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Bioengineered corneas for transplantation and *in vitro* toxicology

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1. ABSTRACT

Bioengineered corneas have been designed to replace partial or the full-thickness of defective corneas, as an alternative to using donor tissues. They range from prosthetic devices that solely address replacement of the cornea's function, to tissue engineered hydrogels that permit regeneration of host tissues. In cases where corneal stem cells have been depleted by injury or disease, most frequently involving the superficial epithelium, tissue engineered lamellar implants reconstructed with stem cells have been transplanted. *In situ* methods using ultraviolet A (UVA) crosslinking have also been developed to strengthen weakened corneas. In addition to the clinical need, bioengineered corneas are also rapidly gaining importance in the area of *in vitro* toxicology, as alternatives to animal testing. More complex, fully innervated, physiologically active, three-dimensional organotypic models are also being tested.

2. INTRODUCTION

Bioengineered corneas are designed to restore human vision and comprise devices that replace part or the full-thickness of damaged or diseased corneas. They range from fully synthetic prostheses to tissue engineered hydrogels that allow some regeneration of the host tissues. In addition, there are also bioengineered lenticules that may be implanted into the cornea to improve vision. These implants correct refractive errors of the eye, and as such may be possible alternatives to spectacles, contact lenses, or laser surgery procedures. More recently, however, with European Directive 76/768/EEC and reports of contaminated contact lens solutions leading to infections, there has been heightened interest in the development of *in vitro* models of human corneas for safety and efficacy testing.

2.1. The cornea as a target organ for repair and regeneration

The average human cornea is about 500 μm thick centrally and about 750 μm peripherally (1). It can be viewed as a largely collagenous matrix containing cells (a

stroma) sandwiched between an outer epithelium and an inner endothelium. A unique property of the cornea is its optical clarity, which accounts for over 70% of the light that is transmitted to the retina for vision. Corneal clarity is now believed to result from a combination of refractive index matching, and the presence of structural components well below the wavelength of visible light (2, 3). Another unique property of the cornea is that it is avascular, as well as immune privileged (4).

The corneal epithelium consists of stratified, non-keratinizing epithelial cells, which are approximately 50 μm thick. The basal layer of the epithelium proliferates to replace the superficial cells lost at the anterior surface (5), which are subsequently replenished by a population of corneal stem cells that reside within the corneal/scleral limbus (6). Several anti-inflammatory and anti-microbial factors are secreted by the epithelium as an insoluble mucous layer which aids in stabilizing the tear film (7). Biochemically, the stroma consists mainly of type I collagen (13.6%), 0.9% glycosaminoglycans, and 80% water, making the stroma resemble a hydrogel. Stromal cells, or keratocytes, are localized in lamellar networks within this hydrogel. Lastly, the endothelium, which is a single cell layer, functions to regulate the hydration level of the corneal stroma, which helps to maintain central corneal thickness. Sodium/potassium ATPase pumps within the endothelium circulate aqueous humor between the anterior chamber and stroma. A detailed review of the corneal layers is given in (8).

The function of the cornea is dependent upon its optical clarity. When loss of clarity, due to disease or damage is irreversible, corneal blindness results. The World Health Organization's (WHO) definition of blindness includes individuals who have a visual acuity of 3/60 or less. That being said, it is estimated that there are 50 million people worldwide who possess bilateral blindness, as well as at least 150 million people who have impaired vision in both eyes (9, 10). While cataracts are responsible for almost half the cases of blindness, corneal damage and disease due to various etiologies is the next largest culprit, with corneal trachoma affecting roughly 5 million people worldwide (9). Furthermore, it is estimated that corneal ulceration and ocular trauma is responsible for 1.5 to 2 million new cases of corneal blindness annually (10). At present, transplantation of human donor tissue is the only widely acceptable treatment.

Depending upon the nature of the damage, replacement of one or more corneal layers is performed using corneal allografts or progenitor cell grafts (*e.g.*, limbal stem cell grafts). In other conditions such as keratoconus, where stromal matrix is damaged or weakened, attempts to strengthen the matrix by *in situ* crosslinking have been attempted.

In this review, we discuss some of the better known strategies aimed at restoring the light transmission and protective functions of the cornea. This includes the development of tissue substitutes and carriers for stem cell delivery, and *in situ* strengthening of the cornea.

2.2. Corneal substitutes for *in vitro* testing

The superficial location of the cornea makes it a target for accidental exposure to chemicals and compounds that could cause irritation or permanent vision loss. Hence, compounds destined for human use need to be screened for safety, in addition to efficacy.

In recent times, interest in tissue engineered corneas as *in vitro* replacements for animals in the field of toxicology has escalated. This is in light of the ban on consumer product testing using animals that is currently in place in Europe (European Union Directive 76/768/EEC), which is expected to expand worldwide. Previously, animal based toxicological tests have been used to study potentially irritating and toxic substances, in order to assure the production of safe commodities (11). This is important in the context of the cornea, due to its exterior location, and the damage which can result in opacification, scarring, inflammation and angiogenesis. In order to design an *in vitro* alternative for the cornea, it is ideal to contain the same biochemical, morphological, physiological, and even genetic components found in the native cornea. This review focuses only on 3-dimensional *in vitro* models.

