Topical Cryopreserved Amniotic Membrane and Umbilical Cord Eye Drops Promote Re-Epithelialization in a Murine Corneal Abrasion Model

Sean Tighe,1,2 Hamid-Reza Moein,3 Lorraine Chua,1 Anny Cheng,1,2,4 Pedram Hamrah,3 and Scheffer C. G. Tseng1,4

1TissueTech, Inc., Miami, Florida, United States
2Florida International University, Herbert Wertheim College of Medicine, Miami, Florida, United States
3Center for Translational Ocular Immunology, Department of Ophthalmology, Tufts Medical Center, Tufts University School of Medicine, Boston, Massachusetts, United States
4Ocular Surface Center and Ocular Surface Research & Education Foundation, Miami, Florida, United States

Corneal abrasion leading to epithelial defects can be caused by a multitude of factors including mechanical trauma, ocular surface foreign bodies, chemical burns, contact lens wear, and dryness due to tear film deficiency.1 Prolonged or persistent corneal epithelial defects threaten vision as they increase the likelihood of microbial infection and stromal ulceration and scarring.2 To mitigate corneal morbidity and potential blindness, it is important to promote prompt epithelialization during wound healing.

Clinically, amniotic membrane (AM) grafts have been used to treat a wide variety of corneal disorders including corneal scars, burns, infections, autoimmune processes, and postsurgical trauma.3 The clinical efficacy of AM in these conditions has been attributed to its anti-inflammatory, antiangiogenic, and antiscarring effect that together promote corneal re-epithelialization.4–5 When the fresh AM tissue is appropriately processed and preserved, it retains the natural biologic and functional components6–7 and can be used as either a tissue to be sutured, as an inlay, or as a temporary patch graft as an onlay to cover the damaged ocular surface. However, the former must be done in the operating room, while the latter only covers the central cornea and may obscure patients’ vision.

Recently, we have identified an active matrix component, termed the HC-HA/PTX3 complex,8 that is responsible for exerting AM’s aforementioned actions (reviewed in Ref. 5) and found in the umbilical cord (UC) at significantly greater quantities.7 In fact, UC has been shown to be significantly more effective in promoting IL-10 (anti-inflammatory cytokine) and inhibiting IL-12 (proinflammatory cytokine) protein synthesis.9 In order to use the additional concentration of HC-HA/PTX3 found in the UC, mitigate the aforementioned disadvantages of using AM as a sheet, and facilitate the manufacturing scale up from one donor placenta, we have developed eye drops containing morselized cryopreserved amniotic mem-
brane and umbilical cord (AMUC). Our recent preliminary study has shown that topical AMUC is effective in promoting healing of corneal epithelial defects in four patients presenting with cicatricial ocular diseases. To better understand its therapeutic potential, we herein report the safety and efficacy of AMUC in promoting corneal epithelialization and restoring corneal regularity in a murine model of corneal abrasion.

**Materials and Methods**

**Preparation of AMUC Drops**

Amniotic membrane and umbilical cord drops were prepared as previously described from frozen human placenta obtained from eligible pregnant women following elective cesarean section delivery under full informed consent in compliance with American Association of Tissue Banks Standards. The AMUC tissues were aseptically processed in accordance with current Good Tissue and Manufacturing Practices using the proprietary CRYOTEK method (TissueTech, Miami, FL, USA). After separation from other placental tissues, the AM and UC from the same donor were morselized in a Cuisinart CBT-700 blender (East Windsor, NJ, USA) at a ratio of 1:3 (wt/vol, AMUC weight [g]: 0.9% saline volume [mL]) along with 1.25 μg/mL Amphotericin B (Mediatech Manassas, VA, USA). The morselized mixture was packaged into ophthalmic tubes. The final concentration of AMUC drops was 25% tissue weight/saline volume.

**Animals**

C57BL/6 male mice, 6- to 10-weeks-old, were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA) and were handled according to the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal protocol (No. B2015-73) was approved by the Institutional Animal Care and Use Committee at Tufts University and Tufts Medical Center (Boston, MA, USA). Up to five mice were kept in one cage at a time with their ears tagged according to a standard method for numerical identification. All mice were monitored daily for body weight and physical activity, of which the latter was graded as 0 = normal, 1 = reduced, 2 = poor, and 3 = moribund.

