

# Tissue-Cultured Human Cord Lining Epithelial Cells in Treatment of Persistent Corneal Epithelial Defect

Nguyen Dinh Ngan<sup>1,2</sup>, Hoang Minh Chau<sup>3,4</sup>, Pham Ngoc Dong<sup>3</sup>, Le Xuan Cung<sup>3</sup>, Nguyen Thu Thuy<sup>3</sup>, Phan Toan Thang<sup>5</sup>, Than Van Thai<sup>6</sup>, Vu Thi Nga<sup>7</sup>, Nguyen Duy Bac<sup>8\*</sup>

<sup>1</sup>Department of Ophthalmology, 103 Military Hospital, Hanoi, Vietnam; <sup>2</sup>Department of Ophthalmology, Vietnam Military Medical University, Hanoi, Vietnam; <sup>3</sup>Department of Cornea and external diseases, Vietnam National Institute of Ophthalmology, Hanoi, Vietnam; <sup>4</sup>Department of Ophthalmology, Hanoi Medical University, Department of Ophthalmology; <sup>5</sup>Department of Surgery, Yong Loo Lin school of Medicine, National University of Singapore, Singapore; <sup>6</sup>NTT Hi-tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam; <sup>7</sup>Institute for Research and Development, Duy Tan University, 03 Quang Trung, Danang, Vietnam; <sup>8</sup>Vietnam Military Medical University (VMMU), Hanoi, Vietnam

## Abstract

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**\*Correspondence:** Nguyen Duy Bac. Vietnam Military Medical University (VMMU), Hanoi, Vietnam. E-mail: [nguyenduybac@vmmu.edu.vn](mailto:nguyenduybac@vmmu.edu.vn)

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**BACKGROUND:** Persistent corneal epithelial defect (PED) is a consequence of many ocular surface disorders. Although many therapies have been suggested, the treatment of this disease have faced a lot of difficulties up to now. The transplatation of cultivated amniotic epithelial cells sheets is the new promised method for PED. Cord lining epithelial cells (CLECs) are epithelial cells of amniotic membrane of umbilical cord, so these cultivated cells sheet may be good for treating PED

**AIM:** To evaluate the efficacy of the transplantation of cultivated CLECs sheets in treatment of PED and analyze some influential factors of this therapy.

**METHODS:** A prospective interventional case series with transplantation of tissue-cultured human CLECs in 37 PED eyes in Vietnam National Institute of Ophthalmology.

**RESULTS:** Thirty four of 37 eyes were healed with the cells transplantation and 22 eyes of them healed within a week postoperatively. There were normal corneal scars and normal corneal epithelial cell (by impression cytology detection) on transplantation site in all 31 successful cases. The other successful eyes were done lamellar keratoplasty (respectively in 1 month, 3 months, 6 months and 27 months postoperatively) to investigate the histopathology of the CLECs transplant site. The histopathological images showed normal corneal scar and there was no appearance of CLECs in transplant site.

**CONCLUSION:** tissue-cultured human CLECs transplantation is a quite safe and effective treatment for persistent corneal epithelial defect. The CLECs may help the epithelial healing at early stage but do not exist at transplant site for a long time.

## Introduction

Persistent epithelial defect (PED) of cornea occurs when the corneal epithelium fails to regenerate steadily over a corneal wound within due course (usually less than 2 weeks in normal corneas), although the wound has been intensively treated [1]. The potential causes of PED are myriad and its mechanisms remain unclear, related to multiple factors and hard to determine specifically. Therefore, no specific therapy for this disease has been available up to now [1]

Many therapies for PED have been suggested, such as: anti-inflammation, artificial tear, nutritious solution for ocular surface, soft contact lens, tarsorrhaphy, keratoplasty [1]. However, some PEDs were even failed to heal and the visual functions were threatened in spite of extensive treatments [2], [3], [4], [5]. Amniotic membrane transplantation has been recently employed in PED with promised result, nonetheless the success rate were varied in many studies (from 31.4% to 90%) [2], [6], [7]. Moverover, using amniotic graft may reduce the corneal transparent and visual acuity. Stem cell technologies have been applied to treat PEDs in some special

situations, and supposed a new promised therapy for this disease. Cultivated epithelial autografts (limbal stem cells, oral mucosal cells) or cultivated epithelial allografts (amniotic epithelial cells) have been tried in some rough cases with promised results [7], [8], [9], [10]. Parmar DN, et al., (2006) firstly successfully performed the trial of using tissue-cultured amniotic epithelial cells for 3 refractory PED cases [7].

