·Basic Research·

Transplantation of tissue-engineered human corneal epithelium in limbal stem cell deficiency rabbit models

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Abstract

• AIM: To evaluate the biological functions of tissue-engineered human corneal epithelium (TE-HCEP) by corneal transplantation in limbal stem cell deficiency (LSCD) rabbit models.

• METHODS: TE-HCEPs were reconstructed with DiI-labeled untransfected HCEP cells and denuded amniotic membrane (dAM) in air-liquid interface culture, and their morphology and structure were characterized by hematoxylin-eosin (HE) staining of paraffin-sections, immunohistochemistry and electron microscopy. LSCD models were established by mechanical and alcohol treatment of the left eyes of New Zealand white rabbits, and their eyes were transplanted with TE-HCEPs with dAM surface outside by lamellar keratoplasty (LKP). Corneal transparency, neovascularization, thickness, and epithelial integrality of both traumatic and post transplantation eyes were checked once a week by slit-lamp corneal microscopy, a corneal pachymeter, and periodic acid-Schiff (PAS) staining. At day 120 post surgery, the rabbits in each group were sacrificed and their corneas were examined by DiI label observation, HE staining, immunohistochemistry and electron microscopy.

• RESULTS: After cultured for 5 days on dAM, HCEP cells, maintaining keratin 3 expression, reconstructed a 6-7 layer TE-HCEP with normal morphology and structure. The traumatic rabbit corneas, entirely opaque, conjunctivalized and with invaded blood vessels, were used as LSCD models for TE-HCEP transplantation. After transplantation, obvious edema was not found in TE-HCEP-transplanted corneas which became more and more transparent, the invaded blood vessels reduced gradually throughout the monitoring period. The corneas decreased to normal thickness on day 25, while those of dAM eyes were over 575μm in thickness during the monitoring period. A 4-5 layer of epithelium consisting of TE-HCEP originated cells attached tightly to the anterior surface of stroma was reconstructed 120 days after TE-HCEP transplantation, which was similar to the normal control eye in morphology and structure. In contrast, intense corneal edema, turbid, invaded blood vessels were found in dAM eyes, and no multilayer epithelium was found but only a few scattered conjunctiva-like cells appeared.

• CONCLUSION: The TE-HCEP, with similar morphology and structure to those of innate HCEP, could reconstruct a multilayer corneal epithelium with normal functions in restoring corneal transparency and thickness of LSCD rabbits after transplantation. It may be a promising HCEP equivalent for clinical therapy of corneal epithelial disorders.

• KEYWORDS: tissue-engineered human corneal epithelium; limbal stem cell deficiency rabbit; lamellar keratoplasty; human corneal epithelial cells; denuded amniotic membrane; reconstruction

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INTRODUCTION

Human corneal epithelium (HCEP), a multi-cellular layer located at the anterior surface of cornea, is the first defence barrier of cornea and crucial for maintaining corneal transparency and absorbing oxygen/nutrients [1]. Human corneal epithelial cells are in dynamic equilibrium state that is maintained by a population of unipotent limbal epithelial stem cells (LESCs) [2,3]. Severe damage in the limbal region, by chemical burns, thermal burns, multiple surgical procedures, microbial infection and so on, may lead

to limbal stem cell deficiency (LSCD) with characteristics of neovascularization, poor epithelialization, conjuncticalization and corneal opacity [4,5].

Autograft LESC transplantation has been used as an ideal therapy method for LCSD patients [6]. Recently, the improved transplantation of *in vitro* cultured limbal tissue autografts on amniotic membrane (AM) for successful clinical implementation of LSCD has been reported [7,8]. The disadvantages of autologous transplantation is its feasibility restricted only to patients with unilateral LSCD, while surgical trauma to the other healthy eye remains a great concern to most of LSCD patients. Then an alternative approach is the transplantation of cultivated allogeneic (allogeneic) LESCs [9,10]. But a new problem is that *in vitro* culture of LESCs is very difficult and time-consuming because of the rapidly differentiating nature of the cells, and the availability of donor corneal tissue for their preparation is limited [11]. Therefore, untransfected HCEP cell lines can provide a readily available source of HCEP cells for long-term studies on development of new therapies for corneal epithelial disorders [12-14].

