Outcomes of Penetrating Keratoplasty Following Autologous Cultivated Limbal Epithelial Stem Cell Transplantation

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Key Words. Penetrating keratoplasty • Limbal stem cell • Transplantation • Limbal stem cell deficiency • Graft survival • Visual outcomes

ABSTRACT

The purpose of this study is to investigate the outcomes of penetrating keratoplasty (PKP) following autologous cultivated limbal epithelial stem cell transplantation (CLET). A prospective, single center, interventional cohort study investigating patients with unilateral total limbal stem cell deficiency (LSCD) treated with CLET who underwent PKP. Patients with confirmed corneal re-epithelialization > 6 months post-CLET, and with best-corrected visual acuity (BCVA) < 0.3 logMAR were offered PKP. CLET survival assessed by slit lamp, corneal impression cytology (CIC), and in vivo confocal microscopy. Confirmation of corneal re-epithelialization by histological and immunocytochemical (ICC) examination of trephined corneal buttons. Mean change in best-corrected visual acuity (logMAR) following PKP and PKP survival at 12 months were calculated. Twenty patients underwent PKP. Mean time of PKP was 19 months (range 11–41 months, SD 7.26) post-CLET. Median follow-up time post-PKP was 15 months (range 1–32, SD 10.2). CIC and ICC of all corneas confirmed corneal re-epithelialization before PKP. Mean pre-PKP BCVA was 1.46 (range 0.3–2.7, SD 0.94) improving to a mean post-PKP BCVA of 0.74 (range 0–2.7, SD 0.87); mean improvement in BCVA post-PKP of 36 letters (95% CI 15.0–57.1, p = .002). Kaplan-Meier mean graft survival was 90.9% (95% CI 50.8–98.7) at 12 months. We recommend a two-stage approach with CLET followed by PKP >12 months later. Patients experienced a significant improvement in BCVA following PKP. PKP did not have a detrimental effect on CLET survival. PKP survival post-CLET is better than that reported for high risk PKP.

SIGNIFICANCE STATEMENT

A clear cornea is essential for good vision. In severe ocular surface diseases, such as chemical or thermal burns, there can be damage to limbal stem cells, resulting in limbal stem cell deficiency (LSCD). The ocular surface in patients with unilateral total LSCD with autologous limbal stem cell transplantation was successfully restored; however, these patients often require corneal transplantation to restore their sight. This study demonstrates that these patients have an excellent visual outcome and graft survival following corneal transplantation. A two-stage procedure was advocated in first restoring the ocular surface followed by corneal transplantation later.

INTRODUCTION

Limbal Stem Cell Deficiency

A clear cornea is essential for good vision. In severe ocular surface diseases, such as chemical or thermal burns, there can be damage to limbal stem cells, resulting in limbal stem cell deficiency (LSCD). Limbal stem cells (LSCs) are located within crypts in a region known as the limbus, which forms the junction between the peripheral cornea and the sclera [1–4]. These cells are responsible for the continuous repair and renewal of the corneal epithelium. LSCD results in the loss of corneal transparency due to conjunctivalization (superficial scarring and vascularization) of the cornea, often associated with deep stromal opacity. LSCD can be unilateral or bilateral depending on whether one eye or both eyes are affected. Clinically, LSCD can be classified as mild/partial, in which only a limited part of the limbus is involved; or severe/total, in which more than two quadrants of the limbus are affected combined with central corneal involvement resulting in
Mild/Partial LSCD usually presents with a sectorial conjunctival area of the cornea; patients tend to experience relatively mild ocular surface symptoms, with decreased vision only if the visual axis is involved. These patients often do not require surgical treatment. In severe cases, however, the visual axis is often involved, leading to very low vision. This, in combination with pain and photophobia due to recurrent epithelial defects and chronic ocular surface inflammation, can make the patient functionally blind [7].

**Treatment of Total Limbal Stem Cell Deficiency**

Total LSCD can be treated by replacing the limbal stem cell (LSC) population. This is accomplished either by using whole-tissue grafts or by transplanting ex vivo cultured limbal cells (or cells from other source tissues) [8–10]. In the past 19 years, treatment of severe total LSCD has been mainly achieved by transplantation of ex vivo expanded LSCs cultured from a small biopsy of the limbus from a healthy contralateral eye (autologous, in unilateral cases) [1, 11–15], from a donor (allogeneic, in bilateral cases) [16], or from ex vivo cultivated oral mucosal autograft (EVOMAU) [17, 18]. The deeply scarred cornea can then be replaced by corneal transplantation (penetrating or lamellar keratoplasty) often as part of a two-stage approach [19, 20]. While ex vivo expanded stem cell or tissue transplantation alone may improve vision in these patients, a combination of both procedures, that is, stem cell and corneal transplantation, is often necessary to achieve optimal visual recovery. Corneal transplantation without prior regeneration of the corneal epithelium by stem cell transplantation inevitably fails [21] in patients with severe or total LSCD.

