



Urea-De-Epithelialized Human Amniotic Membrane for Ocular Surface Reconstruction

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Key Words. Cell culture • Conjunctiva • De-epithelialization • Human amniotic membrane • Ocular surface reconstruction • Tissue engineering

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Received September 15, 2018; accepted for publication December 20, 2018; first published March 13, 2019.

<http://dx.doi.org/10.1002/sctm.18-0201>

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ABSTRACT

The conjunctiva is a clear tissue covering the white part of the eye and lines the back of the eyelids. Conjunctival diseases, such as symblepharon, cause inflammation, discharges, and photophobia. The treatment often requires excision of large parts of conjunctiva. Tissue engineering of conjunctival cells using human amniotic membrane (HAM) denuded of its epithelium as a basement membrane scaffold has been shown to be effective for covering conjunctival defects. However, most epithelial denudation protocols are time-consuming and expensive or compromise HAM's basement membrane structure and matrix components. We have previously described a method to de-epithelialize HAM using ice-cold urea (uHAM). In this report, we used this method to provide tissue-engineered constructs with cultivated conjunctival epithelial cells on uHAM in two patients, one with a giant conjunctival nevus and the other with a large symblepharon. Autologous conjunctival epithelial cells harvested from incisional biopsies of these two patients were cultured on the uHAM scaffold. The transplantation of tissue-engineered constructs to patients' ocular surface immediately after the removal of lesions showed successful reconstruction of the ocular surface. Postoperatively, there were neither recurrence of lesions nor epithelial defects throughout the follow-up (up to 7 and 19 months, respectively). This report highlights the translational potential of an efficient and inexpensive method to prepare de-epithelialized HAM as a basement membrane scaffold for cell-based tissue-engineered treatments of ocular surface disorders. *STEM CELLS TRANSLATIONAL MEDICINE* 2019;8:620–626

SIGNIFICANCE STATEMENT

Ocular surface diseases often require excision of large conjunctival areas. Tissue engineering of conjunctival cells requires a bare scaffold to promote cell growth. Human amniotic membrane (HAM) is a common scaffold option for tissue engineering with surgical purposes. So far, the available protocols for HAM epithelial denudation present a number of problems. Ice-cold urea has been successfully used to de-epithelialize HAM for conjunctival epithelial explant culture. The authors believe that this efficient and inexpensive method will facilitate and mainstream a minimally invasive cell-based approach for the reconstruction of extensive ocular surface wounds.

INTRODUCTION

The conjunctiva is the mucous membrane that lines the inner aspect of the eyelid and the sclera. It is the largest component of the ocular surface and consists of a stratified nonkeratinized columnar epithelium resting on the connective tissue [1]. The epithelial cells have tight junctions that connect adjacent cells and form a layer that serves as a protective barrier from external injury [2]. The goblet cells in the conjunctiva produce mucin that aid in lubricating the eye and contribute to ocular surface immune surveillance [3].

Chemical burns, mucous membrane pemphigoid, trachoma, tumors, pterygium, and Stevens-Johnson syndrome can impair conjunctival architecture and physiology [1]. Extensive ocular surface diseases (OSDs) involving the conjunctiva cause sight-threatening complications, such as chronic dry eye, corneal infections, and corneal and scleral melting [4]. Conjunctival reconstruction can be achieved by direct closure of wounds, if the defects are small, or with autologous conjunctival or amniotic grafts for larger defects (e.g., following resection of large tumors, recurrent pterygia, symblephara)

[5, 6]. However, there are issues with these surgical approaches. For autologous grafts, there is a limitation of donor graft size [7]. Amniotic membrane alone is only a temporary solution with poor long-term outcomes, because postoperative epithelialization depends on the viability of the surrounding tissue [8]. Oral and nasal mucosa have been tested as alternatives to replace the diseased conjunctiva [9, 10]; however, complications with donor tissue harvesting, keratinization, and cosmetic appearance can affect outcomes [11].

A recent approach to ocular surface rehabilitation is grafting a bioengineered construct that combines cultivated conjunctival cells on a biological scaffold [12, 13]. The *ex vivo* culture of conjunctival epithelium has been successfully established with the cultivated cells retaining their mature phenotype and functional characteristics similar to those found *in vivo* [14]. Various clinical trials have shown the effectiveness of cultivated conjunctival epithelium transplantation (CCET) for OSDs, achieving faster epithelial healing, prompt resolution of inflammation, and shorter recovery time [13, 15].

To construct a tissue-engineered graft, conjunctival epithelial cells have to be cultivated on carriers [14]. Different substrates, such as amniotic membrane, other biological carriers, and synthetic materials, have been studied in animal and clinical trials. Among the biomaterials, human amniotic membrane (HAM) is one of the most widely used scaffolds [16]. It consists of a mechanically strong basement membrane with a porous matrix, rich in growth factors, hydrated proteoglycans, and glycoproteins, allowing excellent cell adhesion and growth [17]. In addition to its anti-inflammatory and antimicrobial activity, HAM is nonimmunogenic and can facilitate wound healing and re-epithelialization [18].

