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Therapeutic efficiency of tissue-engineered human corneal endothelium transplants on rabbit primary corneal endotheliopathy*

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Abstract: To evaluate the therapeutic efficiency of tissue-engineered human corneal endothelia (TE-HCEs) on rabbit primary corneal endotheliopathy (PCEP), TE-HCEs reconstructed with monoclonal human corneal endothelial cells (mcHCECs) and modified denuded amniotic membranes (mdAMs) were transplanted into PCEP models of New Zealand white rabbits using penetrating keratoplasty. The TE-HCEs were examined using diverse techniques including slit-lamp biomicroscopy observation and pachymeter and tonometer measurements in vivo, and fluorescent microscopy, alizarin red staining, paraffin sectioning, scanning and transmission electron microscopy observations in vitro. The corneas of transplanted eyes maintained transparency for as long as 200 d without obvious edema or immune rejection. The corneal thickness of transplanted eyes decreased gradually after transplanting, reaching almost the thickness of normal eyes after 156 d, while the TE-HCE non-transplanted eyes were turbid and showed obvious corneal edema. The polygonal corneal endothelial cells in the transplanted area originated from the TE-HCE transplant. An intact monolayer corneal endothelium had been reconstructed with the morphology, cell density and structure similar to those of normal rabbit corneal endothelium. In conclusion, the transplanted TE-HCE can reconstruct the integrality of corneal endothelium and restore corneal transparency and thickness in PCEP rabbits. The TE-HCE functions normally as an endothelial barrier and pump and promises to be an equivalent of HCE for clinical therapy of human PCEP.

Key words: Tissue-engineered human corneal endothelium, New Zealand white rabbits, Penetrating keratoplasty, Primary corneal endotheliopathy, Transparency

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1 Introduction

Human corneal endothelium (HCE), a single layer of flat hexagonal cells, bounds the anterior chamber supported by stroma. HCE is essential for maintaining human vision; it provides both nutrients and oxygen for the cornea, maintaining corneal transparency and thickness. The cells of the HCE (hereafter HCECs) lose the ability to proliferate after birth. Once HCECs are injured, the wound can heal only through the enlargement and migration of the adjacent cells. When the cell density is below a threshold for maintaining the physiological functions of HCE, primary corneal endotheliopathy (PCEP) occurs (Schierholter and Honegger, 1975). There are about 1 million PCEP suffers in China and 12 million globally, and the number is increasing continuously (Wei *et al*., 1993; Xie and Shi, 2007). At present, the only way of curing PCEP is by cornea transplantation (Capella, 1971). Unfortunately, the availability of

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healthy donor corneas is extremely limited (Liu and Zhang, 2006; Xie and Shi, 2007). Keratoplasty of tissue-engineered human corneal endothelium (TE-HCE) offers an alternative approach for recovering the lost vision of PCEP suffers (Liu and Zhang, 2006).

In vitro construction of TE-HCEs was first attempted by Raphael *et al*. (1992). Diverse types of cells and various scaffold carriers have been combined previously and a therapeutic efficiency of about one week of maintenance of corneal transparency has been attained in rabbit (Aboalchamat *et al*., 1999; Ishino *et al*., 2004; Koizumi *et al*., 2008; Fan *et al*., 2009b; Proulx *et al*., 2009). Unfortunately, all the previous trials fell well short of a clinical therapy for PCEP; the immortalized seed cells were either potentially tumorigenic or limited in number after primary culture or 4–5 rounds of subculture (Fan *et al*., 2007; 2009a). Since 2009, we have successfully constructed TE-HCEs using a non-transfected HCE cell line as the source of seed cells and modified denuded amniotic membranes (mdAMs) as the carrier frames. The TE-HCE, normal in morphology and structure, maintained the corneal transparency of rabbits for more than 39 d after transplantation (Fan *et al*., 2009b; 2010a). A new type of TE-HCE has also been constructed using monoclonal HCECs (mcHCEC) and mdAMs, which maintained the corneal transparency of rabbits for more than 100 d after transplantation (Fan *et al*., 2010b).

