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CLINICAL STUDY

Transplantation of cultivated oral mucosal epithelial cells for severe corneal burn

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Abstract

Purpose **To access the feasibility of using cultivated oral mucosal epithelial cell transplantation (COMET) for the management of severe corneal burn.**
Methods **COMET was performed to promote re-epithelialization in two eyes with acute alkaline burn and one eye with chronic alkaline burn, and to reconstruct the ocular surface in two eyes with chronic thermal burn. Autologous oral mucosal epithelial cells obtained from biopsy were cultivated on amniotic membrane. Immunoconfocal microscopy for keratins and progenitor cell markers was performed to characterize the cultivated epithelial sheet. Following transplantation, the clinical outcome and possible complications were documented. The patients were followed for an averaged 29.6 ± 3.6 (range: 26–34) months.**
Results **Cultivated oral mucosal epithelial sheet expressed keratin 3, 13, and progenitor cell markers p63, p75, and ABCG2. After COMET, all the corneas became less inflamed, and the corneal surface was completely re-epithelialized in 6.0 ± 3.2 (range: 3–10) days in all but one patients. Microperforation occurred in one patient, and a small persistent epithelial defect developed in another. Both were solved uneventfully. In all patients, superficial corneal blood vessels invariably developed, and to further improve vision, conjunctivo-limbal autografting ($N = 3$) and/or penetrating keratoplasty ($N = 3$) were performed subsequently. The vision of all patients showed substantial improvement after additional surgeries.**
Conclusions **This study showed the potential of COMET to promote re-epithelialization and reduce inflammation in acute corneal burn, and to reconstruct the corneal surface in chronic burn. COMET may, therefore, be considered an alternative treatment for severe corneal burn.**
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Keywords: oral mucosa; amniotic membrane; cultivation; cornea; burn

Introduction

In the past 15 years, the use of preserved human amniotic membrane (AM) has contributed significantly to a better management of corneal burn.[1] In the acute stage of burn, the use of AM dressing (AMD) has been reported to reduce inflammation[2] and protect the remaining corneal epithelial stem cells, thereby promoting corneal wound healing.[3,4] In addition, transplantation of AM (AMT) as a graft to the damaged corneal surface has been reported to facilitate re-epithelialization,[5] reduce inflammation, and melting,[6] thereby stabilizing the cornea.[7] However, in very severe burn especially when the cornea is devitalized and is surrounded by ischaemic sclera, either AMD or AMT alone is insufficient to promote wound healing.[5] In that situation, tenonplasty should be performed first to promote vascularization to the sclera.[8] Nevertheless, in the absence of corneal epithelial stem cells, re-epithelialization of the cornea by conjunctival epithelium is a lengthy process, and is often interfered by recurrent epithelial defect.[9] Persistent epithelial defect of the cornea not only predisposes to microbial infection, but also to sterile ulceration or even perforation. Therefore, promotion of re-epithelialization is the most important issue for the management of acute corneal burn.

Recently, cultivated oral mucosal epithelial cells transplantation (so called 'COMET') has

shown encouraging results in reconstructing corneal surface affected by burn or autoimmune diseases.[10–14] Autologous in origin, this technique avoids the need for long-term immunosuppression. In this study, we reported our results of using COMET for the management of acute and chronic corneal burn. We found that especially in the acute stage, COMET promoted re-epithelialization and stabilized the corneal surface, and facilitated further reconstruction surgeries.

Materials and methods

There were five patients with severe limbal stem cell deficiency (LSCD) caused by burn. The LSCD was manifested by chronic inflammation, persistent epithelial defect, and at least 180 degrees of pannus ingrowth. The severity of corneal neovascularization (NV) was quantified by NIH image tool Image J, and expressed as percentage of corneal area occupied by NV.[15] The calculation was repeated three times to obtain the mean and SD.

Cell culture

Informed consent was obtained from all patients in accordance with the tenets of the Declaration of Helsinki for research involving human subjects. The cell culture protocol was similar to that described by Nakamura *et al*,[10] but with some modifications in that: (1) A larger 6×6 mm buccal mucosal tissue was harvested. (2) The tissue was treated with 100 μl of 1.2 IU dispase II in PBS at 37°C for 1 h. Then the tissue was transferred to another 35 mm dish, and treated with 75 μl of 0.25% trypsin-EDTA solution at 37°C. After every 3 min, the cells were collected by gently scrapping the tissue with a blunt spatula, and the trypsin solution containing the cells was collected. The procedure was repeated at least three times, and the cell-containing trypsin solution was then neutralized with 10 ml SHEM with 5% foetal bovine serum. Following centrifugation, the cells were resuspended with 1.0 ml full medium, and plated onto a 25 mm culture insert overlaid with denuded AM,[10] which in turn was cocultured with mitomycin C pretreated 3T3 fibroblasts in a six-well plate. Unlike earlier report, the culture was not air-lifted.

