DISSERTATION

Tissue Engineering of Heart Valves:
Antigen Removal from Xenogeneic Tissue Scaffolds

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SURPERVISION BY SHIORI ARAI ENTITLED TISSUE ENGINEERING OF HEART VALVES: ANTIGEN REMOVAL FROM XENOGENEIC TISSUE SCAFFOLDS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

TISSUE ENGINEERING OF HEART VALVES:

ANTIGEN REMOVAL OF XENOGENEIC TISSUE SCAFFOLD

Tissue-engineered heart valves hold the promise of an ideal heart valve substitute by using appropriate and functional cells and scaffolds. An ideal heart valve should be durable, non-immunogenic, non-thrombogenic, resistant to infection and capable of regeneration and growth. Xenogeneic tissues are potential candidates for scaffolding of tissue-engineered heart valves. Anionic detergent-based decellularization has been employed to eliminate xenogeneic tissue immunogenicity. The present studies were performed to develop a technique to detect antigenic proteins in xenogeneic tissue scaffolds, to evaluate the efficacy of antigen removal of current detergent-based decellularization of xenogeneic tissues, to develop novel techniques to enhance antigen removal, and to address issues related to the cytotoxic effects of sodium dodecyl sulfate (SDS).

To develop a technique that identifies antigens in bovine pericardium (BP) or porcine aortic valve conduit (PAV), immune serum was obtained from rabbits that were injected untreated BP or PAV every two weeks. Immunoblot assay was performed on untreated BP or PAV with the rabbit immune serum. Immunoblot assay identified multiple bands and the banding density increased with 14 day through 70 day post-immune serum. This suggested a specific

acquired immunity in rabbits towards xenogeneic tissue with time. The immunoblot-based assay developed in this study was capable of surveying a broad range of potential soluble protein antigens in xenogeneic bioscaffolds.

Detergent-based decellularization was evaluated for its efficacy on removal of antigens from BP or PAV using the developed immunoblot assay. BP or PAV were treated with hypotonic solution, 0 to 0.5% SDS or sodium deoxycholate (SD) followed by aqueous washout. Higher concentrations of detergent improved antigen removal in BP with SDS or PAV with SD; however, neither SDS- nor SD-decellularization resulted in complete removal of antigens based on the developed immunoblot assay.

A novel method for enhancing antigen removal from xenogeneic bioscaffolds was developed. This method, termed solid-phase tissue electrophoresis (TE), was based on the concept of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. TE was performed at 0, 60 or 120V after tissue treatment with various concentrations of SDS. Both SDS concentration and TE treatment significantly enhanced antigen removal from PAV based on two-way ANOVA (p=0.001 or p=0.025 respectively). A significant SDS concentration-TE treatment interaction was not detected. Treatment with 1% SDS and 120V followed by aqueous washout resulted in apparent complete removal of antigens to levels below the detection limit of the immunoblot assay. .

To evaluate cytotoxicity of SDS, ovine vascular cells were cultured with various concentrations of SDS for 48 hours. SDS concentrations >10μM reduced the total cell number, while concentrations >100μM reduced the

percentage of live cells of ovine vascular cultured cells. SDS concentrations were measured in the washout solution of SDS-treated BP. SDS leached from SDS-treated BP at concentrations that are potentially cytotoxic. The extent of SDS leaching was dependent on the SDS concentration used for tissue treatment, and diminished over 96 hours.

In conclusion, significant issues were identified with current detergent-based decellularization methods for xenogeneic bioscaffolds with regard to the completeness of antigen removal and cytotoxic effects of detergents. TE may address one of these issues by significantly enhancing antigen removal from detergent-treated xenogeneic bioscaffolds.

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CHAPTER I

INTRODUCTION

Valvular heart disease is a pathological change on the heart valve apparatus due to congenital or acquired conditions. It interferes with the normal circulation and causes volume or pressure overload on either the left, right or both ventricles. Excess load on the heart can lead to heart failure which is a cause of high morbidity and mortality both in humans and animals. While medical treatment alleviates signs of heart failure, surgical correction is the only way to treat a damaged valve apparatus in order to prevent heart failure. Surgical correction can be performed by repair or replacement of the damaged valves, however, none of current options have not been a perfect solution. Thus some patients with valvular heart disease may have to experience multiple surgeries throughout their lives, which increase the mortality rate.

The field of tissue engineering developed in an effort to create a substitute for normal tissues. It seeks a means to create, repair, replace or regenerate organ function by using combinations of cells and scaffolds under an appropriate physiological environment either *in vitro* or *in vivo* (Figure 1.1). Tissue-engineered heart valves hold the promise to replace functional valves by achieving the following goals;

1. durability

2. non-immunogenicity
3. non-thrombogenicity
4. growth

None of the currently available heart valve prostheses accomplish these goals. A tissue-engineered heart valve is theoretically a viable implant that could achieve these goals.

Xenogeneic tissues are candidate scaffolds for tissue engineering of heart valves. They are readily available, can be implanted with less *in vitro* manipulation compared to synthetic materials, and have the potential to regenerate. A crucial issue when utilizing xenogeneic tissues is that tissue immunogenicity needs to be minimized before implantation. Xenogeneic tissues must undergo a process termed "decellularization" before they can be used as a biological scaffold for tissue engineering (Figure 1.1). The goals of the decellularization process are to remove antigens that would otherwise trigger an immune response, to preserve the biomechanical properties of the tissue, and avoid creating a cytotoxic environment that could inhibit or prevent recellularization. After decellularization, the tissue must undergo "recellularization" process in order to be viable.

The goal of achieving complete antigen removal has been hampered by lack of suitable methods for assessing the presence of antigens within xenogeneic tissues. Chapter IV describes the development of a novel immunodetection assay developed to identify the presence of antigenic proteins in xenogeneic tissues. Chapter V presents the results of studies that assess

current detergent-based decellularization methods for their ability to remove antigenic proteins from candidate xenogeneic tissues. Chapter VI presents the results of a novel method for antigen removal from xenogeneic tissues termed solid-phase tissue electrophoresis. Chapter VII addresses the issue of residual detergent leaching from SDS-treated xenogeneic tissue and its potential cytotoxic effect on subsequent recellularization.

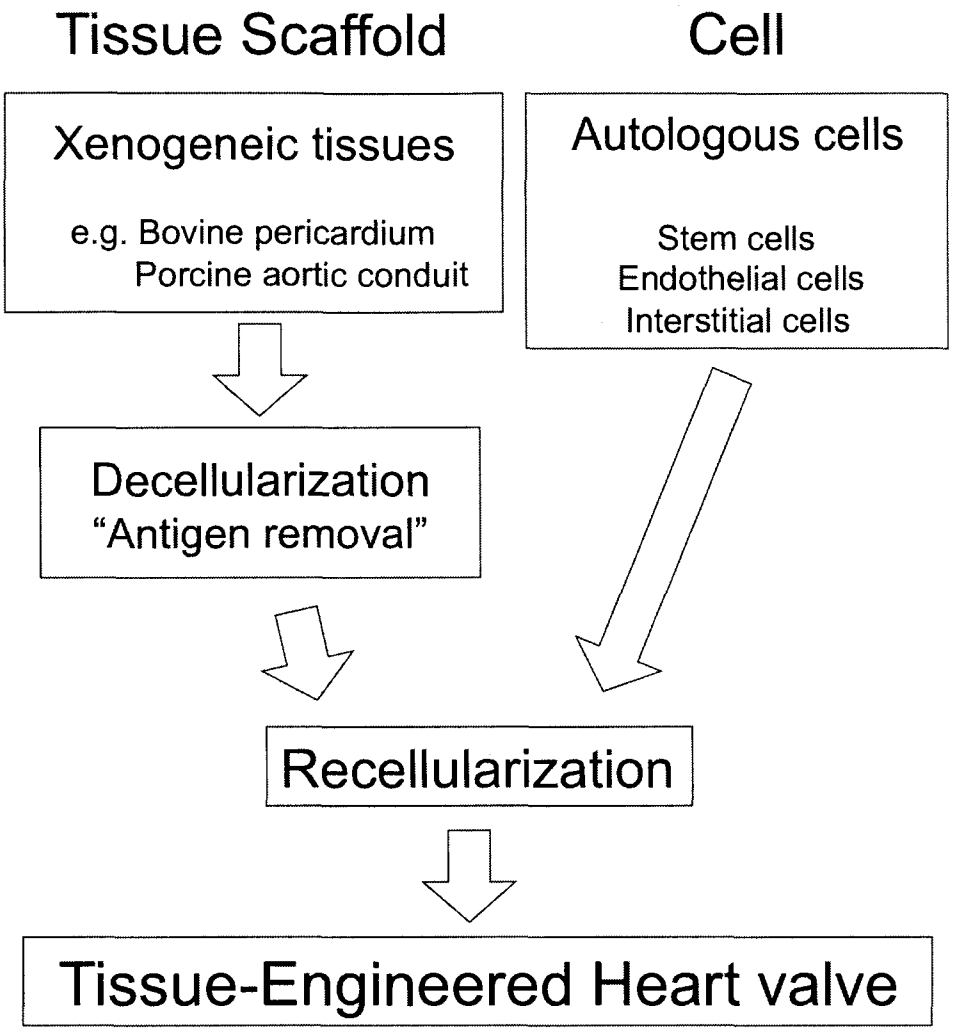

Figure 1.1 Concept of tissue-engineered heart valves. The major components of a tissue-engineered heart valve are a scaffold and cells. One possible scaffold is a xenogeneic tissue. A xenogeneic scaffold must undergo a process of "decellularization" to removal tissue antigens. The cellular component would likely come from autogenous stem cells that differentiate into both valve endothelial cells and valve interstitial cells. "Recellularization" of the scaffold could be undertaken *in vitro* prior to implantation or *in vivo* after implantation.

CHAPTER II

REVIEW OF THE LITERATURE

Significance of Valvular Heart Disease and Heart Valve Surgery

Valvular heart disease is one of the common heart diseases in humans. It can cause heart failure which carries a high mortality in population in the United States.[1] Currently 5 million people are in heart failure and 287,000 people die annually in the United States.[1] The 1.1 million hospitalizations for heart failure in 2004 amounted to nearly $29 billion in hospital charges.[2] Valvular heart disease is the etiology in 5.8% of heart failure and is the third most common concomitant disease.[3] In animals, valvular heart disease is also an important heart disease. Several reports described that valvular heart disease is the most commonly seen cardiovascular disease in dogs and it has accounted for approximately 40% in canine population.[4, 5] In one report, the incidence of valvular heart disease was reported to be 75% of the cardiovascular disease seen in dogs.[6]

Currently, there is no medical therapy to treat morphologically damaged heart valves and surgical treatment is often required.[7] Horstkotte *et al.* reported that the survival rate is very low in patients who have mitral regurgitation without surgical correction compared with patients who had surgery.[8] Thus, surgery is inevitable for patients with valvular heart disease. In human patients, valve repair surgery is recommended for patients with valve annular dilation, pliable valves, chordal rupture or perforation of the leaflet. Valve replacement surgery is

preferred in patients especially with aortic or pulmonic valve disease or patients who have morphologically severely deformed valve in any of four valves.[7] The biggest advantage of valve repair is that no prosthesis is required. Therefore, prosthesis-related complications such as hemorrhage, thromboembolic events, or prosthesis failure can be avoided.[9, 10] However, the outcome of the valve repair surgery is dependent on surgeon's skills and therefore is not consistent. In one report by Scherptong *et al.*,[11] valve repair surgery was associated with a high rate of recurrent regurgitation suggesting that valve repair surgery is an unpredictable palliative procedure. Al-Radi *et al.*[12] also reported that reoperation rate in patients with valve repair surgery was approximately 10% higher than that with valve replacement surgery.

Heart valve replacement surgery offers a predictable result and can be a curative treatment. Historically, valve replacement surgery was considered to have lower survival rate compared to valve repair surgery. However, recent studies reported similar survival rate between heart valve repair and replacement surgery.[11, 13, 14] Wang *et al.*[14] also showed in their study that the five-year freedom from the repeat mitral valve surgery was 100% in patients who had valve replacement; whereas patients who had repair surgery showed about 90% freedom from reoperation. This evidence supports the idea that valve replacement surgery is a feasible procedure.

Limitations of Current Prostheses

Currently available heart valve prostheses are imperfect. The ideal heart valve substitute has not yet been achieved. Several types of valve prostheses

have been developed and include mechanical valves, glutaraldehyde-fixed tissue valves, and allograft valve.

Mechanical Heart Valve: Mechanical valve consists of an artificial valve apparatus made by carbon or titanium and sewing ring cuff that surrounds the apparatus, which is made by polyester such as polytetrafluorethene (Teflon) or polyethylene terephthalate (Dacron). Because of its chemical characteristic, mechanical valves have excellent long-term durability. However, it is still a foreign material and it is highly thrombogenic.[10] To avoid thromboembolic events, lifetime anticoagulation therapy is required for mechanical prostheses. Patients with mechanical valves are at risk of complications associated with anticoagulation therapy. A study by Hammermeister *et al.*[15] demonstrated a high incidence of hemorrhage in patients with anticoagulation therapy after mechanical valve replacement and 19% of these patients with hemorrhage episodes resulted in death. Increased risks of abortion in pregnant patients who had anticoagulation therapy with mechanical valve prosthesis were also described in a study.[16] In veterinary patients, Orton *et al.*[17] reported that a long-term outcome after mechanical valve replacement was disappointing due to a difficulty of anticoagulation therapy in dogs. While mechanical heart valves could provide the best durability among currently available heart valve prostheses, complications associated with thrombosis and lifetime anticoagulation therapy make them less than ideal.

Glutaraldehyde-fixed Xenograft Heart Valve: Glutaraldehyde-fixed xenograft prostheses (i.e. bioprosthetic heart valves) consist of xenogeneic

tissues cross-linked with aldehydes, a polymer stent and sewing ring. Xenogeneic tissues such as porcine aortic valve and bovine pericardium are mainly used because of their availability and they can be implantable with minimum morphological manipulation *in vitro*. Since these xenogeneic tissues would otherwise elicit immune rejection,[18, 19] they are fixed with glutaraldehyde to mask the antigenic proteins and reduce the tissue immunogenicity.

Glutaraldehyde-fixed xenograft prostheses can overcome some of the drawbacks of mechanical prostheses. Chronic anticoagulation therapy is not necessary because they are less thrombogenic. Ninety days of anticoagulation therapy after valve replacement with glutaraldehyde-fixed xenograft prosthesis has been a standard recommendation based on Heras's study.[20] In their study, the thromboembolic incidence in patients who had a valve surgery without postoperative anticoagulation therapy was extremely high for the first ten days and became significantly lower 90 days after surgery.

A major drawback of glutaraldehyde-fixed xenograft prostheses is a lack of long-term durability. The average valve durability is reported to be between 10 and 15 years and the valves need to be replaced due to structural damage such as inflammatory pannus, thrombus formation, leaflet tear/degeneration, fibrosis, and calcification.[21-24] Although the structural damage found in glutaraldehyde-fixed xenograft heart valve could be multifactorial issues, a lack of cell repopulation due to toxicity of glutaraldehyde[25-27] that prevents the tissues from healing themselves[28] and prevents formation of a non-thrombogenic surface are thought to be key factors.[27, 29] Further, issues have been raised concerning the

host immune response to glutaraldehyde-fixed xenogeneic tissues.[30-33] Dahm *et al.*[32] demonstrated that glutaraldehyde-fixed bovine pericardium induced both cell and antibody mediated responses. In their study, T-lymphocytes were collected after implantation of glutaraldehyde-fixed tissues. These T-lymphocytes were incubated with extract of glutaraldehyde-fixed tissues. After 72 hours, increased DNA synthesis and proliferation of T-lymphocytes were observed suggesting that glutaraldehyde-fixed tissues stimulated T-lymphocyte activation. This study also demonstrated the higher binding affinity against glutaraldehyde-fixed tissues with immune serum. Several studies suggest that this immune reaction against glutaraldehyde-fixed tissues may strongly be associated with calcification of glutaraldehyde-fixed tissues, which leads the failure of bioprosthesis.[31] Human *et al.*[31] demonstrated a humoral immune response against glutaraldehyde-fixed xenogeneic tissues. Their study demonstrated that the calcification of glutaraldehyde-fixed tissues was severe when valves were treated with immune serum compared to pre-immune serum. One report also has shown that implantation of glutaraldehyde-fixed porcine aorta elicits an immune response and the degree of tissue calcification was correlated with the immune response.[31] This evidence suggests that glutaraldehyde treatment does not completely mask tissue antigenicity and contributes to prosthesis degeneration. Thus, currently available glutaraldehyde-fixed xenogeneic heart valve prostheses are not ideal.

Allograft Heart Valve: Cryopreserved allograft heart valves were first proposed as a viable heart valve substitute with the hope that they would improve in valve durability. Allograft heart valves have excellent hemodynamics,

are not rigid to conform well to the valve annulus of the recipients and have very low incidence of infection.[34-38] Freedom from endocarditis was 93, 91, 89 and 89% at 5, 10, 15, 20-year respectively.[39] Further, the risk of recurrent endocarditis was lower when allograft heart valve is used as compared to mechanical valve or glutaraldehyde-fixed heart valves.[35, 36, 40, 41] Allograft heart valves do not require routine anticoagulation therapy after surgery due to its low incidence of thromboembolic complications.[37, 42-44] Freedom from thromboembolism was nearly over 90% at 10 years after surgery.[38, 39] Da Costa *et al.*[42] reported 100% freedom at 5 years from thromboembolism in their patients. The freedom from life-long anticoagulation therapy, and more importantly from the complication of anticoagulation therapy is one of the desirable properties of allografts.

A major drawback of allograft heart valves is lack of availability. The average number of heart valves prepared for allograft is between 70 and 150 annually at each institution[42, 45, 46] while the number of patients who required valve replacement was 104,000 in 2006.[1, 47] Lack of durability is also an issue in use of allograft heart valves. Structural failure of allograft valve includes rupture of cusp and leaflet, commissural tear and calcification.[45, 48] Freedom from reoperation from structural failure decreases with time[44, 45, 49, 50] and was ranged between 79 and 92% at 10 years, 55 and 76% at 15 years, 35 and 50% at 20 years.[38, 39, 45, 48, 51] Thus, allograft heart valve has a high risk of failure requiring reoperation beyond 15 years. Thus, allografts have failed to achieve the hoped for improvement in durability.

The causes of degeneration of the allograft valve remain debatable. Possible causes include lack of viability and immune rejection. Questions remain about whether allograft heart valves are viable depending on the storage methods.[37] It remains undetermined whether cells are viable at the time of surgery and whether a lack of viability causes heart valve dysfunction. In general, fresh allograft valves harvested from heart transplant recipients or brain-dead multiorgan donors are considered viable at harvest. They can be stored at 4 °C but need to be used within few days to maintain their viability. Allograft valves can be cryopreserved within 2 to 3 days of donor death to maintain their viability unless used as fresh tissues.[52] Viability decreases to about 50% of normal tissues when cryopreserved,[53] valves stored at 4°C with antibiotics for later use can be implanted as allograft tissues, but it is now known that they do not maintain viability. Lund *et al.* described a freedom from failure for viable (fresh) versus non-viable (4°C stored) allograft valves at 10 and 15 years of 71 % and 71% versus 61% and 32%, respectively.[39] In addition, they reported that the overall survival at 15 years was 77% in patients receiving viable allograft whereas the survival of patients with non-viable allograft was 46%. Although the viability may be a crucial determinant of allograft tissue degeneration, it is difficult to obtain the fresh allograft tissues when patients are awaiting. Whether allograft heart valve is viable or not, allogeneic cells in the valve can be detrimental. Allograft heart valves are transplanted assuming that a lack of blood vessels in normal heart valves prevents them from rejection. However, studies have shown that allograft heart valve can elicit immune reaction both *in vitro* [54] and *in vivo*.[54-

[64] Fischlein *et al.*[63] demonstrated that patients with blood-type incompatible grafts had a more activation of lymphoblasts in systemic circulation as compared with blood-type compatible grafts and it was seen within three days of surgery. In Smith's study,[58] human lymphocyte antigens (HLA) specific antibody was also found in patients received allograft heart valves. It has been a debate whether this cellular and humoral response corresponds to valve degeneration, however, Pompilio *et al.*[65] demonstrated the correlation between the increase of antibody response and the structural deterioration in allograft tissues. In their study, panel-reactive antibody (PRA) was followed for 72 months after surgery to measure HLA specificities. Structural deterioration was observed when the peak PRA is more than 80% or when PRA is consistently greater than 70% for 72 months. Based on this study, immune rejection was considered to be strongly associated with allograft failure. Although the number of population in their study was low, the evidence shown in the study suggested that the current allograft is not a life-long solution due to lack of durability caused by immune reaction.