Direct penetrating keratoplasty (PKP) has been tried, in which the intrinsic corneal endothelium and Descemet's membrane of rabbits were stripped off and then the TE-HCE was transplanted. Such a trial has not been reported for PCEP rabbits. To evaluate the therapeutic efficiency of TE-HCEs on PCEP rabbits, PKP with previously constructed TE-HCEs (Fan *et al*., 2010b) was attempted in PCEP rabbits and its therapeutic efficiency was evaluated both in vivo and in vitro.

2 Materials and methods

2.1 Materials

Corneas from a woman (26 years old) who died from a cerebral hemorrhage were provided, with permission of her next of kin, by the Affiliated Hospital of Medical College, Qingdao University, and used to establish an HCEC line. This usage was approved by the Medical Ethics Committee of the hospital, and the privacy of the patient was protected in compliance with the Helsinki Declaration. Healthy New Zealand white rabbits without eye diseases (about 2.0–2.5 kg in body weight) were obtained from the Animal Laboratory of the Shandong Eye Institute of Shandong Medical Academy (Qingdao, China). All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for use of animals in ophthalmic and vision researches. The C3B mcHCEC line (2*n*=46) was cloned from a non-transfected HCE cell line established previously in our laboratory. mdAMs were prepared from fresh amniotic membranes by denuding epithelium and then coating proteins. TE-HCEs were constructed using DiI-labeled mcHCECs and mdAMs in 20% fetal bovine serum-Dulbecco's modified Eagle medium/nutrient mixture F-12 (1:1, v:v) (FBS-DMEM/F12) medium at 37 °C in a 5% CO_2 incubator as described previously (Fan *et al*., 2010a).

2.2 Preparation of PCEP rabbit models

After anaesthetization with ether, the corneal endothelia of the right eyes of 9 New Zealand white rabbits were scraped away with metal scrapers, yielding PCEP rabbit models. The edema and turbidity of the scraped corneas were monitored daily. The left eyes of the rabbit testers were left untouched. Once the endothelium-scraped corneas became opaque, the right eyes were used as PCEP models for TE-HCE transplantation.

2.3 TE-HCE transplantation

PCEP rabbits were divided into three groups each comprising three rabbits. The right eyes of Group 1 were transplanted with TE-HCEs (TE-HCE eyes), those of Group 2 were used for only PKP with nothing transplanted (PKP eyes) and those of Group 3 were left non-transplanted (PCEP eyes). All left eyes were used as the normal controls (control eyes). TE-HCEs were transplanted with their mdAMs touching the corneal stroma. PKP was carried out using the methods described previously (Fan *et al*., 2010b).

2.4 In vivo examination of rabbit corneas

After transplantation, the exterior appearance,

corneal edema and immune rejection of rabbit eyes were monitored daily. Corneal transparency was examined with a KJ5D slit-lamp biomicroscope (Kangjie Medical Instrument Co., Ltd., Suzhou, China), corneal thickness was measured using an SW-1000P pachymeter (Souer Electronic Technology Co., Ltd., Tianjin, China), and eye pressure was measured with a TONO-PEN AVIA® applanation tonometer (Reichert Inc., USA) at intervals of 3–7 d.

2.5 Examination of transplanted corneas

Two hundreds days after transplantation, rabbits were euthanized with ether and their corneas were sampled. After being rinsed twice with phosphate-buffered saline (PBS), each cornea was cut into four pieces, equal in area. The first piece was stained with 1% alizarin red and examined using a Nikon E200 microscope. It was then examined for DiI-label of HCECs using a Nikon TE2000-U fluorescent microscope; the second piece was used to make paraffin sections stained with hematoxylin-eosin (HE) for observation under a Nikon E200 microscope; the third piece was used for observation with a JSM-840 scanning electron microscope (SEM; JEOL, Tokyo, Japan), and the last piece was used for observation with an H700 transmission electron microscope (TEM; Hitachi, Tokyo, Japan). The number of corneal endothelial cells was counted using eyepiece graticules with the cell density calculated and averaged based on five random graticules.

2.6 Statistical analysis

The observation values were described as mean± standard error (SE) from triplicate samples with the statistical significance calculated with analysis of variance (ANOVA; single factor). A probability (P) of <0.05 was accepted as the significance threshold level.

