13

Ocular Surface Reconstitution

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1. Introduction

1.1 The ocular surface – anatomy and pathology

The corneal epithelium, conjunctival epithelium, and the lacrimal system constitute the ocular surface. A healthy corneal epithelium is essential for corneal health and visual function. The corneal epithelium is a 5- to 6-cell-thick layer that provides a defensive barrier against pathologic organisms. It exists in a dynamic equilibrium, with superficial cells being constantly shed into the tear film. Populations of pluripotent stem cells reside in the palisades of Vogt at the human corneoscleral limbus and generate transient amplified cells that centripetally migrate toward the central cornea. These transient amplified cells undergo terminal differentiation into epithelial cells and repopulate the corneal epithelium, i.e. the XYZ hypothesis (Thoft et al., 1983). Severe ocular surface disorders, such as infection, keratoconjunctivitis sicca, Stevens-Johnson syndrome, ocular cicatricial pemphigoid or chemical/thermal injuries, can progress to corneal scarring, conjunctivalization, neovascularization, or stromal melts. Depletion of the limbal stem cells may follow, resulting in impaired vision or eventual corneal blindness. According to the World Health Organization, corneal disorders, e.g. trachoma or onchocerciasis, constitute a significant cause of loss of vision and blindness in the world (Thylefors et al., 1995).

The conjunctiva is a thin, transparent, mucus membrane, overlying a thin vascular stroma. It is divided into three geographic zones: bulbar, forniceal, and palpebral. The conjunctival nonkeratinized stratified epithelium contains mucin-producing goblet cells, which are important for tear film stability. Additionally, the conjunctiva participates in the ocular surface antimicrobial defense via the conjunctiva-associated lymphoid tissue, as well as secretory antimicrobial peptides, such as defensins (Haynes et al., 1999). Disorders of the conjunctiva include elastotic changes, fibrovascular proliferation, malignancies, and autoimmune conditions such as Stevens-Johnson syndrome or cicatricial pemphigoid. Complications include dysfunctional tear syndrome, keratinization, symblepharon formation, eyelid disfigurement, and eyelash misalignment. Patient discomfort, cosmetic imperfection, increased risk of infection, and visual impairment are some notable concerns.

A normal tear film is essential for maintenance of the corneal and conjunctival epithelia. Composed of three layers, mucin, aqueous and lipid layers, the human tripartite tear film has antimicrobial, epitheliotrophic, mechanical, and optical properties. A wide range of physiologic or pathologic conditions, such as biologic aging, hormonal changes, chemical or thermal injuries, chronic inflammation, or autoimmune disorders, may disrupt the tear film and trigger a deleterious cascade, injuring ocular surface epithelia. Furthermore, suboptimal

lacrimal functions may result in poor surgical outcomes, especially after penetrating keratoplasty or limbal stem cell transplantation.
Traditionally, the eyelids and lacrimal gland were excluded from the definition of the ocular surface. It is evident that visual function and epithelial health would not be feasible without these structures. The eyelids are essential for ocular surface protection and tear film maintenance. Untreated eyelid deformities, lid malpositions, or eyelash misalignments can precipitate detrimental consequences to the integrity and function of the ocular surface epithelia. Thus, a functional ocular surface requires structurally and functionally intact eyelids and lacrimal gland.

2. Ocular surface reconstitution

In severe ocular surface disorders, the management strategies entail symptomatic relief, reconstitution of the anatomic and physiologic ocular surface, and treatment and prevention of recurrence of the causative condition. Here we will discuss strategies to restore the conjunctival epithelium, corneal epithelium, and lacrimal function. Figure 1 illustrates the management strategies. Injury or inflammation causes severe ocular surface disorder with conjunctival scarring, limbal stem cell deficiency, corneal opacity with neovascularization, lacrimal dysfunction, disorganized lashes, and lid malposition (a). Mainstay treatment options include antibiotics, anti-inflammatory agents, lubrication, and amniotic membrane transplantation, as well as removal of lashes and correction of lid changes (b). As progress is made in science and tissue bioengineering, tissue replacement and regeneration may be feasible to restore the ocular surface and vision (c).

2.1 Conjunctival tissue reconstitution

2.1.1 Suppression of cicatricial changes

Commonly, ocular surface diseases limited to the conjunctiva progress to excessive cicatricial changes and loss of normal epithelial anatomy. Cicatricial changes to the conjunctival epithelium generally result from poorly controlled fibroblastic activities, e.g. tissue injuries or persistent inflammation. In addition to disrupting the tear film, cicatricialization of the conjunctiva has important implications in glaucoma surgeries, where availablility of healthy conjunctiva is essential for good surgical outcomes. A widely adopted therapeutic strategy is pharmacologic suppression of the inflammatory cascade and the fibroblast activation pathway using corticosteroids and antimetabolites.
Recently, research efforts have been directed toward transforming growth factor beta (TGF-β) and its involvement in fibroblast proliferation. TGF-β is a multifunctional cytokine, which plays an important role in tissue repair and regeneration. After injury, TGF-β triggers a complex cascade involving monocyte and leukocyte chemotaxis, induction of angiogenesis, control of production of cytokines and inflammatory mediators, deposition of extracellular matrix materials, and prevention of their enzymatic degradation (Border & Ruoslahti, 1992; Massagué et al., 1992). Excessive TGF-β activity has been associated with exuberant fibrotic changes in the eye and other organs. In a murine model, TGF-β was associated with formation of granulation tissue (Roberts et al., 1986) and increased inflammatory cell activity, as well as with exuberant extracellular collagen type-III deposition (Siriwardena et al., 1999). Using immunohistochemistry, Razzaque et al (2003) found increased accumulations of type-I and type III collagens and heat shock protein 47, a collagen-binding protein in fibrotic conjunctiva of patients with ocular cicatricial pemphigoid compared to normal subjects. Up-regulation of these proteins was also detected when ex-vivo

