

# Midterm Outcomes of Autologous Cultivated Limbal Stem Cell Transplantation With or Without Penetrating Keratoplasty

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**Purpose:** To report the midterm outcomes of autologous limbal stem cell transplantation cultivated on amniotic membrane (AM) with or without subsequent penetrating keratoplasty (PKP) in patients with total unilateral limbal stem cell deficiency (LSCD).

**Methods:** Eight eyes of 8 consecutive patients with unilateral total LSCD underwent autologous limbal stem cell transplantation cultivated on AM. Four eyes underwent subsequent optical PKP. Main outcome measures were corneal vascularization and transparency.

**Results:** The patients were followed for  $34.0 \pm 13.5$  months (6–48 months). Seven cases had a stable corneal epithelium with marked decrease in opacification and vascularization. Progressive sectorial conjunctivalization was evident in all cases with subsequent PKP at the last follow-up. Primary failure was observed in one case because of exposure.

**Conclusions:** Transplantation of autologous stem cells cultivated on AM with or without subsequent PKP seems to be an effective way for visual rehabilitation in total LSCD. More work with more cases and longer follow-up are needed to optimize this procedure to provide and maintain an adequate supply of limbal stem cells in these patients.

**Key Words:** cornea, limbal stem cell deficiency, cultured cells, stem cell transplantation, penetrating keratoplasty

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Epithelial stem cells located at the limbus represent the ultimate source for corneal epithelial renewal and repopulation.<sup>1–3</sup> These poorly differentiated slow cycling cells have great capacity for colonogenic expansion and error-free division with a long life span. These cells, which act as a barrier against corneal conjunctivalization, are dependent on factors such as limbal stroma (stem cell niche), normal tear production, and normal conjunctival vasculature.<sup>4–6</sup>

When limbal stem cells are dysfunctional or deficient, limbal stem cell deficiency (LSCD) develops. It may be partial or total depending on the extent of limbal involvement and the underlying disease process. The clinical hallmark of LSCD is conjunctivalization of the corneal surface accompanied by recurrent and persistent epithelial defects, chronic inflammation, and scarring secondary to destruction of the basement membrane and ulceration of the cornea.<sup>4–7</sup>

Its surgical management depends on laterality and severity of corneal involvement. Partial LSCD could be managed using AM transplantation or sectorial conjunctival epitheliectomy.<sup>8–10</sup> In partial or total unilateral cases, conjunctival limbal autograft (CLAU) is a good choice. In bilateral total LSCD, keratolimbal allograft surgery, or living-related conjunctival limbal allograft are surgical alternatives.<sup>11</sup> Recently, transplantation of limbal epithelial stem cells cultivated on a carrier such as amniotic membrane (AM) or transplantation of ex vivo cultured autologous oral mucosal epithelial cells has been considered as alternative procedures to treat LSCD.<sup>12</sup> Because stem cell deficiency is sometimes accompanied by severe corneal stromal opacity and/or corneal endothelial dysfunction, most patients require penetrating keratoplasty (PKP) for visual rehabilitation. Herein, we report our midterm surgical outcomes with special emphasis on the results of subsequent PKP in these cases.

## MATERIALS AND METHODS

In this interventional case series, 8 eyes of 8 patients (all males) with unilateral total LSCD because of chemical or thermal burn underwent autologous cultivated limbal stem cell transplantation. The study was conducted at Labbafinejad Medical Center, Shahid Beheshti University (Medical Campus) with the collaboration of Royan Institute, Tehran, Iran, during 2004–2008. It was approved by the Institutional Review Board and Ethics Committee of the Ophthalmic

Research Center of Shahid Beheshti University and Royan institute. The risks and advantages of the surgery were completely explained to the patients, and an informed consent was obtained from all patients. All surgeries were performed by 2 surgeons (A.B.-R. and M.-A.J.).

Diagnosis of LSCD was based on characteristic clinical findings confirmed by the presence of corneal goblet cells on impression cytology. Typical clinical manifestations included complete vascularization and opacification of the cornea, corneal conjunctivalization, chronic ocular surface inflammation, and poor epithelial integrity (persistent or recurrent epithelial defects).

