Xeno-Free Autologous Cultivated Oral Mucosal Epithelial Transplantation for Bilateral Ocular Surface Burns: Clinical Outcomes and Immunohistochemical Analysis

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Abstract

Purpose: To report the clinical and phenotypic findings following autologous cultivated oral mucosal epithelial transplantation in eyes with ocular surface burns in a retrospective case series. Methods: This study included 19 eyes of 18 patients with bilateral limbal stem cell deficiency following ocular burns treated between 2007 and 2010. All patients underwent an oral mucosal biopsy, following which the oral epithelium was cultivated on de-epithelized human amniotic membrane using a xeno-free explant culture technique. A monolayer of cultivated oral epithelium was transplanted onto the patient’s ocular surface. Post-operative ocular surface stability, corneal avascularity and visual improvement were assessed. From five eyes that subsequently underwent keratoplasty or keratoprosthesis surgery, the excised corneal tissue was subjected to histopathology and immunohistochemical analysis. Results: The mean follow-up was 22.3 months. All transplanted eyes showed superficial corneal vascularization by 3 months. A stable ocular surface was seen in 7 (37%) eyes at the end of one year. Vision did not improve in 12 (63%) eyes, vision improved from hand motions to counting fingers in 6 (32%) eyes and to 20/125 in one (5%) eye. Histopathology of excised corneal tissue showed six to eight layers of epithelial stratification and absence of goblet cells. Immunohistochemical analysis of the transplanted epithelium showed expression of p75, p63, suprabasal K19 and K3 and absence of K12, K14. Conclusions: Clinical outcomes of autologous cultivated oral mucosal epithelial transplantation in eyes with ocular surface burns were poor and the transplanted cells maintained the oral phenotype on the corneal surface.

Limbal stem cell deficiency (LSCD) is a rare cause of corneal blindness which results from physical, chemical or immunological damage to the corneal epithelial stem cells located at the limbus1,2. In unilateral cases LSCD can be treated by either conventional or cultivated autologous limbal transplantation from the unaffected fellow eye3,4. However, in bilateral cases there is no autologous source for limbal stem cells and either a living or a cadaveric allogeneic donor is required5. An alternative to allogeneic limbal grafting, which necessitates long-term systemic immuno-suppression, is transplantation of autologous epithelium from non-ocular sources.

The possibility of oral mucosa being used as a substitute for limbal epithelium was considered because of the phenotypic semblance between the two epithelial lineages6,7. Animal trials and preliminary human trials also demonstrated that the ex-vivo cultivated oral mucosa could be a suitable therapeutic alternative to limbal epithelium in eyes with LSCD8-12. However the cell culture protocols described for cultivating oral mucosal cells for human transplantation utilized various animal derived or xeno-biotic materials13-19. Use of xeno-biotic materials in cell culture for clinical use is undesirable as it carries the risk of transmitting known or unknown infections to the transplant recipient20. To avoid xeno-biotic usage, we developed a xeno-free technique of culturing oral mucosal cells,6 adopted from our standardized protocol for limbal epithelial cultivation21, which has been used successfully to treat over 500 eyes with unilateral LSCD22-24. In this study we report the clinical outcomes and immunohistochemical findings in eyes with LSCD following ocular surface burns,
treated by xeno-free autologous cultivated oral mucosal epithelial transplantation (COMET).

Methods:

Patients: At the LV Prasad Eye Institute, Hyderabad, India autologous COMET was offered as an alternative to allogeneic cultivated limbal epithelial trans-plantation, between October 1, 2007 and November 1, 2010, to patients with bilateral and total LSCD (defined clinically as 360° superficial corneal vascularization, diffuse fluorescein staining of the corneal surface with or without persistent epithelial defects, conjunctivalization of the corneal surface and absence of limbal palisades of Vogt) following ocular surface burns. The Institutional Review Board approved of this pilot study for 20 eyes (LEC 06003) and recommended the following exclusion criteria to be applied before enrolment: (a) patients with LSCD due to unknown causes or causes other than ocular surface burns; (b) patients who had bilateral but partial LSCD; (c) patients with total LSCD, but with dry eye disease (Schirmer's test without anesthesia of <10 mm at 5 minutes) or keratinisation of the ocular surface epithelium; (d) patients with no visual potential as determined by clinical examination and electrophysiological testing (flash visual evoked potential and flash electroretinogram); (e) patients with untreated concurrent ocular problems, such as glaucoma and infection.