HAM is routinely frozen in cryoprotective solution prior to clinical use to allow timely donor screening for transmissible diseases. This renders the amniotic epithelial cells no longer viable. Various studies have shown that cells grown over HAM with intact epithelium maintain stemness and progenitor phenotypes, whereas tissue culture on HAM denuded of epithelium showed better explant outgrowth, stratification, and cell junction formation [19]. Because the primary goal of conjunctival reconstruction is to re-epithelialize and stabilize the ocular surface, there is a need for efficient epithelial repopulation, stratification and goblet cell differentiation. Hence, the de-epithelialization of HAM is a crucial step for conjunctival epithelial cell expansion [20, 21].

HAM epithelial denudation can be achieved with a variety of protocols using chemical and enzymatic agents [22, 23]. However, the effectiveness in removing epithelial cells varies among these methods. Moreover, when enzymes are used, the harsh digestion process can also affect the integrity of basement membrane, extracellular matrix protein, and growth factor content [17, 21]. In this case study, we showed the clinical outcomes of two patients receiving CCET on urea-denuded HAM in ocular surface reconstruction.

MATERIALS AND METHODS

Operation Information

Informed consent was obtained from patients under the tenets of the Declaration of Helsinki. A corneal specialist (J.M.) conducted the ophthalmic examinations, biopsies, and transplantation

of tissue-engineered constructs at the Singapore National Eye Centre. Clinical grade donor amniotic membranes were obtained from Singapore Eye Bank. A cell biologist (G.Y.) was responsible for HAM preparation and explant cultures at the Singapore Eye Research Institute.

HAM Preparation and Urea Denudation

Fresh human fetal amnion was isolated from placenta (mother aged below 40 years) after elective cesarean section, with written consent and protocol approved from SingHealth Centralized Institutional Review Board (CIRB 2015/2607). The amnion was processed, transported, and stored according to the U.S. Centers for Disease Control and Prevention Guidelines. Mandatory serology tests for transmissible diseases were negative before clinical use, following the routine standard operational procedure. The HAM was stored frozen in Dulbecco's modified Eagle's medium/glycerol (50:50 vol/vol; Sigma-Aldrich, St. Louis, MO, USA) for 6 months before use. After thawing, HAM pieces were washed with sterile Dulbecco's phosphate buffer solution ($\text{Ca}^{++}/\text{Mg}^{++}$ free, Lonza 17-512F, cGMP, Singapore) to clear glycerol. HAM denudation was performed as described previously. In brief, it was immersed in ice-cold 5 M urea (Sigma-Aldrich) in phosphate-buffered saline for 5 minutes, followed by gentle scraping using a cotton tip to remove the loosened amniotic epithelium. The denuded HAM was rinsed extensively with phosphate-buffered saline to remove all cell debris and urea. It was then flattened on a sterile nitrocellulose frame (interior 4×4 cm size; autoclaved at 121°C at 100 kPa for 15 minutes) with basement membrane side facing up and conditioned with culture media for 30 minutes before use for cell culture.

Conjunctival Explant Culture and Tissue-Engineered Construct

The conjunctival biopsy was performed in two cases in the operation theater by harvesting approximately 2×2 mm of healthy conjunctival tissue. In the giant nevus case, caution was taken to ensure the harvest biopsy did not contain any melanocytic cells. For the patient with symblepharon, biopsy was taken outside of the scarred region. The tissue was immediately stored in a sterile vial containing balanced salt solution, double boxed, and transferred at room temperature to laboratory for processing. The samples were washed in Hank's balanced salt solution ($\text{Ca}^{++}/\text{Mg}^{++}$ free, Lonza 10-543Q, cGMP, Singapore) containing penicillin 300 $\mu\text{g}/\text{ml}$, streptomycin sulfate 300 $\mu\text{g}/\text{ml}$ (Lonza, 17-602E, cGMP, Singapore), and 7.5 $\mu\text{g}/\text{ml}$ amphotericin B (Lonza 17-836E, cGMP, Singapore) for 3 times with careful clearing of blood traces and stromal debris. The samples were trimmed to 0.5×0.5 mm blocks and evenly placed on the basement membrane side of the urea-denuded HAM (uHAM). The culture medium was Defined Keratinocyte Serum-Free Medium with bovine pituitary extract (Thermo Fisher Scientific 10,744,019, Waltham, MA, USA), containing 1% recombinant insulin human (FeF Chem 66,001, ISO9001, Koge, Denmark), 10 ng/ml recombinant human epidermal growth factor (Cellgenix 1,016-050, GMP, Freiburg, Germany), 0.5 $\mu\text{g}/\text{ml}$ hydrocortisone (Sigma, Fluka PHR1014, USP), 5% Human Serum AB (Lonza 14-490E, cGMP), 0.9 mM calcium chloride (CaCl_2 , Sigma-Aldrich), 100 U/ml penicillin/100 $\mu\text{g}/\text{ml}$ streptomycin sulfate, and 2.5 $\mu\text{g}/\text{ml}$ amphotericin B. After 24 hours for initial attachment, the blocks from the biopsies were fully immersed in culture medium, which was replenished every 3 days. Regular documentation of cell