Cord lining epithelial cells (CLECs) are epithelial cells of amniotic membrane of umbilical cord. These cells have been isolated and proved to be progenitor/stem cells. CLECs were differentiated to be some type of epithelium by some invitro study [11], [12], [13]. Zhou Y, MacAry PA, et al., have shown many markers of corneal and limbal epithelial cells in CLECs and the safety of CLECs human transplantation [12], [14]. Reza HM, (2011) has differentiated CLECs to corneal epithelial cells and transplanted them to rabbit eyes. The results showed that rabbits' cornea after CLECs transplatation had no limbal stem cells deficiency signs and remained transparent with normal histology structures up to 10 weeks postoperatively. These studies have suggested the CLECs application in treatment of ocular surface diseases, especially PEDs – one of the difficult treated corneal diseases [11]. From this hypothesis we carried out the prospective study to evaluate the efficacy of the transplantation of tissue-cultured CLECs in treatment of refractory PEDs.

## Materials and Methods

### Subjects

In this study, 37 patients with PED in Vietnam National Institute of Ophthalmology were enrolled from December 2010 to May 2014. All patients were followed up at least 36 months. All our cases had been failed to healed with other treatment therapies. The descemetocoele or perforated ulcer, corneal ulcer with inflammatory infiltrative site or abscess in deep stroma, the PEDs with total limbal stem cells deficiency or the eyes with eyelid malformation were excluded from the study.

This study was approved by the institutional review board of Vietnam National Institute of Ophthalmology, and informed consent was obtained from all patients.

### The tissue-cultured CLECs preparation

The cells sheets were cultured in the laboratory of Department of surgery, National University of Singapore. The isolated CLECs were evaluated for the quality and expression of surface markers, and stored in single-cell suspension on

culture dish with the density of  $3-4 \times 10^4$  cells/cm<sup>2</sup> at -196°C. Then, they were taken out of storage tanks and thawed at 37°C in water bath. And the cells were supplemented with PTTe-1 medium to the falcon tissue culture inserts (12-well plates) at density around 10000 cells/cm<sup>2</sup>. The next step, the culture plates were put straight into 37°C/5% CO<sub>2</sub> incubator. The fresh culture media was replaced every 2 days until the cells grew into a continuous surface on the scaffold (from 5 to 7 days), consists of 1-2 cell layers. Finally, the cultured CLECs were determined the markers of cornea-like epithelial cells (CK3, CK12). For transportation, the Falcon tube (50ml) with cultured fluid contained 2 wells storage in -80°C.

### The tissue-cultured CLECs transplantation

The PEDs was cleaned by crescent knife to prepare transplantation site. The cells sheet with scaffold was cut to the shape and size of the ulcer. Then, the sheet was placed with the epithelium side down, and covered with soft contact lens. Two crossed Vicryl 8/0 sutures were placed over the contact lens to sclera for pressing and fixing the cells sheet. In necessary case, the combined surgeries such as hypopion lavage, pterygium excision and conjunctival autograft were done. Second cells transplantation was performed when the PEDs were ameliorated but not completely healed 4 weeks after the first surgery. The fixation sutures were removed to check the healing of the defect 7 days after surgery.

Postoperative medications included topical moxifloxacin 0.5% (Vigamox; Alcon Laboratories, Inc. Fort Worth, USA) four times daily and sodium hyaluronate 0.1% (Sanlein, Santen Pharmaceutical Company) six times daily.

The result of cells sheet transplantation was evaluated by two criteria; the time of healing and the quality of the corneal transplantation site. The treatment success meant the PED was completely healed after one or more transplatation, the failure meant the PED was not healed after transplatation or the corneal epithelial defect was recurrently appeared in 4 weeks after surgery and was not able to regraft.

The quality of the transplantation site on cornea was clinical evaluated and catergorized into 3 grades: *good* - the epithelial surface was smooth, the cornea was transparent and the anterior segment could be seen clearly. There is no neovessel at transplantation site; *moderate*-the epithelial surface was not smooth, but there is no epithelial defect. The cornea was mild opaque, but the pupil was able to see. There were neovessels in anterior stroma at transplantation site; *bad*-the epithelial surface was rough and there is epithelial defect or corneal recurrent erosion. The cornea was very opaque and the anterior segment could not be seen. There were many neovessles at at transplantation site, some of them were in posterior stroma.