All patients had a comprehensive ophthalmic examination including measurement of uncorrected visual acuity, best spectacle-corrected visual acuity, slit-lamp biomicroscopy, intraocular pressure (IOP) measurement, and fundoscopy before surgery. B-scan ultrasonography was performed on eyes that had severe haziness of ocular media to rule out gross posterior segment pathologies. Adequacy of tear film was evaluated using (1) tear meniscus height (normal,  $\geq 0.5$  mm) and (2) Schirmer test (*a*) without anesthesia (normal tear level  $\geq 25$  mm) and (*b*) with anesthesia (normal tear level  $\geq 15$  mm). The findings were documented using digital corneal photography (Imagenet and SL-8Z; Topcon, Tokyo, Japan).

Epithelial transparency and superficial vascularization of the cornea were independently graded by 2 of the authors (B.E. and P.B.) before and 3 and 6 months after surgery from 1+ to 4+, given that the preoperative condition of each patient was considered as 4+. For more accuracy, they used both slit-lamp findings and digital corneal photographs. In cases without subsequent keratoplasty, the grading was repeated 12 months after surgery. Mean of grading by 2 examiners was considered as the final grade. Improvement of at least 1+ at both defined clinical parameters was considered as clinical success.

If corrected visual acuity was  $\leq 20/200$ , subsequent PKP was performed for visual rehabilitation. In eyes with subsequent keratoplasty, the lack of conjunctivalization in the central 5 mm of the cornea was considered as clinical success. Appearance of a peripheral superficial vascularized corneal pannus was considered as corneal conjunctivalization, which was supported by late fluorescein staining and confirmed by impression cytology. Progression of the conjunctivalization to the central corneal area was considered as failure. Impression cytology was performed before and 6–9 months after corneal transplantation.

### Impression Cytology

After instilling anesthetic drop (tetracaine 0.5%) into the conjunctival sac of the diseased eye, excess moisture was cleared. Trapezoid end-pointed small strip papers were cut using a cellulose acetate filter (47 mm, pore size 0.45  $\mu\text{m}$ ) (Schleicher & Schuell Microscience GMBH, Dassel, Germany). Four 5  $\times$  5 mm-cut papers were applied on the superior, inferior, nasal, and temporal quadrants or recipient–donor junctions, based on the standard method for a few seconds with gentle pressure using the blunt edge of a forceps according to Tseng modified method.<sup>13</sup> Cellulose papers were applied onto the eye so that the paper straddled either the limbal area or the cornea recipient–donor junction.

After sampling, the filter paper with the specimen was fixed in a cytology fixative containing glacial acetic acid,

formaldehyde, distilled water, and ethyl alcohol in a 1:1:6:14 volume ratio and within a labeled 24-well container. In the pathology laboratory, after rehydrating in 70% alcohol, the specimens were stained with a combination of periodic acid–Schiff and Papanicolaou staining.<sup>7,13</sup> Finally, the papers were cleared in xylene, and each paper was mounted with a DPX mountant (Depex-Polystyrene dissolved in xylene) on a glass slide with the epithelial cells facing up. The prepared slides were examined with a light microscope (BX43; Olympus, Tokyo, Japan) attached to a digital camera (DP12; Olympus) by one ophthalmopathologist (M.R.K.).

### Tissue Screening

All AM donors had a negative history for transmissible diseases. They were screened for human immunodeficiency viruses I and II, human T-cell leukemia–lymphoma I and II, hepatitis B and C, and syphilis before use. All AMs were prepared, procured, and processed at Royan Institute.

### Limbal Biopsy in the Healthy Eye

Under local anesthesia, a superficial small limbal tissue (30% deep) was harvested from the superior limbus of the healthy eye. It was 1 mm in tangential diameter and was extended from the sclera 1 mm posterior to the limbus into 1 mm of clear cornea with a conjunctival mantle. The limbal biopsies were transported to the tissue bank of Royan Institute in phosphate-buffered saline (PBS) and processed immediately for final cultivation over the AM. Both puncta of the fellow diseased eye was cauterized at the time of limbal biopsy.

