Stepwise Surgical Approach for in vivo Expansion of Epithelial Stem Cells to Treating Severe Acute Chemical Burns with Total Limbal Deficiency

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To describe the clinical outcome of a new surgical treatment for the acute stages of severe corneal burn injury and its complications, a prospective study of five acute corneal burn patients with severe limbal damage was performed. Amniotic membrane transplantation (AMT) and conjunctival limbal autograft (CLAU) was performed at the acute stage of corneal burn injury to reconstruct the damaged ocular surface (step I). Three to six months later, the opaque central part of the amniotic membrane containing in vivo grown corneal stem cells were removed and re-transplanted to the defect created after the removal of pseudopterygium (step II). All injured eyes were successfully treated, but in one eye with marked stromal lysis, three-layered AMT and penetrating keratoplasty with re-transplantation of in vivo grown corneal stem cells was performed. In the former cases, visual acuity was greatly improved more than three lines (ranging from 3 to 12 lines). In short, re-transplantation of in vivo grown corneal stem cells after AMT and CLAU is a recommendable modality for restoring a stable corneal epithelium of a severely burned ocular surface in the acute stage and can be considered a preventative measure for avoiding late onset complications.

Key words: amniotic membrane transplantation, chemical burn injury, in vivo grown corneal stem cells

INTRODUCTION

Following acute chemical burns, the cornea frequently manifests several pathologies including persistent epithelial defects, stromal ulceration, and intense inflammation, which collectively may result in severe visual loss. Inflammatory cytokines and oxidative stress may play a role in tissue destruction and ulceration. These will result in significant ocular morbidity and a poor long-term visual prognosis. For mild burns, conventional therapies such as pressure patch, topical antibiotics, a mydriatic-cycloplegic, topical steroids, collagenase inhibitors, and therapeutic soft contact lenses may be available. But these procedures do not adequately address severe burn injuries. The management of total corneal epithelial defect, ongoing inflammation, and stromal ulceration continues to pose a clinical challenge.

Amniotic membrane (AM), the innermost layer of the placenta, consists of a thick basement membrane and an avascular stroma. Amniotic membrane trans-
plantedation (AMT) is currently being applied to a widening spectrum of ophthalmic diseases. It can be used as a substrate for reconstructing corneal and conjunctival surfaces damaged by various ocular surface disorders. In our previous study, we described AM patching as an effective method for treating acute alkali burns. However, severe cases (burn grade ≥ 3, Roper-Hall classification) frequently progress to visual loss due to vascularization, scarring of the stroma or an overgrown pannus that covers the whole cornea. Also, because of the considerable risk of complications, no effective therapy has yet been developed.

Therefore, a new strategy for managing severe corneal burn and preventing late complications should be researched. Here, we report the re-transplantation of in vivo grown corneal stem cells on AM after AMT and CLAU for a new surgical approach to treating severe corneal burn with total limbal deficiency.

**PATIENTS AND METHODS**

**Patients**

We studied 5 patients (5 eyes) with whole corneal epithelial defects and severe limbal damages from acute cornea burn injuries (burn grade ≥ 3). They were managed at the Ophthalmology Department of Chung-Ang University. Two patients had alkali burns, 2 had acid burns, and the remaining 1 patient had a thermal burn. Surgery for these patients were approved by the institutional review boards. The cause of injury, burn grade, surgical procedures, follow-up period, visual acuity, and final outcome of each patient was obtained and recorded. Surgical success was defined as a clear cornea with no complications and improved visual acuity. Failure was defined as persisting corneal epithelial defects, visual loss, corneal opacity or neovascularization formation.

**Human amniotic membrane preparation**

Human amniotic membrane tissue was obtained, processed and preserved as previously reported, but with minor modification. In brief, a human placenta was obtained shortly after elective cesarean deliveries. All human amniotic membrane donor tissues were tested and were serologically nonreactive for the human immunodeficiency virus, human hepatitis type B and C and syphilis. Under a lamellar flow hood, the placenta was cleaned of blood clots with sterile saline solution containing 50 μg/ml penicillin, 50 μg/ml gentamicin, 100 μg/ml neomycin and 2.5 μg/ml amphotericin B. The amniotic membrane was separated from the remaining chorion by blunt dissection and flattened onto a nitrocellulose membrane with the epithelium/base- ment membrane surface up. The paper with the adherent amniotic membrane was stored at −70°C in a sterile vial containing DMEM (Dulbecco’s Modified Eagle Medium, Gibco) and glycerol in a 7:3 (v/v) ratio prior to transplantation. When used, the amniotic membrane was removed from the storage medium and peeled from the nitrocellulose filter paper, after which it was transferred to the eye and fitted to cover the wounded cornea.