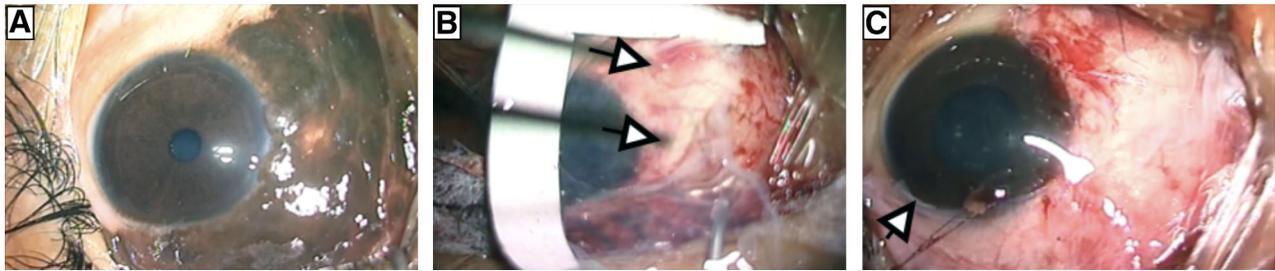


Figure 1. Tissue-engineered cultivated conjunctival epithelial transplantation in case 1. (A): Preoperation with giant conjunctival nevus. (B): Transfer of uHAM-CCET (arrowheads) over the bare sclera after lesion excision. (C): uHAM-CCET graft (arrowhead) secured in place with 10–0 nylon sutures and fibrin glue.

culture was conducted twice weekly using digital single lens-reflex camera (EOS 4000D; Canon, Tokyo, Japan) and phase contrast microscopy (Eclipse TS100; Nikon, Tokyo, Japan). After 18 to 21 days, the confluent monolayer culture was replaced with stratification medium (same culture medium with 1.2 mM CaCl_2) for 7 days, and the uHAM-CCET construct was ready for transplantation.

Immunofluorescence

Samples were fixed in freshly prepared neutral-buffered 3% paraformaldehyde (Sigma-Aldrich) for 20 minutes at room temperature. After washing with phosphate-buffered saline (PBS; 0.1 M, Life Technologies), the samples were treated with 50 mM ice-cold ammonium chloride (Sigma-Aldrich) to quench free reactive aldehydes. They were permeabilized and blocked with 0.15% saponin (Sigma-Aldrich), 2% bovine serum albumin (Sigma-Aldrich), and 5% normal goat serum (Invitrogen). The samples were then incubated with primary antibodies against human epitopes of Ki67 (Sigma-Aldrich AB9260, 1 $\mu\text{g}/\text{ml}$), MUC5AC (Sigma HPA040615, 5 $\mu\text{g}/\text{ml}$), cytokeratin 4 (CK4, Acris BM559, 2 $\mu\text{g}/\text{ml}$, Rockville, MD), and CK19 (Millipore MAB3238, 2 $\mu\text{g}/\text{ml}$, Burlington, MA) for 2 hours at room temperature. After PBS washes, the samples were incubated with appropriate fluorescence conjugated immunoglobulin G secondary antibody (Jackson ImmunoRes, West Grove, PA) for 1 hour in the dark. They were then mounted with FluoroShield containing 4',6-diamidino-2-phenylindole (Santa Cruz Biotechnology, Santa Cruz, CA) for nuclear contrast staining and visualized under fluorescence microscopy (Axiomager Z1; Zeiss, Oberkochen, Germany).

Electron Microscopies

Samples were fixed with 3% glutaraldehyde (EM Sciences, Hatfield, PA) in sodium cacodylate buffer (Sigma-Aldrich) and then in 1% aqueous solution of osmium tetroxide (EM Sciences), and processed for Epon-Aradite embedding. After staining with 3% uranyl acetate (EM Sciences) and lead citrate (prepared from lead nitrate and sodium citrate, Sigma-Aldrich), ultrathin sections were examined under transmission electron microscopy (TEM; JEOL 2100, Tokyo, Japan) at 80 kV. For scanning electron microscopy (SEM), the fixed samples were dehydrated with an alcohol series of increasing concentration, critical point dried, and sputter-coated with gold alloy (10 nm thick) and viewed under Quanta 650 field-emission gun-scanning electron microscope (FEI, ThermoFisher) at 20 kV.

Tissue-Engineered Construct Transplantation

In both cases, the uHAM-CCET construct was trimmed to match the wound size, placed with epithelium-side-up, and secured to the resected conjunctival margins with interrupted 10/0 polygalactin sutures and fibrin glue. All surgical steps were documented, including the preoperative size of the lesion (Fig. 1A), the positioning and tailoring of the uHAM-CCET size after wound excision (Fig. 1B), and final aspect after suturing (Fig. 1C). The remaining uHAM-CCET pieces were sent back to the laboratory, fixed, and processed for immunofluorescence and electron microscopies. Both patients were prescribed with dexamethasone (1 mg/ml, Alcon, Fort Worth, TX) and levofloxacin (1 mg/ml, Santen Pharmaceuticals, Osaka, Japan) eye drops four times daily for a minimum of 3 months.