### Preparation of Human AM and Culture of Limbal Biopsies

AMs were obtained under sterile conditions from healthy delivering women undergoing elective cesarean sections. The tissues were processed as previously reported.<sup>14</sup> Briefly, the tissue was washed with PBS containing ofloxacin (0.3%) and gentamicin (50  $\mu\text{g}/\text{mL}$ ), then flattened onto nitrocellulose paper with the epithelium/basement membrane side up and cut into pieces of approximately 3  $\times$  3 cm. The AM pieces were stored in PBS containing 1.5% dimethyl sulfoxide at  $-70^\circ$  centigrade for up to 5 months. Before using, AM pieces were thawed, washed with PBS, and incubated in ethylenediaminetetraacetic acid 0.2% at 37°C for 15 minutes to eliminate cellular adhesions, followed by gentle scraping in 5% ammonium chloride to remove the epithelium without breaking the basement membrane. Acellularity of AM was confirmed by a phase contrast inverted microscope (CKX 41; Olympus). The denuded AM was washed with PBS and then attached, basement membrane side up, to the bottom of a cell culture insert from which the base had been removed.

The limbal biopsy was irrigated 3 times in Dulbecco's modified Eagle medium and F12 (DMEM/F12) containing amphotericin B (1.25  $\mu\text{g}/\text{mL}$ ) and gentamicin (50  $\mu\text{g}/\text{mL}$ ). Excess conjunctiva was removed from the biopsy under a stereomicroscope. The remaining tissue was irrigated in the above-mentioned solution and then incubated in dispase II (1.2 U/mL) in Hanks-buffered salt solution (without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) for 5–10 minutes at 37°C and 5%  $\text{CO}_2$ . The tissue was

then irrigated by DMEM/F12 medium containing 5% human serum albumin.

The limbal epithelial cells were cultured as previously described.<sup>14</sup> Briefly, the limbal biopsy specimens were inoculated onto the basement membrane side of the denuded AMs and cultured in DMEM/Hams F12 (1:1) supplemented with 5% fetal bovine serum (FBS), 0.5% dimethyl sulfoxide, 2 ng/mL human epidermal growth factor, 5  $\mu$ g/mL insulin, 5  $\mu$ g/mL transferrin, 5 ng/mL selenium, 0.5  $\mu$ g/mL hydrocortisone, 30 ng/mL cholera toxin, 50  $\mu$ g/mL gentamicin, and 1.25  $\mu$ g/mL amphotericin B. Cultures were incubated in a humidified incubator in 95% air and 5% CO<sub>2</sub>. The cultures were maintained for 10 days, and the medium was replaced every 2 days. At day 10, the limbal biopsies were removed from AM and cultivation was continued for an extra 4 days. Cellular phenotype and the process of stem cell migration of the cultured cells were evaluated by an inverted phase contrast microscope. When cell sheets were confluent in an expansion of approximately 2 × 2 cm, they were washed in serum and cholera toxin-free corneal epithelium culture medium for a maximum of 24 hours and then transported to the operating room for immediate transplantation.

### Transplantation Surgery

Under general anesthesia, a 360-degree limbal peritomy was performed. Subconjunctival scar tissue and excess Tenon layer were dissected and removed. Conjunctiva was recessed 3–5 mm posterior to the limbus. The fibrovascular scar tissue (corneal pannus) covering the cornea was dissected and stripped off. The AM with overlying cultivated stem cells was placed epithelial side up on the bared surface of the cornea and adjacent sclera. It was sutured onto the recipient episclera with several tangential long-bite separate 10-0 nylon sutures. The conjunctiva was closed over the periphery of the graft using a running 10-0 nylon suture. Finally, the ocular surface was covered with an overlay of AM epithelial side down acting as a patch to protect the transplant. At the end, lateral tarsorrhaphy was performed.

