Decellularized Human Cornea for Reconstructing the Corneal Epithelium and Anterior Stroma

BY

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THESIS

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DEDICATION

Dedicated to my wonderful loving parents, Majid and Shafqat, for their constant support, encouragement, and unconditional love.

Dedicated also to my dear husband, Suleman, for his patience and understanding.

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First of all, I would like to thank God for giving me life direction, strength and helping me to accomplish my PhD work. Thank you for giving me guidance and support through all the difficulties.

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LIST OF ABBREVIATIONS

H & E	Hematoxylin and Eosin
TAC	Transient amplifying cells
LSCs	Limbal stem cells
LSCD	Limbal stem cell deficiency
TGF	Transforming growth factor
IL	Interleukin
PDGF	Platelet derived growth factor
EGFR	Epidermal growth factor receptor
PDGFR	Platelet derived growth factor receptor
IGF	Insulin-like growth factor
LIF	Leukemia inhibitory factor
bFGF	Basic fibroblast growth factor
KGF	Keratinocyte growth factor
HGF	Hepatocyte growth factor
KGFR	Keratinocyte growth factor receptor
ECM	Extracellular matrix
PCL	Poly (ε-caprolactone)
PVA	Polyvinyl alcohol
PEUU	Poly (ester urethane) urea
PMMA	Poly (methyl methacrylate)
KPro	Keratoprothesis

LIST OF ABBREVIATIONS

FDA	US food and drug administration
PHEMA	Poly (2-hydroxyethyl methacrylate)
BM	Basement membrane
HCECs	Human corneal endothelial cells
HKLs	Human keratoplasty lenticules
SDS	Sodium dodecyl sulfate
PBS	Phosphate buffered saline
PEG	Poly (ethylene glycol)
NaCl	Sodium chloride
DAPI	4',6-diamidino-2-phenylindole
PI	Propidium iodide
PFA	Paraformaldehyde
PAS	Periodic Acid Schiff
MHC	Major histocompatibility complex
SEM	Scanning electron microscope
TEM	Transmission electron microscope
HLA	Human leukocyte antigen
GAGs	Glucosaminoglycans
DMEM	Dulbecco's modified Eagle's media
FBS	Fetal bovine serum
TBS	Tris buffered saline
BSA	Bovine serum albumin

LIST OF ABBREVIATIONS

ALDH1	Aldehyde dehydrogenase 1
FITC	Fluoroscein isothiocyanate
KSFM	Keratinocyte serum free media
CM	Conditioned media
NaOH	Sodium hydroxide

SUMMARY

The optical clarity of the cornea is highly dependent on the integrity of its primary layers including the outermost epithelial, the middle stromal, and the innermost endothelial layers. The epithelium plays a pivotal role in maintaining the corneal surface healthy as it forms first barrier to the environmental insults. There has been enough evidence that damage to epithelial cells renders stromal cells (keratocytes) exposed that eventually causes destruction of these keratocytes and makes the cornea opaque.

A number of tissue engineering approaches have been investigated for the reconstruction of the whole cornea or its individual layers as an alternative to using cadaver corneal tissue for transplants. In recent years, decellularized organ matrices have drawn attention as they provide a more natural environment for the growth and differentiation of cells. In this study, we undertook a tissue engineering approach to evaluate methods for removing cells from human cadaver corneas while maintaining the integrity of the basement membrane and the stromal matrix.

Our results have shown that corneas decellularized with NaCl plus nucleases method resulted in removal of the cellular components as evident with H and E and nuclear staining (DAPI). Positive staining for the basement membrane proteins; collagen type IV, fibronectin and laminin suggested that the decellularization did not have deletarious effect on basement membrane both in the central and limbus region. The integrity of the extracellular matrix evaluated by scanning and transmission electron microscopy demonstrated no gross changes in the collagen fibers in decellularized corneas except that the collagen fibers were slightly larger due to swelling. The decellularization process caused significant swelling of the corneal tissue,

however, following deturgescence with glycerol or optisol GS, the stroma was optically clear indicating no gross change to the lamellar organization. The effect of decellularization method on stiffness of the cornea investigated by comparing the elastic modulus showed no significant difference in the elastic modulus between the control and the decellularized corneas measured by Student's t-test.

The ability of decellularized cornea to support cell growth was also studied. Phenotypic evaluation of the corneal epithelial cells grown on decellularized corneas revealed that they can differentiate and express the corneal specific keratin 12 similar to that seen in human cadaver corneas. To examine the level of differentiation of basal epithelial cells, the expression of markers typically associated with limbal stem and progenitor cells also evaluated by immunofluorescence. The basal epithelium was found to express DeltaNp63and ABCG2 indicating that the construct supported a "limbal" (undifferentiated) phenotype in the basal layer. Proliferation was noted in the basal epithelial layer as evident by Ki67 staining. Corneal fibroblasts grown on NaCl plus nuclease decellularized corneas attached and exhibited native spread morphology and demonstrated positive staining for vimentin. An increase in expression of ALDH1, a marker of corneal keratocytes suggested that majority of the fibroblasts had partially reverted to a keratocyte phenotype over time.

For in vivo study, a decellularized graft was transplanted on a rat cornea ablated with excimer laser showed graft epithelialization as demonstrated by a reduction in flourescein staining over time.

In summary, this is the first study on decellularization of human cornea with which re-cellularization with both corneal epithelial cells and fibroblasts was demonstrated. The novel method for decellularization has potential applications in corneal and limbal tissue engineering, where a biopsy may be used to grow both epithelial cells and stromal cells to reconstruct the corneal epithelium with a stromal niche.

I. INTRODUCTION

1. Background

1.1 Cornea Anatomy and Function

The eye is a complex organ, divided into three large tissue layers. The cornea, the first tissue layer that light hits before entering into the eye, is composed of multiple layers of cells. The light then reaches another important tissue called the 'Lens' which is transparent and has a spherical shape. After passing through the lens, the light bends and reaches the 'Retina', which is present at the back of the eye. All three layers have a consistent and uniform arrangement to maintain the integrity of the eye (Pinnamaneni and Funderburgh, 2012).

The cornea, which is the outermost layer of the eye, provides a protective barrier to maintain the ocular integrity. It is composed of five distinct layers (Figure 1). From anterior to posterior the first layer is called the epithelium which consists of 5-7 layers of epithelial cells. Underneath the epithelium there is a thin acellular layer called Bowman's Layer. The third layer, stroma, consists of cells called keratocyte and the matrix, makes up most of the cornea. The stroma lies on a thick membrane called Descemet's membrane, which is devoid of any cells. The posterior most part is a single cell layer thick endothelium, consisting of endothelial cells (Almubrad, and Akhtar, 2011; Patel et al., 2001; Schmoll et al., 2012).

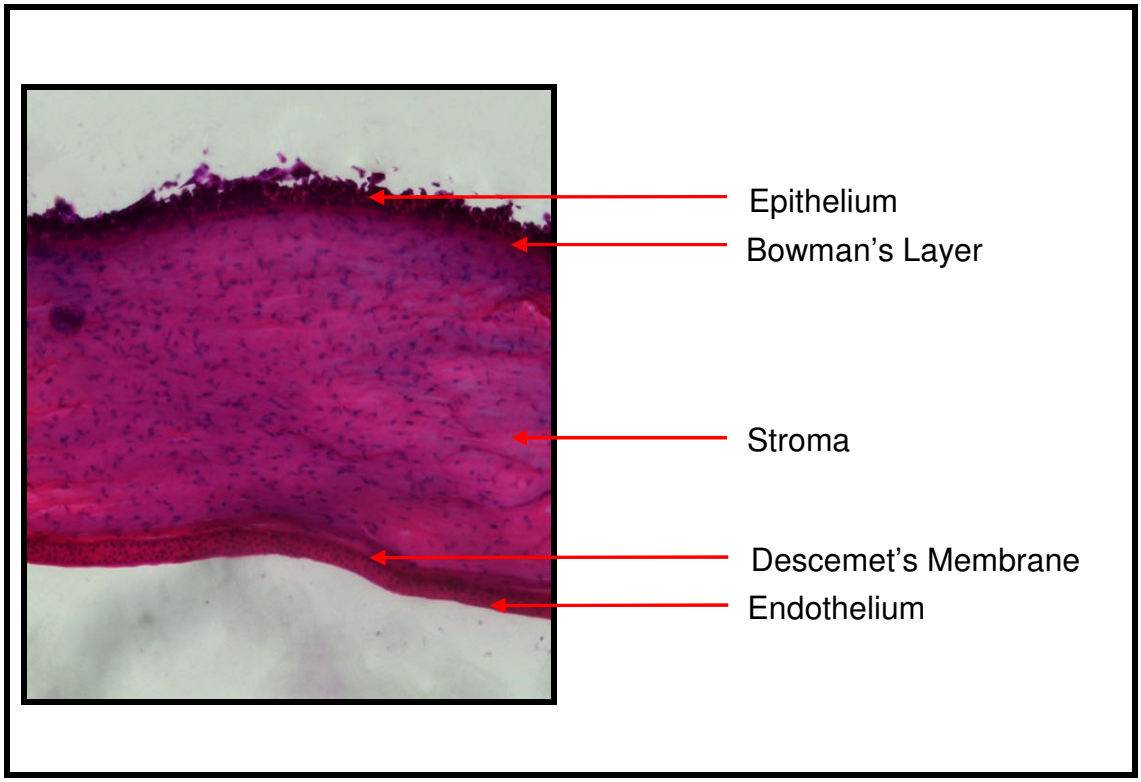

Figure 1. Section of the human cornea. Human corneal section about 10µm thick stained with Hematoxylin and Eosin (H & E).

1.2 Corneal Epithelial (Limbal) Stem Cells

The cornea consists of several epithelial layers that are not only phenotypically distinguished but also behave differently too. The less differentiated epithelial cells are the basal epithelial cells, which are attached to the basement membrane by hemidesmosomes and to each other by desmosomes. The most undifferentiated cells are present in the limbus region and are called the 'limbal stem cells' (LSCs). These stem cells undergo continual self-renewal and generate an intermediate cell type known as transient amplifying cells (TAC). These TACs migrate upwards toward the surface and then further divide and differentiate terminally into corneal epithelial cells (Lavker et al., 2004). The limbal stem cells are necessary for the constant regeneration of the corneal epithelium.

Limbal stem cells, like any other stem cells, are located in a specialized microenvironment, (aka 'niche'), which supports and protects their population from environmental insults and maintains the 'stemness' of the stem cells. Limbal stem cells are located exclusively in the limbus, the boundary between the cornea and the sclera. It has been shown that limbal epithelial cells in the limbus extend from the surface of the cornea into the underlying stroma and form pockets known as 'limbal epithelial crypts', or 'palisades of Vogt'. These crypts are of variable lengths in the human cornea. Limbal crypts that are shorter than 40um are termed 'minor crypts' and the ones larger than 40um are termed 'major crypts'. (Shanmuganathan et al. 2007) (Molvaer et al., 2012)

1.3 Limbal Stem Cell Deficiency (LSCD)

The integrity of the corneal epithelium depends on the presence of self-renewing limbal stem cells. These limbal stem cells, or their microenvironment where they reside, can be damaged due to various factors such as mechanical or chemical insults. They may also be damaged or compromised by immune diseases, like 'Stevens-Johnson Syndrome', resulting in a serious ocular condition known as 'Limbal Stem Cells Deficiency' (LSCD) (Grueterich et al., 2003). LSCD is associated with persistent epithelial defects, neovascularization and severe loss of vision if left untreated.

1.4 Factors Important for the Integrity of Epithelial Cells

Epithelial-Keratocyte Interaction

Epithelial cells are regulated by a number of factors. Epithelial-stromal cells interactions play a vital role in the function of epithelial cells and epithelial stem cells in a variety of the tissues. On the ocular surface, there are three major patterns of cytokine interaction, potentially involved between the epithelium and keratocytes of the cornea and limbus, that are important for maintaining both epithelial cells and keratocytes (Figure 2).

(1) Type I: Transforming growth factor (TGF)-α, interleukin (IL)-1β, and platelet-derived growth factor (PDGF)-B are released by corneal epithelial cells to act on

keratocytes by binding to their respective receptors present on stromal keratocytes; epidermal growth factor receptor (EGFR), IL-1R, and PDGFR-β.

(2) Type II: Insulin-like growth factor (IGF)-I, TGF-β1, -β2, leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and their receptors are expressed by both epithelium and fibroblasts and therefore mediate both epithelial and keratocytes activity.

(3) Type III: Keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) are released by keratocytes to stimulate corneal epithelial cells through their respective receptors on epithelial cells; KGFR and c-met (Li and Tseng, 1995).

This back and forth signaling between keratocytes and epithelial cells plays a pivotal role in maintaining both cell types. Studies on the growth of limbal epithelial cells have also shown that when co-cultured with fibroblasts, grown on fibroblast feeder layer, or cultured in fibroblast conditioned media, they express better growth and proliferation potential as compared to when cultured without fibroblasts (Tseng et al. 1996).

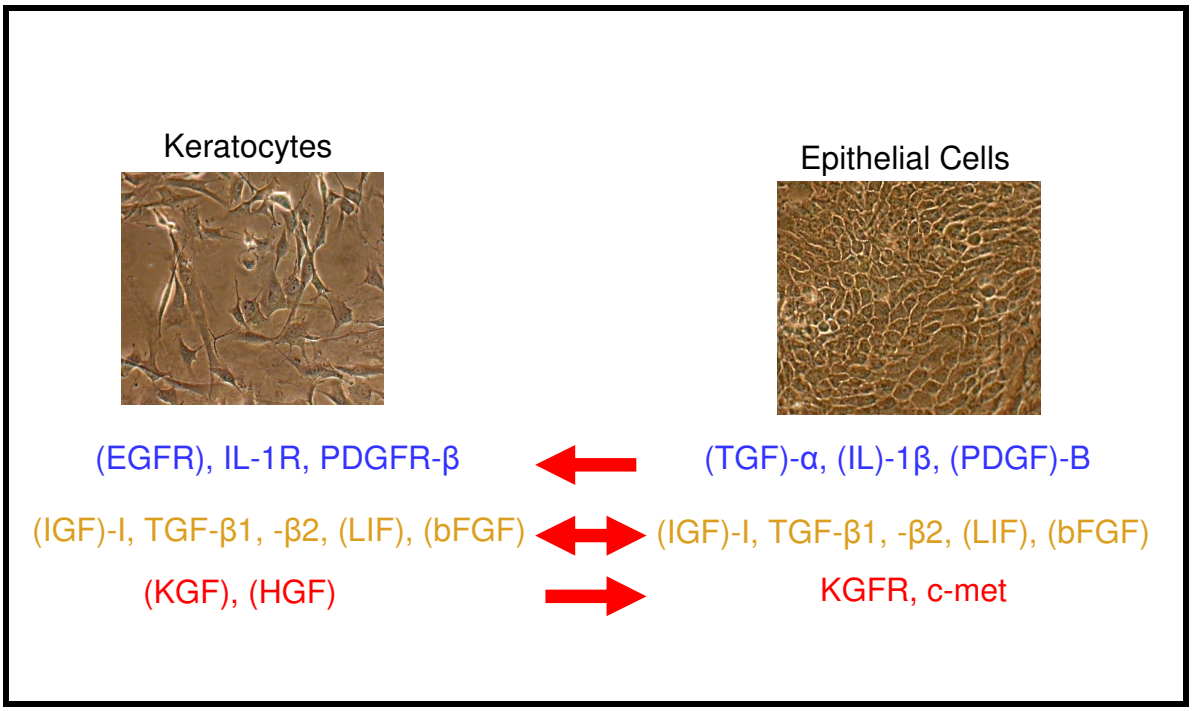

Figure 2. Cytokines interaction between keratocytes and epithelial cells. The three major pattern of cytokine interaction potentially involved between the epithelium and keratocytes of the cornea.

Extracellular Matrix

Another factor regulating the fate of epithelial cells and limbal stem cells is the extracellular matrix (ECM). ECM is a biologically active scaffold comprises of a meshwork of proteins, proteoglycans, and glycosaminoglycans. In addition to providing mechanical strength and elasticity to tissues, ECM components have also been associated with the regulation of cell proliferation and differentiation (Lin and Bissell, 1993). The ECM thus provides an appropriate microenvironment for stem cells and differentiated cells and facilitates repair and regeneration during wound healing.

The ECM of corneal stroma supports keratocytes which in turn support epithelial cells and limbal stem cells, while the ECM of epithelial basement membrane has a direct effect on both epithelial cells and the limbal stem cells. Corneal stroma comprises of about 90% of the total corneal thickness and is important in maintaining the mechanical shape and the structure of the cornea. Corneal stroma comprised of highly organized sheets of connective tissue that are made up of mostly collagen fibers arranged parallel to each other with keratocytes embedded in them. These sheets of connective tissue are stacked orthogonally with respect to each other to allow proper refraction of the light and maintain the transparency of the cornea. (Last et al. 2012)

Keratocytes, which originate from the neural crest, are present in the stroma, embedded in collagen fibers. In adult, these keratocytes remain in a quiescent state in the stroma under normal physiological conditions. Upon injury, these keratocytes transform into a fibroblast phenotype and migrate into the damaged region and replicate. These

transformed cells secrete new extracellular matrix during the stromal remodeling phase. After wound healing, these fibroblasts undergo apoptosis and are replaced by the keratocytes to achieve transparency (Carlson et al., 2003).

2. Tissue Engineering

Tissue engineering is defined as an "interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue or whole organ function'' (Langer and Vacanti, 1993).

It is believed that ancient Egyptians may have been the first ones to practice Tissue Engineering in1500 B.C. There is a description in the *Papyrus of Ebers*, on how skin wounds can be treated using honey, lint, and grease. Honey provides anti-bacterial and anti-inflammatory properties that are produced by honeybees. While the lint was used as a scaffold to facilitate healing of the wound, and the grease to seal the wounded surface to protect it from disease causing microorganisms present in the environment (Nahmias and Yarmush, 2009).

There are three basic components of a biological tissue, referred to as the Triad of Tissue Engineering (Figure 3). These components are scaffolds, signals, and the cells. Scaffold mainly consists of ECM such as collagen fibers, proteoglycans, and glycoproteins in the form of fibers and fibrils. Depending on the type of tissue, there is a special set of cells important for maintaining the functionality of that specific tissue.

These cells perform biological functions such as proliferation, differentiation, apoptosis, migration, etc. by receiving biochemical cues from signaling molecules such as proteins. All three components are crucial for the success of any tissue engineered scaffold (Langer and Vacanti, 1993).

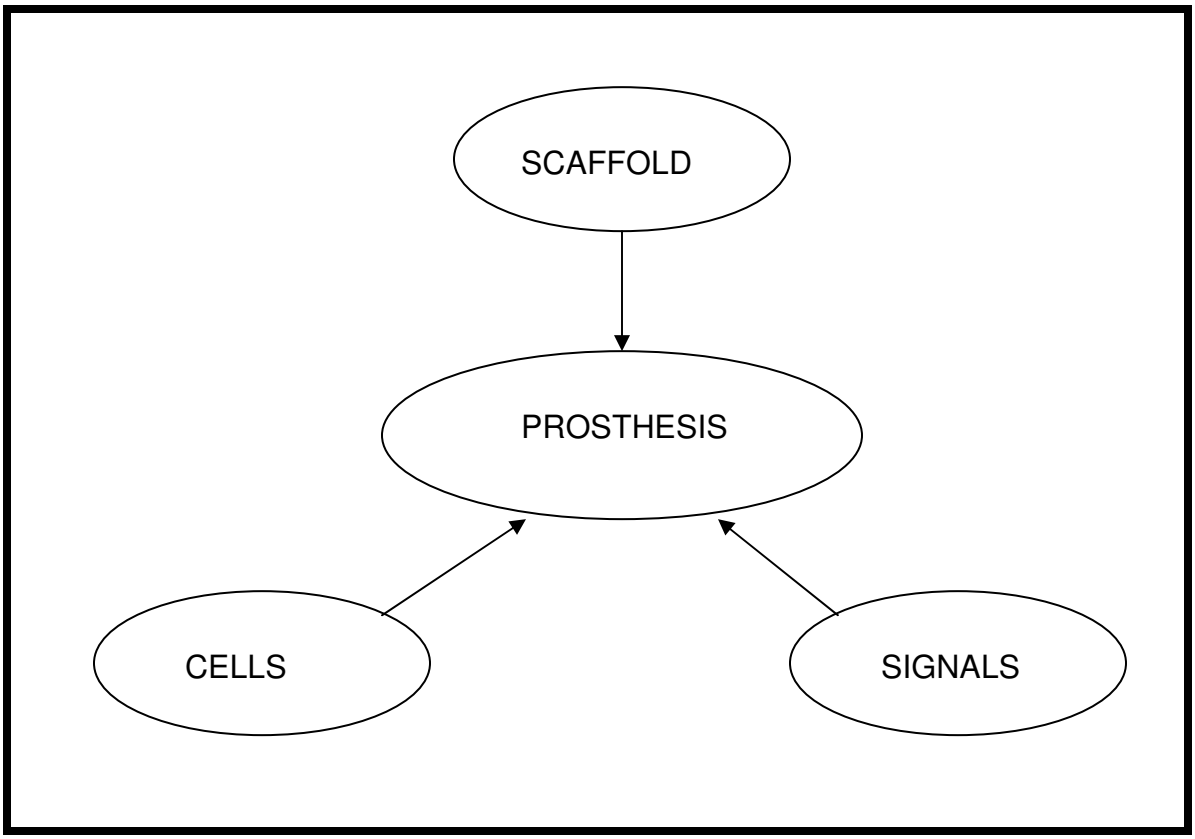

Figure 3. The tissue engineering triad. The components of the triad of tissue engineering are cells, signals and scaffolds.

2.1 Approaches to Corneal Transplantation

There are estimated about 10 million cases of vision loss worldwide associated with cornea. About 30,000 to 40,000 corneal grafts are performed annually in the US. Although, the cornea is an immune-privileged site, the corneal rejection rate starts below 10% and over the long term it increases to 25-50% (George and Larkin 2004; Nishida et al., 2004; Williams et al., 1992; Miri et al., 2010). Thus, the long term success of the corneal grafts is still limited by rejection. Due to shortage of corneas for transplantation, researchers have tried to tissue engineer corneas using both natural and synthetic materials, as a scaffold, to grow corneal cells (Hicks et al., 1997). The engineered cornea, regardless whether it is developed with natural or synthetic material, must fulfill the functions of the cornea it replaces. It must be transparent, provides protection against chemical, mechanical, and microbial insults, be non-immunogenic, and have a similar refractive index to allow proper refraction of light.

The limited supply and the variable quality of the donor cornea are significant problems that could be overcome by developing an artificial cornea. Different biomaterials are being sought for their potential to replace the cornea (Figure 4). A great advantage of using synthetic or natural material for developing an artificial cornea is the ability to form a product in a uniform manner that is repeatable, gives identical products, and can be manufactured at a large scale. There is a rising need to investigate different materials/devices that can replace the function of the cornea, in whole or in part.

2.2 Synthetic Scaffolds

There is a lot of research being done on using synthetic material to develop artificial cornea or individual layers of the cornea. Synthetic materials that have been studied include polymers, such as gelatin hydrogels (Mimura et al., 2008), electrospun poly (ε-caprolactone) (PCL) nanofibers that are surrounded by polyvinyl alcohol (PVA) hydrogel disk (Bakhshandeh et al., 2011), nano-hydroxyapatite/poly (vinyl alcohol) hydrogel skirt with poly (vinyl alcohol) hydrogel clear transparent center (Fenglan et al., 2007), and poly(ester urethane)urea (PEUU) (Wu et al., 2012). While many of these show some promising results, they also have shortcomings, and are currently not in the form that could be used in clinical studies.

Currently, there are two synthetic corneas approved by FDA for Transplantation, Boston Keratoprosthesis (KPro) and AlphaCor.

KPro is one of the most commonly used keratoprosthesis in the U.S. that has both synthetic and natural components (Gomaa et al., 2010). Though KPro is FDA approved and is currently being used clinically, there are some side effects associated with it. One of the main side effects of Boston KPro is the development of glaucoma in some of the patients. It is suggested that due to its more rigid texture as compared to the native cornea, it elevates intraocular pressure and causes glaucoma (Carlsson et al., 2003). Other main risks associated with the Boston Kpro are the risks of melting, infection, and extrusion, given the lack of proper integration with the host.

The second FDA approved synthetic cornea is AlphaCor. AlphaCor is made with the synthetic polymer, poly(2-hydroxyethyl methacrylate) (PHEMA), which has been used extensively in ocular devices such as contact lenses. AlphaCor is a modified version of KPro that incorporated a softer polymer to avoid the complications associated with the rigidity of the Boston Kpro device (Crawford et al., 2002).

The main limiting factor for these artificial devices is the lack of epithelialization and/or poor epithelialization. The epithelium forms a barrier that protects the eye from environmental insults. An absence of epithelium and/or poor epithelium renders the eye susceptible to infections (Myung et al., 2007). Poor healing between an artificial implant and a recipient cornea could result in infection and/or inflammation of the tissue. If these conditions are not fixed, eventually the implant gets extruded. Due to this shortcoming, these artificial corneas are used only in patients with more complicated situations and are not good candidates for the cadaver donor corneas.

2.2 Natural Scaffolds

Compared with the synthetic scaffolds, natural scaffolds are more promising to use due to their ability to support cell growth without the use of additional adhesion proteins. Some of the natural materials include amniotic membrane (Chen et al., 2012; Tsujita et al., 2012; Nubile et al., 2011), collagen-chondroitin sulfate foams (Vrana et al., 2008), fibrin- 0.1% agarose gels (Ionescu et al., 2011), silk film from the cocoon of the silk worm Bombyx mori (Lawrence et al., 2009), and collagen (Fagerholm et al., 2010; Gendron et al., 2011).

A comparative analysis of the composition of basement membranes of human limbus epithelium and amniotic membrane epithelium showed that these two membranes share many of the vital proteins (Fukuda et al., 1999; Endo et al., 2004; Cooper et al., 2005). Because of the extensive similarities for the types of proteins in an amniotic membrane and limbal basement membrane, many researchers are optimizing conditions for using amniotic membrane for replacement of the damaged cornea.

While amniotic membrane has shown to support ex vivo expansion of limbal epithelial stem cells, there is still resistance to adopting it universally given that its mechanical and optical properties are not optimal, which makes it disadvantageous to be used as a scaffold for corneal epithelial transplantation.

Recently, Fagerholm et al. (2010) developed biosynthetic corneas made up of clinical-grade recombinant human collagen type III made in yeast and then cross-linked with a chemical cross-linking agent and molded into an implantable form. These corneas were extensively tested in animal models. Now, they are conducting a phase I clinical trial study where they transplanted these artificial corneas into nine patients having keratoconus and one patient with corneal infection. These grafts were monitored for a period of two years and evaluated for nerve innervations, sensitivity, tear film production, growth of epithelium on the graft, and infiltration of the host keratocyte cells. Through multiple tests, they proved that these corneas closely mimic natural corneas and were able to restore all the essential elements of a natural corneal tissue. However, at close examination, they found that the graft was epithelialized, but the epithelium was missing in some patches at the site of sutures and this caused some irregularity and roughness to

the surface of the graft. However, the authors suggested using less disruptive sutures for the future study, which would solve this problem. This study showed very promising results and could be used as a possible alternative to human donor tissue for transplantation (Fagerholm et al., 2010).

