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# Clinical outcomes of xeno-free allogeneic cultivated limbal epithelial transplantation for bilateral limbal stem cell deficiency

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## ABSTRACT

**Purpose** To report the clinical outcomes of allogeneic cell-based therapy for bilateral corneal blindness due to limbal stem cell deficiency (LSCD).

**Methods** This retrospective study included 28 eyes of 21 patients, at least 8 years of age, with bilateral and total LSCD, treated between 2001 and 2010. A limbal biopsy was obtained from the eye of an adult living related donor. The limbal epithelial cells were cultivated in the laboratory using a xeno-free explant culture technique and transplanted onto the recipient eye after 10–14 days. All transplant recipients received topical and systemic immunosuppressants.

**Results** At a mean follow-up of 4.8±2.8 years, 20 (71.4%) eyes maintained a completely epithelised, avascular and stable corneal surface, and among them 13 (46.4%) eyes subsequently underwent a penetrating keratoplasty (PK). The Kaplan–Meier survival rate of the PK allograft was 76.9±11.7% at 1 year with a median survival of 3.3 years. Visual acuity improved to 20/60 or better in 19 (67.8%) eyes. No donor or recipient eyes developed serious ocular complications.

**Conclusions** Allogeneic cultivated limbal epithelial transplantation, followed by PK when needed, can successfully restore the ocular surface and improve vision in patients with corneal blindness due to bilateral LSCD.

Stem cells of the corneal epithelium are located at the limbus in specialised structures called the palisades of Vogt.[1 2] Rarely, severe inflammatory or traumatic damage to the limbal stem cells causes corneal epithelial dysfunction, progressive conjunctival overgrowth over the corneal surface, loss of corneal clarity and blindness.[2] However, this pathological process can be reversed by transplanting the limbal epithelium from a normal eye onto the diseased eye.[2 3] In unilateral cases, the healthy fellow eye can act as the autologous donor, while in bilateral cases an allogeneic donor is required, either living or cadaveric.[4] Living donors are preferable as limbal cells obtained from cadavers have a lower proliferative rate in vitro[5] and a poorer corneal epithelisation rate in vivo.[6] Also, expanding the donor limbal cells as an epithelial sheet in the laboratory (ex vivo) before transplantation,[7 8] as opposed to direct transplantation, offers significant advantages in terms of reducing both the amount of donor tissue required and the corneal epithelisation time after transplantation.[9]

Although numerous groups have described different laboratory techniques for ex-vivo cultivation of limbal cells,[8] only a few of the reported protocols are completely free of animal-derived products or xenobiotics.[8 10] In fact, there are no published reports on the clinical application of allogeneic limbal epithelial cells cultivated ex vivo using xeno-free culture protocols. To fill this gap in the existing literature, this study describes the long-term clinical outcomes and complications of xeno-free allogeneic cultivated limbal epithelial transplantation in patients with bilateral limbal stem cell deficiency (LSCD).

## METHODS

### Patient selection

A retrospective chart review of 552 patients who underwent cultivated limbal epithelial transplantation at the L V Prasad Eye Institute, Hyderabad, India, between 1 April 2001 and 1 April 2010 was carried out. The inclusion criteria for this study were: patients who were 8 years or older when they developed corneal blindness and patients who underwent allogeneic limbal transplantation for bilateral and total LSCD (defined as 360° superficial corneal vascularisation, diffuse fluorescein staining of the corneal surface with or without persistent epithelial defects, conjunctivalisation of the corneal surface and absence of limbal palisades of Vogt). Patients who underwent autologous limbal transplantation (n=527) and cases in which fetal bovine serum (FBS) was used for limbal culture (n=4) were excluded from the study. Patients with dry eye disease (Schirmer's test without anaesthesia of <10 mm in 5 min), patients with no visual potential as determined by clinical examination and electrophysiological testing (flash visual evoked potential and flash electroretinogram) and patients with untreated ocular comorbidities, such as glaucoma and infection, were not considered for surgery.

### Data collection

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

### Patient demographics

During the entire study period 28 eyes of 21 patients underwent allogeneic cultivated limbal epithelial transplantation. The mean age at the time of surgery was 27.9±14.7 years with a male to female ratio of 4.25 : 1. The median time period between the initial injury and allogeneic cultivated limbal epithelial transplantation surgery was 38 months (range 9–432). All eyes had total LSCD with 360° of corneal neovascularisation. Other preoperative clinical characteristics of the transplanted eyes are summarised in table 1.

