Glycosaminoglycan Stabilization in Bovine Pericardium

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Glutaraldehyde crosslinked bovine pericardium has been used for fabrication of bioprosthetic heart valves as well as cardiac patches for soft tissue repair. However, calcification and limited mechanical stability result in shortened life for the prostheses. Previous research has shown that glutaraldehyde crosslinking does not stabilize glycosaminoglycans (GAGs) and that GAGs are lost from porcine bioprosthetic heart valves[1]. Bovine pericardial tissue is composed of an amorphous network of collagen and elastin fibers, proteoglycans, and GAGs. The GAGs of bovine pericardium include dermatan sulfate, chondroitin sulfate, and hyaluronan [2].

It is hypothesized that Glut does not stabilize GAGs in pericardium and loss of GAGs may play a role in the degenerative failure of pericardial valves. Also stabilizing GAGs in the extracellular matrix of the pericardial valves may improve their function and extend their life. The objectives of this study were to 1) characterize the structure of bovine pericardium and determine the contribution of GAGs to the structure and mechanical behavior of the tissue; 2) determine the effectiveness of glutaraldehyde fixation in the retention of GAGs; 3) determine the effectiveness of GAG stabilizing fixation of Neomycin enhanced carbodiimide crosslinking chemistry; 4) determine the stability of GAGs in vivo and correlation with calcification.
DEDICATION

This work is dedicated to my family. To my grandfather, whose quiet strength I admire. He has a prosthetic heart valve, and he means more to me than any statistic. To my parents, who have always believed in and encouraged me. Thank you for loving me and nurturing my curiosity in science. To my twin sister who has always been there for me, even when we are separated by distance.
ACKNOWLEDGMENTS

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CHAPTER ONE

INTRODUCTION

1.1 Valvular Heart Disease

Almost 79.4 million people in the United States have one or more cardiovascular disease, which is almost one in every three Americans. For the year 2007 it was expected to cost $431.8 billion dollars for medical treatment of cardiovascular disease in the United States alone [3]. Cardiovascular disease includes high blood pressure, coronary heart disease, stroke, and congestive heart failure. Valvular heart disease is one such disease that can lead to heart failure and death. In 2002, nearly 99,000 inpatient valve procedures were performed in the United States [3].

The heart and cardiovascular system provide oxygen and nutrient transport to the body as well as removal of metabolic waste. The heart possesses four valves to control the unidirectional flow of blood to the heart and lungs. Two semi-lunar valves are located at the outlets of the left and right ventricle, the Aortic and Pulmonary valves respectively. As the ventricles contract, pressure increases, and the valve leaflets are passively pushed upward away from the center of the lumen allowing blood to be expelled from the ventricle during ventricular systole. During ventricular diastole the cusps passively fall back into the lumen, supporting the blood flowing in from the atria. Also two atrio-ventricular valves, the Mitral and Tricuspid, are positioned at the inlets of the left and right ventricles respectively. These valves control of blood flow into the ventricles from the atria, and are more complex than the semi-lunar valves. The atrio-
ventricular valves have chordae tendineae inserted into the free edge of the cusp and are connected to the papillary muscles and myocardium along the ventricle wall. As the ventricle contracts, the papillary muscles contract pulling the chordae tendineae to pull the valve leaflets down and together to prevent the back flow of blood from the ventricle and allowing the atria to fill [4].

Historically, rheumatic heart disease was the most common cause of valvular disease. After 1950 penicillin was prescribed to treat streptococcal infection, preventing rheumatic fever, and decreasing the occurrence of mitral valve stenosis [5]. The majority of valve procedures performed today continue to be the mitral and aortic valves. Theses procedures address valvular stenosis and insufficiency. Stenosis is defined by obstruction of blood flow, and valve insufficiency describes both regurgitation (back flow) and incompetence[6].

Mitral stenosis is still predominately caused by rheumatic fever, but can also be due to congenital conditions or calcification. Since the use of antibiotics mitral valve insufficiency has become a more prominent problem[6, 7]. Mitral valve regurgitation is due to myxomatus degeneration (mitral valve prolapse) or deposition of mucopolysaccarides in the spongiosa, increasing cuspal thickness. Dilation and calcification may also occur, further altering the structure of the valve. The increase in cuspal thickness inhibits the valve from functioning normally, as the cusps can not align to close the valve and support the blood filing the atria. The development of mitral valve prolapse is associated with acquired disorders leading to cuspal degeneration, as well as genetic disorders of connective tissue such as Marfan syndrome[4]. Only 11% of
patients with mitral valve prolapse require surgical intervention. Undiagnosed, however, asymptomatic patients have a 33% risk of heart failure and other adverse effects [6].

Aortic stenosis is the most prevalent valvular condition. Blockage of blood flow through the aorta can be a result of a congenital condition, or acquired by rheumatic heart disease and calcification[6]. Calcification of degenerative valves is frequently the cause for valve replacements in patients over 70 years of age. With calcific aortic stenosis the aortic valve area is dramatically reduced, limiting the blood flow from the left ventricle as well as increasing the pressure gradient. To compensate for the changes the left ventricle becomes hypertrophic. Other possible causes of aortic stenosis include fungal endocarditis and atherosclerosis in patients with extremely elevated cholesterol levels [4].

Aortic insufficiency, less prevalent than aortic stenosis, is present as acute and chronic aortic regurgitation. Acute regurgitation is typically the result of infective endocarditis, dissection of the aorta, systemic hypertension, or trauma. Hypertension can be controlled by pharmacotherapy, reducing the degree of regurgitation, only if permanent damage as not occurred. Valve replacement or repair is needed when there are indications for congestive heart failure. Chronic aortic regurgitation is most commonly caused by aortic root dilation, followed by congenital bicuspid valve, rheumatic fever, other congenital conditions affecting the connective tissue, and syphilis. Valve replacement depends on the severity of regurgitation. Vasodilators may be given prior to surgical replacement or alone in less severe cases to reduce the peripheral artery resistance and therefore degree of regurgitation volume[4].
1.2 The Native Heart Valve

Healthy, normal valves of the heart allow flow of blood in one direction without obstruction, allowing regurgitation, thromboembolism, or extreme stress to the leaflets. Proper function of the heart requires all components of the valve to be structurally intact and work in unison[6]. The semi-lunar valves (aortic and pulmonary) each consist of three fibrous cusps, attached at the aortic or pulmonary root, with equal space between the attachments of adjacent cusps called commisures. The ventricular surface of the free edge of each cusp contains a small nodule, which meet at the center when the valve is closed to provide resistance to mechanical load due to the pressure difference across the valve. The cusps also provide support under mechanical load by stretching and the interaction of the extracellular matrix of the individual layers of the cusp [4, 6] The aortic heart valve leaflet structure, as shown in Figure 1, is composed of three layers, the fibrous layers of the fibrosa and ventricularis are separated by the spongiosa.

The ventricularis, the layer facing the ventricular cavity, is composed of collagen with elastin fibers arranged in the radial direction through the cusp. The abundance of elastin in the ventricularis provides the elasticity needed for the cusp to contract against the wall when the valve is open reducing the surface area. Elastin also provides the tension needed to close the valve, stretching the cusp to increase the coaptation area. Circumferentially aligned collagen fibers of the fibrosa run parallel to the free edge of the cusp, provides strength as well as stiffness to each cusp to maintain the curvature and prevent the back flow of blood. The central layer of the cusp, the spongiosa, is comprised of loosely arranged collagen fibers and glycosaminoglycans. The spongiosa,
while not a source of strength in the cusp, adjusts to changes in orientation of the cusp throughout the cardiac cycle. The interactions of the network of fibers in the cusp serve to transmit the load produced by the ventricular pressure to the fibrous annulus and vessel wall. Maintenance of this extracellular matrix is accomplished by the valvular interstitial cells. Continuous synthesis, degradation, and re-organization of the ECM by matrix degrading enzymes such as matrix metalloproteinases ensure the durability and functionality of the valve [6].

![Figure 1: Internal Structure of Aortic Valve Leaflet [8]](image)

**1.2.1 Collagen**

The most abundant component of the extracellular matrix is collagen which is present in many tissues such as tendon and skin. Collagen is the tensile resistant component of the ECM and self aligns in the direction of tension [9]. It is present in the
heart valve leaflet as collagen types I, III, and IV. Collagen I is the primary element of the fibrosa as interweaving, parallel fibers. It also exists in smaller amounts in the ventricularis and spongiosa. Collagen III is located in modest amounts in all three layers as intertwined, wavy fibers. Collagen IV is also found in smaller amounts throughout the leaflet [10].

1.2.2 Elastin

Elastin fibers interwoven with collagen are prominent in the inflow layer of the ventricularis. Fibrosa and Spongiosa layers contain smaller amounts of intertwined elastin fibers[10]. The radially aligned elastin fibers place tension on the collagen of the ventricularis, and act as a spring returning the collagen to their original position after loading has been removed as indicated below in Figure 2 [8].

![Collagen-Elastin Matrix](image)

**Figure 2: Collagen-Elastin Matrix** [11]
1.2.3 Glycosaminoglycans

Glycosaminoglycans (GAGs) are linear polysaccharides of repeating disaccharide units. One monosaccharide is an amino sugar, and the other is typically uronic acid. GAGs are negatively charged and hydrophilic making them able to absorb water. The GAG chains are covalently attached to a serine residue on a core protein, resulting in molecules called proteoglycans [12]. Proteoglycans, and their negatively charged GAG side chains, are present in all three layers of the heart valve leaflet at varying levels depending on the proteoglycan. However, they are more concentrated in the spongiosa layer. The proteoglycan distribution is dependent on the type of GAG side chain as well as the type of collagen present [10]. There are three types of proteoglycans of different lengths in native heart valve tissue. The smallest proteoglycan decorin is aligned along collagen and has chondroitin and dermatan sulfate GAG side chains. Perlecan is found along the cell surface and elastin fibers and contains heparin sulfate GAG side chains. Versican, the largest proteoglycan present, also contains chondroitin and dermatan sulfate GAG side chains. Hyaluronate is a GAG that is not bound to a protein core [13].

The mechanical role of glycosaminoglycans in heart valve tissue is still being established. Vyavahare, et al. have shown that fatigue of collagen films and glutaraldehyde crosslinked porcine aortic bioprostheses results in loss of GAGs as well as structural changes in collagen. Mechanical testing following fatigue demonstrated a reduction in flexural rigidity indicating a loss of the natural stiffness and susceptibility to failure [14]. Removal of GAGs from cusps results in decreased cusp thickness, water content, as well as decreased ability to re-hydrate [1]. Fatigue is not the only mechanism
resulting in GAG loss. Naturally there is a high turn-over rate of GAG synthesis[15]. There are also large quantities of GAG degrading enzymes in natural cusp tissue [16].

