Constructing a Novel Three-Dimensional Biomimetic Corneal Endothelium Graft by Culturing Corneal Endothelium Cells on Compressed Collagen Gels

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Abstract

Background: Endothelium allotransplantation is the primary treatment for corneal decompensation. The worldwide shortage of donor corneal tissue has led to increasing pressure to seek an alternative for surgical restoration of corneal endothelium. Compressed collagen (CC) gels have excellent biocompatibility, simple preparation course and easy to be manipulated. This study aimed to form a new biomimetic endothelium graft by CC.

Methods: We expanded bovine corneal endothelial cells (B-CECs) on laminin-coated CC to form a biomimetic endothelium graft. Scanning electron microscope was used for ultrastructural analysis and tight junction protein ZO-1 expression was tested by immunohistochemistry.

Results: The biomimetic endothelium graft, we conducted had normal cell morphology, ultrastructure and higher cell density $(3612.2 \pm 43.4$ cells/mm$^2)$. ZO-1 localization at B-CECs membrane indicated the bioengineered graft possess the basic endothelium function.

Conclusions: A biomimetic endothelium graft with B-CECs expanded on CC sheet was constructed, which possessed cells' morphology similar to that of *in vivo* endothelial cells and specific basic function of endothelium layer. This method provided the possibility of using one donor's cornea to form multiple uniformed endothelium grafts so as to overcome the shortage of cadaveric cornea tissue.

Key words: Bioengineer Graft; Compressed Collagen Gel; Cornea Endothelium

INTRODUCTION

Cornea endothelial cells (CECs) are nonrenewable *in vivo* after birth. Once the CECs density drops below a certain level (<500/mm^2), the CECs left can no longer pump out enough water to keep corneal transparency leading to corneal stromal edema, clouding and vision loss, which is the so-called corneal decompensation.[1] Nowadays, the primary therapy for corneal decompensation is endothelium allotransplantation. However, the resource of donated corneal allograft is limited and far from sufficient compared with the huge requirement,[2] especially in developing countries. A recent global survey reported that the number of patients waiting for corneal transplantation is 12.7 million, but only 1.4% of them got the chance to accept surgery.[3] The endothelial graft needs to be renewed if the number and function of donor CECs on the allograft again decrease under the threshold, this usually happens

around 10 years after endothelium allotransplantation thus a patient may receive several transplant operations throughout lifetime. Currently, endothelial grafts are 1:1 replacement of cadaveric tissue and the total amount of transplanted CECs from an adult donor can no more than 3000/mm^2. In addition, allograft rejection and postoperative inflammation accelerate CECs loss after transplantation. All of these sharpen the contradiction of cornea endothelium replacement tissue shortage.

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Constructing a biomimetic corneal endothelium graft with abundant CECs amount and accessible cost budget is a potential way to overcome this difficulty, as *in vitro* CECs could expand and remain cell phenotype and function in a few passages.[4] Through the years, a number of studies have been done to reduce endothelial to mesenchymal transformation by optimizing culture medium composition and culture substrate.[5-7] This technique would allow one donor cornea to potentially treat multiple patients. Collagen gels have been considered as a promising material to mimic cornea Descemet's membrane and stroma for its good biocompatibility and acceptable price.[8,9] However, the hydrated properties make this material weak and hard to manipulate which largely limited its application. While compressed, collagen (CC) gels are mechanically stronger and denser than the conventional type and its ultrastructure correspond more with nature cornea stroma.[10,11] Our previous work have shown its capability to support the generation of human corneal epithelial cell surface layer.[12] Here, bovine corneal endothelial cells (B-CECs) were used to investigate whether CC can be a suitable substrate to construct a biomimetic endothelium graft.

METHODS

Ethical approval
This study adhered to the tenets of the 1964 *Declaration of Helsinki* and has got the permission by Ethics Committee (Peking University Third Hospital Medical Science Research Ethics Committee).

