A 3D Tissue Engineering Model to Study Mitral Valve Annulus Calcification under Diabetic Conditions

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A 3D TISSUE ENGINEERING MODEL TO STUDY MITRAL VALVE
ANNULUS CALCIFICATION UNDER DIABETIC CONDITIONS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
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by
Erin James
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Accepted by:
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ABSTRACT

The most complex heart valve is the mitral valve (MV). Many pathologies can affect the MV, including stenosis, regurgitation, prolapse, and mitral annulus calcification (MAC). MAC is chronic degeneration of the annulus, which is the fibrous, saddle-shaped “ring” that can contract and relax with the myocardium. The prevalence of MAC is around 15% but increases in patients with other cardiovascular diseases and risk factors. It is also thought to increase in patients with type 2 diabetes, but MAC has not been properly characterized within this population because of confounding factors such as cardiac disease and kidney disease.

The goal of this project is to reveal the interactions between the mitral valve annular cells, valvular endothelial cells (VECs) and valvular interstitial cells (VICs), that lead to calcification. To study the effects of diabetic conditions on MAC and the MV, we investigated 2D and 3D cell cultures of VECs and VICs. We had two aims for this project: 1) investigate diabetes-induced alterations of mitral valve cells in 2D cell culture under static and dynamic conditions and 2) explore diabetes-induced modifications of mitral valve cells and extracellular components in 3D cell culture under dynamic conditions.

The Flexcell compression system was used to study 2D dynamic cell culture, and a tissue engineered MV and MV bioreactor were used for 3D cell culture. The tissue engineered MV was made by seeding a decellularized porcine MV with porcine VECs and VICs. This valve was placed in the bioreactor with high glucose cell culture media, and it ran for seven days under diabetic conditions. Samples from the 2D cell cultures as
well as the bioreactor were analyzed using immunofluorescence and western blotting. Proteins of interest included osteocalcin, a calcium-binding protein typically found in bone, α-smooth muscle actin, and glycated proteins containing carboxymethyl lysine.

Results show that osteocalcin and carboxymethyl lysine expression increased in the diabetic model. This suggests that there may be a relationship between diabetes and MAC.
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CHAPTER ONE
INTRODUCTION AND BACKGROUND

1.1 Mitral Valve

The heart is one of the main components of the cardiovascular system, along with blood and blood vessels. Its function is to pump blood all throughout the body. To do this, blood must flow through the four chambers of the heart: the right atrium, right ventricle, left atrium, and left ventricle. Heart valves separate chambers of the heart from each other as well as the ventricles from major arteries. Two heart valves, the pulmonary valve, and the aortic valve act as a border between the ventricle and either the aorta or pulmonary artery. Two different heart valves, the tricuspid valve, and the mitral valve separate each atrium from its corresponding ventricle.

1.1.1 Function

The mitral valve is located between the left atrium and left ventricle. It acts as a barrier for blood passing through the heart. The main function of the mitral valve is to allow unidirectional blood flow from the left atrium to the left ventricle during diastole. The heart muscles contract during systole, and when this occurs, the mitral valve closes, preventing regurgitation, or backflow, of the blood. If the mitral valve does not function properly, then it could become regurgitant or stenotic, or narrowed. There are four major components that work together to ensure the mitral valve is functioning properly: the mitral annulus, mitral valve leaflets, chordae tendineae, and papillary muscles. The anatomy of the mitral valve is illustrated in Figure 1.
The mitral annulus is the structure that separates the left ventricle from the left atrium. It is a fibrous, saddle-shaped “ring” with an anterior and posterior region\(^4\). The posterior annulus is located towards the back of the heart while the anterior annulus is located towards the front of the heart. The anterior annulus is anchored into the surrounding heart tissue, and the posterior annulus is loosely connected to the tissue\(^4\). This allows the annulus to contract and relax with the myocardium. The shape of the mitral annulus reduces stress placed on the tissue. This structure is innervated, and it acts as a passage for blood vessels to the mitral leaflets\(^4\).

The mitral valve is the only heart valve that has two leaflets\(^4\). These leaflets, particularly the anterior leaflet, are comprised of crisscrossed collagen strands. The junction of the leaflets is called a commissure. **Figure 2** shows how the leaflets fit together when the mitral valve is closed.
as well as where the commissures are located. It is evident in Figure 2 that each leaflet has a unique shape. The anterior leaflet has a trapezoidal shape while the posterior leaflet has a crescent shape\(^4\). The anterior leaflet is larger than the posterior leaflet, and it is tightly connected to the anterior portion of the mitral annulus\(^4\). Both leaflets are made up of collagen fibers, and they each are divided into three sections. These sections are referred to as scallops and are small bumps on the leaflets; the three scallops are lateral (A1, P1), central (A2, P2), and medial (A3, P3)\(^4\). The scallops are depicted in Figure 2. The leaflets connect and form two commissures: the anterolateral commissure, where A1 connects to P1, and the posteromedial commissure, where A3 connects to P3\(^4\). It has been shown that mitral valve leaflets open in less than 100 milliseconds (ms), and they will open roughly 3 billion times during someone’s life\(^4\). Therefore, it is important that the leaflets stay healthy so that they can function properly.

**Figure 2.** Labeled mitral leaflets\(^{3,4}\).
The chordae tendineae are fibrous strings that connect the papillary muscles and the anterior and posterior leaflets\(^4\). There are two types of chordae tendineae: primary (marginal) chordae and secondary (basal) chordae. Primary chordae attach to the free edges of the mitral leaflets\(^4\). The secondary chordae attach to the posterior mitral leaflet body and the anterior leaflet edge\(^4\). Because the collagen in secondary chordae is folded tightly, it is thicker and extends further than primary chordae. The largest and thickest secondary chordae is known as the strut chordae; this is inserted into the anterior leaflet\(^4\). The collagen fibrils in primary chordae have a lower density and less folding which limits how far these chordae can stretch\(^4\). Chordae tendineae are able to adapt to the changing loading conditions in the heart.

The final component of the mitral valve is the papillary muscles. These muscles originate in the left ventricle wall and extend into the cavity\(^4\). The chordae tendineae protrude from the heads of the papillary muscles to different parts of the mitral leaflets. There are two papillary muscles, lateral and medial, and they get their names from where they attach to the commissures\(^4\). These muscles can be seen in Figure 1. The lateral papillary muscle has one head and two blood supplies, while the medial papillary muscle has two heads and only one blood supply\(^4\). The papillary muscles help control and maintain the opening and closing of the mitral valve. The leaflets’ prolapse is prevented because of the relationship between the papillary muscles and chordae tendineae. The mitral leaflets are kept under outwardly directed tension because of the orientation and distance between each papillary muscle head\(^4\).

