneca, SC. The hearts were put in ddH$_2$O over ice while dissection bins and tools were set up. The aortic valve was carefully harvested above the valve up to the sino-tubular junction, leaving a 5 – 1- mm of length on the two coronary arteries, and ensuring to retain a thin layer of septum muscle and membranous septum on the ventricular side of the valve. The retained muscle layer and membrane at the ventricular side of the valve was termed the "skirt". The figures below show the portion that was dissected in relation to the heart anatomy and the resultant valve.

Figure 28: The image on the left highlights the area of the aortic valve that was harvested. The middle image is the harvested valve showing the "skirt" at the bottom and aortic root at the top. The image on the right is the view from the ventricular side showing the "skirt".

The harvested valves were then decellularized following an established protocol previously developed in the lab by Lee Sierad. All washes and incubations were conducted on a shaker plate. First, the valves were subjected to a series of hypotonic ddH$_2$O washes. Native porcine cellular components and fragments were removed in a 0.05 M NaOH wash followed by a two day incubation in a decell solution consisting of a collection of detergents (50mM Tris, 0.25% SDS, 0.5% DOC, 0.5% Triton x100, 0.2% EDTA in ddH$_2$O and pH balanced to 7.4±0.05). The decell solution was then rinsed from the valves by undergoing a series of rinses and washes in ddH$_2$O changes, incubated in 0.02% sodium azide for 20 hours, rinsed in ddH$_2$O changes, washed in 70% ethanol changes, rinsed in ddH$_2$O changes, incubated in 0.02% sodium azide for 20 hours, and then incubated in 1XDPBS changes. The valves were then removed of DNA and RNA components by incubating for 2 days in 2 changes of prewarmed DNase/RNase solution at 37°C. The valves were then rinsed of the DNase/RNase solution and the removed nuclear components through a series of 1XDPBS rinses and incubation in 0.02% sodium azide for 24 hours. All following steps were conducted sterilely by using aseptic technique, sterile tools and bottles, and sterile filtered solutions. The valves were sterilized in a 2 hour incubation in 0.1% peracetic acid and then rinsed and incubated in a series of changes of 1XDPBS for 24 hours.

1.5.2 Harvested Valve PGG Fixation and Neutralization

In order to prevent leaflet folding or bunching, the leaflets were stuffed with sterile cotton balls soaked in sterile filtered 0.15% PGG solution. The cotton stuffed valves were put in a jar filled with 0.15% PGG solution, the jar was covered in foil to protect from light, and the valves were incubated in the solution for 21 hours on a shake plate. Following the PGG incubation, the cotton was removed and the valves were washed and incubated in sterile 1XDPBS changes for 24 hours. The PGG was neutralized by incubating the valves in 50:50 DMEM/FBS plus 1% PSA on the shake plate overnight.

1.5.3 Harvested Valve Surface Modification with Fibrin Glue and Substrates

The valves were prepared to fit in the seeding chamber by inserting them in mounting rings designed by Lee Sierad. This preparation was done prior to surface medication treatment so the surface treatment would not be disrupted afterward. To mount the valves, the "skirt" was crushed between two 3D printed rings. To fully crush the tissue between the rings and hold the skirt securely in place, the 3D printed rings with the crushed tissue were placed between to larger metal rings with a heavy duty spring and metal rings were threaded together. For cell seeding it is desired to have the cusps and inside of the valve completely closed off so a small concentrated volume of cell solution can be retained in the area of interest. In order to create this seal, the coronary arteries were sutured shut.

The valves are now ready for surface modifications. The fibrin glue was prepared as detailed in section 1.4.2. In order for the gel to form on the tissue only and not

immediately upon mixing the thrombin with the fibrinogen, the thrombin and fibrinogen mixture was made at a ratio of 1:5 thrombin to fibrinogen to reduce gelation time and the mixture was kept on ice. Using a paintbrush for even coverage of gel on the tissue, the gel was spread on the prewarmed valves so the fibrin would gel when in contact with the tissue. The paintbrush was dipped in ice water between applications so a clump of gel would not form on the brush. The gel was applied on both sides of the cusps, and the sinuses in 3 applications allowing 3 – 5 minutes between applications for the gel to preset. The valves were put back in the oven at 40^oC for 1.5 hours to allow the gel to fully polymerize.

After gel polymerization, 2 of the valves were incubated in 5ug/mL of laminin solution and the other 2 valves were incubated in 5ug/mL of fibronectin solution for 48 hours at 37^oC (solutions prepared as described in section 1.3.2). To completely seal the interior of the valve, a clear silicone membrane was sutured to the bottom of the aortic root (this step was not be performed when the valve was mounted in the rings and the coronaries were sutured because it blocks access to the ventricular side of the leaflets which was necessary when painting on the fibrin glue). The culmination of these preparations and modifications to the valves are shown in the figure below.
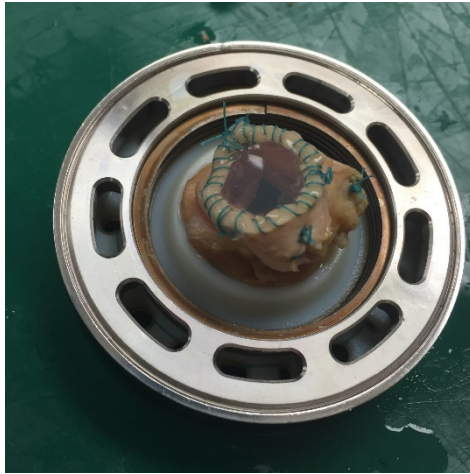
Figure 29: Harvested aortic valve mounted in rings, coronary arteries sutured, and silicone membrane sutured to seal off the aortic side of the valve.

1.5.4 Endothelial Cell Seeding of Harvested Valve in Dynamic Seeder

The prepared and mounted valve was placed in an acrylic seeding chamber. The mounting rings were specially designed to fit snuggly and rest on a lip at the top of the seeding chamber so the valve is secured in place with the aortic root protruding into the center of the chamber and the ventricular side of the valve facing upwards. After the valve was put in place in the chamber, 23×10^6 of passage 8 hCAECs were suspended in 40 mL of culture media and 10 mL of the cell suspension was pipetted into each of the valves. The suspension was carefully observed to ensure it was staying in the interior of the valve and not leaking into the bottom of the chamber. Additional culture media was used to fill the valve until the media reached the top of the cusps. To create a tight seal between the lid and the chamber, an O-ring was placed around 3D printed lids (Designed by Lee Sierad) and pressed into the acrylic chamber. For sterile media exchange, 2 claves (show in blue in Figure 31) were attached to the ports extending from the lid via luer-loc mechanism. To fill the small

remaining volume between the lid and the top of the valve, a syringe filled with

culture media was attached to one of the claves and a male luer was attached to the

other to allow air to exit as the chamber is filled with media. The media was injected

into the chamber via the syringe until excess media came out of the adjacent open

clave.
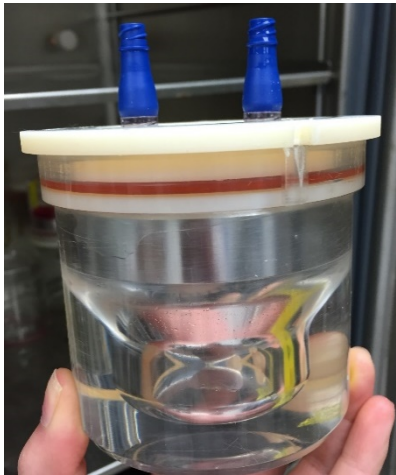
Figure 30: Seeding chamber with ring mounted valve inside and the 3D printed lid attached and claves (blue) attached for media exchange.
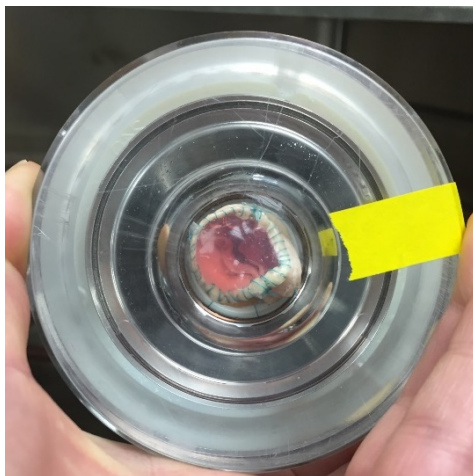
Figure 31: View from the bottom of the seeder of the sealed off valve filled with cell solution.

In order to achieve uniform cell coverage on the all surfaces of the valve which

has an intricate anatomy, a device was used that continuously rotates the chamber.

This device was developed by a previous student in the lab, Richard Pascal, and was

termed "the rotator". The rotator consists of a metal frame that connects to a

stepper motor. The rotator was wired to a data acquisition system (DAQ) that is

controlled by LabVIEW so it can be programmed to rotate at a set rotations per

minute value (rpm) and for set increments of time. The rotator secures into a shake

plate, but note the shake plate was not turned on for these studies. After the valves

were filled with the cell solution, the chambers were secured in the rotator's metal

frame and set to rotate axially with respect to the lumen of the valve according the

following protocol in Table 1 below at 37°C. To ensure each cusp has adequate

chance of having cells attach, the rotating protocol was set to allow each of the 3

cusps to incubate on the bottom side of the chamber during the static steps. Steps 3

– 7 were repeated for an additional 8 hours. At this time, the chambers were taken

out of the rotator, the media was changed on the valves, and they were then placed

in the incubator with the lids down to prevent media from leaking through the

silicone membrane on the aortic side. The valves incubated in this position for 2

days.

Table 1: Series of rotations and static incubations for uniform endothelial cell coverage

Step	Rotations/min	Time	Stop Position
1	2 rpm	2 hours	Cusp 1 down: 0°
2	0 rpm	50 min	---
3	2 rpm	10 min	Cusp 2 down: 120°
4	0 rpm	50 min	---
5	2 rpm	10 min	Cusp 3 down: 240°
6	0 rpm	50 min	---
7	2 rpm	10 min	Cusp 1 down: 0°

Figure 32: Rotator system consists of the metal frame where the seeding chambers can be secured and is connected to a stepper motor that is controlled by LabView. The system resides in the 37C incubator for cell viability.
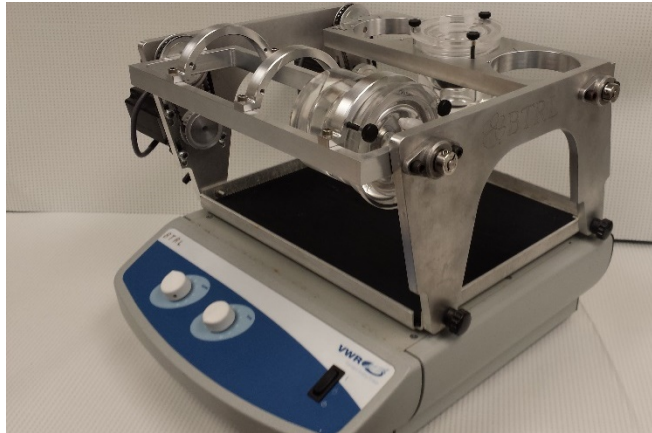
Figure 33: The metal frame of the rotator is secured to a shake plate. There are 2 different attachments for the chambers to connet in to depending on the desired rotation orientation (axially or end-over-end). Both attachements are shown.