Tissue-engineered Heart Valve

Concept of tissue engineered heart valve: The field of tissue engineering has emerged as an effort to create living substitutes for normal tissues. It seeks a means to create, repair, replace or regenerate tissue and organ function by using combinations of cells and scaffolds under an appropriate physiological environment either *in vitro* or *in vivo* (Fig 1.1). Autologous cells such as endothelial cells[66-71] or interstitial cells[67, 72] could be candidates to reseed a heart valve scaffold. Endothelial cells have variety of functions associated with

mechanisms of immune reaction, inflammation and angeogenesis[73] and have been used in tissue engineering.[27, 72, 74-80] In addition, they prevent the initiation of coagulation cascade by presenting tissue factor pathway inhibitors, protein thrombomodulin and heparin sulphate proteoglycans on their surface.[81] They inhibit platelet activation[27, 77, 82, 83] and they regulate mechanical function of the valve leaflets.[84] Interstitial cells are a component of cell population of heart valves that play a role in valve remodeling (i.e. production of extracellular matrix), angiogenesis and cell proliferation.[85-89] They also excrete fibronectin in response to injury and actively participate to repair process.[90] Thus, it would be necessary to repopulate tissue-engineered heart valves with interstitial cells to provide functional heart valves. Several recent studies have demonstrated repopulation of interstitial cells onto various scaffolds *in vitro* and *in vivo,* and synthesis of extracellular matrix components such as glycosaminoglycan, collagen and elastin was confirmed.[27, 66, 67, 76, 91-97]

Tissue-engineered heart valves hold the promise of a functional heart valve replacement by using appropriate and functional cells and scaffolds. By precisely going through each step of the process, a tissue-engineered heart valve could be durable, non-immunogenic, non-thrombogenicity, resistant to infection and capable of regeneration. None of the currently available heart valve prostheses have these properties. In order to achieve this goal, a scaffold that provides the structural component for cells will be a crucial.

Current Approaches to Scaffold of Tissue Engineered Prosthesis:

Scaffolds used for tissue engineering of heart valves require sufficient structural

integrity to withstand functional stress. In addition, the scaffolds should provide cells with appropriate environment to repopulate and should degrade overtime to be replaced by host tissues. Two types of scaffolds for tissue engineering of heart valve have currently been employed; synthetic polymeric scaffolds and naturally-derived xenogeneic tissue scaffolds.

Synthetic Polymer Scaffolds: Polymeric scaffolds can be made by polyglycolic acid,[67, 98-103] polycarbonate urethane,[104] polylactic acid,[105-107] poly-L-lactic acid,[101, 105, 108, 109] polycaprolactone,[110-113] or polyhydroxyoctanoate.[114, 115] Synthetic polymeric scaffolds are readily available, cause minimum immune reactions and some studies using polymeric materials have demonstrated an excellent durability of polymeric valve scaffold. Sachweh and Daebritz studied a durability of polymeric valve prostheses[104]. In their *in vitro* studies, the prostheses tolerated more than 600 million cycles in fatigue test suggesting 16 to 26 years of normal function of human heart (average; 38 million cycles/year) and it is similar to the durability of mechanical heart valves. One of the hurdles that synthetic materials need to overcome is a capability to provide cells with sufficient environment to adhere, to synthesize extracellular matrix, and to repopulate. In Zund's study,[76] human fibroblasts were collected and seeded on polyglycolic acid mesh for three weeks. They described that the human fibroblasts adhered and proliferated on the scaffold and the tissue structure was similar to normal human tissues. Schmidt *et al.*[100] used polyglycolic acid mesh as a scaffold and incubated myofibroblasts obtained from human umbilical cords on the mesh in a pulsatile bioreactor for 28 days. On histological and

immunohistological examination, vimetin and smooth muscle α-actin were detected on repopulated cells in addition to the synthesis of collagen and glycosaminoglycan. Mol *et al.*[98] combined polyglycolic acid and cells harvested from human saphenous veins. They reported that after four weeks tissue formation and mechanical properties increased with time. Comparable studies have been performed in many studies.[67, 101, 107, 109] In spite of these positive results, limitations of the use of polymeric materials for tissue engineering have emerged. Engelmayr *et al.*[101] developed tissue-engineered heart valves using polyglycolic acid and poly-L-lactic acid scaffold with smooth muscle cells. Despite encouraging collagen and glycosaminoglycan production, formation of elastin was not detected on both histology and biochemical assay after three weeks of incubation *in vitro.* An *in vivo* study by Shinoka *et al.*[103, 116] showed problems of thickening on leaflets of their tissue-engineered heart valves which suggests a possible excess of extracellular matrix.[72] Polycarbonate urethane used in Sachweh's study had great durability *in vitro,* however, thickening of leaflet and restricted motion of leaflet caused regurgitation *in vivo* study.[104] Further, calcification was found close to the commissures on the tissue-engineered valves in few animals. A tear of leaflet and thrombus formation was also observed in this study. One animal who received this valve scaffold developed thrombus on the leaflet and was found to have myocardial infarctions at necropsy. Sodian *et al.*[117] reported some critical issues in the use of polymeric scaffolds. In their study, polyhydroxyoctanoate was co-cultured with cells harvested from carotid artery and jugular vein about two weeks. After 17 weeks

of implantation of the tissue-engineered heart valves in lambs, the valves were explanted and examined by light microscopy and environmental scanning electron microscopy. The surface on the scaffold was smooth equivalent to native tissues and no thrombus formation was seen. However, a confluent endothelial cell lining could not be observed either by recipient or reseeded cells even after 17 weeks. Moreover, contrary to their expectation, the scaffold did not degrade after 17 weeks.

Xenogeneic Tissues Scaffolds: Another approach to create a tissue-engineered scaffold is to use naturally derived scaffold. Xenogeneic tissues currently used to in glutaraldehyde-fixed bioprosthetic valves such as bovine pericardium and porcine aortic valves are potential candidates for scaffolding of a tissue-engineered heart valves. Such bioscaffolds are readily available, can be implantable with less *in vitro* manipulation compared to synthetic materials because of their similarity to natural heart valves, and have a potential to regenerate. The major issue that must be addressed before xenogeneic tissues can be used as a scaffold is the reduction or elimination of tissue immunogenicity before implantation.

The process of eliminating immunogenicity of unfixed xenogeneic scaffolds has been termed "decellularization". The concept of decellularization is based on the assumption the antigenicity resides predominately in the xenogeneic cells of the native tissues. Decellularization is a technique to remove cells in order to reduce tissue immunogenicity while maintaining the mechanical integrity of tissue scaffolds.[118] Decellularization protocols have incorporated

physical and chemical treatments, or combination of both. Physical treatments use hypo/hyper tonic solution,[119-121] snap freezing[122] or mechanical agitation.[74, 123-125] Treatment with hypotonic or hypertonic solutions to cause disruption of cell membranes by changing the osmotic pressure in cells. Rapid freezing of tissues is also applied for the same reason and to cause intracellular ice crystals that disrupt cell membranes. These methods are often used prior to chemical treatments to facilitate chemical exposure. Mechanical agitation is simultaneously used with hypotonic/hypertonic or chemical treatments to improve an efficacy of cell lysis and removal of cellular remnants. Chemical treatments included enzymatic and detergent treatments. Enzymatic treatment uses trypsin,[123, 126] DNAse and/or RNAse,[121, 127, 128] which assist the removal of cells and cell debris by cleaving the targeted bindings. Detergents used in decellularization include inonic,[121-123, 126, 129, 130] non-ionic[80, 121-123, 131, 132] and zwitterionic.[133] These detergents solublize cytoplasmic components and cell membranes, and disrupt lipid-lipid, lipid-protein or protein-protein interactions. Decellularization has a potential to remove cellular materials and to introduce a xenogeneic tissue scaffolds with minimum tissue immunogenicity.

Current Methods for Decellularization of Xenogeneic Bioscaffolds

Combinations of physical and chemical treatments are mainly used in decellularization of xenogeneic tissues based on efficacy of morphologic removal of cells from tissues. Among the various of combination protocols, treatments that utilizes detergents have generally been shown to give superior results over enzymatic treatments.[121, 123, 130] Anionic detergents are most often used, with

sodium dodecyl sulfate (SDS) and sodium deoxycholate (SD) showing the greatest efficacy for complete cell removal from xenogeneic tissues.[96, 121, 130, 133] In Booth's study, porcine aortic valves were incubated with hypotonic solution followed by 0.1% SDS treatment or 0.5% SD. No cell fragments were observed based on histological examination and there were no morphological changes on the extracellular matrix after the treatment.[133] Similarly, an absent of cells in xenogeneic tissues were observed in the treatment with hypertonic-0.5%SDS solution,[122] SDS added RNase and DNase,[123] SDS or SD following with hypotonic solution[121] or 1% SDS or 1% SD solution.[130] On the contrary, non-ionic (i.e. Triton-X 100)[121, 128] and zwitterionic (i.e. CHAPS) treatments have resulted in insufficient cell removal.[133] Grauss *et al.*[128] used 1% Triton X-100 to decellularize rat aortic valve and observed no change in cellularity in comparison to the normal group. Increasing the concentration of Triton X-100 did not show improvement in decreasing cellularity. 0.05% trypsin was further added to Triton-X100 and the combination treatment achieved complete decellularization. However this treatment caused severe damage on extracellular matrix showing a complete loss of elastin and glycosaminoglycan, disorganization of the collagen fibers, and fragility on the edge of the scaffold that disabled it for sewing. Booth *et al.* compared variety of reagents such as Triton-X100, SDS, CHAPS, Tween20, SD and MEGA10 for decellularization of porcine aortic valve.[133] They reported that all the reagents except SD and SDS found to be incapable of complete decellularization.

Immune Response to Current-Generation Decellularized Xenogeneic Bioscaffolds

Xenograft rejection includes hyper acute rejection (HAR), acute humoral xenograft rejection, acute cellular xenograft rejection and chronic rejection. Galactosyl α-1,3-galactose (αGal) is a carbohydrate residue and is responsible for HAR. αGal is expressed on endothelial cells of non-primate mammals and New World monkeys but is absent in humans, apes and Old World monkeys.[134] Once such xenoepitope is recognized by antigen presenting cells, major histocompatibility molecules class I and class II are presented on their surface to CD8+ and CD4+ T cells respectively. Activated CD4+ T cells (helper T cell) will then divide into Th (helper T cell) 1 cell and Th2 cell. Th1 cells activate macrophages which function as phagocytes and produce metalloproteinases which degrade extracellular matrix and recruit inflammatory cells. In addition, Th1 cells activate CD8+ cells. Th2 cells stimulate B cells to proliferate and promote production of immunoglobulins. The series of immune response further stimulate the production of inflammatory cytokines and eicosanoids and activate endothelial cells. Activation of endothelial cells triggers the complement cascade resulting in platelet activation, coagulation and disruption of vascular endothelial cells.[18, 19] Thus, vascularized xenografts undergo rapid graft destruction in HAR.

Reports of a rapid failure of the first generation of decellularized xenogeneic tissues have emerged. SynerGraft® heart valve was introduced as a tissue-engineered decellularized xenogeneic heart valve prosthesis.[135]

SynerGraft® heart valve was constructed by porcine aortic valve decellularized with hypotonic lysis, nuclease digestion and sequential washing process. SynerGraft® was implanted in four children and the implanted valves were functioning postoperatively. However, SynerGraft® valve was rapidly degenerated and caused graft rupture. One child died due to graft rupture on 7th day of surgery. Two children died with severe and sudden degeneration of the graft 6 weeks and a year after surgery. Fourth valve was explanted in two days after surgery. Gross morphology of the explanted valves exhibited severe inflammatory response characterized by dense fibrous tissues, rupture of the graft and perforation of the leaflet. Histological examination of these valves showed severe foreign body type reaction and incomplete acellularity of the grafts. Interestingly, Kasimir *et al.* reported later a presence of αGal epitope in SynerGraft® tissues.[83, 136]

Second generation of decellularized xenograft tissues treated with a detergent was introduced and demonstrated satisfactory midterm results.[137] However, they also have failed in a similar manner as seen in SynerGraft® heart valve failure.[138] *In vitro* study also revealed that decellularized xenogeneic tissues induce inflammatory response. Kasmir *et al.*[83] demonstrated that extraction of decellularized xenogeneic tissues attracted less human monocytes *in vitro* as compared to untreated tissue, however, there were more human monocytes than negative control. Rieder *et al.*[139] also assessed the effect of decellularization whether it reduces the degree of migration of lymphocyte, leukocyte, monocyte and granulocyte. Migration of monocyte and lymphocyte

were decreased whereas granulocyte migration was still higher toward the extract of decellularized xenogeneic tissues which corresponds to findings seen in early inflammatory reaction. Although decellularized tissues may elicit less immunogenicity in contrast to untreated tissues, these studies suggest that the problem of antigenicity of decellularized xenogeneic tissues has not been solved. Interestingly, αGal has been detected in detergent-treated xenogeneic tissues as well as SynerGraft® depending on a treatment used in a study.[121]

Recent studies suggested a presence of non-αGal proteins in xenogeneic tissues and reported that they are strongly associated with xenograft rejection including delayed xenograft rejection.[140-142] Griffiths *et al.*[143] study revealed thirty-one potential xenoantigens in bovine pericardium that reacted with immunized rabbit serum. Byrne *et al.*[144] also found fourteen cytoplasmic xenoantigens and six xenoantigens including extracellular matrix proteins in baboons who received porcine tissues. Although the clinical significance of potential xenogeneic antigens has not been determined, it is likely that there is more than tissue antigenicity goes beyond the αGal moiety. These antigens need to be measured and eliminated before unfixed xenogeneic bioscaffolds can be successfully implanted.

Need for Validation of Decellularization Techniques

The idea of decellularization comes from a study in 1950's.[145] The primary purpose of their study was to remove "immunologically reactive proteins" from xenogeneic vessels. Histological examination was performed to determine cellularity based on the assumption that the removal of cells would decrease the

amount of antigenic proteins from tissues resulting in reduction of the immunogenic response. Since then, the histological examination has been a gold standard to evaluate the efficacy of decellularization technique.[70, 72, 119, 122, 128, 130, 132, 133, 146-150] Hematoxylin and eosin staining is mainly used in histological examination to determine the cellularity in tissues. Hematoxylin stains the cell nuclei to purple color by binding to nucleotides through ionic bonds and Eosin stains cytoplasmic compartments and extracellular matrix to pink. On light microscopy, cell nuclei can be clearly and easily detectable. Thus this staining technique has been applied in many years as a standard technique to evaluate "acellularity" in decellularization technique. Transmission electron microscopy has also been used because of its ability to evaluate not only the cellularity but also the architecture of the extracellular matrix. Kim *et al.*[122] used transmission electron microscopy to demonstrate the acellularity and the intactness of the decellularized tissues. This technique further advanced studies of the efficacy of the decellularization and added an ability to evaluate adverse effects of decellularization on the extracellular matrix.[80, 124, 147] Scanning electron microscopy[80] or laser scanning microscopy[82, 123, 136] have also been used to evaluate the efficacy of decellularization by using a higher resolution than the light microscopy could produce. However all of these studies depend on evaluation of cellular morphology and do not directly assay for antigens in the tissues.

Recent studies have utilized direct measurement of presumed or potential antigens through immunohistochemistry or immunofluorescent methods to

evaluate the efficacy of decellularization techniques.[121, 151] Vimentin,[151-154] smooth muscle α-actin,[7, 72, 121, 153] CD31,[152] or von Willebrand factor[72] is used as a cell marker to detect interstitial cells or endothelial cells. Goncalves *et al.*[121] demonstrated the importance of protein-based techniques to evaluate the efficiency of decellularization methods. They compared the efficacy of decellularization technique by means of histology, immunohistochemistry and western blotting in decellularized bovine pericardium. In their study, they found residual proteins in histologically decellularized tissues. Kasimir *et al.*[83] also found a similar result that even acellularity was confirmed histologically, a specific protein can be detectable by using protein-based assay. Goncalves *et al.*[121] revealed different efficiencies of the treatments depending on the protein being assayed. They observed that αGal, but not major histocompatibility type I and smooth muscle α-actin, was removed by certain treatments in decellularized bovine pericardium. These studies have raised questions about the appropriateness of morphology-based methods in evaluating the efficiency of decellularization of xenogeneic bioscaffolds. The concept of "antigen removal" is replacing "decellularization" as the principal endpoint for achieving a non-immunogenic bioscaffolds. Biological assays that measure the efficiency of antigen removal from candidate xenogeneic bioscaffolds are needed.

Decellularization and Cytotoxicity

Decellularization protocols not only need to address removal of cells/antigenic proteins, but also need to address a potential cytotoxicity of reagents that might prevent recellularization of tissue-engineered heart valves.

In decellularization process, several reagents have been utilized either as single or in combination with other reagents. SDS has shown the great efficacy of removal of cells and specific antigens,[96, 121, 130, 133] however concerns about the cytotoxicity of SDS that could prevent repopulation of cells on a candidate scaffold have been raised. Goncalves *et al.*[121] described in their preliminary study that it was impossible to recellularize vascular cells on SDS-treated tissues and suggested a potential cytotoxic effect of SDS. A study by Rieder *et al.*[94] also described a potential cytotoxic effect of SDS. In their study, human vascular endothelial cells were implanted on SDS-treated tissues. No cell took place but a cell lysis was seen within 24 hours. On the other hand, Knight *et al.*[93] reported that mesenchymal progenitor cells had a capability to infiltrate SDS-treated heart valve leaflet in 24 hours. This apparent disagreement regarding of SDS cytotoxicity has yet to been clarified in further studies. Thus, questions remain about the potential cytotoxicity of decellularization treatments, especially those that employ ionic detergents. Future decellularization methods of xenogeneic bioscaffolds will not only need to accomplish efficient antigen removal, but will need to be compatible with subsequent recellularization of the bioscaffold.

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CHAPTER III

STATEMENT OF HYPOTHESES AND OBJECTIVES

A tissue-engineered heart valve has a potential to provide an ideal heart valve prosthesis. One possible approach to scaffolding a tissue engineered heart valve is by use of a "decellularized" xenogeneic tissue. The goals of the decellularization process are: complete removal of tissue antigens, preservation of biomechanical properties, and avoidance of cytotoxic effects. Questions have been raised regarding both the completeness of antigen removal and the cytotoxic effects of current detergent-based decellularization methods. Thus, the following hypotheses were formulated:

Hypothesis 1: Xenogeneic tissues injected into rabbits will induce an acquired immune response. Immune serum from injected rabbits can be used in an immunoblot assay to assess the completeness of antigen removal from xenogeneic scaffolds.

Hypothesis 2: Current detergent-based decellularization methods do not achieve complete antigen removal from candidate xenogeneic bioscaffold tissues

Hypothesis 3: Solid-phase tissue electrophoresis enhances detergent-based decellularization of xenogeneic bioscaffold tissues

Hypothesis 4: SDS leaches from SDS-treated xenogeneic tissues at levels that are cytotoxic to cells

In order to address the above hypotheses, the following study objectives were developed:

Objective 1: to develop an immunoblot assay that is capable of detecting a broad range of potential protein antigens in xenogeneic tissues

Objective 2: to evaluate the efficacy of anionic detergent-decellularization on antigen removal from xenogeneic tissue scaffolds

Objective 3: to demonstrate proof of the concept that solid-phase tissue electrophoresis can enhance antigen removal from xenogeneic tissue scaffolds

Objective 4: to determine if SDS leaches from SDS-treated xenogeneic tissues at levels that could inhibit recellularization of the tissue.

CHAPTER IV

DEVELOPMENT AND VALIDATION OF AN IMMUNOBLOT METHOD FOR DETECTING ANTIGENS IN CANDIDATE XENOGENEIC BIOSCAFFOLDS

INTRODUCTION

Tissue scaffolding is an important process in developing tissue-engineered heart valves. Tissue scaffold needs to be durable, non immunogenic, non thrombogenic, resistant to infection and capable to regenerate. Xenogeneic tissue is a potential candidate for tissue scaffolding because its similar disposition as natural heart valve and its wide availability allow simple manipulation *in vitro* and accessibility. However, concerns remain regarding tissue immunogenicity when xenogeneic tissues are used to produce tissue-engineered heart valve.[1, 2]

Decellularization is a technique to remove cellular components from tissues which are otherwise elicit severe immune rejection.[3] Efficacy of decellularization has been assessed by light microscopy,[4-16] transmission electron microscopy,[8, 14, 17, 18] laser scanning microscopy,[5, 17, 19] and immunohistochemistry toward specific antigens.[20, 21] However, none of these techniques allow evaluation of the presence of "antigenic proteins". Therefore, development of a sensitive and robust immunoblot-based assay is in need in order to detect soluble protein antigens in xenogeneic scaffold tissues.

A specific hypothesis addressed by this study is that xenogeneic tissue extract induces acquired immune response in rabbits. If this hypothesis is correct, specific antigenic proteins in xenograft tissue extracts can be detected by immunoblot assay with rabbit immune serum and the degree of detection will be increased with time as acquired immune response is stimulated.

