Stabilization of Vascular Elastin by Treatment with Tannins

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STABILIZATION OF VASCULAR ELASTIN
BY TREATMENT WITH TANNINS

A Dissertation
Presented to
the Graduate School of
Clemson University

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

by
Jason C. Isenburg
December 2006

Accepted by:
Dr. Naren Vyavahare, Committee Chair
Dr. Dan Simionescu
Dr. Martine LaBerge
Dr. Lawrence Grimes
ABSTRACT

Elastin is an important component of the extracellular matrix that endows cardiovascular tissue with the ability to extend and recoil repetitively. It has been shown that elastin may be vulnerable to enzymatic degradation and calcification in certain pathological cases. As a result, elastin degeneration may be associated with the failure of tissue-derived cardiovascular devices, such as bioprosthetic heart valves, as well as the development and progression of aortic aneurysms, a disease associated with progressive connective tissue degeneration of the arterial wall. Therefore, an elastin stabilizing agent may be beneficial for both applications. As such, our objectives for elastin stabilization are two-fold: (1) in the case of tissue-derived cardiovascular implants, to develop a tissue pretreatment targeting elastin stabilization that may significantly extend the clinical durability of such devices, and (2) to hinder the progression of aortic aneurysm formation by limiting elastin degradation with the delivery of therapeutic elastin stabilizing agents. For both of these applications, we propose to stabilize elastin using tannins, a class of naturally derived plant polyphenols known to interact with elastin.

In studies presented here, we have confirmed that tannins bind to elastin, resulting in improved resistance to elastolytic degradation. This binding is effective within treated porcine aortic wall, as suggested by the preservation of elastin through in vitro and in vivo studies. Tannin-mediated elastin stabilization also correlated into a decreased propensity of aortic tissue to calcify in vivo. These data indicate that tannins may be used to improve the chemical fixation or pretreatment step used with tissue-derived cardiovascular implants, thus increasing the longevity of these devices. In addition, using
an animal model to induce abdominal aortic aneurysm formation, we were able to show that elastin stabilization (via tannin treatment) may be effective in inhibiting aneurysm formation and progression. The potential use of tannins as a novel therapy for abdominal aortic aneurysms is significant, as the only current treatment for this potentially fatal pathology is surgical repair (bypass with a stent graft) or outright surgical replacement, options which possess substantial drawbacks.

Future studies are needed to better evaluate the long-term efficacy and binding interactions between tannins and tissue. Additionally, the in vivo efficacy of tannin-mediated elastin stabilization for both bioprosthetic heart valves and abdominal aortic aneurysms should be tested in large animal models, such as sheep and/or pigs. If successful, this concept of elastin stabilization could have significant clinical implications by improving the longevity of tissue-derived cardiovascular devices, and by revolutionizing the management and treatment of abdominal aortic aneurysm patients.
DEDICATION

I would like to dedicate this work to my wife, Mary, and daughter, Allie. Their everlasting love, support, patience, and smiles have made this journey an enjoyable one. I would also like to recognize my parents and sister, who have been not only great teachers, but great friends as well. Without their encouragement, this work would not be possible.
ACKNOWLEDGEMENTS

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In addition, I would like to thank all of the past and current members of the Cardiovascular Implant Research Laboratory. Their knowledge and friendship have been the driving forces behind my great time here at Clemson. I would particularly like to thank Dr. Michael Bailey, Dr. Agneta Simionescu, Dr. Josh Lovekamp, and Dr. Qijin Lu for their expertise and advice over the years. Thanks also to Nishant Kiramchandani and Anthony Greer, who as summer students, contributed to a portion of the work presented here.

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CHAPTER 1
INTRODUCTION

Elastin is an extracellular matrix protein which provides numerous connective tissues with resilience or elasticity. In general, it is well understood that elastin is fairly stable against proteolysis, as accounted for by its high insolubility and extremely low metabolic turnover (with a half-life on the order of decades). Despite this perception, there are distinct cases where elastin degradation and/or an imbalance of elastin-degrading enzymes have been observed, namely within (1) tissue-derived cardiovascular devices, such as bioprosthetic heart valves, and (2) the development and progression of aortic aneurysms, a disease associated with progressive connective tissue degeneration of the arterial wall.

Cardiovascular biomaterials derived from glutaraldehyde fixed animal tissues are often used to surgically replace diseased or damaged human heart valves. Although these devices improve the lives of hundreds of thousands of patients annually, they often fail due to issues of tissue degeneration and calcification. Elastin and collagen are the two major extracellular matrix components found within tissue-derived cardiovascular biomaterials. While it is recognized that collagen fibers are adequately stabilized or crosslinked by glutaraldehyde fixation, elastin is apparently not protected from degeneration once implanted. This is likely due to the vulnerability of glutaraldehyde treated elastin towards the action of matrix-degrading enzymes, as elastin lacks the amine groups necessary for reaction with glutaraldehyde. Therefore, in regards to bioprosthetic heart valves, the objective of this work is to develop a safe and effective procedure that
stabilizes elastin in such a way as to render these bioprostheses more resistant to biological degeneration. The expectation is that this approach will extend the durability of tissue-derived cardiovascular biomaterials beyond their present limits, as the average bioprosthetic heart valve currently lasts just 10 years.\textsuperscript{2,3}

Abdominal aortic aneurysms are cardiovascular pathologies associated with impaired aortic wall integrity, subsequently leading to an abnormal ballooning of a segment of the artery. In general, aneurysm patients are asymptomatic, which can be detrimental as aneurysm progression can lead to further dilatation of the artery and eventual fatal rupture. Abdominal aortic aneurysms, which are apparently increasing in frequency among the population, have been cited as one of the top ten causes of death among older males.\textsuperscript{4} In fact, studies have indicated that as many as 5\% of men over age 50 have such an aneurysm.\textsuperscript{5} The dilatation observed within aneurysmal tissues is associated with deterioration of the arterial architecture. This degeneration of the aortic wall is due to elastin degradation, one of the defining characteristics of an abdominal aortic aneurysm. As such, it is logical to assume that the progression of this disease could be greatly reduced by local delivery of pharmacological agents capable of stabilizing elastin. Such a technology could significantly increase the longevity of aneurysm patients and substantially delay (or potentially eliminate) the requirement for surgical interventions. The idea of elastin stabilization may present a treatment option for patients with small or early-stage aneurysms, a group which represents the largest percentage of patients with a known aneurysm.\textsuperscript{6} As aneurysm therapies stand now, the only option for these patients is an imaging surveillance program; once the aorta reaches a critical diameter, surgical repair or replacement is performed. Such an approach is
risky, however, as 10% of aneurysm ruptures occur before reaching this critical diameter.\(^\text{6}\)

For these applications, we propose to investigate tannins as novel agents that stabilize elastin. Tannins are naturally derived plant polyphenols, which are known to specifically bind to hydrophobic regions in proline-rich proteins such as elastin and collagen.\(^\text{7}\) Tannins, specifically tannic acid, are also commonly used in electron microscopy, sometimes in combination with glutaraldehyde, for ultrastructural demonstration of elastin fibers, suggesting that tannins bind to elastin.\(^\text{8,9}\)

The work presented in the following chapters is outlined in Figure 1.1. To briefly summarize, the motivation of this project stems from the fact that although there is no known elastin stabilizing technology, elastin is susceptible to enzymatic degradation in some distinct pathological cases. Treatment with tannins can circumvent this vulnerability to elastolysis, as we demonstrated that these polyphenolic compounds bind to and stabilize pure elastin against the action of degradative enzymes. This idea of elastin stabilization was extended to investigate its feasibility and efficacy within our proposed applications, as a pretreatment for tissue-derived cardiovascular devices and as a therapy for inhibiting aneurysm development. Studies on the structural requirements and safety of tannin-elastin interactions revealed that tannic acid derivatives, specifically pentagalloyl glucose, may be the ideal elastin stabilizing agent.
Figure 1.1 A schematic highlighting the primary studies performed for this project.

(TA = tannic acid, PGG = pentagalloyl glucose)

References


CHAPTER 2

LITERATURE REVIEW

2.1 Heart Valve Prostheses

Heart valves, consisting of two or three opening leaflets or cusps (depending on the location of the valve), control the one-way flow of blood through the four chambers of the heart. The right and left sides of the heart each consist of two chambers and two valves (Figure 2.1). On the right side, the tricuspid valve regulates blood flow between the right atrium (the upper chamber) and the right ventricle (the lower chamber). The pulmonary valve is an outgoing valve that allows for flow from the right ventricle to the pulmonary arteries, which carry blood to the lungs to acquire oxygen. Oxygen-rich blood is then returned to the heart to the left atrium. Similar to the tricuspid valve on the right side, the mitral valve controls flow between the left atrium and left ventricle. Both the tricuspid and mitral valves, classified as atrioventricular, possess a support system which anchors the cusps into the base of their respective ventricles. The primary structural entity of this support system is the chordae tendineae, a series of cord-like tendons which actively help to prevent backflow into the atria during ventricular contraction. Once in the left ventricle, blood flow is pumped through and regulated by the aortic valve out to the aorta, allowing the distribution of oxygen-rich blood throughout the body. After being circulated, blood returns to the right atrium via the vena cava, thus completing the cycle.

The mitral and aortic valves are under a larger amount of stress due to higher pressure in the left side of the heart, and thus require a more frequent need for
replacement among these valves. This is particularly true for the aortic valve, which will be the primary focus of this section on replacement or prosthetic heart valves.

Figure 2.1 Cross-section of the heart, including all four valves.¹

2.1.1 Anatomy and Function of the Aortic Valve

The aortic valve facilitates the one-way flow of blood that is pumped from the heart to the rest of the body. The human aortic valve consists of three leaflets which open and close passively due to pressure differentials between the left ventricle and the aorta. Structurally, the leaflets of these valves are primarily composed of collagen, elastin, and glycosaminoglycans (GAGs). These components are distributed in an anisotropic pattern, comprising a structure with three layers: the fibrosa, spongiosa, and ventricularis (Figure 2.2).²⁻⁴ This three-layered composite structure makes cuspal tissue extremely soft
and pliable when unloaded yet strong enough to bear loads when closed. The fibrosa layer is located at the outflow surface (the aortic side of the cusp tissue) and composed predominantly of collagen fibers aligned in the circumferential direction, parallel to the unattached, free edge of the cusp. These collagen fibers contain macroscopic crimps that give rise to corrugations on the outflow surface of the valve when it is open during systole. The corrugations disappear during diastole as the cusps straighten, allowing elongation of the layer with minimal stress.³

![Figure 2.2 Cross-section of aortic valve cusp.](image)

The ventricularis layer exists at the inflow surface of the valve and is composed primarily of elastin. These fibers are arranged in the radial direction, providing the tissue with a great deal of extensibility. This permits the expansion of the cusp during diastolic closing of the valve. Similarly, elastin recoils during the systolic phase, causing a slight reduction in size during valve opening.⁶
The shear stresses and compressive forces acting on the cuspal tissue during flexion are managed by the action of the centrally located spongiosa layer. This layer consists largely of GAGs and loosely arranged collagen fibers. The hydrophilic GAGs, due to the absorption of water, form a gel within this center layer, which works to minimize the shear stress which results from the opposing movement of the cusp’s two outer layers during flexion. The spongiosa layer is also responsible for the absorption of compressive forces acting upon the valve under diastolic conditions.

Functionally, the three anisotropic layers of the valve structure are crucial to the durability and function of the valve. It should be noted, however, that the outer endothelial cell layer of this structure is also crucial to its interaction with the blood. The layer of endothelial cells covering the surfaces of the native valve allow for a minimal amount of damage to blood components, as well as guard against the infiltration of undesirable biological molecules such as cholesterol and lipids.

### 2.1.2 The Need for Aortic Valve Replacement

Diseased heart valves are generally classified as either stenotic or regurgitant. Valvular stenosis is due to a loss of mobility of one or more valve cusps, resulting in the partial or complete blockage of the valve orifice, causing increased resistance to blood flow through the valve. In contrast, valvular regurgitation (or insufficiency) is the inability of the valve cusps to fully close, potentially resulting in undesirable backflow of blood. Both conditions can result in a reduced amount of blood being distributed throughout the body and/or an increased workload on the heart itself. The most common pathology observed in diseased heart valves is age-related (or degenerative) calcification (Figure 2.3), resulting in valvular stenosis. Other conditions responsible for valvular
disease include rheumatic fever and bacterial infection, which may induce tissue inflammation and fibrosis, potentially producing stiff valve tissue that may become stenotic. Marfan syndrome, a genetic connective tissue disorder, can lead to aortic insufficiency even within younger patients. In addition, a number of congenital defects that hinder normal valve formation may lead to valvular stenosis or insufficiency.

**Figure 2.3** Outflow surface of normal (A) and calcified (B) aortic valves in their closed positions. Mineral deposits on the cuspal tissue are denoted by arrows.9

To date, there is no prevalent drug therapy for diseased heart valves. Therefore, in these cases of valvular damage or disease, surgical intervention in the form of valve repair or valve replacement is required. The most common repair technique involves minimally invasive valvuloplasty performed on stenotic valves. A balloon catheter is deployed upon the diseased valve to assist in mechanically shifting the valve cusps, potentially breaking apart areas of heavy calcification. Although potentially beneficial, valvuloplasty patients are presented with risk of embolism (due to release of debris) or
valvular insufficiency (due to mechanically-induced trauma to the cusp) as a result of the procedure.\textsuperscript{11} Other methods of valve repair involve “patching” a torn or physically damaged leaflet with a segment of autologous or xenogenic tissue such as pericardium.\textsuperscript{12,13}

Although such options for valve repair exist, the current therapy of choice for treating diseased heart valves is clearly surgical replacement using heart valve prostheses. It is estimated that approximately 275,000 valve replacement surgeries are performed worldwide each year.\textsuperscript{14} This accounts for approximately 15\% of all adult cardiac surgeries.\textsuperscript{15} These replacements, which have been widely used for decades, are typically categorized as mechanical valves or valves made from biological tissues, commonly referred to as bioprosthetic heart valves (BHVs). Briefly, mechanical valves are known for their biological inertness and longevity, but present the risk of thromboembolism to the recipient; therefore, chronic anticoagulation therapies are typically required for recipients of these valves. On the other hand, tissue-derived valves do not usually entail long-term administration of anticoagulant medication, however they are susceptible to degeneration and calcification, thus limiting their durability.

### 2.1.3 Options for Heart Valve Replacement

#### 2.1.3.1 Mechanical Heart Valves

The evolution of mechanical heart valves has seen three generations of designs: the caged ball, the tilting disc (or leaflet), and the tilting bileaflet designs.

The era of commercially available mechanical valves began with the implementation of the Starr-Edwards caged-ball design in the early 1960s. (It should be
noted that the Hufnagel descending aortic ball valve was the first design to be used in the 1950s, however its application as a “commercially available” device may be disputed with only about 200 patients receiving this style of valve.\textsuperscript{16,17} The design, a methacrylate ball and tube secured with nylon rings, resulted in a high rate of thromboemboli, but displayed excellent wear resistance and paved the way for more successful devices with similar concepts.) The Starr-Edwards valve originally implemented a methacrylate cage housing a silicon rubber ball which served as the mobile occluding element of the valve. The modern version of the Starr-Edwards valve (Edwards Lifesciences; Irvine, CA) consists of a heat-cured silicon occluder and a cage covered with Teflon fabric. Although various modifications such as these have been made, the original idea behind the design is still in use. In fact, it is approximated that 80,000 Starr-Edwards valves have been implanted as aortic valve replacements, with an even greater population receiving them as mitral valve substitutes.\textsuperscript{17}

While these devices were relatively successful in the clinical setting, their susceptibility to thromboembolism and wear drove the search for a more suitable mechanical valve design. As such, the tilting disc valves made their debut with the Bjork-Shiley flat-disc valve in 1969. As the name suggests, the tilting disc valves consist of a single disc which acts as the occluder, with an outer “cage” or struts serving to retain the occluder. This tilting disc valve experienced great success for nearly two decades, accounting for more than 150,000 aortic valve replacements in that time span.\textsuperscript{17} The most common tilting disc valves currently in use implement a pyrolytic carbon disc retained by titanium struts, such as the Medtronic Hall valve (Figure 2.4A; Medtronic; Minneapolis, MN).\textsuperscript{18}
The use of tilting disc valves naturally led to the next generation of mechanical prostheses, the tilting bileaflet design. The Gott-Daggett bileaflet valve was first introduced in 1963, but was taken off the market shortly after due to suboptimal flow characteristics. However, this led to the implementation of the most widely implanted prosthetic valve to date, the St. Jude Medical bileaflet valve (St. Jude Medical Inc.; Minneapolis, MN). Since its inception in the late 1970s until 2003, approximately 800,000 of these valves have been used to replace diseased aortic valves.\textsuperscript{17} Its fluid profile, along with the implementation of pyrolytic carbon-coated leaflets and struts, resulted in enhanced hemodynamics and biocompatibility. Various valve manufacturers have joined in the crusade for the ultimate bileaflet mechanical valve, resulting in a number of commercially available bileaflet designs. One recent development in these designs is the employment of “floating hinges”, which allow the surgeon to properly orient the leaflets in the most favorable position for fluid flow, as implemented in the
Bicarbon valve (Figure 2.4B) by Sorin Biomedica (Saluggia, Italy) and the Edwards MIRA valve by Edwards Lifesciences (Irvine, CA).18

2.1.3.2 Tissue-Derived Heart Valves

As with many biological materials used in the medical device industry, tissue-derived valves can be broken down into three categories based on their origin: autografts, homografts (allografts), and xenografts. As one might expect, the designs behaving most similarly to the native valve are the pulmonary autograft and the human allograft valves. The pulmonary autograft replacement, referred to as the Ross procedure and first performed in the late 1960s, entails the transplantation of the patient’s own pulmonary valve into the aortic valve position.19 The pulmonary valve is then replaced with a cryopreserved pulmonary valve from a cadaver (homograft). Since the replacement for the aortic valve is autologous, it results in improved hemodynamics and creates the potential for the replacement tissue to become a living, growing (and thus dynamic) tissue, making it an attractive valve substitute for younger patients. Along with its ability to potentially remodel, the autografts typically do not require long-term anti-coagulation and provide solid durability. Drawbacks of these autografts include the lack of suitable pulmonary valves for all patients, with potential complications arising from differences in size and geometry between the native pulmonary and aortic valves.20 In addition, with two valves being excised, the Ross procedure is not suitable for all patients due to the severity of the procedure. Along these lines, the Ross procedure is considered the most technically demanding valve replacement technique for surgeons.21

Because of these complications, surgeons often opt to perform valve replacement surgery in younger patients using a cryopreserved cadaveric human aortic or pulmonary
valve known as an allograft or homograft. Because this type of replacement valve is obtained through a tissue donor, there is no need for a double valve replacement as in the case of the Ross procedure. In addition, aortic allografts present significant geometrical, hemodynamic, and structural advantages over the use of a pulmonary valve in the aortic position. However, in spite of these advantages, the possible immunologic response to donor tissue and the limited, if any, donor-cell viability following tissue procurement, preservation, and subsequent implantation are thought to decrease the long term durability of these valves, especially in pediatric patients. These deficiencies, along with the insufficient supply in both numbers and sizes of allograft valves often necessitate the use of mechanical valves with anticoagulation therapy in young patients.

Xenograft valves, also called bioprosthetic heart valves, fabricated from chemically pretreated porcine aortic valves or bovine pericardia have been in use since the late 1960s. Chemical pretreatment, often referred to as fixation, of these xenografts is required in order to make the prosthetic devices resistant to enzymatic attack upon implantation and to reduce immunity of the xenogenic tissue. Ongoing improvements of these bioprosthetic heart valves (BHVs) have focused on fixation at different pressures, as well as pretreatments to help mitigate the propensity of the xenograft tissue to calcify, a major problem which will be discussed in greater detail later in the text. First generation BHVs were fixed at high pressure with glutaraldehyde, but with no anti-calcification treatments. Glutaraldehyde is a highly reactive water soluble dialdehyde that has been widely used as a tissue fixative for xenografts due to its ability to crosslink extracellular matrix proteins (specifically collagen), sterilize tissue, and reduce tissue antigenicity. Many modern designs of BHVs utilize very low (or zero)
pressure fixation with glutaraldehyde and/or include other chemical treatments that are intended to prevent or reduce calcification. While glutaraldehyde has been used for decades as a BHV fixative, it certainly has its drawbacks which will be addressed later in the text; however, a clear-cut superior alternative has yet to be found.

Historically, the majority of BHVs that have been clinically utilized are stented valves; that is, they consist of chemically fixed tissue cusps (collected from porcine aortic valves or produced from bovine pericardia) mounted onto either a metal or plastic stent with three posts (struts) and surrounded by a sewing ring at the base. In contrast, a number of contemporary BHV designs are “stentless”, consisting of the porcine aortic valve dissected with a portion of the aortic wall intact. The absence of a synthetic support structure (i.e., a stent) in these designs is thought to allow for more natural hemodynamics and a larger orifice area, allowing larger valves to be implanted in a given root. Examples of both stented and stentless designs are shown in Figure 2.5. Potential complications of stentless BHVs are derived from the fact that a greater amount of xenogenic tissue is exposed after implantation. For instance, the portion of aortic wall which accompanies these devices has been shown to be highly susceptible to calcific degeneration when implanted without the proper anti-calcification measures.\textsuperscript{23,24} In addition, stentless valves are more difficult to implant, which results in longer surgery times; therefore, care must be taken in deciding which patients are suitable for such a procedure. It should be noted that since stentless valves consist of a significant portion of aorta, elastin is a component of great concern within these devices.
Figure 2.5 Examples of stented (A) and stentless (B) BHV designs.  

2.1.4 Goals of Heart Valve Prosthesis Design

The goal of prosthetic heart valve design is to provide a durable substitute for one or more natural heart valves that are no longer optimally functional. As previously described by others and originally presented by Harken et al, the ideal heart valve replacement should follow the “Ten Commandments of Satisfactory Prosthetic Heart Valves” as adapted and outlined in Table 2.1. Heart valve substitutes should be generally biocompatible (nonthrombogenic, resistant to infection, and chemically inert); durable; easy to implant; non-obtrusive (allowing full opening and closure); and noise free to the patient. With the exception of a “noisy” replacement valve, the absence of one or more of these properties may lead to potential device failure. As such, to truly understand the requirements of an exceptional heart valve substitute, it is appropriate to investigate the mechanisms by which current prosthetic valves fail. Since our primary focus in regards to heart valve replacements is ultimately a treatment targeted at elastin stabilization, the
remainder of this literature review will predominantly focus on tissue-derived bioprosthetic heart valves.

Table 2.1  The Ten Commandments of Satisfactory Prosthetic Heart Valves

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<tr>
<td>Nonthrombogenic</td>
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<td>Chemically inert (and nonhemolytic)</td>
</tr>
<tr>
<td>Offer little to resistance to flow</td>
</tr>
<tr>
<td>Prompt closure</td>
</tr>
<tr>
<td>Complete closure</td>
</tr>
<tr>
<td>Durable</td>
</tr>
<tr>
<td>Practical and relatively easy to implant</td>
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<tr>
<td>Capable of permanent implantation</td>
</tr>
<tr>
<td>Proper healing at the implant interface</td>
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<tr>
<td>Noise-free to the patient</td>
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2.1.5  Mechanisms of Bioprosthetic Heart Valve Failure

Glutaraldehyde treated bioprosthetic heart valves (BHVs) comprise more than half of all valve replacements, and this percentage appears to be increasing in favor of these tissue-derived devices. While these BHVs improve the lives of thousands of patients each year, their suboptimal performance is difficult to ignore. In fact, it has been estimated that about half of all implanted valve bioprostheses fail (that is, result in death or require re-operation) within 10 years. This loss of function can be the result of several problems, including infection, prosthesis-related thromboembolism, non-structural dysfunction, and structural deterioration or degradation.
2.1.5.1 Valvular Endocarditis

Infective endocarditis is a serious problem in which the majority of cases are fatal, necessitating a great deal of precaution concerning sterility. Endocarditis occurs at least two to three times more often during prosthetic valve replacement than with other open heart surgeries. This high risk of infection is primarily due to the insertion of an intracardiac foreign body and the numerous opportunities for infection during surgery and postoperatively. The most common mechanism for failure of infected prosthetic valves is dehiscence (loss of implant attachment due to rupture at the suture line). Although these cases are somewhat rare, patients with early-onset valvular endocarditis unfortunately experience an extremely high rate of mortality, estimated as high as 80%.

2.1.5.2 Prosthesis-Related Thromboembolism

When speaking of thromboembolism risk in patients receiving replacement heart valves, the focus automatically shifts to mechanical heart valves, and rightfully so. Due to a combination of unnatural hemodynamics and material-blood interactions, mechanical heart valves have the increased risk of cause thrombosis formations upon implantation. The natural cusps of tissue-derived valves provide a flow profile more similar to that of the native valve, making thromboembolism in these cases an uncommon event. Although rare, the possibility of thrombosis in patients receiving BHVs does exist, particularly in the first few months after implantation. Just as the mechanical valves go through an initial adaptation period just after implantation when thromboembolism risk is at its peak, stented tissue-derived valves experience this same precarious interval. For this reason, short-term administration (usually up to 3 months post-surgery) of anticoagulant medication is often still prescribed for these patients. Among BHV recipients, it appears
that those receiving stented BHVs are at a slightly higher risk for thrombosis in comparison to patients receiving stentless BHVs.\textsuperscript{31}

2.1.5.3 Non-Structural Dysfunction

Non-structural dysfunction includes problems such as tissue (pannus) overgrowth, paravalvular leakage, hemolysis, and any other host tissue responses that might be detrimental to the function of the implant. Unlike many of the other forms of BHV failure, these show little specificity for the implant type, occurring at approximately the same rate in both tissue-derived and mechanical prostheses.\textsuperscript{32} This is particularly true for pannus overgrowth, which is the overgrowth of the tissue around the sewing ring of the implanted valve during implant integration. A potentially serious problem, pannus overgrowth often compromises the ability of prosthetic valve cusps to open and close properly.

Non-structural dysfunction can also derive itself from the interaction between the surface of the replacement valve and blood. As such, replacement valve designs must account for the risk of hemolysis, the destruction of red blood cells on the foreign surfaces of valves caused by the vulnerability of these blood cells to mechanical shearing. This can ultimately lead to a depleted capability of the blood to transport oxygen to and from the body’s tissues. Similar to the risk of thromboembolism, tissue-derived valves fare much better than mechanical valves in this respect, primarily due to the ability of the more natural cusps to open completely, thus creating less hemodynamic disturbance in the blood flow.

Leakage around the outside of the valve (at the interface between the replacement valve and native tissue) may also have serious consequences. Referred to as paravalvular
leakage, this malady defeats the purpose an operational heart valve by simply allowing blood to flow around a closed prosthesis, resulting in undesirable blood backflow. Fortunately, paravalvular leakage is extremely rare unless the valve recipient is inflicted with valvular endocarditis.\textsuperscript{18}

2.1.5.4 Structural Dysfunction

The most common form of failure for tissue-derived valves is structural dysfunction, a mechanism of failure often separated into two categories: calcific and noncalcific.\textsuperscript{32} Noncalcific structural dysfunction includes all forms of mechanical and material degradation not involving cuspal mineralization. Calcific failure, as the name implies, involves the calcification of the tissue cusps due to the deposition of calcium phosphate crystals.\textsuperscript{33,34} This process of crystal of formation shares similarities with bone formation and results in tissue hardening and the subsequent loss of valve mechanical properties, ultimately causing tissue degeneration and failure.

Noncalcific Structural Dysfunction

There is considerable evidence that degradation of the extracellular matrix components (independent of calcification) may lead to mechanical issues and ultimately be responsible for the failure of bioprosthetic heart valves. The constant cyclic burden of high pressure differentials across heart valves makes them extremely vulnerable to mechanical failure, or noncalcific structural dysfunction. This is commonly manifested through tears or perforations at highly stressed regions such as the cuspal commissures and points of maximal flexion. Implanted valve tissue has been shown to undergo a 50% reduction in mechanical strength after only four years of implantation.\textsuperscript{35} The alteration in
the behavior of collagen fibers during valve flexion as well as the loss of the valve’s ability to remodel its extracellular matrix can result in abnormal cuspal flexion. Long-term fatigue has been shown to damage the microstructure of extracellular collagen fibrils, thus making them more prone to failure by contributing to the reduction in bending strength of the valve tissue. From a biochemical standpoint, increased levels of matrix-degrading enzymes such as matrix metalloproteinases (MMPs), which will be discussed in greater detail later in the text (Section 2.2.1.3), have been observed in clinically explanted bioprosthetic valves.

The reduction in the amount of glycosaminoglycan (GAG) molecules is also thought to be involved in this process, altering the natural strain-absorbing cuspal configuration. The loss of these GAG molecules might also be responsible for the presence of interlayer shearing, resulting in delamination, fracture, and loss of collagen fibers from the functioning valve.

Elastin fibers, which provide tissue with resilience and serve to dampen the abrupt loading and unloading cycles experienced by valve leaflets, are also interconnected with the collagen fibers within these tissues. While it is unclear if their degradation leads directly to noncalcific structural dysfunction, the mechanical role of elastin in heart valves has been widely investigated and reported. This role will be discussed in greater detail later in the text (Section 2.3.1).

Calcific Structural Dysfunction

Calcification can occur independently or in conjunction with mechanically-induced abrasions or tears (noncalcific dysfunction). The majority of implanted BHVs undergo some degree of calcification, with the following three phases: nucleation,
deposition, and accumulation leading to structural failure. The mechanisms that lead to this type of valvular dysfunction are incompletely understood. \(^3\) Several factors influence the likelihood of deposition, which can be divided into three categories: host factors, implant-related factors, and mechanical stress-related factors.

The most significant and most frequently recognized host factor associated with the promotion of implant calcification is recipient age. Calcification occurs in only 10% of BHV patients aged 65 and over within ten years of implantation. This is in extreme contrast to young patients (<35 years of age), of whom nearly all require reoperation and replacement within five years of implantation, most often due to calcification. \(^2\) The contribution of age toward the rate of calcification is thought to be the result of comparatively high levels of osteocalcin (a bone-associated protein linked to tissue mineralization) as well as enhanced parathyroid hormone and vitamin D metabolism in the younger population. \(^4\)

Implant-related factors affecting calcification include glutaraldehyde pretreatment as well as the integrity of certain tissue components such as collagen, proteoglycans, elastin, and phospholipids. \(^2,4\) The relationship between glutaraldehyde crosslinking of BHVs and in vivo calcification is the most widely discussed implant-related factor, with several theories attempting to explain the correlation between the two. The first, and most widely accepted of these, is through the devitalization of the interstitial cells of valve tissue. \(^3,4\) This action results in the loss of the cell’s ability to sustain the energetically expensive action of the membrane calcium pumps that maintain low intracellular calcium concentrations. The outcome is an influx of calcium into the phosphate-rich cell body, resulting in the nucleation of hydroxyapatite, the primary
structural element of calcium-phosphate crystals. It is apparent, however, that this is likely not the only mechanism through which glutaraldehyde affects implant calcification. The crosslinking of acellular collagenous biomaterials has also resulted in a calcified matrix, revealing that the amount of calcification may be proportional to the level of crosslinking. Additionally, it is theorized that the binding of the amine groups of lysyl side chains within the collagen molecule by glutaraldehyde might result in a net negative charge among collagen’s amino acids, exposing negatively charged carboxyl groups for binding to positive calcium ions. Furthermore, residual free aldehydes (those aldehyde groups which are loosely bound or unreacted) have been implicated as a major mechanism for the promotion of calcification.

The exact role of extracellular matrix components in calcification is still unclear, although several theories exist for their involvement. Studies have shown that collagen fibers may potentially act as sites for crystal nucleation in undersaturated solutions of calcium and phosphate, thereby serving as a catalyst for the formation of hydroxyapatite. The likelihood of collagen’s involvement in the initial phase of calcification could be small, but it may participate in the accumulation and enlargement of the hydroxyapatite formation. Proteoglycans are thought to play a role in the prevention of the hydroxyapatite nucleation process via multiple mechanisms, including serving as a protector of collagen molecules. The staggered nature of collagen molecules creates a series of empty gaps, which are occupied by proteoglycans. Seemingly, by filling these holes, proteoglycans interfere with hydroxyapatite nucleation and protect the collagen from calcification. In addition, the glycosaminoglycan components of these molecules may serve to chelate calcium, thereby preventing binding with extracellular
phosphate in the nucleation of hydroxyapatite crystals.\textsuperscript{7} The link between glycosaminoglycan loss and calcification is noteworthy since glutaraldehyde has no apparent stabilizing effect on these molecules.\textsuperscript{48}

Similar to the lack of reactivity with glycosaminoglycans, glutaraldehyde fixation also has an extremely limited effect on elastin.\textsuperscript{49} Elastin, a structural protein responsible for tissue recoil, is abundant in the aortic wall (making it an extremely vital component of stentless BHVs), and although present in small amounts, works as a functional component of aortic valve cusps.\textsuperscript{39} It has been shown that elastin may be susceptible to deterioration in BHVs, which likely leads to subsequent initiation and proliferation of calcium deposition.\textsuperscript{24,50,51} While the exact mechanisms of elastin-oriented calcification are not fully understood, it is hypothesized that elastin’s vulnerability to this pathology begins with the degradation of its own protective coating.\textsuperscript{34} This degradation renders particular areas of the elastin molecule susceptible to calcium binding, thus leading to hydroxyapatite formation. Furthermore, it appears that elastin degradation can actively stimulate the manifestation of osteogenic proteins and genes, which ultimately leads to increased propensity to calcification.\textsuperscript{52,53}

Phospholipids have also been implicated in calcification, however, their role is unclear but suspected to be electrostatic in nature. This is supported by evidence suggesting that implants devoid of lipids contain much lower amounts of calcium and are therefore less likely to undergo calcification.\textsuperscript{44,54}

It is also believed that mechanical stress acts as a catalyst for the formation of calcific deposits within implanted BHVs. Hydroxyapatite deposition most often occurs in areas under the highest levels of stress and/or flexure such as the cuspal
As with many issues involving cardiovascular implant calcification, the exact correlation between mechanical stress and tissue mineralization is not completely understood. Studies have shown that implants in the mitral position are more likely to calcify than those in the aortic position, presumably due to the higher stresses incurred by the mitral valve when closed, thus linking mechanical forces and mineralization. Higher mechanical stresses have also been theorized to contribute to the increased rate of calcification in younger patients. This apparent synergism between calcific and noncalcific structural dysfunction underlines the importance of maintaining the structural integrity of the tissue components within replacement heart valves. For instance, degradation of elastin could lead to both calcific and noncalcific mechanisms of failure.