AM, with properties of facilitating epithelialization, anti-angiogenic activity and low or no immunogenicity, has been considered as an extremely useful biomaterial for ophthalmologcial surgery [15,16]. Recently, HCEP cells from an utHCEPC01 cell line, together with epithelium-denuded AM (dAM), were successfully utilized in reconstruction of tissue-engineered human corneal epithelium (TE-HCEP) which had almost the same morphology and structure to HCEP *in vivo* [17]. But there is no report on TE-HCEP transplantation in LSCD rabbit models. In this paper, we first report the transplantation of TE-HCEP, reconstructed with utHCEPC01 cells and dAM, into LSCD rabbit models by lamellar keratoplasty (LKP) to evaluate its biological functions.

MATERIALS AND METHODS

Materials New Zealand white rabbits without eye diseases, weighing 2.0kg-2.5kg, were purchased from Lukang experimental animal center (Jining, Shandong, China). Untransfected HCEP cells, from the previously established utHCEPC01 cell line [13], were cultured in 10% fetal bovine serum (FBS) (HyClone, Logan, Utah)-Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12, 1:1) medium (pH 7.2) (Invitrogen, Carlsbad, CA). Fresh AMs were obtained from Shandong Eye Institute of Shandong Medical Academy, Qingdao, China, and denuded to obtain dAMs according to Fan *et al* [18].

Methods

***In vitro* reconstruction of TE-HCEP** The passage 80 HCEP cells at logarithmic phase were collected using 0.25% trypsin (Sigma-Aldrich) with the density of cell suspension adjusted to 1.0×10^7/mL with 10% FBS (HyClone)-DMEM/F12 medium (pH 7.2) (Invitrogen). Into each of a dAM-paved culture insert in a 6-well plate, 500μL cell suspension was added and cultured at the same conditions as described above for 12 hours. Then 0.8mL 10% FBS-DMEM/F12 medium (pH 7.2) was added and air-liquid interface cultured for 5 days. The morphology and growth status of the cells were monitored daily and the medium was refreshed daily.

Characterization of reconstructed TE-HCEP The surface morphology of reconstructed TE-HCEP was examined with an Eclipse TS100 inverted microscope (Nikon, Tokyo, Japan) and a JSM2840 scanning electron microscope (SEM) (JEOL, Tokyo, Japan). The histology of reconstructed HCEP was examined with paraffin section and hematoxylin-eosin (HE) staining. The multilayer structure of reconstructed TE-HCEP and its dAM attachment status were examined with a H700 transmission electron microscope (TEM) (Hitachi, Tokyo, Japan). The cellular keratin 3 expression of TE-HCEP was examined with mouse anti-human keratin 3 monoclonal antibodies (Santa Cruz Biotechnology) and FITC-conjugated goat anti-mouse IgG antibody (Biosynthesis Biotechnology) in freeze sections, and observed under a Ti-S fluorescent microscope (Nikon).

Establishment of LSCD rabbit models The left eyes of 6 rabbits were used for LSCD traumatic surgery, and all the right eyes were used as normal control. After each rabbit was anesthetized by intramuscular injection, corneal limbus of its left eye was removed circularly, ranged from 2mm inner to 2mm outer, 100μm-150μm in depth. After covered by 75% alcohol sponge for 90 seconds, the center of the corneal epithelium was shaved by scalpel. The post-surgical eye was treated with 0.3% levofloxacin drops, tobramycin and dexamethasone ointment(Alcon-Couvreue,Puurs, Belgium) 3 times a day, and its corneal opacity, neovascularization, epithelial integrality was monitored once a week by a slit-lamp corneal microscope (Nikon, Tokyo, Japan), epithelial fluorescence staining and cyto-imprinting by periodic acid-Schiff (PAS) staining during a period of 4 weeks.

TE-HCEP transplantation into LSCD rabbit eyes The LSCD rabbits were divided into 2 groups randomly, 3 rabbits in each group. The left eyes (LSCD eyes) of one group were transplanted with TE-HCEP and those of the other group were transplanted with dAM, all the right eyes were used as normal controls. Each of LSCD rabbits was anesthetized by intramuscular injection with 1% tetracaine hydrochloride before LKP surgery. After removed the conjunctiva and exposed the sclera, the pathological tissues and hyperplasia on the surface of the cornea were cut off into a smooth planting bed. Then a reconstructed TE-HCEP or dAM sheet, washed by phosphate-buffered saline (PBS, pH7.4), was placed onto the surgical site with HCEP cells faced inwards. The implants were firstly fixed on sclera with