MATERIAL AND METHODS

Rabbit Immune Serum

Untreated bovine pericardium (BP) or porcine aortic valve conduit (PAV) were minced and homogenized for 1 min on ice in a solution of 10 mM Tris HCl (pH 8.0), 100 KIU/ml aprotinin, 1 mM dithiotheritol, 2mM $MgCl_2$, 10mM KCl, 0.5mM Pefabloc in nanopure water. New Zealand White rabbits (2 rabbits for each tissue) were injected subcutaneously with tissue homogenate every 2 weeks for 10 weeks. Tissue homogenate was injected with Freund's adjuvant on the initial inoculation and incomplete Freund's adjuvant on subsequent inoculations. Serum was collected every two weeks up to 70 days and stored at -80 °C until used.

Protein Extraction

Proteins were extracted from untreated and treated BP and PAV for analysis. Equal wet weights of tissues were minced, placed in a solution of 0.1% SDS, 10 mM Tris HCl (pH 8.0), 100 KIU/ml aprotinin, 1 mM dithiotheritol, 2 mM $MgCl_2$ 10 mM KCl and 0.5 mM pefabloc. Tissue suspensions were shaken on ice for 1 hour and centrifuged at 17,000g for 20 min. The supernatant was used as water-soluble proteins. The pellet was re-suspended in a solution of 1.25%

SDS, 10 mM Tris HCl (pH 8.0), 100 KIU/ml aprotinin, 1 mM dithiotheritol, 2 mM $MgCl_2$ 10 mM KCl and 0.5mM pefabloc, shaken on ice for 1 hour, and centrifuged at 17,000g for 20 mins. The supernatant was used as lipid-soluble proteins. The supernatants were concentrated using Centricon (Millipore) for 1 hour at 6,500g for BP or Amicon (Millipore) for 15 mins at 7,500g for PAV and saved. Protein concentrations were determined by DC Protein Assay (Bio-Rad).

Immunoblot

Protein fractions were mixed with an equal volume of sample buffer (Invitrogen) and reduced in 5% (v/v) β-mercaptoethanol (Gibco) at 95°C for 3 minutes. Equal volumes of protein solution were loaded on 4-12% 1.5 mm Tris-glycine gels (Invitrogen). Gels were run for 1.5 hours at a constant 125 V at room temperature.

Proteins were transferred to 0.2 µm pore size nitrocellulose membranes (Invitrogen) at a constant 25V at room temperature for 1.5 hours. Membranes were washed for 5 minutes in Tris-buffered saline with 1% Tween20, blocked with 5% BSA for 1 hour at room temperature, and left at 4 °C overnight. Membranes were incubated with rabbit 70-day immune serum for 2 hours at room temperature on a shaker using a 1:100 titer for BP and 1:1000 titer for PAV. Membranes were washed with Tris-buffered saline with 1% Tween 20, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) for BP or swine anti-rabbit IgG (DAKO) for PAV. Membranes were washed again with Tris-buffered saline with 1% Tween 20. Signals were developed by Supersignal

West Pico/Femto (6:1) chemiluminescent substrate (Pierce) using Hyperfilm ECL (Amersham Biosciences).

Data Analysis

Replicate gels were run for each soluble protein extract. Optical density was measured on each film using Adobe Photoshop software (version 7.0) and shown as row number.

RESULTS

Pre-immune (day 0) rabbit serum did not produce immune banding on immunoblot analysis of soluble proteins extracted from untreated BP (Figure 4.1-2) or PAV (Figure 4.3-4). Multiple immune bands were produced by 14 day post-injection serum against BP and PAV. Banding pattern intensity increased with each succession of 14, 28, 42, 56, and 70 day post-injection antiserum against BP and PAV suggesting specific acquired immunity toward xenogeneic soluble proteins from both tissues.

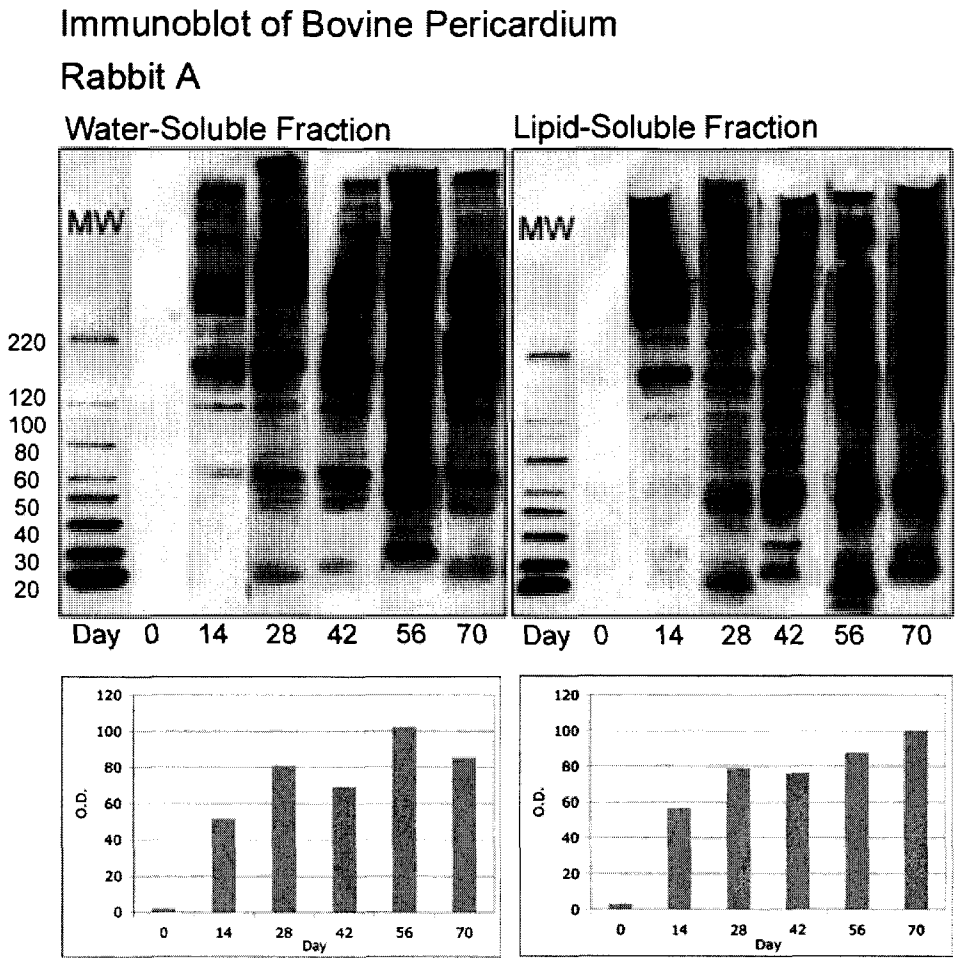

Figure 4.1 Immunoblot analysis of water- and lipid-soluble proteins extracted from untreated bovine pericardium (BP) using immune serum from rabbit A immunized with BP tissue homogenate. Blots were incubated with pre-immune (0 day), or 14, 28, 42, 52, or 70 day post-immune serum. Optical density (O.D.) of each immune banding was shown in graph. Molecular weight (MW) was shown as kD.

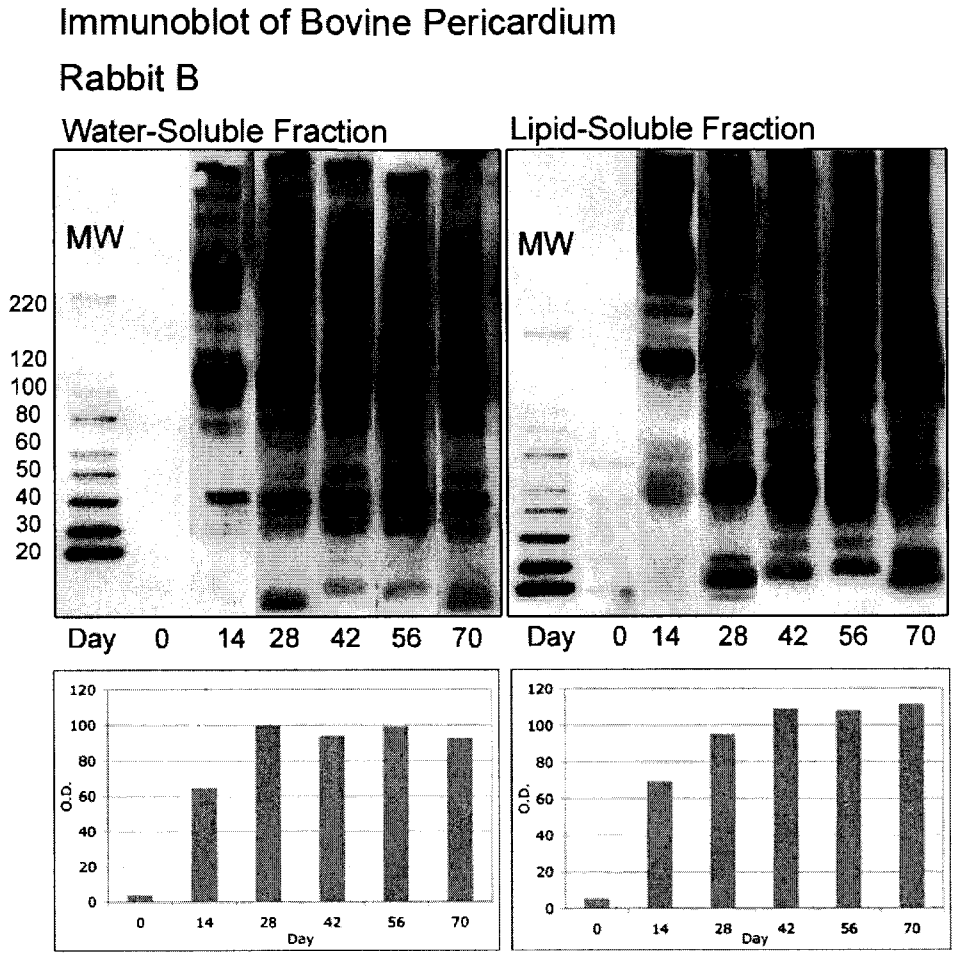

Figure 4.2 Immunoblot analysis of water- and lipid-soluble proteins extracted from untreated bovine pericardium (BP) using immune serum from rabbit B immunized with BP tissue homogenate. Blots were incubated with pre-immune (0 day), or 14, 28, 42, 52, or 70 day post-immune serum. Optical density (O.D.) of each immune banding was shown in graph. Molecular weight (MW) was shown as kD.

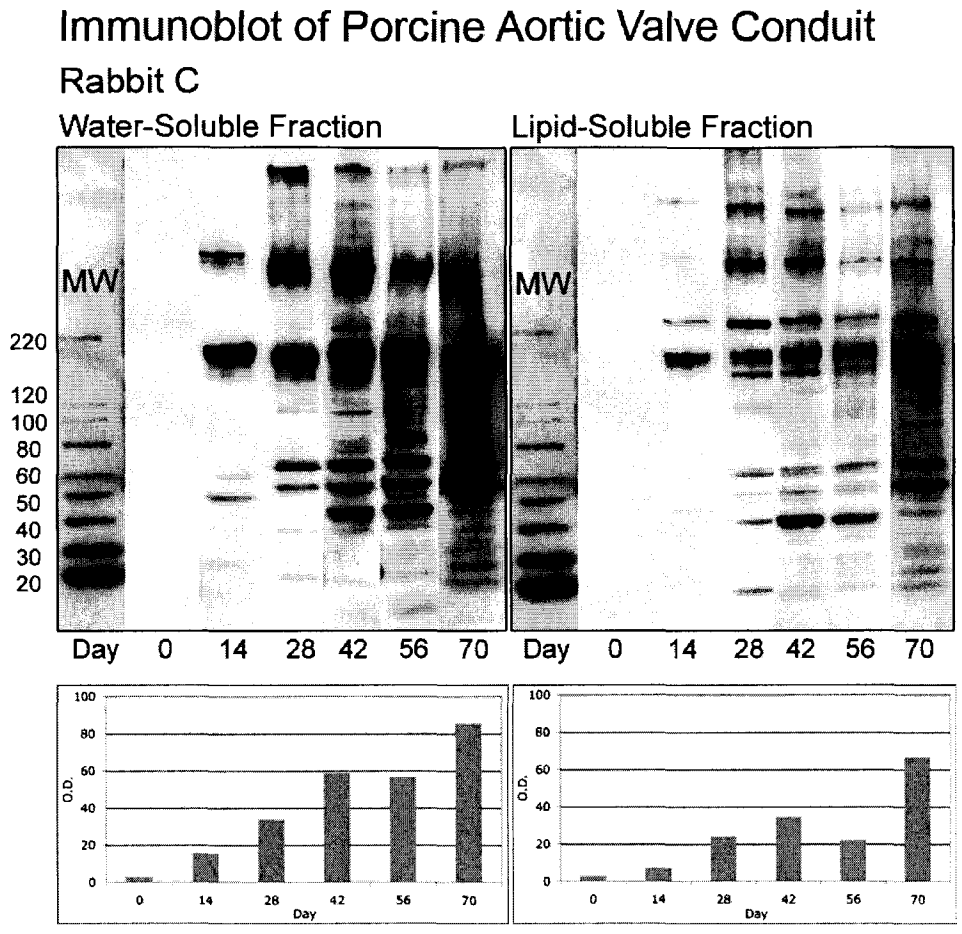

Figure 4.3 Immunoblot analysis of water- and lipid-soluble proteins extracted from untreated porcine aortic valve conduit(PAV) using immune serum from rabbit C immunized with PAV tissue homogenate. Blots were incubated with pre-immune (0 day), or 14, 28, 42, 52, or 70 day post-immune serum. Optical density (O.D.) of each immune banding was shown in graph. Molecular weight (MW) was shown as kD.

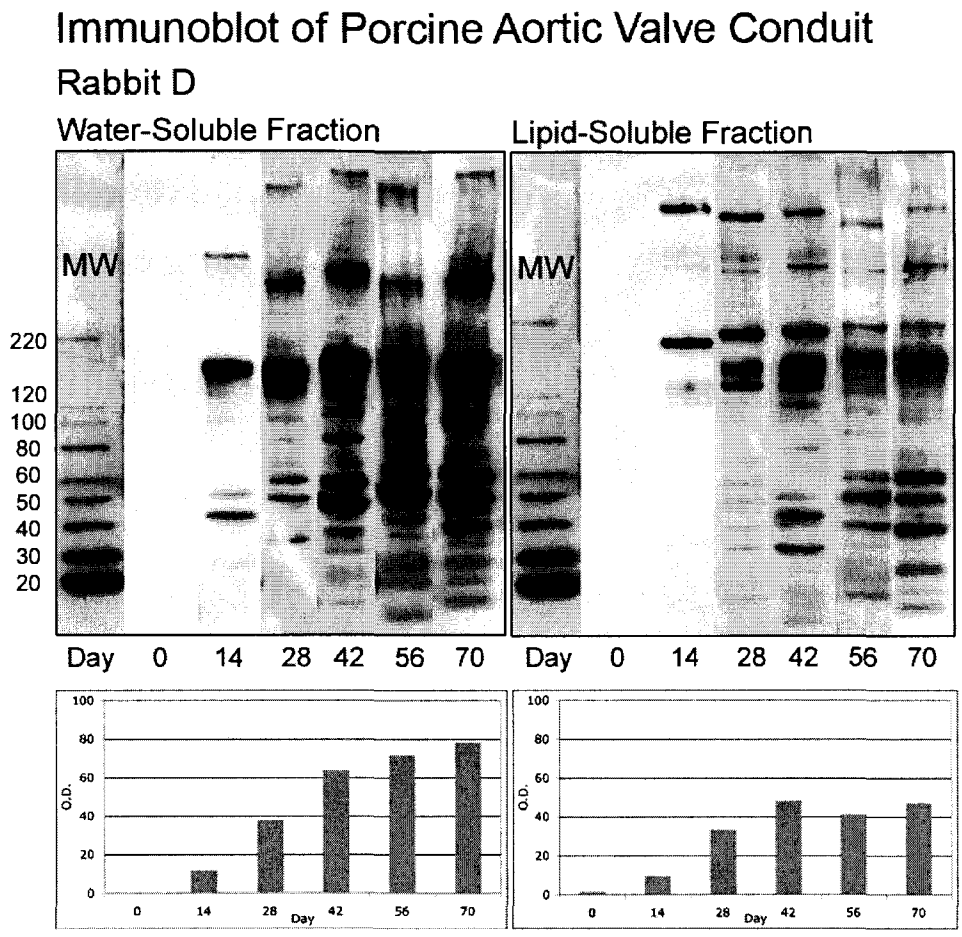

Figure 4.4 Immunoblot analysis of water- and lipid-soluble proteins extracted from untreated porcine aortic valve conduit (PAV) using immune serum from rabbit D immunized with PAV tissue homogenate. Blots were incubated with pre-immune (0 day), or 14, 28, 42, 52, or 70 day post-immune serum. Optical density (O.D.) of each immune banding was shown in graph. Molecular weight (MW) was shown as kD.

DISCUSSION

Advantages of a xenogeneic bioscaffold approach to tissue engineering of heart valve include its immediate implantability with minimum *in vitro* morphogenesis, appropriate mechanical properties, and the provision of a suitable environment for cell differentiation and orientation. A crucial issue when utilizing xenogeneic tissues is the elimination of tissue immunogenicity to avoid host reaction. Detergent-based "decellularization" of xenogeneic tissues is one approach to scaffolding a tissue engineered heart valve construct. Although histological examination is used to evaluate acellularity,[4, 6-11, 13-16, 22, 23] there is no universally accepted standard for determining the completeness of antigen removal from decellularized xenogeneic bioscaffolds. Several studies document that morphologic acellularity does not assure complete antigen removal from decellularized xenogeneic bioscaffolds. Galactosyl α-1,3-galactose epitope, known to mediate hyperacute and chronic immune rejection in vascularized xenografts, is not completely removed from decellularized xenogeneic bioscaffolds.[19, 20, 24] A recent report described that greater than 30 extractable proteins from bovine pericardium are capable of eliciting a xenogeneic immune response.[25] Byrne *et al.*[26] also reported 14 porcine antigens including cytoskeleton and extracellular matrix proteins after cardiac xenotransplantation in baboons. Thus results from an assay that targets only a few specific antigenic proteins should be carefully interpreted whether tissue immunogenicity is reduced or not. A report from Ueda *et al.*[18] demonstrated a complete removal of soluble-proteins after decellularization treatment in porcine heart valves.

Although this technique somewhat gives an idea whether there are residual soluble-proteins, it does not demonstrate whether these proteins are antigenic. From the standpoint of developing tissue-engineered heart valve, it is not necessary to remove proteins that do not elicit immune response and it is even important to leave such proteins in a scaffold that may be essential for functional scaffolds. Thus a development of an assay that detects broad range of antigenic proteins that elicit immune response is important.

This study reported a development of an immunoblot-based assay that is both sensitive and capable of surveying a broad range of potential soluble protein antigens in xenogeneic bioscaffolds. This approach could form the basis of a robust standard for assaying the completeness of antigen removal achieved by decellularization treatments of xenogeneic bioscaffolds. However, the immunoblot assay reported here only detects extractable soluble protein antigens and thus does not evaluate the potential role that insoluble matrix proteins might play in immune rejection of xenogeneic bioscaffolds.

In conclusion, rabbit anti-tissue immune serum immunoblot-based assay of extractable proteins is a sensitive and robust method that is applicable for evaluating the completeness of antigen removal from decellularized xenogeneic bioscaffolds.

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CHAPTER V

EFFICIENCY OF ANIONIC DETERGENTS IN REMOVNING ANTIGENS FROM CANDIDATE XENOGENEIC BIOSCAFFOLDS

INTRODUCTION

One approach to scaffolding a tissue-engineered heart valve is through the "decellularization" of xenogeneic tissues such as bovine pericardium and porcine aortic valves. Such an approach has the advantages of requiring minimal pre-implantation morphogenesis and biomechanical appropriateness, however concerns about the short- and long-term immunogenicity of decellularized xenogeneic bioscaffolds remain.[1-6] Treatment of xenogeneic bioscaffolds with anionic detergents such as sodium dodecyl sulfate (SDS) and sodium deoxycholate (SD) is currently favored over other decellularization methods.[7-12] None of currently available assay detects residual antigenic proteins after decellularization. Evaluation of the completeness of protein antigen removal from SDS- or SD-treated bovine pericardium (BP) or porcine aortic valve conduit (PAV) is necessary. A specific hypothesis addressed by this study is that antigenic proteins exist in histologically decellularized xenogeneic tissue scaffolds.

MATERIAL AND METHODS

Decellularization

BP and PAV were obtained postmortem from healthy animals, transported under aseptic conditions in phosphate buffered saline (PBS) with 10KIU/ml aprotinin, 0.1% EDTA, 1% antibiotic solution (100 U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin B), and stored at -80 °C until used.

