**Title: Experimental models of corneal endothelial cell therapy and translational challenges to clinical practice.**

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**Review highlights**

* Recent literature has shown significant advances in the field of cell therapy research for corneal endothelial disorders.
* Most corneal endothelial cell therapystudies have been performed in rabbits with only limited evidence from clinical studies.
* There is a need to trace the fate of transplanted CE cells, in order to develop effective storage and delivery strategies.

**Abstract**

The human corneal endothelium (CE) is a post-mitotic monolayer of endothelial cells, thought to be incapable of *in vivo* regeneration. Dysfunction of the CE is a commonly cited indication for corneal transplantation, with corneal blindness being the fifth most common cause of blindness globally. In 2012 alone 184,576 corneal transplants were performed in 116 countries (Gain, *et al.*, 2016). Presently, outcomes following human corneal transplantation have been reported to have over 97% success rate in restoring the recipient’s vision (Patel, et al., 2019). However, the continuing demand for cadaveric human corneas has driven research into alternative sources of CE and with the advent of protocols to produce cultured hCECs there is now the potential for cell therapy to regenerate the damaged CE. This review aims to examine the merits and limitations of different types of human and animal models used so far to test the concept of CE cell therapy.

**Key words and phrases:**

Corneal endothelium; cell therapy; corneal endothelial cell transplantation; Fuchs corneal dystrophy

**List of Abbreviations**

|  |  |
| --- | --- |
| APCM | Acellular porcine corneal matrix |
| Asc-2P | L-ascorbic acid 2-phosphate |
| AV-SF | Aloe vera gel and silk fibroin scaffold |
| BCS | Bovine calf serum |
| BCVA | Best corrected visual acuity |
| bFGF | Basic fibroblast growth factor |
| BM-EPCs | Bone marrow-derived endothelial progenitor cells |
| BMHAM | Basement membrane of human amniotic membrane |
| CCT | Central corneal thickness |
| CE | Corneal endothelium/Corneal endothelial |
| CS | Calf serum |
| CECs | Corneal endothelial cells |
| CEC-like cells from ESC | Corneal endothelial cell-like cells from embryonic stem cells |
| ChS | Chondroitin sulfate |
| CX-43 | Connexin-43 |
| dAM | Denuded amniotic membrane |
| DM | Descemet’s membrane |
| DMEK | Descemet’s membrane endothelial keratoplasty |
| DMEM | Dulbecco’s modified Eagle’s medium |
| DNase | Deoxyribonuclease I |
| DSAEK | Descemet’s Stripping Automated Endothelial Keratoplasty |
| ECD | Endothelial cell density |
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| EGM-2 | Endothelial growth medium-2 |
| ESCs | Embryonic stem cells |
| EU | European Union |
| FACS | Fluorescence-activated cell sorting analysis |
| FCED | Fuchs’ corneal endothelial dystrophy |
| FCS | Foetal calf serum |
| FBS | Foetal bovine serum |
| FESEM | Field Emission Scanning Electron Microscopy |
| FGF | Fibroblast growth factor |
| FGF-2 | Fibroblast growth factor 2 |
| H&E | Haematoxylin and eosin stain |
| hCECs | Human corneal endothelial cells |
| HCEP | Human corneal endothelial progenitors |
| hEGF | Human epidermal growth factor |
| hESCs | Human embryonic stem cells |
| HM | Hand movements |
| HPCTS | Hydroxypropyl chitosan |
| ICE | Iridocorneal endothelial syndrome |
| IGF | Insulin-like growth factor |
| IOP | Intraocular pressure |
| logMAR | Logarithm of the Minimum Angle of Resolution |
| MEM | Eagle's Minimal Essential Medium |
| MCECs | Monkey corneal endothelial cells |
| MSCs | Mesenchymal stem cells |
| MSC-CM | Human bone marrow-derived mesenchymal stem cell conditioned medium |
| NCS | Newborn calf serum |
| NGF | Nerve growth factor |
| NH4OH | Ammonium hydroxide |
| OCT | Optical coherence tomography |
| PAPCM | Posterior acellular porcine corneal matrix |
| PBS | Phosphate-buffered saline |
| PKP | Penetrating keratoplasty |
| PL | Perception of light |
| PR | Projection of rays |
| PPCD | Posterior polymorphous corneal dystrophy |
| qPCR | Quantitative real-time polymerase chain reaction |
| RAFT | Real architecture for 3D tissues |
| RCECs | Rabbit corneal endothelial cells |
| RCEC-iron | RCEC that had been exposed to spherical iron powder |
| RNase | Ribonuclease A |
| ROCK | Rho-associated protein kinase |
| RPMI | Roswell Park Memorial Institute |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| SAD | Sodium alginate dialdehyde |
| SCGS | Spherically Curved Gelatin Sheet |
| SDS | Sodium dodecyl sulphate |
| SEM | Scanning electron microscopy |
| SFM | Serum-free medium |
| SPM | Superparamagmetic spheres |
| TBS | Trisbuffered saline |
| TEM | Transmission electron microscopy |
| TGF-β | Transforming growth factor-beta |
| UK | United Kingdom |
| VA | Visual acuity |
| VEGF | Vascular endothelial growth factor |
| ZO-1 | Zonula occludens-1 |

**1. Introduction**

Across the globe, there is a significant disease burden caused by corneal endothelial dysfunction. This has contributed to substantial rates of corneal blindness, the fifth leading cause of blindness globally. Human corneal endothelial cells are generally considered post-mitotic and are, therefore, incapable of replacing damaged or lost cells via mitosis. Studies on donor human corneal endothelium have shown that CE cells do not exit the cell cycle and are arrested in G1 phase due to contact inhibition and TGF-β expression (Joyce et al., 1996; Joyce et al., 2002). Normally, humans have a sufficient density of corneal endothelial cells to last for a lifetime. However, excessive cell loss may occur as a result of previous ocular surgeries, or because of some pathological conditions, such as Fuchs’ corneal endothelial dystrophy (FCED), posterior polymorphous corneal dystrophy (PPCD), Iridocorneal endothelial syndrome (ICE), herpes viral infections, trauma, or raised intraocular pressure, which could cause CE decompensation. As a result of CE decompensation, the cornea may become swollen and oedematous leading to loss of transparency and corneal blindness.

Currently, the mainstay of clinical treatment is endothelial keratoplasty (EK), a technically challenging procedure that is hampered by a shortage of suitable and available human donor material (Gain et al., 2016). In recent clinical practice, Descemet’s Stripping Automated Endothelial Keratoplasty (DSAEK) and Descemet’s Membrane Endothelial Keratoplasty (DMEK) are the preferred methods for treating corneal endothelial disorders (Melles, et al., 2002; Thomas, et al., 2013). These procedures are generally very efficient at restoring normal vision and show very good long-term results (Rajan, 2014). However, the preparation of DSAEK and DMEK transplants requires whole corneas from cadaveric donors, which are in high demand globally. At the time of writing this article there is a global donor shortage, which limits the treatment of corneal blindness especially in resource-poor parts of the world. As a result of this shortage of donors, there is a need for novel approaches to be developed that could potentially eliminate the need for donor tissue. The lack of quality, limited numbers of donors, and surgical complexity has promoted significant research interest in corneal endothelial cell therapies to circumvent the regular use of human donor corneas, and potentially avoid their use altogether. A few non-transplant requiring treatment methods have emerged recently, such as the use of Rho-associated coiled-coil forming kinase (ROCK) inhibitor eye drops to promote CE cell adhesion and proliferation (Meekins, et al., 2016; Okumura, et al., 2016a). More recently, however, some researchers are challenging the notion about the lack of *in vivo* capacity of the CE to regenerate itself and have proposed a non-transplant requiring treatment that only involves surgically removing the diseased central CE and DM and allowing the peripheral CE to renew and regenerate the central part that was removed (Van, et al., 2017).