2.1.6 Limiting Structural Dysfunction in Bioprosthetic Heart Valves

In addition to reducing tissue antigenicity, chemical pretreatments of BHV tissue primarily exist to perform one of the following preventive functions: (1) resist enzymatic and chemical degradation, or (2) serve as an anti-calcification therapy. In other words, their purpose is to limit noncalcific and/or calcific structural dysfunction, respectively.

2.1.6.1 Glutaraldehyde Fixation

As early as 1967, researchers discovered that collagenous biomaterials could be stabilized by reaction with aldehydes. Initial investigations into tissue fixation focused on two common fixatives, glutaraldehyde and formaldehyde. Experiments suggested that although formaldehyde was successful as a tissue fixative, it did not result in the same resistance to degradation as glutaraldehyde. Formaldehyde has only one active aldehyde group in its chemical structure, while glutaraldehyde is a dialdehyde. The
The difunctionality of glutaraldehyde makes it capable of forming extremely stable crosslinks between lysine groups of protein molecules, thereby increasing the tissue’s resistance to degradation.

Glutaraldehyde crosslinking is successful in making certain components of the tissue, namely collagen, resistant to enzymatic and chemical degradation by the formation of stable crosslinks within and between the molecules of the extracellular matrix. Moreover, these crosslinks reduce tissue antigenicity, thereby limiting implant rejection. Glutaraldehyde is also capable of killing both bacteria and spores, making it a successful sterilizing agent.59

The fixation reactions involving glutaraldehyde are dependent upon concentration, temperature, pH, and the composition of the glutaraldehyde solution (i.e. the amount of monomer and polymer components).59,60 The functional groups taking part in this reaction are the amines of peptide lysyl residues and the two aldehydes of glutaraldehyde. Many forms of this reaction are possible, some of which may contribute to the unusual long-term stability of the crosslinks formed. The proposed crosslinking structures of glutaraldehyde can be both short and long, allowing crosslinks to form both intramolecularly (within individual collagen fibers), as well as intermolecularly (between adjacent proteins molecules), resulting in the stabilization of the implant tissue.

While glutaraldehyde treated BHVs provide a valve substitute that is sterile, non-antigenic, and fairly resistant to collagen-specific degradation, this pretreatment is certainly not without fault, as indicated by their limited durability. Structural changes undergone within and between collagen fibers impose a variety of alterations in the mechanical properties and flexural behavior of the replacement tissue. Additional
drawbacks of glutaraldehyde fixation include incomplete fixation of certain extracellular matrix (ECM) components (other than collagen) and cell lysis, which results in the exposure of intracellular components to the extracellular environment.

The altered mechanical properties of BHVs due to glutaraldehyde crosslinking are frequently implicated in the susceptibility of these implants to failure.3,61-67 Bioprosthetic valves are subjected to three types of stress: tensile, compressive, and shear. These all act in a cyclic manner, loading and unloading the tissue as the heart beats nearly 30-40 million times per year. For this reason, the mechanical properties and the fatigue resistance of this tissue are extremely important factors to consider in design. Despite these considerations, glutaraldehyde fixation has been shown to cause an increase in tissue stiffness, possibly resulting in abnormal flexure patterns during valve function.3,64

Another major shortcoming of glutaraldehyde fixation is its inability to stabilize non-collagenous components of the ECM. Many of the molecules making up the ECM lack the amine functionalities necessary for crosslinking with aldehydes, most notably elastin and glycosaminoglycans (GAGs), leaving them susceptible to enzymatic degradation.49,68,69

In regards to elastin, this vulnerability is significant considering it is one of the major ECM components of the aortic segment of stentless BHVs and the ventricularis layer of the valvular cusp structure. Elastin degeneration could lead to loss of elastic recoil and deterioration of mechanical properties,40,70 potentially resulting in clinical failure of implanted cardiovascular prostheses. The inability of glutaraldehyde to stabilize elastin is also significant with respect to calcification, as elastolysis and the presence of elastin-degrading enzymes, specifically matrix metalloproteinases (MMPs),
have been linked to calcium deposition. In regards to GAGs, the hydrophilicity of these molecules contributes greatly to the shear behavior of valve tissue, an action occurring primarily in the spongiosa layer. Failed glutaraldehyde treated BHVs have been shown to have a decreased amount of GAG molecules, furthering their implication in valve failure. These facts suggest that elastin and GAG degeneration in glutaraldehyde treated BHVs may be important clinically.

Natural heart valves are certainly dynamic structures, a quality which is lost by “locking” the tissue into place by glutaraldehyde fixation. Through the formation of extremely stable crosslinks within the extracellular matrix, the ability of structural collagen fibers to deform in response to applied forces is diminished. Under normal circumstances, the fibers making up the ECM are constantly rearranging within the surrounding ground substance in order to optimize the valve’s mechanical properties for the current phase of the cardiac cycle. The loss of this ability further contributes to the suboptimal mechanical properties of glutaraldehyde treated bioprosthetic tissue.

Similarly, glutaraldehyde pretreatment also leads to a loss of cell viability within the BHV tissue. The interstitial cells existing within all three layers of the heart valve structure serve a key role in its function by maintaining the mechanical integrity of the tissue as it undergoes constant fatigue. These cells work to continually replenish the ECM in response to the dynamic loads and environment. The loss of this cellular capability is largely responsible for the gradual degradation of glutaraldehyde crosslinked bioprosthetic heart valves. Endothelial cells also play an important function in the prevention of valve related complications, but are adversely affected by glutaraldehyde fixation. These cells serve as a blood interface throughout the cardiovascular system.
preventing the initiation of the coagulation cascade. By causing the denuding of this endothelial layer in cuspal tissue, glutaraldehyde is responsible for potential thromboembolic events which may result from the contact of blood with subendothelial components.

2.1.6.2 Anti-Calcification Pretreatments and Alternative Fixatives

In addition to glutaraldehyde’s effect on the viability of different cells and biostability of ECM components, the fixative has also been implicated as a direct contributor to the pathogenesis of calcification. This is thought to occur secondary to many of the problems previously discussed, such as cell death. For instance, free residual aldehydes (which are toxic) following fixation are thought to be promoters of calcium deposition.46,47 As a result, many BHVs now incorporate anti-calcification pretreatments, some which work in conjunction with glutaraldehyde and others which have been proposed as replacements to glutaraldehyde (that is, tissue fixatives which are less likely to calcify than glutaraldehyde).

As mentioned, residual aldehydes and “free” glutaraldehyde (that which is not fully bound) are believed to be linked to calcification. Therefore, one strategy to mitigate calcification is to neutralize these free aldehyde groups with compounds that have reactive amines, thus “capping off” or quenching these aldehydes. The most notable of these neutralizing agents are common amino acids such as glutamine, homocysteic acid,46 and lysine.74,75 Other anti-calcification agents used in conjunction with glutaraldehyde include amino-oleic acid76 and amino-biphosphonates.77 While it is suspected that both of these compounds work as aldehyde neutralizing agents, other mechanisms may also play a role. For instance, it has been hypothesized that amino-oleic acid may work to
limit the diffusion of calcium ions into valve-related tissue. Similarly, amino-
bisphosphonates, which are widely used in orthopedic research as well, are known to
disrupt crystal formation, thus hindering the production of hydroxyapatite. Aluminum
chloride, ethanol, and detergents are also among the growing class of anti-calcification
treatments that are used in combination with glutaraldehyde. Rather than working to
“neutralize” glutaraldehyde, as in the case of some of the aforementioned amino acid
treatments, these agents are active toward other components of the tissue such as elastin
and phospholipids. The use of aluminum chloride is particularly noteworthy for the
aortic portion of stentless BHVs, as this compound apparently binds to elastin and in
doing so, delays the onset of elastin-oriented calcification. In addition to aluminum,
other trivalent metal ions, namely ferric chloride, have also exhibited promising anti-
calcific properties. Ethanol is often used to treat BHV cusps for its ability to extract
cell lipids, which are a major proponent of calcification. In addition, this ethanol
pretreatment has been shown to have an effect on the conformation of collagen fibers,
which is believed to be beneficial towards calcification prevention.

A number of anti-calcification therapies have been used without glutaraldehyde
fixation and have shown to successfully inhibit or delay tissue mineralization, however
none are in clinical use to date. The term “anti-calcification therapy” may be misleading,
as the primary purpose of the majority of these compounds is not necessarily to prevent
calcification. Rather, they are meant to be alternative crosslinking agents and since they
do not implement glutaraldehyde, they also result in reduced propensity to calcify. The
most popular of these alternative fixatives includes the use of carbodiimides as well as
fixations triggered by the oxidation of a light-sensitive dye (PhotoFix®, Carbomedics;
Austin, TX). In regards to carbodiimides, 1-ethyl-3-(3-dimethyl aminopropyl)-
carbodiimide (EDC) is used in conjunction with n-hydroxysuccinimide (NHS), which
acts as an enhancer to the reaction. By coupling the amine and carboxyl groups within
the tissue, this treatment creates amide bonds or bridges between proteins, thus
crosslinking the tissue. Photo-oxidation fixation relies on the use of a photoactive
dye, whose activation leads to the formation of a catalyst. This catalyst aids in the
conversion of amino acids within the tissue, subsequently allowing for crosslink
formation with nearby amino acids. These BHV pretreatments, along with a number
of others including epoxies, diisocyanates, acyl azide, and genipin have resulted in
reduced calcification and cytotoxicity (in comparison to glutaraldehyde treated tissue) in
animal models. Although these alternatives appear somewhat promising, questions
remain as to their ability to crosslink collagen to the same degree as glutaraldehyde. As
such, glutaraldehyde-fixed devices are still the conventional choice for commercially
available BHVs, often used in conjunction with one or more of the aforementioned
anticalcification treatments.

2.2 Abdominal Aortic Aneurysms

2.2.1 Definition and Pathological Characteristics

Aneurysms are vascular pathologies associated with impaired integrity of the
blood vessel wall, subsequently leading to an abnormal ballooning of a segment of the
artery (Figure 2.6). They occur most commonly in the aorta (thoracic aneurysm if in the
chest, abdominal aneurysm if in the abdomen) or in the blood vessels in the brain
(cerebral aneurysm).
Abdominal aortic aneurysms (AAAs), which are the most common, typically occur in the infrarenal abdominal aorta; that is, the portion of aorta between the renal artery branches and the iliac bifurcation. In general, AAA patients are asymptomatic, which can be detrimental as aneurysm progression can lead to further dilatation of the artery and eventual fatal rupture. AAAs, which are apparently increasing in frequency among the population, have been cited as one of the top ten causes of death among older males. In fact, studies have indicated that as many as 5% of men over the age of 50 have an AAA.

While smoking is the most common risk factor for AAAs, strong correlations have also been observed for genetic predisposition, hypertension, age, atherosclerosis, and sex. For instance, those patients with a family history of AAAs have an increased risk of about 30% and are apparently more likely to experience aneurysm rupture.
Furthermore, even though men are 10 times more likely to have an AAA, women are about 3 times more likely for that AAA to rupture.97,98

Although aneurysms were once believed to be caused solely by a late degenerative phase of atherosclerosis, their pathogenesis is now considered to be more multifactorial.92 The risk factors listed above, such as genetic predisposition, hypertension, immunologic factors, hemodynamic factors, and defects of arterial components can also play a role in aneurysm development. However, it should be noted that atherosclerosis can still significantly contribute to this “multifactorial” theory behind aneurysm formation. For instance, atherosclerotic lipid and calcium deposits within the arterial wall can elicit an inflammatory response, thus stimulating secretion of elastolytic enzymes. These subsequently induce and maintain progressive destruction and weakening of aortic structural proteins, which eventually results in aneurysmal dilatation. As such, AAA development is typically characterized by aortic dilatation, an overall degeneration of the arterial architecture, the presence of matrix-degrading enzymes, and inflammatory infiltration.

2.2.1.1 Aortic Dilatation

Normal infrarenal aortic diameters are about 1.7 cm in men over the age of 50. Conventionally, any aorta 3 cm in diameter or larger is considered aneurysmal. According to the American Academy of Family Physicians, an aneurysm is a “permanent focal dilatation of an artery to 1.5 times its normal diameter”.99 As this statement implies, aortic dilatation is the primary characteristic by which aneurysms are defined. As a result, aortic diameter is the sole means of diagnosing and monitoring AAAs. The observed dilatation is associated with changes in aortic wall mechanical properties.100
which can be further attributed to other characteristics of AAA formation, namely loss of extracellular matrix components such as elastin and collagen.

2.2.1.2 Degeneration of Extracellular Matrix Components

The mechanical properties exhibited by the aortic wall rely on the extracellular matrix components, elastin and collagen. Therefore, the loss of structural integrity of the aneurysmal aortic wall is primarily explained by degeneration (and remodelling in general) of these matrix components.101,102

It is well understood that elastin, which provides resilience or elastic distensibility, absorbs the majority of normal load experienced by the abdominal aorta under physiological conditions. At the same time, aortic collagen functions as a “safety net”, assisting in bearing a portion of the load during periods of heightened aortic distension and high pressures.103 In a healthy aorta, these two proteins work in concert to provide a three-dimensional network which simultaneously endows the aorta with distensibility and strength.

Accelerated elastin degradation has been established as a primary trait of AAA and is thought to be essential in the formation and progression (expansion) of these pathologies.104,105 It has been estimated that there is a 75% loss of medial elastin content in explanted human aneurysmal aortas as compared to normal aortic tissue.105 Studies have also shown that the diseased tissue from AAA patients exhibit elastic lamellae disruption or fragmentation and increased levels of elastase activity.105-107 This lack of elastin within the aneurysmal aorta contributes to its inability to maintain its original size and shape, resulting in the typical “balloon” associated with aneurysms.
On the other hand, loss of collagen has been linked to aneurysm rupture.\textsuperscript{104} In regards to collagen, the term “loss” may be misleading since it has been found that the collagen content within aneurysms actually increases as the aneurysm grows.\textsuperscript{108} This increase in collagen is likely a compensatory response due to loss of elastin and collagen, increased mechanical load on the existing collagen fibers, and an overall increase in tissue remodelling. The increased cyclic load has been shown to induce collagen synthesis by nearby vascular smooth muscle cells.\textsuperscript{109} In addition to medial smooth muscle cells, collagen is also laid down by adventitial fibroblasts. These compensatory mechanisms do well in keeping the diseased aorta functional as long as possible; however, they obviously have a threshold. The ultimate strength of the diseased aorta relies on collagen; as such, it is reasonable to assume that rupture occurs when collagen degradation (mediated by matrix-degrading enzymes) exceeds this compensatory collagen production.

\subsection{2.2.1.3 Matrix Metalloproteinase Activity}

Various enzymes are believed to play a role in the development and progression of AAAs. Included in this group are cathepsins,\textsuperscript{110} serine proteases,\textsuperscript{111} and plasminogen activators.\textsuperscript{112} Cathepsins and serine proteases are capable of some of the proteolytic activity similar to that which is described and observed within aneurysmal tissue. Plasminogen activators assist in transforming plasminogen to plasmin, a general protease that is capable of activating matrix metalloproteinases (MMPs). While these different enzymes play a role in the aforementioned proteolysis associated with aneurysms, MMPs are apparently the group of enzymes most closely related to arterial wall weakening and AAA development.\textsuperscript{113}
MMPs are a family of enzymes that play an important role in extensive tissue remodelling, specifically by degrading extracellular matrix components such as elastin and collagen. MMPs are characterized from other proteases by their requirement for zinc binding to their active site. They are secreted in latent form and because of their potential proteolytic (and thus destructive) nature, MMP activation is deeply regulated on multiple levels: intracellular gene transcription, extracellular activation of the latent enzyme, and inhibition by endogenous tissue inhibitors of metalloproteinases (TIMPs). In general, MMPs consist of three domains: (1) the propeptide domain, including the cysteine switch which is important in maintaining latent form, (2) the catalytic domain, which possesses the zinc binding site, and (3) the C-terminal domain, which is the varying portion across different MMPs. TIMPs inhibit MMPs by irreversibly binding to the active form of the protease in a 1:1 molar ratio. Consequently, the overall proteolytic environment is determined by the relative amounts or ratios of MMPs and TIMPs.

While MMPs are prevalent in pathological disorders, they are also important in normal physiological processes such as angiogenesis, bone remodelling, embryogenesis, and many others. Some of the cardiovascular diseases in which elevated levels of MMPs are observed include intimal hyperplasia, atherosclerosis, heart valve disease, and aortic aneurysms.

Specific to aneurysms, the most widely investigated MMPs are those capable of elastolytic activity: MMP-2 (72 kDa gelatinase), MMP-9 (92 kDa gelatinase), and MMP-12 (macrophage metalloelastase). The role of such enzymes has been validated by experimental results as well as studies on explanted human AAAs. It is believed that MMP-2 plays a significant role in initial aneurysm formation, as it has been found in high
concentrations within small or early-stage aneurysms in experimental animal models and human tissue. This trend of elevated MMP-2, which is commonly secreted by smooth muscle cells and fibroblasts, has been observed in the form of increased enzyme activity and increased mRNA expression.

The other gelatinase, MMP-9, has also been found to contribute to AAA development. MMP-9, which is capable of degrading elastin (but to a somewhat lesser degree than MMP-2), is exhibited in human aneurysmal tissue at levels 10 times greater than normal aorta. This same study revealed that the predominant source of MMP-9 overexpression was localized to macrophages. MMP-9 is of utmost importance as it is the most abundant elastolytic enzyme found within aneurysmal tissues and possesses strong collagenolytic capabilities as well.

The roles of MMP-2 and MMP-9 on aneurysm formation have been further authenticated with the implementation of knockout mice models. MMP-2 and MMP-9 knockout mice were extremely resistant to experimental aneurysm formation, displaying virtually no increase in aortic diameter (less than 10%) in both cases. This study revealed that the absence of either gelatinase offered protection toward the tissue’s matrix, thus preventing aneurysm formation. As a result, an interaction between these two MMPs is apparently required to properly elicit aneurysm formation; in other words, the enzymes work in concert within this pathology.

In regards to MMPs, one of the primary catalysts for aneurysm formation is apparently an imbalance between MMPs and their inhibitors, TIMPs. Expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA was elevated in human aneurysmal aorta versus normal aorta. The key to this study, however, was the large increase in the ratio
between MMPs and inhibitors, suggesting an imbalance that favors proteolytic
degradation. Similar results, also on human cadaveric tissues, have been published
confirming an extreme increase in the MMP-2 to TIMP-2 ratio (for protein) for
aneurysmal aorta.\textsuperscript{122} In general, all TIMPs possess the ability to inhibit multiple different
MMPs, however, it appears that TIMP-1 possesses an affinity to inhibit MMP-9 while
TIMP-2 is important in regulating MMP-2 activity.\textsuperscript{108} Mice deficient in the gene for
TIMP-1 exhibited an increased susceptibility to AAA formation, indicative of the
protective effects of these endogenous MMP inhibitors.\textsuperscript{123}

Aside from MMP-2 and MMP-9, other MMPs have been investigated for their
part in aneurysm formation and development, such as MMP-1, MMP-8, MMP-12, and
MMP-13. MMP-1 is an interstitial collagenase that is increased in human aneurysms and
in addition to possessing the ability to directly digest collagen, can also cleave latent
MMP-9 into its active form.\textsuperscript{124} MMP-8, a relatively powerful neutrophil collagenase, is
elevated within human AAA tissue.\textsuperscript{125} This enzyme has been connected to
atherosclerosis and, more recently, shown to be in abundance along with MMP-9 at the
site of aneurysm rupture.\textsuperscript{126} MMP-12 knockout mice displayed somewhat improved
resistance to aneurysm formation in an experimental model.\textsuperscript{127} It is important to note,
however, that the effect of MMP-12, a macrophage metalloelastase, knockout was not
nearly as significant as compared to MMP-2 and MMP-9 knockout studies.\textsuperscript{120,127} MMP-
13, also referred to as collagenase-3, is also overactive in aneurysmal aorta.\textsuperscript{128} This
collagenase is localized to smooth muscle cells (SMCs) and is adept in activating other
latent MMPs. The origins and substrates for these MMPs is summarized in Table 2.2.
Table 2.2 Matrix Metalloproteinases (MMPs) Associated with Aneurysm Formation\textsuperscript{115}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Alternate name</th>
<th>Primary substrate(s)</th>
<th>Source/origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Collagenase-1</td>
<td>Collagen (type I, III)</td>
<td>Inflammatory</td>
</tr>
<tr>
<td>MMP-2</td>
<td>72 kDa gelatinase A</td>
<td>Collagen (type IV), elastin</td>
<td>SMCs, fibroblasts</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Neutrophil collagenase</td>
<td>Collagen (type I, III)</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>MMP-9</td>
<td>92 kDa gelatinase B</td>
<td>Elastin, collagen</td>
<td>Macrophages, SMCs</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Macrophage elastase</td>
<td>Elastin</td>
<td>Macrophages</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase-3</td>
<td>Collagen (type IV)</td>
<td>SMCs</td>
</tr>
</tbody>
</table>

2.2.1.4 Inflammation

A notorious attribute of abdominal aortic aneurysms is the consistent presence of an inflammatory infiltrate. This inflammation is characterized by the presence of macrophages and lymphocytes.\textsuperscript{104,107} One study on human AAA specimens revealed that approximately 80% of aneurysms had major inflammatory infiltrates.\textsuperscript{129} The use of powerful immunosuppressants has also been shown to at least partially prevent experimental aneurysm formation in rodents.\textsuperscript{130}

Although the exact source of this inflammation is unclear (and likely varies from patient to patient), potential causes include an autoimmune response\textsuperscript{131} or an infection due to \textit{chlamydia pneumoniae}.\textsuperscript{132} The intense inflammatory reaction is more likely induced by products of matrix degradation, specifically elastin peptides. These peptides apparently serve as a chemotactic agent for infiltrating macrophages through interactions with the 67 kDa elastin laminin receptor.\textsuperscript{133} Vascular smooth cells exposed to elastin peptides in vitro exhibited an increased expression for this receptor as well as an increase in MMPs.\textsuperscript{134}
Inflammatory cells are a common source for many of the aforementioned MMPs implicated in aneurysm formation, most notably MMP-9. As such, it is likely that these cells release a cascade of pro-inflammatory cytokines that results in the activation of MMPs and other proteolytic enzymes. 115 Such cytokines have been found to be elevated in aneurysm patients, including interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α. 119 With this in mind, it is reasonable to assume that there is likely a complex interaction between matrix degradation, the production of matrix-degrading enzymes, and the recruitment of inflammatory cells.

2.2.1.5 Summary

Abdominal aortic aneurysms are clearly complex pathologies that cannot be explained by one particular phenomenon. Instead, they are likely due to a multifactorial process characterized by enzymatic degradation and inflammation. Figure 2.7 shows a hypothesized path for aneurysm formation. While this is one viable path, it is important to note that the chronology of these events may vary significantly from case to case.
For the set of circumstances shown in Figure 2.7, initial matrix degradation (due to aging, angiogenesis, protease/inhibitor imbalance, or atherosclerosis) may result in the release of elastin peptides. These peptides will elicit an intensified inflammatory response. This elevated inflammation likely causes the release of more MMPs, such as MMP-9 which has been primarily localized to macrophages. Elevated MMPs cause even more matrix degradation, and the subsequent release of more degradation products. This triggers the intertwined ongoing cascade of MMP production, matrix degradation, and inflammation, eventually leading to enough tissue degeneration to allow for aneurysmal dilatation.

Figure 2.7 Hypothesis for mechanisms of abdominal aortic aneurysm formation.
2.2.2 Diagnosis and Screening

The majority of abdominal aortic aneurysm patients are asymptomatic. As a result, aneurysms are most often discovered incidentally via imaging processes such as ultrasonography, computed tomography (CT) scans, magnetic resonance imaging (MRI) scans, and chest X-rays. It should be noted that in cases of very large AAAs, physical palpation of the patient’s abdomen can reveal the presence of arterial dilatation. However, this detection method is severely limited in its accuracy, particularly with obese patients\textsuperscript{135} and those patients with early stage (3.0 to 3.9 cm) aneurysms.\textsuperscript{136} However, late stage aneurysms (greater than 5.0 cm) are palpable 76\% of the time.\textsuperscript{136}

As stated earlier, aortic diameter is the primary means of diagnosing and monitoring aneurysm formation and progression. Aortic diameters that are 3 cm or larger are considered aneurysmal and merit further observation. Frequency of surveillance is dependent on the size of the aneurysm, as outlined in Table 2.3. Screening and monitoring are most often performed by abdominal ultrasound, which is relatively inexpensive, non-invasive, and does not require contrast media. CT imaging is more expensive and requires intravenous contrast agents, but its image provides more insight and accuracy in regards to the size and three-dimensional shape of the aneurysm. Figure 2.8 shows an example of a 3D CT scan of an AAA in a human.
Table 2.3  Recommended Surveillance for Abdominal Aortic Aneurysm Patients

<table>
<thead>
<tr>
<th>Diameter of aneurysm</th>
<th>Frequency of follow-up observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 3 cm</td>
<td>No further testing</td>
</tr>
<tr>
<td>3 to 4 cm</td>
<td>Every 12 months</td>
</tr>
<tr>
<td>4 to 4.5 cm</td>
<td>Every 6 months</td>
</tr>
<tr>
<td>Greater than 4.5 cm</td>
<td>Referral to vascular specialist</td>
</tr>
</tbody>
</table>

Figure 2.8  Three dimensional CT scan of an abdominal aortic aneurysm.

Unfortunately, ruptured aneurysms are associated with an extremely high mortality rate. This, along with the fact that the majority of AAA patients are asymptomatic, has led to the possibility of routine screening for asymptomatic adults. In a “consensus statement” made by a number of American vascular specialists in 2004, they recommended that the following groups be screened for AAAs even if
asymptomatic: all men 60 to 85 years of age, all women 60 to 85 years of age with cardiovascular risk factors, and both men and women over 50 years of age with a family history of AAA.\textsuperscript{137} Recently, this suggestion came to partial fruition in the United States, as the SAAAVE Act was passed into legislation.\textsuperscript{138} This law provides for aneurysm screening as a Medicare benefit for high-risk males (those with a family history of AAAs and those who smoke). The significance of this new legislation is profound in that it will allow many more aneurysms to be detected and at an earlier stage. The need for such a screening program has been justified with similar clinical studies. For instance, the Multicenter Aneurysm Screening Study Group in the United Kingdom utilized a very large sample size of asymptomatic men (approximately 67,000) and found that approximately 5\% of those subjects had an abdominal aortic aneurysm.\textsuperscript{93} Furthermore, the screening decreased aneurysm-related mortality by 50\% in comparison to a control group of patients who were not screened.

\textbf{2.2.3 Current Options for Treatment or Repair}

Currently, the primary methods of AAA treatment are surgical repair of the diseased aorta in order to prevent rupture. For this reason, elective surgery is not suggested until the risk of rupture exceeds the risk of operative mortality. Through various trial studies, it has been established that an aortic diameter of 5.5 cm should serve as the threshold of AAA surgical repair. In one study, moderate aneurysms (4 to 5.5 cm) were electively repaired, but with minimal benefits. Mortality was not improved improved in these patients as compared to those that underwent normal imaging surveillance without surgery.\textsuperscript{139} In a different study, the UK Small Aneurysm Trial reaffirmed that the benefits of elective surgery performed on small or moderate diameter
aneurysms (less than 5.5 cm) did not outweigh the surgical risks. Once the aortic diameter surpasses 5.5 cm, however, risk of rupture rises from 1% to 10%; thus, the 5.5 cm threshold. This limit has not been validated for females with AAAs, which is noteworthy since it has been observed that the risk of rupture within women is greater and is more likely to occur at a smaller diameter than men.

In the cases where surgical repair is required and performed, two options exist: open and endovascular repair. Open aneurysm repair (Figure 2.9) is the more conventional therapy in which the diseased or aneurysmal portion of the aortic wall is dissected away and replaced with an artificial mesh vascular graft. This method, which is well established, requires little to no radiologic follow-up after repair. Due to the severity of this open procedure, however, it is limited in that it is not appropriate for many older patients and those presented with other pre-existing cardiovascular issues. When used in an “elective” situation, this procedure yields an operative mortality of 4% to 6%.

Figure 2.9 Schematic of open surgical aneurysm repair.
More recently, endovascular repair of abdominal aortic aneurysms has been gaining in popularity. Like many less invasive technologies, this procedure involves gaining access to the lumen of the abdominal aorta through small incisions in the femoral vessels. With the help of a guide wire, endovascular stent grafts are then implanted percutaneously at the site of aneurysm as shown in Figure 2.10. These devices, consisting of a cloth graft with a stent “exoskeleton”, are deployed across the diseased portion of aorta, thus creating a bypass for blood flow through the prosthetic lumen of the stent graft.\textsuperscript{99} While this method of repair is less invasive and thus more suitable for those patients who cannot handle major vascular surgery, these devices are unfortunately anatomically appropriate for only 30% to 60% of aortic aneurysms.\textsuperscript{95} The endografts also require close postoperative monitoring as CT scans are typically suggested at 1, 6, and 12 months after repair, and annually after that. One of the primary reasons for this precautionary approach is the risk of endoleaks (and the subsequent potential for graft migration) for these devices, occurring in 10% to 20% of cases.\textsuperscript{142} Endoleaks allow the flow of blood into the aneurysm sac, resulting in increased risk for aneurysm expansion and/or rupture. Although this less invasive technology is promising, its complications and lack of long-term data make its overall application as an aneurysm treatment suboptimal.
2.2.4 Animal Models

Most samples of human aneurysms come from patients who have reached the late stage threshold (5.5 cm or greater), and subsequently had their diseased aorta removed and replaced with a graft. Additionally, aneurysmal tissues are often collected from cadavers whose mortality is associated with aneurysm rupture. From a research standpoint, these tissues provide little insight into the pathogenesis of abdominal aortic aneurysms since they are collected at such a late stage. Subsequently, little information can be gained on potential pathways for inhibiting aneurysm formation and/or progression from these samples. This underlines the importance of reproducible animal models for aortic aneurysms, which can allow for examination and potential inhibitory treatments of early-stage pathogenic processes.
2.2.4.1 Genetically Engineered Mice

Due to their relative low cost and the ability to manipulate specific genes, genetically engineered mice have become widely used subjects for aneurysm animal models. Certain mutations have been found to make mice naturally predisposed to AAA formation. For instance, blotchy mice have a genetic mutation which ultimately results in reduced levels of lysyl oxidase, an enzyme which facilitates the natural crosslinking of elastin and collagen fibers. As a result of these extracellular matrix defects, these mice have a predisposition to form AAAs which are histologically characterized by elastin fragmentation and disrupted smooth muscle layers.\textsuperscript{143,144} Aneurysms found in these animals are typically in the thoracic rather than abdominal region.

Mice deficient in low density lipoprotein (LDL) receptors and apolipoprotein E (apoE), a protein responsible for lipoprotein transport, are also prone to aneurysm formation.\textsuperscript{145,146} These genetic defects lead to hyperlipidemia and are often used with special diets to produce hypercholesterolemia as well. These models were originally used for atherosclerosis research, but have more recently been used as an aneurysm model as well. Aneurysms within these altered mice will occur naturally, but are most often found in the suprarenal abdominal aorta, while human aneurysms are typically in the infrarenal region.