Tissue treatments were performed at room temperature under gentile agitation. BP and PAV were subjected to 4 hours of hypotonic cell lysis treatment by immersion in deionized water with aprotinin 10KIU/ml, 6.5% (v/v) Tris-buffer (Sigma), 0.1% EDTA and 1% antibiotics. Tissues were then treated for 24 hours in PBS with or without sodium dodecyl sulfate (SDS) or sodium deoxycholate (SD) at concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5%. Lastly, tissues were washed in PBS with aprotinin 10KIU/ml, 0.1% EDTA and 1% antibiotics for 96 hours with complete change of the washing solution every 24 hours.

Protein Extraction

Proteins were extracted from untreated and treated BP and PAV for analysis. Equal wet weights of tissues were minced, placed in a solution of 0.1% SDS, 10 mM Tris HCl (pH 8.0), 100 KIU/ml aprotinin, 1 mM dithiotheritol, 2 mM $MgCl_2$ 10 mM KCl and 0.5 mM pefabloc. Tissue suspensions were shaken on ice for 1 hour and centrifuged at 17,000g for 20 min. Supernatant was used as water-soluble proteins. The pellet was re-suspended in a solution of 1.25% SDS, 10 mM Tris HCl (pH 8.0), 100 KIU/ml aprotinin, 1 mM dithiotheritol, 2 mM $MgCl_2$

10 mM KCl and 0.5mM pefabloc, shaken on ice for 1 hour, and centrifuged at 17,000g for 20 mins. The supernatant was used as lipid-soluble proteins. The supernatants were concentrated using Centricon (Millipore) for 1 hour at 6,500g for BP or Amicon (Millipore) for 15 mins at 7,500g for PAV and saved. Protein concentrations were determined by DC Protein Assay (Bio-Rad).

Histology

Treated tissues were fixed in buffered 10% formaldehyde solutions, dehydrated, and embedded in paraffin and sections were stained by Hematoxylin and Eosin stains to determine the cellularity.

Immunoblot

Protein fractions were mixed with an equal volume of sample buffer (Invitrogen) and reduced in 5% (v/v) β-mercaptoethanol (Gibco) at 95°C for 3 minutes. Equal volumes of protein solution were loaded on 4-12% 1.5 mm Tris-glycine gels (Invitrogen). Gels were run for 1.5 hours at a constant 125 V at room temperature.

Proteins were transferred to 0.2 µm pore size nitrocellulose membranes (Invitrogen) at a constant 25V at room temperature for 1.5 hours. Membranes were washed for 5 minutes in Tris-buffered saline with 1% Tween20, blocked with 5% BSA for 1 hour at room temperature, and left at 4 °C overnight. Membranes were incubated with rabbit 70-day immune serum for 2 hours at room temperature on a shaker using a 1:100 titer for BP and 1:1000 titer for PAV. Membranes were washed with Tris-buffered saline with 1% Tween 20, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) for BP or

swine anti-rabbit IgG (DAKO) for PAV. Membranes were washed again with Tris-buffered saline with 1% Tween 20. Signals were developed by Supersignal West Pico/Femto (6:1) chemiluminescent substrate (Pierce) using Hyperfilm ECL (Amersham Biosciences). Replicate gels were run for each soluble protein extract.

Data Analysis

Optical density was measured on each film using Adobe Photoshop software (version 7.0). Optical density results for treated tissues were expressed as a percent of the optical density of protein extracts from untreated control tissues. Results were analyzed by one-way ANOVA (JMP version 5.1). Values of $p<0.05$ were considered significant.

RESULTS

Histology

Hypotonic lysis and washout treatment alone did not eliminate detectable tissue cellularity on histological analysis of either BP or PAV. Distinct cellularity disappeared from BP treated with 0.025% or higher concentrations of SDS. Remnant hematoxylin staining (nuclear material) did not completely disappear from BP until the tissue was treated with 0.5% SDS (Figure 5.1). Distinct cellularity disappeared from PAV leaflets with 0.1% or higher concentration of SDS and aortic wall of PAV treated with 0.5% SDS (Figure 5.2-3). Remnant hematoxylin staining was still apparent in both valve leaflets and aortic wall of PAV treated with 0.5% SDS treatment (Figure 5.2-3). In SD group, histological

decellularization was found to be 0.5% in BP (Figure 5.4) whereas cells remained in both PAV leaflet and wall (Figure 5.5-6).

Immunoblot Analysis of Detergent-Treated Tissues

Hypotonic lysis and washout alone decreased, but did not eliminate, soluble protein antigen detection from BP or PAV. Treatment with SDS decreased detectable water- and lipid-soluble protein antigens from BP over 0.025% compared to hypotonic lysis alone ($p<0.05$) (Figure 5.7). There was no significant difference over 0.025%. Treatment with SDS did not decrease detectable water- and lipid-soluble protein antigens from PAV compared to hypotonic lysis alone (Figure 5.8). Treatment with SD enhanced removal of water-soluble protein antigens from PAV over 0.05% compared to hypotonic lysis alone ($p<0.05$) (Figure 5.9) but not water- and lipid-soluble protein antigens from BP and lipid-soluble protein antigens from PAV (Figure 5.10).

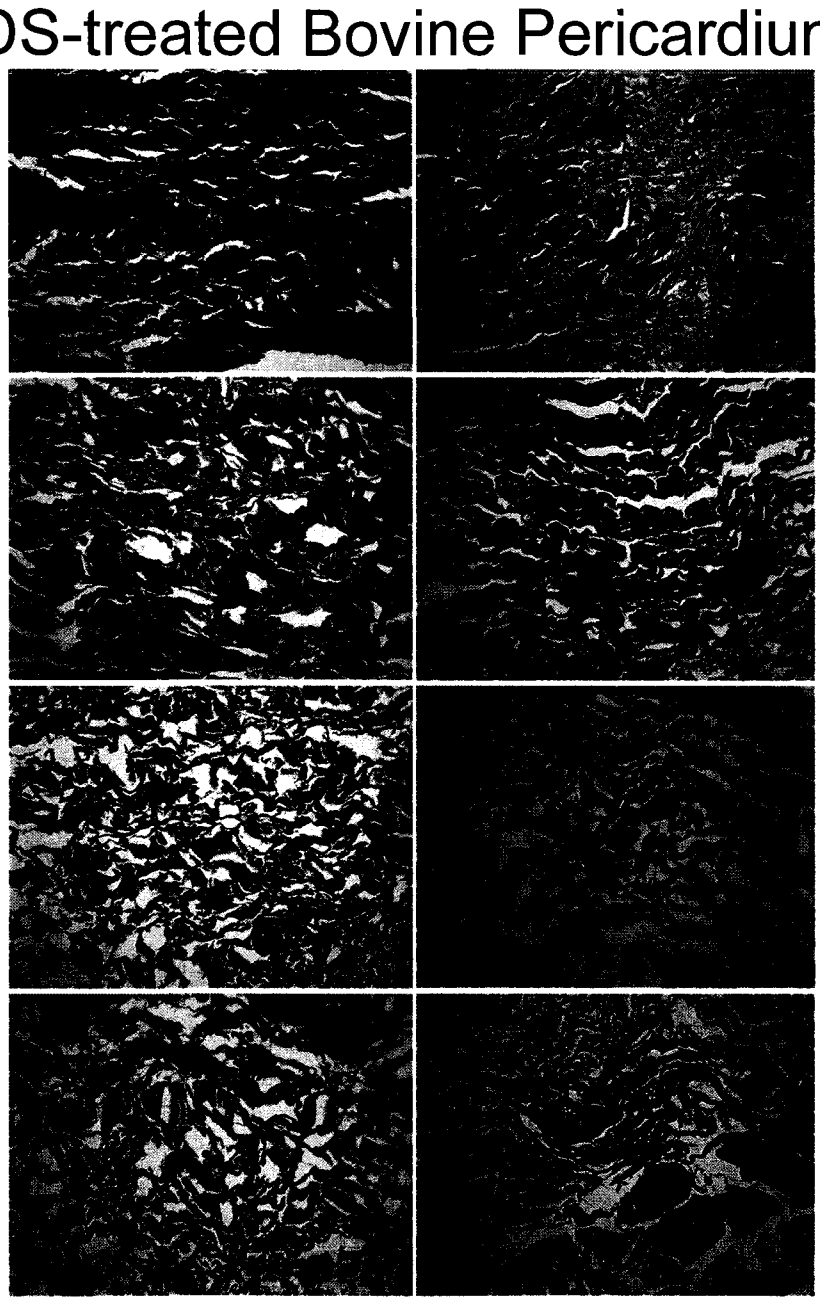

Figure 5.1 Histological examination on bovine pericardium with or without treatment. Treatment included hypotonic and sodium dodecyl sulfate (SDS) (0 to 0.5%) solution followed by aqueous washout treatment. Hematoxylin-eosin staining (X400).

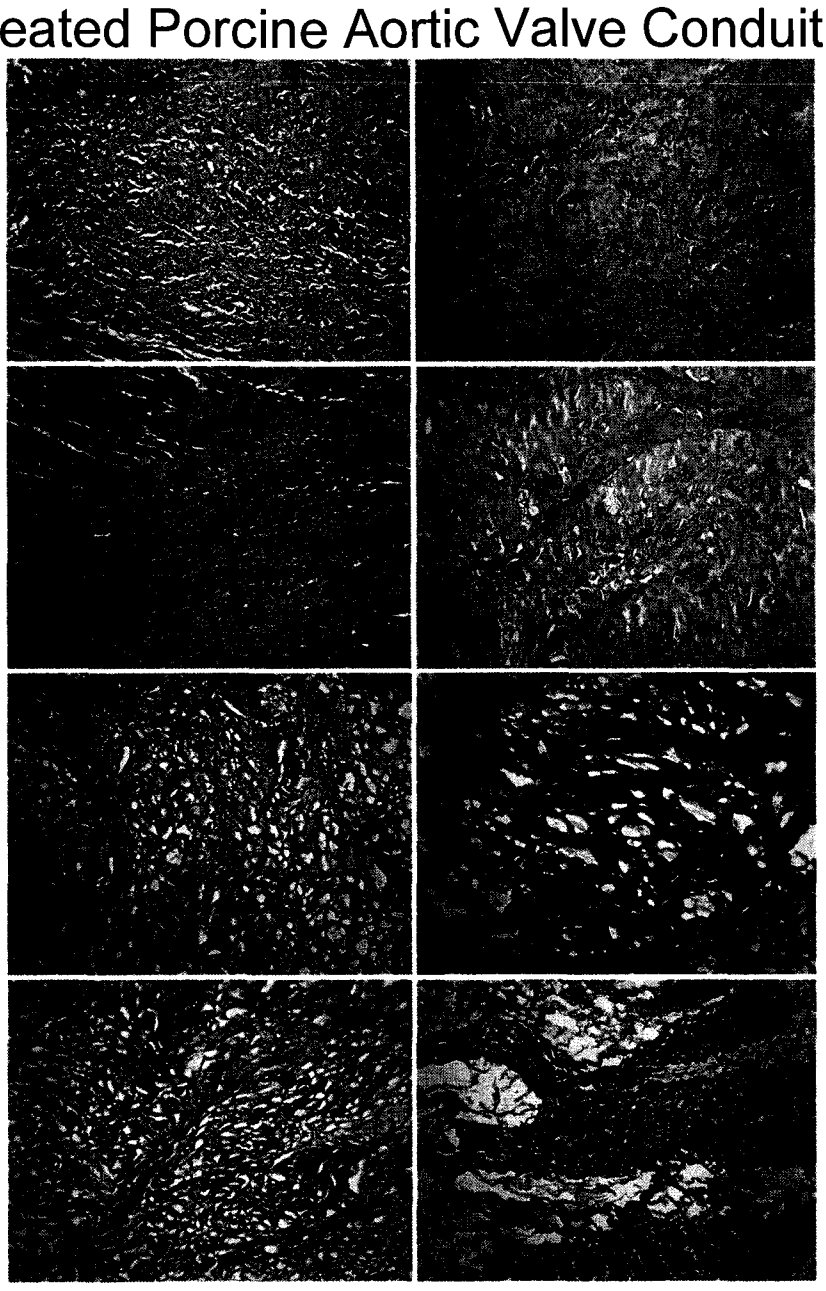

Figure 5.2 Histological examination on porcine aortic valve conduit wall with or without treatment. Treatment included hypotonic and sodium dodecyl sulfate (SDS) (0 to 0.5%) solution followed by aqueous washout treatment. Hematoxylin-eosin staining (X400).

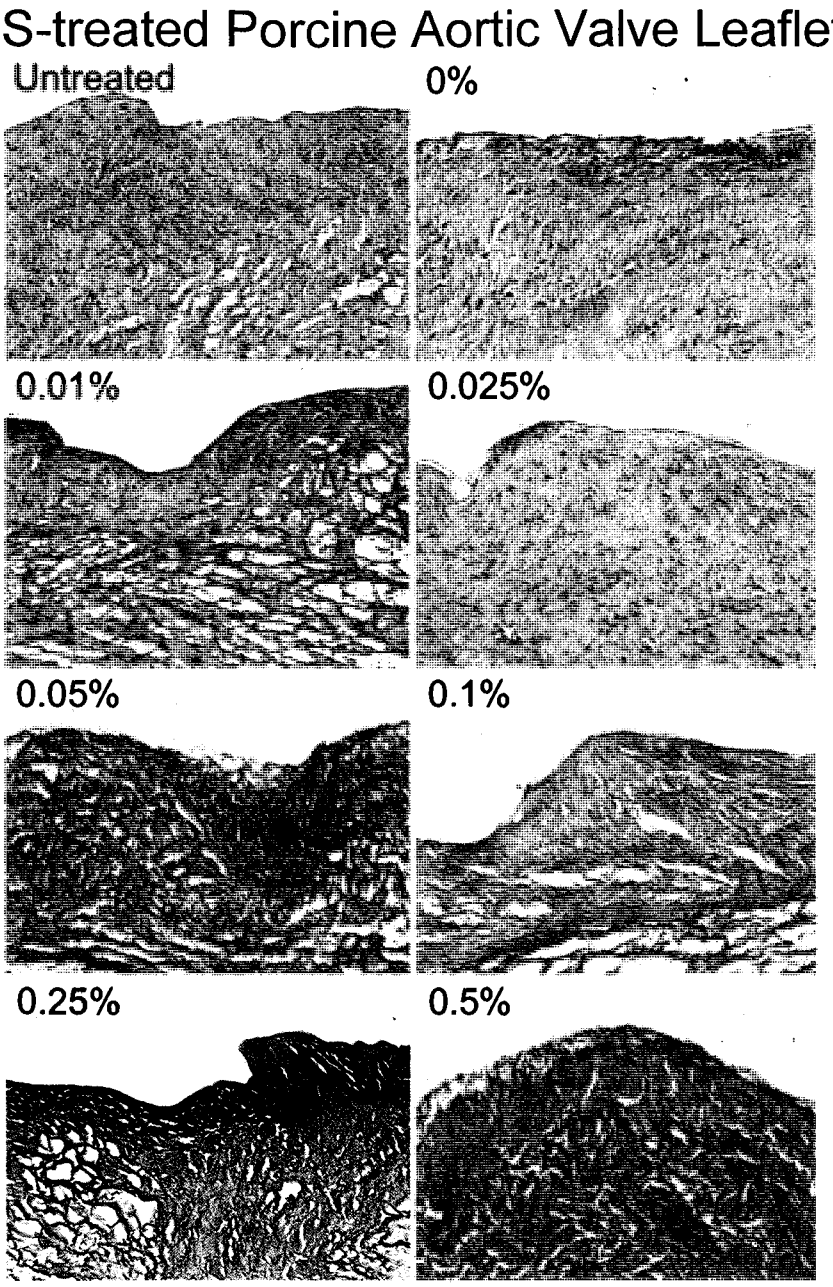

Figure 5.3 Histological examination on porcine aortic valve leaflet with or without treatment. Treatment included hypotonic and sodium dodecyl sulfate (SDS) (0 to 0.5%) solution followed by aqueous washout treatment. Hematoxylin-eosin staining (X400).

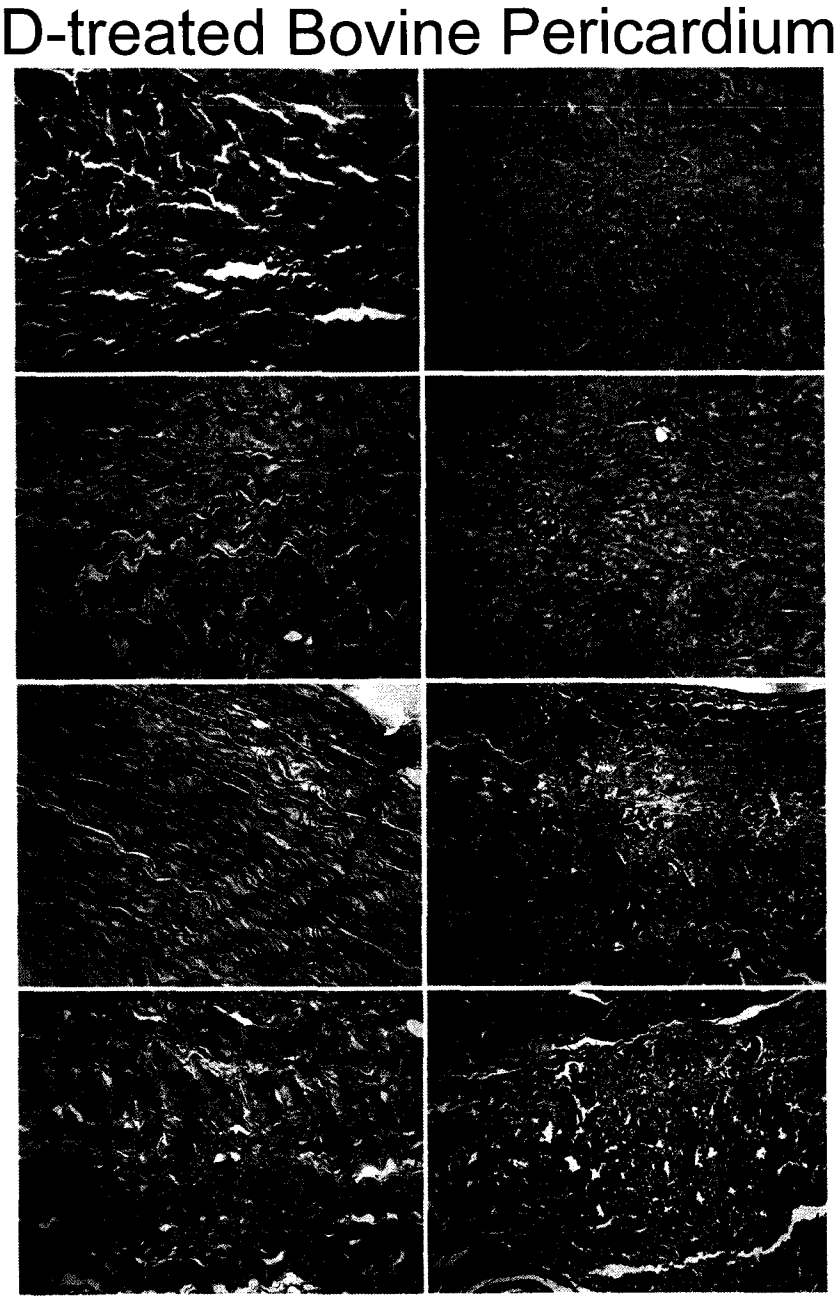

Figure 5.4 Histological examination on bovine pericardium with or without treatment. Treatment included hypotonic and sodium deoxycholate (SD) (0 to 0.5%) solution followed by aqueous washout treatment. Hematoxylin-eosin staining (X400).