Data Collection: The data retrieved from the medical records included age and sex of the patient, type and date of injury, details of prior ocular procedures, Snellen's best spectacle corrected visual acuity (BCVA) and at each follow-up visit, presence or absence of lid abnormalities, dry eye disease, symblepharon, degree of limbal involvement, intra-operative surgical details, post-operative complications, duration of follow-up and status of ocular surface at each visit (slit-lamp findings including fluorescein staining).

Surgical Technique of Oral Mucosal Biopsy: All patients underwent an oral examination by a physician to rule out any contraindications to a mucosal biopsy. The patients were advised 5% povidone-iodine mouth wash twice daily for 3 consecutive days prior to the biopsy. After confirming satisfactory oral hygiene, an oral mucosal biopsy of 3 x 3 mm was obtained under local anesthesia (2% xylocaine sub-mucosal infiltration) from the inner surface of the patient's lower lip. The biopsied area was left bare and the patient was advised to continue the mouth wash for one week following the biopsy. The oral biopsies were performed by one surgeon (MR).

Technique of Oral Mucosal Epithelial Cultivation: The tissue was transported to the laboratory in human corneal epithelium (HCE) medium, which has been described previously.21-24 Briefly, HCE medium composed of minimal essential Eagle's medium (Sigma, cat. no. M0644) with alpha modification/Nutrient mixture (Sigma, cat. no. I2643), HAM's F12 medium (1:1) containing 2 mM L-glutamine (Sigma, cat. no. G6392), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma, cat. no. P4333), 2.5 µg/mL amphotericin B (Sigma, cat. no. A2942), 10 ng/mL human recombinant epidermal growth factor (Sigma, cat. no. E9644) and 5 µg/mL human recombinant insulin (Sigma, cat. no. I2643) along with 10% (vol/vol) autologous serum. Under strict aseptic conditions, human amniotic membrane (hAM) was prepared and preserved by our eye bank, measuring 3 x 4 cm was de-epithelialised using TrypLE (Invitrogen, cat. no. 12604) and 0.25% EDTA (Sigma, cat. No. E5134) solution after incubating at 37°C for 30 minutes. The patient's oral mucosal tissue was divided into small pieces after separation from the underlying connective and minor salivary glands. The tissue bits were explanted over the de-epithelialized hAM, epithelial side-up. A similar parallel culture was also prepared as a backup. A submerged explant culture system was used without a feeder cell layer was used. The culture was incubated at 37°C with 5% CO2 and 95% air in HCE media (Thermo Fisher Scientific, model: 371). The growth was monitored daily under an inverted phase contrast microscope (Olympus, CX40) and the HCE medium was changed every other day. The culture was transplanted when a monolayer of the cells growing from the explants became confluent, typically in 15 to 19 days. The laboratory cultures were performed by one experienced cell biologist (SG).

Technique of Cultured Oral Mucosal Epithelial Transplantation: Any symblepharon which prevented adequate separation of the lids was released to permit the insertion of a wire speculum (no additional surgery to treat the symblepharon was performed). A peritomy was performed and the corneal fibrovascular pannus was excised, fixed in 10% formaldehyde solution and sent for histopathological analysis. The hAM and monolayer of cultivated oral mucosal epithelial cells was spread over the cornea, epithelial side up. Using a sutureless technique the graft was secured to underlying ocular surface with fibrin glue (TISSEEL™ Kit from Baxter AG, Austria) and
the margins of the graft were tucked under the surrounding conjunctival edge. Bandage contact lenses were not applied at the end of surgery. The transplantations were performed by the one experienced ocular surface surgeon (VSS).

**Postoperative Treatment Regimen:** All the recipient eyes received topical prednisolone acetate 1% eye drops 8 times daily, tapered gradually based on the level of inflammation and ciprofloxacin 0.3% eye drops 4 times daily in the first post-operative week or until complete epithelization was noted.

**Follow-up Schedule:** All patients were seen on post-operative day one, at one week, at six weeks, and thereafter every six to eight weeks. Each examination included a complete history, including any new ocular or systemic symptoms, a complete ocular examination including fluorescein staining, and any signs of neovascularization or surface instability. The post-operative clinical assessment was performed by one ocular surface specialist (SB).