1.1.3 Composition

The extracellular matrix (ECM) is “rich in biochemical and biophysical factors that can direct cellular function”\(^1\)\(^2\), and it is a major factor in the biomechanical function of the mitral valve\(^6\). The ECM also acts as the site for signaling molecules to initiate different processes\(^6\).
There are three levels of ECM in a healthy mitral valve; they are the fibrosa layer, the spongiosa layer, and the ventricularis layer. The fibrosa layer is mainly composed of collagen fibers which provide strength to the mitral valve. Elastin is the main component of the ventricularis layer. Elastin provides the valve with the ability to stretch and relax, an important feature for the mitral valve so that blood can flow properly through the heart. Loose connective tissue that is high in proteoglycans (PGs) and glycosaminoglycans (GAGs) can be found in the spongiosa layer, and these factors help the surrounding layers movements and motions. The ECM is critical to proper function of the mitral valve, and it is vital that the ECM is always balanced. In healthy adults, VICs maintain mitral ECM homeostasis; these cells intervene and help during ECM remodeling in response to the normal aging of the mitral valve. The layers of the ECM can be seen in Figure 3.

**Figure 3.** Comparison of the aortic valve (A) and mitral valve (B) structure.
As previously mentioned, collagen is a major component of the extracellular matrix. There are 28 members of the collagen family, but type I, type III and type V are the most abundant in the mitral valve\(^6\). Collagen provides the stiffness and strength to the valve and helps prevent valvular prolapse. Roughly 605 collagen fibrils crosslink to form one collagen fiber in a healthy mitral valve\(^6\). Under biaxial stretch, individual collagen fibrils pack together, but when they are not stretched, the fibers relax and form a collagen crimp\(^6\). The collagen crimp resorbs shock caused by a biaxial stretch.

Proteoglycans are typically found in connective tissue, and they consist of glycosaminoglycans attached to a core protein\(^6\). PGs are polar molecules that are interspersed in collagen fibrils\(^6\). Because of their interactions with other ECM components, they can endure high compressive loads\(^6\). GAGs consist of repeating disaccharide units, and four major types exist in the mitral valve: hyaluronan, chondroitin sulfate (CS), dermatan sulphate (DS), and heparin sulfate/heparin (HS)\(^6\). Versican, the highest expressed PG in the mitral valve, and hyaluronan, the highest expressed GAG, work together to loosen the mitral valve’s matrix in order to make the cell proliferation and migration processes easier during valve remodeling\(^6\). Versican and hyaluronan thrive in the spongiosa layer where they can bind water and aide in tissue elasticity\(^6\). Research has shown that PGs and GAGs are often cause mitral valve abnormalities or disfunction\(^6\).

Elastin fibers are known for their ability to undergo a considerable amount of deformation and return to the original shape. It is important that the mitral valve has plenty of elastin fibers with this function in order to stretch and respond to the hemodynamic environment of the heart\(^6\). Elastin fibers found in the ventricularis layer of the ECM are typically organized in continuous sheets and positioned along the circumferential axis and radial axis\(^6\).
Two main cell types are found in all cardiac valves, including the mitral valve: valvular endothelial cells (VECs) and valvular interstitial cells (VICs)\textsuperscript{12}. VECs line the surface of the mitral leaflets, while VICs are found throughout the mitral leaflets. Biochemical signals, matrix proteins, and matrix remodeling enzymes are secreted by both cell types in order to maintain tissue homeostasis\textsuperscript{12}. These cells tend to help the mitral valve, as they can attempt to repair the valve in response to injury\textsuperscript{12}. Their interaction with each other is vital in maintaining the integrity of the valve\textsuperscript{12}.

VICs are the most common cell type in heart valves, and they maintain the structural integrity of the mitral valve\textsuperscript{12,13}. They typically maintain a quiescent phenotype, but they can become activated; activated VICs are more contractile and help repair and remodel the valve and ECM\textsuperscript{13}. However, activated VICs can lead to calcification\textsuperscript{13}. The number of VICs present in the mitral valve decreases with age which results in the degeneration of collagen. This can lead to mitral valve prolapse and regurgitation\textsuperscript{12}.

VECs attempt to repair the MV in response to disease by undergoing the endothelial to mesenchymal transition (EMT) where fibroblasts are acquired to aid in tissue regeneration\textsuperscript{6}. This is especially helpful as one ages as this can restore the valve with VICs\textsuperscript{14}. Because of this, the levels of VICs are said to be maintained by VECs\textsuperscript{14}. One study showed that the number of VECs that underwent EMT increased with larger leaflets which suggests that EMT plays a significant role in mitral regurgitation and in response to leaflet tethering\textsuperscript{15}. VECs have been known to express osteoblastic markers, like osteocalcin (OC), especially in valves with rheumatic disease\textsuperscript{14}. 
1.1.3.1 Biomarkers of VICs and VECs

α-smooth muscle actin (αSMA) is a protein that marks for smooth muscle cells and myofibroblasts and contributes to mechanical tension\textsuperscript{16}. This protein is often found in activated VICs as they respond to injury or unusual forces\textsuperscript{15}. Research has proven that VICs express αSMA during mitral valve prolapse, and its expression tends to increase as the disease progresses\textsuperscript{17}. There are also some VICs that can differentiate into osteoblasts and express osteogenic markers like OC; this may be the beginning of calcification in the valve\textsuperscript{15}.

E-selectin is a cell adhesion molecule. It is expressed in endothelial cells, including VECs, and it plays a role in inflammation\textsuperscript{18}. It is an important protein in endothelial cell activation and dysfunction\textsuperscript{18}.

1.2 Mitral Valve Pathology

There are many disorders that affect the mitral valve including mitral valve prolapse (MVP), mitral regurgitation (MR), mitral annular calcification (MAC), and mitral stenosis. Different factors affect each of these disorders, but it is common for any of these to be affected by abnormalities in the mitral valve structures.

1.2.1 Mitral Valve Prolapse (MVP)

Mitral valve prolapse is one of the most prevalent valvular abnormalities\textsuperscript{19}. There are multiple names for MVP, including Barlow syndrome, billowing leaflet syndrome, floppy valve syndrome, and mucinous degeneration\textsuperscript{8}. A normal mitral valve will billow slightly into the left atrium to allow for blood to flow into the left ventricle, and a “floppy” mitral valve is an exaggerated form of a billowed valve\textsuperscript{19}. Mitral valve prolapse occurs when the mitral leaflets do not coapt or fasten together, and it could lead to other disorders such as mitral regurgitation\textsuperscript{19}. 
One major cause of mitral valve prolapse is myxomatous degeneration\(^8\). The middle layer of the mitral leaflets, or the spongiosa layer, is made up of myxomatous material\(^1^9\), and this material can become elongated causing the mitral leaflets and chordae tendineae to stretch\(^8\). The stretching of these components does not allow the mitral valve to properly close and can displace the leaflets, causing prolapse\(^8\). It has also been shown that the mitral annulus increases in size during mitral valve prolapse\(^2^0\); however, the leaflets go through the most changes during MVP. The loose, myxomatous material of the leaflets becomes prominent\(^1^9\), with an increase in the proteoglycans\(^8\). Fragmentation of collagen occurs, allowing the mitral leaflets to lose their structure and become billowed\(^8\). Mitral valve prolapse is one of the leading causes of mitral regurgitation, another major disease affecting the mitral valve\(^2^1\). **Figure 4** illustrates normal blood flow through the heart versus blood flow through a heart with MVP and regurgitation.