Figure 3: Glycosaminoglycan structure

1.3 Heart Valve Replacements

Diseased heart valves require either surgical repair or replacement. The ideal replacement would function similar to the native valve, allowing uninhibited forward flow when open, but preventing backward flow (regurgitation) when closed. The replacement valve would also not cause turbulent blood flow, but limit hemolysis and the coagulation cascade. The prosthetic valve should be biocompatible, having non-thrombogenic blood contacting surfaces. Ease of implantation with low morbidity rates is essential. Durability for the patient lifetime is critical to avoid repeat operation because of device failure. Finally, the ideal prosthetic valve would not affect the daily
activity of the patient. The quality of life for the patient would be improved if no anticoagulation therapy was necessary, no noticeable sounds were produced by the valve, and if there was a low risk of endocarditis[17-19].

**Table 1:** Characteristics of the ideal heart valve replacement[18, 19]

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<td>Non-thrombogenic</td>
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<tr>
<td>Infection resistant</td>
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<tr>
<td>Chemically inert and non-hemolytic</td>
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<tr>
<td>Extended durability</td>
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<tr>
<td>Easily implanted</td>
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<td>Appropriate healing at prosthesis interface</td>
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Current replacements are limited to mechanical or tissue valves. Both have specific advantages and disadvantages and depending on the patient’s age, sex, and health one type may be better suited than the other. There are concerns of durability for both young and old patients, as well as the patients’ ability to take anti-coagulation medication [20]. There are multiple mechanical and bioprosthetic valves currently approved by the FDA for use as shown in Tables 2 and 3.
1.3.1 Mechanical Heart Valve Replacements

Mechanical heart valves were introduced in the 1950s with the design of the ball in cage valve. The most notable being the Starr-Edwards ball valve. The initial design has remained almost unchanged except for iterations in the design for example the Lucite cage was replaced by a satellite metal cage with a silicone-elastomer ball. The first ball in cage valve implantation occurred in the 1960’s, and now with over 250,000 Starr-Edwards valves implanted worldwide as of 2003 [21]. Advantages of the caged ball design include that no audible sound is produced by the valve opening and closing. Disadvantages however, are that turbulent blood flow is induced by the valve and therefore necessitates anticoagulation therapy. The high profile of the cage also causes complications as it extends into the ventricle or aorta depending on implant site[17]. With the advent of new designs including the tilting disk and bileaflet, use of the Starr-Edwards valve has been discontinued in the United States, but it is continued to be used in less developed countries due to the reasonable cost [21].

Table 2: FDA Approved Mechanical Heart Valves

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In the late 1960’s mechanical valve design experienced advancement with the use of pyrolytic carbon and the concept of the tilting disk to replace the ball-in-cage valve. The Bjork-Shiley tilting disk valve was the first of its kind but now three tilting disk valves have been approved by the FDA for use in the United States [21]. The disk of each consists of graphite which is coated with pyrolitic carbon. The disk is housed in a circular ring with a sewing cuff as shown in Figure 4.

The latest and most commonly implanted mechanical valve is the bileaflet valve. Initial prototypes were developed in the late 1960’s. The bileaflet valves currently used include the St. Jude Medical and CarboMedics valves which were first implanted in 1977 and 1986 respectively [21]. These offer a wider opening angle than the tilting disk design, but still have the common complication of thrombosis necessitating anticoagulation therapy[17].

The ultimate choice for heart valve prosthesis depends on the patient conditions and surgeon’s preference. Although mechanical valves have better durability, there is still a risk for thrombo-embolism and life long anticoagulation treatment is required. For
children with bioprosthetic heart valves the chances for calcification are much greater, as well as either valve will not grow with the patient making repeat surgery necessary[20].

1.3.2 Bioprosthetic Heart Valve Replacements

Mechanical valves require life-long anticoagulation, and have altered hemodynamics, while tissue valves have hemodynamics similar to the native valve and typically require anticoagulation only temporarily after surgery. In the 1960’s allografts began the use of tissue valves for replacement of diseased tissue. Carpentier, in 1972 developed the technique for using chemically treated tissue for valve replacement, establishing the bioprosthetic valve. There are currently three approved types of bioprosthetic valves: bovine pericardial valves, porcine aortic valves (with and without stents), and allografts. FDA approved bioprosthetic valves are listed in Table 3 [17].

**Table 3: FDA Approved Bio-prosthetic Heart Valves**

| Bioprosthetic | Stented (Porcine) | Angell - Shiley (AS) xenograft  
|              | Stented (Bovine)  | Edwards S.A.V.  
|              | Stentless         | Medtronic Standard & Mosaic  
|              | Homografts        | GJM Biocor & Epic  
|              |                   | Carpentier - Edwards Perimount  
|              |                   | Ionescu - Shiley Standard  
|              |                   | CryoLife O'Brien & SynerGraft  
|              |                   | Edwards Prima Plus  
|              |                   | Medtronic Freestyle  
|              |                   | SJM Toronto SPV & Quattro  
|              |                   | CryoLife Homograft |
The benefits of bioprosthetic grafts include decreased thrombogenicity, no sound produced by the valve movement, and only short-term anti-coagulation is required. Despite these advantages tissue valves are not ideal because they do not last over 15 – 20 years. Failure occurs uniformly across bioprosthetic valve types due to structural deterioration, leaflet wear, and calcification[17].

Figure 5: Edwards Life Sciences Bioprosthetic Valves, 1) Bovine pericardial stented valve, 2) porcine stented valve, and 3) porcine stentless valve (www.lifeisnow.com)

First generation porcine bioprosthetic valves include the Carpentier-Edwards and Hancock (Medtronic) valves, which were available in the 1970’s. Fresh porcine aortic valves were chemically treated in glutaraldehyde at high pressure to maintain valve coaptation and were then mounted on stents. The second generation porcine xenografts (Carpentier-Edwards duraflex and Hancock II) improved long-term durability by treating tissue with anto-mineralization agent, and fixing at lower pressures to not distort the collagen fiber architecture[17, 20, 22-25].

Bovine pericardial bioprosthetics first prototype was the Ionescu-Shiley valve in 1970, but due to accelerated deterioration implantation was stopped. Carpentier improved the valve by mounting the tissue to a flexible stent, and modifying the
mounting procedure to reduce shear stress and tears. From this development came the Carpenter-Edwards Perimount that is currently available[17].

Tissue valves can be either allograft or bioprosthetic which are constructed from porcine valves or bovine pericardium. While having improved hemodynamics, these valves fail within 15 years because of calcification and loss of mechanical durability[26]. The devices are sufficient for elderly patients with a shorter life expectancy; however, the current devices are insufficient for pediatric patients or patients whose valves must last much longer than 15 years[17].

1.4 Pericardial Tissue

1.4.1 Components and Structure

Bovine pericardium is used as a biomaterial in numerous prostheses such as cardiac patches, replacement chordae tendinae in mitral valves, reconstruction of ligaments in the knee and hand, and most prominently as leaflets for heart valves [27]. Pericardial tissue is the sac surrounding the heart which provides a natural barrier to infection for the heart and prevents adhesions to the surrounding tissue. The pericardium also serves mechanical roles by preventing over dilation of the heart, maintaining the correct anatomical position of the heart, and regulating the pressure to volume ratio in the left ventricle during diastole[27-29]. The structure of the tissue determines its behavior under loading in both conditions physiologic to the pericardium and as a prosthetic device. The two layer serous membrane is divided by the pericardial cavity which contains a lubricating fluid. The outer layer, the parietal pericardium, is easily removed
from the heart and as such is the portion used in bioprostheses. The parietal pericardium is attached to the sternum by sterno-pericardial ligaments and the diaphragm by loose fibrous tissue. Removal of fatty cells and connective tissue leaves the fibrosa, the portion which provides the mechanical properties to the tissue[27].

![Figure 6: Structure of Pericardial Tissue](image)

The fibrosa is composed of multiple layers of collagen bundles which vary in direction with elastin interwoven through the layers. It is interesting to note that in human fetus pericardial tissue the collagen fibers are straight and delicate; as elastin develops the collagen becomes wavy. With age, as the tissue becomes less organized, the collagen fibers lose their waviness [30]. This as well as other studies indicate the dynamic nature of pericardial tissue, in that the structure is modified to compensate for
mechanical loading in the normal physiologic conditions. Loading as well as environmental and other factors result in structural variation between animals as well as within the sac [27]. Pericardial tissue is dynamic in that its structure is modified to compensate for mechanical loading in its normal physiologic conditions. Therefore there are environmental and other factors that affect the tissue structure resulting in variation between animals, as well as within the sac [27, 31].

Variations within the sac and amongst animals include differences in fiber alignment, extensibility, and thickness. These and other factors have been examined and categorized noting preferred areas of selection [32-37]. With some variation, the tissue over the left ventricle has been suggested by many studies to be the most optimum as this area also experiences the greatest mechanical stress [33, 34]. However individual analysis of the tissue structure would provide more uniform and accurate selection of tissue by alignment [33, 36].

1.4.2 GAGs in Pericardial Tissue

The pericardial matrix is 95% laminated collagen fibers (types I and III) interwoven with elastin (4%) and small proteoglycans with glycosaminoglycans (~1%).[38, 39]. Proteoglycan containing dermatan sulfate which is 4-sulfated, is the most abundant in the pericardial tissue[40, 41]. Other GAG disaccharides found in the tissue include hyaluronic acid, and 0-sulfated glycosaminoglycans [2]. The number sulfated corresponds to the position on the carbon ring of the sulfate group. The repeating disaccharides are covalently bound to a core protein (except hyaluronan). The sulfate and
carboxyl groups of GAGs are negatively charged rendering the tissue hydrophilic. This hydrophilicity may plan an important role in the water content and subsequent viscoelasticity of the tissue [2]. Viscous fluids always experience movement as a response to shear forces. Viscoelastic solids typically exhibit signs of stress relaxation and creep with a distinct hysteresis loop. Hysteresis is present when the response on the stress strain curve varies between loading and unloading, indicating the ability to dissipate mechanical energy[27].

Figure 7: GAG Disaccharides: (A) hyaluronic acid, (B) dermatan sulfate, (C) chondroitin sulfate, (D) heparan sulfate, and (E) keratan sulfate.

The mechanical role of GAGs in the pericardium has been investigated by Mavrilas et al. After enzyme removal of GAGs from glut fixed pericardium, the tissue was tested under dynamic tensile testing. However, the difference in hysteresis was not significant after removal of GAGs. However this could be due to incomplete removal of GAGs as only 17% of the total GAGs were removed [2].
1.4.3 Mechanical Properties of Bovine Pericardium

With regard to mechanical durability, further evaluation of pericardium as a biomaterial has been investigated to determine the safety of a potential chemical treatment or origin of the tissue. Many testing methods have been utilized to evaluate bovine pericardial tissue properties which include uniaxial tensile testing of ultimate strength [42], high strain rate testing at both small and large deformations [43], shear testing [44], compressive buckling[45], flexural mechanical response by three-point bending [46], and dynamic cyclical uniaxial loading for extended duration [47]. The multidirectional fibers are the cause of the anisotropic property of the tissue. As a result mechanical loading in one direction will have varying outcomes depending on the alignment and orientation of the test selection[48-50].

