Comparison of amniotic membrane transplantation with and without cultured limbal epithelium for persistent corneal ulcers

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ABSTRACT
This study compared the clinical outcomes of amniotic membrane transplantation with and without cultured limbal epithelial transplantation (CLET) for persistent corneal ulcers. The study included 30 patients that were assigned to either human amniotic membrane transplantation (AMT) only or a two-step cultured limbal epithelial transplant (CLET) with amniotic membrane carrier. The patients underwent a comprehensive ocular examination, Cochet-Bonnet esthesiometry, impression cytology, and anterior segment optical coherence tomography (AS-OCT). Slit-lamp images before and after treatment were analyzed using ImageJ to compare the fibrovascular tissue area. The state of epithelialization was assessed by fluorescein staining on day 30. Mean age of patients was 64 ± 20.39 in the AMT group and 55.17 ± 14.51 in the CLET group (p = 0.16). Visual acuity improved significantly in both groups after treatment, but the improvement was significantly greater in the CLET group (p = 0.022). Impression cytology demonstrated PAS+ cells in the corneal epithelium in 9 patients from the CLET group and 10 patients in the AMT group (p = 0.704). Cochet-Bonnet esthesiometry increased significantly after treatment with a similar rate between groups (p = 0.435). Improvement with reduction of the fibrovascular tissue area was achieved in both groups (p < 0.01 and p = 0.001 in the CLET and AMT group, respectively). Independent samples T-test demonstrated a greater reduction in the CLET group (p = 0.028). Kaplan-Meier analysis of epithelialization demonstrated higher success rate in the CLET group (p = 0.006). Persistent corneal ulcers could be associated with partial limbal stem cell deficiency (LSCD). In refractory cases, CLET should be the preferred treatment option with more sustainable results regarding corneal epithelialization and stromal scarring.

Introduction
Corneal opacity is one of the leading causes of blindness according to the World Health Organization (WHO) [1]. In addition to low vision, corneal disease decreases the quality of life due to constant pain, tearing, light sensitivity, redness and aesthetic appearance of the affected eye.

Since its first ophthalmic application in the 1940s, amniotic membrane transplantation has become an integral part of ocular surface disease treatment [2]. Among its many properties are the anti-angiogenic, anti-inflammatory, anti-fibrotic, and anti-microbial effects, as well as factors regulating and promoting epithelialization [3]. There are numerous transplantation techniques either as an inlay (graft), onlay (patch) or a combination of the two, single or multilayered depending on the depth of the ulcer and required effect. Despite the high success rate, there are conditions for which AM transplantation proves inadequate with incomplete epithelialization and opaque scarring [4].

Recently, another technique based on advances in tissue engineering has gained popularity. Cultured limbal epithelial transplantation has been found effective in cases of limbal stem cell deficiency, defined as persistent epithelial defects, corneal conjunctivization, neovascularization and scarring, and chronic inflammation. Most studies include cases of chemical burns, Stevens-Johnson’s syndrome, ocular cicatricial pemphigoid and aniridia with an overall success rate of 76% [5]. The method is still under extensive investigation with a wide variety of culture protocols and surgical approaches [6]. Current advances aim at avoiding the potential concerns associated with culturing and transplantation techniques. The main objective is to use protocols free of animal products, which need
to ensure maximal clonal expansion while preserving undifferentiated phenotype and proliferative potential of the cells rendered.

The purpose of this study is to compare the clinical outcomes of amniotic membrane transplantation with and without cultured limbal epithelium for persistent corneal ulcers.

**Subjects and methods**

This was a prospective comparative study that began in May 2016 and continued through December 2020 in a tertiary referral center. Statistical analysis of the data was performed using IBM SPSS v23. The tests used include Mann-Whitney U-test, Wilcoxon signed rank test, chi-squared test, T-test.

**Ethics statement**

Informed consent was obtained from all individual participants included in the study prior to treatment. All procedures involving human participants were performed in accordance with the 1975 Helsinki declaration and its later amendments or comparable ethical standards.

**Study design**

Thirty patients with persistent corneal ulcers were included. Persistent corneal ulcers were defined as epithelial defects with stromal loss, thickened edges, surrounding epithelial irregularities (marked with fluorescein) and surrounding stromal edema that did not heal for over 3 months with optimal conventional therapy including lubricants, topical/local antibiotics and steroids and/or therapeutic contact lenses.

