Original Article

Transplantation of cultured rhesus monkey vascular endothelial cells to allogeneic cornea concomitant with stripping of Descemet's membrane

Qin Zhu, Min Wu*, Xiaomei Sun[1], Wenjia Zhang, Zhulin Hu, Hai Liu*

Context: In cases of damaged corneal endothelium cells (CECs) of the eye, transplantation of cultured vascular endothelial cells (VECs) may be a viable method to restore transparency. **Aims:** To evaluate the viability of replacing damaged primate CECs with cultured allogeneic VECs. **Subjects and Methods:** Rhesus monkey VECs (RMVECs) were cultured and proliferating cells were labeled with bromodeoxyuridine (BrdU) *in vitro*. RMs of the experimental group (n = 6) underwent manual Descemettt membrane stripping with transplantation of RMVECs labeled with BrdU; those in the control group received manual Descemetnt membrane stripping without transplantation. Postoperative evaluations included the transparency and appearance of the corneal graft; distribution and ultrastructural changes of RMVECs on the inner surface of the cornea using scanning and transmission electron microscopy, and immunohistological identification of BrdU. **Results:** At 90 days postsurgery, the corneal grafts of the monkeys in the experimental group retained better transparency than those of the controls, without corneal neovascularization or bullous keratopathy. A layer of cells with positive BrdU staining was found on the posterior surface of the treated corneas in the experimental group, while there was no VEC structure in corneal grafts from the monkeys of the control group. **Conclusions:** RMVECs can grow on the posterior surface of the cornea without Descemet's membrane. Cultured and transplanted RMVECs appeared similar in ultrastructure. VECs can provide a barrier to maintain corneal dehydration and transparency to some extent.

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The transplantation of the cultured corneal endothelial cells (CECs) has been reported in various animal models,[1-8] but the cell resource is very limited, and substitute carriers easily fall off the posterior surface of the cornea.[9,10] Therefore, it is imperative to find a promising substitute for human CECs. Due to share many characteristics with CECs, human vascular endothelial cells (VECs) become an ideal candidate.[11-15] Here, we used primate CECs as seed cells and transplanted them to the cornea concomitant with the stripping of Descemet's membrane by centrifugal precipitation. Our purpose was to investigate the possibility of replacing damaged primate CECs with allogeneic VECs.

Subjects and Methods

Cell culture and labeling of DNA with bromodeoxyuridine

Rhesus monkey (RM) choroid-retina VECs (cell line RF/6A; RMVECs) were purchased from the American Type Culture Collection, cultured in 1640-medium (Gibco, USA) supplemented with 10% fetal calf serum, L-glutamine (final concentration 2 mM), and antibiotics (50 U/mL penicillin, 50 μg/mL streptomycin, 12 μg/mL amphotericin B), and then incubated in a humidified atmosphere at 37°C in 5% carbon dioxide (CO_2). The culture medium was changed every other day. When the RF/6A cells reached confluence in the cell culture bottle, they were rinsed in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS), trypsinized with 0.05% trypsin-ethylenediaminetetraacetic acid for 3 min at 37°C, and passaged at ratios of 1:2.

Cells were then cultured in complete medium containing required amino acids[16] (40 ug/mL each), casamino acids (200 ug/mL), and bromodeoxyuridine (BrdU) (10 ug/mL BrdU, Sigma, USA), or identical medium without BrdU, for 72 h. When cells reached confluence, the layer was trypsinized, and the suspended endothelial cells were gently centrifuged (200 ×g, 5 min, 20°C) in sterile glass centrifuge tubes. The trypsin solution was decanted, and cells were resuspended at a final concentration of 2×10^5 cells/mL in the complete culture medium.

Cell climbing film

To perform BrdU labeling and observe VECs using scanning electron microscopy (SEM), we made cell climbing films

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using RMVECs before transplantation. In brief, RF/6A cell suspensions, with or without BrdU labeling, were directly grown on coverslips for 4 h following gentle addition of the medium. They were placed in an incubator at 37°C and 5% CO_2 for 24 h. The slide was removed, and cells were then fixed in 4°C acetone for 10 min, removed, and air dried at room temperature.