The tearing strength of pericardium under uniaxial loading indicates that the tissue damage occurs with or without tears by de-lamination of the layers, shear, and rupture of the tissue [42]. High strain rate testing provides better analysis of the viscoelastic properties of fixed pericardium with evaluation at both small and large deformations [43]. Shear testing indicates that glut fixed bovine pericardium has a high resistance to shear possibly due to the increased stiffness and disruption of collagen fibers caused by glut fixation [44]. Compressive buckling of pericardium showed that greater compressive forces are required to cause collapse as compared to porcine leaflets, because of the dense layering of collagen in pericardium [45]. Three-point bending found that native and glut fixed bovine pericardium were more stiff when flexed in the direction perpendicular to the preferred fiber orientation, which would suggest that the
flexural properties of pericardium are due mainly to the cross links between fibers and not the actual collagen fibers [46]. Cyclical loading provides an indication of the tissues’ response to fatigue and suggest the importance of the original collagen orientation [47].

1.5 Bioprosthetic Valve Failure

1.5.1 Non-Calcific Deterioration

The majority of bioprosthetic valves fail either because of structural deterioration, calcific deterioration, or both. Structural deterioration of prosthetic valves leads to stenosis and regurgitation which requires undesirable immediate surgical treatment and repeat procedures. Non-calcific deterioration in many cases is a result of valve design or material selection. Results also indicate that damage to collagen fibers and tearing of cusps can occur independently of calcification [51, 52]. Early pericardial bioprostheses experienced failure after punctures and tears in the tissue near points of attachment on the stent [53, 54]. Abrasion of the tissue on struts of the stent or Dacron coating cause regions of damaged collagen bundles [55]. This abrasion and shearing of tissue also results in tearing of the valve cusp at contact with suture which produces points of concentrated stress [27, 56]. Modifications to the design of pericardial valves improved the resistance to tears at the commisures with the Carpentier-Edwards stented bovine pericardial valve which secures the cusp from inside the frame of the stent [57]. Other valve design changes include lower profile stents that are more flexible [18]. Although design alterations can improve resistance to tearing, failure due to deterioration has not been eliminated.
The presence of tears and structural degeneration is also due to material wear. The dynamic loading of cusps results in damage to the extracellular matrix [55]. The cyclical loading of valve cusps, during opening and closing, results in buckling and rupture of collagen fibers [58]. Although porcine bioprostheses tend to be more susceptible to buckling as pericardial tissue is more dense and lacks the loose network of the spongiosa found in the aortic cusp, which collapses due to compressive forces [45]. Mechanical loading also accelerates the enzyme degradation of bovine pericardial tissue resulting in damage to the matrix from two sources [59]. This furthers the idea that one single act does not typically cause the failure of a valve. Degeneration and ultimate failure of prosthetic valve tissue is a multifaceted occurrence, with many factors affecting the durability and lifetime of a specific valve.
Shearing of the layers of prosthetic valves may also play a role in tears of cusps and valve failure [27]. The viscoelasticity of the tissue affects the mechanical properties of the tissue and its resistance to shear. Recently GAGs have been studied in relation to their role in valve mechanics and failure. Examination of failed implanted porcine aortic valves revealed a loss of GAGs [60]. Fatigue of glut fixed porcine valves also showed a loss of GAGs and damage to collagen fibers [61]. The removal of GAGs from porcine tissue has permanent affects on the mechanical properties of the tissue [62].

1.5.2 Calcification

Calcific structural deterioration of bioprosthetic valves is slightly more prevalent than non-calcific deterioration[57]. The progression of calcification results in compromised tissue structure. The accumulation of calcium typically occurs because non-viable cells of the prosthetic valve are not able to sustain the pre-existing lower concentration of calcium in the cell. Calcium binds to the phospholipids membrane of organelles creating mineralization. Extracellular matrix components of collagen and elastin as well as debris can provide additionally locations for calcium nucleation [57]. The presence of glutaraldehyde in the tissue makes the tissue more susceptible to mineral deposition [63, 64]. It is also important to note that calcification of elastin occurs whether it is crosslinked or not, while calcification of collagen is dependent on the presence of aldehyde cross linking [61, 65].

Mineralization of the tissue begins with small deposits and progresses to where tissue thickness can double after 112 days of implantation [66]. As the deposits grow,
nodules can pierce through the tissue surface inducing further structural damage [66]. The effects of calcification and structural deterioration may be a cooperative progression. The mechanical stress caused by loading and unloading of cusps has resulted in increased calcification [67, 68]. Calcium deposits have also been found in areas of high movement and stress such as the commissural region [64, 66]. The disruption of the tissue matrix can expose and produce new sites for calcium deposition, while the damage from calcification results in increased structural disruption [18]

![Image](image.jpg)

**Figure 9:** Calcified Bovine Pericardial Bioprosthetic Heart Valve [26]

Pathological mineralization of the prosthetic tissue resembles that of the physiological calcification of bone. Deposition of calcium phosphate, closely related to hydroxyapatite, at the cell membrane as well as calcification of the extracellular matrix occurs [18, 66]. Key molecules in bone formation include osteopontin, osteonectin, and
Osteocalcin. Osteopotin, an acidic calcium binding phosphoprotein [69, 70], has been found in calcified bioprosthetic valves as well as calcified native heart valves [71-73].

Studies of cartilage have initiated the theory that GAGs and proteoglycans may play a role preventing tissue mineralization by binding calcium and preventing the nucleation of hydroxyapatite [74]. One hypothesis is that the loss of GAGs may result in voids or areas available for calcium nucleation [75]. In bioprosthetic tissue, removal of GAGs resulted in increased calcification of bovine pericardium [76]. Preservation, stabilization and addition of GAGs has also proved to inhibit calcification [77-79]. However, other studies have indicated no prevention of mineralization with alternate stabilization of GAGs [80, 81]. Thus the role of GAGs in bioprosthetic valve calcification remains unknown.

1.6 Glutaraldehyde Fixation

Glutaraldehyde (1, 5-pentanedialdehyde) (Glut) fixation has been the standard since tissue bioprosthestic valves became clinically available in 1970. Glut crosslinking forms Schiff base bonds with the primary amines of amino acid residues lysine and hydroxylysine on collagen, while other extracellular matrix components lack the amine functionalities required for crosslinking [18, 82]. These degradation inhibiting bonds render the tissue more stable against mechanical loading. Glut also improves the tissue antigenicity and makes the tissue thromboresistant eliminating the need for anticoagulation treatment [18, 82]. To some extent Glut will sterilize the tissue against bacteria, fungi, and viruses, but depends on the concentration, pH, temperature and
duration of the fixation [83]. At low concentrations Glut has poor anti-bacterial and anti-fungal properties [84]. Disadvantages of Glut crosslinking include the cytotoxic effects and the ability to induce calcification [31]. Physical changes and autolysis are also complications with the use of Glut [18, 82]. Treated bioprosthetic tissue varies from native healthy tissue in that there is a loss of surface endothelium or mesothelium, bovine interstitial cells undergo autolysis, collagen bundles loosen, and there is a loss of GAGs [85]. Glut crosslinking does not stabilize GAGs and they are lost after storage from porcine aortic heart valves [1]. Glut fixation eliminates the tissues’ natural ability to remodel and scavenge cell debris making it more susceptible to mineralization. In complete binding also leaves aldehyde residuals which are cytotoxic. Glut’s ability to induce calcification and ultimate failure is the main challenge with bioprosthetic heart valves that remains to be eradicated.

1.7 Anti Calcification Technologies

There are three main approaches to anti-calcification treatment, systemic therapy, local therapy or drug delivery, and modification biomaterials. Systemic therapy by diphosphonates has been explored; however, they interfere with physiologic calcification in normal bone growth [86]. Local delivery of ethane hydroxybisphonate was successful at reducing calcification with limited undesirable effects [87, 88].
1.7.1 Modification of Biomaterials

Modification of biomaterials is the main approach taken to prevent calcification either by treating the glut fixed tissue or using an alternative fixation. Anti-calcification modifications to glut fixed tissue include the use of diphosphonates [87], metal ions such as aluminum and iron [89-91], amino-oleic acid [92, 93], surfactants such as sodium dodecyl sulfate to remove acidic phospholipids [94], and incubation in ethanol [14, 95]. Ethanol pre-treatment prevents calcification by removing phospholipids and cholesterol, but permanently alters the conformation of the collagen [14, 95]. More recent research shows that the use of octanol storage after glut fixation preserves the collagen triple helical structure unlike ethanol, but does not compare the ability to reduce calcification [96]. Multiple stage anti-calcification fixation with ethanol pretreatment, glut fixation, followed by detoxification with L-glutamic acid monosodium salt and extraction of lipids by chloroform and methanol resulted in a significant reduction of calcification with less visible changes to the structure and improved stability [97]. Decellularization is another means of reducing calcification by removing the entire cell as mineralization begins in the phospholipid membranes of non-viable interstitial cells [39, 98]. Removal of cells may only reduce initial calcification as host cells infiltrate the tissue and become devitalized.

1.7.2 Alternative Fixation Chemistries

Modified and alternative fixations have sought to find better means for sterilizing bioprosthetic tissue without the toxic effects of Glut. The key requirements for such
Fixations include that they must be effective in preventing adverse effects, systemic and local toxicity, thrombosis, infection, immune response, and structural deterioration. Fixation must also not alter normal valve hemodynamics [18]. Modification to the fixation process to include lysine treatment neutralizes glut residues and reduces calcification [99-102]. Alternative crosslinking chemistries include the use of epoxides, carbodiimides, and acyl azides which reduce the calcification of tissue [18, 95, 103-106]. Triglycidylamine (TGA), a poly-epoxide, treated tissue has improved stability and biomechanical properties compared to glut fixed tissue, but still some calcification [107]. TGA fixation in conjunction with 2-mercaptoethylidene-1, 1-bisphosphonic acid treatment eliminates calcification in long term implants [108]. Other novel means of fixation of tissue for implantation include photo-oxidative crosslinking [109-111] and ultraviolet light treatment [112].