Patients

Case 1

A 14-year-old Indonesian male presented with an extensive nasal melanocytic lesion in his left eye that covered approximately half of the bulbar conjunctiva (Fig. 2A). He noted a growth of the lesion during puberty. There were no complaints of pain, itching, or bleeding of the lesion; the remaining ophthalmic assessment was also unremarkable, with uncorrected visual acuity (UCVA) of 6/7.5. Surgical incisional biopsies were taken from five different areas, all of which revealed cysts and benign melanocytes; there was no overt atypia or mitotic activity, which supported the diagnosis of benign conjunctival melanocytic nevus. Surgery was subsequently conducted with a complete excision of the lesion with a safety margin of 2 mm. Cryotherapy was performed at the margin of the resected conjunctiva as an additional step of the procedure before grafting a uHAM-CCET construct onto the bare sclera.

Case 2

A 36-year-old Chinese male presented with a severe symblepharon that extended from the inner aspect of the lower eyelid to the inferior limbus in both eyes, with greater severity in the right. Scarring of the lower bulbar conjunctiva and fibrous, keratinized tissue that covered approximately 2.5 mm of the inferior corneolimbal area was noted. There was also fornix shortening and scarring (Fig. 2D). The patient had a history of working at an industrial site with exposure to toxic fumes. However, he denied any mechanical or chemical trauma, eye surgeries, or any autoimmune disorders. The rest of his ophthalmic examination was unremarkable, and his UCVA was 6/6.

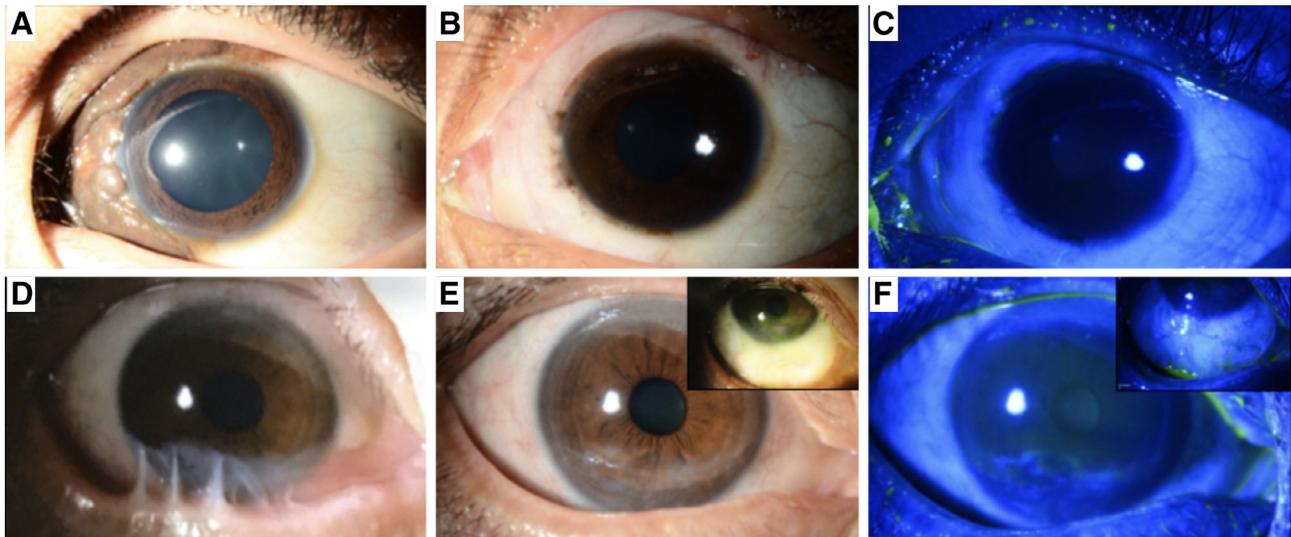


Figure 2. Pre- and postoperative slit-lamp biomicroscopic images of cases 1 (A–C) and 2 (D–F). **(A):** Preoperative for case 1. Giant melanocytic conjunctival nevus extending from superior and inferior bulbar conjunctiva toward the caruncula. **(B):** 3 months after operation. Transparent and vascularized conjunctiva covering the ocular surface resembling a normal aspect as compared with the opposite untouched bulbar conjunctiva. Mild sub-Bowman opacities underneath the intact epithelium were present in the previous scarred area. **(C):** Fluorescein vital dye staining. The absence of green fluorescence area demonstrated the intact regenerated epithelium. **(D):** Preoperative for case 2. Large symblepharon with scarring at the inferior bulbar and tarsal conjunctiva and fibrotic adherence to the inferior corneo-limbal areas. **(E):** 19 months after operation. Transparent and vascularized inferior bulbar conjunctiva without any signs of scarring or epithelial defect. **(F):** Fluorescein vital dye staining. The regenerated epithelium was intact as shown by the absence of green fluorescence area. Insets in (E, F) showed a free inferior fornix, without tissue adhesions or scarring.

An incisional conjunctival biopsy was performed outside the area of the affected conjunctiva and a histopathological examination with direct immunofluorescence was negative for ocular mucous membrane pemphigoid. An oral and maxillofacial surgeon specialist harvested a mucosal graft from the hard palate prior to the eye surgery. Complete excision of the symblepharon was performed with a debridement of the corneal epithelium to release all tissue adhesions. To reconstruct the tarsal plate, a hard palate graft was placed and secured at the inferior tarsal plate with 10/0 polygalactin sutures before the uHAM-CCET was attached to the bulbar surface.