**Figure 4.** Normal heart versus heart experiencing MVP and regurgitation\(^1^0\).
1.2.2 Mitral Regurgitation (MR)

Flaws and deformities in any of the mitral valve’s four structures, along with mitral valve prolapse, are the main causes of mitral regurgitation\textsuperscript{19}. Some other causes include myxomatous degeneration, rheumatic heart disease, infective endocarditis, connective tissue disease, congestive heart failure, and cardiomyopathy\textsuperscript{8}. In a functional heart, blood flows from the left atrium through the mitral valve and into the left ventricle. During mitral regurgitation, blood flows backwards from the left ventricle to the left atrium because the mitral valve leaflet cannot close firmly\textsuperscript{22}.

Because there are many causes of this valvular disease, it can be divided into primary and secondary mitral regurgitation\textsuperscript{22}. Primary mitral regurgitation is known as degenerative or organic whereas secondary mitral regurgitation is known as functional or ischemic\textsuperscript{22}. Some common causes of primary MR include papillary muscle rupture, MVP, and leaflet perforation\textsuperscript{22}. Secondary MR arises due to remodeling or abnormalities in the left ventricle, often caused by cardiomyopathy\textsuperscript{22}.

As previously mentioned, abnormalities of the mitral valve’s four structures can lead to MR, and this is typically found in primary MR. Valvular leaflets can become shortened, deformed, or rigid, causing MR, and this usually occurs in patients with rheumatic heart disease\textsuperscript{19}. Other diseases like infective endocarditis and trauma to the heart can cause perforations and destruction to the leaflets which will cause MR\textsuperscript{19}. The chordae tendineae may rupture spontaneously or because of other diseases such as infective endocarditis\textsuperscript{19}. The chordae tendineae connects the papillary muscles to the leaflets, so if they lengthen or rupture, then the leaflets cannot open and close properly. The chordae connected to the posterior leaflets tend to rupture or become dysfunctional more frequently than the chordae connected to the anterior
leaflet, and most of the time, increased mechanical strain is the reason for this\textsuperscript{19}. Rupturing of the papillary muscles is rare, but if it occurs, the contraction of the valve can no longer be controlled, leading to MR\textsuperscript{22}. Myocardial infarction can lead to extreme MR because it causes necrosis of papillary muscles\textsuperscript{19}. There are two main abnormalities of the mitral annulus: dilation and calcification\textsuperscript{19}. Dilation of the annulus occurs during any heart disease that also dilates the left ventricle\textsuperscript{19}. Mitral annulus calcification is a common abnormality, but severe calcification can lead to MR; calcification may immobilize the leaflets so that they do not close tightly, leading to backflow of blood\textsuperscript{19}.

\textit{1.2.3 Mitral Annulus Calcification (MAC)}

Mitral annulus calcification is a degenerative process that occurs in the annulus of the mitral valve, and it affects the posterior annulus more than the anterior annulus\textsuperscript{5}. This abnormality is one of the most common identified during an autopsy\textsuperscript{19}. At one point, MAC was considered an age-related process, but it has been shown recently that other mechanisms and factors may contribute to the development of MAC\textsuperscript{5}. Some of these factors include cardiovascular diseases like coronary artery disease (CAD), mitral valve disease, and arrhythmias\textsuperscript{5}.

Calcification of the annulus can lead to immobilization of the mitral leaflets; if the leaflets are unable to move properly, coaptation of the leaflets will be affected\textsuperscript{19}. Without the coaptation of the leaflets, severe mitral regurgitation may occur. If a large amount of calcification occurs on the annulus, the mitral valve opening might become narrowed, which is a disease known as mitral stenosis\textsuperscript{19}. There are many imaging methods used to identify MAC, and echocardiography was once considered the gold standard method\textsuperscript{5}. Now, scientists are limiting the use of echocardiography to detect MAC because it cannot always distinguish between
calcification and dense collagen\textsuperscript{5}. Echocardiography may still be used in some cases, but other technologies, like electron-beam computed tomography (CT), cardiac CT, and multislice (spiral) CT have been found to be more effective. A cardiac CT image of MAC is shown in Figure 5.

\textbf{Figure 5.} White arrows indicate MAC in a cardiac CT image\textsuperscript{5}.

\subsection*{1.2.4 Mitral Stenosis (MS)}

Stenosis means narrowing, and mitral valve stenosis, or mitral stenosis, is the narrowing of the mitral valve. There are many causes of mitral stenosis including rheumatic heart disease, infective endocarditis, and severe mitral annular calcification\textsuperscript{8}. The most prominent cause is rheumatic fever, which leads to rheumatic heart disease\textsuperscript{19}. Rheumatic fever can lead to four different types of fusion within the mitral valve: commissural, cuspal, chordal, and combined\textsuperscript{19}. If commissural or cuspal fusions occur, the leaflets will no longer open to their full ability. This
results in the valve opening to become narrowed, causing mitral stenosis. Fusion of the chordae tendineae can result in the shortening and thickening of other mitral valve structures including the leaflets\textsuperscript{19}. An example of mitral stenosis can be seen in Figure 6.

1.3 Diabetes

Diabetes mellitus (DM) occurs because of flaws in insulin secretion or action, resulting in hyperglycemia, or high blood glucose\textsuperscript{23}. There are three types of DM: type 1, type 2, and gestational. Type 1 DM is an autoimmune disease that destroys pancreatic beta cells\textsuperscript{23}. These cells synthesize and secrete insulin, so their destruction leads to a decrease in insulin production. Genetic disposition, as well as certain environmental factors, impact the etiology of type 1
Pancreatic beta cells continue to produce insulin in type 2 DM, but cells become resistant to the insulin, causing blood glucose levels to rise. Type 2 diabetes accounts for roughly 90-95% of the diabetic population and is typically diagnosed in adults. Insulin shots are not required for type 2 diabetes patients because their pancreas can still produce insulin, unlike those with type 1 diabetes. However, the pancreas can become overworked as it constantly tries to reduce blood glucose levels by producing more insulin; this can result in the death of beta cells, leading to type 2 diabetes patients requiring insulin shots. Gestational diabetes occurs in pregnant women who did not have a problem with high blood sugar before the pregnancy. There is an increased risk of adverse outcomes in the mother, fetus, or newborn baby when hyperglycemia occurs during pregnancy.

1.3.1 Advanced Glycation End Products (AGEs)

Hyperglycemia has been shown to cause injury to cells by increasing the presence of advanced glycation end products (AGEs). Many diabetic complications worsen due to advanced glycation including nephropathy, retinopathy, and neuropathy because AGEs will accumulate in the kidneys, retina, and blood vessels. Three stages occur during the formation of these end products: early, intermediate, and late. Early-stage glycation involves a reaction between glucose and a free amino group to form an unstable compound known as a Schiff base. The Schiff base is rearranged to form an Amadori product, a more stable compound. The intermediate stage begins with the degradation of the Amadori product to form different dicarbonyl compounds. Finally, AGEs are formed during the late stage of glycation. AGEs can accumulate on proteins, causing them to impede their function. One of the most common AGEs is N-carboxymethyl lysine (CML), and it is believed to represent protein damage caused by diabetes. The AGE formation process is shown in Figure 7.
1.3.2 Diabetes and MAC

The etiology of MAC is unknown, but research has shown that there are relations between MAC and diabetes. However, linking diabetes to MAC has proven to be difficult due to confounding factors, including cardiovascular disease and kidney disease. Qasim et al. studied the prevalence of MAC in type 2 DM patients that have no kidney or cardiovascular disease. They determined that the amount of time the patient had diabetes was associated with the presence and severity of MAC. They also discovered that older patients and females have a greater chance of developing MAC. However, whether or not diabetes expedites the progression of MAC needs to be investigated further.