### Postoperative Management and Follow-up

Postoperative medical treatment included a topical steroid (betamethasone 0.1%), antibiotic (chloramphenicol 0.5%), and preservative-free artificial tears (Artelac; Bausch & Lomb, Rochester, NY). Topical steroid was tapered gradually based on the severity of ocular surface inflammation and discontinued after 1.5–2 months. The antibiotic drop was continued until complete epithelialization. Artificial tears were used for lubrication as needed. All patients received systemic prednisolone 1 mg·kg<sup>-1</sup>·d<sup>-1</sup>, which was typically tapered off over 4–6 weeks.

Follow-up visits were scheduled on days 1, 3, and 7; weekly for up to 1 month; every 2 weeks up to 3 months; and monthly up to 1 year. Thereafter, the patients were regularly visited every 3 months. In each follow-up visit, a complete eye examination with special attention to corneal epithelial integrity, vascularization, and transparency was performed. Epithelial healing was followed under the overlay AM using fluorescein dye waiting for a few minutes for penetration of the fluorescein under the AM.

Based on the criteria mentioned above, corneal transplantation was performed using Hessburg-Barron vacuum trephine system in a standard manner as needed at least 6 months after stem cell transplantation. Interrupted suturing using 10-0 nylon was used.

## RESULTS

Eight eyes of 8 patients (all males) with unilateral total LSCD were operated on (Table 1). They were injured by an acidic (n = 2) or alkaline (n = 3) chemical agent or thermal burn (n = 3). The mean age at the time of surgery was 35.9 ± 17.8 years (20–65 years). The mean interval between ocular damage and surgery was 5.6 ± 5.7 years (1–15 years). The mean follow-up was 34.0 ± 13.5 months (6–48 months). Four subsequent optical PKPs were performed on 4 eyes to improve visual acuity (12, 6, 8, and 6 months after surgery).

All of the overlay AMs were ruptured and finally dissolved 2–3 weeks after the surgery. Epithelial defect healed in 7 eyes during first 2 weeks. One eye (case 5) had tectonic PKP because of persistent epithelial defect (epithelial defect longer than 14 days), leading to corneal perforation because of a small upper eyelid notch and infrequent blinking causing chronic exposure. He had upper eyelid reconstruction and entropion repair before surgery. Despite multiple conservative and surgical interventions (including autologous serum drops, lateral and medial tarsorrhaphies, heavy lubrication, frequent patching periods, and conjunctival flap), his eye was finally covered by oral mucosa to prevent recurrent corneal perforation (Table 2).

Corneal transparency and superficial vascularization, which were graded as 4+ before surgery, changed to a mean of 2.4 ± 0.5 (2+ to 3+) and 2.3 ± 0.5 (2+ to 3+) 3 months; 2.0 ± 0.6 (1+ to 3+) and 2.1 ± 0.6 (1+ to 3+) 6 months after surgery. It was 1.8 ± 0.3 (1.5+ to 2+) and 2.2 ± 0.8 (1.5+ to 3+) in cases without subsequent keratoplasty 12 months after the surgery, respectively.

Epithelial defect healed in all 4 cases with subsequent optical PKPs during 2 weeks. We had 3 cases of success in these eyes. Total progressive corneal conjunctivalization was observed in 1 case (failure). In successful cases, a slowly progressive corneal conjunctivalization was observed sparing the central 5 mm of cornea. Impression cytology confirmed conjunctivalization in these 4 eyes.

Ocular hypertension was observed in one case with subsequent PKP, which was refractory to medical treatment (case 6). A cataract was observed in one case because of chronic steroid usage and previous ocular surgeries (case 6). We had 5 episodes of endothelial rejection in 3 PKPs. One eye had 3 episodes of rejection, which was considered a high-risk graft, and was kept on systemic mycophenolate mofetil (Cellcept; Hoffmann-La Roche, Inc, Nutley, NJ). Although, it was later discontinued because of hepatic toxicity.