1.5.5 Cellular Adhesion, Coverage, and Viability Analysis

After the 2 day static incubation, the valves were removed from the chambers and each of the cups as well samples from the sinuses were dissected from the valves. One cusp from the fibronectin samples and one cusp from the laminin samples were imaged with Live/Dead (section 1.3.5) to analyze cell viability and cell spreading. One set of fibronectin and laminin cusps was fixed in Karnovsky's fixative for scanning electron microscopy to see the cell morphology (described in section 1.5.6 below). One set was fixed in 4% PFA and then imaged with DAPI to see the distribution of nuclei (section 1.1.5). Another set was fixed in 4% PFA for H&E staining to analyze the distribution of the cells across the scaffold surface using the modified processing protocol for hydrogels (section 1.4.6). IHC was also done to analyze the functioning and expression of the endothelial cells (section 1.3.6).

For IHC, the sections were stained for endothelial cell marks CD31 (cluster of differentiation 31), vWF (von Willebrand Factor), and VE-cadherin (vascular

endothelial cadherin). The previously described IHC protocol was used. Anti-CD31 (rabbit) was used at a concentration of 1:50, anti-vWF was used at 4 ug/mL (rabbit), and VE-cadherin (rabbit) was used at a concentration of 1:50.

1.5.6 Statistical Analysis of Fibronectin and Laminin Treatments Using ImageJ

Using the cusps stained with DAPI, both the inflow and the outflow sides of the cusps (1 from each of the 4 valves) were fluorescently imaged from corner to corner, ensuring cells were not included in duplicate frames. The number of images taken totaled to 272. The images were then imported into ImageJ software. In ImageJ the images were converted to 8-bit to increase the contrast between the fluorescing cells and the background. The threshold was then adjusted until the program highlighted each individual cell as accurately as possible. The 8-bit conversion and threshold settings were then applied to every image and the "analyze particles" function was used to count the number of cells were detected in each image and record the data in a spreadsheet. A student's t-test ($\alpha=0.05$) was used to analyze the differences between the sides and treatments of the cusps.

1.5.7 Scanning Electron Microscopy Sample Preparation and Imaging

Scanning electron microscopy was used to image the surface morphology. Following the removal of the seeded valves from the chambers, one cusp from the fibronectin sample and one cusp from the laminin sample were carefully cut away from the valve and stored in Karnovsky's fixative (2.5% gluteraldehyde, 2% formaldehyde in 0.1 cacodylate, pH=7.4). Prior to SEM analysis samples were

dehydrated in a series of ethanol dilutions. This was followed up by a critical point

drying step using Hexamethyldisilazane (HMDS). Once fully dehydrated, samples

were mounted, platinum sputter coated in a Hummer 6.2 Sputter Coater shown in

Figure 29 at the Clemson AMRL facility and imaged using the Hitachi S-4800

microscope.

Figure 34: Anatech USA Hummer 6.2 Sputter Coater

1.6 **Study 6: Application of fibrin glue hydrogel and laminin substrate solution surface modifications on decelled and harvested porcine native valves, dynamic seeding, and in vitro testing for cellular retention analysis**

After concluding that the combination of laminin and fibrin glue resulted in the

best cell attachment in study 5, this final study was conducted to analyze the surface

modification method for cellular retention by exposing an endothelialized valve to

physiological shear stress in the bioreactor.

1.6.1 Valve Preparation, Surface Modification, and Endothelial Seeding

Valves were harvested, decellularized, fixed in 0.15% PGG, neutralized, crushed into a mounting ring, coated with fibrin glue, and incubated in 5ug/mL of laminin solution as detailed in study 5. The valve was seeded in the rotator with 5.5×10^6 passage 4 human aortic endothelial cells (hAECs) and rotated according to the protocol in Table 2. Steps 9 – 10 were repeated for 24 hours.

Table 2: Rotating protocol for endothelial seeding in study 6

Step	Rotations/min	Time	Stop Position
1	2 rpm	2 hours	Cusp 1 down: 0°
2	0 rpm	50 min	---
3	2 rpm	10 min	Cusp 2 down: 120°
4	0 rpm	50 min	---
5	2 rpm	10 min	Cusp 3 down: 240°
6	0 rpm	50 min	---
7	2 rpm	10 min	Cusp 1 down: 0°
8	0 rpm	50 min	---
9	2 rpm	2 hours	Cusp 2 down: 120°
10	0 rpm	50 min	---

1.6.2 In Vitro Testing in the Heart Valve Bioreactor

After the valve was removed from the seeder, it was placed in the heart valve bioreactor design previously described. The flow rate and pressures were gradually increased over 5 days to reach physiological pressures. The ramp up protocol is described in Table 3.

Table 3: Ramp up protocol over 5 days for seeded valve in the bioreactor

Duration	Systolic/Diastolic Pressure (mmHg)	Stroke Volume (mL)
26 hours	5/2	(not measured)
16 hours	7/3	(not measured)
10 hours	10/5	33
14 hours	13/7.5	38
9 hours	15/12	46
16 hours	17.5/12.5	49
10 min	20/13.5	55

1.6.3   Cellular Adhesion, Coverage, and Viability Analysis

After reaching physiological pressure for 10 minutes, the valve was removed from the bioreactor and the cusps were dissected. One cusp was fixed in 4% PFA and then stained and imaged with DAPI. Another cusp was stained and imaged with Live/Dead. The last cusp was reserved for cryo-sectioning and H&E staining since cryo-sectioning is gentler on tissues and is more likely to preserve the hydrogel. This sample was fixed in 4% PFA for 3 hours, incubated in 15% sucrose solution in 1XDPBS and then a 30% sucrose solution to prevent ice artifact formation in the samples when they are frozen. The sample was then gradually infiltrated with cyro-section freezing medium (OCT) by soaking in 1XDPBS, 50:50 1XDPBS/OCT, and finally 100% OCT. The infiltrated sample was then frozen at -20°C in a cryo-block embedded in OCT. The embedded cusp was sectioned in 10 um sections using a cyro-section microtome and placed directly on a glass side. The slides were put in freezer (-20°C) before staining with H&E following a shortened staining protocol. After staining the slides were coverslipped and imaged using a light microscope.

Chapter 2: Experimental Results and Discussion

2.1   Study 1: Static seeding of TE valve with incremental rotation in seeder for analysis of cellular adhesion

The results of study 1 showed that sufficient cellular coverage can be achieved on a tissue-engineered valve made of PGG fixed bovine pericardium and seeded with human adipose derived stem cells. From the DAPI images shown in Figure 30, cells were able to attach to the pericardium and create complete coverage on the surface. From the H&E stained cross sections in Figure 31, a monolayer of cells can be seen on the scaffold's surface. The H&E sections, taken at 3 different locations of the cusp, also shows cell attachment was present along the entire surface of the cusp. These images do reveal, however, cell attachment was greater at the center of the cusp compared the outer edge/corner of the cusp. The DAPI images and H&E images were taken from 2 different cusps which additionally implies all three of the cusps were adequately covered with cells. It was a concern that cells would prefer and settle on the surfaces at the bottom of the seeder which is why the valve was rotated every 6 hours along with the addition of new cells. Altering the position of the valve seemed to allow a more uniform cellular distribution. No empty spots were found on the valve cusps.
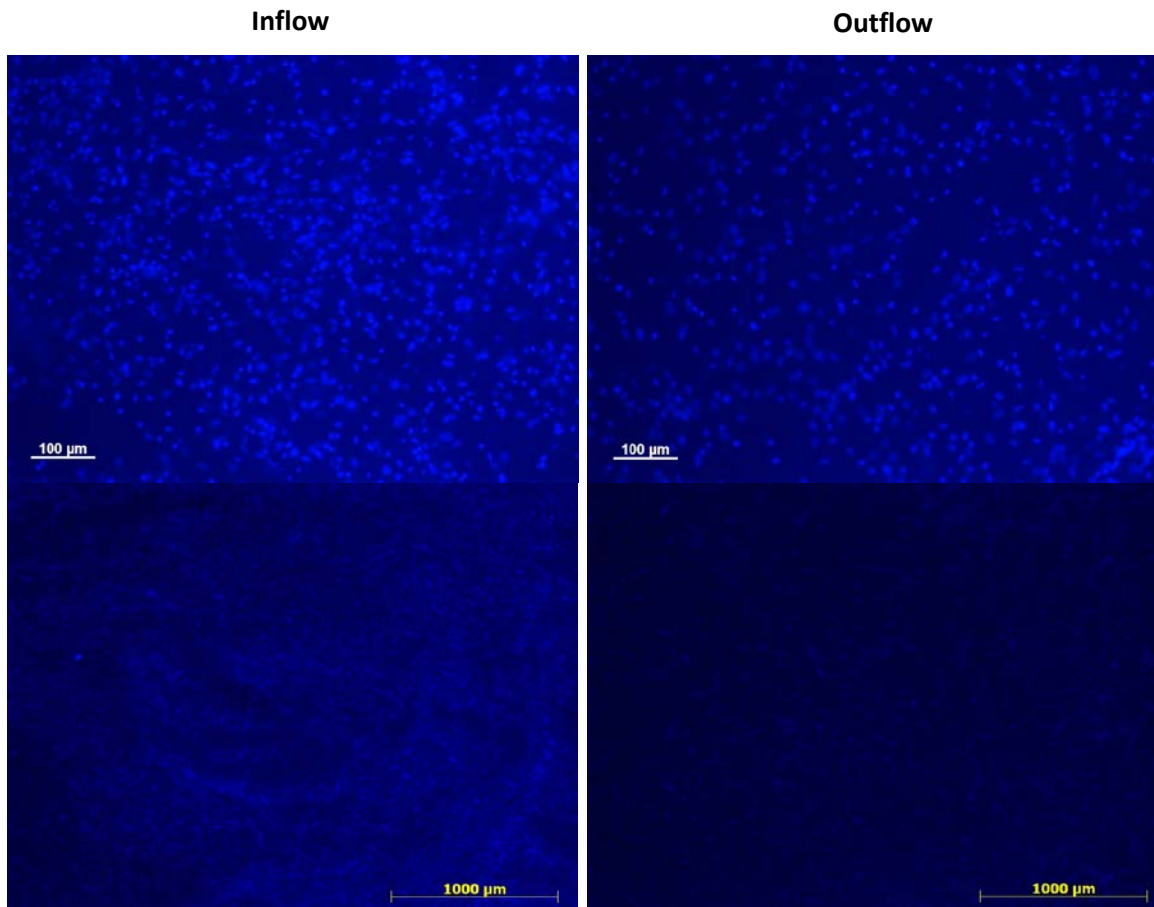
Inflow

Outflow

Figure 35: Nuclear distribution on the inflow (left) and outflow (right) surfaces of TE valve statically seeded and stained with DAPI. Taken at 10x (top) and 2.5x (bottom).
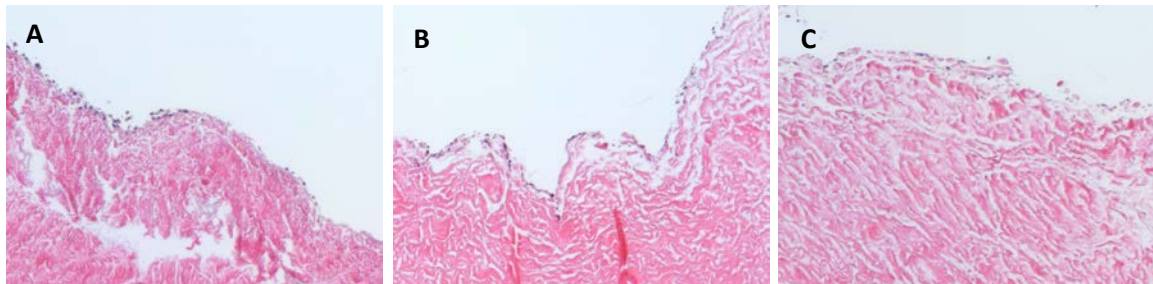
Figure 36: H&E stain showing seeded HADSCs on the surface of TE valve cusp cross sections taken from the center of the leaflet (A), one-third of the way through the leaflet (B), and the outer edge of the leaflet (C).