The design of any tissue replacement strategy requires a detailed understanding of the function that was performed by the native tissue. So far, all of these artificial corneal substitutes are not close to ideal: if they fulfill one criteria, they compromise on some of the other components such as collagen texture, arrangement of collagen fibers, optimal porosity to allow diffusion of nutrients and migration of cells, or the stiffness of the tissue. Also, for normal functionality of the cornea, it is impossible to develop an artificial cornea containing a complete set of proteins as in a normal cornea. Hence, due to these shortcomings, long term stability and functionality of these artificial corneas still have not been attained.

3. Donor Corneas

The gold standard treatment for replacing the damaged cornea is the surgical replacement of the cornea with donated cadaver human cornea. In 2011, more than 46,000 corneal transplants were performed in the United States. The first corneal transplant was performed in 1905. Due to increased awareness of cornea donation over years, the number of grafts made available in 2011 was around 67,000 as compared to 59,000 in 2010. Even with this increased number of available corneal grafts, the need for corneal tissue is more than what is available (Eye Bank Association of America).

The corneas that are donated are not all appropriate for transplantation. There are some corneas unsuitable for transplantation such as from donors suffering from infections or have transferable diseases such as HIV or hepatitis. After careful medical evaluation, the donor corneas are distributed for transplantation or sent for use in research and education (Eye Bank Association of America). Successful transplantation depends on many factors including the quality of the donor cornea as well as the patient's health and eye condition.

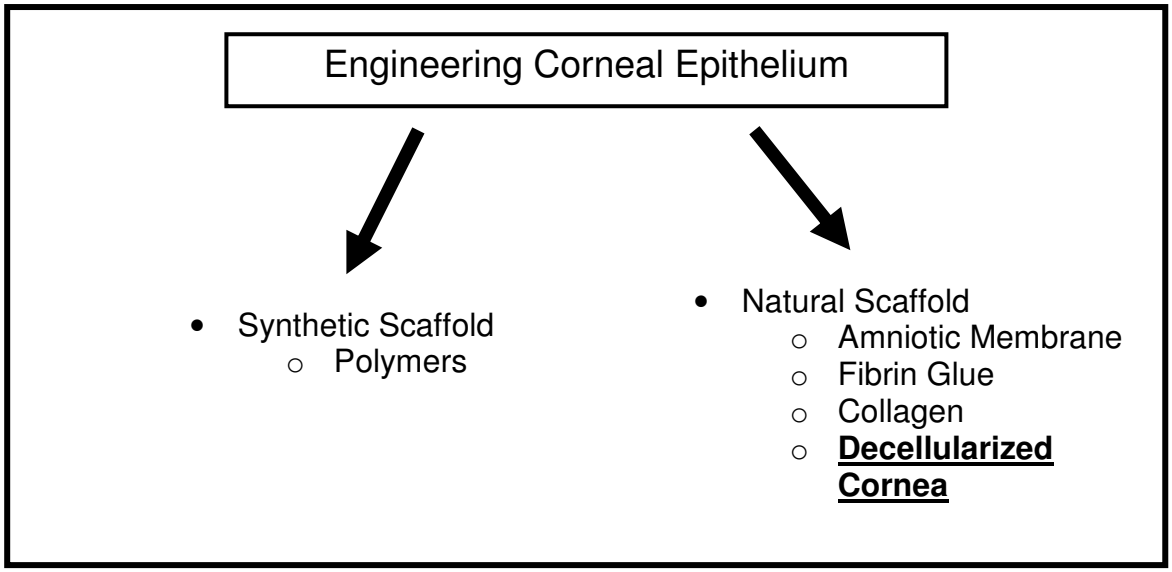

Figure 4. Approaches to engineering corneal epithelium. Examples of both synthetic and natural scaffolds for reconstruction of corneal epithelium for the purpose of transplantation.

3.1 Decellularized Cornea for Transplantation

Successful transplantation depends on the quality and availability of the donor cornea as well as the patient's underlying condition. Decellularized corneal matrices would provide an alternate to patients whose eyes are at high risk for rejecting donor corneas. These high-risk patients often have a vascularized corneal bed that is more prone to graft rejection. In an effort to overcome the problem of donor tissue rejection, researchers have developed decellularized corneas that have drawn lots of attention in recent years for their potential to be used as an alternative to donor corneas for transplantation.

3.2 Significance of Decellularization and Preservation of Basement Membrane

The idea behind using decellularized cornea is to develop a scaffold that mimics the natural physiological condition of the cornea and is biochemically and mechanically similar to the native cornea, which is critical for successful integration into the host tissue and also for maintaining the functionality of the cornea. The extracellular matrix is preserved among species and is not recognized as a foreign object by the host and thus should not induce an immune response. However, donor cells are immunogenic and therefore these cells must be removed prior to transplantation. The extracellular matrix (ECM) of any tissue contains a network of proteins such as collagen, growth factors, chemokines, glycoproteins and proteoglycans. Similarly, corneal ECM carries proteins that are essential in providing structural and physiological support to the tissue and the cells. A highly specialized form of ECM is the basement membrane that contain unique

set of proteins that play critical role in cellular growth, differentiation, morphology, proliferation, survival and migration of the cells in that special tissue. The corneal stroma is composed of a uniform layout of collagen fibers along with other proteins and any defect in this arrangement results in damage to the cells and eventually to the tissue. Therefore, the best scaffold to grow the corneal cells to develop an artificial human cornea is the human cornea itself.

The decellularized cornea can be transplanted without cells but to facilitate the integration of the graft with the host tissue, repopulating the graft with the host corneal cells prior to the transplantation could be performed. In order to make a suitable matrix for tissue engineering cornea, the scaffold should fulfill three main requirements. First of all, it should be devoid of any cell components to avoid any immunological and inflammatory response due to foreign cell components that act as antigens. Secondly, following de-cellularization, the basement membrane (BM) of the epithelial cells should be preserved as it plays key roles in many corneal cellular functions. Epithelial basement membrane is a thin sheet like structure present underneath the epithelium. Epithelial basement membrane like other BMs, is in contact with the cells and is responsible for regulating various cell processes including facilitating migration of epithelial cells, adhesion of basal epithelial cells, their proliferation and differentiation and prevents epithelial cells from undergoing apoptosis. In general, basement membranes are highly crosslinked and express about 50 proteins with collagen type IV making up for 50% of all the proteins (Kalluri, 2003). Finally, the third requirement is that, it should support cells when repopulated with recipient cells.

3.3 Studies on decellularized cornea

Due to the convenient availability of animal corneas, there are studies by several researchers that involve the use of porcine corneas as a decellularized scaffold for transplantation on rabbit eyes. In all these studies, after decellularization, the graft was transplanted to the rabbit eye and evaluated for growth and migration of the host epithelial cells and keratocytes into the graft (Zhang et al., 200; Xu et al., 2008; Oh et al., 2008; Amano et al., 2008; Xiao et al., 2011; Du, and Wu, 2011; Hashimoto et al., 2010; Sasaki et al., 2009; Fu et al., 2010; Zhang et al., 2007).

Some decellularization studies looked into the ability of the scaffold to support cells growth only *ex vivo*. For example, a study by Zhang et al. investigated using rat limbal stem cells on decellularized dog cornea to see if it supports cell growth *ex vivo* (Zhang et al., 2012). Another study aimed to evaluate the potential of using decellularized bovine corneas as a scaffold for ex-vivo expansion of human corneal endothelial cells (HCECs) (Bayyoud et al., 2012). However, the goal of that study was different from ours, as it focused on the posterior cornea to grow endothelial cells while our focus was the anterior cornea to grow epithelial cells.

Due to the easy access and availability of animal tissues, research is currently being done on animal tissues. Although to get preliminary data, the use of animal tissues is helpful, but for the purpose of clinical testing, the use of such tissues could be a problem. First of all, the approval to initiate clinical trial could be challenging due to safety concerns. There have been concerns that the transplant of xenogenic tissue

including cornea could cause transmission of diseases (Takeuchi et al., 2005; Langat, and Mwenda, 2000; Kim et al., 2011; Michaels et al., 1997).

There is only one study on the use of human corneal tissue for epithelial tissue engineering. They developed a "hemicornea" from human keratoplasty lenticules (HKLs) that are currently used for anterior lamellar keratoplasty surgeries. However, in this study, the authors only removed the corneal epithelium and left keratocytes in the stromal matrix. Later, they plated corneal epithelial cells on the hemicorneal graft and evaluated for the reconstruction of the epithelium. Their main focus was to use the matrix as a template to grow epithelium that mimics normal corneal epithelium organized and stratified into four to five cell layers, with basal cells expressing stem cell markers (Barbaro et al., 2009). They did not perform complete removal of cells which could cause an immune response when transplanted to treat the damaged eye. Secondly, this group did not look into the feasibility of transplanting and biocompatibility of the 'hemicornea' in vivo, in an animal model. The aim of this study was not to develop a decellularized scaffold to reconstruct corneal epithelium, instead, to use the underlying healthy stroma to reconstruct epithelium.

4. Purpose and Significance of the Study

The use of cadaver donor corneas for treating corneal conditions carries risk of immune rejection and requires long-term application of immunosuppressive medications. Synthetic devices such as KPro and AlphaCor often result in poor integration and/or development of other eye complications such as causing glaucoma by increasing

intraocular pressure. Natural scaffolds can integrate into the host tissue, but are not devoid of shortcomings. For example, fibrin gel scaffold is not porous enough to allow proper diffusion of nutrients and migration of the cells. Research on decellularized corneas from animal source show encouraging results but safety concerns remain. Therefore, there was a need to develop a novel method to engineer cadaver corneas to be used in humans that are safe, transparent, and non-immunogenic in an effort to replace damaged corneas.

4.1 Statement of overall hypothesis and Objective

We proposed to create a transplantable biologically functional cornea by engineering a cadaver cornea for the treatment of corneal diseases. We hypothesized that *tissue engineering application of the corneal epithelium would be optimized and success achieved with the establishment of the proper stromal microenvironment (niche) to provide the necessary support to the transplanted epithelial cells.*

Therefore, we proposed to develop a decellularized cadaver corneal construct that was capable of supporting the growth and differentiation of human corneal epithelial and stromal cells by implementing the following specific aims:

4.2 Specific Aims

Aim 1. To decellularize cornea while preserving extracellular matrix proteins

1.1 Decellularization of human corneas

1.2 Evaluation of the basement membrane and extracellular matrix

Aim 2. To reconstruct anterior stroma in decellularized cornea

2.1 Repopulation of decellularized cornea with corneal fibroblasts

2.2 Repopulation of decellularized cornea with corneal epithelial cells

Aim 3. To test reconstructed anterior stroma construct *in vivo*

3.1 Testing of engineered anterior stroma construct in an animal model

The schematic of the experimental design is explained below:

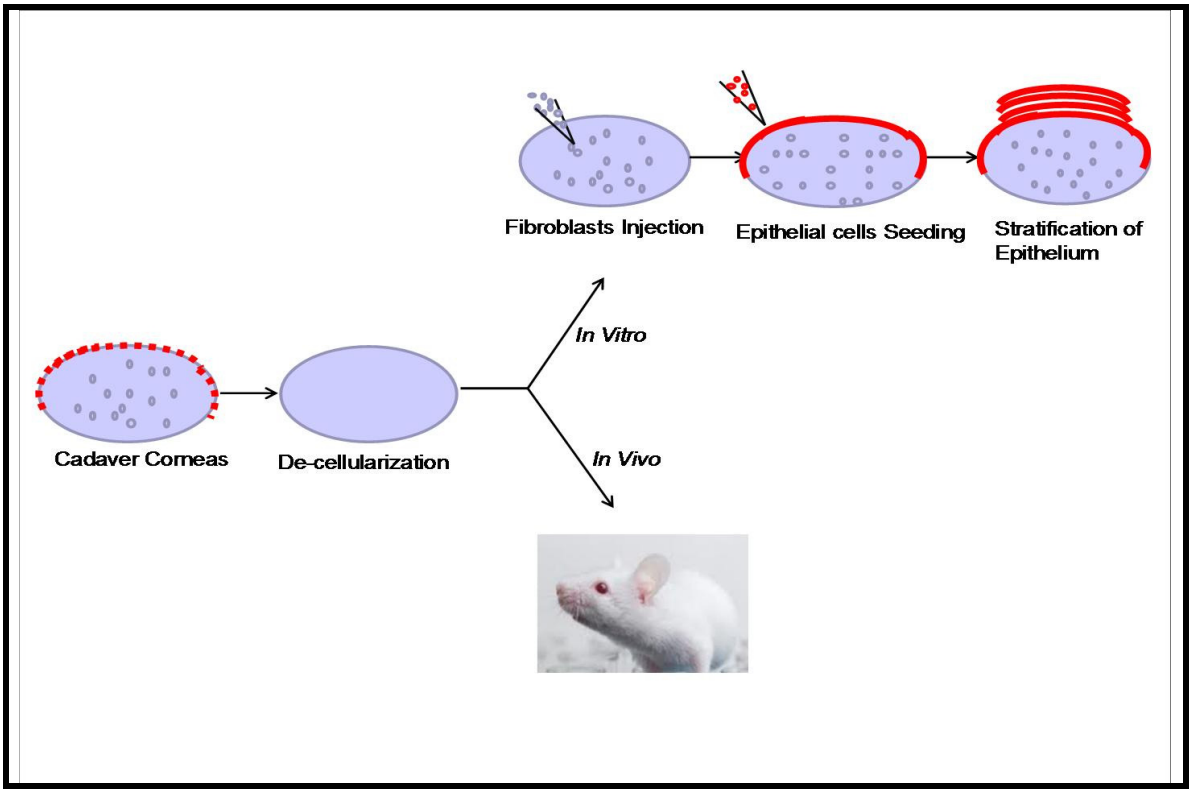

Figure 5. Schematic of the experimental design for engineering anterior cornea.

II. PREPARATION AND CHARACTERIZATION OF DECELLULARIZED HUMAN CORNEA

1. Introduction

The optical clarity of the cornea is highly dependent on the integrity of its primary layers including the outermost epithelial, the middle stromal, and the innermost endothelial layers. A number of tissue engineering approaches have been investigated for the reconstruction of the whole cornea or its individual layers as an alternative to using cadaver corneal tissue for transplants (De Miguel et al., 2010; Han et al., 2002; Griffith et al., 1999; Carlsson et al., 2003; Watanabe et al., 2011; Sumide et al., 2006).

Reconstruction of the corneal epithelium has been studied extensively with tissue engineered corneal epithelium now in clinical use in selected academic centers worldwide (Kawashima et al., 2007; Thanos et al., 2010; Meller et al., 2010; Pauklin et al., 2010; Colabelli et al., 2010; Basu et al., 2011; Marchini et al., 2012).

Both synthetic and biological matrices have previously been tested for corneal tissue engineering (Li et al., 2003; Shimmura et al., 2003; Chirila, 2001). In recent years, decellularized organ matrices have also drawn attention as they provide a more natural environment for the growth and differentiation of cells when compared to synthetic scaffolds (Ott et al., 2008). In the cornea, decellularized xenograft matrices have been studied primarily for stromal replacement. (Fu et al., 2010; Wu et al., 2009; Xu et al., 2008; Hashimoto et al., 2010; Zhou et al., 2010; Sasaki et al., 2009; Oh et al., 2009). In this study, we undertook a tissue engineering approach to evaluate methods for removing cells from human cadaver corneas while maintaining the integrity of the basement membrane and the stromal matrix. The optimized protocol resulted in a decellularized

cornea that is fully capable of supporting the growth and differentiation of human corneal epithelial and stromal cells *in vitro*.

2. Material and Methods

2.1 Decellularization of human cadaver corneas

Human donor corneoscleral buttons that had been stored in Optisol GS media and deemed unsuitable for transplantation were obtained from the Illinois Eye Bank, Chicago, IL. Most corneas had either passed their expiration date or had low cell counts which made them undesirable for clinical use. The mean age of the donors was 50 $\pm$ 25 years. The following five different methods were employed to remove cells from the human cadaver corneas. All experiments were repeated a minimum of 3 times.

Method 1 (Detergents):

Corneas were placed in 50 mL centrifuge tubes with sodium dodecyl sulfate (SDS) or Triton X-100 in concentrations ranging from 0.1-1% at room temperature for 24 h under shaking. To remove the detergents, the corneas were washed with phosphate buffered saline (PBS) three times, each for 24 h at 4^{o}C on a rotating shaker.

A slight variation of the detergent based method was also adapted from Daniels et al., (2005), where corneas were treated with 1% SDS for 12 h after which they were rinsed with PBS 3-5 times for 2 h, in 75% ethanol for 12 h, and in PBS for 3-5 times for 1 h, all at room temperature under shaking (Daniel et al., 2005; McFetridge et al., 2004).

Method 2 (Liquid nitrogen):

Corneas were placed in 50 mL centrifuge tubes, snap frozen with liquid nitrogen, and were left in the tube with the cap closed tight to create a hypoxic environment. The corneas were then incubated in the same tubes for 7 days at room temperature (Amano et al., 2008).

Method 3 (Poly(ethylene glycol)):

Corneas were treated with poly (ethylene glycol) (PEG) MW 1,000 and MW 8,000. PEG is an amphiphilic polymer that damages the cell membranes. The rationale for using PEG is to avoid using detergents. For our purpose, the decellularization protocol adapted from Uchimura et al., (2003) was modified to 45 min PEG exposure at room temperature under shaking. To remove PEG, corneas were washed with PBS three times as described above.

Method 4 (Osmotic gradient plus detergents):

Tissues were subjected to a stepwise treatment in which a combination of hypotonic and hypertonic buffers along with Triton X-100 and SDS was used. This procedure, adapted from a study on carotid arteries by Roy et al., (2005) was modified to preserve the extracellular matrix and remove cells from the cornea. In the first step, the cornea was treated with hypotonic buffer containing 10.0 mM Tris (pH 8.0) for 24 h at 4^{o}C under continuous shaking. After 24 h the cornea was placed in hypertonic buffer containing 1% Triton X-100, 1.5 M KCl, and 10.0 mM Tris (pH 8.0) under the same

conditions. In the third step, hypertonic buffer was replaced by an extraction buffer containing 1% SDS and 10.0 mM Tris (pH 9.0) for 24 h. To remove SDS, the cornea was incubated in 1% Triton X-100 at 37^{o}C for 30 min under shaking. Finally the cornea was washed extensively with PBS change every 24 h for up to 72 h under the same temperature and shaking conditions.

Method 5 (NaCl plus nucleases):

Corneas were subjected to non-surfactant treatment involving incubation in 1.5 M sodium chloride (NaCl) solution for 48 h with NaCl change after 24 h (Gonzalez-Andrades et al., 2010). This method was modified in the second step by treating the corneas with DNAse 5 U/mL and RNAse 5 U/mL for 48 h. Corneas were then washed with PBS for 72 h with PBS change every 24 h. The decellularization procedure was carried out at room temperature under continuous agitation.

2.2 Deturgescence of Decellularized Corneas

Corneas following decellularization were dehydrated to remove excess fluid to regain transparency by incubating in two different solutions. After decelllularization, swollen corneas were stored in Optisol-GS for at least 24 h or until needed for the experiment to remove extra fluid. Optisol-GS is currently the solution of choice for storage of corneas used in Eye banks. The main component of Optisol-GS is chondroitin sulfate and dextran that protect the cornea from swelling during storage.

The second solution that was tested to remove fluid was glycerol which is commonly used for long-term storage of tissues. To test the toxicity of glycerol, decellularized corneas were incubated in different concentrations of glycerol ranging from 5% to 100%. After 24 h of incubation, these corneas were placed on fibroblasts cultured in a 12-well plate. The experiment was carried out for one week and media was not changed during the study. After one week, the fibroblasts were stained with crystal violet to visualize the degree of cell viability.

2.3 Histologic Evaluation of Decellularized Corneas

The removal of cellular components was investigated by routine histology and using the nuclear stains 4',6-diamidino-2-phenylindole (DAPI) and/or propidium iodide (PI). Decellularized corneas were embedded in optimal cutting temperature (OCT) medium, sectioned with a cryostat and fixed in 4% paraformaldehyde (PFA) solution in PBS for 20 min at room temperature. Following fixation, the tissue sections were permeabilized with 0.5% Triton-X-100 in PBS for 30 min at room temperature and stained with PI or DAPI. Periodic Acid Schiff (PAS) staining was also used to evaluate the integrity of the basement membrane. Fluorescent images were captured with a Zeiss Axioscope2 upright phase contrast and epifluorescence microscope. The integrity of the extracellular matrix was investigated with a hematoxylin and eosin (H and E) stain and scanning electron microscopy. The H and E stain was used to observe the corneal structure as well as the degree of decellularization. The presence of residual cellular/membranous material was assessed by immunostaining for human MHC class I antigen (HLA-A, B, C).

For scanning electron microscopy studies, the corneas were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde (PFA) in PBS, pH 7.4, at room temperature for 2 h, then washed in PBS buffer, pH 7.4, 3 times for 5 min each. For secondary fixation, samples were fixed in 2% osmium tetraoxide in PBS for 1 h at room temperature, and dehydrated in graded ethanol series: 25%, 50%, 75%, 95% each for 15 min and finally in 100% ethanol with 3 changes for 5 min each. Samples were critical point dried using a CPD 030 - Balzers Critical Point Dryer (BAL-TEC). Samples were mounted on an aluminum stub and coated with gold/palladium with a thickness of approximately 275 Angstrom using sputter coater (Denton Desk IV Sputter Coater). Scanning electron microscope JEOL (Japan Electron Optics Laboratory) 5600LV SEM was used to visualize and compare the anterior surface morphology and the arrangement of the collagen fibers network of control and decellularized corneas.

For transmission electron microscopy, samples were primary fixed in 2% PFA and 2.5% glutaraldehyde in sodium cacodylate then secondary fixed with 1% osmium tetroxide at 4^{o}C. Dehydration with ethanol was performed as described for SEM followed by infiltration with 1 part Spurr's resin to 1 part 100% ethanol over molecular sieves, followed by embedding with fresh resin and polymerization. Polymerized blocks were sectioned at 90nm on a Reichert-Jung Ultracut E ultramicrotome then placed on 200 hex mesh parlodion/carbon coated nickel grids, dried, and stained with 4% uranyl acetate, followed by Reynold's lead citrate. Stained grids were imaged with a JEOL 1200EX transmission electron microscope. The size and distance between collagen fibers were measured for a minimum of 50 fibers for each TEM section.

For periodic acid Schiff (PAS) staining, frozen sections were incubated in 0.5% periodic acid for 10 min at room temperature. The sections were then washed in running water for 5 minutes and then treated with hydrochloric acid Schiff's reagent for 7 min warmed at room temperature. This is followed by 5 min wash with running water.

2.4 Mechanical Testing of Decellularized Corneas

To characterize the mechanical properties of NaCl plus nucleases treated cornea, uniaxial tension test and tension to rupture tests were performed. In this experiment an MTS machine is used to perform tensile tests of specimens (Figure 6). The cornea was cut in 6-8mm strips and fastened between two aluminium clamps. In biological tissues, stress increases much faster with increasing strain at first, and after a number of cycles, it becomes steady. To record steady state data, preconditioning of the cornea at 20% was done for the initial period of adjustment. A 44N load cell was used to stretch the tissue and elongation was recorded. In all the samples, tension conditions were set at 50% and at a frequency of 0.1 Hertz. From the data collected, a tension stress-strain curve was plotted to measure Secant Elastic Modulus to draw a line between any two points on a stress-strain curve and measuring the slope of that line. Secant Elastic Modulus is also called stress-strain ratio and is a close approximation of the Elastic Modulus in the elastic range. In this study, the slope of the stress-strain curve was compared between control and decellularized corneas to determine the stiffness of the corneas.

In order to keep the solution conditions the same, control corneas were kept in PBS for 1 week to allow swelling to match up with the decellularized corneas. The experiment was carried out in water bath to avoid variability in the data due to drying out

of the tissue during the experiment. Statistical analysis was performed using a Student's t-test.

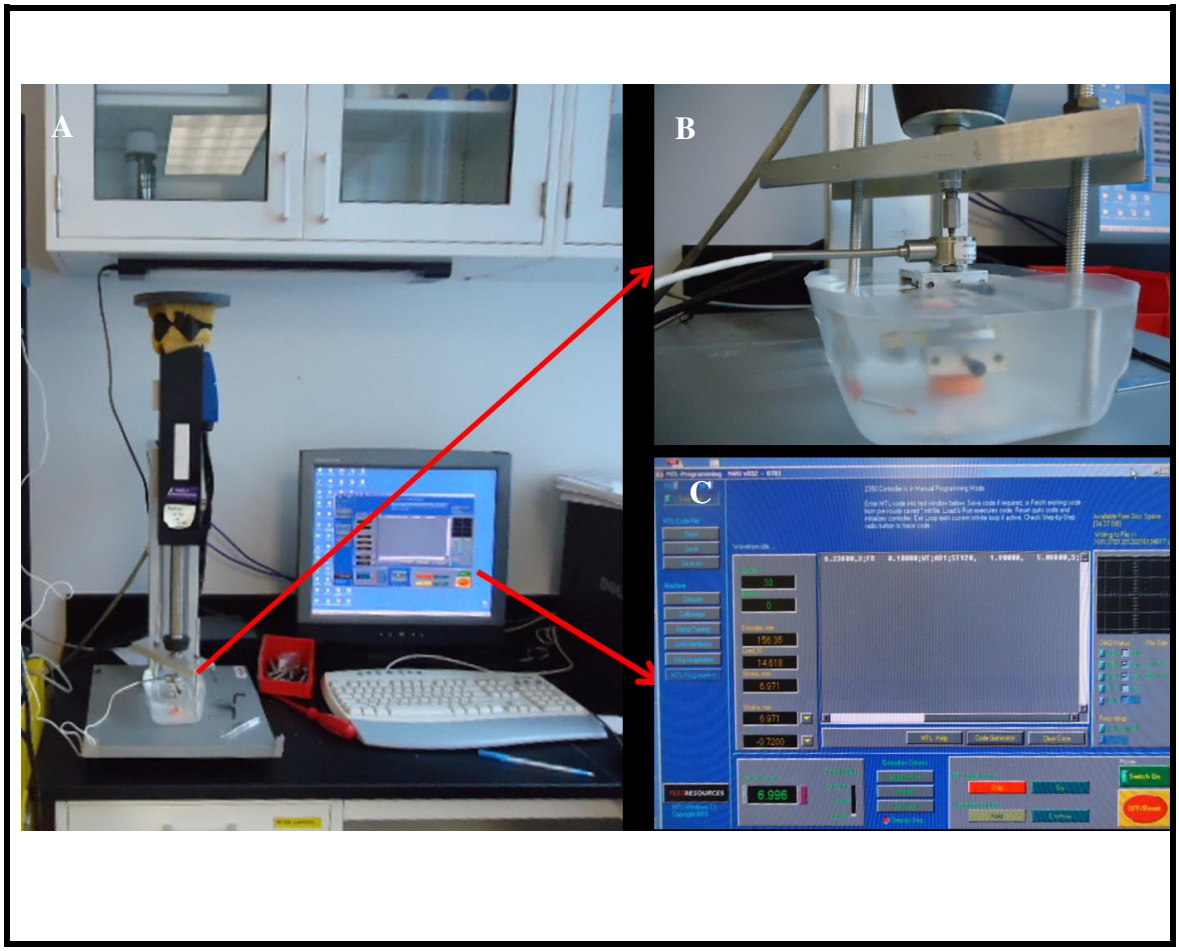

Figure 6. Mechanical testing apparatus and set up. (B) The experiment was carried out in water bath to avoid drying out of the tissue during the experiment and (C) MTS software was used to generate data.