### Case Reports

#### Case 1

A 42-year-old man who had suffered from severe chemical burn in his left eye 15 years ago presented with extensive corneal opacity and vascularization with a visual

TABLE 1. Demographic Data

Case	Age (yrs)	Eye	Cause of LSCD	Time Interval Between Burn and Surgery (yrs)	BSCVA			Subsequent PKP/Month After	BSCVA After PKP	Follow-up (mo)	Duration of Epithelial Healing (d)	Previous Surgeries	Final Outcome
					Before	After	Before PKP						
1	42	OD	Thermal	15	HM	20/80	20/200	Yes/12 m	20/40	48	5	PKP + ECCE + PC IOL	Success
2	20	OS	Thermal	4	CF@2.5 m	20/200	20/120	No	—	48	13	No	Success
3	23	OS	Alkaline	1	CF @ 30 cm	20/120	20/120	No	—	36	10	Laser epilation + entropion repair + forniceal reconstruction + KLAL + PKP	Success
4	22	OD	Alkaline	5	HM	20/200	20/80	No	—	28	7	Forniceal reconstruction +	Success
5	26	OD	Thermal	1	HM	20/120	CF @ 1	Yes/7 m	—	40	PED	AMT + entropion repair + LKP + forniceal reconstruction + upper lid notch repair	Primary Failure
6	29	OS	Alkaline	14	HM	20/80	20/200	Yes/6 m	20/80	36	7	No	Success
7	60	OS	Acid	1	CF @ 1	CF @ 2.5	CF @ 2.5	Yes/8 m	20/200	30	12	Forniceal reconstruction + ECCE	Failure
8	65	OD	Acid	4	HM	20/200	20/200	Yes/6 m	20/60	6	10	No	Success

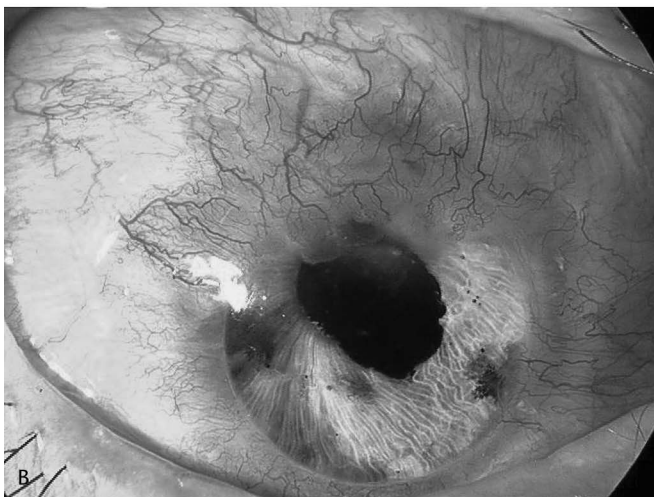
AMT, amniotic membrane transplantation; BSCVA, best spectacle-corrected visual acuity; CF, count finger; ECCE, extracapsular cataract extraction; HM, hand motion; KLAL, keratolimbal allograft surgery; LKP, lamellar keratoplasty; LP, light perception; PED, persistent epithelial defect; PC IOL, posterior chamber intraocular lens.

acuity of hand motion (Fig. 1A). There was a history of corneal transplantation, cataract extraction, and intraocular lens implantation 4 years after the injury. Right eye was normal with visual acuity of 20/20. The patient underwent transplantation of autologous limbal stem cells cultivated on AM. There was an inferior progressive conjunctivalization with extension toward the central cornea 9 months after the surgery confirmed by impression cytology (IC). One year after the stem cell transplantation, corneal re-graft was performed in the left eye. Six months after re-graft, the transplanted cornea was clear. At the 9-month visit, there were punctate corneal epithelial erosions at the superior quadrant where a superficial vascularized area was noted at the donor-recipient area. Impression cytology disclosed conjunctivalization in the superior and nasal quadrants. At 17-month follow-up visit, the best spectacle-corrected visual acuity was 20/80, and the patient was asymptomatic. There were foci of punctate corneal epithelial erosions together with superficial interface vascularization and mild epithelial haze at the superior region (Fig. 1B).