2.2.4.2 Angiotensin II Induced Aneurysms

Systemic delivery of angiotensin II to hyperlipidemic (LDL receptor or apoE deficient) mice results in aneurysm formation\textsuperscript{147,148} The delivery of angiotensin II is typically regulated by osmotic minipumps. Although its mechanisms of action are not necessarily clear, recent research suggests that accumulation of macrophages in the aortic
media may act as the primary causal event.\textsuperscript{149} This attraction of macrophages, mediated by increased cytokine activity,\textsuperscript{150} results in elastin degradation likely due to increased protease recruitment. Interestingly, this rodent model shows preference towards aneurysm formation in males over females, similar to the trend observed in human AAAs.\textsuperscript{151}

Downsides of this model include the location of aneurysm formation, as it consistently forms only in the suprarenal region. In addition, dilatations within the angiotensin II model appear to be associated with very large thrombus formation rather than the luminal dilatation and wall thinning observed in human aneurysms.\textsuperscript{148}

2.2.4.3 Chemical Injury Aneurysm Models

Elastase Perfusion

It has been established that elastin degradation is one the primary characteristics of aneurysm development. As such, the use of elastase to induce AAAs in animals has been widely used since its introduction in 1990.\textsuperscript{152} While primarily used on rats, it has also been attempted on larger animals such as dogs.\textsuperscript{153} Within this model, the abdominal aorta is clamped off near the renal arteries and near the iliac bifurcation, thus isolating an infrarenal segment of aorta. Using a small catheter, this segment is perfused with pancreatic elastase, typically for 2 hours at a rate of approximately 1 mL/hour in rats.\textsuperscript{154} Over the course of a few days, the aorta is characterized by inflammatory infiltrates (dominated by macrophages and T cell lymphocytes) and increased protease activity.\textsuperscript{155,156} In addition, disruption of the elastic lamellae is seen immediately following infusion with elastase.\textsuperscript{143} This abrupt elastin degradation, along with
perfusion-induced mechanical forces, causes an instantaneous change in diameter in the range of a 30% to 70% increase.\textsuperscript{157-159} While this model results in reproducible aortic dilatation associated with AAA formation, one may argue this immediate diameter change is not truly representative of the somewhat slow progression of human AAA development.

Elastin peptides, the products of elastin degradation, also induced aneurysm formation in rats when infused through the infrarenal aorta in the same fashion, which lends insight into the mechanisms of the elastase perfusion model.\textsuperscript{160} The use of elastase periadventitial (directly applied to the outside of the artery rather than through luminal perfusion) to provoke aneurysm formation in rats has also been met with moderate success.\textsuperscript{161} However, it also appears to cause significant immediate dilatation and its ability to form “true” aneurysms has been debated.\textsuperscript{162}

Calcium Chloride

The calcium chloride (CaCl\textsubscript{2}) model is based on the perivascular or periadventitial application of a high concentration calcium chloride solution for 15 minutes, a method originally used to induce aneurysms in rabbit carotid arteries.\textsuperscript{163} More recently, this model has been used on abdominal aorta of rabbits,\textsuperscript{164} mice,\textsuperscript{165} and rats.\textsuperscript{166} The CaCl\textsubscript{2}-based chemical injury model has also been adapted to rat abdominal aorta to study mechanisms of vascular calcification.\textsuperscript{167}

This model, which has been utilized in rats for our aneurysm experiments, was chosen for its ability to \emph{progressively} elicit elastin degradation, inflammation, elastin-oriented calcification, elevated MMP levels, and arterial dilatation, which are all characteristics of human AAA development and progression. Using this model on mice,
studies have shown that MMP-deficient mice exhibited a decreased susceptibility to calcium chloride-induced AAA formation\textsuperscript{120}; therefore, asserting that the perivascular CaCl\textsubscript{2} model relies heavily on MMP activity to induce AAA formation. In addition, the aforementioned model allows for easy periadventitial application of potential AAA therapies. Ultimately, this perivascular model was beneficial over other aneurysm models for its ability to mimic the morphologic aspects of human AAA progression without the major mechanical damage apparently induced by the elastase perfusion model. For instance, using calcium chloride, mice did not exhibit an immediate increase in aortic diameter after one week; however, significant increases were observed after two weeks with progressive expansion continuing in following weeks.\textsuperscript{165} This slower progression of aneurysm development closely imitates the morphology of human AAA development.

2.2.5 Pharmacological Strategies

For the many patients who have a small or early stage aneurysm, there is unfortunately no current option or therapy (other than imaging surveillance). In these cases, the aortic diameter is periodically monitored until it reaches a critical threshold (typically 5.5 cm), at which point surgical repair or replacement is performed as described above. This “wait and see” approach is not without risk, however, as 10% of the abdominal aortic aneurysms that rupture do so at diameters less than 5 cm.\textsuperscript{119} Therefore, alternative pharmacological treatments targeted at limiting aortic expansion may be helpful in reducing incidence of rupture and circumventing the need for surgical repair.
2.2.5.1 Beta Blockers

Beta blockers, or beta-adrenergic antagonists, are conventionally used for hypertension, but have also proven to be effective in limiting the rate of aortic expansion in aneurysmal patients. Propanolol, the most noteworthy beta blocker, was originally shown to reduce aneurysm-related deaths in a turkey model from 44% in control animals to just 1%. These agents are known to lower blood pressure, which would logically benefit the aneurysmal aortic wall from a mechanical viewpoint. However, it has also been shown to stimulate lysyl oxidase activity, thus theoretically enhancing the natural crosslinks of collagen and elastin. Although initially promising, three randomized human trials of propanolol administered to small AAA patients revealed that this drug did not inhibit aneurysm expansion in comparison to patients receiving a placebo. Furthermore, the drop-out rates of subjects within these studies were overwhelmingly high (between 40% and 70%) due to symptoms of impaired pulmonary function and an overall reduction in quality of life.

2.2.5.2 Anti-Angiotensin II Agents

Angiotensin-converting enzyme (ACE) inhibitors block the conversion of angiotensin I to angiotensin II, which is a major vasoconstrictor. For this reason, ACE inhibitors, like beta blockers, are used for the treatment of hypertension. In addition to improving high blood pressure, ACE inhibitors apparently demonstrate some degree of MMP inhibition by likely interacting with the zinc binding site, as well as having a significant effect on connective tissue remodeling following vascular wall injury. Furthermore, humans taking ACE inhibitors for hypertension exhibit increases in markers for collagen III production, which may be a sign of a strengthened aortic wall.
beneficial effects carried over to the rat elastase perfusion model, where ACE inhibitors were able to inhibit experimental aneurysm formation. The effect of ACE inhibitors on human aneurysms has not yet been verified by randomized trials.

2.2.5.3 Anti-Inflammatory Agents

Since inflammation is one of the primary traits of aneurysmal tissues, and has even been suggested as the possible root cause of these pathologies, it is logical to assume anti-inflammatory agents may be useful in treating AAAs. The reduction in inflammation provided by corticosteroids correlated into improved elastin preservation, thus resulting in improved resistance to aneurysm formation. While the use of these particular anti-inflammatory agents would not be appropriate due to their effect on immunosuppression and wound healing, these studies opened the door for the potential use of related anti-inflammatory agents as an aneurysm therapy.

In a small case study with human patients, the use of non-steroidal anti-inflammatory drugs decreased the rate of aneurysm growth (1.5 mm/year vs. 3.2 mm/year in controls). The anti-inflammatory drug used was indomethacin, which apparently limits prostaglandin-E₂, whose synthesis has been linked to aortic aneurysm growth. Unfortunately, a number of anti-inflammatory agents have been taken off the market due to risk factors. Indomethacin, which may elicit gastrointestinal side effects, could potentially face a similar fate.

2.2.5.4 Matrix Metalloproteinase Inhibitors

Doxycycline, a member of the tetracycline family has been studied extensively for its ability to inhibit MMPs and, therefore, as a potential pharmacological treatment for
aneurysms. Doxycycline is effective toward MMPs on multiple levels: (1) it suppresses
MMP mRNA synthesis,\textsuperscript{180} (2) it inhibits extracellular activation of the proenzyme,\textsuperscript{119} and
(3) through non-specific direct inhibition, it can block the zinc binding sites along the
MMP’s catalytic domain.\textsuperscript{181} In addition to MMP inhibition, tetracyclines also possess
antibacterial properties.

In the initial aneurysm animal study by Petrinec et al (utilizing the elastase
perfusion model in rats), doxycycline improved susceptibility to aortic dilatation while
preserving the elastin.\textsuperscript{157} As expected, MMP activities were downregulated in those rats
receiving doxycycline treatment. These results were confirmed in the murine angiotensin
II aneurysm model as well.\textsuperscript{182}

In humans, studies have shown that the doses and serum levels of doxycycline
that are required to inhibit MMP activity are tolerable.\textsuperscript{183} Furthermore, significant levels
of the drug were found in the aortic wall of patients given an intravenous bolus of
doxycycline.\textsuperscript{184} It was concluded that the levels found in these tissues was enough to
inhibit MMP-9 activity. A small preliminary trial by Thompson et al revealed that an
oral dose of doxycycline (twice a day for seven days) significantly reduced MMP-2 and
MMP-9 expression in aortic wall, thus opening the door for a larger randomized
study.\textsuperscript{185} In the only reported human randomized trial to date, doxycycline apparently
reduced aortic expansion rates (1.5 mm/year vs. 3 mm/year in controls), however, there
was no statistical significance over the 18 month follow-up period.\textsuperscript{186}

MMP and protease inhibitors hold great promise as an AAA therapy. However,
caution should be taken in regards to their efficacy and safety. The “biological
redundancy” of the different MMPs may allow for other members of the MMP family to
compensate when one is being inhibited. In addition, these enzymes are essential for many normal physiological processes such as wound healing, angiogenesis, and embryonic development; therefore, their systemic inhibition could be perilous. This possibility has recently been foreshadowed by the prevalence of musculoskeletal side effects in patients undergoing systemic MMP inhibitor therapy.187

2.3 Elastin

2.3.1 Structural Features and Function

Elastin is a crucial insoluble extracellular matrix component of many tissues, including blood vessels, skin, lung, and ligamentum nuchae. Of particular interest to our research is the elastin found in arterial tissue and in the ventricularis layer (that layer of the valve leaflet closest to the heart) of valve cusp tissue. Depending on its location and size, elastin can constitute greater than 50% of an artery’s dry weight.188

The monomeric unit of elastin, tropoelastin, is a soluble ~70 kDa protein synthesized by fibroblasts and smooth muscle cells. Tropoelastin is crosslinked with desmosine and isodesmosine by the action of lysyl oxidase to form mature elastic fibers, which are oriented in vascular tissue to form concentric rings of elastic lamellae through the medial layer as shown in Figure 2.11. Embedded between these lamellae are layers of smooth muscle cells. The protein structure of elastin is rich in proline and glycine, however is not glycosylated and consists of very little free lysine. Mature elastin, which is insoluble and primarily hydrophobic in nature, is unusual in that it experiences very little metabolic turnover, as evidenced by its nearly 70-year biological half-life.101 The inability of elastin to revitalize itself is significant since this means elastin degradation
(such as that observed in tissue-derived cardiovascular devices and abdominal aortic aneurysms) is not likely to be accompanied by new elastin synthesis. Therefore, an elastin stabilizing agent for these cases would be extremely beneficial.

Figure 2.11 Distribution of elastic lamellae through a cross-section of arterial wall.\textsuperscript{189}

Based on present knowledge of elastin, we believe that an elastin-stabilizing agent (specifically tannins, which are introduced and described below in Section 2.4) may be able to bind to potential enzyme cleavage sites on the elastin molecule. A schematic depicting the main features of the elastin molecule is shown in Figure 2.12. The secondary structure of tropoelastin is characterized by an alternance of $\beta$-sheets, which are hydrophobic areas susceptible to elastases, and $\alpha$-helical crosslinking domains that offer resistance to proteases.\textsuperscript{190} Most elastin-degrading enzymes, such as MMPs, neutrophil elastase, and pancreatic elastase preferentially act on hydrophobic areas in the
As such, elastin stabilization should be targeted toward these areas along the elastin molecule.

**Figure 2.12** Structural features of elastin showing the repetitive alternance (square brackets) of $\beta$-hydrophobic domains and $\alpha$-crosslinking areas.

Developmentally, elastin is essential to arterial morphogenesis, as mutations or deficiencies in the elastin gene have resulted in supravalvular aortic stenosis and other arterial occlusive diseases.\textsuperscript{188,193} This may be related to the fact that vascular elastin plays a role in regulating the proliferation, differentiation, and migration of smooth muscle cells.\textsuperscript{194} In regards to its function, elastin is generally known more for its mechanical contribution. In many biological tissues, including blood vessels, elastin bears mechanical loads at low strains until that load reaches a threshold, at which point the stiffer collagen fibers assume the duty. As its name implies, elastin provides the tissue with elasticity or resilience. In arteries, the concentric elastin layers allow the tissue to stretch repetitively under the hemodynamic forces of blood flow without plastic
deformation. Because of elastin, the tissue is able to recoil back to its original shape, size, and configuration (Figure 2.13). This compliance is particularly important in the aorta, where the vessel’s recoil is the driving force behind blood flow into other branching vessels, such as the coronary arteries.

![Figure 2.13 Depiction of elastin recoil.](image)

Similarly, elastin fibers within heart valve leaflets serve to dampen the abrupt loading and unloading cycles experienced by these tissues. Studies have shown that elastin in both human and porcine cusps is like a “sponge”, creating an intertwining framework in which tubular collagen bundles pass through. As described above, elastin is able to withstand very large deformations, particularly in comparison to collagen. Cusp tissue, whose extracellular structure is dominated by collagen, undergoes a significant amount of deformation. Therefore, it is reasonable to assume that elastin plays a major role in the biomechanics of this tissue despite being overshadowed, in
regards to overall content, by collagen. For instance, it has been shown that this elastic fiber network absorbs tissue forces, thus allowing for slower valve closure.\textsuperscript{39} It has been theorized that this three-dimensional network of elastin and collagen works by allowing elastin to bear the tension during the initial phases of loading while collagen fibers begin to straighten. The less-extensible collagen fibers begin taking on a more significant load once they straighten. The most significant function of elastin takes place during unloading, in which elastin serves to return the collagen bundles back to their original (undeformed) state.\textsuperscript{5,38} This has been the observed case within native aortic valve cusps, so its exact correlation to cusps within fixed BHVs is somewhat less clear. However, it makes sense that any damage to elastin within these BHV tissues may be damaging to the implant. Collagen would have to bear abrupt loads in conditions it was not necessarily meant to withstand, potentially leading to material/implant fatigue.\textsuperscript{40}

In addition to its mechanical contribution, the significance of elastin degradation is described in greater details below.

\textbf{2.3.2 Elastin Degradation}

Due to its insolubility, natural desmosine and isodesmosine crosslinks, and extremely long biological half-life, elastin is generally perceived to be resistant to degradation. However, there are a specific set of enzymes, matrix metalloproteinases (MMP-2, MMP-9, and MMP-12), which are capable of degrading elastin. The activity of these enzymes is a clinical reality, as we have already discussed their presence within diseased native heart valves, explanted bioprosthetic heart valves, and abdominal aortic aneurysms. Elastin degeneration has also been implicated in other vascular pathologies, such as atherosclerosis and coronary restenosis.\textsuperscript{194}
Elastin degradation results in the release of soluble elastin peptides. These peptides are not simply passive by-products of the degradation process; rather, it has been demonstrated that they are active in protease production, chemotaxis, cellular proliferation, and various other biological activities. The release of elastin peptides can result in even more matrix degradation, as it has been shown that interactions between these peptides and smooth muscle cells increase expression of the elastin laminin receptor (ELR). This binding with ELR, a 67 kDa receptor found on a number of cell types, subsequently results in the promotion of greater MMP synthesis both at the mRNA and protein levels. Numerous studies have confirmed this correlation between upregulated MMP activity and the presence of elastin peptides. The use of luminally-perfused elastin peptides as an aneurysm animal model, which elicits elevated MMP levels and matrix degradation at the site of perfusion, also solidifies the biological power of these peptides.

In addition to MMP activity, elastin peptides have been shown to have varying effects on a broad range of cell types, including smooth muscle cells, fibroblasts, endothelial cells, macrophages, neutrophils, lymphocytes, and leukocytes. Of particular importance to many vascular diseases is the activation of vascular smooth muscle cells into a proliferative and migratory phenotype. This activation, which again is mediated by interactions between elastin peptides and ELR, is associated with an abrupt increase in intracellular calcium and has been implicated as a primary contributor to neointimal occlusion. The extreme bioactivity of elastin peptides underlines the clinical significance of elastin degradation and the subsequent need to protect elastin from degeneration.
2.4 Tannins

2.4.1 Chemical Structure and Properties

Based on their known properties, we propose to investigate tannins as novel agents that stabilize elastin from degradation. Tannins are naturally derived plant polyphenols typically divided into the following categories: condensed tannins, complex tannins, and hydrolysable tannins. Hydrolysable tannins are further classified into gallotannins, which are esters of gallic acid, and ellagitannins, which are esters of hexahydroxydiphenolic acid. For our application, we have focused on the use of gallotannins, particularly tannic acid (TA) and pentagalloyl glucose (PGG). Such gallotannins are known to specifically bind to hydrophobic regions in proline-rich proteins such as elastin and collagen and form a shell of multiple hydrogen bonds with surrounding proteins. Tannins, particularly TA, are also commonly used in electron microscopy, sometimes in combination with glutaraldehyde, for ultrastructural demonstration of elastin fibers, suggesting that tannins possess an affinity for elastin. Other advantages of tannins include the ability to partially inhibit enzyme activity, provide antibacterial and anti-inflammatory effects, and effectively reduce protein antigenicity.

The chemical structure of tannic acid, displayed in Figure 2.14, is characterized by a D-glucose molecule esterified at all five hydroxyl moieties by gallic acid (3, 4, 5-trihydroxybenzoic acid), yielding a pentagalloyl glucose “core”. This core molecule is further derivatized to produce five “tails” of gallic acid residues extending from the original glucose. The exact chemical structure of TA is somewhat ill-defined as each one of these tails may consist of anywhere between 2 and 10 galloyl residues. The
structures depicted in Figure 2.14 show variations of deca-galloyl glucose, a common representation of TA.

Another member of the gallotannin family of particular interest for our application is pentagalloyl glucose (PGG). PGG, shown as an isolated chemical structure in Figure 2.15 but also highlighted by the grey circle in Figure 2.14, is essentially the core of tannic acid. This relative of TA has a well-defined chemical structure, consisting of just one gallic acid residue bound to each hydroxyl moiety of the central glucose molecule, in contrast to the tails of multiple gallic acids found in TA.

**Figure 2.14** Composition and structure of tannic acid.
2.4.2 Previous Medical Applications

Decades ago (first reported in 1925), tannic acid was used as a standard topical treatment of burns due to its ability to reduce inflammation and bind to tissues, consequently fixing burn toxins into the wound area and preventing their further distribution throughout the patient’s body.\textsuperscript{210} This practice was relatively short-lived, however, due to issues of hepatotoxicity. More recent literature has questioned the negative effects of TA, however, since (1) the observed hepatotoxicity was independent of TA dosage, and (2) a high incidence of liver damage is seen even in those burn patients never treated with tannic acid.\textsuperscript{211} It is believed that the majority of the inferior results for this application were likely due to inadequate preparations (poor purity) of TA and/or the chemical instability of TA.

While the topic of TA and hepatotoxicity has been and still is heavily debated, a significant amount of research has recently been dedicated to assessing the validity of
these issues and the use of potential alternatives, such as TA “mimics”\textsuperscript{212,213} and high purity TA.\textsuperscript{211} The proposed mimics are synthetic dendrimers consisting of a varying number of galloyl moieties. These dendrimers were shown to have activities similar to TA but with improved chemical stability.\textsuperscript{212} Likewise, the use of high purity TA resulted in improved chemical stability and thus decreased hepatotoxicity;\textsuperscript{211} however, it should be noted that these results contradict previously reported studies.\textsuperscript{214} The correlation between the chemical structure of tannic acid, its instability, and potential toxicity will be clarified later in Chapter 6.

Studies have also shown that TA may be used as a (partial) collagen crosslinking agent, as exhibited by increased denaturation temperature of tannin-crosslinked dermal collagen.\textsuperscript{215} These results are consistent with studies showing stabilization of collagen with catechin, a plant-derived polyphenol unrelated to the gallotannin family.\textsuperscript{216} It is important to point out, however, that while the degree of collagen crosslinking was improved as compared to untreated collagen in these cases, it did not come near to matching the properties of glutaraldehyde, a common collagen crosslinker.

The pharmacological properties of tannins have been investigated for their anticarcinogenic, antimitagenic, antiviral, and antimicrobial capabilities.\textsuperscript{217} Indirectly, many tannin-containing food and drinks, such as green tea and red wine, have also been associated with these properties. For instance, the inverse correlation between green tea consumption and frequency of (gastric) cancer has been widely investigated and documented.\textsuperscript{218} This same relationship was exhibited for any diet rich in vegetables and fruits, which contain an abundance of polyphenolic tannins. In specific tumor studies on animals, TA and other green tea polyphenols inhibited skin tumors in mice, a trend which
was observed in different cases of tumor induction.\textsuperscript{219,220} Even oral administration of green tea polyphenols in very low doses inhibited formation of colonic tumors in rats.\textsuperscript{221} This beneficial effect of TA and/or green tea polyphenols has been demonstrated extensively in a number of animal models and tissues.\textsuperscript{217} Although the exact mechanism of action is not clear, it is hypothesized that these anticarcinogenic and antimutagenic properties are likely related to the antioxidative traits of tannins, thus protecting cells from oxidative damage.

Tannins have also been shown to inhibit growth of a number of filamentous fungi, yeasts, and bacteria, therefore exhibiting antimicrobial properties. This includes the ability to at least partially inactivate viruses such as strains of influenza and herpes, with inhibitory effects towards human immunodeficiency virus (HIV) as well.\textsuperscript{221} The capacity of tannins to inhibit the growth of microorganisms such as these is likely due to their ability to form complexes with numerous substrates, such as enzymes and metal ions. In both cases, this may result in inactivation of these substrates, which are required for microbe survival.

\section*{2.5 References}


CHAPTER 3  
PROJECT RATIONALE  

3.1 Hypothesis  

Due to its insolubility and low metabolic turnover, elastin is generally perceived to be stable and resistant to proteolysis. However, there are pathological cases where degradation of elastin is observed, such as within tissue-derived cardiovascular devices (bioprosthetic heart valves) and abdominal aortic aneurysms.

Glutaraldehyde, the conventional pretreatment for bioprosthetic heart valves, essentially does not react with elastin, thus leaving this pivotal extracellular matrix component vulnerable to enzymatic attack once implanted. Therefore, a pretreatment for these devices targeted at stabilizing elastin (analogous to the use of glutaraldehyde as collagen stabilizing fixative) may be beneficial in extending the overall longevity of these devices. This is important as we believe the indifference towards elastin could contribute to their less-than-ideal performance.

Elastin degradation is a primary hallmark of abdominal aortic aneurysm formation and progression. Elastin and the general architecture of the aorta degrades in these cases, resulting in progressive expansion associated with aneurysmal tissues. As such, it is logical to assume that by stabilizing the elastin within these diseased arteries, that we may be able to halt or limit the development of this potentially fatal pathology.

For both of these applications, we hypothesize to stabilize elastin using tannins, a class of naturally derived plant polyphenols known to interact with elastin.
3.2 Specific Aims

**Aim 1: Identify polyphenolic tannins as potential elastin-stabilizing agents.**

Elastin, which is vulnerable to enzymatic degeneration, may be stabilized with polyphenolic tannins. Time-dependent binding studies between pure elastin and tannins were completed, as well as subsequent studies on tannin-treated pure elastin and its ability to resist in vitro enzymatic digestion.

**Aim 2: Evaluate the feasibility of tannic acid as an elastin-stabilizing agent for tissue-derived cardiovascular implants, such as bioprosthetic heart valves.**

Tannic acid treatment of aortic wall may be beneficial in rendering the tissue resistant to enzymatic degradation and calcification, which could extend the clinical durability of these tissue-derived devices. The properties of tannin-treated aortic wall were evaluated and compared with tissue treated with glutaraldehyde, a common tissue pretreatment for bioprosthetic heart valves.

**Aim 3: Assess the toxicity of tannic acid treated tissues and investigate potential (less toxic) alternatives.**

Tannic acid, which was once used as a burn wound treatment, may elicit toxic effects.\(^1\,^2\) As a result, toxicity of tannic acid treated tissues was evaluated and compared to tannin derivatives such as pentagalloyl glucose, which is essentially the core structure of tannic acid. Due to improved chemical stability, pentagalloyl glucose may be a more viable option as an elastin stabilizing agent.

**Aim 4: Investigate polyphenolic tannins as therapeutic agents for abdominal aortic aneurysms.**

The formation or progression of aortic aneurysms, a disease associated with elastin degeneration, may be inhibited with site-specific treatment of elastin stabilizing agents. Using an animal model to induce aortic aneurysm formation,
site-specific tannin treatment was applied perivascularly and evaluated for its ability to hinder aneurysm formation and progression (separately) by monitoring changes in aortic diameter and elastin integrity.

3.3 Clinical Significance

3.3.1 Elastin Stabilization in Bioprosthetic Heart Valves

To date, there is no prevalent drug therapy for diseased heart valves; therefore, surgical replacement is the primary option. It is estimated that approximately 275,000 valve replacement surgeries are performed annually, with tissue-derived bioprosthetic heart valves constituting about half of these. To these devices are typically made from porcine aortic valve tissue or bovine pericardium. In order to make these xenogenic tissue devices more resistant to enzymatic degradation, they are pretreated prior to being implanted into a human. Glutaraldehyde, the most common pretreatment or fixative used for this purpose, stabilizes collagen within these valves. Collagen is the prevalent component of valve cusps and possesses a large amount of lysine, meaning many amine groups are available for binding with glutaraldehyde. While glutaraldehyde demonstrates an excellent capacity to bind with and crosslink collagen, it has virtually no stabilizing effect on elastin. This is likely due to elastin’s lack of amine groups necessary for reaction with glutaraldehyde. In addition to glutaraldehyde, there is no pretreatment or fixative available which claims to bind to elastin.

This indifference towards elastin could be detrimental since elastin degradation (and the resulting products, elastin peptides) can lead to calcification, altered mechanical properties, intensified inflammation, and stimulation of protease production. The issue
of elastin degradation is especially noteworthy for stentless bioprosthetic heart valves, which include a portion of the elastin-rich aortic wall. For all bioprosthetic heart valves (stented and stentless), elastin is also present within the cusps. Elastin degradation may contribute to the suboptimal performance of these devices, as it has been estimated that about half fail within 10 years of implantation.\textsuperscript{8,9} The development of a pretreatment targeted specifically at stabilizing elastin, just as glutaraldehyde is used to crosslink collagen, may beneficial in extending the clinical durability of bioprosthetic heart valves.

\textbf{3.3.2 Elastin Stabilization as a Treatment for Abdominal Aortic Aneurysms}

Patients with small or early stage aneurysms (aorta less than 5 cm in diameter) comprise the largest percentage of all AAA patients.\textsuperscript{10} This number is likely to increase dramatically with the advent of “blanket” screening of asymptomatic subjects. In these cases of small AAAs, no treatment exists (other than imaging surveillance) until the aortic diameter reaches the 5.5 cm threshold, thus requiring surgery. This “wait and see” approach is not without risk, however, as 10\% of the abdominal aortic aneurysms that rupture do so at diameters less than 5 cm.\textsuperscript{10} Therefore, alternative pharmacological treatments targeted at limiting aortic expansion may be helpful in reducing incidence of rupture and circumventing the need for surgical repair.

As a result, the long-term goal of this project is to slow the evolution of degenerative processes associated with abdominal aortic aneurysms. The unique properties of polyphenolic tannins may allow for the development of a novel site-specific strategy based on stabilizing elastin against enzymatic degradation. If successful, this technology could offer more effective therapies for treating aortic aneurysms, either (1) in conjunction with endovascular stent grafts, or (2) as a stand-alone treatment, including
for aneurysms at the early and moderate stages for which there is no current option other than medical surveillance.

3.4 References


CHAPTER 4
TANNINS AS ELASTIN STABILIZING AGENTS

4.1 Introduction

Elastin is an extracellular matrix protein which endows tissue with resilience or the ability to recoil repetitively. In addition to tissue mechanics, preserving elastin is also important since the by-products of elastin degradation have been associated with undesirable effects, such as tissue calcification and recruitment of inflammatory cells and proteases. Despite this, virtually no effort has been made to stabilize elastin against the action of proteolytic enzymes. We propose to do this with tannins, a class of naturally derived plant polyphenols. Tannins were chosen for their ability to bind to proline-rich proteins such as elastin. Furthermore, they are often used in electron microscopy as an elastin-specific mordanting agent, indicating that an interaction between elastin and tannins exists.

In the studies presented in this chapter, we provide evidence that tannic acid, a polyphenolic tannin, binds to pure elastin in a time-dependent pattern and this binding increases the resistance of elastin to enzymatic degradation. Throughout these studies, tannin treated elastin is compared to that treated with glutaraldehyde. Glutaraldehyde is a common tissue treatment used for its ability to stabilize and crosslink collagen. As shown here, however, glutaraldehyde does not protect pure elastin from enzymatic degradation.
4.2 Methods

4.2.1 Materials

Porcine hearts were obtained from local USDA approved slaughterhouses. Ultra pure type VII collagenase, tannic acid, trinitrobenzenesulfonic acid, and other chemicals were of highest purity available and obtained from Sigma (St. Louis, MO). Elastin purified from bovine neck ligament, ultra pure pancreatic elastase, and elastin-orcein were from Elastin Products Company Inc. (Owensville, MO). Glutaraldehyde was obtained from Polysciences Inc. (Warrington, PA).

4.2.2 Elastin-Orcein Study

Pilot experiments utilized commercially available bovine neck insoluble elastic fibers labeled with orcein (elastin-orcein), which is routinely used as a substrate for pancreatic elastase. Glutaraldehyde (Glut) fixed elastin substrate was prepared by treating elastin-orcein with 0.6% Glut in 50 mM Hepes buffered saline, pH 7.4, overnight at room temperature. Following treatment, samples were centrifuged at 5000 rpm for 15 minutes, followed by washing with ddH₂O, dialyzing against ddH₂O, and finally suspending the Glut-treated elastin substrate in elastase buffer (100 mM Tris buffer, pH 8.0, 1 mM CaCl₂, 0.02% NaN₃) at a final concentration of 20 mg/ml. Similarly, elastin-orcein substrate was treated with 0.3% tannic acid (TA) in Hepes buffer, rinsed, dialyzed and resuspended as above. As a control, elastin-orcein was incubated overnight in 50 mM Hepes buffered saline (without Glut or TA), washed, dialyzed and resuspended at a final concentration of 20 mg/ml. Elastase assays were performed by separately incubating untreated, Glut-treated, and TA-treated elastin substrates (n=3) with pure
pancreatic elastase (0.1 Units/mL in elastase buffer) at 37°C for 96 hours. Following elastase digestion, samples were centrifuged at 12,000 rpm and the amount of digested elastin was assayed by measuring the supernatant OD at 570 nm (maximal absorption wavelength of orcein). The release of orcein dye in solution allowed for direct quantification of elastin degradation. As background controls, corresponding substrates incubated in buffer without elastase were used. Values are expressed as % degradation, using untreated elastin-orcein (completely degraded after 96 hours) as a positive (100%) control. All samples were assayed in triplicates.

4.2.3 Preparation and Characterization of Pure Aortic Elastin

Ascending porcine aorta was collected fresh at the slaughterhouse and transported to the laboratory on ice. After cleaning fat and adherent tissues, aorta was cut into 2 mm x 4 mm strips and thoroughly rinsed in cold saline. Aortic elastin was purified by a sequence of extractions that included sodium hydroxide (NaOH) treatment and collagenase digestion with slight modifications. Aortic strips were suspended in 100 mM NaOH (60 strips in 100 ml) and incubated for 14 hours at 37°C on a shaker at 180 rpm. This step extracts all cellular material, non-collagenous components, and some of the collagen, leaving elastin intact. The strips were rinsed thoroughly in ddH2O and then incubated on a shaker at 180 rpm for 16 hours at 37°C with collagenase (0.5 Units / mg wet tissue) in 50 mM Tris buffer, 10 mM CaCl2, pH 8.0. The collagenase preparation was pre-adsorbed with elastin fibers to remove residual elastolytic activities. This final digestion step completely removes residual collagen, leaving behind pure aortic elastin. Elastin strips were rinsed thoroughly in ddH2O and stored frozen at -80°C.
4.2.4 Purity Analysis of Aortic Elastin

The porcine elastin preparation (and standard bovine neck elastin for comparison) were subjected to N-terminal amine group analysis\(^6\) and hexosamine assay.\(^7\) In addition, sodium dodecyl sulfate extracts prepared from elastin were subjected to polyacrylamide gel electrophoresis and silver staining for detection of contaminating proteins.\(^8\) For histological studies, paraffin embedded samples were sectioned and stained with Movat’s pentachrome.

4.2.5 Optimization of Elastase Digestion

In order to develop a screening method for elastin stabilization, we developed and optimized an in vitro assay that investigates resistance to porcine pancreatic elastase. This test is analogous to the widely used assay for resistance to collagenase, which is utilized as a barometer for collagen crosslinking or stabilization.