RESULTS

Tissue Engineering of CCET

The conjunctival epithelial biopsy adhered to the uHAM in 4–6 hours and generated visible explants after 2–3 days (Fig. 3A). The primary colonies expanded, merged, and propagated to a confluent monolayer sheet over 3–4 weeks (Fig. 3B, 3C), and cells displayed predominantly cobblestone appearance, typical of epithelial morphology and lack of any slender fibroblastic cell types (Fig. 3D). After stratification, multilayered epithelium was seen under TEM (Fig. 3E). The epithelial cells were anchored to neighboring cells through cell junctions (tight and adherens) and desmosomes (Fig. 3F), and closely adhered to the basement membrane via hemi-desmosomes (Fig. 3G). They did not show any signs of cellular abnormality, such as alterations of mitochondria and endoplasmic reticulum or induction of lysosomes and intracellular vesicles (Fig. 3E–3H). Toward the superficial layer, some cells exhibited short apical microvilli (Fig. 3H). Under SEM, the polygonal-shaped epithelial cells were closely packed together with clear interface between cells, and the apical surface was covered by microvilli (Fig. 3I). Immunofluorescence showed that

the conjunctival epithelial sheet positively expressed proliferating cell antigen Ki67 (Fig. 3J), CK19 (epithelial marker; Fig. 3K), CK4 (conjunctival epithelial marker; Fig. 3L), and mucin glycoprotein MUC5AC (Fig. 3M).

Clinical Results

Case 1

The ocular surface was stable at the first postoperative visit, without any epithelial defect, and it remained stable thereafter. In the 2nd-month postoperative visit, the aesthetic aspect of the operated eye was similar to the untreated contralateral eye, with minimal conjunctival scarring and no epithelial defect on the ocular surface. The patient remained satisfied, and the visual acuity was stable. He had no complaints at his last follow-up, 7 months after surgery (Fig. 2B, 2C).

Case 2

Complete conjunctival epithelial healing was achieved within 3 weeks. There was a persistent small corneal epithelial defect of less than 1.5 mm at the inferior nasal periphery of cornea, which was treated with bandage contact lenses and lubricants. In the 5th-month postoperative visit, there were no signs of ocular surface inflammation or epithelial erosions (Fig. 2E, 2F). At his last follow-up visit, over 19 months after surgery, the patient's UCVA was unchanged, with a completely epithelialized and stable ocular surface without any signs of symblepharon recurrence.

DISCUSSION

We have previously described a de-epithelialization protocol for HAM using a brief 5-minute treatment with ice-cold 5 M urea, followed by gentle scraping [23]. This method is nontoxic