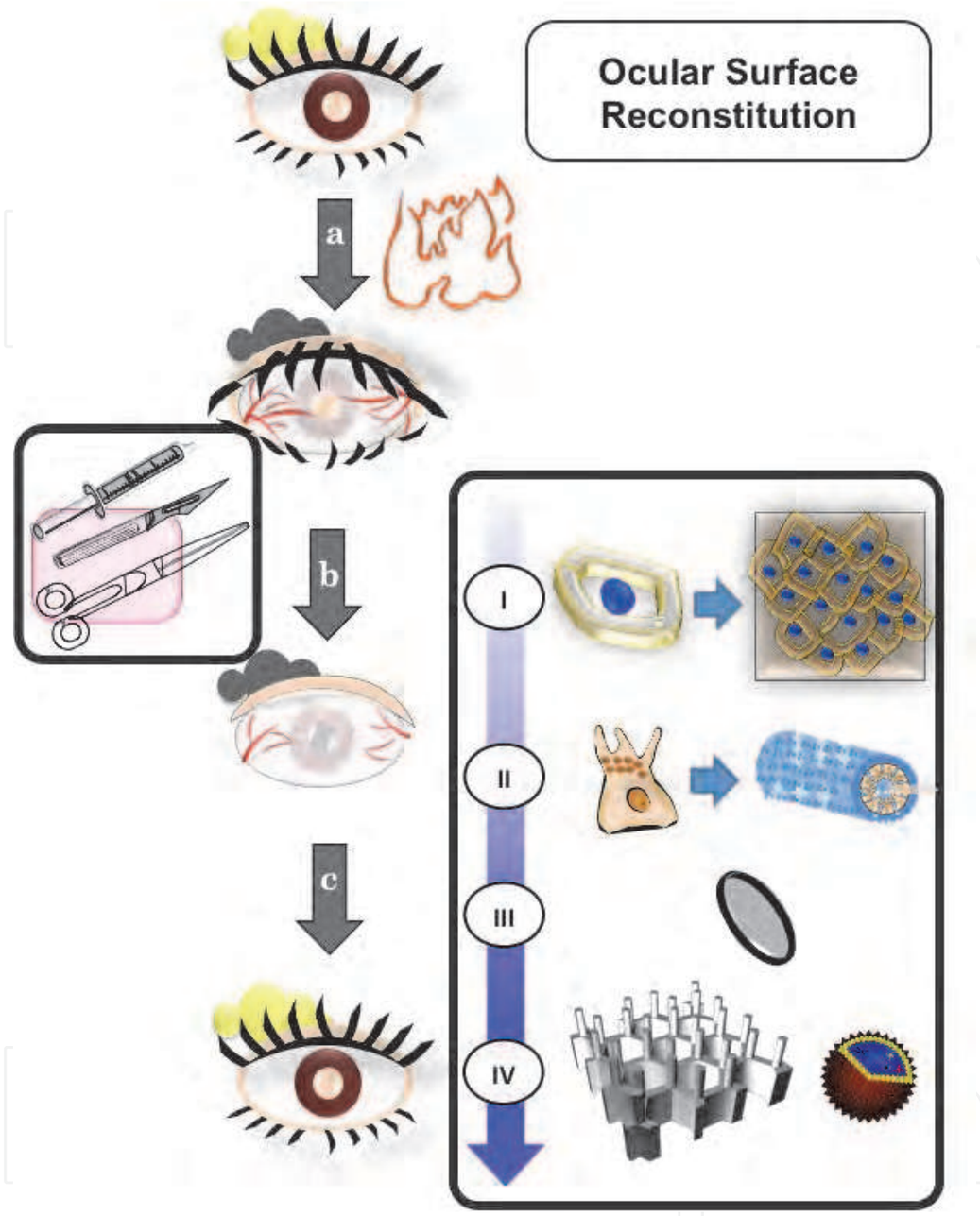

Fig. 1. Ocular Surface Reconstitution: (a) ocular surface destruction, (b) preliminary management – antibiotics; anti-inflammatory; amniotic membrane; eradication of microtrauma sources; etc. (c) ocular surface reconstitution – tissue transplant ± bioengineered substratum (I); lacrimal gland function restoration (II); corneal transplant or keratoprosthesis (III); regenerative medicine, targeted drug delivery, (IV); oculoplastic reconstruction, etc. (Adapted from Nguyen et al., 2008)

conjunctival fibroblasts were treated with TGF-β. Elevated levels of bone morphogenetic protein-6 and activin A, both part of the TGF- β superfamily, have been reported in scar tissue after glaucoma incisional surgeries, and their mRNA expression was

immunolocalized to epithelial cells, vascular endothelia, stromal fibroblasts, and macrophage-like cells using real-time polymerase chain reaction (Andreev et al., 2006). It is suggested that these multifunctional growth factors participate in tissue wound healing, and altered expression of these factors may play a role in conjunctival scarring.

It is, therefore, hypothesized that modulation of TGF-β activity may help control the formation of scar tissue. Indeed, this has been demonstrated in animal models and clinical trials. CAT-152 is a human monoclonal antibody capable of neutralizing the growth factor TGF-β2. In in vitro and in vivo models, CAT-152 was shown to inhibit conjunctival scarring (Cordeiro et al., 1999; Thompson et al., 1999). In a rabbit model, SB-431542, a potent and selective inhibitor of the TGF-β1 activin receptor-like kinase, decreases subconjunctival deposition of collagen in rabbits undergoing glaucoma filtration surgery (Xiao et al., 2009). It is thought that SB-431542 inhibits phosphorylation of Smad2 stimulated by TGF-β, abrogating fibroblast transdifferentiation and type-I collagen synthesis. A prospective randomized placebo controlled clinical study of patients undergoing trabeculectomy showed that subconjunctival injection of CAT-152 appears to be safe in human undergoing trabeculectomy (Cordeiro 2003; Siriwardena et al., 2002). Successful and safe suppression of TGF- β superfamily activities may modulate fibroblast proliferation and cicatricial changes to the conjunctiva.

An alternative approach is stimulation of tissue regrowth and reduction of the conjunctival wound contraction mediated by modified contractile fibroblasts using biocompatible porous matrices (Gabbiani et al., 1971; Majno et al 1971). In a guinea pig skin model, highly porous, cross-linked collagen-glycosaminoglycan copolymer matrix had been reported to induce partial morphogenesis of skin when seeded with dermal and epidermal cells. After grafting on a full-thickness skin wound model (Yannas et al., 1982, 1989), the seeded matrix regenerated mature epidermis and near physiologic dermis, which was distinct from scar tissue and able to prevent wound contraction, compared to non-seeded matrix. For ophthalmic applications, porous collagen-glycosaminoglycan copolymer matrix was shown to decrease wound contraction in a full-thickness defect in a rabbit conjunctival model (Hsu et al., 2000). In animal glaucoma filtration surgery model, implantation of biodegradable porous collagen matrix in the subconjunctival space was associated with less initial scar formation and maintenance of long-term intraocular pressure control (Chen et al., 2006). In this study, Masson trichrome and β-smooth muscle actin immunocytochemistry established reduction of myofibroblast population in the eye having bioactive matrix implant compared to control. The authors concluded that the collagen matrix may simulate a more physiological structure for tissue regrowth (Chen et al., 2006; Hsu et al., 2000). Scleral buckle composed of biodegradable collagen-glycosaminoglycan copolymer had been reported to be safe and effective in a rabbit model (Wu et al., 2008). Similar results have been demonstrated with modified porous poly(lactide-co-glycolide) scaffold as well (Lee et al., 2003). These findings provide insight into cellular behaviors and assist in the understanding of conjunctival tissue bioengineering.