2.2 Study 2: Static seeding of TE valve with incremental rotation and exposure to shear stress for analysis of cellular retention

Compared to study 1, half concentration of PGG was used in study 2 to increase the pliability of the pericardium. The pericardium fixed in 0.15% PGG in the first study was very thick and difficult to suture into the mount. Since PGG is a cross-linker, decreasing the concentration, decreases the rigidity of the tissue. The seeding method was repeated with an even higher concentration of cells, so it is assumed there was a sufficient layer of cells on the valve's surface prior to putting the valve in the bioreactor. However after the valve was put in the bioreactor and exposed to very low pressures (12mmgHg/4mmHg) and low stroke volume (1.43 mL), the cells were no longer able to remain attached to the pericardium's surface. Figure 32 shows the pericardium surface after removal from the bioreactor and imaged with DAPI. Almost no cells can be seen. The shear stress was too great for the HADSCs statically incubated on pericardium for 24 hours. It is thus implied the cells were not adequately attached to the surface prior to in vitro testing. Inadequate cellular adhesion and retention could be attributed to the fact the cells used were not differentiated endothelial cells. Native endothelial cells create a strong network and communicate with each other and the underlying cells and their surrounding environment. In vivo, endothelial cells produce and reside in a rich layer of extra-cellular matrix which provides mechanical support and attachment sites. The endothelial cell's relationship with its surroundings could be associated with the cell's ability to adhere to the basal lamina. Without the presence of this extracellular

matrix and the differentiated endothelial cells, it is possible the cells were not able

to establish adequate attachment to the pericardium.
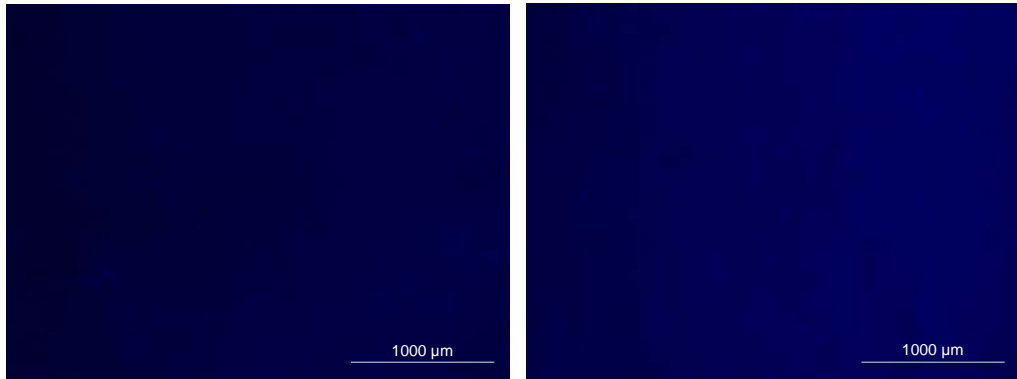
Figure 37: Nuclei distribution on the surface of inflow (left) and outflow (right) sides of a cusp taken from seeded TE valve after in vitro testing and stained with DAPI. Taken at 2.5x.

2.3 Study 3: Static seeding of bovine pericardium tissue samples with endothelial cells and with added adhesive protein substrates for increased cellular adhesion

After finding insufficient cellular attachment and retention in study 2 using

HADSCs seeded on pericardium, this study used known endothelial cells drop

seeded on pericardium incubated in adhesive protein solutions, fibronectin and

laminin. These protein substrates are found in native extra-cellular matrix and

provide as attachment sites for endothelial cells to the basal lamina. Fetal bovine

serum (FBS) was used as a control, as this is a component added in normal culture

media so all samples were exposed to FBS after cells were seeded and media was

added. In the Calcein AM fluorescently labeled images in Figure 33, the 2.5x images

show in conclusive results, as all sample appeared to have adequate coverage. In the

10x images, it is seen that the cells seeded on the pericardium treated with laminin

(A), display more spreading out compared to the other samples. Cell spreading out

is a sign the cells are interacting with the environment and neighboring cells rather than staying in a spherical balls.

The cross sections analyzed with H&E, does not show conclusive results as very few cells (nuclei are stained dark purple), appear in any of the samples. This could be attributed to the presence of only a few number of cells therefore when 0.05 um cross sections were taken, not many cells were captured in the sections. Some cells could have also been removed in the tissue processor which is rough on samples as it infiltrates them with paraffin. The surface of the pericardium is very uneven as seen in Figure 34. The pericardium scaffold has a "cotton-candy" topography which may have prevented adequate attachment and creation of the endothelial cell network amongst the neighboring cells.

IHC was also done to determine the presence of the adhesive protein substrates after culturing cells on the treated scaffolds. The immunohistchemical staining with anti-laminin on both the control sample (FBS) and the laminin treated sample revealed positive stain for laminin presence in both samples. The staining showed laminin was present within the samples, and slightly present on the surface, meaning either the laminin solution was able to travel within the pericardium or laminin existed within the scaffold even prior to treatment.
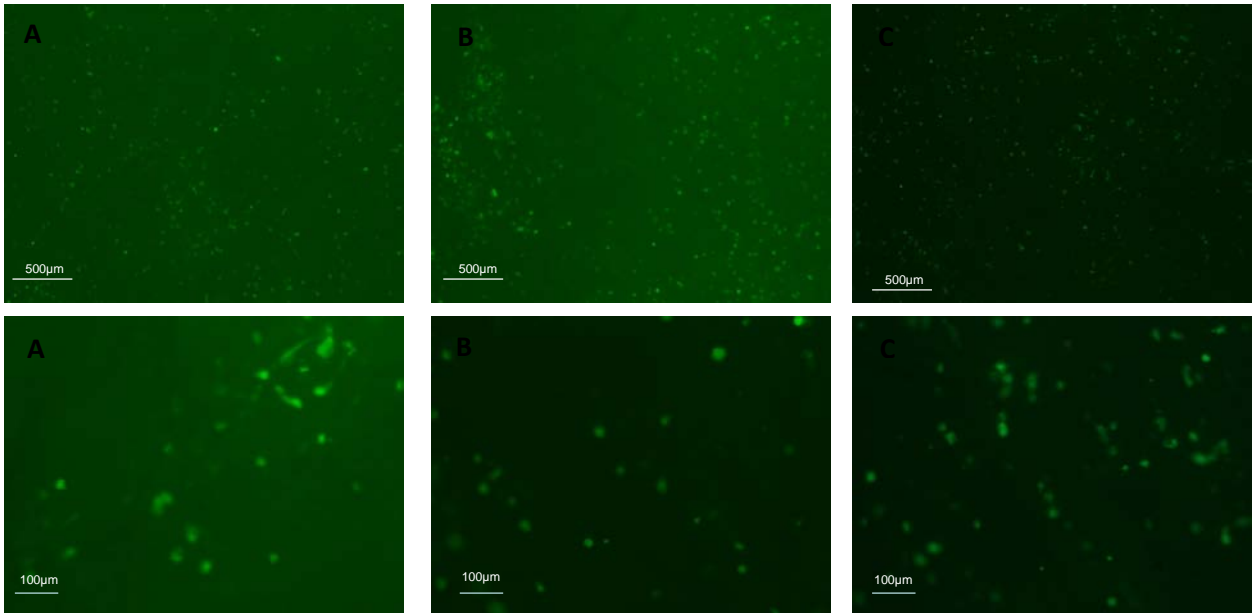
Figure 38: Calcein AM stained pericardium scaffolds treated with laminin (A), fibronectin (B), and FBS for control (C) showing endothelial cell distribution and spreading out on the surface. Top row imaged at 2.5x and the bottom row at 10x.
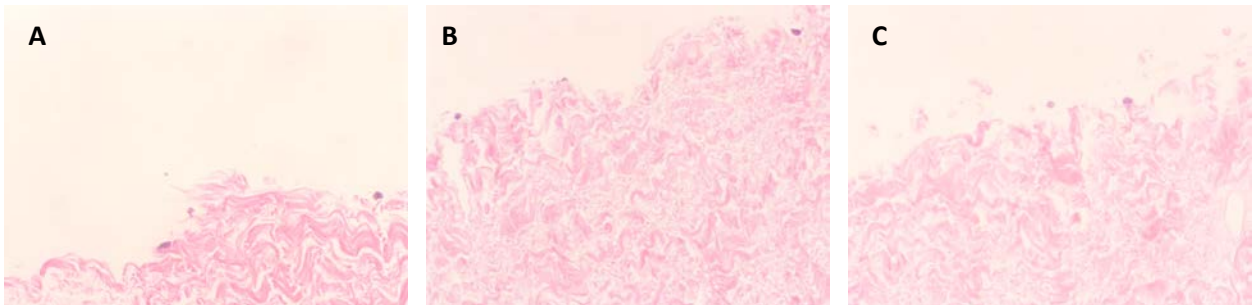
Figure 39: Cell distribution on cross sections of pericardium scaffolds treated with laminin (A), fibronectin (B), and control sample incubated in FBS (C) and seeded with endothelial cells and taken at 10x.
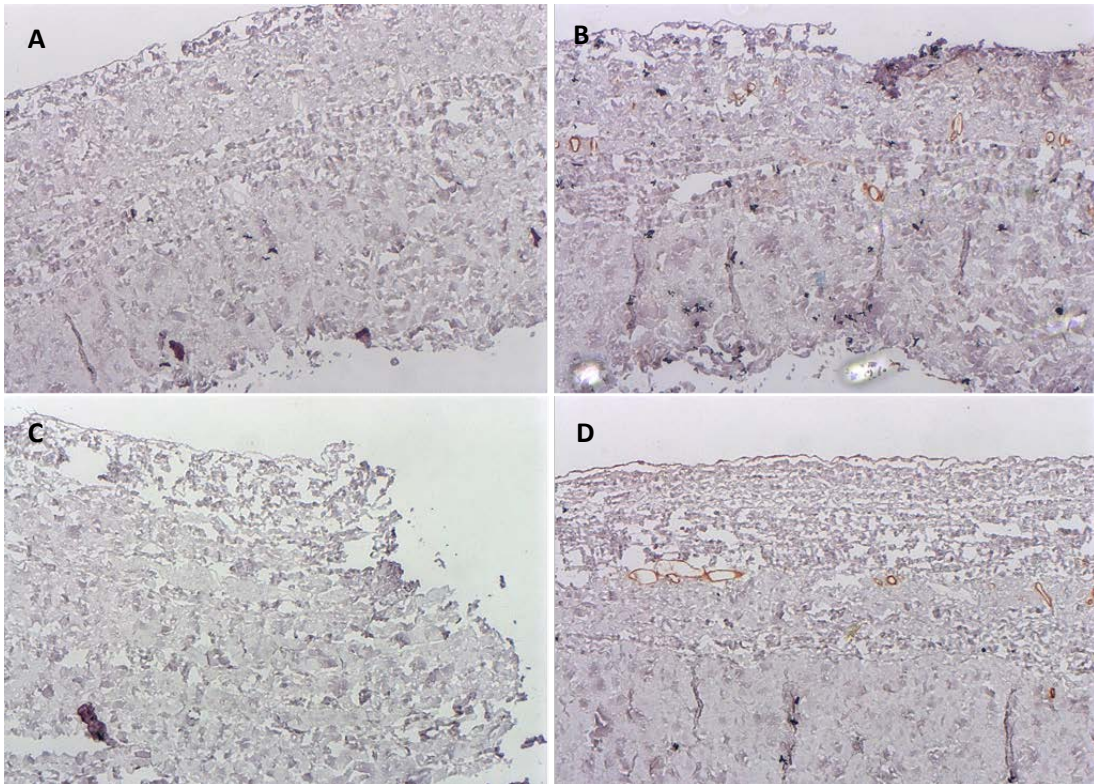
Figure 40: staining with anti-laminin showing the presence of laminin in cross sections of scaffolds treated with laminin and FBS for control. (A) FBS negative control, (B) FBS sample tagged with anti-laminin, (C) laminin sample negative control, and (D) laminin.
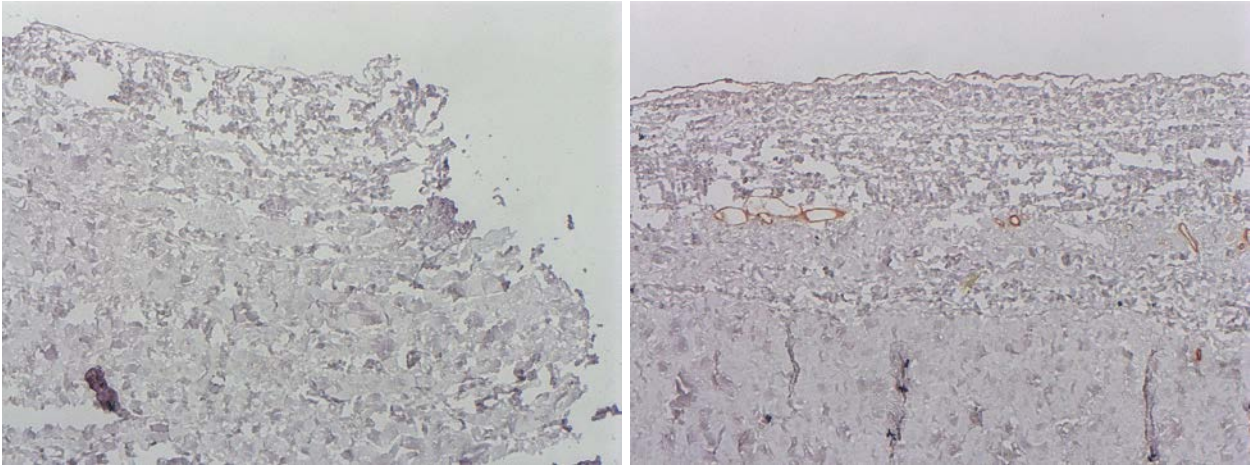
Figure 41: IHC staining with anti-laminin on laminin incubated and seeded scaffolds imaged at 20x. Both images show cross sections tagged with the antibody.