The role of GAGs in preventing bioprosthetic tissue calcification is not entirely known, but studies have found that with preservation of GAGs there is a reduction in tissue calcification. Alternative crosslinking with the addition of glycosaminoglycans has proved to reduce calcification [113, 114]. GAG targeted fixation has been accomplished by pre-treating tissue with sodium periodate which resulted in reducing calcification[77]. Further studies have shown that with carbodiimide/N-hydroxysuccinimide (EDC/NHS) treatment GAGs were preserved, but there calcification was still significant [80]. With the addition of neomycin sulfate pretreatment to EDC/NHS glut crosslinking, significantly more GAGs were retained in porcine tissue, however calcification was not completely reduced [81].
CHAPTER TWO
EXPERIMENTAL PLAN

2.1 Research Hypothesis

Previous studies have shown that Glut cross-linking does not stabilize glycosaminoglycans (GAGs) and that GAGs are lost from porcine bioprosthetic heart valves [1]. We hypothesize that Glut does not stabilize GAGs in pericardium and loss of GAGs may play a role in the degenerative failure of pericardial valves. Also stabilizing GAGs in the extracellular matrix of the pericardial valves may improve their function and extend their life. To evaluate these hypotheses, this work was divided into four specific aims.

2.2 Research Aims

Aim 1: To characterize bovine pericardial tissue structure, and to determine the contribution of glycosaminoglycans to the structure and mechanical behavior of the tissue. Characterization of the extracellular matrix and GAGs of the tissue at four specific sites was conducted by histological staining. The site specific GAG content and disaccharides present were also assessed.

The structure of the matrix and presence of GAGs was examined through transmission electron microscopy. Total GAG and hexosamine values were also quantified. The role of GAGs was evaluated through mechanical testing before and after treatment with GAG degrading enzymes.
Aim 2: To determine the effectiveness of glutaraldehyde fixation in retention of glycosaminoglycans. The retention of glycosaminoglycans after fixation and storage in Glut as well as the resistance to GAG degrading enzymes were quantified.

Aim 3: To determine the effectiveness of glycosaminoglycan stabilizing fixation. The effect of Neomycin trisulfate on the stability of glycosaminoglycans was examined. Also stability and resistance to enzyme were evaluated after fixation and storage. Finally, the effect of Neomycin on pericardial tissue properties including the water content, collagen stability, and elastin stability were investigated.

Aim 4: To determine the stability of glycosaminoglycans in vivo and correlation with calcification. Subdermal implantation of cross-linked tissue was performed to evaluate the calcification of tissue as well as the retention of glycosaminoglycans.
CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

Ammonium acetate, neomycin trisulfate salt hydrate, glutaraldehyde (50% stock), hyaluronidase (from bovine testes, type IV-s, 3,000–15,000 U/mg), chondroitinase ABC (from Proteus vulgaris, lyophilized powder, 50–250 U/mg), D(+) glucosamine-HCL, collagenase Type VII from Clostridium histolyticum, 1-9-dimethylmethylen blue (DMMB) were all purchased from Sigma-Aldrich Corporation (St. Louis, MO). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), n-hydroxysuccinimide (NHS) were acquired from Pierce Biotech (Rockford, IL). Acetyl Acetone, p-dimethyl aminobenzaldehyde, and 4-(2-hydroxyethyl)-1-piperazinoethanesulfonic acid (HEPES) were bought from Fisher Scientific (Fair Lawn, NJ). 4-morpholinoethanesulfonic acid hydrate (MES) hydrate was purchased from Acros Organics, NJ. Fresh bovine pericardial tissue was obtained from a local abattoir, Snow Creek Meat Processing, Seneca, SC.
3.2 Methods

3.2.1 Bovine Pericardium Collection and Fixation

Fresh bovine hearts, with pericardium intact, were collected from a local abattoir. The pericardial tissue was dissected removed from the heart by cutting along the base of the heart, leaving the remaining tissue intact, and transported to the laboratory in un-buffered saline. The tissue was rinsed, and trimmed of external fat in the laboratory.

Glutaraldehyde fixed bovine pericardium (GLUT) was prepared by fixing the fresh tissue (within 4 hours of collection) in 0.6% glutaraldehyde. Fresh pericardium was placed in 0.6% glutaraldehyde in 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered saline solution at a pH of 7.4 for 24 hours. The tissue was then transferred to 0.2% glutaraldehyde in HEPES buffered saline for storage at room temperature.

Carbodiimide treated bovine pericardium (EDC) was fixed using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide HCL (EDC) and N-Hydroxysuccinimide (NHS). Fresh pericardium was immersed in a 30mM EDC/ 6mM NHS solution buffered with 50mM MES at pH 5.5 for 24 hours at room temperature. After 24 hours fixation the tissue was rinsed, fixed and stored with the standard GLUT fixation.

Bovine pericardium was treated with glycosaminoglycan stabilizing agent, Neomycin Trisulfate (NEO) for 1 hour. Fresh tissue was covered with 1mM Neomycin in – buffer at a pH of 7.4. After one hour the tissue was rinsed and then fixed in 30mM
EDC/ 6mM NHS for 24 hours, followed by standard fixation and storage in glutaraldehyde.

For site specific studies the fresh bovine pericardium was oriented based on the pericardiodiaphragmatic attachments and the sternopericardial ligaments and cut into four regions as shown below in figure 10 [34]. The four regions were defined to be Posterior Left Ventricle (PV), Posterior Right Ventricle (PR), Anterior Right Ventricle (AR), and Anterior Left Ventricle (AL).

![Figure 10: Schematic diagram of bovine pericardial excision and orientation [34]](image)

### 3.2.2 Enzymatic Removal of Glycosaminoglycans

Pericardial tissue was removed from storage solutions and samples were punched out using a 5/16” diameter die. The pericardial disks were then rinsed in 100mM Ammonium Acetate buffer (pH 7.0). GAGs were removed from the tissue by incubating the samples in 1.2 ml of 10 U/ml hyaluronidase and 0.2 U/ml high purity chondroitinase ABC in 100mM Ammonium Acetate buffer. Undigested samples as controls were placed in 1.2mL of Ammonium Acetate buffer. All samples were shaken at 650 RPM for 48
hours at 37°C. After incubation, the samples were rinsed three times in distilled water. The tissue was then frozen at -80°C for one hour and then lyophilized for at least 24 hours.

### 3.2.3 Quantification of Glycosaminoglycans by Hexosamine Assay

Lyophilized tissue was weighed to obtain the dry tissue weight. Samples were then hydrolyzed in 2 ml of 2M HCl for 20 hours at 95°C. After hydrolysis, the samples were dried under nitrogen gas flow in a boiling water bath until only a brown residue remained in the glass tube. Glucosamine HCL standards (0 – 200 µg/sample) were prepared in 1M NaCl for a total volume of 2 ml. The sample hydrolysates were also dissolved in 2 ml of 1 M NaCl. 2 ml of 3% Acetylacetone in 1.25 M Sodium Carbonate was added to all samples and standards and heated for 1 hour at 96°C. After cooling to room temperature, 4 ml of 100% ethanol was added, and followed by the addition of 2 ml of Ehrlich’s reagent (0.18M p-dimethylaminobenzaldehyde in 50% ethanol containing 3N HCL). The samples were kept for 45 minutes at room temperature before measuring optical absorbance at 540 nm on a microplate spectrophotometer (µQuant, Biotek Instruments Inc., Winooski, VT). The total hexosamine content was calculated by comparing the absorbance values to a set of linear D(+) -glucosamine standards (0-200 per 2 ml). All sample values were normalized by the dry tissue weight. Six samples per group were used unless stated otherwise.
3.2.4 Quantification of Glycosaminoglycans by Uronic Acid Assay

Pericardial GAGs were extracted and quantified from 5/16” diameter samples by using a uronic acid assay[115, 116]. GAG enzyme treated and un-treated samples were frozen in liquid nitrogen and pulverized into a small pellet. The pellet was then frozen at -80°C for one hour and then lyophilized for at least 24 hours. Lyophilized tissue was weighed to obtain the dry tissue weight. GAGs were extracted from the tissue in 1ml of 0.5N sodium hydroxide at 4°C while shaking at 750 rpm for 24 hours, followed by the addition of 0.5 ml of 30% Trichloroacetic Acid to precipitate proteins while shaking at 500 rpm at 4°C for 24 hours. The GAG suspension was then centrifuged for 12 minutes at 12,000xG to remove the tissue and precipitate proteins. The supernatant (3500 MW) was dialyzed against several changes of water at room temperature for 24 hours to remove TCA. The remaining GAG solution was transferred to a glass test tube by adding 10% Cetylpyridinium Chloride (final concentration of 5%) and incubated at 37°C for 24 hours to precipitate the GAGs. The GAG precipitate was collected by centrifuging the suspension at 2000xG for 15 minutes and dissolved in 3ml of 10% sodium acetate. GAGs were reprecipitated with 12 ml of 100% ethanol at -20°C for at least 48 hours. The pure GAG extract was finally collected by centrifugation at 2000xG for 15 minutes and dried in hood overnight or under nitrogen at room temperature.

The extracted GAGs were quantified colorimetrically to detect uronic acid, which is a component of all GAGs except keratin sulfate[115]. The dried GAGs in the unknown samples were dissolved in 1 ml of benzoic acid saturated water, followed by the addition of 5ml of Borax decahydrate (25mM) in 36 N Sulfuric Acid mixed in a dry ice
and acetone bath. After vortexing to ensure good mixing the unknown samples were placed in a boiling water bath for 10 minutes, and cooled to room temperature. After adding 0.2 ml of carbazole reagent (0.125% carbazole in 100% ethanol), samples were placed back into the boiling water bath for 15 minutes, and cooled to room temperature. Optical absorbance was measured using a microplate spectrophotometer (µQuant, Biotek Instruments Inc., Winooski, VT). Quantitative values for uronic acid content were found by comparing the unknown samples with a set of glucuronolactone standards (0 – 25 µg per ml) prepared similarly except that the carbazole reagent was added before heating, and then heated once for 25 minutes. All values were normalized to the dry tissue weight for comparison. Six samples per group were used (n=6).

