

# Coculture of autologous limbal and conjunctival epithelial cells to treat severe ocular surface disorders: Long-term survival analysis

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**Background:** Cultivated limbal epithelium for reconstruction of corneal surface is a well-established procedure; however, it is not adequate for damage which also extensively involves the conjunctiva. In severe cases of ocular surface damage that warrant additional conjunctival transplantation apart from cultivated limbal stem cell transplantation, we describe the long-term survival of a novel method of cocultivating autologous limbal and conjunctival epithelium on a single substrate. **Materials and Methods:** Forty eyes of 39 patients with severe limbal stem cell deficiency and conjunctival scarring or symblepharon underwent transplantation of autologous cocultivated epithelium on human amniotic membrane. A ring barrier was used to segregate the central limbal and peripheral conjunctival epithelia *in vitro*. Patients were followed up at regular intervals to assess stability of the ocular surface, defined by absence of conjunctivalization into the central 4 mm of the cornea and absence of diffuse fluorescein staining. Penetrating keratoplasty (PKP) was subsequently performed, where indicated, in patients with surface stability. **Results:** The cumulative survival probability was 60% at 1 year and 45% at 4 years by Kaplan–Meier analysis (mean follow-up duration: 33 ± 29 months, range: 1–87 months). Best-corrected visual acuity improved to greater than 20/200 in 38% eyes at the last follow-up, compared with 5% eyes before surgery. Immunohistochemistry in five of the corneal buttons excised for PKP showed an epithelial phenotype similar to cornea in all five. **Conclusions:** Synchronous use of cultured limbal and conjunctival epithelium offers a feasible alternative and a simpler one-step surgical approach to treat severe ocular surface disorders involving limbus and conjunctiva.

**Key words:** Cocultivated epithelium, conjunctiva, cornea, limbal stem cells, ocular surface

Cultivated limbal epithelial transplantation (CLET) for corneal resurfacing is now the mainstay of treatment for limbal stem cell deficiency (LSCD) following injury or other ocular surface disorders, with minimal biopsy required for cultivation compared with direct transplantation.<sup>[1–3]</sup> Protein-rich human amniotic membrane (HAM) serves as both a substrate for cultivation and a biological patch that facilitates healing of the ocular surface in most cases of conjunctival involvement.<sup>[4–6]</sup>

In severe cases with near-total LSCD and extensive conjunctival scarring (with or without symblepharon), additional conjunctival transplantation may also be required in addition to CLET. Conjunctival transplantation was performed as an autograft or living-related allograft.<sup>[7,8]</sup> In recent years, the *in vivo* cultivation of conjunctival cells and transplantation of cultivated conjunctival epithelium for conjunctival disorders have been demonstrated.<sup>[9–11]</sup> This led us to develop a coculture of central limbal and peripheral conjunctival cells on a single HAM to facilitate the reconstruction of the entire ocular surface in a simpler one-step surgery and reduce the size of biopsy

from both sources.<sup>[12]</sup> The outcome of such an autologous transplantation of cocultivated limbal and conjunctival epithelium in a patient who presented with severe bilateral acid injury was very encouraging, based on our case report.<sup>[12]</sup>

The feasibility of autologous coculture prompted us to evaluate this procedure in a larger case series. Herein, we present the results of the long-term survival of autologous cocultivated epithelial transplantation in a group of 40 eyes of 39 patients with severe ocular surface disorders.

## Materials and Methods

Before clinical transplantation, the technique of “cocultivated epithelium” was first standardized by *in vitro* culture and characterization of limbal and conjunctival tissues, obtained from patients undergoing eye surgery who provided consent for such a biopsy. The protocol was approved by the institutional review board and the research adhered to the tenets of the Declaration of Helsinki. The biopsy and explant culture technique have been described in detail in our earlier articles on cultivated epithelial transplantation.<sup>[13,14]</sup> In brief, the limbal and conjunctival biopsy was fragmented and placed on a de-epithelialized HAM. The membrane was flooded with culture medium supplemented with 10% fetal bovine serum for standardization purposes and patient’s autologous serum for clinical transplantation. The culture was incubated at 37°C in 5% CO<sub>2</sub> for 2 weeks.