**Corneal Abrasion and Treatment**

The mouse model of corneal epithelial abrasion was created and modified from the methodology previously described. In brief, mice were anesthetized on day 0 with intraperitoneal injections of ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture. The cornea was analyzed with a slit lamp for any pathology and mice with any epithelial defect, scar, or neovascularization were excluded from the study. Topical 0.5% proparacaine hydrochloride (Akorn, Lake forest, IL, USA) ophthalmic solution was then applied. In order to standardize the epithelial defect size, a 2-mm trephine was used to mark the right corneas of each mouse. Then 5 μL of 0.1% (1 mg/mL) fluorescein solution was used to mark the area of trephination and washed with PBS. Next an Algebrush (The Alger Company, Inc., Lago Vista, TX, USA) was used to remove the corneal epithelium within the area of the trephination. Each cornea was then imaged with white light and blue filter right after for baseline imaging. Animals were kept warm with the aid of heating pads to facilitate thermoregulation during anesthesia recovery period.

The right eye of each mouse received 10 μL of AMUC (the treatment group, n = 24) or 10 μL of vehicle, 0.9% NaCl with 1.25 ug/mL Amphotericin B (the control group, n = 24), three times a day, every 6 hours (at 8:00 AM, 2:00 PM, and 8:00 PM, respectively), for 6 days. These 48 animals were subdivided into two sessions (i.e., 12 animals each group per session undergoing the same experiment). Postoperative pain was managed by subcutaneous injections of sustained release Buprenorphine (1.0 mg/kg; ZooPharm, Fort Collins, CO, USA).

**Measurement of Corneal Regularity and Epithelial Defect Size**

All mice were evaluated at 12 and 24 hours, and daily (every morning at 8:00 AM) post abrasion. Corneal epithelial regularity was assessed in the treated eyes by a Zeiss microscope (model: Stemi 305, Jena, Germany), which provides an illumination ring (roughly 1-mm diameter) projected onto the corneal surface. The distortion of the ring was used to estimate the corneal surface irregularity using a 5-point scale based on the number of distorted quarters in the reflected ring: 0 = no distortion (complete smoothness); 1 = distortion in one quarter of the ring; 2 = distortion in two quarters; 3 = distortion in three quarters; 4 = distortion in all four quarters; and 5 = severe distortion. At each time-point, images were taken by a Canon digital camera (×2.1 digital magnification; Melville, NY, USA) mounted on top of the microscope’s binocular. All images were coded and analyzed in a masked fashion.

The size of the corneal epithelial defect was then evaluated by applying 0.1% fluorescein solution and illuminated by blue filter light and imaged by the same camera and digital zoom (×2.1) mounted on a slit-lamp Topcon SL-1E (×16 magnification; Tokyo, Japan). All images were taken by the same total magnification (×33.6) with the same focal point (i.e., with a standardized distance). All images were shuffled and analysis was conducted in masked fashion using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) to measure the size of the epithelial defect. Complete (100%) epithelialization was defined by the lack of any fluorescein-staining.