3.2.5 Tensile Testing

Fresh pericardial tissue was cut into dogbone-shaped samples with dimensions (30mm length x 5 mm width x ~ .5 mm thickness). Half of the samples were digested by GAG degrading enzymes as described earlier. Ten samples per group were tested (n=10). The samples were then placed in the grips of the MTS (Synergie 100) machine and cycled five times up to 10% strain at a speed of 0.2 mm/s. Displacement and load were recorded, and were then used to calculate and plot the stress and strain for cycles 2 through 5. The modulus was calculated as the slope from the linear region (0-2%) of the stress-strain curve. Hysteresis was also calculated as:

\[ h = \frac{(\text{loading} - \text{unloading area})}{\text{loading area}} \]

34
3.2.6 Flourophore-Assisted Carbohydrate Electrophoresis

Assessment of the GAG disaccharides was performed based on the methods of Calabro [117]. Fresh pericardial tissue was cleaned, rinsed in de-ionized water, and samples were punched out using a 5/16” diameter circular die. Four punches per sample were minced, frozen and lyophilized in pre-weighed 1.5 ml centrifuge tubes. The dry weight was recorded after 24 hours of being lyophilized, as 10 to 12 mg of dry tissue was needed. For every 3 mg of dry tissue 1ml of 100 mM Ammonium Acetate Buffer (pH 7.0), and 100 µl of Proteinase K (10mg/ml) were added. The tissue incubated at 60°C for 16 hours, and boiled for 10 minutes to stop digestion. Samples were then centrifuged at 1200 rpm for 10 minutes to separate supernatant. A volume of 200 µl was aliquoted per sample and placed into the filter portion of a Microcon YM 3 tube (Fisher) and centrifuged at 9000 xG for 25 minutes. The retentate was recovered in 100 µl of Ammonium Acetate Buffer (pH 7.0), and the protein amino-acids (flow-through) discarded. The GAG solution was then digested with hyaluronidase and chondroitinase ABC (5mU/sample) for 22 hours at 37°C, and boiled for 10 minutes to stop. The samples were centrifuged again at 9000 xg for 25 minutes through Microcon YM 3 filters, and the flow-through or disaccharides were retained. Chondroitin Sulfate and Hyaluronic Acid disaccharide mixture was prepared as standards (1000 pmoles). To the lyophilized GAG standards and samples, 40 µl of Aminoacridone fluorotag (20mM AMAC in 3:17 HAc: DMSO) were added and kept at room temperature for 15 minutes. A volume of 40 µl of 1.25 M NaCNBH₃ was added before incubated at 45 °C for 2 hours. Monosaccaride running buffer and gel (kit – company name here) were prepared and
stored at 4 °C overnight. Loading buffer (20 µl) and tracking dye (2 µl) were added to samples (20 µl) before placing 18 µl into each well. The gels were run at 500 V for 80 minutes at 4 °C. The fluorescent bands were then photographed under ultraviolet light, and lanes were analyzed by densitometry. Two samples per group were used.

### 3.2.7 Water Content

The water content of fresh and fixed bovine pericardium, before and after enzymatic digestion of GAGs, was determined by weight analysis. After enzyme incubation, the samples were rinsed, blotted gently to remove excess water, and wet weight was recorded. The tissue was then lyophilized for 24 hours and the dry weight was measured. The percent water content (WC) was calculated as:

\[
\% \text{ WC} = \left[\frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}}\right] \times 100
\]

### 3.2.8 Collagen and Elastin Stability

The collagen stability in cross-linked bovine pericardium was determined by measuring the resistance to collagenase as well as the thermal denaturation temperatures \(T_d\) for tissues fixed with GLUT, EDC, and NEO respectively. Differential Scanning Calorimetry (DSC) (Model DSC 7, Perkin-Elmer, Boston, MA) was performed to quantify \(T_d\) \((n=3\) per group\). Each sample was heated at a rate of 10°C/minute from 20 to 110°C, and the temperature at the endothermic peak was recorded \((T_d)\).

The resistance to collagenase was determined after fixation, samples were taken of 5/16” in diameter from the sheets of pericardial tissue and rinsed with water. Samples
were then lyophilized and weighed to attain the initial dry weight prior to digestion with collagenase. The collagenase enzyme (type VII) was prepared at 75 U/ml in a buffer of 50 mM Tris buffer, 10 mM CaCl₂, and 0.02% Sodium Azide (NaN₃) at a pH of 8.0. Each sample was placed in a volume of 1.2 ml of the collagenase solution for 48 hours at 37°C while shaking at 650 RPM. The resistance to elastase was determined in a similar manner. Each sample was placed in 1.2 ml of 5 U/ml elastase dissolved in a buffer of 100mM Tris, 1 mM CaCl₂, and 0.02% Sodium Azide (NaN₃) at a pH of 7.8. for 24 hours at 37 °C while shaking at 650 RPM. After freezing and lyophilizing the tissue, the dry weight was once again recorded as the final weight. The weight loss was calculated as follows:

\[
\text{Weight loss} = \left(\frac{\text{Initial Weight} - \text{Final Weight}}{\text{Initial Weight}}\right) \times 100
\]

3.2.9 Subdermal Implantation

Bovine pericardial tissue fixed in GLUT, EDC and NEO (n=12/fixation group) was cut into small diameter circles, and rinsed thoroughly with sterile saline prior to implantation. Male juvenile Sprague-Dawley rats (33-40 g, Harlan Laboratories, Indianapolis, IN) were placed under general anesthesia by inhalation of 2-4% isofluorane gas. A single, small incision was made in to the dorsal aspect of each rat, from which two subdermal pockets were formed. A single pericardial tissue sample was placed into each pocket and then the incision was closed with surgical staples. The rats were humanly euthanized after 21 days using CO₂ asphyxiation; samples were removed and prepared for analysis. The following diagram (Figure 11) illustrates how the tissue was
prepared and analyzed. All animals received humane care in compliance with protocols approved by Clemson University Animal Research Committee and the National Institutes of Health (Publication No. 86-23, revised 1996). Samples for calcium analysis and GAG quantification were immediately frozen on dry ice after sacrifice. Samples for histological studies were placed in 10% alcoholic acid formalin at ambient temperature. Analysis of GAG content was carried out on a section of each explanted sample by hexosamine quantification as previously described.

**Figure 11:** Subdermal implant samples; this schematic indicates how each sample was divided for study. Hexosamine was performed on tissue for GAG quantification. Calcium analysis was also performed on the tissue, and Histology was also performed to examine the tissue calcification and GAG content qualitatively.

### 3.2.10 Calcium Analysis

Calcium analysis was completed on one section of the lyophilized explanted tissue after obtaining the dry tissue weight for normalizing the data. The dried tissue was hydrolyzed in 1ml of 6N Ultrex II hydrochloric acid for 8 hours in a boiling water bath. After hydrolysis, the samples were dried under a continuous flow of Nitrogen gas, and re-
suspended in 1 ml of 0.01 N Ultrex HCl. Hydrolyzed samples were then centrifuged before making the appropriate dilutions in an Atomic Absorption Matrix (0.3N Ultrex HCl and 0.5% Lanthanum Oxide). The calcium content of each sample was acquired by atomic absorption spectroscopy on Perkin Elmer A Analyst200 Atomic Absorption Spectrometer.

### 3.2.11 Transmission Electron Microscopy

To examine the extracellular matrix at the fibril level and to observe GAGs transmission electron microscopy analysis was performed on fresh as well as fixed tissue. Tissue preparation was executed as described by Simionescu [118]. Briefly small 1 mm2 fragments of tissue were cut and stored in Karnowsky’s fixative (2.5% glutaraldehyde EM grade, 2% formaldehyde histology grade, in 0.1M cacodylate buffer, pH 7.4) at room temperature. The tissue was then placed in 5-10 ml of 2% glutaraldehyde EM grade, 0.2% toluidine blue in 0.1M cacodylate buffer (pH 7.4) overnight at 4°C. Samples were then rinsed in 0.1% Toluidine Blue in 0.1M cacodylate buffer pH 7.4 at room temperature and then treated with 1% OsO4 (EM grade) in 0.1M cacodylate buffer with 0.1% Toluidine Blue (pH 7.4) for 60 minutes at room temperature. After treatment with OsO4, the tissue was rinsed with 0.1M cacodylate buffer (pH 7.4) and dehydrated through an increasing series of ethanol. The samples were then transferred to propylene oxide and embedded in PolyBed 812. Ultra-thin sections (100nm) were cut from the middle portion of samples, collected on copper grids, and stained with 2% uranyl acetate followed by Hainaichi Lead stain. Grids were examined on a Hitachi H7000.
transmission electron microscope at 100kV. Toluidine blue stains all GAG species effectively, and appear as fine filaments at the TEM level [118].

3.2.12 Histological Analysis

Representative samples were taken from fresh, fixed as well as explanted fixed bovine pericardium, and fixed in alcoholic acid formalin for 24 hours, embedded with paraffin, and sectioned at a thickness of 5 µm. To qualitatively examine the extracellular matrix components, Gomori’s One Step Trichrome stain was used. Elastin fibers were observed by staining with Verhoeff’s stain for Elastic Tissue. Alcian blue staining with Brazilliant!® nuclear fast red counterstain were used to detect GAGs. Quantitative calcium analysis was also performed on explanted tissue from subdermal implant study by staining with Alizarin Red and Fast Light Green counterstain by Dahl’s Method for Calcium. A Zeiss Axioskop 2plus microscope was used with SPOT Advanced software to obtain histological images.

3.2.13 Statistical Analysis

The results presented are reported as a mean with the standard error of the mean (SEM). Comparisons between groups were done by analysis of variance (ANOVA) as well as two-tailed student’s t-test of unequal variance. Significant differences were defined with p< 0.05.
4.1 Bovine Pericardial Tissue Structure

4.1.1 Site Specific Characterization

The structure of the pericardial extracellular matrix was evaluated at four specific sites overlying the posterior left (PL), posterior right (PR), anterior right (AR) and anterior left (AL) ventricles as previously described. Histological staining specific for elastin, collagen and glycosaminoglycans was done as shown in figures 12, 13 and 14 respectively. Elastin fibers are interwoven with collagen bundles and account for only a portion of the matrix as seen in figure 12. Staining with Gomori’s trichrome confirms the presence of collagen (green) as the main component of the extracellular matrix. The collagen appears discontinuous in several sections as the bundles are in cross section. No significant differences between sites could be distinguished with only qualitative evaluation.

Alcian blue stain for glycosaminoglycans was conducted to qualitatively assess the GAG content of bovine pericardium by site. Blue areas are clearly seen on the edges of the sections in figure 12, but this could be due to edge affects. Some diffuse blue can also be seen throughout the tissue, but quantitative studies are needed for verification of the GAG content.
Figure 12: Verhoff’s van Gieson stain for elastin of bovine pericardial tissue. A & B (PR), C & D (PL), E & F (AR), G & H (AL) at 200x and 400x magnification.
Figure 13: Gomori’s trichrome stain for collagen of bovine pericardial tissue A (PR), B (PL), C (AR), D (AL) at 200x magnification.

Figure 14: Alcian blue stain for glycosaminoglycans of bovine pericardial tissue A (PR), B (PL), C (AR), D (AL) at 200x magnification.
The hexosamine content was quantified in the posterior and anterior regions above the right and left ventricles before and after enzyme treatment with Chondroitinase and Hyaluronidase for 48 and 96 hours. In the first case, fresh non-digested pericardial tissue was found to have significantly greater hexosamine content in the posterior regions as compared to the anterior regions as shown below in Figure 15. There was no difference between groups after treatment with GAG degrading enzymes (p<0.05).