The use of cultured corneal endothelial cells for transplantation has been explored since the 1970s and has been shown to be a promising method for restoring normal CE function (Jumblatt, et al., 1978; Gospodarowicz, et al., 1979 and Jumblatt, et al., 1980). However, the clinical application of this technique still remains limited by the restricted proliferative capacity of the human corneal endothelial cells (hCECs) as shown during *in vitro* culture, and the associated morphological transformation to a spindle-shaped phenotype. In order to study and test the potential of cell-based therapies to restore corneal endothelial cell function, a suitable model is needed that allows assessment of the post-cell therapy outcomes and determines the safety of the procedure. This would allow researchers to accurately compare the effects of cell therapy on corneal thickness and transparency in decompensated corneas, and enable them to test whether cell therapy is capable of restoring normal CE function. In order to fully understand the current state of research in this area, literature search was undertaken to collate and review information pertaining to the different models used for corneal endothelial cell transplantation testing.

**2. *Ex vivo* models of corneal cell therapy**

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**2.1 *Ex vivo* animal corneal endothelial cell therapy models**

Studies on animal corneal endothelial cell therapy utilise cadaveric animal corneas. A search of the literature in PubMed revealed 7 studies using *ex vivo* animal based corneal endothelial cell therapy models. The most prevalent recipient species used in these studies is bovine – reported 3 times, followed by rabbit – reported 2 times and porcine – reported 2 times. A single study reported using two recipient species in their model (bovine and rabbit). The most commonly used species, as a source of donor material is human, reported 6 times, whereas the use of bovine donor CE was reported only once. These *ex vivo* studies uniformly reported that *in vitro* cultured corneal endothelial cells can be successfully transplanted into various *ex vivo* cultured animal corneas and demonstrate a capacity to attach and form a monolayer of flattened cells that maintained expression of corneal endothelial cell markers ZO-1 and Na+/K+-ATPase (Table 1).

**Table 1 – Corneal endothelial cell transplantation in *ex vivo* cultured animal corneas or various substrate carriers, presented in chronological order of publication.**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Recipient corneas** | **Species for cultured cells** | **Cell culture medium** | **Substrate carrier** | **Seeding density** | **Method for inducing CE dysfunction** | **Transplantation technique** | **ROCK inhibitor used** | **Functional assessment method** | **Outcome** | **Follow-up period** | **References** |
| Bovine and Rabbit corneal buttons (11 mm) | Bovine (passage 2-3) | DMEM, 10% CS, 5% Dextran, 50μg/ml Gentamycin, and 2.5μg/ml Fungizone. 100ng/ml FGF was added every other day. | N/A | 2.5x104 to 3x105 cells per button | CE removed by sweeping with a cotton swab. | Cell suspension transplantation | None | Alizarin red staining | Flattened morphology and attachment to DM | 24 hours | Gospodarowicz and Greenburg, 1979 |
| Rabbit | Human (passage 101) | DMEM/F12, 20% BCS, 10 ng/ml bFGF, 10ng/ml EGF, 50μg/ml N-acetylglucosamine hydrochloride, 50 μg/ml glucosamine hydrochloride, 0.8mg/ml ChS, 50μg/ml oxidation-degradation products of ChS, 50μg/ml carboxymethyl-chitosan, 5ng/ml bovine ocular extracts, 10% (v/v) culture supernatant of HCS cells at logarithmic phase | Denuded amniotic membrane (dAM) | 1.67×106 cells/ml with 20% FBS-containing DMEM/F12 medium | CE scraped away with metal scrapers. DM intact. | Seeding of cell suspension | None | Light microscopy, Alizarin red staining, Immunofluorescence assay, Electron microscopy | Maintained expression of marker proteins, cell-junction proteins and membrane transport proteins. Had excellent biocompatibility to dAM | 116 hours | Fan, et al., 2011a and 2011b |
| Porcine eyes | 1. Human (passage 2-3)  2. Immortalized human cell line (B4G12) | F99, 5% FBS, 10ng/ml bFGF, 20 mg/ml Asc-2P, 20 mg/ml bovine insulin, 2.5 mg/ml transferrin, 0.6 ng/ml sodium selenite | Plastic compressed collagen type I (RAFT) | 2000-4000 cells/mm2 for 4-14 days | Not specified | Pull-through/air-bubble technique similar to DSAEK | None | OCT, histology, immunoflu-orescent staining, electron microscopy | RAFT transplantation, using Tan EndoGlideTM insertion system. Well-integrated endothelial layer present. | No incubation. Only the ease of transplantation using Tan EndoGlideTM insertion system was demonstrated. | Levis, et al, 2012 |
| Decellularized porcine corneas | Human (passage 1) | DMEM⁄ F12, 5% FCS, 100 U⁄ml penicillin G, 100μg/ml streptomycin sulphate, 1.25g⁄ml amphotericin B, 0.1ng⁄ml EGF, 1.0ng⁄ml bFGF,1.0μg⁄ml hydrocortisone | N/A | 103cells⁄ml | 9.0 mm corneal buttons decellularized by a series of washes and incubation with EDTA, aprotinin, SDS, TBS, DNase and RNase | Injection of cell suspension | None | Immunolocalization of type I collagen, keratocan, lumican, cytokeratin-3 and type VIII collagen | Decellularized porcine corneas were successfully repopulated with human corneal cells | 14-30 days | Yoeruek, et al., 2012 |
| Decellularized Bovine Corneal Posterior Lamellae | Human (Primary) | DMEM/F12, 5% FCS, 100U/mL penicillin G, 100μg/mL streptomycin sulphate, 1.25g/mL amphotericin B, 0.1ng/mL EGF, 1.0μg/mL hydrocortisone | N/A | 5 × 104 cells | 5.0 mm posterior lamellae button decellularized by a series of washes and incubation with EDTA, aprotinin, SDS, TBS, DNase and RNase | Cell seeding | None | Immunohistochemistry of ZO-1, CX-43, Na+/K+-ATPase, Na+/HCO3−carboanhydrase, collagen type VIII, collagen type IV and cytokeratin-3 | Monolayer formation and positive expression of ZO-1, CX-43, Na+/K+-ATPase, Na+/HCO3−carboanhydrase, collagen type VIII, cytokeratin-3 | 1-14 days | Bayyoud, et al, 2012 |
| Cadaver bovine eye (n=3) | Human cadaver corneal endothelial precursor cells (passage 1) | DMEM/F12, 20 ng/mL EGF, 40ng/mL bFGF, B-27, 100U/mL penicillin, 100 mg/mL streptomycin, 250ng/mL amphotericin B | Nanocomposite Gel Sheet | 2.57 × 105 cells for 3 corneas | Native DM/Endothelium were left intact | Cell injection between the endothelium and the Nanocomposite gel sheet. | None | Histological observation | The injected cells had a successful engraftment | 7 days | Parikumar, et al., 2014 |
| Artificial collagen mass and equine collagen membrane | Human (Primary) | DMEM, 50U/mL penicillin, 50mg/mL streptomycin, 10% FBS, and 2ng/mL bFGF | Artificial collagen mass and equine collagen membrane | N/A | Artificial collagen mass was used so no need to induce decompensation | Cell seeding | None | Optical coherence tomography | DM is very important in proper functioning of hCECs | 48 hours | Tsaousis, et al., 2016 |