For establishing cocultures, six to eight fragments of limbal explants were placed within the central 15 mm of the amniotic membrane and four to eight fragments of conjunctival explants

### Access this article online

#### Website:

www.ijo.in

#### DOI:

10.4103/0301-4738.99840

### Quick Response Code:



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**Manuscript received:** 05.04.11; **Revision accepted:** 07.03.12

in a circular manner at the periphery of the membrane.<sup>[12]</sup> A ring-shaped barrier made of Perspex (specially designed for the study) with a thickness of 0.3 mm, an internal diameter of 1.5 cm, and height of 0.8 cm was placed at the center of the de-epithelialized amniotic membrane [Fig. 1], so as to segregate the growth from the two explants. The limbal explants were placed inside the ring, while the conjunctival explants were placed outside the ring. The plates were observed for cell growth every day both within and outside the ring. A monolayer of closely packed cells was observed in 10–15 days of culture.

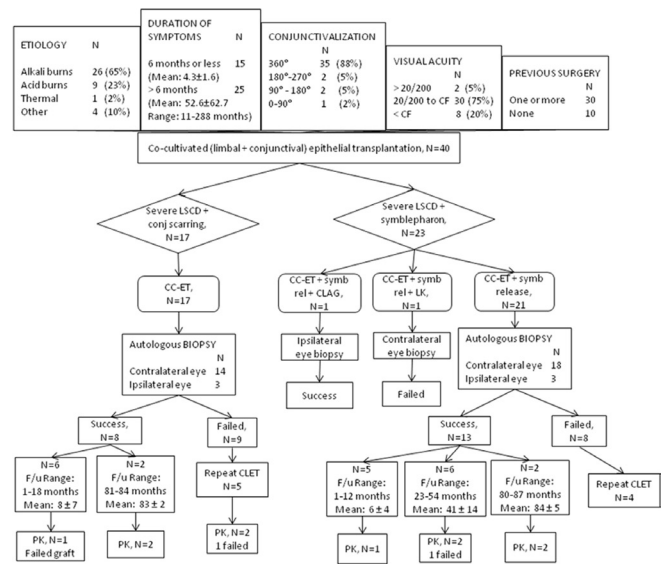
After 2–3 weeks of growth, the conjunctival cultures and coculture (with and without barrier) were stained with hematoxylin and eosin (H and E) and Periodic Acid Schiff's (PAS). Goblet cell count per 1000 cells was done and recorded in conjunctival cultures and cocultures with barriers. Limbal cells were characterized for the markers CK3, CK14, CK19, and ABCG2, while conjunctival cells were characterized for CK19, CK3, and MUC5AC. A BrdU pulse chase experiment was performed on limbal epithelial cultures in order to determine the number of label-retaining progenitor cells. The details of this procedure have been elaborated by Fatima *et al.*<sup>[13]</sup>

During the period of study (June 2001 to November 2006), 477 autologous CLET procedures were performed at our institute with a diagnosis of limbal stem cell deficiency, with informed consent. Of these, 47 eyes of 46 patients who would have otherwise required additional conjunctival transplantation in addition to auto-CLET, for severe ocular surface damage extensively involving the conjunctiva, such as scarring or symblepharon, underwent cocultivated limbal and conjunctival epithelium transplantation.

Data from the medical records of the 46 patients were reviewed retrospectively for primary etiology, previous surgeries, preoperative, and postoperative best-corrected visual acuity (BCVA), degree of conjunctivalization and symblepharon, type of biopsy, complications, and final outcome. Seven patients were excluded from the study, of which one patient did not have follow-up data beyond day 1, four patients had allografts, and two had inaccurate perception of light and surgery was for cosmetic purposes. The spread of preclinical characteristics such as acuity, conjunctivalization and symblepharon, number of eyes with acute or chronic presentation, history of previous surgery, and type of biopsy (whether ipsi- or contralateral) as well as the surgical procedure are shown as a flow chart in Fig. 1.

The surgical technique used for cocultivated epithelial transplantation has been described in detail in our earlier case report.<sup>[12]</sup> Following the removal of pannus and symblepharon release if any, the cocultivated epithelial sheet on HAM was placed on the ocular surface, cell side up, and gently spread over the cornea and limbus and anchored with the help of fibrin tissue sealant (Tisseel kit; Baxter AG, Vienna, Austria). Patients with stromal scarring in the visual axis leading to decrease in vision were considered for PKP at a later date. Histopathological reports of recipient corneal buttons were also reviewed for those patients who had subsequent penetrating keratoplasty (PKP).