Cultured vascular endothelial cells transplanted to denuded Descemet's membranes of rhesus monkey corneas

The experiment was approved by the Local Institutional Care and Animal Research Committee. Animals were handled in a strict accordance with the Association for Research in Vision Ophthalmology Statement on the use of Animals in Ophthalmic and Vision Research. Twelve RMs were randomly divided into two treatment groups ($n = 6$, each). Animals of both groups were given a manual Descemet's membrane stripping. In addition, the RMs in the experimental group were transplanted with RMVECs during the stripping; those in the control group ($n = 6$) did not receive these cells. All surgical procedures were performed by a single surgeon.

For the manual Descemet's membrane stripping, all RMs were anesthetized with an intramuscular injection of ketamine hydrochloride (5–10 mg/kg body weight). Viscoelastic material (sodium hyaluronate [Aiwei]; Bausch and Lomb Shandong Chia Tai Freda Pharmaceutical Group, Shandong, China) was injected through the paracentesis to fill the anterior chamber. A corneal button 7 mm in diameter was obtained by trephination. A cotton ball soaked in 75% alcohol was placed on the corneal endothelium, and folds of Descemet's membrane were visible after 1 min. The Descemet's membrane with corneal endothelium was then stripped under the operating microscope.

In the experimental group, the cultured RF/6A cells were transplanted to the denuded corneal button of the recipient by centrifugation. A continuous 10-0 nylon monofilament suture was used to join the host and corneal button. An air bubble, usually one-fourth to one-third of the total anterior chamber volume, was injected into the anterior chamber for graft support. The eye was injected subconjunctivally with a mydriatic solution (0.15 mL), 2.5 mg of dexamethasone, and 0.15 mL of gentamicin (40 mg/mL) and then taped shut to prevent evaporation until the animal recovered from the anesthesia. Control animals underwent manual Descemetta membrane stripping without transplantation of cultured VECs. The operated eye received daily applications of antibiotic-hydrocortisone and atropine ointments, and twice-weekly injections of dexamethasone (2.5 mg).

Microscopic evaluation after transplantation

After transplantation, the corneal appearance was examined by slit lamp biomicroscopy every day for the 1st week, once a week thereafter for the 1st month, and then once a month for 3 months. Three months after transplantation, the corneal buttons were removed and examined by SEM and transmission electron microscopy (TEM). The corneal buttons were immersed in 2.5% glutaraldehyde in PBS, washed 3 times in PBS, postfixed for 2 h in 2% osmium tetroxide, and washed another 3 times in PBS. They were then dehydrated serially in 50%, 70%, 90%, and 100% ethanol solutions for 10 min at each concentration.

For SEM, the samples were further dried with CO_2 in a critical point dryer and gold coated by ion sputtering before examination under an SEM at an accelerating voltage of 10 kV. For TEM, specimens were embedded in epoxy resin, and ultrathin sections (50-70 nm) were collected on copper grids. Counterstaining was with aqueous uranyl acetate and phosphotungstic acid (1 h each) followed by Reynolds' lead citrate (20 min). Specimens were examined in a TEM.

Immunohistochemistry

Immunocytochemical examination of the RF/6A cells and the corneal buttons were performed at 90 days postsurgery. Cells and corneal buttons were fixed with 4% paraformaldehyde in PBS for 10 min. After washing with PBS, the fixed specimens were then permeabilized in 0.3% Triton X-100 in PBS (PBST) for 15 min and blocked with 4% bovine serum albumin (BSA) in PBS for 30 min. This was followed by incubation with the specific primary mouse anti-human monoclonal antibodies (Abcam, U.K.) diluted in BSA/PBST for 2 h and with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibodies (Abcam, U.K.) (1:100) for 20 min at room temperature in dark. Unbound excess labels were removed by rinsing the samples in PBS. The samples were viewed under a fluorescence microscope.

Histology

Corneal buttons were fixed in 10% formalin for 24 h and then embedded in optimal cutting temperature (OCT) compound and stored at –20°C. Frozen OCT-embedded sections were cut into 8-μm thick sections, placed on silane-coated microscope slides, stained with hematoxylin and eosin (H and E), and observed with light microscopy.

Results

Distribution and attachment of cultured rhesus monkey vascular endothelial cells on corneal button

After 4 h of culture, RMVECs attached and spread on the cell culture bottle. The RMVECs grew readily toward each other, forming intercellular bridges. As cell density increased, RMVECs eventually appeared fusiform or polygonal, then reached confluence and formed a monolayer with contact inhibition after 5 days of cultivation at 37°C. RMVECs distributed uniformly and had the typical elongated spindle shape [Fig. 1a].