3. Results

3.1 Decellularization of Human Corneas

Human corneas decellularized with different methods were initially screened for the removal of cells with H&E staining, as well as the nuclear stain DAPI (Figure 7). Complete removal of cells was observed for corneas treated with the NaCl plus nuclease treatment method as well as the osmotic gradient and 1% SDS processing methods. The use of liquid nitrogen, and poly (ethylene glycol) (PEG 1000 or 8000) did not result in complete removal of cells and cellular debris. The presence of residual cellular/membranous material was assessed by immunostaining for human MHC class I antigen (HLA-A, B, C) (Figure 8). HLA staining was positive for liquid nitrogen, poly (ethylene glycol) (PEG 1000), and triton X-100, while for 1% SDS, osmotic gradient, poly (ethylene glycol) (PEG 8000), and NaCl plus nuclease treatment the staining was negative suggesting the tissue is not immunogenic.

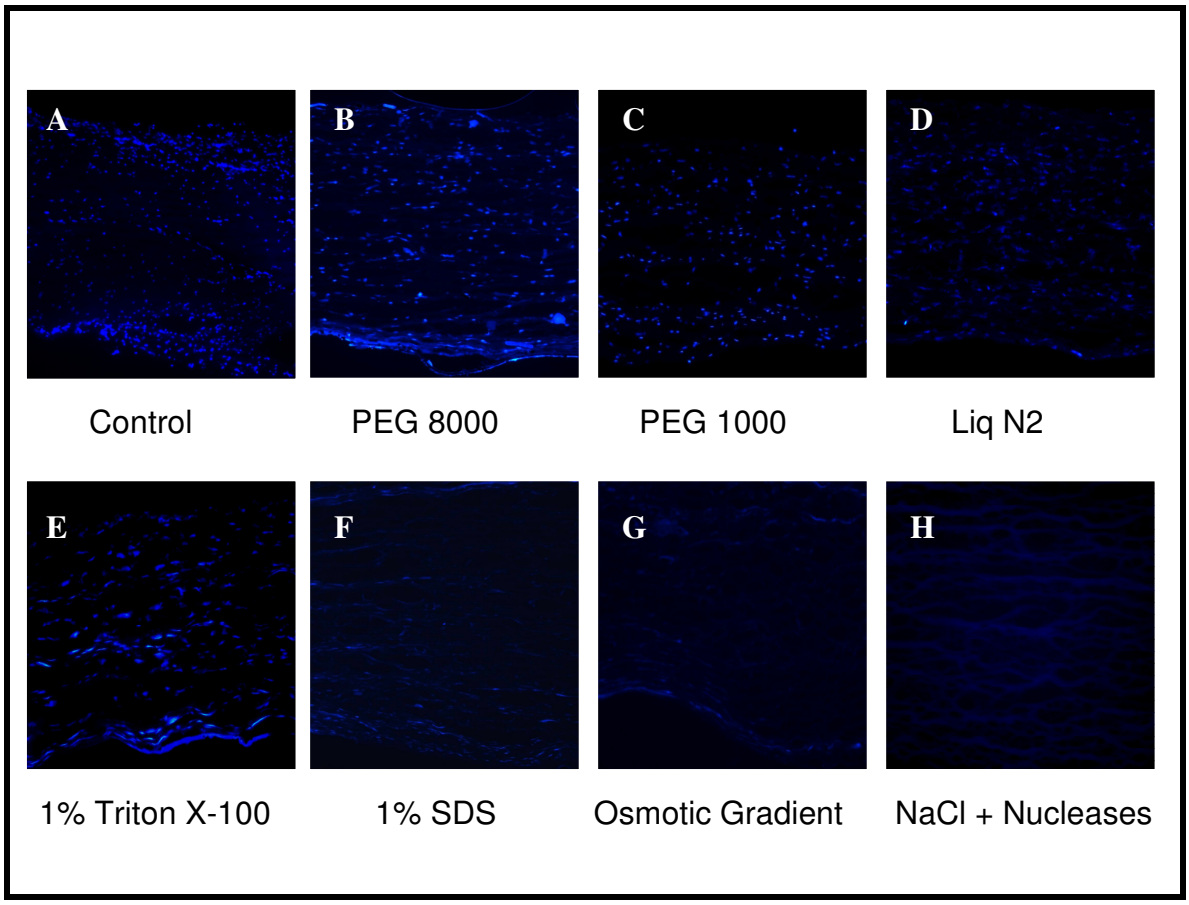

Figure 7. Evaluation of removal of cells. DAPI staining of corneal sections treated with different decellularization methods. (B, C, D, E) 1% Triton X-100, liquid nitrogen, PEG 1000, and PEG 8000 treatment methods did not remove cells while, (F, G, H) 1% SDS, osmotic gradient, and NaCl + nucleases methods resulted in successful removal of cellular components.

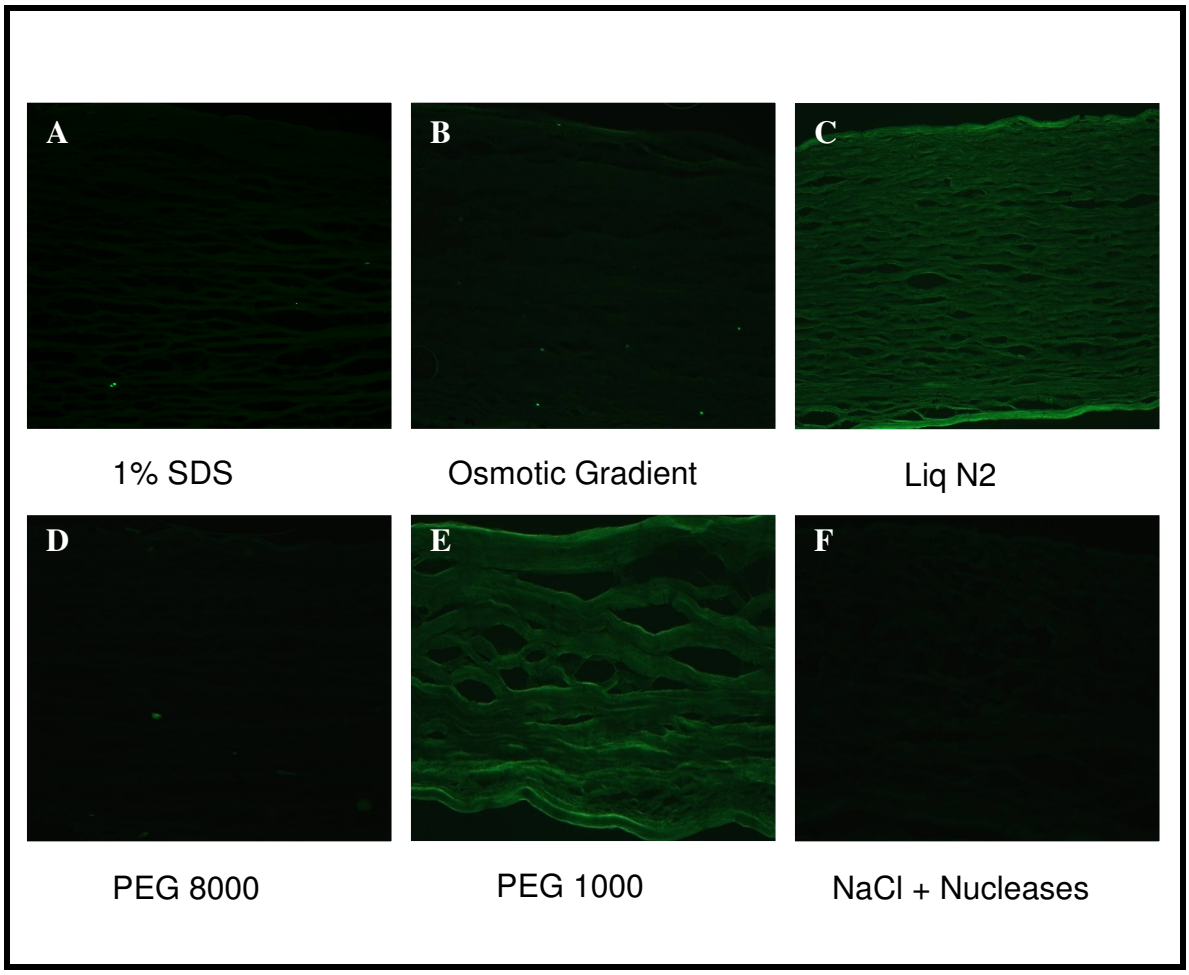

Figure 8. Evaluation of removal of immunogenic antigens. HLA staining of corneal sections treated with different decellularization methods. (A, B, D, F) 1% SDS, osmotic gradient, PEG 8000, and NaCl + nucleases methods resulted negative for HLA staining while, (C, E) liquid nitrogen and PEG 1000 treatment methods showed positive staining for HLA staining.

3.2 Evaluation of Extracellular Matrix

The epithelium basement membrane was evaluated with PAS staining (Figure 9) and immunostaining for collagen type IV (not shown for all the treatment methods). PAS stains carbohydrate in tissues and is routinely used in histologic studies to identify basement membranes that contain high proportions of glycoproteins/carbohydrates.

On PAS staining, the epithelial basement membrane of corneas treated with NaCl plus nucleases appeared intact and comparable to the untreated corneas. Likewise, on immunofluorescence, there was no difference in the staining for collagen type IV, laminin, and fibronectin between the NaCl plus nuclease treated and fresh cadaver corneas (Figure 10). The basement membrane in the limbal region was evaluated more specifically using a monoclonal antibody against α1 chain of collagen type IV (Figure 10). There was also no difference compared to control, suggesting that the treatment did not have a deleterious effect on the basement membrane of the limbal region (Figure 10). The detergent based methods in particular SDS, was found to induce damage to the basement membrane by PAS staining in certain areas.

The decellulrization process generally caused significant swelling of the corneal tissue, however, following deturgescence with glycerol the stroma was optically clear indicating no gross change to the lamellar organization (Figure 10). Glycerol decellularized corneas regained their transparency and showed no cytotoxicity to the cells at low concentrations (Figure 11). However, at high glycerol concentrations of 80%, 90% and 100%, cell death was more significant. There was also some loss of cells due to

washing during the staining procedure as seen in the 0% glycerol case (control). Normal cornea contains some fluid in it, therefore, to avoid over dehydration 50% glycerol was used to remove excess fluid out of the tissue. 50% glycerol with 50% basal media (DMEM) is used for storing many biological tissues for long term storage. In this study, the ability of the glycerol treated cornea to support cell growth was not investigated, therefore, for the corneas used for grafting, optisol-GS was used which is currently used to store corneas at Eye bank.

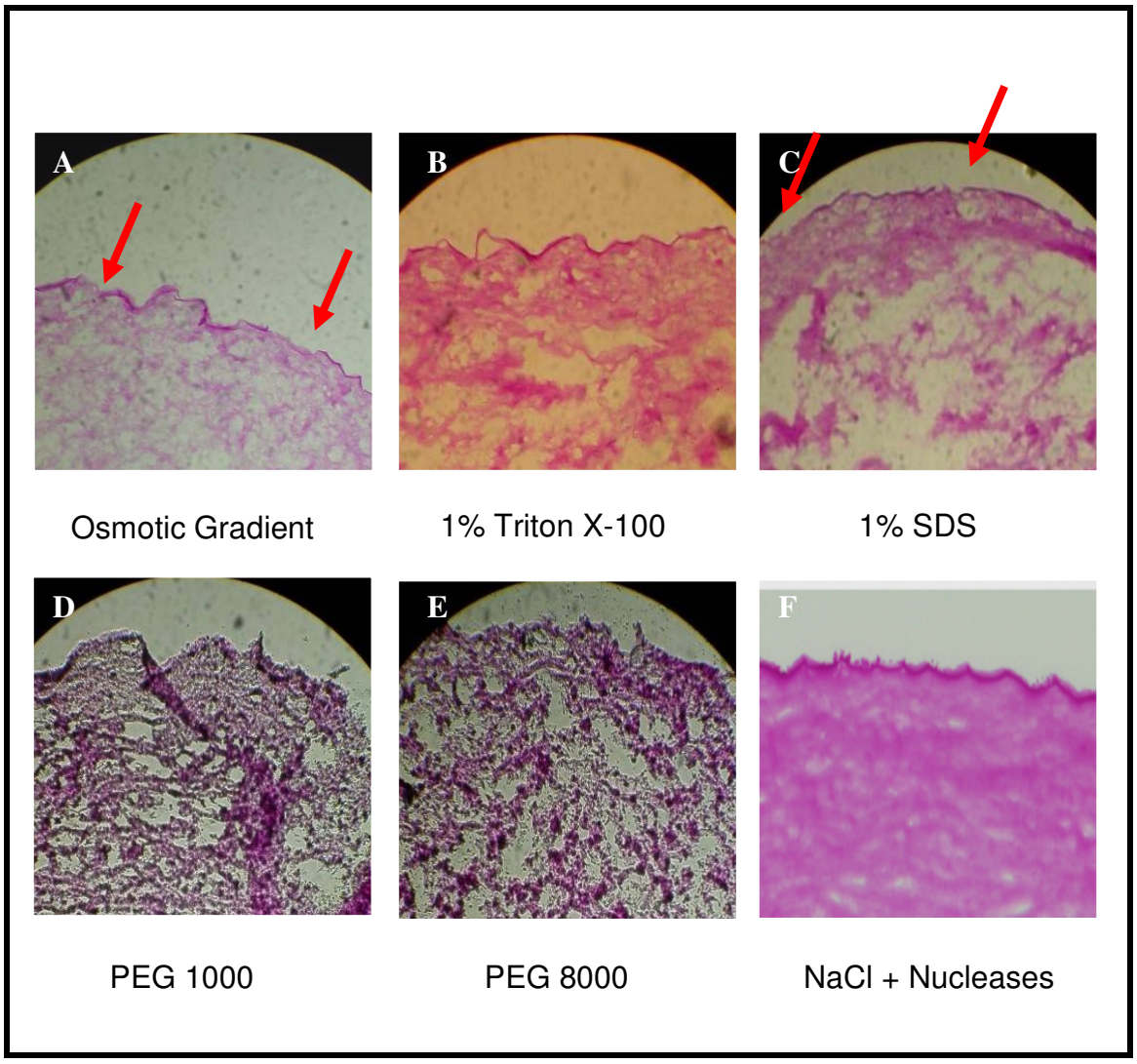

Figure 9. Evaluation of epithelium basement membrane. PAS staining of corneal sections treated with different decellularization methods. (A, C) Corneas treated with osmotic gradient and 1% SDS had inconsistent basement membrane integrity. Arrows show areas where the basement membrane is damaged. (B, F) Triton X-100 and NaCl + nucleases resulted in intact basement membrane. (D, E) Both PEG (1000, 8000) treatments resulted in damage to the epithelium basement membrane along with significant damage to the extracellular matrix.

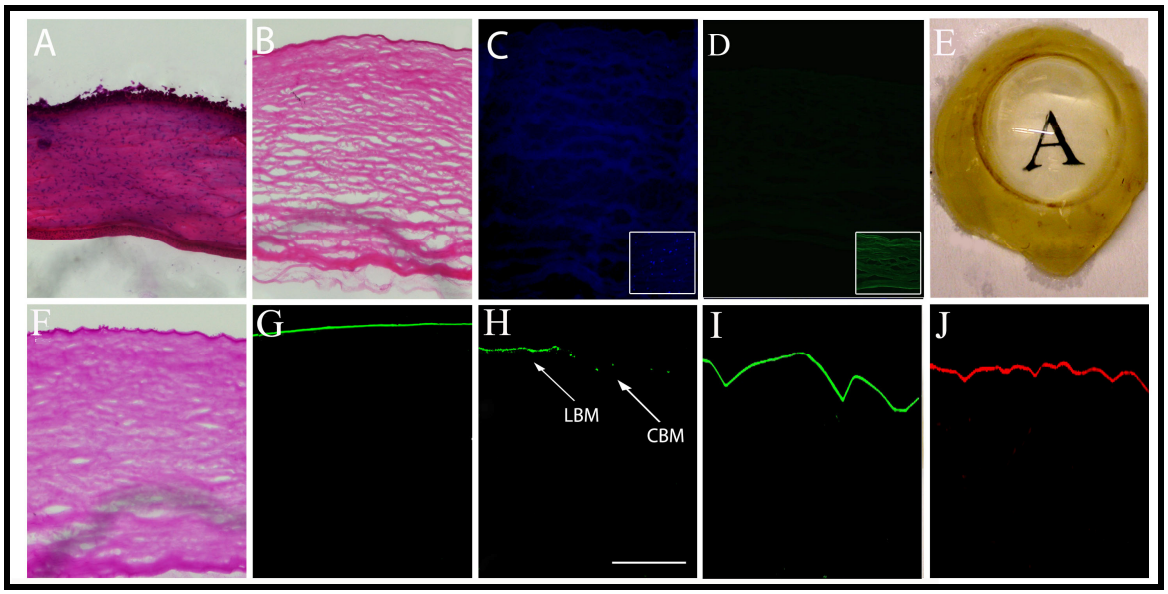

Figure 10. Histological analysis of corneas exposed to the NaCl plus nuclease decellularization method. Hematoxylin and eosin staining in (A) control cornea and (B) decellularized cornea. (C) DAPI staining of NaCl decellularized cornea demonstrating absence of nuclei (inset showing incomplete decellularization with liguid nitrogen method), (D) HLA staining of NaCl decellularized cornea demonstrating no HLA (inset showing positive HLA staining with liquid nitrogen method), (E) Optically clear NaCl plus nuclease decellularized cornea after deturgescence with glycerol. The presence of the epithelial basement membrane in decellularized cornea was observed by (F) PAS staining and (G) by immunostaining against collagen type IV, (H) the alpha1 subtype of collagen type IV which is specific for the limbal basement membrane (LBM) (arrow), central corneal basement membrane (CBM) (arrow), (I) fibronectin, and (J) laminin staining. (H) Scale Bar = 50 mm. NaCl, sodium chloride; DAPI, 4',6-diamidino-2-phenylindole; HLA, human leukocyte antigen; PAS, periodic acid Schiff.

Table 1. Results of Corneal Decellularization Using the Tested Methods.

Decellularization Method	DAPI	Basement Membrane	HLA Staining
SDS	-	+/-	-
Triton X-100	+	+	+
Liquid Nitrogen	+	+	+
PEG (1000)	+	+	+
PEG (8000)	+	+	-
Osmotic Gradient	-	+/-	-
NaCl + Nucleases	-	+	-

Table 1. DAPI: ‘‘ + ’’ = presence of nuclei/DNA, ‘‘ - ’’ = absence of nuclei/DNA; basement Membrane: ‘‘ + ’’ = completely intact basement membrane, ‘‘ + / - ’’ = areas of basement membrane disruption; HLA: ‘‘ + ’’ = presence of membranous/cellular material by HLA staining, ‘‘ - ’’ = absence of HLA staining.SDS, sodium dodecyl sulfate; PEG, poly(ethylene glycol); NaCl, sodium chloride; DAPI, 4',6- diamidino-2-phenylindole; HLA, human leukocyte antigen.

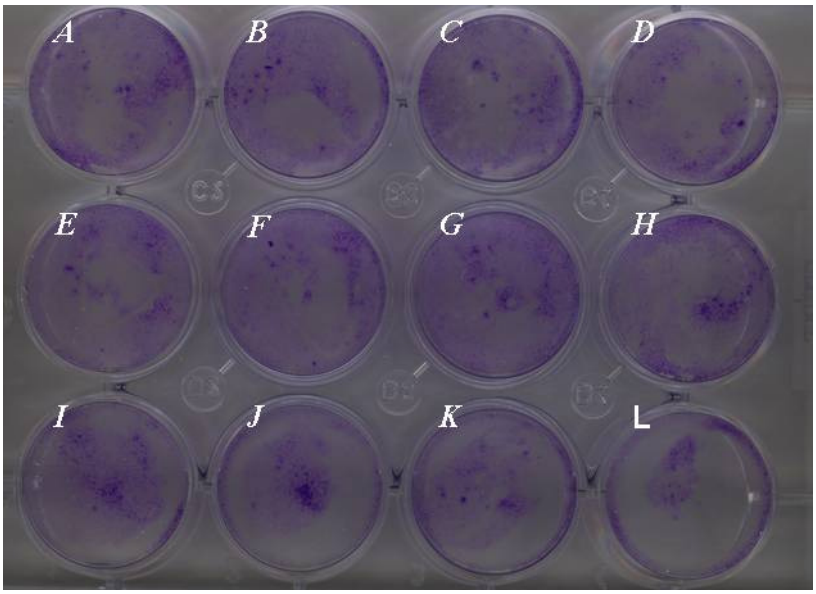

Figure 11. Optimization of glycerol concentration for achieving corneal deturgence. Decellularized corneas were treated with different concentrations of glycerol to remove excess fluid. Decellularized corneas treated with (A) 0% (control) (B) 5% (C) 10% (D) 20% (E) 30% (F) 40% (G) 50% (H) 60% (I) 70% (J) 80% (K) 90% and (L) 100% glycerol concentration were placed in each well of a 12-well plate, containing 80% confluent corneal fibroblasts, to evaluate the cytotoxicity of increasing glycerol concentration. Glycerol decellularized corneas regained their transparency and showed no cytotoxicity to the cells at low concentrations. However, high glycerol concentrations of 80%, 90% and 100% showed more cell death.

The effect of the NaCl plus nuclease treatment on the integrity of the matrix was further evaluated by scanning and transmission electron microscopy (Figure 12). Epithelial cells seen in control corneas were absent in decellularized corneas with preservation of the limbal matrix and epithelial basement membrane following decellularization. No gross changes in the collagen fibers were noted in decellularized corneas except that the collagen fibers were slightly larger due to swelling as noted above (Figure 13). Rapid stromal swelling is also observed in ex vivo stored corneas for longer time in normal situation (Meek, and Quantock, 2001; Ruberti, and Klyce, 2001).

To maintain structural and functional integrity of the cornea, a normal orientation of the fiber network is very important. Disruption of this network eventually leads to degeneration of the cornea, and cause irregularities inside the stroma as well as on the surface of the cornea and disrupts the normal curvature that results in compromised vision. For our study, the diameter of the collagen fibers and the distance between these fibers were measured in images taken under a transmission electron microscope (Figure 13). The mean diameter of the collagen fibers was 377 ± 42 Å in normal compared to 499 ± 26 Å in decellularized corneas ($P < 0.001$). There was no significant difference in the distances between the fibers (276 ± 33 Å vs. 297 ± 70 Å). Overall, the NaCl plus nuclease decellularization procedure did not appear to alter the ultrastructure of the cornea (Figure 13).

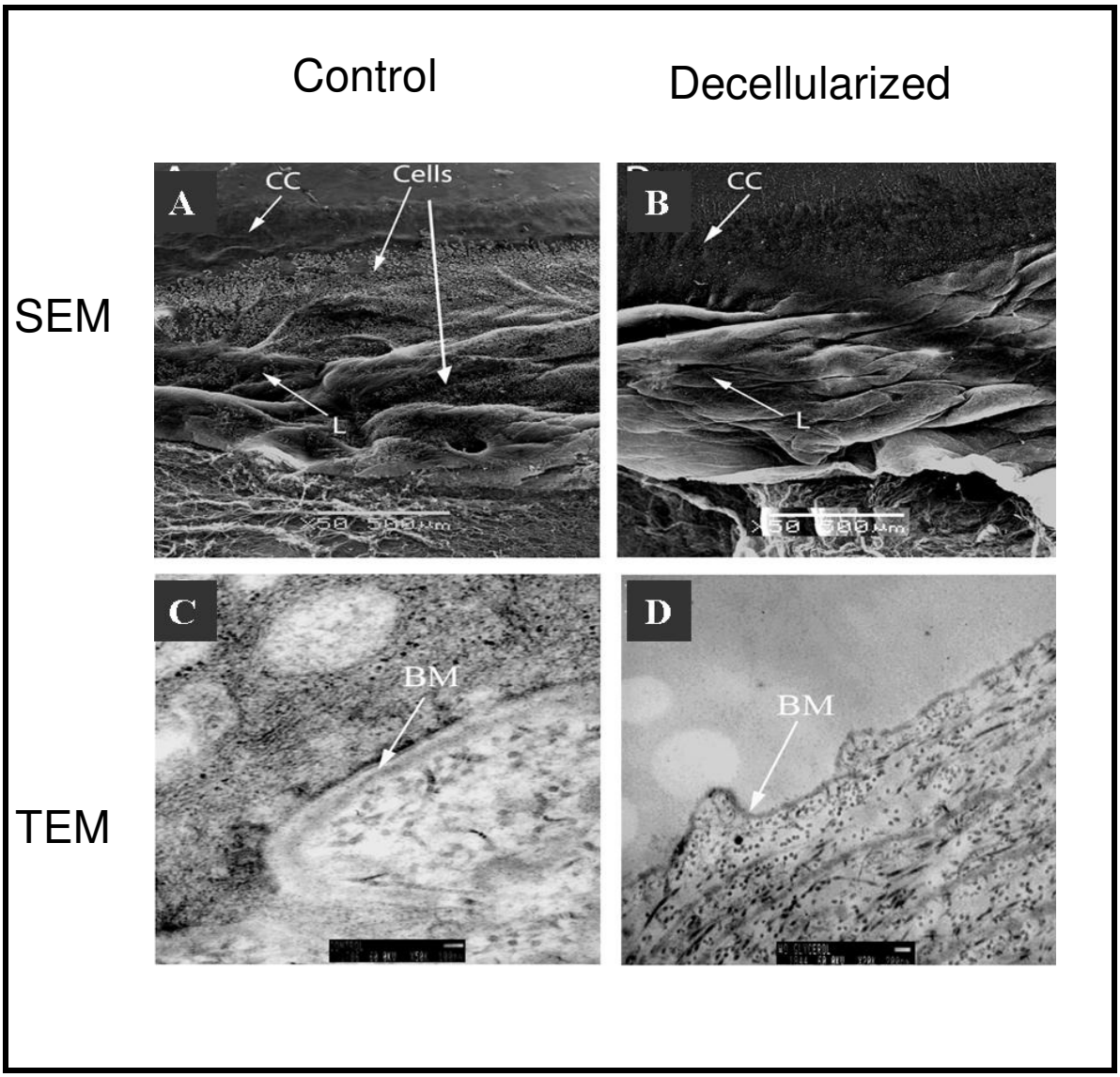

Figure 12. Electron micrographs to show removal of cells and integrity of basement membrane. (A, B) SEM micrographs to show removal of cells. Anterior surface of the limbus in (A) control and (B) NaCl plus nuclease decellularized corneas. No significant difference is observed in the anterior surface of the central cornea (CC), and limbal (L) region. (C, D) TEM micrographs to show integrity of the basement membrane. Epithelial basement membrane (BM), (arrow) is shown in (G) control and (H) NaCl plus nuclease decellularized cornea.