**2.2 *Ex vivo* human corneal endothelial cell therapy models**

The following studies utilised corneas dissected from whole eyes of deceased human donors. Through a literature search in PubMed a total of 9 articles were found that reported using an *ex vivo* human model for testing corneal endothelial cell transplantation of *in vitro* cultured human corneal endothelial cells. 8 out of the 9 studies identified used a single human donor source of CE and 1 of the studies reported using cultured human CE (up to the tenth passage) and porcine CE (Table 2). This method for cell transplantation testing showed the plausibility of a cell-therapy approach for treating corneal endothelial disorders. Researchers have had success in demonstrating efficient cell attachment, morphology and monolayer formation post-transplantation and restoring of normal CE function based on the corneal de-swelling measured by ultrasound pachymetry or OCT (Table 2). The use of human donor and recipient tissues also avoids the inherent between-species variation of animal studies, allowing more accurate modelling of endothelial cell biology and proliferative potential relative to the use of animal tissues. The *ex vivo* human studies collated in table 2 report a short term follow up ranging in length from 8 hours to 15 days, potentially reflecting the challenge of long term maintenance of these models in *ex vivo* conditions, which may limit their translational potential in human trials. Although there was a positive outcome associated with every study, Amano et al., (2005) reported lower Na+/K+ ATPase pump function, which may limit the translational potential of using human primary cell cultures as donor material. In addition to this challenge, the use of *ex vivo* human corneas as a model means that these studies lack the ability to model the effect of intraocular pressure and immune-mediated responses that would be encountered in a living human eye.

**Table 2 – *Ex vivo* cultured human corneas used for corneal endothelial cell transplantation studies, presented in chronological order of publication.**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Recipient corneas** | **Species for cultured cells** | **Cell culture medium** | **Seeding density** | **Method for inducing CE dysfunction** | **Transplantation technique** | **ROCK inhibitor used** | **Functional assessment method** | **Outcome** | **Follow-up period** | **References** |
| Human (n=41) | 1.Human (up to passage 10)  2.Porcine (Primary and passage 1) | - | 150,000 – 700,000 cells per cornea | (1) Mechanically, with a cotton swab; (2) chemically, with 0.04 μM ammonium hydroxide solution; (3) physically, by freezing to -80oC. | Seeding of cell suspension | None | SEM | Monolayer formed. FGF enhanced morphology | 4 weeks | Böhnke, et al., 1999 |
| Human (n=26) | Immortalized human cell line (HCEC-12) | F99 [1:1 Ham's F12 and M199], 5% NCS, 20μg/ml Asc-2P 20μg/ml bovine insulin, 2.5μg/ml transferrin, 0.5ng/ml sodium selenite, 10ng/ml FGF | 5,000 or 50,000 or 200,000 or 500,000 cells/200μl | Native CE had undergone complete necrosis during organ culture. DM was intact. | Seeding of cell suspension | None | Perfusion experiment, Confocal microscopy, Trypan blue/Alizarin red staining, SEM | 293±53 – 2293±377 cells/mm2 density. Normal corneal thickness | 8-12 hours | Aboalchamat, et al., 1999 |
| Human (n=7) | Human (Primary or passage 1) | OptiMEM-I, 8% FBS, 40ng/mL FGF, 5ng/mL EGF, 20ng/mL NGF, 20μg/mL Asc-2P, 0.005% human lipids, 200mg/L calcium chloride, 0.08% ChS, 1% RPMI-1640 vitamin solution, 50μg/mL gentamicin, antibiotic/antimycotic solution diluted 1/100 | 2.5-5x105 cells/mL | DM denuded of CE by treatment with 0.02N of NH4OH and 0.5% sodium deoxycholate, and then washed five times with 5 mmol/L EDTA | Seeding of cell suspension | None | TEM, Immunolocalization of (ZO-1) | Cells formed a monolayer and expressed ZO-1 | 7 - 14 days | Chen, et al., 2001 |
| Human | Human (passage 5) | DMEM, 15% FBS, 30mg/L L-glutamine, 2.5mg/L Fungizone, 2.5mg/L doxycycline, 2ng/mL bFGF | 2 x 105 cells in 2ml | CE scraped off from fresh human corneas with a sterile cotton swab. | Seeding of cell suspension | None | Ussing chamber, Alizarin red and trypan blue staining, Light and electron microscopy | Morphology and cellular density similar to hCEC *in vivo*, pump function was lower than in normal corneas | 1 day | Amano, et al., 2005 |
| Human (n=5) | Human (passage 2, incorporated with SPMs) | Opti-MEM-I, 8% FBS, 200 mg/mL Calcium chloride, 0.08% CS, 20μg/mL Asc-2P, 100 μg/mL Pituitary extract, 5 ng/mL EGF, 20ng/mL NGF, 10 mL/L Insulin-Transferrin-Selenium A Supplement, 20mL/L RPMI Vitamin Solution, 10mL/L Antibiotic/antimycotic | 300,000 – 1,000,000 cells per cornea | DM/Endothelium removed from the cornea of the anterior segment under an operating microscope | Seeding of cell suspension | None | CM-DiI-labelling, Histology, TEM | Cell attachment and monolayer formation | 3-7 days | Patel, et al., 2009 |
| Human (120-200 μm decellula-rized stroma) | Human (passage 4-5) | EGM-2, EGF, VEGF, FGF, IGF, hydrocortisone, gentamicin, amphotericin-B, 10% FBS | 130 cells/mm2 | Decellularization achieved by incubation in detergent solution for 72h followed by extensive washing with PBS | Seeding of cell suspension | None | SEM, histology, and immunocytoch-emistry | Na+/K+-ATPase and ZO-1 expression | 14 days | Choi, et al., 2010 |
| Human | Human umbilical cord blood mesenchymal stem cells | OptiMEM-1, 8% FBS, 40ng/mL FGF, 5ng/mL EGF, 20ng/mL NGF, 20μg/mL Asc-2P, 0.005% human lipids, 200mg/L calcium chloride, 0.08% ChS, 1% RPMI-1640 vitamin solution, 50μg/mL gentamicin, antibiotic/ antimycotic solution diluted 1/100 | 5×105 cells/ 400μl | Mechanical scrape wound made in the CE in an X-shaped pattern using a capsule polisher and DM was intact. The damaged CE was either removed or left on the DM. | Seeding of cell suspension | None | Immunocytochemistry, qPCR, light microscopy | Expressed ZO-1, N-cadherin and endothelial-like phenotype | 14 days | Joyce, et al., 2012 |
| Human corneal button with DM (n=15) | Human hESCs | Differentiation medium - including 5 μmol/L of ROCK inhibitor Y-27632 | 120,000 cells/cornea | 7.5-8.0mm button. CE removed using an ocular stick and DM was left intact | Seeding of cell suspension | 5 μmol/L of ROCK inhibitor Y-27632 was used in cell culture but none was used for transplantation | Immunohistochemistry | hESCs differentiate into corneal endothelial-like cells | 15 days | Hanson, et al., 2017 |
| Human (n=11) | 1. Human (Primary)  2. Immortalized human cell line (HCEC-12) | OptiMEM-I, 8% FBS, 5ng/mL EGF, 20mg/mL Asc-2P, 200mg/L calcium chloride, 0.08% CS, 50mg/mL gentamicin | 260,000-310,000 cells/ 200μL | From the trabecular meshwork margin the DM/Endothelium were scored 360 degrees circumferentially using a Sinsky hook | Seeding of cell suspension | None | Anterior Segment OCT, immunocytochemistry, histology | Normal corneal thickness | 12 hours | Rolev, et al., 2018 |