2.4 Study 4: Static seeding of bovine pericardium tissue samples with endothelial cells treated with fibronectin and laminin protein substrates and PureCol, Q.Gel, and Fibrin Glue hydrogels for increased cellular adhesion

This study aimed to increase endothelial cell attachment by providing an environment that more closely mimics the in vitro surroundings. While the protein substrates were added in the previous study, there still lacks an extra-cellular matrix (ECM) or basal lamina to provide binding sites for the endothelial cells to attach. 3 different hydrogels were used in this study to mimic the ECM, providing an even surface for attachment and communication as well as mechanical support that may have been lacking in the previous studies. After a 3 day incubation of the surface modified pericardium with drop seeded endothelial cells, it can be seen in Figure 37 the cells were attaching and spreading out with the fibrin glue samples, the Q.Gel with laminin and without substrate samples, and the scaffold only treated with laminin. The histological samples at the 3 day time point were again

inconclusive, as it appears the cells were again washed away in the processor or during the rinsing steps prior to fixing the tissue.

After the 7 day incubation, almost no cells were seen in the Calcein AM tagged images and black spots were observed on the scaffolds. These black spots are thought to be PGG coming of the tissue, as the tissue in this experiment was not neutralized in 50:50 DMEM/FBS prior to seeding on accident. The media was changed at the 3 day time point, and as shown in Figure 38 the media on the scaffolds was yellow by day 7 indicating an acidic pH likely due to the PGG.

The histological cross sections in Figure 41 shows the gel on the surfaces well. The fibrin glue formed a straight line across the surface of the pericardium. Hematoxylin (dark purple) stains negatively charged compounds, such as DNA or RNA. The fibrin glue and the Q.Gel with fibronectin treated sample shows cells on the surface that was stained by the hematoxylin. The same thing is seen in the fibrin glue and laminin treated sample but not to the same degree. The collagen from the PureCol can also be seen on the surface in the cross sections but no cells were observed. The scaffolds with the synthetic ECM, Q.Gel, is also seen in the histology. The Q.Gel actually appears to have infiltrated the pericardium by about 100 um and is stained by the hematoxylin in both the 3 day and 7 day histology samples. The samples that were not treated with either of the hydrogels, show no cellular attachment in the histology images.