Cell isolation and culture
Bovine eyes received from local slaughterhouse were processed within 3 h after enucleation. Circular resect the whole cornea 1 mm outer the corneal limbus to get corneal explants. B-CECs were isolated and cultivated as previously described.[13] Wash the corneal explants 3 times with ice-cold phosphate-buffered saline (PBS) containing 2% penicillin-streptomycin and 50 µg/ml gentamicin. Strip Descemet's membrane with abacterial surgical forceps under dissecting microscope. Add trypsin to the strips and incubated for 10 min under 37°C to isolate B-CECs from Descemet's membrane then centrifuged (300 ×g, 5 min). B-CECs were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin-streptomycin on gelatin-coated 6-well dish at 37°C in incubator containing 5% CO_2. The fresh medium was replaced every 2 days. B-CECs grown to form a confluent monolayer in 5–7 days of incubating and were used between the second and the fourth passages. All purchased from Fisher Scientific, UK.
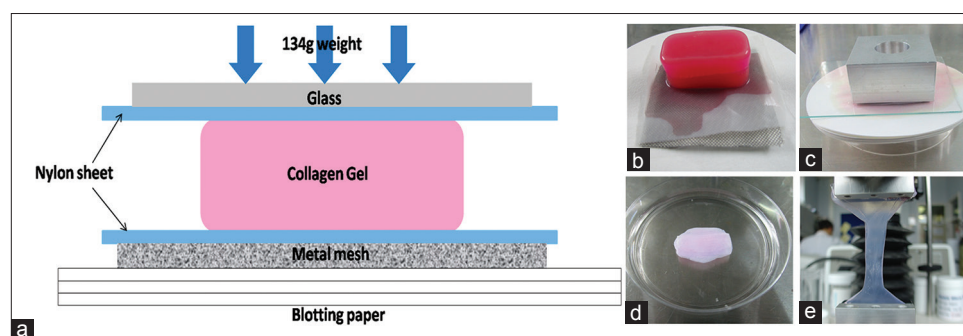
Preparation of compressed collagen gels
Mix 4 ml rat-tail Type I collagen (First Link Ltd, UK), 1 ml modified Eagle's minimum essential medium (Gibco, UK), and 0.5 ml sodium hydroxide (1 mol/L; Fisher, UK). Cast the solution into a rectangular molds (33 mm × 22 mm × 8 mm), then gelling at 37°C, 5% CO_2 for 30 min to form the conventional uncompressed collagen gel. The CC gel was construct by compressing the conventional collagen gel between two nylon mesh (50 µm mesh size) layers under 134 g pressure for 5 min at room temperature to eventually get a compressed sheet, shown in Figure 1. This method has been used in our previous study.[11,12] The mechanical properties were assessed by a Texture Analyser (Stable Micro Systems, UK), CC samples were clamped to the machine and pulled in opposite vertical directions until broken at a test speed of 0.1 mm/s.[14] Young's modulus was calculated using the formula: $E = (F/A)(L/\Delta L)$ (A: unstressed cross-sectional area; F: The force; L: Unstressed length; and ΔL: Change in length).[10]

Culture of bovine corneal endothelial cells on biomimetic substrate
Transfer the CC gel into a 12-well tissue culture insert with polycarbonate membrane base (Invitrogen, Fisher Scientific, UK). The surface of CC was coated with laminin (1.5 µg/cm²) for 2 h to enhance cell adherence ability. B-CECs were seeded onto CC surface at a density of 1×10^9/ml (0.5 ml per well) and incubated at 37°C, 5% CO_2 for 7 days on the cells reaching confluence then the biomimetic monolayer endothelium sheets were ready for further examination.[15] Cell density was measured by counting cell numbers in at least four fields of view from four different CC constructs seeded with cells. The number of cells per mm² was then calculated.

Scanning electron microscopy
B-CECs on CC were examined by scanning electron microscope (SEM). Samples for SEM examination were fixed in 2.5% glutaraldehyde solution for 2 h at 4°C and washed

Figure 1: Preparation of compressed collagen gel. (a) Schematic diagram. (b) Uncompressed collagen gel. (c) Compressing progress. (d) Biomechanical test of compressed collagen gel. (e) Mechanical properties test for compressed collagen gel.