Characterization of cultivated oral mucosal epithelial cells

Remnants of the cultures after transplantation were embedded in OCT, and 5 μm frozen sections were stained with anti-keratin 3 (1 : 100; Millipore), anti-keratin 13 (1 : 100; abcam), anti-p75 (1 : 100; Millipore), anti-p63 (1 : 150; Millipore), and anti-ABCG2 (1 : 100; Calbiochem) at 4°C overnight, then stained with secondary antibodies conjugated to FITC or Cy3 (1 : 200) at room temperature for 30 min. The slides were then counterstained with PI (for FITC) or DAPI (for Cy3), mounted and observed with a laser confocal microscopy (TCS SP2-MP system; Leica). Negative controls were performed using an irrelevant monoclonal antibody (anti-digoxigenin; Roche Applied Science) to replace primary antibodies.

The surgical procedures were similar to earlier reports in pannas removal, excision of Tennon's capsule, topical mitomycin C soaking, suturing of the graft with 10-0 Vicryl, and protecting the graft with a soft contact lens. The postoperative medication included preservative-free dexamethasone 1 mg/ml every 3 h, 0.3% ciprofloxacin four times a day, and Tobradex ointment (both from Alcon) once per day. BSS with or without 20% autologous serum was also given for lubrication. If necessary, systemic prednisolone (1 mg/kg/day) and cyclophosphamide (100 mg/day) were given only in short duration after operation (1–2 months), mainly to control postoperative inflammation. After discharge, the patients were regularly followed. As required by the *Eye* Journal, we certify that all applicable institutional and governmental regulations concerning the somatic cell therapy were followed.

Results

Characterization of cultivated oral mucosal epithelial cells

The cultivated oral mucosal epithelial cells were generally 2–5 cells thick, and the nuclei were mostly elongated (Figure 1a). The epithelium was full-thickness keratin 3 (Figure 1b) and suprabasal keratin 13 positive (Figure 1c). The staining for p75 was found solely in the cytoplasm of basal epithelial layer (Figure 1d), and that for p63 in the nuclei of the same layer (Figure 1e). In contrast, the ABCG2 staining was cytoplasmic and more prominent in the basal layer (Figure 1f). No positive signal was observed in any of the negative control staining (Figure 1c and e; insert). These results suggested that the cultured cells were keratinocytes, and were likely to contain progenitor-like cells.

Surgical outcome

Since April 2006, COMET was performed in the acute stage ($N = 2$) and chronic stage ($N = 3$) of burn. The mean follow-up was 29.6 ± 3.6 (range: 26–34) months. The results are summarized in Table 1, and the following is the concise clinical course of individual patients:

Patient 1
A 27-year-old man suffered from thermal injury to both eyes, with 360 degrees of peri-limbal ischaemia, total