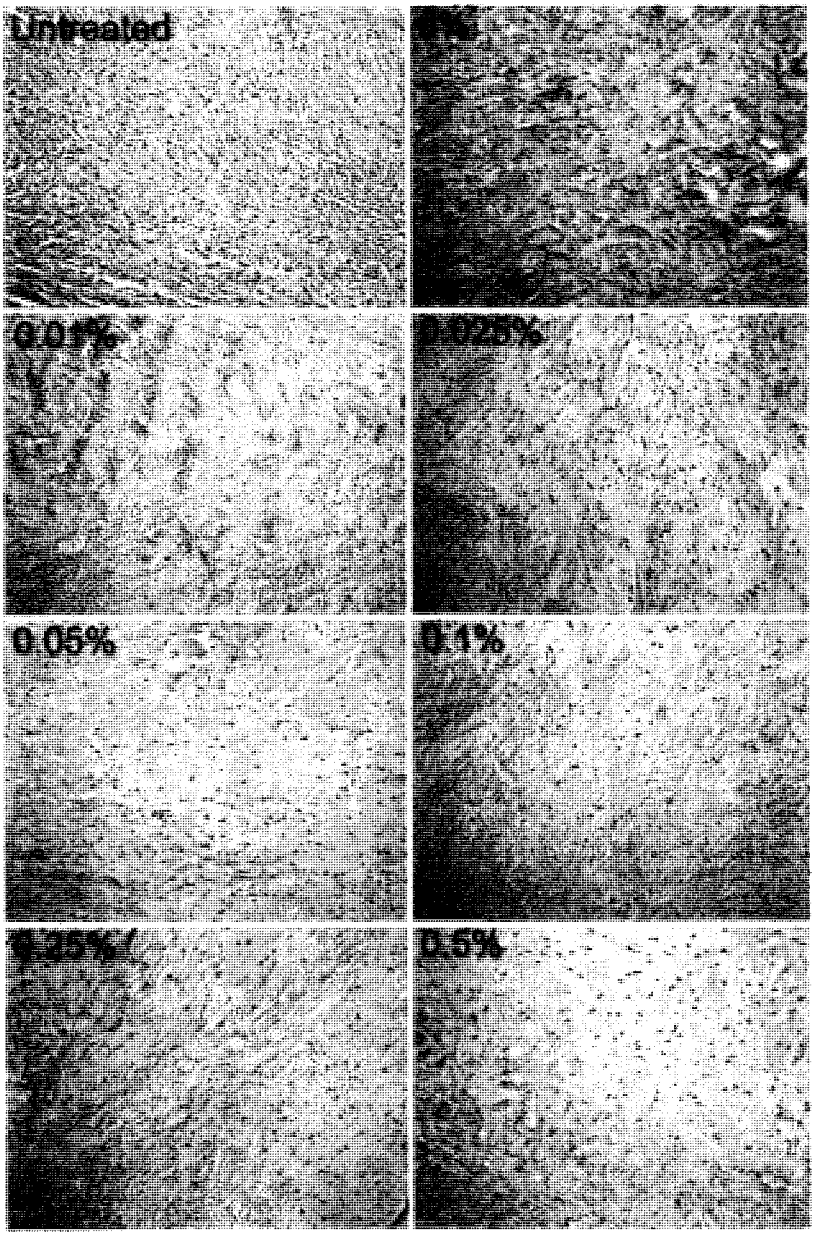

Figure 5.5 Histological examination on porcine aortic valve conduit wall with or without treatment. Treatment included hypotonic and sodium deoxycholate(SD) (0 to 0.5%) solution followed by aqueous washout treatment. Hematoxylin-eosin staining (X400).

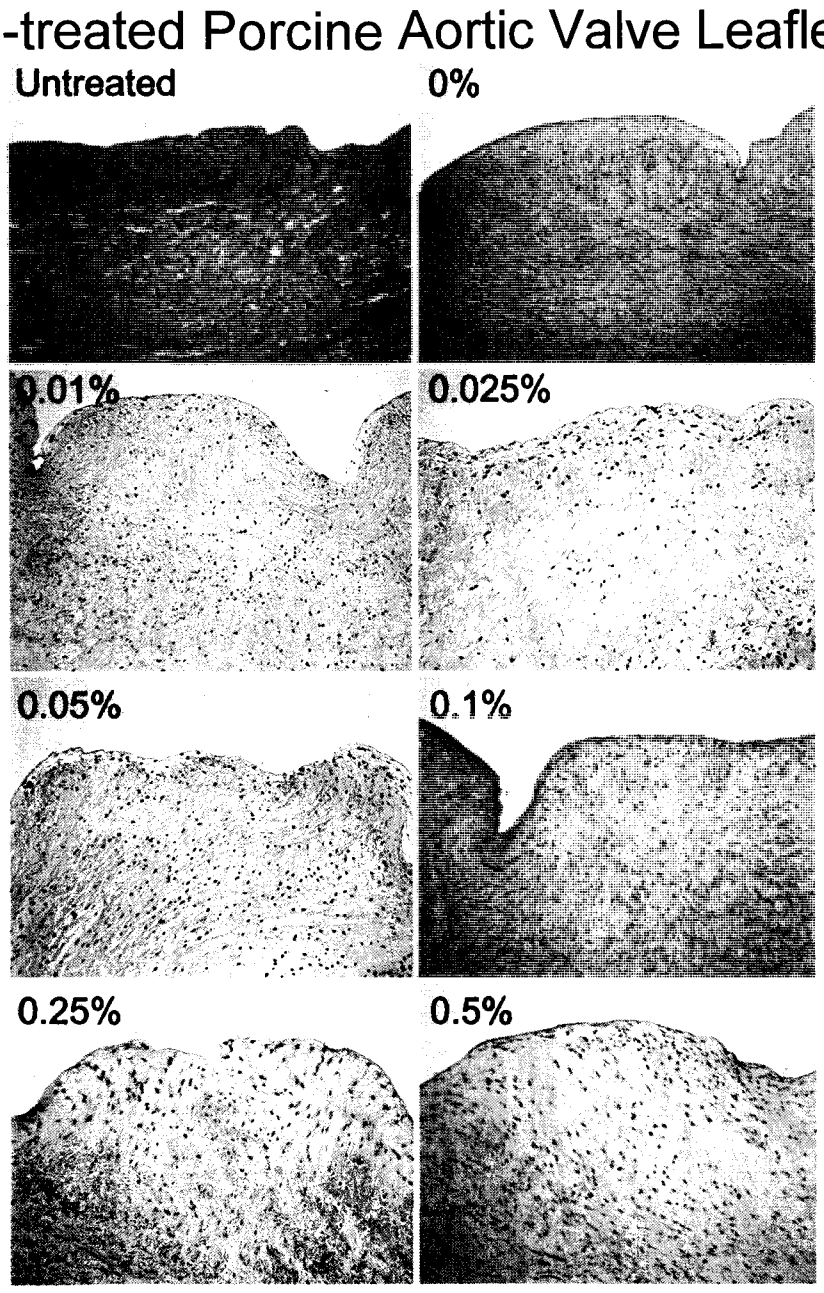

Figure 5.6 Histological examination on porcine aortic valve leaflet with or without treatment. Treatment included hypotonic and sodium deoxycholate (SD) (0 to 0.5%) solution followed by aqueous washout treatment. Hematoxylin-eosin staining (X400).

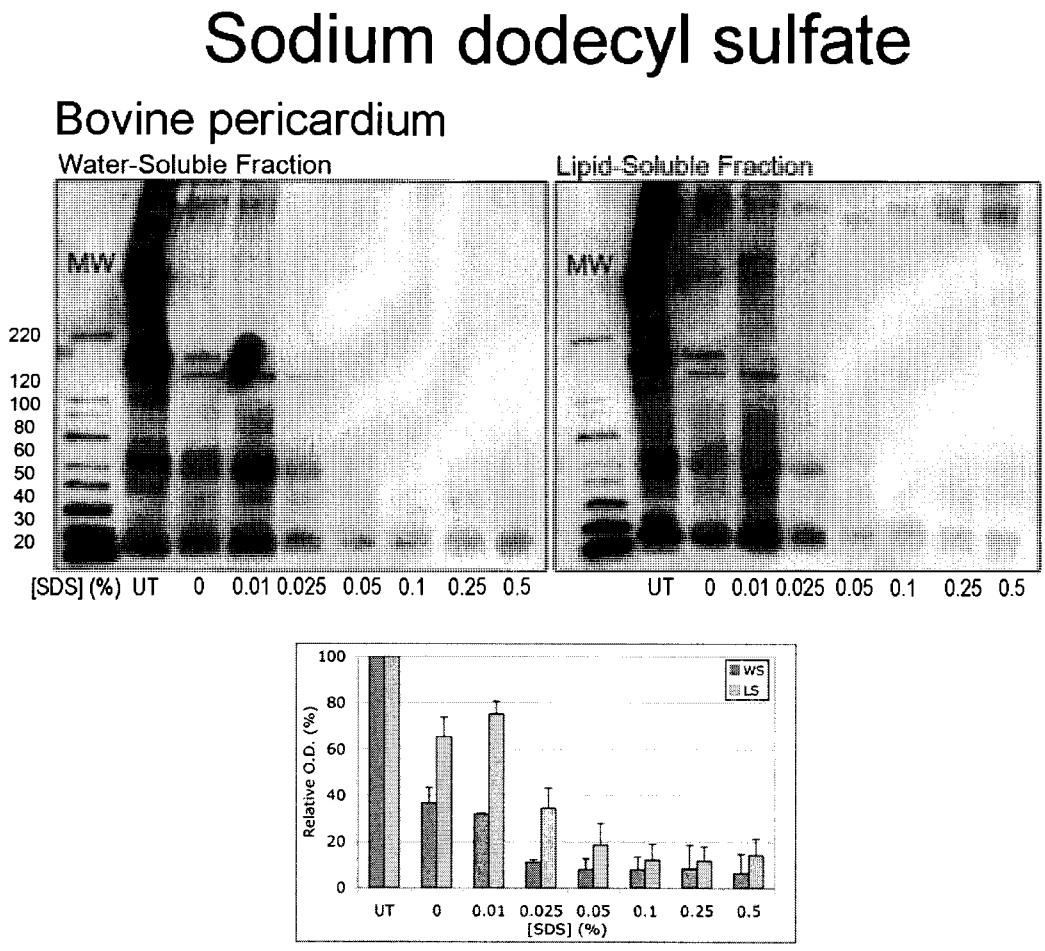

Figure 5.7 Immunoblot analysis of protein extracts (WS: water-soluble proteins, LS: lipid-soluble protein) from untreated and sodium dodecyl sulfate (SDS)-treated bovine pericardium using 70 day post-immune serum. Relative optical density (below) from immunoblot analysis expressed as a percent of optical density of untreated (UT) control. Values are mean ± S.D. Molecular weight (MW) was shown as kD. SDS treatment decreased detectable water- and lipid-soluble protein antigens from BP over 0.025% compared to hypotonic lysis alone ($p<0.05$). There was no significant difference over 0.025%.

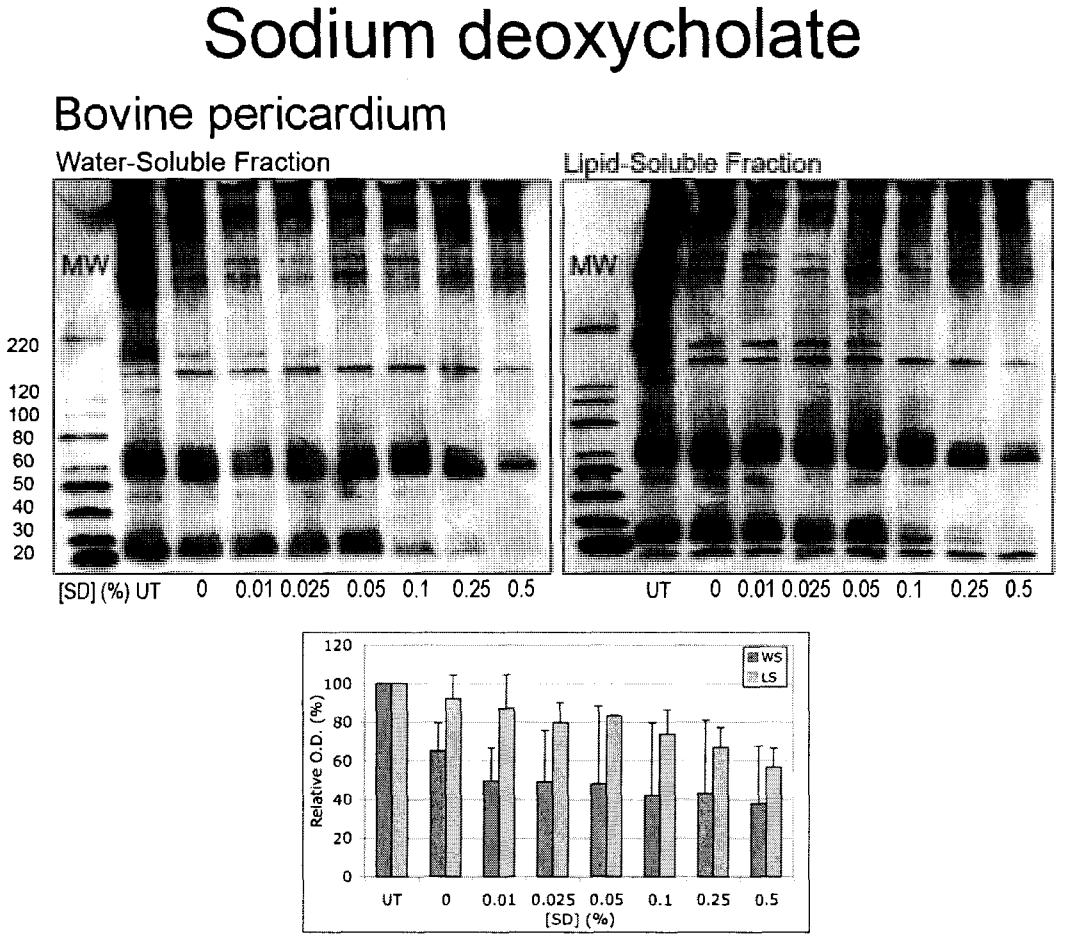

Figure 5.8 Immunoblot analysis of protein extracts (WS: water-soluble proteins, LS: lipid-soluble protein) from untreated and sodium deocycholate (SD)-treated bovine pericardium using 70 day post-immune serum. Relative optical density (below) from immunoblot analysis expressed as a percent of optical density of untreated (UT) control. Values are mean ± S.D. Molecular weight (MW) was shown as kD. There was no significant difference in treated tissues (p>0.05).

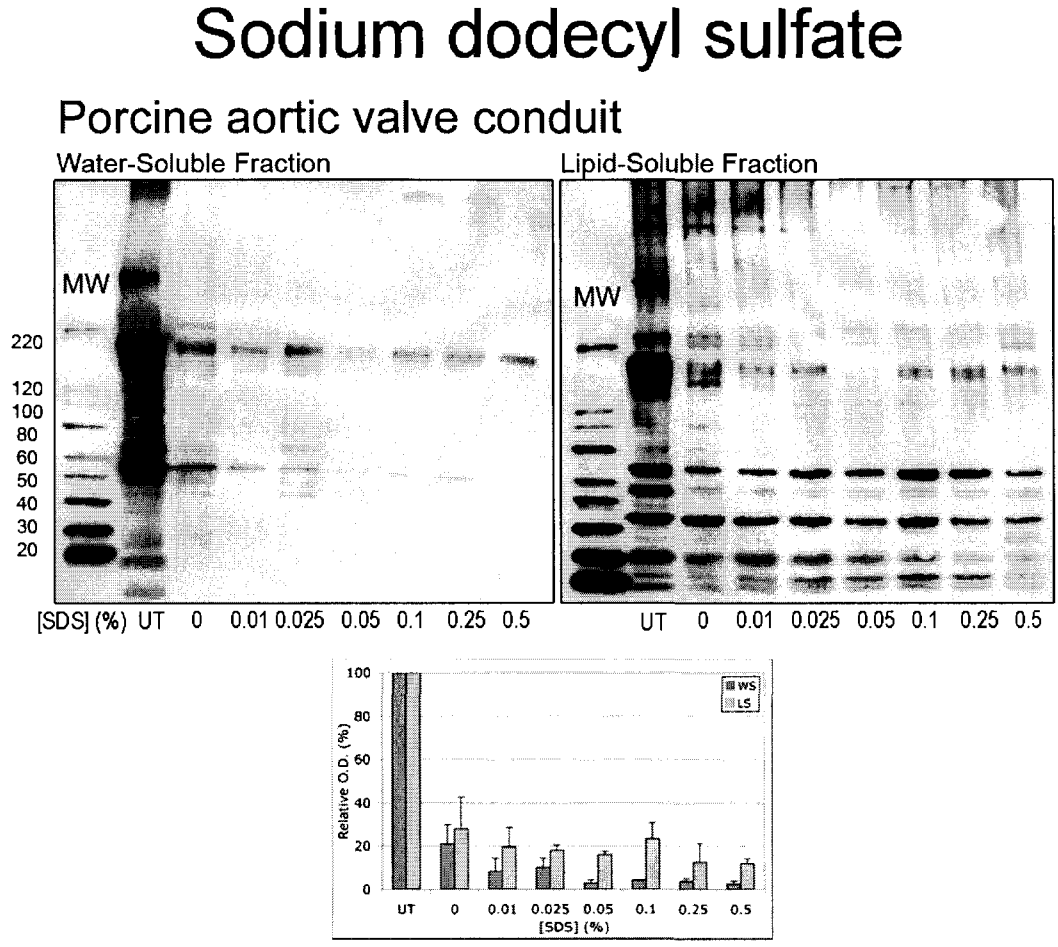

Figure 5.9 Immunoblot analysis of protein extracts (WS: water-soluble proteins, LS: lipid-soluble protein) from untreated and sodium dodecyl sulfate (SDS)-treated porcine aortic valve conduit using 70 day post-immune serum. Relative optical density (below) from immunoblot analysis expressed as a percent of optical density of untreated (UT) control. Values are mean ± S.D. Molecular weight (MW) was shown as kD. SDS treatment did not decrease detectable protein antigens compared to hypotonic lysis alone (p>0.05)

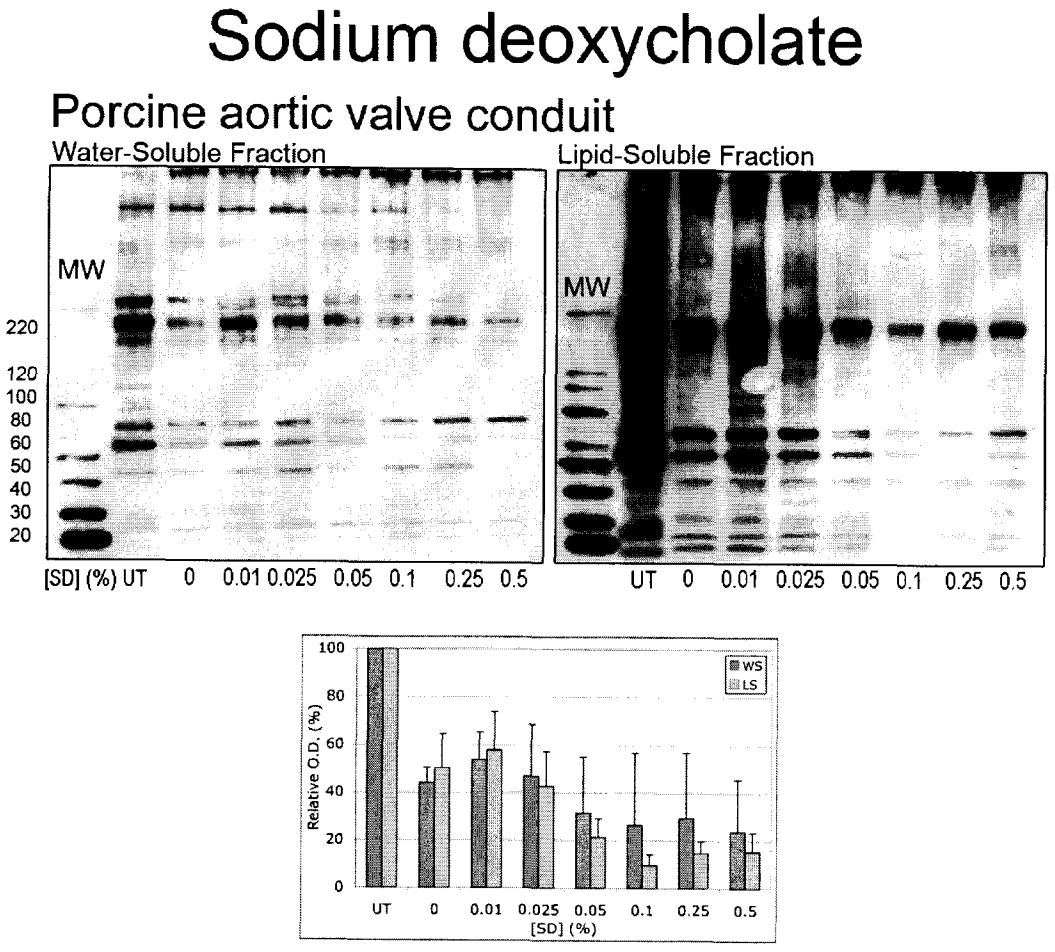

Figure 5.10 Immunoblot analysis of protein extracts (WS: water-soluble proteins, LS: lipid-soluble protein) from untreated and sodium deocycholate (SD)-treated porcine aortic valve conduit using 70 day post-immune serum. Relative optical density (below) from immunoblot analysis expressed as a percent of optical density of untreated (UT) control. Values are mean ± S.D. Molecular weight (MW) was shown as kD. There was a significant difference in water-soluble protein antigens over 0.05% compared to hypotonic lysis ($p<0.05$) whereas there was no significant effect on lipid-soluble protein antigens ($p>0.05$).

DISCUSSION

Advantages of a xenogeneic bioscaffold approach to tissue-engineering of heart valves include its availability, biomechanical and structural appropriateness, and suitability for implantation without a need for extensive *in vitro* morphogenesis. Biological scaffolds are more likely to provide an appropriate matrix environment to guide differentiation of seeded cells toward appropriate phenotypes than synthetic matrices. The principle barrier to the use of unfixed xenogeneic tissues as a scaffold for tissue engineering is the elimination of antigenicity to avoid host reaction. Further, antigen removal from xenogeneic bioscaffolds must be accomplished without substantially altering the biomechanical properties of the graft or creating a cytotoxic environment within the graft.