Most large studies on stem cell transplantation have focused primarily on the outcomes of the stem cell transplantation [22]. To the best of our knowledge, very little research has been conducted or published to date (none in the U.K.) on the outcomes of corneal transplantation following LSC transplantation [19, 20], particularly with respect to the long-term survival of corneal grafts after LSCT, or the potential impact of corneal transplantation on the grafted stem cells. Similarly, there are no established guidelines regarding the timing of the two-stage transplantations and/or whether they can be combined into a single-stage procedure [11, 12].

We aimed to investigate the outcomes of penetrating keratoplasty (PKP) in our cohort of patients who have received a CLET and to determine the overall graft survival in these patients, who would be deemed high risk due to previous conjunctivalization and neovascularization both superficially and at the deeper corneal stromal level. In addition, we also investigated the potential impact of PKP on LSC survival following previous CLET.

**Materials and Methods**

We designed a prospective, single center, intervention cohort study involving all patients in our Medical Research Council (MRC) U.K.-funded phase II clinical trial for the treatment of unilateral total LSCD with autologous cultivated limbal epithelial stem cell transplantation (CLET, n = 23). Consecutive patients presenting to the Eye Department at the Royal Victoria Infirmary, Newcastle upon Tyne, U.K., with unilateral total LSCD who met the inclusion criteria (specifically, with no other associated ocular pathology as per slit-lamp biomicroscopy, tonometry, fundoscopy, B-scan ultrasound and electrodiagnostic testing) for our phase II clinical trial were recruited between June 2012 and January 2015 and followed up for 36 months post-CLET. The study was carried out in accordance with the principles of the Declaration of Helsinki, with approval from the local Research Ethics Committee (11/NE/0236), MHRA Clinical Trial Authorization (17136/0254/001-0001) and under an HTA licence (11122). All patients gave full informed consent to participate in the clinical trial and to have the procedures carried out. The results of the CLET phase II clinical trial will be presented in due course. In this study, we only report the clinical outcomes of the patients who subsequently underwent PKP after CLET.

**Autologous Limbal Stem Cell Transplant**

All patients underwent CLET using the Newcastle method, previously described by Kolli et al. [1]. Briefly, a clinical diagnosis of unilateral total LSCD was confirmed in all subjects by corneal impression cytology (CIC) (i.e., cytokeratin profiling) and in vivo confocal microscopy (IVCM - HRT3, Heidelberg, Germany). A limbal biopsy was taken from the healthy fellow eye at most commonly the 12 o’clock position, but occasionally the 6 o’clock position. This was immediately transferred to the Good Manufacturing Practice (GMP) biomanufacturing facilities and plated onto a sheet of human amniotic membrane (HAM) that had been wrapped around a glass coverslip and trapped between a second glass coverslip. The explant culture was incubated in limbal epithelium medium (a 3:1 solution of Dulbecco’s modified Eagle’s medium and Ham’s F12 nutrient medium, supplemented with 10% autologous serum, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 1.4 ng/ml tri-iodothyronine, 24 μg adenine, 8.4 ng/ml cholera toxin, 10 ng/ml epidermal growth factor, and 1% penicillin-streptomycin) at 37°C and 5% CO2. The medium was exchanged every 2–3 days until a >90% confluent monolayer of epithelial cells was seen to have populated the HAM epithelial surface. After the successful ex vivo expansion of LSCs was confirmed, patients underwent a superficial keratectomy, followed by transplantation of the HAM with the overlying ex vivo expanded ALSCs. A second HAM, epithelium side up, was used to act as a protective bandage over the ALSCs. Both HAM were sutured into place with separate 10-0 nylon sutures and a bandage contact lens (22 mm) was placed at the end of the procedure. Postoperative drops regimen consisted of preservative free prednisolone acetate 1% drops two hourly (tapered down to QDS by three months and OD from six months postoperatively), autologous serum eye drops 50% two hourly (continued indefinitely) and chloramphenicol 0.5% drops four times a day (continued until the removal of the bandage contact lens and conjunctival sutures at 8 weeks postoperatively, after the superficial HAM had melted). Success of CLET was determined by clinical assessment, IVCM and CIC performed at 6-monthly intervals post-CLET.

