THESIS

TISSUE ELECTROPHORESIS FOR GENERATION OF PORCINE ACELLULAR DERMAL MATRICES

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ABSTRACT

TISSUE ELECTROPHORESIS FOR GENERATION OF PORCINE ACELLULAR DERMAL MATRICES

Background: Acellular dermal matrices have several applications including treatment of burns, reconstructive surgery, and treatment of chronic ulcers. Xenogeneic acellular dermal matrices have the advantage of increased availability compared to matrices derived from human cadavers (i.e. allogeneic dermal matrices), however they have a higher potential for generating an inflammatory response in the recipient. One approach to creating an acellular dermal matrix is through chemical and detergent-based processes collectively known as decellularization. Concerns regarding the completeness of soluble protein and antigen removal associated with current detergent-based decellularization treatments have been raised. The aim of this study was to compare the efficacy of a standard detergent-based decellularization and a novel electrophoresis-based method at removing soluble protein and protein antigens.

Hypothesis: I hypothesized that tissue electrophoresis would enhance the removal of soluble proteins and protein antigens from porcine dermis compared to a standard detergent-based decellularization protocol.

Methods: Skin was harvested from 6 pig cadavers. A portion of skin from each pig was assigned to four treatment groups:

1. Epidermis removal without sodium dodecyl sulfate (SDS) (positive or untreated control)
2. Epidermis removal with 0.5% SDS (epidermis removal control)
3. Epidermis removal with 0.5% SDS and standard 0.5% SDS decellularization treatment with a 6 h passive diffusion washout period

4. Epidermis removal with 0.5% SDS and Tissue Electrophoresis-based decellularization (0.5% SDS, 2% agarose gel, and 0.5 Amp) for 6 h

The completeness of soluble protein and antigen removal was evaluated by SDS-PAGE and immunoblot analysis, respectively. Rabbit anti-porcine and human IgG serums were the primary antibodies for immunoblot analysis.

Results: Tissue electrophoresis decellularization increased removal of soluble proteins from porcine dermis when compared to standard passive detergent-based decellularization, based on SDS-PAGE analysis. Antigen removal, based on immunoblot analysis, was increased compared to untreated dermis, but was not significantly different between standard detergent-based and tissue electrophoresis-based decellularization treatments.

Conclusion: Tissue electrophoresis enhances removal of soluble proteins from porcine dermis compared to standard detergent-based decellularization. This enhanced removal of soluble proteins may translate into reduced inflammatory response to xenogeneic acellular dermal matrices implanted into humans. Optimization of electrophoretic parameters may further increase the efficiency of tissue electrophoresis as a decellularization method.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

1.1: DERMAL MATRIX – DEFINITION & CLASSIFICATION

Dermis is one of three layers of the skin and gives skin its mechanical strength. Decellularized dermis is generated from human or animal cadaveric skin by removing the other skin layers (epidermis and hypodermis) and then treating the dermis to remove the native cellular component. Decellularized dermis yields a sheet of extracellular matrix (ECM) which is an appealing biomaterial for use in tissue engineering, regenerative medicine and reconstructive surgery. Additionally, ECM sheets can be used as a reinforcing 'patch' to repair complicated defect geometries and large damaged areas because of their physical properties (pliability, flexibility, and retractility).The ability of a dermis to promote cell adhesion and proliferation as well as its favorable physical properties make it appealing as a tissue engineering platform as well as a biomaterial for reconstructive surgery.

Located between epidermis and hypodermis, the dermis is comprised of fibroblasts and dense ECM. The ECM is a complex network of various combinations of proteoglycans, hyaluronic acid, collagen, fibronectin, and elastin. ECM may assist healing by stimulating cell proliferation and differentiation, guiding cell migration, and modulating cellular response. Additionally, the ECM is responsible for the dermis' mechanical integrity and elasticity. Below the dermis is the hypodermis, which consists of loose connective tissue comprised primarily by adipose tissue. The epidermis consists primarily of cells and is responsible for the barrier function of skin.

Dermis can be harvested from human or animal tissues. The source of the tissue dictates its classification. The primary terms used to define the tissue source are autograft, allograft, and xenograft. A tissue obtained from one site and transplanted to another on the same patient is

known as an autograft. An allograft is obtained from a donor of the same species as the recipient. In contrast, a transplant from a species different from the recipient is a xenograft (i.e. dermis transplanted from pig to human).

One approach to making a tissue-engineered dermis for use in humans is through 'decellularization' of xenogeneic tissues such as porcine dermis. The advantages of using a xenogeneic dermis include its biomechanical and structural appropriateness, and availability. Porcine dermis is a good candidate for use in humans because the arrangement and structure of their collagen matrix is similar. Porcine tissue is readily available and easily harvested, and while human derived dermis would be great to use, the main disadvantage of relying on human derived dermis is that the availability of the tissue is dependent on the organ bank [1].The limited availability of human tissue makes purchasing human dermis quite expensive. The use of a tissue-engineered dermal matrix is one of the alternative solutions that could alleviate the gap between organ supply and demand.

Dermal matrices, commonly referred to in the literature as: dermal substitutes, acellular dermis, and biologic scaffolds, are a group of wound treatment materials derived from allogeneic or xenogeneic skin, that assist in wound closure and tissue reconstruction. A dermal matrix is made using a three-step method. First the epidermis is removed using a chemical process. The dermis then undergoes a decellularization process consisting of osmotic lysis of the cells, treatment with detergent to solubilize cell membranes and dissociate DNA from proteins, and finally a wash out treatment to remove any residual cellular elements or chemicals. Because of the dynamic nature of the wound healing process, it is important that the structure of the dermal substitute allows for proper interactions between cells, extracellular matrix (ECM) and growth factors that reconstitutes tissue after injury [2]. The native dermal skin layer is primarily composed

of ECM which plays an important role in tissue regeneration and is composed of proteoglycans, hyaluronic acid, collagen, fibronectin, and elastin. Not only does the ECM provide structural support for cells, it also creates a reservoir of active molecules that can be rapidly mobilized following injury to stimulate cell proliferation and migration [3].

The importance of the ECM in wound healing has been recognized by the tissue-engineering field and has thus led to the development of wound products that aim to replace it. Comprised of a reconstituted or natural collagen matrix, these dermal matrices aim to mimic the structural and functional characteristics of native ECM [4]. The three dimensional structure of the matrix provides a temporary support into which cells can migrate and proliferate in an organized manner, which in turn leads to tissue regeneration and ultimately wound closure.

Scaffolds for reconstructive surgery can be biological or synthetic, or a combination of both. Scaffolds may be acellular or cellular. The latter may be generated by "recellularizing" the scaffold with the patient's own cells to minimize immune rejection.

- Biological tissue
    - Animal (i.e. porcine, bovine)
    - Human (i.e. cadaveric skin)
- Synthetic materials
- Composite materials (contains two or more components and may be biological or synthetic)

Decellularized biological matrices provide a structure for tissue remodeling, while the removal of viable cells minimizes or prevents an inflammatory or immunogenic response. The removal of cellular matter leaves a structure for tissue remodeling [5].

Cellular matrices, matrices that contain living cells, may be of biological, synthetic, or composite origins. It is important to note that these matrices are first decellularized, and then, through the use of tissue engineering approaches, the matrix is combined with *in vitro* cultured cells to generate functional tissues. Cellular matrices are clinically advantageous in that they reduce the healing time for acute and chronic wounds and promote tissue remodeling.

The acceptance of a biological transplant is dependent on how well the manufacturing process is at removing not only cells, but also antigens. An antigen is a substance that induces the production of antibodies. If the host body recognizes an antigen as foreign or potentially harmful, the immune system will try to neutralize it by eliciting a humoral response or by cell mediated processes.

1.2: CLINICAL APPLICATIONS

The dermal matrix, essentially a collagen sheet, lends itself to a variety of clinical uses. It has been shown to be effective in three main contexts: as a wound dressing, as a tissue replacement, and in reconstructive surgeries.

Wound Dressing: Acellular dermal matrices may be used as a wound dressing. While most wound dressings need to be changed frequently, acellular matrices provide a scaffold for tissue repair and therefore must remain on the wound for a sufficient length of time. The two most common areas where we see dermal matrices as a wound dressing is in the treatment of burns and chronic ulcers (diabetic foot, venous leg, and pressure ulcers).

The skin is the largest organ of the human body. It is the first line of defense against infection, prevents dehydration and helps regulate body temperature through changes in blood flow and sweat production. Skin loss can occur for many reasons including: genetic disorders, acute trauma, chronic wounds or even surgical interventions, but one of the most common

reasons for skin loss is thermal trauma. Severe thermal trauma at a molecular level constitutes a disruption in the native structures of proteins and other macromolecules. When proteins come into contact with enough heat, the intermolecular bonds are broken and proteins denature into a lower energy configuration from which it may not spontaneously return to its native conformation [6]. This type of burn is defined as a full-thickness burn and is characterized by the complete destruction of epithelial-regenerative elements. Severe burns often result in the patient being unable to regenerate his skin. Thus full-thickness wounds, if left untreated, would 'heal' by contraction, leading to cosmetic and functional defects. Currently, the clinical 'gold standard' in full-thickness injury treatment is split-thickness autologous skin grafting [7]. This involves harvesting epidermis with a superficial part of the dermis from an undamaged skin donor site and applied to the full-thickness wound.

Each year 696,000 people in the United States visit the emergency room due to burns [8], which results in 50,000 hospitalizations [9]. Consider a patient with 50% total body surface area (TBSA) full-thickness wounds. This patient would only have 50% of undamaged skin left that could be used for split-thickness skin harvesting. The harvesting of skin from the donor would add to the total wound size and result in a wound area covering 100% of the body. In extensive injuries, donor sites may be extremely limited and alternative approaches must be explored. The use of acellular dermal matrices as a wound dressing has seen significant success in the treatment of burn victims [10, 11, 12, 13, 14].

