Change of Recipient Corneal Endothelial Cells After Non-Descemet’s Stripping Automated Endothelial Keratoplasty in a Rabbit Model

Jen-Pin Sun,1-3 Fung-Rong Hu,1,4 Yan-Ming Chen,5 Hsiao-Sang Chu,1 and Wei-Li Chen1,4

1Department of Ophthalmology, National Taiwan University Hospital, Taipei, Taiwan
2Department of Ophthalmology, National Taiwan University Hospital Hsin-Chu Branch, Hsinchu, Taiwan
3Rayming Eye Clinic, New Taipei City, Taiwan
4Center of Corneal Tissue Engineering and Stem Cell Biology, National Taiwan University Hospital, Taipei, Taiwan
5Department of Ophthalmology, E-Da Hospital/I-Shou University, Kaohsiung, Taiwan

Correspondence: Wei-Li Chen, Department of Ophthalmology, National Taiwan University Hospital, 7 Chung-Shan South Road, Taipei, Taiwan; chenweili@ntu.edu.tw.

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PURPOSE. We used a rabbit model to evaluate the interface embedded between the recipient corneas and transplanted donor corneal discs after non-Descemet's stripping automated endothelial keratoplasty (nDSAEK).

METHODS. Unilateral DSAEK and nDSAEK surgeries were performed on New Zealand white rabbits. In vivo confocal microscopy was performed to show: the changes in corneal endothelial cells embedded between the recipient corneas and the transplanted donor corneal discs (CEEB); and the interface opacity by z profile. Immunohistochemistry were performed to evaluate the functional change of CEEB at post-nDSAEK 3 months. Transmission electron microscopy was performed to evaluate the morphology of CEEB after nDSAEK at post-nDSAEK 1, 3, and 6 months.

RESULTS. In vivo confocal microscopy showed a time-dependent decrease in the density of CEEB at postoperative 1, 2, or 3 months (P < 0.01). Interface opacity was higher in the nDSAEK group than the DSAEK group at all examination points, but the difference was statistically insignificant. At 3 months after surgery, the CEEB were negative for Na+/K+-ATPase staining. Staining with TUNEL showed apoptotic changes in some areas. During a 6-month observation, the CEEB showed a time-dependent thickening and loss of uniform thickness of cellular morphology. At 3 and 6 months post nDSAEK, extensions of the cellular processes into the donor graft stroma combined with intracellular vacuoles containing collagen-like materials were also found.

CONCLUSIONS. After non-Descemet's stripping automated endothelial keratoplasty, the CEEB showed decreased density, loss of pump function, apoptosis and changed morphology. However, the interface opacity was not significantly greater compared with DSAEK eyes.

Keywords: DSAEK, nDSAEK, corneal endothelial cells, in vivo confocal microscopy, transmission electron microscopy

Dysfunction of the corneal endothelial cells and subsequent corneal edema can be caused by various etiologies, such as pseudophakic bullous keratopathy, Fuchs’ endothelial dystrophy, corneal graft failure, viral corneal endothelitis and congenital diseases. For decades, penetrating keratoplasty was widely accepted as the main procedure to treat corneal endothelial dysfunction.1–3 This procedure is also the most successful solid organ transplantation with the lowest rejection rate. The drawbacks of penetrating keratoplasty include marked refractive errors (including myopia, hyperopia, and irregular astigmatism), the risk of expulsive hemorrhage during operation, suture-induced problems and possible wound disruption with trauma, even long after surgery.4–7 Over the past several years, new techniques replacing only the diseased corneal layers have become popular. Among these, Descemet’s stripping automated endothelial keratoplasty (DSAEK) provided a successful way to treat corneal endothelial dysfunction.8–13 As DSAEK became a popular replacement for penetrating keratoplasty, a modified DSAEK technique, termed non-nDSAEK, has been proposed.14–16 This technique eliminates the procedure of stripping the Descemet’s membrane, usually performed in DSAEK before implanting the posterior lamellar graft, and is particularly recommended for non-Fuchs’ type bullous keratopathy.14–17 The advantages of nDSAEK are the diminished risk of damaging the recipient posterior stroma during Descemet’s membrane stripping, and the shorter surgical time. However, it may increase the risk of corneal disc dislocation, as well as subclinical corneal abnormalities, as shown by in vivo confocal microscopy.15 Both DSAEK and nDSAEK have been proven quite effective in treating corneal endothelial dysfunction without guttata, with rapid visual recovery and minimal postoperative astigmatism.14–16