2.1.2 Conjunctival tissue substitutes

2.1.2.1 Autologous mucosal transplantation

Other therapeutic options for conjunctival tissue reconstitution include autologous tissue grafts and bioengineered conjunctival substitutes. Similar to any epithelia, the conjunctival epithelium is in a dynamic self-renewal equilibrium. Epithelial cells with high mitotic

capacity are distributed predominantly in the bulbar and forniceal conjunctiva in humans (Pellegrini et al., 1999; Tsubota et al., 2002). In autologous conjunctival autografts, e.g. for pterygium excision, the conjunctival autograft typically successfully replenishes the donor bed. However, severe tissue deficiency necessitates transplantation of other mucosal tissues. Other mucosal tissues having structure analogous to the conjunctiva, i.e. nonkeratinized stratified squamous epithelium with goblet cells, include nasal, esophageal, vaginal, and tracheal mucosa. Gibson and colleagues (1986) demonstrated the feasibility of transplanting oral epithelium onto the ocular surface in a rabbit model. In a case report, oral mucosa was used in combination with a modified Gunderson conjunctival flap to treat ocular surface burn injury (Cheng & Chang, 2006). In a series of patients with severe bilateral mucus deficiency syndrome after severe lye, acid, or heat burns, radiation, or systemic mucosal disease, autologous nasal mucosal grafts from the nasal conchae were transplanted to cover the ocular surface (Naumann et al., 1986). Symptomatic and visual improvements were reported. Long-term follow-up, using impression cytology and hematoxylin and eosin and periodic acid-Schiff staining of biopsied tissue, demonstrated that functional goblet cells persisted in autologous nasal mucosa for up to 10 years after transplantation (Wenkel et al., 2000). Another study also reported similarly promising outcomes for patients with cicatricial ocular surface secondary to lye injury (Kim et al., 2010).

2.1.2.2 Cultivated bioengineered tissues

Currently, a variety of substrates are readily available for cultivation of conjunctival tissue, e.g. murine-derived 3T3 fibroblast feeder layer, human amniotic feeder layer, and serum-free media. Irradiated 3T3 murine feeder cells are widely utilized in a number of cell culture systems because these immortalized 3T3 cells secrete growth factors to support cellular proliferation (Todaro & Green, 1963). Although the murine-derived feeder layers provide satisfactory support for tissue growth and maintenance, the tissue is unsuitable for human use because of several considerations. The xenoantigenic nonhuman sialic acid Neu5Gc produced by the human embryonic stem cells using this culture system may trigger cell killing in vivo if exposed to human serum containing circulating antibodies specific for Neu5Gc (Martin et al., 2005; Rheinwald 1980). Other concerns include transmission of murine diseases or contamination with virus or prion agents (Boneva et al., 2001; Ramarli et al., 1995).

Consequently, alternatives are being explored. The human amniotic feeder layer, derived from human placentas, has been found to promote clonal growth of human limbal epithelial progenitors (Chen et al., 2007; Tanioka et al., 2006)). Such bioengineered tissues would be suitable candidates for replacement of native conjunctival tissue as the human amniotic membrane is a stable and elastic substratum that can be integrated into the ocular surface. The anti-inflammatory properties of human amniotic membrane would be especially advantageous in the case of inflammatory cicatricial changes, as any trauma to the recipient bed would reactivate or incite the inflammatory cascade that may inflict further injury to both the host and the graft. Other substrates include ultrathin poly(β-caprolactone) membrane, silicone, and others (Ang et al., 2006; Selvam et al., 2006; Yoon et al., 2007). To minimize the biohazard of using xenobiotic materials, serum-free media are being investigated as well. One study demonstrated good epithelization following transplantation of serum-free autologous cultivated conjunctival epithelium on human amniotic membrane in pterygium excision (Ang et al., 2005). Tissue cultivation will be discussed in detail below.

2.2 Corneal epithelium reconstitution

In humans, the known organs that demonstrate immunologic privilege are the eyes, brain, testicles, placenta, and fetus. The anterior chamber immunologic privilege and avascularity of the cornea significantly contribute to the high success rate of corneal allografts, e.g. penetrating keratoplasty, which can be performed without HLA-typing (Nguyen et al., 2007, 2008, 2010). However, the risks of allogeneic graft rejection increase significantly in patients with severe ocular surface disorders or limbal stem cell deficiency. In severe cases, management of corneal blindness involves treatment of the ocular surface disorder, restoration of limbal stem cells, development of corneal tissue equivalents, or use of a keratoprosthesis.

2.2.1 Human amniotic membrane transplantation

Now widely employed in burn management and ophthalmology, the human amniotic membrane found its first applications as surgical dressing and tissue graft in dermal burns and as a strategy to impede tissue adhesion in mucosal injuries (Davis, 1910; Stern, 1913). Subsequently, its utility was expanded to reconstruction of the oral cavity, bladder, and vagina, as well as tympanoplasty and arthroplasty. De Rotth (1940) first reported the suitability of amniotic membrane in ocular surface reconstruction where he applied the amniochorion to repair symblepharon. A suboptimal surgical outcome was observed because the chorion component can incite inflammation and scarring. The amniotic membrane enjoyed limited use until the 1990s when its properties were better understood and its ophthalmic applications became widely adopted.

In humans, the amniotic membrane, which lines the inner surface of the placenta, is composed of a basement membrane and a subjacent stromal matrix devoid of vascular and lymphatic vessels, as well as nerve fibers. It has many unique features, including suppression of inflammation and promotion of tissue healing. Its basement membrane assists in induction of adhesion and migration of epithelial cells for wound healing (Azuara-Blanco et al., 1999; Dua et al., 2004; John et al., 2002; Kim & Tseng 1995; Shimazaki et al., 1998). Modulation of the inflammatory cascade is realized by suppression of interleukin alpha and interleukin 1 beta in epithelial cells (Solomon et al., 2001). Release of growth factors such as epidermal growth factor, keratinocyte growth factor, or basic fibroblast growth factor (Koizumi et al., 2000) and inhibition of proteinase activity (Kim et al., 2000) promote tissue survival. The amniotic membrane also effectuates reduction of scar tissue formation by reducing polymorphonuclear infiltration (Park & Tseng, 2000) and suppressing the TGF-β signaling system and myofibroblast differentiation of normal fibroblasts (Lee et al., 2000; Tseng, 1999).

The available literature supports its use as an epithelial surrogate after excision of large ocular surface neoplasias (Espana et al., 2000; Gündüz et al., 2006; Tomita et al., 2006), reconstruction of conjunctival ocular surface (Solomon et al., 2003; Zhou et al., 2004; Oberhansli et al., 2005; Tseng et al., 2005), excision of pterygia (Ang et al., 2007; Nakamura et al., 2006), severe or refractory neurotrophic corneal ulcers (Chen et al., 2000; Ivekovic et al., 2002; Khokhar et al., 2005), bacterial keratitis (Barequet et al., 2008), and symptomatic alleviation following acute ocular burns (Tamhane et al., 2005). A prospective noncomparative interventional case series study reported that nonpreserved human amniotic membrane appears useful for ocular surface reconstruction following excision of extensive ocular surface neoplasia, such as squamous cell carcinoma, malignant melanoma

and conjunctival-orbital lymphangioma (Gündüz et al., 2006). No immune graft rejection was encountered. For pterygium excision, complete epithelialization, early resolution of ocular inflammation, and no recurrence of pterygium had been reported following orthotopic transplantation of sterilized, freeze-dried amniotic membrane, over a follow-up period of 14 months (Nakamura et al., 2006). Caution is advised, however, in using amniotic membrane transplant for primary pterygium excision in patients with sufficiently recruitable conjunctiva. Randomized prospective studies comparing conjunctival autograft versus amniotic membrane transplant for pterygium showed a higher recurrence rate in the amniotic membrane transplant group (Essez et al., 2004; Luanratanakorn et al., 2006; Tananuvat & Martin, 2004). A recent study (Baraquet et al., 2008) demonstrated the use of human amniotic membrane in an animal model to assist in the healing of bacterial keratitis. In this rat model of Staphylococcus aureus keratitis, histopathologic examination revealed the least corneal haze and neovascularization in the cefazolin/amniotic membrane combination group, compared to the normal saline and amniotic membrane, and cefazolin without amniotic membrane transplantation groups.