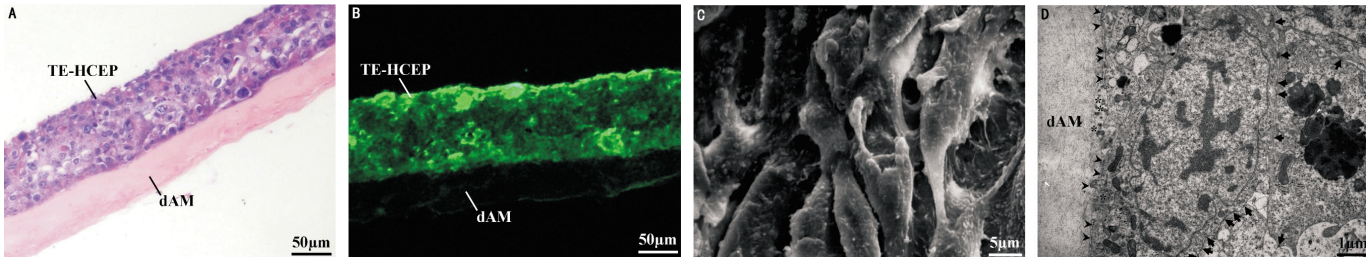

Figure 1 TE-HCEP reconstructed *in vitro* at day 5 A: HE staining; B: Keratin 3 expression; C: SEM; D: TEM.

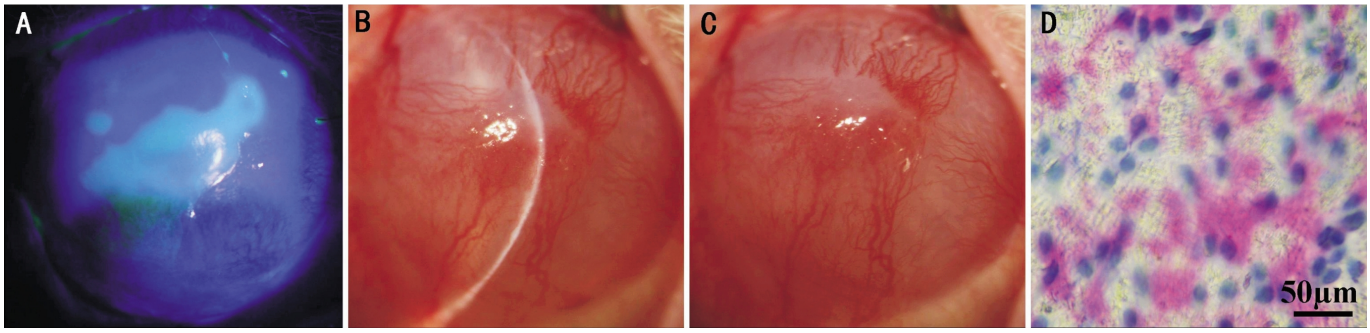

Figure 2 Photographs of traumatic LSCD rabbit eyes A: Fluorescence staining, day 7; B: slit-lamp photos, day 28; C: slit-lamp based external eye image, day 28; D: PAS staining, day 28.

10-0 nylon suturing in four corners, and sutured in another eight positions equably. Then excessive parts of nylon thrums were cut off. Post-surgical treatment included 0.3% levofloxacin drops, tobramycin and dexamethasone ointment given three times daily.

Evaluation of transplantation efficiency One week post LKP surgery, dAMs were removed from TE-HCEP transplanted corneas, and corneal edema, transparency, in-growth of blood vessels, thickness and epithelial integrality of transplanted rabbits were monitored about once a week by a slit-lamp corneal microscope (Kangjie Medical Instrument Ltd., Suzhou, China), a corneal pachymeter (Tomey, Tokyo, Japan), and fluorescence staining, respectively. At day 120 postoperative, the rabbits in each group were sacrificed and their corneas were detected by DiI fluorescence, HE staining, keratin 3 immunohistochemical staining and TEM observation with the same methods as described above.

RESULTS

***In vitro* reconstruction of TE-HCEP** After air-liquid interface cultured for 5 days, the HCEP cells formed a 6-7 layers' epithelium-like structure with a continuous layer of flattened apical cells (Figure 1A). The cells of TE-HCEP expressed keratin 3 genes on dAM (Figure 1B), indicating that they reserved the inherent properties of HCEP cells. And the cells were cobblestone in shape and rich in microvilli on apical surface (Figure 1C). The cells constructed intercellular junctions including desmosomes (arrows) and cell-dAM junctions (arrow heads), and those, adjacent to dAM, secreted a lot of vesicles (*) on the boundary of cell and dAM (Figure 1D).