Figure 7. AGE formation.
1.4 Mitral Valve Tissue Engineering

Current methods to repair diseased heart valves include mechanical or bioprosthetic heart valve replacements. Both solutions have many limitations: mechanical valve replacements require lifelong use of anticoagulant medication, and bioprosthetic valve replacements are less durable and need more frequent valve replacements. Because of this, the field of tissue engineering and regenerative medicine has been rapidly growing recently. The goal of tissue engineering is to repair or replace damaged organs or tissues using biological substitutes; a combination of cells, scaffolds, and bioreactors is used to achieve this goal. In vitro tissue-engineered models also utilize cells, scaffolds, and bioreactors to mimic the in vivo environment or act as disease models.

1.4.1 Cells

There are many different cell sources used in heart valve tissue engineering. Autologous cells are harvested from the patient and provide the best biocompatibility as they do not induce an immune response\textsuperscript{29}. Xenogenic and allogeneic cell sources can also be used. Allogeneic cells are the best option if cells cannot be harvested from the patient. The risk of rejection is a concern when using allogeneic cells, but genetic altering can reduce this risk\textsuperscript{29}. Xenogenic cells are more likely to trigger an immune response from the patient and have the potential to transfer viruses from the host animal to the patient\textsuperscript{29}.

Stem cells are the future of tissue engineering. They have two distinctive characteristics that make them good cell sources for heart valve tissue engineering: self-renewal and differentiation. After harvesting autologous stem cells from the patient, a donor, or an animal, they can be expanded in vitro and then seeded onto a scaffold\textsuperscript{30}. Mesenchymal stem cells and endothelial progenitor cells are two types of stem cells used in heart valve tissue
Mesenchymal stem cells can be derived from different tissues including bone marrow and adipose tissue. A living trileaflet valve has been assembled in vitro using bone marrow-derived mesenchymal stem cells. Adipose tissue-derived mesenchymal stem cells express similar characteristics as endothelial progenitor cells, including the ability to differentiate into endothelial cells. One concern regarding the use of stem cells is the creation of teratomas, a germ cell tumor.

### 1.4.2 Scaffolds

Even with more advancements in stem cell research, the preferred method of creating a tissue-engineered heart valve is using acellular scaffolds. Selecting the appropriate material for a scaffold is essential as the scaffold will provide structure and support for the attachment and growth of cells. There are two main approaches to developing a scaffold: pre-made porous scaffolds and decellularized extracellular matrix (ECM) from allogenic or xenogenic tissue.

Pre-made porous scaffolds are usually made from natural or synthetic biomaterials. ECM obtained from allografts or xenografts is the primary source of biological scaffolds, as they contain proteins, such as collagen or fibrin, and other organic polymers. One significant advantage to natural scaffolds is their excellent biocompatibility which allows cells to attach and proliferate. However, they cannot stabilize physically or mechanically, making them unavailable for specific load-bearing applications. A solution to this limitation is to crosslink the material or develop a composite with a synthetic material. Polymers such as polyglycolic acid (PGA) and polylactic acid (PLA) are currently used as materials for synthetic scaffolds. It is easier to control these materials' mechanical and physical properties, allowing the scaffolds to be tailored to specific environments. A drawback to synthetic scaffolds is their biocompatibility, leading to difficulty in cell attachment and growth. Processes, such as coating a synthetic
scaffold with collagen, have been developed to modify surface and bulk properties to improve biocompatibility. This approach to creating scaffolds allows for a wide variety of biomaterials to choose from as well as determining specific microstructure and architecture of the scaffold. However, pre-made porous scaffolds lead to inconsistencies in cell distribution.

ECM derived from allogenic or xenogenic sources produces scaffolds most like natural tissue. Decellularization of a tissue removes all cellular antigens from that tissue while preserving the ECM components and structure. The likelihood of an immune response decreases when the cellular antigens are removed. Not only are decellularized scaffolds biocompatible, but they also contain growth factors that facilitate cell growth. However, there is a concern about the homogeneity of cell distribution after cell seeding into a decellularized scaffold, as well as insufficient removal of the cellular components.

1.4.3 Bioreactors

A bioreactor allows for a controlled in vitro environment that mimics the in vivo chemical and mechanical environment of the heart valve. They can be used in different ways: to prepare a scaffold for implantation, to study the pathophysiology of a valve, and to test potential treatments in a simulated environment.

1.5 Current Solutions to Mitral Valve Disease

1.5.1 Replacement

Mitral valve diseases such as regurgitation, stenosis, or MAC generally require surgery to repair or replace the valve. Mitral valve replacement is currently limited to valve pathologies that are difficult or impossible to repair. If pathologies such as severe MAC and progressive cardiomyopathy are present, valve replacement is preferred to reduce the adverse effects of the surgical repair. Mitral valve replacement is also used in patients who could not tolerate
reintervention surgeries to repair the valve\textsuperscript{7}. Current valve replacement options include mechanical valves and bioprosthetic valves. There are three major types of mechanical valves: caged ball, tilting-disc, and bileaflet. Examples of mechanical and bioprosthetic valves can be seen in Figure 8. Mechanical replacement valves offer excellent durability, which leads to a reduction in reoperations\textsuperscript{7,37}. There is a risk of thrombosis on the surface of the mechanical valve due to high shear stresses resulting from blood flow around the prosthetic\textsuperscript{37}. This leads to the most prominent disadvantage of mechanical valves: the requirement of lifelong anticoagulation medication\textsuperscript{7,37}. Bioprosthetic valves are usually made from stented or stentless porcine or bovine tissue\textsuperscript{37}. They do not require lifelong anticoagulation medication because there is a lower risk of thrombosis\textsuperscript{37}. However, they are significantly less durable than mechanical valves, and deterioration of the bioprosthetic valve leads to an increase in reoperations\textsuperscript{37}. The lifetime risk of reoperation for bioprosthetic valves is around 25\%, while the risk for mechanical valves is about 3\%\textsuperscript{37}. After considering the risk of reoperation percentages for each type of valve replacement, patients older than 60 to 65 years are recommended to receive bioprosthetic valves. Patients younger than 60 to 65 years are advised to receive a mechanical valve\textsuperscript{37}. 
Another mitral valve replacement method is transcatheter mitral valve replacement (TMVR). As the mitral valve has a complex shape and anatomy, this procedure can be complicated to perform. This procedure may introduce serious complications such as thrombosis and valve sealing. TMVR is typically utilized to replace valves that suffer from mitral regurgitation or stenosis. The replacement valves are designed in a manner that does not necessarily account for severe MAC. These valves include the Sapien valve by Edward Lifesciences, a balloon-expandable valve used initially for aortic valve replacement, the Melody valve by Medtronic, and the Lotus valve by Boston Scientific. While a TMVR device specifically designed to treat MAC patients does not exist yet, two current clinical trials are testing transcatheter valves in MAC patients. The SUMMIT trial is studying the Tendyne valve.
by Abbott\textsuperscript{38}. The Apollo trial studying the Intrepid valve has previously excluded MAC patients, but it recently expanded its exclusion criteria to allow MAC patients to participate in the study\textsuperscript{11,38}.