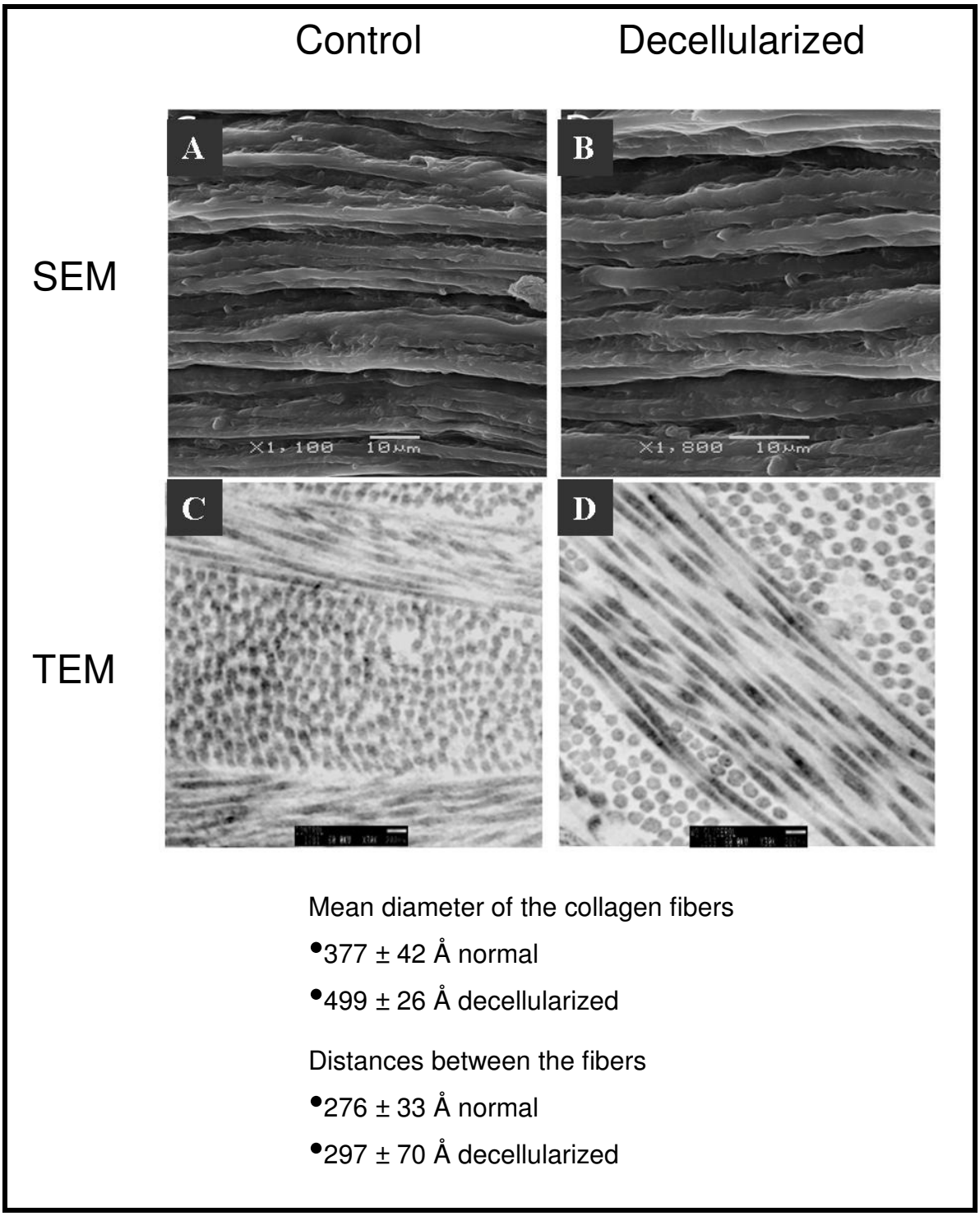

Figure 13. Electron micrographs to show integrity of the collagen fibers. (A, B) Scanning electron micrographs. On cross-section of cornea, the collagen fibrils appear similar between (A) control and (B) NaCl plus nuclease treated corneas. (C, D)Transmission electron micrographs. Collagen fibers in (C) control and (D) NaCl plus nuclease decellularized cornea.

3.4 Mechanical Testing of Decellularized Corneas

A tensile test was performed for control and decellularized corneas using an MTS machine (Figure 14). Seven control and decellularized corneas with NaCl plus nucleases method were compared. Statistical analysis performed using a Student's t-test showed no significant difference in the elastic modulus between the control and the decellularized corneas (Figure 15). However, it must be noted that our result had large standard deviation and variability in the data collected, and overall, the study was under powered. One of the reasons for having a lot of variation in the value of elastic modulus among the group is because the sample consisted of corneas of donor from different ages and medical conditions. The mechanical properties of cornea are dependent on the age. The cornea becomes stiffer as the patient ages due to a decrease in the elasticity of the tissue.

To measure ultimate tensile strength, tension is measured at the point of rupture of the tissue. For this test 44N load cell was used to stretch the cornea. However, this force was not enough to rupture the cornea. Therefore, no data was collected for the tension to rupture test.

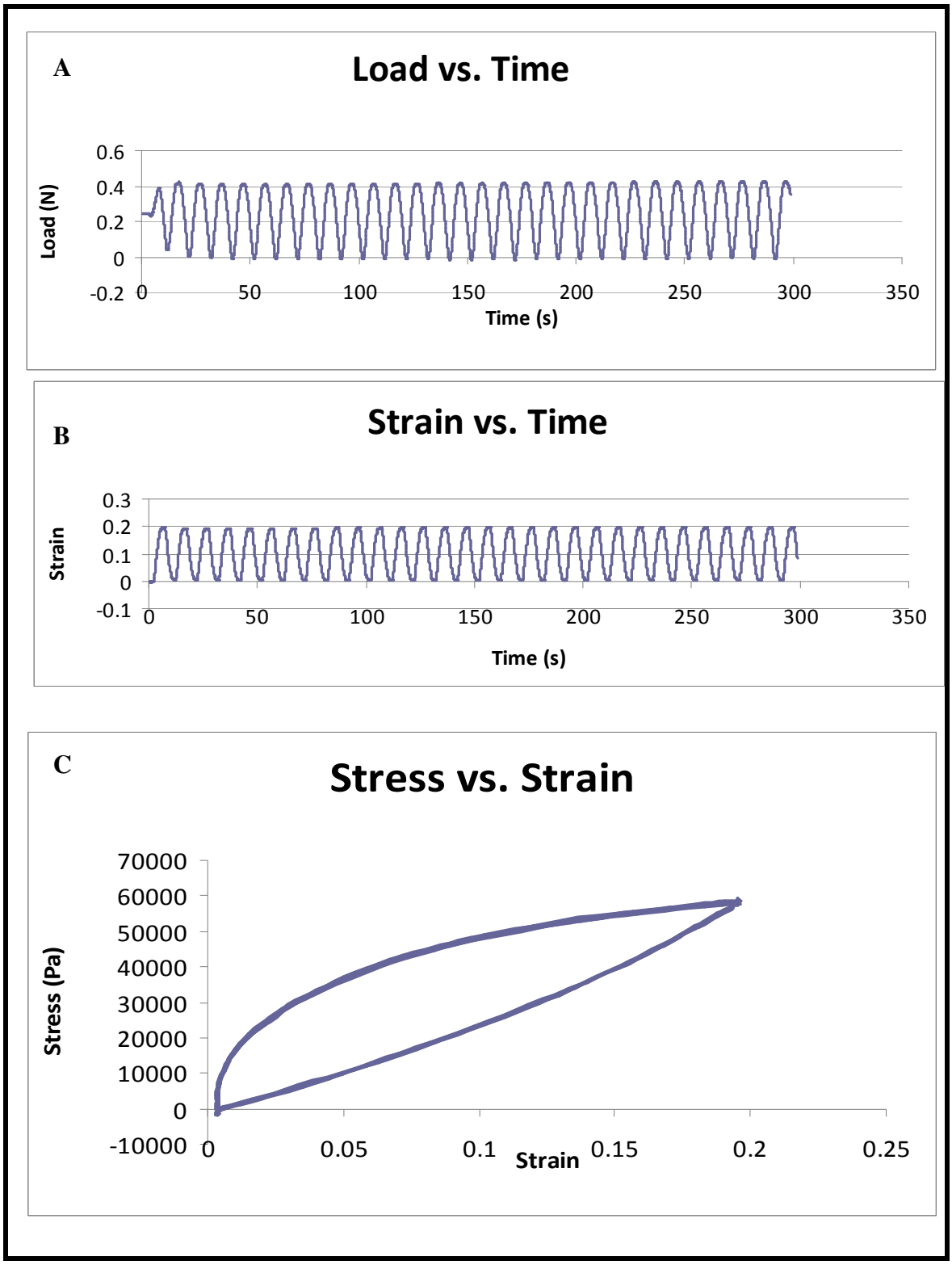

Figure 14. Stress-strain curve. (A) Load vs. time curve (B) Strain vs. time curve (C) Stress-strain curves during loading and unloading showing low hysteresis.

A

Elastic Modulus of Control vs. Decellularized Cornea	
Control	**Decellularized**
111677	90759
107983	107068
131359	77611
264306	204525
269690	96855
59941	137185
80611	15584
P – Value: 0.397226	

B

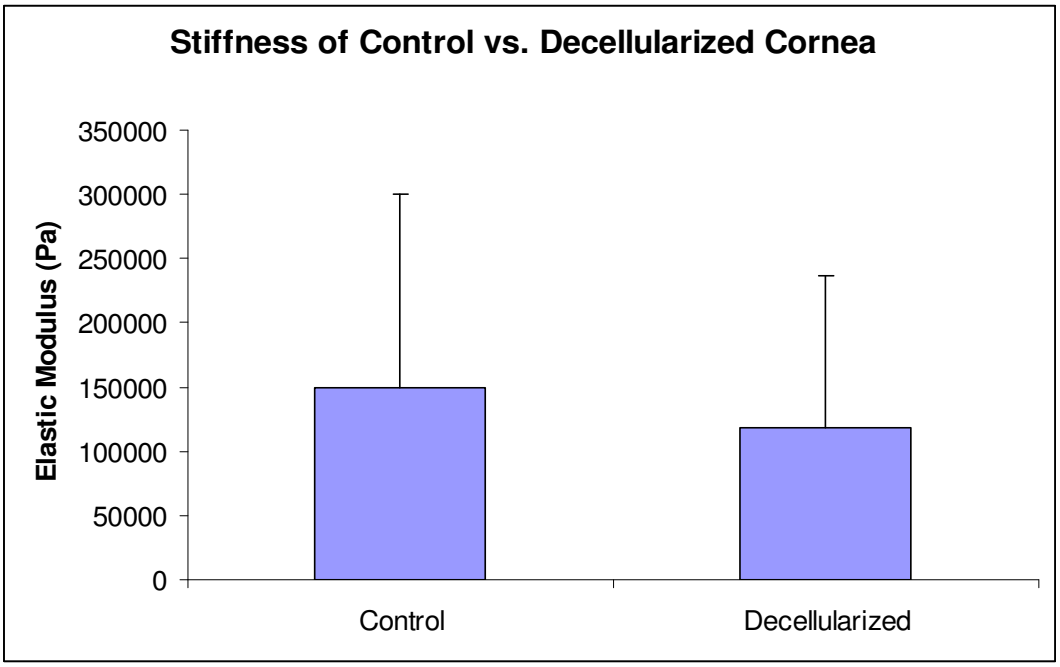

Figure 15. Elastic modulus of control and decellularized corneas. Seven control and decellularized corneal samples were tested.

4. Discussion

The purpose of this aim was to develop a decellularized human cornea as a suitable scaffold for tissue engineering of the corneal epithelium and its underlying stroma. The rationale behind this approach was that a tissue construct that best recreates the corneal and limbal microenvironment would likely offer the most optimal substrate for survival and function of the tissue engineered corneal epithelium. This may be particularly important since in many clinical situations the pathology or damage is extended beyond the epithelium and often includes the extracellular matrix and cells within the anterior stroma. A decellularized human cornea could potentially provide a matrix that is nearly identical to the native cornea on which appropriate cells can be repopulated to reconstruct the corneal epithelium with the underlying stromal support.

To decellularize any organ or tissue, either physical, chemical or a combination of both methods is utilized. Any treatment method to remove the cells from a tissue disrupts the native architecture of the extracellular matrix. Therefore, the goal of decellularization was to minimize the disruption of the extracellular matrix while maximizing the retention of mechanical and biological properties of the tissue. Physical methods to remove cells include agitation, sonication, mechanical pressure, or freezing and thawing. All these methods cause cell membrane lysis and release of the nuclear content into the matrix which is then removed by washing the tissue multiple times with fluids such as saline, water, or the culture medium. For thin tissues such as membrane, physical treatment might be enough to decellularize, however, for more complex, thicker tissues, a chemical treatment must be added to achieve complete removal of cells. These chemical methods

include using trypsin, ionic solutions, and detergents that penetrate through the tissue and reach the deeply embedded cells into the tissue and cause disruption of the cell membranes and the intercellular and extracellular adhesions. The main challenge after decellularization with the chemical treatment is the removal of the residual chemical prior to repopulating with cells and transplantation. Some of the chemicals such as detergents like SDS is very difficult to remove from the tissue, especially from the compact tissues and organs and requires aggressive rinsing of the tissue (Gilbert et al., 2006).

In this study a total of five methods were tested for their effectiveness in cellular removal, preservation of the extracellular matrix, and the ability of the resulting decellularized cornea to support the growth of corneal cells. Among the various detergent and non-detergent based methods, a process using NaCl, DNAse and RNAse was found to provide the most superior results with complete decellularization and minimal disturbance of the basement membrane and the ultrastructure of the cornea. The mechanism of action of this method is to first cause cell lysis by putting the cornea in a hypertonic solution made with 1.5 M NaCl. This solution caused shrinking of the cells and ultimately disruption of the cell membrane and release of the nuclear materials, DNA and RNA. To cleave off DNA and RNA, NaCl was replaced with enzymes DNAse and RNAse. Finally, to remove the resultant cellular remnants from the tissue, cornea was washed with PBS. All this process was done on an orbital shaker to facilitate chemical exposure to the deeper layers of the tissue, and for removal of cellular debris.

The use of liquid nitrogen and PEG (1000, 8000) resulted in incomplete removal of cells from corneas. The rationale for using PEG and liquid nitrogen was to avoid using

detergents, which usually cause more destruction to the matrix. PEG is an amphiphilic polymer having both hydrophilic and hydrophobic parts. For the purpose of removing cells from the tissue, PEG can actively interact with the cell membrane and destabilizes it. It is biocompatible and nontoxic to humans and hence already used in many pharmaceutical drugs. It is also currently used in eye drop lubricants (Ota et al., 2007). For this study, PEG with two molecular weights 1000 and 8000 were used. The viscosity of the polymer increases with increasing the molecular weight of the polymer, so PEG 1000 is less viscous and PEG 8000 has longer side chains that make it more viscous. Both of these PEG 1000 and PEG 8000 were not effective in removing cells from the cornea. Corneal sections treated with PEG showed a lot of damage to the extracellular matrix when stained with Periodic Acid Schiff. Sections stained with HLA immunostaining showed positive staining for PEG 1000 and negative for PEG 8000.

Liquid nitrogen was poured directly on the cornea to rapidly freeze it and cause lysis of cell membrane by the formation of intracellular ice crystals. This also required rinsing of the tissue to remove cellular debris. However, this rapid freezing did not completely remove cells. It also showed positive HLA staining. Further study to evaluate whether this rapid freezing caused fracture of the ECM was not performed.

The use of detergents, while successful in removing cellular materials, rendered the corneas unsuitable for epithelial cell attachment afterwards. This was consistent with a previous study on decellularized human cornea using 2% Triton X-100 and 0.1% NH_4OH after which laminin (basement membrane protein) was no longer detectable in the epithelial basement membrane (Choi et al., 2010). With triton X-100 treatment, some

of the cells were retained in the cornea. Triton X-100 is non-ionic detergent that disrupts lipid–lipid and lipid–protein adhesions while leaving protein–protein interactions intact. SDS is ionic detergents and removes cells by solubilizing cytoplasmic and nuclear cellular membranes. It is also known to remove glucosaminoglycans (GAGs) from the tissue (Gilbert et al., 2006). GAGs are long unbranched polysaccharides containing repeating disaccharide units. Their extended chain length imparts high viscosity to the solution. GAGs are located primarily on the surface of cells or in the extracellular matrix. They have rigid structure that provides structural integrity to cells and provides passageways between cells, allowing for cell migration. Some of the examples of GAGs are hyaluronate, chondroitin sulfate, heparin sulfate, heparin, dermatan sulfate, and keratin sulfate. Different GAGs are present in different parts of the body and perform vital biological functions. In cornea, keratan sulfate is present, which is responsible for maintaining even spacing of collagen type I fibrils to allow proper refraction of light. Defect in keratin sulfate causes distortion of the arrangement of fibrils and causes corneal opacity (Esko et al., 2009). However, loss of GAGs from the cornea was not evaluated in this study.

SDS also has a tendency to denature proteins. SDS in comparison with Triton X-100 treatment consistently and effectively removed the cells completely from the cornea. We showed in this study that SDS successfully removed the cells from the cornea, but did not support re-epithelialization ex vivo, probably due to denaturing of the proteins (Chapter 3). Also, both detergents caused significant damage to the extracellular matrix as shown by the PAS staining.

Besides biochemical cues that are provided by proteins and other signaling molecules, physical cues are also important for integrity of any tissue and organ. It has been shown that the behavior of cells can be regulated by topographical cues (Au et al., 2007; Curtis, and Wilkinson, 1997; Lim, and Donahue, 2007; Jones et al., 2012). For successful engineering of the cornea, both biochemical and mechanical properties need to be optimized. In the cornea, collagen is a major component of the extracellular matrix and comprises predominantly of type I and V collagens in the stroma (Newsome et al., 1982). The fibrils are aligned in parallel sheets within a layer and are surrounded by proteoglycans. These layers are stacked orthogonally with respect to each other. This orientation of fibrils having regular size and spacing between them is important for minimizing light scattering and therefore, transparency of the cornea (Farrell et al., 1973).

There are many eye disorders associated with the damage or disruption of ECM fibril network. Collagen fiber disruption causes abnormal corneal refraction and vision is degraded. Keratoconus is one such disease where the normal collagen-fibril network is disrupted due to weakening of the adhesions. This loosening of collagen mesh is accompanied with increased intraocular pressure that pushes the cornea outward causing it to protrude out. Corneas having keratoconous are significantly weaker than that of normal corneas (Andreassen et al., 1980; Nash et al., 1982). There are many other disorders associated with weakening of the stroma, therefore, for developing suitable substitute for the cornea, mechanical properties need to be adjusted.

In this study, to compare stiffness of the decellularized with the native cornea, we used tensile testing. Several different methods have been used to determine the elastic

modulus of the cornea and a wide range of values has been published (0.01–11.1 MPa) (Lombardo et al., 2012; Knox et al., 2011; Elsheikh et al., 2007; Hjortdal, 1996; Hoeltzel et al., 1992; Jue, and Maurice, 1986; Liu, and Roberts, 2005; Nash et al., 1982; Nyquist, 1968; Wollensak, and Iomdina, 2009; and Zeng et al., 2001). The wide range of values for the elastic modulus suggests that there are various external factors, such as the type of storage media, temperature, humidity, swelling or dehydration of the tissue, and testing techniques that may affect the viscoelastic property of the cornea in the experimental environment.

The two commonly used testing methods to measure elastic modulus are tensile testing, which involves pulling on a strip of the cornea and bulge testing which involves applying pressure behind the cornea and measuring the deflection of the cornea as a function of pressure. For this study, tensile testing method was employed. The behavior of viscoelastic materials is dependent upon conditions such as temperature and hydration therefore these parameters were kept constant throughout the experiment.

Biological tissues that are soft in nature, such as the cornea, are viscoelastic materials and exhibit a complex stress-strain relationship that is dependent on time, temperature, and frequency of the loading and unloading, which means that the rate at which a load is applied changes the measured value for the Young Modulus. The stress-strain relationship of the cornea is non-linear and that makes calculating the elastic modulus difficult. During cyclic loading/unloading in viscoelastic material some energy is stored and some is lost. Therefore, for the cornea, the true elastic modulus is called the 'Dynamic Modulus' which is the vector sum of stored modulus and damping modulus

(loss modulus). For pure elastic material, the phase shift between stress and strain is equal to 0, and for the pure viscous material the phase shift between stress and the strain is equal to 90^{o}, and for the viscoelastic material like the cornea, the phase shift is between $0\text{-}90^{o}$. The damping modulus is calculated by incorporating this phase shift (Stolz et al., 2004). However, for the data we collected, the hysteresis curves were fairly symmetrical and had low damping (small phase shift), which indicated that these corneas are mostly elastic under the applied strain. Therefore, for comparison purposes between decellularized and control corneas, the damping modulus was not accounted for and the stored modulus part was considered to be the close approximation to the elastic modulus.

There are many methods published for calculating the elastic modulus using stress-strain hysteresis curves (Stolz et al., 2004; Polfik et al., 1996; Pattin et al., 1996). The alternative methods for determining the modulus of the material are tangent modulus or a secant modulus. In this study, the secant elastic modulus was calculated in the elastic range of the loading curve. This is done by drawing a line through the center of the length of the loading curve and the slope of that line gives secant elastic modulus. In tangent modulus, the instantaneous rate of change of stress as a function of strain is calculated. This is done by drawing a line at any point on a stress-strain curve and the slope of that line gives tangent modulus at that instantaneous position (Buzard, 1992).

5. Conclusion

The decellularization method using NaCl plus nucleases treatment successfully removed the cells from the human cornea while preserving the integrity of the

extracellular matrix, basement membrane, and the stiffness of the cornea. The decellularized corneas could have potential application in corneal tissue regeneration and could be potentially used as an alternative to donor corneas for the transplantation purposes.

III. ABILITY OF DECELLULARIZED HUMAN CORNEA TO SUPPORT CELL GROWTH *(IN VITRO)*

1. Introduction

Epithelial cells play a pivotal role in the maintenance of the healthy corneal surface. They form the first barrier to the environmental insults and damage to these cells results in corneal vascularization. There has been a body of evidence that damage to epithelial cells make stromal cells (keratocytes) exposed, that eventually causes destruction of these keratocytes and make the cornea opaque.

For the treatment of damaged epithelium, in most clinical applications, a small population of corneal epithelial stem/progenitor cells from the limbus are expanded in culture on a substrate, typically a basement membrane such as human amniotic membrane and transplanted to the damaged corneal surface (Tsai et al., 2000). The main clinical indication for the use of tissue engineered corneal epithelium is the treatment of limbal stem cell deficiency, a condition where the epithelial stem cells responsible for renewing the corneal epithelium have been lost. The early clinical results appear promising, especially when autologous engineered epithelium is used and immune mediated rejection is avoided (Sharpe et al., 2007). However, success with tissue engineered corneal epithelium is not always attained and in some cases despite a successful initial transplant, a normal corneal epithelium is only temporarily restored (Kenyon, and Tseng, 1989). Although, there are many factors that contribute to the clinical failure of the tissue engineered epithelium, it seems that one of the major issues is the lack of an appropriate *in vivo* environment that can support the long-term survival and function of the corneal epithelium and its stem cells.

Besides factors essential for the health of the corneal epithelium such as a healthy tear film, proper eyelid closure, and the absence of destructive inflammation, the function and survival of the epithelium is also highly dependent on the structural and biochemical support from the underlying stroma. Previous studies in the cornea as well as other epithelial tissues have clearly demonstrated the importance of the interactions between the epithelium and its underlying layers (Li et al., 1995). In the cornea, epithelial-stromal interactions have been found to play a critical role in regulating epithelial proliferation and differentiation both during normal and wound healing states (Liu, J., et al. 1999; Li et al., 1995; Wang et al., 2007; Melles et al., 1995; Wilson et al., 2001).

Based on this information, we developed a decellularized corneal construct that can be used for both epithelium and anterior stromal reconstruction. The main advantage of using decellularized cornea over other natural or synthetic scaffolds is its highly complex native structure that best supports tissue repair and regeneration.

2. Material and Methods

2.1 Human Corneal Epithelial Cell Culture

Human corneal epithelial and fibroblast cultures were initiated from fresh cadaver eyes obtained from the Illinois Eye Bank as described (Djalilian et al., 2008). To isolate limbal epithelial cells, the limbus was separated from the central cornea with an 8 mm trephine. The limbal ring was incubated in 2 mg/mL Dispase II (Invitrogen) in PBS for 1 h at 37°C. The epithelial sheets were peeled off and digested in 0.25% trypsin-EDTA at

37°C for 30 min. Cells were washed and plated on the epithelium basement membrane side of the decellularized corneas at a density of 1 x 10^6 cells/cornea. To induce stratification after 2 weeks, the media was reduced to allow growth at the air-liquid interface.

2.2 Optimization of Media Conditions for Epithelial Cells

Every cell type requires certain nutrients for its optimal growth. To keep cells alive and healthy for longer periods of time *in vitro*, and to have desired proliferation, migration and differentiation, medium must be supplemented with several factors. A number of media conditions were tested to grow epithelial cells. A commercially available medium keratinocyte serum free media (KSFM) was compared with CM (designed by mixing KSFM plus DMEM plus Ham's F12 plus fibroblasts conditioned media) both for *in vitro* and *ex vivo* epithelial cultures.

2.3 Human Corneal Fibroblasts Cell Culture

To isolate corneal fibroblasts, the epithelium and Descemet's membrane were first removed after incubation in dispase as described above. The remaining stroma was then cut into 1mm x 1mm pieces and incubated in 0.1% collagenase (Sigma) in DMEM at 37°C for 1.5 h on a shaker. The isolated keratocytes were expanded and differentiated into fibroblasts by culturing in DMEM supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin and 100 µg/mL streptomycin. After 1-2 passages, the cells were trypsinized and resuspended in DMEM and 10% FBS. A total of 1x10^6 fibroblasts

cells were resuspended in 70 µL of media and injected into the stroma of each decellularized cornea. This was done using a 25g needle on a 1 mL syringe that was advanced into the mid-stroma then moved back and forth and side to side a few times to create a potential space into which the cells were injected.

2.4 Cell Viability and Proliferation

LIVE/DEAD Viability/Cytotoxicity kit (Molecular Probes) was used to visualize live and dead cells in the cornea. The cornea was rinsed with PBS once and then incubated for 30 min at room temperature in calcein AM (2 µM) and ethidium homodimer (4 µM) solution. After incubation, the cornea was rinsed in PBS three times to remove the excess solution. The tissue was then placed on a glass bottom dish and imaged using a Leica SP2 confocal microscope at excitation/emission of 495 nm/515 nm for live cells and excitation/emission of 495 nm/635 nm for dead cells.

Proliferation in the epithelium was assessed by immunostaining for Ki67. Fibroblast proliferation within the stroma was measured using CellTiter 96® AQueous One Solution Cell Proliferation (MTS Assay, Promega). MTS Assay is used to measure the number of viable cells in a solution. This assay measures mitochondrial activity of the cell by the conversion of MTS reagent (tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium) into a colored formazan product that is soluble in tissue culture medium. It is a colorimetric assay where the quantity of formazan production is directly proportional to the number of living cells. Briefly, equal number of fibroblasts were injected into decellularized corneal stromal

pieces then cultured up to 4 weeks. At one week time points, the corneal pieces were removed from culture and incubated in an MTS solution for 3 h. The solution was collected and the calorimetric changes were measured by a spectrophotometer at 492 nm.

