Cultivated Limbal Epithelial Transplantation in Children With Ocular Surface Burns

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**Importance:** Although several reports are available on the use of conventional and cultured limbal epithelium using various substrates in the treatment of limbal stem cell deficiency (LSCD), the patient populations studied have been largely adults. Thus, to our knowledge, the outcomes of this procedure exclusively in a pediatric population have not been reported previously.

**Objective:** To report the outcomes of autologous ex vivo cultivated limbal epithelial transplantation (CLET) in pediatric patients with LSCD after ocular burns.

**Design and Setting:** A retrospective, interventional case series of patients treated at the L. V. Prasad Eye Institute, Hyderabad, India.

**Participants:** Children up to 15 years with LSCD secondary to chemical or thermal injury who underwent CLET from April 1, 2001, through June 31, 2010, with a follow-up of at least 1 year, were included in the study.

**Intervention:** After a limbal biopsy specimen obtained from a healthy area of the limbus, the limbal epithelial cells were cultured on a denuded human amniotic membrane substrate using a xeno-free explant culture technique. A monolayer of cultivated epithelial cells along with the amniotic membrane was transplanted on the patient’s affected eye after pannus excision. In cases of failure, the same procedure was repeated.

**Main Outcomes and Measures:** Ocular surface stability and visual improvement were the primary and secondary outcome measures, respectively. Success was defined as a stable corneal epithelium without conjunctivalization. Eyes with conjunctivalization and persistent epithelial defects were classified as failures.

**Results:** Of the 107 eyes of 107 patients included in this study, 73 eyes (68.2%) underwent 1 and 34 eyes (31.8%) underwent 2 autologous CLET procedures. At a mean follow-up of 3.4 years, 50 eyes (46.7%) achieved completely epithelialized, avascular, and stable ocular surfaces. At the final visit, 58 eyes (54.2%) had improvement in visual acuity of 0.2 or more logMAR units.

**Conclusions:** Autologous CLET was successful in restoring the ocular surface and improving vision in almost half of the children blinded by ocular burns.


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The corneal epithelial surface is maintained in a transparent state by the corneal epithelial stem cells, which are believed to be located at the basal layer of the limbal epithelium. Any physical, chemical, thermal, or immunologic insult to the limbal region can result in permanent damage to the corneal epithelial stem cells, leading to limbal stem cell deficiency (LSCD). This deficiency results in loss of corneal clarity and a decrease in vision, often leading to unilateral and bilateral corneal blindness. With the advent of different techniques of limbal stem cell transplantation, it has become possible to treat eyes with blindness due to LSCD. There are several articles about the use of conventional and cultured limbal epithelium using various substrates in the treatment of LSCD. However, the patient population includes largely adults, with few pediatric cases. Thus, to our knowledge, the outcomes of this procedure exclusively in a pediatric population have not been reported previously. It is known that the ocular inflammatory responses to iatrogenic trauma can be more severe in pediatric patients compared with adults, which, in turn, can jeopardize the final outcome of the surgical procedure. In this study, we describe the clinical outcomes of autologous ex vivo cultivated limbal epithelial transplantation (CLET) in children with unilateral LSCD after ocular surface burns, using a standardized xeno-free protocol of limbal cell culture.
PATIENTS

The study was approved by the institutional review board of L. V. Prasad Eye Institute, Hyderabad, India, and was conducted in strict adherence to the tenets of the Declaration of Helsinki. Prior written informed consent was obtained from all guardians. A retrospective medical record review of all the patients undergoing autologous CLET from April 1, 2001, through June 31, 2010, was performed. All patients up to 15 years with unilateral partial or total LSCD secondary to chemical or thermal burns and a follow-up of 1 year or longer were identified. Limbal stem cell deficiency was clinically diagnosed based on the presence of some or all of the following clinical features: partial or complete conjunctivalization of the cornea, loss of corneal transparency, persistent corneal epithelial defects, superficial neovascularization, absence of limbal palisades of Vogt, limbal ischemia, and chronic inflammation. Patients who underwent limbal transplantation for causes other than chemical or thermal burns, those with less than 1 year of follow-up, and those with no visual potential as evaluated by flash visual evoked potential and flash electroretinogram, in which CLET was performed for cosmesis only, were excluded. None of the patients were noted to have dry eye.