	Fibrin Glue	PureCol	Q.Gel	No Gel
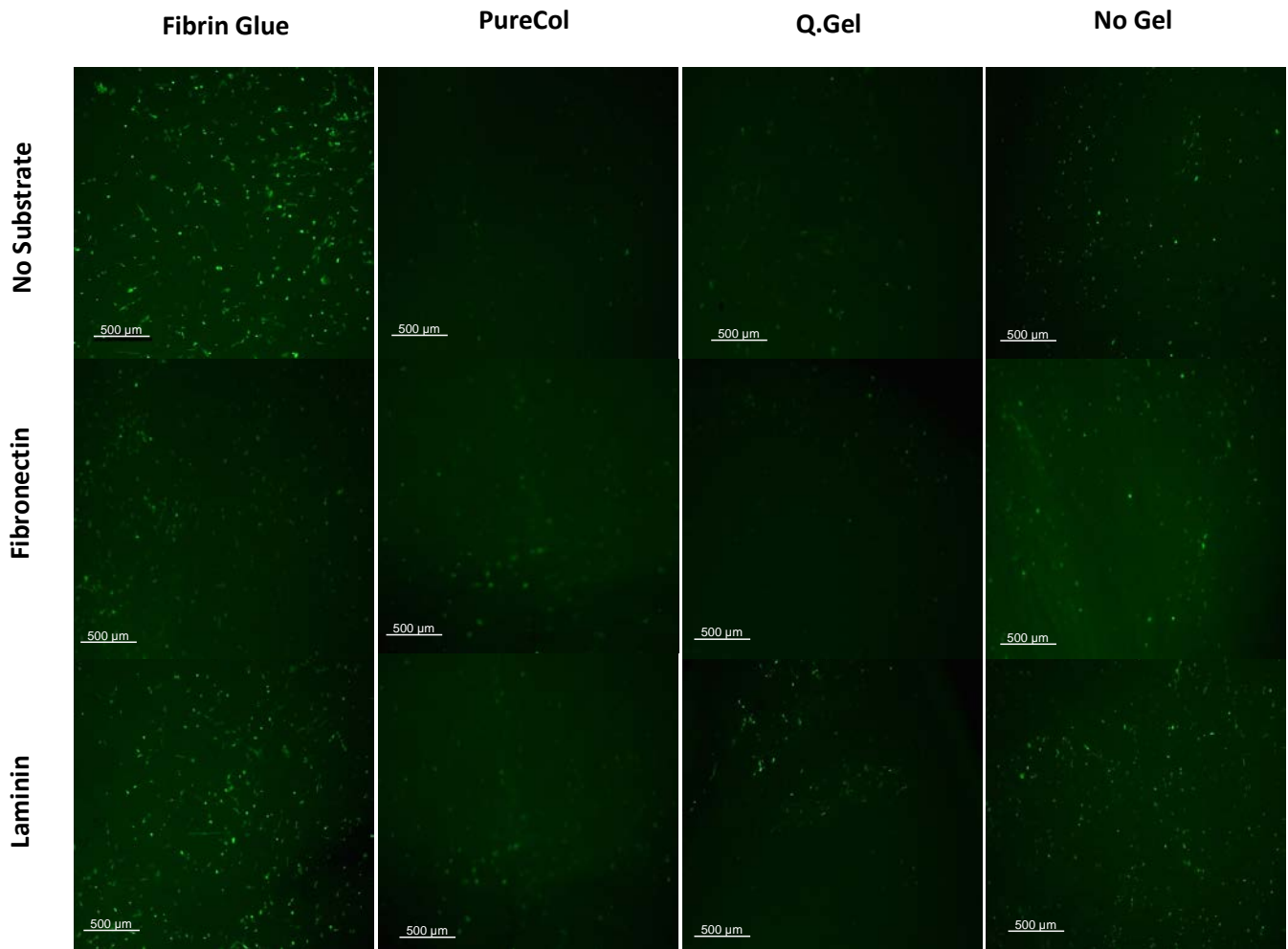
Figure 42: Calcein AM stained seeded scaffolds treated with a combination of hydrogels and protein substrates showing cellular attachment and spreading after incubating for 3 days. Taken at 2.5x.
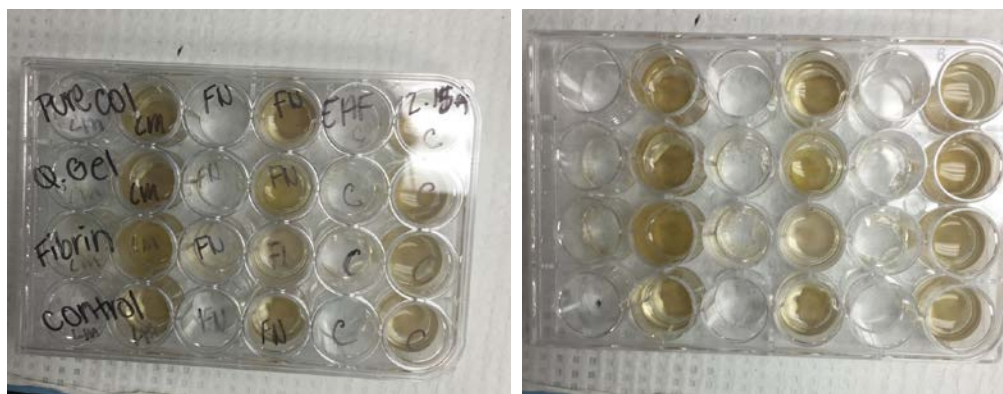
Figure 43: 24 well plate with the seeded scaffolds in culture media at day 7 of incubation (3 day samples have already been removed).
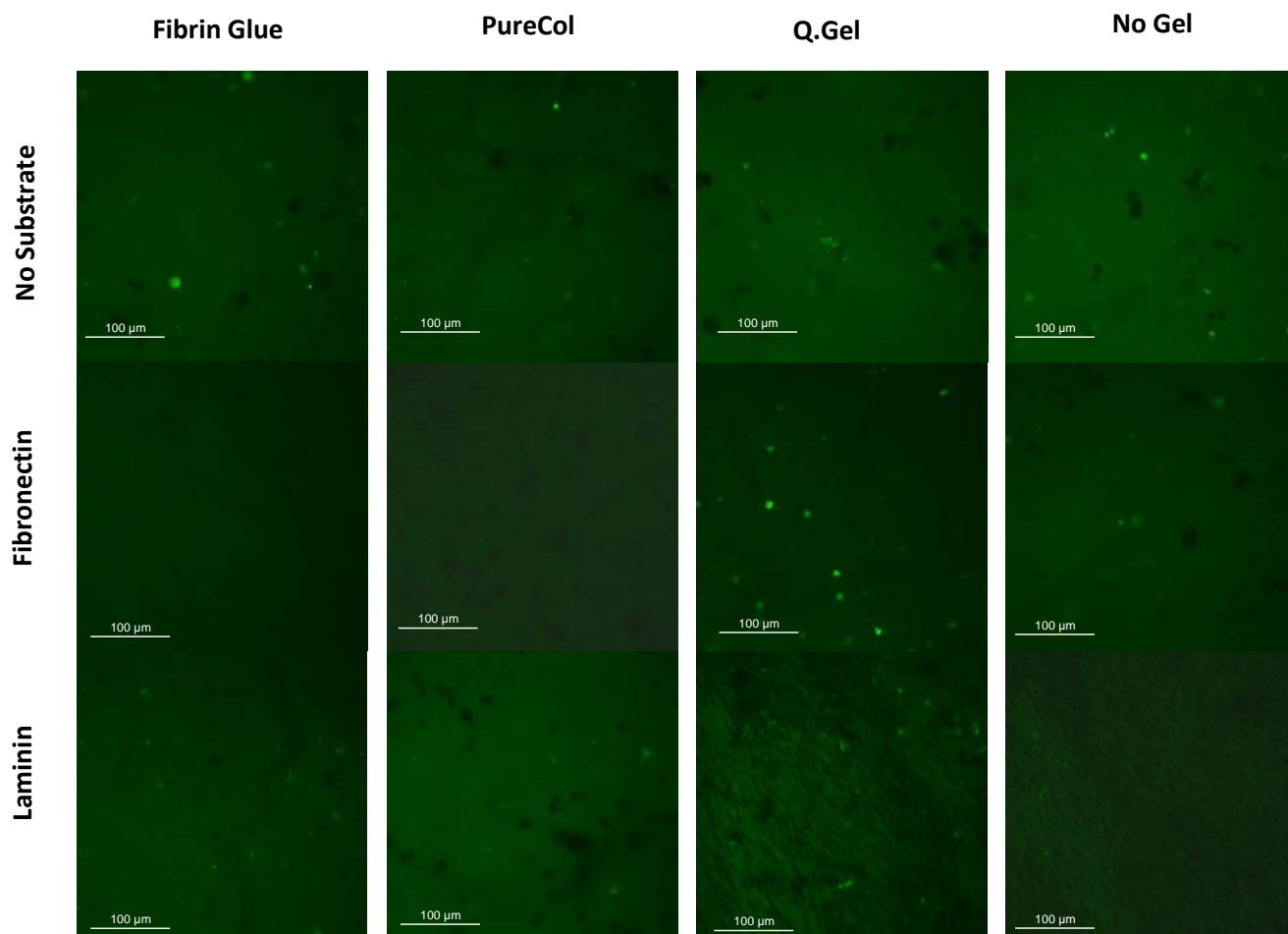
Figure 44: Calcein AM stained seeded scaffolds treated with a combination of hydrogels and protein substrates showing cellular attachment and spreading after incubating for 7 days. Taken at 10x.
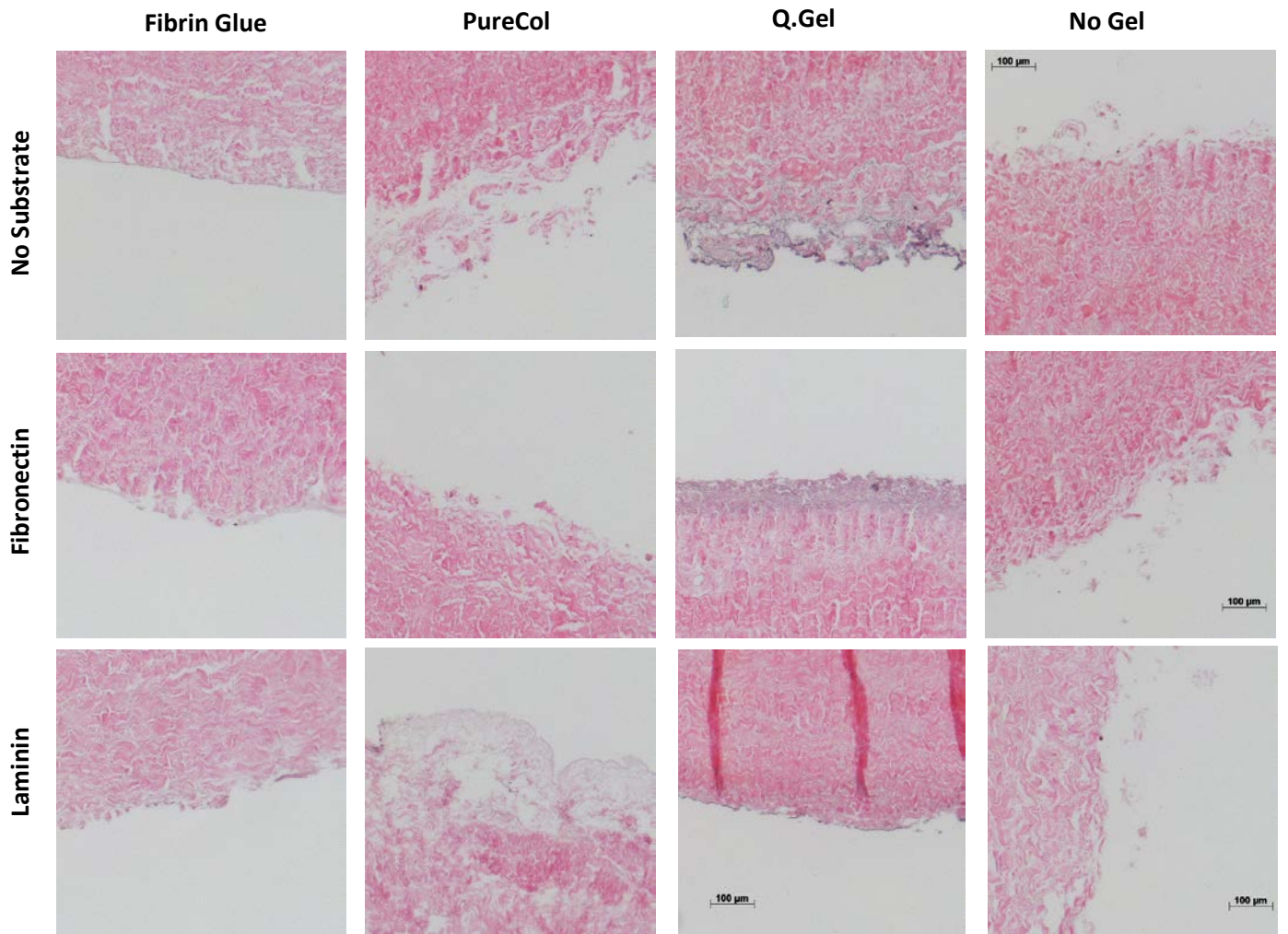
Figure 45: Cross sections of paraffin embedded cusps taken from porcine valve surface modified with hydrogels and protein substrates and seeded with endothelial cells stained with H&E after 3 day incubation. Taken at 10x.
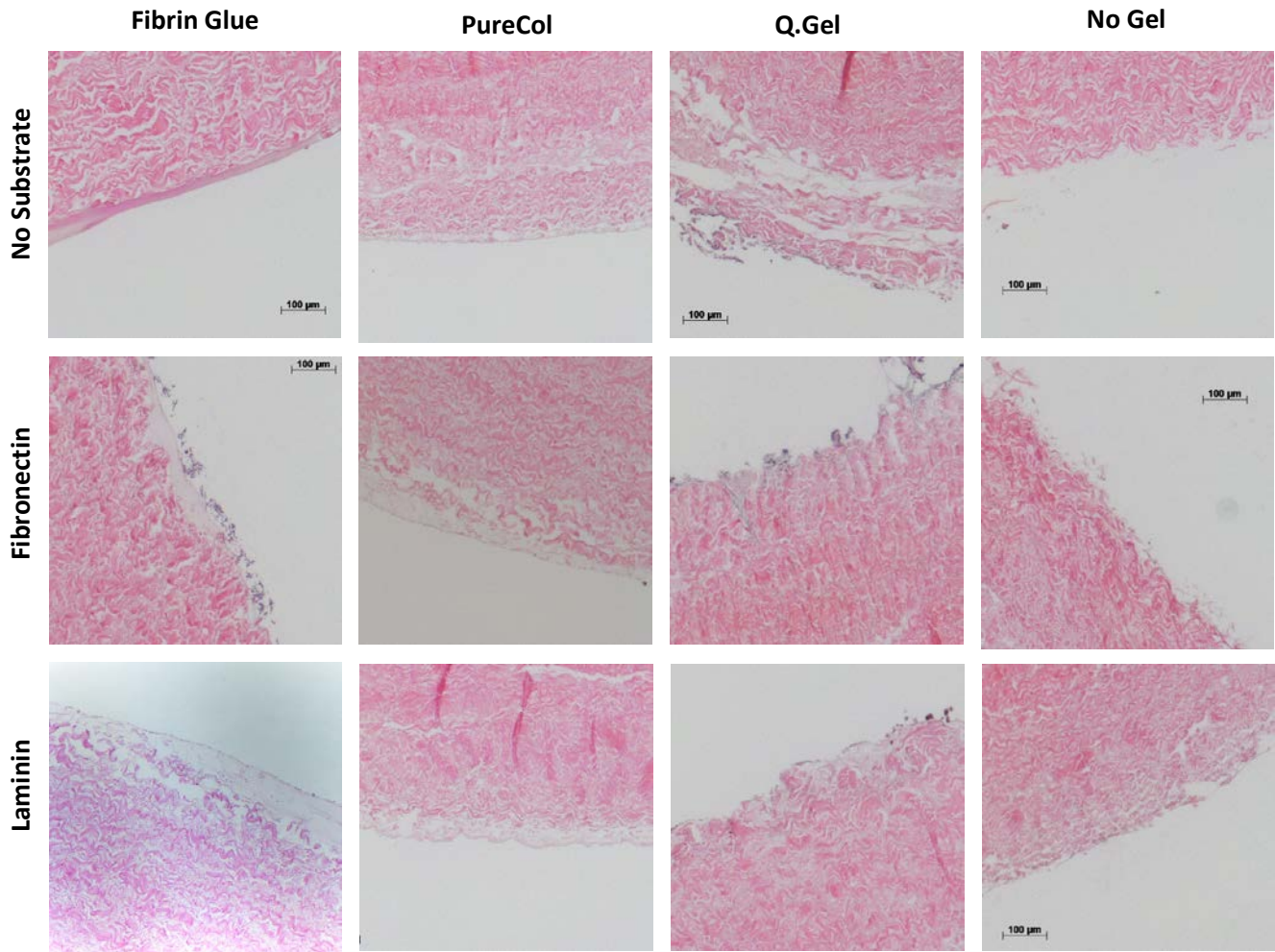
Figure 46: Cross sections of paraffin embedded cusps taken from porcine valve surface modified with hydrogels and protein substrates and seeded with endothelial cells stained with H&E after 7 day incubation. Taken at 10x.