After manual Descemet's membrane stripping, corneal stromal fibers were on the denuded corneal button without endothelium [Fig. 1b]. The cultured RMVECs were uniformly distributed on the corneal button following centrifugation [Fig. 1c].

Clinical observations after surgery

At 7 days postsurgery, in the RMs of the experimental group there was obvious conjunctival hyperemia, mild corneal opacity, and edema with normal anterior chamber depth and visible iris [Fig. 2a]. No neovascularization of the cornea or iris was found, and the pupil diameter was ~3 mm. RMs of the control group showed obvious conjunctival hyperemia, total corneal opacity, and edema, while the anterior chamber depth was normal and the iris was visible. The pupil diameter was ~4 mm [Fig. 2b].

At 14 days postsurgery, in the RMs of the experimental group conjunctival hyperemia was reduced and corneal opacity was dense, while the anterior chamber depth was still normal and the iris was visible [Fig. 2c]. In the control monkeys, corneal

opacity was also heavier, anterior chamber became dimly visible, the pupil was fuzzy, and the iris invisible [Fig. 2d].

At 30 days postsurgery, in the experimental group the conjunctival hyperemia had disappeared, corneal transparency increased, and no iris neovascularization was found [Fig. 2e]. In controls, conjunctival hyperemia remained and the corneal endothelial decompensation cannon keratopathy (enucleation of the eye contentsline ophthalmectomy) appeared with a high degree of edema in the corneal stroma [Fig. 2f].

At 60 days postsurgery, in the experimental RMs, a slightly lighter anterior chamber was found in two animals, and the diameter of the pupil of these two animals was dilated to ~5 mm [Fig. 2g].

At 90 days postsurgery, in the experimental group mild conjunctival hyperemia and corneal opacity with a small amount of new blood vessels were found with a shallow anterior chamber, unclear iris glimpse, and visible pupil on the horizon with a diameter of ~5 mm.

The current clinical observations indicate that RMVECs can maintain partial transparency of the cornea, but cannot completely replace the function of CECs [Fig. 2h].

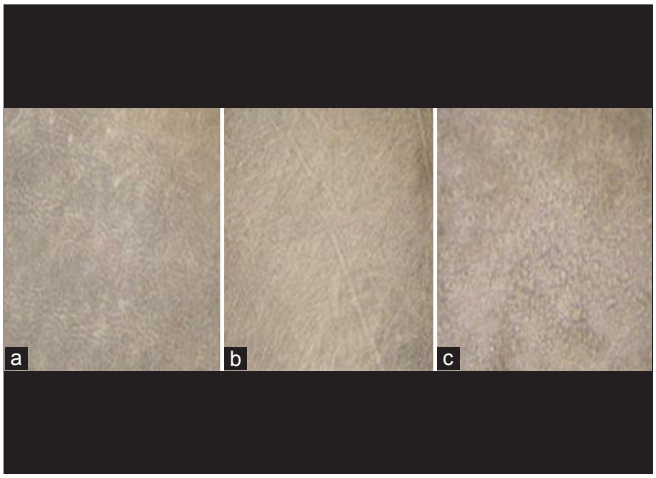

Figure 1: Corneal buttons observed under a phase-contrast microscope. (a) Rhesus monkey vascular endothelial cells distributed uniformly. (b) After manual Descemet's membrane stripping, corneal stromal fibers were on the denuded corneal button without endothelium. (c) After centrifugation, the cultured rhesus monkey vascular endothelial cells homogeneously distributed on the corneal button

Observation with scanning electron microscopy of the corneal surface and the trabecular meshwork after transplantation of rhesus monkey vascular endothelial cells

SEM revealed that CECs of the graft were hexagonal as normal, and there were tight connections between cells [Fig. 3a]. Occasionally, leukocyte and red blood cell deformation were found on the collagen surface [Fig. 3b]. At 30 days postsurgery, in the experimental group cultured RMVECs were attached to the corneal surface, and no tight connections between cells were observed. The cell gap and a small number of exposed collagen fibers were visible. No obvious white blood cells or cellular debris were on the cell surface [Fig. 3c and d]. There were a large number of white blood cells in the trabecular meshwork with red blood cells and cell debris clogging [Fig. 3e]. In the control group, there were no residual cells on the cornea after manual Descemet's membrane stripping. The bare surface of the posterior cornea was a collagen fiber-like structure, with fibers arranged in parallel and of uneven thickness, and there were visible gaps between the collagen fibers.

At 60 days postsurgery, the number of RMVECs and the number with irregular polygon morphology had significantly increased, and cell membrane integrity had improved [Fig. 3f-h].