**Immunohistochemistry and Hematoxylin & Eosin (H&E) Staining**

Corneas were harvested at the end of the study, then were fixed in acetone, frozen in optimal cutting temperature compound (OCT), and stored at −80°C until analysis. Frozen OCT blocks were either sectioned (6 μm) and mounted on glass slides or melted in room temperature for immunohistochemistry. Cornea sections were blocked with 5% donkey serum, 0.3% Triton X-100 in Tris-buffered saline (TBS) and stained with Keratin 12 primary antibody (Abnova, Jhongli, Taiwan) for 1 hour at room temperature. Sections were washed with TBS and incubated for 1 hour in secondary antibody (Rhodamine Red-X [RRX] AffiniPure Donkey Anti-Rabbit IgG; Jackson ImmunoResearch, Inc., West Grove, PA, USA). Whole-mount corneas were blocked in 3% BSA containing 1% anti-FcR monoclonal antibody for 1 hour at room temperature first and then incubated overnight at 4°C on the shaker with conjugated fluorescent antibodies against CD45 (pan leukocyte marker; PE anti-mouse CD45; Biolegend, San Diego, CA, USA) and beta-III tubulin (neuronal marker, NorthernLights NL657; R&D systems, Minneapolis, MN, USA). Corneas were washed three times in PBS for 10 minutes each, flattened with three radial incisions, and mounted on a glass slide. A mounting media containing 4% diamidino-2-phénylindole (DAPI) (nuclear staining) (Vectorshields; Vector Laboratories, Inc., Burlingame, CA, USA) was used before cover slipped.
FIGURE 1. A representative case to illustrate rapid epithelialization and restoration of corneal smoothness by AMUC eye drops. Representative pictures illustrate and compare epithelial defect at baseline (at time of epithelial debridement) and different time points after abrasion between AMUC- and vehicle-treated mice. For each animal, the corneal epithelial defect was evaluated using 0.1% fluorescein and cobalt blue light and corneal smoothness was evaluated via regularity of a reflected light off the corneal surface.
Hematoxylin and Eosin (H&E) staining was performed using an automatic histology stainer (Shandon Varistain Gemini; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA), according to the manufacturer’s protocol. Frozen cornea section slides were water washed first to remove the OCT then loaded inside the machine. Slides were stained with hematoxylin for 2 minutes and then were incubated with clarifier for 1 minute. Slides were then incubated with bluing reagent for 1 minute. Washed slides were then incubated for 1 minute in 95% alcohol and stained with eosin for 2 minutes. During the staining procedure, the slides were washed with running water before adding a new reagent. Hematoxylin and Eosin-stained slides were dehydrated in a 95% and 100% ethanol, respectively. Finally, the slides were dipped in xylene, and cover slipped.

Fluorescent-labeled slides were imaged using a confocal microscope (Nikon A1r; Nikon Instruments, Inc., Melville, NY, USA). Three different fields from the peripheral cornea and one central field were imaged in each cornea. Captured whole-stack images were analyzed with IMARIS software version 8.0 (Bitplane AG, Zurich, Switzerland) to calculate the number of CD45 (pan leukocyte marker)-positive cells in the whole cornea and Ki-67 (epithelial cell proliferation marker)-positive cells in the epithelium. The number of cells in peripheral corneal fields were averaged for each cornea. Three-dimensional (3D) images of corneal nerves were rendered using IMARIS software and stromal versus subbasal nerves were separated according to their anatomic location in the cornea. Stromal and subbasal corneal nerve images were traced separately using NeuronJ13 (in the public domain, http://www.imagescience.org/meijering/software/neuronj/), a free semiautomatic software. Total nerve length in each cornea was measured by averaging total nerve length in three different peripheral field images and in one central field image. Total nerve length in periphery and central cornea was reported as nerve density in millimeters per millimeters squared.

Hematoxylin and Eosin-stained slides were imaged using a bright field microscope (Nikon Eclipse E800). Four sections were selected and 2 to 4 areas from each section were imaged. Representative sections were selected and the thinnest and thickest part of the epithelium plus the area of epithelium were measured in each cornea section by using Imagej software.15

### Statistical Analysis

Summary of the data were reported as mean ± SD. Descriptive statistics for continuous variables were analyzed using SPSS software, version 24.0 (SPSS, Inc., Chicago, IL, USA). Differences between the AMUC and control group were analyzed by the Student’s t-test, and the χ² test. Correlation between parameters was analyzed by linear regression using Excel (Microsoft Corp., Seattle, WA, USA). A P value less than 0.05 (P < 0.05) was considered statistically significant.

### Results

In this study, we demonstrated that topical AMUC drops were effective in promoting corneal epithelial wound healing and restoring corneal surface regularity when compared with the vehicle control. Such a difference was illustrated in the composite of a representative case (Fig. 1).