**Surgical techniques**

Patients were anesthetized with a peribulbar block supplemented by intravenous sedation. The abnormal corneal epithelium and the superficial fibrovascular scar tissue were debrided by blunt dissection, and conjunctival peritomy was performed at 360°. The AM was then spread over the whole cornea with the basement membrane side facing up, and secured by a purse-string 10-0 nylon running suture onto the surrounding conjunctiva. In cases of marked stromal tissue loss and corneal thinning, multilayered AM grafts were applied to fill the ulcer cavities. The autologous limbal-conjunctiva tissue was harvested from the patient’s contralateral normal eye (2-3 mm × 6-10 mm) and autografted to the limbal area of the burned eye with interrupted circumferential sutures to anchor the graft to the underlying AM (Fig. 1). Finally, a large piece of the AM was applied over the entire cornea as a temporary patch (Step I). After 3 days, the temporary amniotic membrane patch (TAMP) was removed from the cornea.

Three to six months after the aforementioned step I operation, late onset complications such as pseudopterygium and central AM opacification may still occur. If this is the case, step II procedure is performed to deal with these complications and to
Fig. 1. Schematic drawing showing the procedure of step I operation: Amniotic membrane transplantation to the whole cornea with limbal-conjunctival autograft (CLAU), anchoring with 10-0 nylon suture.

Fig. 2. Schematic drawing showing the procedure of step II operation: Note the re-transplantation of in vivo grown limbal epithelial cells on amniotic membrane to the site of removed pseudopterygium. The defect created after the removal of pseudopterygium (Fig. 2). The last piece is used for light and electron microscopic examination. AM patching was also applied to cover those denuded corneal surfaces.

Postoperatively, antibiotic eyedrops, corticosteroid eyedrops, and sodium hyaluronate eyedrops were administered four times a day. During the same period, autologous serum drops and artificial

Table 1. Patients data and surgical outcome

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex/ Age</th>
<th>Eye</th>
<th>Underlying disease</th>
<th>Burn Grade</th>
<th>Follow-up Period (month)</th>
<th>Surgical management</th>
<th>Period between step I and II (month)</th>
<th>Visual acuity</th>
<th>Preoperative</th>
<th>At last Follow-up</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M/43 OD</td>
<td>Alkali burn (Lime)</td>
<td>4</td>
<td>20</td>
<td>AMT+CLAU +TAMP</td>
<td>Remov-IGSA and reT+TAMP</td>
<td>4</td>
<td>HM</td>
<td>20/20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 M/35 OS</td>
<td>Alkali burn (Ammonia)</td>
<td>3</td>
<td>28</td>
<td>AMT+CLAU +TAMP</td>
<td>Remov-IGSA and reT</td>
<td>3</td>
<td>0.04</td>
<td>20/30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 M/38 OS</td>
<td>Acid burn (Sulfuric acid)</td>
<td>3</td>
<td>31</td>
<td>AMT+CLAU +TAMP</td>
<td>Remov-IGSA and reT+TAMP</td>
<td>3</td>
<td>0.08</td>
<td>20/40 Mild ectasia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 M/40 OD</td>
<td>Acid burn (Sulfuric acid)</td>
<td>4</td>
<td>39</td>
<td>Multi-layered AMT+CLAU</td>
<td>PKP+ECCE+IOL+reT-IGSA +TAMP</td>
<td>6</td>
<td>HM</td>
<td>20/50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 M/39 OD</td>
<td>Thermal burn (Boiled water)</td>
<td>4</td>
<td>22</td>
<td>AMT+CLAU +TAMP</td>
<td>Remov-IGSA and reT+TAMP</td>
<td>4</td>
<td>LP (+)</td>
<td>20/60</td>
<td></td>
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</table>

tears were administered nine times a day.