The United States consistently ranks third on the list of countries with the highest diabetic populations. It is estimated that there were as many as 17.7 million cases in 2000 and it is projected to be around 30.3 million cases by 2030 [15]. Those afflicted with diabetes face a lifetime risk as high as 25% of developing a foot ulcer [16]. Diabetic wounds of the lower extremity are

slow to heal and have a significant impact on the patient quality of life. It is estimated that it costs approximately $28,000 to treat a diabetic foot ulcer during the 2 years after diagnosis [17]. Dermal matrices are used, not only for diabetic ulcers, but also for similar chronic wounds such as pressure and venous leg ulcers. Figure 1 below shows the application of the dermal matrix Apligraf to a venous leg ulcer. If a foot ulcer is left untreated, it could result in amputation. Dermal matrices have also shown success in treating diabetic lower extremity wounds that are unresponsive to traditional wound management therapies [7, 11,18].

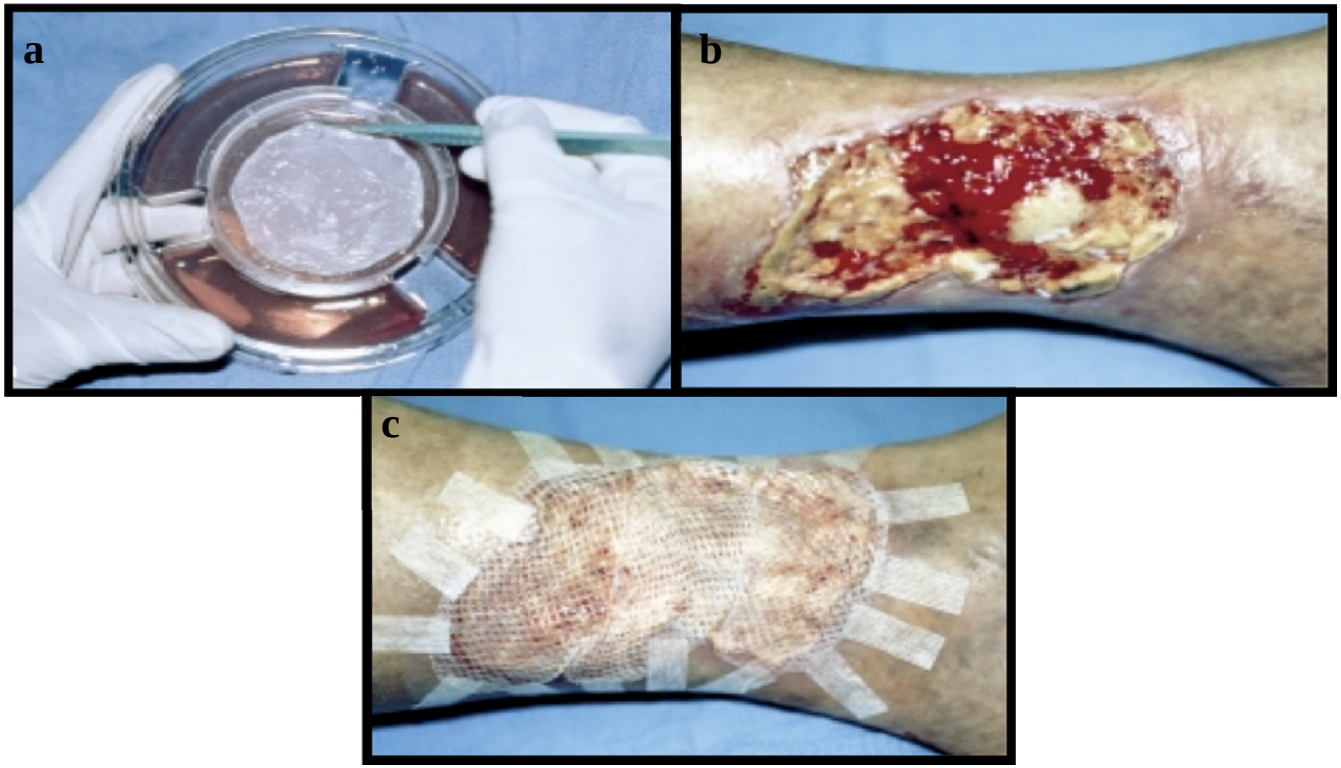

Figure 1: (a) The white disk is the Apligraf matrix suspended in nutrient medium. (b) Venous leg ulcer prior to Apligraf application. (c) Venous leg ulcer after Apligraf applicaiont.[19]

Tissue Replacement: Cellular dermal matrices, matrices containing autologous cells, have been used in a number of procedures that aim to replace target tissues. Cellular matrices have also been used to treat burns, chronic leg ulcers, pressure ulcers, and corneal replacements [19]. The advantage of a matrix containing cultured cells from the patient is the provision of permanent wound coverage, a decreased requirement for donor sites, rapid coverage of the wound, faster pain relief, and a better functional and cosmetic outcome. The disadvantages are the requirement for a skin biopsy, a 3 week delay for *in vitro* cell cultivation, and high cost.

Acellular dermal matrices can also promote cellular ingrowth ***in situ.*** These matrices, over time, induce a cellular response from the recipient which allows for the matrix to become integrated into the patient. The matrix is said to be 'remodeled' when local cells proliferate with in the matrix. Cellular matrices allow for constructive remodeling of the matrix. The host cellular response that these matrices induce allow for the repair and reconstruction of the lower urinary tract, dura mater, esophagus, muculoteninous structures, and blood vessels [20].

Reconstructive Surgery: It is becoming more common to see dermal matrices being used in reconstructive surgeries. The dermal matrix is used as a soft tissue implant in a number of surgeries including: skin cancer excision, scar revision, oral resurfacing, cleft palate repair, oculoplasty, hernia repair, and breast reconstruction [21].

Dermal matrices have become popular for hernia repair and it is estimated that approximately 200,000 ventral hernias of the abdominal wall are repaired annually in the United States [22]. Disruption of the abdominal wall and loss of its integrity may result from traumatic injury, infection, or surgical incision or resection. The most commonly used method for the treatment of a hernia involves the use of synthetic implants [23]. Synthetic implants, such as the commonly used polypropylene mesh, causes adhesions that can lead to bowel obstruction,

perforation, infertility, and chronic pain [22]. Health care professionals have started to adopt the use of biologically derived dermis for use in hernia repair due to some of the shortcomings of the current method. Biologically derived dermal matrices have been shown to cause fewer bowel adhesions and be a viable option for use in hernia repair. Alloderm, a freeze-dried extracellular tissue matrix derived from donated cadaver skin, has been extensively used in abdominal wall and breast reconstruction repairs [24]. Figure 2 shows Alloderm used to repair an abdominal wall fascial defect.

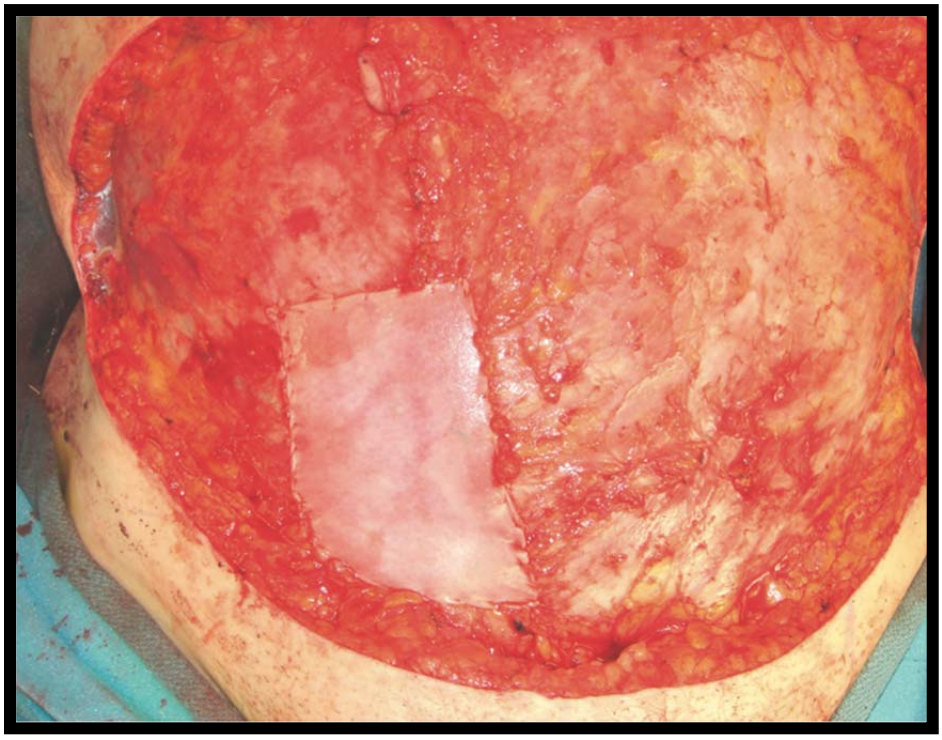

Figure 2: Repair of abdominal wall fascial defect using Alloderm [18].

In 2008, approximately 182,460 women were diagnosed with breast cancer in the United States [25]. Many of those patients either chose or required a mastectomy. Dermal matrices have become popular for use in breast reconstruction procedures. In fact, there has been nearly a decade of experience with dermal matrices to assist with implant-based, post-mastectomy breast reconstruction and revisionary breast surgery [26]. The most common breast reconstruction procedure, after mastectomy, is tissue expansion and placement of implants. Dermal matrices have garnered popularity for use in breast reconstruction because of its mechanical and cellular properties. Dermal matrices, serving as a scaffold for cellular ingrowth and revascularization, allows for ready incorporation of the graft with minimal inflammation. Additionally, the high tensile strength and low modulus of elasticity allows tension to be applied to the graft during expansion without graft failure [26]. Furthermore, dermal matrices have been shown to be safe for prosthetic reconstruction considering the low complication, revision, and explant rates. Another advantage is that dermal matrices achieve aesthetic outcomes that more closely resemble patients' natural breast [26, 27, 28, 29].There are a large number of commercially available dermal matrices on the market (Table 1).