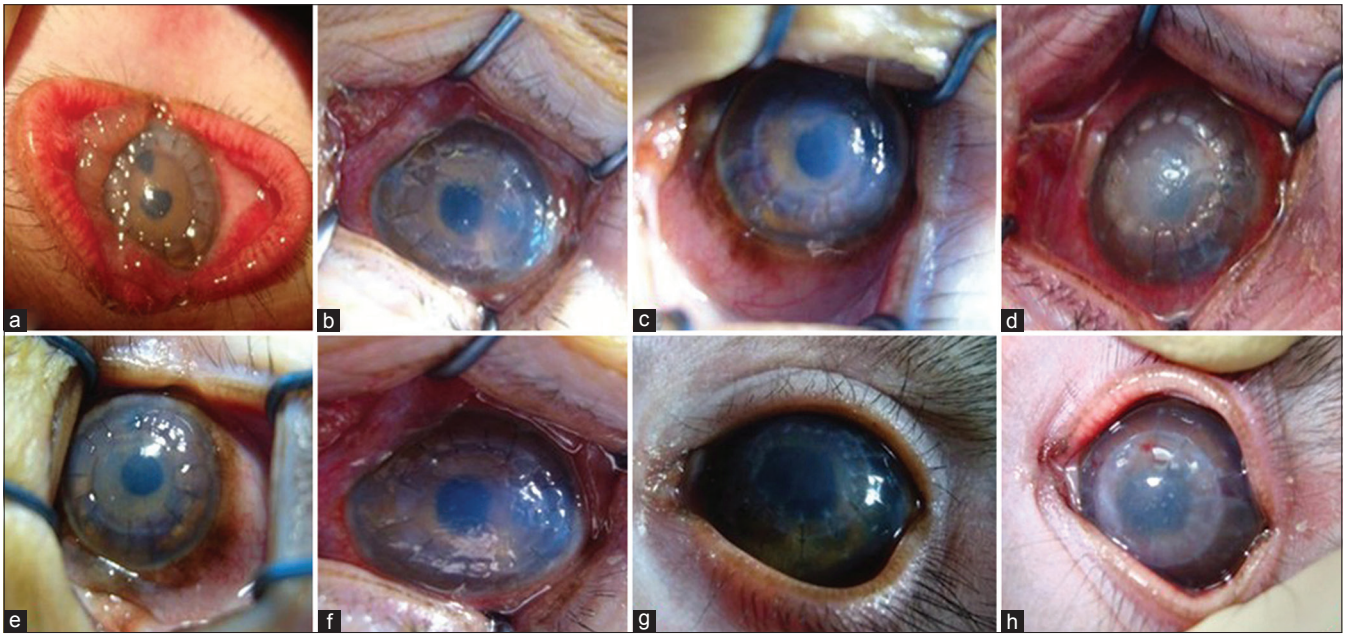

Figure 2: Clinical observation of corneal transparency after surgery. The corneal grafts of the monkeys of the experimental group retained better transparency than those of the controls, without corneal neovascularization or bullous keratopathy. Fig. 3a, 3c, 3e, 3g, 3h respectively to show at the 7d ,14d ,30d ,60d ,90d after DSAEK of experimental group. Fig. b, d, respectively to show the 7d,14d, 30d after DSAEK of control group