Complete ocular surface coverage using amniotic membrane was performed in patients with severe acute chemical and thermal burns to protect and promote conjunctival regeneration and to prevent complications of burns, such as symblepharon formation and corneal melt (Joseph et al., 1991). However, Joseph and colleagues did not observe a positive association between amniotic membrane transplantation and overall success rate of ocular surface restoration or preservation of ocular integrity in patients with severe acute burns, whether used alone or in conjunction with other surgical procedures. Using laser scanning in vivo confocal microscopy, Nubile et al (2001) discovered two different mechanisms of adhesion: when the amniotic membrane behaved as a patch, instead of as a graft, the epithelial cells migrated under the membrane, culminating in membrane disintegration; when the amniotic membrane acted as a graft, it became the basement membrane matrix, integrating with the superficial corneal stroma and allowing epithelial cell migration and proliferation (Nubile et al., 2001; Resch et al., 2006). This behavior potentially explains the poor outcome observed by Joseph et al (1991). Accordingly, care should be taken during transplantation to allow the membrane to be a graft, thus promoting epithelial migration and proliferation. Zhou et al (2004) noted that severity of the symblepharon, severity of decrease in lacrimal function, and a decreased amount of remaining healthy conjunctiva are poor prognostic factors for long-term outcomes of amniotic membrane transplantation for conjunctival surface reconstruction. A higher rate of failure was associated with preoperative use of topical steroid in patients receiving amniotic membrane transplantation for persistent epithelial defect and conjunctival repair (Saw et al., 2007). However, the preoperative use of steroids may simply be an indication of the severity of the disease.

Traditionally, sutures are used to anchor the amniotic membrane to the ocular surface; however, there is a recent shift toward bioadhesive transfixation instead. There are a number of motivations for this shift: suture fixation requires a greater amount of surgical expertise and dexterity; sutures can cause corneal irritation and scarring; membrane shrinkage can result in graft loss; and sutures need to be extricated. For conjunctival autograft following pterygium excision, the biocompatibility, safety, and efficacy of fibrin bioadhesives was substantiated in a prospective randomized, interventional case series (Uy et al., 2005). In a rabbit model, Szurman et al (2006) demonstrated that fibrin glue provided a better surgical outcome than sutures for amniotic membrane transplantation. In the

bioadhesive group, histology revealed a smooth fibrin layer at the graft-host interface and a continuous, stratified layer of cytokeratin-3 expressing corneal epithelial cells on the membrane surface. The authors found that suture fixation tends to promote membrane contraction, raised membrane edges, and epithelial ingrowth into the submembrane space (Szurman et al., 2006). These findings were essentially corroborated in another study (Sekiyama et al., 2007). The fibrin bioadhesive biodegrades within 2 weeks, whereas the amniotic membrane persists for at least 12 weeks, providing sufficient time for epithelialization (Sekiyama et al., 2007). Sutureless transplantation with fibrin glue or ProKera™ is reportedly a safe and easy method that avoids complications related to sutures and shortens the time of surgery (Kheirkhah et al., 2006).

2.2.1.1 Disadvantages of human amniotic membrane

Amniotic membrane only provides a supportive substratum for epithelial stem cells to proliferate and migrate to restore the ocular surface. Thus, transplantation of the amniotic membrane without cultivated epithelial cells for true limbal stem cell deficiency may not be curative. Without the limbal stem cells and corneal epithelial cells, the cornea may not maintain avascularity and clarity because of secondary conjunctivalization and neovascularization of the cornea. In a retrospective case study, Maharajan et al (2007) reported the correlation between a higher success rate of ocular surface reconstruction using amniotic membrane transplantation and the availability of stem cells.

There are some minor disadvantages of amniotic membrane transplantation. As already noted, suture fixation of amniotic membrane requires surgical dexterity to manipulate the membrane on the ocular surface. As a substrate for stem cell expansion, the surface with stem cells is transfixed face-up on the corneal surface. This position has some theoretical drawbacks: delayed migration of epithelial cells to the corneal surface before cellular repopulation, attrition of epithelial cells from exposure in patients with severe dry eye syndrome or ocular surface disorders, or mechanical displacement of the tissue. Another disadvantage is the association with corneal calcific opacification. In a large series of amniotic membrane transplantation for persistent epithelial defect, partial limbal stem cell deficiency and conjunctival reconstruction, Anderson and colleagues (2003) reported a significant (12.8%) rate of corneal calcification, occurring 3-17 weeks postoperatively. Risk factors were preoperative corneal calcification in either eye and postoperative use of phosphate-containing eye drops. A more recent study suggests that the use of phosphate-buffered eye drops, commonly found in artificial tear or topical corticosteroid formulations, favors the formation of insoluble crystalline calcium phosphate hydroxyapatite deposits in the stroma in the presence of epithelial keratopathy (Bernauer et al., 2006). Studies using an animal model confirmed these findings as well (Schrage et al., 2010). The authors discouraged the use of phosphate-buffered eye drops as excessive use of such eye drops may cause corneal calcification, which may necessitate corneal graft surgery for subsequent visual rehabilitation.

2.2.2 Epithelial cell transplantation

Treatment of limbal stem cell deficiency includes transplantation of cultivated limbal stem cells. The unique properties of amniotic membrane make it an attractive candidate as a substratum for stem cell expansion. Tsubota and colleagues (1996) were among the first to introduce the combined transplantation of allograft limbal tissue and amniotic membrane in

patients with ocular cicatricial pemphigoid, where a short-term success rate of 86% was observed. Koizumi et al (2000) reported successful transplantation of cultivated corneal epithelium using acellular amniotic membrane as a substrate for ex vivo expansion of the epithelial cells. Nakamura et al (2004, 2006) proposed the application of sterilized, freeze-dried amniotic membrane as a substrate for cultivating autologous corneal epithelial cells for ocular surface reconstruction. In a rabbit model, the authors reported no significant difference between freeze-dried and cryopreserved amniotic membrane. Corneal epithelium cultivated well and reepithelialization of the grafted cornea was demonstrated postoperatively (Nakamura et al., 2004, 2006).