2.5 <u>Study 5: Application of fibrin glue hydrogel and protein substrate surface modifications on decelled and harvested porcine native valves, dynamic seeding, and cellular adhesion and uniform coverage analysis</u>

Since the previous studies were conducted on sections of tissue samples, the purpose of study 5 was twofold: to evaluate the fibrin glue surface modification for uniform cell coverage on the surface of heart valve and to conclude if the endothelial cells have a preference for the laminin or the fibronectin. Study 5 compares the cusps from 4 types of valves – a decelled valve, a native valve, a valve treated with

fibrin glue and fibronectin, and a valve treated with fibrin glue and laminin. Figure 42 shows cross sections of each of these samples stained with DAPI. The native cusp is completely infiltrated with cells both on the surface, endothelial cells, and within the cusp, interstitial cells. The decelled cusp shows no cells. The cusps treated with fibrin glue and laminin shows a few endothelial cells on the surface as well as the cusps treated with fibrin glue and fibronectin.

The surfaces of the treated cusps were also stained with DAPI. The fibrin glue gel allowed the cells to reside in different planes which made it difficult to capture an image showing the all of the cells on the surface in one image. Figure 43 shows these images. It was also observed that the outflow sides of the cusps had more cells than the inflow sides of the cusps. This is probably due to the increased incubation time where the valves sat statically with the outflow side up. Overall, greater cellular attachment was observed on the valves treated with fibrin glue and laminin compared to the valves treated with fibrin glue and laminin. Calcein AM reveals that the cells were viable after both treatments, although most cells were observed on the inflow side of the laminin sample. An image was taken on the stereomicroscope with Calcein AM stain to get a better representative image of the distribution of cells on the surface, since this microscope images the surface in 3-dimensions. The image from the cusp modified with fibrin glue and laminin in Figure 47 shows a uniform cell distribution.

Scanning electron microscopic images of the outflow sides of the valves, showed more cells on the cusp treated with fibrin glue and laminin compared to the one

treated with fibronectin. Both samples however, showed cell spreading meaning the cells were interacting well with the environment.

IHC analysis revealed a positive stain for von Willebrand factor on the surface of both the fibronectin and the laminin treated cusps. VE-cadherin also stained positive on the fibronectin treated sample and only slightly positive on the laminin treated sample. Anti-CD31 did not show very positive results in either sample, but it also was very weak in the native cusp meaning the antibody used may not have worked or the antigen retrieval step did not adequately expose the CD31 markers. As expected the decelled cusp did not stain positive for any of the endothelial cell markers. The presence of these markers, demonstrates the presence and proper expression of endothelial cells on the surfaces of the cusps after being modified with fibrin glue, protein substrates, and seeded with endothelial cells in a rotating device.

Statistical analysis between the 2 valves treated with fibrin glue and fibronectin and the 2 valves treated with fibrin glue and laminin showed that more cells were found on the inflow sides of the cusps compared to the outflow sides of the cusps in all samples. Additionally there was statistical significance between the different treatments as well. Many more cells were found on the cusps treated with laminin compared to the cusps treated with fibronectin. This is believed to be attributed to the fact that laminin is a major component of the basement membrane that serves to organize the intricate network of the basement membrane. Therefore laminin may attach the endothelial cells better. While fibronectin is only an adhesive protein

substrate that is associated with the basal lamina but is not necessarily a part of the