Establishment of LSCD pathological model One week after traumatic injury, brush-like capillary blood vessels grew across the limbus into the cornea. And corneal epithelial surface was not intact by fluorescence staining (Figure 2A). After 4 weeks, the whole surface of cornea was covered with blood vessels, and the cornea was entirely opaque (Figure 2B, C). Positive PAS staining indicated that goblet cells intruded into the cornea (Figure 2D). The rabbit eyes with typical LSCD characteristics were used for TE-HCEP transplantation.

Transparency and thickness of transplanted corneas Corneal edema was not found in TE-HCEP transplanted LSCD rabbit eyes, and the corneas became more and more transparent with only a little vascular ingrowth throughout the monitoring period (Figure 3). In contrast, intense corneal edema developed in dAM-transplanted rabbit corneas which were turbid. And abundant vascular ingrowth was found throughout the monitoring period (Figure 4).

The corneal thickness of TE-HCEP transplanted LSCD rabbit eyes decreased significantly at day 12 and returned to normal thickness at day 25 post surgery, while that of dAM-transplanted eyes was much thicker throughout the monitoring period with a thickness of 575μm even at day 120 postoperative (Figure 5).

Histological structures of transplanted corneas On day 120 after surgery, the rabbits were scarificed and their corneal histological structures were examined. It was found that the cells in the transplanted areas of corneal epithelia of TE-HCEP transplanted eyes emitted DiI fluorescence (Figure 6A), while those of corneal epithelia of dAM-transplanted eyes did not (Figure 6E). And a 4-5