3. CORNEAL SUBSTITUTES FOR TRANSPLANTATION

3.1. Completely synthetic corneal replacements: keratoprostheses

Although this review focuses on tissue engineering, for completeness and as a comparison, corneal prostheses will be briefly discussed. Keratoprostheses (KPro's) were developed to restore minimal function – light transmission – and as such, were the first artificial corneas developed. As several recent, comprehensive reviews are available on keratoprostheses (12-14), in this review, we focus only on a few representative KPro's that are currently used in clinical trials and more recent, significant developments.

The majority of KPro designs are based upon a transparent central optic, surrounded by a skirt to provide stable anchorage through integration into the host tissue. Majority of skirts are designed to permit cellular integration of the host tissue through fibroblast in-growth, which would promote extracellular matrix (ECM) (mainly collagen) deposition to provide firm anchorage. Various materials have been employed in skirt design, maintaining proper strength to allow for suture placement. When designing the anterior surface of the device, consideration has been made to promote epithelialization, as well as to inhibit downgrowth of epithelial cells into the stroma/implant interface. As well, the posterior surface can be modified to inhibit cellular attachment and proliferation to prevent retroprosthetic membrane formation, which leads to corneal opacification. The materials used in KPros should also allow sufficient oxygen permeation and nutrient permeability in order to maintain the cellular components (15). Most importantly, the synthetic materials used in the device should be non-immunogenic nor mutagenic, to allow graft acceptance.

The osteo-odonto keratoprosthesis (OOKP), developed by Strampelli (16), consists of autologous tissue derived from tooth and bone which surrounds a central poly(methyl methacrylate) PMMA optic. Before implantation, the osteodental skirt is pre-implanted into the buccal mucosa to allow colonization of fibroblasts to support integration when implanted ocularly. This KPro has been one of the most successful, as it has a low extrusion rate, due to the excellent integration of the skirt material (mostly hydroxypatite) with the host tissue (17). Complications associated with this KPro include retroprosthetic membrane formation, glaucoma and decentration of the central optic, due to absorption of the osteodental skirt (18).

Another KPro that has been developed utilizing PMMA is the Boston KPro, or the Dohlman-Doane KPro, in which there are two types, with the most common one being the single collar button. This design consists of a front plate that is 5.5-7 mm in diameter, connected by a 3.5 mm stem piece to a 7 mm back plate. This KPro is used in patients who have had multiple graft rejections, including those with chemical burns, congenital glaucoma and herpetic keratitis (19).

The Seoul-type KPro consists of an optic surrounded by a skirt, with additional haptics for increased mechanical biostability post implantation. The haptics are secured to the eye through suturing of the skirt to the cornea, as well as fixation to the sclera. The optics are comprised of a PMMA surface modified with poly(ethylene glycol) (PEG), while the skirt was made initially of poly(tetrafluoroethylene) (PTFE) but more recently of either poly(propylene) or poly(urethane). The haptics are designed using polypropylene monofilaments. A recent study was published that followed nine patients at 62 months post operative, who had severe intractable ocular surface disease. Average visual acuity preservation time was 31.6 months. However, complications associated with this device included retroprosthetic membrane formation, extrusion and retinal detachment. Regardless of the skirt materials used in the study, tissue melt occurred with full skirt exposure at a mean interval of 12.9 months, necessitating mucosal, amniotic membrane or scleral grafting to compensate. In all the cases where KPro exchange was required due to polyurethane skirt degeneration, retinal detachment occurred, suggesting retinal management techniques are required for long term survival, as well as exchange (20).

The current generation of the original Chirila KPro has undergone multicentre clinical trials. The first generation consisted of a PHEMA hydrogel core-skirt model that was sutured in place through a traditional penetrating keratoplasty technique, which has given rise to the current two-stage lamellar procedure. The newer device that has gained market approval is known as the AlphaCor™ KPro. This device consists of an interpenetrating network of transparent PHEMA, with an opaque sponge skirt also made of PHEMA. The skirt differs in water content, resulting in a KPro that does not have any glued joints, or mechanical junctions (21). The surgical procedure consists of implanting the KPro in an intrastromal pocket via removal of the central posterior corneal lamellae, and closed over with a conjunctival flap. After three months, the flap and anterior corneal lamellae are removed, exposing the central optic. While this device has been shown to have low complication rates in normal healthy eyes, it was also meant for eyes that were already pathologic, in patients that had experienced multiple graft failures, or were not eligible for conventional donor tissue transplants. Complications with these KPro's include corneal melting, formation of retroprosthetic membranes and optic depositions, as well as rare cases of device extrusion. Contraindications for this device include abnormal tear film, as well as uncontrollable high intraocular pressure. Topical administration of medroxyprogesterone has been shown to limit corneal melting of the device. In the most recent report on the clinical outcomes of AlphaCor™ implantation, it has been stated that HSV-1 (herpes simplex virus 1) infection is no longer considered a contraindication for implantation (22).