Fifteen subjects received human amniotic membrane transplantation (AMT) only and fifteen underwent a two-step cultured limbal epithelial transplant (CLET) with amniotic membrane as a carrier. Patients were randomly allocated to groups using Microsoft Excel 2016. Patients underwent a comprehensive ocular examination including visual acuity (VA), IOP, biomicroscopy, ocular surface fluorescein staining and ophthalmoscopy (whenever possible). Data from patients with very low vision was quantified using the results described by Schulze-Bonsel et al. [7]. Infectious etiology was ruled out based on negative microbiological and/or molecular tests.

Impression cytology samples from all participants were collected using cellulose-acetate membrane filters after topical anesthesia. Hematoxylin, eosin, Papanicolau and Periodic acid Schiff (PAS) stains were used to visualize cellular changes.

Images were acquired using slit-lamp photography from all patients before treatment and 30 days postoperatively. Results were analyzed by an observer masked to the treatment and time point. The area with fibrovascular scar tissue was marked and calculated automatically by software ImageJ v.1.53e (Figure 1).

Cochet-Bonnet esthesiometry was used to determine the central corneal sensitivity threshold before and one month after treatment.

Anterior segment optical scans were performed prior to and 30 days after treatment to assess corneal epithelium integrity, stromal reaction and tissue loss using a spectral domain OCT (3D OCT-2000FA, Topcon). The site of limbal biopsy was selected based on limbal scans from areas with preserved palisades of Vogt. Changes in stromal tissue and epithelial recovery were evaluated after treatment.

**Amniotic membrane transplantation technique**

Standard human amniotic membrane transplantation included adapting the donor tissue to the ocular surface epithelial side up with 10/0 nylon interrupted limbal suture. All patients received a topical combination of antibiotic and steroid for 1 week following the procedure.

As a first step of our approach to CLET, a minimally invasive limbal biopsy was taken from the affected eye with simultaneous amniotic membrane transplantation. The harvested autologous limbal stem cells were cultured in a dedicated facility in compliance

![Figure 1. Digital slit lamp image of a patient with selected fibrovascular area after setting a scale in pixels based on corneal diameter. The area was calculated automatically by the software.](image-url)
with Good Medical Practice guidelines following the manufacturer’s instructions (CnT-Frame, CELLnTEC Advanced Cell Systems AG, Bern, Switzerland). An intact AM was used as a substrate. The graft was inserted in a submerged explant culture system with no additional bovine/human serum or 3T3 fibroblast feeder layers. Cells were cultured for 14 days at 37°C in a humidified incubator with 5% CO₂ in air. An inverted phase contrast microscope (Olympus EP50, Olympus LS) was used to assess cell confluence and morphology. The second step in our CLET technique was to spread the central portion of the prepared graft containing the cultured limbal epithelial cells over the entire corneal surface of the affected eye, epithelial side up and adapt it with 10/0 nylon interrupted limbal suture. The rest of the amniotic membrane was applied as a second layer (amniotic membrane patch) and fixed to the conjunctiva with 8/0 interrupted sutures. Success criteria were defined as complete epithelialization of the corneal surface, reduction of the fibrovascular scar tissue and improvement in visual acuity. Fluorescein stain was used to determine the completion of epithelialization on day 30. Assessment for adverse reactions was made including infection, progressive corneal thinning and failure of the transplant defined as persistent epithelial defects, neovascularization and corneal opacity, as well as detrimental effects of the limbal biopsy on the donor eye condition.

**Results**

The mean age of the patients was 64 ± 20.39 years (mean ± SD) in the AMT group and 55.17 ± 14.51 years (mean ± SD) in the CLET group (p = 0.16). Fifteen male and 15 female patients were included in the study. The male-to-female ratio between the groups was similar using the chi-squared test (p = 0.27, \( \chi^2 = 1.2 \)). All patients reported subjective symptoms of impaired vision, tearing, photosensitivity, pain and redness for at least 3 months with no improvement by conventional treatment.

The etiology of the ulcers included post herpetic complications (n = 6), neurotrophic keratopathy (n = 7), toxic keratopathy (n = 3), ocular rosacea (n = 2), corneal melting in rheumatoid arthritis (n = 3), multiple ocular surgeries (n = 4), stroke with Bell’s palsy (n = 4), local irradiation for nasopharyngeal carcinoma (n = 1).