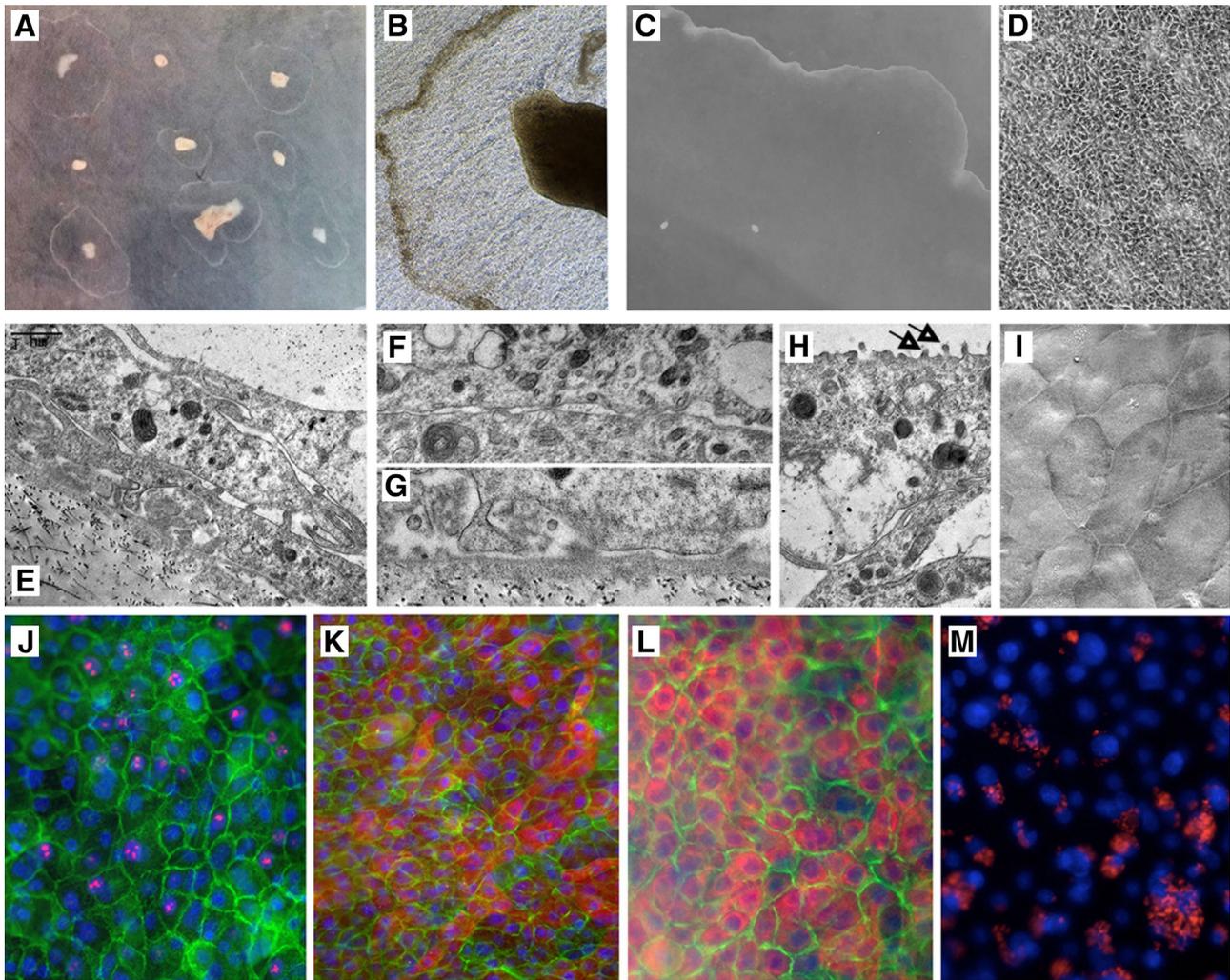


Figure 3. Cultivated conjunctival epithelium on urea-denuded HAM. Explant culture of conjunctival biopsies on the uHAM. **(A):** Overview of several explants at day 4 culture at $\times 4$ magnification using DSLR. **(B):** $\times 10$ magnification under contrast phase microscopy. **(C):** Border of epithelial outgrowth after culture for 14 days at $\times 2$ magnification using DSLR. **(D):** Cells at confluent density at $\times 20$ magnification under phase contrast microscopy. **(E–H):** Transmission electron micrographs of cultivated conjunctival epithelium with stratification (E), cell-to-cell contacts (F), cell-basement membrane contacts (G), and superficial layer with short microvilli (H). **(I):** Scanning electron micrograph showing the cobblestone appearance of cultivated conjunctival epithelial cells. **(J–M):** Immunofluorescence of cultivated conjunctival epithelium showing the expression of nucleolar Ki67 (J, red fluorescence), epithelial cytokeratin CK19 (K, red fluorescence), conjunctival-specific cytokeratin CK4 (L, red fluorescence), and mucin MUC5A2 (M, red fluorescence). Samples in (J–L) were stained with phalloidin (green fluorescence).

and preserves the basement membrane structure, stromal composition, and growth factor content. This case series showed two patients with extensive OSD who received uHAM-CCET. The patients had satisfactory visual and aesthetic outcomes, and the reconstructed ocular surface was stable throughout a mid-term follow-up period of up to 19 months. There were no signs of recurrence. At the time of transplantation, the confluent epithelia generated on uHAM expressed typical conjunctival epithelial markers (CK4, CK19, MUC5AC), as well as multilayered intact epithelial structure.

Large areas of conjunctival lesions requiring reconstruction, such as symblephara, large neoplastic lesions, and recurrent pterygia, may cause a risk of ocular surface instability [24]. In such cases, CCET can improve wound healing and re-establish both the anatomy and homeostasis of the ocular surface. Our protocol showed that a small biopsy of conjunctival tissue (less than 2×2 mm size) was sufficient to generate conjunctival epithelium on

denuded HAM of 4×4 cm in dimension for transplantation use. The successful acquisition and expansion of small conjunctival biopsies highlights the minimal invasiveness of this approach. This can be particularly useful in patients with extensive OSD, in which only judicious and small-scale harvesting of healthy conjunctiva is possible [25].

In most tissue engineered-based treatments, the cultivated cells require a carrier for surgical delivery and also to serve as an appropriate scaffold that facilitates cell growth and establishes cell-to-cell contact and signaling [8]. This enables the cells to effectively form an intact epithelium [16]. HAM is one of the most popular bioscaffolds used in tissue engineering because of its strong basement membrane with hydrated proteoglycans and glycoproteins, as well as its spongy matrix, which is rich in growth factors [16]. When HAM is frozen in cryoprotective solution with 50% glycerol, the viability of amniotic epithelial cells is adversely affected, whereas the basement membrane structure and integrity

is maintained [17]. The freeze-thaw method and/or harvest site will affect HAM transparency. For instance, when compared with the corneal transparency, freeze-dried HAM retained up to 85% transparency, whereas when it was freeze-thawed and harvested distally, it was 83% as transparent as a normal cornea [26]. The intact HAM has been shown to support stemness and progenitor phenotypes for limbal epithelial cell culture [23]. In contrast, the cells cultured on the denuded AM displayed better repopulation, stratification, and epithelial hallmarks, such as cell junction formation [27]. Use of HAM is associated with certain disadvantages, including the risk of transmission of communicable diseases and biological variability of AM components from different donors [26]. In spite of these, HAM remains the gold standard substrate for ocular surface reconstruction.