4.2.5.1 Elastase Concentration

Strips of lyophilized pure aortic elastin (~10 mg/strip) were suspended in 1 ml of 0, 1, 5, 10 or 20 Units/mL of elastase solutions prepared in 100 mM Tris buffer, 1 mM CaCl\(_2\), 0.02% NaN\(_3\), pH 7.8, and incubated at 37°C for 20 hours with shaking at 600 rpm. Samples were centrifuged for 10 minutes at 10,000 rpm (4°C), rinsed in ddH\(_2\)O, lyophilized, weighed, and percentage of elastin degradation calculated from dry weights before and after exposure to elastase.

4.2.5.2 Elastase Digestion Time

Lyophilized pure aortic elastin strips were incubated with 20 Units/mL of elastase in buffer (as above; 100 mM Tris, 1 mM CaCl\(_2\), 0.02% NaN\(_3\), pH 7.8) for 0, 2, 6, 24 or 48
hours at 37°C with shaking at 600 rpm. Samples were rinsed, lyophilized, weighed and percentage of elastin degradation was calculated based on dry weights before and after elastase as described above.

4.2.6 Tannic Acid Binding with Pure Aortic Elastin

Pure aortic elastin strips (2 mm x 4 mm) were suspended in 1.5 mL of an 8 mg% tannic acid (TA) solution prepared in 50 mM Na₂HPO₄ buffered saline, pH 5.5. A second group consisted of pure aortic elastin suspended in a solution containing 8 mg% TA and 16 mg% glutaraldehyde (TA+Glut) in same buffer. As controls, TA solution and the mixture of TA and Glut were incubated without exposure to samples of elastin. Samples were incubated at room temperature and at defined time intervals (0, 20, 40, 60, 120, 360 minutes and 24 hours). Following each respective time interval, samples were retrieved and analyzed for tannin content in solution according to the Folin-Denis method for total phenols, as originally described by Rosenblatt⁹ and modified by Mole.¹⁰ For this phenol quantification assay, samples were mixed with a tungstate-phosphomolybdate reagent, followed by addition of a saturated sodium carbonate solution and ddH₂O. After 10 minutes at room temperature, OD at 760 nm was measured in a microplate spectrophotometer. Standard curves were constructed with TA in the range of 0 to 8mg% and with TA (0 to 8mg%) in mixtures with Glut (16 mg%). Glut interference with the TA color reaction was minimal (no statistical differences found for all data points). Finally, the elastin strips were rinsed in ddH₂O and lyophilized. The amount of TA bound to pure aortic elastin was calculated from the differences between initial TA concentration in solution and concentration of TA in solution after incubation with elastin.
strips. Results were expressed as μg of TA bound by 1 mg dry elastin. All samples were assayed in triplicates.

4.2.7 Effect of Tannic Acid pH on Elastin Stabilization

Strips of pure aortic elastin were treated with solutions of 0.3% tannic acid for 4 days at room temperature. The effect of solution acidity on elastin stabilization was investigated at pH 5.5 and pH 7.4. For pH 5.5, TA was dissolved in 50 mM Na₂HPO₄ buffered saline. For pH 7.4, TA solution was in 50 mM Hepes buffered saline. As controls, buffer solutions (with no TA) were made for each pH using either Na₂HPO₄ for pH 5.5 or Hepes for pH 7.4. After incubating the pure elastin in one of these four groups, samples were rinsed (3 times, 1 hour each in ddH₂O) and the elastase digestion assay was performed with 20 Units/mL of elastase dissolved in 100 mM Tris buffer, 1 mM CaCl₂, 0.02% NaN₃, pH 7.8 at 37°C for 48 hours with shaking at 600 rpm. Dry weights before and after enzyme digestion were used to calculate percent digestion or mass loss.

4.2.8 Effect of Tannic Acid Concentration on Elastin Stabilization

Lyophilized pure aortic elastin strips were suspended in 0, 0.003, 0.03, 0.3 and 0.8% tannic acid solutions in 50 mM Na₂HPO₄ buffered saline, pH 5.5, at room temperature. A second series of elastin strips were incubated in 0.6% Glut solutions containing 0, 0.003, 0.03, 0.3 and 0.8% TA in same buffer. After 48 hours, samples were rinsed in ddH₂O, lyophilized, and weighed. The treated elastin samples were then incubated with 20 Units/mL of elastase dissolved in 100 mM Tris buffer, 1 mM CaCl₂, 0.02% NaN₃, pH 7.8 at 37°C for 48 hours with shaking at 600 rpm. Samples were rinsed, lyophilized, weighed, and percentage of elastin degradation calculated from weights
before and after exposure to elastase. The “used” elastase solutions were recovered after incubation with treated elastin and routinely assayed for elastase activity using elastin-orcein as a substrate to rule out potential inhibitory effects of TA that may have leached from elastin samples.  

4.2.9 Kinetics of Tannic Acid-Mediated Elastin Stabilization

Lyophilized pure aortic elastin strips were treated with 0.3% tannic acid in 50 mM Na₂HPO₄ buffered saline, pH 5.5, at room temperature. A second series of elastin strips were incubated in a mixture of 0.6% Glut and 0.3% TA in this same buffer. Samples were incubated for 1, 2, 4, and 7 days at room temperature. At designated time intervals, samples were rinsed in ddH₂O, lyophilized, and weighed. Samples were then incubated with 20 Units/mL of elastase dissolved in 100 mM Tris buffer, 1 mM CaCl₂, 0.02% NaN₃ pH 7.8 at 37°C for 48 hours with shaking at 600 rpm. Samples were rinsed, lyophilized, weighed and percentage of elastin degradation was calculated from weights before and after exposure to elastase. The elastase solution recovered after incubation with treated elastin was assayed for elastase activity with elastin-orcein as described above.

4.2.10 Data Analysis

Results are expressed as means ± standard error of the mean (SEM) with n=6 for each experimental group, unless otherwise noted. Statistical analyses of the data were performed using the Student’s t-test and probability values (p) for significance were calculated, with p<0.05 considered significant.
4.3 Results

4.3.1 Elastin-Orcein Study

In a pilot experiment, we tested the ability of Glut and separately TA to stabilize elastin-orcein against the action of elastase (Figure 4.1). Orcein dye, which is originally bound to the elastin, is released into solution upon cleavage of elastin; therefore, spectrophotometric measurement of the dye in solution can be directly correlated to elastin degradation. As expected, untreated elastin was completely degraded after 96 hours (~100% degradation). Glut-treated elastin-orcein was also almost degraded in its entirety (93.7%). These values for Glut were not statistically different from those of native elastin (p>0.10) indicating that Glut does not effectively stabilize elastin, meaning this treatment leaves elastin extremely vulnerable to enzymatic degradation. In contrast, only about 35% of TA-treated elastin-orcein (p<0.05) was degraded by elastase, indicating that a significant portion of elastin was protected from enzymatic activity by treatment with TA. These data indicate that TA has an excellent potential as an elastin-stabilizing agent.
Figure 4.1 Tannic acid (TA) mediated stabilization of elastin-orcein against the action of elastase. Note the complete degradation of Glut-treated elastin-orcein and protection by TA (p<0.05 for Glut vs. TA).

4.3.2 Purity of Porcine Aortic Elastin

Selection of proper elastin purification methods is important because preparations may differ in purity and integrity and thus may compromise binding, stabilization, and digestion results. For our studies, we selected a sequence of extraction steps that result in intact, highly purified elastin. The method does not involve excessive heating and thus may conserve the natural configuration of elastin. The porcine aortic elastin preparation contained $10.12 \pm 1.6$ moles of amines per mole of elastin, indicating that the preparation procedure induced minimal degree of random peptide cleavage. These values were not statistically different from those of the historical standard of bovine neck ligament elastin (data not shown). Hexosamine levels were also minimal (0.25% of total weight), indicating relative absence of glycosaminoglycans or matrix glycoproteins in the aortic
elastin preparation. In addition, aortic elastin contained undetectable protein impurities, as evidenced by electrophoretic analysis of extracts followed by silver staining (data not shown). Histological analysis with Movat’s pentachrome stain confirmed the absence of cell remnants and collagen fibers in the aortic elastin preparation (Figure 4.2). The fact that the untreated aortic elastin preparation was completely digested by elastase (see results below in section 4.3.3) was also an excellent indication that the preparation was pure.

![Figure 4.2](image)

**Figure 4.2** Histology of fresh porcine aorta (A) and the pure aortic elastin preparation used in this study (B). Note the absence of smooth muscle cells (red), collagen (yellow), and ground matrix (blue) in the purified elastin. Original magnification, 100x.

### 4.3.3 Stabilization of Porcine Aortic Elastin

To harness the potential of tannins as elastin stabilizing agents, we investigated the optimal conditions required for TA-mediated stabilization of elastin purified from porcine aorta. This was tested using an elastase digestion assay which we developed. To optimize this assay, we first investigated the appropriate concentration of elastase (Figure 4.3) and time (Figure 4.4) required for complete degradation of pure aortic elastin. The
results show that a minimum of 10 units of porcine pancreatic elastase per mL are required for 24 hours in order to get complete degradation of ~10 mg dry pure aortic elastin. To ensure complete degradative capabilities were reached, we selected to use 20 Units/mL of elastase for 48 hours for further studies.

In order to determine the kinetics of TA binding to elastin, we incubated elastin strips with TA and separately with mixtures of TA and Glut (TA+Glut) and assayed the concentration of TA in solution for up to 24 hours (Figure 4.5). The TA concentration for this binding study was selected so that it fits within the linear portion of the Folin-Denis assay standard curve. For TA and Glut mixtures, we used a ratio of 1:2 (TA:Glut), as this is a standard ratio used in tissue processing for electron microscopy. Levels of TA in control solutions incubated without elastin remained constant throughout the study, indicating that the solutions were stable. However, within 1 hour of incubation with elastin, the amount of TA in solution decreased by ~50%, decreasing to 10% after 6 hours, and to less than 3.5% of initial TA at 24 hours, clearly indicative of TA binding to elastin. TA binding values were also normalized to dry weight of elastin strips and are depicted in Figure 4.5. The resulting kinetics show a rapid binding within the first 6 hours, leveling off afterwards at ~3 mg of TA per mg dry elastin. Interestingly, the binding rate of TA from the mixtures of TA and Glut was higher than that of TA alone up to 6 hours of incubation (Figure 4.6; p<0.05 for all time points between 20 minutes and 6 hours), thus suggesting that Glut may facilitate or accelerate the binding of tannins to elastin.
Figure 4.3 The minimum elastase concentration needed to obtain complete digestion (>99% mass loss) of untreated pure aortic elastin is 10 Units/mL.

Figure 4.4 The minimum time of elastase incubation needed for complete digestion (>99% mass loss) of untreated pure aortic elastin is 24 hours.
Figure 4.5  Kinetics of tannin-elastin interactions. Tannic acid (TA) concentration in solution (µg TA/mL) decreases in the presence of aortic elastin (EL) as to compared to controls without added elastin (CTRL).

Figure 4.6  Cumulative binding of tannic acid (TA) to pure aortic elastin (µg TA per mg dry elastin). ●-● indicates statistically different values (all data points up to 6 hours) for TA vs. TA+Glut (p<0.05).
Tannic acid treatment was performed at pH 5.5 and pH 7.4 to determine the more suitable condition for elastin stabilization. Elastase digestion results revealed that TA at pH 5.5 was significantly improved over that at pH 7.4 (p<0.05; Figure 4.7). These experiments also revealed that TA is not stable in solution (long-term) at pH greater than 6. This instability was associated with the appearance of a brown precipitate, which was apparently ineffective as an elastin-stabilizing agent. Since TA stability was significantly improved at pH 5.5 over a long period of time, we chose this pH for present studies.

**Figure 4.7** Digestion of pure aortic elastin after varying the pH of tannic acid (TA) solutions revealed that elastin stabilization was more effective at pH 5.5.
To determine the optimal TA concentration for elastin stabilization, pure aortic elastin was exposed to increasing TA concentrations and tested for resistance to elastase (Figure 4.8). Control elastin preparations, including Glut-treated elastin, were completely degraded. Treatment with 0.003% TA did not apparently stabilize elastin and 0.03% TA offered minimal stabilization (p>0.05). Meanwhile, concentrations of 0.3% TA and higher reduced the susceptibility towards elastase by ~50%. Statistical analysis showed that digestion values for elastin treated with 0.8% TA were not different from those of 0.3% TA-stabilized elastin (p>0.10). Mixtures of increasing TA concentrations prepared in 0.6% Glut were more effective than TA alone (p<0.05 for 0.3% TA), suggesting that Glut enhances TA-mediated stabilization of elastin. For further stabilization studies, we selected to use concentrations of 0.3% TA and 0.3% TA in 0.6% Glut.

Figure 4.8 Digestion of pure aortic elastin treated with varying concentrations of tannic acid (TA) with and without glutaraldehyde (Glut). The combination (TA+Glut) was slightly more effective in stabilizing elastin than TA alone (p<0.05).
The time needed for TA treatment to stabilize pure elastin was investigated for TA alone as well as mixtures of TA and Glut (Figure 4.9). All elastin samples treated in Glut were completely digested by elastase, irrespective of the length of Glut treatment. Stabilization of elastin by TA, as well as by TA and Glut increased with time, reaching a relatively constant value after 4 days (Figure 4.9). These results indicate that stabilization of pure elastin may occur within 4 days of treatment with TA or mixtures of TA and Glut.

![Graph](image)

**Figure 4.9** Treatment time of tannic acid (TA) needed to obtain elastin stabilization. Note that glutaraldehyde (Glut) treated elastin was vulnerable to digestion in all cases.

Gravimetric digestion data were further confirmed by histology with Movat’s pentachrome stain. As seen in Figures 4.10A and 4.10B, the following general trends across all studies were validated visually: Glut treatment leaves elastin fibers susceptible to enzymatic degradation, while tannin treatment alleviates this vulnerability. In fact,
analysis of elastase digested, Glut-treated elastin was not possible due to complete (~100%) degradation. We therefore selected to expose Glut-treated elastin to elastase for just 1.5 hours, an interval which we found to yield about 50% digestion and mass loss (Figure 4.4). Partially degraded Glut-fixed elastin exhibited extensive fraying and massive loss of fibers (Figure 4.10A). Elastase treatment of TA+Glut stabilized elastin revealed excellent preservation of aortic structure, without massive loss of elastin integrity (Figure 4.10B), as seen in the similarity to pure elastin which was never exposed to enzyme (Figure 4.2B).

**Figure 4.10** Glutaraldehyde fixed aortic elastin exposed to elastase shows clear signs of fiber degradation after 1.5 hours of incubation (A), while elastin stabilized with a mixture of tannic acid and glutaraldehyde exhibits nearly complete resistance to elastase degradation for up to 48 hours (B).

Movat’s pentachrome stain, original magnification, 100x.

### 4.4 Discussion

The long-term aim of this project is to develop a method for elastin stabilization which may be applied to improve tissue-derived cardiovascular devices (such as
bioprosthetic heart valves) and/or treat abdominal aortic aneurysms. Due to the complex composition of the cardiovascular and vascular tissues associated with these applications, it was considered imperative to undertake a fundamental study using pure elastin as a model for elastin-containing tissues. For this purpose we purified elastin from porcine aorta using established procedures and developed an accelerated degradation assay using pancreatic elastase. Optimal conditions for complete enzymatic digestion of pure aortic elastin were found to be 48 hours of incubation with 20 units of pancreatic elastase at 37°C with vigorous shaking (Figures 4.3 and 4.4). These conditions constituted a reliable in vitro model of elastin degeneration. Since the degree of elastin degradation in vivo is not fully known, this model could be considered an accelerated screening system that allows for evaluation of elastin stabilization strategies.

The lack of basic knowledge regarding the use of tannic acid (TA) for elastin stabilization also prompted us to investigate the stability of TA solutions and optimal conditions required for interactions between TA and elastin, such as pH of TA solutions (Figure 4.7), TA concentration (Figure 4.8), and time of TA treatment (Figure 4.9).

The first finding of our studies was that treatment of elastin with glutaraldehyde (Glut) had practically no effect on its susceptibility to elastase. This was shown initially in a pilot experiment using orcein-labeled bovine neck elastin, a traditional elastase substrate (Figure 4.1) and in subsequent experiments using aortic elastin (Figures 4.8, 4.9, and 4.10). Elastin stabilization by Glut could not be improved by increasing incubation time for up to 7 days (Figure 4.9) or by altering the pH of the Glut solution (data not shown). It was previously shown that extremely low levels of radioactive Glut incorporated into elastin in vitro at pH 7.4, providing evidence that Glut does not
effectively react with elastin.\textsuperscript{14} This lack of stabilization by Glut may be explained by the fact that elastin contains an extremely low number of reactive amine groups suitable for reaction with Glut.\textsuperscript{15} Glut was used in these studies not only as a kind of negative control, but also because Glut is widely used in the preparation of commercially available tissue-derived cardiovascular devices. Therefore, we have demonstrated that these preparations are ineffective toward the elastin within these tissues.

By comparison with Glut, treatment of elastin-orcein (Figure 4.1) and pure aortic elastin (Figures 4.7, 4.8, 4.9, and 4.10) with TA was efficient in preventing elastase-mediated degradation, indicating that TA has excellent potential as a stabilizing agent. Binding studies clearly showed that TA binds to pure elastin in a time-dependent pattern (Figures 4.5 and 4.6). A calculated $3 \, \mu g$ of TA were found to bind to $1 \, mg$ of dry elastin within 24 hours, which yielded a calculated value of $\sim 1$ mole of TA bound per mole of elastin (considering the molecular weight of elastin as 70 kDa and that of TA as 1.8 kDa for a decagalloyl derivative) under current experimental conditions. Interestingly, when TA was used in mixture with Glut, the kinetics of TA binding to elastin were enhanced within the first 6 hours of incubation (Figures 4.5 and 4.6), suggesting that a beneficial interaction between TA and Glut may occur during treatment of elastin. From studies presented in Figures 4.8 and 4.9, it also appeared that the use of TA in combination with Glut might be beneficial for elastin stabilization. While this compatibility between TA and Glut may be important for the development of a tissue pretreatment for cardiovascular devices, it is important to note that TA, even when used alone, also provided a significant level of stabilization in all cases as compared to untreated and Glut-treated elastin.
The mechanisms of TA-mediated elastin stabilization against the action of elastase are not known. One potential explanation could be that TA that may have leached from treated elastin samples is working by inhibiting elastase activity. As a result, we tested elastase solutions (recovered after incubation with treated elastin) using an elastin-orcein assay and showed no change in activity as compared to fresh elastase solutions (data not shown), ruling out this possibility.

Based on present knowledge of elastin composition, we speculate that TA protects elastin by binding to potential elastase cleavage sites on the elastin molecule. A schematic depicting the main features of the elastin molecule was previously shown in Figure 2.12. The secondary structure of tropoelastin is characterized by an alternance of β-sheets, hydrophobic areas susceptible to elastases, and α-helical, crosslinking domains. Most elastolytic enzymes, such as matrix metalloproteinases, neutrophil elastase, and pancreatic elastase preferentially act on hydrophobic areas in the elastin structure. Therefore, it is tempting to speculate that TA, which has a known affinity for hydrophobic regions, stabilizes elastin by binding to these (hydrophobic) potential elastase cleavage sites, thus preventing enzyme-substrate interactions. This hypothetical relationship between TA and elastin is schematically illustrated in Figure 4.11. The bonds between TA and elastin are shown as hydrogen bonds, since it is known that TA interacts with proteins by creating a vast “shell” of hydrogen bonds.
Figure 4.11 Hypothetical interactions between TA and elastin. Elastin is composed of alternating hydrophilic crosslinking regions (1) and hydrophobic domains (2). The naturally derived desmosine crosslinks (3) connect mainly hydrophilic regions of the elastin molecule. Hydrophobic areas are susceptible to elastase cleavage and are also the potential target of TA stabilization. The hydrophobic “core” of the TA molecule (circular structure), likely incorporates itself into hydrophobic areas (4), while the hydroxyl moieties of TA establish multiple hydrogen bonds (dashed lines) with neighboring elastin molecules (5), resulting in improved elastin stabilization.

4.5 Conclusions

Glutaraldehyde (Glut) treatment is entirely ineffective in protecting elastin from enzymatic degradation, but treatments with tannins such as tannic acid (TA) can circumvent this vulnerability. TA binds to vascular elastin and this binding significantly increases its resistance to enzymatic degradation. As a result, TA may hold great promise for (1) improving the long-term performance of cardiovascular implants such as
bioprosthetic heart valves, and (2) developing a therapy for limiting the progression of abdominal aortic aneurysms based on elastin stabilization.

4.6 References


CHAPTER 5
ELASTIN STABILIZATION IN BIOPROSTHETIC HEART VALVES

5.1 Introduction

Chemically preserved biological tissues have been used in the form of bioprosthetic heart valves (BHV) for several decades. The intended result of this preservation or fixation is to reduce the xenogenic tissue’s antigenicity and to form crosslinks within the extracellular matrix, resulting in a biomaterial that is more resistant to enzymatic degradation. Glutaraldehyde (Glut) has been widely used as a tissue fixative for BHVs due to its ability to crosslink matrix molecules, sterilize tissue, and reduce tissue antigenicity. Thousands of Glut-fixed BHVs are used for surgical replacement of diseased human valves annually, yet over half of these treated BHVs fail within 10 years of implantation due to tissue degeneration and calcification.¹,²

While Glut fixation is generally perceived to adequately crosslink the collagen component of BHVs, it is unable to stabilize other important constituents of the ECM, particularly elastin. The elastin molecule is known to contain very few free amine groups required for crosslinking with Glut, which leaves it highly susceptible to enzymatic degradation. This vulnerability is significant considering elastin is one of the major ECM components of the aortic segment of stentless BHVs and the ventricularis layer of the valvular cusp structure. Elastin degeneration could lead to loss of elastic recoil and deterioration of mechanical properties, potentially resulting in clinical failure of implanted cardiovascular prostheses.³,⁴ It has also been shown that pure elastin, even in the absence of Glut fixation, is susceptible to degradation and calcification after
implantation in animal models.\textsuperscript{5,6} These facts suggest that elastin degeneration in BHVs and tissue-derived cardiovascular implants may be important in the clinical setting and that there is a need for a fixation procedure that specifically enhances the stability of elastin towards enzymatic degradation.

We propose to do this with tannins, a class of naturally occurring plant polyphenols known to interact with proteins. As presented in the previous chapter, Glut did not adequately stabilize pure elastin, whereas tannins (specifically tannic acid, TA) bound to pure aortic elastin in a time-dependent pattern which resulted in improved resistance to elastase. In addition, when TA was used in mixture with Glut, the kinetics of TA binding to elastin were enhanced, which translated into improved elastin stabilization. In the studies presented here, this work has been extended to evaluate the feasibility and applicability of TA as a pretreatment or fixative for bioprosthetic heart valves. To do so, we investigated the efficacy of TA to stabilize elastin within porcine aortic wall. We demonstrate that TA does not impair the ability of Glut to adequately crosslink aortic collagen and that by protecting elastin from enzymatic attack, TA may also inhibit calcification of Glut-treated aorta.

In addition, we evaluated the efficacy of tannin treatment when used in conjunction with metals, specifically aluminum chloride and ferric chloride. Aluminum and iron, which are suspected to bind to elastin, have been previously investigated as anti-calcification pretreatments for BHVs.\textsuperscript{7,8} Furthermore, tannins have a strong binding affinity for metal ions such as these,\textsuperscript{9} so the effects of the two agents together (tannins and anti-calcification metal ions) were examined as a potential BHV pretreatment.
5.2 Methods

5.2.1 Materials

Tannic acid, type I bacterial collagenase (277 U/mg), azo dye-impregnated collagen, aluminum chloride, ferric chloride, and other chemicals were of highest purity available and obtained from Sigma (St. Louis, MO). Glutaraldehyde (EM grade, 8% solution) was acquired from Polysciences, Inc. (Warrington, PA). High purity porcine pancreatic elastase (135 U/mg) and elastin-orcein were obtained from Elastin Products Company, Inc. (Owensville, MO).

5.2.2 Tissue Preparation and Fixation Procedures

Ascending porcine aorta (supravalvular segments, approximately 3 cm in height) was collected fresh at local USDA-approved slaughterhouses and transported to the laboratory on ice in saline (9 g NaCl per liter). All tissues were harvested from healthy male pigs, six to eight months of age, and weighing approximately 200 pounds. Aorta was cleaned of fat and extraneous tissue, rinsed exhaustively in cold physiological saline, and cut into 4 mm x 4 mm squares. Dumbbell shapes (40 mm long, 10 mm wide at each end, 5 mm wide in center) were also cut for mechanical testing.

For the majority of studies, samples were treated by one of two methodologies: (a) glutaraldehyde (Glut) fixation, or (b) Glut treatment followed by tannic acid (Glut/TA). For Glut treatment, aortic samples were fixed with 0.6% glutaraldehyde in 50 mM Hepes buffered saline, pH 7.4, overnight at room temperature, followed by 0.2% glutaraldehyde in same buffer for 7 days at room temperature, as described previously.\(^\text{10}\) Glut/TA treatment consisted of the fixation steps outlined in the Glut protocol, followed
by TA treatment (0.3% tannic acid in 0.6% glutaraldehyde in 50 mM Na₂HPO₄ buffered saline, pH 5.5 for 4 days at room temperature). For each treatment, 50 mL of fixative or solution were used for every 6 aortic samples (4 mm x 4 mm). As controls, fresh untreated samples were also used in all experiments except in the case of subdermal implantation.

5.2.3 Tannin Binding to Porcine Aortic Wall

Tannic acid (TA) was first investigated for its ability to bind to porcine aortic wall using a phenol-specific histological stain. Porcine tissue was left untreated (fresh), treated with glutaraldehyde alone (Glut), or treated with Glut/TA. Samples were sectioned (6 µm) and then stained with 10% ferric chloride (FeCl₃) in methanol and counterstained with 1% light green. The ferric chloride in this case stains phenols, such as tannins, black.

5.2.4 Elastin Stabilization of Porcine Aortic Wall

Resistance to elastase was evaluated using the enzyme digestion assay previously optimized by our group for pure aortic elastin. Samples of porcine aorta were either left untreated (fresh) or fixed with one of the two aforementioned treatments (Glut or Glut/TA, n=6 per group), rinsed 3 times (1 hour each) in 100 mL ddH₂O at room temperature, and lyophilized to record dry weight. Each sample (~15 to 25 mg dry weight) was incubated with 1.0 mL of elastase (20 U/mL) dissolved in 100 mM Tris buffer, 1 mM CaCl₂, 0.02% NaN₃, pH 7.8, for 48 hours at 37°C with orbital shaking at 650 rpm. Samples were then centrifuged (10000 rpm, 10 minutes, 4°C) and supernatants retained for enzyme assay (see below). Tissue samples were individually rinsed 3 times
in 1 mL ddH₂O, centrifuged (as above), lyophilized to obtain dry weight after elastase, and the percent of digested tissue was calculated. To evaluate the possible direct inhibitory effects of Glut and/or TA (leaching from the fixed tissues) on elastase activity, the enzyme solutions retrieved after elastase treatments were checked for activity using an elastin-orcein assay.\textsuperscript{11}

Elastin stabilization after elastase treatment was also verified by histology. Tissue samples randomly retrieved from each group were placed in Karnovsky’s fixative (2.5% glutaraldehyde, 2% formaldehyde in 0.1 M cacodylic acid buffer, pH 7.4), dehydrated through graded alcohols, and embedded in paraffin. Sections (5 \( \mu \)m) were stained with Verhoeff van Giesson’s procedure (elastin fibers stain black while collagen fibers stain red or pink) to assess the integrity of aortic elastin fibers.

5.2.5 Collagen Crosslinking Studies

The thermal denaturation temperatures (\( T_d \)), common indicators of collagen crosslinking density,\textsuperscript{12} were measured in samples from each treatment group (n=3) using a differential scanning calorimeter (DSC) (Perkin-Elmer DSC 7; Boston, MA). The treated aortic wall samples (approximately 2 mm x 2 mm) were sealed in aluminum pans, heated at a rate of 10°C/min from 20°C to 110°C, and \( T_d \) determined as the temperature measured at the endothermic peak. This observed endothermic peak occurs at the temperature where collagen fibers unravel or denature, resulting in a measurable release of energy. Therefore, a higher denaturation temperature correlates into improved collagen crosslinking.

As an additional test of collagen crosslinking, samples were tested for resistance to collagenase. Samples of porcine aortic wall were either left untreated (fresh) or fixed
with Glut or Glut/TA as outlined above (n=6), rinsed 3 times (1 hour each) in 100 mL ddH₂O, and lyophilized to record dry weight. Samples (~15 to 25 mg dry weight) were immersed in 1.2 mL of type I collagenase (150 U/mL) dissolved in 50 mM Tris buffer, 10 mM CaCl₂, pH 7.4, and incubated at 37°C with orbital shaking at 650 rpm for 24 hours. Following this exposure to collagenase, samples were centrifuged (10000 rpm, 10 minutes, 4°C), individually rinsed 3 times in 1 mL ddH₂O, lyophilized to obtain dry weight after collagenase, and the percent of digested tissue was calculated. The “used” collagenase solutions were salvaged in order to test for enzyme activity using an azoic dye-impregnated collagen assay, similar to the elastin-orcein assay used to check for elastase activity.

5.2.6 Mechanical Testing

Porcine aorta was cut into dumbbell shapes in the circumferential direction of the aorta and either left untreated (fresh) or fixed by the Glut or Glut/TA methodologies as described above. Stress-strain analysis was performed on each group (n=3) at a constant uniaxial velocity of 0.2 mm/sec using a 10-pound load cell on a Vitrodyne V1000 material testing apparatus (Liveco, Inc.; Burlington, VT). Results are displayed as stress-strain curves in the range of 0 to approximately 80% elongation or strain.

5.2.7 Rat Subdermal Implantation

Male juvenile Sprague-Dawley rats (25-35 days old, weighing ~50 g, Harlan Laboratories; Indianapolis, IN) were sedated with acepromazine (0.5 mg/kg, Ayerst Laboratories; Rouses Point, NJ) and maintained on 2% isoflurane throughout surgery. A small incision was made on the backs of the rats and three subdermal pouches were
created. Glut and Glut/TA-treated samples of aortic wall (4 mm x 4 mm) were implanted into the subdermal pouches (30 implants per group). The rats were euthanized by CO₂ asphyxiation at 7 and 21 days and capsule-free samples were retrieved for calcium analysis, histology, and elastase digestion. The animal protocol was approved by the Animal Research Committee at Clemson University and NIH guidelines for the care and use of laboratory animals (NIH publication #85-23 Rev. 1985) were observed throughout the experiment.

For calcium analysis, explants (n=10 per group per time point) were rinsed in saline, cut approximately in half, and lyophilized to obtain dry weight (10 to 15 mg each). The samples were then individually hydrolyzed in 6 N hydrochloric acid (HCl) at 95°C for approximately 8 hours. Following hydrolysis, they were dried under a continuous stream of nitrogen gas (~45 minutes) and subsequently reconstituted in 1.0 mL of 0.01 N HCl. These tissue hydrolysates were diluted in the range of 20-fold to 50-fold in an atomic absorption matrix (0.5% lanthanum oxide in 0.3 N HCl) and assayed for calcium content using atomic absorption spectrophotometry.¹⁴

Using these same tissue hydrolysates, desmosine content within Glut and Glut/TA-treated 21 day explants was examined using radioimmunoassay by our collaborator Dr. Barry Starcher.¹⁵ Desmosine, an amino acid unique to elastin, may be used to express elastin content. As controls, tissue hydrolysates from non-implanted Glut and Glut/TA-treated aorta were used to calculate percent of desmosine lost due to the in vivo model.

The other half of the explants (n=10), which were not acid hydrolyzed, were lyophilized and used for elastase digestion. Details of this in vitro enzyme digestion were
performed as described above (20 Units/mL porcine pancreatic elastase, 48 hours, 37°C, 600 rpm) and dry weights recorded in order to calculate percent mass loss. For histology studies, explanted samples were placed in Karnovsky’s fixative and paraffin embedded sections (5 μm) were stained using Alizarin red for calcium deposits and hematoxylin and eosin for general morphology.

5.2.8 Long-Term Stability of Tannic Acid Treatment

An in vitro system was implemented to test the propensity of TA to leach out from the treated tissue and to evaluate the effect of this leaching on resistance to elastase. Aortic wall samples (n=6) treated with one of the fixation procedures (Glut or Glut/TA) were rinsed 3 times (1 hour each) in 100 mL ddH$_2$O, then shaken reciprocally at 120 rpm in phosphate buffered saline (PBS) with 0.02% NaN$_3$, pH 7.4 for 14 days at 37°C (50 mL per 6 tissue samples). After subjecting tissues to this system, samples were rinsed, lyophilized to record dry weight, and subjected to the elastase digestion assay as described above. For comparative purposes and as a possible indicator of elastin stability in vivo, Glut and Glut/TA-treated aortic samples were implanted subdermally in juvenile rats for 7 and 21 days (as described above) and explants analyzed for resistance to elastase. This would allow us to not only test for stability of TA interactions in vivo, but to also assess the accuracy and validity of our in vitro leaching model.