Corneal abrasion created a comparable initial epithelial defect size of 3.15 ± 0.03 mm² in the AMUC treatment group and 3.14 ± 0.03 mm² in the control group (Fig. 2, P = 0.2). After abrasion, the AMUC treatment group had a significantly smaller defect area than the control group at 12 hours (P = 0.002), day 1 (P = 0.016), and day 2 (P = 0.04) (Fig. 2). Consequently, the time to achieve complete corneal epithelialization was 3.15 ± 1.44 days for the AMUC group, but 4.00 ± 1.63 days for the control group (P = 0.06). Moreover, the AMUC treatment group had a higher incidence of complete healing at each time-point. In fact, 4.2% of the AMUC group had healed within 12 hours, whereas none of the control group had healed. No additional mice had healed by day 1 in any group but by day 2, 41.7% of the AMUC group experienced complete healing, whereas only 20.8% of the control group had completely healed (P = 0.213).

Spearman correlation showed that the epithelialization was significantly correlated with time (r = −0.8, P < 0.001), treatment groups (r = 0.17, P < 0.001), and corneal surface regularity (r = −0.11, P = 0.04). A statistical model was then established by multivariable linear regression to determine the impact of various factors on corneal re-epithelialization and it was identified that time (B-value, −0.47; 95% confidence interval [CI], −0.51 to −0.43, P < 0.001), treatment groups (B-value, −0.24; 95% CI, −0.4 to −0.078, P = 0.004), and corneal regularity (B-value, −0.1; 95% CI, −0.18 to −0.03, P = 0.009) were significant predictive factors. The predictive equation for
corneal epithelial defect size was: $2.39 \pm 0.47 \text{ (healing days)} - 0.24 \times \text{treatment group (AMUC group, 1; control group, 0)} - 0.1 \times \text{corneal regularity (distorted quarter as 0 to 5)}. $

To determine mechanistic understanding of the above findings, Ki-67 staining was used to demonstrate corneal epithelial proliferation (Fig. 4A). Results showed the number of Ki-67-positive cells was significantly greater in the AMUC group compared with the control group in central ($P = 0.025$) but not peripheral ($P = 0.624$) corneal epithelium. Because corneal nerves are known to play a role in epithelial proliferation and differentiation, we also evaluated stromal and subbasal nerve densities by immunostaining of corneal flat mounts (Fig. 5). Confocal microscopy showed total nerve density was higher in the AMUC group compared with the control group and this was shown in both the stromal and subbasal nerve plexus, but the difference did not reach a statistical difference. The increased epithelial proliferation and nerve density also coincided with decreased inflammation in the AMUC group (Fig. 4B). Inflammatory cell infiltration as judged by CD45+ cells was significantly reduced in the peripheral ($P = 0.022$) but not in the central ($P = 0.384$) cornea. Lastly, H&E and K12 staining confirmed partial restoration of normal stratified corneal epithelial layers in both groups without any significant differences (Fig. 6).

As a way of gauging the well being of animals undergoing the aforementioned treatments, we also monitored the body weight and physical activity of the mice every day throughout the study. The results showed that there was no statistically significant difference in the body weight change between AMUC-treated mice and the controls at any time-point. However, compared with the baseline, both groups experienced a statistically significant body weight loss by Day 3 ($P < 0.05$). After Day 3, the mice in both groups regained the body weight each day. Only the control group experienced a significant weight loss on days 2, 4, and 5 compared with baseline. We also did not observe any difference in their physical activity during the entirety of the experiments although one mouse in the control group had reduced activity at 12 hours post abrasion.