### Surgical technique of limbal biopsy

Donors underwent a general physical examination and haematological screening for HIV, hepatitis B and C, and syphilis. A limbal biopsy was taken from first-degree living-related donors (parents or adult siblings). The procedure involved careful dissection of a 2×2 mm piece of limbal epithelium with 0.5 mm of clear corneal stromal tissue at the limbus from the donor under strict aseptic conditions. Limbal tissue that contained limbal epithelial cells at the pigmented line (palisades of Vogt) and a part of the corneal stroma was excised.[10–13]

### Technique of limbal cultivation and transplantation

The standardised laboratory technique of limbal cultivation using a xeno-free explant culture technique and the surgical technique of limbal transplantation have been described in detail previously,[10–13] and are provided in the supplementary appendix (available online only). Very briefly, explants were cultured in a media cocktail that included autologous serum from the donor and growth factors, making them xeno free.

### Postoperative topical treatment regimen

Both donor and recipient eyes received topical prednisolone acetate 1% eye drops eight times a day, tapered gradually based on the level of inflammation, and ciprofloxacin 0.3% eye drops four times a day in the first postoperative week or until complete epithelisation was noted.

**Table 1** Demographic characteristics of eyes undergoing allogeneic cultivated limbal epithelial transplantation for bilateral and total LSCD

| Characteristic | N (%) |
|---|---|
| Visual acuity at presentation | |
| Worse than 20/200 | 26 (93) |
| 20/200 | 2 (7) |
| Aetiology of limbal stem cell deficiency | |
| Acid burns | 8 (29) |
| Alkali burns | 8 (29) |
| Chronic ocular allergy | 4 (14) |
| Chronic contact lens wear | 2 (7) |
| Ocular cicatricial pemphigoid | 2 (7) |
| Stevens–Johnson syndrome | 1 (3.5) |
| Idiopathic | 1 (3.5) |
| Thermal injury | 1 (3.5) |
| Blast injury | 1 (3.5) |
| Previous ocular surgery | |
| Amniotic membrane grafting | 11 (39) |
| Lid surgery | 4 (14) |
| Limbal transplantation | 4 (14) |
| Penetrating keratoplasty | 2 (7) |

LSCD, limbal stem cell deficiency.

### Postoperative systemic treatment regimen

Systemic immunosuppressants were administered to all recipients after adequate counselling about possible adverse reactions. Baseline haematological investigations, hepatic and renal parameters were obtained. These parameters were reassessed every 4–6 weeks. The routine immunosuppressive protocol was to start oral ciclosporin therapy systemically in a dosage of 5–7 mg/kg 48 h before surgery, along with methylprednisolone, 1 g intravenously, for the first three consecutive postoperative days. During the postoperative period, ciclosporin was tapered to the maintenance dosage of 1.5–2 mg/kg over 4–8 weeks. Patients also received oral prednisolone, 1 mg/kg, which was tapered on a weekly basis to the maintenance dosage of 5 mg/day. The patients were continued on the maintenance dose, until the graft failed or the patients developed signs of drug-related toxicity.

### Follow-up schedule and additional surgery

Both donors and recipients were seen on postoperative day 1, at 1 week, at 2–4 weeks and thereafter every 6–8 weeks. At each follow-up visit a comprehensive ophthalmic examination of both eyes was performed. Penetrating keratoplasty (PK) was done 12 months or later after successful limbal transplantation if the BCVA was worse than 20/60 and attributed to corneal stromal scarring.

### Primary outcome measure

The primary outcome measure was the success of limbal transplantation, defined clinically as a completely epithelised, avascular and clinically stable corneal surface (figure 1A,B). Failure was defined as the recurrence of LSCD in the form of superficial corneal vascularisation, conjunctivalisation or persistent epithelial defects (figure 1C,D). Survival time was calculated in months from the date of limbal transplantation to the date of failure or the date of last follow-up depending on the clinical outcome.

### Secondary outcome measures

The secondary outcome measure was success of PK, defined clinically as a clear corneal graft. Failure was defined as loss of corneal clarity for more than 2 months. Survival time was calculated in months from the date of PK to the date of failure or the date of last follow-up depending on the clinical outcome. Other outcome measures were improvement in BCVA at last follow-up or before undergoing PK compared to baseline; and complications in both donor and recipient eyes.

### Histopathology and immunohistochemical analysis

The ocular surface pannus excised during limbal transplantation and the corneal button excised during PK was sent for histopathology and immunohistochemical analysis. The detailed laboratory protocols are described in the supplementary appendix (available online only).