3 times for 10 min with distilled water. Samples were then postfixed with 1% osmium tetraoxide for 2 h and washed with distilled water 1 time before gradient dehydrated at critical point followed by AuPd sputtering. SEM (FEI Quanta FEG 600, Oregon, USA) were used to observe the samples.[16]

Immunohistochemistry and imaging

The biomimetic monolayer endothelium sheets were rinsed in PBS, embedded, and frozen at −80°C. 7–10 μm thick cryostat sections were collected onto polylysine-coated slides and air-dried for 2 h before immunolabeling. The sheets were then fixed in 100% methanol for 15 min then 100% acetone for 5 min at −20°C, after that incubated with 1% (w/v) bovine serum albumin (Sigma-Aldrich, UK) at room temperature to block nonspecific binding sites. Primary antibody against ZO-1 (1:50, Chemicon, UK) were added onto the sheets and incubated overnight at 4°C. Fluorescein isothiocyanate-labeled secondary antibody (1:50, Sigma-Aldrich, UK) were subsequently incubated with the sheets for 1 h at room temperature.[12] For the last step, the sheets were costained with propidium iodide (Sigma-Aldrich, UK). The immunolabeled endothelium sheets were observed under fluorescence microscopy (Carl Zeiss Meditec, Germany).

RESULTS

Structure features of biomimetic endothelium graft

The biomimetic endothelium models were manufactured by culturing B-CECs on CC, which Young's modulus was 1724.2 ± 45.4 kPa. Seven days after seeding, B-CECs adhered to CC and reached confluence. The morphology of B-CECs and structure characterizations of the sheets are shown in Figure 2. B-CECs formed a monolayer on CC, shown in Figure 3, vertical section. The cells were closely aligned and uniformed with a cell density of 3612.2 ± 43.4 cells/mm^2 on average.

Ultrastructure of bovine corneal endothelial cells on compressed gel

We used SEM to observe the ultrastructure and the surface of this *in vitro* endothelium sheets. Numerous microvilli and cell borders interdigitated flaps were presented, tight cell-cell junction can be identified as well, which are the specific structures of endothelium cells,[17,18] shown in Figure 4. Most of the B-CECs showed a hexagonal shape, which corresponding to the physiological phenotype of endothelium cells.