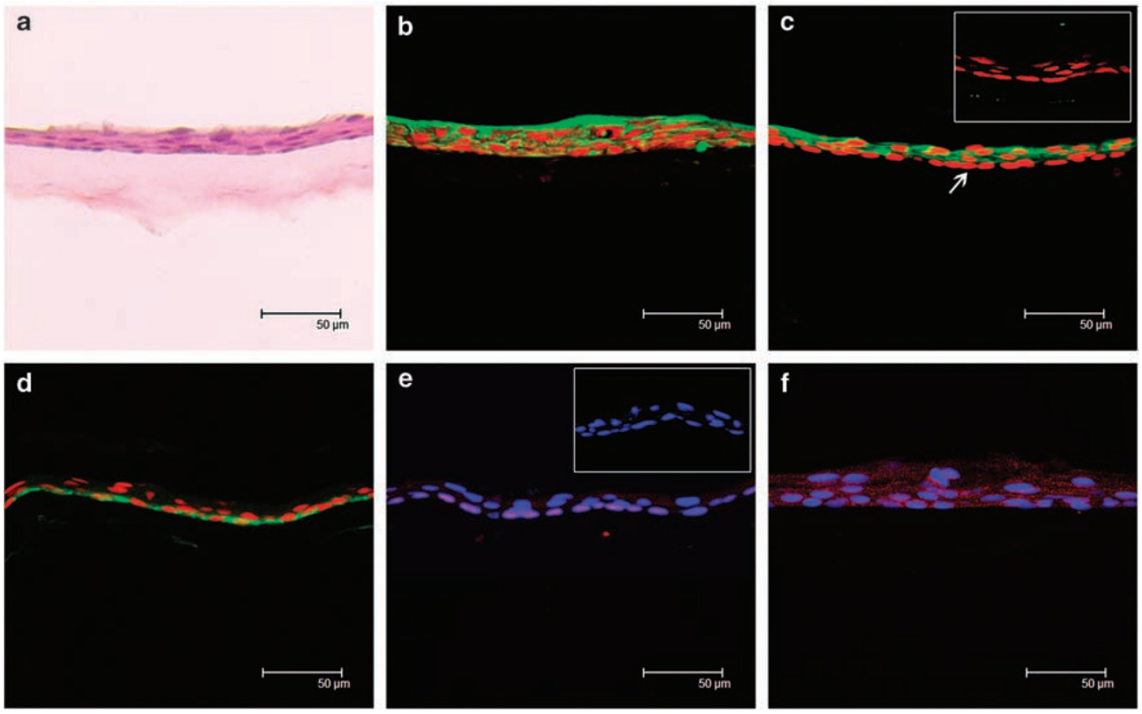

Figure 1 Immunoconfocal microscopy for human oral mucosal epithelial cells cultivated on denuded amniotic membrane (AM) under submerged condition. HE staining showed 2–5 layers of stratified epithelium with elongated nuclei on AM (a). Full-thickness cytoplasmic keratin 3 staining (b). Suprabasal keratin 13 staining (c). (note that K13 staining in the basal layer was negative (c; arrow; insert: negative control)). Cytoplasmic p75 staining in the basal layer (d). p63 staining in the nuclei of basal epithelium (e; insert: negative control). Cytoplasmic ABCG2 staining, which was more prominent in the basal layer, and was also more obvious between the cell–cell junctions (f; green: FITC; red: Cy3; blue: DAPI).

corneal, and bulbar conjunctival epithelial defect OD (Figure 2a). Injury to the left eye was less severe. To promote re-epithelialization, AMD (Figure 2b) followed by AMT (Figure 2c) were performed, and the upper lid defect was repaired by hard palate cartilage graft and retroauricular skin graft. Fifteen months after the injury, the cornea was still covered by dense fibrovascular tissue with symplepharon (Figure 2d). Following COMET, the cornea was promptly re-epithelialized (Figure 2e) without epithelial defect 4 days postoperatively. However, as the residual corneal stroma was very thin, a 1×1 mm descementocele developed and then perforated. A small patch graft sealed the hole successfully (Figure 2f).

Afterwards, although superficial NV persisted, the cornea and limbus became much less inflamed (Figure 2g). *In vivo* laser confocal microscopy showed that transplanted cells appeared to be larger (Figure 2h), with brighter and smaller nuclei, whereas the normal corneal epithelial cells in the left eye appeared to be smaller (Figure 2i). Penetrating keratoplasty (PKP) combined with cataract extraction was performed 10 months after COMET. Following PKP, although the epithelium layer was not as transparent as genuine corneal epithelium, and some NV still existed, the best-corrected visual acuity (BCVA) could reach 20/200 (Figure 2j).

Patient 2

An 18-year-old man suffered from alkaline burn OS caused by cement spillage. At admission, more than half of the limbus was ischaemic with total corneal epithelial defect (Figure 3a). Despite twice AMD, corneal inflammation persisted, and pannus began to invade the cornea (Figure 3b). There was no improvement nearly 2 months after injury (Figure 3c). After COMET, the ocular surface soon became quiescent, the cornea was completely re-epithelialized in 1 week (Figure 3d and e). His BCVA improved from CF/60 cm to 20/200, limited by NV and residual corneal opacity (Figure 3f). Confocal microscopy revealed that transplanted oral mucosal epithelial cells in OS (Figure 3g) were morphologically very similar to corneal epithelial cells in OD (Figure 3h). To further improve vision, the patient received conjunctivolimbal autografting (CLAU) 22 months after COMET, and his final vision improved to 20/40 (Figure 3i).

Table 1 Demographic data of patients who received cultivated oral mucosal epithelial cell transplantation (COMET)

No.	*Sex*	*Age*	*Eye*	*Diagnosis*	*Previous surgeries*	*Initial VA*	*Mean percentage of corneal NV*[a]	*Re-epithelialization in days*	*Complication*	*Subsequent surgeries*	*BCVA*	*Follow-up (months)*
1	M	27	OD	Chronic thermal burn	AMD, AMT	HM	97.9 ± 0.2	4	Microperforation	PKP + ECCE, AMD	20/200	34
2	M	18	OS	Acute alkaline burn	AMD	CF/60	49.3 ± 1.9	7	—	CLAU	20/40	33
3	M	25	OD	Acute alkaline burn	Tenonplasty, AMD, AMT	CF/20	21.9 ± 1.6	105	Small persistent epithelial defect	CLAU, ECCE + PCIOL	20/60	27
4	M	55	OS	Chronic thermal burn	AMT	CF/1M	55.9 ± 0.5	3	—	ECCE + PCIOL, PKP	20/80	28
5	M	56	OS	Chronic alkaline burn	CLAU, PKP, ECCE	HM	61.6 ± 1.2	10	—	CLAU, PKP	20/400	26