LIMBAL BIOPSY, CULTURE, AND TRANSPLANTATION

Our technique for cultivation of limbal epithelium on denuded human amniotic membrane has been previously described in detail.64 The limbal biopsy specimen is obtained from a healthy area of the limbus from the ipsilateral or contralateral eye. It is cultivated on deepithelialized amniotic membrane by a simple feeder cell-free explant culture method that generates a monolayer of the corneal epithelium within 10 to 14 days, and stratification of the cultured epithelial monolayer occurs in vivo. Fetal calf serum was used until 2002, after which autologous serum was used. Cultures that covered more than 50% of the 2.5 × 5-cm human amniotic membrane are considered adequate for clinical use.

Our surgical technique of CLET involves dissection of the ocular surface pannus 2 to 3 mm behind the limbus if this landmark is visible with the help of blunt-tip spring scissors or conjunctival scissors and a No. 15 blade on a Bard-Parker handle. A combination of sharp and blunt dissection from the periphery toward the central area is performed to clear the cornea. Symblephara are released, taking due care not to injure the extraocular muscles and other tissues. Cultivated limbal epithelium on human amniotic membrane is brought to the ocular surface, cell side up, and the membrane containing the cultured cells is gently spread over the cornea and limbus without damaging or dislodging the cells. The membrane is secured to the ocular surface with 10-0 monofilament nylon material sutures or fibrin glue (Tissel Kit; Baxter AG). In cases with severe corneal thinning, additional lamellar or penetrating keratoplasty is performed.

Postoperatively, all patients were treated with 1% prednisolone acetate eye drops 8 times daily tapered to once a day in 4 to 6 weeks and 0.3% ciprofloxacin hydrochloride eye drops 4 times daily for 1 week or until the surface epithelium healed. Visual rehabilitation with keratoplasty procedures, glasses, or contact lenses was performed when indicated, and amblyopia management was started in appropriate cases.

OUTCOME MEASURES AND DATA COLLECTION

The stability of the ocular surface was the primary outcome measure, which was assessed clinically, based on the absence of recurrent breakdown of the corneal epithelium and absence of conjunctivalization. Failure was defined by the presence of conjunctivalization and persistent corneal epithelial defects that caused ocular surface instability. The improvement in visual acuity was a secondary outcome measure.

The details collected from the medical records of the patients included demographics; cause of the LSCD; time since injury; details of prior intraocular or extraocular operations; ocular alignment; visual acuity; anterior and posterior examination details, such as eyelid abnormalities, symblepharon, extent of conjunctivalization, and corneal scarring; optic disc findings; ultrasound B-scan findings (when posterior segment evaluation was not possible clinically); surgical details; complications; and postoperative examination findings. The follow-up was recorded at postoperative day 1, 2 weeks, 6 weeks (± 1 week), 3 months (± 2 weeks), 6 months (± 1 month), 1 year (± 2 weeks), and annually thereafter. Examination with the patient under short-term general anesthesia was performed for children with poor cooperation. Visual acuity was recorded by age-appropriate charts. In younger children in whom visual acuity was recorded as fixing and following light, a logMAR value of 1.78 was considered for analysis.

STATISTICAL ANALYSIS

Collected data were entered on a Microsoft Excel spreadsheet. Statistical analysis was performed using statistical software R, version 2.14.1 (GNU General Public License). Kaplan-Meier survival analysis was performed to evaluate the survival of autologous CLET. Univariate analysis was performed to evaluate the effect of preoperative characteristics, such as age at presentation, cause of injury, duration between injury and CLET, extent of conjunctivalization and symblepharon, number and type of prior surgical interventions performed, and type of limbal transplantation performed (CLET alone or combined with keratoplasty) on survival of CLET. These variables were subsequently fitted in a multivariate model (Cox proportional hazards regression model using the Akaike information criterion with forward and backward elimination) to estimate the adjusted hazard ratio (HR). A log-rank test was used to compare the survival probability with each variable. The Shapiro-Wilk test was used to assess the distribution of the visual acuity data. A 1-sided paired-sample Wilcoxon signed rank test was used to compare the preoperative and postoperative visual acuity. P < .05 was considered statistically significant.