To detect any abnormal cells on transplanted site, the impression cytology was done 1 month, 3 months and 6 months postoperatively in success cases. To know whether CLECs were stayed at transplanted sites, as well as partially understand the epithelial healing mechanisms of defect, some male CLECs graft were used in female cornea. Then we performed keratoplasty for those cases to take the cornea for searching for CLECs. The SRY (the specific marker of Y chromosome) were tested in the cell-received cornea by PCR to determine the existence of CLECs on transplanted site by Genetics and Molecular biology laboratory of Vietnam Military Medical University.

## Results

### The characteristics of studied patients and PEDs before surgery

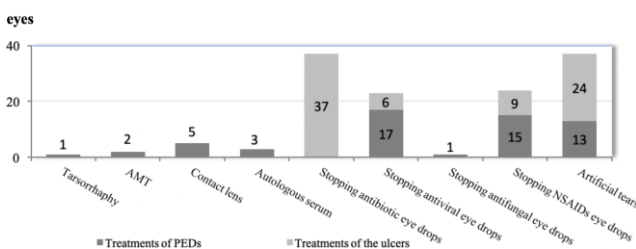
There were 37 patients (26 male, 11 female) with the age from 21 to 86 years old (average  $59.6 \pm 19.8$  years old). All patients have unilateral corneal ulcer.

The original causes of the corneal ulcers were described in Table 1. Most of the causes were infection (26/37 eyes) with 14 herpetic eyes (37.8%).

**Table 1: Original causes of the corneal ulcers**

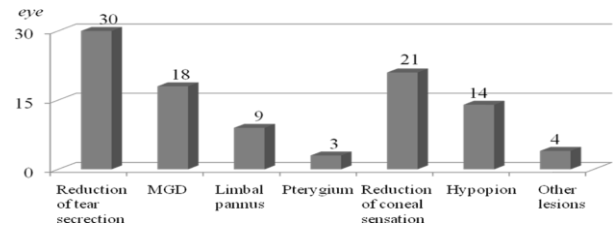
Causes	Quantity	Percentage
Infection	14	37.8
Herpes	11	29.7
Bacteria	1	2.7
Fungi	3	8.1
After surgery	1	2.7
After radiation	2	5.4
Eye trauma	1	2.7
Sjogren syndrome	4	10.9
Unidentified causes	37	100
Total		

The period time of the corneal defects were from 4 weeks to 28 weeks ( $9.9 \pm 5.2$  weeks) and the period time of the PEDs were from 2 weeks to 13 weeks ( $5.0 \pm 2.9$  weeks). All patients in our study were refractory with medical treatment (stopping corneal toxic eye drop, using artificial tear, using contact lens...). There were 2 recurrent PEDs after AMT (Figure 1).



**Figure 1: The previous treatments of PEDs; (AMT: amniotic membrane transplantation; NSAIDs: non steroid anti-inflammation drugs).**

The size of the PEDs were from 11% to 65% corneal area (average  $33.9 \pm 14.9$  % corneal area). The depth of PEDs were mostly less than two third of corneal thickness (33/37 eyes). The combined ocular lesions were shown in Figure 2.



**Figure 2: Combined ocular lesions; (MGD: meibomian gland dysfunction).**

### The results of tissue-cultured CLECs transplantation

The success rate of our study was 91.9% (34/37 eyes). There were 31 cases completely epithelialized in 2 weeks, 2 cases in 4 weeks, only one case with healing time more than 4 weeks and need second surgery. In 3 cases that failed to heal with transplantation, there was only one case recurred epithelial defect a week after healing.

We found no association between healing time and some PEDs characteristics (original causes, the period time of the defects and the PED, the size of defect). However, the correlation between the time of epithelial healing and the tear secretion was 0,36 ( $p < 0,05$ ). The healing time of eyes with the reduction of cornea sensation was longer than the healing time of the others ( $p < 0,05$ ). The healing time of eyes that were both reduction of tear secretion and MGD ( $10.1 \pm 5.5$  days) were significant longer than the others ( $7.8 \pm 4.1$  days) ( $p < 0,05$ ).

**Table 2: The quality of the transplantation sites**

Transplantation site Follow-up time	Good	Moderate	Bad	Total
1 month (34 eyes)	12	19	3	34
3 months (33 eyes)	20	11	2	33
6 months (32 eyes)	25	7	0	32
12 months (30 eyes)	25	5	0	30
36 months (30 eyes)	24	5	1	30
The last checked time (30 eyes)	26	4	1	30

(At the end of the study, 30 eyes were followed up from 36 to 64 months, average  $45 \pm 7.7$  months, the others were done corneal transplant as the schedule).

One eye recurred herpes keratitis at corneal transplantation site 17 months postoperatively. After successful treated, the corneal scar was hazy with stroma neovascularization, so the transplantation site was classified as bad quality.