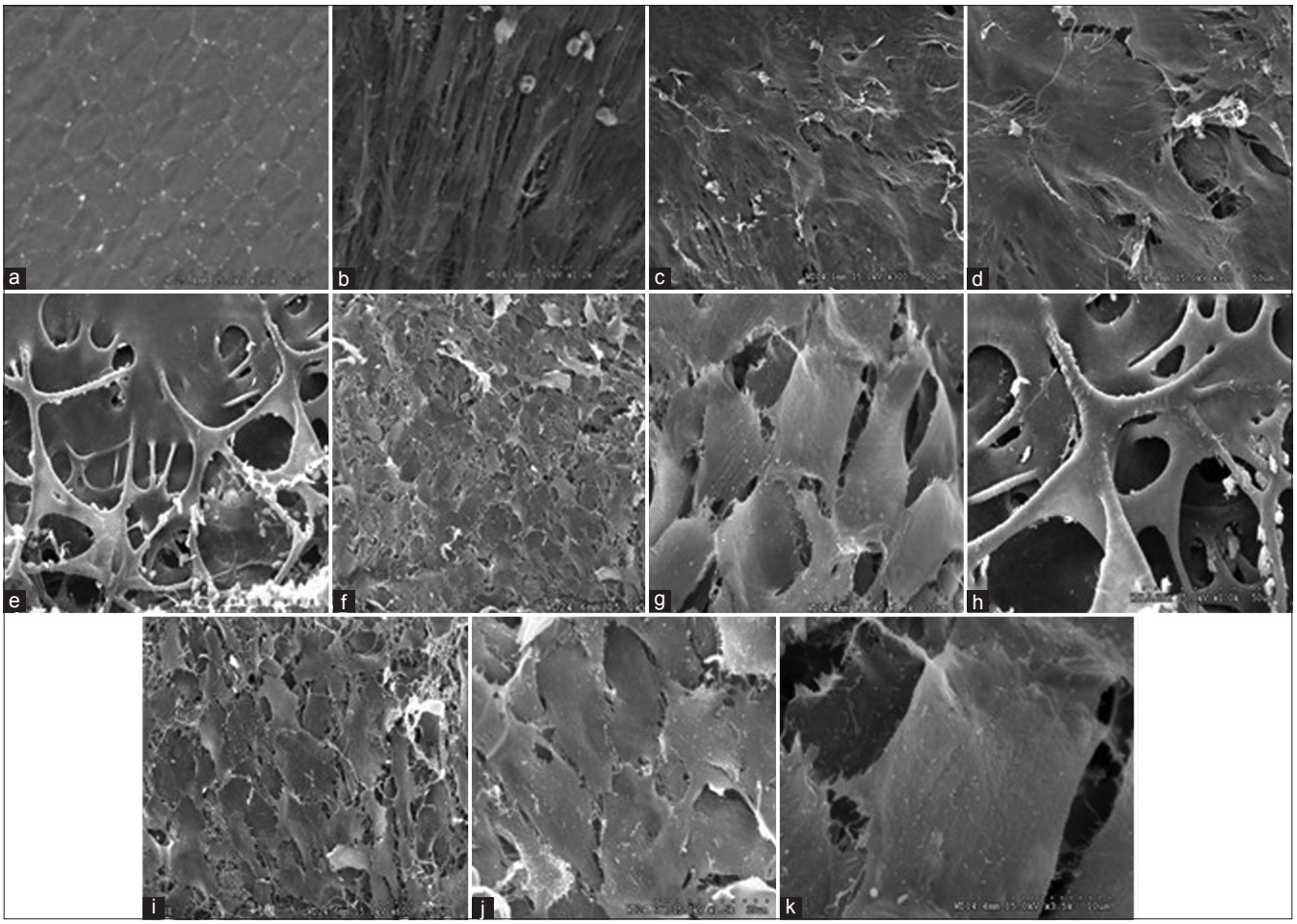

Figure 3: Observation via scanning electron microscopy of internal corneal surface and trabecular meshwork after the transplantation of rhesus monkey vascular endothelial cells. Scanning electron microscopy revealed that rhesus monkey vascular endothelial cells of irregular shape were uniformly distributed on the inner surface of the cornea and growing well, a small number of white blood cells were between the rhesus monkey vascular endothelial cells, and cellular debris existed in the trabecular meshwork. (a) Normal corneal ultrastructure. (b) Corneal tissues after DSAEK in the control group. (c and d) At 30 d after DSAEK, (e,f,g and h) At 60 d after DSAEK. (i, j and k) At 90 d after DSAEK

At 90 days postsurgery, the RMVECs density continued to increase, the big laminated RMVECs attached in the internal surface of cornea plants, and no apparent white blood cells were found. This indicated that the transplanted RMVECs grew well on the posterior surface of the cornea [Fig. 3i-k].

Observation of the corneal surface after transplantation of rhesus monkey vascular endothelial cells via transmission electron microscopy

Before transplantation, the RMVECs cultured *in vitro* and observed via TEM were approximately circular, and the microvilli on cell surfaces were of various lengths. The majority of cells had a tight junction structure. The nucleus was oval, located in the center of the cell, with finely granular chromatin of uniform distribution. The cytoplasm was rich in organelles with visible pinocytotic vesicles and no obvious swelling of mitochondria or dilation of endoplasmic reticulum [Fig. 4a-d].