Another group (23) has tested a KPro that is comprised of a central optic made of silicone, surrounded by an opaque skirt of PTFE. The BioKPro III is inserted in a similar fashion to the AlphaCor™, through a two-stage process. Initially the implant is covered with either a conjunctival or buccal mucous membrane back, and three months later the central optic is exposed. Follow up occurred between 18-48 months, with failure in six of seven patients due to extrusion, although it is noted that all the patients had extensive corneal pathologies, including thermal injury, chemical injury, congenital rubella, measles keratitis and Steven's Johnson syndrome. Also, with the one implant that was not extruded, the patient recorded mucous accumulation on the optic which required clearing. The authors' observed that more work needed to be performed on this implant in order for it to be a viable option (23).

Recent developments include a KPro developed by Storsberg and co-workers at the Fraunhofer Institute for Applied Polymer Research, Germany. The "new artificial cornea adheres to eye cells without needing to be held in place by sutures - a major advantage over other artificial corneas, which can cause inflammation and infection". According to the German team, the skirt portion is coated with a "special protein" that has been described to "connect to the natural cells of the eye". The optic, however, is cell free (http://www.rsc.org/chemistryworld/News/2007/October/18100701.asp).

3.2. Keratoprostheses with cell coverage

Researchers believe it is important for the epithelium to cover the KPro, in order to maintain the tear film, prevent infection as well as extrusion of the implant (24). Materials such as PMMA, poly(vinyl alcohol) (PVA), and PHEMA are utilized for traditional KPro's. As these materials are non-cell adhesive, improvements have been made to modify the ability of corneal cells to adhere, and migrate over the surface. Naturally occurring extra

cellular matrix proteins and other cell adhesive peptides have been grafted onto the KPros to alleviate this problem.

Sheardown *et al.* (25) covalently attached cell adhesion peptides from laminin and fibronectin, including laminin derived YIGSR, and its synergistic peptide PDSGR, as well as fibronectin based RGDS and PDSGR. These were attached to poly(dimethyl siloxane) (PDMS) surfaces. It was found that a surface modification with multiple peptides had a synergistic effect on corneal epithelial cell attachment, when compared to single peptides on their own (25).

Jacob *et al.* (26) investigated the coupling of cell adhesion peptides and various cytokines to polymethacrylic acid-co-2-hydroxyethyl methacrylate (PHEMA/MAA). The bioactive factors included fibronectin, laminin, substance P, IGF-1 (insulin-like growth factor 1) and RGD. They tested whether corneal epithelial cell growth rate and adhesion were increased when the bioactive factors were directly coated on the surfaces, or if they were tethered through PEG spacers. It was found that the spacer molecules provided the correct microenvironment for epithelial cells to proliferate, compared with little to no epithelial growth on surfaces only coated with the bioactive factors (26). It is also of importance to note that the peptides and factors are exposed to biodegradation, and effort must be taken to ensure long term attachment of the epithelial cells on the surface is maintained.

The Stanford keratoprosthesis has been developed based on a mechanically enhanced core and skirt method. It consists of a double network of PEG and poly(acrylic acid) (PAA) as its central optic, allowing for the overgrowth of corneal epithelial cells. The use of a double network of polymers increases the mechanical strength, optical transparency and biocompatibility of the polymer. The Stanford KPro has also been designed using polymer technology that allows for microperforation of the outer skirt, to further enhance integration with the host tissue. As well, a photochemical surface modification strategy has been utilized to tether bioactive factors to specific sites of the polymer to promote cell adhesion (13, 27). The KPro has been shown to be tolerated well in animal models leading up to six weeks, and further studies are ongoing (28).

3.3. Self-assembled corneal substitutes

Germain and co-workers (29) at the Laboratoire d'Organogenèse Expérimentale (LOEX) use a self-assembly approach for a corneal substitute. Ascorbic acid is used to stimulate the secretion of collagen and other ECM molecules by dermal fibroblast cells. Resulting sheets of matrix macromolecules are stacked together to form a stroma, allowed to further integrate in culture, and then an epithelium is seeded on top of the stack. These constructs show excellent corneal morphology and cells express appropriate tissue-specific markers. The primary drawback is the time needed to produce enough transplantable material rapidly for transplantation. More recently, Carrier *et al.* (30) followed up with a new self-assembled model comprising a stroma consisting of human corneal and dermal fibroblasts. The authors contend that the combination of the corneal and dermal fibroblasts were more conducive to the formation of a well-differentiated epithelium that showed higher re-epithelialization rates than just corneal fibroblasts alone. They showed that this model reproduced the microanatomy of the native human cornea. In addition, this model was able to reproduce a mechanistically accurate wound healing process. This suggests that this model could be used as a tool for studying wound healing, screening bioactive factors that could modulate wound healing or as a pre-screen prior to animal testing (30).

Guo *et al.* (31) characterized the ECM macromolecules deposited by primary human corneal fibroblasts in such self-assembled corneal substitutes. The average culture took four weeks to produce a multi-layered construct about 36 μm thick. These constructs were highly cellular and are morphologically similar to the stroma of mammalian corneas, with multiple, parallel layers of cells and small fibrillar ECM arrays. The fibrils and collagen were approximately 27 to 51 nm, with a mean of 38.1 ± 7.4 nm, compared to the 31 ± 0.8 nm reported in adult human corneas (32).