5.2.9 Effect of Metals on Tannic Acid Treatment

Samples of porcine aortic wall were treated with aluminum chloride, ferric chloride, or one of these metals in combination with glutaraldehyde and/or tannic acid, as outlined in Table 5.1. Once treatments were complete, samples were rinsed in ddH$_2$O (3
rinses, 1 hour each), lyophilized to record dry weight, and exposed to the elastase digestion assay as described above (n=6). Since metals such as aluminum and iron are expected to bind very strongly with polyphenolic tannins, we investigated the long-term stability of TA binding with tissue in the presence of these metal ions. Once treated with one of the aforementioned methodologies, samples were exposed to the prolonged in vitro model with vigorous shaking as described above in Section 5.2.8. The treated samples of aortic wall were shaken reciprocally at 120 rpm in phosphate buffered saline (PBS), 0.02% NaN₃, pH 7.4 for 14 days at 37°C (50 ml per 6 tissue samples). After 14 days, these samples were subjected to the elastase digestion assay (n=6) to investigate the potential effects of instability or reversibility of metal-tannin-tissue interactions. As an alternative model for investigating stability of the interactions with tissue, aluminum-treated samples were also implanted in a rat subdermal implant model (see below) and exposed to elastase digestion following explantation at 7 and 21 days. The resulting values for resistance to enzyme were subsequently compared to those exposed to the 14 day in vitro leaching system.

Since aluminum chloride has been widely proposed as an anti-calcification treatment for the aortic portion of bioprosthetic heart valves, samples treated with aluminum (G/Al) or aluminum and tannic acid (Glut/Al/TA) were also utilized in the rat subdermal model to investigate in vivo tissue calcification. Capsule-free explants were retrieved at 7 and 21 days (n=10 per time point), hydrolyzed in 6 N hydrochloric acid (HCl), dried under nitrogen, reconstituted in 1.0 mL of 0.01 N HCl, and analyzed for calcium content using atomic absorption spectrophotometry as described above.
Table 5.1 Descriptions of tissue treatment groups.

<table>
<thead>
<tr>
<th>Group Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glut</td>
<td>0.6% glutaraldehyde (1 day) followed by 0.2% glutaraldehyde (7 days), in 50 mM Hepes buffered saline, pH 7.4</td>
</tr>
<tr>
<td>Glut/TA</td>
<td>Glut (as listed above) followed by 0.3% tannic acid (4 days) in 50 mM Na₂HPO₄ buffered saline containing 0.6% glutaraldehyde, pH 5.5</td>
</tr>
<tr>
<td>Al</td>
<td>0.1 M aluminum chloride in ddH₂O (4 hours)</td>
</tr>
<tr>
<td>Glut/Al</td>
<td>Glut cycle followed by 0.1 M aluminum chloride (4 hours)</td>
</tr>
<tr>
<td>Glut/Al/TA</td>
<td>Glut cycle followed by aluminum chloride, then 0.3% tannic acid (4 days) in 50 mM Na₂HPO₄ buffered saline containing 0.6% glutaraldehyde, pH 5.5</td>
</tr>
<tr>
<td>Fe</td>
<td>0.1 M ferric chloride in ddH₂O (4 hours)</td>
</tr>
<tr>
<td>Glut/Fe</td>
<td>Glut cycle followed by 0.1 M ferric chloride (4 hours)</td>
</tr>
<tr>
<td>Glut/Fe/TA</td>
<td>Glut cycle followed by aluminum chloride, then 0.3% tannic acid (4 days) in 50 mM Na₂HPO₄ buffered saline containing 0.6% glutaraldehyde, pH 5.5</td>
</tr>
</tbody>
</table>

5.2.10 Data Analysis

Results are expressed as means ± standard error of the mean (SEM). Statistical analyses of the data were performed using single-factor analysis of variance (ANOVA). Subsequently, differences between means were determined using the least significant difference (LSD) with an alpha value of 0.05.
5.3 Results

5.3.1 Effect of Tannic Acid on Porcine Aortic Wall

The ability of tannic acid (TA) to bind to intact porcine aortic wall was investigated using a histology stain specific for phenols. This technique allows us to see if/where TA is incorporating itself into the tissue following fixation, as indicated by a distinct black stain. Expectedly, fresh tissue (data not shown) and glutaraldehyde treated tissue resulted in virtually no black staining (Figure 5.1A). Meanwhile, a large amount of TA apparently incorporated itself into the tissue, as suggested by the large amount of black seen in Figure 5.1B. The majority of the (black) stain is seen along the elastin fibers, providing evidence that tannins primarily bind to the elastin within aortic wall.

![Figure 5.1](image.png)

**Figure 5.1** Phenol-specific stain shows incorporation of tannins into Glut/TA-treated porcine aortic wall (B) as compared to glutaraldehyde-treated tissue (A). Phenols such as TA stain black as denoted by arrow in (B). Original magnification, 200x.

Resistance to elastase digestion was tested using fresh aortic samples and aorta treated with Glut or with the sequential treatment of Glut followed by TA (Figure 5.2). While Glut-treated aorta was evidently less susceptible to elastase degradation than fresh
aorta (p<0.05), the conventional Glut treatment still left the tissue extremely vulnerable (greater than 60% mass loss). Treatment with Glut/TA dramatically increased resistance of aorta to elastase, yielding values 15 times lower than those of Glut alone (p<0.05). The quantitative elastase digestion values were also confirmed by histology (Figure 5.3). Almost no aortic elastin fibers were preserved after elastase treatment of the Glut-fixed aorta (Figure 5.3B), while the majority of elastin fibers remained intact for the Glut/TA group after enzyme digestion (Figure 5.3D), indicating excellent elastin preservation by Glut/TA. All enzyme solutions retrieved after elastase treatment of tissue samples displayed similar activities using an elastin-orcein assay (data not shown) suggesting that Glut and/or TA that may have leached from the fixed tissues had no direct inhibitory effects on elastase activity.

**Figure 5.2** Efficacy of tannic acid as elastin-stabilizing agent for porcine aortic wall. Aorta treated with glutaraldehyde and tannic acid (Glut/TA) was significantly more resistant to elastase-mediated degradation than Glut alone (p<0.05).
Figure 5.3 Histology of aortic samples before and after elastase. Glut treated aorta before (A) and after elastase (B); Glut/TA-treated aorta before (C) and after elastase (D). Elastin fibers remained primarily intact within the Glut/TA-treated tissue (D). VVG stain, original magnification, 200x. Elastin fibers (stained black) are indicated by white arrows.

In order to evaluate the potential inhibitory effects of TA on Glut fixation of aortic collagen, the thermal denaturation temperature ($T_d$) and resistance to collagenase of treated aortic samples was analyzed. As anticipated, aorta fixed with Glut had a marked increase in $T_d$ as compared to that of fresh aorta (p<0.05, Table 5.2). Treatment of aorta with Glut/TA yielded slightly higher $T_d$ values but these were not statistically different that those of Glut-fixed aorta (p>0.10).
Table 5.2 Denaturation temperatures of treated porcine aortic wall samples.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Thermal Denaturation Temperature ($T_d$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>$68.37 \pm 0.67^\circ C$</td>
</tr>
<tr>
<td>Glut</td>
<td>$90.43 \pm 0.27^\circ C$</td>
</tr>
<tr>
<td>Glut/TA</td>
<td>$92.92 \pm 0.93^\circ C$</td>
</tr>
</tbody>
</table>

As expected, resistance to collagenase digestion showed that Glut treatment had a stabilizing effect on aortic collagen in comparison to fresh aorta ($p<0.05$, Figure 5.4). Treatment with the Glut/TA sequence appeared to significantly improve the stability of aortic collagen as compared to Glut alone ($p<0.05$), as indicated by the virtually complete preservation of tissue mass after collagenase (undetectable mass loss). All enzyme solutions retrieved after collagenase treatments displayed similar activities using an azocollagen assay (data not shown), suggesting that Glut and/or TA that may have leached from the fixed tissues had no direct inhibitory effects on the activity of the collagenase enzyme. These results indicate that TA does not impede, and may even assist in, the collagen crosslinking ability of Glut.
Resistance to collagenase revealed that glutaraldehyde (Glut) maintained its ability to crosslink collagen even in the presence of tannins (Glut/TA). In fact, mass loss values for Glut/TA-treated aorta were essentially zero, suggesting that TA may even enhance the ability of Glut to protect collagen from enzymatic degradation.

Stress-strain analysis was performed on samples from each group (n=3) and representative plots are shown in Figure 5.5. Expectedly, as indicated by the greater slope of the stress-strain curves, Glut fixation reduced distensibility and stiffened aorta. Treatment with Glut/TA apparently produced a stiffer material than both fresh and Glut-treated aorta, clearly indicating that TA interacts with elastin fibers within the aortic tissue.
Figure 5.5 Mechanical properties of porcine aorta treated with glutaraldehyde (Glut) and a combination of Glut and tannic acid (Glut/TA). As compared to untreated aorta (Fresh), all stabilization treatments reduced distensibility of aortic tissue.

The propensity of tannic acid to inhibit calcification of Glut-fixed aortic wall was studied in a well-established rat subdermal model. The calcium analysis performed on subdermal explants from two different time points is shown in Figure 5.6. For both 7 and 21 day time points, the calcium content was approximately three times lower for Glut/TA in comparison with Glut alone (p<0.05). Explants were examined by histology with Alizarin red staining to confirm the presence of calcium deposits and study their distribution (Figure 5.7). The abundance of calcium deposits in the Glut samples (Figures 5.7A and 5.7B) and the lower numbers of deposits within Glut/TA explants (Figures 5.7C and 5.7D) validates the values obtained by calcium analysis (Figure 5.6). Hematoxylin and eosin staining did not reveal significant differences in tissue structure or host response between the two groups (data not shown). The weight gain in the rats
implanted with Glut/TA treated aorta as compared to Glut samples did not differ significantly indicating that TA treatment did not adversely influence rat growth (data not shown). Overall, these results indicate that tissue stabilization by TA may partially inhibit calcification of Glut-fixed aortic wall.

Figure 5.6 Calcification of treated aorta in the rat subdermal implant model. For both time points, treatment with tannic acid (Glut/TA) yielded approximately three times less calcium than glutaraldehyde (Glut) alone (p<0.05).
Figure 5.7 Calcium deposition in subdermally implanted aorta. Representative sections (lumen is at right) of glutaraldehyde-treated aorta explanted at 7 days (A) and 21 days (B). Glut/TA treatment of aorta yielded few detectable calcification deposits after 7 days (C) and only moderate amounts within 21 days of implantation (D). Alizarin red stain, calcium deposits (red) indicated by arrows, original magnification, 100x.

Samples explanted from the rat subdermal implant model at 21 days were also assayed for desmosine content. Desmosine is an amino acid specific to elastin and can therefore be used to express relative elastin content. After 21 days, Glut-treated aorta lost more than 35% of its original pre-implant desmosine, confirming the presence of in vivo elastin degradation (Figure 5.8). Meanwhile, Glut/TA-treated tissue lost about half as much desmosine as compared to Glut (Figure 5.8, p<0.05). These values endorse the conclusions drawn from our in vitro elastase digestion studies. As such, we can conclude that TA treatment significantly protects elastin from in vivo degradation.
Figure 5.8 Percent of desmosine lost in aortic wall implants after 21 days in the rat subdermal model. Loss of desmosine can be correlated to elastin loss or degradation, suggesting that tannins (Glut/TA) provide a significant amount of elastin stabilization in vivo (p<0.05).

The reversibility of tannic acid binding to aortic tissue was investigated using an accelerated in vitro system and an in vivo animal model (designed to stimulate “leaching” of unbound or reversibly bound TA). Once exposed to these models, samples were subjected to the elastase digestion assay (Figure 5.9). After 14 days of continuous and vigorous shaking in PBS, elastase digestion results revealed that the Glut/TA-treated group became somewhat more vulnerable to enzyme treatment (Figure 5.9) than those samples not exposed to prolonged shaking (unimplanted controls in Figure 5.9; p<0.05). This partial loss of elastin stabilizing capabilities is an indication that a small fraction of the tissue-bound TA may have been loosely attached to the aortic extracellular matrix components. It is also important to note that even with the aggressive shaking, the
Glut/TA group still exhibited a 3-fold stronger resistance to elastase than aorta treated with Glut alone (p<0.05), suggesting that a substantial amount of TA remains tightly bound within the tissue.

For comparative purposes, we also studied the in vivo stability of interactions between tannic acid and porcine aorta. This was done by implanting treated tissues subdermally into juvenile rats for 7 and 21 days, the same model used to investigate in vivo calcification. Following completion of these time points, explants were subjected to the elastase digestion assay (Figure 5.9). For both 7 day and 21 day explants, the resistance to elastase of the Glut/TA group was significantly different from non-implanted controls, yet still improved over that of Glut alone (p<0.05). It is apparent that any leaching of TA from the Glut/TA treated aorta may have reached a plateau within 7 days of implantation, as indicated by the small change in elastase resistance from 7 to 21 days. These results indicate that a significant portion of TA is strongly bound within porcine aorta; however, partial reversibility or instability of these interactions may be a concern, as this was observed both in vitro and in vivo.
Figure 5.9 In vitro and in vivo reversibility of tissue stabilization. For the in vitro model, samples were treated and aggressively shaken to stimulate TA to leach from the tissue and then tested for resistance to elastase. Glut and Glut/TA tissues were also implanted subdermally for 7 and 21 days in juvenile rats and explanted samples tested for resistance to elastase. Both in vitro and in vivo models revealed that a portion of the incorporated TA may be reversibly bound. As compared to controls, the tannin-treated groups consistently exhibited improved resistance to enzyme (p<0.05).

5.3.2 Effect of Metals and Tannic Acid on Porcine Aortic Wall

Tannins, with their ability to stabilize elastin against enzymatic degradation, and metals (such as aluminum and iron), with their ability to inhibit calcification, were investigated to see if an improved bioprosthetic heart valve pretreatment could be developed by combining these technologies. Samples of porcine aortic wall were treated with Glut, TA, aluminum chloride, and/or ferric chloride (Table 5.1). In vitro elastase results (Figure 5.10) revealed that aluminum, which is believed to bind to elastin fibers,
did not protect elastin whether used alone (Al) or in conjunction with glutaraldehyde (Glut/Al; p>0.10 versus Glut alone). The implementation of Al within the glutaraldehyde and tannic acid fixation procedure (Glut/Al/TA) slightly improved the tissue’s resistance to elastase, although this difference was not significant (p>0.10 versus Glut/TA; Figure 5.10). In all cases, extremely similar results were reflected in experiments involving ferric chloride in place of aluminum chloride (Figure 5.11). For instance, the iron treatment had little to no effect on resistance to elastase when used alone or with Glut (Fe, Glut/Fe), and slightly improved resistance when used in combination with Glut and TA, although not to a significant level (Glut/Fe/TA; Figure 5.11). Together, these results suggest that: (1) aluminum and iron, despite inhibiting elastin-oriented calcification, do not protect elastin from enzymatic degradation, and (2) aluminum and iron do not hinder the elastin stabilizing capabilities of tannins when used in a stepwise treatment procedure.

**Figure 5.10** Resistance to elastase revealed that aluminum (Al) had no effect on elastin stabilization (p>0.10 for Glut vs. Glut/Al). Similarly, the addition of Al to tannin treatment (Glut/Al/TA) did not significantly change elastin stabilization properties.
Figure 5.11  Similar to the results obtained for aluminum, ferric chloride treatment resulted in very minimal effects on resistance to elastase (p>0.05 for Glut vs. Glut/Fe, Glut/TA vs. Glut/Fe/TA).

Despite the apparent inefficacy of these metal ions in regards to elastin stabilization, we also examined their effect on the long-term tissue binding between tannins and tissues. Metal ions, which bind very strongly to tannins, may be beneficial in reducing some of the reversibility issues of TA-tissue interactions displayed in Figure 5.9. To investigate this, we exposed tissue to the same in vitro (14 days of continuous, vigorous shaking at 37°C in PBS) and in vivo models (7 and 21 day subdermal implants in juvenile rats). Note that ferric chloride-treated aortic wall was only exposed to the in vitro system, as this tissue was not implanted. Results from the in vitro model showed that aluminum or iron treatment significantly curtailed the amount of TA leaching from the tissue, as evidenced by its retention of elastin stabilizing qualities after 14 days (p<0.05 for Glut/TA vs. Glut/Al/TA or Glut/Fe/TA). In fact, both of these metal iron
treatments resulted in virtually no changes in resistance to elastase after undergoing the accelerated 14 day in vitro model: from 6.0% digestion immediately after fixation to 7.4% for aluminum, and from 5.1% to 6.3% for iron (p>0.10, Figure 5.12). Although promising, these results apparently may be an indication of the in vitro model’s limitations. When exposed to an in vivo model, the elastin stabilizing properties of the Glut/Al/TA group were very similar to those from tissue without aluminum (Glut/TA; p<0.05). That is, they also exhibited a partial reversibility in their interactions with the tissue. As was the case with Glut/TA treated tissue, this reversibility for aluminum treated samples appeared to level off, as 7 and 21 day data were similar (p>0.10).

Figure 5.12 The use of aluminum (Glut/Al/TA) or iron (Glut/Fe/TA) appeared to delay or hinder TA leaching from the tissue, as evidenced by minimal changes in digestion after 14 days in the in vitro model. However, implant studies revealed that these metal ions were ineffective in limiting this partial reversibility in vivo. (# denotes that no implant studies were performed for Glut/Fe/TA.)
The effect of aluminum on tissue calcification was investigated through the juvenile rat subdermal implant model. Aluminum with glutaraldehyde, a commercially utilized fixation and anti-calcification procedure, yielded less calcification than Glut (as expected) and Glut/TA treated aorta at both time points (Figure 5.13). In comparison to Glut/TA, however, this data was statistically significant only at the 21 day time point (p<0.05). Interestingly, tissue treated with both aluminum and tannic acid (Glut/Al/TA) exhibited significantly higher calcium deposition than both Glut/Al and Glut/TA at both time points (p<0.05, Figure 5.13). Therefore, it appears that the combination of the two treatments may negate their efficacy in regards to limiting or hindering tissue mineralization.

Figure 5.13  Aluminum, which is known to limit tissue mineralization, did not hinder the propensity of aortic wall to calcify in the presence of glutaraldehyde and tannic acid (Glut/TA vs. Glut/Al/TA).
5.4 Discussion

One of the long-term objectives of this work is to create an elastin stabilization procedure for cardiovascular implants, namely bioprosthetic heart valves. In preliminary experiments presented in Chapter 4, we showed that tannic acid (TA) binds to pure aortic elastin and, in doing so, increases its resistance to enzymatic degradation. We also demonstrated that the binding kinetics and efficacy of TA solutions are enhanced in the presence of glutaraldehyde (Glut) at pH 5.5. In the studies presented here, we chose this Glut/TA mixture for studies on efficacy of TA-mediated elastin stabilization in porcine aorta. The ability to crosslink aortic collagen and effects on mechanical properties were also studied. In addition, an in vivo model was used to investigate the effect of TA stabilization on calcification. We evaluated the reversibility of interactions between TA and aortic elastin using in vitro and in vivo models. Finally, we tested the efficacy of tannin treatment of aortic wall when used in conjunction with anti-calcific metal ions.

Conventional fixation methodologies for cardiovascular implants, such as Glut\textsuperscript{1} and others,\textsuperscript{16-18} have typically focused on collagen crosslinking. These fixation techniques, most notably Glut, have proven to be efficient in crosslinking the collagen component, as suggested by their improvements in resistance to collagenase and increased thermal denaturation temperatures.\textsuperscript{19} However, to our knowledge no claim has been made pertaining to their ability to stabilize elastin. Elastin’s exceptionally low number of reactive amine groups in comparison to collagen make elastin a poor target for fixation by Glut. This ineffectiveness has been confirmed earlier by the minimal incorporation of radioactive Glut into purified elastin in vitro.\textsuperscript{5}
It was already exhibited that pure aortic elastin treated with Glut is not protected from elastase-mediated degradation. In present studies, we demonstrate that this same vulnerability to enzymatic degradation is present within Glut-treated aortic wall (Figure 5.2). Even prolonged treatment with Glut (up to 8 weeks), yielded tissue that was still significantly susceptible to elastase digestion (data not shown). This lack of stabilization by Glut is noteworthy since elastin is the most prevalent extracellular matrix protein within aortic wall. Although it is not as abundant within the valve leaflets, elastin is also present within the cusp structure and crucial to their mechanical properties. Therefore, it would be advantageous to develop a method for improved elastin stabilization in cardiovascular implants.

The process by which elastin degradation occurs within cardiovascular implants is not fully understood, although it is tempting to speculate that elastin-degrading enzymes play a dominant role in this degeneration. Studies indicate that there is a relationship between enzymatic degradation of elastin and the presence of matrix metalloproteinases (MMPs), a class of enzymes responsible for extracellular matrix remodeling. Consequently, elastase treatment was used as an in vitro model of elastin degradation by MMPs. The use of high purity pancreatic elastase is analogous to the widely accepted method of utilizing collagenase to study the collagen crosslinking ability of a fixative. Just as resistance to collagenase digestion is used as an indicator of collagen fixation, elastin stabilization can be analyzed by investigating resistance to elastase digestion.

Using a histology stain specific for tannins, we saw visual confirmation that tannic acid apparently bound to porcine aortic wall, with a specific concentration of tannins accumulated on or around the elastin fibers (Figure 5.1). By using the elastase
digestion assay, we saw that this binding correlated into extremely efficient protection of aortic elastin (and the aorta in general) from enzymatic degeneration. This was verified gravimetrically (Figure 5.2) and by histology (Figure 5.3). As mentioned in the previous chapter and portrayed in Figure 4.10, it is our hypothesis that tannins, which have a known affinity for hydrophobic regions, stabilize elastin by binding to potential elastase cleavage sites, which are also hydrophobic in nature.

The compatibility between Glut and TA was addressed by investigating thermal denaturation temperatures and resistance to collagenase of treated porcine aorta. The addition of TA as a fixation step had virtually no effect on the thermal denaturation temperature of Glut-fixed aorta indicating that TA does not hinder Glut fixation of collagen in the aorta (Table 5.2). As the same time, TA did make the Glut-treated samples significantly more resistant to collagenase, producing a biomaterial which was virtually un-degradable by collagenase (Figure 5.4). These results suggest that TA, besides binding to elastin, may also bind to Glut-fixed collagen and render it more resistant to enzymatic attack. It was therefore apparent that TA does not hinder Glut fixation of aortic collagen, nor does Glut impede TA stabilization of elastin. Moreover, TA improves the ability of Glut-fixed aortic tissue to resist both elastase and collagenase.

The mechanisms of these synergistic effects of Glut and TA on tissues are not known, but they may be due to: a) binding to different sites (Glut binds to amine groups, while TA has high affinity for hydrophobic domains in matrix proteins) or b) chemical interactions between Glut and TA that may yield a new unknown reaction product that acts as a superior elastin and collagen fixative. Although the exact nature of this product is unknown, the reaction of Glut with TA may form acetals, which could enhance the
crosslinking ability of Glut. This synergy and chemical compatibility between the two fixatives is important in that it may enable the implementation of TA into current Glut fixation procedures for bioprosthetic heart valves.

By definition, the application of any effective tissue fixative will yield mechanical properties different from those of fresh aorta and also could be used to approximate efficacy of fixation. By interacting with the aortic matrix components, TA treatment produces a material which is less distensible than Glut-fixed aorta (Figure 5.5). These results clearly indicate that TA interacts strongly with elastin and possibly with collagen components of aortic tissues. The functional implications of these changes in mechanical properties require further investigation using accelerated fatigue testing and intra-circulatory implants in large animals.

Calcification of cardiovascular implants is one of the most crucial problems associated with the failure of bioprosthetic heart valves. Although the exact mechanisms of implant calcification are not fully understood, the link between calcification and the integrity of elastin fibers has been under great investigation. Elastin-oriented calcification has been associated with increased activities of elastin-degrading MMPs; furthermore, site-specific delivery of MMP inhibitors significantly reduced elastin-mediated calcification, suggesting a strong correlation between elastolysis and calcium deposition. Therefore, by protecting elastin from enzymatic degradation, it is reasonable to speculate that elastin-oriented calcification may also be inhibited. This hypothesis was partially confirmed by our results, which clearly documented the ability of TA (which effectively prevents elastin degradation) to significantly reduce the propensity of Glut-fixed aorta to calcify in vivo (Figures 5.6 and 5.7). While no particular claim is being
made for tannins as an anti-calcification treatment, it is important to note the apparent correlation between elastin stabilization by TA and inhibition of calcification.

Using samples from this same model, it was discovered that glutaraldehyde treated aorta lost approximately 35% of its desmosine, or elastin (Figure 5.8). Although it has been expected that Glut treatment leaves elastin vulnerable to enzymatic degradation, this finding is significant since in vivo loss of elastin within treated bioprosthetic heart valve tissue has not previously been shown quantitatively (to our knowledge). These results further solidify the need for an elastin stabilizing technology for these tissues, especially considering samples were implanted for just 21 days in a non-circulatory model. As hypothesized, the addition of tannins to the Glut fixation procedure, as in the case of Glut/TA, significantly reduced the amount of in vivo elastin degradation. Not only does this provide more evidence for the efficacy of tannins as elastin stabilizing agents, but it also validates many of our in vitro studies, such as tannin binding and resistance to elastase, as we have demonstrated elastin stabilization is a potential reality in vivo as well.

As an additional test of tannic acid efficacy and safety, the long-term stability of TA binding to aortic tissue was investigated by prolonged shaking in vitro in PBS for 14 days and by implantation in the rat subdermal model for up to 21 days (Figure 5.9). After being subjected to these “accelerated degeneration” systems, samples were tested for resistance to elastase. While the conditions of the in vitro shaking model are meant to imitate physiological conditions (pH 7.4, 37°C, PBS), the difference in elastase digestion results between this group and the rat subdermal explants was statistically significant (p<0.05), suggesting that additional unknown biological factors may have played a role.
within the implant model. In the case of both model systems, TA-treated tissue became significantly more vulnerable to elastase as compared to tissue immediately exposed to enzyme. However, resistance to elastase apparently reached a plateau after 7 days in vivo indicating that only a portion of the TA was susceptible to leaching, possibly only those TA molecules which were not tightly bound or in excess. However, it is notable that TA treatment yielded a biomaterial that was significantly more resistant to elastase digestion than Glut-treated aorta in all cases (Figure 5.9). These two models have evident limitations, and definitive answers regarding the long-term stability of TA require more extended studies in fatigue testers and circulatory implants. The potential causes and effects of this partial reversibility will be investigated in greater detail in Chapter 6.

The use of aluminum and iron, metal ions which have been shown to resist aortic calcification when used as a tissue pretreatment, were tested for their effect on tannin fixation. We hypothesized that the use of tannins and metal ions together may be useful since: (1) tannins bind very strongly with such metal ions creating a very stable metal-tannin complex, and (2) the anti-calcific metal ions are believed to interact directly with elastin. Therefore, it is reasonable to assume that this complex may serve as a more stable “bridge” linking tannic acid to elastin, thus limiting the partial reversibility between tannins and tissue. This seemed to be the case using our long-term in vitro model for binding reversibility, as we observed that the aluminum and iron (with TA) treated tissues exhibited virtually no changes in susceptibility to enzymatic degradation after being vigorously shaken for 14 days (Figure 5.12). As already mentioned, this same model caused Glut/TA treated tissue to be approximately twice as susceptible to enzymatic degradation after 14 days of shaking. This effect, however, did not carry over
to in vivo studies, as resistance to elastase was similar for Glut/Al/TA explants in comparison to Glut/TA explants (Figure 5.12). Likewise, in vivo tissue calcification of Glut/TA samples was not improved (and was actually made worse) by adding the aluminum treatment step. This incompatibility between tannins and aluminum may be attributable to (1) competitive binding between the two agents, and (2) an ineffective complex resulting from tannin-aluminum interactions. In other words, when tannins bind to aluminum, they may be blocking or “capping off” the aluminum ions, thus partially preventing their ability to inhibit calcification as they normally would. Such a conclusion is purely speculation, however, especially considering the anti-calcification mechanisms of aluminum chloride treatment have not been well established. That being said, due to improved tissue calcification as compared to Glut/Al/TA, tannins appear to show more promise as an elastin-stabilizing agent when used without metal ions such as aluminum. More studies investigating this relationship, and the use of ferric chloride in vivo, are required to gain a better understanding of each agent’s mechanism of action.

5.5 Conclusions

Glutaraldehyde (Glut) fixation does not adequately protect vascular tissues from degradation and calcification. The addition of tannic acid (TA) to the Glut fixation process dramatically increases the biostability of Glut-fixed aortic wall without hindering collagen crosslinking. TA is chemically compatible with Glut fixation and efficiently complements the beneficial effects of Glut fixation. This stabilization of elastin significantly decreases calcification of treated aorta implanted in the rat subdermal model. Studies on the stability of interactions between tannins and aortic wall show that a portion of TA originally bound to the tissue may be partially reversible. Aluminum and
iron, metals shown to inhibit tissue calcification, were mostly ineffective in enhancing the beneficial effects of tannin treatment. The present study suggests that the use of TA as an elastin-stabilizing agent for bioprosthetic heart valves is both practical and feasible, and has a strong potential to extend the clinical durability of these cardiovascular devices.

5.6 References


CHAPTER 6
SAFETY AND STRUCTURAL REQUIREMENTS OF TANNIN-MEDIATED
ELASTIN STABILIZATION

6.1 Introduction

It has been shown that pure elastin, with or without chemical fixation processes, may be vulnerable to enzymatic degradation\(^1\) and calcification\(^2,3\). Since these are common problems associated with the failure of tissue-derived cardiovascular devices and the progression of vascular pathologies like abdominal aortic aneurysms, a technology targeting elastin stabilization may be beneficial.

In work presented within Chapters 4 and 5, we have demonstrated that tannic acid (TA), a naturally occurring plant polyphenol, binds to elastin and in doing so, protects it from in vitro digestion by elastase. In addition, treatment of porcine aorta with TA resulted in improved resistance to degradation and in vivo calcification in a rat subdermal model.\(^4\) However, we have also provided evidence for the partial instability of TA after long-term interaction with vascular elastin. This undesirable property may limit long-term elastin stabilization and may also contribute to implant toxicity, as it has been shown that the by-products of TA hydrolysis are toxic.\(^5,6\) Such effects could obviously limit the safety, and therefore clinical applicability, of TA treated devices.

Tannic acid is composed of a central glucose molecule derivatized at its hydroxyl groups with one or more galloyl residues (Figure 6.1, and also portrayed in Figure 2.14). We hypothesized that the polyphenolic nature of TA, along with its relatively hydrophobic “core” and hydrophilic “shell” are features responsible for the elastin-
stabilizing action of TA. We also believe that the partial instability of TA-elastin interactions may be due to hydrolyzable ester (depsidic) bonds (highlighted in Figure 6.1) within the structure of TA. In present studies, we evaluated the elastin-stabilizing potential and cytotoxicity of the individual components of TA as well as derivatives of TA, such as acetylated TA (AcTA), pentagalloyl glucose (PGG, grey circle in Figure 6.1), free gallic acid (Gall) and glucose (Glc). This study provides evidence that the main structural groups essential to the TA-elastin interactions are polyphenolic hydroxyl groups. In addition, we show that PGG, the core of tannic acid, possesses elastin-stabilizing characteristics similar to TA, yet is much less cytotoxic than TA. As a result, PGG could exhibit more promising potential as an elastin-stabilizing agent for cardiovascular bioprostheses and abdominal aortic aneurysms.

**Figure 6.1** Chemical structure of tannic acid (TA), consisting of a central glucose molecule esterified at all five hydroxyl moieties with two gallic acid molecules. The shaded circle highlights pentagalloyl glucose (PGG), the core structure of TA.
6.2 Methods

6.2.1 Materials

The following chemicals were purchased from the noted suppliers and used in current studies: glutaraldehyde (50% solution), acetic anhydride (98%), and tannic acid (Sigma-Aldrich; St. Louis, MO); pyridine (99%), diethyl ether, and glucose (Acros Organics; Morris Plains, NJ); ethyl acetate (EM Science; Gibbstown, NJ); gallic acid (MP Biomedicals, Inc.; Aurora, OH); high purity porcine pancreatic elastase (EC134, 135 U/mg) and elastin-orcein (Elastin Products Company, Inc.; Owensville, MO); Dulbecco’s modified Eagle media (DMEM) and fetal bovine serum (FBS) (Cellgro; Herndon, VA); penicillin-streptomycin for cell culture (Invitrogen; Carlsbad, CA); CellTiter 96® AQ™ One Solution Reagent, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) (Promega; Madison, WI); LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes; Eugene, OR).

6.2.2 Tissue Preparation

Ascending porcine aortas (supravalvular segments, approximately 3 cm in length) were collected fresh at a local USDA-approved slaughterhouse and transported to the laboratory on ice. Aorta was cleaned of fat and extraneous tissue, rinsed exhaustively in cold physiological saline, and cut into 4 mm x 4 mm squares for elastase digestion and cytotoxicity testing and into rings for residual stress analysis (see below, Section 6.2.7). For tensile testing, dumbbell shapes (40 mm long, 10 mm wide at each end, 5 mm wide in center) were used, with the long axis of the dumbbell being parallel to the circumferential direction of the aorta.
Details of tissue treatment groups, which are discussed in the following sections, are also summarized in Table 6.1.