Postoperatively, all patients were treated with prednisolone acetate 1% eye drops in tapering frequency and ciprofloxacin hydrochloride 0.3% eye drops. The patients were seen on



**Figure 1:** Flow chart of clinical preoperative characteristics in patients and results of surgical intervention (N=40 eyes) LSCD = Limbal stem cell deficiency, LK = Lamellar keratoplasty, CC-ET = Co-cultured epithelial transplant, PK = Penetrating keratoplasty, Symb rel = Symblepharon release, CLET = Cultivated limbal epithelial transplantation

postoperative day 1, 1 week, 2 weeks, 5 weeks, and thereafter 2 or 3 monthly until last follow-up. Each examination included a complete evaluation of recipient and donor sites for any signs of neovascularization or surface instability and assessment of visual acuity. Survival was defined as the duration of stable ocular surface following cocultivated epithelial transplantation at last visit, including conjunctivalization not encroaching into the central 4 mm of the cornea and sparing the visual axis. Failure was defined as progressive conjunctivalization of the cornea or graft, diffuse fluorescein staining, vascularization of the limbus, and a persistent epithelial defect. The outcome was evaluated at months 1, 3, 6, 12, 18, 24, 28, 36, 48, 60, 72, and 84.

The data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL). Kaplan–Meier (K-M) survival analysis was performed to estimate the cumulative survival probability of cocultivated epithelial transplantation. The effect of possible risk factors on the survival of cocultivated epithelial transplant was studied using the Cox proportional hazard survival regression at an alpha level of 0.05. The survival plots in K-M analysis were also used to determine the probability of symblepharon following cocultivated epithelial transplantation.

**Results**

Eight isolated conjunctival cultures and 22 cocultures were made from limbal and conjunctival tissues harvested from 26 patients (15 male and 11 female, mean age: 49 years, range: 6–80 years) who underwent cataract surgery or CLET. Eleven of these were studied with the use of barriers. Growth was observed in all the explant cultures within 2–4 days, expanding to a monolayer in 2 weeks.

In cocultures without barriers, the whole mount preparation of the limbal and conjunctival fragments revealed a confluent growth and could not be distinguished from each other as a monolayer. The goblet cell count could not be done in view of the confluence of limbal and conjunctival cultures.

In the presence of the barrier, the stained areas represent the cultured cells, whereas the ring of unstained area represents the area where the ring was placed and therefore was totally devoid of cell growth, thus showing efficient barrier function [Fig. 2]. The cells within the ring showed epithelial cells only while the cells outside showed both epithelial cells and goblet cells that were evident by PAS stain which shows the intracytoplasmic mucin. Mean goblet cell percentage in cocultures with barrier was 0.43% (range 0.1–1.4%). PAS stain showed PAS-positive cells mostly at the periphery.

Immunostaining of cultured conjunctival cells was positive for CK19 (expressed by conjunctival epithelium and basal cells) and mucin marker MUC5AC and negative for cornea specific CK3 antibody [Fig. 3a-c]. The limbal culture in coculture showed cells positive for CK3, CK14, and ABCG2 [Fig. 3d-f]. The BrdU pulse-chase experiments showed  $2.3 \pm 2.4\%$  of label-retaining limbal cells after 30 days in culture. This was further substantiated by the presence of 2.4% ABCG2-positive limbal epithelial cells.

Clinical data on the ocular surface reconstruction with autologous cocultivated limbal and conjunctival epithelial transplantation were reviewed for 40 eyes of 39 patients (mean age:  $16.8 \pm 9.3$  years, age range: 3–36 years, 30 male and 9 female) with a mean follow-up period of  $33.4 \pm 29.2$  months (range: 1–87 months). The most common etiology for LSCD was chemical injury, occurring in 35 of 40 eyes (85%). The pre- and postoperative outcomes of two patients are shown in Fig. 4.