RESULTS

DEMOGRAPHICS AND PREOPERATIVE CHARACTERISTICS

A total of 236 pediatric transplantations were performed during the study period. We included 107 eyes of 107 patients after applying the exclusion criteria. The mean (SD) age of the patients at the time of surgery was 7.5 (3.72) years (range, 2-15 years). The most common cause of LSCD was alkali injury, especially with lime, noted in 89 eyes (83.2%). In the remaining eyes, acid injury, injury with unknown chemicals, or thermal injuries were the causative factors. A total of 92 eyes (86.0%) had total LSCD, whereas 15 eyes (14.0%) had partial LSCD.
(involving to 180° to 330° of the limbus). Operations performed before CLET (≥1 per eye) included 3 eyelid procedures (tarsorrhaphy and ectropion correction), 57 ocular surface procedures (amniotic membrane, conjunctival autograft, symblepharon release, and limbal allograft transplantation), and 9 cornea operations (penetrating or lamellar keratoplasty). These procedures were performed at our institute or outside prior to presentation to us and included those performed for the management of the acute stage of chemical injury. The Table summarizes the preoperative characteristics, with the study population divided into 2 age groups. No significant difference was noted in the preoperative characteristics among the 3 groups. Ocular alignment examination revealed orthotropia in 73 patients and a manifest strabismus in 22 patients. Of these, 22 eyes had exotropia, 4 eyes had esotropia, and 5 eyes had an additional vertical component of strabismus. Ocular alignment could not be assessed in 12 patients because of poor cooperation.

**SURGICAL PROCEDURE**

The limbal tissue for cultivation was harvested from the contralateral eye in almost all cases, except in 3 cases of partial LSCD, where the tissue was harvested from the same eye from an area of healthy limbus. The mean duration between the injury and CLET was 15.5 months (median, 8 months; range, 1-108 months). Early intervention at 1 month was planned in 2 children with limbal ischemia. Symblepharon release was performed along with CLET whenever indicated. A simultaneous lamellar keratoplasty for corneal thinning or penetrating keratoplasty for corneal perforation was performed in 8 eyes (7.5%).

**PRIMARY OUTCOME**

At a mean (SD) follow-up of 41.2 (26) months (range, 12-118 months), success was achieved in 40 eyes (37.4%) (12 [30.0%] in those 6 years or younger and 28 [70.0%] in those older than 6 years), partial recurrence of conjunctivalization was observed in 13 eyes (12.1%) (7 [53.8%] in those 6 years or younger and 6 [46.2%] in those older than 6 years), and complete conjunctivalization was observed in 54 eyes (50.5%) (28 [51.9%] in those 6 years or younger and 26 [48.1%] in those older than 6 years). Univariate Cox proportional hazards regression model revealed that duration between injury and CLET was noted to be the only significant factor affecting survival. The survival rate in eyes undergoing surgery at or earlier than 4 months was significantly lower compared with those operated on after 4 months (HR, 0.38; 95% CI, 0.23-0.65; \( P = .002 \)). The multivariate Cox proportional hazards regression model revealed that in addition to this factor, eyes undergoing previous surgical procedures, such as amniotic membrane grafting before CLET, had better CLET survival (coefficient, 0.58; HR, 1.79; 95% CI, 1.00-3.18; \( P = .04 \)). Kaplan-Meier analysis revealed an overall probability of survival of the transplant of 45.0% at 24 months and 33.0% at 48 months. At 24 months, the survival probability in eyes operated on at or earlier than 4 months was 12.8%, which was significantly lower than the 54.0% in eyes operated on later than 4 months (coefficient, −1.26; HR, 0.28; 95% CI, 0.15-0.53; \( P = .001 \)) (Figure 1). Of the cases with failure of CLET, 34 (31.8%) underwent an additional CLET. This procedure was performed at a mean duration of 18.5 months (range, 3.5-53 months) after the first surgery. Of those, 10 transplantations (29.4%) were successful, 7 (20.6%) had partial recurrence, and 17 (50.0%) had complete recurrence of conjunctivalization. Thus, ocular surface stability was achieved in 50 cases (46.7%) after either 1 or 2 transplantations.