The baseline visual acuity was found similar in the two groups using Mann-Whitney U-test with a median of 0.003 ± 0.0052 (median ± SEM) in the CLET group and 0.05 ± 0.0077 (median ± SEM) in the AMT group (p = 0.734). Wilcoxon signed rank test showed significant improvement in postoperative VA in the CLET group to a median of 0.05 ± 0.0387 (p = 0.001) and the AMT group to 0.05 ± 0.0103 (p = 0.01). However, using Mann-Whitney U-test the difference in the rate of improvement in the two groups was statistically significant (p = 0.022) with a greater degree in the CLET group.

Impression cytology demonstrated PAS+ cells in the corneal epithelium in 9 patients from the CLET group and 10 patients in the AMT group. Chi-square test demonstrated the rate of PAS+ cells to be similar (p = 0.704). There were signs of inflammatory infiltration and xerosis in all patients, which had progressed to keratinization of the epithelium in five cases from the CLET group and six cases from the AMT group (p = 0.704) (Figure 2).

Median esthesiometry for the CLET group was 200 ± 22.696 (median ± SEM) and 200 ± 23.504 (median ± SEM) for the AMT group. Mann-Whitney U test showed comparable baseline esthesiometry in the two groups (p = 0.703), which improved significantly after treatment (p = 0.024 in the CLET group, p = 0.046 in the AMT group on Wilcoxon signed rank test). The rate of improvement was found to be similar between the two groups using Mann-Whitney U-test (p = 0.435).

The mean area of the corneal fibrovascular tissue in the CLET group was 38.77 ± 17.33 (mean ± SD) and 34.30 ± 20.09 (mean ± SD) in the AMT group (p = 0.52, T-test). Statistically significant improvement was achieved with reduction of the area with fibrovascular tissue in both groups using paired samples T-test.

![Figure 2. Impression cytology sample with signs of keratinization with copper discoloration of the whole area and a few PAS+ cells on the corneal surface (arrows) (PAS, hematoxylin, PAP, x100).](image-url)
with mean area $16.23 \pm 13.78$ ($p < 0.01$) and $22.64 \pm 14.96$ ($p = 0.001$) in the CLET and AMT group, respectively. Independent samples T-test demonstrated statistically significant differences in the reduction of fibrovascular tissue area between the two groups with greater decrement in the CLET group ($p = 0.028$) (Figure 3).

AS-OCT demonstrated extensive loss of epithelium, moderate stromal hyperreflectivity and edema surrounding the defect. Limbal scans revealed slightly altered contour with sectoral loss of palisades in 28 of all cases. Two patients from the CLET group had significant changes in limbal structure with flattened palisades due to multiple intraocular surgeries (Figure 4). Postoperative OCT showed restored epithelial integrity in all patients from the CLET group and in 8 from the AMT group. There was hyperreflective scar tissue formation within the stroma of all patients, which prevented further stromal loss (Figure 5).

The success criteria were met in 8 out of 15 cases in the AMT group and in all patients in the CLET group. Kaplan Meier survival analysis demonstrated the difference was statistically significant ($p = 0.006$). Follow-up of the cases with incomplete epithelialization showed four patients with delayed but complete epithelialization at two months, but four patients needed additional treatment with a second amniotic membrane transplantation and other available options (including regenerative agents).

No adverse reactions to CLET as previously defined were recorded.
Discussion

Nonhealing corneal ulcers present a specific challenge in ophthalmic practice. Amniotic membrane transplantation has been established as a gold standard in the treatment of ocular surface disease, including persistent corneal ulcers [8]. Conversely, cultured limbal epithelial transplantation is still under investigation with numerous reports of novel protocols and favorable results [9]. Despite the obvious advantages of CLET, its propriety should be determined on an individual basis due to its high value and resources required.

Since its introduction by Pellegrini in 1997 [10], CLET has proven successful for ocular surface disease including mainly chemical burns, Stevens-Johnson syndrome, ocular cicatricial pemphigoid, aniridia-associated keratopathy. Success rates vary between 76 and 100% [9]. Early-on in the development of the technique a lot of different protocols arose using DMEM/HAM's F12 based media with multiple feeder layers, usually from 3T3 mouse fibroblasts [11]. To provide growth factors, bovine or patient sera were used. Currently, the main focus of the technique is to exclude animal products that bear the risk of infectious contamination and immunogenicity [12]. With progress in bioengineering, growth factors are now safely produced using xenofree technology. This led to the implementation of biologically safe protocols without any xenobiotic products. The culture protocol used in our study is in accordance with the current Good Manufacturing Practice guidelines under xenofree conditions.