At 90 days postsurgery, in the experimental group the RMVECs were closely attached to the corneal stroma, with visible desmosome connections between adjacent RMVECs. Nuclei were long and spindle-shaped with a uniform distribution of chromatin. The cells had a large number of cytoplasmic organelles, endoplasmic network, Golgi complex, and lysosomes. Ultrastructurally, Weibel–Palade bodies were observed that were endothelial cell-specific [Fig. 4e-f]. In the control group, the corneal surface consisted of a homogeneous fiber-like substance and no cell-like structures were found [Fig. 4g].

Through observation with TEM, it appeared that abundant cytoplasmic organelles existed in the RMVECs cultured *in vitro*, with many of the biological characteristics of VECs *in vivo*.

Corneal tissue sections with hematoxylin and eosin staining

In the experimental group, the posterior surface of the corneal graft was visible within a single cell growth, and closely attached to the corneal stroma without a homogeneous elastic layer-like substance between the cells and matrix [Fig. 5]. In the control group, the corneal posterior surface was smooth, and no cell-like substances were found. H and E staining showed that RMVECs did not secret elastic fibers to help the formation of Descemet's membrane.

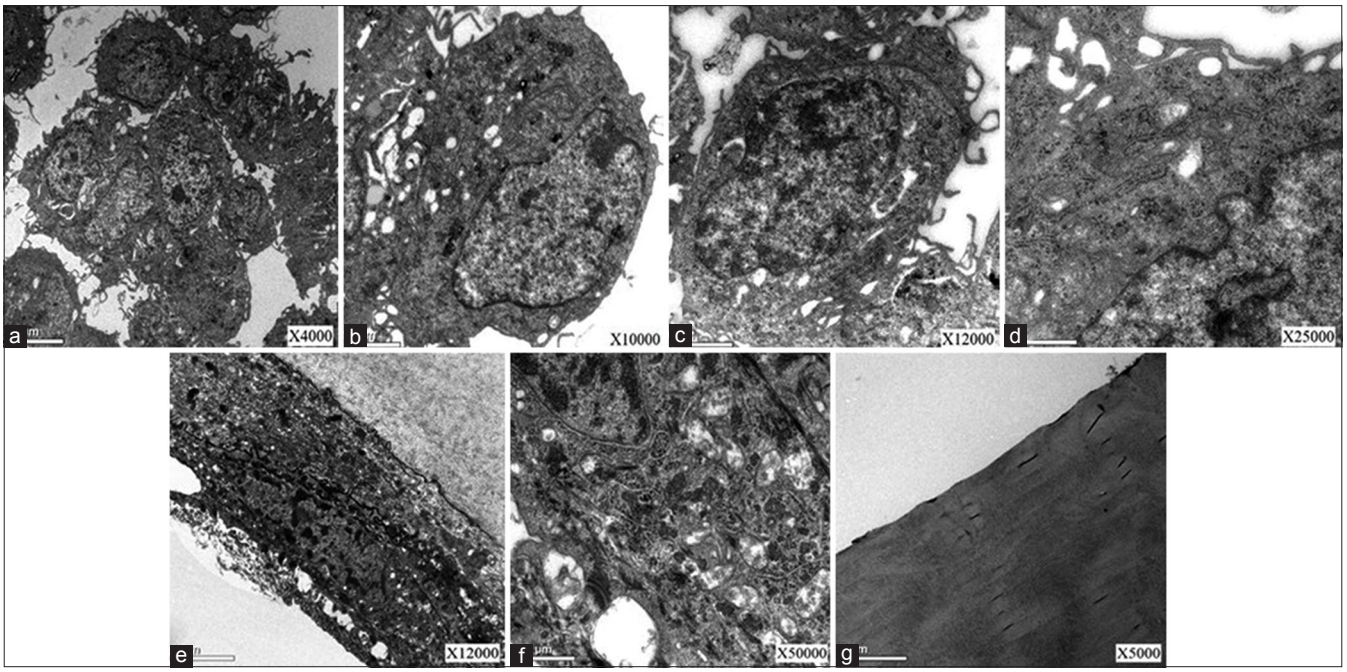

Figure 4: Observation via transmission electron microscopy of vascular endothelial cells. A large number of desmosome connections were observed between rhesus monkey vascular endothelial cells. Abundant organelles appeared in the cytoplasm of rhesus monkey vascular endothelial cells. There was no vascular endothelial cell structure in corneal grafts from the monkeys of the control group. a, b, c and d: Under the TEM RMVECs cultured in vitro. e and f: Under the TEM RMVECs structure in corneal grafts. g: TEM of control group