The cumulative survival of cocultivated epithelial transplantation decreased from 92% to 60% in the first year. The decrease is more gradual after the first year, from 60% to 56% in the second year, and 45% at the end of 4 years [Fig. 5a, K-M survival plot]. Variables that were analyzed for any effect on survival of the cocultivated epithelial transplant using Cox regression are shown in Table 1. The duration of symptoms had

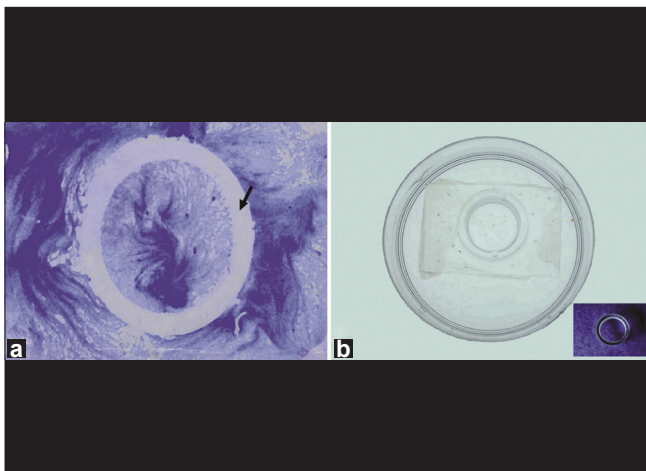
a significant effect on survival [Fig. 5b], indicating a smaller probability of survival in the group with less than 6 months duration (HR = 3.83,  $P = 0.039$ ).

The number of eyes having ambulatory BCVA ( $>20/200$ ) improved from 2 out of 40 (5%) eyes preoperatively to 15 out of 40 eyes ( $\sim 38\%$ ) postoperatively at the last follow-up with a mean duration of  $33 \pm 29$  months. The other clinical parameters, namely, etiology, presence of symblepharon, type of biopsy, and previous surgery, did not have a significant effect on the survival of cocultivated transplant [Table 1, Cox regression,  $P > 0.05$ ]. While acid injury had a greater hazard risk for rejection (HR = 1.81) than alkali injury (HR = 0.60), the difference in survival was not statistically significant (log rank test,  $P = 0.837$ ).

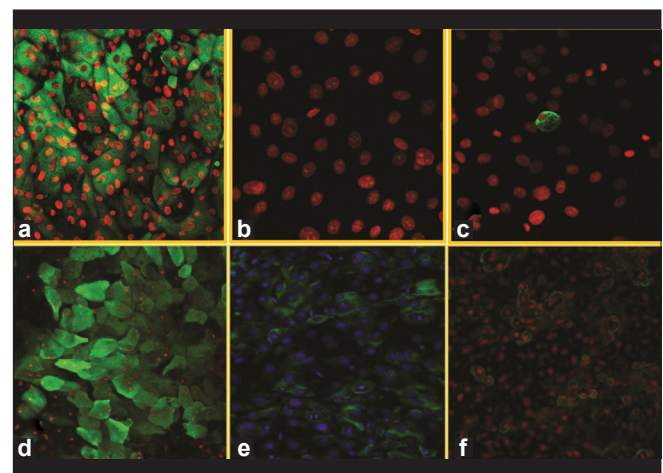
The probability of not having a symblepharon occurrence or recurrence following cocultivated transplant decreased steeply from 87% to 46% between 1 month and 18 months after the surgery (K-M analysis). After that, it reached a plateau with a 39% probability of survival at 48 months.

Ten eyes underwent PKP at a mean duration of  $11.8 \pm 6.4$  months (range: 4–23 months) following cocultivated epithelial transplantation with a mean follow-up of  $42.8 \pm 31.7$  months (range: 2–81 months). PKP was successful in seven eyes with clear grafts at the last follow-up, while the remaining three failed due to corneal graft rejection. Case details of the 10 eyes of patients who underwent PKP are shown in Table 2.

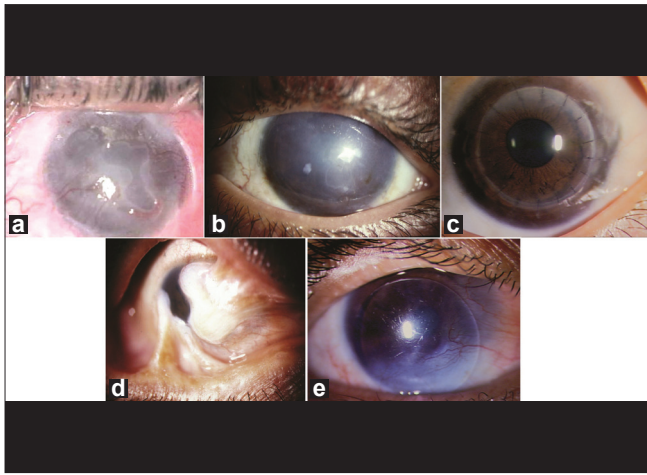
Histological evaluation of 7 of the 10 recipient corneal buttons showed a well-stratified three- to five-layered epithelium. Epithelium was uniform in three of the seven buttons, one showed areas of hyperplasia, two had focal areas of denudation, and one had focal thinning with basal cell edema. All corneal buttons had vascularized stromal scarring



