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 ± 43.4 cells/mm²). 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
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. Through the years, a number of studies have been done to reduce endothelial to mesenchymal transformation by optimizing culture medium composition and culture substrate. 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. 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. Our previous work have shown its capability to support the generation of human corneal epithelial cell surface layer.

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. 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% CO₂. The fresh medium was replaced every 2 days. B-CECs grown to 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% CO₂ 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. 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. Young’s modulus was calculated using the formula: $E = \frac{F}{A} \cdot \frac{L}{\Delta L}$ (A: unstressed cross-sectional area; F: The force; L: Unstressed length; and $\Delta L$: Change in length).

**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 × 10⁶/ml (0.5 ml per well) and incubated at 37°C, 5% CO₂ for 7 days on the cells reaching confluence then the biomimetic monolayer endothelium sheets were ready for further examination. 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 with 2.5% glutaraldehyde solution for 2 h at 4°C and washed...
3 times for 10 min with distilled water. Samples were then postfixed with 1% osmium tetroxide 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/mm² 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.

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**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.
Cornea endothelium is a monolayer of hexagonal cells forming the corneal inner surface, as well as the barrier between cornea and aqueous humor. 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/mm² at birth to 2000–2500/mm² in adults, and once the cell density drops below 500/mm², 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), 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.

The thickness of posterior cornea grafts dissected by microkeratome in DSAEK is not that predictable. While DMEK grafts preparation requires much more training and suffer a higher failure rate, McCauley et al. 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 predictable in both sheets’ thickness and cell density. However, the graft has also been improved to form multiple uniformed endothelium grafts to overcome the shortage of cadaveric cornea tissue.

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. Bioengineering an endothelium 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.

**REFERENCES**
1. Tan DT, Dart JK, Holland EJ, Kinoshita S. Corneal transplantation. Lancet 2012;379:1749-61. doi: 10.1016/s0140-6736(12)60437-1.
2. Brunette I, Roberts CJ, Vidal F, Harissi-Dagher M, Lachaine J, Sheardown H, et al. Alternatives to eye bank native tissue for corneal stromal replacement. Prog Retin Eye Res 2017;59:97-130. doi:
Adhesion, migration, and proliferation of cultured human corneal epithelial cells isolated from paired donor corneas. PLoS One 2011;6:e28310. doi: 10.1371/journal.pone.0028310.

Yamaguchi M, Eshara N, Shima N, Kimoto M, Funaki T, Yokoo S, et al. Adhesion, migration, and proliferation of cultured human corneal endothelial cells by laminin-5. Invest Ophthalmol Vis Sci 2011;52:679-84. doi: 10.1167/iovs.10-5555.

Gao Y, Zhou Q, Qu M, Yang L, Wang Y, Shi W, et al. In vitro culture of human fetal corneal endothelial cells. Graefes Arch Clin Exp Ophthalmol 2011;249:663-9. doi: 10.1007/s00417-010-1547-y.

Choi JS, Kim EY, Kim MJ, Giegenack M, Khan FA, Khang G, et al. In vitro evaluation of the interactions between human corneal endothelial cells and extracellular matrix proteins. Biomed Mater 2013;8:014108. doi: 10.1088/1748-6041/8/1/014108.

Wray LS, Orwin EJ. Recreating the microenvironment of the native cornea for tissue engineering applications. Tissue Eng Part A 2009;15:1463-72. doi: 10.1089/ten.tea.2008.0239.

Mi S, Connon CJ. The formation of a tissue-engineered cornea using plasticly compressed collagen scaffolds and limbal stem cells. Methods Mol Biol 2013;1014:143-55. doi: 10.1007/978-1-62703-432-6_9.

Hadijanayi E, Mudera V, Brown RA. Close dependence of fibroblast proliferation on collagen scaffold matrix stiffness. J Tissue Eng Regen Med 2009;3:77-84. doi: 10.1021/term.08.0176.

Mi S, Chen B, Wright B, Connon CJ. Plastic compression of a collagen gel forms a much improved scaffold for ocular surface tissue engineering over conventional collagen gels. J Biomed Mater Res A 2010;95:447-53. doi: 10.1002/jbm.a.32861.

Feng Y, Foster J, Mi S, Chen B, Connon CJ. Influence of substrate on corneal epithelial cell viability within ocular surface models. Exp Eye Res 2012;101:97-103. doi: 10.1016/j.exer.2012.05.005.