HAM denudation can be achieved using chemical and enzymatic agents. The use of dispase, trypsin, ethylenediaminetetraacetic acid (EDTA), thermolysin, and ethanol has shown variable efficiencies and treatment time (from a few minutes to hours). They can also affect the basement membrane integrity and the collagen and matrix protein content, as well as the loss of growth factors [20–22]. We have reported a gentle, nontoxic, and efficient protocol using ice-cold urea to de-epithelialize HAM that resulted in a bioscaffold with intact basal lamina and smooth basement membrane surface [23]. The treatment of ice-cold urea creates an osmotic gradient that loosens cell-to-cell and cell-basement membrane contacts, enabling an efficient removal of dead epithelial cells from AM basement membrane by gentle scraping. Similar effect has been reported with other agents, like EDTA, but the complete de-epithelialization can take hours [21, 23].

Several clinical reports have demonstrated the safety and efficacy of HAM as a scaffold for conjunctival reconstruction. In 2002, Scuderi et al. reported the use of cultivated conjunctival cells on petroleum gauze as a scaffold for ocular surface reconstruction following the excision of conjunctival nevus, xeroderma pigmentosum, and iatrogenic symblepharon [28]. However, no stability data on the patient follow-up were presented. A larger case series of CCET and HAM had shown the successful management of giant nevi, large pterygium, leaking trabeculectomy blebs, and superior limbic keratoconjunctivitis with full epithelialization within 12–14 days and a reported stability of 11.6 months [29]. Sangwan et al. reported a different approach using both limbal and conjunctival cells cultured on denuded HAM for treating a case of extensive symblephara after a chemical burn. They showed an improvement in visual acuity and reestablishment of ocular surface with a follow-up longer than 1 year [30].

The use of new biomaterials has raised interest in the field of tissue engineering, and new candidates to substitute HAM as a scaffold such as polylactide-co-glycolide, poly ϵ -caprolactone,

and other extracellular matrix-protein-containing membranes are under investigation [31]. *in vitro* studies have shown the improvement of cell growth and goblet cell differentiation [32]. Although these synthetic polymer membranes could have the advantages of higher mechanical strength and biodegradability, the lack of transparency and challenges of fine-tuning the hydrophobicity could affect the biocompatibility, such as cell adhesion, proliferation, and viability [25].

CONCLUSION

This is the first clinical report to show that CCET on denuded HAM prepared by an efficient and inexpensive method of ice-cold urea has achieved satisfactory visual and aesthetic outcomes for patients presenting OSD. However, our work has limited sample size, which renders any statistical inference unreliable. Recruitment of more cases is ongoing. This case series demonstrated that uHAM-CCET is a safe and feasible approach and has translational potential for ocular surface reconstruction.

ACKNOWLEDGMENTS

We thank Singapore Eye Bank for the help in amnion collection (consent taking and tissue processing). We also acknowledge SingHealth Advanced Bioimaging Core Facilities and Electron Microscopy Unit, National University of Singapore, for confocal and electron microscopies. This study was funded by Singapore National Research Foundation under its Translational and Clinical Research (TCR) program (NMRC/TCR/008-SERI/2013), National Medical Research Council, Ministry of Health, Singapore.

AUTHOR CONTRIBUTIONS

F.B.: data collection, analysis and interpretation, manuscript writing, final approval of manuscript; G.H.-F.Y.: conception and design, data collection, analysis and interpretation, manuscript writing, final approval of manuscript; M.F., H.S.O., Y.-C.L., X.-Y.S.: data collection, analysis and interpretation, final approval of manuscript; S.Y.S.: provision of study material and patients, final approval of manuscript; J.S.M.: conception and design, financial support, provision of study material and patients, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

REFERENCES

- 1 Van Buskirk EM. The anatomy of the limbus. *Eye (Lond)* 1989;3:101–108.
- 2 Fischbarg J, Diecke FP, Iserovich P et al. The role of the tight junction in paracellular fluid transport across corneal endothelium. Electro-osmosis as a driving force. *J Membr Biol* 2006;210:117–130.
- 3 Drosophila 12 Genomes Consortium, Clark AG, Eisen MB et al. Evolution of genes and genomes on the *Drosophila* phylogeny. *Nature* 2007;450:203–218.
- 4 Dart JK. The 2016 Bowman Lecture Conjunctival cures: Scarring conjunctivitis 30 years on. *Eye (Lond)* 2017;31:301–332.
- 5 Sotozono C, Inagaki K, Fujita A et al. Methicillin-resistant *Staphylococcus aureus* and methicillin-resistant *Staphylococcus epidermidis* infections in the cornea. *Cornea* 2002;21(suppl 7):S94–S101.
- 6 Honavar SG, Bansal AK, Sangwan VS et al. Amniotic membrane transplantation for ocular surface reconstruction in Stevens-Johnson syndrome. *Ophthalmology* 2000; 107:975–979.
- 7 Fuest M, Liu YC, Coroneo MT et al. Femtosecond laser assisted pterygium surgery. *Cornea* 2017;36:889–892.
- 8 Behaegel J, Ni Dhubghaill S, Koppen C et al. Safety of cultivated limbal epithelial stem cell transplantation for human corneal regeneration. *Stem Cells Int* 2017;2017: 6978253.
- 9 Inatomi T, Nakamura T, Kojyo M et al. Ocular surface reconstruction with combination of cultivated autologous oral mucosal epithelial transplantation and penetrating keratoplasty. *Am J Ophthalmol* 2006;142:757–764.