![Figure 15: Site specific hexosamine content after 48 hours of enzyme digestion](image)

For the second case, half of the tissue was treated with GAG degrading enzymes for 96 hours to determine a baseline non-GAG related hexosamine value (Figure 16). However, due to considerable variability there were no statistical differences between groups or within groups after enzyme (n=5 and 6, p<0.05).
To further investigate the GAG content at specific sites in the pericardium, fluorphore-assisted carbohydrate electrophoresis was conducted on GAGs extracted from the tissue. Light bands of the gel correspond to Hyaluronic Acid (HA), 0-sulfated (0-S), 6-sulfated (6-S) and 4-sulfated disaccharides (Figure 17). The most prominent band corresponds to 4-sulfated disaccharides. This could be both chondroitin sulfate and dermatan sulfate. Densitometry based on relative density units (RDU) results confirm that this 4-sulfated GAG content is significantly greater than the other GAG types present (Figure 16-A). With an average of 6956 RDU/mg of dry tissue, 4-sulfated glycosaminoglycans make up 80% of the total GAG content. HA, 0-sulfated and 6-sulfated GAGs combined are 20% of the GAG content. (Figure 18-B) There was no significant difference between the anterior right, anterior left, posterior right or posterior left regions (p<0.05).
Figure 17: FACE Electrophoresis Light bands indication hyaluronic acid, 0-sulfated, 6-sulfated, and 4-sulfated disaccharides in fluorophore-assisted carbohydrate electrophoresis.

Figure 18: (A) Relative densitometry units per mg of dry tissue for GAG types in fresh bovine pericardial tissue. (B) Average relative density units RDU/mg in fresh pericardium.
4.1.2 Glycosaminoglycans in Fresh Bovine Pericardium

To quantify the total GAG content of fresh bovine pericardium excluding non-GAG related hexosamines, uronic acid assay was performed before and after enzyme digestion. With 13.5± 1.64 µg of uronic acid per 10 mg of dry tissue, and 8.56 ± 2.00 µg of uronic acid per 10 mg of dry enzyme digested tissue (Figure 19). The 37% decrease in GAG content, although not significant (p<0.05), indicates that complete GAG removal is not obtained by Chondroitinase ABC (p. vulgaris) and Hyaluronidase after 48 hours of incubation. When compared to Figure 20, the uronic acid content is approximately 13 % of the total hexosamine content with 103.04±1.64 µg/10 mg of hexosamine in the same tissue. Also of note is there is no significant difference after treatment with enzyme suggesting incomplete digestion (p<0.05).

Figure 19: Micrograms of Uronic Acid per 10 mg of dry pericardial tissue after treatment with Hyaluronidase and Chondroitinase
To further examine the presence of GAGs in bovine pericardium, the fresh tissue was examined under transmission electron microscopy. Fresh tissue was treated with Toluidine blue, a GAG specific stain, and examined at different magnifications. Few select locations were found to have darkly stained thin GAG fibers. GAGs were found in the space between fibers, bordering elastin fibers, as well as connecting collagen fibers as shown in Figure 21. Many GAG fibers were found at the surface of elastic fibers, amongst elastin-associated microfibrils (Figure 21 A, B, and D). GAGs also appear as bridges or connections between adjacent collagen fibers (Figure 21 E and F). In some cases the collagen fibers appear in cross-section with GAG fibers connecting neighboring fibers (Figure 21 A and C).
**Figure 21:** TEM of Fresh Pericardial Tissue  
A) Collagen fibers and elastin fringe with thin spindle shaped fibers 30,000X  
B) Elastin fibers with fringe associated GAG small fibers 20,000X  
C) 15,000X  
D) Elastin fringe with thin spindles (GAG) 20,000X  
E) 15,000X  
F) Same location as E at 40,000X magnification.
Resistance to tensile loading was conducted to evaluate the mechanical role of GAGs in fresh bovine pericardium. Fresh pericardium, half of which was treated with GAG degrading enzymes, was loaded to 10% strain and the modulus was calculated from 0 to 2% of the linear region as described earlier. Sample stress strain curves are shown in Figure 22 show the extreme cases in each group as an indication of the variability. The modulus was slightly lower for fresh non-digested tissue. Hysteresis, a measure of viscoelasticity, was slightly lower after enzyme digestion. There was, however, no significant difference in modulus or hysteresis with or without GAG enzyme incubation.

![Sample Stress Strain Curves](image)

**Figure 22:** Sample Stress Strain Curves (A & B) Fresh Pericardium Tensile Cycling Sample (C & D) Enzyme Digested Pericardium Tensile Testing Sample

**Table 4:** Modulus and Hysteresis of Fresh Pericardium

<table>
<thead>
<tr>
<th></th>
<th>Modulus (MPa)</th>
<th>Hysteresis</th>
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<tbody>
<tr>
<td>Fresh</td>
<td>0.036 ± 0.003</td>
<td>0.32 ± 0.073</td>
</tr>
<tr>
<td>Digested</td>
<td>0.043 ± 0.007</td>
<td>0.28 ± 0.079</td>
</tr>
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</table>
4.2 Glutaraldehyde Fixation Effectiveness

To evaluate the stability and retention of GAGs in glutaraldehyde fixed pericardial tissue, the GAG content was assessed 14 days after fixation. FRESH and GLUT fixed tissue was incubated in GAG degrading enzymes. After enzyme digestion both groups showed a significant decrease in GAG content (p<0.05) with a loss from 81.02 ±6.70 µg/ 10 mg to 59.06 ± 3.50 µg/ 10 mg for fresh tissue and 81.63 ± 3.53 µg/ 10 mg to 59.06 ± 3.59 µg/ 10 mg for GLUT fixed tissue. There was no significant difference between groups, indicating that glutaraldehyde cross linking does not stabilize GAGs against enzyme degradation.

![Bar chart showing GAG stability after storage in Glutaraldehyde for 14 days](image)

**Figure 23:** GAG stability after storage in Glutaraldehyde for 14 days
To confirm GAG loss in the pericardial tissue, uronic acid assay was performed. This method quantifies the uronic acid portion of the GAG as there are non-GAG related hexosamines present in the tissue. As shown in the figure below the uronic acid content is much less than the hexosamine content at 11 µg/10 mg of tissue of non-digested tissue. After enzyme digestion a 40% decrease is seen (6 µg/10 mg, p<0.05).

![Figure 24: Retention of GAGs in GLUT fixed pericardium after enzyme digestion](image)

GAGs are not initially stabilized by glut fixation. To examine if GAGs continue to be degraded over time the Gag content was quantified at 2 times points in the same tissue at 14 days, and 5 months in GLUT. A significant loss of GAGs occurred between 14 days after fixation and 5 most storage (from 81.6 to 53.4) a 35% reduction. After treating the stored tissue with GAG degrading enzymes the GAG content was decreased even further to 35.6 µg/10 mg of dry tissue.
Neomycin trisulfate with EDC fixation followed by storage in GLUT was examined as an alternative fixation for stabilization of GAGs. Three concentrations of neomycin were used to stabilize the tissue. After quantifying the GAG content, there was no significant different between the groups, with 85 to 90 µg of hexosamine per 10 mg of dry tissue. 1mM NEO was selected because there was no significant difference.

To assess the stability and retention of GAGs, the hexosamine content was measured before and after treatment with enzyme. Although significant GAG loss occurred in fresh and glut fixed tissue, no significant loss occurred in the NEO fixed tissue, with 75 µg of hexosamine as compared to 59. Neomycin is able to retain and stabilize GAGs against enzyme digestion.
Figure 26: Effect of Neomycin Concentration

Figure 27: GAG stability with NEO and resistance to enzyme digestion
After 5 months storage in glut the GAG content of NEO fixed tissue did decrease but not as much as in GLUT fixed tissue. After enzyme digestion of 5 months storage the NEO fixed tissue showed no significant decrease in GAG content from undigested tissue at 5 months, while GLUT fixed tissue had a significant loss in GAG content. NEO has improved GAG retention after 5 months storage.

![Figure 28: GAG stability with NEO after 5 months of storage in Glut](image)

GAG stabilization is improved by using NEO, but the properties of the tissue should not be negatively affected. The water content indicates the tissue hydration and viscoelastic potential. The tissue was exposed to GAG degrading enzymes to possibly correlate the stabilization of GAGs with water content. However, there was no significant difference between the GLUT, EDC, and NEO fixations. In comparison with fresh tissue, the water content was significantly greater in NEO fixed tissue after enzyme digestion (p<0.05) indicating improved hydration with the stabilization of GAGs.
Stability of elastin and collagen in pericardial tissue was evaluated to ensure that the properties of the tissue are not negatively altered. Tissue fixed with GLUT, EDC, and NEO was treated separately with collagen and elastin degrading enzymes. The tissue was weighed before and after treatment and the percent mass retained was calculated as described earlier. There was no significant difference in percent mass retained between any of the groups. Almost 99 to 100 percent of the tissue was retained after treatment with collagenase and 96 to 97 percent after elastase indicating stable crosslinking of both collagen and elastin. The stability of collagen was further examined by measuring the thermal denaturation temperature by differential scanning calorimetry. The denaturation temperature was significantly lower for GLUT fixed tissue than NEO indicating improved crosslinking of collagen in pericardial tissue with NEO fixation.
Transmission electron microscopy was also performed on crosslinked pericardium to evaluate the presence of GAGs in the extracellular matrix, as well as the integrity of the tissue. In Glut fixed tissue, few sites were found with dark staining fibers. The majority of GAG fibers were found in the interfibrillar space as shown in figure 30. Some of the GAG fibers were found connecting adjoining collagen fibers. Reduced quantities of GAGs were observed at the surface of elastin as compared to the abundance in fresh pericardial tissue (Figure 30 C and D).

For comparison, for EDC/NHS crosslinked pericardium was also evaluated by TEM. GAGs were found in the space between extracellular matrix fibers as well as bridging adjacent collagen fibers. Few were also found on the surface of elastin with the elastin associated microfibrils. At higher magnification the characteristic spindle shape of the GAG fibers can be seen.

**Table 5:** Collagen and Elastin resistance to enzyme after crosslinking chemistry

<table>
<thead>
<tr>
<th>Fixation</th>
<th>% Mass Retained (Collagenase)</th>
<th>% Mass Retained (Elastase)</th>
</tr>
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<tbody>
<tr>
<td>GLUT</td>
<td>101.97 ± 1.90</td>
<td>96.54 ± 0.85</td>
</tr>
<tr>
<td>EDC</td>
<td>99.37 ± 0.86</td>
<td>96.62 ± 1.24</td>
</tr>
<tr>
<td>NEO</td>
<td>99.09 ± 0.58</td>
<td>96.96 ± 0.99</td>
</tr>
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</table>

**Table 6:** Thermal denaturation temperature after fixation

<table>
<thead>
<tr>
<th>Fixation</th>
<th>Average Td (°C)</th>
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</thead>
<tbody>
<tr>
<td>GLUT</td>
<td>89.14 ± 0.40</td>
</tr>
<tr>
<td>EDC</td>
<td>93.37 ± 0.44</td>
</tr>
<tr>
<td>NEO</td>
<td>92.26 ± 0.53</td>
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</table>
Figure 30: Glutaraldehyde fixed bovine pericardium transmission electron microscopy images A) Thin filaments (darkly stained area) surrounded by collagen fibers at 15,000X; B) same as A at 30,000X magnification; C) Thin spindles darkly stained with collagen fibers at 15,000X; and D) same as C at 30,000X magnification.