AMD = amniotic membrane dressing; AMT = amniotic membrane transplantation; BCVA = best corrected visual acuity; CLAU = conjunctivo-limbal autografting; NV = neovascularization; PKP = penetrating keratoplasty; VA = visual acuity; HM = hand motion; CF = counting finger; ECCE = extracapsular cataract extraction; PC-IOL = posterior chamber intraocular lens.
[a]Severity of corneal NV is expressed as NV area/total corneal area (in percentage). The calculation was repeated three times to obtain the mean and SD.

Patient 3

A 25-year-old man suffered from alkaline burn OD with 360 degrees of limbal ischaemia. Initially, he was admitted to another hospital, where conjunctivotenonplasty, AMT, and AMD were performed. However, after 2 months, there was little improvement, and therefore he was referred to our hospital. At admission, there was still prominent perilimbal scleral necrosis in more than half of the limbus, and the cornea was marble-like (Figure 4a). His vision was CF/20 cm, and elevated IOP was controlled medically. AMT followed by AMD were performed again; unfortunately the procedures failed to promote re-epithelialization and 2 months later, the transplanted AM (Figure 4b) and the corneal stroma started to melt (Figure 4c), therefore COMET was performed (Figure 4d). Postoperatively, an epithelial defect about 1 × 1 mm persisted for more than 3 months, then gradually healed at postoperative 105 days (Figure 4e). Fourteen months after COMET, the cornea became clearer, but superficial blood vessels still existed (Figure 4f and g), so he underwent CLAU to reconstruct the inferior limbus. 10 months after CLAU, his vision improved to 20/60 after uneventful cataract surgery (Figure 4i).

Patient 4

A 54-year-old man suffered from old thermal burn OS caused by smelting iron. There was a direct adhesion between the cornea and inferior bulbar conjunctiva. He was referred to our hospital, and underwent release of ankyloblepharon and reconstruction of lower fornix with AMT. Unfortunately, symblepharon gradually recurred later. To reconstruct the conjunctival surface, the patient underwent release of symblepharon and COMET. Re-epithelialization was complete within 3 days after COMET. As his vision was still impaired by corneal opacity and irregularity even after cataract surgery, he received PKP 26 months after COMET. At present, his BCVA reaches 20/80.

Patient 5

A 56-year-old man suffered from alkaline burn OS. He was admitted to another hospital where five AMTs, CLAU, cataract extraction, and PKP were performed. Unfortunately, all the procedures failed to stabilize the corneal surface, so he was referred to our hospital. At admission, there were symblepharon, persistent epithelial defect, dense infiltration, and heavy NV in the cornea. The patient then underwent release of symblepharon and COMET. After surgery, the cornea became smooth without epithelial defect 10 days after COMET. Although initially the cornea was free of NV, blood vessels gradually invaded the whole cornea, and