The sequential changes in the Descemet’s membrane and recipient corneal endothelial cells embedded between the donor and recipient corneal stroma (CEEB) in nDSAEK have
seldom been reported. Hatanaka et al.\textsuperscript{17} used a rabbit model to demonstrate that 2 weeks after nDSAEK, the CEEB were positive for Na\textsuperscript{+}/K\textsuperscript{+}-ATPase but assumed a marked different morphology from healthy endothelial cells. To the best of our knowledge, the long-term structural changes in the corneal endothelial cells and Descemet’s membrane and the relative changes in interface corneal opacity in DSAEK and nDSAEK in animal models have not yet been reported. It this study, we aimed to observe the changes in function and structure of the CEEB after nDSAEK in a rabbit model. We also used in vivo confocal microscopy to compare differences in interface opacity between DSAEK and nDSAEK.

**METHODS**

**Surgical Procedure**

New Zealand white rabbits (female, 3.0–3.5 kg, 6 months old) were used in this study. Use, care and treatment of all animals followed the regulation of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures were approved by the Committee for Animal Research of the National Taiwan University Hospital. All surgical procedures and in vivo confocal microscopic observations were performed with animals under general anesthesia induced by intramuscular injection (Zoletil 20 mg/kg; Virbac Animal Health, Carros, France) and 4 mg/kg of xylazine (Rompun; Bayer Animal Health, Leverkusen, Germany). The eyes were topically anesthetized with 0.5% proparacaine hydrochloride (Alcaine; Alcon, Fort Worth, TX, USA) before manipulation. The right eye of each animal was used for the experiments and the left eye was not treated. Fifteen recipient eyes (six for DSAEK and nine for nDSAEK) were included. The corneas were inspected under a surgical microscope, and observations were recorded every 2 weeks after surgery for 5 to 6 months (transmission electron microscopy). For donor preparation, six rabbits (12 eyes) were euthanized with intravenous injection of 240 mg/kg thiamylal sodium (Shinlin Singseng Pharmaceutical, Taoyuan, Taiwan). The same agents were also used for euthanizing rabbits at the end of the experiments.

The preparation of donor grafts was performed immediately before the DSAEK or nDSAEK procedures. First, all donor eyeballs received corneal pachymetric measurement (OcuScan; Alcon) to determine the corneal thickness. A lamellar corneal cut was performed using a laser system (IntraLase; Abbot Medical Optics, Santa Ana, CA, USA) with the posterior donor discs aimed at 120 μm. Anterior segment optical coherence tomography (Visante; Carl Zeiss Meditec AG, Oberkochen, Germany) was performed to confirm graft thickness of 120 ± 20 μm. The corneas were then trephined to obtain donor discs at 8-mm diameter. These graft tissues were then implanted unilaterally into the right eyes of the recipient rabbits using either DSAEK or nDSAEK techniques.

In the recipient eyes immediately before the DSAEK and nDSAEK surgeries, lensectomies were performed. At surgery, pupils were dilated with topical phenylephrine and tropicamide (Mydrin M, Santen Pharmaceutical Co., Ltd., Osaka, Japan); tropicamide (Mydrin P, Santen Pharmaceutical Co., Ltd., Osaka, Japan); and dexamethasone, neomycin, and polymyxin B ointment (Maxitrol; Alcon) and were maintained with the right side of the face facing upward for 30 to 60 minutes.

**In Vivo Confocal Microscopy**

In vivo confocal microscopy was performed on the right eye of six subjects post operation, and 1, 2, and 3 months after DSAEK and nDSAEK with the Heidelberg Retinal Tomograph (HRT-3) equipped with a Rostock Cornea Module (Heidelberg Engineering GmbH, Heidelberg, Germany). This instrument uses a ×60 water-immersion objective lens (Olympus Europa GmbH, Hamburg, Germany) and a 670-nm diode laser as a light source, resulting in an image dimension of 400 × 400 μm\textsuperscript{2} and a transverse resolution of 1 μm.\textsuperscript{15} Before examination, one drop of vidisic gel (HanBul Pharm Co., Ltd., Seoul, Korea) was applied to the surface of a sterile disposable plastic cap (Tomo-cap, Heidelberg Engineering GmbH) on the front lens of the microscope. The surface of the plastic cap (Heidelberg Engineering GmbH) was positioned on the central cornea. Images at the central cornea were examined layer by layer. The scanning was performed continuously from the posterior surface of the cornea back to the anterior surface. At least five examinations per eye were performed.