1.5.2 Repair

TMVR is not always suitable for replacing a valve with MAC because the heart will not heal as well due to a significant paravalvular leak (PVL)\textsuperscript{11}. Instead, there are other methods to repair a valve with MAC. Decalcification of the annulus provides better healing and less PVL, but there is a risk of weakening the annulus\textsuperscript{11}. Focal decalcification is a common technique to treat focal MAC, but for severe cases of MAC, other methods are preferred\textsuperscript{11}. Two decisions must be made before determining the appropriate technique: should the valve be repaired or replaced, and how will the calcified annulus be addressed\textsuperscript{11}?

Superior outcomes have been achieved by repairing the annulus rather than replacing the entire valve regarding survival and left ventricle function\textsuperscript{11}. An annuloplasty reinforces the annulus, but MAC creates problems when trying to mount the annuloplasty ring\textsuperscript{11}. However, if repair is the best method to treating MAC, the annuloplasty ring must be mounted. If the annulus is not decalcified, an annuloplasty ring can be mounted outside the calcium. If decalcification of the annulus occurs, the posterior leaflet must be detached before reconstruction of the annulus can take place. The leaflet and ring are then reattached to the annulus\textsuperscript{11}.

There are three techniques utilized for annular reconstruction. The first is the Carpentier method which involves using a dissected atrial muscle flap to cover the deconstructed annulus\textsuperscript{11}. Tirone David and colleagues reported the next technique. This involves using a bovine pericardium strip to reconstruct the posterior annulus trigone to trigone\textsuperscript{11}. The final technique
uses the anterior leaflet to patch the deconstructed posterior annulus\textsuperscript{11}. All three techniques require some level of annular decalcification before beginning reconstruction of the annulus.

If the mitral valve is overtaken with a severe case of MAC, stenosis, and mild regurgitation, then a complete bypass of the valve might be required. This can be done by inserting a graft with a valve to bypass the left atrium\textsuperscript{39} or connecting a terminally valved conduit to the left atrium\textsuperscript{3}. **Figure 9** depicts both methods of bypassing the mitral valve.

**Figure 9.** Mitral valve bypass. (A) depicts graft to bypass valve and (B) depicts a terminally valved conduit connected to the left atrium\textsuperscript{11}. 
Abbott developed the MitraClip (MC) in 2003 as a minimally invasive way to repair a mitral valve that suffers from mitral regurgitation\textsuperscript{40}. The MitraClip is a transcatheter valve repair technique in which a clip is attached to the mitral valve to aid it in closing. There are several complications that may arise which may lead to reintervention procedures\textsuperscript{40}. These complications include thrombosis, acute kidney injury, and functional or structural device failures\textsuperscript{40}.
CHAPTER TWO
MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 2D Static Cell Culture

Porcine valvular endothelial cells (pVECs) and porcine valvular interstitial cells (pVICs) were used in this study. The cells were cultured under standard conditions in Dulbecco’s Modified Eagle Medium (DMEM), which has a glucose content of 1 g/L, with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Ab/Am). High glucose (HG) media was made using D-(+)-Glucose Monohydrate to bring the glucose concentration to 5.5 g/L. Each cell type was cultured using normal glucose (NG) media and high glucose, or diabetic, media. The pVECs and pVICs were plated at cell density of 5000 cells/cm^2 and kept in an incubator at 37°C with 5.0% CO₂.

2.1.2 2D Dynamic Cell Culture

The Flexcell FX-5000C Compression System was used for dynamic cell culture. This system consists of a controller system that allows the air flow to be controlled. The air flows into a baseplate which houses four BioFlex 6-well plates. These plates have a flexible, silicon bottom. There are holes in the baseplate that allows the air to travel beneath each well plate, forcing the silicon membrane to move up. The membrane became flat once the air pressure released. pVICs and pVECs were grown in T-75 cell culture flasks until there were 1.2 million cells (200,000 cells per well) for each condition (pVICs in NG, pVICs in HG, pVECs in NG, and pVECs in HG). Each cell condition was subcultured onto their own collagen type IV treated BioFlex plate. The BioFlex plates were then placed in the baseplate, and the entire system was set in the incubator. The Flexcell was run for 3 days at a pressure of 120 mmHG and a cyclic pressure
waveform at 1 Hz. At the end of this dynamic cell culture, immunofluorescence and protein extraction was performed.

**Figure 10.** (A) Flexcell set up in incubator. (B) Baseplate with four BioFlex 6-well plates.

**Figure 11.** Diagram of how the load is applied in the Flexcell Compression System.⁹
2.1.3 Tissue-engineered Mitral Valve and Bioreactor

2.1.3.1 Decellularization of Scaffolds

Porcine hearts were retrieved from Snow Creek Meat Processing. Mitral valves were dissected from the hearts, and they went through the decellularization process immediately. The valves were incubated overnight in deionized water at 4°C. The following day, they went through three 15-minute rinses of deionized water under agitation before incubation in 0.05M NaOH for two hours at room temperature. They were then placed in decellularization solution overnight at room temperature. This solution consisted of 20 mM Tris, 0.2% Sodium Dodecyl Sulfate (SDS), 1% Triton X-100, 1% Deoxycholic Acid, sodium salt, and 0.4% ethylenediaminetetra-acetic acid and was pHed to 7.4. The valves were kept in this solution for 5 days with a solution change every day. They went through ten 15-minute deionized water rinses and a 20-minute 70% ethanol rinse before being incubated in an RNAse/DNAse solution for two nights at 37°C under agitation. There was a 24-hour solution change, and then the valves were sterilized with 0.1% peracetic acid in phosphate-buffered saline (PBS).
2.1.3.2 Seeding of the Scaffolds and Assembly of the Bioreactor

A preliminary study was performed to determine the effects of high glucose on valvular cells. pVICs and pVECs were cultured and used to seed the decellularized mitral valve scaffold. Seeding began once 10 million pVECs and 14 million pVICs were available. A mixture of thrombin and fibrinogen was added to the cells; pVECs received 500 μL of thrombin and 500 μL of fibrinogen while pVICS received 250 μL of thrombin and 250 μL of fibrinogen. pVECs got a higher concentration of the mixture to get the cells to gel faster and stick to the valve. The pVICs were injected into all parts of the leaflets, and the pVECs were dropped onto the leaflets. The valve was then rolled in the mixture to ensure it was coated in enough cells and then placed in the incubator for 30 minutes without any media. The valve was placed in a rotator in the incubator, and it rolled in NG media overnight. The following day, the mitral valve bioreactor

Figure 12. Mitral valve after one day in decellularization solution.
was assembled in a sterile field, and the seeded mitral valve was put in the bioreactor. Diabetic media was prepared and consisted of DMEM, 10% FBS, and 2% Ab/Am with the glucose concentration at 5.5 g/L. This media was loaded into the bioreactor. The bioreactor contained a ventricular chamber, atrial chamber, compliance chamber, and the seeded mitral valve. It ran for 7 days with a pressure of 120 mmHG.