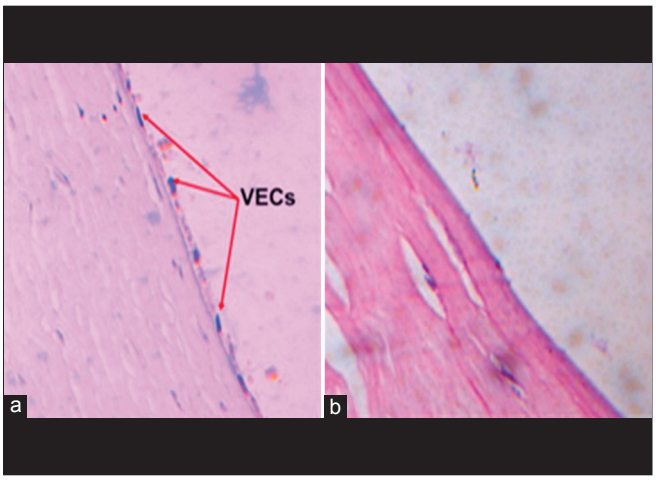

Figure 5: H and E staining of corneal grafts. (a) The experimental group showed that a monolayer of cells grew in the internal surface of corneas. (b) The control group: The internal surface of corneas was smooth, and no cell-like substances were found

Anti-bromodeoxyuridine monoclonal antibody immunohistochemical staining

After the anti-BrdU immunohistochemical staining, the nuclei of RMVECs were stained dark brown and yellow with visible lumps and granulated uneven coloring; the cytoplasm was lighter in color, and a nonuniform yellow or light brown. In the negative control group of RMVECs without BrdU, the nuclear and cytoplasmic staining were negative [Fig. 6].

In the experimental group, a deeply BrdU-stained monolayer cell with brownish yellow was observed on the cornea, which indicated the successful transplantation of RMVECs with marked BrdU on the surface of corneal grafts. The BrdU stain of corneal tissues in the control group was negative, and no cell structure colored brownish yellow was found. This suggests that the cells on the posterior surface of the cornea were VECs cultured *in vitro*, but not the CECs proliferated from the surrounding healthy CECs.

Discussion

In this study, we proved that RMVECs, cultured and proliferative *in vitro* and labeled with BrdU, could be transplanted to allogeneic corneas via manual Descemet's membrane stripping by centrifugation. RMVECs were not transplanted in the control group, and in that group there was no evidence of activation of the fluid barrier or Na^+-K^+ pump in the corneal endothelium; the aqueous humor quickly entered into the corneal stroma which led to corneal edema and visible bullous keratopathy. In addition, it was also proved in this study that healthy CECs surrounding the remnants at the 3-4 mm incision lacked the regenerative ability and could not recover through proliferation their normal CEC functionality to compensate for the central injury.

In the experimental group, corneal opacity gradually decreased during the first postoperative month, indicating that about 1 month from transplantation of RMVECs onto the posterior corneal surface was required for cell layer formation via growth connections. After forming the cell layer, RMVECs could participate in restoring barrier and pumping functions.

We suggest three reasons for the progressive opacities of the corneal grafts in the experimental group. First, the maintenance of corneal transparency is mainly by the way of the avascular cornea, neatly arranged fibers, and the