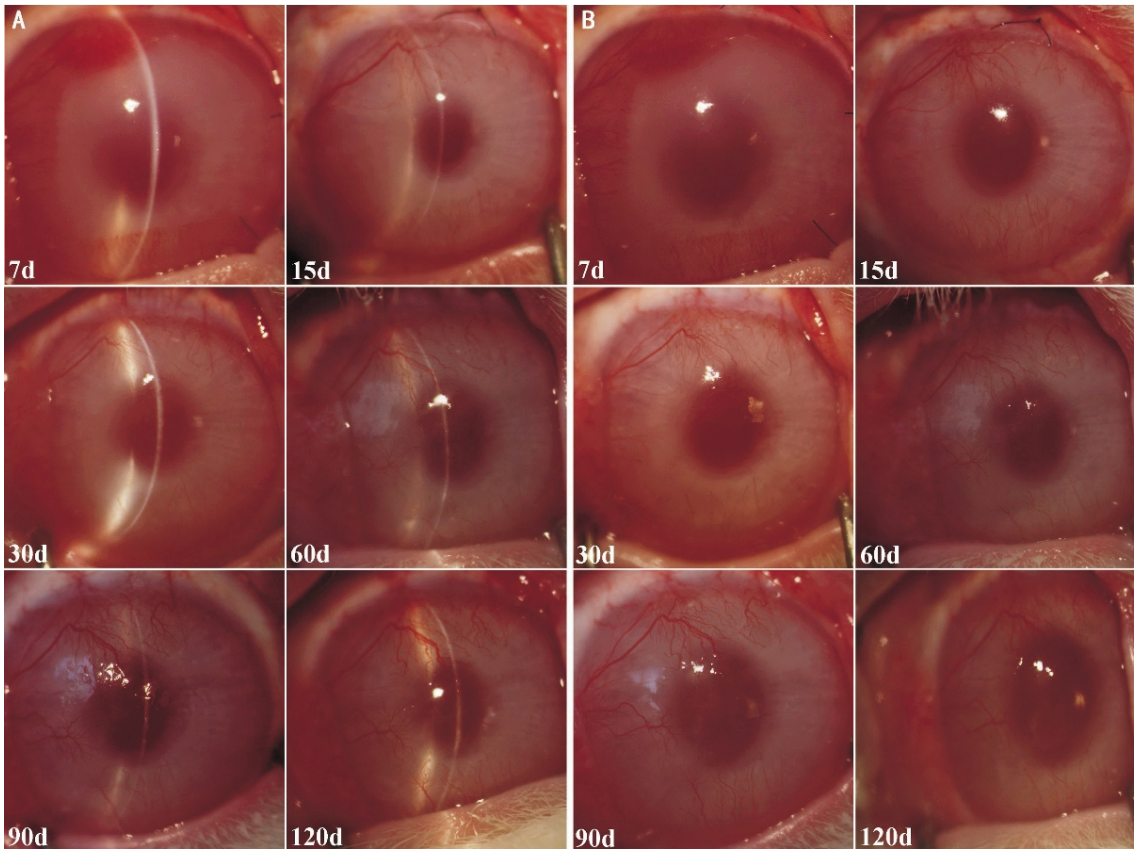

Figure 3 Photographs of TE-HCEP transplanted rabbit corneas A: slit-lamp micrograph; B: slit-lamp based external eye image.

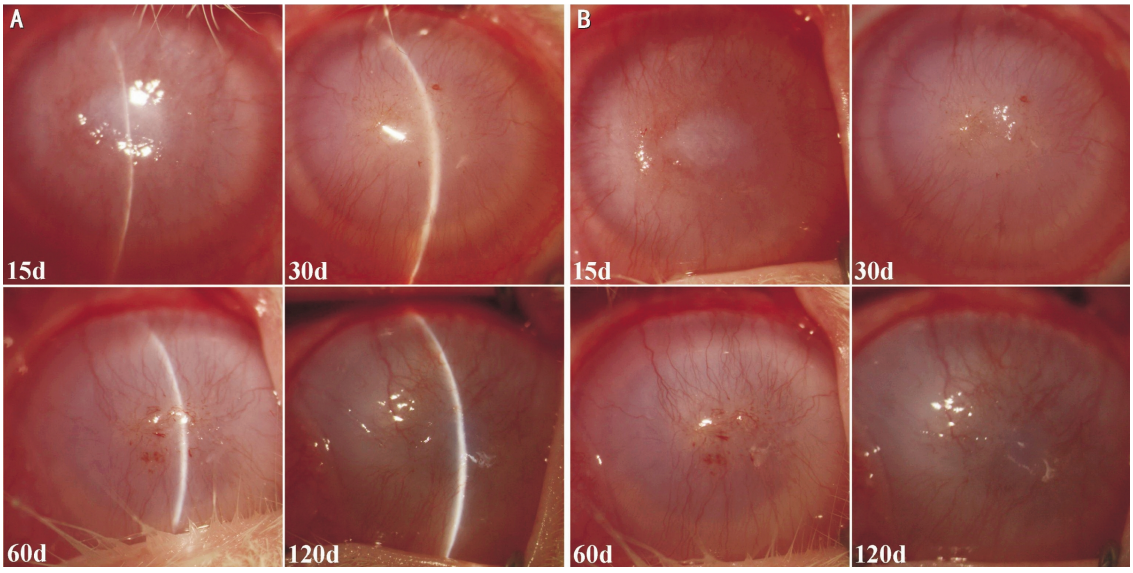

Figure 4 Photographs of dAM-transplanted rabbit corneas A: slit-lamp micrograph; B: slit lamp based external eye image.

layers' epithelium-like structure was reconstructed by the cells, maintaining positive expression of keratin 3, in the TE-HCEP transplanted eyes (Figure 6B, C), while no multilayer epithelium was found in the dAM-transplanted eyes but only a few scattered conjunctiva-like cells appeared with no DiI fluorescence (Figure 6F,G). Under TEM, the HCEP cells in the TE-HCEP transplanted areas of corneal epithelium had normal ultrastructure and excellent epithelial integrality with numerous intercellular cell junctions including desmosomes (arrow) established (Figure 6D), while those in dAM-transplanted areas of corneal epithelium

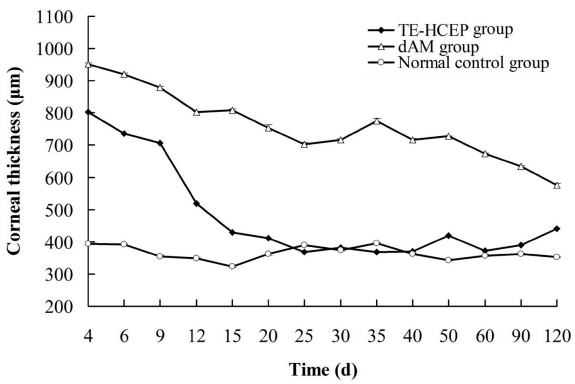

Figure 5 Corneal thickness after transplantation.