**Primary and Secondary Outcome Measures:** Based on the clinical appearance of the corneal surface an impression of success or failure of therapy was made. Success was defined as a totally epithelized, stable and avascular corneal surface. Failure was defined as appearance of any superficial corneal vascularization (even if the corneal surface was epithelized and stable), epithelial defects lasting more than two weeks and conjunctival overgrowth on the cornea (conjunctivalization). The secondary clinical outcomes were improvement in BCVA from baseline and ocular and oral complications.

**Additional Surgery:** Either penetrating keratoplasty (PK) or Boston Type 1 keratoprosthesis was performed in eyes with a stable ocular surface (irrespective of superficial vascularization) but poor visual improvement attributed to corneal stromal scarring. The corneal tissue excised during PK or keratoprosthesis surgery was fixed in 10% formaldehyde and processed for histopathology and immunohistochemistry analysis as described below.

**Hematoxylin-Eosin, Periodic Acid Schiff (PAS) staining:** The pannus excised at the time of COMET, the unused back-up culture and the corneal button excised at the time of keratoplasty/ keratoprosthesis were fixed in 10% buffered formalin, embedded in paraffin and serial sections of 5 µm thickness were taken on silane-coated glass slides. Sections were deparaffinized, rehydrated with distilled water and stained with hematoxylin and eosin and Schiff's reagent and observed under light microscope.

**Immunohistochemistry:** The primary antibodies, anti-K3/12, anti-K14 and anti-K19 were procured from Chemicon, anti-p75 from Abcam, anti-p63 from Thermo Scientific, anti-Ki67 from Dako, while the anti-K19 and immunohistochemistry (IHC) developing reagents were purchased from BioGenex. The serial sections of the unused back-up culture and the excised corneal tissue were de-paraffinized and rehydrated, blocked for endogenous peroxidase using 3% H2O2 in methanol. Antigen retrieval was done using citrate buffer (pH-6.0) in a microwave for 15 minutes and allowed to cool to room temperature. Blocking was done using 2.5% bovine serum albumin in 1x phosphate buffered saline (PBS) before primary antibody incubation at room temperature for one hour. HRP-conjugated secondary antibody incubations and IHC developing was done as per manufacturer's instructions (BioGenex). The samples were counterstained with hematoxylin, mounted in a resinous mounting medium and observed under a light microscope. Cadaveric human conjunctival and corneal tissue obtained from the eye bank and oral mucosal tissue obtained from voluntary human donors (SG, SB, MR, GKV, VSS) were used as controls. The histopathology and immuno-histochemical analysis was performed and interpreted by one experienced ocular pathologist (GKV).

**Result:**

**Demographics:** During the entire study period 19 eyes of 18 patients with bilateral and total LSCD following ocular surface burns underwent autologous COMET. The mean age at the time of surgery was 23.7± (12.5) years with male to female ratio of 2.8:1. The median time period between the initial injury and autologous COMET was 34 months (range: 6 to 240) months. Other pre-operative clinical characteristics of the transplanted eyes are provided in Table 1.

**Biopsy, Ex-vivo Cultivation and Transplantation:** Three patients underwent biopsy and trans-plantation under general anaesthesia, whereas others were operated under local anaesthetics. No anaesthetic or intra-operative complications occurred during either biopsy or transplantation. Following the biopsy no donor site complications were noted. The mucosal defect created on the lower lip following the oral biopsy completely healed by one week. In the laboratory, a confluent monolayer of cells formed on the denuded-hAM in a mean duration of 19.3 days (range 15 to 27 days). No cultures showed microbial contamination or inadequate growth.
Table 1: Preoperative clinical characteristics of the transplanted eyes