### Statistical analysis

All statistical analyses were performed using MedCalc statistical software V.11.5.1.0 (MedCalc Software, Mariakerke, Belgium). Kaplan–Meier survival analysis was performed to estimate the probability (reported as percentage with standard error) of limbal or PK allograft survival.

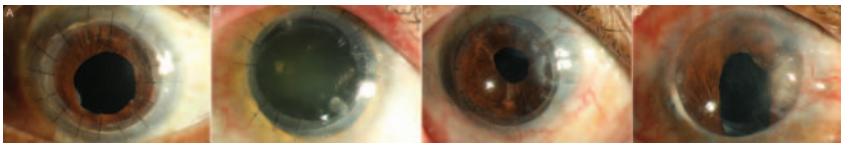

**Figure 1** Postoperative clinical photographs of four eyes that underwent allogeneic cultivated limbal epithelial transplantation, followed by penetrating keratoplasty (PK) for limbal stem cell deficiency (LSCD). (A) Left eye of a 21-year-old man with bilateral acid burns, 14 months after PK and 26 months after limbal transplantation showing a stable surface and a clear graft, with all sutures intact. (B) Left eye of a 40-year-old woman with bilateral alkali burns, 2 years after PK and 40 months after limbal transplantation, showing a stable surface, a clear central graft and the scar of a resolved suture infiltrate at 4 o'clock. (C) Right eye of a 34-year-old man with bilateral acid burns, 3 months after PK and 18 months after limbal transplantation showing early recurrence of LSCD between 12 and 3 o'clock. (D) Right eye of a 14-year-old girl with bilateral alkali burns, 2 years after PK and 3 years after limbal transplantation showing recurrence of LSCD superiorly between 7 and 4 o'clock.

## RESULTS

### Primary outcome

At a mean follow-up of 4.8±2.8 years (range 1–9.5), 20 (71.4%) eyes maintained a completely epithelised, avascular and stable corneal surface. The Kaplan–Meier survival rate of allogeneic limbal transplantation was 76.4±8.7%, 70.5±8% and 63.9±8.9% at 1, 2 and 3 years and thereafter (figure 2).

### Outcomes of PK

Thirteen (46.4%) eyes underwent PK following allogeneic cultivated limbal epithelial transplantation. The median duration between allogeneic cultivated limbal epithelial transplantation and PK was 14 months (range 12–22). The Kaplan–Meier survival rate for PK grafts was 76.9±11.7% at 1 year, with a median survival of 3.3 years (figure 3).

### Visual outcomes

BCVA improved from 20/200 or worse in all 28 recipient eyes preoperatively to 20/60 or better in eight (28.5%) eyes, 20/70 to 20/160 in four (14.3%) eyes and remained worse than 20/200 in 16 (57.2%) eyes at 1 year postoperatively. Among the eyes with BCVA worse than 20/60, 13 (46.4%) eyes underwent PK and postoperatively the BCVA improved to 20/60 or better in 11 (84.6%) of 13 eyes. Limbal transplantation and/or PK was thus successful in restoring 20/60 or better BCVA in 19 (67.8%) of the 28 eyes in this study.

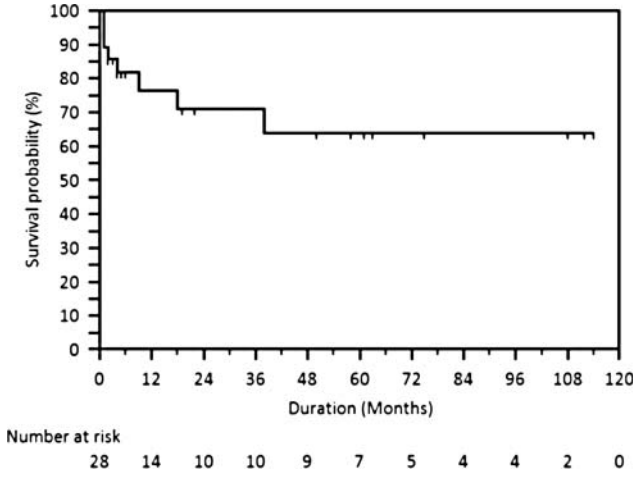


**Figure 2** The Kaplan–Meier survival rate of allogeneic cultivated limbal epithelial transplantation in 28 eyes with total limbal stem cell deficiency was 76.4±8.7%, 70.5±8% and 63.9±8.9% at 1, 2 and 3 years and thereafter. Failure of limbal transplantation occurred in eight eyes, of which seven occurred between 1 and 9 months and one each occurred at 18 and 36 months after limbal transplantation.