**3. *In vivo* animal models of corneal endothelial cell therapy**

These studies involved the use of live animals in laboratory research. Through a literature search in PubMed, a total of 35 articles were found that reported using an *in vivo* animal model for testing corneal endothelial cell transplantation of *in vitro* cultured corneal endothelial cells (Table 3). The most prevalent recipient animal species was rabbit - reported 21 times (60%), followed by primate – 7 times (20%), feline – 5 times (14%), and rat - 2 times (6%). Out of the 35 studies that were retrieved, 3 studies used more than one species as a source of cultured corneal endothelial cells for transplantation (Table 3). The most prevalent species used for *in vitro* corneal endothelial cell culture and transplantation was human - reported 14 times (37%), followed by rabbit - 10 times (26%), 5 primate (13%), 5 feline (13%), 2 bovine (5%), 1 murine (3%) and 1 rat (3%). 18 (51.4%) of the studies identified reported the use of a substrate carrier in the delivery of donor-cultured corneal endothelial cells while 17 (48.6%) used no substrate carrier. The most common transplantation technique is cell injection, utilised by 16 studies (45.7%). The use of a composite sheet is reported by 10 studies (28.6%) and the use of a surgical or combination transplantation technique is employed by 9 studies (25.7%). Overall, most of the studies report positive outcomes in restoring corneal clarity and thickness in the recipient animal models, demonstrating that *in vitro* cultured corneal endothelial cells retain their functional potential and are able to restore normal CE function after transplantation. Three of the studies do report adverse reactions in animal recipients, such as persistent corneal oedema and immune rejection (Koizumi et al., 2007; Ishino et al., 2004 and Tchah, 1992).