Quantification of corneal interface haze after DSAEK and nDSAEK was done by a procedure modified from our previous publications.\textsuperscript{18–20} Briefly, the z-scan system was used for five measurements at 4-μm z-intervals, where +8, +4, +0, −4, and −8 μm from the CEEB layer, were obtained and averaged for analysis. The method to quantify the corneal opacity was modified by Seeberger's method, in which the corneal opacity in each layer was reflected by image brightness.\textsuperscript{21} Briefly speaking, the selected images were saved as JPEG files. The files were opened in ImageJ software (http://imagej.nih.gov/ij/, provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The pixel value of the selected central area of each image was used to quantify the reflectivity (interface opacity) of each image. The reflectivity (interface opacity) quantified by our method showed a strong agreement with the data obtained from a confocal microscope (Confoscan 3; Nidek Technologies Srl, Albignasego, Italy) z-scan system by simple linear regression model ($R > 0.98$, $P < 0.001$) in preliminary testing. To determine the cell density, the three clearest images of the corneal endothelial layer were selected. In each image, a clear continuous area of cells was selected and at least 50 cells were counted (Fig. 1). The measurement of cell density and interface opacity was analyzed by two independent observers. The average result of six rabbits in each group was used for comparison.
Recipient Corneal Endothelial Cells After nDSAEK

**Immunohistochemistry**

At 3 months after surgery, three animals in each group (DSAEK and nDSAEK) were killed, and the corneoscleral buttons were used for immunohistochemistry. The corneal specimens for immunohistochemistry were embedded at −80°C. Cornea sections were processed according to our previous publications and incubation with 1:100 diluted mouse anti-Na⁺/K⁺-ATPase antibody (Anti-Na⁺/K⁺-ATPase α-1, clone C464-6; Millipore Corp., Billerica, MA, USA). Sections were then incubated with 1:2000 diluted AlexaFluor 488-conjugated goat anti-mouse IgG (Invitrogen Corp., Carlsbad, CA, USA). They were subsequently washed and mounted on glass slides with medium containing 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) (Vectashield mounting media; Vector Laboratories, Burlingame, CA, USA). The results were inspected with confocal laser scanning microscopy (Zeiss LSM 510 Meta Confocal Laser Microscope; Carl Zeiss Microscopy). The images were obtained under the scanning option of “DIC/transmitted light and double channel fluorescence” and were processed and assembled using Zeiss LSM Image Browser Version 4.2 (Carl Zeiss). TUNEL staining (Apo-BrdU DNA Fragmentation Assay Kit; BioVision, San Francisco, USA) was used to evaluate the apoptosis of CEEB cells. The results were inspected with a fluorescence microscope (Olympus, Tokyo, Japan).

**Transmission Electron Microscopy**

At 1, 3, and 6 months after nDSAEK, excised corneas (from three eyes at three different time points) were fixed in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer for 2 hours at 4°C. After several washes, the corneal tissues were fixed with 1% osmium tetroxide, dehydrated in alcohol of different concentrations, and embedded in 100% Epon. Semithin sections (1-μm thick) were stained with toluidine blue for observation at the light microscope level, whereas ultrathin sections (80-nm thick) were collected on uncoated copper grids for transmission electron microscopy.

The sections were examined under an electron microscope (Hitachi H-1200; Hitachi, Tokyo, Japan).

**Statistical Analysis**

Results of CEEB density and interface opacity were presented as mean ± standard deviation. Statistical analysis comparing the results between the groups was performed by Mann-Whitney U test (SPSS, Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

**RESULTS**

The Donor Lamellar Discs Were Successfully Transplanted to Rabbit Eyes in Both the DSAEK and nDSAEK Groups

Both DSAEK and nDSAEK were successfully performed on rabbit eyes. The operated corneas were clear in both groups from postoperative 7 days to 6 months. All transplanted corneal discs were well attached to the recipient posterior corneal stroma during the observation period.