**ADDITIONAL OPERATIONS**

Significant scarring in the visual axis necessitated a keratoplasty procedure for visual rehabilitation. An optical penetrating keratoplasty was performed in 16 patients (15.0%), of which 10 grafts (62.5%) survived at the final follow-up. This procedure was performed at a mean

![Figure 1. Kaplan-Meier survival plots of cumulative probability of survival of cultivated limbal epithelial transplantation (CLET) in eyes undergoing early surgery (≤4 months after chemical injury) vs late surgery (>4 months).](image-url)
duration of 12 months (range, 1.7–40.7 months) after successful CLET. Three eyes (2.8%) underwent optical lamellar keratoplasty and 2 grafts survived. Figure 2 shows clinical photographs of eyes after alkali injury and the outcomes after surgical intervention. For eyes with dense amblyopia, keratoplasty was not considered. Histologic studies of the excised corneal buttons in patients undergoing penetrating keratoplasty revealed a phenotypically cornealike epithelium in all cases. In 8 eyes (7.5%) with moderate to severe recurrence of symblepharon, a symblepharon release procedure combined with amniotic membrane grafting alone or with conjunctival limbal autograft was performed. Six eyes (5.6%) had ptosis repair, and 2 eyes (1.2%) underwent squint correction. Phototherapeutic keratectomy was performed for residual scarring in 1 eye.

SECONDARY OUTCOME

At the final visit, 58 eyes (54.2%) had improvement in visual acuity of more than 0.2 logMAR units. A best-corrected visual acuity of more than 0.7 logMAR units (20/200) was achieved in only 27 eyes. Among patients with a successful outcome and those with partial peripheral recurrence of conjunctivalization, the mean (SD) presenting visual acuity was 1.6 (0.5) logMAR units, which significantly improved to 0.8 (0.6) at the final follow-up (P = .002). Only 1 eye had worse vision due to glaucomatous disc damage in this group. Twelve patients were advised to undergo patching therapy, which failed to improve vision in most patients because of amblyopia or nonadherence to treatment.

ADVERSE EVENTS

The most common postoperative complication was microbial keratitis. This complication was noted in 7 eyes (6.5%) as microbial keratitis, suture, or graft infiltrate. The infection was caused by gram-positive cocci in 6 cases and by gram-negative bacilli in 1 case. All patients were treated with fortified topical antibiotics, resulting in resolution of the infection in 5 eyes. However, in 2 eyes, therapeutic keratoplasty was performed and both grafts failed. Panophthalmitis developed in 1 eye, resulting in a phthisical eye. Corneal allograft rejection was noted in 4 eyes (3.7%), leading to graft failure. Inflammatory granuloma was noted in 4 eyes (3.7%), of which 2 were treated medically with topical steroids, and in 2 eyes surgical excision was performed. Thinning of the cornea required tissue adhesive application in 2 eyes (1.9%). An increase in intraocular pressure requiring topical antiglaucoma medications was noted in 1 eye. Hemorrhage under the amniotic membrane needed evacuation in 1 eye. One or more complications were noted in the same eye. The donor eye remained healthy in all cases. Evisceration with ball implant was performed in 1 phthisical eye for cosmesis.

COMMENT

Pediatric LSCD constitutes 40% of the LSCD referrals to our institute. Almost all cases of alkali injury were unintentional, with either calcium hydroxide available in the form of bleaching powder or edible calcium hydroxide available as a chuna packet, a common additive to chewing tobacco. The injuries were caused when the chuna packets burst open while playing, rubbing eyes after handling bleaching powder, or a splash of liquid that contained bleaching powder. This finding indicates the need for increased public awareness about the potential ocular damage and need for prevention of domestic chemical injuries. In addition, manufacturers and vendors need to comply with safety protocols established for manufacturing and sale of chemicals.

The use of autologous limbal tissue in the technique we describe avoids the need for systemic immunosup-
pression used for keratolimbal allograft, thus making autologous CLET an ideal procedure in pediatric patients.

Conjunctival limbal autograft is a popular surgical intervention for total unilateral LSCD. However, it involves excision of at least 90° to 240° of conjunctival-limbal tissue from the healthy eye. This procedure not only is associated with complications of the conjunctival-limbal autograft itself but may also result in complications, such as partial LSCD and filamentary keratitis, in the donor eye. Although improved ocular surface stability has been reported in 80% to 100% of patients undergoing conjunctival limbal autograft, the number of cases reported is rather small. Autologous CLET has the advantage of using a small amount of limbal tissue, thus reducing donor site complications. Because of the extensive experience with this procedure at our center, we even used it to treat partial LSCD.