To assess corneal fibroblasts migration in the decellularized stroma, a pocket was made in the center of stroma with the microtome. Equal number of fibroblasts was gently injected into the pocket using pipette tip. The corneas were incubated for 4 h at 37°C. After 4 h, once the cells have adhered to the stroma, more media was added to each cornea to avoid drying out. At one week time points, corneas were sectioned and stained with DAPI to evaluate the migration of cells from the injection site towards anterior and posterior of the cornea.

2.5 Immunostaining

For immunostaining, human corneas in OCT were cut into 10 μm sections, fixed in 4% PFA for 20 min followed by permeabilization in 0.3% Triton-X in Tris Buffered Saline (TBS) for 10 min at room temperature. The sections were blocked with 10% donkey serum with 1% bovine serum albumin (BSA) in TBS for 1 h at room temperature. The primary antibodies included the goat polyclonal anti-collagen type IV (1:100, Southern Biotech), mouse monoclonal anti-collagen type IV alpha 1 (1:100, DSHB), goat anti-keratin 12 (1:100, Santa Cruz Biotechnology), mouse anti-ABCG2 (1:100, Santa Cruz Biotechnology), mouse anti-p63 (1:100, Santa Cruz Biotechnology), rabbit anti-Ki67 (1:50 Epitomics), mouse anti-human HLA Class I (1:50, Biolegend), and goat anti-ALDH1 (aldehyde dehydrogenase 1) (1:50 EMD Chemicals). The primary antibody was

incubated either overnight at 4°C or for 2 h at room temperature. A fluoroscein isothiocyanate (FITC)-conjugated donkey anti-goat, anti-rabbit or anti-mouse IgG (1:300-400, Jackson ImmunoResearch) or rhodamine conjugated anti-goat IgG was applied for 1 h at room temperature in the dark. For negative control, the primary antibody incubation step was omitted or instead isotype control was used. Slides were coverslipped with mounting medium with or without DAPI and images were visualized using a Zeiss Axiovert fluorescence microscope, and photographed with an AxioCam (Carl Zeiss, Thornwood, NY) camera.

3. Results

3.1 Optimization of Media Conditions for Epithelial Cells

Corneal epithelial cells were grown in different media conditions, but the best results were obtained with combination of keratinocyte serum-free medium (KSFM; Invitrogen) along with DMEM, Ham's F12, and Fibroblast Conditioned Medium (CM). The growth of epithelial cells in CM is compared with the current gold standard medium KSFM both *in vitro* and *ex vivo* cultures (Figure 16). Epithelial cells were evaluated for a period of 3 weeks. For *in vitro* evaluation, corneal epithelial cells were grown on tissue culture plastic while for *ex vivo* comparison, epithelial cells were grown on a decellularized cornea. The epithelial cells grown *in vitro* in CM, showed a higher proliferation as compared to KSFM by gross examination with a microscope. The level of differentiation was based on the size of the cell and nucleus to cytoplasm ratio (N/C ratio). N/C ratio determines the maturity of the cell and less differentiated cells exhibit

high nucleus to cytoplasm ratio (N/C ratio). Epithelial cells grown in CM exhibited larger nucleus and overall smaller cell size as compared to cells grown in KSFM.

For *ex vivo* study, corneal epithelial cells were grown on an anterior surface of decellularized cornea. Cells were grown for a period of 3 weeks in CM and gold standard media; KSFM. After 3 weeks, whole cornea stained with calcein AM and ethidium homodimer solution showed almost intact stratified sheets of epithelium grown in CM. On the other hand, KSFM, very few individual epithelial cells dispersed throughout the anterior surface were observed.

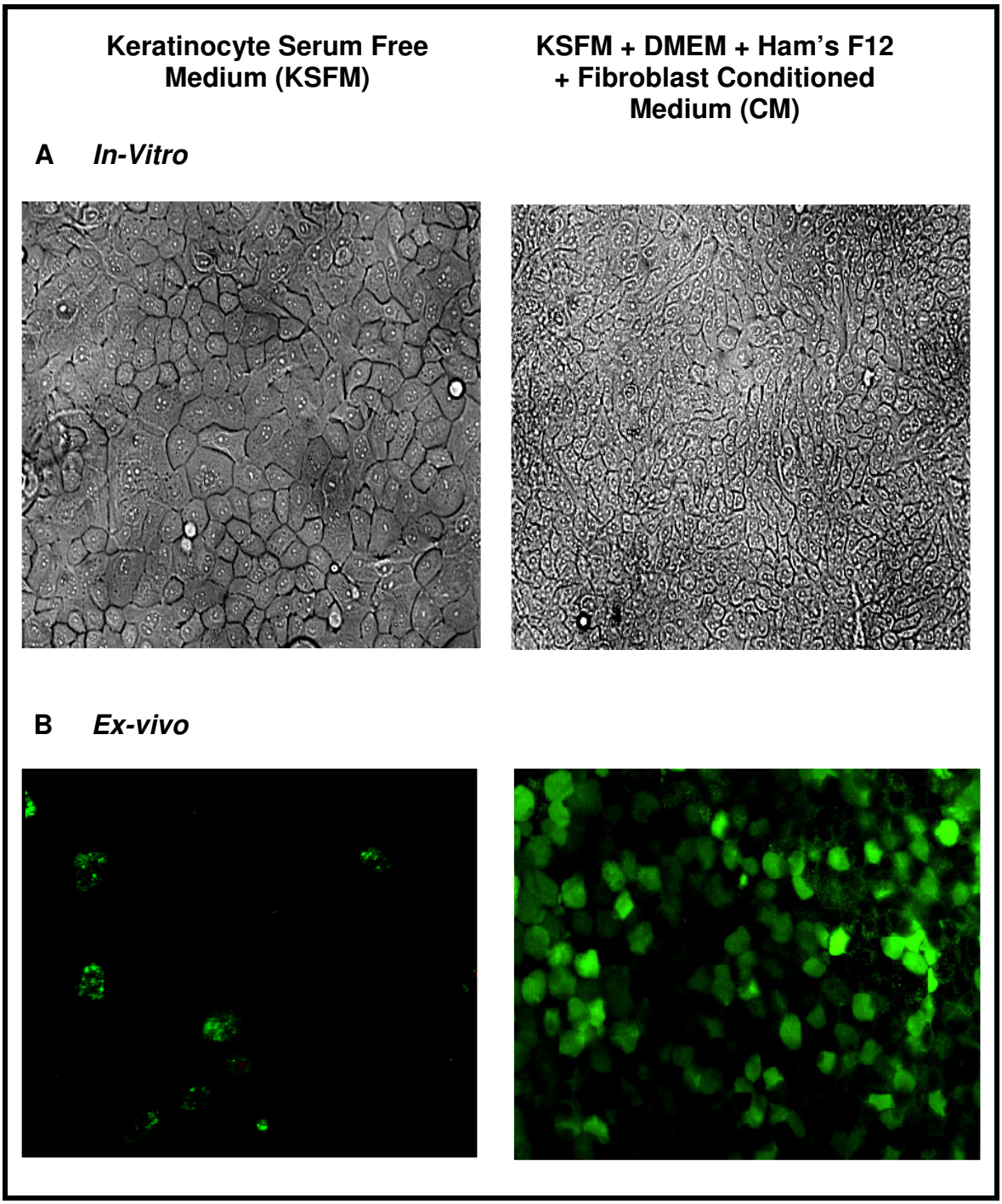

Figure 16. Optimizing media conditions for corneal epithelial cells. (A) *In Vitro* culture of epithelial cells for 3 weeks in KSFM and CM. CM showed better cell growth and less differentiated cells as compared to KSFM. (B) *Ex vivo* culture of epithelial cells for 3 weeks in KSFM and CM also showed that CM supported epithelial cells while cells grown in KSFM didn't survive.

3.2 Ability to Support Cell Growth

The ability of the decellularized corneas to support corneal epithelial and fibroblast cell growth was further evaluated in culture (Figure 17). Limbal epithelial cells were plated on decellularized corneas. In NaCl plus nuclease decellularized corneas, the epithelial cells readily attached and by the end of week 2, became nearly confluent as revealed by the Live/Dead Assay (Figure 17). Stratification of the epithelium was successfully induced by raising the tissues to the air-liquid interface. Corneas decellularized using detergent based methods, in particular SDS (Methods 1 and4), did not support epithelial growth, due to poor attachment of the cells (data not shown).

Corneal fibroblasts were injected into NaCl plus nuclease decellularized corneas. The cells attached and exhibited native, spread morphology. The viability assay performed up to 3 weeks revealed that the cells remained alive with evidence of proliferation (based on cell density) for the duration of the study (Figure 17). The fibroblasts appeared to migrate into the more anterior layers (closer to the epithelium) of the stroma (Figure 17). Corneas decellularized by detergent based methods also supported the growth of fibroblasts (data not shown).

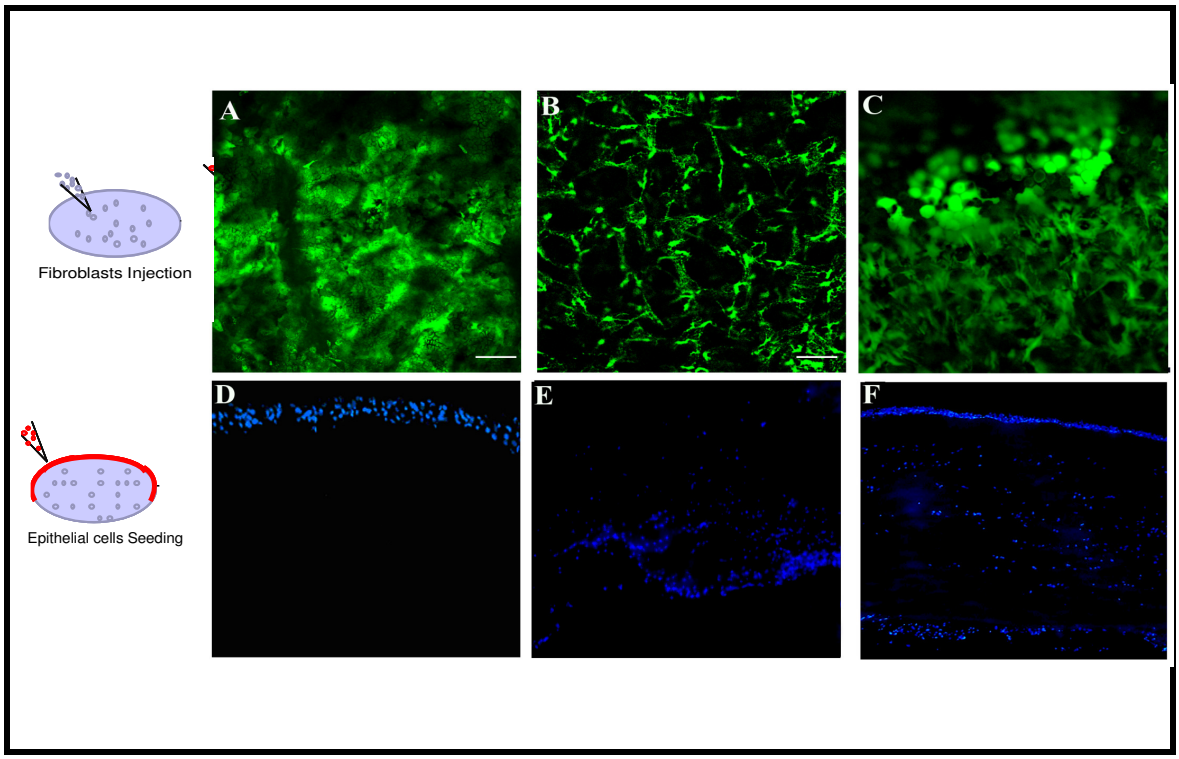

Figure 17. Cell viability and cytotoxicity assay and DAPI staining in NaCl plus nuclease treated corneas. The tissue was stained with calcein AM and imaged en face with confocal microscopy: (A) Corneal epithelial ells cultured for 2 weeks over decellularized cornea, demonstrating viability and differentiation of the superficial layer. (B) Corneal fibroblasts injected into the stroma of decellularized corneas and cultured for 5 days demonstrating viability, and (C) A crosssectional image showing viable fibroblasts in the stroma and stratified epithelium on the anterior of cornea. DAPI staining of the cross sections demonstrating (D) stratified epithelium and (E) Fibroblasts after 2 weeks in culture, and (F) corneal fibroblasts 5 weeks after injection of fibroblasts and 3 weeks after plating epithelial cells. Scale Bar = 300 mm for A, B, C and 100 mm for D, E, and F.

Phenotypic evaluation of the corneal epithelial cells grown on decellularized corneas revealed that they can differentiate and express the corneal specific keratin 12 similar to that seen in human cadaver corneas (Figure 18). To examine the level of differentiation of basal epithelial cells, the expression of markers typically associated with limbal stem and progenitor cells was also evaluated by immunofluorescence. The basal epithelium was found to express DeltaNp63and ABCG2, indicating that the construct supported a "limbal" (undifferentiated) phenotype in the basal layer (Figure 18). Proliferation was noted in the basal epithelial layer as evidenced by Ki67 staining (Figure 18).

The fibroblasts grown on posterior of the cornea were stained with vimentin at day 1. The de-differentiation of fibroblasts in the decellularized corneal stroma over time was evaluated with the expression of ALDH1, a marker of corneal keratocytes. This was performed in corneas ranging from 1 to 4 weeks after injection of fibroblasts. As early as 1 week, 64 % ± 12% of the cells were found to be expressing ALDH1, suggesting that the majority had partially reverted to a keratocyte phenotype (Figure 19). The percentage of ALDH1 staining did not differ significantly at subsequent time points examined (range 59%-87%), indicating no correlation with time in culture (P = NS by Chi-square).

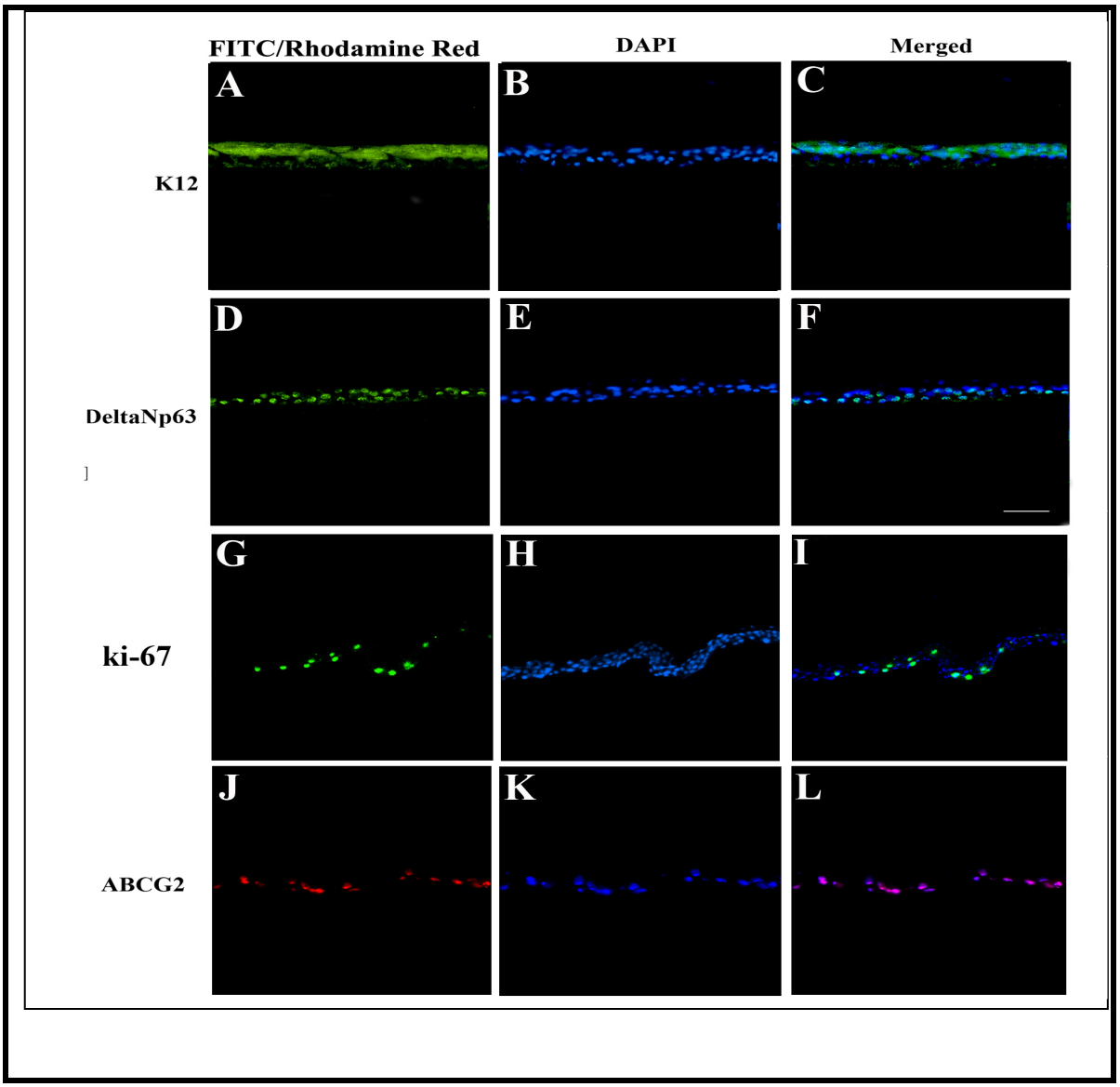

Figure 18. Phenotypic evaluation of epithelial cells. Corneal epithelial cell were grown over NaCl plus nuclease decellularized corneas for 2 weeks and then raised to the air-liquid interface to induce differentiation (A–L). (A–C) Corneal specific cytokertain 12 expression is noted in all the superficial epithelial cells. The basal epithelium expresses stem/progenitor cell markers including (D–F) DeltaNp63 (J–L) ABCG2 and proliferation marker (G–I) Ki-67.

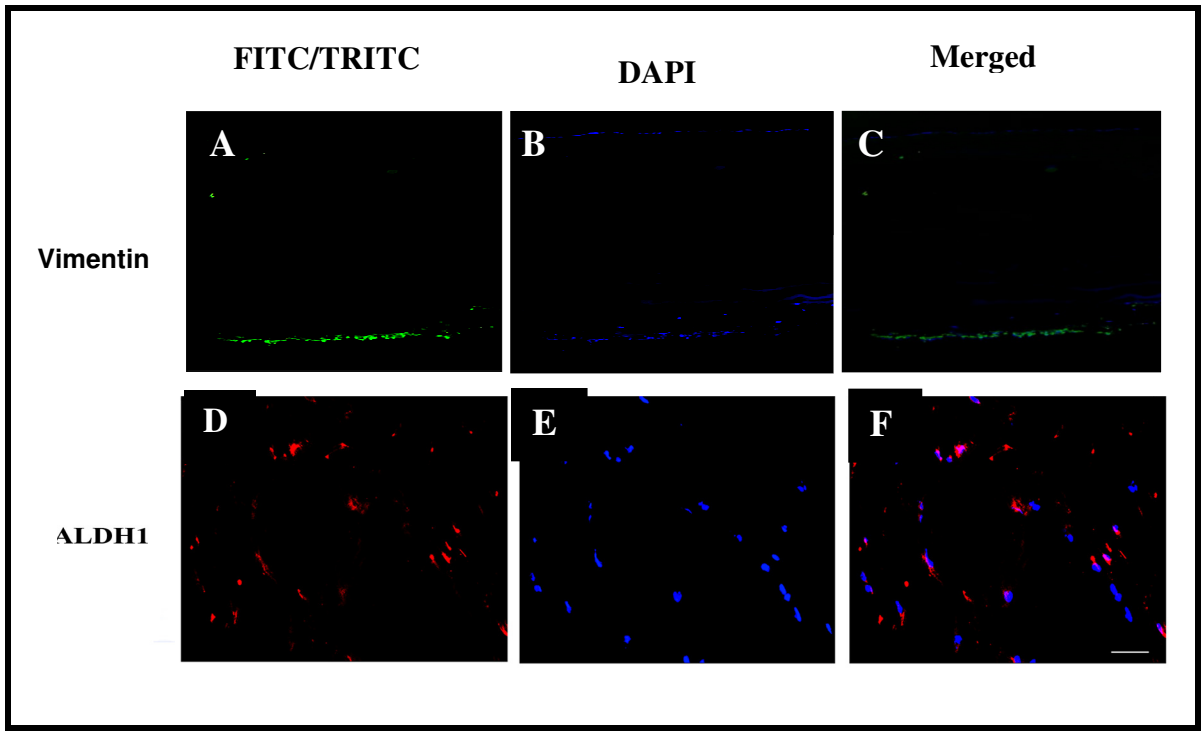

Figure 19. Phenotypic evaluation of corneal fibroblasts. (A-C) Corneal fibroblasts were grown on the posterior surface of NaCl plus nuclease decellularized corneas for 1 day expressing positive staining for vimentin. (D-F) Corneal fibroblasts grown in decellularized cornea after 1 week, demonstrating ALDH1 staining. Scale Bar = 50 mm. ALDH1, aldehyde dehydrogenase 1.

The migration of the fibroblasts in the stroma was determined by staining the corneal sections with DAPI staining. Images at one week time points showed an increase in migration of the fibroblast from the injection site toward the anterior and posterior stroma during the 4 week study, as noted by histology and MTS assay (Figure 20). Also, the rate of proliferation of corneal fibroblasts, measured by MTS Assay, showed a steady increase in proliferation over a period of 4 weeks (Figure 20).

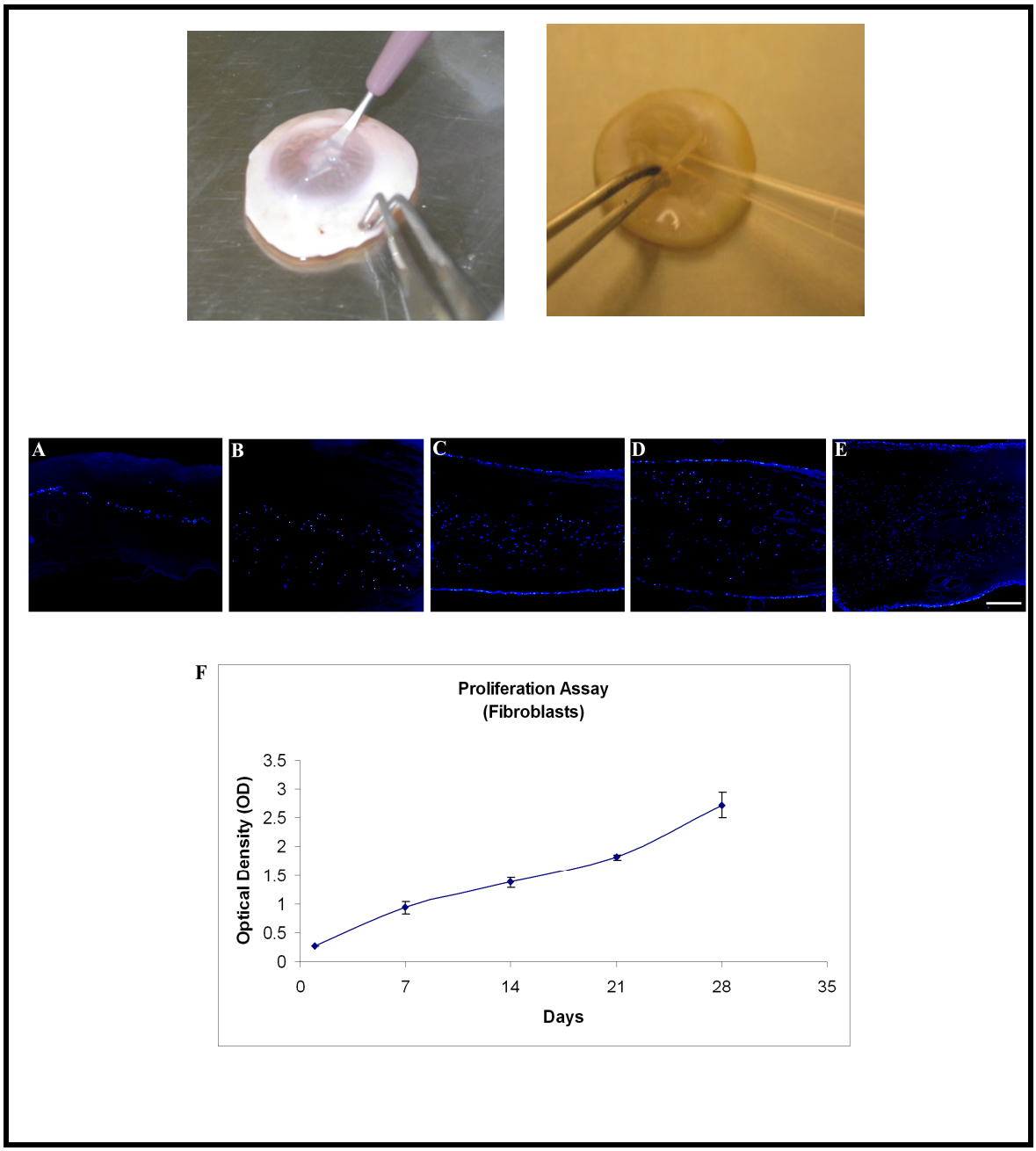

Figure 20. Corneal fibroblasts proliferation and migration assay. Corneal fibroblasts grown in a pocket made in stroma for (A) 1 day, (B) 7 days, (C) 14 days, (D) 21 days, and (E) 28 days, stained with DAPI demonstrate proliferation and migration of cells over time. (F) An MTS Assay showing proliferation of cells over a period of 4 weeks. Scale Bar = 100 mm.

4. Discussion

There are several aspects of this study which are worth highlighting. In this study, stromal cells were included as supporting cells for the epithelial cells. Most tissue engineering applications of the corneal epithelium have primarily included the underlying matrix for supporting the epithelium. Several recent studies have considered the importance of the stromal cells in corneal epithelial tissue engineering, and have developed experimental approaches that incorporate stromal cells in the tissue construct (Choi et al., 2010; Barbaro et al., 2009; Pang et al., 2010; Phu et al., 2010).

Our study is the first to use a decellularized human cadaver cornea instead of a xenograft cornea. Decellularized human corneas, while eliminating the theoretical risk of zoonotic infections, are also less likely to induce immunologic responses in a human host, which may potentially be seen with xenogenic grafts (Kasimir et al., et al. 2006). Corneal tissues which are structurally intact but are unsuitable for transplantation are good candidates for such applications.

Previous studies have highlighted the importance of corneal and limbal fibroblasts in corneal epithelial proliferation, differentiation, and wound healing (Espana et al., 2003). There is a bi-directional signaling between corneal keratocytes/fibroblasts and epithelial cells. The epithelium regulates the function of corneal fibroblasts, and the corneal fibroblasts regulate epithelial cells function through a complex network of signaling molecules. (Wilson et al., 1999, 2001; Nishimura et al., 1998; Szerenyi et al., 1994; Hong et al., 2001).