GAG stability was also evaluated by TEM for Neomycin crosslinked pericardium. GAGs were also only visible in select locations of the tissue indicating variable staining. Dark stained fibers were found along the surface of elastin fibers as shown in Figure 32. At higher magnification the spindle shaped fibers can clearly be seen in the interfibrillar space. The same location of the tissue can be seen in Figure 33, at 3 different magnifications. GAGs are shown in the space between fibers as well as connecting neighboring fibers. The anisotropic behavior of the tissue can also be seen in the varying direction and alignment of the fibers.
**Figure 31:** EDC/NHS crosslinked bovine pericardium as imaged under transmission electron microscopy. A) Darkly stained thin spindles (GAGs) intertwined between collagen fibers at 15,000X; B) same as A at 30,000X magnification. C) More thin filaments (GAGs) surrounded by collagen fibers at 15,000X; D) same location as C at 30,000X magnification.

**Figure 32:** NEO treated bovine pericardium TEM images. Thin dark staining GAG fibers A) at 15,000X and B) 30,000X magnification.
**Figure 33:** NEO treated bovine pericardium examined under transmission electron microscopy. Thin darkly stained fibers (GAGs) are found between collagen fibers at A) 15,000X, B) 30,000X and C) at 50,000X.
4.4 GAG Stability in vivo and Correlation with Calcification

In vivo GAG stability was examined by quantifying the hexosamine content of implanted and non-implanted tissue. There was no significant difference in hexosamine content after implantation for 21 days. This result was confirmed qualitatively by histological study with Alcian blue stain. Overall only a diffuse blue is seen, similar to the staining of fresh pericardial tissue.

![Image of hexosamine content before and after subdermal implantation](image.png)

**Figure 34:** Hexosamine content before and after subdermal implantation
Figure 35: Un-implanted and implanted cross-linked bovine pericardium stained with Alcian blue for GAGs A) GLUT un-implanted; B) GLUT implanted; C) EDC un-implanted; D) EDC implanted; E) NEO un-implanted; F) NEO implanted all at 25X magnification.
In an effort to correlate any role of GAGs with prevention of calcification, the calcium content of the tissue was evaluated quantitatively and qualitatively. Atomic absorption of diluted hydrolyzed tissue indicates that the calcium content was significantly lower in EDC crosslinked pericardium in comparison to NEO, but there was no significant difference from GLUT fixed tissue (p<0.05). There were 65 µg of calcium per mg of dry tissue with 51 and 38 µg of calcium in GLUT and EDC fixed tissue respectively.

Qualitative analysis of the implanted tissue for calcium was achieved by staining with Alizarin red. The central region of all samples was dark red indicating the presence of calcium in the tissue regardless of fixation method.

Figure 36: Calcium content of implanted cross-linked bovine pericardium
Figure 37: Implanted cross-linked bovine pericardium stained with Alizarin red for calcification A) GLUT at 25X; B) GLUT at 100X; C) EDC at 25X; D) EDC at 100x; E) NEO at 25X; F) NEO at 100x.
CHAPTER 5
DISCUSSION

Glycosaminoglycans are found in many tissues of the body including cartilage and heart valve leaflets. They provide hydration and resistance to mechanical loading in the extracellular matrix [119]. Research on the presence of GAGs in pericardium has suggested that GAGs may play in important role in the dynamic mechanical properties of bovine pericardium [2]. Their presence in bioprosthetic heart valves has been extensively studied by our group. Studies have shown that Glut does not stabilize GAGs in porcine aortic valves, and that GAGs continue to be lost over time and as well as after accelerated fatigue testing [1, 61]. Alternative fixation with carbodiimide based crosslinking in conjunction with Neomycin trisulfate has shown to improve the stability and resistance of GAGs in porcine aortic valves [81]. As such, these indicate that the loss of GAGs may play a role in the degenerative failure of pericardial valves and their preservation could improve the durability and life of the valves.

5.1 Bovine pericardial tissue structure

5.1.1 Site Specific Characterization

The site specific GAG content was evaluated in two separate pericardial tissue sacs after 48 and 96 hours. In the first case there were significantly more GAGs in the posterior regions of the left and right ventricle. However, after enzyme digestion of 48
hours there was no difference in hexosamine content between areas. In the second pericardial sac the tissue was treated with enzyme for 96 hours, but there were no significant differences in GAG content based on the origin of the tissue. Also the overall hexosamine content was higher in the second sac with values ranging from 66.54±10.64 to 88.76±4.72 µg of hexosamine per 10 mg of dry tissue as compared to 53.52±3.24 to 72.74±2.05 µg in the first sac. This indicates that the initial hexosamine content could vary between 53 and 88 µg/10 mg depending on the tissue. This variation should be taken into consideration with a higher sample number to accommodate the large possible variability.

Numerous studies have examined the extracellular matrix components of the pericardium with inter- and intra-sac variability, however, research has only been conducted on the primary load bearing components of elastin and collagen. One study found that the extensibility varied from site to site within the pericardial sac and was due to the density of elastin in the tissue. They also found no significant difference in tissue thickness between positions or sacs [27, 35]. Simionescu et al further mapped bovine pericardial tissue by examining the fiber orientation, suture holding power, and thickness[34]. With these criteria, they determined that the regions overlying the left ventricle and superior aspect of the right ventricle were suitable for heart valve fabrication. Another more quantitative method of Small Angle Light Scattering (SALS) was developed to map the fiber direction and orientation [120]. Based on there research the area overlying the left ventricle was the most ideal site for bioprosthetic use [32, 33, 120]. Although variability between sacs was highlighted as such one specific site will
not have identical properties from sac to sac. It was suggested that the tissue be pre-sorted based on fiber alignment where homogeneity and uniformity are crucial [32].

Several studies have attempted to map the tissue structure of bovine pericardium to determine an ideal site for tissue selection; however they present varying conclusions as to the area overlying the left ventricle [33, 34] and right ventricle [37]. The variability of the matrix components, density, and alignment could be due to many factors including breed, age, sex, weight, and environment of the animal [27, 31]. By increasing the sample number and or using paired samples, inter- and intra-sac variability can be accounted for.

Alcian blue stain for glycosaminoglycans was conducted to qualitatively assess the GAG content of bovine pericardium by site. Blue areas are clearly seen on the edges of the sections in figure 12, but this could be due to edge affects. Some diffuse blue can also be seen throughout the tissue. The limited stain may also be due to the small quantity of GAGs present, perhaps immunostaining specific for dermatan sulfate and chondroitin sulfate would elicit better qualitative results [121-124].

Fluorophore-assisted carbohydrate electrophoresis of bovine pericardial tissue by area further confirmed the presence of GAGs in fresh tissue. Results also support hexosamine data indicating there is no significant difference in GAG content between sites. The most prominent GAG dissacharide present was 4-sulfated with over 80%. These could be both chondroitin 4-sulfate and dermatan sulfate. Low amounts of 0-sulfated and 6-sulfated dissacharides were present with only slightly higher presence of hyaluronic acid. This correlates with published data which found the presence of a low
molecular weight dermatan sulfate proteoglycan [40, 41]. Another, more recent, study found dermatan sulfate, chondroitin sulfate and hyaluronic acid present in bovine pericardium. 4-sulfated GAGs also dominated, however they found an absence of 6-sulfated GAGs [2]. This variation could be due to variability in the tissue.

5.1.2 Glycosaminoglycans in Fresh Bovine Pericardium

GAG content

The GAG content was found by uronic acid irrespective of the site. The fresh undigested content was 13.5 µg of uronic acid per 10 mg of dry tissue (Figure 19). Comparing with published data, 11.78 µg of uronic acid were found, however this value was based on 10 mg of wet tissue [2]. After enzyme digestion the uronic acid content decreased by only 37%, indicating that Chondroitinase ABC (p. vulgaris) and Hyaluronidase are not completely effective at degrading GAGs in bovine pericardium. The GAGs in bovine pericardium, as presented earlier, are predominately Chondroitin 4-sulfate and dermatan sulfate. Very small amounts of hyaluronic acid are present. The activity of Chondroitinase ABC (p. vulgaris) is only 34 – 40% for dermatan sulfate [125]. Chondroitinase ABC from Flavobacterium heparinum has a 100% activity with Dermatan, Chondroitin 4, and 6-Sulfate.

The uronic acid content when compared to corresponding hexosamine values from the same tissue can provide an approximation of the baseline non-GAG related hexosamine values. With nearly 103.04 µg of hexosamine per 10 mg, the uronic acid content at 13.5 µg was only 13% of the total hexosamine content. Overall, the fresh
tissue GAG content values are much lower than those found in porcine aortic valves, with 170 to 240 µg of hexosamine per 10 mg of dry tissue [1, 80, 126], and 150 µg of uronic acid per 10 mg of dry tissue [1]. These results indicate that the majority of hexosamine dissacharides present in the tissue are non-GAG related. Due to variability between different pericardial sacs, a constant baseline of non-GAG hexosamine remains to be found.

**Visualization of GAGs in the ECM**

Transmission electron microscopy of fresh pericardial tissue found few areas of clearly stained GAGs. This could be due to incomplete staining or indication of the structure of the tissue. The use of toluidine blue was selected because of its effectiveness in porcine aortic tissue [118] however cuproline blue would provide better visualization of GAGs in pericardium [41, 127].

**Biomechanical Role of GAGs**

The biomechanical role of GAGs in pericardium was investigated through cyclic tensile testing; however no significant conclusions could be made. There were significant variations in the overall stiffness of the tissue within groups due to variability in the collagen fiber alignment and anisotropy of the pericardial tissue. After digesting the tissue with Chondroitinase ABC (p. vulgaris) and hyaluronidase, no significant difference was found in the hysteresis or modulus. The role of GAGs in pericardium was studied by Mavrila et al by dynamic mechanical tensile testing. They also found no significant difference in mechanical properties by removing GAGs [2]. These results could be due to incomplete digestion of the dermatan sulfate in the tissue or limited by the testing
Flexural three-point bending of pericardium has reported to evaluate the mechanical properties of bovine pericardial tissue and the effects of various crosslinking, however, the effect of GAG loss or preservation has not been examined [46].