Figure 13. (A) Seeding of the mitral valve. (B) Rotator set up with seeded mitral valve.
Figure 14. Bioreactor in sterile cell culture hood.
Figure 15. (A) Labeled bioreactor setup. (B) Top view of mitral valve in bioreactor.
2.2 Histological Methods

2.2.1 Tissue Sectioning

Tissue samples from the bioreactor run as well as a fresh and decellularized mitral valve were fixed in 10% formalin for 24 hours prior to tissue processing. Once the tissues were processed, the samples were embedded in paraffin for sectioning. A microtome was used for tissue sectioning where 5 μm sections were cut from each sample and placed on a microscope.

Figure 16. Diagram of how the valve was cut for different testing following the bioreactor run. Image created using BioRender.com.
slide. The slides dried for 2 hours at 56°C to ensure all water was removed. The slides could then be used for histological staining.

2.2.2 Hematoxylin and Eosin Stain

Slides were deparaffinized, cleared, and hydrated in two rinses of xylene, 100% ethanol, and 95% ethanol. After a one-minute rinse in distilled water, the slides were stained with hematoxylin for seven minutes. They were then dipped in clarifier five times and stained with bluing reagent for one minute. Following a one-minute rinse in tap water, the slides were stained with eosin for 30 seconds. The slides were rehydrated in 95% and 100% ethanol and placed in xylene until the coverslip was applied.

2.2.3 Masson’s Trichrome Stain

Slides were deparaffinized, cleared, and hydrated in rinses of xylene, 100% ethanol, 95% ethanol, and 50% ethanol. They were then kept in Bouin’s fixative for one hour at 56°C. Next, the slides were washed in running water and placed in Weigert’s Iron Hematoxylin working solution for 10 minutes. They were washed in running water for 10 minutes and placed in Biebrich Scarlet Acid Fuchsin for 2 minutes. The slides were then placed in Phosphotungstic Phosphomolybdic acid for 15 minutes and Aniline Blue solution for 5 minutes. After a quick rinse in distilled water, the slides were placed in 1% Acetic acid for 2 minutes. They were placed in 95% and 100% ethanol and cleared in xylene before a coverslip was applied.

2.2.5 Immunohistochemistry (IHC)

Slides were deparaffinized and rehydrated the following solutions: xylene, 100% ethanol, 95% ethanol, 70% ethanol and 50% ethanol. To unmask the antigen, sections were incubated for 30 seconds in drops of 0.1% proteinase K. The slides were then rinsed twice in TBS for five minutes each. Sections were incubated with drops of 0.3% H₂O₂ for five minutes. Next, to
permeabilize the tissue, the slides were rinsed twice in 0.025% Triton for five minutes. Then, the slides were rinsed twice in TBS for five minutes each and incubated in normal blocking serum for one hour in a humid chamber. The humid chamber consisted of a slide rack in a large Pyrex dish with water filling the sides of the rack and bottom of the dish, and the dish was covered with saran wrap. Drops of the primary antibody were applied to the sections; the negative control received drops of TBS instead of the primary antibody. The slides were incubated overnight at 4°C in a humid chamber. The primary antibody was tapped off, and the slides were rinsed in TBS for five minutes before the secondary biotinylated antibody was applied for 30 minutes in the humid chamber. Following two five-minute rinses of TBS, drops of the ABC complex was applied to the sections for 30 minutes. After two rinses of TBS, the sections were developed with diaminobenzidine tetrahydrochloride (DAB) and rinsed in tap water for at least five minutes. The slides were then counterstained with hematoxylin for 2 minutes. Finally, they were dehydrated and cleared using ethanol and xylene before a coverslip was applied.

2.3 Immunofluorescence (IF)

Immunofluorescence was performed on all four cell culture conditions from both static and dynamic cell culture.

2.3.1 IF on Static Cell Culture

pVICs were subcultured into two 12-well plates, one with HG media and one with NG media, so that there were 20,000 cells per well. pVECs were also subcultured into two 12-well plates at the same plating density. These well plates were placed in the incubator for two weeks, and the cell culture media (CCM) was changed every three days. To begin the IF process, the CCM was removed from each well, and the cells were rinsed with warm PBS. The cells were fixed with a solution containing 4% paraformaldehyde for 20 minutes at room temperature and
then permeabilized in 0.3% Triton X-100 in PBS for 10 minutes. Next, the cells were blocked with 5% bovine serum albumin and 0.05% Triton X-100 in PBS for one hour. After the blocking solution was removed, the cells were stained with the primary antibody and left overnight at 4°C. The antibodies used for pVICs were osteocalcin (OC) (abcam, ab13420), CML (R&D systems, MAB3247), and α-smooth muscle actin (αSMA) (abcam, Ab5694). The antibodies used for pVECs were OC, CML, and e-selectin (bioss, bs-1273R). The next day, the cells were stained with the secondary Alexa Fluor antibody followed by a DAPI stain. Images were taken immediately.

2.3.2 IF on Dynamic Cell Culture

The same procedure was followed for immunofluorescence on the dynamic cell culture. Osteocalcin (bioss, bs-4917R) was used, but all other antibodies were the same. Images were taken the day after the secondary antibody had been applied to the cells. Three wells from each BioFlex plate were used for IF – one well for each antibody.

2.4 Western Blot

2.4.1 Protein Extraction for Cultured Cells

For the cells grown under static conditions, pVICs and pVECs were subcultured onto 6-well plates (40,000 cells per well) and kept in the incubator for two weeks. After the CCM was removed and the wells were rinsed with PBS, 500 μL of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) and 5 μL of protease inhibitor were added to each well. Cells were then scraped with a cell scraper, and the scraped cells and proteins were transferred to a centrifuge tube. The mixture was homogenized for 45 seconds and stored at -20°C until it was time to perform the BCA assay to quantify the protein content.
Three wells from the dynamic cell culture were going to be used for protein extraction; however, the CCM evaporated from two wells from each plate, so proteins were extracted from only one well from each condition. The same protocol was followed for these cells.

Thermo Scientific’s BCA protein assay kit was used to determine the protein concentration in the samples. This required pipetting 25 μL of each sample and standard into a 96-well plate and adding 200 μL of the working reagent to each well. After a 30 minute incubation at 37°C, the absorbance was measured at 562 nm on a plate reader.

2.4.2 Protein Extraction for Tissue Samples

Protein extraction was performed on a tissue sample taken from the MV that was in the bioreactor. The tissue was frozen in liquid nitrogen and then pulverized. RIPA buffer was added to the pulverized tissue and homogenized. The homogenates were centrifuged at 12,000 x g for 15 minutes. A BCA assay was performed on the collected supernatant to determine the protein concentration.