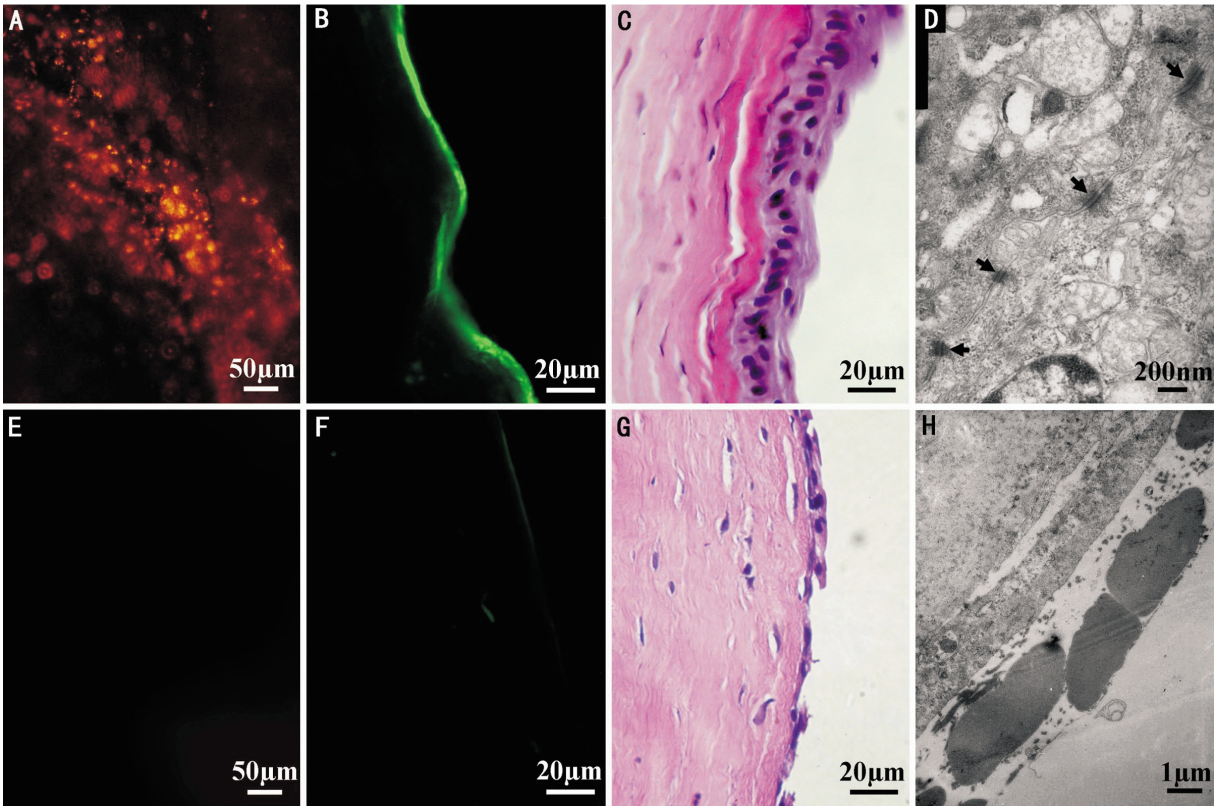

Figure 6 Corneas from TE-HCEP (A-D) and dAM (E-H) transplanted LSCD rabbits at day 120 A, E: DiI fluorescence; B, F: keratin 3 expression; C, G: HE staining; D, H: TEM.

had only a few scattered conjunctiva-like cells, exhibiting poor epithelial integrality (Figure 6H).

DISCUSSION

HCEP cell lines as a useful tool provides a readily available source of HCEP cells for *in vitro* reconstruction of TE-HCEP [14,17]. Due to the availability of untransfected HCEP cells and a novel TE-HCEP was successfully reconstructed in our laboratory [13,17], TE-HCEP, reconstructed with DiI-labeled untransfected HCEP cells and dAM in air-liquid interface culture, were transplanted into LSCD rabbit models by LKP and its biological function were evaluated in this study.