### Ocular and systemic complications

Among the 28 eyes, failure of limbal transplantation occurred in eight (28.6%) eyes, of which six (75%) failures occurred between 1 and 9 months and one (12.5%) each occurred at 18 and 36 months after limbal transplantation (both these eyes had undergone PK, figure 1 C,D). Six (75%) of these eight eyes developed progressive corneal vascularisation and conjunctivalisation, while two (25%) eyes developed persistent epithelial defects with corneal melting. Epithelial rejection episodes occurred in both eyes of one patient, both of which were reversed medically and the corneal surface remained epithelised until 9.5 years surgery. Among the 13 eyes undergoing PK, failure of the PK graft occurred in nine (69.2%) eyes: three (33.3%) eyes experienced irreversible endothelial rejection followed by persistent graft oedema; two (22.2%) eyes each developed recurrence of LSCD and suture-related graft infiltrates, and one (11.1%) eye each had traumatic graft dehiscence and late endothelial failure. During the study period, two (7.1%) eyes developed ocular hypertension requiring topical and/or oral aqueous suppressants but no eyes required glaucoma surgery. One patient developed generalised malaise and oral ulcers, which was attributed to systemic immunosuppression,

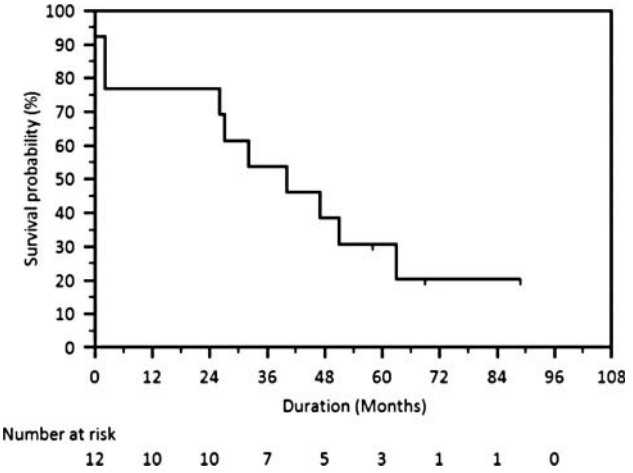


**Figure 3** The Kaplan–Meier survival rate for penetrating keratoplasty (PK) grafts was 76.9±11.7% at 1 year with a median survival of 3.3 years when PK was performed 12 months after allogeneic cultivated limbal epithelial cell transplantation in 13 eyes with total limbal stem cell deficiency (LSCD). Among the 13 eyes undergoing PK, failure of the PK graft occurred in nine (69.2%) eyes: three (33.3%) eyes experienced irreversible endothelial rejection followed by persistent graft oedema; two (22.2%) eyes each developed recurrence of LSCD and suture-related graft infiltrates, and one (11.1%) eye each had traumatic graft dehiscence and late endothelial failure.

and one patient experienced an Addisonian crisis that abated with the discontinuation of oral steroids. Due to the low incidence of systemic side-effects, statistical correlation with the dose of immunosuppressants was not possible. The donor sites re-epithelised within 10–14 days without developing conjunctivalisation or any ocular surface deficit.

### Histology and immunohistochemistry

Haematoxylin and eosin and periodic acid–Schiff (PAS) staining of the pannus excised during limbal transplantation showed eight to 10 layer thick stratified columnar epithelium with the presence of goblet cells and underlying loose fibrovascular stromal tissue (figure 4A). These findings were consistent with the clinical impression of LSCD. Haematoxylin and eosin staining of the corneal buttons excised during PK (figure 4B) showed a five to six cell layered squamous epithelium with basement membrane. No remnants of the human amniotic membrane were seen. Goblet cells were not observed on PAS staining, Bowman's membrane was absent and variable stromal scarring was noted. The Descemet's membrane and endothelial complex was noted to be normal in all eyes. Immunohistochemical examination of the excised corneal tissues showed that the epithelial cells expressed both p63 and cornea-specific marker K12 (figures 4C,D).