**Figure 2:** (a) Whole mount preparation of cultured epithelial cells on human amniotic membrane (barrier removed after culturing) showing the growth of limbal cells in the centre and conjunctival cells at the periphery outside the ring barrier and a clear circular zone, devoid of cells (arrow). [H and E,  $\times 100$ ], (b) Setup for the coculture of conjunctival and limbal tissues on a single HAM, on a Petri plate with limbal explants inside the ring barrier and conjunctival explants outside the barrier. (Inset: self-designed ring-shaped barrier made of Perspex)



**Figure 3:** Conjunctival epithelial cells on human amniotic membrane showing (a) CK19 positivity with green (FITC labeled) cytoplasmic staining and red (PI labeled) nuclei [ $\times 200$ ]. (b) CK3 negative with only red nuclei [ $\times 200$ ]. (c) Presence of goblet cells positive for MUC5AC as shown by green cytoplasmic staining and red nuclei [ $\times 200$ ] Limbal epithelial cells showing. (d) CK3 positivity with green cytoplasmic staining and red nuclei [ $\times 200$ ]. (e) CK14 positive with green cytoplasmic staining and blue (PI labeled) nuclei [ $\times 200$ ]. (f) Positive ABCG2 with green membrane staining and red nuclei; FITC = Fluorescein iso thio cyanide, PI = Propidium iodide

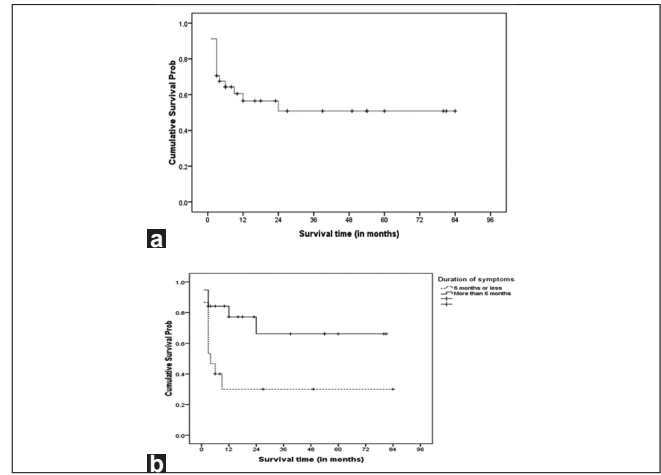


**Figure 4:** (a) Left eye of Patient A showing LSCD with 360° conjunctivalization 6 months after lime injury. (b) Stable ocular surface in patient A, 1 month after cocultivated limbal and conjunctival epithelial transplantation with biopsy from contralateral eye. (c) Left eye of patient A showing clear graft 2 years postpenetrating keratoplasty (d) Right eye of patient B, 38 months after acid injury, diagnosed as LSCD with extensive symblepharon. (e) Right eye of patient B, 2.5 years following cocultivated transplantation with symblepharon release surgery, showing scarring inferonasally. LSCD = Limbal stem cell deficiency, LSCT = Limbal stem cell transplantation, BCVA = Best-corrected visual acuity

**Table 1: Effect of various clinical factors on the survival of cocultivated epithelial transplantation, analyzed using Cox (proportional hazards) regression method**

	N	Hazard ratio	95% CI	P value
Type of autologous transplant				
Contralateral	33	1.16	0.2-7.5	0.88
Ipsilateral	7			
Duration of symptoms				
6 months or less	15	3.83	1.1-13.7	0.039
More than 6 months	25			
Etiology of LSCD				0.35
Alkali burns	25	0.60	0.2-1.6	0.32
Acid burns	9	1.81	0.7-4.6	0.21
Other causes	6			
Previous surgeries				
One or more surgeries	30	0.80	0.3-2.5	0.70
None	10			
Symblepharon				
Present	23	0.59	0.2-1.8	0.35
None	17			

and two also showed corneal thinning. Immunohistochemistry with monoclonal antibodies (AE5) against cytokeratin CK3 was performed in six corneal buttons, of which five showed a positive reaction and one had presence of residual amniotic membrane.