The HCEP cells, from untransfected utHCEPC01 cell line, were used in TE-HCEP reconstruction, and they formed a 6-7 layers' epithelium-like structure with a confluent layer of flattened apical cells rich in microvilli on dAM after air-liquid interface cultured for 5 days. HCEP cells constructed numerous intercellular junctions and maintained positive keratin 3 expression. All these indicate that the reconstructed TE-HCEP, with good integrality, has almost the same morphology and structure as that of innate HCEP. These characteristics of reconstructed TE-HCEP were similar to those reported previously [17].

The LSCD eye, established by alkaline burning [19-21], mechanical removing [22] or combined method [23,24], is a basic pathological model for evaluating biological functions of TE-HCEP *via* corneal epithelium transplantation [25]. Since the alkaline burning and combined method often result in unpredictable subsequent lesions and are difficult to control, we used modified mechanical removing method to establish LSCD rabbit models in this study. The LSCD rabbit models with entirely corneal opaque, conjunctivalization and neovascularization were established. And continuous succedent observation also indicated that the limbal stem cells of the LSCD rabbit corneas were irreversibly destroyed, losing its compensative capacity, and it is difficult to re-epithelize and leading to persistent corneal epithelial defects. Thus it is rational to believe that the established LSCD rabbit models were suitable for TE-HCEP transplantation.

Transplantation of reconstructed TE-HCEP is valuable in long-term studies on understanding the development of new therapies for corneal diseases and damages [14,17]. With successfully established LSCD rabbit models, the reconstructed TE-HCEP was transplanted into LSCD rabbit eyes by LKP in the present study. By 120 days after transplantation, no obvious edema was found, and the TE-HCEP transplanted cornea, with only a little vascular ingrowth, became more and more transparent, while dAM-transplanted cornea maintained edema, opacity and neovascularization throughout the monitoring period. The thickness of TE-HCEP transplanted cornea decreased with time and returned to normal at day 25, while the thickness dAM-transplanted cornea, even decreased with time, was

much thicker than that of TE-HCEP transplanted cornea. The thickness decreasing of dAM-transplanted cornea might be due to the digestion and absorption of dAM with time. On day 120 after TE-HCEP transplantation, a 4-5 layer of epithelium-like structure was reconstructed from HCEP cells with DiI label. The cells, maintaining keratin 3 expression, formed numerous intercellular cell junctions including desmosomes. In contrast, the corneal surface of the dAM-transplanted LSCD eye only developed a monolayer epithelium, which is highly conceivable as a result of conjunctival epithelial invasion. These results indicate that the TE-HCEP exhibits normal biological function after transplanted into LSCD rabbit models, probably as a result of constant re-epithelization, which may represent transient amplifying HCEP cells[26].

In conclusion, the *in vitro* reconstructed TE-HCEP, from untransfected HCEP cells and dAM, has similar structures and functions to those of rabbit corneal epithelia, and can reconstruct *in vivo* a corneal epithelium-like structure and maintain long-term corneal transparency and thickness of LSCD rabbits. The reconstructed TE-HCEP can be used promisingly as an HCEP equivalent for clinical therapy of corneal epithelial disorders.

REFERENCES

1 Kinoshita S, Adachi W, Sotozono C, Nishida K, Yokoi N, Quantock AJ, Okubo K. Characteristics of the Human Ocular Surface Epithelium. *Prog Retin Eye Res* 2001;20(5):639-673

2 Dua HS, Shanmuganathan VA, Powell-Richards AO, Tighe PJ, Joseph A. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *Br J Ophthalmol* 2005;89(5):529-532

3 Stepp MA, Zieske JD. The corneal epithelial stem cell niche. *Ocul Surf* 2005;3(1):15-26

4 Tseng SCG, Chen JJY, Huang AJW, Kruse FE, Maskin SL, Tsai RJF. Classification of conjunctival surgeries for corneal diseases based on stem cell concept. *Ophthalmic Clin North Am* 1990;3:595-610

5 Tseng SC. Regulation and clinical implications of corneal epithelial stem cells. *Mol Biol Rep* 1996;23(1):47-58

6 Kenyon KR. Limbal autograft transplantation for chemical and thermal burns. *Dev Ophthalmol* 1989;18:53-58

7 Sangwan VS, Matalia HP, Vemuganti GK, Fatima A, Ifthekar G, Singh S, Nutheti R, Rao GN. Clinical outcome of autologous cultivated limbal epithelium transplantation. *Indian J Ophthalmol* 2006;54(1):29-34