The conventional cultivation methods for mucosal epithelial tissues involve the use of xenobiotic materials such as fetal bovine serum (FBS) and murine-derived 3T3 feeder cells. Given the risks of zoonotic infection and xenoantigenic materials as discussed above, FBS-free culture systems have been developed, but these have reduced efficacy for cell propagation. Ang et al (2006) introduced the use of human serum for in vivo and ex vivo expansion of human conjunctival epithelial cells on amniotic membranes. Cultivated epithelial cells on amniotic membrane substrates using autologous serum showed complete corneal epithelialization, retained corneal clarity, and improved visual acuity in patients with severe limbal stem cell deficiency (Nakamura et al., 2006). Autologous serum expansion and FBS expansion appeared to have comparable proliferative capacities with equivalent morphologies (Nakamura et al., 2006). Autologous serum had also been used to cultivate oral epithelial cells for patients with severe ocular surface disease with promising outcomes (Ang et al., 2006).

Murine 3T3 feeder cells are typically used in these cultivation systems to maintain stemness of the stem cells. However, these xenobiotic feeders cannot be used for transplantation in humans. Accordingly, other culture systems, such as human amniotic epithelial cells, human embryonic fibroblasts or feeder cell-free, are being investigated for feasibility as the feeder cells. Chen and colleagues (2007) compared murine 3T3 cells and human amniotic epithelial cells, and found that, although murine 3T3 feeder cells demonstrated faster initial proliferative capacity, human amniotic epithelial feeder cells retained evidence suggestive of undifferentiated state for more generations. Lai et al. (2007) similarly concluded that human amniotic epithelial cells are suitable for cultivation of mouse embryonic stem cells, compared to mouse embryonic fibroblast feeder cells. Under both FBS-supplemented and serum-free conditions, human embryonic fibroblast feeder cells was found to support expansion of human limbal epithelial cells as well as 3T3 feeder cells (Notara et al., 2007). A feeder cell-free system had been introduced by Yokoo et al. in 2008. Of note, the authors found that, compared to conventional medium containing 10% FBS cocultured with 3T3 fibroblast feeder cells, cells grown in serum-, feeder cell- and bovine pituitary extract (BPE)-free culture medium demonstrated higher colony forming ability, equivalent proliferative capacity, and similar morphology. Two weeks after transplantation onto rabbit corneas, these cells showed histologic findings suggestive of normal corneal epithelium (Yokoo et al., 2008). These autologous cultivation or serum- feeder cell- and BPE-free systems would theoretically reduce the risk of allograft rejection and transmission of xenobiotic infectious agents, and the need for long-term corticosteroid and immunosuppressive therapy may be obviated. Adoption of a standardized protocol to ensure availability of tissues with consistent constituents and consistent clinical outcomes would be desirable (Hopkinson et al., 2006).

2.2.3 Other substrates for epithelial cell cultivation

Alternative materials are being investigated. Rama et al (2001) investigated the application of a fibrin substrate for cultivating autologous limbal stem cells. Good epithelization and regression of inflammation and vascularization were reported following transplantation onto corneas damaged by total limbal stem cell deficiency (Pellegrini et al., 1999; Rama et al., 2001). These findings were corroborated by another group (Han et al., 2002). A more recent study comparing fibrin bioadhesive substrate and amniotic membrane for cultivation of corneal epithelial sheets showed that both had similar levels of colony-forming progenitor cells, while more differentiation was observed in the fibrin group (Higa et al., 2007). A thermoresponsive surface, which allows easy harvest of intact transplantable corneal epithelial cell sheets from ex vivo expansion of limbal stem cells, has been developed (Nishida et al., 2004). Without transplantation of the substratum, e.g. amniotic membrane, theoretically, this epithelial sheet will rapidly repopulate the corneal surface, without the time delay for migration. Using this technique, Nishida et al (2004) performed ex vivo fabrication of autologous oral mucosal tissue and transplantation on a small group of four patients with bilateral total corneal stem cell deficiencies with reasonable success. Lai et al (2007) also demonstrated successful transplantation of bioengineered human corneal endothelial cell monolayer sheet grafts, cultivated on the thermoresponsive surface then attached to gelatin hydrogel discs, into denuded rabbit corneas. Restoration of corneal clarity was observed, compared to controls.

An innovative approach to cultivating cells on contact lens has recently been introduced (Di Girolamo et al., 2007). It was found that successful explant cultures could be made using either superior forniceal conjunctiva or superior limbal epithelial biopsies placed on a siloxane-hydrogel extended wear contact lens. These lenses were subsequently inserted onto the eyes of three patients with limbal stem cell deficiency. The patients were found to have a stable transparent corneal epithelium with no recurrence of conjunctivalization or corneal vascularization along with improvements in both best-corrected visual acuity and symptom scores. It is theorized that the close proximity between the therapeutic contact lens and the ocular surface facilitates cell migration from their artificial substratum to replenish the damaged ocular surface. Di Girolamo et al also postulated that the diffusion of secretory factors from the contact lens niche would promote corneal wound healing, inhibit angiogenesis, and rescue or activate any remaining limbal stem cells. Research is now being conducted to create a plasma polymer surface that can coat CL surfaces that would not only allow for appropriate attachment and growth of the epithelial cells but also transfer of the cultured cells onto the denuded corneal surface once inserted (Deshpande et al., 2010). This technology is similar to that being used in patients with extensive burn injuries and chronic nonhealing diabetic ulcers for transfer of autologous keratinocytes (Moustafa et al., 2004, 2007; Zhu et al., 2005). Both rabbit and human corneal cells were able to attach and proliferate well on acrylic acid-coated contact lens surfaces, and there could be reliable transfer of epithelial cells to rabbit corneas (Deshpande et al., 2010). Other substrates for ex vivo cell expansion being investigated are composite membranes of alginate polymeric membrane coated with chitosan, NaOH-surface-modified poly(epsilon-caprolactone), three-dimensional collagen-proteoglycan-based scaffolds, and human anterior lens capsule (Ang et al., 2006; Galal et al., 2007; Lai et al., 2007; Ma et al., 2007; Oztürk et al., 2006; Torbet et al., 2007). Though studies have not yet been performed in humans, these results are indeed exciting in the realm of corneal stem cell therapy.

2.2.4 Autologous non-ocular tissues

As discussed in the conjunctival section, when available, transplantation of autologous tissues is the treatment of choice to obviate the risks of allograft rejections despite the presence of the anterior chamber immunologic privilege. In cases of bilateral severe limbal stem cell deficiency, oral mucosal transplantation is an adequate alternative. Kinoshita and Nakamura proposed a two-step process for patients at increased risks for allograft rejection or patients having low tolerance for immunosuppressive therapies. Here, the autologous oral mucosal epithelial progenitor cells undergo ex vivo expansion prior to transplantation (Kinoshita & Nakamura, 2004). Ex vivo expansion of a carrier-free sheet is more beneficial than a direct transplantation of an autologous buccal mucosal graft because the latter typically contain opaque subepithelial fibrous tissue. A long-term study reported good epithelial coverage, regression or stabilization of corneal neovascularization, and improved visual acuity in a cohort of patients with severe ocular surface disorder (Inatomi et al., 2006; Nakamura et al., 2010). The main mechanisms of graft failure were loss of transplanted cultivated oral epithelial cells, leading to persistent epithelial defect, followed by invasion of neighboring conjunctival epithelial cells, infiltration of inflammatory cells, and neovascularization. And as expected, good graft survival, intact ocular surface integrity, and no inflammatory cell infiltration were observed in successful cases, (Nakamura et al., 2007). Another study also showed good results using human buccal mucosa cultivated on thermoresponsive surface with mitomycin-treated 3T3 feeder cells for patients with chronic conjunctival inflammation and limbal stem cell depletion (Nishida et al., 2004). Ex vivo expansion of embryonic stem cells or bone marrow-derived mesenchymal stem cells have been investigated with promising preliminary results (Homma et al., 2004; Ma et al., 2006; Oh et al., 2008).