Guo Y, Liu Q, Yang Y, Guo X, Lian R, Li S, et al. The effects of ROCK inhibitor Y-27632 on injectable spheroids of bovine corneal endothelial cells. Cell Reprogram 2015;17:77-87. doi: 10.1089/cell.2014.0070.

Mi S, Khutoryanskii VV, Jones RR, Zhu X, Hamley IW, Connon CJ, et al. Photochemical cross-linking of plasticly compressed collagen gel produces an optimal scaffold for corneal tissue engineering. J Biomed Mater Res A 2011;99:1-8. doi: 10.1002/jbm.a.33152.

Palchesko RN, Funderburgh JL, Feinberg AW. Engineered basement membranes for regenerating the corneal endothelium. Adv Healthc Mater 2016;5:2942-50. doi: 10.1002/adhm.201600488.

Mi S, Chen B, Wright B, Connon CJ. Ex vivo construction of an artificial ocular surface by combination of corneal limbal epithelial cells and a compressed collagen scaffold containing keratocytes. Tissue Eng Part A 2010;16:2091-100. doi: 10.1089/ten.tea.2009.0748.

Waring GO, Bourne WM, Edelhauser HF, Kenyon KR. The corneal endothelium. Normal and pathologic structure and function. Ophthalmology 1982;89:531-90. doi: 10.1016/s0161-6420(82)34746-6.

Sumide T, Nishida K, Yamato M, Ide T, Hayashida Y, Watanabe K, et al. Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces. FASEB J 2006;20:392-4. doi: 10.1096/fj.04-30356e.

DelMonte DW, Kim T. Anatomy and physiology of the cornea. J Cataract Refract Surg 2011;37:588-98. doi: 10.1016/j.jcrs.2010.12.037.

Melles GR, Ong TS, Ververs B, van der Wees J. Descemet membrane endothelial keratoplasty (DMEK). Cornea 2006;25:987-90. doi: 10.1016/j.ico.2005.12.038.

Dapena I, Ham L, Mellers GR. Endothelial keratoplasty: DSEK/DSAEK or DMEK – the thinner the better? Curr Opin Ophthalmol 2009;20:299-307. doi: 10.1097/ico.0b013e328328d18.

Chen ES, Terry MA, Shamie N, Hoar KL, Friend DJ. Precise tissue in Descemet’s stripping automated endothelial keratoplasty donor characteristics and early postoperative complications. Ophthalmology 2008;115:497-502. doi: 10.1016/j.ophtha.2007.11.032.

Thiel MA, Kaufmann C, Dedes W, Bochmann F, Becht CN, Schipper I, et al. Predictability of microkeratome-dependent flap thickness for DSAEK. Klin Monbl Augenheilkd 2009;226:230-3. doi: 10.1055/s-0028-1109243.

Vianna LM, Stoeger CG, Galloway JD, Terry M, Cope L, Belfort Jr., et al. Risk factors for eye bank preparation failure of Descemet membrane endothelial keratoplasty tissue. Am J Ophthalmol 2015;159:829-34. doi: 10.1016/j.ajo.2015.01.030.

McCaulley MB, Price MO, Fairchild KM, Price DA, Price FW Jr. Prospective study of visual outcomes and endothelial survival with Descemet membrane automated endothelial keratoplasty. Cornea 2011;30:315-9. doi: 10.1097/ICO.0b013e3181e571b.

Alarcon EI, Vulesevic B, Argawal A, Ross A, Bejjani P, Podrebarac J, et al. Coloured cornea replacements with anti-infective properties: Expanding the safe use of silver nanoparticles in regenerative medicine. Nanoscale 2016;8:6484-9. doi: 10.1039/c6nr01339b.

Okumura N, Kukatani K, Numata R, Nakahara M, Schlötzer-Schrehardt U, Kruse F, et al. Laminin-511 and -521 enable efficient in vitro expansion of human corneal endothelial cells. Invest Ophthalmol Vis Sci 2015;56:2933-42. doi: 10.1167/iovs.14-15163.

Levis HJ, Peh GS, Toh KP, Poh R, Shortt AJ, Drake RA, et al. Plastic compressed collagen as a novel carrier for expanded human corneal endothelial cells for transplantation. PLoS One 2012;7:e50993. doi: 10.1371/journal.pone.0050993.

Palchesko RN, Lathrop KL, Funderburgh JL, Feinberg AW. In vitro expansion of corneal endothelial cells on biomimetic substrates. Sci Rep 2015;5:7955. doi: 10.1038/srep07955.
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。