In Vivo Laser Confocal Microscopy Examination Demonstrated the Time-Dependent Decrease of CEEB Density in nDSAEK, and the Similar Severity of Interface Opacity Between DSAEK and nDSAEK

All rabbit eyes receiving nDSAEK showed a time-dependent decrease in the density of CEEB (Figs. 1, 2A), with preoperative scores of 3089 ± 347/mm²; postoperative 1-month scores of 2350 ± 410/mm², postoperative 2-month scores of 2249 ± 468/mm²; and postoperative 3-month scores of 1860 ± 260/mm² (all $P < 0.01$). The corneal endothelial cells not only decreased in density, but the arrangement became clumpy and irregularly distributed (Fig. 1). The morphology of the cellular nuclei also lost the normal kidney shape, and became irregular. During the whole observation period, no inflammatory cells or highly reflective particles were observed in human patients were observed at the donor–recipient interface, or in the adjacent donor or recipient corneal stroma. The interface opacity of nDSAEK was higher than DSAEK at all postoperative time points, but the difference was not statistically significant (Fig. 2B).

Immunohistochemistry Showed Different Healing Patterns of the Interface Between DSAEK and nDSAEK

Histological sections of corneas after DSAEK showed well-attached grafts with a thin and poorly identified stroma-stroma interface at 3 months after operation (Fig. 3A). In the nDSAEK group, by contrast, residual recipient Descemet’s membrane and CEEB could be clearly identified at the same time points (Fig. 3B). Immunohistochemistry suggested that the CEEB in the nDSAEK group lost Na⁺/K⁺-ATPase protein expression at 3 months after operation (Figs. 3C, 3D). Some cells that were TUNEL-positive were found among the CEEB (Fig. 4), indicating that some cells were undergoing apoptosis.

Transmission Electron Microscopy Observation of CEEB in nDSAEK Eyes

Although the donor corneal lamellar discs in the nDSAEK group adhered well to the recipient endothelium/Descemet’s layer, time dependently distinct morphological changes in
CEEB were found clearly under transmission electron microscopy (Fig. 5). A time-dependent increase and irregular change of cellular thickness in CEEB was found from postoperative months 1 to 6. The cells lost their normal uniform thickness, and the thickness of cell–cell contact areas decreased. At postoperative month 6, the cellular nuclei changed their morphology from a large flat shape into a smaller and thicker one. In addition, the protrusion of cellular process into donor corneal stroma was also found at postoperative months 3 and 6. At postoperative months 3 and 6, several intracellular vacuoles containing collagen fiber–like material, which have similar size and shape as the collagen fibers in the adjacent donor corneal stroma, were found (Fig. 5).

**DISCUSSION**

Although DSAEK and nDSAEK are popular surgical procedures with high success rates, little is known about the relative postoperative results of these two procedures, especially in terms of interface structure and opacity.\(^{15,17}\) Such comparison is important since the postoperative graft adherence onto the recipient cornea, the postoperative corneal clarity, and the fate of the CEEB may significantly influence postoperative results.

Normal corneal endothelial cells are made of closely interdigitated, nonproliferative cells arranged in a mosaic pattern of mostly hexagonal shapes. Several junctional complex structures are present, and the interconnected endothelial cell layer provides a “leaky” barrier to the aqueous humor. These cells have a large nucleus and contain abundant cytoplasmic organelles, which demonstrate an active metabolic character and the ability of secretion. The most important physiologic function of these cells is to regulate the water content of the corneal stroma through \(\text{Na}^+\)/\(\text{K}^+\)-ATPase, which is located on the corneal endothelial cell membrane. Human corneal endothelial cells have no proliferative ability, and may lose their density due to disease or aging.\(^{24–27}\)

CEEB were found clearly under transmission electron microscopy (Fig. 5). A time-dependent increase and irregular change of cellular thickness in CEEB was found from postoperative months 1 to 6. The cells lost their normal uniform thickness, and the thickness of cell–cell contact areas decreased. At postoperative month 6, the cellular nuclei changed their morphology from a large flat shape into a smaller and thicker one. In addition, the protrusion of cellular process into donor corneal stroma was also found at postoperative months 3 and 6. At postoperative months 3 and 6, several intracellular vacuoles containing collagen fiber–like material, which have similar size and shape as the collagen fibers in the adjacent donor corneal stroma, were found (Fig. 5).