Bovine corneal endothelial cells expressed ZO-1 on basement membrane model

Immunohistochemistry results are presented in Figure 3. The ZO-1, a tight junction protein necessary for pump function, expression can be detected in the bioengineered endothelium sheets. ZO-1 expression on cell membrane indicate that B-CECs transfer from Descemet's membrane to CC may still remain the key function of endothelial cells.
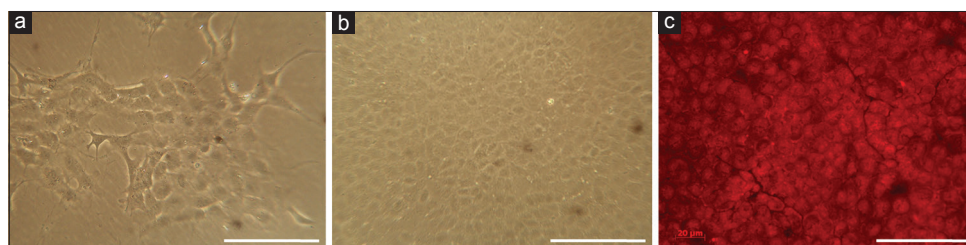
Figure 2: Structure of endothelium graft model. (a) B-CECs adherent to CC. (b) B-CECs reached confluence on CC. (c) PI staining for a confocal section. Scale bar = 50 μm. B-CECs: Bovine corneal endothelial cells; CC: Compressed collagen; PI: Propidium iodide.
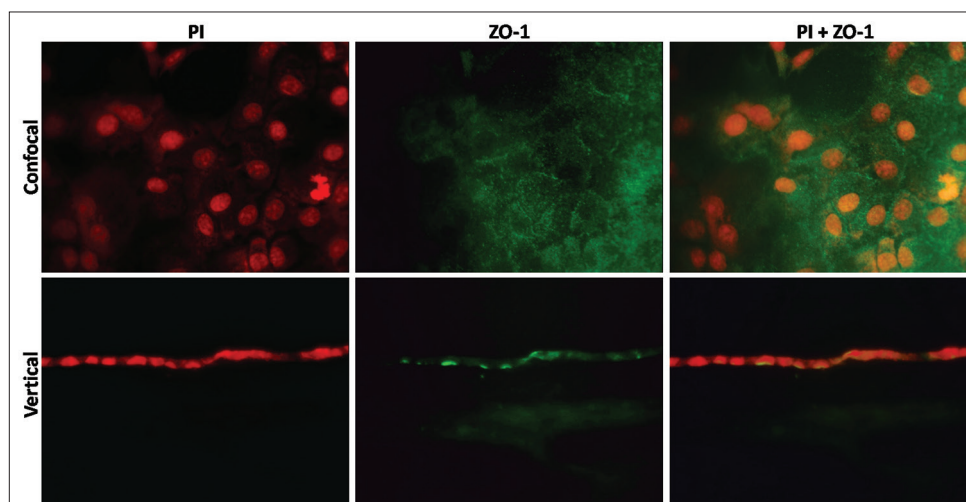
Figure 3: ZO-1 staining for confocal and vertical sections of bioengineered endothelium graft. Green = ZO-1, Red = PI. Scale bar = 50 μm.
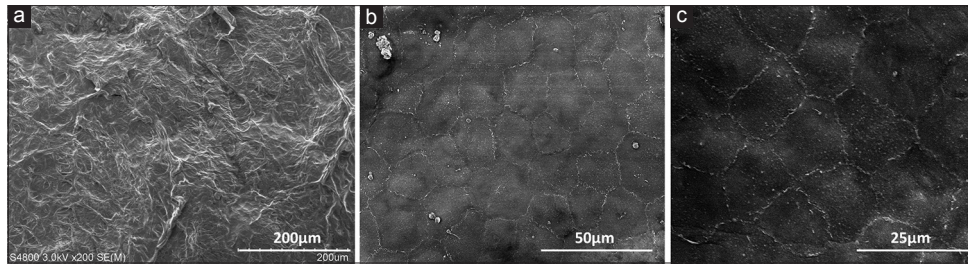
Figure 4: SEM of the compressed collagen gel and endothelium graft model. (a) Surface of compressed collagen gel. (b) Surface of endothelium graft model. (c) Tight junction and microvilli of B-CECs. B-CECs: Bovine corneal endothelial cells; SEM: Scanning electron microscope.

DISCUSSION

Cornea endothelium is a monolayer of hexagonal cells forming the corneal inner surface, as well as the barrier between cornea and aqueous humor.[19] CECs pump water from corneal stroma to anterior chamber actively to maintain cornea's transparency and relative dehydration status and they do not divide after birth *in vivo*. The density of CECs declines gradually throughout lifetime, from approximately 3500–4000/mm^2 at birth to 2000–2500/mm^2 in adults, and once the cell density drops below 500/mm^2, cornea would loss its transparency causing visual function deficiency. Descemet's membrane, which consists of Type IV collagen, is secreted by the endothelial cells. It is 3 μm at birth and increases in thickness with age to reach 30–40 μm in the elderly.