Boston keratoprosthesis, which is being considered for unilateral LSCD, has benefits of quick visual rehabilitation, no concerns for astigmatism management and graft rejection, and achievement of binocular function in eyes with good visual acuity. However, the need for long-term use of a bandage contact lens and topical antibiotics to prevent tissue necrosis and secondary infection makes it a less preferred option in developing countries because of the lack of hygiene and low adherence to antibiotic use.

Simple limbal epithelial transplantation involves use of a 2 × 2-mm donor limbal tissue, which is cut into several pieces, glued to the amniotic membrane, and placed on the ocular surface after pannus dissection. It has been recently described as an alternative to CLET, but long-term outcomes are awaited.

Lack of elaborate data about outcomes of CLET in a pediatric population makes direct comparison of our results difficult. In recently published studies about outcomes of CLET in a large study population of predominantly adults, the success rate varies from 68% to 80%. Our success rate of 46.7% after 1 or 2 transplantsations is lower than that of the reported studies, highlighting the poorer outcomes in children, despite similar causes of LSCD. In addition, the cumulative survival probability of 45.0% at 2 years is lower than the 68% in previous studies. The causes of failure of limbal transplantation are known to be multifactorial and may be due to the loss of stem cells during transplantation because of a hostile in vivo microenvironment.

Postoperative inflammation may play a significant role in the outcome of CLET. The topical steroids were tapered in 6 weeks in our study. Use of oral steroids can be considered in cases with severe inflammation. Whether the use of oral steroids would increase the survival of CLET needs to be further explored. The severity of the initial insult and its management may also influence the outcome of CLET. Eyes with prior surgical interventions, such as amniotic membrane grafting for acute chemical injury, had better survival of CLET in our series, emphasizing the importance of appropriate management of chemical injury in the acute phase. Certain aspects that contribute to the severe LSCD at presentation in our group may be predominance of alkali injuries; lack of anesthesia facilities in small towns, making emergency management difficult and thus resulting in residual lime embedded in the conjunctiva or cornea in some cases; delay in referral to higher centers; and lack of adherence to instillation of medications. Eyes undergoing CLET at or before 4 months from the time of injury had a higher failure rate in our series, indicating that low-grade smoldering inflammation may persist for prolonged periods, especially in cases with acute alkali injury. Hence, we recommend that CLET be considered only after 4 months from the time of chemical injury.

Despite achieving a stable ocular surface, the visual improvement was not encouraging. Severe injury that resulted in significant corneal scarring and late presentation after the injury often led to deprivation amblyopia. Contact lens trial and optical keratoplasty were considered for residual corneal scarring in appropriate patients. In those patients with delayed presentation, dense amblyopia, and strabismus, where visual outcome was not expected to significantly improve after keratoplasty, intervention was deferred. Lack of adherence to spectacle wear and patching therapy also contributed to poor visual outcomes.

In eyes with significant residual corneal scarring, keratoplasty should be considered once the ocular surface stabilizes at approximately 6 to 8 weeks to prevent stimulus deprivation amblyopia and optimize visual recovery in younger children. A simultaneous penetrating keratoplasty, along with CLET, may be beneficial in pediatric patients with extensive stromal damage because it is a 1-step procedure and provides faster visual rehabilitation. However, in 6 of 8 eyes in which a simultaneous lamellar or penetrating keratoplasty was performed with CLET for corneal thinning or perforation, the grafts failed and microbial keratitis developed in 3. In addition, primary corneal graft failure was noted in 1 eye despite successful CLET. These findings are similar to those of a recently published study in which CLET combined with simultaneous keratoplasty had an increased risk of microbial keratitis and rejection compared with CLET alone. Thus, in eyes where tectonic support in the form of keratoplasty is anticipated, a 2-stage procedure where a volume augmentation by keratoplasty is performed first, followed by CLET, may be considered.

The present study is limited because it is retrospective, with subjective clinical criteria for outcome evaluation and lack of data on ocular alignment in some patients. Nevertheless, this study with a large sample size highlights the utility of this technique in this unique pediatric age group.

In conclusion, unintentional injury with calcium hydroxide was the most common cause of LSCD in our series. Autologous CLET by a simple, xeno-free, explant culture technique achieved ocular surface stability in almost half the patients. Management of LSCD in children remains challenging, with poorer outcomes than in adults and amblyopia limiting the visual outcome. Timely intervention in pediatric patients to achieve ocular surface stability, followed by visual rehabilitation and amblyopia management, is essential to maximize outcomes.

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Conflict of Interest Disclosures: None reported.

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