2.4.3 Western Blot

The Thermo Scientific Western Blot kit was used. Concentrations of the protein samples and non-reducing sample buffer were determined from the BCA assay. Samples were then prepared and boiled for 5 minutes to allow denaturing of the proteins. While the samples were boiling, the electrophoresis chamber was assembled. This included the chamber filled with electrophoresis buffer and two gels; the entire assembly was surrounded by an ice bath to prevent the chamber from overheating. Once the samples finished boiling, 40 μL of each sample was loaded into a lane of the gel. The gel was run for 35 minutes at 200 V. While the gel was running, transfer preparation began. This included soaking two cassettes, four filter pads, and four filter papers in transfer buffer. The polyvinylidene fluoride (PVDF) membranes were cut out
using a sizing tool and activated in 100% methanol for 20 seconds followed by a 2 minute was in water. The membranes were then placed in transfer buffer until it was time to build the “gel sandwich”. Once the electrophoresis step ended, the gels were removed and placed in transfer buffer for 15 minutes. Then, the gel sandwich could be assembled; the stacking order of the sandwich was: clear side of cassette, filter pad, filter paper, PVDF membrane, gel, filter paper, filter pad, and black side of cassette. The sandwiches were placed in a transfer cell which consisted of an electrode module, ice pack, stir bar, and transfer buffer. The transfer cell was also placed in an ice bath, and it ran for 90 minutes at 90 V. Once the transfer step was complete, a blocking buffer was added to the membranes where they incubated for one hour at room temperature. The membranes were then incubated in a diluted primary antibody mixture overnight at 4°C. The next day, the membranes were washed in a tris-tween solution before being incubated for one hour in an HRP detection reagent mixture. Following two tris-tween washes, the membranes were rinsed in the ECL working solution for five minutes and then imaged using the Bio-Rad imager.

2.5 Live/Dead Assay

After the bioreactor run ended, different parts of the seeded mitral valve were used for different experiments. A live/dead stain was performed on one part of the posterior leaflet. The live/dead assay kit is used to determine cell viability. The LIVE/DEAD Viability/Cytotoxicity Assay Kit by Molecular Probes was used. The section of leaflet being tested was placed in a 6-well plate with 3 mL media. After the live/dead solution was prepared (0.5 μL Calcein AM per 1 mL PBS and 2 μL ethidium homodimer-1 per 1 mL PBS), the media was removed from the well plate, and the tissue was rinsed with PBS. The solution was added to the well plate, and it was
incubated for 20 minutes at 37°C. The tissue was imaged immediately after it was removed from the incubator.
CHAPTER THREE

RESULTS

3.1 IF on Cultured Cells

Cells that were cultured both statically and dynamically were stained with immunofluorescence to visualize specific antibodies. The proteins of interest for pVECs were OC, CML, and e-selectin, and the proteins of interest for pVICs were OC, CML, and αSMA. All images were taken at 10x magnification. Blue represents the cell nuclei (DAPI stain) while Alexa Fluor 488 stained the protein of interest green, and Alexa Fluor 594 stained the protein of interest red.

3.1.1 pVECs

Expression of CML was detected in both static and dynamic pVEC cell culture. Images of pVECs cultured in both NG and HG are shown in Figure 17. It appears that CML expression was increased in HG media for both static cell culture and dynamic cell culture. AGEs such as CML will be present under normal conditions, but the images indicate that there is an increase in the number of AGEs under hyperglycemic conditions.

<table>
<thead>
<tr>
<th>Normal Glucose</th>
<th>High Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static A</td>
<td>Dynamic B</td>
</tr>
<tr>
<td></td>
<td>Static C</td>
</tr>
<tr>
<td></td>
<td>Dynamic D</td>
</tr>
</tbody>
</table>

**Figure 17.** IF staining for CML.
OC was expressed in both NG and HG pVECs cultured in the Flexcell (Figure 18 C and D). OC expression is not visible in the static groups. When running IF on cells, there is a specific dilution ratio specified by the manufacturer of the antibody. The dilution for OC used in an IF on cultured cells is about 1:200, so 5 μL of the antibody was needed. We believe that the proper dilution was not achieved for OC as we were running low on this particular antibody. This could be the reason why this protein did not fluoresce for both HG and NG cultured cells (Figure 18 A and B). However, it could be stated that a mechanical stimulation, like one from the Flexcell, is needed for a strong expression of OC on cultured cells.

Figure 18. IF staining for OC.
The expression of E-selectin was similar for cells grown in NG and HG media (Figure 19 A vs. B and C vs. D). Expression was similar between the statically cultured cells and dynamically cultured cells. This means that the mechanical stimulation provided by the Flexcell system did not contribute to E-selectin expression in pVECs.

![Image of IF staining for E-selectin](image)

**Figure 19.** IF staining for E-selectin.

The corrected total cell fluorescence (CTCF) was determined using ImageJ software. The graph resulting from these calculations is shown in Figure 20. It is evident that the high glucose group for each antibody produced a stronger fluorescence than the normal glucose groups.
Figure 21 shows the graph created using the CTCF values for pVECs cultured under dynamic conditions. For both CML and osteocalcin, the high glucose groups produced a stronger fluorescence than the normal glucose groups. However, for E-selectin, the cells cultured with normal glucose media produced a stronger fluorescence.

Figure 20. Graph of corrected total cell fluorescence (CTCF) of pVECs under static conditions and table including the values from the graph.

<table>
<thead>
<tr>
<th></th>
<th>HG</th>
<th>NG</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML</td>
<td>5173120</td>
<td>3511450</td>
</tr>
<tr>
<td>OC</td>
<td>656302.8</td>
<td>47076.07</td>
</tr>
<tr>
<td>E-sel.</td>
<td>2757736</td>
<td>632361.8</td>
</tr>
</tbody>
</table>

Figure 21. CTCF graph for pVECs under dynamic conditions and table including the values from the graph.
3.1.2 pVICs

Expression of CML was seen in pVICs grown in NG and HG media as well as under static and dynamic conditions (Figure 22). The expression was similar between the NG and HG of the static conditions. However, for the dynamic conditions, it appears that HG condition produced a stronger CML expression than the NG group. Based on this result, it seems that diabetic conditions produce a higher number of AGEs. Mechanical stimulation, on the other hand, does not affect the number of AGEs present as both static and dynamic groups expressed CML.

Figure 22. IF staining for CML.
Figure 23 shows the results of IF staining for OC on pVICs. The OC did not stain well for the static group. We believe this is due to the insufficient dilution ratio that resulted from not having enough antibody when running the experiment. There was also little difference seen between the NG and HG groups of the dynamic cell culture. This means that pVICs may not produce a higher number of osteogenic markers under diabetic conditions. This indicates that calcification of the mitral valve annulus results from osteogenic markers expressed in the endothelial cells and not the interstitial cells.
The cells cultured in HG media under both static and dynamic conditions express more αSMA than those cultured in NG media (Figure 24). Activated VICs express αSMA more than quiescent VICs, and these results suggest that VICs become activated under diabetic conditions. The static, NG group expressed a higher amount of αSMA than was expected; this could be because of the large number of cells present, as the blue represents cell nuclei. αSMA contributes to mechanical tension in cells, and it may be expressed more in diabetic conditions. Hyperglycemia reduces the contractility of cells, so more αSMA would need to be produced in VICs to allow for proper mechanical tension and contractility.