Numerous studies have demonstrated the success of CLET, but few compared the results with other techniques for limbal stem cell grafting. Sharma et al. [13] compared amniotic membrane transplantation and cultured limbal transplantation for the treatment of partial limbal stem cell deficiency with similar outcomes for both techniques. This would suggest that in cases of partial LSCD, AMT should be the procedure of choice due to its availability and lower economic value. However, in a study by Borderie et al. [14] compared to classical limbal tissue transplantation, autologous cultured limbal stem cells had better long-term success, safety and improvement in vision.

In our study, we compared amniotic membrane transplantation and cultured limbal epithelial transplantation in cases with persistent corneal ulcers. It is our belief that regardless of the exact etiology, there is a certain point when non-healing corneal ulcers deplete the stem cell reservoir at the limbus and exceed the cornea’s regeneration abilities. This was supported by impression cytology and AS-OCT findings, pointing to partial limbal stem cell deficiency (LSCD) in some of our patients.

We found that VA improved significantly in both groups, but the rate of improvement was significantly greater in the CLET group. These results point to a better outcome of CLET over AMT in cases of persistent ulcers.

We demonstrated that persistent ulcers were associated with abnormal corneal sensitivity probably due to the direct loss of nerve fibers as well as the toxic effect of multiple topical medications and altered quality of the tear film. The insult to corneal innervation highlights the complex pathophysiology of persistent ulcers.

Corneal sensitivity increased significantly in both groups with a similar rate. In previous studies, increment in corneal sensitivity was accompanied by higher corneal nerve density as measured by in vivo confocal microscopy [15,16]. Recovery of corneal innervation as a component of the stem cell niche could lead to improved epithelialization process.

Amniotic membrane integrates with the corneal stroma, reducing inflammation and therefore subsequent scarring, simultaneously providing optimal environmental conditions for limbal stem cell proliferation and migration [17]. Furthermore, studies have demonstrated that cultured limbal stem cells secrete multiple growth factors that modify the wound healing process in the whole cornea including the stroma [18,19].

Comparison between fibrovascular tissue area before and after treatment demonstrated improvement in both groups. However, the patients in the CLET group demonstrated significantly greater rate of reduction in corneal scarring, which could explain the improvement in VA. Preventing extensive corneal scarring could be considered as another advantage of CLET over AMT. This might be the result of a synergistic effect of amniotic membrane properties and limbal stem cell secretome.

The patients included in our study had non-healing corneal ulcers refractory to conventional treatment. AS-OCT demonstrated sectoral loss of palisades of Vogt in most patients, which could be a sign of partial LSCD. Our initial hypothesis was that CLET would lead to better outcomes due to the replenishment of the epithelial stem cells, providing optimal conditions for niche function combined with the effects of the amniotic membrane. Although previous studies found that partial LSCD could be managed with AMT alone [13], our results point to a better outcome with CLET. All patients in the CLET group achieved the success criteria, but only 8 out of 15 in the AMT group. Kaplan-Meier analysis proved this to be significant.
Therefore, in certain cases with persistent corneal ulcers CLET should be the preferred technique. Our results correspond to the reported overall success rate of CLET. Notably, CLET has been found less effective when treating autoimmune ocular surface disease with uncontrollable inflammation [20] and aniridia-associated keratopathy either due to genetic defects in patient’s autologous limbal stem cells or to the use of allogeneic stem cells [21]. None of the patients included in our study was diagnosed with a similar condition, therefore the success rate is expectedly high.

The limitations of our study are the small sample size as well as the complexity of patient randomization in a double-blind way, since the CLET was performed in two stages. We perceive the lack of confocal microscopy a limitation as well, since it could give more insight into the wound healing process.

Conclusions
Persistent corneal ulcers could be associated with partial LSCD. Amniotic membrane transplantation is a well-established technique and has been shown to be effective in cases of partial LSCD. However, in refractory cases, CLET should be the preferred treatment option with more sustainable results regarding visual acuity, corneal epithelialization and stromal scarring.

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Disclosure of interest
All authors declare that they have no relevant financial or non-financial interests to disclose regarding the publication of this paper.

Data availability
Data supporting the findings of this study are available at all times from the corresponding author upon reasonable request.

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