**Table 3 – *In vivo* animal models of corneal endothelial cell transplantation, presented in chronological order of publication.**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Recipient animal** | **Species for cultured cells** | **Cell culture medium** | **Substrate carrier** | **Seeding density** | **Method for inducing CE dysfunction** | **Transplantation technique** | **ROCK inhibitor used** | **Functional assessment method** | **Outcome** | **Follow-up period** | **References** |
| **Rabbit (n=4)** | Rabbit-RCEC-iron (passage 3-4) | Low glucose DMEM, 15% FBS, 2·5mg/1 fungizone, 2·5mg/1 doxycycline, 2ng/m1 bFGF | None | 1x105 cells/200μl | Cryo-injury of the center and the eight peripheral regions of the cornea | Cell injection (magnetic attraction) | None | Pachymeter, Tonometer, Slit-lamp examination, Histology, CM-Dil-labelling | Clear corneas | 8 weeks | Mimura, et al., 2003 |
| **Rat (n=4)** | Human (passage 4-5) | DMEM, 15% FBS, 2.5mg/l fungizone, 2.5mg/l doxycycline, and 2ng/ml bFGF | Denuded 4mm rat corneal buttons coated with 0.01% human plasma fibronectin | 1x106 cells/300μl | CE gently scraped off with a sterile cotton swab | PKP | None | Slit-lamp examination, Pachymetry, CM-Dil-labelling, Histology, Alizarin red and trypan blue staining | Thin and transparent grafts | 28 days | Mimura, et al, 2004a |
| **Rabbit (n=4)** | Human (passage 4-5) | Low-glucose DMEM, 15% FBS, 2.5mg/l amphotericin B, 2.5mg/l doxycycline, and 2 ng/ml bFGF | Collagen type I sheet coated with fibronectin | 1x106 cells in 300μl | 6.0mm excision of Descemet's membrane (DM) | Collagen sheet transplant | None | Slit-lamp examination, Pachymetry, Tonometry, Dil-labelling, Potential difference and short-circuit current measurement, Histology | Thin and transparent grafts | 28 days | Mimura, et al., 2004b |
| **Rabbit (n=4)** | Rabbit-RCEC-iron (passage 3-4) | Low glucose DMEM, 15% FBS, 2·5mg/1 fungizone, 2·5mg/1 doxycycline, 2ng/m1 bFGF | None | 5x105 cells/200μl | Cryo-injury of the center and the eight peripheral regions of the cornea | Cell injection (magnetic attraction) | None | Pachymeter, Pneumatic tonometer, Slit-lamp examination, TEM, Histology | Corneal transparency | 12 months | Mimura, et al., 2005a |
| **Rabbit (n=6)** | Rabbit (sphere-forming precursors) | DMEM/F12, B27, 20 ng/mL EGF, 20ng/mL bFGF | None | 1x107 cells | Cryo-injury of the center and the eight peripheral regions of the cornea | Cell injection | None | Pachymeter, Pneumatic tonometer, Slit-lamp examination, Ussing chamber, Immunocytochemistry, RT-PCR, Histology, Dil-labelling | Corneal transparency | 28 days | Mimura, et al., 2005b |
| **Rabbit (n=12)** | Human (passage 2) | Opti-MEM, 15% FBS, 40ng/ml of bovine pituitary FGF, 5ng/ml EGF, 20ng/ml NGF, 50μg/ml gentamicin, 1% antibiotic/antimycotic | Bio-degradable gelatin carrier | 4x104 cells/cm2 | Central 7.0mm of CE removed with a silicone tipped cannula | Gelatin / cell sheet construct transplant | None | Slit-lamp examination, Pachymetry, SEM, Immunostai-ning | Thin and transparent grafts | 28 days | Hsiue, et al., 2006 |
| **Rabbit** | Human (passage 5) | DMEM 10% FBS, 30μg/mL L-glutamine, 100 units/ml penicillin, 100μg/ml streptomycin, 2ng/ml bFGF | 7.0 mm rabbit corneal button | 3x106 cells per dish | 7.0 mm graft beds prepared from the central corneas by trephination | Corneal button/cell sheet graft | None | Slit-lamp examination, Pachymetry, SEM, BrdU-labeling, Light microscopy and immunofluo-rescence staining, Na+/K+ATP-ase activity | Thin and clear corneas | 7 days | Sumide, et al, 2006 |
| **Primate (n=3)** | Primate (passage 3-5) | DMEM, 10% FBS, 50 U/ml penicillin, 50μg/ml streptomycin, 2ng/ml bFGF | Collagen type I | 5-10x102 cells/mm2 | CE mechanically scraped with a 20-gauge silicone needle over ~9.0-mm area (the diameter of the cornea is ~10 mm) | Collagen carrier inserted and attached to DM by air injection. | None | Slit-lamp examination, Pachymetry, Specular microscopy, Alizarin red S staining, Light microscopy, Immunocyto-chemistry, Dil-labelling, TEM, SEM | Corneal transparency | 6 months | Koizumi, et al, 2007 |
| **Primate (n=1)** | Primate (passage 3-5) | DMEM, 10% FBS, 50 U/ml penicillin, 50μg/ml streptomycin, 2ng/ml bFGF | None | 8x104 cells in 50μl | CE mechanically scraped with a 20-gauge silicone needle over ~9.0-mm area (the diameter of the cornea is ~10 mm) | Cell-suspension injection | None | Pachymeter, Specular microscopy, Slit-lamp examination, Alizarin red staining, TEM, SEM, Immunocytochemistry | Corneal oedema | 6 months | Koizumi, et al, 2007 |
| **Rabbit (n=8)** | Human (passage 2) | Opti-MEM, 15% FBS, 40ng/ml FGF, 5ng/ml EGF, 20ng/ml NGF, 20μg/ml Asc-2P, 0.005% human lipids, 0.2mg/ml of calcium chloride, 0.08% ChS, 1% RPMI 1640 vitamin solution, 50 μg/ml of gentamicin, and 1% antibiotic/antimycotic | Gelatin carrier | 4x104 cells/cm2 | 7.00 mm of  CE was scrapped gently with a silicone tipped cannula | Cell sheet / gelatin construct transplant | None | Slit-lamp examination, Pachymetry, Tonometry, PKH26 labelling, Histology, TEM, Western blotting | Normal corneal thickness | 168 days | Lai, et al., 2007 |
| **Rabbit** | Human (passage 4-5) | DMEM/F12, B27, 20ng/ml EGF,c40ng/ml bFGF | None | 150 DiI-labeled hCEC spheres | Cryo-injury of the center and the eight peripheral regions of the cornea | Cell injection | None | Pachymeter, Pneumatic tonometer, Slit-lamp examination, Histology | Normal corneal thickness | 28 days | Mimura, et al., 2007 |
| **Feline (n=25)** | Feline (passage 3) | DMEM, 50 U/ml penicillin, 50 g/ml streptomycin, 8% FBS, 2.5ng/ml bFGF | BMHAM | 5.0x103 cells/mm2 | Excising a 7.50-mm corneal button in the centre of the cornea | 7.5mm cat corneal button/  BMHAM construct | None | Slit-lamp examination, Pachymetry, Light and electron microscopy, Trypan blue and alizarin red staining | Transparency and normal corneal thickness | 6 weeks | Wencan, et al., 2007 |
| **Feline (n=20)** | Feline (Primary) | Iscove’s medium, 20% FCS | None | 200,000 cells/200μl | Cryo-injury with a tip 10 mm in diameter | Cell injection | None | Histology and SEM | A healthy corneal endothelial monolayer | 30 days | Kiełbowicz, et al., 2010 |
| **Feline (n=4)** | Human CECs derived from BM-EPCs and foetal CECs (passage 1-2) | DMEM/F12,  10% FBS | 7.5mm porcine corneal acellular matrix | 2.0x103 cells/mm2 | 7.5-mm diameter defect was created in the CE and DM by gentle mechanical scraping using a blunt pinhead | Transplantation of the lamellar construct and attachment with an air bubble | None | Slit-lamp examination, Pachymetry, Dil-labelling, TEM, SEM, RT-PCR, Immunocytochemistry, Histology | Clear grafts | 28 days | Shao, et al., 2011 |
| **Rabbits (n=12)** | Rabbit (passage 1) | DMEM/F12, 20% FBS, antibiotics | None | 1x107 cells/ml | Cornea cut off like a “C”. The CE scrapped and the DM exposed | Cell injection and encapsulation by SAD and HPCTS solution | None | Slit-lamp examination, Histology, SEM | Clear grafts | 60 days | Liang, et al, 2011 |
| **Rat (n=8)** | Immortalized human cell line (B4G12) | 1.Leaching liquid from APCM  2.Human endothelial-SFM, 10ng/ml bFGF | None | 8.5x104 cells or 3000 cells/mm2 | Cryo-injury with a brass dowel | Cell injection | None | Slit-lamp examination, Specular microscopy, Pachymetry, Alizarin red staining, Histology, Immunofluorescent staining | Restored corneal transparency | 1 month | Ju, et al, 2012a |
| **Rat (n=15)** | CECs derived from rat neural crest cells | DMEM/F12 (3:1), 10% FBS | None | 8.5x104 cells (3000 cells/mm2) | Cryo-injury with a brass dowel | Cell injection | None | Slit-lamp examination, Pachymetry, Immunofluorescent staining, RT-PCR, Flow cytometry, Histology, Alizarin red staining | Clear grafts with normal thickness | 28 days | Ju, et al., 2012b and 2012c |
| **Rabbit (n=12)** | Rabbit (passage 3-5) | DMEM, 10%  FBS, 50 U/mL penicillin, 50μg/mL streptomycin, 2 ng/mL bFGF | None | 2x105 cells/200μl | CE mechanically scraped with a 20-gauge silicone needle | Cell injection +/- 100μM Y-27632 | 100μM Y-27632 ROCK inhibitor | Slit-lamp examination, Pachymetry, Dil-labelling, Histology, Alizarin red staining, Immunofluorescent staining | Y-27632 only produced corneal transparency | 14 days | Okumura, et al., 2012 |