5.2 Glutaraldehyde Fixation Effectiveness

The use of glutaraldehyde to process and sterilize bioprosthetic valves continues to be the conventional treatment despite the failure of valves by calcification and structural deterioration. Glut is also limited in its ability to stabilize GAGs against enzyme digestion and loss after long term storage. After digestion significant decreases in GAG content were found for both fresh and Glut fixed bovine pericardium after 14 days of fixation. Even further loss was found after storage in Glut for 5 months. Similar results were found in porcine aortic bioprosthesis [1]. As the uronic acid content or total GAGs in glut fixed pericardium is approximately 12 μg/10 mg of dry tissue, the decrease in hexosamine is much greater than the uronic acid content. This suggests that even non-GAG related hexosamines are lost and other extracellular matrix components are becoming denatured. If Glut fixed tissue valves remain on the shelf even 5 months GAG loss will occur prior to implantation.

5.3 GAG Stabilizing Fixation Effectiveness

5.3.1 GAG Content

The loss of GAGs from porcine bioprothethic valves has been shown to reduce the mechanical durability of the tissue [1]. Stabilization of GAGs was achieved by
alternative crosslinking with carbodiimide fixation, however carbodiimide was not able to retain all GAGs [80]. 1-Ethyl-3-(3 dimethylaminopropyl) carbodiimide (EDC) activates carboxylic groups of GAGs for reaction with free amine groups. The addition of N-hydroxysuccinimide (NHS) increases the number of crosslinks, and limits hydrolysis by increasing the number of activated carboxylic groups. These activated carboxyl groups form amide crosslinks with free amine groups of collagen [128]. The carboxylic groups of the uronic portion in GAGs are the active sties fro enzyme degradation [125, 129]. Carbodiimide crosslinking does not stabilize all GAGs as some carboxyl residues remain available for enzyme degradation [80].

The incorporation of Neomycin (1mM) with EDC/NHS crosslinking has previously been evaluated for stabilization of GAGs in porcine aortic bioprostheses [81]. Three concentrations of NEO were evaluated to determine the stability of GAGs after treatment with hyaluronidase and chondroitinase. There was no significant difference in hexosamine content at a factor of 10 less and greater than 1mM, as such 1mM was used for further testing. However, lower concentrations may be feasible based on the reduced quantity of GAGs and sites for binding in the tissue. Neomycin trisulfate, a sulfated oligosaccharide, has been demonstrated to prevent the degradation of hyaluronic acid by various hyaluronidases [130]. Neomycin also has amine functionalities to react with the remaining GAG residues. Neomycin coupled with carbodiimide and glut fixation shows improved stability of GAGs after enzyme as well as after five months storage in glut. This data is supprted by earlier studies that present that NEO prevents the degradation of GAGs in porcine aortic tissue [81].
5.3.2 Tissue Properties after GAG Stabilization

Glutaraldehyde has exhibited the ability to reduce the hydration of aortic cusps, and hydration of porcine leaflets has been demonstrated to have an affect on the internal shear properties of the tissue with increased hysteresis and stress relaxation [131-133]. Although pericardial leaflets do not have distinct layers like the spongiosa which can accommodate large shear strain, the tissue exhibits similar shear modulus in fresh tissue as compared to porcine cusps. After glut fixation the shear modulus of pericardium increased similar to porcine aortic valves [44]. The water content of pericardial tissue was measured as an indication of the tissue hydration, which showed no significant difference after fixation. Enzyme digestion was performed to examine if a loss of GAGs would affect the hydration capability of the tissue. However, only fresh tissue experienced a decrease in water content with GAG loss. This varies from other data indicating that the water content of porcine cusps decreased with fixation [134]. This variation could be due to variation in tissue structure. By stabilizing GAGs in the tissue, the bulk and or bound water content was not affected in pericardial tissue.

The structural integrity of pericardial tissue is vital to the performance of bioprosthetic heart valves. Collagen fibers form bundles which provide the architectural framework and mechanical strength of the tissue. The collagen network of pericardium is structure in which the orientation of undamaged collagen is key to the mechanical properties and durability of the tissue [46, 47]. Degeneration of the collagen and elastin network leads to mechanical failure as well as increase the potential for calcification [135]. The results presented here indicate that after fixation all groups have improved or
similar stability of collagen and elastin as Glut fixed pericardium. After treatment with collagenase and elastase there was no significant difference between fixation groups. The percent mass retained after collagenase was higher for pericardial tissue than that of similar studies of porcine cusps [80]. The thermal denaturation temperature also provides information as to the stability of collagen. The temperature at which collagen is denatured occurs once the triple helical structure becomes a random coil. The temperature is higher with increasing intra-helical and intermolecular crosslinking [136]. Glut fixed pericardium had a significantly lower denaturation point, indicating a higher degree of crosslinking after carbodiimide and neomycin treatment. After treatment with carbodiimide and neomycin cusps also had a slightly higher denaturation temperature compared to just Glut, but the difference was not significant [81]. The values found are within the same rants as those found by other studies with pericardium [136] and porcine cusps [80]. After treatment with carbodiimide and neomycin cusps also had a slightly higher denaturation temperature compared to just Glut, but the difference was not significant [81].

Evaluation of the ultra structure by transmission electron microscopy did not indicate any significant differences between fixation groups for the GAG content or structure. Staining with Toluidine blue revealed small filaments near collagen fibrils as well as some near the surface of elastin. Differences were apparent between fresh and crosslinked tissue. Fewer GAGs were found near elastin fibrils; however this could be due to inadequate staining. Study of the dermatan sulfate proteoglycan in bovine pericardium presented similar images as reported here. Staining with Cuprolinic blue
revealed small fibers associated with collagen fibrils sometimes resembling spikes [41]. In depth evaluation of the GAG content in porcine cusps found damage in the extracellular matrix, and loss of GAGs between and along the surface of collagen fibers after Glut fixation [118].

5.4 In Vivo GAG Stability and Correlation with Calcification

Bioprosthetic valves implanted in the body ultimately fail due to calcification and structural deterioration. Examination of failed valves has shown fragmentation of collagen bundles, mineralization of the tissue, damage as a result of mechanical fatigue, and GAG loss [18, 60]. It is suggested that GAGs present in the connective tissue inhibit calcification of the extracellular matrix by occupying space in the matrix, and binding free calcium ions preventing the formation of calcification [135, 137]. The rat subdermal model has been the standard means of evaluating the potential of a biomaterial for calcification in the body [138, 139]. Numerous studies have been conducted to evaluate the role of GAGs in calcification of bioprosthetic valve tissue. GAGs have been removed, stabilized, and added to the prosthetic heart valve tissue in attempt to evaluate the role of GAGs in calcification. Extraction of proteoglycans from BP resulted in lower thermal stability and increased calcification [140]. Grafting hyaluronic acid to the structure of glutaraldehyde fixed bovine pericardium reduced calcification [79]. Periodate stabilization of endogenous GAGs and crosslinking of exogenous Chondroitin Sulfate resulted in significant reduction of calcification in bovine pericardium [78]. In the present study GAGs were stabilized with neomycin and EDC pre-treatment, however
there was no reduction in calcification. Earlier studies by our group on porcine tissue treated with periodate and EDC found similar results in that there was no significant difference in calcium content as compared to glut [80]. Also in the present study the calcium content was significantly higher in tissue treated with Neomycin as compared to EDC/NHS treated tissue. Neomycin pre-treatment of porcine aortic tissue, however, showed a reduction in calcium content compared to glut after implantation [81].

Correlation of the GAG content with the calcium content is not conclusive. There was no change in GAG content in any of the groups after 21 days. Previous studies have found increased hyalurondase and chondroitinase activity and significant GAG loss in subdermal implanted porcine aortic cusps after 21 days [16, 80, 126]. It is possible that with longer implantation times substantial GAG loss may occur as the GAG disaccharides found in bovine pericardial tissue are different from those of the porcine aortic leaflet as well as having different structure.

Although the role of GAGs in the prevention of calcification remains unclear, it is certain that bioprosthetic valve tissue calcifies despite the stabilization of GAGs. The methods presented here all involve the continued use of Glut, as such it can be assumed that GAG stabilization has a limited potential to prevent the calcification of tissue and toxicity caused by Glut.
CHAPTER 6

FINAL CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Advances in the design of bioprosthetic heart valves are such that at optimum conditions, will fail after 10-15 years. Glutaraldehyde has dominated as the single fixative used for commercially available tissue valves. These studies indicate that:

- GLUT fixation alone does not stabilize GAGs in bovine pericardium from enzyme degradation or loss in storage.
- Neomycin combined with EDC/NHS has enhanced standard Glut cross linking to include the stabilization of GAGs.
- Tissue properties of NEO fixed pericardium are not negatively altered after fixation.
- Tissue treated with Glut still has significant levels of calcification even with the addition of NEO and EDC/NHS.
6.2 Recommendations for Future Studies

Tissue variability has widely been explored as to the optimum site of pericardial tissue for bioprosthetic use, however no investigations to date have included analysis of the proteoglycan and glycosaminoglycan content variability. Future studies could investigate more in depth the content by using multiple pericardial sacs from bovine of equivalent age, sex, and weight.

Limited visualization of GAGs was achieved by Alcian blue with histology and Toluidine Blue with TEM. The limited stain may be due to the small quantity of GAGs present, future evaluation of bovine pericardium should include immunostaining specific for dermatan sulfate and chondroitin sulfate [121-124].

The role of GAGs in pericardial bioprosthetics remains to be elucidated. Physiologically relevant mechanical testing of pericardium with intact extracellular matrix as compared to completely degraded GAGs is needed. The use of alternative enzyme may ensure complete removal. Flexural three point bending and evaluation of shear properties could lead to a better understanding as to the role of GAGs in bioprosthetic heart valves.

Neomycin, a natural hyaluronidase inhibitor, did not completely inhibit degradation of GAGs in pericardial tissue. Hyaluronic acid accounts for only 6% of the total GAGs in the tissue. The mechanism by which GAGs are maintained may be because of the NEO amine functionalities and its ability to bind the remaining carboxyl groups of the GAGs to collagen. Further studies could include evaluation an alternative
to NEO which will have similar amine functionalities for binding carboxyl groups on GAGs, to determine if NEO is an enzyme inhibitor in pericardial tissue.

NEO showed the ability to stabilize GAGs after storage and against enzyme, but once implanted valves will be subjected to a variety of mechanical stresses such as shear, tension, compression. Accelerated fatigue testing is the next logical investigation to determine if the durability of pericardial bioprosthetic heart valves is improved by the stabilization of GAGs.

Prevention of calcification was not achieved with the addition of NEO to traditional Glut treatment. Future studies should combine NEO fixation with anti-calcification treatments such as ethanol incubation for prevention of calcification. Studies could also evaluate the effects of GAG stabilization on the tissue without the use of Glut which is known to cause calcification.
REFERENCES


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