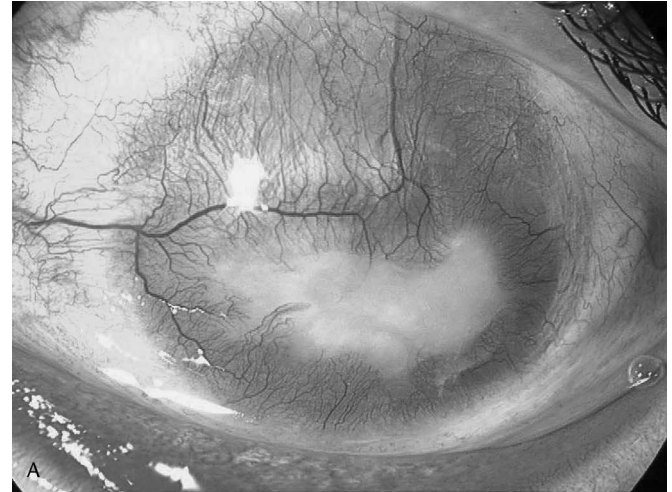
### Case 6

A 29-year-old-man, a victim of an alkaline chemical injury 14 years ago, presented with severe corneal opacity and vascularization of the left eye (Fig. 2A). Vision of the right and left eyes was 20/20 and hand motion, respectively. The injured eye underwent cultivated stem cell transplantation. Visual

acuity improved to counting fingers at 75 cm along with moderate decrease in corneal vascularization and opacity 3 months after transplantation. Because of deep corneal stromal opacification, PKP was performed 6 months later. One month after PKP, visual activity improved to 20/120. Six weeks later, IOP rose to 32 mm Hg, which was controlled by topical timolol and systemic acetazolamide. Cup to disc ratio was  $0.3 \times 0.3$ . Systemic acetazolamide was discontinued because of elevated liver enzymes and replaced by topical dorzolamide 2%. Eight weeks after surgery, the patient developed epithelial rejection, which was treated with topical steroids. Four months after surgery, IOP rose to 30 mm Hg again, brimonidine 0.2% (Allergan, Inc, Irvine, CA) and latanoprost 0.005% (Pfizer, Inc, New York, NY) were added to his therapeutic regimen. Five months after PKP, visual acuity improved to 20/80 but IOP went out of control. The patient underwent Ahmed glaucoma valve surgery. Five months after Ahmed glaucoma valve, corneal endothelial and subepithelial rejections were observed, which were aggressively treated with topical betamethasone. He had 3 attacks of corneal endothelial rejection. There was a slight progressive vascularization from the graft periphery 8 months after PKP. Considering multiple episodes of corneal endothelial rejection and progressive corneal conjunctivalization, systemic cyclosporine 300 mg (Sandimmune; Novartis Pharma Stein AG, Stein, Switzerland) daily was started. One month later, it was replaced by



**FIGURE 1.** Clinical photographs of the left eye of case 1. A, Preoperative view showing extensive corneal vascularization and opacification. B, Seventeen months postoperative view showing limited and progressive conjunctivalization of superior part of the corneal graft.



**FIGURE 2.** Clinical photographs of the left eye of case 6. A, Preoperative view displaying corneal conjunctivalization and vascularization. B, Twenty months post PKP slit-lamp photograph of the left cornea demonstrating limited conjunctivalization. Note central clear cornea.

mycophenolate mofetil 2 g/d because of elevated liver enzymes. Two months later, the liver enzymes critically rose again and so it was discontinued. After that, the patient was put on fluorometholone 0.25% drop once daily and cyclosporine 2% drop. One year after PKP, impression cytology confirmed corneal conjunctivalization in superior and inferior temporal quadrants. About 20 months after PKP, vascularization and conjunctivalization were moving on but it had not reached the central 5 mm of the cornea (Fig. 2B). At the last visit, visual acuity maintained on 20/80 and IOP was 16 mm Hg without any glaucoma medication.

## DISCUSSION

Our study suggests that cultivated stem cell transplantation seems to be an effective way for rehabilitation of vision in patients with unilateral total LSCD. The long-term results are indefinite. Although visual acuity improved

significantly in the case of subsequent PKP, we observed slowly progressive vascularization and conjunctivalization on the graft at midterm follow-up.