Table 1: Currently commercially available dermal matrices. allo,allogeneic; xeno, xenogeneic; synth, synthetic. Note: all cell seeded scaffolds were derived from an allogeneic cell source.

brand name/manufacturer	graft type		scaffold source	scaffold material
	cell-free	cell-seeded scaffold		
AlloDerm LifeCell Corporation, Branchburg, NJ, USA	x		Allo	human acellular dermis
Integra Dermal Regeneration Template Integra neuroSciences, Plainsboro, NJ, USA	x		xeno+synth	polysiloxane, bovine cross-linked tendon collagen, GAG
BioBrane/BioBrane-L UDL Laboratories, Inc., Rockford, IL, USA	x		xeno+synth	silicon film, nylon fabric, porcine collagen
TransCyte (Dermagraft TC) Advanced BioHealing, Inc., New York, NY and La Jolla, CA, USA		x	xeno+synth	silicon film, nylon mesh, porcine dermal collagen
Apligraf Organogenesis Inc., Canton, Massachusetts, CA,USA		x	Xeno	bovine collagen
PolyActive HC Implants BV, Leiden, The Netherlands		x	Synth	polyethylene oxide terephthalate/polybutylene terephthalate

1.3: PROPERTIES OF AN IDEAL MATRIX

Despite all efforts, there is currently no dermal matrix on the market that is perfect. From a clinical standpoint, the tissue must be safe for the patient, be clinically effective, and be convenient in handling and application. Furthermore, an ideal substitute must not be toxic, immunogenic or cause excessive inflammation, and should have no or a low level of transmissible disease risk. Additionally, the matrix should retain its structural integrity and be biocompatible. The substitute should be able to support the reconstruction of normal tissue, with similar physical and mechanical properties to the target tissue. The substitute should also be cost-effective, readily available, user-friendly and possesses a long shelf life [7, 18, 30, 31].

1.4: DERMAL MATRICES AND THE IMMUNE RESPONSE

It wasn't until the 1960's that any serious attempts to utilize xenografts clinically were reported [32]. In these experiments, chimpanzee kidneys were transplanted into patients with renal failure [33]. The term xenograft refers to a transplant of tissue between animals of different species. When xenografts were first being used in humans, a number of immunological barriers to the implants success were identified. As advances in immunology were made, the field of xenotransplantation grew. We begin to see porcine skin being grafted onto burn patients as early as 1978 [34].

Pigs are phylogenetically more unrelated to humans than non-human primates. Because the evolutionary distance between a pig and human is so vast, the immunologic barriers are more difficult to overcome than human to non-human primate. In fact, *in vitro* studies have shown that the immune response to xenogeneic cells is more vigorous than that elicited by allogeneic cells [35]. Although non-human primates are phylogenetically closer than other species to humans, they are not considered to be a suitable source for clinical xenotransplantation because of ethical

issues, high risk of cross-species transmission of diseases to humans, difficulties in breeding, and organ size disparities [36]. Pigs are considered by many researchers to be the preferred source animal species for xenotransplantation. Additionally, pigs have similar organ size and physiology to humans.

Based on experimental findings in which untreated pig tissues were implanted into nonhuman primates, a three-phase rejection process develops. The established nomenclature of the different types of xenograft rejection includes hyperacute rejection (HAR), acute vascular rejection (AVR) and its major component acute humoral xenograft rejection (AHXR), and chronic rejection. This nomenclature refers to the time course and predominant mechanism of rejection. HAR develops within minutes to hours after transplantation and can destroy the transplant in a short period of time [37]. HAR is the first barrier to xenotransplantation and if it is somehow overcome, there may still be other less aggressive processes that work to reject the implant in a matter of days such as AVR/AHXR. This process is characterized by the progressive infiltration of xenograft by natural killer cells and macrophages, the activation of endothelial cells, and by the marked aggregation of platelets [38]. Furthermore, if AVR/AHXR can be averted, the cell-mediated rejection becomes active. This process resembles the rejection process of allotransplantation since it is associated with the structural differences of major histocompatibility (MHC) molecules between different species [39].

The innate immune system, along with natural antibody-producing B cells, natural killer (NK) cells, macrophages and complement, as well as adaptive immune responses from T and B cells are responsible for rejection of xenografts.

Hyperacute Rejection: In the pathogenesis of HAR, xenoreactive antibodies react with antigens of the non-primate mammal's and New World monkey's endothelium and lead to the

destruction of the implant via the activation of the complement cascade. Histologically, HAR is characterized by diffuse interstitial hemorrhage, edema and thrombosis of small vessels. The primary antigen recognized by these antibodies is the Galα1-3Galβ1-4GlcNAc (α-gal) oligosaccharide, which is abundantly expressed on the endothelial cells of non-primate mammals and New World monkeys. The antibodies recognizing α-gal are termed anti-α-gal. Inconveniently, the xenoreactive antibodies naturally occur in high concentrations in humans, apes, and Old World monkeys [37].

Humans and nonhuman primates do not express the α-gal epitope on their cells, while the majority of animals studied to date do. The absence of α-gal expression in humans and nonhuman primates is related to defects of the α1,3galactosyl-transferase gene that catalyzes the assembly of the α-gal molecule in other animals [37]. Since bacteria as well as other pathogens express α-gal, it is assumed that the appearance and high concentration of these xenoreactive antibodies may be related to the defense against bacteria [40]. It is interesting to note that these xenoreactive antibodies are not present at birth, but develop a few weeks or months thereafter [41], possibly in association with the bacterial colonization of the gastrointestinal tract. Thus the presence of xenoreactive antibodies is advantageous for the immunity against bacteria, but detrimental in xenotransplantation.

Approximately 1% of circulating immunoglobulins in humans (both IgG and IgM) are xenoreactive antibodies [35]. Human Immunoglobulin G (IgG) is a class of antibodies that makes up approximately 75% of serum immunoglobulins in humans and is the most abundant antibody found in circulation. Other classes of antibodies include: IgA, IgD, IgE, and IgM. IgG molecules are synthesized and secreted by plasma B cells and bind to antigens to protect the host from infection. Human IgG is known for its ability to bind to a diverse set of antigens, but presents a

challenge in xenotransplantation. These antigens view the foreign tissue as a threat and works to induce rejection of the tissue. Because human IgG contains a high percentage of "natural antibodies" to porcine tissues and antigens including α-gal, it is important that decellularization methods used to treat porcine dermis remove these antigens in order to prevent a hyperacute rejection process against the tissue after implantation.

Acute Xenograft Rejection: For those xenografts that manage to overcome HAR, there may still be a second phase of rejection that typically occurs within days, referred to as AVR. In this phase of rejection, elicited xenoreactive antibodies directed at non-α-gal xenoantigens play a major role, in concert with activated complement and coagulation systems (AHXR) [42]. The pathological mechanism of AVR resembles that of HAR, but usually includes infiltration by innate immune cells such as NK cells, macrophages, and neutrophils. Xenoreactive T cells are known to contribute to rejection by participating in the T-Cell-dependent elicited B-cell antibody response to α-gal and non-α-gal xenoantigens.

AVR/AHXR can be induced by the presence of very low levels natural and elicited antibodies. Additionally, it has been shown that AHXR can develop in primates that receive organs from α-gal-deficient pigs, which indicates that xenoreactive antibodies specific for non-α-gal antigens can also cause this type of rejection [36].

Chronic Xenograft Rejection: The phenomenon of chronic rejection in xenografts is not well known due to the difficulties in overcoming acute forms of rejection. Studies have found that incompatibilities between the recipient coagulation proteins and porcine endothelial cell-associated anticoagulant components may result in chronic procoagulant state of the xenograft endothelium, and is thought to play a role in chronic xenograft injury [43, 44]. The mechanisms and actions of the various types of rejection are shown in figure 3.

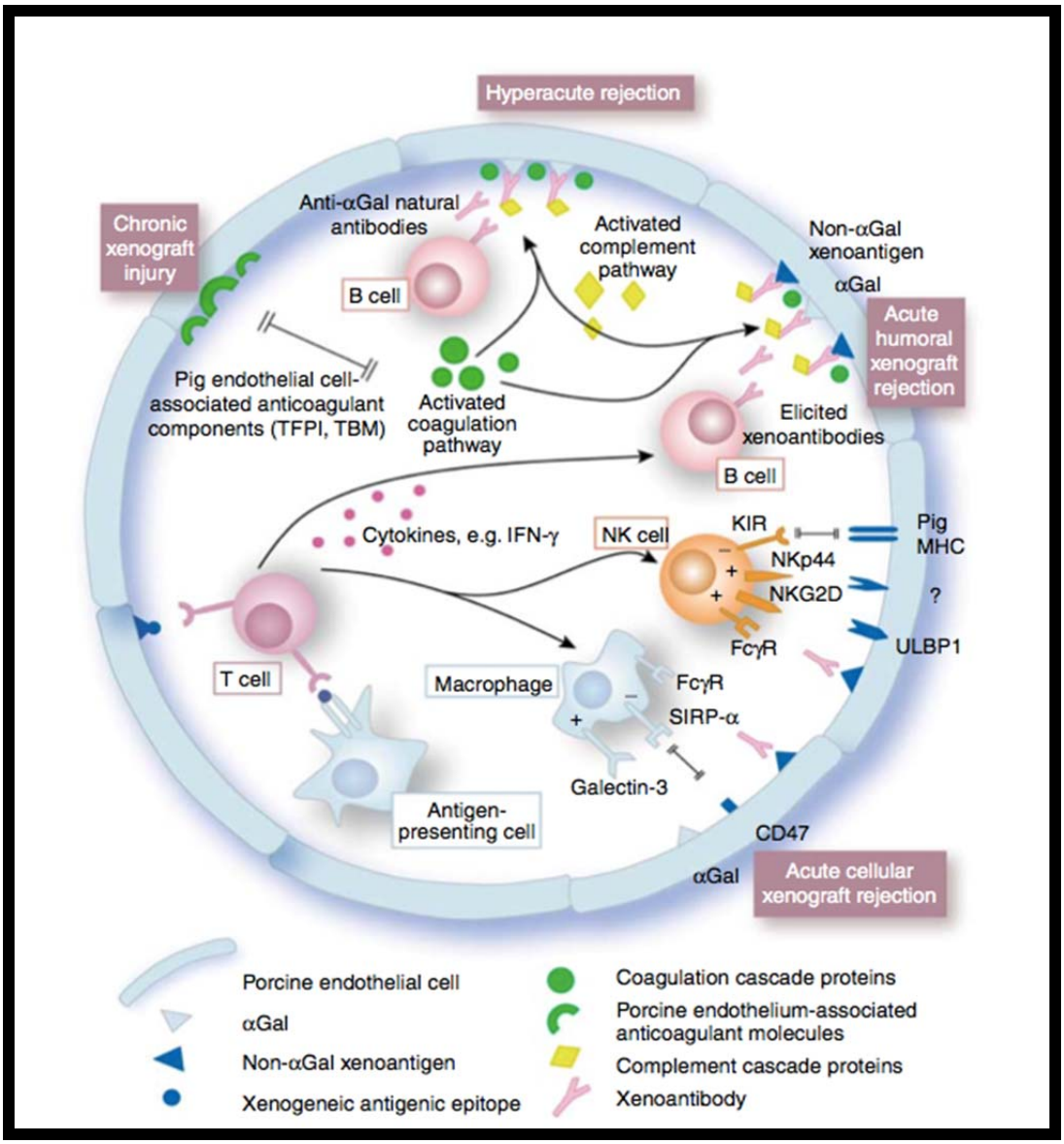

Figure 3: Current understanding of the mechanisms involved in rejection of porcine xenografts in non-human primates [35].