| **Primate (n=2)** | Primate (passage 3-5) | DMEM, 10% FBS, 50 U/mL penicillin, 50μg/mL streptomycin, 2 ng/mL bFGF | None | 2x105 cells/200μl | CE mechanically scraped with a 20-gauge silicone needle over ~9.0-mm area (the diameter of the cornea is ~10 mm) | Cell injection +/- 100μM Y-27632 | 100μM Y-27632 ROCK inhibitor | Slit-lamp examination, Pachymetry, Dil-labelling, Histology, Alizarin red staining, Immunofluorescent staining | Corneal transparency was achieved in both +/- 100μM Y-27632 ROCK | 3 months | Okumura, et al., 2012 |
| **Feline (n=3)** | Human | DMEM/F12, 20% FBS | dAM | 3.22×106 cells | 7.0-mm central cornea was trephined | Lamellar keratoplasty | None | Slit-lamp examination, Pachymetry, Dil-labelling, Alizarin red staining, Histology, SEM, TEM | Clear grafts with near-normal thickness | 104 days | Fan, et al., 2013 |
| **Primate (n=3)** | Primate | Low-glucose DMEM, 15% FBS, antibiotics, 2ng/mL bFGF, 0.3mM Asc-2P | SCGSs | 4000 cells/mm2 | Central CE scraped (8 mm in diameter) with a 20-gauge silicone needle. Central DM peeled off (4 mm in diameter) | Transplantation of MCEC-SCGS constructs (4mm of central DM removed) | None | Slit-lamp examination, Pachymetry, Sodium Fluorescein and Protein Permeability  Assay, Immunocyto-chemistry, Histology, PKH26 labelling | Restored corneal transparency. Fibroblast-like cells migrated between gelatin sheet and stroma. | 28 days | Kimoto, et al., 2014 |
| **Rabbit (n=10)** | Human CEC-like cells from ESCs | DMEM/F12, 20% knockout serum replacement, 200mM l-glutamine, 10mM nonessential amino acids, 14.3M β-mercaptoethanol, and 8ng/ml bFGF | PAPCM lamella +DM | 2x105 cells/mm2 for 4h | 7.5mm of the DM/Endothelium peeled off with a sharp hook | CEC-like cells/ PAPCM transplant | None | Slit-lamp examination, Specular microscope, OCT, Immunofluo-rescent staining, FACS, Western blotting, RT-PCR | Corneal thinning | 28 days | Zhang, et al., 2014 |
| **Primate (n=6)** | Monkey vascular endothelial cells | 1640-medium, 10% FCS, 2 mM L-glutamine,50U/mL penicillin, 50μg/mL streptomycin, 12μg/mL amphotericin B | Denuded corneal button | 2 × 105 cells/mL | 7.0 mm corneal button was trephined | DSAEK | None | Slit lamp examination, SEM and TEM, Immunohistochemistry, Histology | Transparency without corneal neovascularization or bullous keratopathy | 90 days | Zhu, et al., 2015 |
| **Rabbit** | Rabbit | MSC-CM | None | 5.0 × 105 cells/200μl DMEM | Mechanically scraping CE from the DM with a 20-gauge silicone needle | Cell injection with 100μM  Y-27632 | 100μM  Y-27632 ROCK inhibitor | Slit-lamp microscopy, Contact specular microscopy, Fluorescence staining | Restored transparency and normal corneal thickness | 2 weeks | Okumura, et al., 2015 |
| **Rabbit (n=2)** | Rabbit | Optimem I, 8% FBS, 20μg/ml Asc-2P, 0.08% ChS, 200mg/l calcium chloride, 10U/ml penicillin and 10μg/ml streptomycin, 5ng/ml EGF | Human purified type I collagen membranes | Confluent cell monolayer | CE removed with a 30-gauge needle | DMEK | None | OCT, Histology, Immunostai-ning, Light and electron microscopy | Maintained transparency | 6 weeks | Vázquez, et al., 2016 |
| **Rabbit (n=6)** | Rabbit (passage 2) | EGM-2 with EGF, VEGF, FGF, IGF, hydrocortison-e, gentamicin, amphotericin-B, 10% FBS | AV-SF scaffold | 1.9 × 104 cells | Bent 20 G needle and a sinskey hook were used to scrape and peel DM in a circular shape with a diameter of 7 mm | Implantatio-n of AV/SF film scaffold (6.0 mm diameter) | None | Corneal photographs, FESEM, RT-PCR, Histology, Immunohist-ochemistry | Corneal transparency | 4 weeks | Kim, et al., 2016 |
| **Rabbit** | Human (passage 3) | Low-glucose DMEM with 15% FBS, antibiotics, 2ng/mL bFGF, 0.3 mM Asc-2P | Collagen sheet +/- Viscoat® | 40,000 cells/mm2 for 3 weeks | CE scraped with a silicone sleeve, then 6.0-mm descemetorhexis with a 27-gauge needle | Collagen sheet transplant | None | Live/dead assay, Immunocyto-chemistry | Viscoat® improved cell viability of the transplanted hCECs | 14 days | Yamaguchi, et al., 2016 |
| **Primate** | Primate (passage 2-8) and Human (passage 2-5) | Primate – DMEM, 10%  FBS, 50 U/mL penicillin, 50μg/mL streptomycin, 2 ng/mL FGF-2;  Human – MSC-CM | None | 5x105 cells in 200μl | CE completely scraped from DM with a 20-gauge silicone needle | Cell injection +/- 100μM Y-27632: Primate (n=8) Human (n=10) | 100μM Y-27632 ROCK inhibitor | Slit-lamp examination, pachymetry | Y-27632 promoted corneal transparency | 7-14 days | Okumura, et al., 2016b |
| **Feline (n=8)** | Feline (passage 2) | DMEM, 10% FBS, 5ng/mL hEGF, 25lg/mL bovine pituitary extract, 25lg/mL gentamicin sulfate,100 IU/mL penicillin G | None | 2x105 – 1x106 | Central CE (7-mm diameter) or entire CE (18-mm diameter) removed. DM intact. | Cell injection with Y-27632 | 100μM or 350μM Y-27632 ROCK inhibitor | Slit-lamp, pachymetry, CEC morphometry, histology, electron microscopy, and function and wound healing–related protein immunostaining | Incompletely functional CE | 1 month | Bostan, et al., 2016 |
| **Rabbit (n=4)** | Human (passage 1) | Dual media approach | Thin corneal stromal carriers | 3,000 cells/mm2 | 7.0 mm of the DM was stripped and removed | Tissue-engineered endothelial keratoplasty | None | Slit lamp, AS-OCT, tonometer, *in vivo* confocal, Immunohistochemistry, trypan blue and alizarin red staining, SEM | Complete reversal of corneal blindness | 28 days | Peh, et al., 2017 |
| **Rabbit (n=10) and Primate (n=2)** | Human:  1.CEC-like cells from skin-derived precursors;  2. Immortalized human cell line (B4G12) | 1.DMEM/F12 (3:1), 1% penicillin/streptomycin, 50μg/mL fungizone, 2% B27 supplement, 40 ng/mL bFGF, 20ng/mL EGF  2.B4G12 - human endothelial-SFM | None | 2.0x105 or 4.0x105 cells/100μl or 50μl | CE mechanically scraped with a 20-gauge silicone needle over ~9.0-mm area (the diameter of the cornea is ~10 mm) | Cell injection with 3.2μg (rabbit) or 1.6μg (primate) of Y-27632 | 3.2μg (rabbit) or 1.6μg (primate) of Y-27632 ROCK inhibitor | Slit-lamp microscope, tenonometer, Visante OCT, confocal microscope, and non-contact specular microscopy  Dil signal, H&E staining and immunofluorescent staining | Restored corneal transparency | Rabbit – 3-7 days  Primate – 1-3 months | Shen, et al., 2017 |
| **Rabbit (n=4)** | Murine skin-derived precursors | Proliferation and differentiation media | Type I atelocollagen sheets | 1 x 106 cells per cm2 | 8.0 mm central cornea was excised by Hessburg-Barron Vacuum Trephine | Collagen sheets placed on corneal buttons | None | Slit-lamp microscopy, ultrasound pachymeter, IOP | Transplanted corneas maintained corneal transparency and thickness | 8 days | Inagaki, et al., 2017 |
| **Rabbit (n=3)** | Human (passage 3-5) | Low glucose DMEM, 15% FBS, 2.0ng/mL bFGF, 100 ng/mL Asc-2P, 1 μM TGF-β inhibitor SB431542, antibiotic/ antifungal agents | Vitrigel carrier | 1.3 x 106 cells/well (12-well plate) | 8.0 mm DM removed using a reverse Sinskey hook | Vitrigel/ hCEC graft transplant | None | Slit lamp, pachymeter, immunocytochemistry | Reduced corneal thickness and restored transparency | 14 days | Yoshida, et al., 2017 |
| **Rabbit (n=2)** | Human (passage 1) and Rabbit (passage 1) | Optimem I, 8% FBS, 0.3mM Asc-2P, 200 mg/L calcium chloride, 0.04% ChS, 10U/mL penicillin, 10μg/mL streptomycin, 20ng/mL NGF, and 5ng/mL EGF | Silk fibroin film | 100,000 cells/cm2; passage 1 | CE removed with a 30-gauge needle | DMEK | None | AS-OCT, Histology, Immunocytochemistry, | Restored corneal transparency and thickness | 6 weeks | Vázquez, et al., 2017 |
| **Rabbit (n=6)** | Rabbit (passages 2-3) | DMEM, 10% FBS, 50U/mL penicillin, 50μg/mL streptomycin, 2ng/mL FGF-2 | None | 5.0 x 105 RCECs in 200μl DMEM | CE mechanically scraped from DM with a 20-gauge silicone needle. With or without 4.0 mm descemetorhexis. | Cell injection with 100μM Y27632 ROCK inhibitor | 100μM Y27632 ROCK inhibitor | Slit-lamp microscopy, ultrasound pachymeter, contact specular microscopy, IOP, TEM | Final ECD and CCT similar with or without DM | 14 days | Okumura, et al., 2018 |