### 6.2.3 Acetylated Tannic Acid

The hydroxyl groups of tannic acid were replaced with acetyl groups as described by Hagerman\(^7\) and Porter,\(^8\) thus producing acetylated tannic acid (AcTA). Briefly, a 50:50 mixture of acetic anhydride and pyridine was added dropwise to tannic acid (10 mL per 2 g of TA). Upon mixing this solution with water, a solid formed which was thoroughly rinsed with a series of washes in dilute acetic acid (to remove pyridine) and water. The resultant solid, consisting of AcTA, was then lyophilized and the loss of free hydroxyl groups (normally represented by a peak at \(\sim3400\ \text{cm}^{-1}\)) was confirmed using Fourier transform infrared (FTIR) spectroscopy analysis.

Samples of porcine aorta were treated in a solution of 0.3% acetylated tannic acid and compared with fresh aortic wall or tissue treated with Glut and/or TA, as described in Table 6.1. The resulting groups (Fresh, Glut, TA, Glut/TA, AcTA, Glut/AcTA; \(n=6\) per group) were then exposed to porcine pancreatic elastase digestion (see below, Section 6.2.7) to assess elastin-stabilizing capabilities of each treatment.
<table>
<thead>
<tr>
<th>Group ID</th>
<th>Treatment details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glut</td>
<td>0.6% glutaraldehyde (1 day) followed by 0.2% glutaraldehyde (7 days), in 50 mM Hepes buffered saline, pH 7.4</td>
</tr>
<tr>
<td>TA</td>
<td>0.3% tannic acid (1.78 mM, 4 days) in 50 mM Na₂HPO₄ buffered saline, pH 5.5</td>
</tr>
<tr>
<td>Glut/TA</td>
<td>Glut (as listed above) followed by 0.3% tannic acid (4 days) in 50 mM Na₂HPO₄ buffered saline containing 0.6% glutaraldehyde, pH 5.5</td>
</tr>
<tr>
<td>AcTA</td>
<td>0.3% acetylated tannic acid (4 days) dissolved in a mixture of dimethyl sulfoxide:isopropanol:ddH₂O (3:3:1)</td>
</tr>
<tr>
<td>Glut/AcTA</td>
<td>Glut followed by 0.3% acetylated tannic acid dissolved in a mixture of dimethyl sulfoxide:isopropanol:ddH₂O (3:3:1) with 0.6% glutaraldehyde</td>
</tr>
<tr>
<td>Gall</td>
<td>8.9 mM gallic acid (4 days) in 50 mM Na₂HPO₄ buffered saline, pH 5.5</td>
</tr>
<tr>
<td>Glut/Gall</td>
<td>Glut followed by 8.9 mM gallic acid (4 days) in 50 mM Na₂HPO₄ buffered saline containing 0.6% glutaraldehyde, pH 5.5</td>
</tr>
<tr>
<td>Glc</td>
<td>1.78 mM glucose (4 days) in 50 mM Na₂HPO₄ buffered saline, pH 5.5</td>
</tr>
<tr>
<td>Glut/Glc</td>
<td>Glut followed by 1.78 mM glucose (4 days) in 50 mM Na₂HPO₄ buffered saline containing 0.6% glutaraldehyde, pH 5.5</td>
</tr>
<tr>
<td>PGG</td>
<td>0.15% PGG (4 days) in 50 mM Na₂HPO₄ buffered saline, pH 5.5, containing 20% isopropanol</td>
</tr>
<tr>
<td>Glut/PGG</td>
<td>Glut followed by 0.15% PGG with 0.6% glutaraldehyde (4 days) in 50 mM Na₂HPO₄ buffered saline, pH 5.5, containing 20% isopropanol</td>
</tr>
</tbody>
</table>
6.2.4 Individual Components of Tannic Acid

The individual components of tannic acid, gallic acid (Gall) and glucose (Glc), were used to treat porcine aortic wall. Gallic acid treatment of porcine aorta was performed at a concentration of 8.9 mM in order to create equimolar amounts of unbound gallic acid in comparison to TA. Likewise, the concentration of Glc used (1.78 mM) correlated to equimolar amounts of glucose in TA (one glucose per tannic acid molecule). Similar to studies with acetylated tannic acid, fresh samples and those fixed with Glut and/or TA were also used for comparative purposes. The subsequent tissue groups (Glut, Glut/TA, Gall, Glut/Gall, Glc, Glut/Glc; Table 6.1) were exposed to in vitro elastase digestion to investigate elastin fiber stability.

6.2.5 Pentagalloyl Glucose Synthesis

Pentagalloyl glucose (PGG) was prepared from tannic acid as outlined by Hagerman et al.\textsuperscript{9} Initially, TA was methanolyzed using a solution of 70% methanol in acetate buffer (0.1 M acetic acid, pH 5.0). Methanolysis was performed at 65°C for 15 hours. Following this incubation period, the reaction was stopped by immediately raising the solution’s pH to 6.0. Methanol was then removed by rotary evaporation and replaced with ddH\textsubscript{2}O. A series of separate extractions involving diethyl ether (3 extractions) and ethyl acetate (3 extractions) were performed. Following each set of extractions, the resulting products were rotary evaporated to remove the ether and ethyl acetate, respectively. Once cooled to 4°C, a precipitate resulted which was rinsed in a series of washes with dilute methanol (thrice in 2% methanol in ddH\textsubscript{2}O) and ddH\textsubscript{2}O (once). The resulting precipitate was centrifuged after each rinse, and the end product ultimately lyophilized. The purity of PGG was confirmed by matrix-assisted laser
desorption/ionization (MALDI) mass spectroscopy and nuclear magnetic resonance (NMR). PGG, which was insoluble in water, was dissolved in isopropanol, then diluted in buffered saline (0.145 M NaCl, 50 mM Na₂HPO₄, pH 5.5) to yield final concentrations of 0.15% PGG in a 20% isopropanol solution. This concentration of PGG, which is approximately half the molecular weight of TA (depending on the length of the gallic acid “tails” extending from TA), was used to create molar concentrations of PGG equivalent to TA.

PGG, alone or in conjunction with Glut, was used to treat aortic wall samples, as described in Table 6.1 and compared with fresh tissue and that treated with Glut and/or TA. Once treated, elastin stability experiments (resistance to elastase) were performed, as well as tests for mechanical properties and cytotoxicity.

6.2.6 Efficacy of Pentagalloyl Glucose

6.2.6.1 Elastin Stability

Resistance to elastolysis was evaluated using the elastase digestion assay that we developed to test for elastin stability. Samples of porcine aorta (4 mm x 4 mm) were either left untreated (fresh) or fixed with one of the aforementioned treatments (Glut, TA, Glut/TA, PGG, Glut/PGG, as described in Table 6.1; n=6 per group), rinsed 3 times (1 hour each) in 100 mL ddH₂O at room temperature, and lyophilized to record dry weight. Each sample (~15 to 25 mg dry weight) was incubated with 1.0 mL of porcine pancreatic elastase (20 U/mL in 100 mM Tris buffer, 1 mM CaCl₂, 0.02% NaN₃, pH 7.8) for 48 hours at 37°C with orbital shaking at 650 rpm. This optimized assay results in nearly complete removal of unstabilized elastin fibers, as exhibited in Chapter 4. Samples were
then centrifuged (10000 rpm, 10 minutes, 4°C) and supernatants retained to assess enzyme activity (see below). Residual tissue samples were rinsed 3 times in 1 mL ddH₂O, centrifuged (as above), lyophilized to obtain dry weight, and the percent of digested tissue after elastase was calculated. To evaluate the possible direct inhibitory effects of any of the chemicals (that may have leached from the treated tissues) on elastase activity, the elastase solutions retrieved were checked for activity using an elastin-orcein assay.¹¹

The in vitro system previously used to test the stability of TA-elastin interactions (the propensity of TA to leach out from the treated tissue) was also implemented to evaluate the stability or reversibility of PGG binding. Aortic wall samples (n=6) were treated with Glut or Glut/PGG, as described in Table 6.1. Following treatment, they were rinsed 3 times (1 hour each) in 100 mL ddH₂O, then shaken reciprocally at 120 rpm in phosphate buffered saline (PBS), 0.02% NaN₃, pH 7.4 for 14 days at 37°C (50 ml per 6 tissue samples). After subjecting tissues to this system, samples were rinsed, lyophilized to record dry weight, and subjected to the elastase digestion assay as described above.

### 6.2.6.2 In Vivo Calcification

Tissue calcification was investigated using a standard accelerated in vivo model, which was also presented in studies within Chapter 5. For this model, male juvenile Sprague-Dawley rats (25-35 days old, weighing ~50 g, Harlan Laboratories; Indianapolis, IN) were sedated with acepromazine (0.5 mg/kg, Ayerst Laboratories, Rouses Point, NJ) and maintained on 2% isoflurane throughout surgery. A small incision was made on the backs of the rats and three subdermal pouches were created. Glut and Glut/PGG treated samples of aortic wall (4 mm x 4 mm; treatment descriptions in Table 6.1) were
implanted into these subdermal pouches (16 implants per group). The rats were euthanized by CO₂ asphyxiation at 7 and 21 days and capsule-free samples were retrieved for calcium analysis and histology. The animal protocol was approved by the Animal Research Committee at Clemson University and NIH guidelines for the care and use of laboratory animals (NIH publication #85-23 Rev. 1985) were observed throughout the experiment.

For calcium analysis, explants (n=8 per group per time point) were rinsed in saline, cut approximately in half, and lyophilized to obtain dry weight (10 to 15 mg each). The samples were then individually hydrolyzed in 6 N hydrochloric acid (HCl) at 95°C for approximately 8 hours. Following hydrolysis, they were dried under a continuous stream of nitrogen gas and subsequently reconstituted in 1.0 mL of 0.01 N HCl. These tissue hydrolysates were diluted in the range of 30-fold to 100-fold in an atomic absorption matrix (0.5% lanthanum oxide in 0.3 N HCl) and assayed for calcium content using atomic absorption spectrophotometry.¹²

For histology studies, explanted samples were placed in Karnovsky’s fixative and paraffin embedded sections (5 μm) were stained using Alizarin red for calcium deposits and hematoxylin and eosin for general morphology.

6.2.7 Mechanical Testing

6.2.7.1 Residual Stress Measurements

Porcine aorta was cut transversely into ring segments approximately 1 cm in height. The rings were kept intact (unopened) and left untreated (Fresh) or treated with Glut, Glut/TA, Glut/PGG, TA, or PGG (n=5 per group) as described in Table 6.1. After
fixation was completed, the aortic rings were immersed in ddH$_2$O with the cross section of the aorta facing upward, allowing free movement of the sample. As described by Gratzer and Lee$^{13}$ and Liu and Fung,$^{14}$ the rings were cut once in the radial direction, allowed to “relax” and open for 15 minutes under water, and then digitally photographed. These digital photographs were used to calculate the opening angle of each aortic ring graphically using Adobe Photoshop 7.0.

6.2.7.2 Tensile Testing

Porcine aorta was cut into dumbbell shapes in the circumferential direction and tensile testing was performed, as described in Chapter 5. Briefly, samples were either left untreated (fresh) or fixed by Glut, Glut/TA, Glut/PGG, TA, or PGG. Stress-strain analysis was performed on each group (n=4) at a constant uniaxial strain rate of 0.2 mm/sec using a 10-Newton load cell on a Synergie 100 testing apparatus from MTS Systems Corporation (Eden Prairie, MN). For each sample, elastic modulus was calculated as the slope of the stress-strain curve between 0 and 5% strain.

6.2.8 Cytotoxicity Testing of Tissue Extracts

The cytotoxicity of treated aorta was tested using rat skin fibroblasts exposed to tissue extracts, similar to that described in ISO 10993-5, a set of testing standards for evaluating the safety of medical devices. To obtain these extracts, aortic wall was treated with Glut, Glut/TA, Glut/Glc, Glut/Gall, or Glut/PGG as described in Table 6.1. After fixation, samples were rinsed 3 times (1 hour each) in 100 mL ddH$_2$O, and then underwent orbital shaking at 120 rpm in phosphate buffered saline (PBS) with 0.02% sodium azide, pH 7.4 for 14 days at 37°C (50 mL per 6 tissue samples, 4 mm x 4 mm).
This wash solution of PBS, with its potential extracts included, was the agent specifically applied to cells and tested for cytotoxicity. This method (vigorously washing in PBS for 14 days) was previously used to test the propensity of tannins to leach from the treated tissue, as described above.

Primary rat skin fibroblasts were isolated from the skins of adult male Sprague-Dawley rats (Harlan Laboratories; Indianapolis, IN) using a cell explant method. For our studies, the fibroblasts were seeded onto 24-well plates (50,000 cells per well) in 1 mL Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained in a humidified incubator at 37°C with 5% CO₂. After 24 hours, the media was removed and replaced with one of the PBS extracts diluted 10-fold in fresh culture medium. PBS and ethanol (EtOH; 70% in ddH₂O) were also used as negative and positive cytotoxicity controls, respectively. The cells were incubated with these solutions for 2 hours and then rinsed with 1 mL PBS. At this point, the viability of the cells was assessed by LIVE/DEAD® assay, in which live cells fluoresce green and dead cells fluoresce red. Digital micrographs of cells were taken 45 minutes after exposure to the LIVE/DEAD® reagent. Separately, cell viability and metabolism was also measured by MTS assay (n=3 per group for each test) using MTS reagent diluted 5-fold in culture medium. After 90 minutes, absorbance was read at 490 nm in a microplate reader. This absorbance is proportional to dehydrogenase enzyme activity within metabolically active cells, thus creating the correlation between absorbance and cell viability. Samples were blanked to controls containing media and MTS reagent without cells.
6.2.9 Data Analysis

Results are expressed as means ± standard error of the mean (SEM). Statistical analyses of the data were performed using single-factor analysis of variance (ANOVA). Subsequently, differences between means were determined using the least significant difference (LSD) with an alpha value of 0.05.

6.3 Results

6.3.1 Acetylated Tannic Acid

To investigate the mechanisms of binding between tannic acid and elastin, the hydroxyl groups of TA were replaced by acetyl groups. The complete loss of these hydroxyls was confirmed by FTIR analysis, specifically by the disappearance of a broad peak at 3400 cm\(^{-1}\) (Figure 6.2). Samples of porcine aortic wall were treated with acetylated tannic acid (AcTA) and tested for resistance to elastase. The elastase digestion values were compared with those obtained from untreated aorta as well as samples treated with Glut and/or TA (Figure 6.3). As previously exhibited in Chapter 5, Glut-treated aorta was slightly less susceptible to elastase digestion than fresh aorta (p<0.05), but the conventional Glut treatment still yielded a tissue that lost approximately 70% of its dry mass via enzyme digestion. The implementation of TA, particularly in the case of Glut/TA, decreased the susceptibility of elastin to enzymatic degradation (p<0.05). In the absence of hydroxyl groups (as in AcTA), TA lost its ability to resist elastase-mediated digestion, as evidenced by the nearly seven-fold increase in mass loss of Glut/AcTA-treated aorta versus Glut/TA-treated aorta (p<0.05). These data suggest that the phenolic hydroxyl groups of TA are required to achieve stabilization of vascular elastin.
Figure 6.2 FTIR scans of normal tannic acid (TA) and acetylated tannic acid. The broad band at 3400 cm$^{-1}$ (black arrow) is representative of the outer hydroxyl groups of TA. The absence of this band within acetylated TA confirms removal of these hydroxyl groups and subsequent acetylation.
By modifying the hydroxyl groups of TA by acetylation (AcTA), TA lost its ability to provide the tissue with resistance to elastase digestion both alone (AcTA) and in combination with glutaraldehyde (Glut/AcTA) in comparison to TA and Glut/TA, respectively (p<0.05 for AcTA vs. TA and Glut/AcTA vs. Glut/TA).

6.3.2 Individual Components of Tannic Acid

To better understand the requirements for elastin stabilization, the individual components of TA were also used to treat aortic samples and tested for resistance to elastase digestion. The simple alcohols, gallic acid (Gall) and glucose (Glc), appeared to have no stabilizing effect on the tissue, neither alone or in combination with Glut, in comparison to TA (Figure 6.4; p<0.05). Note that the percent mass loss values for Glut/Gall and Glut/Glc alone are similar to those obtained for samples treated with Glut alone (p>0.05). Since elastase-mediated digestion indicated that Gall, Glc, and AcTA did not assist in preventing elastin degradation, we focused our studies on investigating
pentagalloyl glucose (PGG), the core polyphenol of TA, as a potential elastin-stabilizing agent and its comparison with TA.

**Figure 6.4** Efficacy of individual tannic acid components as elastin-stabilizing agents.

Neither gallic acid (Gall) nor glucose (Glc), whether alone or in conjunction with glutaraldehyde (Glut/Gall, Glut/Glc), exhibited elastin-stabilizing capabilities as compared to Glut/TA (p<0.05 for all Gall or Glc groups vs. Glut/TA).

6.3.3 Pentagalloyl Glucose

6.3.3.1 Pentagalloyl Glucose: Elastin Stabilizing Efficacy

The purity of pentagalloyl glucose (PGG) was verified by MALDI mass spectroscopy and NMR (data not shown). The implementation of PGG as a fixative or pretreatment clearly enhanced the ability of the tissue to resist elastase-mediated degradation in comparison to glutaraldehyde alone (Figure 6.5; p<0.05). Results from elastase digestion of PGG-treated aorta were similar to those treated with tannic acid.
alone (p>0.10). In addition, the combination of glutaraldehyde and PGG (Glut/PGG) treatment yielded tissue just as resistant to enzymatic degradation as that treated with Glut/TA (p>0.05), providing further evidence that PGG stabilizes aortic wall to the same degree as TA. For all elastase degradation studies performed, enzyme solutions were retrieved after digestion of tissue samples and displayed similar activities using an elastin-orcein assay (data not shown). This implies that none of the leachable components resulting from the treatments/fixatives had direct inhibitory effects on the activity of the enzyme.

![Figure 6.5](image)

**Figure 6.5** Efficacy of pentagalloyl glucose (PGG) as an elastin-stabilizing agent. Aortic samples treated with PGG resisted elastase-mediated degradation just as well as those treated with TA, both alone (TA vs. PGG; p>0.10) and in combination with glutaraldehyde (Glut/TA vs. Glut/PGG; p>0.10).

We previously observed in Chapter 5 that TA-treated tissues lost a portion of their elastin stabilizing capabilities when exposed to a long-term vigorous shaking model (14
days in PBS, 120 rpm), likely indicative of a partial reversibility in the TA-tissue interactions. Although it was believed that PGG may improve upon this with its improved chemical structure and stability, a similar trend was observed for Glut/PGG-treated tissues when exposed to the same prolonged model (Figure 6.6). Approximately twice as much PGG-treated tissue was digested by elastase following this in vitro “leaching” model as compared to PGG-treated tissue exposed to elastase immediately following fixation. However, the digestion values for tissues subjected to the 14 day in vitro shaking model were significantly lower for PGG-treated aorta as compared to TA-treated aorta (p<0.05). Additionally, as was the case for Glut/TA, Glut/PGG treatment yielded a tissue that was much more resistant to enzymatic degradation that Glut alone even when exposed to the prolonged model (p<0.05 for Glut vs. Glut/PGG).

Figure 6.6 Following exposure to a prolonged model of vigorous shaking, PGG appeared to exhibit the same partial reversible binding that was observed for TA. It should be noted, however, that the values for PGG (for “14 days in vitro”) were statistically different in comparison to TA (p<0.05).
6.3.3.2 Pentagalloyl Glucose: In Vivo Calcification

The addition of PGG to the fixation step, as in Glut/PGG treated tissue, significantly reduced in vivo tissue calcification in the rat subdermal model (Figure 6.7). These differences were statistically significant from tissue treated with Glut alone, both for 7 and 21 day explants (p<0.05). These calcium results are similar to those found for TA treated tissue, which also revealed a decreased propensity to calcify as compared to Glut (Chapter 5). Alizarin red staining of 21 day explants confirmed the quantitative results shown in Figure 6.7, revealing that Glut treated aorta (Figure 6.8A) had more calcium deposits than Glut/PGG treated aorta (Figure 6.8B). These results suggest that PGG-mediated elastin stabilization translated into improved resistance to in vivo calcification as well.

![Graph showing calcium analysis on treated aorta following 7 days or 21 days implantation in the rat subdermal model.](image)

**Figure 6.7** Calcium analysis on treated aorta following 7 days or 21 days implantation in the rat subdermal model. Similar to treatment with tannic acid, the use of PGG resulted in significantly reduced calcification as compared to glutaraldehyde alone (Glut) at both time points (p<0.05), most likely due to PGG’s ability to stabilize elastin.
Figure 6.8 Calcium deposition within 21 day explants was confirmed by histological analysis with Alizarin red staining (calcium deposits = red). Glutaraldehyde treated tissue (A) showed significantly more calcium deposits than Glut/PGG treated aorta (B).

6.3.3.3 Pentagalloyl Glucose: Mechanical Testing

In order to further investigate the interaction between elastin and polyphenolic tannins, mechanical properties such as elastic modulus and opening angle (residual stress) were evaluated for untreated aorta (Fresh) and tissues treated with Glut, TA alone, PGG alone, Glut/TA, and Glut/PGG. For tensile testing experiments, a representative stress-strain graph from each group is displayed in Figure 6.9, while the calculated elastic moduli are shown in Table 6.2. Expectedly, stress-strain analysis revealed that fresh aorta was markedly less stiff than all other groups that underwent fixation (p<0.05), particularly for those groups treated with glutaraldehyde. Treatment with Glut/TA and Glut/PGG significantly increased the modulus (and subsequently the stiffness) of the tissue in comparison to Glut alone (p<0.05), indicating that TA and PGG both bind to elastin fibers within the aortic tissue. Overall, TA and PGG treatment resulted in tissues with similar stiffness and distensibility (p>0.05 for both cases of TA alone vs. PGG alone and Glut/TA vs. Glut/PGG), suggesting their interactions with aortic wall are similar.
Figure 6.9  Representative stress-strain behavior of porcine aorta treated with glutaraldehyde and/or tannins. As compared to fresh aorta, all stabilization treatments reduced distensibility of the tissue. The implementation of TA and PGG into the Glut fixation process (Glut/TA and Glut/PGG) significantly increased tissue stiffness. With or without Glut, treatment with TA and PGG yielded comparable mechanical properties, suggesting TA and PGG interact with the tissue similarly.

Table 6.2  Elastic moduli of treated porcine aortic wall.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Elastic modulus (Average ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>0.17 ± 0.01 MPa</td>
</tr>
<tr>
<td>Glut</td>
<td>1.28 ± 0.04 MPa</td>
</tr>
<tr>
<td>Glut/TA</td>
<td>4.85 ± 0.30 MPa</td>
</tr>
<tr>
<td>Glut/PGG</td>
<td>4.61 ± 0.40 MPa</td>
</tr>
<tr>
<td>TA</td>
<td>0.85 ± 0.07 MPa</td>
</tr>
<tr>
<td>PGG</td>
<td>0.89 ± 0.13 MPa</td>
</tr>
</tbody>
</table>
In regards to opening angle, the implementation of any one of the fixatives (Glut alone, tannins alone, or Glut and tannins combined) drastically reduced the opening angle of aortic rings as compared to fresh tissue (Figure 6.10; p<0.05). A representative picture of an opened aorta from each treatment group is shown in Figure 6.11. Fresh aorta nearly opened to a straight line (~180°) while Glut-fixed tissue possessed an opening angle approximately 5 times less. All groups fixed with tannins, whether alone or in conjunction with Glut, were relatively similar and opened significantly less than Glut alone (p<0.05), further solidifying the idea that TA and PGG have a similar effect on aortic elastin fibers.

![Opening Angle Chart]

**Figure 6.10** Opening angle measurements of fresh and fixed aorta. The opening angles of fresh aortic rings were significantly larger than the treated samples. In addition, rings treated with tannins opened less than those treated with Glut alone (p<0.05). TA and PGG, both of which interact with aortic wall elastin, exhibited similar opening angles when used to treat tissue.
Figure 6.11 Representative opening angle pictures from each group showing aortic rings after cutting and unaided opening due to internal residual stress.

6.3.4 Cytotoxicity Testing

The cytotoxicity of tissue extracts obtained after fixation was assessed both qualitatively (Live/Dead assay) and quantitatively (MTS assay). Results of the Live/Dead assay revealed that the cells exposed to extracts from Glut, Glut/Glc, Glut/Gall, and Glut/PGG-treated tissue remained viable after 2 hours of exposure (Figure 6.12). In addition, it appeared that the very small concentration of sodium azide used in the wash solutions had little to no effect on cytotoxicity in this time frame, as suggested by the presence of live cells in the PBS control group. However, cells which were exposed to extracts from Glut/TA-treated aorta experienced a notable amount of cell death. As expected, the positive control (EtOH) essentially left no viable cells remaining.

Quantitative analyses from the MTS assay confirmed Live/Dead results (Figure 6.13). The absorbance values for cells exposed to Glut extracts were comparable to those from the PBS control group (p>0.05). Likewise, Glut/PGG extracts were relatively
similar to the PBS group (p>0.05), suggesting that neither Glut nor PGG treatments produce tissue that may leach toxic components in these conditions. On the other hand, Glut/TA extracts yielded an absorbance of nearly half that of these groups (p<0.05), suggesting that TA is nearly twice as toxic as PGG at equimolar concentrations.

**Figure 6.12** Cytotoxicity of extractables obtained from treated aorta. Qualitative Live/Dead assay (live cells green, dead cells red) shows that application of Glut/TA extracts resulted in fewer live cells than all other groups, with the exception of the positive ethanol control (EtOH). Note the large number of live cells associated with Glut/PGG, indicating virtually no cytotoxic effects. Original magnification, 200x.
Figure 6.13  Quantitative viability assay of rat skin fibroblasts after being exposed to extractables from fixed tissues. MTS results are reported as absorbances at 490 nm, which are proportional to cell viability. Glut, Glut/Glc, Glut/Gall, and Glut/PGG extracts were not more toxic than the PBS negative control (p>0.05). However, Glut/TA extracts resulted in about half the number of viable cells in comparison to Glut/PGG (p<0.05).

6.4 Discussion

Our long-term goal is to develop a fixation or stabilization technology targeted at elastin. Ultimately, we believe this could (1) benefit the longevity of tissue derived cardiovascular implants by presenting a more effective pretreatment or fixation process for these devices, and (2) be developed as a therapy to halt the progression of abdominal aortic aneurysms. In Chapters 4 and 5, we have previously shown that tannic acid (TA) may be a suitable candidate for this stabilization as it binds to both pure aortic elastin and intact aortic wall. For the purpose of a bioprosthetic heart valve pretreatment, the use of TA has been shown to be effective in the presence of glutaraldehyde (Glut), the
conventional fixative used for these devices historically and currently, without hindering the beneficial attributes of Glut cross-linking. In addition, Glut/TA-treated aortic wall, when implanted in the rat subdermal model as presented in Chapter 5, exhibited significantly less calcification than Glut-treated aortic wall, most likely due to the elastin-stabilizing capabilities of TA. While these studies promote TA as an excellent stabilizing agent, little was known about the interactions between TA and elastin. Furthermore, partial instability in the binding between TA and vascular elastin was observed in the data presented in Chapter 5. This undesirable property may limit the efficacy of elastin stabilization and contribute to possible implant toxicity.

Therefore, the aim of this study was to investigate the structural requirements of tannins as elastin-stabilizing agents, and to evaluate the efficacy and safety of TA treatment of porcine aorta. To assess the significance of phenolic hydroxyl groups, we examined the ability of TA to maintain its elastin-stabilizing qualities when its hydroxyl groups were replaced by acetyl groups (as in the case of AcTA). In the absence of its hydroxyl groups, TA essentially lost all elastin-stabilizing capabilities, as evidenced by the poor resistance to elastase digestion exhibited by AcTA (Figure 6.3). Along these lines, similar tests were performed on the simple components of TA (gallic acid and glucose) to determine if one particular factor was responsible for elastin stabilization (Figure 6.4). Similar to results obtained for AcTA, Gall and Glc provided no resistance to elastase-mediated degradation. The fact that AcTA had no effect on resistance to elastase implies that multiple polyphenolic hydroxyl groups are a requirement for elastin binding and stabilization. It is important to note that Gall, which possesses similar phenolic hydroxyl groups, is apparently not able to bind to elastin in the same fashion as
the polyphenolic tannin, TA. Glucose, which also has its own hydroxyl groups, similarly had no effect on resistance to enzymatic degradation. When considered together, these results suggest that the presence of polyphenolic hydroxyl groups is a clear requirement for an elastin-stabilizing agent.

Pentagalloyl glucose (PGG), consisting of a central glucose molecule and one gallic acid bound to each hydroxyl moiety, could also be considered a component of tannic acid. In contrast to the other components of TA, PGG treatment of aortic wall provided a level of elastin stabilization, as indicated by resistance to elastase, similar to that of TA (Figure 6.5). The structure of PGG is comparable to TA with its outer hydroxyl groups; however, PGG possesses only one layer of gallic acid residues on each “tail” extending from the core, suggesting that elastin stabilization is independent of the length of these tails (as measured by the number of gallic acid residues).

Since PGG proved to be effective in protecting elastin from elastase-mediated degradation, further tests were performed to compare the properties of TA-treated aorta versus PGG-treated aorta. Specifically, we investigated the long-term stability or reversibility of PGG-tissue interactions, the propensity of PGG-treated aorta to calcify in vivo, and the effects of PGG on tissue mechanical properties.

By exposing Glut/TA treated tissue to a prolonged in vitro model with vigorous shaking (14 days in PBS, 120 rpm), we previously observed in Chapter 5 that the interactions between TA and tissue were partially reversible. As this model progressed, the TA-treated tissue became more vulnerable to enzymatic degradation, indicative of a portion of the TA leaching from the tissue and, therefore, no longer providing as much elastin stabilization as was originally observed. PGG, which we believed may partially
circumvent this reversibility due to an improved chemical structure, also became more vulnerable to elastase-mediated digestion with time and vigorous shaking (Figure 6.6). Although this was not desirable, the overall results are still encouraging since elastase digestion of tissue treated with Glut/PGG and exposed to the prolonged in vitro model revealed that: (1) Glut/PGG treatment still exhibited significantly improved elastin stabilization as compared to Glut alone, and (2) Glut/PGG yielded tissue slightly more resistant to elastase that Glut/TA following this long-term model.

In addition to resistance to elastase, Glut/PGG was also similarly effective in regards to resisting in vivo tissue calcification as compared to Glut-treated aorta (Figures 6.7 and 6.8), a trend which was also seen in Chapter 5 for Glut/TA-treated implants. This is likely directly related to the ability of PGG and TA to preserve elastin, and therefore resist elastin-oriented calcification. This is especially noteworthy since calcification is one of the primary modes of failure of bioprosthetic heart valves; furthermore, it is also often observed as a pathological characteristic of abdominal aortic aneurysms.

The idea behind tannin-mediated elastin stabilization and other treatments (such as Glut) is to bind to components of the extracellular matrix; since these components (namely elastin and collagen) play a significant role in tissue biomechanics, it is natural to assume that successful fixation will result in a change in mechanical properties in comparison to fresh or native tissue. This is especially true in the case of Glut treatment, which results in significantly increased stiffness in comparison to native tissue. In present studies, we investigated tissue distensibility (via elastic modulus) and residual stress (via ring opening angles). In the case of elastic modulus, Glut treatment yielded a tissue that was significantly less distensible than fresh tissue, as expected and noted
previously in Chapter 5. Meanwhile, treatment with Glut/TA or Glut/PGG yielded the least amount of distensibility (Figure 6.6). This change in elastic modulus for Glut/TA and Glut/PGG treated tissues can likely be attributed to a high degree of binding to elastin, which does not occur with Glut alone. The decreased distensibility observed in Glut/TA or Glut/PGG treated tissues may be undesirable for bioprosthetic heart valve tissue, although its exact implications are unclear. Glut has long been vindicated for its effect on tissue stiffness within these devices, however, continues to be widely used today.\textsuperscript{15,16} Porcine aorta treated with TA or PGG alone was more distensible (less stiff) than Glut treated tissue, but less distensible than fresh aorta. The fact that TA alone and PGG alone both increased stiffness (and strength) in comparison to fresh tissue may be important in regards to using these tannins as a therapy for abdominal aortic aneurysms. The weakened architecture associated with aneurysmal tissues may benefit from this increased stiffness and strength.