The advantages of these autologous tissues are a significant reduction of xenobiotic materials in the transplant, a decreased risk of infection, a minimized risk of allograft immunologic rejection, and a reduced need for immunosuppressive therapy. However, some authors still used the 3T3 murine feeder cells. Also, it would be intriguing to look at long-term restoration of corneal avascularity and clarity as these tissues are not corneal epithelium and do not possess innate limbal stem cells.

2.2.5 Corneal tissue substitutes

Severe ocular surface diseases not only affect the epithelium but also involve the corneal stroma as well. Thus, reconstitution of the ocular surface may provide symptomatic relief but not visual rehabilitation. Tissue bioengineering is required to regenerate corneal tissue equivalents that integrate into the host and restore vision. A number of substrates have been proposed as three-dimensional growth scaffolds for ocular tissues: irradiated acellular non-human cornea disc (Zhang et al., 2007), fish scale-derived scaffold (Lin et al., 2010), collagen-copolymer extracellular matrix (Li et al., 2003; Liu et al., 2006), silk film biomaterials (Lawrence 2008), and electrospun chitosan-graft-poly (β-caprolactone)/poly (β-caprolactone) nanofibrous scaffolds for retinal progenitor cells (Chen et al., 2011).

Since corneal stroma is composed of lamella of collagen fibrils, many investigators developed collagen-surface modified scaffolds to promote host tissue integration, repair and regeneration. Of note, Myung and colleagues (2007, 2008) used photolithography to construct a three dimensional collagen type I - poly(ethylene glycol)/poly(acrylic acid) "core and skirt" corneal prosthesis, which can support surface reepithelization and stromal

integration. In a rabbit model, the authors observed that corneal epithelium forms a confluent layer on the scaffold. The surface modification of collagen also assisted in the integration of the seeded corneal fibroblasts into the hydrogel skirt. Li et al (2003) performed lamellar keratoplasty using collagen-copolymer extracellular matrix scaffold in pigs. The authors reported increased procollagen synthesis and nerve regeneration, compared to control. Similar findings were reported by Liu et al (2006) using corneal substitutes made of cross-linked porcine type I collagen and water-soluble carbodiimides, in rabbit and minipig models. Using recombinant human collagens cross-linked with carbodiimide and hydroxysuccinimide, Merrett et al (2008) constructed bioengineered corneal equivalents for transplantation into minipigs. These synthetic corneal implants were found to be optically clear, with good epithelial coverage. Importantly, the authors also demonstrated repopulation of the synthetic stroma with host corneal stromal cells, as well as reinnervation with similar nerve density compared to allograft.

3. Ocular surface prosthetic devices

As discussed above, contact lenses are being investigated as a stem cell reservoir and delivery vehicle. Therapeutic applications of contact lenses extend to other uses as well, for symptomatic alleviation of aberrant ocular surface and drug delivery. Many individuals suffer severe eye discomfort and pain that is unresponsive to the mainstay treatment options. In these patients, the ocular surface can be improved by an ocular surface prosthetic device - not for limbal stem cell delivery but to create a microenvironment that supports the corneal surface, i.e. the Prosthetic Replacement of the Ocular Surface Ecosystem or PROSE, developed by Perry Rosenthal. The device itself is a fluid-ventilated, gas-permeable polymer with optic and haptic portions situated over the sclera that are linked to a transitional zone that suspends the lens over the cornea without contact. The unique design of this prosthesis provides a constant aqueous layer over the cornea surface, which contributes to healing of the corneal tissue and subsequent relief of symptoms (Rosenthal et al., 2000; Shepard et al., 2009)). Studies have demonstrated corneal healing as soon as 6 hours after device placement (Takahide et al., 2007). Stason and colleagues (2010) reported improved visual acuity and visual function in a significant number of these patients. Better quality of life has been reported as well (Romero-Rangel et al., 2000).

The most common route of drug delivery for the ocular surface is topical. This modality has some limitations, however, including rapid clearance from the ocular surface, short duration of therapeutic dosage, and systemic absorption (Bourlais et al., 1998; Ciolino et al., 2009; Ghate et al., 2008; Gulsen et al., 2004; Patton et al., 1978; Saettone et al., 2002). Accordingly, the use of contact lenses as drug delivery vehicles has been investigated. Sedlavek (1965) in Czechoslovakia first studied the use of soft contact lenses for drug delivery; since then many different drugs and technologies have been successfully tried using this technique (Sedlavek, 1965). The optimal design for a contact lens drug delivery system is one that allows for zero-order release kinetics, along with being comfortable and biocompatible (Ciolino et al., 2009). With these criteria in mind, the soft hydrogel lenses were the first lenses to be used in this capacity. The hydrogel lenses have high water content and large intermolecular pores, which allow them to absorb water soluble drugs and release them with a large initial pulse and then gradually thereafter (Jain, 1988). To maintain first-order kinetics, the hydrogel lens requires regular instillation of drops at intervals of 2-4 hours or reapplication of a soaked lens (Jain & Batra, 1970). This approach is suboptimal as drug

uptake and release kinetics are dependent on the equilibrium solubility of the drug present in the lens matrix (which is small for most of the hydrophobic drugs [Gulsen et al., 2004]), the inconvenience of frequent dosing, and the waste of drug remaining in the soaking solution.

Efforts to overcome these limitations focus on lens designs that allow for a sustained release of therapeutic dosage. One idea involves the use of nanoparticles in which the ophthalmic drug could be encapsulated and then dispersed in the lens material (Gulsen et al., 2004). These so called "drug-laden hydrogels" were found to release therapeutic levels of drugs for a few days with the ability to manipulate drug delivery rates by varying the load of nanoparticles in the gel (Gulsen et al., 2004). This discovery allowed for lenses that could release drugs over extended periods of time and thus behaved in a zero order kinetic fashion. However, although this was exciting, the development of a sustained, long-term drug delivery system that functions under the physiological temperature, pH and salinity of the eye continues to be problematic (Ciolino et al., 2009). Most recently, research is being conducted on the development of prototype lenses that incorporate a dual polymer system such that there is a polymer film containing the drug compounds; the film is then coated with a transparent polymer that is used in contact lenses (Ciolino et al., 2009). The results with this new lens showed that there could be controlled release with zero-order kinetics for over 4 weeks (Ciolino et al., 2009).