## DISCUSSION

Table 2 presents a comparison of previous studies on allogeneic cultivated limbal epithelial transplantation with the current study.[6 9 12 14–23] Having used FBS before October 2002,[12] the authors subsequently developed a feeder-free explant culture system, using autologous human serum and humanised growth factors for limbal cultivation.[10] Over the course of the past decade the authors have characterised the cultured cells,[24] standardised the xeno-free cultivation technique,[10] and shown how the transplanted monolayer of limbal cells forms a normal stratified corneal phenotype in vivo.[12 13] The authors have had the clinical experience of performing more than 500 autologous cultivated limbal epithelial transplantations;[13] and they have reported success rates of 71% and 66%, respectively, with primary and repeat procedures in eyes with severe ocular burns.[11 25] In contrast to previous studies on autologous procedures, this study focused on patients with bilateral LSCD who underwent allogeneic cultivated limbal epithelial transplantation.

This study found that the transplantation of allogeneic limbal epithelial cells cultivated using a xeno-free explant culture technique was successful for long-term ocular surface restoration in 71.4% of the 28 recipient eyes. Previous studies have shown that the success rate of allogeneic cultivated limbal epithelial transplantation ranges from 50% to 100% (table 2).[6 9 12 14–23] However, the indications for surgery, sample size, and follow-up duration vary widely among different studies.[8] Baylis *et al*[8] found no significant differences in the clinical outcomes of cultivated limbal epithelial transplantation based on the source of donor tissue (autologous or allogeneic), culture technique (explant or suspension) or indication for surgery. Similarly, in the authors' experience the overall success rates of allogeneic and autologous limbal transplantation using the explant culture technique were almost identical (71.4% vs 71%).[11] In addition to being clinically effective and free of animal-derived products, the cultivation technique was also extremely reliable, ex-vivo expansion being successful in every case. The authors have previously shown that inadequate growth or contamination is seen in less than 1.5% of the cultures in their system.[11]

To the authors' knowledge (Pubmed search using the terms 'limbal transplantation', 'PK' and 'graft survival') this is also the largest study reporting the PK allograft survival rate after allogeneic cultivated limbal epithelial transplantation. It has been reported that 18–38% of eyes undergoing limbal transplantation require one or more PK procedures for visual improvement.[8] In this study, 46% of eyes undergoing allogeneic limbal transplantation needed PK and the 1-year PK allograft survival rate (76.9%) was far better compared to that reported in eyes without previous transplantation (33.3–46.2%),[2 13] or in eyes with other high-risk factors for PK allograft failure (32–67%).[12 13] This two-staged approach of limbal transplantation for ocular surface stabilisation followed by corneal replacement for visual improvement is considered to be beneficial for the long-term success of both procedures, compared to single-staged limbal and corneal transplantation.[13]

Notwithstanding the strengths of this study, the limitations of the study must also be borne in mind when interpreting the results. First, although all patients had bilateral affliction, the aetiology was heterogeneous. Therefore, the results are extrapolatable only to cases similar to those included in this study rather than bilateral LSCD in general. Second, although all donors were first-degree relatives, the authors did not perform human leucocyte antigen (HLA) typing and thus graft survival as a function of the number of HLA mismatches could not be assessed. Third, the authors did not perform any DNA analysis post-transplantation to elucidate if the surviving corneal epithelium belonged to the host, the limbal donor or the PK donor. It is therefore not clear whether the transplanted allogeneic cells continued to proliferate on the corneal surface or simply provided a hospitable milieu for the rejuvenation of quiescent host cells. If the latter is true then immunosuppression need not have been continued indefinitely. Fourth, although the authors used strict clinical definitions for the outcomes, it can be argued that this lacked objectivity. Better objective methods

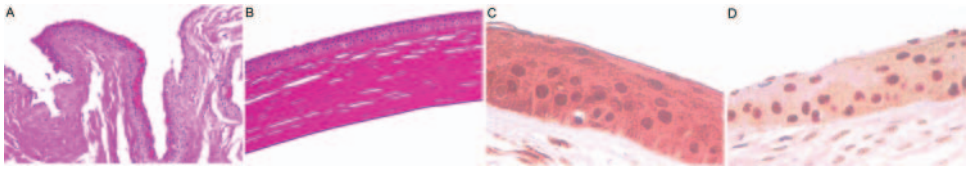


**Figure 4** Photomicrographs of the ocular surface pannus and corneal tissue excised from the right eye of a patient with bilateral limbal stem cell deficiency (LSCD) following acid injury. (A) Periodic acid–Schiff (PAS) staining of the pannus excised from the cornea during limbal transplantation shows eight to 10 layer thick stratified columnar epithelium with presence of goblet cells and underlying loose fibrovascular stromal tissue. These findings are consistent the clinical impression of LSCD. (B) PAS stained section of the corneal button excised during penetrating keratoplasty following successful allogeneic limbal transplantation shows a five to six cell stratified epithelium with basement membrane. Goblet cells are not observed, Bowman's membrane is absent and variable stromal scarring is noted. The Descemet's membrane and endothelial complex is noted to be normal. Immunohistochemical examination of the same corneal tissue shows that the epithelial cells express p63 (C) and cornea-specific marker K12 (D).