RESULTS

The preoperative data, clinical presentation, and surgical outcome are summarized in Table 1. The average age was 39 ± 2.9 (ranging from 35-43) years. The mean follow-up period was 28 ± 7.6 (ranging from 20-39 months) months. Every subject had severe corneal burn injuries (Burn grade 3: 2 eyes, grade 4: 3 eyes), and AMT using CLAU (Step 1) was successfully completed in all cases without any acute complications. Using an electron microscope, we observed that such expanded epithelium on AM consisted of poorly differentiated corneal epithelium and incompletely formed hemidesmosomes in specimens taken at one month. However, at 3 months, it exhibited a well-differentiated multilayered corneal epithelium with firm hemidesmosomes and adhesion complexes (Fig. 3). Central corneal opacity and peripheral pseudopterygium developed within 6 months after Step I in all cases. The mean interval between Step I and Step II was 4 ± 1.2 (ranging from 3-6 months) months. All eyes healed successfully with epithelialization 3 to 7 days after Step II, with improved visual acuity (better than 20/100).

Representative cases

Alkali Burn. Case 1. A 43-year-old male suffered a lime burn in his right eye 14 days before his referral. Slit lamp biomicroscopy revealed persistent corneal ulceration with severe limbal damage (burn grade 3.5) (Fig. 4A). His visual acuity was hand
Fig. 4. A: Slit lamp view of a 43-year-old male presented with a diffuse corneal edema and ulceration after alkali burn. B: Two weeks after step I procedure (AMT with CLAU), the ocular surface was epithelialized, and maintained relatively clear. C: 3 days after step II procedure, in vivo grown limbal epithelial cells graft (arrow) was stable. D: Three month after step II procedure, clear cornea was evident and vision was much improved (20/20).

The central ulcer was carefully debrided. AMT, CLAU, and TAM (temporary amniotic membrane patch) were performed as the first step of the operation in reconstructing the damaged ocular surface (Fig. 4B). Satisfactory re-epithelialization of the transplanted AM appeared 6 days after the operation, and his visual acuity was improved to 20/30. Four months after the operation, he complained of blurred vision. A slit lamp examination revealed central cornea opacification associated with peripheral pseudopterygium invasion. We thus performed the second step of the operation by removing the central part of the in vivo grown corneal stem cells on AM and re-transplanting it to where the pseudopterygium was removed (Fig. 4C). TAM was performed to cover the denuded corneal surface and was removed 2 days later. The epithelium on the wounded cornea was completely healed 3 days after the operation. The follow-up examination after 3 months showed a transparent and stable ocular surface with improved visual acuity of 20/20 (Fig. 4D).

Acid Burn. Case 4. A 40-year-old male suffered an accident in which his right eye was exposed to 90% sulfuric acid. He had a peripheral wedge-shaped breakage and persistent epithelial defects with stromal melting (burn grade 4). He was initially managed in a local clinic for 20 days with conventional methods and without operation. Multilayered (3 layers) AMT were applied to cover the groove-like thinning of the cornea, and CLAU was performed simultaneously. Six months later, the transplanted AM and cornea were opacified with minimal peripheral neovascularization and cataract
Fig. 5. A: Slit-lamp biomicroscopic view of a 37-year-old male who had 90% sulfuric acid burn. 6 months after the step I procedure, corneal opacity with peripheral neovascularization developed. B: PKP; ECCE+IOL; retansplantation of IGEA and TAMP were applied to the patient. A stable ocular surface with improved visual acuity (V:A = 0.2) was evident at the end of follow-up (39 months).

development (Fig. 5A). Penetrating keratoplasty, extracapsular cataract extraction, and intraocular lens implantation, combined with the removal and re-transplantation of the central part of in vivo grown corneal stem cells on AM were performed. TAMP was applied to cover the whole cornea. After 39 months of follow-up, the patient’s visual acuity was improved to 20/60 with a clear, smooth, intact corneal epithelial surface (Fig. 5B).