3 Results

3.1 Establishment of rabbit PCEP model

Obvious corneal edema and white turbidity of scraped eyes appeared in the three groups of PCEP rabbits once the corneal endothelium was scraped away. The eyes became opaque after 3 d, appearing as identical white porcelains (Fig. 1 in p. 496). This indicated that PCEP models of rabbits had been established successfully, in readiness for TE-HCE transplantation.

3.2 Appearance of transplanted rabbit eyes

No obvious signs of corneal edema or immune rejection were found in the TE-HCE eyes or PKP eyes (Fig. 2), indicating that PKP and transplantation of TE-HCE had not caused severe corneal edema and immune rejection.

3.3 Corneal transparency

Corneas of the transplanted eyes became transparent 3 d after TE-HCE transplantation and the transparency increased with time, reaching normal levels after 30 d. Corneal transparency was maintained for 200 d prior to histopathological examination (Fig. 3a). In contrast, PKP eyes remained turbid and the white turbidity worsened with time (Fig. 3b). The turbidity of PCEP eyes without transplantation was almost the same as that of PKP eyes (data not shown).

3.4 Corneal thickness and eye pressure

The corneas of PCEP, PKP, and TE-HCE eyes were thicker than those of the control eyes during the early stages following transplantation. From Day 15, corneas of TE-HCE eyes became thinner and thinner with time, reaching the thickness of those of the control eyes 156 d after transplantation (Fig. 4a). In contrast, the corneal thickness of PCEP eyes remained almost unchanged, being 2.41-fold thicker than that of the control eyes. The corneal thickness of PCEP eyes was almost the same as that of PKP eyes (data not shown). The corneal thickness of TE-HCE eyes was significantly different from that of either PKP eyes or PCEP eyes (P<0.01).

The eye pressures of TE-HCE, PKP, and control eyes fluctuated with time (Fig. 4b). There was no obvious relationship between the eye pressures of TE-HCE, PKP, and control eyes.

3.5 Origin of corneal endothelial cells in transplants

The corneal endothelial cells in the transplanted area did not originate from autogenous proliferation; those in the transplanted areas of TE-HCE eyes emitted DiI fluorescence while those of PKP and control eyes did not, as observed 200 d after transplantation (Fig. 5).

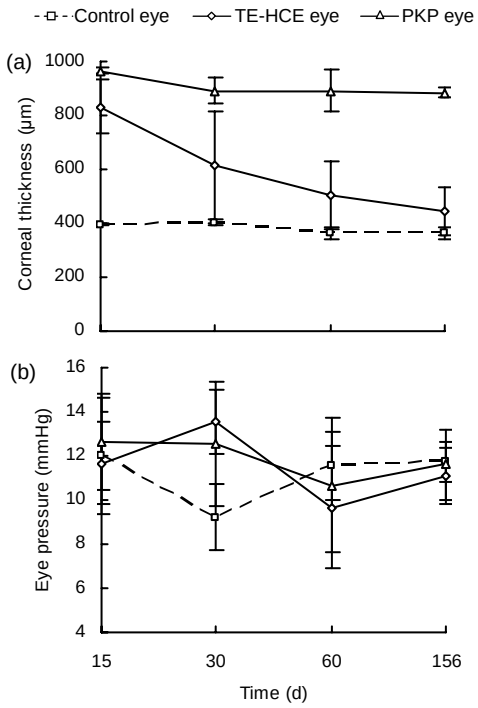


Fig. 4 Changes in the corneal thickness (a) and the eye pressure (b) of transplanted rabbit eyes

3.6 Cell junctions established by corneal endothelial cells in transplanted eyes

Abundant intercellular junctions were established and intact corneal endothelia were reconstructed in TE-HCE eyes. The corneal endothelial cells were either hexagonal or polygonal in shape (Fig. 6). The average cell density of the corneal endothelia of TE-HCE eyes was (1913.33±94.52) cells/mm^2, similar to that of the control eyes [(2173.33±61.10) cells/mm^2]. The corneal endothelium reconstructed from TE-HCE transplants was almost the same as the control in terms of both morphology and cell density.