Humoral rejection: Hyperacute rejection (HAR) is mediated by antibodies directed against α-gal. When anti-α-gal antibodies bind, complement and coagulation systems are activated and lead to endothelial damage and thrombosis. Elicited xenoreactive antibodies directed to α-gal and non-α-gal xenoantigens are central in the development of acute humoral xenograft rejection (AHXR). Cellular rejection: Binding of xenoreactive antibodies to endothelial cells results in antibody dependent cell-mediated cytotoxicity by natural killer (NK) cells and macrophages. Acute vascular rejection (AVR) is mediated by a combination of AHXR and cellular mechanisms. Lastly, incompatibilities between the recipient coagualtion proteins and porcine endothelial cell associated anticoagulant components are believed to result in a chronic procoagulant state of the xenograft endothelium, and is thought to play a role in chronic xenograft injury.

Natural vs. Specific Immunity: The immune system is composed of two major subdivisions, the non-specific immune system (natural immune system) and the specific immune system. The natural immune system is the first line of defense against invading pathogens while the specific immune system acts as a second line of defense and protects against re-exposure to the same pathogen. Although both these immune systems function to protect against invading organisms, they differ in a number of ways. One of the major differences between the two systems is reaction time. The natural immune system, for the most part, is constitutively present and ready to be mobilized upon infection. In other words, the natural immune system has a fast reaction time. The specific immune system, however, requires some time to react to a pathogen. Another major difference is the specific immune system is antigen specific, and as such only reacts with the organism that induced the response. Conversely, the natural immune system is not antigen specific and reacts to a diverse set of antigens.

1.5: DECELLULARIZATION OF PORCINE DERMAL MATRICES

It is important that xenografts be treated in a manner to make them nonimmunogenic when implanted into a human. A successful decellularization treatment would eliminate graft rejection. As described in the previous section, if untreated xenografts were to be put in a human, the implant would undergo hyperacute, acute, and chronic immune rejection. The main goal of treating a xenograft is to remove not only the cells, but also the antigens that would elicit an immune response.

Decellularization vs. Antigen Removal: Decellularization is a term that refers to a process by which cells are removed from a tissue. Initial attempts at decellularization focused on cell removal, but recent studies have demonstrated inadequate removal of antigens in tissues following decellularization [45, 20, 46, 47, 48, 49, 50, 51]. As discussed earlier, most antigens are located

on the cell membrane, thus it would be best to remove cells and thereby reduce the probability of a graft being rejected. By removing the cells, we are essentially removing the incompatibility of the tissue because xenoantigens are located on the cells. After removing the cells, the matrix proteins that are left are those that have been conserved in nature and will not differ significantly between species. Although a tissue may appear to be devoid of cells, cellular debris containing antigens may be spread throughout the tissue. The inability to remove antigens would result in an immune response upon implantation. Current decellularization approaches work under the assumptions that xenoantigens are only found within the cell and removal of cells as determined under light microscopy is equivalent to bioscaffold non-immunogenicity. As a result of recent findings, the focus has shifted from decellularization to antigen removal in the production of dermal matrices. By focusing on antigen removal rather than cell removal, investigators are better able to produce bioscaffolds with less immunogenicity. An antigen removal approach focuses on removing the barriers to a successful xenotransplant, the antigens. Antigen removal targets the major immunogenic components including cells and their lipid membranes, and membrane associated antigens [52].

Current Decellularization Methods: In an effort to overcome antibody-mediated xenograft rejection, a standard decellularization method has been developed by dermal matrix manufacturers. The main goal of this process is to remove or inactivate the antibodies responsible for causing the rejection while maintaining the structure and function of the source tissue. The process of xenogenic graft decelluarization first starts with the harvesting of the tissue. Common tissue sources include: bovine pericardium, porcine small intestine submucosa, or porcine dermis.

The standard decellularization process in the literature follows three main steps. The first step in the process is osmotic lysis of cells within the tissue. Lysis is performed using sterile water, a hypotonic/hypertonic or detergent solution [53, 54, 55]. The osmotic solutions have a dual purpose, they work to lyse cells, but also help rinse cell residue from within tissue following lysis. Next, detergents are used to solubilize cell membranes and dissociate DNA from proteins. The final step involves a wash out to remove any residual cellular elements or chemicals. Currently, this method requires multiple days to achieve greater than 90% decellularization.

The most common detergents used are sodium dodecyl sulfate (SDS), Triton-X 100, and sodium deoxycholate. SDS is a highly ionic detergent with an anionic hydrophobic ligand and works to solubilize parts of the cell that are not water soluble. SDS appears to be more effective than Triton X-100 for removing nuclei from dense tissues and organs while preserving tissue mechanics [56, 57]. The use of SDS in a decellularization protocol can make the difference between complete and incomplete cell nuclei removal [58].

Some manufacturers cross-link their products after decellularization using either gluteraldehyde or hexamethylene diisocyanate. Crosslinking creates links between individual strands of collagen and works to stabilize the implant and prevent destruction by collagenase, but renders the matrix nonresorbable [59]. The advantage of crosslinking is that it stabilizes the collagen implant to prevent tissue destruction by inflammatory cells. A disadvantage to the crosslinking process is that the rejection response may not be completely eliminated. Additionally, crosslinking prevents natural ingrowth by native cells, thereby retarding remodeling and ultimately leading to graft breakdown. Crosslinking may produce bonds that are very short and inflexible between collagen strands. These short bonds may inhibit cell migration and vascular regeneration, while residual chemicals in the product may produce an inflammatory

response causing the matrix to be nonresorbable. Crosslinking helps to decrease the immune response by the host, but would not allow any cells to proliferate within the graft.

There is growing concern within the xenotransplantation community regarding the immunogenicity of decellularized xenogeneic matrices. Several independent studies have shown that the current decellularized dermal matrices are subject to immune mediated rejection [20, 46, 47, 48, 49, 50]. This suggests that the gold standard method of decellularization may not be as effective at preventing an immune response as once thought.

Rejection of decellularized matrices may have arisen due to a focus on removal of viable cells instead of identification and removal of antigenic proteins. As it stands, dermal matrix manufacturers characterize a tissue as successfully decellularized based on light microscopic or histological examination. As mentioned earlier, elimination of cells from a tissue does not assure adequate removal of xenoantigenic proteins, nor does it mitigate an immune response. There is abundant evidence in the literature that decellularized xenografts elicit an immune response [60, 61, 62, 20].

A notable example of a decellularized xenograft matrix being rejected occurred in 2001. Synergraft, an acellular, unfixed porcine aortic valve-based construct, decellularized using the gold standard method was implanted into four male children varying in age from 2.5 to 11 years. Three children died, two suddenly after 6 weeks and 1 year after implantation due to a severely degenerated implant. The third died on the 7th day due to Synergraft rupture. The fourth graft was explanted 2 days after implantation. All four grafts showed severe inflammations that eventually lead to structural failure. Histology demonstrated a severe immunological response[48]. The deaths of these children bring to light the need for a decellularization method that not only removes cells, but also ensures the removal of protein antigens.

Tissue Electrophoresis: Tissue electrophoresis is a novel method for decellularization or antigen removal of xenogeneic scaffold tissues. Tissue electrophoresis (TE) is essentially a four-step method which involves hypertonic 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. The basis for this method is similar to that of SDS-polyacrylamide gel electrophoresis (PAGE). The SDS binds and imparts a uniform negative charge to proteins, which in turn causes them to migrate predictably in an electric field. By actively 'pulling the cells' out of the tissue, we can expedite the entire process. Previous studies have demonstrated 'proof of concept' of TE as a decellularization method for human dermis and aortic valves [51]. This novel method actively 'pulls' cells and their associated antigens out of the target tissue.

CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

The overall goal of this research is to evaluate tissue electrophoresis as a method of decellularization or antigen removal porcine dermal matrix for human applications. To that end, I hypothesize that tissue electrophoresis enhances the removal of extractable soluble protein and protein antigens and antigenicity compared to standard detergent-based decellularization methodologies. The specific aims of this study were:

1. To compare the efficiency of soluble protein removal in porcine dermis between a standard detergent decellularization with passive washout and a tissue electrophoresis (TE)-based decellularization protocol by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
2. To compare the efficiency of protein antigen removal from porcine dermis between standard detergent-based and TE-based decellularization protocols based on immunoblot analysis with rabbit anti-porcine immune serum (acquired immunity) or human IgG (natural antibodies).

CHAPTER 3: EXPRIMENTAL DESIGN AND METHODS

3.1: EXPERIMENTAL DESIGN - GROUPS AND NUMBER OF REPLICATES

Porcine skin was collected from 6 pigs and divided into 4 treatment groups with each pig contributing a portion of skin to each treatment group. Four treatment groups each consisting of 5 x 5 cm sections of porcine skin were compared to evaluate the efficacy of protein and antigen removal. Each pig contributed one section of skin to each treatment group (n = 6 / treatment group). The 4 treatment groups were:

1. Epidermis removal without SDS (positive or untreated control)
2. Epidermis removal with 0.5% SDS (epidermis removal only control)
3. Epidermis removal 0.5% SDS and standard 0.5% SDS decellularization treatment with 6 h passive diffusion washout period
4. Epidermis removal 0.5% SDS and Tissue Electrophoresis-based decellularization (0.5% SDS, 2% agarose gel and 0.5 Amp) for 6 h

3.2: STATISTICAL ANALYSIS

Evaluations of overall differences between treatments (based on optical densities of gels) were determined by repeated measures ANOVA with a p-value < 0.05 considered significant. Pairwise comparisons were be determined using the Tukey's test to determine differences across treatment pairs.