<table>
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<tr>
<th>Case</th>
<th>Age (Yrs)</th>
<th>Sex</th>
<th>Eye</th>
<th>Case of Injury</th>
<th>Gap between Injury to Previous Ocular Surgery</th>
<th>VA Pre-COMET</th>
<th>Lid Abnormalities</th>
<th>Symblepharon</th>
<th>PED</th>
<th>VA@3 months</th>
<th>VA@6 months</th>
<th>Surface Stability at 6 mnts</th>
<th>Outcome at 12 mnts (pk/kpro)</th>
<th>Total follow up (COMET-Sx time gap in months)</th>
<th>Outcome at last follow up</th>
<th>VA@Final FU</th>
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M=Male; F=Female; Allo-LT= Allogeneic Limbal Transplantation; PK= Penetrating Keratoplasty; AMG= Amniotic Membrane Grafting; VA=Best Corrected Visual Acuity; HM= Hand Movements; PL= Perception Of Light; CF= Counting Fingers; Kpro= Boston Type 1 Keratoprosthesis; TA+BCL= Tissue Adhesive And Bandage Contact Lens Application; Tarso= Tarsorrhapy; COMET= Autologous Cultivated Oral Mucosal Epithelial Transplantation
Primary Outcome: The mean follow-up was 22.3 (range: 7 to 48) months. Post-operatively on day one and at one week, fluorescein staining was negative over the grafted area and no folding or loosening of the hAM was noted. At six weeks all the grafted eyes had a completely epithelialized and stable corneal surface but absence of peripheral superficial corneal vascularization noted in 16 (84%) of 19 eyes. However, peripheral superficial corneal vascularization was seen in all eyes by three months. Therefore none of eyes met the clinical criteria of success at 3 months and thereafter. In 7 (36.8%) eyes the peripheral vascularization did not progress and the corneal surface was completely epithelialized and stable at 12 months after COMET. In the remaining 12 (63.2%) eyes the central cornea became progressively vascularized or developed persistent epithelial defects with recurrence or worsening of symblepharon between 3 and 9 months of COMET.

Visual Outcomes: Prior to COMET the BCVA ranged from hand movements to perception of light in all eyes. On the last date of follow-up or before undergoing keratoplasty or keratoprosthesis surgery the BCVA had not improved in 12 (63%) eyes, had improved to counting fingers in 6 (32%) eyes and to 20/125 (5%) in one eye.

Additional Surgery: Of the 7 eyes with a stable ocular surface, one eye underwent PK and four eyes underwent Boston type 1 keratoprosthesis surgery for visual improvement. Following PK the corneal graft developed repeated epithelial defects and a permanent tarsorrhaphy had to be performed three months later. Three years after PK the BCVA with an intact tarsorrhaphy was hand movements. The final BCVA in the four eyes that underwent Boston type 1 keratoprosthesis ranged from 20/20 to 20/30 with a maximum follow-up of 26 months.

Histopathology: (a) hematoxylin and eosin and PAS staining of the pannus excised during COMET showed eight to ten layer thick stratified columnar epithelium with presence of goblet cells and underlying loose fibrovascular stromal tissue. These findings were consistent the clinical impression of LSCD. (b) hematoxylin and eosin and PAS staining of the unused back-up oral mucosal culture showed a monolayer of epithelium on a thick eosinophilic membrane. (c) hematoxylin and eosin staining of the corneal buttons excised during keratoplasty or keratoprosthesis surgery following COMET showed a six to eight cell stratified epithelium with basement membrane. No remnants of the hAM were seen. Goblet cells were not observed in PAS staining. A few sub-epithelial blood vessels were also seen in close proximity to the basement membrane both at the periphery and at the centre. Bowman’s membrane was absent and variable stromal scarring was noted. The Descemet’s and endothelial complex was noted to be normal in all eyes.

Immunohistochemistry: (a) Immunohistochemical examination of the unused back-up culture showed: (1) Cytoplasmic K3/K12 expression was seen in all cells; (2) p63 expression was seen in all cells; (b) Immunohistochemical examination of the post-COMET corneal tissues and control corneal and conjunctival specimens showed: (1) K19 being expressed in the basal layer of the epithelial cells of post-COMET corneas, in the basal layer of the limbal epithelium in control corneas, in all layers of the conjunctiva and in the basal cells of the oral mucosa; (2) expression of K14 was absent in post-COMET corneas, absent in control corneas, present in the basal cells of conjunctiva, absent in oral mucosa; (3) Cytoplasmic K3/K12 expression was seen in all epithelial layers of post-COMET corneas, control corneas and oral mucosa but absent in conjunctiva; (4) Cytoplasmic K12 staining was seen only in the control corneal epithelium and was absent in oral, conjunctival and post-COMET epithelium; (5) Ki-67 expression was seen in the supra-basal layer of all specimens; (6) p63 expression was seen in basal and supra-basal layers of the post-COMET corneas, control corneas, conjunctiva and oral mucosa; (7) p75 expression was seen in basal epithelial cells of post-COMET corneas, basal epithelial cells of the limbus in control corneas, basal cells of conjunctiva as well as oral mucosa; (8) CD31 and CD34 expression was seen in sub-epithelial layers of the central and peripheral post-COMET corneas.