3.7 Histopathological examination of corneal endothelium

The corneal endothelial cells formed a compact confluent monolayer on the endothelial surfaces of the corneas of TE-HCE eyes after 200 d (Fig. 7). The HCE cells of TE-HCE eyes were either hexagonal or polygonal in morphology, almost identical to those of the control eyes. The histological structure and thickness of the corneas of TE-HCE eyes were almost the same as those of the control eyes.

3.8 TEM examination of corneal endothelium

The corneal endothelial cells of TE-HCE eyes formed a continuous monolayer endothelium and the cells attached tightly to the Descemet's membrane in vivo (Fig. 8). The ultra-structure of the cells was almost identical to that of the control eyes, containing plenty of mitochondria and rough endoplasmic reticulum.

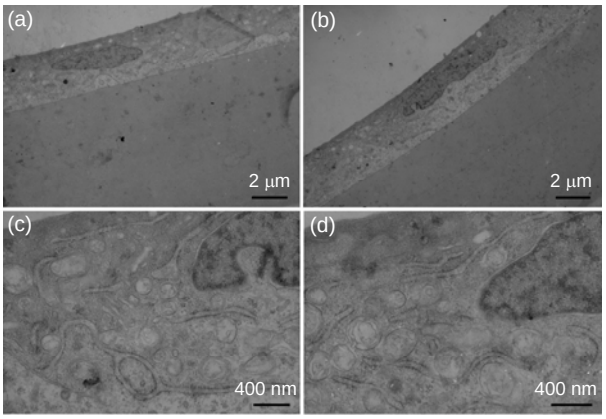
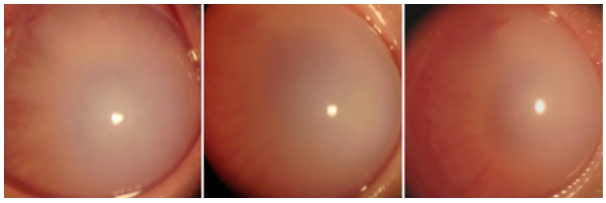


Fig. 8 TEM structures of the rabbit corneas
(a) Cornea of control eye; (b) Cornea of TE-HCE eye; (c) Corneal endothelium of control eye; (d) Corneal endothelium of TE-HCE eye

4 Discussion

To validate the therapeutic efficiency of reconstructed TE-HCE on PCEP rabbits, PKP surgery was performed with the TE-HCE and its therapeutic efficiency was evaluated both in vivo and in vitro in this study. After PKP surgery on successfully established PCEP rabbit models, no obvious signs of corneal edema or immune rejection were found in TE-HCE and PKP eyes, indicating that PKP surgery and transplantation of TE-HCE had not caused severe corneal edema and immune rejection. Similar results were reported previously in TE-HCE transplantation (Fan *et al*., 2009b; 2010b).

TE-HCE transplants were capable of recovering the corneal transparency of PCEP rabbits, maintaining the transparency of corneas for longer than previously reported (Aboalchamat *et al*., 1999; Ishino *et al*., 2004; Fu *et al*., 2006; Koizumi *et al*., 2008; Fan *et al*., 2009b; 2010b; Proulx *et al*., 2009). The corneas of



(a) (b) (c)

Fig. 1 Rabbit PCEP eye model established by scraping away the endothelium
(a) Group 1; (b) Group 2; (c) Group 3

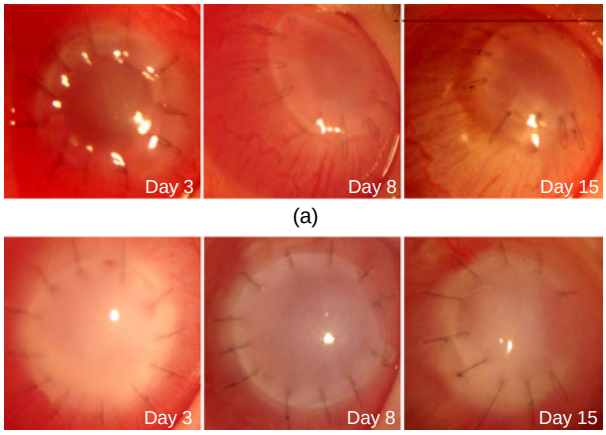


Fig. 2 Rabbit eyes after TE-HCE transplantation (a) and PKP (b)