**DISCUSSION**

The pathogenesis of acute corneal chemical burns is quite complex and often results in long-term complications such as pseudopterygium, corneal opacity, and symblepharon. Several proteinases including metalloproteinase, plasminogen activator, and latent collagenase released from the damaged cornea play pivotal roles in cornea ulceration. Infiltration of polymorphonuclear leukocytes (PMNs) and the various proteinases they release contribute to early inflammatory-mediated irreversible damages.

The epithelial defect facilitates the penetration of PMNs into the corneal stroma from the tear film to elicit acute inflammation, which leads to tissue degradation or scar formation. Therefore, it is desirable to achieve rapid epithelialization, suppress acute inflammation, and prevent tissue degradation and scar formation.

It is well known that AM can inhibit inflammation by regulating protease activity in alkali burned cornea. AM contains several proteinase inhibitors and also suppresses inflammatory genes (iNOS and its related genes and Matrix metalloproteinases — II, VI) in ex vivo keratocyte culture systems. The AM patch has proved to be a viable alternative method for treating chemical burns with minimal limbal damage. But, when the loss of limbal and stromal cells is increased with total corneal epithelial defects, AMT and CLAU should be adopted as described in Step I. This procedure involves two major strategies: The CLAU restores the stem cell population and the AMT restores the damaged basement membrane and stroma.

The value of AM is in its ability to restore an intact basement membrane that is invariably damaged in severe burn injuries to the cornea. Basement membrane is known to support epithelial cell adhesion, differentiation, and migration, and suppresses epithelial cell apoptosis. AM basement membrane is an ideal substrate for supporting the growth of epithelial progenitor cells by prolonging their lifespan and maintaining their clonogenicity. AM also facilitates epithelialization as a bandage contact lens protecting the migrating epithelial cells from the windshield wiper action of the eyelids.

In our study, we found that in vivo expansion of corneal epithelium on AM consisted of well differentiated multilayered corneal epithelium with firm hemidesmosomes and adhesion complex at 3
months. This finding justifies the Step II procedure, in which \textit{in vivo} expanded corneal epithelial cells together with AM can be removed and retransplanted to the defect created after the removal of pseudopterygium. The advantage of a limbal tissue \textit{in vivo} culture is that tightly-attached and well-formed limbal epithelial cells can then be harvested on AM. Details concerning the longevity, motility, and adherence of the autologously \textit{ex vivo} grown epithelial cells of the host eye are yet unknown.\textsuperscript{25-27}

Therapeutic goals in acute corneal burn injuries must be directed towards healing and preventing late complications. Three to 6 months after AMT and CLAU, the opacification of the central part of the AM and the formation of pseudopterygium usually occur. AM always dissolves with corneal epithelial down-growth. Central opacity is caused by epithelial proliferation and activated inflammatory response (Data not shown). The dissolved AM components induced an inflammatory reaction and initiated the proliferation of more epithelial cells with neovascularization. The growing pseudopterygium may also lead to inflammation and contributes to the central opacity.

Previous studies showed the stroma of AM to contain a unique matrix component that suppresses TGF-\(\beta\) signaling and prevents the proliferation and myofibroblast differentiation of normal human corneal and limbal fibroblasts\textsuperscript{28} in addition to normal conjunctival and pterygium body fibroblasts.\textsuperscript{29} AM suppresses the mixed lymphocyte reaction, considered to be the \textit{ex vivo} equivalent of the delayed hypersensitivity reaction.\textsuperscript{30} The expression of proinflammatory cytokines, including interleukin-1\(\alpha\) and -1\(\beta\), are reduced when corneal epithelial cells are cultured with, rather than without, AM.\textsuperscript{31} These actions may explain why this procedure prevents the recurrence of pterygium\textsuperscript{32-34} and why corneal neovascularization is mitigated.\textsuperscript{19}

Considering the poor ocular prognosis and complications of corneal opacity, neovascularization, and visual loss, this new surgical treatment may prove to be the future treatment of choice for patients with corneal epithelial defects and severe limbal damage in the acute stages of severe burn injuries. Our report also shows the beneficial effects of \textit{in vivo} cultured corneal epithelial cells on human amniotic membrane as a sheet for reconstructing the damaged ocular surface.

**REFERENCES**