3.4. Biomimetic corneal implants

Since type I collagen is the dominant biopolymer found in the human cornea (70% dry weight), and is amenable to modification, it has been investigated for its use as a scaffold for artificial cornea construction. Type I collagen scaffolds have been used previously to culture human corneal stromal fibroblasts. It was observed that the cell scaffold interactions caused a change in the mechanical properties of the gels, as well as the permeability of the gels when cultured *in vitro* (33, 34). This indicated the remodeling of the matrix by the stromal cells changed the properties to be more like the natural stroma. Resultant tissue had a lamellar like microstructure following 21 days of incubation, as compared to its initial spongy structure.

The authors have developed various collagen-based corneal substitutes. They have been successfully implanted as either deep lamellar grafts or full thickness implants into mice, rabbits, guinea pigs, dogs and pigs. . These gels can be fabricated to the appropriate dimensions and curvatures, which allow for transmission of 90% or higher of white light. Collagen sources have included both porcine and bovine extracted collagens, as well as the more current use of recombinant human collagen. To make the gels mechanically strong enough to permit suturing and resist biodegradation, crosslinking, co-polymerization and development of interpenetrating networks have been used. We recently demonstrated that a simple type I collagen-based corneal stroma mimic (fabricated by crosslinking porcine collagen with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS)) could be successfully implanted into mini-pigs with stable host-graft integration (35). Without the use of post operative steroids, the

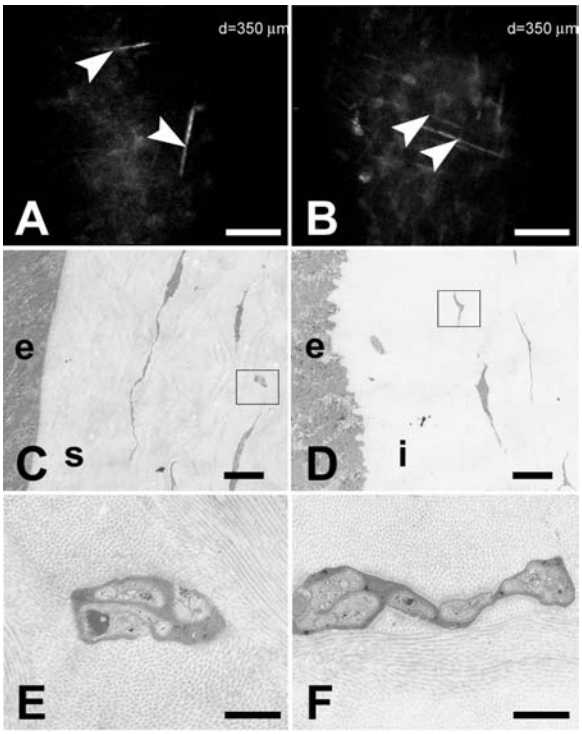

Figure 1. *In vivo* confocal microscopy (IVCM) and TEM images of pig corneas at 12 months post-operative. (A-B) IVCM images. Regenerated nerves (arrowheads) can be seen sub-basally in both non-surgical (A) and operated cornea (B) in the deep stromal layer. (C-F) Postoperative regeneration of nerves in the tissue engineered cross sections of control and implanted corneas by transmission electron microscopy (TEM). (C) Unoperated cornea visualized using transmission electron microscopy showing normal epithelial stromal boundary and nerve fibre (boxed). The operated eye shows similar epithelial/stromal boundary (D), as well as re-innervation at 12 months (boxed). (E) Magnification of boxed inset from the control eye showing intact nerve. (F) Magnification of boxed inset from the operated eye showing a regenerated nerve, show the staining of light and dense vesicles. (A-B) Scale bars are 50 µm. (C-D) Scale bars are 5 µm. (E-F) Scale bars are 1 µm. (e) denotes epithelium, (i) denotes implant.

implants resisted any adverse inflammatory response, and were not rejected. At 12 months post implantation, the implants were integrated and optically clear. Histology showed that a regenerated, stratified, epithelium covered the surface of the implant, with proper barrier function and tear film restoration. Stromal cells were observed to enter into the implant. Of importance, it was observed that corneal nerves had re-innervated the implant, through *in vivo* confocal microscopy (IVCM), as well as through transmission electron microscopy (TEM) (Figure 1). Nerve density found in the implanted corneas was at levels similar to the contralateral control. Mechanically, corneas harvested from implanted eyes mimic normal pig corneas in key mechanical properties. Generally, mechanical properties of implants were found to be either similar or superior to the ones used for control pig corneas (Table 1).

Implants fabricated with recombinant human collagen showed very similar results (36). Using recombinant human collagen is advantageous as it avoids the risk of disease transmission from animal extracted collagen, as well as a possible immune response. This is important as the ultimate goal is human clinical trials. Using type III recombinant human collagen, we have achieved mechanical properties of up to 1.7 MPa tensile strength,with 14% elongation at break and an elastic modulus of about 20 MPa (36). In the same study, we noted that remodeling of the pig corneas had occurred, and the cornea stromas at 12 months post-operative showed a lamellar arrangement.

In a different study, detailed TEM analysis of collagen fibrils in the implants at 12 months post-operative showed that collagen fibril distribution, and alignment is similar to the untreated control corneas (37). Furthermore, collagen fibrils within the remodelled implant area were between 23-28 nm, comparable to the fibril diameter of untreated control corneas and allografts.