The number of successful cases were done impression cytology at 1 month postoperatively was 27 cases, at 3 months was 24 cases and 6 months was 31 cases (exception 3 keratoplasty eyes). The pictures of cytologies showed normal epithelial cells: cuboidal shape, quite uniform size, and round nucleus, the same as cells on normal cornea (Figure 3B, and Figure 1D). There were no abnormal nucleus

in these cells. Some cells at the bad transplantation site were in grade of mild metaplasia (Figure 3F and 3G).

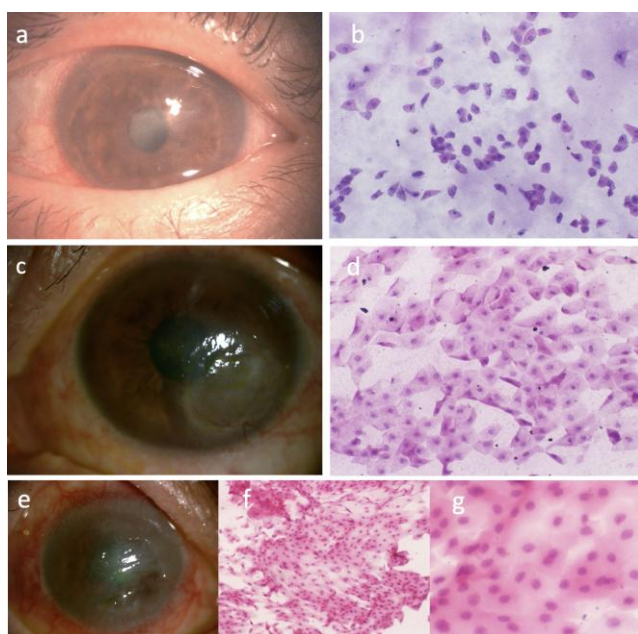


Figure 3: The corneal transplantation site and results of impression cytology; A) good transplantation site 3 months postoperatively; B) impression cytology of same patient (HE, x 100); C) moderate transplantation site 3 months postoperatively; D) impression cytology of same patient (HE, x 200); E) bad transplantation site 1 month postoperatively; F) and G) impression cytology of same patient (HE, x 100 and x 400)

In 4 corneal transplanted eyes, there were 3 females, that were done keratoplasty in 1.5 months, 3 months and 27 months postoperatively, and one male (6 months after surgery). The histology of corneal specimens showed normal epithelium at transplanation sites, but the number of layers increased (about 12-15 layers) (Figure 4). There were no inflammation cells infiltrated beneath the transplanation sites. The PCR test looking for SRY were negative in all 3 female cornea, but the test results of cells sheet and male conea were positive.

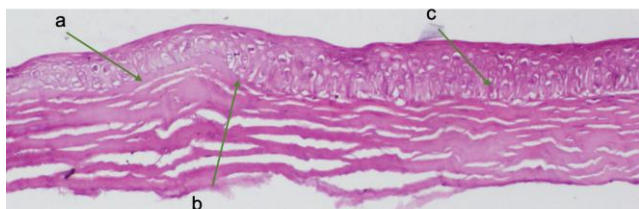


Figure 4: The histology of cell-received cornea (Trichrome-Masson, x 100); A) healthy corneal epithelium with Bowman layer; B) the connection between the healthy cornea and transplantation site; C) the transplantation site without Bowman layer

There were some mild complications in surgical procedure. Thirty eyes were subconjunctival haemorrhage without any interruption of surgical procedure. One eye was severe haemorrhage and the

conjunctiva was inflated, so the contact lens had to be seen trephined to 10 mm in diameter to be easier to fixed.

The postoperative complications were not severe. One contact lens was lost fixation 4 days postoperatively, then the cells sheet was removed and the contact lens was replaced until the cornea was completely healed (12 days after surgery). There were 2 eyes that the cells sheets were slightly moved inspite of good fixation contact lens. After removing the fixation sutures, all these eye were completely epithelial healed.

## Discussion

The mechanism of PEDs were partially specified by original causes of the corneal ulcer, the combined ocular lesions and the previous treatments. In our study, the original causes of two third eyes were infection (26/37 eyes, Table 1), the remarkable combined ocular lesions were the reduction of cornea sensation, dry eye, hypopyon (Fig. 2), so that the two main mechanisms of PED in our patients might be ocular inflammation and neurotropic keratopathy. The addition mechanism was epithelial toxic from eye drops (Fig. 1). The other mechanisms may not be important role in our study group.