### 4. Clinical trials of corneal endothelial cell transplantation

The literature on human trials of human corneal endothelial transplantation is very limited, with a search in PubMed revealing two trials on human CE cell transplantation (Table 4) (Kinoshita, et al., 2018; Parikumar, et al., 2018). It is important to note that both of these trials were performed in two cohorts of patients diagnosed with bullous keratopathy. These clinical trials of transplantation of *in vitro* cultured hCECs over follow-up periods of 18-24 months confirm previous results from *in vivo* and *ex vivo* animal models as well as *ex vivo* human models (Kinoshita, et al., 2018; Parikumar, et al., 2018).

The trials of Kinoshita et al., (2018) and Parikumar et al., (2018) used two different approaches in terms of cell delivery, with Kinoshita et al. employing the use of a cell injection in tandem with a ROCK inhibitor. Whereas Parikumar et al. used a nanocomposite (D25-NC) gel sheet applied over the recipient damaged CE onto which cultured donor human corneal endothelial precursors (HCEP) were injected. The study by Parikumar et al., (2018) reported a much lower seeding density of transplanted corneal endothelial cells relative to the cell injection technique employed by of Kinoshita et al (2018) i.e., 1.6 x 105 cells versus 1×106 or 5x105 cells, respectively, which may be possible due to the trapping and supporting effect of the nanocomposite gel sheet (Table 4). In addition, after the removal of the nanocomposite gel sheet on day three of the treatment protocol, microscopic analysis of the sheets in all three patients revealed no residual HCEPs, suggesting that the majority, if not all of the HCEPs, remained in the patient’s eye or the anterior chamber of the eye. Both of these studies were uncontrolled, meaning it is very difficult to attribute the functional recovery entirely to the use of transplanted CE. The host DM was not removed in the clinical patients undergoing cell injection therapy, and the contribution of residual peripheral host CE cells to functional recovery was not evaluated in these studies. In addition, the use of a ROCK inhibitor and its implications in recovery of corneal function require further assessment.

In terms of clinical functional outcomes, both studies stated successful outcome measures, with both groups reporting subjective functional recovery of corneal transparency (Table 4). Importantly, neither group report any adverse effects in their treated patient cohorts, providing early evidence of the safety of these treatments. It is acknowledged by Kinoshita et al. that there is a theoretical risk of ectopic tumour formation due to the migration of transplanted CE cells into the circulation through the venous drainage of the trabecular meshwork. However, they did not detect any tumour growth in any of their patients at their final follow-up assessment at two years post treatment. The safety of these clinical trials in this regard requires a much longer follow-up period before it can be concluded with certainty that the risk of ectopic tumour formation is indeed low.

A comparison between DSAEK, DMEK and cell therapy is summarized in Table 5. An advantage of cell therapy over DSAEK and DMEK would be a significantly reduced reliance on donor tissue. However, there are more regulation constraints on cell therapy because it is not yet an established clinical procedure and there is a theoretical and unproven risk of ectopic tumor formation post-transplantation. DSAEK and DMEK are well-established clinical procedures and are not limited by these safety regulations. Moreover, before corneal endothelial cell transplantation can be adopted as a clinical procedure it must demonstrate that it’s either equal or superior to DMEK and DSAEK in terms of clinical outcomes. In developed countries availability of donor tissue is not a limiting factor for DSAEK and DMEK. Therefore, in order for cell therapy to be adopted it must show superiority in terms of clinical outcomes, such as rejection rates, late endothelial failure, speed of visual recovery, best corrected visual acuity, and also cost of treatment in comparison to the current treatment methods. Ideally, the long-term clinical outcomes of cell therapy should be compared to DSAEK and DMEK before this novel treatment method can be applied in the clinic.

Many clinical trials for DSAEK and DMEK have been reported (Ang, et al., 2016; Zhu, et al., 2018; Singh, et al., 2017; Stuart, et al., 2018; Price, et al., 2018; Roberts, et al., 2015) and the results generally seem to point out that DMEK provides better outcomes in visual acuity and graft survival with lower graft rejection rates compared with DSAEK. In a retrospective cohort study DMEK showed 97.4% graft survival versus 78.4% in DSAEK, and lower graft rejection rates - DMEK 1.7% versus DSAEK 5.0% at 2 years (Woo, et al., 2019). The Cornea Preservation Time Study (CPTS), which is a randomized, controlled clinical trial reported an even higher graft survival rate for DSAEK with probability of graft failure and of graft rejection of only 1.3% and 3.6% respectively for up to 5 years follow-up showing that DSAEK and DMEK are extremely successful treatment methods (Stulting, et al., 2018; Patel, et al., 2019). Neither Kinoshita et al., (2018) nor Parikumar et al., (2018) reported any adverse immune reactions during the follow-up period, however, a major limitation in the two cell therapy trials was the lack of a methodology to track engrafted cells. Therefore, it was not possible to definitively measure graft survival rates and compare them with DSAEK and DMEK.

To the best of our knowledge no studies so far have compared the visual acuity between cell therapy treatment and DSAEK/DMEK. In their clinical trial Parikumar, et al., (2018) used hand motion as measurement of visual acuity for patients with very low vision so this was not directly comparable to Kinoshita, et al., (2018) or other DSAEK/DMEK clinical trials that we found. Kinoshita, et al., (2018) converted decimal visual acuity to logMAR visual acuity to facilitate statistical analysis and showed BCVA (logMAR) ~0.2 twenty-four weeks after cell therapy and ~0.1 two years later, which seems to be comparable to what Woo, et al., (2019) reported for DMEK: range ~0.11-0.25. However, the available data on clinical corneal cell therapy trials are limited by a small number of patients, short follow up duration and the trial being non-randomised and non-controlled. A single clinical trial comparing all three techniques would be required for a more accurate assessment.