Additionally, a two-way ANOVA was performed with the two independent factors being pig and treatment. A p-value < 0.05 was considered significant.

3.3: PORCINE DERMIS DECELLULARIZATION TREATMENT METHODS

Epidermis Removal: The treatment groups all underwent the same epidermis removal protocol except that Group 1 did not include SDS in the treatment solution. Porcine skin was

harvested and the subcutaneous tissues, fat, and hair were removed using sharp surgical dissection. Samples were placed in a solution consisting of 0.5% sodium dodecyl sulfate (SDS) (w/v) in Hypertonic Epidermis Removal Solution (1 M NaCl, 10 mM Tris pH 7.6, 1 mM EDTA disodium salt, 1% antibiotic/antimycotic, 0.05% Halt Mammalian Protease Inhibitor). The submersed skin remained in a large flat sterile container with SDS-Hypertonic Solution and was treated for 24 h at room temperature with 150 rpm agitation. After the treatment period, the tissue was removed from the solution and the epidermis separated from the dermis with a scalpel.

SDS-Based Decellularization (Passive): Group 3 tissues underwent a SDS based decellularization protocol similar to standard decellularization treatment of porcine dermis for commercial surgical implants. After the epidermis was removed, tissue samples were soaked in diluted 1X Tris base, Acetic acid, and EDTA (TAE) buffer containing 0.5% SDS. The samples were allowed to soak at room temperature for 6 h at 150 rpm agitation.

Tissue Electrophoresis: Group 4 tissues underwent TE decellularization. The samples from this treatment group were placed in a solution of 2% agarose gel with 0.5% SDS (w/v) in diluted (1X) TAE buffer. The volume of the gel solution was approximately a 10:1 ratio of gel solution to tissue volume. The solution was slowly heated to 85 °C until the agarose was completely dissolved. The dermis tissue was blotted dry and placed in warm (50 °C) agarose gel in an appropriate size mold. The tissue-gel complex was allowed to solidify for 1 h at 4 °C. The tissue-gel complex was then placed between filter papers and blotting pads (enough to fit tightly between the electrodes). The sandwich was placed in a horizontal (Western blot) electrophoresis chamber. The chamber volume was filled with running buffer solution consisting of diluted (1X) TAE buffer. The chamber was then closed and the leads placed in the power supply. The power supply was run for 6 h at a constant current of 0.35 A. After electrophoresis was completed, the

tissue-gel complex was removed from the chamber and the tissue physically separated from the gel.

Protein Extraction: Treated tissues were rinsed in phosphate-buffered saline (138 mM sodium chloride, 2.7 mM potassium chloride, 10mM sodium phosphate dibasic, 1.8mM annhydrous potassium phosphate monobasic, pH 7.4), then blotted dry and weighed. These weights were used to calculate the volume of lysis solution that was used (4 mL of lysis solution for each gram of tissue) and was composed of 50 mM Tris HCl pH 8.0, 1X Halt protease inhibitor cocktail, 1 mM dithiothereitol, 2 mM $MgCl_2$, 1 mM Pefabloc, 0.5% SDS, 0.5% CHAPS, and 10 % glycerol. The tissue was finely minced into small pieces (~2 mm^2) and combined with approximately 1.5 mL of glass beads per gram of tissue. The appropriate volume of lysis solution was then added to the tubes containing the minced tissues and glass beads. Tubes were stirred for 1 min and cooled on ice for another minute. This stir and cool process was repeated for a total of 4 times. Tubes were then incubated horizontally at -4°C for 24 h. At the end of the cold incubation, samples were centrifuged at 7,500 xG at 2°C for 10 min. The protein supernatants was recovered and transferred to a 3 kDa molecular weight cut off (MWCO) concentrator tubes. Tubes were then centrifuged for 1.5 h at 15,000 x G at 2°C. The final protein solution was collected, labeled, and stored in -80°C until further use.

SDS-PAGE and Staining: Equal volumes of samples were prepared for SDS-PAGE by mixing 1:1 with sample buffer (63 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.0025% bromophenol blue). Then β- mercaptoethanol was added to a final concentration of 5%. Samples were heated to 95 °C for 3 min, and cooled down. Samples were then carefully loaded onto a 4-12% polyacrylamide gradient mini-gel. Gels were placed in the electrophoresis chamber, isolating cathode from anode. Both electrodes were covered with running buffer (25 mM tris

base pH 8.3, 192 mM glycine, 0.1% SDS). Electrophoresis was run at 135 V and 35 mA for 90 min and inspected for migration of the blue dye to the bottom of the gel. After completion, gels were silver stained as follows. Protein bands were fixed with 100 mL fixation solution (10% glacial acetic acid and 40% ethanol) for 30 min. Bands were then modified with 100 mL sensitizing solution (0.2% sodium thiosulfate, 6.8% sodium acetate, and 30% ethanol) for 30 min. Gels were then washed in ultrapure water 3 times for 5 min. Protein bands were further treated with 100 mL silver reagent (2.5% silver nitrate) for 20 min. Gels were once again washed (twice, 1 min each). Development of the brown color was performed in approximately 5 min with 100 mL developing solution (2.5% sodium carbonate and 0.02% formaldehyde). Color development was stopped with 100 mL of 1.5% EDTA disodium salt. Gels were stored at 4 °C in EDTA solution until imaging. All percentages above are weight per volume.

Immunoblot Analysis with Human IgG: The goal of Human IgG immunoblot analysis was to evaluate natural antibodies that humans have to porcine tissue. This assay would give us a qualitative and quantitative idea of the immunological response a human would have to each of the differently treated porcine tissues.

Samples underwent SDS-PAGE as described in the previous section without the silver staining. After completion of electrophoresis, gels were removed from the chamber and prepared for blotting against nitrocellulose membranes. Protein bands were transferred at 25 V for 2 h in transfer buffer (25 mM Tris base pH 8.3, 192 mM glycine, 20% methanol). The membrane containing protein bands underwent the appropriate immunoreactions as follows. The membrane was first rinsed twice with ultrapure water for 5 min with rotary shaking. Incubate the blot with the primary antibody with gentle shaking for 1 h. The primary antibody (Human IgG) was diluted 1:75 in a solution of 1% instant nonfat dry milk in TBST (10 mM Tris HCl pH 8.0, 150

mM NaCl, 0.025% Tween 20) was added to the blot for 24 h. After incubation the membrane was washed 3 times with TBST for 5 min each with rotary shaking. This was followed by incubating the blot with the secondary antibody (anti-human immunoglobulin G conjugated with horseradish peroxidase) diluted 1:10,000 in TBST for 1 h with rotary shaking. The membrane was washed 3 times with TBST for 5 min each with rotary shaking. Followed by a 5 min wash in ultrapure water. The chimiluminescent reagent (1:1 mix of luminol/enhancer and hydrogen peroxide solution) was added and incubated in the dark for 5 min. The membrane was then imaged in a chemiluminsecent imager.

Production of Rabbit Anti-Porcine Immune Serum: Immunized rabbit serum was generated using two adult New Zealand White rabbits. These rabbits were purchased and acclimated at the Laboratory Animal Resources at Colorado State University for 7 days. Approximately 2 g of untreated porcine tissue were manually minced and homogenized in 10 mL of 10 mM Tris-HCl ph 7.6, 1 mM dithiothreitol, 2 mM magnesium chloride, 10 mM potassium chloride, 1 mM Pefabloc SC, on ice for 1 min. Protein extract was filtered to prevent microbial contamination and had its pH reduced to 7.0 after tissue lysis. A protein extract of approximately 5 mg/mL was diluted 1:1 with Freund's adjuvant for the rabbit vaccinations (complete Freund's adjuvant was chosen for the initial inoculation and incomplete Freund's adjuvant for the boosters). Rabbits were immunized and booster immunizations were planned every 14 days until day 70 (day 0, 14, 28, 42, 56, 70). Approximately 100 mL of blood per rabbit was collected after rabbit euthanasia at the end of the vaccine boosters. Serum was separated using serum activator blood collection tube and stored in aliquots at -80 °C until used.

Immunoblot Analysis with Rabbit Anti-Porcine Immune Serum: Samples underwent similar immunoblot treatment as Human IgG except that the primary was diluted to 1:1000 rabbit

polyclonal serum in 5% milk in TBST and the anti-rabbit secondary was dilute 1:10,000 in 1% milk in TBST for 1 h. Protein band intensity was quantified using optical density measurements of gray pixels by ImageJ software.

CHAPTER 4: RESULTS

4.1: SDS-PAGE

To determine the effectiveness of each treatment in removing soluble protein, a SDS-PAGE was run on tissue from each treatment group. SDS-PAGE analysis of extractable soluble protein showed qualitative (Figure 4) and quantitative (Figure 5) differences in the intensity of the bands between treatment groups. Treatment groups undergoing epidermis removal only (Groups 1 & 2) showed higher levels of extractable protein compared to treatment groups undergoing a decellularization protocol (Groups 3 & 4). Importantly, porcine dermis undergoing TE decellularization treatment (Group 4) showed greater decrease in extractable soluble protein compared to porcine dermis treated with a standard SDS detergent-based decellularization and passive washout (Group 3) suggesting that TE was more efficient at removing soluble proteins compared to standard decellularization methods in the same time period.