In addition to the soft hydrogel lens and their derivatives, collagen shields have also proven to be effective delivery modalities. The collagen shield contact lens is a therapeutic lens composed of non-cross linked porcine collagen with the capability of dissolving on the corneal surface over a period of around 12 hours (Phinney et al., 1988). Studies done using collagen shields soaked with gentamicin and vancomycin produced tear, cornea, and aqueous humor levels that were generally higher or comparable to those achieved through frequent drop administrations (Phinney et al., 1988). Further, collagen shields used for heparin delivery in postoperative rabbit eyes helped to prevent postoperative fibrin formation in eyes at risk for this complication (i.e. eyes requiring surgery for complications of proliferative diabetic retinopathy and glaucoma filtration surgery) (Murray et al., 1990). With time, as bioengineered materials continue to advance, so too will the quality of optimal delivery system contact lenses. Pharmacological contact lenses have the potential to revolutionize the current drug delivery modalities and conquer new ground in the long held battle of medication wastage and systemic absorption side effects.

4. Restoration of lacrimal function

Dry eye syndrome or dysfunctional tear syndrome is a chronic ailment, afflicting up to ten million people in the United States alone, and affecting women twice as often as men (Pflugfelder et al., 2000). As prevalent as it is, currently, there is only one medication (cyclosporine ophthalmic emulsion, 0.05%) approved by the Food and Drug Administration for decreased tear production due to ocular inflammation (Barber et al., 2005; Pflugfelder, 2004). Dry eye syndrome tends to coexist with ocular surface disorder. Importantly, poor lacrimal function correlates with poor surgical outcomes in ocular surface reconstructions, especially stem cell transplantation (Nguyen et al., 2008; Shimazaki et al., 2000, 2007). Temporary remedies, such as artificial tears, hydroxypropyl cellulose ophthalmic insert, punctal plugs, or moisture-chamber spectacles often provide only marginal relief and may interfere with the patient's quality of life. A long-term solution is the construction of an

implantable lacrimal microbiosystem, which would house a colony of monolayer lacrimal acinar cells and produce tear fluid (Selvam et al., 2006, 2007, 2008).

4.1 Artificial lacrimal gland

The parenchyma of the lacrimal gland consists of specialized epithelia: ductal cells and acinar cells. The acinar cells form an acinus structure whose wall is composed of a polarized monolayer of cells. These lacrimal acinar epithelial cells are responsible for production and release of tear proteins such as lactoferrin, growth factors, secretory immunoglobulins, and lysosomal hydrolases into nascent tear fluid (Fullard, 1994). Their apices point toward a central lumen into which tear fluid is secreted. Many acini form a lobule. The ductal cells line the intralobular and interlobular ducts, bringing tear fluid into the eye. An implantable lacrimal microbiosystem would approximate the structure and function of the lacrimal gland. Figure 2 depicts a schematic artificial lacrimal gland using a dead-end tube concept.

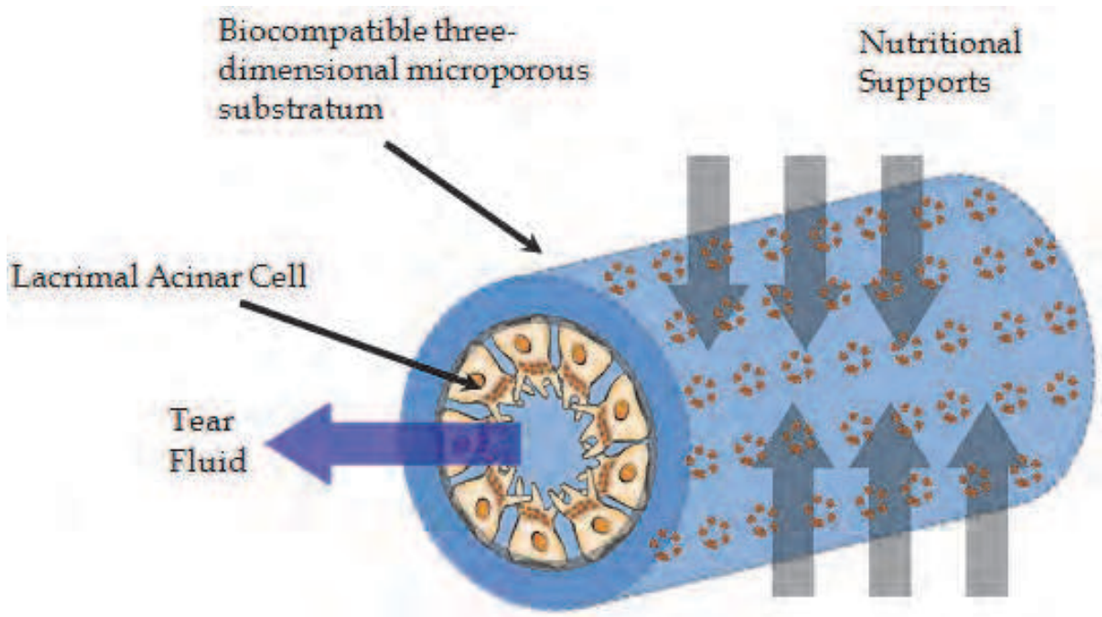

Fig. 2. Schematic of an artificial lacrimal gland where lacrimal acinar cells are seeded in a three-dimensional housing, allowing nutritional support from the environment and egress of tear fluid from the artificial gland.

Development of such devices faces many challenges. As with any implantable devices, a lacrimal microbiosystem must have a biologically compatible encapsulation to prevent rejection or toxicity to the host. However, the lacrimal microbiosystem encapsulation is much more intricate. Instead of a closed system, the enclosure must allow a dynamic bi-directional flux of ions, fluid, nutrients, and materials between the device and the host, while downregulating reactive fibroblastic proliferation in the surrounding environment that may diminish material transfer. Tear production is regulated by a variety of control modalities, such as regenerated intrinsic neurostimulation, extrinsic pharmacologic induction, or electronic excitation. Device construction includes ex-vivo seeding and maturation of the lacrimal acinar cells into a three-dimensional bioengineered scaffold. Strategic placement of pluripotent stem cells may be necessary. The stem cell clusters within

the scaffold would repopulate the lacrimal acinar tissue, as needed. Precise stimuli for regeneration and terminal differentiation would be provided by extrinsic delivery or activation of previously implanted nanovesicles containing plasmids coding for growth factors, for example. As a stem cell device, safeguard mechanisms must be instituted to monitor and prevent autonomous unstructured proliferation and escape of the stem cells, i.e. tumorigenicity.

Recent studies demonstrated monolayer growth of lacrimal acinar cells on Matrigel®, a biocompatible preparation rich in extracellular matrix molecules, i.e. laminin, collagen IV, heparin-sulfate proteoglycans, and growth factors (Selvam et al., 2006, 2007, 2008). Compared to other biocompatible polymeric materials, poly-L-lactic acid (PLLA)-Matrigel® substrate showed superior expression of monolayer acinar cell morphology. Active exocytosis vesicle profile was evident on electron microscopy, and maintenance of protein secretory functions was confirmed by β-hexosaminidase catalytic activity assay. PLLA and PLGA copolymers have a wide range of applications: surgical sutures, scaffolds in bone augmentation and ligament restoration, osteoconductive materials in dental medicine, and components in vascular grafts, urethral stents, and nerve growth conduits. Growth of polarized lacrimal acinar cells on PLLA, which demonstrated the ability to maintain normal monolayer acinar cell morphology, secretory function, and EGF-dependent proliferation, suggests that PLLA may be a good candidate for the development of a tear secretory device. A dead-end tube construct using polyethersulfone has been reported (Li et al., 2006; Liu et al., 2009). After seeding the tube with cultured rat lacrimal acinar epithelial cells, the authors observed that a polyethersulfone dead-end tube may be suitable for attachment and proliferation of these cells, allowing its potential application as an artificial lacrimal gland.