We have implanted lenticules of recombinant human collagen artificial corneas, as inlays, under the Bowman's membrane of a patient (Figure 2A) where it was well tolerated. Phase I human clinical trials are currently occurring in Sweden, consisting of corneal transplantation with recombinant human collagen corneas as deep lamellar grafts (Figure 2B). Early post-operative results show regeneration of cornea epithelium (Figure 2C) and stroma (Figure 2D). This has been observed in the patients with vision loss due only to keratoconus or central scarring. Six month post-operative results show the presence of regenerating nerves (Fagerholm *et al.* MS submitted). Such implants do not cause adverse reactions, and therefore are suitable as temporary grafts or patches. However, longer term monitoring is needed to determine whether or not they will be useful as substitutes for donor tissue. In addition, further modifications are likely needed in order to be useful to a wider range of clinical indications.

We have also shown that synthetic materials can be combined with our collagen based corneal alternatives, in order to enhance interaction with the host cornea. By incorporating a artificial polymer, poly(N-isopropylacrylamide-coacrylic acid-coacryloxysuccinimide) into our collagen corneal alternatives, we were able to modify the gels to include the laminin derived pentapeptide, YIGSR into the matrix (38). Upon deep lamellar keratoplasty implantation into mini pigs, it was observed that the incorporation of YIGSR into the corneal alternatives had a significant effect on nerve regeneration. This was shown through restoration of corneal touch sensitivity within a six week period, compared to allografts.

We also show that corneal substitutes can be fabricated to incorporate micro- or nanoparticles that would release a drug (39).

Table 1. The ratio of mechanical properties of pig corneas harvested from implanted eyes to the ones from control eyes derived from the tensile test

Ultimate tensile stress ratio [Strength], (MPa / MPa)	Ultimate elongation ratio [Elasticity], (% / %)	Elastic modulus ratio [Stiffness], (MPa / MPa)	Energy to break ratio [Toughness], (MPa / MPa)
1.00 +/- 0.19	1.38 +/- 0.10	0.76 +/- 0.04	1.14 +/- 0.26

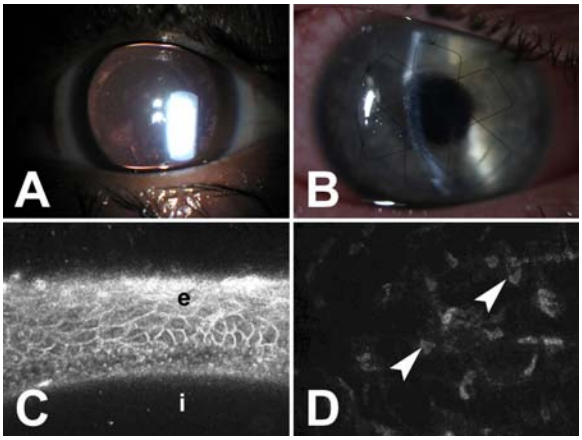

Figure 2. Implanted cornea in human eye. (A) Artificial cornea implanted as an onlay under Bowman's membrane. Arrowhead denotes implant. (B) Artificial cornea implanted as a deep lamellar graft, held in place by overlying sutures. At three months post-operative, the acellular implant (i) has been overgrown with regenerated epithelial cells, (e) as seen in this oblique view of the stratified new epithelium (C). Stromal cells (arrowheads) have migrated into the implant (D).

4. *IN SITU* REPAIR AND ENHANCEMENT OF WEAK CORNEAS

A new treatment for keratoconus, a weakening of the cornea that causes it to bulge outwards, has been developed that approaches the problem from a materials viewpoint. Normally, treatment for keratoconus involves corneal transplantation, however a new method has been developed that utilizes a photosensitizer (Riboflavin) and a light source (UVA) to cross-link the cornea *in situ*. This method has been applied in clinical studies (40-42).

The treatment method developed by Wollensak *et al.* (42) involved abrading the epithelium to allow a riboflavin photosensitizer to diffuse throughout the stroma. This was followed by alternately bathing the eye with the photosensitizer and UVA exposure for a total of 30 minutes. The UVA illuminating power level used in clinical work was set to 3mW/cm^2 at the surface of the eye. This treatment allowed the stroma to be cross-linked to a depth of about 300µm (43). Spoerl *et al.* found the cross-linking was being effected by the photochemical reaction of collagen. This was caused by the production of oxygen radicals by riboflavin and UVA light, inducing a change at the end of an amine group (lysine) (43). After this treatment, the new reactive groups are able to form new covalent bonds.

In the first study, 23 eyes were treated by Wollensak *et al.* (42). All advancing keratoconus progress stopped in the follow-up period, which ranged from three months to four years. 70% of these eyes showed an average regression of 2.01 diopters, and 65% of the eyes indicated a slight improvement in visual acuity.

Caporossi *et al.* (40) treated ten eyes, with similar results, in their clinical study. There was a decrease in keratoconus readings of 2.10 diopters, and an improvement in visual acuity of 3.6 lines. Wang and Swartz also noted a tendency towards more topographic symmetry and a decrease in corneal coma (40).

Seiler *et al.* (41) mention an ongoing international multi-center Phase II clinical study using this new method for treating keratoconus.