8 Kolli S, Ahmad S, Lako M, Figueiredo F. Successful clinical implementation of corneal epithelial stem cell therapy for treatment of unilateral limbal stem cell deficiency. *Stem Cells* 2010;28(3):597-610

9 Nakamura K, Sotozono C, Bentley AJ, Mano S, Inatomi T, Koizumi N, Fullwood NJ, Kinoshita S. Long-term phenotypic study after allogeneic cultivated corneal limbal epithelial transplantation for severe ocular surface diseases. *Ophthalmology* 2010;117(12):2247-2254

10 Chew HF. Limbal stem cell disease: treatment and advances in technology. *Saudi J Ophthalmol* 2011;25:213-218

11 Castro-Muñozledo F. Corneal epithelial cell cultures as a tool for research, drug screening and testing. *Exp Eye Res* 2008; 86(3):459-469

12 Liu J, Song G, Wang Z, Huang B, Gao Q, Liu B, Xu Y, Liang X, Ma P, Gao N, Ge J. Establishment of a corneal epithelial cell line spontaneously derived from human limbal cells. *Exp Eye Res* 2007;84(3):599-609

13 Fan TJ, Xu B, Zhao J, Yang Hs, Wang RX, Hu XZ. Establishment of an untransfected human corneal epithelial cell line and its biocompatibility with denuded amniotic membrane. *Int J Ophthalmol* 2011;4(3):228-234

14 Fan TJ, Yang HS, Hu XZ, Zhao J. Research advances on in vitro reconstruction of tissue-engineered human corneal epithelium. *Shandong Daxue Xuebao* 2010;48(7):7-13

15 Dua HS, Gomes JA, King AJ, Maharajan VS. The amniotic membrane in ophthalmology. *Surv Ophthalmol* 2004;49(1):51-77

16 Gomes JA, Romano A, Santos MS, Dua HS. Amniotic membrane use in ophthalmology. *Curr Opin Ophthalmol* 2005;16(4):233-240

17 Xu B, Fan TJ, Yang HS, Sun A, Zhao J, Ma XY, Hu XZ. In vitro reconstruction and characterization of tissue-engineered human corneal epithelium with seeder cells from an untransfected human corneal epithelial cell line. *Int J Ophthalmol* 2012;5(3):281-285

18 Fan TJ, Zhao J, Wang J, Cong RS, Yang XX, Shi WY, Wang YQ. In vitro reconstruction of tissue-engineered human corneal endothelium and characterization of its morphology and structure. *Int J Ophthalmol (Guoji Yanke Zazhi)* 2010;10(2):225-228

19 Ormerod LD, Abelson MB, Kenyon KR. Standard models of corneal injury using alkali- immersed filter discs. *Invest Ophthalmol Vis Sci* 1989; 30(10):2148-2153

20 Liu HJ, Zhong SZ, Zeng J, Gong XM, Ge J. Autologous limbal autograft for alkali-burned corneas in rabbit:an experimental study. *J First Mil Med Univ* 2003;23(4):344-346

21 Pi YL, Tang WQ, Lu JY, Ying D. Tissue-engineered corneal epithelium transplantation for treatment of corneal alkaline burn in rabbits. *Zhongguo Zuzhi Gongcheng Yanjiu Yu Linchuang Kangfu* 2009;13 (41): 8011-8016

22 Liu XN, Wu J, Zhu XP. Research of corneal epithelial stem cell deficiency anmial models. *Int J Ophthalmol(Guoji Yanke Zazhi)* 2009;9(8): 1583-1584

23 Du Y, Chen J, Funderburgh JL, Zhu X, Li L. Functional reconstruction of rabbit corneal epithelium by human limbal cells cultured on amniotic membrane. *Mol Vis* 2003;9:635-643

24 Ti SE, Anderson D, Toubami A, Kim Charles, Tseng SCG. Factors affecting outcome following transplantation of ex vivo expanded limbal epithelium on amniotic membrane for total limbal deficiency in rabbits. *IOVS* 2002;43(8):2584-2592

25 Dua HS, Joseph A, Shanmuganathan VA, Jones RE. Stem cell differentiation and the effects of deficiency. *Eye* 2003;17(8):877-885

26 Gimeno FL, Lavigne V, Gatto S, Croxatto JO, Correa L, Gallo JE. One-year follow-up of epithelial corneal cell sheet allografts mounted on platelet poor plasma in rabbits. *Mol Vis* 2009;15:2771-2779