basal lamia.
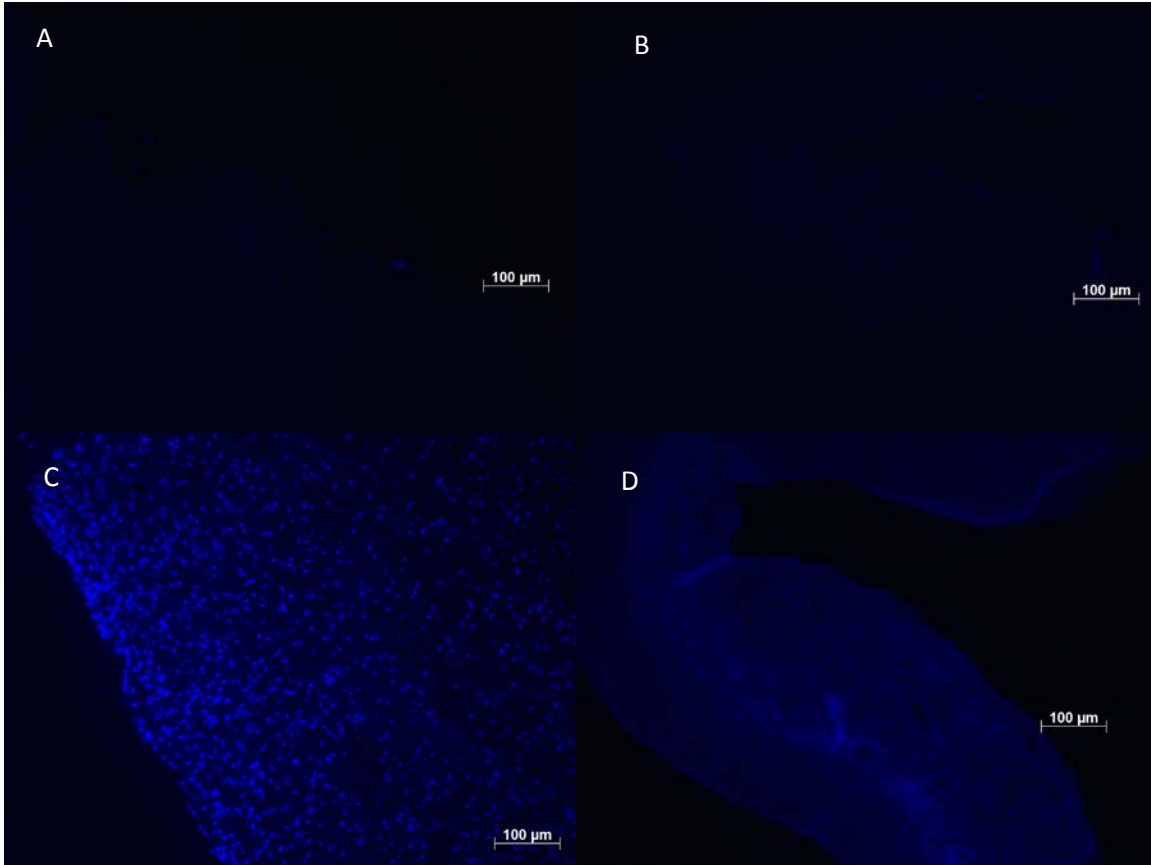
Figure 47: Cross sections of a cusps stained with DAPI taken from: (A) seeded valve treated with fibronectin, (B) seeded valve treated with laminin, (C) native porcine valve, (D) decellularized porcine valve. Taken at 10x.
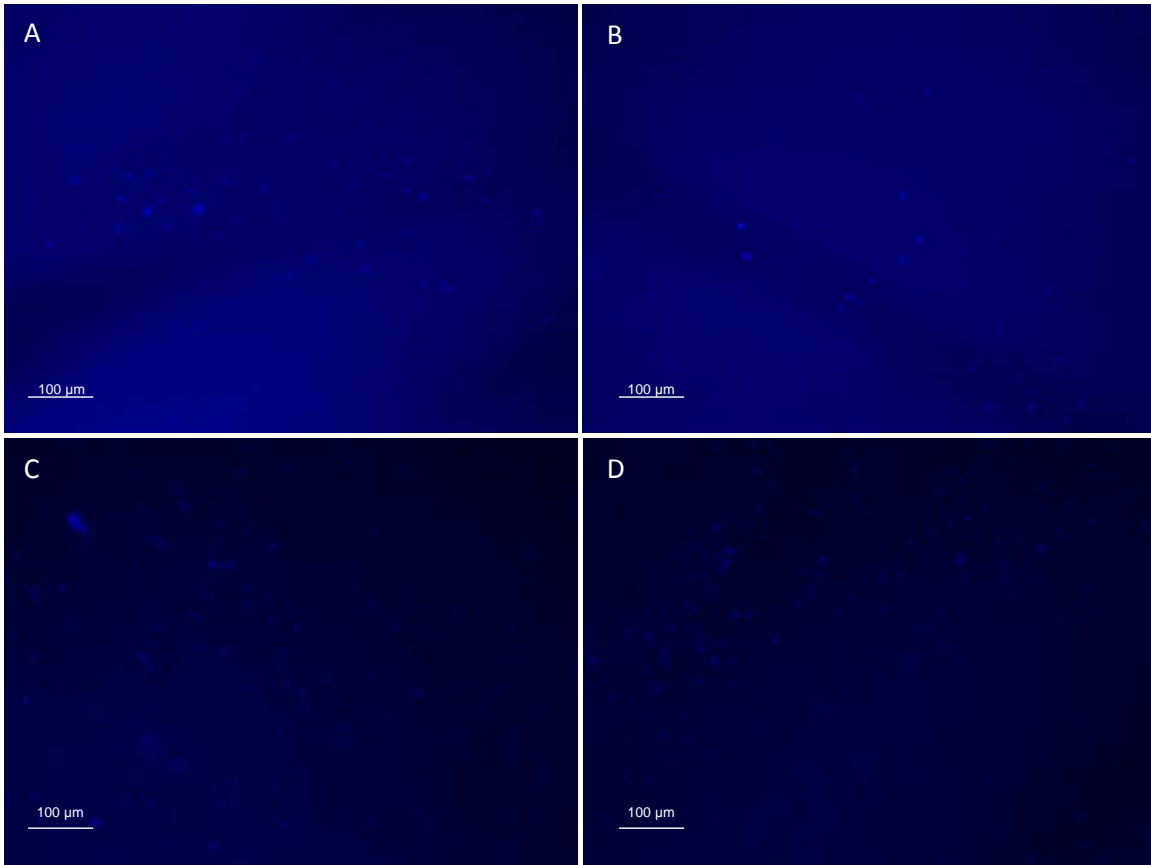
Figure 48: Surfaces of cusps taken from seeded valves treated with fibronectin outflow side (A) and inflow side (B) and treated with laminin outflow side (C) and inflow side (D).
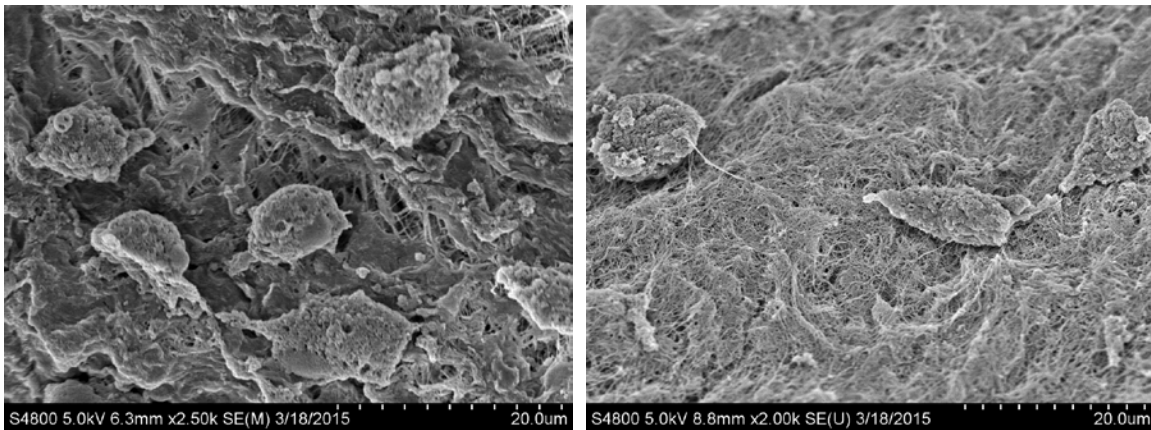
Figure 49: Surface morphology of outflow surface of cusps taken from endothelialzed valves modified with laminin (left) and fibronectin (right) imaged with SEM.
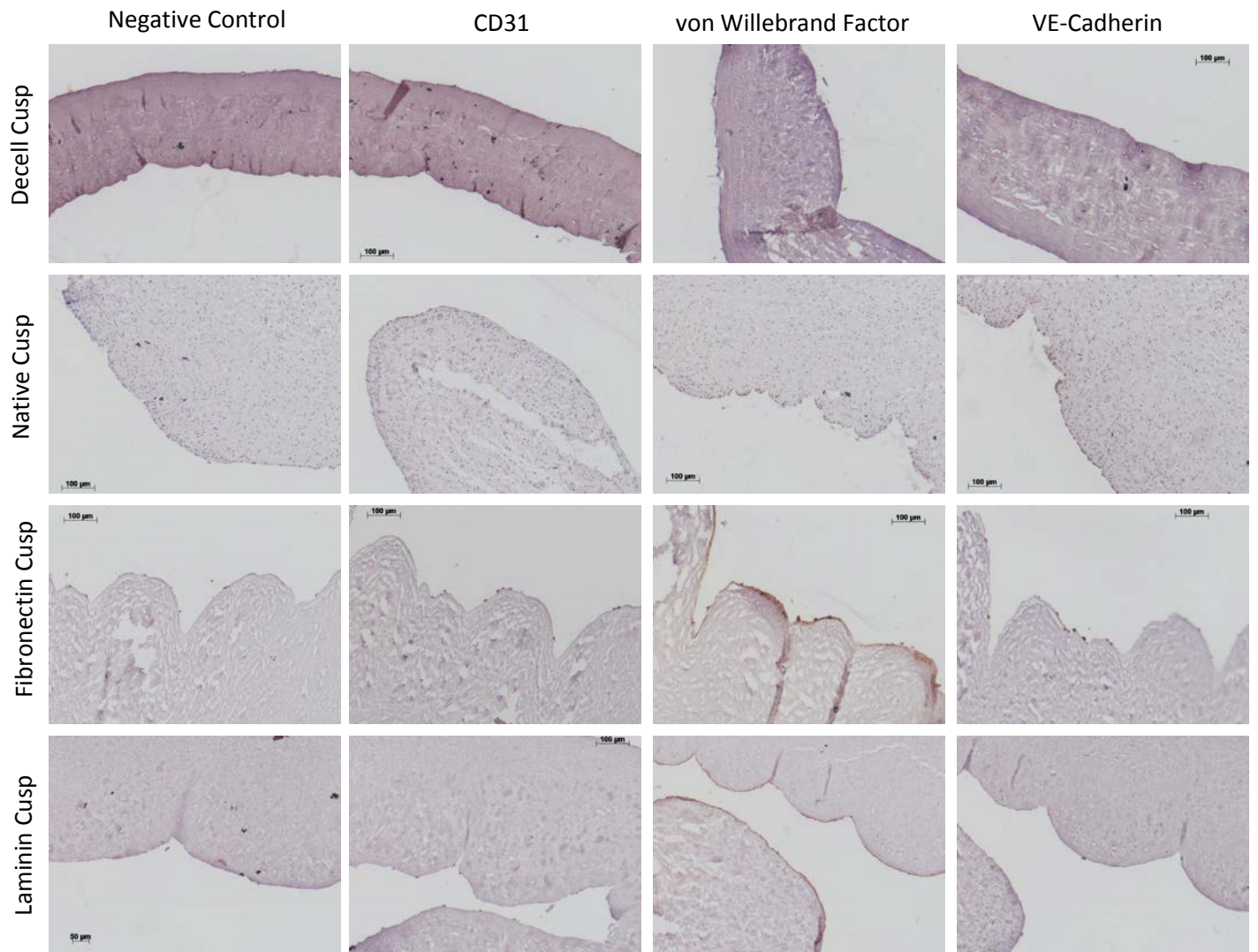
Figure 50: Decelled valve, native valve, and endothelial seeded valves treated with fibronectin and laminin were stained for endoethial cell markers CD31, VE-cadherin, and vWF using IHC. Images taken at 10x.
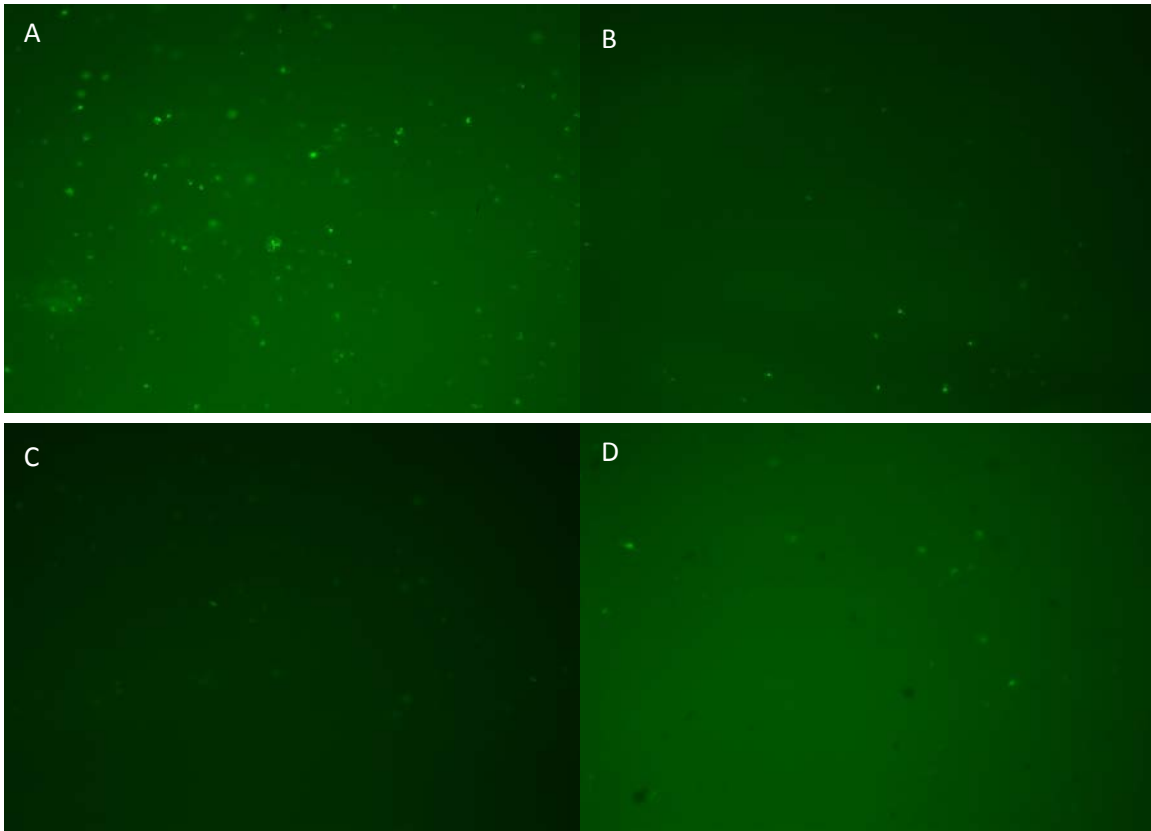
Figure 51: Calcein AM stain showing living cells on the surface of a cusp taken from a valve treated with fibrin glue and protein substrates, laminin (A,B) and fibronectin (C, D), and seeded with endothelial cells. A, C show the inflow side and B, D show the outflow side. Taken at 10x.
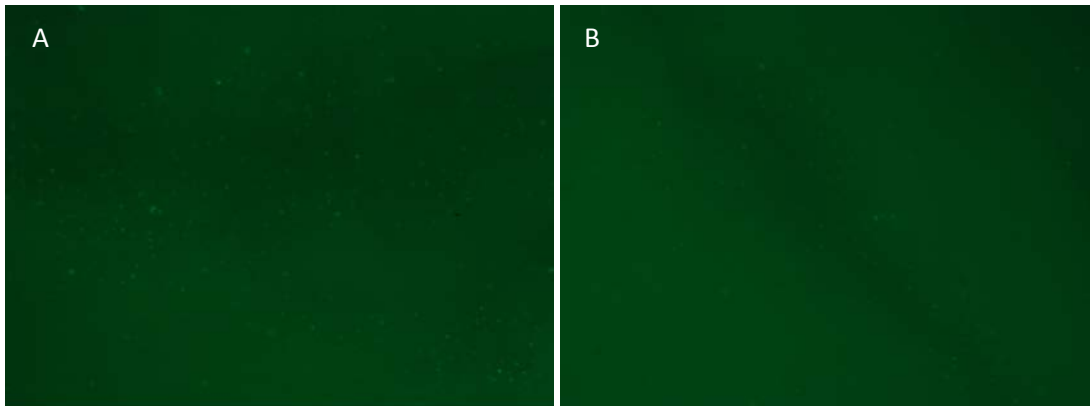
Figure 52: 3D image of calcein AM stain of cusp treated with fibrin glue and adhesive substrates laminin (A) and fibronectin (B) taken on a stereomicroscope with green fluorescent filter.
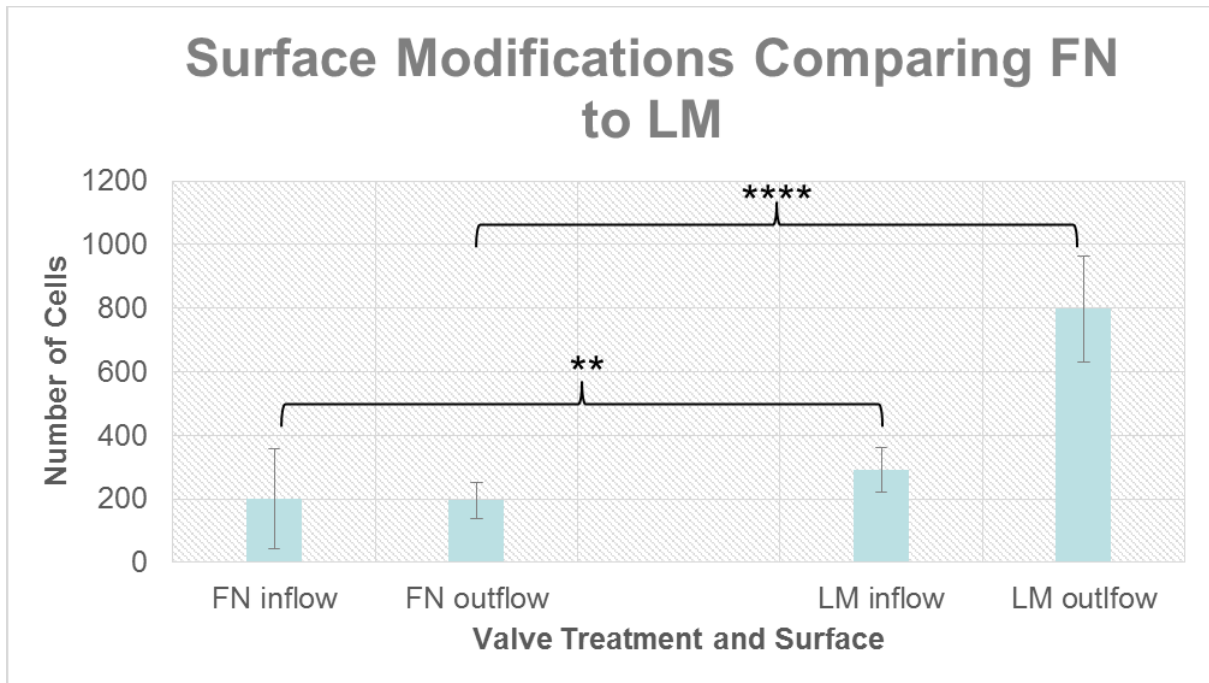
Figure 53: Analytics conducted with ImageJ on 272 total DAPI images of the inflow and outflow cusp surfaces from 2 valves treated with fibronectin (FN) and 2 valves treated with laminin (LM)