4.2 Tear film measurements

Patients with ocular surface disorders typically have an underlying inflammatory process, such as ocular cicatricial pemphigoid, Stevens-Johnson syndrome, Sjögren syndrome, or post chemotherapy. The persistent inflammatory process and dysfunctional tear film lead to poor surgical outcomes. Good preoperative lacrimal function and a healthy conjunctival epithelium are important prognostic factors for limbal stem cell transplantation (Tsubota et al., 1999; Shimazaki et al., 2000). In fact, having a preoperative Schirmer's test > 10 mm correlates to a higher success rate for ocular surface reconstruction. Accordingly, preoperative characterization of tear film is important for ocular surface reconstruction.

Traditionally, tear production and quality are accessed using vital dye stain, tear break-up time, Schirmer's test, or the cotton-thread test. However, these tests have suboptimal repeatability, inter-class correlation, and correlation between the test results and patient symptom (Jordan & Baum, 1980; Lin et al 2005; Machado et al., 2009; Mainstone 1996; Nicols et al., 2004; Saleh et al., 2006; Savini et al., 2008; Schein et al., 1997; Yokoi & Komuro 2004). They are likely to also produce erroneous results by disrupting the natural tear film, affecting tear production, and modifying the tear film structure. Accordingly, recent efforts are directed toward non-invasive optical imaging techniques.

Anterior segment optical coherence tomography (OCT) is a noninvasive technology using a laser to quantitatively measure the tear film and tear meniscus without ocular surface contact or fluorescein dye instillation (Huang et al., 1991, 2008; Ibrahim et al., 2010; Shen et al., 2009; Wang et al., 2006; Zhou et al., 2009). Tear film measurement using OCT showed good correlation with Schirmer's test and patient symptom score. Figure 3 portrays

application of anterior-segment OCT for measurement of lower tear meniscus height, depth, and cross-sectional area. OCT may also be used to measure the tear film or the upper tear meniscus. Other noninvasive technologies include slit-lamp equipped micrometer (Mainstone et al., 1996), slit-lamp photography (Kawai et al., 2007), and reflective meniscometry (Yokoi et al., 1999).

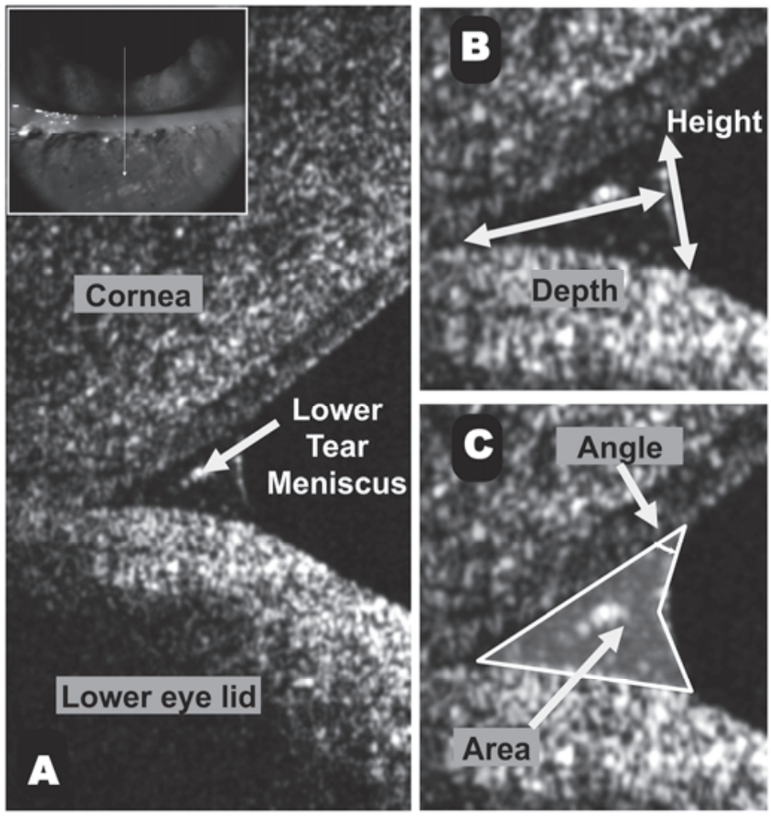

Fig. 3. Lower tear meniscus measurement using anterior segment optical coherence tomography. (A) Anatomy of the lower tear meniscus; the inset illustrates the scan position. (B) and (C) Caliper measurement protocol.

4. Conclusion

The corneal epithelium, conjunctival epithelium, tear film and lacrimal system, and the eyelids constitute the functional ocular surface. Management strategies for severe ocular surface disorders involve treatment and prevention of recurrence of the causative condition, symptomatic alleviation, and reconstitution of the anatomic and physiologic ocular surface. Human amniotic membrane transplantation with or without epithelial graft is a versatile modality for various injuries to the ocular surface epithelia. Other options for reconstitution of the epithelia include suppression of cicatricial changes, autologous mucosal transplantation, or cultivated bioengineered tissues. Motivated by the risk of zoonotic disease transmission or xenobiotic immunogenicity, researchers worldwide are investigating serum-free feeder cell-free culture systems for ex vivo expansion of epithelial tissues for ocular surface applications. The results of these investigations are promising.

Normal lacrimal function is not only important for symptomatic alleviation and optical pursuits; it is also exceedingly essential for surgical outcomes. Preoperative tear film is an important prognostic indicator for ocular surface reconstruction. Strategies for tear film restoration include lubrication, moisture chamber, and artificial lacrimal glands.

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Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications

Edited by Prof. Angelo Carpi

ISBN 978-953-307-268-5
Hard cover, 646 pages
Publisher InTech
Published online 01, August, 2011
Published in print edition August, 2011

This book provides an example of the successful and rapid expansion of bioengineering within the world of the science. It includes a core of studies on bioengineering technology applications so important that their progress is expected to improve both human health and ecosystem. These studies provide an important update on technology and achievements in molecular and cellular engineering as well as in the relatively new field of environmental bioengineering. The book will hopefully attract the interest of not only the bioengineers, researchers or professionals, but also of everyone who appreciates life and environmental sciences.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Pho Nguyen, Shabnam Khashabi and Samuel C Yiu (2011). Ocular Surface Reconstitution, Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications, Prof. Angelo Carpi (Ed.), ISBN: 978-953-307-268-5, InTech, Available from: http://www.intechopen.com/books/progress-in-molecular-and-environmental-bioengineering-from-analysis-and-modeling-to-technology-applications/ocular-surface-reconstitution

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