It is becoming more evident that even the simplest epithelial disruption wound results in keratocyte death and a subsequent stromal response to regenerate the affected area. After any injury, as part of the wound healing mechanism, the keratocytes transform into fibroblasts and migrate into the wound area. This migration is slow and could take a few weeks depending on the depth of keratocytes loss in adjacent layers. These migrating fibroblasts synthesize the repairing matrix. In the next phase, these fibroblasts transform into myofibroblasts, which are involved in contracting the wound area. The extent of fibroblasts to myofibroblasts transformation depends on the severity of the wound. The myofibroblasts have a reversible phenotype; after wound healing, these myofibroblasts either degenerate or transform back into the fibroblast phenotype (Fini, 1999).

In this study, keratocytes were differentiated *in vitro* into fibroblasts mainly because fibroblasts are easier to expand in culture. However, when exposed to corneal stroma, these fibroblasts appeared to revert back into a more keratocyte like phenotype, which may be important in maintaining the transparency of the cornea. In this study, stromal fibroblasts were chosen given their well known interaction with the corneal epithelium, including their production of epitheliotrophic factors (Li et al., 1995; Espana et al. 2003). It should be noted that complete reconstruction of the stromal niche will likely require more than just fibroblasts and epithelial cells, and perhaps may require other cell types, such as neuronal cells. The purpose of this study, however, was not to identify all the necessary cells at this point, but rather to develop a construct which could be used to further study the various cell types that may be necessary.

The differentiation state of the cells that are used to repopulate the underlying stroma may also be important (Carrier et al., 2009). While we envision the final construct to only include a relatively thin anterior stroma, the choice of cell type may still affect its optical clarity. This may not be a critical factor for the limbal area, but more important in the central cornea because these activated fibroblasts during wound healing deposit an opaque scar tissue, which interferes with vision. Future *in vivo* experiments will help determine whether it is necessary to use keratocytes instead of fibroblasts in the construct.

Due to the ease of expansion, most of the studies on seeding the stromal cells on a scaffold for tissue engineering applications involve the use of fibroblasts instead of keratocytes. In culture, freshly isolated keratocytes, under serum-free conditions, maintain a quiescent keratocyte phenotype, but show very little or no proliferation. On the other hand, exposure to serum leads to fibroblast differentiation (Beals et al., 1999; Jester et al., 2007; Scott et al., 2011; Hassell et al., 1992). Studies have shown the use of certain proteins to regulate the expansion of keratocytes *in vitro* without transformation into fibroblasts, such as basic fibroblast growth factor (FGF2), (Jester et al., 1996; Maltseva et al., 2001), 1% platelet-poor horse serum (Jester, and Ho-Chang, 2003), and insulin (Musselmann et al., 2005).

This study also examined to what extent the decellularized human corneas can maintain the epithelial cells in an undifferentiated phenotype, based on the expression of stem cell associated markers. Previous studies on human amniotic membrane have shown that the extracellular matrix helps to preserve cells in a less differentiated state

(Mariappan et al., 2010; Tsai, and Tsai et al., 2010; Grueterich et al., 2003). The decellularization process used in this study appears to preserve the corneal basement membrane and matrix proteins. Notably, the epithelial cells grown over the NaCl plus nuclease decellularized corneas appear to maintain a "limbal stem cell phenotype", as evidenced by the expression of ABCG2 and DeltaNp63. While neither one of these markers are very specific markers of corneal epithelial stem cells, they, nonetheless, confirm previous observations of the corneal/limbal basement membrane and its ability to keep cells in a less differentiated state (Chen et al., 2010).

5. Conclusion

In summary, this is the first study on decellularization of human cornea which has examined recellularization of the cornea with corneal epithelial cells and fibroblasts. Fibroblasts grown in the decellularized corneal stroma showed both active proliferation and migration. Similarly, epithelial cells plated on the anterior of the decellularized cornea proliferated and differentiated into stratified layer similar to the native cornea.

IV. EVALUATION OF DECELLULARIZED CORNEA GRAFTED IN AN ANIMAL MODEL *(IN VIVO)*

1. Introduction

While *in vitro* studies can provide important insights into biological mechanisms, *in vivo* studies provide more convincing evidence of adverse health consequences. The *in vitro* models can not mimic the complicated interactions among cells, tissues, and organs that happen in a human body, therefore, final tests must be done on an animal. Scientists have been solving medical problems, developing new techniques, and treatments for diseases using animal models for biomedical research. Animal models are chosen based on both their anatomy and physiologic similarities with the humans for a specific disease. Rodents such as rats and mice, that have a short life span and are easier to breed in lab, have extensively been used in biomedical research.

In vivo testing is routinely used for biocompatibility studies of a scaffold to collect valuable information without exposing humans to the risk of rejection and infection. This test is done by demonstrating lack of immune response or tissue damage in response to the transplantation of the bioengineered scaffold. The most popular animal models for biocompatibility testing are rats, guinea pigs, rabbits, and mice.

2. Material and Method

2.1 Alkali Injury and Mechanical Epithelium Debridement

Mice were anesthetized with an injection of ketamine hydrochloride (100 mg/kg IP) and xylazine (5 mg/kg IP) hydrochloride intrapertoneally. For local anesthesia, one drop of proparacaine hydrochloride 0.5% was applied to the eye. The state of anesthesia

was monitered by the level of response to pinching the toe, or blinking in response to the administration of eye drop. Under anesthesis, the epithelium removal was tested by mechanical debridement with a 23G needle with blunt tip and varying concentrations of sodium hydroxide (NaOH) ranging from 0.25-1M.

In case of NaOH epithelium removal, a cotton swab soaked in NaOH was gently applied on the cornea for about 30 seconds. For mechanical debridement, 23G needle was used to scrape the epithelium. The tip of the needle was made blunt by scraping on a sand paper. The eyes were then monitored for one week for the severity of inflammation and damage to the cornea.

2.2 Laser Injury Model in a Rat Eye

Rats weighing about 300-500 grams were anesthetized as described above. For epithelium removal, Nidek EC-5000 excimer laser system was used to ablate about 5.0 mm in length and 100 μm deep corneal tissue under anesthesis from a rat eye at the limbal area (junction of cornea and sclera). Thus, the laser had an additional advantage to remove not just epithelium but also the stroma.

2.3 Transplantation of a Decellularized Corneal Graft in a Rat Eye

A lamellar transplantation of human decellularized corneal graft was tested on a rat eye (Figure 21). The process of preparing the graft for transplantation involves a lamellar dissection to separate the anterior half of the cornea, discarding the posterior stroma, Descemet's membrane, and the endothelial cells. After disinfecting the host eye with 10% ophthalmic povodine-iodine, a thin layer of decellularized anterior cornea was dissected from the limbal area with a surgical blade and was grafted onto the rat eye using interrupted 10-0 nylon sutures. In control eyes only laser was done. Immediately after the procedure, antibiotic solution was applied to the eye. The antibiotics were given once every day for the duration of the study. A pilot study was also performed on the feasibility of transplanting decellularized cornea in a mouse eye.

At one week time point, a rat was sacrificed by an intracardiac injection of sodium pentobarbital and its eyes were enucleated, cut into 14 μm sections, and stained with H & E and DAPI to evaluate the graft and infiltration of the host cells into the graft.

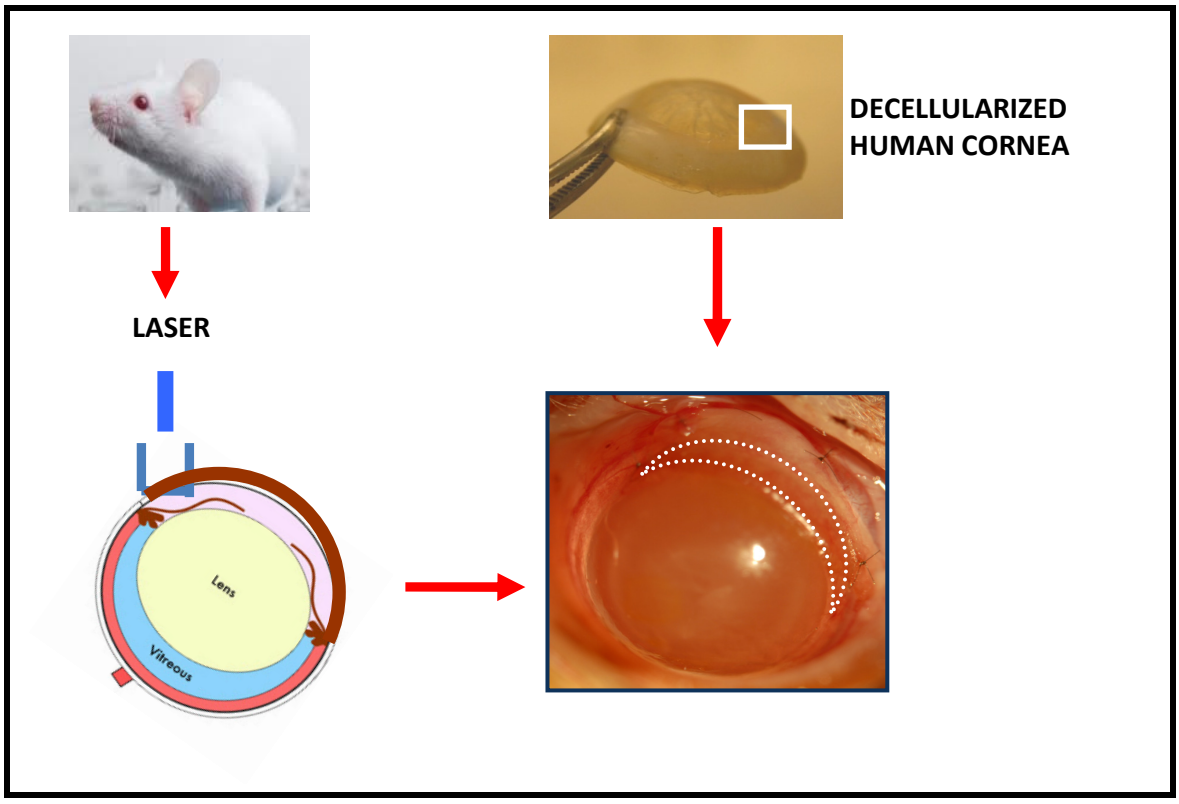

Figure 21. Experimental design for grafting. Human decellularized cornea was grafted on the laser ablated eye.

2.4 Slit Lamp Imaging

The clinical status of the ocular surface was assessed at day 0, 3, and 7 after transplantation, and the degree of opacity due to inflammation and neovascularization was monitored with slit lamp microscope. The rate of epithelialization was assessed by placing a drop of fluorescein on the cornea and recording the area of epithelial healing with slit lamp microscope. Fluorescein is a commonly used stain for detection of epithelium damage as it temporarily stains the areas where the epithelium is missing.

2.5 Staining

After one week of transplantation of the graft, both the grafted and control eyes were enucleated, and sections were stained with hematoxylin and eosin to assess the integration of donor tissue with the host tissue. The sections were also stained with DAPI to check on the status of the host cell migration into the graft.

3. Results

3.1 Evaluation of Alkali Injury and Mechanical Epithelium Debridement

All concentrations of NaOH, tested to remove epithelium, caused significant inflammation in the cornea at day 7, making the cornea opaque (Figure 22).The mechanical debridement method was not destructive, but the results were not consistent. In some experiments, the epithelium was not fully removed as evident by the positive flourescein staining (Figure 22).

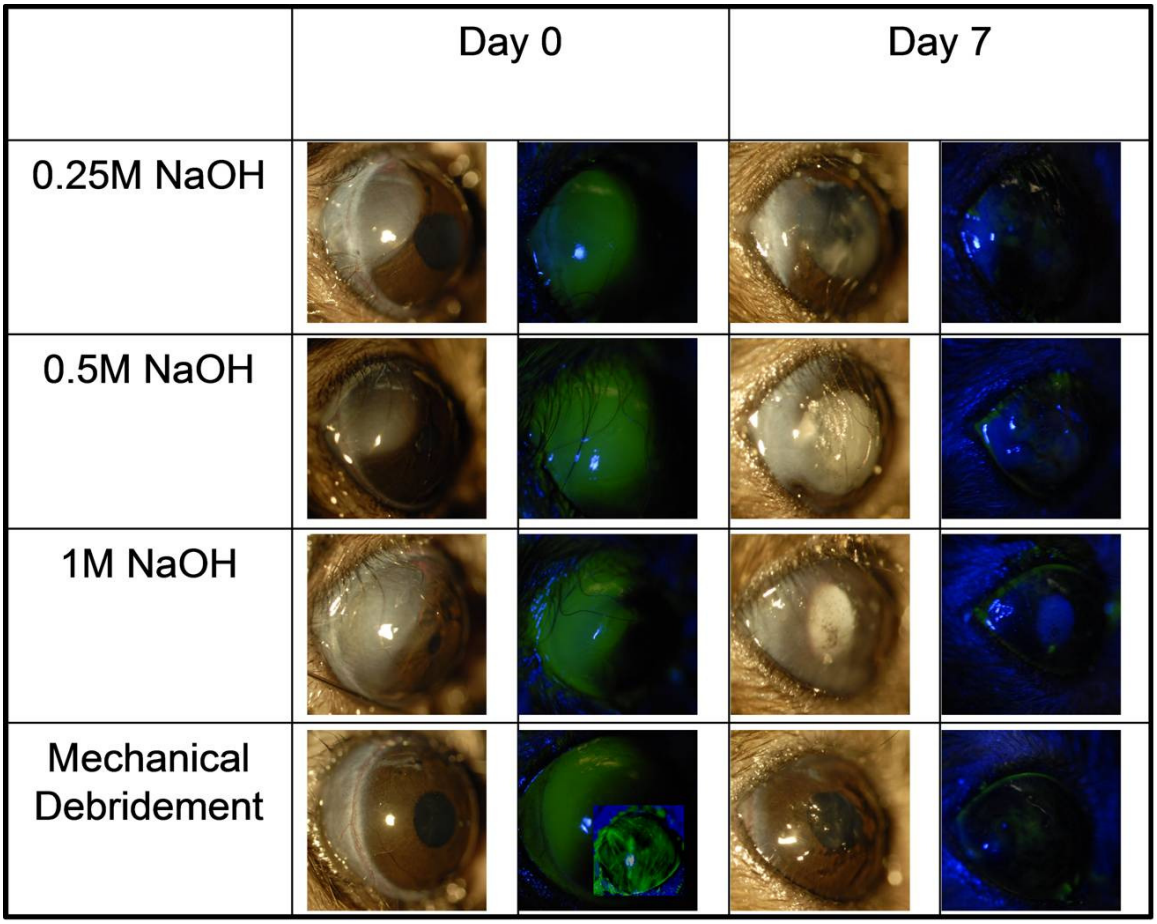

Figure 22. Alkali injury and mechanical debridment. Slit Lamp images with and without flourecein to evaluate NaOH and mechanical injury. Images were taken at Day 0, and 7. Cornea received NaOH (0.25, 0.5, and 1M) resulted in damage deeper into the stroma. At Day 7, epithelium healed but scar developed in the stroma. Mechanical injury was not too destructive and did not result in obvious scar formation but this method did not give consistent result (inset shows incomplete epithelium removal).

3.2 Evaluation of Laser Injury Model in a Rat Eye

Alkali injury and mechanical debridement methods to remove epithelium were also tested in rat eyes and similar results were obtained (data not shown). Alkali injury resulted in much deeper damage in to the stroma, and mechanical injury had inconsistency in results. Therefore, removal of epithelium was tested by a third method, 'Excimer Laser System' in the rat eyes. Upon examination with slit lamp microscope, a positive flourescein staining in cornea after laser injury indicated absence of the epithelium. Also, upon gross inspection, the laser epithelium removal did not seem to cause excessive inflammation in the cornea (Figure 23).

3.3 Evaluation of Graft in a Rat Eye

A decellularized human corneal graft was transplanted on a rat cornea ablated with excimer laser. The laser ablated cornea, with and without graft, were analyzed for a period of one week (Figure 23). At days 3 and 7, in both control and the grafted cornea, there was a significant reduction in the flourescein staining and the surface was completely epithelialized. With a slit lamp microscope, upon gross inspection the eye showed some inflammation. At close examination, the control cornea that received only laser treatment showed more blood vessel formation at the site of injury as compared to the cornea that also received graft. In addition, comparing with control eyes, the eyes that received grafts healed faster and had clearer stroma as the graft acted as a scaffold to facilitate the wound healing.

For histology analysis, the eyes were removed and sectioned. H & E staining revealed a nice stratified epithelium grown on the graft. However, the graft also showed the presence of inflammatory cells in the stroma (Figure 24). Epithelial cells grown on the graft showed positive staining for K12, which is a marker for differentiated corneal epithelium.

A total of 8 grafts were performed. In successful grafts, we observed epithelialization of the graft in one week. But in the earlier cases, where thicker grafts had been used, we observed ingrowth of host corneal epithelial cells into the host–graft junction. The host cornea became opaque and neovascularization occurred, resulting in separation and extrusion of the graft.

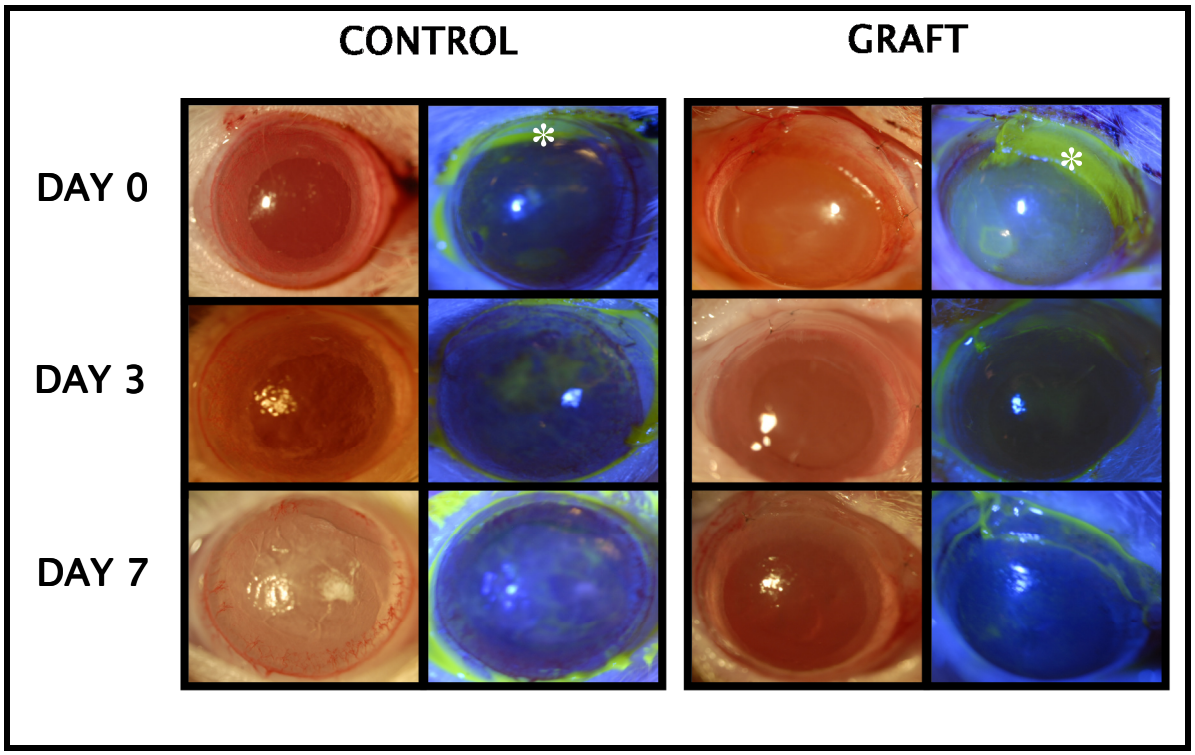

Figure 23. Slit Lamp images with and without fluorescein to evaluate integrity of the graft in the rat eye. Flourescein detects the absence of epithelium, and damage to the epithelium is represented by positive flourescein staining (day 0, asterisk). Images were taken at day 0, 3, and 7. At day 7, the treated area was completely epithelialized in both control and grafted corneas. Cornea without the graft showed blood vessel formation at the site of injury and had irregular epithelium with late fluorescein staining, as compared to the cornea that received the graft. In addition, comparison with control rats, the transplanted rat eyes healed faster and had clearer and smoother stroma.

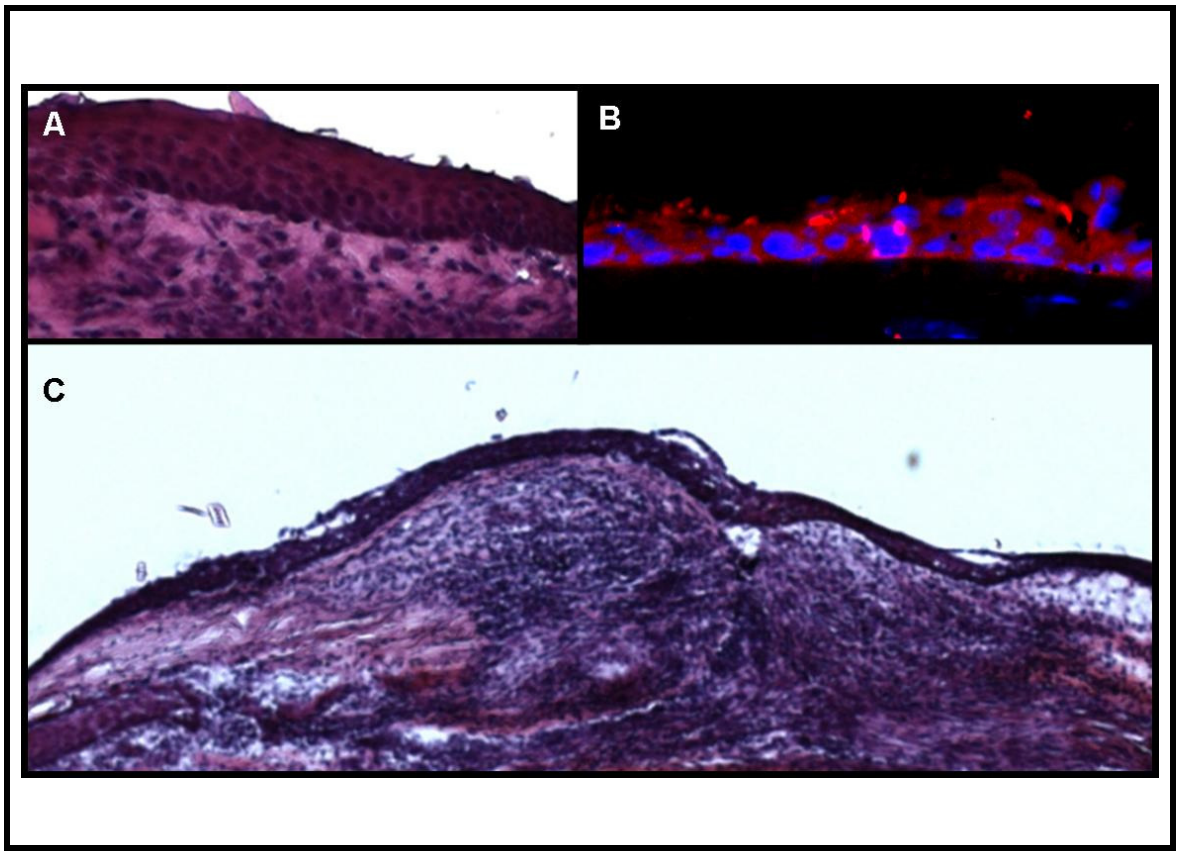

Figure 24. Histological sections of the graft. (A) High magnification image of the graft showing stratified epithelium. (B) Epithelial cells stained with K12 antibody (red), a marker for differentiated corneal epithelium and nucleus stained with DAPI. (C) Low magnification image of the graft stained with H & E shows intensive inflammation.

4. Discussion

For the treatment of damaged cornea, either all the corneal layers are replaced by a donor cornea (penetrating keratoplasty) or selectively only diseased layers of the cornea are replaced by healthy layers while retaining the endothelium (lamellar keratoplasty). Lamellar keratoplasty has some advantages over full thickness corneal replacement as it provides more rapid and predictable visual outcomes, reduced complications and prevent the loss of endothelial cells that have very limited proliferation capacity (Tan et al., 2012). In this study, lamellar transplantation of decellularized human corneal tissue on rats was performed to test biocompatibility of decellularized scaffold and it's efficacy to support epithelial cells *in vivo*.

For lamellar grafting procedures, rabbits are commonly used due to their larger eye size and thicker cornea, in this study, the possibility of grafting was tested in a rat model. Depending on the strain of rat, there is variability in the size of the eye. The mean diameter of the Sprague Dawley rat corneas is about 6.5 mm and the mean cornea stromal thickness is approximately 200 μm (Lee et al., 2004). A pilot study was conducted to assess the feasibility of grafting decellularized human cornea in the rat eye. Several methods such as varying concentrations of NaOH, mechanical debridement in addition to laser ablation were investigated for the removal of epithelium. Chemical removal have shown to cause more damage to the cornea and resulted in more vascularization and inflammation in the cornea. Among the methods tested, laser ablation resulted in more uniform and less destructive injury and was considered comparatively safe and efficient in removing the epithelium.

The laser ablation procedure has been in use for over two decades now and there have been no long term issues associated with using this system. Nidek EC-5000 laser system, used in this study, uses laser of 193nm wavelength, which has been shown to efficiently remove corneal layers while limiting collateral thermal damage to the surrounding tissue (Sakimoto et al., 2006). The components of the corneal stroma such as proteins, glycosaminoglycans and nucleic acid absorb energy of this wavelength, preventing any secondary mechanical shock-waves from transmitting through the eye and thus reduce unwanted damage (Manche et al., 1998).

Initially, more than half of the grafts failed due to the ingrowth of host corneal epithelial cells into the host–graft junction. The host cornea became opaque and neovascularization occurred, resulting in separation and extrusion of the graft. To prevent this ingrowth, the thickness of the graft was reduced which seemed to help graft survival.

Any disturbance to the cornea, in case of our study, disturbance caused by the laser ablation, recruits inflammatory cells to the site of injury. In this study, at one week time point, upon inspection with slit lamp microscope and further with H & E staining revealed stromal inflammation. In the clinical setting, inflammation due to the grafting or any other treatment to the cornea is normal and is controlled by steroids and antibiotics. Irreversible corneal graft rejection is diagnosed when the cornea remains opaque despite the treatment.

Decellularized xenografts have been used in numerous tissue engineering applications. It has been suggested that decellularized tissues are biologically inert and do no induce immune response. This is true only in the case of 100% removal of cells and cellular components. Due to the lack of systematic assessment of decellularization procedure, the amount of retained cellular materials varies among different decellularization techniques. The residual cellular components have the potential to recruit inflammatory cells (Kasimir et al., 2006).

Studies have published on the possible factors such as protein mismatch across the species, which could induce organ/tissue rejection in the host. One such protein is Alpha-gal that has been studied by several groups. Alpha-gal epitope is carbohydrate produced by the cells on glycolipids and glycoproteins in non-primate mammals such as pig and monkey. Antibody to alpha-gal is present in human serum. Once a xenograft containing alpha-gal antibody (from human) is introduced in a recipient having alpha-gal epitope, the quiescent antibody is activated, which in turns activates B cells and results in influx of inflammatory cells, causing hyperacute rejection of the graft. Although, alpha-gal epitope in not present in both rat and human, but the ECM contains many other structural and functional proteins that are part of the native human ECM (donor tissue). These proteins are considered xenogenic in the host animal and have potential to induce immune response. It is more realistic to believe that if our decellularization efficiency was not 100% and if the immunogenic human antigens were left behind in the matrix after decellularization, elevated inflammation could be triggered in the rat eyes after grafting (Keane et al., 2011).