Several studies provide evidence that decellularized xenogeneic bioscaffolds elicit an immune response. *In vitro* contact of decellularized porcine heart valves with human plasma results in IgG deposition and classic complement pathway activation by polymorphonuclear leukocytes which are known to be involved in the early immune rejections.[3] Clinical reports show that first-generation decellularized xenogeneic bioscaffolds implanted in humans caused strong inflammatory responses leading to graft failure.[5, 6] Several studies now document that morphologic acellularity does not assure complete antigen removal from decellularized xenogeneic bioscaffolds. In the previous study, Galactosyl α-1,3-galactose epitope, known to mediate hyperacute and chronic

immune rejection in vascularized xenografts, is not completely removed from decellularized xenogeneic bioscaffolds.[4, 9, 13]

Although several methods for xenogeneic bioscaffold decellularization have been evaluated, currently favored methods generally involve combinations of hypotonic cell lysis, treatment with detergents, and passive aqueous wash out. Anionic detergents such as SDS and SD have resulted in apparent complete cell removal based on morphologic analysis,[8-12, 14] whereas non-ionic and zwitterionic detergents have failed to achieve morphologic decellularization even at higher concentrations.[8, 9, 11, 15] The results of this study show that soluble protein antigens are still detectable in hypotonic lysis, anionic detergent, and aqueous washout-treated xenogeneic bioscaffolds. It seems that the efficacy of SDS and SD is different between bovine pericardium and porcine aortic conduits. However, neither detergent achieved complete antigen removal under the conditions used in this study. It is possible that differences in pH, temperature, or buffer solutions used in other protocols might produce different results than were achieved here. Combination treatments have been advocated for bioscaffold decellularization,[1-4, 14, 16] but this was not evaluated in this study. Although the biological significance of residual antigens detectable by this assay is unknown, it can be said that any retention of protein antigens in decellularized xenogeneic bioscaffolds carries at least a potential for eliciting acute or chronic immune rejections. Further studies should be employed *in vivo* to demonstrate the immunological response against to decellularized xenogeneic tissues to avoid catastrophic immune response to decellularized bioscaffolds. Griffiths *et al.*[17]

identified greater than 30 BP proteins capable of eliciting a xenogeneic immune response in rabbits, which included soluble matrix proteins. Byrne *et al.*[18] also reported 14 porcine antigens including cytoskeleton and extracellular matrix proteins after cardiac xenotransplantation in baboons. The immunoblot assay reported here detects extractable soluble protein antigens and thus does not evaluate the potential role that insoluble matrix proteins might play in immune rejection of xenogeneic bioscaffolds.

In conclusion, hypotonic lysis, SDS or SD detergent treatment, and aqueous washout decellularization of BP and PAV did not completely remove detectable protein antigens under the conditions of this study. Further study is needed to enhance current decellularization protocols to achieve more complete antigen removal from xenogeneic bioscaffold tissues.

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CHAPTER VI

SOLID-PHASE TISSUE ELECTROPHORESIS FOR ANTIGEN REMOVAL FROM XENOGENEIC BIOSCAFFOLDS: PROOF OF CONCEPT

INTRODUCTION

Tissue engineering of heart valve tissues propose a living replacement with characteristics including durability, non-thrombogenicity, resistance to infection and regeneration. One approach to scaffolding a tissue-engineered heart valve is through the "decellularization" of xenogeneic tissues such as porcine aortic valve. Goals of bioscaffold "decellularization" are removal of immunogenicity, maintenance of biomechanical integrity, and compatibility with recellularization. The advantages of a xenogeneic bioscaffold approach include availability and structural and biomechanical similarity allowing minimum morphogenesis *in vitro*. However, concerns about the short- and long-term immunogenicity of decellularized xenogeneic bioscaffolds remain.[1-4] Clinical implantation of first-generation unfixed decellularized xenogeneic tissues into humans have failed because of severe immune rejection of the bioscaffold.[4]

Protocols for decellularization of xenogeneic bioscaffolds consisting of combinations of hypotonic lysis, treatment with anionic detergents such as sodium dodecyl sulfate (SDS) and/or sodium deoxycholate, and passive aqueous washout are currently favored decellularization methods.[5-10] Efficacy of

decellularization has been assessed by light microscopy,[5-7, 9, 11-19] transmission electron microscopy,[9, 15, 20, 21] laser scanning microscopy,[1, 18, 21] and immunoassay of specific known antigens.[10, 22] Questions about whether morphologic assessments of tissue cellularity assure complete removal of antigens from decellularized xenogeneic bioscaffolds have been raised.[1, 10, 23] As a result, the field is moving away from the concept of "decellularization" toward a concept of "antigen removal". An immunoblot-based assay for detecting soluble protein antigens in candidate xenogeneic bioscaffolds was developed in a previous study and it found that hypotonic lysis, ionic detergent, and aqueous washout do not completely remove detectable soluble antigens from candidate bioscaffold tissues.

To improve the efficiency of antigen removal from xenogeneic bioscaffolds, a novel tissue treatment method was developed. It consists hypotonic cellular lysis, treatment with the anionic detergent SDS, embedment of the tissue in a solid-phase gel, and application of electrical current to the tissue-gel complex. This treatment method is based on principles similar to diagnostic immunobloting whereby SDS binds strongly and imparts a uniform negative charge to proteins, which in turn causes them to migrate in an electrical field.

The aim of the present study is to evaluate the efficiency of solid-phase tissue electrophoresis on soluble protein antigen removal from porcine aortic valve conduit.

MATERIAL AND METHODS

Tissue Treatment

Bovine pericardium (BP) and porcine aortic valve conduits (PAV) were obtained postmortem from healthy animals, transported under aseptic conditions in phosphate buffered saline (PBS) with 10KIU/ml aprotinin, 0.1% EDTA, 1% antibiotic solution (100 U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin B), and stored at -80 °C until used. Tissues were sectioned prior to treatment. Tissue treatments were performed at room temperature under gentile agitation. Tissues were subjected to 4 h of hypotonic cell lysis treatment by immersion in deionized water with aprotinin 10KIU/ml, 6.5% (v/v) Tris-buffer, 0.1% EDTA and 1% antibiotics. Tissues were then treated overnight with sodium dodecyl sulfate (SDS) in PBS at various concentrations from 0.01% to 1%.

Solid-Phase Tissue Gel Electrophoresis

Tissues were embedded in 2% agarose or 12 % acrylamide gel with SDS. Concentration of SDS in gel and running buffer was matched to the concentration used to treat the tissue prior to tissue electrophoresis. Solidified tissue gels were placed either in a horizontal gel electrophoresis unit (Bio-Rad) or in a vertical gel electrophoresis unit (Bio-Rad) in Tris-Glycine SDS running buffer run for up to 20 h at 0 V, 60 V, or 120 V at 4°C. Finally, tissues were washed in PBS with aprotinin 10KIU/ml, 0.1% EDTA and 1% antibiotics for 96 h with complete change of the washing solution every 24 h.

Immunoblot Assay for Protein Antigens

Immune serum was generated by injecting homogenized PAV subcutaneously into New Zealand White rabbits every 2 weeks for 10 weeks. Tissue homogenate was injected with Freund's adjuvant on the initial inoculation and incomplete Freund's adjuvant on subsequent inoculations. Serum was collected after 70 days and stored at -80 °C until used.

Soluble proteins were extracted from tissues for immunoblot assay of protein antigens. Equal wet weights of tissues were minced, placed in a solution of 0.1% SDS, 10 mM Tris HCl (pH 8.0), 100 KIU/ml aprotinin, 1 mM dithiotheritol, 2 mM $MgCl_2$ 10 mM KCl and 0.5 mM pefabloc. Tissue suspensions were shaken on ice for 1 h and centrifuged at 17,000g for 20 min. The supernatant was saved. The pellet was re-suspended in a solution of 1.25% SDS, 10 mM Tris HCl (pH 8.0), 100 KIU/ml aprotinin, 1 mM dithiotheritol, 2 mM $MgCl_2$ 10 mM KCl and 0.5mM pefabloc, shaken on ice for 1 h, and centrifuged at 17,000g for 20 min. The supernatants were combined and concentrated using Amicon (Millipore) for 30 min at 7,500g and saved.

Protein fractions were mixed with an equal volume of sample buffer (Invitrogen) and reduced in 5% (v/v) β-mercaptoethanol (Gibco) at 95°C for 3 min. Equal volumes of protein solution were loaded on 4-12% 1.5 mm Tris-glycine gels (Invitrogen). Gels were run for 1.5 h at a constant 125 V at room temperature.

Proteins were transferred to 0.2 µm pore size nitrocellulose membranes (Invitrogen) at a constant 25V at room temperature for 1.5 h. Membranes were

washed for 5 minutes in Tris-buffered saline with 1% Tween20, blocked with 5% BSA for 1 hour at room temperature, and left at 4 °C overnight. Membranes were incubated with rabbit anti-PAV immune serum for 2 h at room temperature on a shaker using 1:1000 titer. Membranes were washed with Tris-buffered saline with 1% Tween 20, incubated with horseradish peroxidase-conjugated swine anti-rabbit IgG (DAKO). Membranes were washed again with Tris-buffered saline with 1% Tween 20. Signals were developed by Supersignal West Pico/Femto (6:1) chemiluminescent substrate (Pierce) using Hyperfilm ECL (Amersham Biosciences).

Histology

Treated PAV were fixed with buffered 10% formaldehyde solutions overnight, dehydrated, and embedded in paraffin. Sections were stained with hematoxylin and eosin stains to examine tissues the cellularity and the morphology of extracellular matrix.

Data Analysis

Three replicate gels were run for each protein extraction. Optical density of immune banding was measured using Adobe Photoshop software (version 7.0). Relative optical density was expressed as a percent of optical density of immune banding for untreated control tissue. Results were analyzed by one-way or two-way ANOVA. Values of $p < 0.05$ were considered significant.

RESULTS

A preliminary experiment was performed to test the migration of proteins from tissues mounted in gel under electric current. Protein migration from SDS-

treated bovine pericardium was observed using both polyacrylamide and agarose gels (Figure 6.1). Protein migration did not occur when tissues were not pretreated with SDS. There was no macroscopical tissue damage after solid-phase tissue electrophoresis (TE).

Immunoblot analysis of TE treated tissues

The effects of TE at three voltages (0, 60 & 120 V) and two SDS or SD concentrations (0.25% & 1.0%) on antigen removal from BP or PAV without aqueous washout are shown in Figure 6.2-4. All treatments in SDS increased protein antigen removal compared to untreated control tissue. Voltage increased protein removal from BP and PAV (p <0.05). Antigen removal was not different between 0.25% and 1.0% SDS (Figure 6.2-3). All treatments in SD increased antige removal compared to untreated control tissue, however, there was no significant difference between TE treated groups (p>0.05) (Figure 6.4). The effect of TE running time (4, 8, 12 hrs) on antigen removal from PAV treated with 0.25% SDS and 120 V before aqueous washout was shown in Figure 6.5. Antigen removal was not enhanced by TE running times greater than 4 h. Washing process improved antigen removal compared with no washing group after TE treatment (p<0.05) (Figure 6.6). The effect of SDS concentration on antigen removal from PAV with and without TE at 120 V for 4 h followed by 96 h aqueous washout was compared (Figure 6.7). Both SDS concentration (p=0.001) and TE (p=0.025) independently enhanced antigen removal from PAV by two-way ANOVA analysis. An interaction between SDS concentration and TE treatment was not detected by two-way ANOVA. The effect of TE on antigen

removal was greatest in PAV treated with 0.05% SDS. Treatment of PAV with TE and 1.0% SDS resulted in apparent complete removal of antigens based on the detection limit of the immunoblot assay.

Histology

Histology of untreated PAV and PAV treated with 0.05% or 1.0% SDS with or without TE is shown in Figure 6.8. Only treatment with 1.0% SDS with TE resulted in complete acellularity of the aortic conduit wall. Some irregularity of the extracellular matrix was observed in PAV treated with 1.0% SDS, however, there was no apparent structural difference in the extracellular matrix between PAV treated with or without TE at the equivalent SDS concentrations level.

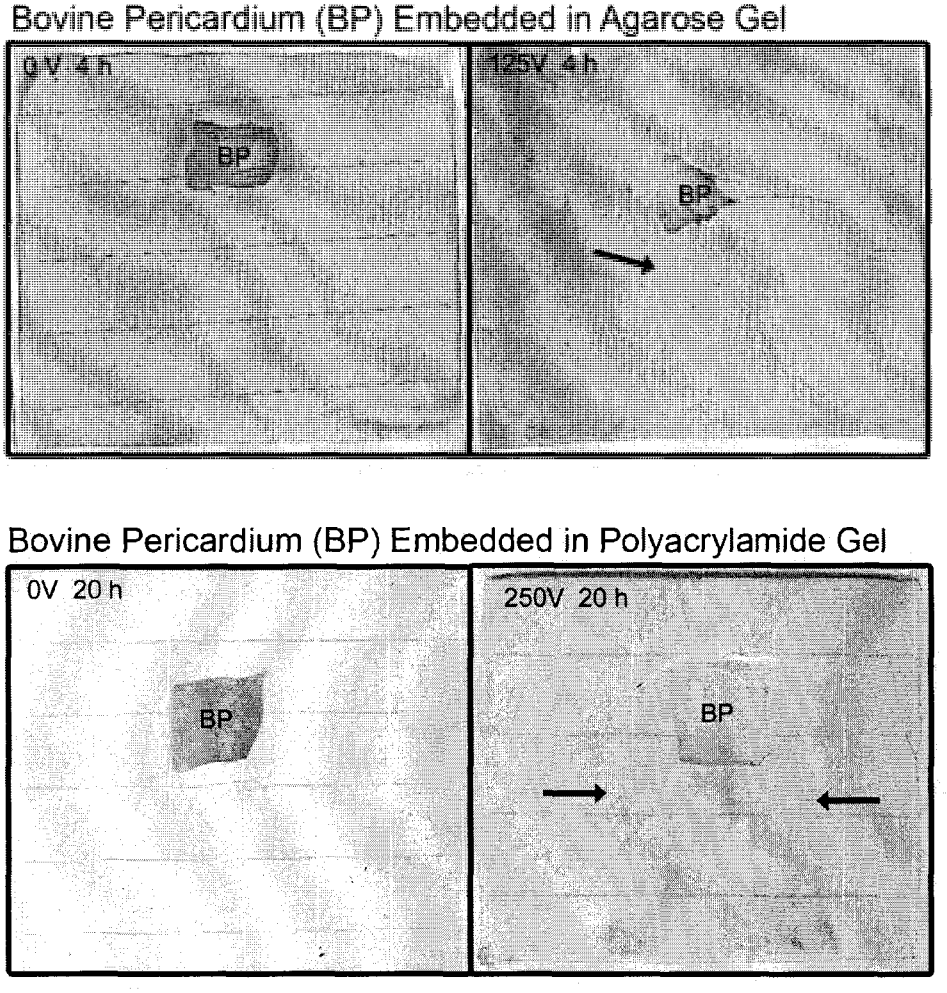

Figure 6.1 Preliminary experiment of solid-phase tissue gel electrophoresis. Bovine pericardium (BP) was mounted in agarose or polyacrylamide gel. Tissue gels were placed in gel electrophoresis unit and were ran for 4 or 20 h at 125 or 250V. Silver staining was performed to detect proteins in tissue gels. Protein streak was observed after tissue electrophoresis as indicated by arrows.

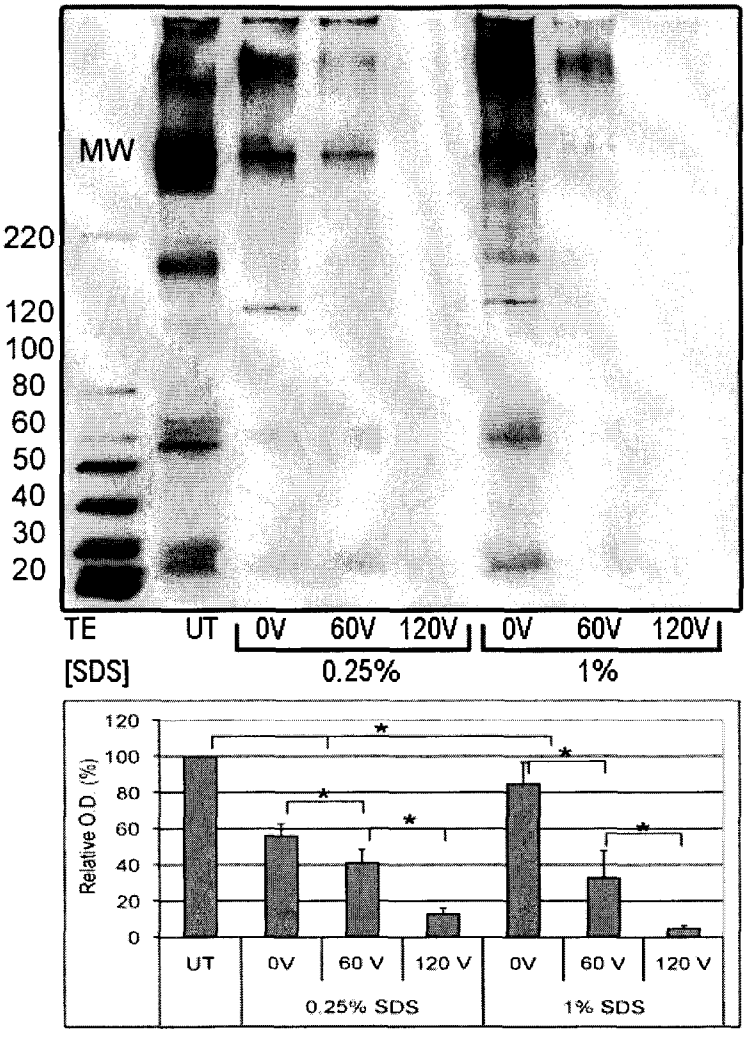

Figure 6.2 Immunoblot analysis of soluble protein antigens extracted from bovine pericardium after sequential treatment with hypotonic lysis, sodium dodecyl sulfate (SDS) (0.25 or 1%) for 24 h, solid-phase tissue electrophoresis (TE) at 0 V, 60 V, and 120 V for 4 h. Relative optical density expressed as a percent of optical density of untreated control (UT). Data are mean ± S.D. (n=3). Asterisks (*) over horizontal bars indicate significant difference (p<0.05) between treatments. Molecular weight (MW) was shown as kD.

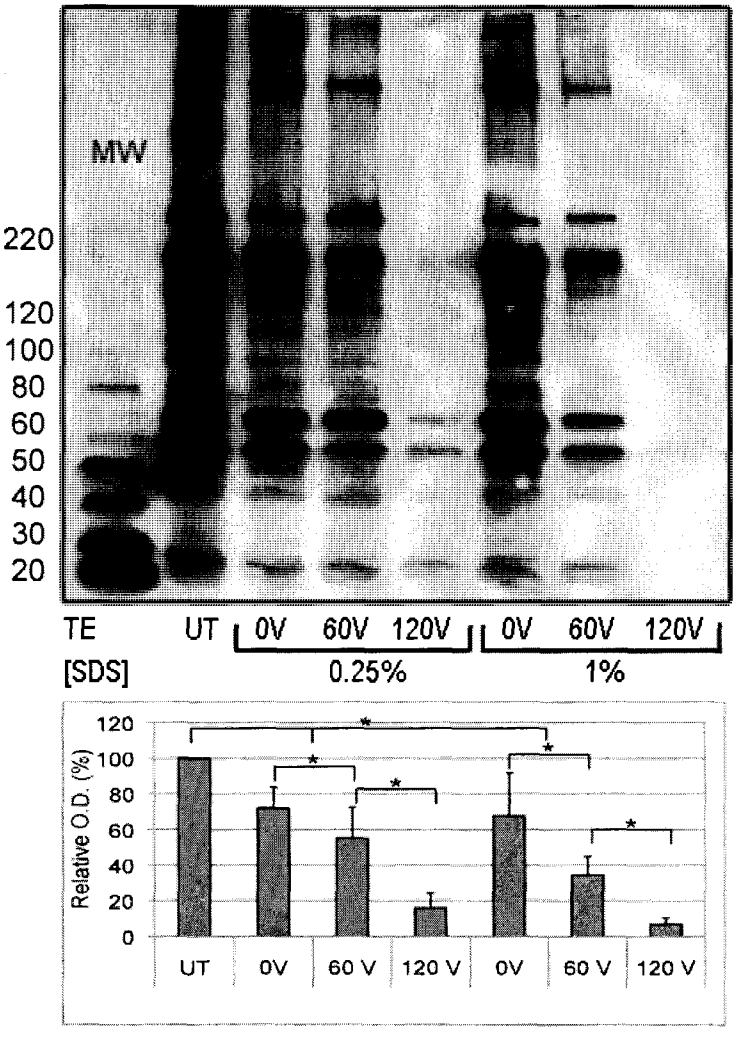

Figure 6.3 Immunoblot analysis of soluble protein antigens extracted from porcine aortic valve conduit after sequential treatment with hypotonic lysis, sodium dodecyl sulfate (SDS) (0.25 or 1%) for 24 h, solid-phase tissue electrophoresis (TE) at 0 V, 60 V, and 120 V for 4 h. Relative optical density expressed as a percent of optical density of untreated control (UT). Data are mean ± S.D. (n=3). Asterisks (*) over horizontal bars indicate significant difference (p<0.05) between treatments. Molecular weight (MW) was shown as kD.