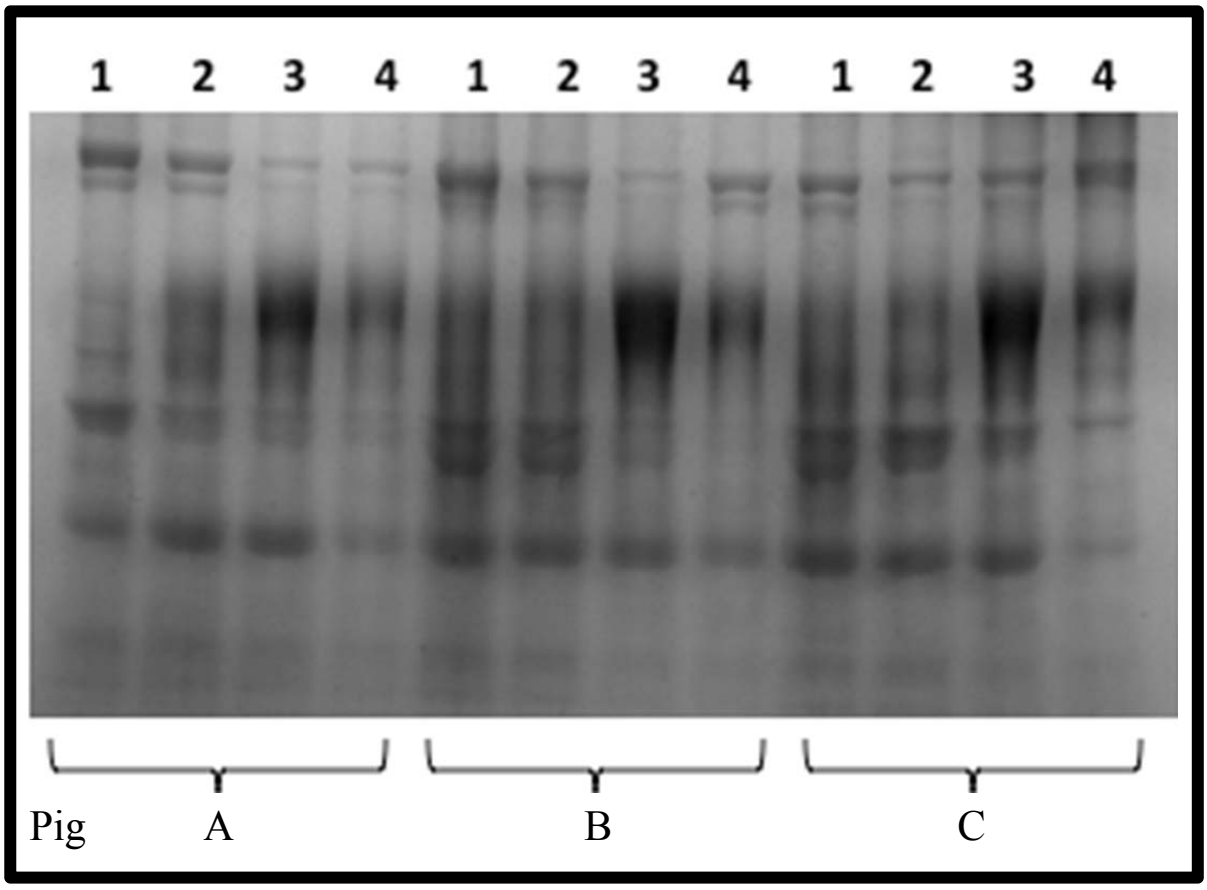

Figure 4: SDS-Page of soluble proteins remaining in tissues after the various treatments.

Treatment Groups:
1 - Untreated Control
2 - Epidermis Removal Control
3 - Passive Decellularization
4 - Tissue Electrophoresis

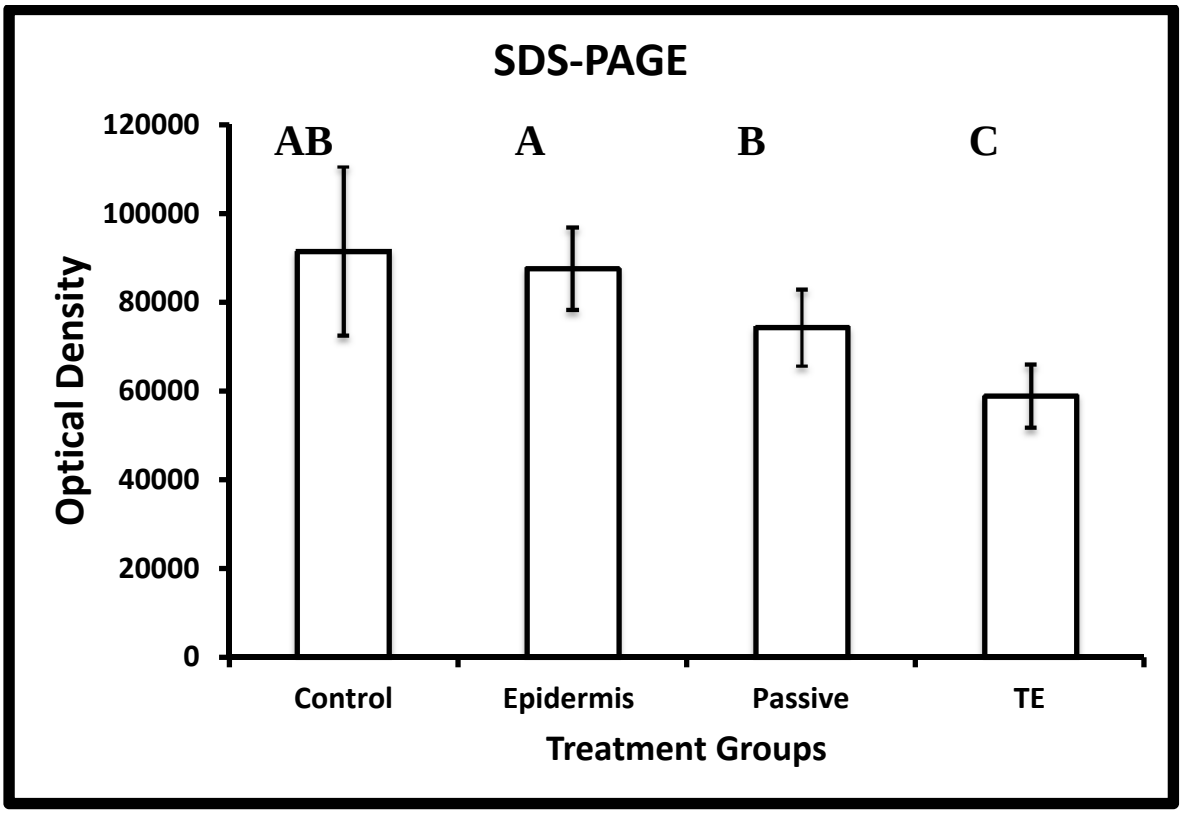

Figure 5: SDS-PAGE Bar graph represents the mean and standard errors for band densities. Means with different letters are significantly different (Tukey's test, $p < 0.05$) (n=6)

4.2: RABBIT ANTI-PORCINE SERUM IMMUNOBLOT (ACQUIRED IMMUNITY)

Based on immunoblot analysis (Figures 6 & 7), both standard SDS detergent-based (Group 3) and TE-based decellularization (Group 4) increased the removal of protein antigens capable of generating an acquired immune response in rabbits compared to the untreated positive control porcine dermis tissues (Group 1). A post-hoc power analysis found the power to be 0.13 (delta = 0.5 and alpha = 0.05).

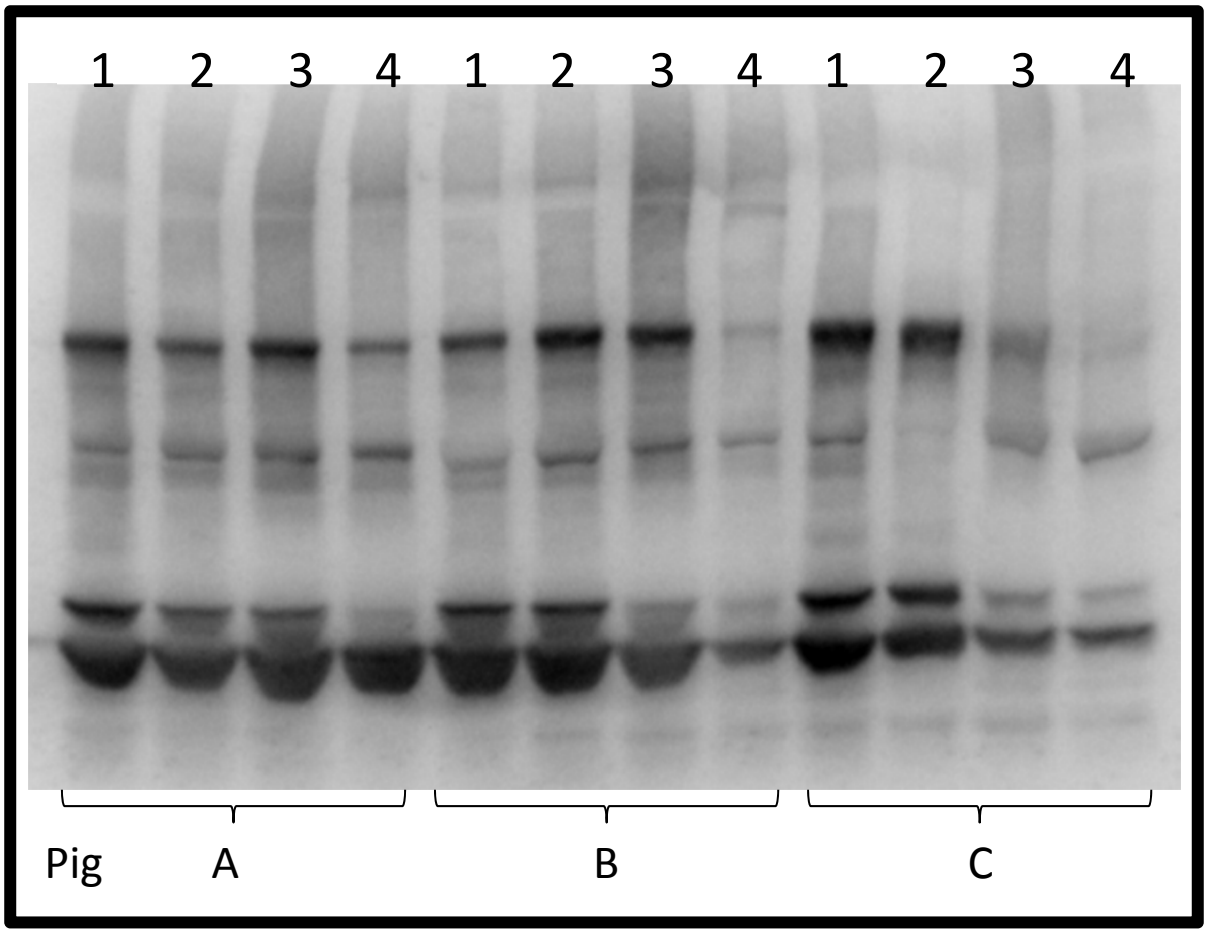

Figure 6: Rabbit polyclonal immunoblot showing antigenic protein from the various treatment groups.