2.6 Study 6: Application of fibrin glue hydrogel and laminin substrate solution surface modifications on decelled and harvested porcine native valves, dynamic seeding, and in vitro testing for cellular retention analysis

This study tested a valve treated and seeded identically as in study 4 with fibrin glue and laminin in vitro in the bioreactor. The pressure were incrementally increased on the valve over a period of 5 days until the systolic pressure reached 20 mmHg, a physiological pressure. An accumulation of fibrin glue was found in one of the cusps and the adjacent sinus after removal from the bioreactor. This cusp was stained with DAPI. DAPI showed some cellular retention after implantation in the bioreactor. Another cusp was imaged with Calcein AM, but no cells were seen (data not shown). The bioreactor however is not a very consistent in vitro test as the

pressure is increased by manually opening the gas release and observing the change in pressure on the output in LabVIEW. It is believed the cells may not have had adequate time to fully attach and be conditioned on the surface before in vitro testing. The objective is for the cells to eventually degrade the fibrin glue as they release plasminogen and then lay down their own extra-cellular matrix during dynamic conditioning in the rotator. When the cells have had time to achieve a uniform layer of ECM to reside and communicate effectively, then they can be tested in an in vitro environment.
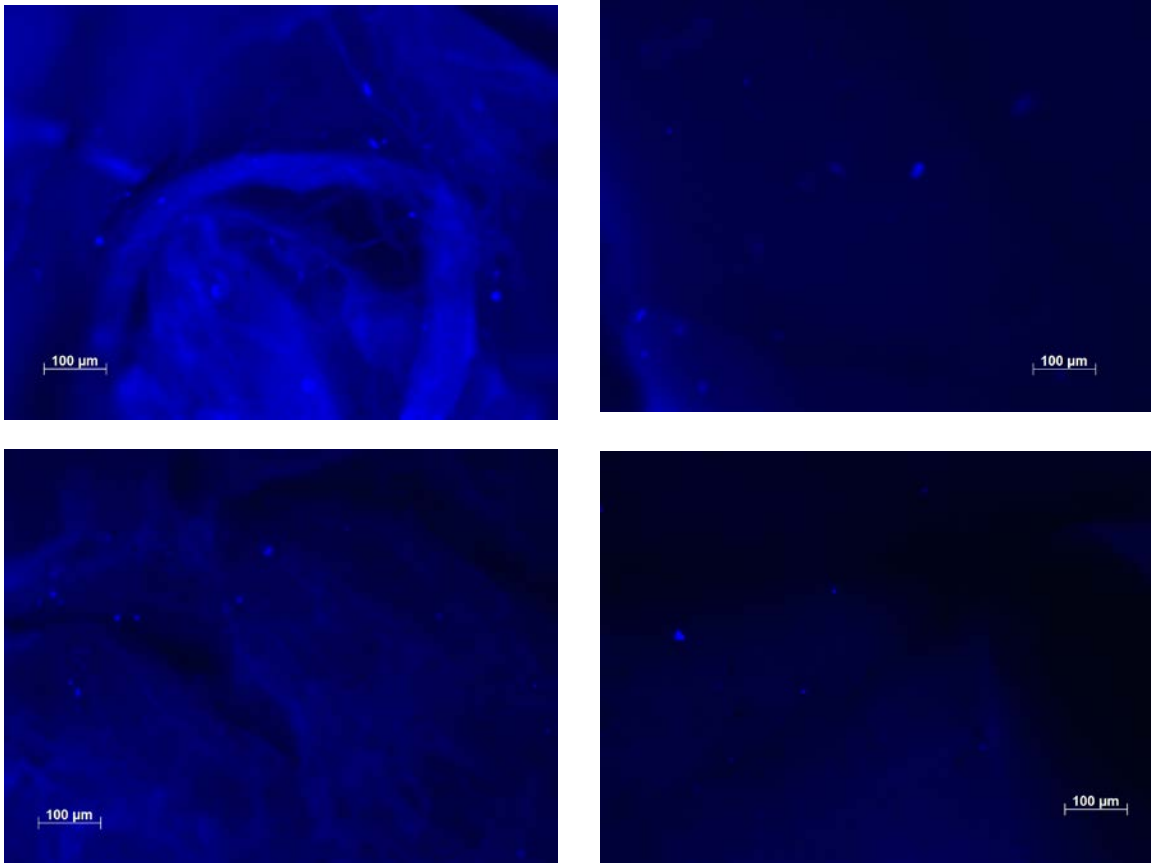
Figure 54: DAPI stain showing the presence of nuclei on valve cusps after surface modifications with fibrin glue and laminin, seeded with endothelial cells, and exposed to physiological shear stress in the bioreactor. Left images are from the outflow side and right images are from the inflow side.

Figure 55: Porcine valve seeded treated with fibrin glue and laminin and seeded with endothelial cells after removal from the bioreactor. One of the coronary sinuses and cusps has accumulated a thickened portion of fibrin glue.

Chapter 3: Conclusions

4.1 <u>Study 1: Static seeding of TE valve with HADSCS and incremental rotation for analysis of cellular adhesion</u>

- Uniform cellular coverage was achieved statically seeding HADSCs on a tissue-engineered valve made of PGG fixed bovine pericardium after 4 rounds of introducing cells to the suspension and altering the position of the valve.

4.2 <u>Study 2: Static seeding of TE valve with HADSCS and incremental rotation followed by exposure to shear stress for analysis of cellular retention</u>

- Cells are not retained after exposing the pericardium valve seeded with HADSCs and incubated for 24 hours to small shear stresses.
- Adequate cellular attachment was not achieved prior to putting the valve in the bioreactor.

4.3 <u>Study 3: Static seeding of pericardium samples with HCAECS and treated with adhesive protein substrates for increased cellular adhesion</u>

- Laminin increased endothelial cell spreading on PGG fixed samples of pericardium after 24 hours more than pericardium samples incubated in fibronectin or fetal bovine serum.
- Endothelial cell uniformity appears and density appears to be the same between the pericardium samples treated with fibronectin, laminin, and FBS.

4.4   Study 4: Static seeding of tissue samples with HCAECS and treated with protein substrates and hydrogels for increased cellular adhesion

- Q.Gel and fibrin glue showed increased cell attachment compared to PureCol and no gel. Fibrin glue appeared to increase cell spreading compared to other gels.

4.5   Study 5: Dynamic seeding of native valve with HCAECS and treated with protein substrates and fibrin glue for cellular adhesion analysis

- Endothelial cell attachment and coverage was observed on decelled and PGG fixed porcine valves treated with fibrin glue and protein substrates and then seeded in a rotating device for 24 hours and statically incubated for 2 days.

- From SEM analysis, endothelial cells were starting to spread out in both the fibronectin and laminin treated valves.

- Immunohistochemistry analysis revealed the cells were expressing von Willebrand Factor on the surface of the cusps after undergoing surface modifications with fibrin glue, protein substrates, and dynamic seeding.

- More endothelial cells attached to the valves modified with fibrin glue and laminin than the valves modified with fibrin glue and fibronectin.

4.6   Study 6: Dynamic seeding of native valve with HAECS and treated with laminin and fibrin glue followed by in vitro testing for cellular retention analysis

- A small number of endothelial cells dynamically seeded on a decelled, PGG fixed porcine valve treated with fibrin glue and laminin for 2 days remained attached

after in vitro testing in a heart valve bioreactor for 5 days reaching pressures of

20 mmHg/13.5 mmHg.

Future Work

- Optimize time of seeding, cell concentration, and adhesive protein concentration. Longer seeding times should be tried to allow for the cells to fully degrade the fibrin glue and make their way to the surface of the scaffold. Cell concentrations should be increased to match concentrations found in literature. Higher concentrations of laminin should be tried.
- Optimize in vitro testing and bioreactor conditioning regiments. The bioreactor pressure should be increased at a much slower rate over the period of a few weeks rather than a few days to allow the cells to become conditioned before being exposed to mechanical forces larger than they can withstand.

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