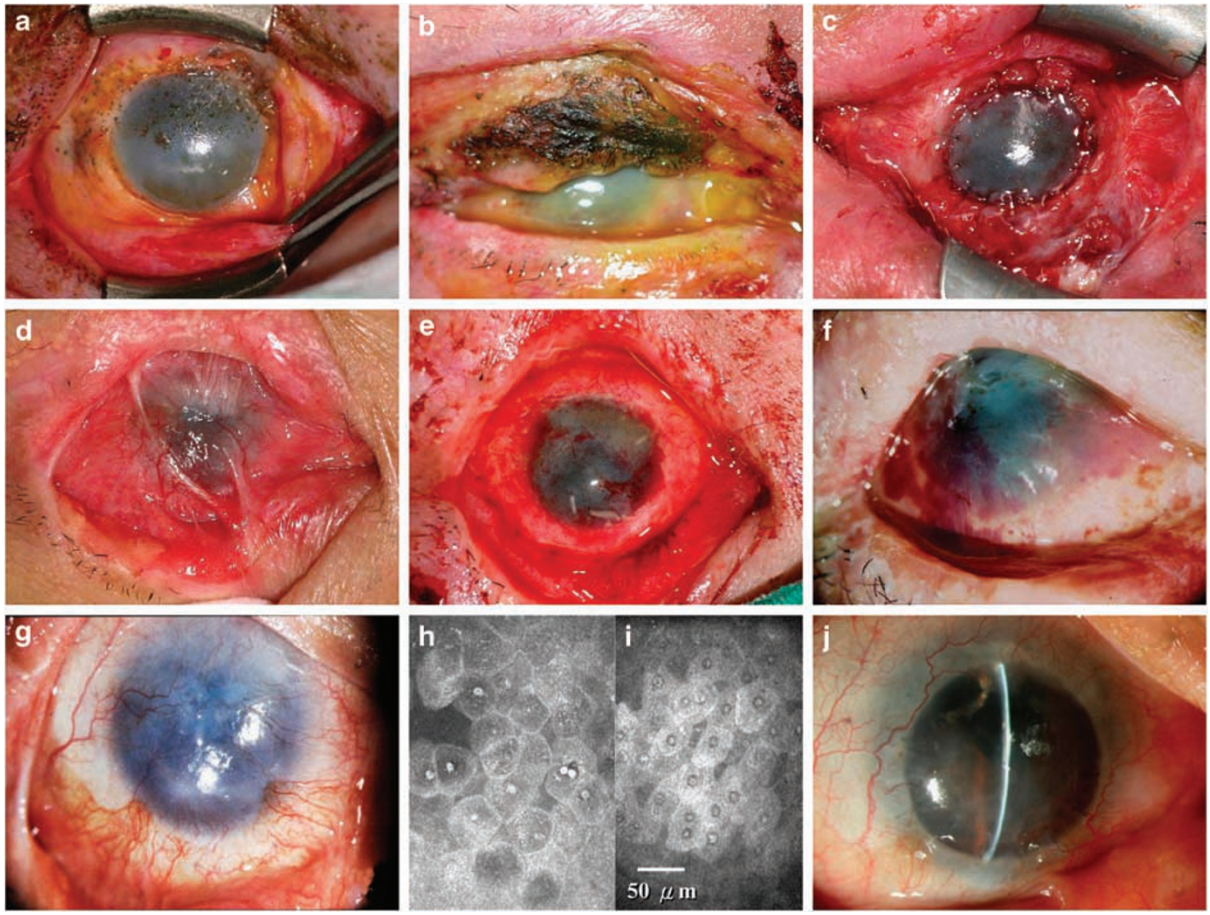

Figure 2 Representative photographs of patient 1. A 27-year-old man suffered from thermal injury to in the right eye with 360 degrees of limbal ischaemia and retained metallic foreign bodies (a). Amniotic membrane (AM) dressing protected the cornea from desiccation before the upper lid was repaired (b). Two months later, the cornea began to melt, and AM transplantation was performed (c). Fifteen months after injury, the ocular surface became stabilized, but the cornea was still covered by dense fibrovascular tissue (d). COMET was performed to reconstruct the ocular surface, with photos taken at the completion of surgery (e) and 2 weeks postoperatively (f). After COMET, although superficial neovascularization persisted, the cornea and limbus remained less inflamed (g). *In vivo* laser confocal microscopy showed transplanted oral mucosal epithelial cells in OD (h) in comparison with the normal corneal epithelial cells in OS (i). Penetrating keratoplasty combined with cataract extraction was performed 10 months after COMET, and the graft remained stable 18 months after PKP (j).

therefore the patient received CLAU 1 year after COMET, and PKP 8 months after CLAU. At post-PKP 3 months, his vision improved from hand motion initially to 20/400.

Discussion

In severe corneal burn, persistent inflammation not only results in poor wound healing, but is also detrimental to the remaining limbal epithelial stem cells. For example, in Patient 2, there was roughly half of the limbus initially not damaged (Figure 3a). However, with persistent severe inflammation, fibrovascular pannus gradually grew from all directions into the cornea, featuring a picture of total LSCD (Figure 3c). For this, we propose that damaging large area of the limbal microenvironment may cause a sustained inflammation and resulting apoptosis of residual limbal epithelial stem cells.[16] On the contrary, regenerating corneal epithelial cells, especially those cultivated on AM, are able to exert an enhanced antiapoptotic/antiinflammatory,[17] and antiangiogenic activity.[18] Therefore, in severe LSCD, it is crucial to promote re-epithelialization so as to disrupt the vicious cycle of chronic inflammation and LSCD.