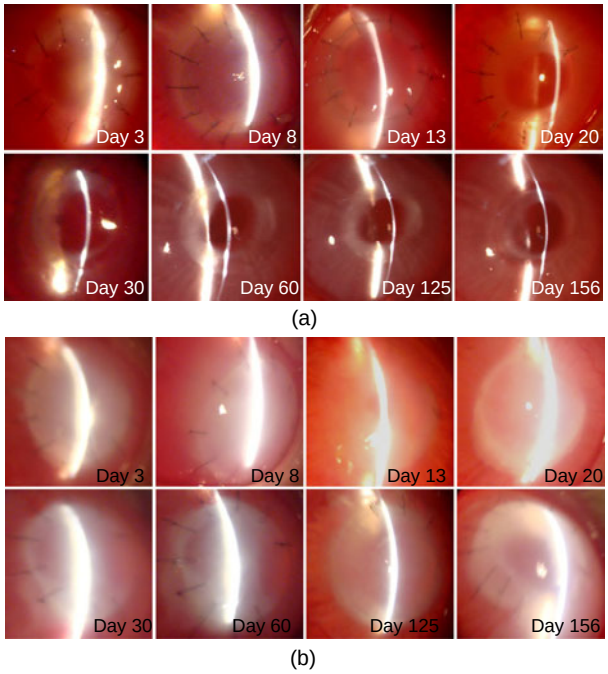


Fig. 3 Rabbit corneas under the slit-lamp biomicroscope after TE-HCE transplantation (a) and PKP (b)

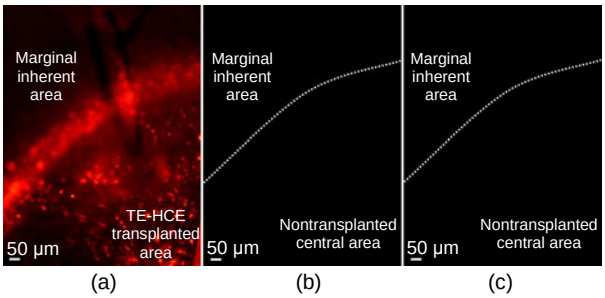


Fig. 5 Fluorescent images of the corneal endothelia of rabbit eyes
(a) TE-HCE eye; (b) PKP eye; (c) PCEP eye

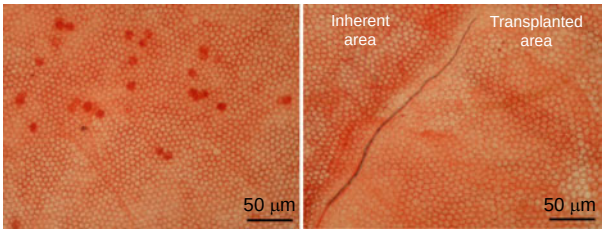


Fig. 6 Alizarin red staining pattern of corneal endothelia of rabbit eyes
(a) Control eye; (b) TE-HCE eye

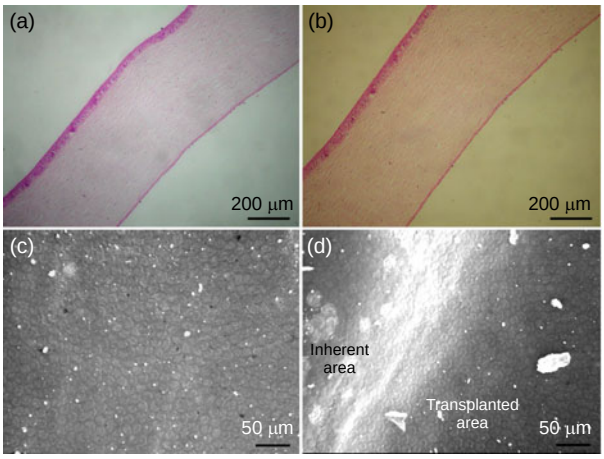


Fig. 7 Histological structures of rabbit corneas
(a) Cornea of control eye, HE staining; (b) Cornea of TE-HCE eye, HE staining; (c) Corneal endothelium of control eye, SEM; (d) Corneal endothelium of TE-HCE eye, SEM