5. TISSUE ENGINEERED SCAFFOLDS FOR DELIVERY OF CORNEAL STEM AND PROGENITOR CELLS

In many cases, damage only occurs to one corneal layer. In general, the outermost epithelial layer that is exposed to the environment may be prone to injury such as chemical burns or dry eye syndrome. However, the stem and progenitor cells that are normally responsible for affecting the repair may also be decimated. As such, there have been various attempts to repopulate the cornea.

During reconstruction of the epithelium, corneal stem cells from the surrounding limbus are isolated either from the undamaged contralateral eye (autograft) or from allogeneic sources. These explants are most frequently seeded on prepared human amniotic membranes, (44) or fibrin substrates (45). Outgrowing cells are allowed to form sheets that are transplanted onto the damaged eye. More recently, the authors and other collaborators have seeded corneal limbal cells onto fully synthetic, crosslinked human recombinant collagen substrates. It has been demonstrated that fully stratified corneal epithelia can be reconstituted on these (Figure 3). We showed that both type I and type III human recombinant collagens supported limbal epithelial proliferation and differentiation (46).

In some patients, both corneal surfaces have been depleted of stem cells. This has been observed in patients with Steven's Johnson syndrome, chemical and thermal injury, pseudo-ocular cicatricial pemphigoid, and idiopathic ocular surface disorder. In these cases, successful autologous reconstruction of the corneal surface by transdifferentiation of oral mucosal epithelium has been performed (47). The oral mucosal cells were cultured on human amniotic membranes, and then transplanted onto 15 eyes in 12 patients. Kinoshita and co-workers further suggested the use of transdifferentiated autologous oral mucosa precursor cells. These cells may be safer for ocular resurfacing than with allogeneic corneal grafts, in particular for younger patients with the most severe ocular surface

disorders. However,

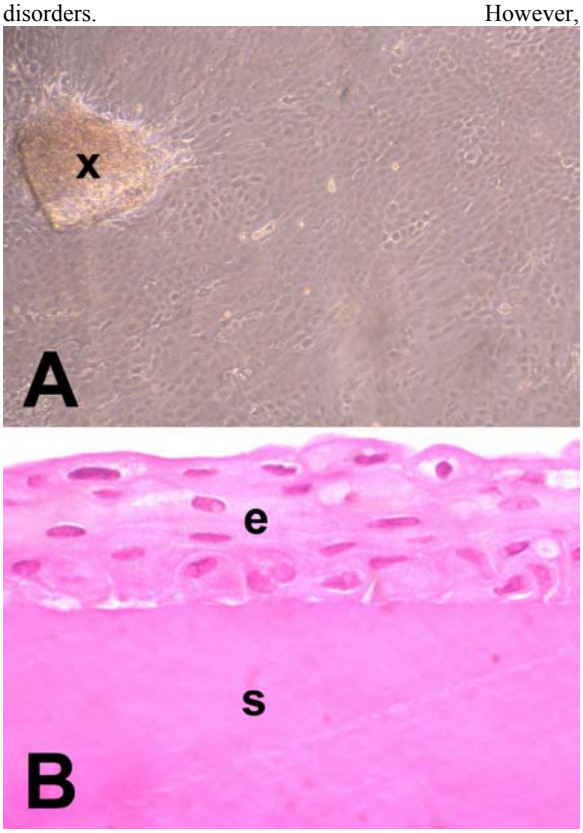

Figure 3. Human limbal stem cells grown on RHC-III Gels. (A) Phase contrast microscopy of human limbal stem cells explanted onto type III recombinant human collagen (RHC-III) artificial corneas after three days growth. (B) Hematoxylin and Eosin staining showing stratified epithelium derived from limbal stem cells following air liquid interface after three weeks. (x) denotes explant, (e) denotes epithelium, (s) denotes scaffold.

it should be noted that all transplanted eyes had some peripheral corneal neovascularization.

Failure of the monolayered corneal endothelium is one of the more common reasons for transplantation, especially in patients with congenital or age-related conditions. Various methods have been tested for corneal endothelial tissue engineering, and transplants of engineered endothelial sheets have been tested in mainly rabbit animal models (48). Rabbits, unlike humans, can rapidly regenerate the endothelium. That being said, human corneal endothelial cells have been grown successfully *in vitro* on a range of supports, including Descemet's membrane (48). In the author's lab, we show that human corneal endothelial cells grow well on amniotic membrane as well as crosslinked type I recombinant human collagen (Figure 4A). However, unlike the limbal cells, we found that corneal endothelial cells proliferate better on type I recombinant human collagen membranes. Poor growth is observed on type III collagen membranes (Figure 4B).

6. *IN VITRO* CORNEAL MODELS

Alternatives for animal research in the field of ocular toxicity testing, has prompted several groups to design tissue engineered *in vitro* corneal alternatives. The Gillette *In vitro* Testing and Research Laboratory have previously created a human corneal epithelial cell line (HCE-T) for *in vitro* test protocols. Fluorescein transepithelial permeability (TEP) is measured via a dose dependent effect of test substances on the HCE-T TEP cell line. The cells are grown on a simplified collagen membrane, and induced to stratify at an air liquid interface, which allows for the formation of tight junctions and a true epithelial barrier (49). The MatTek Corporation has developed a simple three dimensional model based on human foreskin keratinocytes, allowed to stratify in a specialized medium at an air liquid interface shipped in 24-well plates for cellular viability testing using an MTT assay (50). Although these models are becoming popular, they are not able to predict responses to chemicals that may affect the other cell layers of the cornea, or that are dependent upon the interactions of the epithelial cells with other cell types within the cornea.