Similar to those changes observed in elastic modulus, the opening angles (residual stress) of the fixed tissue groups were also drastically different than fresh tissue (Figures 6.7 and 6.8). Opening angles, previously used as an approximation of residual stress within vessel walls,\textsuperscript{14,17} represent the unaided release of the inner circumferential stress, which is likely a result of extended elastic fibers. Glutaraldehyde treatment, which mainly crosslinks collagen fibers, resulted in a tissue that opens much less than fresh tissue, while the tannin treated samples (Glut/TA, Glut/PGG, TA, PGG) clearly yielded the smallest opening angles. By binding to elastin, the implementation of TA or PGG likely fixes or “locks” the orientation of these fibers, resulting in a decreased residual stress. While these inherent residual stresses are believed to be vital to the native aortic
wall, their role in fixed tissues is less clear. Related opening angle experiments have been performed previously to assess alternative fixatives, such as polyepoxy compounds, as well as Glut in various buffers and their effect on elastin. Overall, we believe that our studies on mechanical properties suggest that (1) TA and PGG bind to elastin, thus decreasing the distensibility of the tissue, and (2) the binding mechanisms and efficacy are similar for TA and PGG.

Decades ago, tannic acid was used as a standard topical treatment of burns due to its ability to bind to tissues, consequently fixing burn toxins into the wound area and preventing their further distribution throughout the patient’s body. However, this practice was relatively short-lived due to suspected issues of hepatotoxicity. Since gallic acid is the suspected culprit, this toxicity is likely attributable to poor TA purity (particularly in older preparations of TA) and/or the instability of TA. The root of TA’s instability can be traced to the depsidic ester bonds between gallic acid molecules (Figure 6.1), which are hydrolyzable and can lead to the release of toxic gallic acid residues. While the topic of TA and hepatotoxicity has been and still is heavily debated, a significant amount of research has recently been dedicated to assessing the validity of these issues and the use of potential alternatives, such as TA “mimics” and high purity TA.

For our purposes, the potential toxic by-products of tannic acid stabilization are certainly an important factor to take into account, especially considering that we have previously demonstrated the partial reversibility of binding between TA and aortic wall. By using PGG (which does not possess outer gallic acid residues bound by the depsidic bonds associated with these compounds) it is reasonable to assume that we may be able
to drastically reduce or even eliminate any toxic effects of tannic acid, while maintaining
the elastin stabilization potential of polyphenolic tannins. In the work presented here, we
tested this hypothesis by investigating the cytotoxicity of tissue extracts resulting from
vigorous long-term shaking. In addition to TA and PGG extracts, we also analyzed the
toxicity of Glut/Gall and Glut/Glc extracts, neither of which displayed any significant
toxicity towards the cells as exhibited by both Live/Dead (Figure 6.9) and MTS (Figure
6.10) assays. Since these individual components of TA, gallic acid and glucose, had no
stabilizing effect on the tissue (Figure 6.4), it is likely that they do not bind to the elastin-
rich tissue as TA and PGG do. This likely explains why no toxic effects were observed
in these groups, particularly in the case of gallic acid. While glucose would not be
expected to elicit any toxic effects, free gallic acid, as mentioned above, has been
associated with TA toxicity. However, if Gall does not bind to the tissue to begin with, it
obviously would not be a factor in the tissue extracts we tested for cytotoxicity.
Conversely, PGG apparently did bind to aortic wall, resulting in improved resistance to
elastase (similar to TA), but also exhibited virtually no toxic effects in comparison with
controls. Meanwhile, it was apparent that tissue treated with TA released components
more toxic than those resulting from the PGG treated tissue, as evidenced by the 2-fold
increase in viability for cells exposed to PGG extracts as compared to TA. Similar
studies have been conducted on by-products of TA-treated biomaterials in which their
cytotoxic behavior was confirmed.23

Since PGG has elastin-stabilizing capabilities similar to that of TA and is less
cytotoxic, PGG holds the advantage over TA as a promising elastin-stabilizing agent.
6.5 Conclusions

The main structural groups essential to the interaction between tannic acid (TA) and elastin are polyphenolic hydroxyl groups. Furthermore, the components of TA (gallic acid and glucose) provide no protection from enzymatic degradation individually, but participate synergistically within the polyphenolic tannin structures to generate compounds that bind to elastin. Pentagalloyl glucose (PGG), which is the core of TA, possesses the same unique elastin-stabilizing qualities of TA and endows aortic tissue with similar mechanical properties. PGG, due to the lack of easily hydrolyzable ester bonds between gallic acid residues, is more stable and less cytotoxic than TA, and thus could exhibit potential use as an elastin-stabilizing agent for cardiovascular bioprostheses. For these same reasons, PGG is a more suitable candidate as a therapy for abdominal aortic aneurysms. The safety and efficacy of PGG for this application will be investigated in greater detail in Chapter 7.

6.6 References


CHAPTER 7
ELASTIN STABILIZATION AS A TREATMENT FOR
ABDOMINAL AORTIC ANEURYSMS

7.1 Introduction

Abdominal aortic aneurysms (AAAs) are disorders defined by an increase in aortic diameter which is associated with impaired aortic wall integrity. Progression of this vascular pathology can lead to further dilatation of the artery and eventual fatal rupture. In addition to arterial dilatation, AAAs are typically characterized by an overall degeneration of the arterial architecture, presence of matrix-degrading enzymes, inflammatory infiltration, and often calcification. The loss of structural integrity of the aortic wall is primarily explained by enzyme-mediated degeneration of extracellular matrix components such as elastin and collagen. Notably, diseased tissues obtained from AAA patients exhibit decreased medial elastin content, elastic lamellae disruption or fragmentation, and increased levels of elastolytic activities.1-3 Numerous studies have been undertaken to further investigate the role of proteinases in AAA formation. While serine proteases, cathepsins, and plasminogen activators may also contribute to this proteolysis, matrix metalloproteinases (MMPs) are apparently the group of enzymes most closely related to arterial wall weakening, elastin degeneration, and AAA formation and expansion.4 As a result of its multi-factorial pathogenesis, anti-inflammatory agents, proteinase inhibitors, and genetic and pharmacologic inhibition of MMPs have been tested as potential AAA treatments in experimental animals.5-8 Since long-term, adequate control of local inflammation and MMP activities may be difficult to achieve and may be
accompanied by adverse systemic side effects, therapeutic treatments targeted at directly stabilizing or protecting elastin against the action of elastin-degrading enzymes may be useful.

Based on their known properties, we have investigated tannins as novel elastin-stabilizing agents for this purpose. In work presented in the previous chapters, we have shown that tannins bind to arterial elastin in vitro, rendering tissues resistant to enzymatic degradation, which could be useful in possibly prolonging the durability of bioprosthetic heart valves. Among the different polyphenolic tannins investigated, pentagalloyl glucose (PGG), a compound consisting of a central glucose core with one gallic acid bound to each hydroxyl moiety, has been employed in current studies. By virtue of its elastin-stabilizing properties, we hypothesized that in vivo delivery of PGG to aortic segments would interfere with AAA formation and progression.

To this point, we have shown numerous studies exhibiting the in vitro efficacy of tannins in regards to elastin stabilization of porcine aortic wall, primarily for the purpose of bioprosthetic heart valves. In current studies, PGG was investigated for its effect on aneurysm development within a widely accepted AAA rat model, which will be described in greater detail later in this chapter. In view of this, we performed in vitro efficacy studies of PGG on rat aorta, similar to some of the primary experiments previously done for porcine aorta (ring opening angle and resistance to elastase). Additionally, since PGG will be applied directly to living tissue within the in vivo aneurysm model, it was necessary to evaluate the cytotoxic effects of PGG when directly applied to cells. For all of these in vitro experiments, varying concentrations of PGG were used to target the optimal concentration for efficacy and safety of this application.
PGG was then applied to our in vivo AAA model, allowing us to provide evidence that periarterial treatment with non-cytotoxic levels of PGG hinders aneurysmal dilatation of the abdominal aorta and preserves elastin fiber integrity, providing a platform for potential development of safe and effective treatments for this degenerative disease.

7.2 Methods

7.2.1 Materials

The following chemicals were purchased from the noted suppliers and used in studies presented here: high purity porcine pancreatic elastase (EC134, 135 Units/mg) and elastin-orcein (Elastin Products Company, Inc.; Owensville, MO); Dulbecco’s modified Eagle media (DMEM) and fetal bovine serum (FBS) (Cellgro; Herndon, VA); penicillin-streptomycin for cell culture (Invitrogen; Carlsbad, CA); CellTiter 96® One Solution Reagent, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) (Promega; Madison, WI); Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes; Eugene, OR). Pentagalloyl glucose was synthesized by methanolyis of tannic acid, as described previously in Chapter 6.

7.2.2 In Vitro Studies

7.2.2.1 Cytotoxicity of Pentagalloyl Glucose

Varying concentrations of pentagalloyl glucose (PGG) were applied directly to rat skin fibroblasts (RSFBs) and rat aortic smooth muscle cells (RASMCs) separately to assess toxicity of PGG. It should be noted that these studies, in which cells are directly exposed to solutions of PGG, differ from the cytotoxicity experiments described in
Chapter 6, where extracts from PGG treated tissue (aorta) were tested for their effects on cell viability. This was necessary since PGG would be directly applied to native living tissue in situ within our animal model.

Primary fibroblasts and smooth muscle cells were isolated from the skins and aortae, respectively, of adult male Sprague-Dawley rats (Harlan Laboratories; Indianapolis, IN) using a cell explant method. Each cell type was separately seeded onto 24-well plates (25,000 cells per well) in 1 mL Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained in a humidified incubator at 37°C with 5% CO₂. Media was refreshed after 24 hours, and removed and replaced with one of the PGG solutions 48 hours later (for a total of 72 hours after cells were seeded). For these solutions, PGG was dissolved and diluted in sterile phosphate buffered saline (PBS) to yield final concentrations of 0.03%, 0.06%, 0.10%, 0.12%, or 0.15% PGG. Cells were also incubated with PBS as negative controls and with 70% ethanol as positive (toxic) controls. Cells, both fibroblasts and smooth muscle cells, were directly exposed to these solutions (1 mL per well) for 15 minutes, then rinsed twice with sterile PBS.

Cell viability was assessed by Cell Titer 96® AQueous One Solution Reagent, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-4-sulfophenyl-2H-tetrazolium, inner salt (MTS) assay (Promega, Madison, WI; n=3 per group per cell type) as well as Live/Dead staining (Molecular Probes, Eugene, OR; n=3 per group per cell type). For MTS, cells were exposed to MTS reagent diluted 5-fold in culture media. After 90 minutes of incubation with the reagent, absorbance was read at 490 nm in a microplate reader. This absorbance is proportional to dehydrogenase enzyme activity within
metabolically active cells, thus creating the correlation between absorbance and cell viability. Samples were blanked to controls containing media and MTS reagent without cells. For Live/Dead staining, digital micrographs of cells were taken 45 minutes after exposure to the Live/Dead reagent. Cell viability is expressed visually through this assay, as live cells fluoresce green while dead cells fluoresce red.

7.2.2.2 Opening Angle of Treated Rat Aorta

Fresh rat abdominal aorta (infrarenal) was collected from adult male Sprague-Dawley rats (Harlan Laboratories; Indianapolis, IN) and transported to the laboratory in physiological saline. The tissue was cut transversely into rings segments approximately 2 mm in length. Aortic rings were carefully cleaned of extraneous tissue with the aid of a stereomicroscope. The rings were kept intact (unopened) and left untreated (Fresh) or treated with varying concentrations of PGG (0.03%, 0.06%, 0.10%, 0.30%) dissolved in unbuffered saline (5 mL per aortic sample). Treatments were performed at 37°C and for just 15 minutes in an attempt to simulate the in vivo treatments applied within our aneurym animal model. Following treatment, rat aortic samples were rinsed in saline at room temperature (3 times, 1 hour per rinse). The aortic rings (n=5) were then immersed in ddH2O with the cross section of the aorta facing upward, allowing free movement of the sample. The rings were cut once in the radial direction under a stereomicroscope, allowed to “relax” and open for 15 minutes under water, and then photographed through the stereomicroscope at a magnification of ~20x. The resulting images were used to calculate the opening angle of each aortic ring using Adobe Photoshop 7.0. This opening angle method is similar to that described for porcine aorta in Chapter 6 and has been used by others for both porcine13 and rat14 arteries.
7.2.2.3 Resistance to Elastase

The efficacy of varying concentrations of PGG on rat aorta was tested using a variation of the elastase digestion assay we developed for porcine vascular elastin and aortic wall.\textsuperscript{10,11} Using the fresh abdominal aorta harvested from adult rats, aortic tissue samples (~5 mm in length) were left untreated (Fresh) or treated with 0.03%, 0.06%, 0.10%, or 0.30% PGG. PGG treatments were identical to those described above for opening angle experiments (PGG in unbuffered saline, 15 minutes, 37°C, 5 mL per sample). After treatment, samples were rinsed in saline at room temperature (3 times, 1 hour each) and subsequently lyophilized. The samples of rat aorta (n=5) were then subjected to 1.0 mL of high purity porcine pancreatic elastase (1.0 Unit/mL in 100 mM Tris buffer, 1 mM CaCl\textsubscript{2}, 0.02% NaN\textsubscript{3}, pH 7.8) for 24 hours at 37°C with mild orbital shaking at 400 rpm. Following exposure to this enzyme, elastase solutions were retrieved for desmosine analysis (see below), samples were centrifuged (10000 rpm, 10 minutes, 4°C), and residual tissue was rinsed exhaustively in 100 mL ddH\textsubscript{2}O (3 times, 1 hour each) and ultimately lyophilized. Tissue samples were then individually hydrolyzed in 6 N hydrochloric acid (HCl) at 95°C for approximately 8 hours. After hydrolysis, they were dried under a continuous stream of nitrogen gas (~45 minutes) and subsequently reconstituted in 1.0 mL of 0.01 N HCl.

The resulting hydrolysates (n=5) were assayed for desmosine content using a radioimmunoassay,\textsuperscript{15,16} as described and performed by our collaborator, Dr. Barry Starcher at the University of Texas Health Center at Tyler. Desmosine, an amino acid specific to elastin, can be used to express elastin content. Therefore, preservation or degradation of elastin after exposure to elastase was quantified by this desmosine
analysis. Using these same tissue hydrolysates, the ninhydrin-based method for total amino acid content was used in order to normalize desmosine values to total protein.  

Similarly, desmosine analysis was performed on the retrieved enzyme solutions (n=5) following the elastase digestion assay described above. Identical to the hydrolysis performed on aortic samples, elastase solutions were hydrolyzed with 6 N HCl, dried under nitrogen, and finally reconstituted in 1.0 mL of 0.01 N HCl. The radioimmunoassay for desmosine was again performed on the resulting hydrolysates, with results normalized to total protein in solution.

7.2.2.4 Histology Stain for Tannin Binding

To test for concentration-dependent PGG binding with rat aortic tissue, histology was performed on samples of treated rat aorta using a stain specific for polyphenols such as PGG. Fresh abdominal aorta was harvested from adult rats and transported to the laboratory in physiological saline. Segments of aorta were cut to approximately 2 mm in length and subsequently treated with 0.03%, 0.06%, 0.10%, or 0.30% PGG. Treatments with PGG were consistent with those described above (PGG in unbuffered saline, 15 minutes, 37°C, 5 mL per sample). After PGG treatment, samples were rinsed in ddH₂O at room temperature (3 times, 1 hour each), then stained with ferric chloride (10% FeCl₃ in ddH₂O, 5 minutes), followed by another series of rinses in ddH₂O (3 times, 15 minutes each). The ferric chloride step specifically stains phenols black. Once staining and rinsing were complete, tissue was embedded and frozen in OCT cryosectioning medium (Sakura Finetek, Torrance, CA), sectioned at 6 µm, and counterstained with light green.
7.2.3 Animal Surgeries

7.2.3.1 Experimental Design for In Vivo Aneurysm Studies

Two separate experiments were designed to evaluate the in vivo efficacy of PGG in hindering AAA formation and development in a rat model for aortic aneurysms. The schematic in Figure 7.1 depicts a timeline of each study and the stages of AAA development in the abdominal aorta injury model. For this model, calcium chloride (CaCl2) is applied directly to the rat abdominal aorta, thus causing a chemical injury. This model has been effective in progressively causing aneurysm formation in rabbits, mice, and rats.\textsuperscript{18-22} In addition to progressive expansion of the aorta, the model also elicits other AAA traits (localized inflammation, elastin degeneration, and increased protease activity). Within 28 days after application of CaCl\textsubscript{2} injury, AAA develops and further progresses with time.

In the first experiment, prevention of AAA formation was attempted by periadventitial delivery of PGG just prior to the CaCl\textsubscript{2}-mediated injury (at day 0). Twenty-eight days after application of PGG and CaCl\textsubscript{2}, the animal’s abdominal aorta was evaluated for AAA formation.

In the second experiment, CaCl\textsubscript{2}-mediated injury was performed and AAA allowed to form unhindered. At 28 days after injury, PGG was applied to aneurysmal aorta in an attempt to halt or slow aneurysm progression. AAA development was monitored for another 28 days after PGG treatment.
Figure 7.1 Experimental design for in vivo aneurysm experiments. In Experiment 1, PGG was applied to abdominal aorta immediately prior to CaCl₂-mediated injury and AAA formation evaluated 28 days later. In Experiment 2, PGG was applied to aneurysmal aorta (28 days after CaCl₂-mediated injury) and development of advanced AAA monitored for another 28 days.

7.2.3.2 Experiment 1: Aneurysm Formation

Adult male Sprague-Dawley rats (weighing ~300 g; Harlan Laboratories; Indianapolis, IN) were sedated with acepromazine (0.5 mg/kg; Ayerst Laboratories; Rouses Point, NJ) and maintained under general anesthesia (2% to 3% isoflurane) throughout surgery. The infrarenal aorta between the renal artery and iliac bifurcation was exposed via laparotomy (Figure 7.2) and photographed with a digital camera alongside a 10 mm x 1.1 mm (length x diameter) solid segment of stainless steel wire for initial diameter measurements (see below). For PGG treatment, a strip (1.5 cm x 0.5 cm)
of 8-ply sterile cotton gauze presoaked in 0.03% PGG in saline was placed on top of the abdominal aorta and maintained for 15 minutes. The gauze was then removed and the area rinsed three times with warm saline. The aortas then underwent chemical injury by applying 0.5 M CaCl₂ solution to the abdominal aorta with presoaked gauze for 15 minutes, a treatment which induces aneurysm formation. Throughout all steps of surgery, exposed internal organs were kept moist with periodic application of warm physiological saline. As controls, rat aortas were treated with physiological saline for 15 minutes (rather than PGG), rinsed, and then subjected to CaCl₂. After treating with CaCl₂ (for both PGG and control groups), the gauze was removed, and the incision closed (abdominal intramuscular suture, subcutaneous suture, and final skin closure with surgical staples). The rats recovered and had access to water and chow *ad libitum*.

![Surgical steps for the abdominal aortic aneurysm model (Experiment 1).](image)

**Figure 7.2** Surgical steps for the abdominal aortic aneurysm model (Experiment 1). Black arrows denote abdominal aorta.
The rats (n=12 per group) were anesthetized after 28 days, at which point the abdominal aorta was cleaned of adhesions, exposed, and digitally photographed. The diameters of both the aorta and the steel wire were measured in three areas using SPOT software (Diagnostic Instruments, Sterling Heights, MI) for each rat by two independent investigators blinded to the identity of the samples. The true external aortic diameter was calculated from the ratio of the true diameter of the steel wire and the diameter measured on digital pictures. After photographing the aorta at this day 28 time point, the animals were humanely euthanized by CO₂ asphyxiation. The abdominal aorta was subsequently excised and segments from each aorta processed for analysis of elastin content and integrity, PGG content, extent of calcification and inflammation, and matrix metalloproteinase (MMP) activity, as described below. Upon euthanasia, liver was also collected (n=4 per group) to study effects of PGG on hepatotoxicity. Samples of liver were immediately embedded in OCT cryosectioning medium (Sakura Finetek; Torrance, CA) and frozen on dry ice. Sections (6 µm) were stained with hematoxylin and eosin (H&E) to assess general cell and tissue morphology.

Elastin Integrity Assessment

Following euthanasia, rat aortas from control (saline) and PGG-treated rats (n=12 per group) were collected 28 days after CaCl₂-mediated injury and assayed for desmosine. Desmosine is an amino acid specific to elastin which can be used to express elastin content in tissues.²³,²⁴ Normal rat aortas, which did not undergo surgery, were used as controls (n=6) and similarly assayed for desmosine. Segments of abdominal aorta (~2 mm in length) were lyophilized to obtain dry weight, individually hydrolyzed in 6 N hydrochloric acid (HCl) at 95°C for approximately 8 hours, dried under nitrogen gas
for approximately 45 minutes, and reconstituted in 1.0 mL of 0.01 N HCl. These hydrolysates were analyzed for desmosine content by radioimmunoassay\textsuperscript{15} and results expressed as picomoles of desmosine per mg dry aorta.

Elastin integrity in aortic explants was also verified by histology. Aortic tissue samples were retrieved immediately following euthanasia (n=6), embedded in OCT cryosectioning medium (Sakura Finetek; Torrance, CA), and frozen on dry ice. Sections (6 µm) were stained with Verhoeff van Giesson’s procedure (VVG, Poly Scientific; Bay Shore, NY) for elastic tissue (elastin fibers stain black while collagen stains red/pink) to assess aortic elastin integrity.

Pentagalloyl Glucose Binding Studies

Upon euthanasia, rat abdominal aortas (n=3) retrieved 28 days after PGG treatment were rinsed and lyophilized to obtain dry weight. Segments of the aorta (~2 mm in length) were crushed in liquid nitrogen and PGG extracted with 80% methanol (0.5 mL, 3 extractions, 15 minutes each, at 4°C).\textsuperscript{25} Following each extraction, samples were centrifuged (12000 rpm, 5 minutes, 4°C), extracts collected, and pellets re-extracted with methanol. For each sample, the three resulting extracts were pooled, dried under nitrogen gas at room temperature, and reconstituted in 0.2 mL saline. Samples were then assayed in triplicates for phenol content with the Folin-Denis reaction using a calibration curve with 0 to 150 µg PGG/mL.\textsuperscript{26} Results were normalized to sample dry weights. As controls, rat aortas were treated with 0.03% PGG in situ with presoaked gauze for 15 minutes. Immediately following treatment, aortas were rinsed with saline, rats euthanized, tissue collected, and PGG extracted as described above. These controls
represented the original amount of PGG that would bind to rat aorta at day 0. Samples were also stained for phenols with FeCl₃ as described above.

Calcification Assessment

Twenty-eight days after CaCl₂ injury, rats were euthanized and aortic samples were retrieved for calcium analysis. Samples (n=12) were lyophilized to obtain dry weight, individually hydrolyzed in 6 N HCl at 95°C for approximately 8 hours, dried under nitrogen gas for 45 minutes, and ultimately reconstituted in 1.0 mL of 0.01 N HCl. Calcium content was then measured within these hydrolysates using atomic absorption spectrophotometry as described before. Samples of aorta were also embedded in OCT cryosectioning medium (Sakura Finetek; Torrance, CA), frozen on dry ice, and sectioned (6 µm). These sections were stained with Alizarin red for calcium deposits, with light green as a counter stain, to verify quantitative calcium values.

Immunohistochemical Staining for Inflammation (Macrophages)

Aortic tissues from control (saline) and PGG-treated rats were embedded in OCT cryosectioning medium immediately after euthanasia. Sections (6 µm) were fixed in cold acetone for 5 minutes and incubated with 0.1% proteinase K for antigen unmasking. Following two rinses in Tris-buffered saline (5 minutes each), sections were blocked with 0.3% hydrogen peroxide in 0.3% normal sera in Tris-buffered saline for 5 minutes to neutralize endogenous peroxidase activity. Sections were then incubated with mouse-derived anti-rat monocyte/macrophage monoclonal antibody (Chemicon International; Temecula, CA) diluted in TNB blocking buffer (1:200 dilution) at room temperature for 1 hour. TNB buffer alone was used as a negative control. Following another series of
rinses in Tris-buffered saline, staining (and visualization of the stain) were performed using rat-adsorbed biotinylated anti-mouse IgG secondary antibody (5 µg/mL in Tris-buffered saline; Vector Laboratories; Burlingame, CA), avidin-biotin-horseradish peroxidase complex (Vectastain Elite kit, Vector Laboratories), and diaminobenzidine substrate (DAB Substrate kit, Vector Laboratories). Sections were counterstained with hematoxylin in order to demonstrate cell nuclei. As a positive control for the stain, normal rat spleen, a tissue rich in macrophages, was subjected to this same procedure.

MMP and TIMP Quantification

MMP activities within control (saline) and PGG-treated rat aortas (n=6 per group) retrieved 28 days after CaCl₂ injury were analyzed using gelatin zymography, as described previously.²⁹ Aortic samples were homogenized and proteins were extracted with buffered guanidine-HCl (50 mM Tris, 0.2% Triton X-100, 10 mM CaCl₂, 2 M guanidine-HCl, pH 7.5). Protein within resulting extracts was dialyzed (3500 molecular weight cutoff dialysis tubing) against a buffered solution of 50 mM Tris, 0.2% Triton X-100, pH 7.5, and subsequently quantified with the bicinchoninic (BCA) assay (Pierce; Rockford, IL). For zymography, each lane was loaded with 5 µg of protein. Following staining and development, densities of light bands on a dark background were quantified for MMP-2 (68 and 72 kDa) and MMP-9 (82 kDa) using Gel-Pro Analyzer software (Media Cybernetics, Inc.; Silver Spring, MD) and reported as relative density units (RDU).

Levels of tissue inhibitor of metalloproteinase-2 (TIMP-2) within these same protein extracts (n=6 per group) were analyzed by enzyme-linked immunosorbent assay
(ELISA; TIMP-2 Biotrak Assay kit, Amersham Biosciences; Piscataway, NJ) according to manufacturer’s guidelines and results expressed as ng per mg of protein.

7.2.3.3 Experiment 2: Aneurysm Progression

Adult rats (n=24) were sedated and maintained under general anesthesia as described above for Experiment 1. The infrarenal aorta between the renal artery and iliac bifurcation was exposed via laparotomy, photographed for diameter measurements, treated with 0.5 M CaCl₂ to induce aneurysm formation as described above, and rats closed and allowed to recover. All rats had access to water and chow *ad libitum*. After 28 days, rats were exposed to a second surgery and randomly divided into two groups, PGG treatment group (n=12) and controls (saline; n=12). For this second procedure, rats were anesthetized, the abdominal aorta exposed (Figure 7.3), cleaned of adhesions, and digitally photographed for diameter measurements. In the PGG treatment group, aortas were treated with 0.06% PGG in saline for 15 minutes using same gauze application technique described above. The higher PGG concentration was selected for this experiment (as compared to Experiment 1) because of expected higher degree of elastin degeneration in the pathological (aneurysmal) aortas. In the control saline group, exposed rat aortas were treated with physiological saline for 15 minutes. After rinsing, incisions were closed and rats allowed to recover with free access to food and water. At 28 days after the second surgery (totaling 56 days after initial CaCl₂ injury), all rats were anesthetized, abdominal aorta exposed, cleaned of adhesions, and again photographed for diameter measurements. These photographs allowed us to track diameter changes (and aneurysm development) for each rat, from aneurysm induction (calcium chloride at day 0) to treatment or intervention with PGG (day 28) to our endpoint (day 56).
Figure 7.3 Surgical steps for the abdominal aortic aneurysm model (Experiment 2). For this experiment, a second surgery was performed 28 days after the first in an attempt to halt or slow aneurysm progression. Black arrows denote abdominal aorta.

Five rats from each group (PGG-treated and saline controls) were perfusion-fixed with formalin in order to fix the tissues into place under pressure and evaluate histomorphometry. Following anesthesia and photography (for aortic diameter measurement), heparin (0.2 mL of 1000 Units/mL) was administered through subcutaneous injection. Once the heparin circulated for 20 minutes, the animals were humanely euthanized by CO$_2$ asphyxiation. A catheter inserted into the thoracic aorta allowed the vasculature to be washed/flushed (250 mL saline with 2000 Units/L heparin) and fixed in situ (150 mL of 10% neutral buffered formalin) under 100 mm Hg pressure, to imitate physiological pressure in blood vessels. Flow rates of wash and fixative solutions were both approximately 10 mL/minute. Once perfusion fixation was complete, aortas were digitally photographed again. Those rats which were not perfusion
fixed were euthanized by CO₂ asphyxiation immediately following digital photography of their abdominal aorta.

Samples were collected from each aorta, whether perfusion fixed or not, and processed for assessment of elastin content and integrity, as well as calcium analysis. Histology on the perfusion fixed aortic samples was used for morphometric measurement of external and internal diameters, as described below. All animals received humane care in compliance with protocols approved by the Clemson University Animal Research Committee as formulated by the NIH (Publication No. 86-23, revised 1985).

Histomorphometric Diameter Measurement

For those aortas which were perfusion fixed in situ under physiological pressure (n=5 per group), aortic samples (~3 mm in length) were stored in 10% neutral buffered formalin and processed for histology. Paraffin embedded sections (5 µm) were stained with hematoxylin and eosin (H&E). Low magnification pictures (25x) were used to determine the external diameter, internal diameter, and thickness of the arterial media. These digital measurements were performed using the trace feature in Image-Pro Plus 5.1 (Media Cybernetics; Silver Spring, MD).

Elastin Integrity Assessment

Following euthanasia, rat aortas were collected and assayed for desmosine content (an amino acid specific to elastin) to express elastin content in tissues, as described above. Normal rat aortas, which did not undergo surgery, were again used as controls (n=6). Briefly, segments of abdominal aorta (~2 mm in length) were lyophilized to obtain dry weight, acid hydrolyzed, and ultimately analyzed for desmosine content by
radioimmunoassay. This analysis was performed on all collected rat aortas, whether perfusion fixed or not. Results were expressed as picomoles of desmosine per mg dry aorta. Elastin integrity in aortic explants was also verified by histology using VVG stain for elastin fibers, as described above. In addition to this elastin-specific stain, hematoxylin and eosin (H&E) was also performed to investigate general structure of the aortic tissue.

7.2.4 Data Analysis

Results are expressed as means ± standard error of the mean (SEM). Unless otherwise noted, statistical analyses of the data were performed using single-factor analysis of variance (ANOVA). Subsequently, differences between means were determined using the least significant difference (LSD) with an alpha value of 0.05.

7.3 Results

7.3.1 In Vitro Safety and Efficacy

In anticipation of the animal studies, several studies were performed in vitro to evaluate the effects of a single, 15-minute application of pentagalloyl glucose (PGG) on cell viability and arterial extracellular matrix components, specifically elastin.

Cytotoxicity tests were performed by direct exposure of rat aortic smooth muscle cells and rat skin fibroblasts to increasing concentrations of PGG at 37°C. MTS results, reported as absorbance values which are directly proportional to cell viability, showed that exposure of smooth muscle cells (Figure 7.4) and fibroblasts (Figure 7.6) to PGG concentrations of up to 0.06% had minimal cytotoxic effects. For each cell type, greater than 80% of the cells remained viable after exposure to this concentration of PGG. It
should be noted that exposure to 0.03% and 0.06% PGG, the two concentrations used for in vivo experiments, did not significantly (p>0.10) alter cell viability for smooth muscle cells or fibroblasts. In general, MTS results were confirmed visually by Live-Dead assay (Figure 7.5 for rat aortic smooth muscle cells, Figure 7.7 for rat skin fibroblasts), which fluoresces live cells green and dead cells red.

**Figure 7.4** Rat aortic smooth muscle cells were exposed to varying concentrations of PGG, and cytotoxicity measured using the MTS assay (absorbance values are directly proportional to number of viable cells). Phosphate buffered saline (PBS) and 70% ethanol (EtOH) were used as negative and positive controls, respectively. Cells exposed to 0.03% and 0.06% PGG did not exhibit significantly different viability (p>0.10).
Figure 7.5 Viability of rat aortic smooth muscle cells was visualized with Live/Dead staining (live cells = green, dead cells = red), indicating that PGG up to 0.10% resulted in little to no cell death. PBS = phosphate buffered saline, EtOH = 70% ethanol.

Figure 7.6 Rat skin fibroblasts were exposed to varying concentrations of PGG, and their viability assessed using the MTS assay. Concentrations of PGG up to 0.06% had minimal effects on fibroblast viability. The number of viable cells exposed to 0.03% PGG was not significantly less than those exposed to the negative PBS control (p>0.10).
Figure 7.7 Effect of varying concentrations of PGG on rat skin fibroblast viability was confirmed by Live-Dead staining (live cells = green, dead cells = red). PBS = phosphate buffered saline, EtOH = 70% ethanol.

As a test for elastin stabilization, PGG-treated samples of rat aorta were exposed to an in vitro accelerated model for enzyme-mediated elastolytic degradation. Following digestion with elastase, elastin content within the tissue was determined by desmosine assay (Figure 7.8). As expected, results showed a trend of increasing resistance to elastase with increasing PGG concentrations used for treatment of rat aorta. The samples treated with PGG showed concentration-dependent retention of desmosine (and thus elastin). Concentrations of 0.06% PGG and higher yielded aortic tissue that retained significantly more desmosine than saline controls (p<0.05).
Figure 7.8 To test for elastin stabilization in rat aorta, samples were treated with varying concentrations of PGG for 15 minutes in vitro and then exposed to pure elastase. Elastin content was measured in the aortic tissues by desmosine analysis, revealing that PGG was able to protect elastin from degradation in a concentration-dependent manner.