The graft did seem to induce inflammation which may be in part due to human antigens expressed in the transplanted graft. Despite the inflammation, our results showed that introducing the stromal niche (stromal graft) has a therapeutic effect on healing the epithelium and corneal surface. It was evident that as early as 7 days, the corneal surface after the laser healed better and appeared clearer with very little neovascularization as compared to the control (laser only).

Another potential cause of this inflammation could be the sutures. The sutures used in this study were Nylon, and they are known to induce inflammation and vascularization in the stroma.

Our one week *in vivo* study showed predominantly inflammatory cell infiltration into the stroma with no obvious infiltration of fibroblasts. The keratocytes in the cornea are normally in a quiescent state, which suggests longer time must be required to see any cell growth into the graft. It has been shown that any surgical incision or mechanical stress on the cornea, damages the tissue and the keratocytes within 200-300 μm of the cut undergo apoptosis within few hours after the surgery (Fini, 1999). This creates a region of the tissue devoid of cells surrounding the injury. A study by Carlson et al. on mouse cornea, showed expression of stromal matrix protein synthesized by keratocytes within 12 weeks of wounding by epithelial debridement (Carlson et al., 2003). Another study by Xu-Chu Lin et al. showed infiltration of rabbit keratocytes in decellularized porcine corneal graft after 4 weeks of grafting (Lin et al., 2008). Therefore, a longer study period is required to observe the migration of fibroblasts into the graft.

In this study, the graft was transplanted at the limbus; junction of the cornea and sclera. The reason for choosing the limbal region for grafting is the presence of stem/progenitor cells in this area. These stem cells undergo continual self-renewal and divide and differentiate terminally and migrate upwards toward the surface for healing the epithelium injury (Lavker et al., 2004). The integrity of the corneal epithelium depends on the presence of self-renewing limbal stem cells. There are also other specialized stromal cells (undifferentiated cells) reside in the limbus. Therefore, the idea behind grafting at limbus region is to have a higher stem cells and progenitor cells count that possess high cell proliferation capacity, which would facilitate graft recellularization and integration.

V. Conclusion and Future Work

The decellularization technique used in this study was proved to be successful to support corneal cell growth. *In vitro,* the decellularized construct supported both epithelial cells and fibroblasts, while, the *in vivo* study was able to show that the scaffold promoted the growth of epithelial cells. The graft did seem to induce inflammation, which may be in part, due to residual human antigens present in the transplanted graft. Future animal work may be done using a decellularized rat cornea to reduce the inflammation in the rat eye, caused by the mismatch of antigens. For future studies, another approach could be to introduce rat cells into the decellularized rat cornea, prior to transplantation in the rat eye. We hypothesize that, by growing cells in the graft ex vivo prior to grafting, will give cells more time to synthesize and remodel extracellular matrix.

This in turn, will greatly reduce the time for healing and will help improve the success of the transplantation by preventing immune rejection.

The cell types, used in the stroma, can also have significant impact on the synthesis of extracellular matrix, and on their interaction with the neighboring cells such as epithelial cells and endothelial cells. Therefore, in future, one area of investigation could be the use of mesenchymal stem cells (MSCs) for stromal cell replacement. Previous studies have shown that corneal limbal stromal cells have MSCs properties. These MSCs are immunoprivileged cells, having low expression of class II Major Histocompatibility Complex (MHC-II) on the surface of their cells (De Miquel et al., 2012). In order to evaluate the use of MSCs in replacement of other cell types, studies are conducted to monitor the immune response. In one such study, adult corneal stromal stem cells were directly injected into the mouse corneal stroma and the cornea was evaluated for inflammation. In the study, the mice received three types of treatment: sham, stem cells, and fibroblast injections into the corneas. After injection, all three groups showed rapid influx of neutrophils into the stroma. However, after one week of the study, the corneas that received sham and corneal stem cell treatments, showed a decrease in the level of neutrophils, suggesting a transient inflammation. On the other hand, the cornea that received a fibroblasts injection, resulted in a significant increase in the population of the inflammatory cells (Yiqin Dy et al. 2009).

A weakness of our study is the relatively short *in vivo* study period. This was in part, due to an excessive amount of inflammation, presumably, incited by the human antigens in the rat cornea. In future, by optimizing the use of rat decellularized tissue *in*

vivo, will allow longer time points to be assessed. The novel method for decellularization has potential applications in corneal and limbal tissue engineering, where a biopsy may be used to grow both epithelial cells and stromal cells to reconstruct the corneal epithelium with a stromal niche.

Laser Induced Limbal Stem Cell Deficiency Model

The stem cells of the corneal epithelium are called 'limbal stem cells' and are present in the limbus region, the boundary of the cornea and sclera. Limbal stem cells, like any other stem cells, are located in a specialized microenvironment, (aka 'niche'), which supports and protects their population from environmental insults and maintains the 'stemness' of the stem cells. These stem cells undergo continual self-renewal and differentiate to epithelial cells and migrate upwards toward the surface (Lavker et al., 2004).

It has been shown that limbal epithelial cells in the limbus extend from the surface of the cornea into the underlying stroma and form pockets known as 'limbal epithelial crypts' or 'palisades of Vogt' and reside in them. These crypts are of variable lengths in human cornea. Limbal crypts that are shorter than 40 µum are termed 'minor crypts' and the ones larger than 40 µm are termed 'major crypts' (Shanmuganathan et al., 2007; Molvaer et al., 2012).

The integrity of the corneal epithelium depends on the presence of self-renewing limbal stem cells. These limbal stem cells or their microenvironment where they reside, can be damaged due to various factors such as mechanical or chemical insults. They may

be damaged or compromised by severe injuries such as alkali burns or by immune diseases like 'Stevens-Johnson Syndrome', resulting in a serious ocular condition known as 'Limbal Stem Cells Deficiency' (LSCD) (Grueterich et al., 2003). LSCD is associated with persistent epithelial defects, neovascularization, and severe loss of vision if left untreated.

One of the main advantages of using the Excimer Laser System is the opportunity to control the ablation depth. The laser not only removes epithelium, but also removes the matrix underneath it, so effectively it could destroy the limbal stem cells and the limbal niche when applied on the limbus. Therefore, in future, it will be interesting to investigate the possibility of developing a limbal stem cell deficiency (LSCD) animal model by laser injury.

Our preliminary study has demonstrated, that the decellularization method has maintained the integrity of the limbus. The epithelial cells that were grown on decellularized cornea *ex vivo,* expressed stem cell marker DeltaNp63, and showed higher proliferative potential as evidenced by an increase in Ki-67 expression at the limbus, as compared to the central cornea (Figure 25). Based on our findings that the decellularized construct supported a "limbal" (undifferentiated) phenotype of cells in the limbus, we are interested to study the possibility of reconstructing the limbus. In specific, the aim of our future study is to treat laser induced limbal stem cell deficiency (LSCD) *in vivo*, with the reconstruction of the limbus, by growing limbal stem cells in the decellularized cornea.

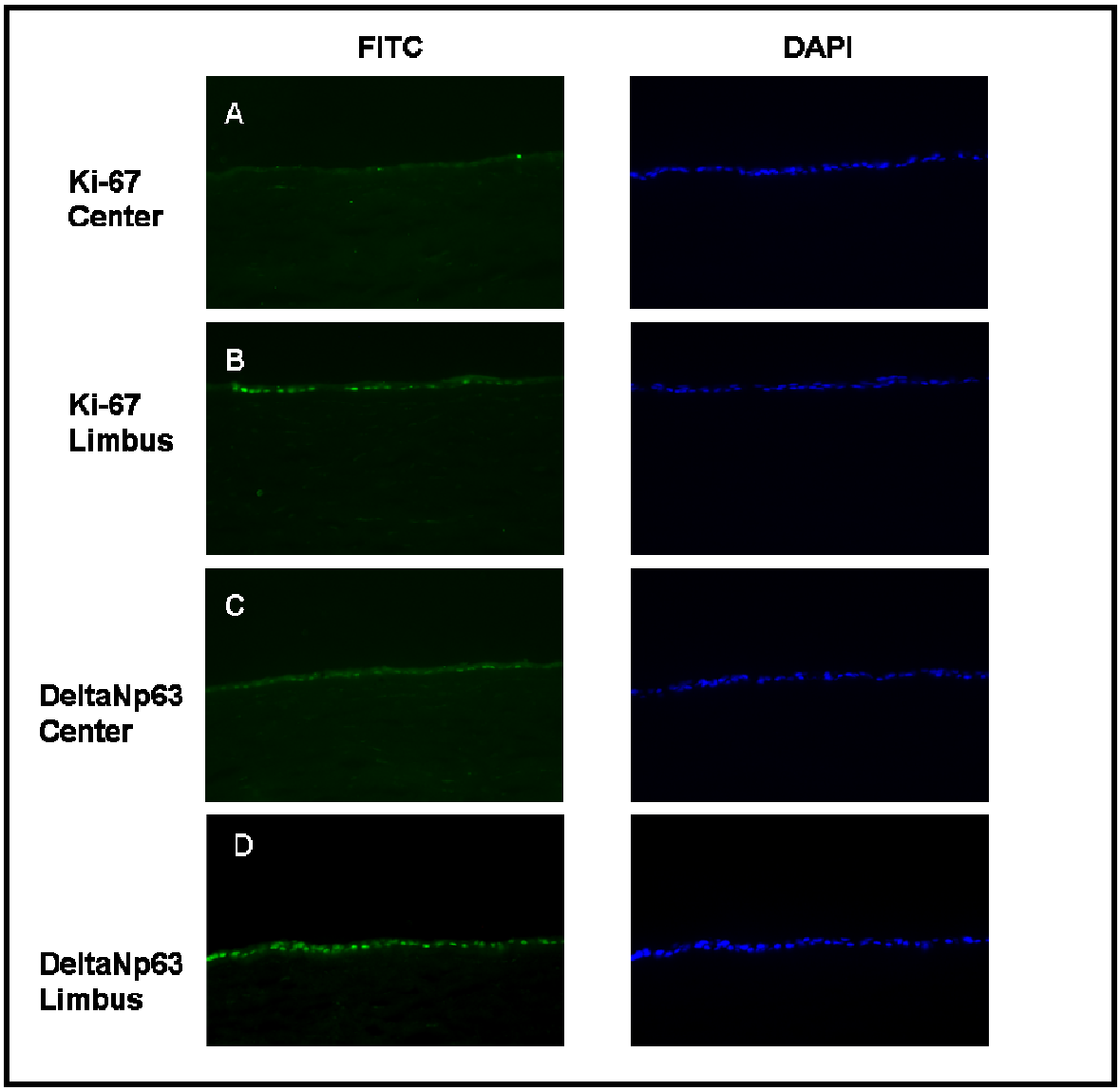

Figure 25. Limbal stem cells supported by decellularized cornea. Limbal stem cells grown on decellularized cornea shows (A, B) higher expression of Ki-67, a marker for proliferation, at the limbus as compared to the central cornea. (C, D) Likewise, there was a higher expression of stem cell marker DeltaNp63 at the limbus as compared to the central cornea.

Our model of 'laser induced limbal stem cell deficiency' is illustrated in Figure 26. This model is based on results and knowledge attained from this study and published literature. Our proposed model involves a 360^{o} total limbal ablation, created with the laser. The depth of the ablation will be optimized to successfully remove all of the stem cells. Once the limbal stem cell deficiency is confirmed *in vivo*, reconstructed limbus by growing limbal stem cells in decellularized cornea will be grafted on the treated eye. The ability of the engineered limbus to reconstruct corneal epithelium *in vivo* will be investigated.

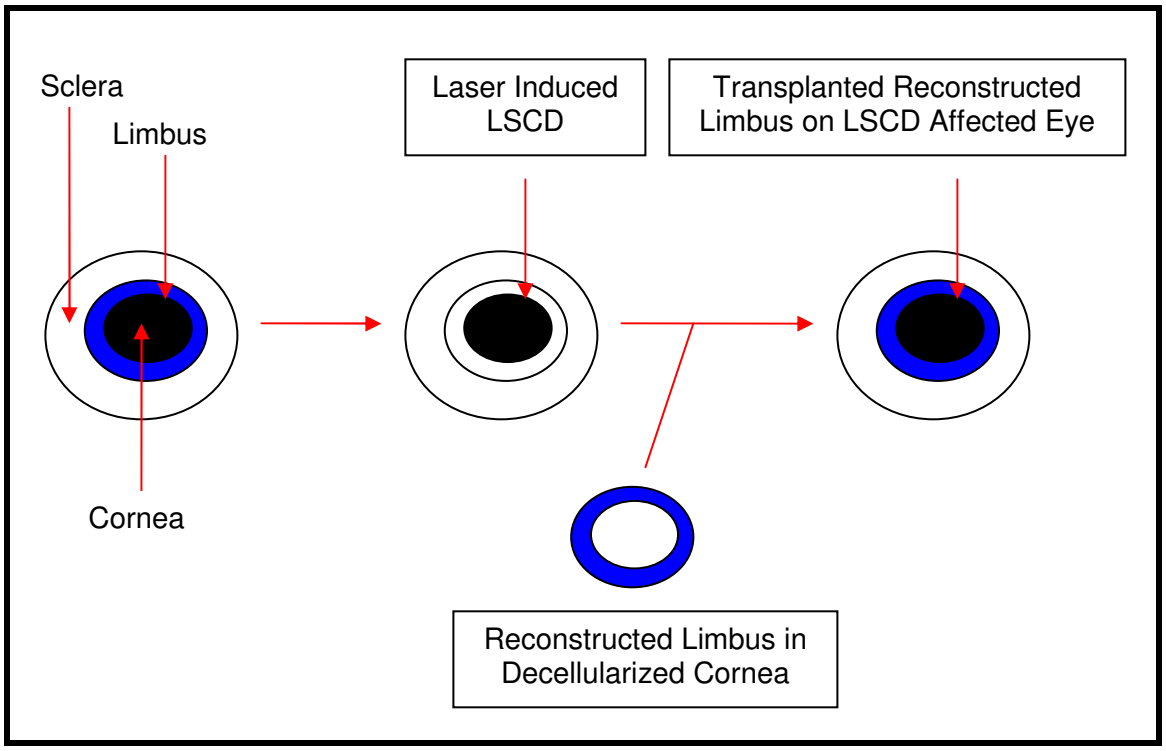

Figure 26. Treatment of LSCD with the engineered limbus. This aim of this study is two-fold. First, to develop a limbal stem cell deficiency (LSCD) in a rat eye using a laser technology, and second, to treat laser induced LSCD with the engineered limbus, reconstructed in the decellularized cornea.

VI. CITED LITERATURE

Almubrad T, Akhtar S. Structure of corneal layers, collagen fibrils, and proteoglycans of tree shrew cornea. Mol Vis. 2011;17:2283-91.

Amano S, Shimomura N, Yokoo S, Araki-Sasaki K, Yamagami S. Decellularizing corneal stroma using N2 gas. Mol Vis. 2008 May 14;14:878-82.

Andreassen TT, Simonsen AH, Oxlund H. Biomechanical properties of keratoconus and normal corneas. Exp Eye Res. 1980 Oct;31(4):435-41.

Angunawela RI, Poh R, Chaurasia SS, Tan DT, Mehta JS. A mouse model of lamellar intrastromal femtosecond laser keratotomy: ultra-structural, inflammatory, and wound healing responses. Mol Vis. 2011;17:3005-12.

Au HT, Cheng I, Chowdhury MF, Radisic M. Interactive effects of surface topography and pulsatile electrical field stimulation on orientation and elongation of fibroblasts and cardiomyocytes. Biomaterials. 2007 Oct;28(29):4277-93.

Bakhshandeh H, Soleimani M, Hosseini SS, Hashemi H, Shabani I, Shafiee A, Nejad AH, Erfan M, Dinarvand R, Atyabi F. Poly (epsilon-caprolactone) nanofibrous ring surrounding a polyvinyl alcohol hydrogel for the development of a biocompatible two-part artificial cornea. Int J Nanomedicine. 2011;6:1509-15.

Barbaro V, Ferrari S, Fasolo A, Ponzin D, Di Iorio E. Reconstruction of a human hemicornea through natural scaffolds compatible with the growth of corneal epithelial stem cells and stromal keratocytes. Mol Vis. 2009 Oct 17;15:2084-93.

Basu S, Mohamed A, Chaurasia S, Sejpal K, Vemuganti GK, Sangwan VS. Clinical outcomes of penetrating keratoplasty after autologous cultivated limbal epithelial transplantation for ocular surface burns. Am J Ophthalmol. 2011 Dec;152(6):917-924.

Bayyoud T, Thaler S, Hofmann J, Maurus C, Spitzer MS, Bartz-Schmidt KU, Szurman P, Yoeruek E. Decellularized bovine corneal posterior lamellae as carrier matrix

for cultivated human corneal endothelial cells. Curr Eye Res. 2012 Mar;37(3):179-86.

Beals M.P, Funderburgh J.L, Jester J.V, Hassell J.R. Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: maintenance of the keratocyte phenotype in culture. Invest. Ophthalmol. Vis. Sci., 40 (1999), pp. 1658–1663.

Buzard KA. Introduction to biomechanics of the cornea. Refract Corneal Surg. 1992 Mar-Apr;8(2):127-38.

Carlson EC, Wang IJ, Liu CY, Brannan P, Kao CW, Kao WW. Altered KSPG expression by keratocytes following corneal injury. Mol Vis. 2003 Nov 21;9:615-23.

Carlsson DJ, Li F, Shimmura S, Griffith M. Bioengineered corneas: how close are we? Curr Opin Ophthalmol. 2003 Aug;14(4):192-7.

Carrier, P., Deschambeault, A., Audet, C., Talbot, M., Gauvin, R., Giasson, CJ., Auger, FA., Guérin, SL., Germain, L. Impact of cell source on human cornea reconstructed by tissue engineering. Invest Ophthalmol Vis Sci 50, 2645, 2009.

Chen Y, Yan XM, Wu HR, Rong B. An experimental study on the fate of the amniotic membrane after amniotic membranetransplantation for acute alkaline burn of rat cornea. Zhonghua Yan Ke Za Zhi. 2012 Jan;48(1):27-32.

Chen, SY., Hayashida, Y., Chen, MY., Xie, HT., Tseng, SC. A new isolation method of human limbal progenitor cells by maintaining close association with their niche cells. Tissue Eng Part C Methods 2010. Dec 23. [Epub ahead of print]

Chirila TV. An overview of the development of artificial corneas with porous skirts and the use of PHEMA for such an application. Biomaterials. 2001 Dec;22(24):3311-7.

Choi HJ, Kim MK, Lee HJ, Ko JH, Jeong SH, Lee JI, Oh BC, Kang HJ, Wee WR. Efficacy of pig-to-rhesus lamellar corneal xenotransplantation. Invest Ophthalmol Vis Sci. 2011 Aug 22;52(9):6643-50. doi: 10.1167/iovs.11-7273.

Choi, J.S., Williams, J.K., Greven, M., Walter, K.A., Laber, P.W., Khang, G., Soker, S. Bioengineering endothelialized neo-corneas using donor-derived corneal endothelial cells and decellularized corneal stroma. Biomaterials. 31:6738, 2010.

Colabelli Gisoldi RA, Pocobelli A, Villani CM, Amato D, Pellegrini G. Evaluation of molecular markers in corneal regeneration by means of autologous cultures of limbal cells and keratoplasty. Cornea. 2010 Jul;29(7):715-22.

Cooper LJ, Kinoshita S, German M, Koizumi N, Nakamura T, Fullwood NJ. An investigation into the composition of amniotic membrane used for ocular surface reconstruction. Cornea. 2005. 24:722–729.

Crawford GJ, Hicks CR, Lou X, Vijayasekaran S, Tan D, Mulholland B, Chirila TV, Constable IJ. The Chirila Keratoprosthesis: phase I human clinical trial. Ophthalmology. 2002 May;109(5):883-9.

Curtis A, Wilkinson C. Topographical control of cells. Biomaterials. 1997 Dec;18(24):1573-83.

Daniel J. Abe K. McFetridge P.S. Development of the human umbilical vein scaffold for cardiovascular tissue engineering applications. ASAIO J 51, 252, 2005.

De Miguel MP, Alio JL, Arnalich-Montiel F, Fuentes-Julian S, de Benito-Llopis L, Amparo F, Bataille L. Cornea and ocular surface treatment. Curr Stem Cell Res Ther. 2010 Jun;5(2):195-204.

De Miquel MP, Fuentes-Julian S, Blazquez-Martinez A, Pascual CY, Aller MA, Arias J, Arnalich-Montiel F. Immunosuppressive properties of mesenchymal stem cells: advances and applications. Curr Mol Med. 2012 Jun 1;12(5):574-91.

Djalilian, A.R., Namavari, A., Ito, A., Balali, S., Afshar, A., Lavker, R.M., Yue, B.Y. J. T. Down-regulation of Notch signaling during corneal epithelial proliferation. Mol Vis 14, 1041, 2008.

Dong Z, Zhou X. Collagen cross-linking with riboflavin in a femtosecond laser-created pocket in rabbit corneas: 6-month results. Am J Ophthalmol. 2011 Jul;152(1):22-27.

Du L, Wu X. Development and characterization of a full-thickness acellular porcine cornea matrix for tissue engineering. Artif Organs. 2011 Jul;35(7):691-705. doi: 10.1111/j.1525-1594.2010.01174.x.

Elsheikh A, Wang D, Pye D. Determination of the modulus of elasticity of the human cornea. J Refract Surg. 2007 Oct;23(8):808-18.

Endo K, Nakamura T, Kawasaki S, Kinoshita S. Human amniotic membrane, like corneal epithelial basement membrane, manifests the alpha5 chain of type IV collagen. Invest Ophthalmol Vis Sci. 2004. 45:1771–1774.

Esko, J., Kimata, K., and Lindahl, L. (2009). Chapter 16 Proteoglycans and Sulfated Glycosaminoglycans. Varki A, Cummings RD, Esko JD, et al. (Eds.), Essentials of Glycobiology. 2nd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.

Espana EM, Kawakita T, Romano A, Di Pascuale M, Smiddy R, Liu CY, Tseng SC. Stromal niche controls the plasticity of limbal and corneal epithelial differentiation in a rabbit model of recombined tissue.Invest Ophthalmol Vis Sci. 2003;44:5130-5

Eyebank Association of America, 2011. U.S. Cornea Transplant Statistics. June 20, 2012. < http://www.restoresight.org/about-us/frequently-asked-questions/>

Fagerholm P, Lagali NS, Carlsson DJ, Merrett K, Griffith M. Corneal regeneration following implantation of a biomimetic tissue-engineered substitute. Clin Transl Sci. 2009 Apr;2(2):162-4.

Farrell RA, McCally RL, Tatham PE. Wave-length dependencies of light scattering in normal and cold swollen rabbit corneas and their structural implications. J Physiol. 1973 Sep;233(3):589-612.

Fenglan X, Yubao L, Xiaoming Y, Hongbing L, Li Z. Preparation and in vivo investigation of artificial cornea made of nano-hydroxyapatite/poly (vinyl alcohol) hydrogel composite. J Mater Sci Mater Med. 2007 Apr;18(4):635-40.

Fini ME. Keratocyte and fibroblast phenotypes in the repairing cornea. Prog Retin Eye Res. 1999 Jul;18(4):529-51.

Fu Y, Fan X, Chen P, Shao C, Lu W. Reconstruction of a tissue-engineered cornea with porcine corneal acellular matrix as the scaffold. Cells Tissues Organs. 2010;191(3):193-202.

Fukuda K, Chikama T, Nakamura M, Nishida T. Differential distribution of subchains of the basement membrane components type IV collagen and laminin among the amniotic membrane, cornea, and conjunctiva. Cornea. 1999. 18:73–79.

Gendron R, Kumar MR, Paradis H, Martin D, Ho N, Gardiner D, Merschrod SE, Poduska KM. Controlled cell proliferation on an electrochemically engineered collagen scaffold. Macromol Biosci. 2012 Mar;12(3):360-6. doi: 10.1002/mabi.201100341.

George AJ, Larkin DF. Corneal transplantation: the forgotten graft. Am J Transplant. 2004 May;4(5):678-85.

Gilbert TW, Sellaro TL, Badylak SF. Decellularization of tissues and organs. Biomaterials. 2006 Jul;27(19):3675-83.

Gomaa A, Comyn O, Liu C. Keratoprostheses in clinical practice - a review. Clin Experiment Ophthalmol. 2010 Mar;38(2):211-24.

Gonzalez-Andrades, M., Cardona, JD., Ionescu, AM., Campos, A., Perez, MD., Alaminos, M. Generation of Bioengineered Corneas with Decellularized Xenografts and Human Keratocytes. Invest Ophthalmol Vis Sci 52, 215, 2010.

Griffith M, Osborne R, Munger R, Xiong X, Doillon CJ, Laycock NL, Hakim M, Song Y, Watsky MA. Functional human corneal equivalents constructed from cell lines. Science. 1999 Dec 10;286(5447):2169-72.

Grueterich M, Espana EM, Tseng SC. Ex vivo expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche. Surv Ophthalmol. 2003 Nov-Dec;48(6):631-46.

Grueterich, M., Espana, EM., Tseng, SCG. Ex vivo expansion of limbal stem cells: Amniotic membrane serving as a stem cell niche. Survey of Ophthalmology 48, 631, 2003.

Han B, Schwab IR, Madsen TK, Isseroff RR. A fibrin-based bioengineered ocular surface with human corneal epithelial stem cells. Cornea. 2002 Jul;21(5):505-10.

Hashimoto Y, Funamoto S, Sasaki S, Honda T, Hattori S, Nam K, Kimura T, Mochizuki M, Fujisato T, Kobayashi H, Kishida A. Preparation and characterization of

decellularized cornea using high-hydrostatic pressurization for corneal tissue engineering. Biomaterials. 2010 May;31(14):3941-8.

Hassell JR, Schrecengost PK, Rada JA, SundarRaj N, Sossi G, Thoft RA. Biosynthesis of stromal matrix proteoglycans and basement membrane components by human corneal fibroblasts. Invest Ophthalmol Vis Sci. 1992 Mar;33(3):547-57.

Henriksson JT, McDermott AM, Bergmanson JP. Dimensions and morphology of the cornea in three strains of mice. Invest Ophthalmol Vis Sci. 2009 Aug;50(8):3648-54.

Hicks CR, Fitton JH, Chirila TV, Crawford GJ, Constable IJ. Keratoprostheses: advancing toward a true artificial cornea. Surv Ophthalmol. 1997 Sep-Oct;42(2):175-89.

Hjortdal JO. Regional elastic performance of the human cornea. J Biomech. 1996 Jul;29(7):931-42.

Hoeltzel DA, Altman P, Buzard K, Choe K. Strip extensiometry for comparison of the mechanical response of bovine, rabbit, and human corneas. J Biomech Eng. 1992 May;114(2):202-15.