Figure 24. IF staining for αSMA.
The CTCF values can be seen in the table in Figure 25 along with the resulting graph.

Osteocalcin produced similar values for the cells cultured in normal and high glucose media with the normal glucose producing a slightly stronger fluorescence. Both CML and αSMA produced a stronger fluorescence in the high glucose groups.

\[\begin{array}{|c|c|c|}
\hline
\text{Antibody} & \text{HG} & \text{NG} \\
\hline
\text{CML} & 7832288 & 6613106 \\
\text{OC} & 2087944 & 2850280 \\
\text{αSMA} & 6306560 & 3099532 \\
\hline
\end{array}\]

**Figure 25.** CTCF graph for pVICs under static conditions and table including the values from the graph.

The graph for the CTCF of pVICs cultured under dynamic conditions shows that all antibodies produced a greater amount of fluorescence in the high glucose groups (Figure 26). αSMA had the largest difference between the high glucose and normal glucose groups.

\[\begin{array}{|c|c|c|}
\hline
\text{Antibody} & \text{HG} & \text{NG} \\
\hline
\text{CML} & 10287859.3 & 2973685 \\
\text{OC} & 4267892.8 & 1492063 \\
\text{αSMA} & 20802082.1 & 4919742 \\
\hline
\end{array}\]

**Figure 26.** CTCF graph for pVICs under dynamic conditions and table including the values from the graph.
3.2 Western Blot

Western blot results for αSMA can be seen in Figure 27. αSMA has a predicted molecular weight of 42 kDa. αSMA was detected in the samples around 42 kDa.

**Figure 27.** Western blot results for αSMA. Lanes: 1 – ladder, 2 – static pVIC, NG, 3 – static pVIC, HG, 4 – static pVEC, NG, 5 – static pVEC, HG, 6 – static pVIC, NG, 7 – static pVIC, HG, 8 – dynamic pVEC, NG, 9 – dynamic pVIC, HG, 10 – dynamic pVIC, NG
The results from the western blot for osteocalcin are seen in Figure 28. The predicted molecular weight for osteocalcin is around 58 kDa. Osteocalcin was detected in each sample around 58 kDa.

**Figure 28.** Western blot results for osteocalcin. Lanes: 1 – ladder, 2 – static pVIC, NG, 3 – static pVIC, HG, 4 – static pVEC, NG, 5 – static pVEC, HG, 6 – static pVIC, NG, 7 – static pVIC, HG, 8 – dynamic pVEC, NG, 9 – dynamic pVIC, HG, 10 – dynamic pVIC, NG
3.3 Diabetic Bioreactor

3.3.1 Live/Dead

A live/dead assay was performed to confirm cell viability following the end of the bioreactor run. Live cells are labeled with calcein and fluoresce green. In Figure 29, two images taken at 2.5x magnification show that many of the seeded cells attached to the MV and grew while in the bioreactor.

![Figure 29. Live/dead images taken at 2.5x magnification.](image)

3.3.2 Histology

Hematoxylin and eosin (H&E) staining was performed on a fresh porcine MV, a decellularized porcine MV, and bioreactor samples. Hematoxylin stains cell nuclei blue while eosin stains ECM and cytoplasm pink. The H&E images for a fresh MV and a decellularized MV are compared in Figure 30. It is evident that the decellularization solution was effective in isolating the ECM.
Samples of the seeded MV were taken after the bioreactor run finished. After H&E staining, cells were seen in the seeded valve (Figure 31). This confirms the findings from the live/dead stain that the valve was seeded properly. A Masson’s Trichrome stain was performed to ensure that collagen had not degraded following the bioreactor run (Figure 32).

**Figure 30.** H&E images of a fresh mitral valve leaflet and a decellularized mitral valve leaflet. (A and B) were taken at 2.5x magnification and (C and D) were taken at 10x magnification.
Figure 31. H&E images of bioreactor sample. (A) was taken at 2.5x magnification and (B) was taken at 10x magnification.

Figure 32. Bioreactor tissue sample stained with Masson’s Trichrome taken 2.5x magnification.
Sections of the seeded MV were stained for osteocalcin following the IHC protocol. **Figure 33A** is the positive control for osteocalcin. Some osteocalcin can be seen along the edge of the sample. **Figure 33B** is the negative control for osteocalcin, and no osteocalcin is seen in this sample.

![IHC osteocalcin staining of bioreactor tissue sample. (A) Positive control for osteocalcin. (B) Negative control for osteocalcin. Both taken at 2.5x magnification.](image)

### 3.3.3 Western Blot

Protein samples from the static 2D cell culture were run again with the bioreactor sample. Results from the western blot for αSMA are shown in **Figure 34**. There was no αSMA detected in the bioreactor sample. αSMA was detected in the static cell culture samples around the predicted molecular weight of 42 kDa.
Osteocalcin was detected in the 2D static cell culture samples as well as the bioreactor sample around the predicted molecular weight of 58 kDa (Figure 35).
Figure 35. Western blot results for osteocalcin. Lanes: 1 – static pVEC, HG, 2 – static pVEC, HG, 3 – static pVEC, NG, 4 – static pVEC, NG, 5 – static pVIC, HG, 6 – static pVIC, HG, 7 – static pVIC, NG, 8 – static pVIC, NG, 9 – bioreactor
CHAPTER FOUR
CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

We have shown that there is an increase in αSMA expression under dynamic 2D cell culture, which indicates the activation of VICs into myofibroblasts. Myofibroblasts are cells involved in increased ECM synthesis and calcification. We detected osteocalcin via western blotting in a bioreactor sample, indicating that activated VICs could secrete bone-like proteins. An increased expression of CML and osteocalcin was seen in diabetic conditions under static and dynamic 2D cell culture. This suggests that there is formation of advanced glycation products in the ECM secreted by activated cells. Overall, we concluded that valvular cell activation induced by high glucose concentration could be detected under static conditions in monolayers. However, modifications in tissue structures leading to calcification need to be evaluated in 3D dynamic conditions that mimic the mechanical and biochemical environment of the valve.

4.2 Future Directions

Many conclusions were made following our experiments. However, we have identified areas requiring further research. For future studies, the time of the bioreactor and Flexcell experiments should be increased to see what effect time has on activation of valvular cells. These experiments should be repeated at least twice more to verify findings and allow for statistical analysis. A nondiabetic bioreactor should be run that used normal glucose media. As previously mentioned, MAC affects the posterior annulus more than the anterior annulus; the reasons for this should be investigated. We also recommend that mechanical forces are gradually applied to a tissue engineered scaffold instead of the jump from cells cultured using the Flexcell to a seeded mitral valve in a bioreactor.
REFERENCES


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