Management of LSCD depends on the extent of involvement (partial or total), laterality (unilateral or bilateral), severity of ocular surface inflammation, presence of symblepharon, tear status, and ocular surface keratinization, in addition to systemic factors such as age and general health of the patient.<sup>15–17</sup> Preliminary measures such as correction of eyelid's structural abnormalities, trichiasis, tear film normalization by emollients, transient or permanent punctal occlusion, and tarsorrhaphy are important and should be considered before performing stem cell transplantation.<sup>4,17</sup> These measures can provide symptomatic improvement in mild cases, especially in the presence of adequate transient amplifying cells (TACs) in the center of the cornea. Undue surgery or administration of eyedrops containing preservatives should be avoided in these cases.<sup>4</sup>

**TABLE 2.** Outcomes of Autologous Cultivated Limbal Stem Cell Transplantation in Patients With Unilateral Limbal Stem Cell Deficiency

Case	Epithelial Haziness				Superficial Vascularization				Impression Cytology		Beginning of Conjunctivalization
	After Surgery (mo)				After Surgery (mo)				Before PKP/Month After the Surgery	After PKP/Time	
	Before	3	6	12	Before	3	6	12			
1	4+	2.5+	1+	—	4+	2+	2+	—	Inf with extension toward the center/9	Sup & Nas/9	9 m
2	4+	3+	2.5+	2+	4+	2+	3+	3+	—	—	—
3	4+	2+	2+	2+	4+	2+	2.5+	2+	LSCD in all quadrants/9	—	—
4	4+	2+	1.5+	1.5+	4+	2+	1+	1.5+	Temp/7	—	—
5	4+	—	—	—	4+	—	—	—	—	—	—
6	4+	2+	2+	—	4+	2+	2+	—	—	Sup & inf tem/12	9
7	4+	3+	3+	—	4+	3+	2+	—	—	LSCD in all quadrants/12	6
8	4+	2.5+	2+	—	4+	3+	2+	—	—	No LSCD/6	—

Inf, inferior; Nas, nasal; Sup, superior; Temp, temporal.

Partial LSCD, especially in the presence of adequate number of TACs, may be cured with AM transplantation alone. In the event of unilateral and total LSCD, transplantation of autologous limbal stem cells by CLAU or conjunctival limbal allograft from a living-related donor is a last resort.<sup>11</sup> The main objective is to continue to supply a new corneal epithelium for a prolonged, if not indefinite, period so that patients can be relieved from annoying photophobia and regain useful visual acuity. These procedures provide more fresh tissue for transplantation. Cultivation of a small part of the limbus may provide epithelial progenitor cells, which might survive for a while on the ocular surface. In eyes with superficial corneal vascularization and pannus, a single procedure is frequent enough. However, if there is a concomitant deep corneal stromal scar, PKP or lamellar keratoplasty is needed to restore vision.<sup>11,18–20</sup> Although, CLAU has been reported to be a very successful procedure, a drawback of it is that because of removal of fairly large segments of limbal tissue, the donor eye is at risk of surgically induced LSCD.<sup>11</sup> Despite this concern, reports regarding subsequent stem cell deficiency in a healthy donor eye are very rare and it seems that in the case of a good patient selection, it is a safe and uneventful procedure.<sup>11</sup> There is no definite agreement on the maximum safe size for harvesting limbal tissue, but it is prudent not to remove large amounts of the limbus.<sup>16,21</sup> Cultivating a small amount of limbal tissue, which has been suggested to overcome this limitation, seems to provide adequate limbal stem cells for treatment of total LSCD.

There have been a number of reports on ex vivo expanded autologous limbal stem cell transplantation with short- and midterm follow-up periods.<sup>22–34</sup> In almost all studies, the authors conclude that this method is a successful procedure to treat unilateral corneal stem cell deficiency. Some key questions still need to be answered. The exact proportion of stem cells present in ex vivo cultured limbal epithelial cell sheets is unclear and needs to be determined. The behavior of limbal epithelial stem cells post transplantation needs to be elucidated. It has been proposed that the success of this treatment relies on the reintegration of exogenous cultured