- 10** Kobayashi M, Nakamura T, Yasuda M et al. Ocular surface reconstruction with a tissue-engineered nasal mucosal epithelial cell sheet for the treatment of severe ocular surface diseases. *STEM CELLS TRANSLATIONAL MEDICINE* 2015;4:99–109.
- 11** Malhotra C, Jain AK. Human amniotic membrane transplantation: Different modalities of its use in ophthalmology. *World J Transplant* 2014;4:111–121.
- 12** Weinberg DA, Tham V, Hardin N et al. Eyelid mucous membrane grafts: A histologic study of hard palate, nasal turbinate, and buccal mucosal grafts. *Ophthalmic Plast Reconstr Surg* 2007;23:211–216.
- 13** Ang LP, Tan DT, Cajucom-Uy H et al. Reconstruction of the ocular surface by transplantation of a serum free cultivated conjunctival tissue equivalent. *Ann Acad Med Singapore* 2004;33(suppl 5):S55–S56.
- 14** Koizumi N, Kinoshita S. Ocular surface reconstruction, amniotic membrane, and cultivated epithelial cells from the limbus. *Br J Ophthalmol* 2003;87:1437–1439.
- 15** Chung SH, Lee JH, Yoon JH et al. Multilayered culture of primary human conjunctival epithelial cells producing MUC5AC. *Exp Eye Res* 2007;85:226–233.
- 16** Zhao C, Tan A, Pastorin G et al. Nanomaterial scaffolds for stem cell proliferation and differentiation in tissue engineering. *Bio-technol Adv* 2013;31:654–668.
- 17** Riau AK, Beuerman RW, Lim LS et al. Preservation, sterilization and de-epithelialization of human amniotic membrane for use in ocular surface reconstruction. *Biomaterials* 2010;31:216–225.
- 18** Forni MF, Loureiro RR, Cristovam PC et al. Comparison between different biomaterial scaffolds for limbal-derived stem cells growth and enrichment. *Curr Eye Res* 2013;38:27–34.
- 19** Gomes JA, Romano A, Santos MS et al. Amniotic membrane use in ophthalmology. *Curr Opin Ophthalmol* 2005;16:233–240.
- 20** Jie J, Yang J, He H et al. Tissue remodeling after ocular surface reconstruction with denuded amniotic membrane. *Sci Rep* 2018;8:6400.
- 21** Hopkinson A, Shanmuganathan VA, Gray T et al. Optimization of amniotic membrane (AM) denuding for tissue engineering. *Tissue Eng Part C Methods* 2008;14:371–381.
- 22** Koizumi N, Rigby H, Fullwood NJ et al. Comparison of intact and denuded amniotic membrane as a substrate for cell-suspension culture of human limbal epithelial cells. *Graefes Arch Clin Exp Ophthalmol* 2007;245:123–134.
- 23** Zhang T, Yam GH, Riau AK et al. The effect of amniotic membrane de-epithelialization method on its biological properties and ability to promote limbal epithelial cell culture. *Invest Ophthalmol Vis Sci* 2013;54:3072–3081.
- 24** Cauchi PA, Ang GS, Azuara-Blanco A et al. A systematic literature review of surgical interventions for limbal stem cell deficiency in humans. *Am J Ophthalmol* 2008;146:251–259.
- 25** Cheng J, Zhai H, Wang J et al. Long-term outcome of allogeneic cultivated limbal epithelial transplantation for symblepharon caused by severe ocular burns. *BMC Ophthalmol* 2017;17:8.
- 26** Cannon CJ, Douth J, Chen B et al. The variation in transparency of amniotic membrane used in ocular surface regeneration. *Br J Ophthalmol* 2010;94:1057–1061.
- 27** Sudha B, Jasty S, Krishnan S et al. Signal transduction pathway involved in the ex vivo expansion of limbal epithelial cells cultured on various substrates. *Indian J Med Res* 2009;129:382–389.
- 28** Eidet J, Dartt D, Utheim T. Concise Review: Comparison of culture membranes used for tissue engineered conjunctival epithelial equivalents. *J Funct Biomater* 2015;6:1064–1084.
- 29** Scuderi N, Alfano C, Paolini G et al. Transplantation of autologous cultivated conjunctival epithelium for the restoration of defects in the ocular surface. *Scand J Plast Reconstr Surg Hand Surg* 2002;36:340–348.
- 30** Tan DT, Ang LP, Beuerman RW. Reconstruction of the ocular surface by transplantation of a serum-free derived cultivated conjunctival epithelial equivalent. *Transplantation* 2004;77:1729–1734.
- 31** Sangwan VS, Vemuganti GK, Iftekhar G et al. Use of autologous cultured limbal and conjunctival epithelium in a patient with severe bilateral ocular surface disease induced by acid injury: A case report of unique application. *Cornea* 2003;22:478–481.
- 32** Ang LP, Cheng ZY, Beuerman RW et al. The development of a serum-free derived bioengineered conjunctival epithelial equivalent using an ultrathin poly(epsilon-caprolactone) membrane substrate. *Invest Ophthalmol Vis Sci* 2006;47:105–112.