6.1. Tissue engineered full thickness models

In an effort to reproduce the true three dimensional structure of the cornea, Zieske *et al.* (51) utilized a stroma comprised of neutralized type I collagen blended with rabbit stromal fibroblasts, and seeded with rabbit corneal epithelial cells. Immortalized mouse endothelial cells were seeded on the reverse side, making a true three dimensional corneal construct (51). Our group sought to fabricate a full thickness cornea using immortalized human corneal epithelial, endothelial and keratocyte cell lines, which reproduced the biochemical and morphological functions of normal human corneas. The tissue engineered artificial corneas were developed around a type I collagen/ chondroitin sulphate stroma, and crosslinked with glutaraldehyde to provide a more physiologically relevant and robust structure. To select appropriate cells for the constructs, immortalized cell lines were screened electrophysiologically through patch clamping,, and tested for proper expression of biochemical markers indicative of the specific corneal cell types. The epithelium was allowed to stratify at an air liquid interface. Constructs were tested for opacity changes in response to irritating chemicals, as well as for changes in the expression of wound healing genes such as c-fos, and IL-1 alpha (52). The limitations of this model were it lacked functional innervation, and could not address its important role.

6. 2. Innervated corneal model

A peripheral nerve supply is necessary for the proper function of various types of innervated tissue (53). Corneal nerve fibres have been known to have important trophic effects on the corneal epithelium. Releasing Substance P (SP) from neurons has been shown to have a synergistic effect with IGF-1 on corneal epithelial migration, adhesion, and wound closure (54). Damage to the corneal nerve supply leads to a condition known as neurotrophic keratitis. This can be caused by trigeminal

nerve damage associated with head surgery, or

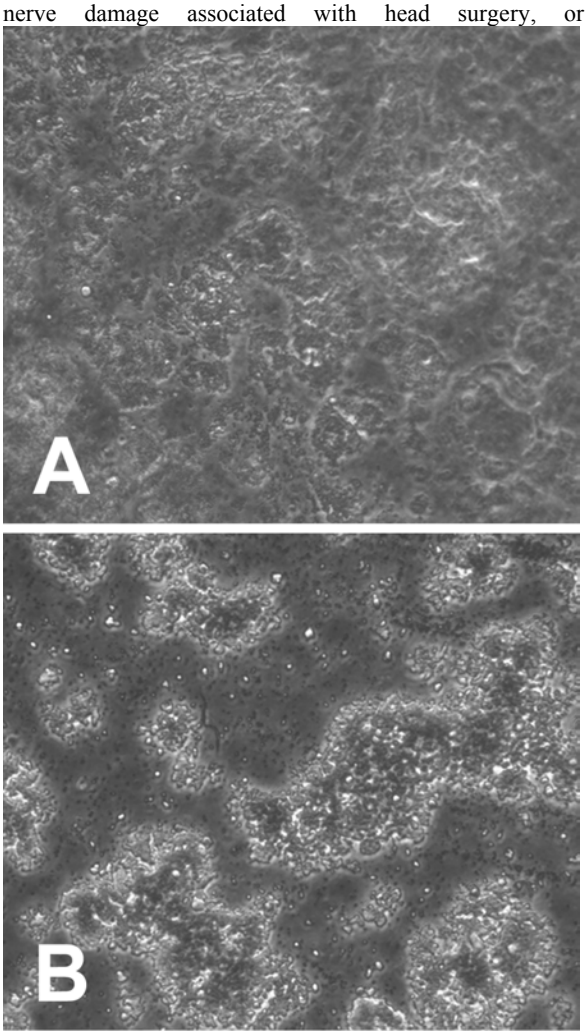

Figure 4. Primary corneal endothelial cell growth on RHC-I and RHC-III gels. (A) Cells cultured on RHC-I artificial corneas show greater coverage compared to RHC-III artificial corneas (B).

keratorefractive procedures such as photorefractive keratectomy and LASIK (laser-assisted *in situ* keratomileusis), leading to transiently mild or severe epithelial alterations (55, 56). Surgical damage of the rabbit trigeminal ganglion leads to significant inhibition of corneal epithelial wound healing, and predisposes the newly healed corneas to recurrent, spontaneous epithelial aberrations (57).

Just as normal corneal function requires innervation, it has been shown that corneal nerves benefit from the presence of corneal cells. Neurotrophic factors secreted by corneal epithelial cells, including nerve growth factor (NGF), and brain derived neurotrophic factor (BDNF), have been shown to aid in guidance and survival of corneal nerves (58, 59). Overexpression of NGF leads to hypertrophy and nerve sprouting, whereas neutralizing antibodies against NGF reduce collateral axon branching (60). Together, it can be seen that the epithelium and neurons in the cornea are mutually beneficial towards each other.