Hong JW, Liu JJ, Lee JS, Mohan RR, Mohan RR, Woods DJ, He YG, Wilson SE. Proinflammatory chemokine induction in keratocytes and inflammatory cell infiltration into thecornea. Invest Ophthalmol Vis Sci. 2001 Nov;42(12):2795-803.

Ionescu AM, Alaminos M, de la Cruz Cardona J, de Dios García-López Durán J, González-Andrades M, Ghinea R, Campos A, Hita E, del Mar Pérez M. Investigating a novel nanostructured fibrin-agarose biomaterial for human cornea tissue engineering: rheological properties. J Mech Behav Biomed Mater. 2011 Nov;4(8):1963-73.

Jester J.V, Lee Y.G, Huang J, Houston J, Adams B, Cavanagh H.D, Petroll W.M. Postnatal corneal transparency, keratocyte cell cycle exit and expression of ALDH1A1. Invest. Ophthalmol. Vis. Sci., 48 (2007), pp. 4061–4069

Jester JV, Ho-Chang J. Modulation of cultured corneal keratocyte phenotype by growth factors/cytokines control in vitro contractility and extracellular matrix contraction. Exp Eye Res. 2003 Nov;77(5):581-92.

Jester JV, Barry-Lane PA, Cavanagh HD, Petroll WM. Induction of alpha-smooth muscle actin expression and myofibroblast transformation in cultured corneal keratocytes. Cornea. 1996 Sep;15(5):505-16.

Jing Zhang, Chen Huang, Yun Feng, Ying Li, Wei Wang. Comparison of beneficial factors for corneal wound-healing of rat mesenchymal stem cells and corneal limbal stem cells on the xenogeneic acellular corneal matrix in vitro.Mol Vis. 2012; 18: 161–173.

Jones RR, Hamley IW, Connon CJ. Ex vivo expansion of limbal stem cells is affected by substrate properties. Stem Cell Res. 2012 May; 8(3):403-9.

Jue B, Maurice DM. The mechanical properties of the rabbit and human cornea. J Biomech. 1986;19(10):847-53.

Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer. 2003 Jun;3(6):422-33.

Kasimir, MT., Rieder, E., Seebacher, G., Nigisch, A., Dekan, B., Wolner, E., Weigel, G., Simon, P. Decellularization does not eliminate thrombogenicity and inflammatory stimulation in tissue-engineered porcine heart valves. J Heart Valve Dis. 15, 278, 2006.

Kawashima M, Kawakita T, Satake Y, Higa K, Shimazaki J. Phenotypic study after cultivated limbal epithelial transplantation for limbal stem cell deficiency. Arch Ophthalmol. 2007 Oct;125(10):1337-44.

Keane TJ, Londono R, Turner NJ, Badylak SF. Consequences of ineffective decellularization of biologic scaffolds on the host response. Biomaterials. 2012 Feb;33(6):1771-81.

Kenyon, KR., Tseng, SC. Limbal autograft transplantation for ocular surface disorders. Ophthalmology 96, 709, 1989.

Khan BF, Harissi-Dagher M, Khan DM, Dohlman CH. Advances in Boston keratoprosthesis: enhancing retention and prevention of infection and inflammation. Int Ophthalmol Clin. 2007 Spring;47(2):61-71.

Kim MK, Wee WR, Park CG, Kim SJ. Xenocorneal transplantation. Curr Opin Organ Transplant. 2011 Apr;16(2):231-6.

Knox Cartwright NE, Tyrer JR, Marshall J. Age-related differences in the elasticity of the human cornea. Invest Ophthalmol Vis Sci. 2011 Jun 17;52(7):4324-9.

Langat DK, Mwenda JM. Potential risks of viral infections in xenotransplantation. Acta Trop. 2000 Sep 18;76(2):147-58.

Langer R, Vacanti JP. Tissue engineering. Science. 1993 May 14;260(5110):920-6.

Last JA, Thomasy SM, Croasdale CR, Russell P, Murphy CJ. Compliance profile of the human cornea as measured by atomic force microscopy. Micron. 2012 Feb 25.

Lavker RM, Tseng SC, Sun TT. Corneal epithelial stem cells at the limbus: looking at some old problems from a new angle. Exp Eye Res. 2004 Mar;78(3):433-46.

Lawrence BD, Marchant JK, Pindrus MA, Omenetto FG, Kaplan DL. Silk film biomaterials for cornea tissue engineering. Biomaterials. 2009 Mar;30(7):1299-308.

Lee JH, Lee HK, Kim JK, Chang JH, Kim SE, Kim EK, Seong GJ, Hong S. Expression of laminin-5 with amniotic membrane transplantation in excimer laser ablated ratcorneas. J Cataract Refract Surg. 2004 Oct;30(10):2192-9.

Li DQ, Tseng SC. Three patterns of cytokine expression potentially involved in epithelial-fibroblast interactions of human ocular surface. J Cell Physiol. 1995 Apr;163(1):61-79.

Li, F., Carlsson, D., Lohmann, C., Suuronen, E., Vascotto, S., Kobuch, K., Sheardown, H., Munger, R., Nakamura, M., Griffith, M. Cellular and nerve regeneration within a biosynthetic extracellular matrix for corneal transplantation. Proc Natl Acad Sci U S A 100, 15346, 2003.

Lim JY, Donahue HJ. Cell sensing and response to micro- and nanostructured surfaces produced by chemical andtopographic patterning. Tissue Eng. 2007 Aug;13(8):1879-91.

Lin CQ, Bissell MJ. Multi-faceted regulation of cell differentiation by extracellular matrix. FASEB J. 1993 Jun;7(9):737-43.

Lin XC, Hui YN, Wang YS, Meng H, Zhang YJ, Jin Y. Lamellar keratoplasty with a graft of lyophilized acellular porcine corneal stroma in the rabbit. Vet Ophthalmol. 2008 Mar-Apr;11(2):61-6.

Liu J, Roberts CJ. Influence of corneal biomechanical properties on intraocular pressure measurement: quantitative analysis. J Cataract Refract Surg. 2005 Jan;31(1):146-55.

Lombardo M, Lombardo G, Carbone G, De Santo MP, Barberi R, Serrao S. Biomechanics of the anterior human corneal tissue investigated with atomic force microscopy. Invest Ophthalmol Vis Sci. 2012 Feb 29;53(2):1050-7.

Maltseva O, Folger P, Zekaria D, Petridou S, Masur SK. Fibroblast growth factor reversal of the corneal myofibroblast phenotype. Invest Ophthalmol Vis Sci. 2001 Oct; 42 (11):2490-5.

Manche EE, Carr JD, Haw WW, Hersh PS. Excimer laser refractive surgery. West J Med. 1998 Jul; 169(1):30-8.

Marchini G, Pedrotti E, Pedrotti M, Barbaro V, Di Iorio E, Ferrari S, Bertolin M, Ferrari B, Passilongo M, Fasolo A, Ponzin D. Long-term effectiveness of autologous cultured limbal stem cell grafts in patients with limbal stem cell deficiency due to chemical burns. Clin Experiment Ophthalmol. 2012 Apr;40(3):255-67. doi: 10.1111/j.1442-9071.2011.02609.x.

Mariappan, I,. Maddileti, S., Savy, S., Tiwari, S., Gaddipati, S., Fatima, A., Sangwan, VS., Balasubramanian, D., Vemuganti, GK. In vitro culture and expansion of human limbal epithelial cells. Nature Protocols 5, 1470, 2010.

McFetridge, P. S., Daniel, J. W., Bodamyali, T., Horrocks, M. and Chaudhuri, J. B. Preparation of porcine carotid arteries for vascular tissue engineering applications. Journal of Biomedical Materials Research Part A 70A, 224, 2004.

Meek KM, Quantock AJ. The use of X-ray scattering techniques to determine corneal ultrastructure. Prog Retin Eye Res. 2001 Jan;20(1):95-137.

Meller D, Pauklin M, Westekemper H, Steuhl KP. Autologous transplantation of cultivated limbal epithelium. Ophthalmologe. 2010 Dec;107(12):1133-8.

Melles, GR., Binder, PS., Moore, MN., Anderson, JA. Epithelial-stromal interactions in human keratotomy wound healing. Arch Ophthalmol 113, 1124, 1995.

Meltendorf C, Deller T, Ackermann H, von Pape U. Corneal intrastromal tissue modeling with the femtosecond laser. Graefes Arch Clin Exp Opthalmol. 2011 Nov; 249 (11) :1661-6.

Michaels MG. Infectious concerns of cross-species transplantation: xenozoonoses. World J Surg. 1997 Nov-Dec;21(9):968-74.

Mimura T, Amano S, Yokoo S, Uchida S, Yamagami S, Usui T, Kimura Y, Tabata Y. Tissue engineering of corneal stroma with rabbit fibroblast precursors and gelatin hydrogels. Mol Vis. 2008;14:1819-28.

Miri A, Al-Deiri B, Dua HS. Long-term outcomes of autolimbal and allolimbal transplants. Ophthalmology. 2010 Jun;117(6):1207-13.

Molvaer RK, Andreasen A, Heegaard S, Thomsen JS, Hjortdal J, Urbak SF, Nielsen K. Interactive 3D computer model of the human corneolimbal region: crypts, projections and stem cells. Acta Ophthalmol. 2012 Jun 8. doi: 10.1111/j.1755-3768.2012.02446.x.

Musselmann K, Alexandrou B, Kane B, Hassell JR. Maintenance of the keratocyte phenotype during cell proliferation stimulated by insulin. J Biol Chem. 2005 Sep 23;280(38):32634-9.

Myung D, Koh W, Bakri A, Zhang F, Marshall A, Ko J, Noolandi J, Carrasco M, Cochran JR, Frank CW, Ta CN. Design and fabrication of an artificial cornea based on a photolithographically patterned hydrogel construct. Biomed Microdevices. 2007 Dec;9(6):911-22.

Nahmias, Y., and Yarmush, M. Tissue engineering application in general surgery. In: Meyer, U., Meyer, T., Handschel, J., and Wiesmann, H.P., eds. Fundamentals of Tissue Engineering and Regenerative Medicine (1st edition). Berlin: Springer, 2009.

Nash IS, Greene PR, Foster CS. Comparison of mechanical properties of keratoconus and normal corneas. Exp Eye Res. 1982 Nov;35(5):413-24.

Newsome DA, Gross J, Hassell JR. Human corneal stroma contains three distinct collagens. Invest Ophthalmol Vis Sci. 1982 Mar;22(3):376-81.\

Nishida K, Yamato M, Hayashida Y, Watanabe K, Maeda N, Watanabe H, Yamamoto K, Nagai S, Kikuchi A, Tano Y, Okano T. Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface. Transplantation. 2004 Feb 15;77(3):379-85.

Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, Adachi E, Nagai S, Kikuchi A, Maeda N, Watanabe H, Okano T, Tano Y. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N Engl J Med. 2004 Sep 16;351(12):1187-96.

Nishimura T, Toda S, Mitsumoto T, Oono S, Sugihara H. Effects of hepatocyte growth factor, transforming growth factor-beta1 and epidermal growth factor on bovine corneal epithelial cells under epithelial-keratocyte interaction in reconstruction culture. Exp Eye Res. 1998 Jan;66(1):105-16.

Nubile M, Dua HS, Lanzini M, Ciancaglini M, Calienno R, Said DG, Pocobelli A, Mastropasqua R, Carpineto P. In vivo analysis of stromal integration of multilayer amniotic membrane transplantation in corneal ulcers. Am J Ophthalmol. 2011 May;151(5):809-822.

Nyquist GW. Rheology of the cornea: experimental techniques and results. Exp Eye Res. 1968 Apr;7(2):183-8.

Oh JY, Kim MK, Wee WR. Lamellar corneal pig-to-rabbit xenotransplantation. Xenotransplantation. 2008 May-Jun;15(3):198-9.

Oh, JY., Kim, MK., Lee, HJ., Ko, JH., Wee, WR., Lee, JH. Processing porcine cornea for biomedical applications. Tissue Eng Part C Methods 15, 635, 2009.

Ota T, Taketani S, Iwai S, Miyagawa S, Furuta M, Hara M, Uchimura E, Okita Y, Sawa Y. Novel method of decellularization of porcine valves using polyethylene glycol and gamma irradiation. Ann Thorac Surg. 2007 Apr;83(4):1501-7.

Ott, H.C., Matthiesen, T.S., Goh, S.K., Black, L.D., Kren, S.M., Netoff, T.I., and Taylor, D.A. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. Nat Med 14, 213, 2008.

Pang, K., Du, L., Wu, X. A rabbit anterior cornea replacement derived from acellular porcine cornea matrix, epithelial cells and keratocytes. Biomaterials 31, 7257, 2010.

Patel S, McLaren J, Hodge D, Bourne W. Normal human keratocyte density and corneal thickness measurement by using confocal microscopy in vivo. Invest Ophthalmol Vis Sci. 2001 Feb;42(2):333-9.

Pattin CA, Caler WE, Carter DR. Cyclic mechanical property degradation during fatigue loading of cortical bone. J Biomech. 1996 Jan;29(1):69-79.

Pauklin M, Fuchsluger TA, Westekemper H, Steuhl KP, Meller D. Midterm results of cultivated autologous and allogeneic limbal epithelial transplantation in limbal stem cell deficiency. Dev Ophthalmol. 2010;45:57-70.

Phu, D., Wray, LS., Warren, RV., Haskell, RC., Orwin, EJ. Effect of Substrate Composition and Alignment on Corneal Cell Phenotype. Tissue Eng Part A 2010.

Pinnamaneni, N. and Funderburgh, J. L. (2012), Concise Review: Stem Cells in the Corneal Stroma. STEM CELLS, 30: 1059–1063. doi: 10.1002/stem.1100

Polfik J, Fardoun F, Degallaix S. Effective and internal stresses in cyclic straining of 316 stainless steel. Materials Science and Engineering A215 (1996) 104-112

Reichard M, Hovakimyan M, Wree A, Meyer-Lindenberg A, Nolte I, Junghans C, Guthoff R, Stachs O. Comparative in vivo confocal microscopical study of the cornea anatomy of different laboratory animals. Curr Eye Res. 2010 Dec; 35(12): 1072-80.

Roy S, Silacci P, Stergiopulos N. Biomechanical properties of decellularized porcine common carotid arteries. Am J Physiol Heart Circ Physiol 289, 567, 2005.

Ruberti JW, Klyce SD. NaCl osmotic perturbation can modulate hydration control in rabbit cornea. Exp Eye Res. 2003 Mar;76(3):349-59.

Sakimoto T, Rosenblatt MI, Azar DT. Laser eye surgery for refractive errors. Lancet. 2006 Apr 29;367(9520): 1432-47

Samra W, and Krueger R. Update on customized excimer ablations: recent developments reported in 2002. Current Opinion in Ophthalmology, 14(4):198–202, 2002.

Sasaki S, Funamoto S, Hashimoto Y, Kimura T, Honda T, Hattori S, Kobayashi H, Kishida A, Mochizuki M. In vivo evaluation of a novel scaffold for artificial corneas prepared by using ultrahigh hydrostatic pressure to decellularize porcine corneas. Mol Vis. 2009 Oct 13;15:2022-8.

Schmoll T, Unterhuber A, Kolbitsch C, Le T, Stingl A, Leitgeb R. Precise thickness measurements of Bowman's layer, epithelium, and tear film. Optom Vis Sci. 2012 May;89(5):E795-802.

Scott SG, Jun AS, Chakravarti S. Sphere formation from corneal keratocytes and phenotype specific markers. Exp Eye Res. 2011 Dec;93(6):898-905.

Shanmuganathan VA, Foster T, Kulkarni BB, Hopkinson A, Gray T, Powe DG, Lowe J, Dua HS. Morphological characteristics of the limbal epithelial crypt. Br J Ophthalmol. 2007 Apr;91(4):514-9.

Sharpe, JR., Daya, SM., Dimitriadi, M., Martin, R., James, SE. Survival of cultured allogeneic limbal epithelial cells following corneal repair. Tissue Eng 13, 123, 2007.

Shimmura, S., Doillon, CJ., Griffith, M., Nakamura, M., Gagnon, E., Usui, A., Shinozaki, N., Tsubota, K. Collagen-poly(N-isopropylacrylamide)-based membranes for corneal stroma scaffolds. Cornea 22, S81, 2003.

Stolz M, Raiteri R, Daniels AU, VanLandingham MR, Baschong W, Aebi U. Dynamic elastic modulus of porcine articular cartilage determined at two different levels of tissueorganization by indentation-type atomic force microscopy. Biophys J. 2004 May;86(5):3269-83.

Sumide T, Nishida K, Yamato M, Ide T, Hayashida Y, Watanabe K, Yang J, Kohno C, Kikuchi A, Maeda N, Watanabe H, Okano T, Tano Y. Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces. FASEB J. 2006 Feb;20(2):392-4.

Szerenyi KD, Wang X, Gabrielian K, McDonnell PJ. Keratocyte loss and repopulation of anterior corneal stroma after de-epithelialization. Arch Ophthalmol. 1994 Jul;112(7):973-6.

Takeuchi Y, Magre S, Patience C. The potential hazards of xenotransplantation: an overview. Rev Sci Tech. 2005 Apr;24(1):323-34.

Tan DT, Dart JK, Holland EJ, Kinoshita S. Corneal transplantation. Lancet. 2012 May 5;379(9827):1749-61.

Thanos M, Pauklin M, Steuhl KP, Meller D. Ocular surface reconstruction with cultivated limbal epithelium in a patient with unilateral stem cell deficiency caused by Epidermolysis bullosa dystrophica hallopeau-Siemens. Cornea. 2010 Apr;29(4):462-4.

Tsai, RJ., Tsai, RY. Ex vivo expansion of corneal stem cells on amniotic membrane and their outcome. Eye Contact Lens 36, 305, 2010.

Tsai, RJF., Li, LM., Chen, JK. Reconstruction of Damaged Corneas by Transplantation of Autologous Limbal Epithelial Cells. N Engl J Med 343, 86, 2000.

Tseng SC, Kruse FE, Merritt J, Li DQ. Comparison between serum-free and fibroblast-cocultured single-cell clonal culture systems: evidence showing that epithelial anti-apoptotic activity is present in 3T3 fibroblast-conditioned media. Curr Eye Res. 1996 Sep;15(9):973-84.

Tsujita H, Brennan AB, Plummer CE, Nakajima N, Hyon SH, Barrie KP, Sapp B, Jackson D, Brooks DE. An ex vivo model for suture-less amniotic membrane transplantation with a chemically defined bioadhesive. Curr Eye Res. 2012 May;37(5):372-80.

Uchimura, E., Sawa, Y., Taketani, S., Yamanaka, Y., Hara, M., Matsuda, H. and Miyake, J. Novel method of preparing acellular cardiovascular grafts by decellularization

with poly(ethylene glycol). Journal of Biomedical Materials Research Part A 67A, 834, 2003.

Vrana NE, Builles N, Justin V, Bednarz J, Pellegrini G, Ferrari B, Damour O, Hulmes DJ, Hasirci V. Development of a reconstructed cornea from collagen-chondroitin sulfate foams and human cell cultures. Invest Ophthalmol Vis Sci. 2008 Dec;49(12):5325-31.

Wang X, Wu Q. Normal corneal thickness measurements in pigmented rabbits using spectral-domain anterior segment optical coherence tomography. Vet Ophthalmol. 2012 Jun 6. doi: 10.1111/j.1463-5224.2012.01041.x.

Wang, LQ., Huang, YF., Huang, JX., Yang, BJ., Chen, B. The interactions of stromal-epithelial in a model of co-culture in the rabbit cornea. Zhonghua Yan Ke Za Zhi 43, 251, 2007.

Watanabe R, Hayashi R, Kimura Y, Tanaka Y, Kageyama T, Hara S, Tabata Y, Nishida K. A novel gelatin hydrogel carrier sheet for corneal endothelial transplantation. Tissue Eng Part A. 2011 Sep;17(17-18):2213-9.

Williams KA, Roder D, Esterman A, Muehlberg SM, Coster DJ. Factors predictive of corneal graft survival. Report from the Australian Corneal Graft Registry. Ophthalmology 1992; 99: 403–414.

Wilson SE, Liu JJ, Mohan RR. Stromal-epithelial interactions in the cornea. Prog Retin Eye Res. 1999 May;18(3):293-309.

Wilson SE, Mohan RR, Mohan RR, Ambrósio R Jr, Hong J, Lee J. The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. Prog Retin Eye Res. 2001 Sep;20(5):625-37.

Wollensak G, Iomdina E. Biomechanical and histological changes after corneal crosslinking with and without epithelial debridement. J Cataract Refract Surg. 2009 Mar;35(3):540-6.

World Health Organization, 2011Vision 2020 Global initiative for the elimination of avoidable blindness: Action plan 2006–2011. June 20, 2012. <http://www.who.int/blindness/Vision2020_report.pdf>

Wu J, Du Y, Watkins SC, Funderburgh JL, Wagner WR. The engineering of organized human corneal tissue through the spatial guidance of corneal stromal stem cells. Biomaterials. 2012 Feb;33(5):1343-52.

Wu, Z., Zhou, Y., Li, N., Huang, M., Duan, H., Ge, J., Xiang, P., Wang, Z. The use of phospholipase A(2) to prepare acellular porcine corneal stroma as a tissue engineering scaffold. Biomaterials 30, 3513, 2009.

Xiao J, Duan H, Liu Z, Wu Z, Lan Y, Zhang W, Li C, Chen F, Zhou Q, Wang X, Huang J, Wang Z. Construction of the recellularized corneal stroma using porous acellular corneal scaffold. Biomaterials. 2011 Oct;32(29):6962-71.

Xu YG, Xu YS, Huang C, Feng Y, Li Y, Wang W. Development of a rabbit corneal equivalent using an acellular corneal matrix of a porcine substrate. Mol Vis. 2008;14:2180-9.

Zeng Y, Yang J, Huang K, Lee Z, Lee X. A comparison of biomechanical properties between human and porcine cornea. J Biomech. 2001 Apr;34(4):533-7.

Zhang C, Jin Y, Nie X, Liu Y, Lei J, Hu D. A comparative study on biocompatibility of acellular corneal stroma materials prepared by serial digestion methods. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi. 2006 Feb;20(2):185-8.

Zhang C, Nie X, Hu D, Liu Y, Deng Z, Dong R, Zhang Y, Jin Y. Survival and integration of tissue-engineered corneal stroma in a model of corneal ulcer. Cell Tissue Res. 2007 Aug;329(2):249-57.

Zhang J, Huang C, Feng Y, Li Y, Wang W. Comparison of beneficial factors for corneal wound-healing of rat mesenchymal stem cells and corneal limbal stem cells on the xenogeneic acellular corneal matrix in vitro. Mol Vis. 2012;18:161-73

Zhou, Y., Wu, Z., Ge, J., Wan, P., Li, N., Xiang, P., Gao, Q., Wang, Z. Development and Characterization of Acellular Porcine Corneal Matrix Using Sodium Dodecylsulfate. Cornea 30, 73, 2010.

VII. VITA

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EDUCATION:

Doctor of Philosophy, Bioengineering, University of Illinois at Chicago, Chicago, IL, August 2012.
Dissertation Project: "Decellularized Human Cornea for Reconstructing the Corneal Epithelium and Anterior Stroma"

Bachelor of Science, Bioengineering, University of Illinois at Chicago, Chicago, IL, Dec 2006.
Senior Design Project: "Control of Gene Exposure and Sequestration by Manipulation of the Cytoskeleton: A Mechanical Role of Gene Control Based on Tensegrity"

TEACHING: EXPERIENCE:

Teacher, Taught Elementary School students Science, Social Studies, English and Mathematics, Guidance Educational Foundation, Chicago, IL, Feb – May 2007.

RESAERCH EXPERIENCE:

Graduate Research Assistant, University of Illinois at Chicago, Chicago, IL, Jan 2008 – Aug 2012.

- Performed *in vitro* and *ex vivo* cell cultures
- Studied corneal epithelial stem cell biology
- Studied corneal fibroblast cell biology
- Performed corneal graft transplantation
- Worked with mouse, rat, and rabbit animal models
- Performed immunostaining of cells and tissues
- Worked with Leica Confocal microscope, Light and Epifluorescent microscopy, Scanning electron microscope, Transmission electron microscope.

OTHER EXPERIENCE:

Personal and house care aids, Metropolitan Asian Services, Chicago, IL, Jan – Apr 2004.

Registration Personnel, Oakton Community College, Skokie, IL, Aug 2002 – Nov 2003.

Bookstore Cashier, Oakton Community College, Skokie, IL, May 2002 – Jul 2003.

PROFESSIONAL MEMBERSHIP:

The Association for Research in Vision and Ophthalmology

Tau Beta Pi Honors Society, Engineering Honors' Society

Society of Women Engineers

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HONORS AND AWARDS:

Eye Bank Association of America (EBAA) - 2010

Chancellor's Supplemental Research Fellowship, UIC - 2010

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Excellence Scholarship, Oakton Community College - 2003

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Shafiq M, Milani B, Majdi M, Makhsous M, Yue B, and Djalilian A. 2012. Evaluation of Decellularized Human Corneal Graft in a Rat Model. The Association for Research in Vision and Ophthalmology. Fort Lauderdale, FL.

Shafiq M, Gemeinhart R, Yue B, and Djalilian, A. 2011. Development of an Acellular Human Cornea to Support the Growth of Corneal Epithelial and Stromal Cells. The Association for Research in Vision and Ophthalmology. Fort Lauderdale, FL.

Parikh K, **Shafiq M,** Amirjamshidi H, Djalilian A. 2009. Role Of Conditioned Media in Corneal Wound Healing. University of Illinois at Chicago. College Of Medicine Research Symposium, Chicago, IL.

Shafiq M, Amirjamshidi H, Djalilian A. 2009. Decellularization of Cadaver Corneas for Ocular Graft Transplantation. University of Illinois at Chicago. College Of Medicine Research Symposium, Chicago, IL.

Shafiq M, Djalilian A. 2009. Development of Limbal Niche for Epithelial Stem Cells Regeneration. University of Illinois at Chicago, Department of Ophthalmology and Visual Sciences, Chicago, IL.

Shafiq M, Yohannan T, Djalilian A. 2009. Development of Limbal Stem Cell Niche for Ocular Surface Transplantation. University of Illinois at Chicago. Student Research Forum, Chicago, IL.

Kumar G, **Shafiq M,** Afshar A, Djalilian A. 2007. Tissue Eengineered Autologous Corneal Grafts, University of Illinois at Chicago, Graduate College Research Symposium, Chicago, IL.

Shafiq M, Mahmood W, Patel R, Patel R, Maniotis A. 2006. Control of Gene Exposure and Sequestration by Manipulation of the Cytoskeleton: A Mechanisl Model of Gene Control. University of Illinois at Chicago Engineering EXPO, Chicago, IL.

PUBLICATIONS:

Shafiq M, Gemeinhart R, Yue B, and Djalilian, A. Decellularized Human Cornea for Reconstructing the Corneal Epithelium and Anterior Stroma. Tissue. Tissue Eng Part C Methods. 2012 May;18(5):340-8.

Amirjamshidi H, Milani BY, Sagha HM, Movahedan A, **Shafiq MA**, Lavker RM, Yue BY, Djalilian AR. Limbal fibroblast conditioned media: a non-invasive treatment for limbal stem cell deficiency. Mol Vis. 2011 Mar 8;17:658-66.