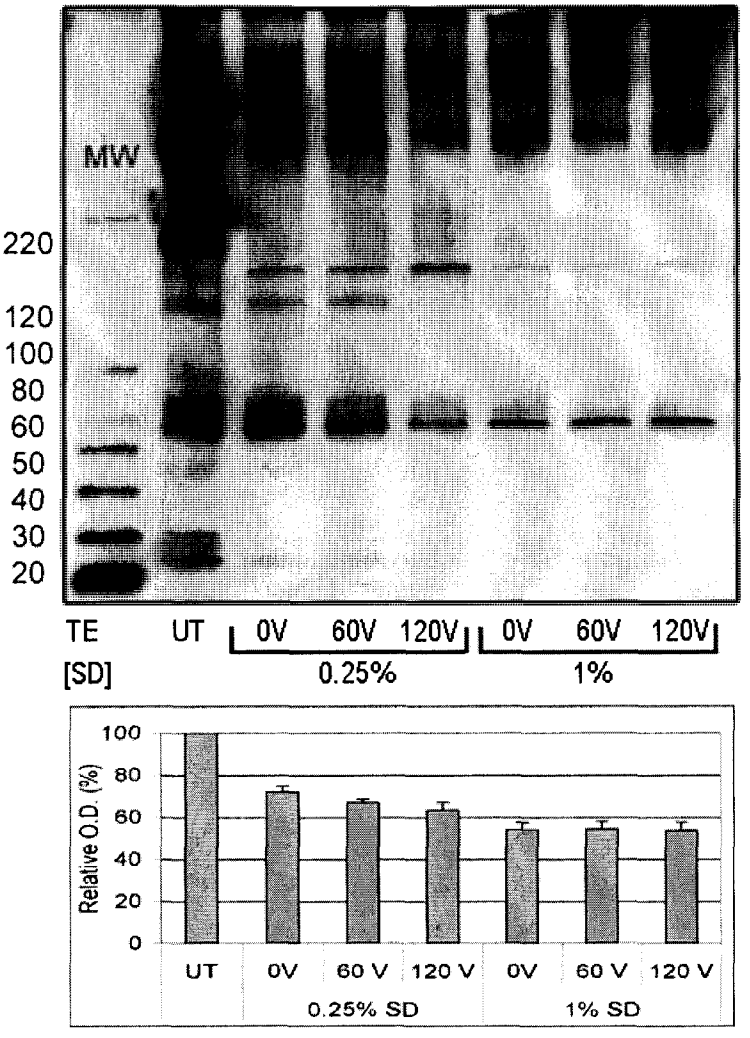

Figure 6.4 Immunoblot analysis of soluble protein antigens extracted from bovine pericardium after sequential treatment with hypotonic lysis, sodium deoxycholate (SD) (0.25 or 1%) for 24 h, solid-phase tissue electrophoresis (TE) at 0 V, 60 V, and 120 V for 4 h. Relative optical density expressed as a percent of optical density of untreated control (UT). Data are mean ± S.D. (n=3). Molecular weight (MW) was shown as kD.

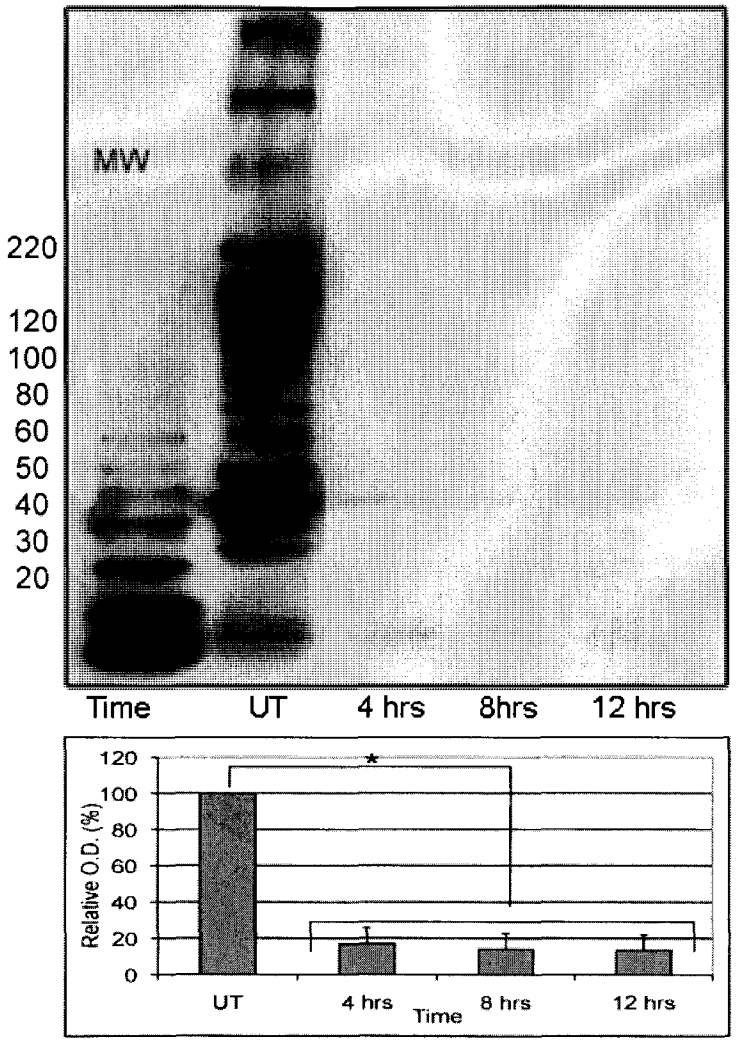

Figure 6.5 Immunoblot analysis of soluble protein antigens extracted from porcine aortic valve conduit after sequential treatment with hypotonic lysis, 0.25% sodium dodecyl sulfate (SDS) for 24 h, solid-phase tissue electrophoresis (TE) 120 V for 4, 8 and 12 h. Relative optical density expressed as a percent of optical density of untreated control (UT). Data are mean ± S.D. (n=3). There was a significant difference between UT and TE groups ($p<0.05$) while antigen removal was not improved greater than 4 h as indicated with asterisk (*). Molecular weight (MW) was shown as kD.

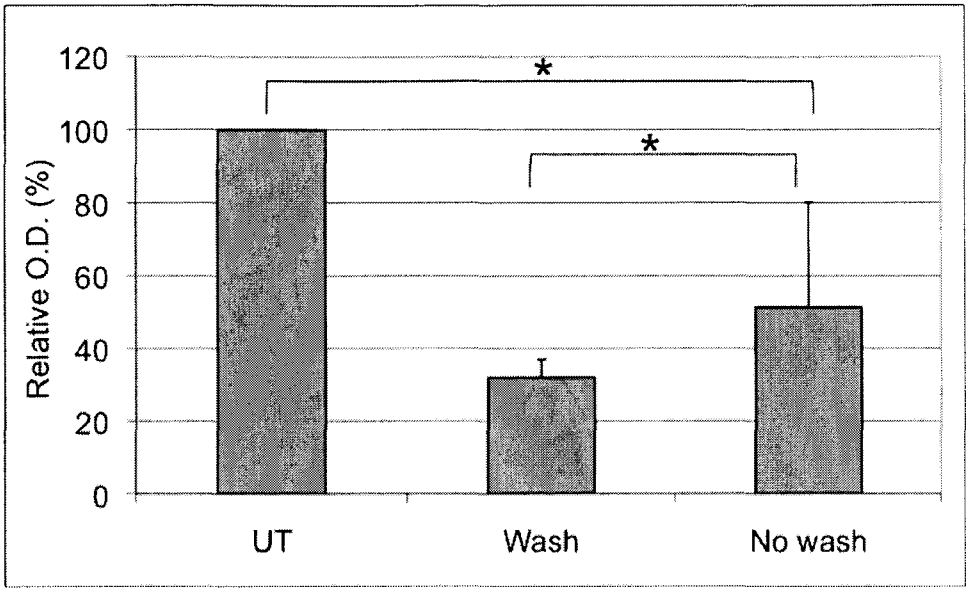

Figure 6.6 Densitometry result comparing washing and no washing group in treated bovine pericardium. Soluble protein antigens extracted from bovine pericardium were separated in two groups, wash or no wash group after sequential treatment with hypotonic lysis, 0.25% or 1% sodium dodecyl sulfate (SDS) for 24 h, solid-phase tissue electrophoresis (TE) 0 or 120 V for 4 h. Relative optical density expressed as a percent of optical density of untreated control (UT). Data are mean ± S.D. (n=3). Asterisk (*) over horizontal bar indicates significant difference ($p<0.05$) between treatments.

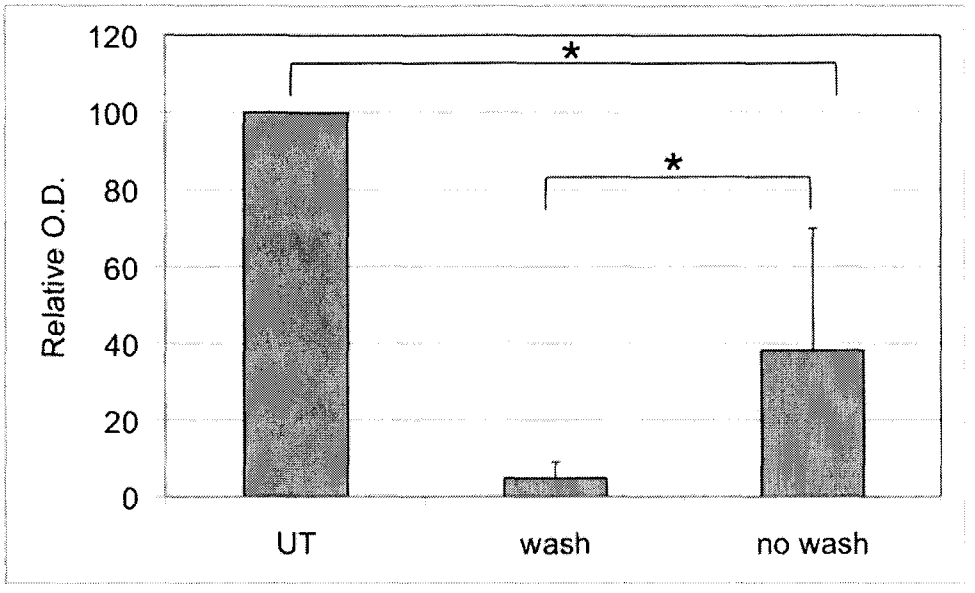

Figure 6.7 Densitometry result comparing washing and no washing group in treated porcine aortic valve conduit. Soluble protein antigens extracted from porcine aortic valve conduit were separated in two groups, wash or no wash group after sequential treatment with hypotonic lysis, 0.25% or 1% sodium dodecyl sulfate (SDS) for 24 h, solid-phase tissue electrophoresis (TE) 0 or 120 V for 4 h. Relative optical density expressed as a percent of optical density of untreated control (UT). Data are mean ± S.D. (n=3). Asterisk (*) over horizontal bar indicates significant difference ($p<0.05$) between treatments.

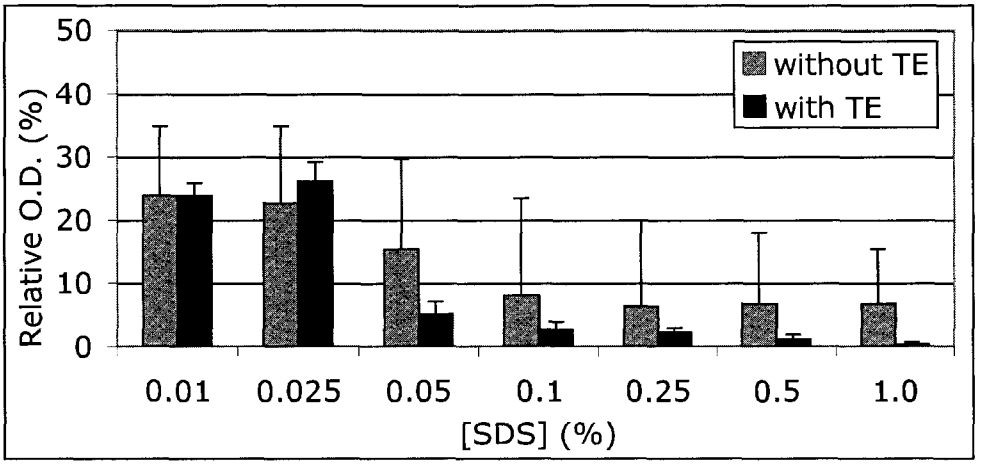

Figure 6.8 Immunoblot analysis of soluble protein antigens extracted from porcine aortic valve conduit (PAV) after sequential treatment with hypotonic lysis, sodium dodecyl sulfate (SDS) for 24 h, with and without solid-phase tissue electrophoresis (TE) at 120 V for 4 h, and passive aqueous washout for 96 h. Optical density (O.D.) of immune banding expressed as percent of optical density of untreated control. Data are mean ± S.D. (n=3). SDS concentration (p = 0.001) and TE (p = 0.025) independently enhanced antigen removal from PAV. A SDS concentration-TE treatment interaction was not detected by two-way ANOVA

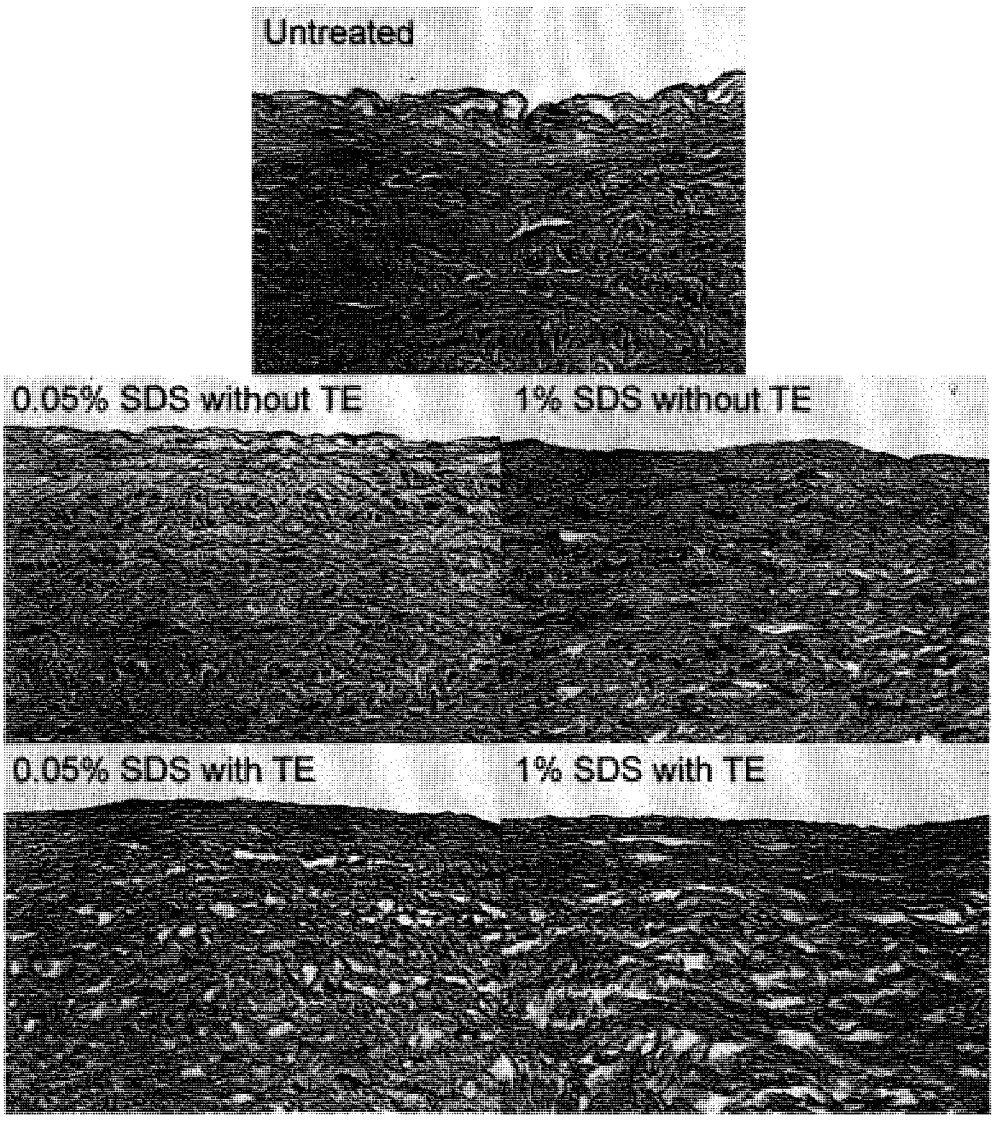

Figure 6.9 Histology of untreated porcine aortic valve conduit (PAV) and PAV treated with sodium dodecy sulfate (SDS) at 0.05% or 1% with or without solid-phase tissue electrophoresis (TE). Hematoxyline-eosin staining (X400)

DISCUSSION

Xenogeneic tissue offers a naturally derived scaffold that can be used in tissue engineering of heart valve. Xenogeneic tissues are more available than allogeneic tissues and require less morphogenesis *in vitro* than synthetic materials. A major hurdle for the use of xenogeneic tissue is the elimination of tissue immunogenicity. Glutaraldehyde-fixed xenograft bioprosthesis has been introduced as heart valve substitutes. They are readily available and have sufficient hemodynamics. Since the tissue proteins are cross-linked with glutaraldehyde, immune reaction is considered to be minimum. Nonetheless, the long-term performance of glutaraldehyde-fixed bioprosthesis is poor due to immune reaction,[24-27] cytotoxicity of glutaraldehyde,[28-30] and a lack of regeneration.

Decellularization of xenogeneic tissues is an essential process in tissue engineering of heart valves based on a biological scaffold (Figure 1.1). The concept of decellularization was developed to reduce tissue immunogenicity while maintaining mechanical integrity and providing an optimal environment for cell regeneration. An assumption of tissue decellularization is that antigens that would incite acute severe immune rejection are largely associated with the native cells of the tissue and are thus removed by decellularization treatment. Although several reports have described complete acellularity[7, 8, 10, 31-33] and the reduction of immunogenicity of decellularized tissues,[34] concerns regarding immune reaction against decellularized xenogeneic tissue scaffolds have been revealed.[1-4] Based on previous studies and a study performed in this dissertation (Chapter

V) it was demonstrated that residual antigenic proteins present in the tissue after decellularization treatment. [1, 10, 23] It is likely that these antigenic proteins play a significant role inducing immune reaction of heart valve substitutes causing tissue deterioration.

Previous studies demonstrate that gel electrophoresis with intact whole tissues is possible.[35, 36] These studies revealed that whole tissues can undergo gel electrophoresis and that the number of proteins detected was higher with whole tissue electrophoresis compared electrophoresis of tissue protein extracts. The present study proposed a new technique termed "solid-phase tissue electrophoresis" (TE) to enhance a detergent-based decellularization technique. TE focused on reducing a broad range of residual antigenic proteins that were remained after anionic detergent-based decellularization. In the present study, tissues were pre-treated with SDS that has a strong binding capacity with proteins. Prior or concurrent treatment of the tissues with SDS imparts a strong negative charge to soluble proteins and protein antigens within the tissue causing them to migrate out of the tissue into a surrounding media when the tissue is placed in an electrical field. Further, TE allowed for a consistent spatial electric current to be applied to tissues by mounting them in a gel and placing it in a stable chamber.

In this study, the combination of SDS-decellularization and TE followed by aqueous washout achieved complete removal of antigenic proteins from PAV when the 1% SDS and 120V were applied. Although the results could be different if aqueous washout was used for entire experiments after TE, TE was a

significant factor to improve antigen removal in xenogeneic tissues even after aqueous washout. SD was not an effective detergent compared to SDS in combination with TE. Cationic detergents were not evaluated with TE in this study. An important issue that was not addressed in this study is the potential effects that TE could have on the mechanical properties of xenogeneic tissue scaffold, especially at high voltage. Based on the histological examination, TE did not appear to cause detrimental damage to the extracellular matrix. As previously reported,[37, 38] higher concentrations of SDS did appear to alter the extracellular matrix. One of the potential advantages of TE is that it may allow effective antigen removal at lower SDS concentration. The durability of TE-treated tissues against functional load needs to be evaluated *in vitro* in order to examine whether TE-tissues has sufficient mechanical properties after the application of electric current.