**FIGURE 2.** The change of CEEB density and interface opacity measured by in vivo confocal microscopy. (A) There is a time-dependent decrease of CEEB density at postoperative months 1, 2, and 3 in nDSAEK group. (B) There is also a time-dependent decrease of interface opacity at postoperative month 1, 2, and 3 in both DSAEK and nDSAEK groups. However, at every time point, there is no significant difference between the DSAEK and nDSAEK groups. Black bars: nDSAEK group. Gray bars: DSAEK groups. \(p\) value: analyzed by Mann-Whitney test. NS, not statistically significant.

**FIGURE 3.** Histological (A, B) and immunohistochemical (C, D) staining. (A) Three months post DSAEK, hematoxylin and eosin (H&E) staining showed a well-attached graft with a thin but poorly identified stroma-to-stroma interface. (B) Three months post nDSAEK, H&E staining showed a clear line of the embedded recipient Descemet’s membrane and CEEB. (C) The bright field image with nuclear counter staining with DAPI demonstrated the donor corneal cells in nDSAEK grafts (thin white arrows), and CEEB (thick white arrows) at postoperative month 3. (D) The donor corneal endothelial cells expressed Na\(^+\)/K\(^+\)-ATPase staining in nDSAEK corneas at postoperative month 3 (thin white arrows). However, there is no Na\(^+\)/K\(^+\)-ATPase expression on CEEB (thick white arrows). Thick black arrows in (A, B): the interface junction between donor and recipient corneas. The images of (C, D) were taken by confocal laser scanning microscopy under the scanning option of “DIC/transmitted light and double channel fluorescence” and the support of commercial software (Zeiss LSM Image Browser, version 4.2; Carl Zeiss Microscopy). Blue in (C, D): DAPI counter staining of cellular nuclei. Red in (C, D): Na\(^+\)/K\(^+\)-ATPase staining. Scale bars: 100 \(\mu\)m (A, B), 50 \(\mu\)m (C, D).
Although limits in the resolution provided by in vivo confocal microscopy did not allow clear identification of the cell membrane and cytoplasm, the change in nuclear morphology could be clearly seen. There was also a time-dependent decrease in the density of corneal endothelial cells. The cell density decreased to 60% at postoperative month 3. Since the TUNEL stain at the same time revealed some apoptotic cells at 3 months after surgery, the CEEB in the nDSAEK group may slowly undergo apoptosis after the surgery, resulting in the loss of density. Unlike in humans, normal rabbit corneal endothelial cells have a high proliferative ability after injury. Theoretically, the rabbit eye is not a perfect model to evaluate corneal endothelial density. However, the CEEB in this study seemed to lose their proliferative ability after being embedded between two stromal layers, which implied the similar healing process of CEEB in human corneas after nDSAEK.

By transmission electron microscopy, we found a time-dependent change of cellular morphology and nuclei in CEEB after nDSAEK. At postoperative month 6, the cellular nuclei demonstrated a significant difference from normal eyes. In addition, the protrusion of cellular process into donor corneal stroma was also found 3 and 6 months post nDSAEK.

Figure 4. Staining with TUNEL. Three months post nDSAEK, there was no cornea cell showing apoptosis in the DSAEK group. However, some TUNEL-positive cells can be found in CEEB. Thick white arrows: TUNEL-positive cells. Thin white arrows: the location of CEEB. Red: PI counterstaining of nuclei. Green: TUNEL-positive cells, which implied apoptotic cells.
combined with the presence of intracellular vacuoles containing collagen fiber–like materials. Although not proven, the CEEB seemed to engulf the collagen fiber in the adjacent tissue. Since the CEEB was embedded in an environment different from its normal condition, it is reasonable to presume that CEEB gained the phagocytic ability to digest the implanted donor corneal stroma, thus can extend its cellular process.

Although the transmission electron microscopy demonstrated that the CEEB were alive by showing the abundant mitochondria, the actively transformed cellular morphology and the phagocytic ability, the CEEB lost its normal physiological functions. Hatanaka et al.\textsuperscript{17} found that the CEEB after nDSAEK still preserved Na\textsuperscript{+}-K\textsuperscript{+} ATPase expression at 2 weeks post operative. However, the CEEB didn’t express such protein in our experiment at postoperative month 3. We presumed that the cells gradually lost their physiological function after the operation.