In Suuronen *et al.* (61), we showed that our previous *in vitro* corneal model could be functionally innervated using a sensory nerve source. Laminin and NGF were crosslinked into the type I rat-tail collagen and chondroitin sulphate hydrogel scaffolds, through glutaraldehyde crosslinking. Both laminin and NGF had previously been shown to promote the differentiation and guidance of nerves (62, 63). Chicken dorsal root ganglia explants (DRG) were selected as the sensory neuron source, and were implanted within the hydrogel scaffold prior to gelling. In order to promote neurite extension, the medium was supplemented with retinoic acid, which has been shown to induce neurite outgrowth from embryonic mouse dorsal root ganglia explants (64). Using innervated constructs the structural morphology found in the human cornea was able to be reproduced. DRGs extended neurites that contained both smooth fibres traversing the corneal stroma, along with smaller beaded fibres that entered the epithelium , which is similar to the structural morphology found in the human cornea (65, 66). As well, transmission electron microscopy showed individual neurite terminals within epithelial cells (61).

The innervated *in vitro* corneal alternatives were tested for neurotransmitter release, as well as functionality. SP, one of the most prominent neurotransmitters found in the cornea, is responsible for the trophic effects of nerves on corneal cells, as well as nerve-stimulated enhancement of epithelial healing in conjunction with other growth factors, such as IGF (8, 67). Upon application of various neurotoxic chemicals, including capsaicin, veratridine, and ouabain, we showed a differential release of SP. Furthermore, we developed a more physiologically relevant toxicological assay (68) that measured the intracellular sodium concentration following exposure to veratridine and ouabain, using a sodium-sensitive dye and 2-photon microscopy. This was based on previous work that revealed a correlation between the amplitude of the nerve response, with the variability of Na^+ current (69). When treated with chemicals, including surfactants, and alcohols, it was found that innervated constructs had a neuroprotective effect on the epithelium. Less cellular death occurred, when compared to non-innervated corneas, evidenced through live/dead analysis of the epithelium. These results helped to confirm the *in vivo* observations of the importance in the function of corneal nerves in maintaining proper epithelium homeostasis (70).

Action potentials were also recorded from surface terminals upon stimulation of the dorsal root ganglia. This showed that the nerves in the tissue engineered constructs were physiologically functional. It was found that the resultant action potentials were lidocaine sensitive, which is similar to those found in guinea-pig corneal polymodal nociceptors (71).

By assessing the degree of cellular death, levels of neurotransmitter release, and the change in internal neuron sodium concentration following exposure to

different sodium transport or channel blockers, the innervated artificial corneal constructs can be used as an *in vitro* alternative to animal testing for ocular irritancy.

Recently, we developed a corneal-scleral model (72). These models were constructed with an avascular cornea surrounded by a pseudosclera, that contains vessel-like structures. Both the sclera and cornea were innervated, and nerves within the sclera formed a ring around the cornea with branches penetrating into the cornea, similar to that found in the normal cornea (73).

7. CONCLUSION

The field of tissue engineering is focused primarily on the construction of biological substitutes that allow the growth of functional and viable cells. Tissue engineered vision correction alternatives have been developed as refractive error correcting inlays and onlays, as well as implants (full thickness penetrating keratoplasty or partial thickness lamellar keratoplasty). When designing a corneal alternative, one must be able to reproduce the desired optical properties, and shape of the initial tissue. As well, design of the implant should facilitate the incorporation of functional ligands to promote cell adhesion and ingrowth. The tissue engineered matrices can either be produced as sterile acellular matrices, or with functional cells included, ready for implantation.

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Abbreviations: ATPase: adenosine triphosphatase, BDNF: brain derived neurotrophic factor, DRG: dorsal root ganglia, ECM: extracellular matrix, EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, HCE-T: human corneal epithelial cell line, HSV-1: herpes simplex virus 1, IGF-1: insulin-like growth factor 1, IKVAV: Ile-Lys-Val-Ala-Val, IL-1: interleukin 1, IVCM: *in vivo* confocal microscopy, KPro: keratoprosthesis, LASIK: laser-assisted *in situ* keratomileusis, NGF: nerve growth factor, NHS: N-hydroxysuccinimide, OOKP: osteo-odonto keratoprosthesis, PAA: poly(acrylic acid), PEG: poly(ethylene glycol), PDMS: poly(dimethyl siloxane), PDSGR: Pro-Asp-Ser-Gly-Arg, PHEMA: poly(2-hydroxyethyl methacrylate), PMMA: poly(methyl methacrylate), PTFE: poly(tetrafluoroethylene), PVA: poly(vinyl alcohol), RGD: Arg-Gly-Asp, RGDS: Arg-Gly-Asp-Ser, RHC-I: recombinant human collagen type I, RHC-III: recombinant human collagen type III, SP: Substance P, TEM: transmission electron microscopy, TEP: transepithelial permeability, UVA: ultraviolet A, WHO: World Health Organization, YIGSR: Tyr-Ile-Gly-Ser-Arg

Key Words: 3D, Artificial, Biomimetic, Collagen, Cornea, Crosslinking, ECM, Extracellular Matrix, Innervation, *In situ*, *In vitro*, Keratoprosthesis, PHEMA, PMMA, Recombinant, Review, Scaffold, Stem Cells, Tissue Engineering, Toxicology, Review

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