**DISCUSSION**

Traditional treatments for epithelial defects include topical antibiotics and corticosteroids, of which the former might interfere with epithelial wound healing and the latter might delay wound healing or cause glaucoma and cataracts. To
circumvent these shortcomings and fulfill such an unmet need, one potential new therapy might be AMUC drops, which were developed under the premise that cryopreserved AMUC grafts have been successfully used to treat a number of ophthalmic indications, as well as in other nonophthalmic clinical indications. In this model of murine corneal abrasion, we noted that topical AMUC indeed promoted corneal epithelialization faster than the vehicle control as evidenced by a significantly smaller corneal epithelial defect at 12 hours ($P = 0.002$), 1 day ($P = 0.016$), and 2 days ($P = 0.04$) post abrasion, a lesser (though not statistically significant) time to complete epithelialization (3.15 ± 1.44 vs. 4.00 ± 1.63 days, $P = 0.06$) (Fig. 2), and a notably higher incidence of complete corneal smoothness (Fig. 3). In fact, Spearman correlation analysis showed that epithelialization was significantly correlated with treatment groups ($P < 0.001$), time ($P < 0.001$), and corneal regularity ($P = 0.04$). These results collectively might suggest a better visual quality outcome with a lower refractive error and a less likelihood of developing microbial infection and other corneal morbidities that are usually accompanied by prolonged or persistent corneal epithelial defects. This notion is also supported by our recent report of successfully treating persistent corneal epithelial defects by topical AMUC in four human patients that presented with cicatricial ocular surface diseases. The accelerated wound healing in the AMUC group was supported by a significant increase of Ki-67$^+$ cells in the central corneal epithelium and a nonsignificant increase of corneal nerve density (Figs. 4, 5).

Previously, it has been reported that corneal epithelial damage, even without stromal involvement, elicits three general phases of healing characterized by an initial lag phase lasting for 6 hours, followed by epithelial (basal and suprabasal) migration and proliferation. In this specific murine model, corneal epithelial injury evokes two waves of neutrophil migration initiated from the limbal vessels within 6 hours and peaking at 12 to 18 hours and 30 to 36 hours, respectively. It has also been shown that $T$ cells are necessary for the localization of these neutrophil and together promote efficient epithelial repair by promoting cytotoxic effects on activated macrophage. Hence, we speculate that one plausible mechanism for AMUC to promote faster epithelialization is through anti-inflammatory actions. This is supported by the finding that CD4$^+$ (inflammatory) cells were significantly decreased in the AMUC group. Additionally, the finding that AM's therapeutic efficacy can be delivered by AMUC drops strongly suggests that the benefit is not based on a structural scaffold alone, but also through a soluble component in the tissue. One such candidate might be HC-HA/PTX3, which consists of high molecular weight hyaluronic acid (HA) covalently linked to heavy chain (HC)-1 and tightly associated with pentraxin 3 (PTX3).
has been shown to possess anti-inflammatory action against activated but not resting neutrophils, macrophages, and lymphocytes. Unlike all conventional anti-inflammatory agents such as glucocorticosteroids, nonsteroid anti-inflammatory agents, cyclosporine/tacrolimus, or various humanized antibodies that specifically target one action from one particular type of inflammatory/immune cell, HC-HA/PTX3 exerts broad anti-inflammatory actions targeting inflammatory cells extending not only from innate but also to adaptive immune responses. HC-HA/PTX3 also mediates a direct antiscarring effect on ocular tissue fibroblasts by suppressing TGF-β signaling, and uniquely maintains the phenotype of limbal niche cells to support the quiescence of limbal epithelial stem cells toward regeneration. Collectively, these actions explain why morselized AMUC drops remain effective in downregulating the inflammatory response and promoting stem cell proliferation toward regenerative healing.

The overall efficacy of AMUC might also be aided by the gel formulation. The AM, and even more so the UC, is composed of a high content of high molecular weight HA that is known to maintain better hydration and lubrication that can protect the ocular surface from dryness and blink-related microtrauma during wound healing. Gel formulations also potentially increase precorneal residence time and improve bioavailability to reduce washout during blinking and lachrymation. These attributes should also be useful in other ocular indications, such as neurotropic keratopathy, burns, dry eye, and post-refractive surgery.

In conclusion, our results show topical AMUC eyedrops are effective in promoting corneal epithelialization and smoothness. We also noted that topical AMUC does not cause any adverse events as evidenced by the lack of any significant change of body weights and physical activities. Collectively, these preclinical safety and efficacy data warrant future controlled clinical trials to determine if topical AMUC represents a novel biologic to mitigate clinical morbidity associated with prolonged corneal epithelial defects.

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