limbal stem cells into the ocular surface and that these cells function to continuously replenish the corneal epithelium. It is interesting that despite the different methodologies employed, the success rate and outcomes are remarkably similar. There are also several studies about successful use of cultivated autologous oral mucosal epithelial transplantation to treat LSCD.<sup>35–40</sup> In a study by Sangwan et al,<sup>41</sup> the authors reviewed medical records of 15 patients with LSCD because of chemical burns who underwent PKP after cultivated limbal epithelium transplantation (autologous, n = 11 and allogenic, n = 4) at a mean interval of 7 months. In their case series, 14 (93%) of the 15 eyes had a successful corneal graft with a stable corneal epithelium. None of the limbal epithelial allografts showed signs of rejection. Finally, they concluded that early results of PKP after cultivated limbal epithelium transplantation were favorable when performed after stabilizing the ocular surface.

There are a few ways to judge the success of this surgery including clinical judgment of ocular surface health by corneal epithelial transparency and degree of vascularization (which is subjective) and laboratory analysis including immunohistochemistry and cytochemical tests. The clinical diagnosis of conjunctivalization over a cornea with previous cultivated stem cell transplantation is very difficult. The corneal epithelium is irregular, and the stroma is opaque and vascularized. Therefore, the optical contrast is not enough to determine the wave of slowly progressive conjunctivalization. However, after lamellar keratoplasty or PKP, in an eye with previous stem cell transplantation (especially the cultivated type), tracking the superficial wave of conjunctivalization and vascularization is more convenient.

We had 4 cases of subsequent optical PKPs with different degrees of progressive conjunctivalization. This confirms the presence of epithelial progenitor cells on the ocular surface, but whether these cells are true stem cells or just TACs has to be identified. We selected fresh donor's corneas with a very good coverage of epithelial cells; therefore, some of the TACs that support corneal clarity might have been transferred by corneal graft. We are not able to definitely judge about the source of corneal clarity whether

it is related to TACs transferred by corneal graft or epithelial progenitor cells cultivated on the AM and transferred to the ocular surface. Nevertheless, it seems that corneal clarity is relatively longer than usual corneal graft without previous stem cell transplantation.

It is well known that stem cell transplantation, especially allogenic or cadaveric types, has a limited survival.<sup>19,42</sup> Stem cells die gradually because of acute or chronic immunologic rejection and/or offending mechanisms such as exposure or tear film instability. In the case of autologous stem cell transplantation (CLAU or cultivated), the same offending mechanisms, with the exception of immunologic rejection, may play a role. Given that epithelial progenitor cells have been transferred on the AM to the ocular surface, progressive conjunctivalization might be attributable to gradual cell attrition and final failure of the graft. This might be because of several factors including unstable tear film, lid margin notching, cicatricial entropion or ectropion, trichiasis, symblepharon, shallow fornices, conjunctival irregularity, and finally improper stem cell niche.

Most of the patients with total LSCD have also associated eyelid structural abnormalities and tear film problems. Stem cells have to permanently be covered by a continuous tear film. It is well known that exposure and dry eye are 2 very important risk factors for survival of stem cell grafts.<sup>4,15</sup> Despite previous reconstructive upper lid surgery, we had one case of primary failure, which was because of chronic exposure resulting from upper lid irregularity and infrequent blinking. Therefore, it is very important to normalize the condition of eyelids and tear film before any kind of stem cell transplantation.

Based on our experience, because of uncertainties regarding the optimized method of cultivation, nature of the cultivated growing cells, indefinite long-term clinical outcomes, cost of these procedures, and fully established effective results of CLAU, we think that cultivation should be performed on the older patients. Probable failure of cultivation with subsequent CLAU may compromise the fellow healthy eye in a young patient.

There are some limitations in this study. We had a limited number of cases with midterm follow-up. We did not perform PKP in all cases. We do not have an objective laboratory judgment about the outcome of the surgery and survival of the stem cells. But we believe that our clinical judgment is more practical because tracking of the conjunctivalization on the graft is easier and more convenient.

In conclusion, cultivated stem cell transplantation on the AM is a partially effective way to improve the health of the injured ocular surface. More work with more cases and longer follow-up are needed to optimize this procedure to provide and maintain an adequate supply of limbal stem cells in these patients.

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