Although the nDSAEK technique has several benefits, the possible interface opacity which may lead to visual impairment is a potential drawback avoided by DSAEK.\textsuperscript{13,15} Kobayashi et al.\textsuperscript{15} used in vivo confocal microscopy to evaluate human patients receiving nDSAEK, and found postoperative haze, interface particles, and needle-shaped materials in all 10 patients during postoperative observation of 1 to 6 months. However, to the best of our knowledge, no head-to-head, qualitative comparison of the interface opacity between DSAEK and nDSAEK has previously been reported. In this study, we used a new generation in vivo laser confocal microscopy device (Heidelberg Engineering GmbH) to observe in vivo findings without killing the animals. This microscopy was powerful enough to provide histologic-like images of corneal microstructures with high resolution. It provides not only satisfactory images, but also, after some modification, qualitative information about cellular density and image reflectivity. In this study, we found higher interface reflectivity in the nDSAEK group than in the DSAEK group at all

**Figure 5.** Transmission electron microscopic findings. (A) Preoperative corneal endothelial cells demonstrated monolayered and flat cellular morphology with flat nucleus. The corneal endothelial cells thickness was uniform and measured around 3.5-\mu m thick. (B) One month post nDSAEK, the CEEB and nucleus shape still demonstrated flat morphology. However, the thickness of the monolayered cells became irregular, measured around 1.0- to 3.5-\mu m thickness. (C) Three months post nDSAEK, the flat morphology of CEEB didn’t change significantly. However, protrusion of cell processes into recipient Descemet’s membrane was found. (D) Inset of (C). Several intracytoplasmic bodies containing collagen fiber–like materials were found. The sizes and shapes of the collagen fiber–like materials were similar to the collagen fibers in the neighbor donor stroma. It could be possible that the CEEB has the phagocytic ability to engulf collagen fibers from neighboring donor stroma, or the cells have the ability to produce collagen fibers and secrete into neighboring donor stroma or recipient Descemet’s membranes. (E) Six months post nDSAEK, the irregular changes of corneal endothelial thickness became evident (1.0–6.0 \mu m) with thickening of nucleus. (F) Inset of (E). The intracytoplasmic bodies containing the collagen fiber-like materials demonstrated in (D) could also be found. The sizes and shapes of the collagen fiber-like materials were also similar to the collagen fibers in the neighbor donor stroma. Long black arrows: nucleus of corneal endothelial cells or CEEB. Short black arrows: collagen fibers in donor stroma. White short arrow: the intracytoplasmic bodies containing the collagen fiber-like materials found in adjacent donor stroma. DS, donor stroma; RD, recipient Descemet’s membrane. Scale bars: 4000 nm (A); 3000 nm (B); 4000 nm (C); 1000 nm (D); 2000 nm (E); 625 nm (F).
postoperative time points as evaluated by in vivo confocal microscope, but the difference was not statistically significant. The results were similar to the external eye photographs taken by surgical microscopy (data not shown), in which both groups showed clear corneas without significant opacity. We may thus conclude that the interface opacity is not a clinically significant complication after nDSAEK compared to DSAEK.

Our findings may contribute new understanding of the wound healing process that takes place in the interface in nDSAEK. Even though nDSAEK is not as popular as DSAEK, the information provided helps elucidate the changes of the embedded corneal endothelial layers, which may be important in further development of corneal endothelioplasty. For example, the CEEB and Descemet’s membrane existed at the edge of the implanted donor corneal tissue if the Descemet’s membrane stripping area is less than the implanted corneal tissue in DSAEK or Descemet’s membrane endothelial keratoplasty. The understanding of the healing process in which our findings afforded should benefit both research and clinical endeavors.

Our study has several drawbacks. First, rabbit corneal endothelial cells are known to be different from human cells in their ability to proliferate, a quality humans lack. However, we found that the CEEB progressively decreased their cellular density in this study, exhibiting a nonproliferative quality similar to human cornea. Second, we used normal rabbit corneas instead of diseased animal corneas as the recipients. While there is no perfect rabbit corneal endothelial disease model, similar studies of nDSAEK also used rabbit eyes in this same experimental model. Third, a 3- to 6-month duration may not be adequate for observing long-term postoperative changes, although the previous study observed the corneas for only 2 weeks. A longer study may provide more information. Fourth, in vivo confocal microscopy and transmission electron microscopy can only provide morphological information, not record functional change or dynamic change. However, such findings are still very informative.

In conclusion, we found that the CEEB after nDSAEK changed significantly in morphology, physiologic function, and cell density after surgery. However, nDSAEK did not result in significantly more interface opacity compared to DSAEK.

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