**Penetrating Keratoplasty**

Following confirmation of successful CLET at 6 and 12 months (+/- 4 weeks) after CLET, patients with logMAR visual acuities worse than the U.K. standard for driving (0.3 logMAR, 6/12...
Snellen BCVA) were offered PKP for visual rehabilitation, to be performed at least 12 months post-CLET.

Conventional PKP was undertaken using donor corneas obtained from the NHS Blood and Transplant Tissue Bank, secured with 12-3-10-0 interrupted and 1-3-11-0 continuous nylon sutures (Ethicon, U.K.). Median PKP size was 8.0 mm (range 7.0–8.5 mm, SD 0.35). Interrupted sutures were selectively removed from 8 weeks postoperatively depending on refraction and corneal topography. All patients were treated post-operatively with preservative free high dose topical steroid (preservative free prednisolone acetate 1%, hourly initially), tapered down to QDS by three months and OD by six months), autologous serum eye drops 50% (often 2 hourly) and a short course topical antibiotic (chloramphenicol 0.5% four times a day for 4 weeks).

In some cases with visually significant cataract, PKP was combined with cataract extraction (with or without intraocular lens [IOL] implantation).

**Outcome Measures and Statistical Analyses**

Patient demographical data were recorded, including age at the time of PKP. CLET survival prior to and after PKP was assessed clinically with slit lamp biomicroscopy and confirmed by IVCM and CIC. Trephined recipient corneal buttons were also examined histologically (particularly for the absence of goblet cells) and with immunocytochemical staining (ICC) for corneal-specific (CK3, CK12) and conjunctival-specific (CK13, CK19) markers.

BCVA (logMAR) was measured pre-PKP and post-PKP at regular intervals (at least 6-monthly). Differences in pre-PKP BCVA and post-PKP BCVA were assessed by paired two-tailed t tests. Statistical significance was determined by p < .05. All rejection episodes (rejection type and time to rejection) or any other post-PKP complications were recorded. Graft failure (defined as a loss of central graft clarity) and time to failure were also recorded. PKP graft survival was calculated using Kaplan-Meier survival analysis.

**RESULTS**

Twenty-three patients underwent CLET for unilateral total LSCD as part of our MRC-funded phase II clinical trial. All patients suffered ocular surface burns: 3 were thermal and 20 chemical. ALSC survival was confirmed by CIC and IVCM in all patients prior to and after PKP. Figure 1 shows IVCM micrographs of the same patient before (A) and after (B) CLET, demonstrating re-epithelialization of the corneal surface.

Twenty of these patients fulfilled the criteria of having a BCVA worse than U.K. driving standard, and underwent PKP (16 males, 4 females; mean age: 42.9 years, range 22–77, SD 13.6). The other three patients, whose BCVA already met the U.K. driving standard post-CLET, required no further intervention. PKP was performed at a mean time of 19 months (range 11–41 months, SD 7.26) post-CLET. All patients were phakic before PKP. Four patients had a combined PKP with extracapsular cataract extraction (ECCE) and IOL implantation in the capsular bag. Two further patients had a subsequent cataract extraction with IOL implantation following their PKP, while one patient remained aphakic following combined PKP with ECCE.

Median follow-up time post-PKP was 15.0 months (range 1–32, SD 10.2). Figure 2 shows sequential color photographs taken of a patient who underwent a PKP procedure for visual rehabilitation following CLET, at baseline (A), 12 months post-CLET (B) and 17 months post-PKP (C).

Clinical slit lamp assessment of the cornea prior to PKP demonstrated a healthy, corneal phenotype epithelium in all cases, that is, a comfortable eye, with no delayed epithelial staining, no epithelial defect, and minimal ocular surface inflammation.

CIC before PKP (Table 1) showed 16 corneas expressing CK3 or CK12 or both, with four expressing neither, but also not expressing CK13 or CK19 (i.e., double negative staining). In these cases, the decision to proceed with PKP was made on the grounds of the slit lamp biomicroscopic assessment of no evidence of LSCD, and absence of Goblet cells (GC) on CIC. Ten corneas co-expressed limited CK13 or CK19, and a further 10 corneas did not express either. There was only one CIC with a few Goblet cells present, but limited to a small peripheral sector. Figure 3 shows examples of CIC in the same patient before and after CLET, with predominantly CK13 positive cells before CLET (A) and predominantly CK12 positive cells after CLET (B).