Amniotics membrane was proven to inhibit inflammatoty reaction, scar creation, and neovascular growth, as well as promote epithelial migration, adhesion when overlay transplantation in PED [6], [15]. These biological efficacies were suggested from many mediator factors (such as EGF, KGF, and HGF, macrophage inhibitory factor, Fas ligand...) inside amniotic epithelial cells and somewhat in membrane stroma [16], [17]. For amniotic membrane graft, these factor may just existed on transplantation site for short time after surgery because of nonviable amniotic epithelial cells and dissovled stroma [15], [18]. So that viable amniotic cells may be supposed more effective in treating PED than amniotic membrane transplantation. Parma (2006) showed succesful corneal epithelial healing in 3 complicated PEDs that had been failed with other therapies. Although the healing mechanism of amniotic epithelial cells transplantation in his study remains unclear, the author presumed the trans-planted cells firstly may provide an initial cellular means to repair the PED and then promote epithelialization by in situ release of anti-inflammatory and healing factors in these cells [7]. Other mentioned mechanism was tissue-cultured cells may also provide a mechanical scaffold promoting subsequent epithelial healing of the cornea. In earlier experiments, He YG had transplanted human amniotic epithelial cells onto denuded rabbit corneal surfaces, these cells had been re-polarized and tightly adhered to the recipient corneal stroma

24h after transplantation. However, the transplantation site was repopulated by host corneal epithelium after 10 days [19].

CLECs were amniotic epithelial cells around umbilical cord, so their microstructure was the same as amniotic cells. They were one kind of stem cell and were able to differentiated to corneal-liked epithelial cells [12], [13], [20]. Moreover, CLECs also were transplanted in rabbits' cornea with total limbal stem cell deficiency with good results. There were 50% cases had no limbal stem cells deficiency signs and the corneas remained transparent with normal histology structures up to 10 weeks postoperatively [11]. Therefore, transplantation tissue-cultured CLECs on PEDs may promote healing in the similar mechanisms of amniotic epithelial cells. In our study, we also found that CLECs were not able to exist long time on transplantation site after surgery. The PCR test for Y chromosome in female corneal specimens showed negative result as soon as 1.5 months postoperatively. Thus, it suggested that the donor CLECs improved the ocular surface through the secretion of factors promoting wound healing, stimulating repopulation of the remaining host corneal epithelial cells. It explained the high success rate in our studied group with most PEDs caused by inflammation and neurotrophic keratopathy.

The most important influential factors of the CLECs transplantation is tear film. The correlations between the tear secretion, situation of MGD and the quality transplantation sites as well as healing time were statistically significant ( $p < 0.05$ ). The healing time were statistically significant longer in the group of reduction of cornea sensation. These factors (tear film, cornea sensation) also contributed to PEDs mechanism, so the medical treatments to ameliorate them before and after CLECs transplantation were also played important role in the result of CLECs surgery.

In our study, the cells sheet with scaffold was placed on the defect with the epithelium side down with the fixation of two Vicryl 8/0 sutures. This method showed the good result: 22/37 eyes complete healing after removing fixation sutures (a week postoperatively), 10 eyes more in 2 weeks postoperatively and 2 eyes healed in 3 weeks. Using directly scaffold of cells sheets instead of collagen shield with concave shape (in Parmar method [7]) made the cultivated procedure simpler and cheaper. However the cultured-well scaffold was flat so it was to difficult to force adherently 100% cells sheets on corneal surface, especilally when the defect was larger than 50% corneal area. Our method to fix the tissue sheet with 2 cross sutures press on soft contact lens is quite easy and simple with good effect. Although these sutures on sclera easily created subconjunctival haemorrhage that made difficult to fix contact lens. We usually used vasoconstriction eye drops to prevent this complication (adrenaline 0.1%).

In conclusion, tissue-cultured human cord lining epithelial cells transplantation is a safe and effective treatment of persistent corneal epithelial defect. This transplantation open a new method in treatment of this complicated diseased. However, more studies with long follow up time were need to re-evaluate the efficacy as well as the mechanism of corneal healing of this cells tranplantation.

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## Conflict of Interests

Assoc. Prof. Phan Toan Thang is the principal investigator of human cord lining epithelial cells. He is a founding director and CSO of CellResearch Corporation Group of Companies in Singapore. Other authors have declared that no competing interests exist.

## Ethical Approval

Subjects voluntarily participate in the research; patient information is confidentiality. Research subjects have the right to end the study at any time. The research was approved by the institutional ethical review board of Vietnam National Institute of Ophthalmology

## Informed Consent

All patients agreed and signed an informed consent form before surgeries

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