TE-HCE eyes became thinner and thinner with the time, while the corneas of PCEP and PKP eyes remained almost unchanged in thickness. Corneal transparency and thickness are important for verifying whether the transplanted TE-HCE can function as a normal endothelial barrier and pump (Davies *et al*., 1976; Macdonald *et al*., 1987; Mergler and Pleyer,

2007). TE-HCE transplants can restore the corneal thickness of PCEP eyes after transplantation. In combination with the recovery of corneal transparency, it can be concluded that the TE-HCE functions normally as the HCE barrier and pump after transplantation. TE-HCE transplants should provide an effective treatment for PCEP. This finding is in accordance with previous reports (Ishino *et al*., 2004; Fan *et al*., 2009b; 2010b).

The eye pressures of TE-HCE, PKP, and control eyes fluctuated from time to time. Therefore, it is not appropriate for evaluating the efficiency of TE-HCE transplantation. This conclusion is in accordance with the report that eye pressure is not stable, changing with age, corneal thickness, and corneal diopter, and between individuals (Wang *et al*., 2008).

Rabbits are unusual in that their corneal endothelial cells remain proliferative throughout their lives (Mimura *et al*., 2005). Determining the origin of corneal endothelial cells in transplants is crucial for verifying the involvement of transplanted TE-HCE in the reconstruction of corneal endothelium (Fan *et al*., 2010b). In the present study, DiI fluorescence observation showed that the corneal endothelial cells in the transplanted area originated from the transplanted TE-HCE, not from autogenous proliferation, while those in PKP and control eyes did not. This finding confirms those of previous studies (Fu *et al*., 2006; Fan *et al*., 2009b; 2010b).

Cell density and intercellular junctions have served as indicators of the integrality and barrier functions of the corneal endothelium (Schierholter and Honegger, 1975; Xie and Shi, 2007; Zhu *et al*., 2008). Alizarin red staining is usually used to show intercellular junctions among corneal endothelial cells (Spence and Peyman, 1976; Fan *et al*., 2010a). In the present study, the results of alizarin red staining showed that abundant intercellular junctions were established and intact corneal endothelia were reconstructed in TE-HCE transplanted corneas. The results of SEM observation showed that the corneal endothelial cells, in hexagonal or polygonal morphology, formed a compact confluent endothelium in TE-HCE eyes, almost identical to those of control eyes. We believe that the TE-HCE transplant is capable of reconstructing an integral corneal endothelium with almost the same morphology and cell density as control eyes, in accordance with previous reports (Doughty, 1998; Fan *et al*., 2009b).

The corneal endothelium is a monolayer of flattened and mitochondria-rich cells that lines the posterior surface of the cornea and faces the anterior chamber of the eye. The corneal endothelium governs fluid and solute transport across the posterior surface of the cornea and actively maintains the cornea in a slightly dehydrated state that is required for optical transparency (Schierholter and Honegger, 1975; Davies *et al*., 1976; Murphy *et al*., 1984). In present study, the results of hematoxylin-eosin staining of paraffin sections showed that a continuous monolayer had been formed on the endothelial surface of corneas in TE-HCE eyes, and the corneal histological structure and thickness of TE-HCE eyes were almost the same as those of control eyes. The results of TEM observation showed that the corneal endothelial cells from TE-HCE formed a continuous monolayer endothelium, the cells attached tightly to the Descemet's membrane, and the ultra-structure of the cells was almost identical to that of control eyes, containing plenty of mitochondria and rough endoplasmic reticula. We conclude that the transplanted TE-HCE can reconstruct a continuous monolayer endothelium, similar in morphology and structure to the native HCE. Similar findings were obtained in our previous report (Fan *et al*., 2009b).

5 Conclusions

TE-HCE transplanted onto PCEP rabbit models can reconstruct the integrality of the corneal endothelium. It restores the corneal transparency and thickness of PCEP rabbit eyes, and functions normally as the endothelial barrier and pump. The TE-HCE promises to serve as a native HCE equivalent for the clinical therapy of human PCEP.

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