Figure 1. In vivo confocal microscopy micrographs showing (A) limbal stem cell deficiency (precultivated limbal epithelial stem cell transplantation [pre-CLET]) with loss of corneal epithelial cells (i.e., conjunctivalization) and (B) post-CLET presence of corneal phenotypic epithelial cells (denoted by arrow).
Histological and immunocytochemistry assessment of all excised corneal buttons (Table 1) showed a healthy corneal epithelial cell layer, generally 3–5 cells thick. All 20 corneal buttons expressed either CK3 or CK12 or both. Five corneas co-expressed limited CK13 or CK19 or both; the remaining 15 did not express either CK13 or CK19. Goblet cells were detected in one cornea, again limited to a small peripheral sector. Figure 4 shows histological sections of the trephined corneal button in a patient at PKP following previous CLET, with predominantly CK12 positive (B) and CK13 negative cells (C).

The final CIC following PKP (Table 1) showed 17 corneas expressing CK3 or CK12 or both, with three not expressing either. Twelve corneas co-expressed CK13 or CK19 in a limited peripheral area of the sample. There were three CIC with few GCs, also limited to a small peripheral sector. There was no significant difference in the rates of expression of CK3/CK12 or CK13/CK19 and presence of GCs on CIC before and after PKP, demonstrating a stable epithelium after CLET, despite PKP.

All patients were phakic at the time of PKP. Three patients had cataract extraction and IOL implant at the time of PKP, one patient had cataract removal at the time of PKP but was left aphakic. Four further patients underwent cataract extraction and IOL implant after PKP; the remaining 12 patients were still phakic at the last follow-up. There was no significant difference in BCVA between the phakic and pseudophakic groups pre-PKP (p = .08, Mann–Whitney test) or post-PKP (p = .31, Mann–Whitney test).

Table 1. Summary showing number of corneas expressing CK3/CK12, CK13/CK19 and showing the presence of goblet cells on corneal impression cytology (CIC) before and after penetrating keratoplasty (PKP), and histological and immunocytochemical analysis of excised corneal buttons for expressions of the same

| Pre-PKP CIC | Corneal buttons | Post-PKP CIC |
|------------|-----------------|--------------|
| CK3+/CK12+ | CK13+/CK19+     | CK3+/CK12+   |
| 16         | 10              | 17           |
| Goblet cells | 1               | 1           |
| Goblet cells | 1               | 3           |

Figure 2. Sequential color slit lamp photos of two patients receiving a penetrating keratoplasty > 12 months post-cultivated limbal epithelial stem cell transplantation (post-CLET). (A) and (D) portray a color photo at baseline showing signs of total limbal stem cell deficiency. Asterisk (*) denotes conjunctivalized corneal surface with neovascularization. (B) and (E) are color photos 12 months post-CLET showing signs of a successful CLET (quiet eye, reasonably clear cornea, but with residual central deep stromal opacity). (C) and (F) show the same eyes at 36 months post-CLET. Arrow points at the limbal explant, which is in situ.

Figure 3. Color micrographs of corneal impression cytology samples with double staining for CK12 and CK13 showing (A) precultivated limbal epithelial stem cell transplantation (pre-CLET) CK13 positive (brown) cells and (B) post-CLET CK12 positive (red) cells. Scale bars are 100 μm.
As shown in Figure 5A, mean pre-PKP BCVA was 1.46 logMAR (range 0.3–2.7, SD 0.94) improving to a mean post-PKP BCVA of 0.74 logMAR (range 0–2.7, SD 0.87, p<.002). This gives a mean improvement in BCVA post-PKP of 36 letters (95% CI 15.0–57.1, p<.002, Fig. 5B). One patient suffered deterioration in vision post-PKP due to blunt trauma, resulting in wound dehiscence, traumatic expulsion of their crystalline lens and vitreous loss, resulting in a loss of 50 letters. Otherwise, no other patient had worse vision post-PKP. In 12 patients (60%), the BCVA post-PKP met the U.K. driving standard (logMAR BCVA 0.3 or better). The reasons for the other eight patients not meeting the U.K. driving standard BCVA are listed in Supporting Information Table A.

Six patients (30%) had a rejection episode (all endothelial rejections), with one of these patients suffering two rejection episodes. Mean time post-PKP to the first rejection episode was 9.29 months (range 2–22, SD 8.50). There were three graft failures, all related to previous rejection episode. Mean time to failure was 14.3 months (range 11–17, SD 3.06). One patient has been subsequently successfully re-grafted, while the other two patients are waiting to be re-grafted. Kaplan-Meier calculated mean graft survival was 90.9% (95% CI 50.8–98.7) at 12 months and 71.6% (95% CI 35.0–89.9) at 32 months (Fig. 6).