Discussion:

COMET emerged with the promise of being an autologous alternative to allogeneic cell based therapy in eyes with bilateral and total LSCD. Based on the experience with ex-vivo cultivation of limbal epithelial cells, we developed an efficient xeno-free explant culture technique of expanding the oral mucosal cells into a transplantable epithelial sheet on denuded hAM. We also noted that the cultivated oral mucosal epithelium had certain phenotypic similarities to limbal as opposed to conjunctival epithelium. These results along with the promising clinical outcomes of COMET reported by other groups encouraged us to attempt this present clinical study in 2007. However, as
elucidated in the results, the clinical experience with autologous COMET for the treatment of chronic ocular burns was extremely disappointing in terms of achieving ocular surface stability, corneal clarity, avascularity and visual improvement.

When we compare this study with the indications, laboratory techniques and clinical outcomes of previous studies on autologous COMET with a sample size of 9 or more eyes, it is noteworthy that: a) none of the previous studies used a xeno-free culture technique; b) the indications for COMET varied widely among different studies; c) all studies used clinical criteria for assessing the outcome of therapy; d) success rates with regards to ocular surface stability ranged from 28.5% to 100% with mean follow-up durations ranging from 12 months to 55 months; and e) all studies reported appearance of peripheral superficial corneal vascularization after COMET. In the context of this heterogeneous data, ocular surface stability achieved in our study (37% in 19 eyes) compares well with that reported by Satake and associates (36% in 11 eyes) and Burillon and associates (44% in 9 eyes) in eyes with ocular surface burns.

A comparison between this study and previous studies on COMET with those on allogeneic limbal transplantation is again difficult, because the indications and sample sizes vary among different studies. Indeed, there are no comparable published studies (with a sample size of five eyes or more) of allogeneic cultivated limbal transplantation in eyes with ocular burns. With regards to keratolimbal allografts, in two series of 16 and 17 eyes with ocular burns among other indications, Solomon and associates and Maruyama-Hosoi and associates reported long-term corneal epithelial stability in 71.3% and 58.8% eyes respectively. Similar to ocular surface stability, the proportion of patients who gained 20/200 or better vision, after keratolimbal allografting (43.5% to 44.6%) was also greater as compared to that after COMET (7% to 30%, Table 2). This limitation of COMET is particularly significant because unlike patients with unilateral LSCD, who usually have good vision in the unaffected eye and may be satisfied with a stable and symptom free ocular surface in the affected eye, the primary need of a patient with bilateral blindness is improvement in vision. Therefore the benefit of COMET of being an autologous therapy not requiring immunosuppression does not outweigh its poor clinical outcomes. In view of these results, currently we do not offer COMET to patients with bilateral LSCD.

Other findings of our study were similar to Chen and associates and Nakamura and associates who performed histopathology and immuno-histochemical analysis in four and six post-COMET eyes, respectively. On histopathology, they found the transplanted epithelium to be five to twelve layers thick without goblet cells or apical microvilli. On immunohistochemistry, they also found that K3 was present in all epithelial layers, K12 was present occasionally at the peripheral portion of corneal tissue, p63 and p75 was present in the basal epithelial layers. These findings along with ours suggest that the transplanted oral mucosal epithelium maintains its original phenotype without any trans-differentiation to the corneal phenotype. Additionally we showed expression of vascular endothelial markers CD31 and CD34 in the sub-epithelial region of the post-COMET corneas to corroborate with the clinical findings of superficial vascularization.

This is the first report on transplantation of oral mucosal cells cultivated using a xeno-free technique of cell culture. Another strength of this study is the homogeneity of the patient cohort; being the largest such study in cases with bilateral ocular burns. Unlike others we used an explant culture technique and transplanted at a monolayer stage, like we do for cultivated limbal epithelial transplantation. It may be argued that these unconventional techniques of cultivation and transplantation may have affected the results. But similar poor outcomes of COMET in ocular burns have been reported with conventional cell culture protocols as well. We also found that the oral epithelium does not convert to a corneal phenotype when transplanted onto the ocular surface and because of the associated vascularization, which is probably essential to its survival, a conjunctivalized ocular surface and one reconstructed after COMET are virtually indistinguishable.

In summary, the findings of our study suggest that transplantation of autologous oral mucosal epithelium cultivated using a xeno-free explant culture system, is unsuccessful in restoring a stable ocular surface and improving vision in eyes with bilateral LSCD following ocular burns. However, our results do not apply to other causes of LSCD or other cell-culture protocols.

Reference:


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