In addition to investigating the tissue, desmosine, which may have been released due to significant elastin degradation, was also assayed within the retrieved enzyme solutions. These results correlated well with those in Figure 7.8, revealing that greater elastin stabilization (with increasing PGG concentrations) resulted in less detected desmosine in solution (Figure 7.9). The amount of desmosine in these “used” solutions was significantly less within the higher concentration PGG groups (0.10% and 0.15%) as compared to saline controls (p<0.05). Although not statistically significant from these controls (p>0.05), aortas treated with 0.03% or 0.06% PGG also appeared to reduce the elastase-mediated release of desmosine into solution.
Figure 7.9 Desmosine analysis on elastase solutions revealed that rat aorta treated with PGG correlated into significantly less desmosine being released into solution as a result of its elastin stabilizing capabilities.

It was believed that this observed elastin stabilization within rat aorta was attributable to PGG’s ability to bind directly to elastin within the tissue. This was confirmed by showing PGG binding to rat aortic elastin using a specific phenol stain (Figure 7.10). Results showed specific staining of elastic fibers, with the intensity of the black stain progressively increasing with increasing concentrations of PGG. Therefore, PGG clearly binds to rat aortic elastin in a concentration-dependent pattern, thus endowing the tissue resistance to elastolytic degradation.
Figure 7.10 For histological confirmation of PGG binding to rat aortic elastin, PGG-treated aortic tissues were stained with a phenol-specific stain (PGG=black) and counterstained with light green. L=lumen, bar=100 µm, arrows point to elastic lamellae.

For further proof of interactions of PGG with arterial elastic fibers, the ring-opening test was performed on PGG-treated rat aorta (Figures 7.11 and 7.12). Native untreated aortic rings, when allowed to open by a single incision, extended to more than 75 degrees, revealing the natural elastic recoil properties of aortic tissues. Rings that were exposed to increasing concentrations of PGG exhibited smaller opening angles, suggestive of the direct interaction of PGG with elastic fibers. With the exception of 0.03% PGG, all PGG-treated tissues opened significantly less than saline controls (p<0.05). The opening angles of aorta treated with 0.03% and 0.06% PGG, the concentrations used in vivo, were not statistically different from one another (p>0.10).
Figure 7.11  To test elastin recoil, aortic rings were treated for 15 minutes with increasing concentrations of PGG and subjected to ring opening analysis. Opening angles were measured graphically, as depicted by the white lines (bottom left corner).

Figure 7.12  Mean opening angles of rat aorta for each treatment group are shown as a function of PGG concentration. The decreased opening angles associated with higher PGG concentrations are likely indicative of increased PGG binding to elastin.
Having established that a 15-minute exposure to PGG solutions of up to 0.06% is not cytotoxic and effectively stabilizes aortic tissue by binding to elastic fibers, two experiments were designed to test the in vivo efficacy of PGG in hindering AAA formation and progression (Figure 7.1). In Experiment 1, prevention of AAA formation was achieved by periadventitial delivery of PGG to healthy abdominal aorta concomitant with the onset of AAA. In a more clinically relevant Experiment 2, PGG was applied to pathologic (aneurysmal) aorta and this treatment significantly hindered chronic AAA progression.

### 7.3.2 Effect of Pentagalloyl Glucose on Aneurysm Formation

In Experiment 1, perivascular application of CaCl₂ to the infrarenal abdominal aorta induced significant changes in aortic diameter at 28 days after injury (Figures 7.13 and 7.14). Comparative measurements of the external aortic diameter of control (saline-treated) rats at day zero and 28 days after surgery (1.395 ± 0.052 mm and 1.939 ± 0.112 mm, respectively) revealed a mean increase in diameter of 42 ± 10 % (p<0.05, n=12). By comparison, aortas that were exposed to PGG exhibited minimal (8 ± 7 %; Figure 7.14) increase in diameter after 28 days (from 1.564 ± 0.064 mm to 1.676 ± 0.097 mm). Figure 7.13 shows representative digital images of control rat aorta at day zero (prior to treatment or chemical injury), saline-treated aorta 28 days after chemical injury, and PGG-treated aorta 28 days after chemical injury.

Considering an arbitrary threshold of 20% diameter increase as aneurysmal in this experimental model, 8 out of 12 rats exhibited aneurysms in the control group (66.7%) and only 2 out of 11 rats were aneurysmal in the PGG group (18.2%, Table 7.1). Chi-squared categorical analysis (with an alpha value of 0.05) revealed that these proportions
were statistically different from one another. These results indicate that PGG application just prior to arterial injury with CaCl₂ effectively hindered development of aneurysms in this experimental model.

Figure 7.13  External diameter of the infrarenal abdominal aorta between the renal artery (top) and iliac bifurcation (bottom) was measured by digital photography at day 0 and 28 days after injury in the control group (saline-treated) and in the PGG group. Black dashed lines were added to aid in identification of aortic anatomy.
Figure 7.14  Mean percent change in diameter at 28 days (relative to day 0) revealed that local delivery of PGG prevented abdominal aortic aneurysm formation. Black arrow denotes time of PGG (or saline) application.

Table 7.1  Aneurysm occurrence (defined as a 20% increase in aortic diameter)

<table>
<thead>
<tr>
<th>Aneurysm Occurrence</th>
<th>% Aneurysmal</th>
</tr>
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<tbody>
<tr>
<td>Saline 8 / 12</td>
<td>66.7</td>
</tr>
<tr>
<td>PGG 2 / 11</td>
<td>18.2</td>
</tr>
</tbody>
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As shown above, such exposure to PGG for 15 minutes had minimal cytotoxic effects on rat smooth muscle cells and fibroblasts. In our experiment, PGG-treated rats did not exhibit significant changes in weight gain during the 28-day test period (mean gain of 42.33 ± 3.47 g in control group vs. 44.25 ± 3.69 g in PGG group, p<0.05, n=12). Liver samples collected from each group did not exhibit any noticeable histological
changes indicative of hepatotoxicity (Figure 7.15). Taken together, these results suggest that periarterial application of PGG did not elicit conspicuous local, organ, or cell toxicity in this experimental model.

![Figure 7.15](image)

**Figure 7.15** Histology on rat liver revealed that PGG treatment had no hepatotoxic effects when delivered locally within the abdominal aortic aneurysm model. Hematoxylin and eosin stain, original magnification 100x.

7.3.2.1 Elastin Integrity

Along with aortic dilatation, perivascular application of CaCl₂ induced major changes in vascular elastin content and integrity as shown by desmosine analysis (Figure 7.16) and histology (Figure 7.17). As compared to non-surgery control aorta collected from age-matched rats, aortic elastin content in the control (saline-treated) group diminished by almost 50%, as suggested by the drastic drop in desmosine content (Figure 7.16). Histology on this same group exhibited characteristic flattening and fragmentation of the elastic laminae at 28 days after injury (Figure 7.17). Conversely, aortas from the PGG group exhibited minimal decrease in elastin content (Figure 7.16) as compared to normal non-surgery control aorta (less than 15% loss of desmosine, p>0.05) and excellent
preservation of elastic laminae integrity and waviness (Figure 7.17), suggesting that PGG delivery effectively prevented elastin degeneration in this animal model.

**Figure 7.16** Desmosine analysis was performed on non-surgery control rat aorta (day 0) and compared to aorta collected 28 days after chemical injury. The PGG group exhibited excellent elastin preservation after 28 days as compared to saline-treated aorta (p<0.05).

Black arrow denotes time of PGG (or saline) application.
Figure 7.17 Elastin integrity at day 0 and day 28 was also evaluated by histology using VVG stain (elastin = black). The natural wavy architecture of elastin fibers remained intact for PGG-treated aorta while saline-treated controls displayed significant elastin fiber fragmentation after 28 days. L = lumen, bar = 50 µm.

7.3.2.2 In Vivo Binding of Pentagalloyl Glucose

The affinity of phenolic tannins towards elastin has been well established in previous chapters, particularly for porcine vascular elastin. In order to validate PGG binding to rat aortic elastin in vivo, we quantified amounts of PGG present in explanted rat aortas (Figure 7.18). As compared to day 0 (1.88 ± 0.6 µg PGG/dry tissue), aortas
explanted 28 days after PGG application contained slightly lower, but not statistically
different amounts of PGG (1.23 ± 0.4 µg PGG/dry tissue; p>0.05), indicating that in vivo
binding of PGG to rat aortic tissues is stable for a minimum of 28 days in this accelerated
animal model.

![Graph](image)

**Figure 7.18** Quantitative analysis of PGG content in explanted aorta shows the presence
of significant amounts of PGG incorporated into the tissue. The majority of this PGG
remained bound even after 28 days in the accelerated animal model (p>0.05 as compared
to day 0).

### 7.3.2.3 Model-Related Pathogenesis

The primary pathogenic characteristics of this experimental model are similar to
those found in human aortic aneurysms, namely medial calcification,\(^{30}\) inflammation,\(^{22}\)
and matrix degeneration.\(^{20,31}\) To investigate the effect of periadventitial PGG application
on these aspects, calcium content, macrophage infiltration, MMP activities, and
expression of TIMP were analyzed in aortas from both groups (PGG-treated and saline-treated controls).

Aortic Calcification

Expectedly, perivascular application of CaCl$_2$ induced substantial tissue calcification after 28 days (Figure 7.19). Alizarin red staining of aorta showed that this calcium was localized mainly in the media (Figure 7.20). Although slightly lower calcium values were observed for PGG-treated aortas, these results were not statistically different from saline-treated controls (p>0.10). In addition, tissue distribution of calcium, as seen by histology, also appeared to be similar for both groups.

![Figure 7.19](image)

**Figure 7.19** Aortic calcification at 28 days after CaCl$_2$ injury was evaluated by analysis of calcium content, which revealed that PGG treatment did not significantly alter this pathological trait of the model (p>0.10).
Calcium analysis results were confirmed by histology with Alizarin red staining (calcium deposits = red). PGG appeared to have little effect on the degree of calcification as compared to saline-treated controls. L = lumen, bar = 100 µm.

**Figure 7.20**

Immunohistochemical (IHC) staining for macrophages revealed comparable inflammatory infiltrates in both groups (Figure 7.21). As a positive control for the stain, sections of spleen were subjected to the same IHC procedure, confirming the brown staining of macrophages (data not shown).

**Inflammation**

Immunohistochemistry for macrophages (positive reaction brown) revealed comparable inflammatory infiltrates in both groups, thus suggesting that PGG does not reduce inflammation. L = lumen, bar = 100 µm.

**Figure 7.21**
Activity of Matrix-Degrading Enzymes

Activities of MMP-2 and MMP-9, enzymes which digest matrix components such as elastin and collagen, were assessed by gelatin zymography. MMP-2 and MMP-9 activities were not different between saline controls and PGG-treated aortas (p>0.10, Figure 7.22). In addition, expression of TIMP-2, a potential inhibitor of the aforementioned MMPs, was also analyzed by ELISA (Figure 7.22). Again, no differences were observed in TIMP-2 for the two groups (p>0.10), suggesting that tissues in both groups were exposed to similar proteolytic environments.

Taken together, these results (calcification, inflammation, and activity of matrix-degrading enzymes) suggest that PGG treatment did not interfere with the pathogenic mechanisms typical to this experimental model. Instead, PGG apparently works by directly binding to and stabilizing elastin.

Figure 7.22 After 28 days, MMP-2 and MMP-9 enzymatic activities, as well as TIMP-2 levels, were not statistically different in the two groups (p>0.10). This data suggests that PGG does not interfere with (inhibit) the MMP activity associated with this model.
7.3.3 Effect of Pentagalloyl Glucose on Aneurysm Progression

7.3.3.1 Aortic Diameter

In Experiment 2, rat aortas were treated with CaCl₂ and abdominal aortic aneurysms (AAAs) were allowed to develop for 28 days. At this time point, a second surgery was performed and PGG was applied to aneurysmal aortas in the PGG group and saline was applied to aneurysmal aortas in the control group. AAA progression was monitored for another 28 days in both groups. Figure 7.23 shows representative pictures of perfusion-fixed aorta from each group (PGG-treated and saline-treated controls) at this time point. Additionally, Table 7.2 displays the number of individual rats from each group whose aortic diameter increased (aneurysm progression), experienced little or no relative change (stationary), or decreased (regression) at day 56 as compared to day 28.

As expected, perivascul ar application of CaCl₂ at day 0 induced a progressive diameter expansion, reaching a mean increase of 47.1 ± 11.8% at 56 days in the saline-treated control group (Figure 7.24). Approximately half of the aneurysmal aortas increased in diameter from day 28 to day 56 in the saline-treated control group (Table 7.2), indicative of chronic AAA progression in this animal model.

By comparison, aneurysmal aortas that were exposed to PGG at day 28 exhibited no increase in mean diameter at 56 days (23.2 ± 4.1% diameter increase relative to day 0) as compared to day 28 mean values (33.6 ± 5.4% diameter increase relative to day 0). In other words, the average diameter of the PGG-treated aorta actually decreased in comparison to its diameter at day 28, although this difference was not statistically significant (p>0.05). The change in diameter at 56 days for saline-treated controls (47.1 ± 5.4%), however, was significantly greater (p<0.05) than that observed for PGG-treated
(23.2 ± 4.1%). It is also noteworthy that 100% (11 out of 11) of aortas in the PGG group maintained the same diameter or exhibited a slight decrease in aortic diameter (56 vs. 28 days; Table 7.2). Overall, these results indicate that PGG application to aneurysmal aortas effectively hindered AAA progression in this experimental model.

Figure 7.23 External diameter of the infrarenal aorta between the renal artery (top) and iliac bifurcation (bottom) was measured by digital photography after perfusion fixation in both groups 56 days after chemical injury (28 days after treatment with PGG or saline). Black dashed lines were added to aid in identification of aortic anatomy.
Figure 7.24  Mean percent change in external aortic diameter at 28 days (time of second surgery and application of PGG or saline, as denoted by black arrow) and 56 days, relative to day 0. Values in the two groups were significantly different at 56 days (p<0.05).

Table 7.2  State of aneurysm development at day 56 following treatment with PGG or saline at day 28. Values represent actual numbers of individual rats that exhibited increased, stationary, or reduced diameters at 56 days, as compared to 28 days.

<table>
<thead>
<tr>
<th>Diameter Increase</th>
<th>Saline</th>
<th>PGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progression (&gt;40%)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Stationary (25% to 40%)</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Regression (&lt;25%)</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>
Using low magnification histology images of perfusion-fixed cross-sections of rat aorta, like those shown in Figure 7.25, morphometric analysis was performed to calculate internal and external diameters of the aortic media, as well as media thickness (Figure 7.26). For external and internal diameter, values were smaller for the PGG group, although this difference was not significant (p>0.05). At the same time, thickness of aortic media was found to be substantially smaller in the PGG treatment group (p<0.05, Figure 7.26). While the meaning of this increased thickness for the saline controls is not well understood, it may be indicative of arterial matrix degeneration, resulting in a loss of tissue “compactness”.

Taken together, these results suggest that PGG treatment of aneurysmal aortas effectively prevented chronic diameter expansion in this animal model.

Figure 7.25  Low magnification pictures taken from each H&E stained aortic cross-section were used for histomorphometric measurement of external and internal diameters, as well as thickness of arterial media using digital imaging. In the representative pictures shown here, PGG-treated aorta possessed a slightly smaller internal diameter, external diameter, and thickness. Bar = 1000 µm.
7.3.3.2 Elastin Integrity

After 56 days, animals were sacrificed and aortas were visually examined for elastin integrity by VVG staining. This elastin-specific stain displayed marked flattening, extensive fragmentation, and complete degeneration of the elastic laminae in extended areas in the saline control group (Figure 7.27). Moreover, overall tissue architecture (H&E stain) was indicative of severe tissue degeneration as outlined by numerous empty spaces, bestowing the aneurysmal aorta with a “spongy” aspect (Figure 7.28). This trait likely coincides with the increased medial thickness and loss of tissue compactness observed during histomorphometric analysis of saline-treated controls, as described above. In contrast, PGG-treated aortas exhibited visibly better preservation of elastic laminae integrity and waviness (Figure 7.27) and overall tissue architecture (Figure 7.28).
Figure 7.27  Aortic elastin integrity was evaluated by histology using VVG stain (elastin = black), showing preservation of the natural wavy architecture of elastin fibers in the PGG-treated samples.  L = lumen, bar = 50 µm.

Figure 7.28  General tissue architecture was also examined by H&E staining. Saline-treated controls, those more prone to aneurysm progression, apparently experienced severed tissue degradation, as suggested by the spongy aspect seen in the aortic wall.  

L = lumen, bar = 50 µm.
Desmosine analysis of aortic tissues at 56 days post-injury in both PGG and saline control groups revealed no significant differences in elastin content ($p>0.05$, Figure 7.29). Moreover, these elastin values were not statistically different from the 28-day values, indicating that the severe elastin degradation observed at 28 days has reached a steady-state and has not changed significantly as AAA progressed from 28 to 56 days. In view of the fact that major elastin degradation (flattening and fragmentation) was seen through histology (Figures 7.27 and 7.28), it may be a case where quantity of elastin was not affected by PGG treatment, but preservation of elastin quality was maintained.

**Figure 7.29** Desmosine analysis on explanted aorta revealed that elastin content in PGG-treated aorta was not different than saline-treated samples ($p>0.05$). Furthermore, 56 day values (for both groups) were not significantly different than 28 day controls, suggesting that the majority of elastin degradation may have occurred prior to intervention with PGG. Black arrow indicates time of intervention with PGG (or saline as control).
7.4 Discussion

Aneurysms arise from irreversible, chronic pathological changes that involve MMP-mediated degeneration of structural matrix components, namely elastin and collagen. The role of MMP/TIMP imbalances in AAA initiation and development have been irrevocably demonstrated by studies using MMP and TIMP-knockout mice.\textsuperscript{20,31-33} Degeneration and loss of elastin are trademarks of AAA development, which apparently lead to progressive dilatation of the aorta. Therefore, the work presented here focused on chemical stabilization of vascular elastin as a novel approach to limit aneurysmal degeneration.

Currently, the only options for treating late-stage AAAs entail surgical procedures such as endovascular stent graft repairs or complete replacement of the diseased section of the aorta with an artificial mesh vascular graft. Non-surgical approaches to suppress aneurysmal degeneration in experimental animals include use of anti-inflammatory agents, proteinase inhibitors, and genetic and pharmacologic inhibition of MMPs. Doxycycline, an antibiotic and MMP inhibitor, showed initial promising results in a clinical study.\textsuperscript{34,35} Recent studies have also reported that the systemic pharmacologic inhibition of c-Jun N-terminal kinase (JNK), an intracellular signaling switch that controls MMP production, might block AAA progression and stimulate aneurysm regression.\textsuperscript{36} Such approaches have been effective when delivered systemically; however, the high doses required for systemic use could ultimately lead to adverse side effects. For instance, doxycycline use in this manner has been associated with muscle pains, cutaneous photosensitivity, and gastrointestinal problems.\textsuperscript{37}
While numerous attempts have focused on proteolytic inhibition for AAA treatment, our novel approach is based on periadventitial delivery of phenolic tannins such as PGG to stabilize elastin and render it resistant to enzymatic degradation. In previous chapters, we have shown that tannins are excellent candidates for such an approach for their ability to bind to and stabilize vascular elastin. PGG, a more stable derivative of tannic acid, was chosen for these studies since its improved structure virtually eliminates any toxic implications that have been associated with tannic acid, as shown in Chapter 6. Our hypothesis was that the ability of PGG to stabilize elastin would extend to rat aorta as well. To verify our hypothesis, we performed a series of in vitro safety and efficacy studies followed by in vivo validation in a rat AAA model.

As demonstrated in the previous chapters, PGG exhibits an outstanding affinity towards elastin, possibly binding to hydrophobic areas, which are known to be susceptible to protease-mediated elastolysis. In current studies we have validated PGG binding to rat aortic elastin in vivo and provided evidence that the binding is stable for a minimum of 28 days in this accelerated AAA experimental model. In previous chapters, we have also provided ample in vitro evidence that binding of PGG to arterial elastin provides an outstanding resistance to proteolytic degeneration.\textsuperscript{10-12} Since the natural elastin turnover is exceptionally low,\textsuperscript{38} we hypothesize that PGG may remain bound to aortic wall (by way of elastin) for extended periods of time after application, sufficient to maintain resistance to enzymes and deter AAA progression.

\textbf{7.4.1 Experiment 1: Pentagalloyl Glucose Hinders Aneurysm Formation}

Traditionally, increase in aortic diameter is considered the defining characteristic of AAA. Numerous investigators studied AAA formation in the CaCl\textsubscript{2} injury model in
mice, rabbits, and rats. In Experiment 1, application of 0.5 M CaCl$_2$ to the rat infrarenal abdominal aorta induced a mean increase in diameter of 42% at 28 days after application. The extent of aneurysmal dilatation observed in our studies is in good correspondence with other published studies using CaCl$_2$ injury, which consistently reported a 25% to 75% increase in diameter in rodents, depending on the time periods evaluated and concentration of CaCl$_2$ used.

Conversely, mean aortic diameter increase in the PGG group was less than 10%, which is traditionally not considered indicative of aneurysm formation. The overall AAA incidence in the PGG group was less than 20%, indicating that in this experimental model, PGG prevented aneurysm formation in more than 8 out of 10 animals. While the detailed mechanisms of this successful approach are not entirely known, it apparently involves PGG-mediated elastin stabilization and effective prevention of proteolytic degeneration.

A second major characteristic of AAA is progressive damage to the arterial elastin component. In our experimental model, periadventitial application of CaCl$_2$ induced a decrease of more than 50% in aortic elastin content at 28 days after application, as compared to non-surgery control aorta. Earlier studies have shown that elastin degeneration starts within the first days after the CaCl$_2$ injury and this chronic process is related to an over-expression of MMPs within the aortic extracellular matrix. These quantitative desmosine results were confirmed by histological analysis of tissue architecture using an elastin-specific stain. Typically, aortic samples that were characterized by low elastin content also exhibited characteristic flattening and
fragmentation of the naturally wavy elastic lamellae. This histological aspect is characteristic of the aortic pathology in CaCl₂-treated aorta.³⁶

Periadventitial treatment of aorta with PGG resulted in remarkable preservation of elastin integrity. Quantitative desmosine results showed that elastin content in the PGG group was not statistically different from the non-surgery controls (p<0.05), indicating little to no elastin degradation. This was accompanied by excellent histological preservation of elastic lamellae integrity and waviness. Other published studies utilizing this experimental model, including the current data shown here, indicate that elastin preservation was consistently associated with absence of aneurysm initiation or progression.²⁰,³¹,³⁶ This indicates that elastin stabilization by PGG treatment may be effective as a potential AAA treatment.

Within days after CaCl₂ application, medial calcification and matrix degeneration occurs in this animal model as a result of increased MMP activities and inflammation within the aortic wall.³⁰ In our studies, application of non-cytotoxic doses of PGG to rat aortas did not alter the calcification or inflammation processes and did not influence the levels of aortic MMPs and TIMP. This is significant, as it provides insight into the working mechanisms of PGG treatment for this application. It suggests that PGG treatment does not directly interfere with model-related pathogenesis; rather, PGG-mediated inhibition of AAA development is apparently due to direct stabilization of elastin and not by inhibition of enzyme activities. Inhibition of MMPs, enzymes which are required for many normal physiological remodeling processes, could lead to undesirable side effects, as mentioned previously.
7.4.2 Experiment 2: Pentagalloyl Glucose Limits Aneurysm Progression

In a more clinically relevant experiment, PGG was directly applied to aneurysmal aortas and AAA progression was followed after this surgical intervention. While most control aneurysmal aortas (periadventitiously treated with saline) continued to expand in diameter, every one of the PGG-treated aneurysmal aortas maintained the same diameter or exhibited a decrease in aortic diameter at the end of the experiment (56 days). In our experiments, diameter expansion in aneurysmal controls was associated with changes in medial thickness and integrity, including severe tissue degeneration. In an experimental setup similar to the studies we are reporting here, Yoshimura et al. reported comparable inhibition of AAA progression after the onset of AAA in rodent animal models, as a result of systemic pharmacologic inhibition of JNK. Since JNK is an intracellular signaling switch that controls MMP production, JNK inhibition may have temporarily inhibited MMP production and thus prevented degeneration of the vascular matrix. To our knowledge, this is the only study (besides our current work) which has shown the ability to halt aneurysm progression by intervening with a treatment weeks after aneurysm onset. Since JNK inhibition apparently works by limiting MMP production and activity, the feasibility of using this technology as a systemic clinical treatment may be limited due to the same unfavorable effects of MMP inhibition.

Histological analysis of aneurysmal aortas at day 56 showed a significant decline in elastin integrity in the control group indicating that elastin degeneration is a chronic pathogenic aspect characteristic to this AAA model. Hallmarks of this process were elastic fiber flattening, fragmentation, and lack of elastic laminae staining in extended areas in almost all samples (5 out of 6). Aortas from the PGG group exhibited improved
preservation of elastic laminae integrity and waviness in most samples (4 out of 6). Desmosine analysis for this experiment revealed little differences for the PGG group as compared to controls, possibly due to high biological variability in this experimental model. Another possible explanation for these conflicting results (histology and desmosine analysis) is that elastin quantity is maintained for both groups, while the quality of elastin within the PGG group is better preserved. Taken together, however, our data suggest that PGG-mediated elastin stabilization in aneurysmal aortas has the potential to significantly hinder elastin degradation and AAA development.

7.5 Conclusions

Maintaining the integrity of the aortic wall is vital in preventing AAA development. Acute localized periadventitial delivery of non-cytotoxic concentrations of PGG inhibits elastin degeneration, attenuates aneurysmal diameter expansion, and hinders development of AAA in an established animal model without interfering with the pathogenic mechanisms typical to this model. PGG binds strongly and specifically to arterial elastin and in doing so, it preserves elastic laminae integrity and architecture despite the presence of high levels of matrix-degrading enzymes. Approaches that target stabilization of the aortic extracellular matrix in aneurysm-prone arterial segments hold great potential towards development of safe and effective therapies for AAAs.

7.6 References


CHAPTER 8
CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

Elastin is an extracellular matrix protein found in abundance within vascular and cardiovascular tissues. Protecting elastin from degeneration associated with matrix-degrading enzymes may be beneficial in (1) improving the clinical longevity of tissue-derived cardiovascular devices such as bioprosthetic heart valves, and (2) halting or limiting development of abdominal aortic aneurysms. These ideas were tested in the work presented here, allowing us to draw the following conclusions:

- Tannins, such as tannic acid and pentagalloyl glucose (PGG), bind to vascular elastin, resulting in improved resistance to elastolytic degradation.¹

- Elastin stabilization may be beneficial as a pretreatment for bioprosthetic heart valves. Tannin treatment for this purpose appeared to be safe, effective, and feasible.²

- Polyphenolic hydroxyl groups, such as those found in tannic acid and PGG, are required for interaction with and stabilization of elastin. PGG, the core of tannic acid, possesses the same unique elastin-stabilizing capabilities of tannic acid and endows aortic tissue with similar mechanical properties. However, PGG is more chemically stable than tannic acid, thus making it less cytotoxic as well.³

- By binding to vascular elastin and protecting it from degeneration, PGG appears to inhibit abdominal aortic aneurysm formation and progression in an
experimental animal model, making it a promising therapeutic agent for aneurysm patients.

8.2 Recommendations

8.2.1 Elastin Stabilization in Bioprosthetic Heart Valves

The studies listed below are recommended for future work in implementing tannins as an elastin stabilizing tissue pretreatment for bioprosthetic heart valves. For these studies, we suggest using PGG rather than tannic acid as a bioprosthetic heart valve pretreatment due to its improved chemical structure and relative lack of toxicity.

1. Examine the effects of tannin treatment on porcine aortic cusp tissue. The majority of the research presented here for bioprosthetic heart valves was on tannin-treated porcine aortic wall. Aorta was used not only because it possesses an abundance of elastin (thus providing an elastin-rich model tissue), but also because it is an important component of stentless bioprosthetic heart valves. That being said, elastin is also present within cusp tissue, just to a lesser degree. Stabilizing elastin within these tissues may still be beneficial towards preserving mechanical properties and resisting tissue calcification. As such, tannin treatment applied to cusps should be investigated for stabilizing efficacy and effect on mechanical properties and in vivo calcification.

2. Further investigate the possibility of creating a tissue pretreatment for these devices with both tannins (PGG) and anti-calcific metal ions (aluminum or iron). Apparently, a small portion of tannic acid which bound to elastin is partially reversible. This same property was observed for PGG as well, although to a somewhat lesser extent. Using our long-term in vitro model, these metal ions (in combination with tannic acid)
appeared to alleviate this reversibility or instability. However, in the case of aluminum, this same preservation of elastin-tannin interactions was not observed for in vivo studies. Further studies on the long-term in vitro and in vivo efficacy of PGG and metal ions (particularly iron) may be beneficial. The relationship between the two could be valuable, as it is well documented that tannins and metals have a very high binding affinity for one another.8

3. Expose tannin-treated bioprosthetic heart valves to long-term cyclic fatigue testing. We have shown that by binding to elastin, tannin treatment alters the mechanical properties of tissue; however, we do not know what effect this will have in the cyclic operation of the valve. Fatigue testing, performed at accelerated rates in the range of 400 to 1200 cycles per minute, can provide insight into the resistance to tissue tears as well as the maintenance (or loss) of extracellular matrix components such as elastin and collagen9,10.

4. Ultimately, tannin-treated bioprosthetic heart valves will need to be implanted in more complex, functional animal models. These models involve actual valve replacement (often in the mitral valve position) in “higher-order” animals such as sheep, canines, calves, goats, and pigs.11 Such tests would provide pivotal insight into the in vivo efficacy and safety of elastin stabilization within a blood-contacting model.

**8.2.2 Elastin Stabilization in Abdominal Aortic Aneurysms**

The following studies are recommended for future work in implementing tannins as a treatment for abdominal aortic aneurysms:
1. Investigate the effect of PGG application on other aneurysm animal models. To date, the only model used to induce aneurysms for our studies has been calcium chloride-mediated injury of rat abdominal aorta. While we have provided evidence that PGG is indeed working by directly stabilizing elastin rather than by inhibiting pathogenic characteristics of the model (such as MMP activities, calcification, and inflammation), it would be beneficial to confirm that this technology is applicable and effective in a variety of models. In addition to perivascularly administering calcium chloride to rats, other well documented options include intraluminal elastase perfusion in rodents, systemic angiotensin-II delivery in mice, and genetically altered (blotchy, copper-deficient) mice. Perivascular delivery of elastase to rabbit aortas has been attempted and reported, but with conflicting results. Ideally, the technology would be extended to higher-order animals such as pigs or canines, however, information on such models is scarce. Luminal elastase perfusion has been attempted on pigs and canines, but did not appear to elicit true aneurysm formation in these cases. The development of such a model in large animals would be extremely helpful in advancing the idea of PGG-mediated elastin stabilization as a treatment for aneurysms.

2. Examine potential delivery methods for PGG applied to abdominal aortic aneurysms. The high doses needed for systemic delivery of PGG would likely not be tolerable (although this is yet to be determined), meaning optimization of a local delivery method for PGG is apparently necessary. The use of PGG as an aneurysm treatment could ultimately fall into one of two categories: (1) in conjunction with endovascular stent grafts, or (2) as a stand-alone treatment, including for aneurysms at the early and moderate stages for which there is no current option other than imaging surveillance.
In regards to endovascular stent grafts, PGG could potentially be bound to these luminal bypass devices and slowly released in the months or years following device implantation. By delivering PGG to the area (as depicted in Figure 8.1A), we may be able to limit any further elastolytic degradation in or around the aneurysmal sac. This would be particularly useful near the neck of the stent graft, potentially reducing distal migration and/or loss of graft fixation which are serious problems with these devices. The efficacy and feasibility of such a combinatorial device needs to be investigated.

As a stand-alone treatment, PGG could be delivered perivascularly through a slow release drug delivery system. Polymers such as polyhydroxybutyrate\textsuperscript{22} and polycaprolactone\textsuperscript{23} have been suggested and used as long-term (greater than one year) drug delivery systems. These are just two examples of the types of polymers that could be incorporated with PGG and developed into a “tube” device that wraps around the diseased aorta, similar to Figure 8.1B. PGG may be able to be delivered as a stand-alone treatment through the lumen as well, with the aid of a non-invasive catheter-driven device which would release a single dose of the elastin-stabilizing agent. Again, the feasibility and efficacy of such delivery devices would need to be evaluated.
Figure 8.1 Conceptual depiction of potential aneurysm products for endovascular (A) and perivascular (B) delivery of PGG to the diseased aorta.

3. Implement a time course study to evaluate in vivo binding of PGG within animal models. Using any of the animal models mentioned above, the amount of PGG which binds, and remains bound, needs to be documented long-term (at least 6 months). This would aid in determining if (or how often) multiple PGG treatments would need to be administered to properly hinder aneurysm progression, as well as potentially providing insight on the necessary concentration of PGG. The use of radiolabelled PGG may be beneficial for this purpose, as it would allow for monitoring of PGG content within the tissue over a given time period. Such studies could initially be done with current experimental methods on rats, but would ideally be implemented with an optimized delivery method for PGG, as described above.
8.3 References