Posterior lamellar techniques, such as Descemet stripping (automated) endothelial keratoplasty (DSEK/DSAEK) and Descemet membrane endothelial keratoplasty (DMEK),[20] have been developed and became an alternative to the traditional full thickness corneal replacement known as penetrating keratoplasty in the early stage of corneal decompensation. In general, thinner graft results in shorter rehabilitation time and better visual function outcome.[21]

The thickness of posterior cornea grafts dissected by microkeratome in DSAEK is not that predictable.[22,23] While DMEK grafts preparation requires much more training and suffer a higher failure rate,[24] McCauley *et al.*[25] even reported a grafts reattachment rate up to 25% after DMEK surgery. Constructing an *in vitro* bioengineered graft can achieve a predictability in both sheets' thickness and cell density. As for CC, this biomaterial has easy production progress and is able to be manipulated to achieve the ideal thickness, which may reach the balance point between decreasing recovery time and reducing grafts dislocation rate. Alarcon *et al.*[26] even successfully added antibiotic properties to collagen implants foreboding the broad application prospect of collagen-constructing grafts. Laminin is coated onto CC to form effective connection and increase grafts cell density, for CECs express laminin receptor and previous experimental study have proved that laminin can promote adhesion, migration, and moderate proliferation of cultured CECs.[5,27] Levis *et al.*[28] seeded CECs onto CC without coated laminin and reached a final graft cell density <2000 cells/mm^2, while in our study, laminin-coated CC can eventually got a graft cell density more than 3600 cells/mm^2.

In our study, B-CECs formed a confluent monolayer on CC. The morphology of cells were similar to *in vivo* status, displayed a homogeneous and hexagonal shape, with the cell density higher than cadaveric graft. The ultrastructural analysis also suggested an integral endothelial layer with tight cell junction and microvilli, which are critical structure for endothelium cell to form a boundary and pump water from corneal stroma into aqueous humor. ZO-1 expression identified in this bioengineered graft further confirmed this observation. ZO-1 is a prominent protein in cell tight junction complex, is also considered to regulate paracellular permeability.[16,29] Bioengineering an endothelial sheet relies on the fact that the CECs can be expanded *in vitro*, but this approach would fail if cells did not maintain functional phenotype. The ZO-1 labeled at B-CECs lateral cell borders indicated that transferred B-CECs still remain the key function which underpins the possibility of constructing a biomimetic endothelium sheet with viability.

To establish a bioengineered graft that can apply to real clinic require further study. Our study is a heterogenic study using bovine cells, human endothelial cells' expansion ability on CC should be tested, as well as the cell function phenotype of transferred human endothelium cell. The animal experiment also needs to be performed to verify the safety and efficiency of graft.

We successfully constructed a biomimetic endothelium graft with B-CECs expanded on CC sheet. The morphology and ultrastructure of cells on biomaterial similar to that of endothelial cells *in vivo*. The graft has also been improved to possess the basic specific function of endothelium layer. This method set the cornerstone of using one donor's cornea to form multiple uniformed endothelium grafts to overcome the shortage of cadaveric cornea tissue.

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Conflicts of interest
There are no conflicts of interest.

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以压缩胶原为基底构建新型三维仿生角膜内皮植片

摘要

背景：异体角膜内皮移植是治疗角膜内皮失代偿的首选方法。在世界范围内，人角膜供体组织供不应求，急需内皮植片替代材料。压缩胶原具生物相容性佳、易于制备、易于加工的特点，是生物工程研究的重要材料。本研究尝试以压缩胶原为内皮细胞支架构建一种新型角膜内皮植片。

方法：分离牛角膜内皮细胞，经体外扩增后接种于Laminin包被后的压缩胶原薄膜上。观察显微镜下植片内皮细胞结构，并应用扫描电子显微镜观察内皮细胞超微结构。免疫组化染色验证内皮细胞紧密连接蛋白ZO-1的表达情况。

结果：在压缩胶原薄片上制备出单层且具有正常内皮细胞六角形态、超微结构和高细胞密度（3612.2 ± 43.4 cells/mm²）的角膜内皮植片，ZO-1正常表达于植片内皮细胞的细胞膜上。

结论：成功构建以压缩胶原为支架的角膜内皮细胞植片，植片内皮细胞具有正常形态和基础细胞功能。这一方法提供了利用一个供体角膜经细胞体外扩增制备多个角膜内皮移植物的可能性，用以克服人角膜组织供体的不足。