**Figure 5:** (a) Kaplan–Meier survival plot for stable corneal surface with no conjunctivalization in the central cornea following cocultivated limbal and conjunctival epithelial transplantation (N = 34 eyes). (b) Comparison of Kaplan–Meier survival based on duration of symptoms (6 months or less, N = 15 and >6 months, N = 19)

### Discussion

There is growing interest in the application of cultivated conjunctival epithelium for conjunctival and corneal deficiencies.<sup>[9-11,15]</sup> This is, to the best of our knowledge, the first report of the clinical application and long-term analysis of cocultivated limbal and conjunctival epithelium, to reconstruct the extensively scarred conjunctiva in one step along with corneal resurfacing. This was similar to the simple cost-effective, feeder cell-free explant culture technique that was used to culture the limbal epithelial cultures.<sup>[13,14,16]</sup> Since using limbal and conjunctival cultures on two different membranes would have been cumbersome and surgically demanding, we hypothesized that the cells could be grown on a single membrane as seen in normal ocular surface. Simulating the natural ocular surface of conjunctiva at the periphery and limbal in the center, we placed the explants in the same manner. However, the confluence of both cells with no demarcation raised the need for a barrier, similar to that provided by limbus in *in situ* condition. This prompted us to develop a ring-shaped barrier that would provide a limbal derived epithelium of 15 mm and a peripheral conjunctival epithelium of 2 cm separated from each other. The stained preparation of the coculture confirmed our hypothesis and showed limbal corneal cells in the center, a cell-free zone in the middle and a peripheral epithelium of conjunctiva that contained goblet cells. The effective role of the barrier, in restricting the *in vitro* growth of limbal cells to the inner ring and conjunctival cells outside the ring, was substantiated by marker-based characterization of limbal and conjunctival cultures.

We restricted the use of cocultivated epithelium to a select group of patients having widespread ocular surface damage with guarded prognosis (47 out of 477 autologous CLET) that would otherwise require additional conjunctival autograft. The cultivated conjunctival epithelium on HAM, unlike amniotic membrane transplantation (AMT) alone, offered a conjunctival surface for conjunctival reconstruction in these cases.<sup>[17]</sup> The

**Table 2: Details of eyes that underwent penetrating keratoplasty (PKP) following cocultivated limbal and conjunctival transplantation**

No.	Age	Sex	Eye	Etiology	Duration (month)	Prev sx	Number of sx	Loss of palisades	Conjunctival scarring	Symblephara (clock hrs)	PK after (month)	F/u duration	Graft at last f/u
1	4	F	OS	Lime	11	AMT	1	360	360	4	9	71	Clear
2	36	M	OD	Acid	12	PKP	1	360	360	—	11	7	Failed
3	6	F	OS	Lime	6	CLAG + misc sx	2	360	360	—	3	81	Clear
4	7	M	OD	Lime	11	Sym rel + AMT	1	360	360	—	10	70	Failed
5	13	F	OS	Lime	38	AMT	1	360	360	1	6	81	Clear
6	26	M	OS	Lime	3	AMT	1	360	360	—	22	59	Clear
7	23	M	OD	Lime	9	AMT	1	360	360	2	8	31	Failed
8	17	M	OS	Lime	72	—	0	360	360	2	10	2	Clear
9	17	F	OD	Lime	48	Sym rel + AMT	1	360	360	5	21	1 day	Clear
10	14	M	OD	Lime	12	AMT	1	360	360	—	14	67	Clear

AMT: Amniotic membrane transplant, Sym rel: Symblepharon release, CLAG: Conjunctival limbal auto graft

alternate surgical technique in these cases is to first reconstruct the conjunctival damage and subsequently perform limbal stem cell transplantation to reconstruct the corneal surface 3–6 months later. This may reduce inflammation and improve the tear film at the time of limbal transplant. However, the risks outweigh the potential benefits as doing staged surgeries would increase cost and time to recovery for the patient.