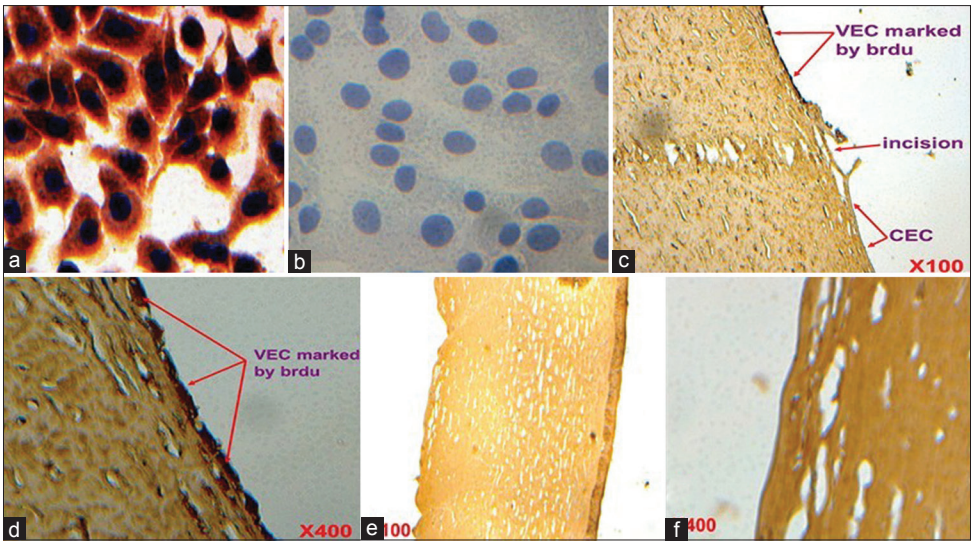

Figure 6: Cell climbing film and immunohistochemical staining of corneal tissues. (a) Rhesus monkey vascular endothelial cells were stained with anti-bromodeoxyuridine antibody. (b) The negative control group without anti-bromodeoxyuridine antibody. (c and d) The corneal tissues of the experimental group showed positive bromodeoxyuridine staining. (e and f) The corneal tissues of the controls showed no bromodeoxyuridine staining

Na^+-K^+-ATPase (Na^+-K + pump) and water channel protein function of CECs.[17] Although surviving RMVECs participated in maintaining the function of the barrier and active pump, which could help preserve corneal transparency, their role might be less than that of the CECs; VECs could not completely replace the CECs pumping function. To fulfill these functions, VECs require the assistance of fibroblasts and smooth muscle cells.[18] Second, *in vivo* the role of VECs in the barrier is closely related to the vascular intima,[18] but the VECs transplanted in this study could not significantly generate an intimal-like substance. Finally, the VECs transplanted in this study were allogeneic, and transplant rejection may have occurred after surgery. This would have led to the injury of the VECs and their functional decline, and corneal graft opacity and edema.

In this study, RMVECs may have been able to distribute uniformly and adhere to the internal graft surface because we used centrifugal precipitation cell transplantation. This method avoids serious graft edema that makes attachment to the wall difficult and also avoids second surgery after temporary implants to replace an artificial cornea.[19] The success of the RMVECs may also be attributed to the flat corneal surface, which is conducive to the adherent growth of VECs. Finally, because the aqueous humor is a plasma ultrafiltrate, and, therefore, its components bear high similarity to blood, adequate nutrition is available for the growth of transplanted VECs.

In the experimental group, SEM showed that some white blood cells and cell debris gathered at the inner surface of the cornea and trabecular meshwork at each time point observed, and corneal neovascularization invaded the periphery of the corneal graft 3 months after surgery; this indicated the postoperative rejection of the transplant. The corneal tissue may be directly destroyed by immune effector cells, or by antibody-dependent cell cytotoxicity targeting the allogeneic tissue.

In this study, we observed via TEM that RMVECs cultured *in vitro* and transplanted into the corneal receptors still retained organelles, the Golgi apparatus, and rough and smooth endoplasmic reticulum. Cultured RMVECs have many of the biological characteristics of VECs *in vivo*, and reliably retain their endocrine functionality after transplantation and subsequent growth. Based on the occurrence of edema in transplanted RMVECs, we hypothesized that Na^+-K^+-ATPase and water channel proteins to some extent participate in maintaining the water balance of VECs but are not as efficient as CECs. This may be associated with ectopic VEC growth, or the loss of the supportive role of smooth muscle cells.

To detect the ability of RMVECs to attach onto the posterior corneal surface, we used BrdU to label and visualize RMVECs. BrdU is an analog of thymidine (a DNA precursor) which can replace thymine in the DNA nucleotide sequence during S phase of the cell cycle and is passed to daughter cells during replication. When made visible by the application of the appropriate antibody via an immunohistochemical protocol, it is a useful indicator of cell proliferation and allows comparison among treatments.[20] Our results proved that proliferating RMVECs labeled with anti-BrdU monoclonal antibody were detectable after manual Descemet's membrane stripping, while the lack of positive BrdU staining suggested that RMVECs transplanted to the corneal surface could not secrete collagen fiber or form a new Descemet's membrane. Vascular intimal formation was the result of the interaction of vascular smooth muscle cells and endothelial cells.

Conclusion

In summary, our results confirmed that the transplantation of RMVECs into allogeneic cornea during manual Descemetic membrane stripping by the method of centrifugation could be performed successfully. RMVECs *in vitro* grew and adhered to the posterior surface of the corneal wall, and provided a barrier and active pump function which maintained corneal dehydration and transparency to a certain extent.

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Conflicts of interest

There are no conflicts of interest.

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