In conclusion, TE enhanced detergent-based decellularization and removed a broad range of residual antigenic proteins from detergent-decellularized xenogeneic tissue scaffolds.

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CHAPTER VII

CYTOTOXICITY AND LEACHING OF SODIUM DODECYL SULFATE FROM DETERGENT-TREATED XENOGENEIC BIOSCAFFOLDS

INTRODUCTION

A tissue-engineered heart valve offers the possibility of a viable implant that could resolve significant thrombosis and durability issues associated with currently available prostheses. Such an implant might even be capable of growth in pediatric patients. Xenogeneic tissues can be a candidate to create tissue-engineered heart valve because of its immediate implantability without a need for extended *in vitro* morphogenesis, appropriate mechanical properties, and the provision of a suitable environment for cell differentiation and orientation. Disadvantage of xenogeneic tissue is its inherent immunogenicity requiring the removal of antigenic proteins. Decellularization has been applied in xenogeneic tissues to remove cellular component of the tissue in order to reduce tissue immunogenicity.[1-7] An ideal decellularization treatment for xenogeneic tissues should remove xenogeneic antigens, maintain the biomechanical properties of the tissue and be compatible with *in vitro* or *in vivo* recellularization by autologous cells.

Sodium dodecyl sulfate (SDS) is an anionic detergent that has consistently emerged as a promising decellularization reagent.[2, 4, 8-13] However, questions have been raised about possible cytotoxicity associated with SDS-treated xenogeneic tissues.[8, 14] This could take the form of free SDS leaching from treated tissue or of residual bound SDS within the treated tissues, or both. A specific hypothesis studied here is that free-SDS is cytotoxic to cultured vascular cells at measurable concentration and SDS leaches into the media of SDS-treated tissues.

MATERIAL AND METHODS

Sodium Dodecyl Sulfate Cytotoxicity

Ovine vascular cells were explanted from the carotid arteries of sheep obtained post-mortem under aseptic conditions. The arteries were washed in phosphate-buffered saline (PBS) with 1% antibiotic-antimycotic solution (penicillin 100 U/ml; streptomycin 10 mg/ml; amphotericin B 0.25 mg/ml) and minced into pieces 3 mm^2 in size. The tissue pieces were placed into 75 mm^2 tissue culture flasks with Dulbecco's Modified Eagle Medium with 1% antibiotic-antimycotic solution and 10% fetal bovine serum. The culture flasks were then incubated in a humidified environment at 37 °C with 5% CO_2. The tissue pieces were removed after several days and the explanted cells grown to 80-90 % confluence. The culture media was changed every third or fourth day. The explanted ovine vascular cells were detached from the culture flasks with 0.25% trypsin in Hank's balanced salt solution with 0.02% (w/v) ethylendiaminetetra-acetic acid (EDTA) and expanded with two passages.

On the third passage, the cells were transferred to six-well culture plates and seeded at a density of 10^5 cells per well. The culture media and conditions were as described above. On the third day, SDS was added to the cell cultures to achieve media concentrations of 0 μM, 10 μM, 50 μM, 100 μM, 500 μM, 1000μM (n= 5 each concentration). The cells were incubated for 48 hours, harvested by trypsin digestion, pelleted by centrifugation, and resuspended in 1 ml of the media. The cell numbers in suspension were counted using a hemocytometer and the total cell number per culture well was calculated. Cell suspensions were mixed with 0.4% trypan blue dye in a 9:1 ratio (cells: dye). The percentage live cell ratio for each cell suspension was determined by counting the number of cells excluding dye from their cytoplasm per 100 cells counted.

Decellularization

Bovine pericardium was obtained post-mortem, cleaned of adipose tissue, washed with sterile PBS solution with Tris-buffered water (pH 7.4), 0.1% (w/v) EDTA, 10 KIU/ml aprotinin and 1% antibiotics-antimycotics solution (penicillin 100 U/ml; streptomycin 100mg/ml; amphotericin B 0.25 mg/ml). Tissues were cut into 1cm^2 squares and stored at - 80°C until used. The bovine pericardium tissue pieces were thawed and weighted just prior to undergoing decellularization. The tissues were treated with Tris-buffered water under gentle agitation at room temperature for 4 hours in order to cause hypotonic lysis of the cells. The tissues were then divided into six groups and treated with PBS only (control group), or one of five concentrations (w/v) of SDS in PBS: 0.01%,

0.025% (0.35mM), 0.05% (1.73mM), 0.1% (3.47 mM), 0.5% (17.34 mM), and 1% (34.67 mM). (n=10 tissue squares per concentration) under agitation at room temperature for 24 hours. Each group of SDS-treated bovine pericardium was washed four times consecutively with 100 ml of PBS under gentle agitation at room temperature for 24 hours each wash. The PBS washing solution was changed each 24 hours and collected for subsequent SDS analysis. All tissues were rinsed with 3 ml of PBS between each wash in order to prevent the transfer of any free SDS. The total washing time for each tissue was 96 hours.

Sodium Dodecyl Sulfate Assay

An assay was developed to measure the SDS anion, dodecyl sulfate (DDS), using attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy. In this method, the concentration of an analyte is determined by measuring its rate of ion exchange into a thin film of an efficient ion-exchange compound that is deposited on the ATR crystal. This measurement is taken by monitoring one or more of the analyte's IR-active vibrational bands. In the present study, the ion-exchange compound was the substituted ammonium salt trimethyl (1,1',3-tris(2-methyl-2-nonyl)-3'-ferrocenyl)ammonium iodide ($NMe_3R^+I^-$). A thin film of $NMe_3R^+I^-$ was deposited on the outer surface (i.e., the surface that would be in contact with the sample) of the diamond ATR crystal by adding a 20 µl aliquot of a 3.0 mM dichloromethane solution (i.e., 60nmol) of $NMe_3R^+I^-$ and allowing the solvent to evaporate.

The ATR probe used was an 18-bounce diamond crystal attached to a React-IR 1000 ATR-FTIR spectrometer (Applied System Inc., Millersville, MD,

USA). The surface-modified probe was immersed in each sample and the increase in absorbance (A) at 1221 cm^{-1} (one of the vibrational SO stretching bands of DDS^-) was monitored over time (t). The concentration of DDS^- in each sample was determined by comparison of the $\Delta A/\Delta t$ value for the linear portion of the A versus t plot for that sample with a calibration curve previously constructed using known concentrations of DDS^- in washing solution. A new thin-film coating of the ion-exchange compound was used for each analysis, and all analyte were performed in triplicate. The final washout SDS concentrations were reported as μM/g of tissue per 100 ml of washing solution. The total amounts of SDS washed out from treated bovine pericardium over 96 hours were calculated and reported as mg/g tissue.

Data Analysis

The effect of SDS concentration on total cell number and percentage live cell ratio was determined using a one-way ANOVA. Differences between the control group and SDS groups were determined with the Tukey-Karmer post-hoc test. $p<0.05$ was considered to be statistically significant. Linear regression was performed and the effective concentration of SDS that caused a t 50% decrease (EC_{50}) in total cell number or percentage live ratio was calculated from regression equations.

RESULTS

Sodium Dodecyl Sulfate Cytotoxicity

The total cell count of cultured ovine vascular cells was decreased ($p<0.05$) at SDS concentrations of 10 μM or higher compared to cells cultured

without SDS (Figure 7.1). Likewise, the percentage live cells was decreased ($p<0.05$) in ovine vascular cells cultured at SDS concentrations $\geq$ 100 μM compared to cells cultured without SDS. The calculated EC_{50} values, based on regression analysis, were 174 μM and 398 μM for the total cell count and percentage live cell ratio respectively (Figure 7.2-3).

Sodium Dodecyl Sulfate Leaching from Decellularized Xenogeneic Tissue Scaffolds

SDS was found to be present in detectable amounts in the washing solution of SDS-treated bovine pericardium (Figure 7.4). The concentration of SDS in washing solution was dependent on the initial treatment concentration of SDS, and diminished with subsequent 24 h washes. SDS was not detected in the PBS-only control treatment group. The total amounts of SDS washed form SDS-treated bovine pericardium over 96 hours were 165.65 ± 18.4μM/g for 1.0 % SDS, 58.7 ± 5.3μM /g for 0.5% SDS, 12.0 ± 0.62μM/g for 0.1% SDS, 2.62 ± 0.1μM /g for 0.05% SDS, and 1.56 ± 0.19μM mg/g for 0.01% SDS.

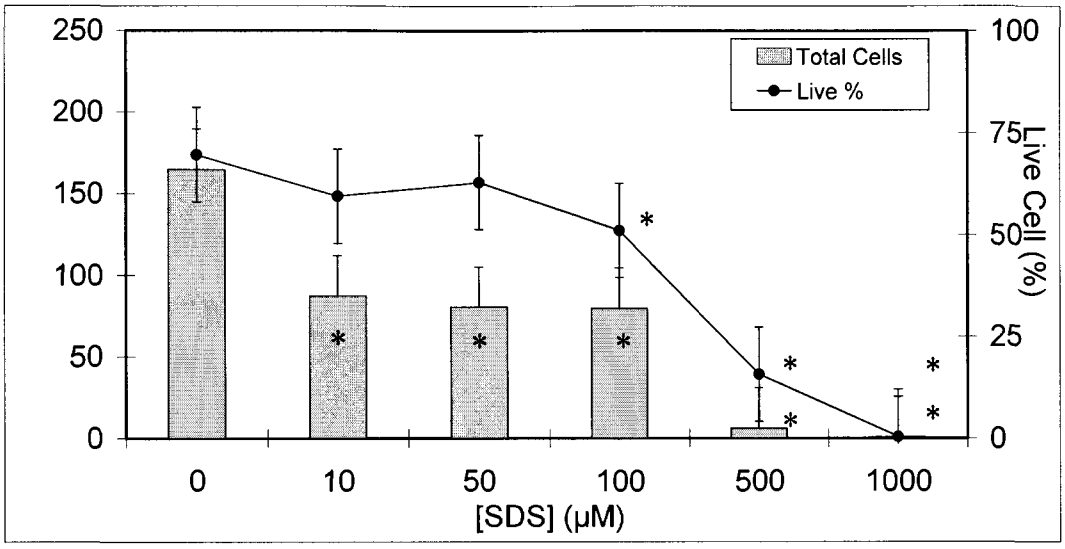

Figure 7.1 Cytotoxicity of sodium dodecyl sulfate (SDS) on cultured ovine vascular cells. Values reported (mean ± SD) are for total cell count ($\times 10^3$) and percentage live cell ratio (%). The total cell count was decreased ($p<0.05$) at SDS concentrations of 10 μM or higher compared to cells cultured without SDS. The percentage live cells were decreased ($p<0.05$) at SDS concentrations ≥ 100 μM compared to cells cultured without SDS.

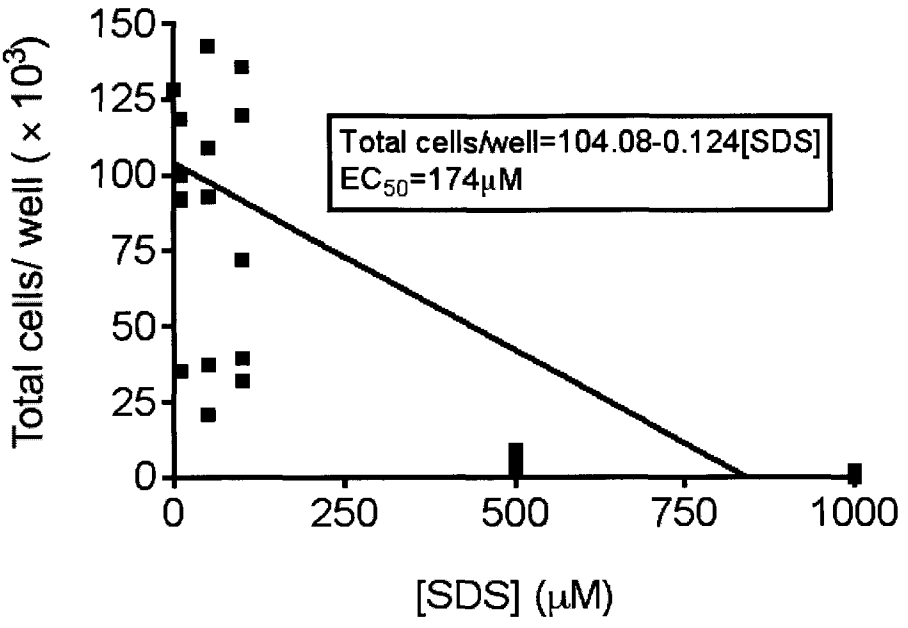

Figure 7.2 Linear regression analysis of total cells/well in different concentrations of sodium dodecyl sulfate (SDS). Calculated EC_{50} was 174μM. R^2 was 0.52 ($p<0.05$).

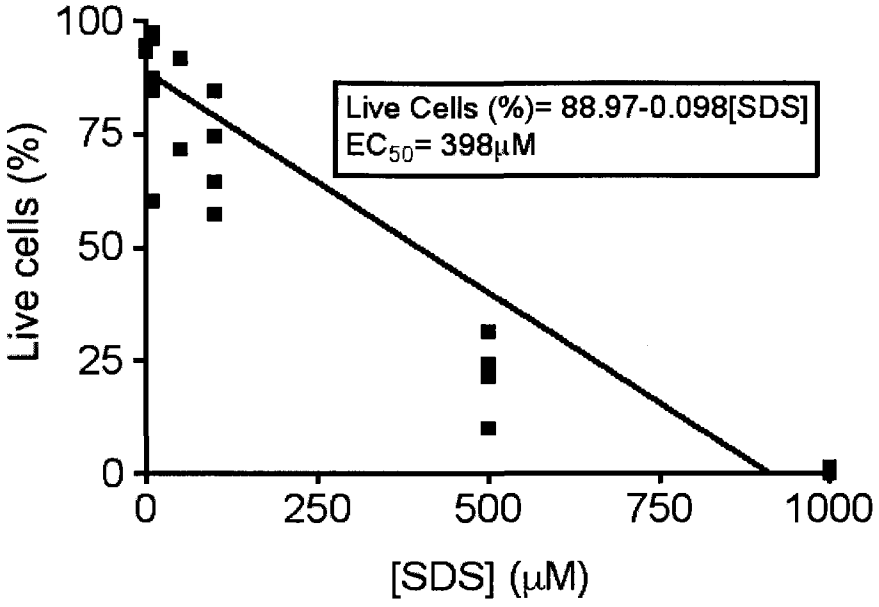

Figure 7.3 Linear regression analysis of live cell % in different concentrations of sodium dodecyl sulfate (SDS). Calculated EC_{50} was 398μM. R^2 was 0.85 (p<0.05)

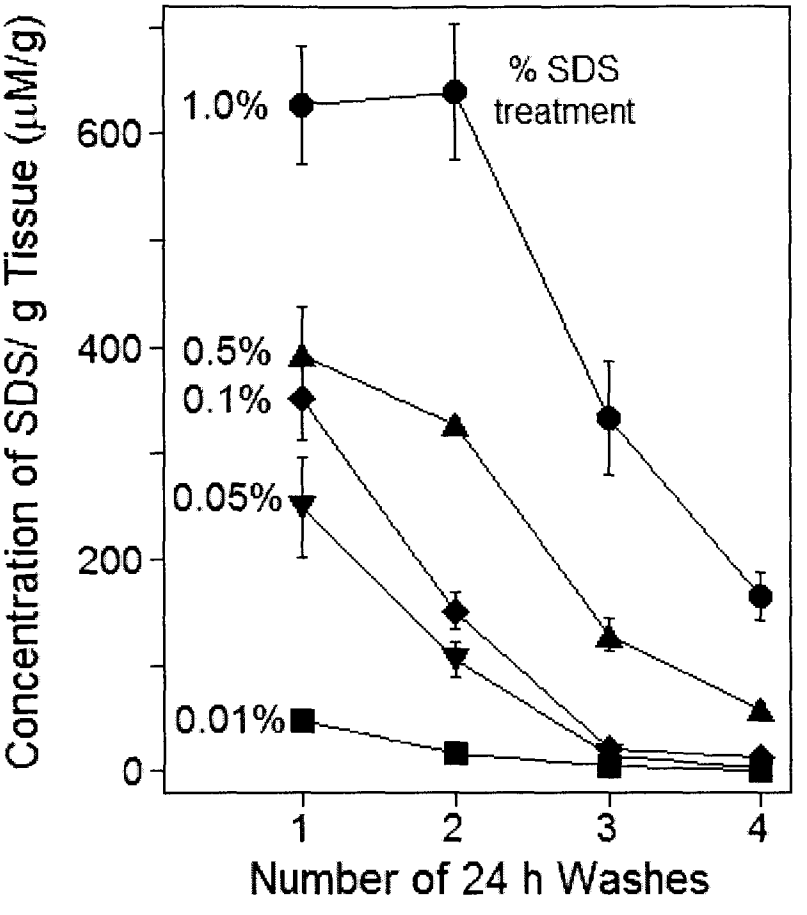

Figure 7.4 Sodium dodecyl sulfate (SDS) washout from treated bovine pericardium. Mean values were shown with standard deviation in μM/1g of treated tissue in 100 ml of washing solution for each four consecutive 24 hours wash periods.

DISCUSSION

The results of the present study confirmed that free SDS leaches from SDS-treated bovine pericardium at concentrations that are potentially cytotoxic. It is reasonable to assume that the same phenomenon occurs in other SDS-treated xenogeneic tissues such as porcine aortic valve conduit. These results offer an explanation for the findings of Rieder *et al.* that media used to store SDS-treated porcine aortic valves may be toxic to cultured vascular cells.[14] The results also showed that the leaching of free SDS into media diminishes considerably over 96 hour periods. Several factors were identified that likely influence the concentration of free SDS in washing or storage media; these include the initial treatment concentration of SDS, the ratio of tissue weight to washing solution volume, and the duration and number of wash changes. Thus, it is possible that while SDS-treated tissues are washed with sufficient volumes and numbers of washes over time, the free SDS concentration may fall below those that are overtly toxic to cultured cells. As such, the present results could be consistent with the results of Wilcox *et al.*,[13] in that SDS-treated porcine valves might not demonstrate a clear cytotoxic effect on cultured vascular cells if the treated tissues were washed with sufficient volumes and numbers of washes over a sufficient period of time. It is important to note that differences in the decellularization protocols such as protease inhibition, treatment with trypsin, and/or the duration, volume and temperature of the washing protocol, may account for differences in results in studies.

The assay reported in the present study measured only free SDS in a bathing media of treated tissues. Any SDS bound to soluble proteins within the media would not be detected by this assay. It is possible that additional SDS leaches in the form of soluble protein-bound SDS. Moreover, the potentially more critical issue of residual SDS being retained within treated tissues was not addressed in this study. However, the results of the present study showed that SDS is absorbed into tissues during treatment and it seems quite unlikely that all SDS absorbed by tissues would freely diffuse into the surrounding media.

In conclusion, SDS has been shown to leach from SDS-treated bovine pericardium at levels that are potentially cytotoxic under the condition studied here. The extent of SDS leaching is dependent on the initial treatment concentration of SDS, and diminishes over 96 hours.

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CHAPTER VIII

SUMMARY AND CONCLUSIONS

Immunoblot assay was developed to evaluate the completeness of antigen removal achieved by decellularization treatments of xenogeneic tissue scaffolds. Immunoblot assay developed in this study was both sensitive and capable of surveying a broad range of potential soluble protein antigens in xenogeneic bioscaffolds. Immune banding was increased in density with time suggesting specific acquired immunity in rabbits toward xenogeneic soluble proteins from both bovine pericardium (BP) and porcine aortic valve conduit (PAV). Using the immunoblot assay developed in this study, hypotonic lysis, sodium dodecyl sulfate (SDS)- or sodium deoxycholate (SD)- detergent treatment, and aqueous washout decellularization was evaluated its efficacy on removing antigenic proteins from BP or PAV. Neither SDS- nor SD-based decellularization completely removed detectable antigenic proteins from BP or PAV. Solid-phase tissue electrophoresis enhanced antigen removal in combination with SDS-decellularization from BP or PAV. Only the combination of SDS-decellularization and TE at 1% SDS and 120V followed by aqueous washout caused apparent complete removal of antigenic proteins from PAV based on the detection limits of the immunoblot assay. SDS is an effective anionic detergent to reduce antigenic proteins from xenogeneic tissue

scaffolds based on the studies presented here, however, SDS leached from SDS-treated BP at levels that are potentially cytotoxic. The extent of SDS leaching is dependent on the initial treatment concentration of SDS, the ratio of tissue weight to washing solution volume, and the duration and number of wash changes.

In conclusion, antigen removal of xenogeneic tissue scaffolds carries an important role in developing an ideal tissue-engineered heart valve. It requires an understanding of biological responses to chemical reactions in tissues and a conceptual understanding of scaffolding techniques. A conceptual approach will be a key to achieving the elimination of tissue immunogenicity in developing an ideal tissue-engineered heart valve.