Treatment Groups:
1 - Untreated Control
2 – Epidermis Removal Control
3 - Passive Decellularization
4 - Tissue Electrophoresis

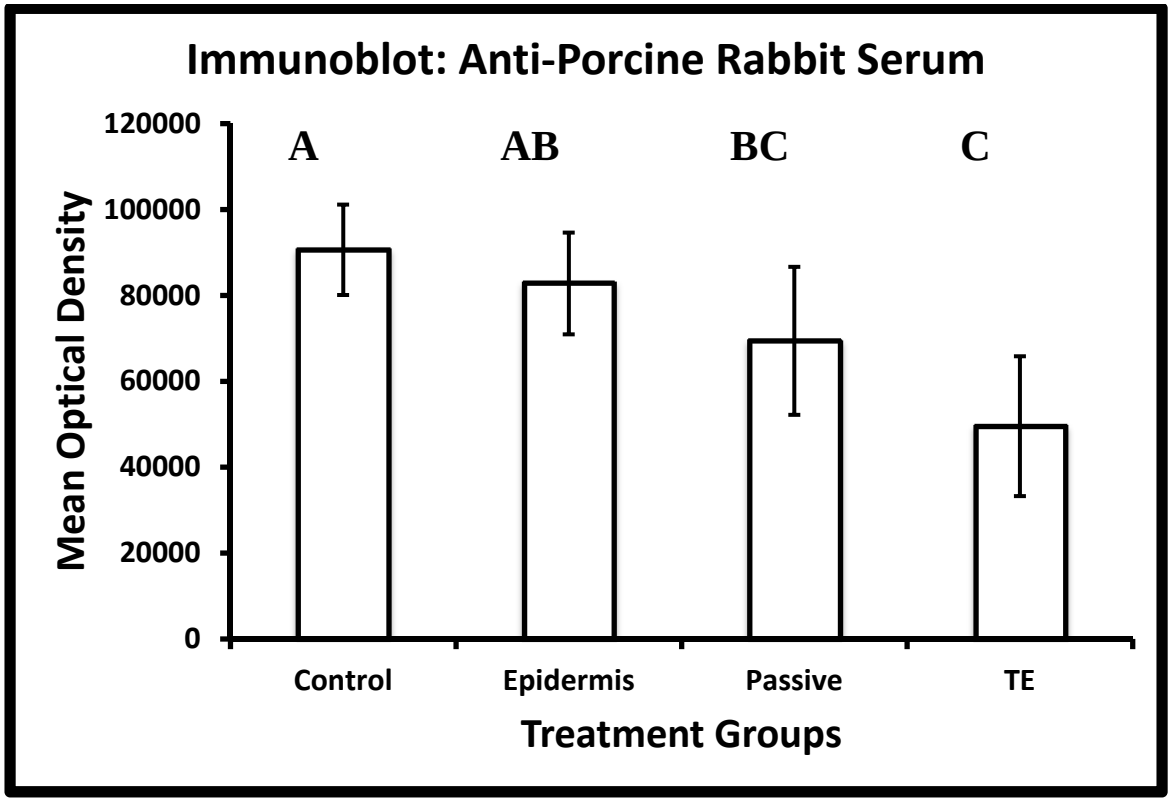

Figure 7: Bar graph represents the mean and standard errors for band densities from immunoblots. Means with different letters are significantly different (Tukey's test, $p < 0.05$) (n=6).

4.3: HUMAN IGG IMMUNOBLOT (NATURAL ANTIBODIES)

Immunoblot analysis with human IgG demonstrated multiple protein bands demonstrating that humans have preexisting antibodies to several porcine dermis proteins (Figure 8). However quantitative analysis of selected band densities show no significant differences between the treatment groups (Figure 9). A post-hoc power analysis was conducted with the following parameters delta = 0.5 and alpha = 0.05. the power was found to be 0.050.

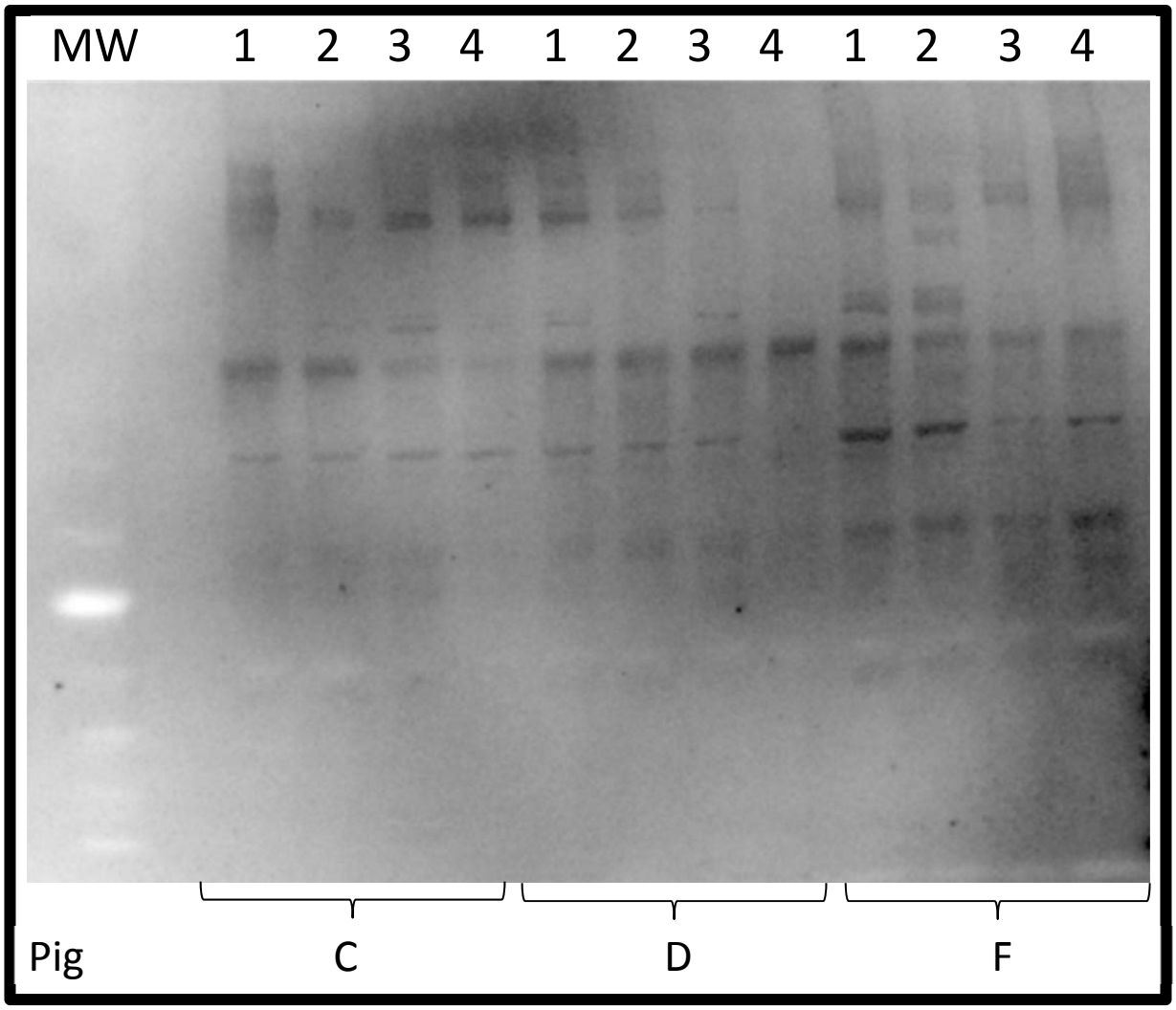

Figure 8: Human IgG immunoglot showing antigentic protein from the various treatment groups.

Treatment Groups:
1 - Untreated Control
2 – Epidermis Removal Control
3 - Passive Decellularization
4 - Tissue Electrophoresis