The cumulative survival with the autologous cocultured transplant in our study was observed to be 60% at 1 year, 56% at 2 years, 45% at 4 years, and 36% after 5 years. Treatment of LSCD with conjunctival limbal autograft or allograft has shown even less success in corneal epithelialization with a cumulative survival of 33% at a mean of 33 months.<sup>[18,19]</sup> The significantly smaller size of conjunctival biopsy required for cultivation of conjunctival cells permits autologous transplantation despite extensive damage to the conjunctiva, as long as a small healthy donor area is available in either eye. The survival of the cocultivated transplant in our case series is expected to be less than that reported with cultivated limbal alone, owing to the above-mentioned reason of this subset of patients having more severe ocular surface damage. Our recently published report of survival of autologous limbal stem cell transplantation in a large case series of 200 patients in the age range of 8–69 years for unilateral LSCD due to chemical injuries showed a 71% survival at  $3 \pm 1.6$  years.<sup>[20]</sup> The duration between onset of symptoms and intervention ranged from 1 day to 7 years.

Epithelial transplantation in the acute phase of ocular surface damage with symptom duration less than 6 months was nearly four times less likely to survive than in the nonacute phase. Rao *et al.* observed similar differences in the outcome of limbal transplants between the acute and chronic stages, attributing the difference to the presence of inflammation and limbal ischemia in the acute stages.<sup>[21]</sup> The alternate possibility is that early surgery minimizes the degree of complications such as fibrovascular pannus and scarring.<sup>[22]</sup> However, observing the trend of better survival with the chronic group,

we routinely wait for at least 6 months postinjury for ocular surface reconstruction whenever possible. Differences have been described between the mechanism of acid and alkali injuries, most acids causing damage to the ocular surface by protein coagulation while most alkalis do so by rapid penetration into the deeper layers.<sup>[22]</sup> However, the difference between acid and alkali injury in the survival of transplanted epithelium is unclear.

The prognosis for PKP in severe chemical injury with vascularized corneal scar is graded by Krachmer as poor with success rate of 50% or less.<sup>[23]</sup> It is worthwhile to note that the success rate of PKP following coculture transplant showed 70% clear grafts at a mean follow-up time of  $43 \pm 32$  months in eyes that had limbal deficiency and conjunctival scarring or symblepharon in addition to vascularized corneal scar. This was also comparable to our previously reported outcome of PKP following stabilization of ocular surface with cultivated limbal transplantation alone, which showed 87% clear grafts at a shorter follow-up duration of 8 months.<sup>[24]</sup> The cocultivated epithelial transplant revealed a three- to five-layered epithelium with a corneal phenotype in the corneal buttons excised for PKP, similar to what was observed after cultivated limbal transplantation.<sup>[25,26]</sup>

The novel technique of coculturing central limbal and peripheral conjunctival cells on a single amniotic membrane was performed with the help of a self-designed physical ring barrier to segregate the *ex vivo* growth of the two cell types. The effective role of the barrier, in restricting the *in vitro* growth of limbal cells to the inner ring and conjunctival cells outside the ring, was substantiated by marker-based characterization of limbal and conjunctival cultures. The confluence of the two cell types in the cultures without barrier further confirms the role of the barrier in preventing the overgrowth of conjunctival cells into the central region.

In conclusion, the composite epithelium generated by cocultivating central limbal and peripheral conjunctival

cells is a novel alternate approach to CLET with additional conjunctival transplantation for patients with severe ocular surface disorders. The technique offers a simpler one-step surgical approach for extended ocular surface reconstruction. This can be considered as an advance in cell therapy and regenerative medicine, demonstrating the feasibility of cultivation and transplantation of two phenotypically different but contiguous epithelia.

## Acknowledgments

We thank Prof D. Balasubramanian for his invaluable assistance with the critical review of the manuscript. This project was supported by grants from the Department of Biotechnology, India, and the Champalimaud Foundation, Portugal.

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**Cite this article as:** Subramaniam SV, Sejal K, Fatima A, Gaddipati S, Vemuganti GK, Sangwan VS. Coculture of autologous limbal and conjunctival epithelial cells to treat severe ocular surface disorders: Long-term survival analysis. *Indian J Ophthalmol* 2013;61:202-7.

**Source of Support:** Nil. **Conflict of Interest:** None declared.

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