In acute corneal burn, AMT has the advantages of reducing pain and promoting re-epithelialization, but it is not necessarily so in very severe burn with scleral ischaemia and extensive inflammation.[5] Koizumi *et al*[19] were the first to report using CCET to promote wound healing in acute Stevens–Johnson's syndrome (SJS) and

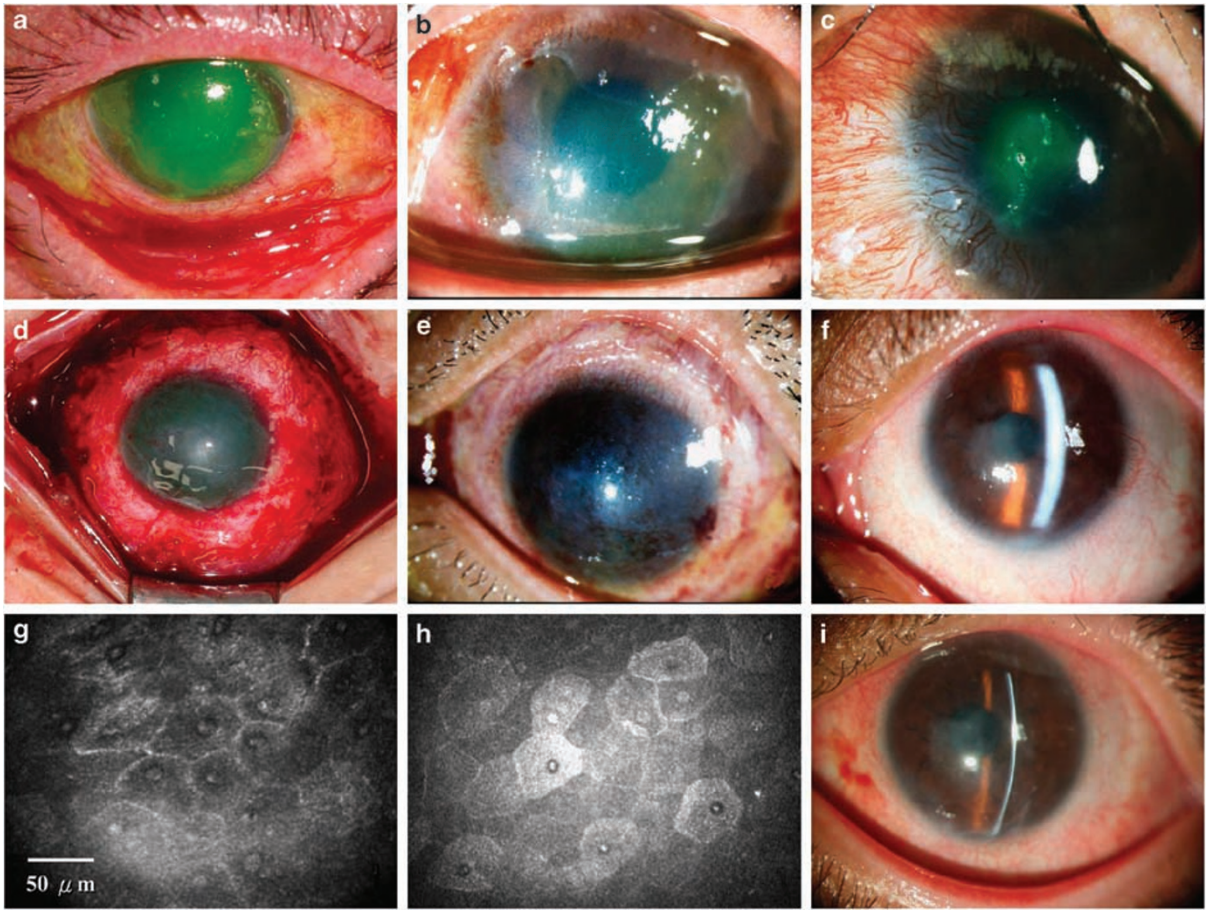

Figure 3 Representative photographs of patient 2. An 18-year-old man suffered from alkaline burn to the left eye with more than 50% limbal ischaemia and total corneal epithelial defect (a). One month later, corneal epithelial defect persisted, and pannus began to invade the cornea (b). Two months after injury, there was 360 degrees of fibrovascular ingrowth with persistent epithelial defect (c). COMET was performed to promote re-epithelialization, with the photo taken immediately after surgery (d). One week after COMET, the ocular surface soon became quiescent (e), however, superficial neovascularization and residual corneal opacity persisted 1 year after COMET (f). *In vivo* confocal microscopy taken 22 months after COMET revealed the transplanted oral mucosal epithelial cells in OS (g) were morphologically very similar to corneal epithelial cells in OD (h). The patient later received conjunctivolimbal autografting, with the photo taken 5 months after surgery (i).