**Table 2** A comparison of the methods and outcomes of the current study with previously reported case series of allogeneic cultivated limbal epithelial transplantation for bilateral LSCD

| Author (year) | Donor source (n) | Culture technique | Feeder cell | Serum | Air-lifting | Xeno free | Success (n/N) (%) | Follow-up (months) |
|---|---|---|---|---|---|---|---|---|
| Current study (2011) | LR (28) | Explant | No | AS | No | Yes | 71.4 | 58 (12–114) |
| Pauklin *et al* (2010)[14] | LR (4), cadaveric (10) | Explant | No | AS | No | No | 50 | 28.5 (9–72) |
| Meller *et al* (2009)[15] | HLA matched donor (1) | Explant | No | AS | No | No | 100 | 31 |
| Shortt *et al* (2008)[16] | Cadaveric (7) | Suspension | No | FBS | No | No | 100 | 9.9 (6–13) |
| Ang *et al* (2007)[9] | Cadaveric (3) | Suspension | Yes | FBS | Yes | No | 100 | 48 |
| Shimazaki *et al* (2007)[6] | LR (8), cadaveric (12) | Suspension<br>Explant | Yes<br>No | FBS<br>AS | Yes<br>Yes | No | 50 | 29 (6–85) |
| Nakamura *et al* (2006)[17] | Cadaveric (7) | Suspension | Yes | AS | Yes | No | 100 | 14.4 (6–20) |
| Daya *et al* (2005)[18] | LR (1), cadaveric (9) | Suspension | Yes | FBS | No | No | 70 | 28 (12–50) |
| Sangwan *et al* (2005)[12] | LR (4) | Explant | No | FBS | No | No | 100 | 15.3 (7–24) |
| Nakamura *et al* (2003)[19] | Cadaveric (3) | Explant | Yes | FBS | Yes | No | 100 | 13 (12–14) |
| Koizumi *et al* (2001)[20] | Cadaveric (13) | Explant | Yes | FBS | Yes | No | 100 | 11.2 (9–13) |
| Koizumi *et al* (2001)[21] | Cadaveric (3) | Explant | Yes | FBS | Yes | No | 100 | 6 |
| Schwabb *et al* (2000)[22] | LR (4) | Suspension | Yes | FBS | Yes | No | 100 | 11.5 (6–19) |
| Schwabb *et al* (1999)[23] | LR (2) | Suspension | Yes | FBS | No | No | 50 | 13 (16–19) |

Although Shimazaki *et al*,[6] Pauklin *et al*[14] and Meller *et al*[15] used human serum without feeder cells for culturing limbal cells for allogeneic transplantation, the use of murine epidermal growth factors precluded these protocols from being completely xeno free.
AS, autologous serum; FBS, fetal bovine serum; HLA, human leucocyte antigen; LR, live related; LSCD, limbal stem cell deficiency.

of outcome assessment include clinical scoring systems, impression cytology or confocal microscopy-based grading systems and symptom score-based questionnaires.

In conclusion, the authors found that their technique of xeno-free allogeneic limbal epithelial transplantation, along with PK whenever needed, was successful in the long-term restoration of the ocular surface and improvement in vision in eyes of patients with bilateral LSCD. Simple and effective techniques of xeno-free limbal cultivation, as described in this study, can eliminate any real or perceived risks associated with the use of animal products and allow wider applicability of cultivated limbal epithelial transplantation.

**Contributors** The corresponding author states that authorship credit of this manuscript was based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. All listed authors met conditions 1, 2, and 3. All persons designated as authors qualify for authorship, and all those who qualify are listed. Each author has participated sufficiently in the work to take public responsibility for appropriate portions of the content. The first and second authors contributed equally to this study.

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**Ethics approval** The study was approved prospectively by the Institutional Review Board and the Institute Committee for Stem Cell Research and Therapy, L V Prasad Eye Institute, Hyderabad, India. The study followed the tenets of the Declaration of Helsinki.

**Patient consent** Obtained.

**Competing interests** None.

**Provenance and peer review** Not commissioned; externally peer reviewed.

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