**DISCUSSION**

To the best of our knowledge, most large studies on autologous LSC transplantation have, understandably, focused primarily on the outcomes of the stem cell transplantation [7, 15, 23–27]. There is a lack of published studies on the long-term outcomes of corneal allografts following limbal (or other source) stem cell transplantation [11, 12, 20, 28–31]. There is also very little information available regarding the potential detrimental effect of corneal transplantation on the survival of previous stem cell transplants. Nor is it known when the optimal time is to perform corneal transplantation with regard to a one-stage versus a two-stage procedure [20].

Autologous CLET successfully reversed total LSCD in all our 20 consecutive cases, as demonstrated by the repopulation of a healthy epithelium with CK3/CK12 positive cells on histological and immunostaining analysis of corneal buttons trephined at the time of PKP. This confirmed the slit lamp assessment that showed clinically normal corneal epithelium without signs of LSCD prior to PKP. We have demonstrated a good correlation between pre-PKP CIC and the trephined recipient cornea in terms of the expression of CK3/CK12. However, there was a greater proportion of CIC showing CK13/CK19 expression, both before and after PKP, owing to likely contamination from the surrounding conjunctiva, as the majority of the trephined recipient corneas did not express these conjunctival markers.

Basu et al. demonstrated that a two-stage procedure with an initial CLET followed by PKP is associated with significantly better clinical outcomes compared with a single-stage procedure [20]. Patients with total LSCD typically suffer with pain and blurred vision as their primary symptoms. Kolli et al. showed that CLET, the first step in this pathway, is effective in reducing pain, but results in limited visual benefit due to deep central stromal scarring in most patients [1]. We have shown that most patients then require a corneal allograft for
visual rehabilitation. As per our current study design, we performed a two-stage procedure in all patients.

In this study, all 20 eyes had a BCVA worse than U.K. driving standard (0.3 logMAR). Indeed, the mean pre-PKP BCVA was 1.46 logMAR, but this improved to a mean post-PKP BCVA of 0.74 logMAR, with 12 of the 20 patients (60%) achieving BCVA better or equal to the U.K. driving standard at a median follow-up of 15 months. This is comparable to Basu et al.’s reported outcome of 15/21 (71.4%) of patients achieving a BCVA of 20/40 or better [20]. All our patients had an improvement in BCVA, with a statistically significant mean gain of 36 letters.

Six eyes from six patients suffered at least one endothelial graft rejection episode (30%), with 50% leading to graft failure despite intensive treatment, although one patient only presented for treatment 10 days after the onset of rejection symptoms. This rate of rejection is higher than that reported for PKP generally and reflects the fact that these are grafts in a higher risk cohort of patients due to their past ocular pathology and the presence of corneal stromal neovascularization. Although at the time of PKP the eyes were clinically quiet in terms of ocular surface inflammation, and this could be deemed high risk grafts [20, 34]. Basu et al. described a two-stage approach as being advantageous in terms of corneal graft survival, and they defined this as having a PKP at least 6 weeks following CLET [20]. Following our phase I clinical trial [1], our protocol has been to delay PKP until at least 12 months following CLET to ensure an eye that is completely quiet in terms of ocular surface inflammation, and this could explain our favorable graft survival rate at 1 year.

There was no evidence that PKP had a detrimental effect on LSC survival following previous CLET in the same eye, demonstrated by slit lamp biomicroscopy showing an absence of signs of recurrent LSCD. This was confirmed by the lack of a significant change in the expression of CK3/CK12, CK13/CK19, or the presence of GCs on CIC before and after PKP. To the best of our knowledge, other studies have not yet reported on the impact of corneal transplantation following previous LSC transplantation on LSC survival.

**CONCLUSION**

In summary, our results support Basu et al.’s study favoring a two-stage approach to visual rehabilitation in patients with total LSCD who underwent CLET in the same eye. However, in contrast to Basu et al., we recommend a delay of at least 12 months following CLET before proceeding with PKP, aiming to have a very quiet eye before corneal transplantation. This is supported by our favorable corneal graft survival outcomes at 1 year. Patients with previous CLET remain a slightly high-risk group for corneal allografts, reflected by their survival rates being slightly worse than low-risk PKP, but better than published data for high-risk PKP. To the best of our knowledge, we are the first to report on the outcomes of CLET following PKP.

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**AUTHOR CONTRIBUTIONS**

G.S.F.: conception and design, administrative support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; B.S-C. and H.S.M.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; O.J.B.: conception and design, administrative support, collection and/or assembly of data, final approval of manuscript; M.L.: conception and design, financial support, administrative support, final approval of manuscript; F.C.F.: conception and design, financial support, administrative support, provision of study material or patients, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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**Figure 6.** Graph showing Kaplan-Meier calculated PKP graft survival. Dotted line denotes 95% confidence intervals. Abbreviation: PKP, penetrating keratoplasty
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