chemical burn.[20] By performing CCET, the cornea soon became re-epithelialized, and the vision was dramatically improved. However, as the cells were from irrelevant cadaveric donors, the recipients had to be immunosuppressed. There has been no consensus regarding how long the recipient should be immunosuppressed after allogeneic limbal stem cell transplantation. Most likely. it will require a prolonged, if not indefinite period of time.[21]

Nakamura *et al*[10,12,13] had reported using COMET for the treatment of ocular surface diseases.The technique is especially valuable for bilateral LSCD caused by autoimmune diseases, such as SJS and cicatricial pemphigoid. In addition to AM, temperature responsive culture dish was used as a novel substrate to grow the epithelial cells, as was reported by Nishida *et al*.[11,22] In this study, we verified that by promoting re-epithelialization and reducing inflammation, COMET inhibits corneal melting and stabilizes the corneal surface, which can dramatically reduce the hospitalization time required for acute severe burn. In chronic burn, ours and earlier studies also showed that COMET is able to reconstruct the conjunctival as well as the corneal surface. In both situations, after COMET, the corneal clarity as well as the vision substantially improved, and more importantly, the patients were free from the complications of transplantation rejection. As we found that the oral mucosal wound generally healed rapidly, we harvested larger tissue than previously described (6×6 *vs* 3×3 mm) in the hope that more epithelial progenitor cells can be preserved in the graft. As are shown in the Results, the full-thickness keratin 3