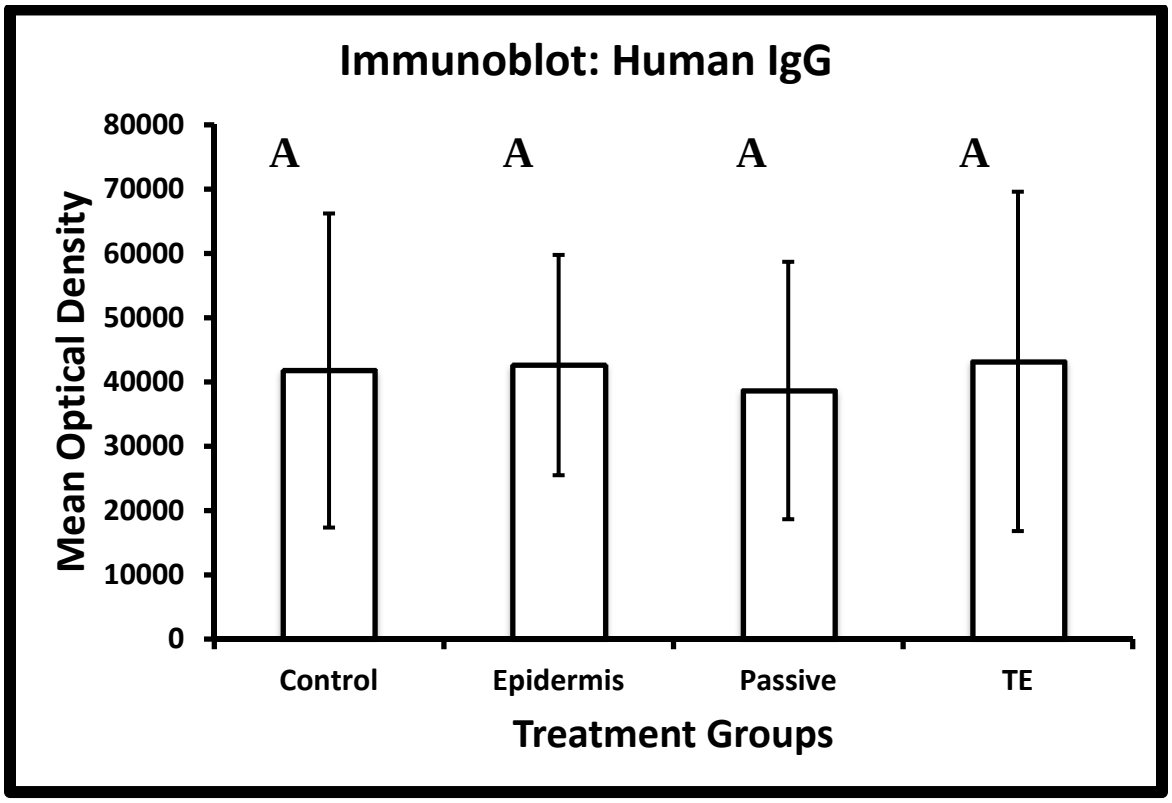

Figure 9: Human IgG Immunoblot bar graph representing mean and standard errors for band densities. Means with different letters are significantly different ($p<0.05$) (n=3).

4.4: TWO-WAY ANOVA

In order to determine whether the difference in protein extraction was due to the type of treatment or the pig that was used, a two-way ANOVA was performed. The two independent variables in the ANOVA were treatment and pig. The table below shows the results of the two-way ANOVA.

Table 2: Two-way ANOVA. A p-value <0.05 is considered significant.

Pig	P<0.120
Treatment	P<0.005
Pig*Treatment	P<0.933

Thus, two-way ANOVA identified a treatment effect that was independent of the pig that was treated.

CHAPTER 5: DISCUSSION

Previous studies of decellularized bioscaffold tissues for tissue engineering have relied on the microscopic appearance or absence of cells in the tissue on histologic examination to determine efficacy. Previous studies from this laboratory have provided evidence that histologic evaluation of cellularity does not ensure complete removal of cellular proteins from a scaffold tissue [45, 51, 61]. SDS-PAGE and immunoblot analysis represent novel methods for providing qualitative and quantitative information about the removal of protein antigens from xenogeneic bioscaffolds tissues after decellularization treatments. This study utilized these methods to compare a novel decellularization method based on active removal of negatively charged SDS-protein complexes in an electrical field with standard detergent-based decellularization in porcine dermis to create less reactive acellular dermal matrices.

5.1: SDS-PAGE

The goal of the SDS-PAGE experiments was to compare the efficacy of decellularization treatments on the removal of soluble proteins. The results supported the hypothesis that tissue electrophoresis-based decellularization was more effective at removing soluble proteins over a wide range of molecular weights (MW). This suggests that active migration of anionic detergent-protein complexes under an electrical field enhances removal of soluble protein compared to passive diffusion based methods over the same period of time.

As expected gels from the untreated control groups appear heterogeneous consistent with the presence of proteins with a wide range of MW. Interestingly, even though overall gel density was decreased by passive detergent-based decellularization treatment, density was increased in the mid-range range portion of the gel compared to gels from untreated control tissues. This suggests that passive detergent-based decellularization may actually cause the release and

increased availability of mid-range MW proteins from cells within the tissue but is not efficient at removing them from the tissue. Tissue electrophoresis-based decellularization treatment produced gels with the least amount of overall density and the gels were homogeneous suggesting that this treatment was most efficient at removing proteins with a wide range of MW. However, tissue electrophoresis did not extract all of the soluble proteins. This suggests there is still considerable room for optimizing the method. Various parameters that may be optimized include voltage, SDS concentration, treatment time, and density/porosity of the tissue electrophoresis gel.

5.2 RABBIT SERUM (ACQUIRED IMMUNITY)

Immunoblot analysis using rabbit serum generated against porcine tissue was performed to evaluate efficiency of removal of proteins capable of generating a xenogeneic immune response, i.e. xenogeneic protein antigens. The results from untreated control porcine tissues demonstrate that many proteins in porcine tissue are capable of generating an acquired antibody immune response when implanted across species. .None of the bands on the immunoblot would be representative of an α-gal antigen because both rabbits and pigs express the α-gal epitope. By using the rabbit serum, we are able to identify distinct bands that likely represent strongly antigenic proteins between pig and rabbit. It is likely that there are more protein antigens that a human would respond to. Using rabbit anti-porcine immune serum likely represents a conservative assay, as humans would react to at least these protein antigens.

Both passive detergent-based and tissue electrophoresis-based decellularization treatments were shown to be more effective at removing antigenic proteins when compared to untreated control tissues. Tissue electrophoresis produced the least overall dense gels; however the difference between standard detergent-based treatment and tissue electrophoresis treatment

did not reach statistical significance. Neither treatment removed all detectable antigenic proteins reinforcing the need for continued optimization of the tissue decellularization methods in general. Tissue electrophoresis method may allow more room for further optimization because of the many parameters that could affect this method.

The post-hoc power analysis showed that the experiment was underpowered. A sample size of 6 pigs yielded a power of 0.13. It would have taken a sample size of 100 to achieve a statistical power of 0.80.

5.3: HUMAN IGG (NATURAL IMMUNITY)

Human IgG was purchased from a commercial source and presumably had no previous immune exposure to porcine dermis. Therefore the antigenic protein bands on the immunoblot represent protein antigens that a human would have natural antibodies against. Thus the goal of this assay was to identify and determine the effect decellularization treatments on the removal of protein antigens that humans have natural antibodies against. Natural antibodies are generated when the recipient species is exposed to antigen prior to implantation of the donor tissue, usually through the environment. To my knowledge this method of evaluating protein antigens that elicit a natural antibody response has not been previously reported. Unlike rabbits, humans do not express the α-gal epitope therefore it is likely that one or more of the antigenic bands on the immunoblot is represent proteins with the α-gal moiety. The presence of several bands on these immunoblots suggests the possibly of protein antigens capable of eliciting a natural antibody response in humans that have not yet be identified or characterized. Surprisingly this immunoblot assay did not show differences between any of the treatment groups. It is possible that natural protein antigens, including α-gal, are particularly difficult to remove by current decellularization methods. It is also possible that technical difficulties associated with running this assay,

including the lack of a known concentration of specific antibodies in the commercial IgG product that was not intended for immunoblotting, make this a less than optimal assay for assessing decellularization. Lastly, this assay was performed on 3 rather than 6 samples and therefore may have been underpowered. Examination of the results of 3 samples did not suggest that running additional samples would likely change the result based on virtually no differences between treatment groups.

This study has several limitations. The tissue electrophoresis treatment was based on a single set of parameters and it is not known if the parameters chosen were the most optimal for decellularization. Parameters such as voltage, SDS concentration, gel density, and treatment time could be optimized to yield a more efficient soluble protein and antigenic protein removal process. It is vital that the optimal parameters be chosen so as to maximize protein antigen removal while minimizing deleterious effects to the tissue. The results of this study are based on a single type of evaluation, all based on extraction of the soluble protein fraction from the tissue and different variations of gel electrophoresis. Other methods for evaluation of decellularization could include light and/or electron microscopy to evaluate for cellular remnants and immunohistochemistry analysis for the presence of specific known antigens such as α-gal. Decellularization treatments should be optimized for other parameters including maintenance of biomechanical integrity and biocompatibility for future recellularization. Lastly the study is likely underpowered and increasing the number of samples might have yielded significant differences based on rabbit anti-porcine immunoblotting. Because of the technical difficulties in working with human IgG, only 3 samples were tested as opposed to 6. This decreased the power of analysis for the human IgG studies. Future studies would need to optimize and validate this assay before the results would be fully meaningful.

In addition to the protein quantification done in this study, there are a number of experiments that would give valuable information. The use of scanning electron microscopy (SEM) would bring to light morphological differences in the collagen fibers between the various treatment groups. The biocompatibility of the tissues could be confirmed by evaluation using an *in vitro* cytotoxicity test using fibroblasts. The test would yield a better understanding on the effect the various treatments have on the ability of human fibroblasts to proliferate on the tissue. Fibroblasts would be used as a qualitative indicator based on a morphological examination of cell damage and growth rates when in direct contact with the various treated tissue. Lastly, a biomechanical assessment of the tissues would give us valuable information on how the various treatments affected the strength of the tissue. A biomechanical assessment such as uniaxial tension tests could be used to gain valuable information on the biomechanical properties of the tissue before and after treatment.

The post-hoc power analysis showed that the experiment was underpowered. A sample size of 3 pigs yielded a power of 0.05. It would have taken a sample size of 100 to achieve a statistical power of 0.80.

5.4 TWO-WAY ANOVA

As can be seen from Table 2, the pigs had no effect on the amount of protein seen on the gels. It is seen that the type of treatment had a significant effect on the amount of protein observed on the gels. Furthermore, the interaction between the different pigs and different treatments was not significant. These results suggest that it is indeed the different types of treatments rather than the biological composition of the different pigs that is responsible for the differences in protein extract.

CHAPTER 6: SUMMARY AND CONCLUSIONS

In summary, this study compared standard detergent-based decellularization with a novel tissue electrophoresis decellularization method in their ability to remove both soluble and antigenic proteins from porcine dermis. The novelty of this method lies in its use of an electric field and negatively charged detergent to actively remove proteins from porcine dermis. Tissue electrophoresis decreased the overall amount of extractable protein. There was a trend toward greater removal of acquired protein antigens by tissue electrophoresis, but no difference between treatments in the removal of natural protein antigens. These studies demonstrate that no treatment method evaluated is completely efficient at removing soluble proteins and antigens from acellular porcine matrices. With further optimization, tissue electrophoresis is a promising method for decellularization of xenogeneic bioscaffolds.

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