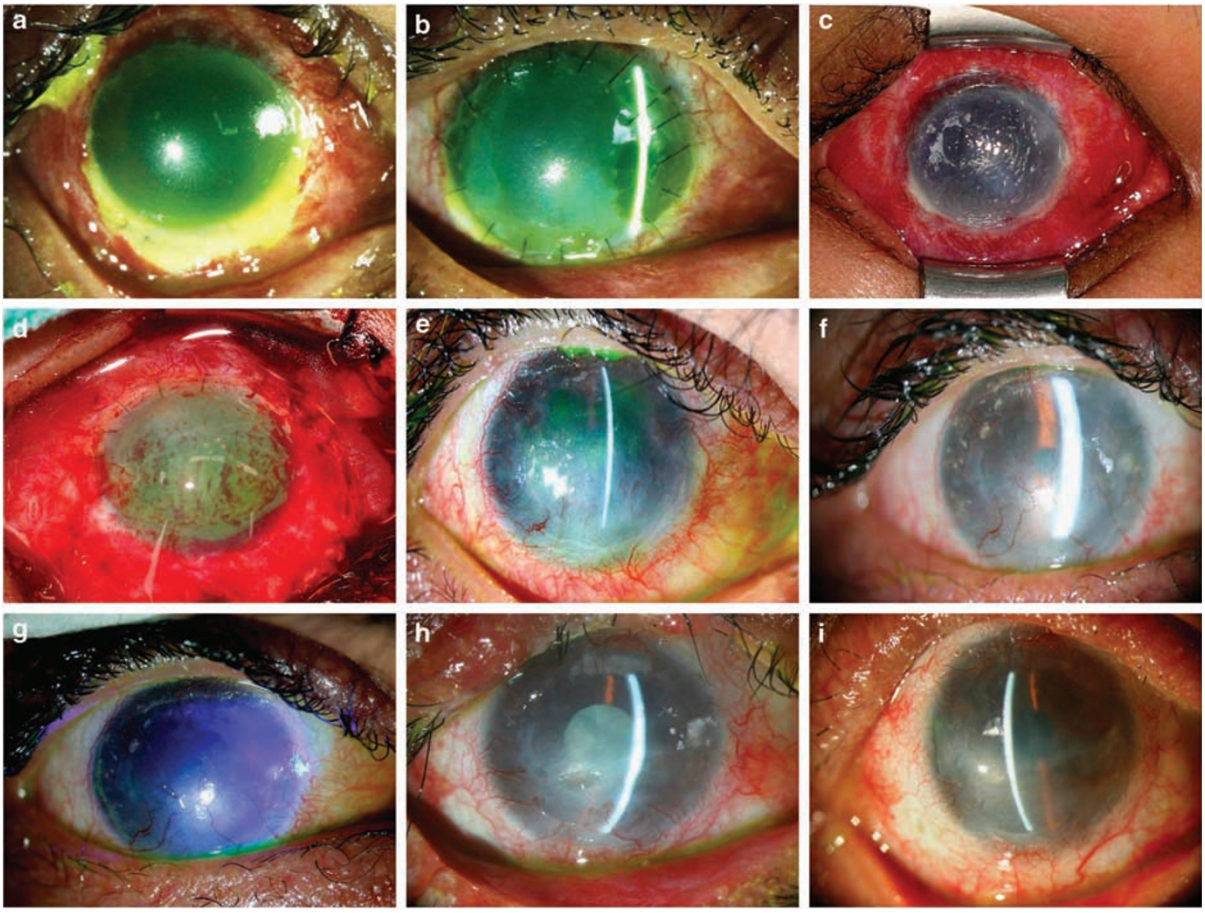

Figure 4 Representative photographs of patient 3. A 25-year-old man suffered from alkaline burn of right eye with 360 degrees of limbal ischaemia. Perilimbal scleral necrosis and total corneal epithelial defect persisted despite conjunctivotenonplasty, amniotic membrane (AM) transplantation, and AM dressing (a). Repeated AM transplantation followed by AM dressing failed to promote re-epithelialization, and 2 months later, the transplanted AM began to melt (b). COMET was performed to prevent further melting, with photos taken before (c) and after surgery (d). Four months after COMET, the cornea was totally re-epithelialized, yet the cornea was still opaque (e). Fourteen months after COMET, the cornea became clearer, but superficial blood vessels were still present in central cornea (f and g). Conjunctivolimbal autografting (CLAU) was performed to reconstruct the inferior limbus, with photos taken 1 month after CLAU (h) and 3 months after cataract extraction and PC-IOL implantation (i).

staining and suprabasal keratin 13 staining confirm that the cultured cells were oral mucosal epithelial cells, but not fibroblasts.[23,24] As the cultures were not air-lifted, epithelial stratification was less then reported earlier,[25,26] and the cells appeared generally flatter. Interestingly, transcription factor p63,[11] and notably low affinity NGF receptor p75,[27] the progenitor cell markers known to be expressed by oral mucosal epithelial cells were both found in the cultures, along with the expression of ABCG2. Expression of ABCG2 by corneal epithelial progenitor cells has been documented;[28] however, its role as a progenitor cell marker for oral mucosal epithelial cells has yet to be determined. Nevertheless, these findings suggest that the cultures might contain progenitor-like cells. Currently, we are examining the corneal tissues obtained after PKP or CLAU to see whether these cells can exist long after transplantation in the graft.

However, unlike successful CCET, which can result in a quiescent and avascular corneal surface, even after successful COMET, the epithelium seemed to attach less firmly to the corneal stroma. This might be due to less-differentiated epithelium from submerged culture, and may explain why a small epithelial defect persisted in patient 3 for more than 3 months. Also, various degrees of corneal NV were still evident after COMET. In our experience, the blood vessels may even reach the central cornea (Figures 2g and 4f). For this, Sekiyama *et al*[29] suggested that decreased secretion of thrombospondin (TSP)-1 and increased secretion of fibroblast growth factor (FGF)-2 by cultivated oral mucosal epithelial cells may be responsible for the inferior antiangiogenic

acitivity following COMET. Their finding was echoed by Kanayama *et al*[30] that cultivated oral mucosal epithelial cells exhibited greater *in vitro* angiogenic activity and increased secretion of fibroblast growth factor-2 (FGF-2), but not vascular endothelial growth factor (VEGF), played a major role. Compared with corneal epithelial cells, the inferior antiangiogenic effect and epithelial barrier function of oral mucosal epithelial cells may result in less satisfactory epithelial transparency and regularity, thus limiting the visual outcome after COMET, which is the main limitation of this technique.

Despite the presence of blood vessels, we observed that the severity of conjunctival inflammation decreased dramatically after COMET. To eliminate the blood vessels, CLAU was performed in the convalescent stage in patients 2, 3, and 5. The reason why conventional CLAU, but not CCET, was performed in our study was because both techniques were still considered investigational by the Department of Health, Taiwan, and only one novel technique can be performed in a single clinical trial. Nevertheless, the size of conjunctivolimbal grafts can be reduced when CLAU is combined with AMT, thereby decreasing damage to the donor site.[31] We propose that in the acute stage of burn, it is improper to use healthy limbal tissue from the non-injured eye for reconstruction, because the chance of graft failure is high due to severe inflammation. In this study, we found that early intervention rather than patient's age was associated with a better visual outcome after COMET, and we propose that COMET can be a practical technique to accelerate wound healing and save vision in the acute stage of severe burn. If the other eye is intact, once the corneal surface becomes quiescent, conventional CLAU or autologous CCET can then be performed to further improve corneal clarity. Alternatively, the combined use of anti-VEGF and COMET might result in a better antiangiogenic effect.

In summary, this study showed the potential of COMET to promote re-epithelialization and reduce inflammation in acute corneal burn, and to reconstruct the conjunctival and corneal surface in chronic burn. Free from rejection and repeatable, COMET may be considered an alternative treatment for the management of severe corneal burn.

Acknowledgements

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