**Table 4 - Clinical trials of human corneal endothelial cell therapy.**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Recipients** | **Species and type of cultured cells** | **Cell culture medium** | **Seeding density** | **Native DM/Endothelium** | **Transplantation technique** | **ROCK inhibitor used** | **Assessment method** | **Primary outcomes** | **Visual acuity** | **Follow-up period** | **References** |
| Human (n=11) | Human corneal endothelial cells (hCEC) - Passage 2-3 | Modified Opti-MEM-I Reduced Serum Media | 1×106 or 5x105 cells/300μl + ROCK inhibitor | A silicone needle (Inami) was used to remove the abnormal ECM on the patient’s DM or the degenerated hCECs in an 8-mm diameter area of the central cornea (or both). | Cell injection using ROCK inhibitor | 100 μM ROCK inhibitor Y-27632 | Contact specular microscopy; slit-lamp; corneal thickness measurement, VA | Cell density 947 to 2833 cells/mm2 and corneal thickness <630μm | Improvement in BCVA of two lines or more was recorded in 9 of the 11 treated eyes at 2 years with a mean post-op logMar acuity of 0.05 (n=11) from preoperative average acuity of LogMar 0.80 | 24 weeks and 2 years | Kinoshita, et al., 2018 |
| Human (n=3) | Human corneal endothelial progenitor cells (HCEP) - Primary | DMEM/F-12, 20ng/mL EGF, 40ng/mL bFGF, B-27, 100U/mL penicillin, 100mg/mL streptomycin and 250ng/mL amphotericin B | 1.6 x 105 HCEP | The native DM/Endothelium were left intact | Cell injection using a nanocomposite (D25-NC) gel sheet as a supporting structure | None | Corneal clarity, VA | Bullae in the cornea disappeared by the 3rd-11th post-operative day in three patients | At 18 months follow-up VA improved from PL+/PR+ to HM+ in one patient, from HM+ to 6/60 in another while in the third patient, VA didn’t change | 18 months | Parikumar, et al., 2018 |

**Table 5 - Comparison between DSAEK/DMEK and cell therapy clinical trials.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Treatment method** | **Donor demand** | **Probability of graft failure** | **Graft rejection rates** | **Visual acuity** | **Regulation constraints** |
| **DSAEK/DMEK** | One donor per recipient eye | DSAEK - 1.3% for up to 5 years follow-up (Patel, et al., 2019)  DMEK - 1.7% in a follow-up ranging from 5.7 to 68 months (Deng, et al., 2018) | DSAEK - 3.6% (Stulting, et al., 2018)  DMEK - 1.9% in a follow-up ranging from 5.7 to 68 months (Deng, et al., 2018) | DMEK has better BCVA measured in logMAR than DSAEK.  Woo, et al., (2019) reported BSCVA for DMEK range ~0.11-0.25 and DSAEK ~0.2-0.39. | None. It is an established clinical procedure with evidence base. |
| **Corneal Endothelial Cell therapy** | One human donor could provide cells for multiple recipients | Data not available and no established methods for tracking cell engraftment | No immune rejection has been reported and data not available | Parikumar, et al., used Snellens acuity of HM as measurement. Kinoshita, et al., used BCVA measured in Decimel/logMAR showing BCVA (logMAR) ~0.8 before transplantation, ~0.2 twenty-four weeks after and ~0.1 two years later. | Yes, and is yet to gain regulatory approval for wide spread use. |

**5. Discussion**

Cell therapy approaches may potentially revolutionize the field of corneal transplantation, with rapid improvements in visual recovery and significantly reduced donor tissue requirements relative to the use of conventional surgical treatments utilising cadaveric human donor corneas. The use of *ex vivo* and *in vivo* human and animal models has enabled researchers to elucidate the cell culture methodologies that allow successful propagation and transplantation of CE. However, CE therapies in current research model systems do not faithfully and fully recapitulate the biology and immunology of the human cornea, making it difficult to predict the immune response to CE therapy in human clinical trials. The limitations are described below.

**5.1 The species-specific CE proliferation characteristics of animal models**

Although the use of animal models is the most common approach to modelling CE therapies, the use of animal models is not without observational bias, with significantly more studies being performed in the rabbit relative to other species (Tables 1 and 3). This use of rabbit may lead to observations that are not directly applicable to humans as rabbit corneal endothelium is substantially more capable of regenerating and proliferating relative to primate and human corneas (Van Horn and Hyndiuk, 1975; Mimura, et al., 2005c; Graham, et al., 2000). CE has been shown to possess proliferative capacity in rabbits (Van Horn, et al., 1977; Mimura, et al., 2005c) and rats (Tuft, et al., 1986). This increased regenerative potential of the rabbit native CE makes these corneas and species relatively unsuitable to truly assess the effect of transplanted CE cells. The CE of a number of other animal models, such as cats (Van Horn, et al., 1977) and primates (Van Horn and Hyndiuk, 1975) seem to possess similar non-proliferative characteristics as the human CE. This similarity in cellular proliferative characteristics potentially makes the cat and primate models more suitable species for cell therapy testing compared to rabbit and rat models, but they are difficult to procure and maintain in animal experimentation, and have significant ethical implications.

**5.2 Use of *in vitro* cultured human corneal endothelial cells for transplantation**

Human corneal endothelial cells have a limited proliferative capacity. When isolated and cultured in vitro they tend to undergo a morphological transformation to a fibroblastic phenotype and loose their typical hexagonal morphology or they become senescent. Researchers have been using different culture medium confirmations to try and tackle these two problems and have shown some success but a standardized culture protocol has yet to be established for long-term maintenance in culture. Additionally, early passage hCECs are considered more likely to retain their functional capacity and normal morphology and are, hence, preferred by researchers for transplantation testing. In all the studies reported in this review that used hCECs for transplantation the use of primary or passage 1 cells was reported nine times; passage 2-5 was reported fourteen times; passages up to 10 were reported once; and over 10 passages were reported once. One study reported on establishing an un-transfected continuous human corneal endothelial cell line up to 101 and 224 passages from a 26 years old deceased female donor with the subcultures lasting 3 years (Fan, et al., 2011b). The authors used the following culture medium: DMEM/F12, 20% FBS, antibiotics, 10 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml epidermal growth factor (EGF), 50 μg/ml N-acetylglucosamine hydrochloride, 50 μg/ml glucosamine hydrochloride, 0.8 mg/ml chondroitin sulfate, 50 μg/ml oxidation-degradation products of chondroitin sulphate, 50 μg/ml carboxymethyl-chitosan, 5 ng/ml bovine ocular extracts and 10% (v/v) culture supernatant of human corneal stromal (HCS) cells at logarithmic phase. Such high proliferative potential could have been achieved due to the use of corneal stromal cells-conditioned medium (CSC-CM). Zhu, et al., (2016) reported that CSC-CM was able to stimulate CEC proliferation better than bone marrow -derived endothelial progenitor cells (BEPCs), and bone marrow-derived mesenchymal stem cells (BMSCs). However, a major limitation of the study is that other researchers have not managed to reproduce such high passage number of un-transfected hCECs yet. Moreover, the functional potential of these extended passages were reported and not tested in animal or *in vitro* corneal models.

This disparity in the number of cells passages shows that the majority of culture conditions currently in use are unable to maintain the cells for a large number of passages. The majority of researchers seem to be able to achieve up to 0-5 passages only. Since the CECs, CSCs and corneal epithelial cells are all present in the different layers of the cornea and secrete factors that might affect each other’s proliferation and wound healing properties the use of CSC-CM should be tested further, and possibly in combination with corneal epithelial cell-conditioned medium. This might mimic the natural environment of the hCECs where they are able to communicate with the stromal cells and the corneal endothelial cells and could lead to enhanced proliferation capacity. Therefore, further research is required to optimize the culture conditions and to standardize the cell culture protocols, specifically to address long term *in vitro* maintenance of HCE cells with documented functional properties in order to assist translation to clinical studies.

**5.3 Corneal endothelial cell density and the use of supporting materials**

There seems to be a big variation in the numbers of transplanted cells that different studies have used with a range of cell seeding densities from 1x103 cells/mL to 1x107 cells/mL. An important question to be addressed is to determine the minimum number CE cells required to achieve a significant functional recovery. In addition, the method of delivery and seeding density could feasibly influence the functional recovery of the recipient donor cornea and as it stands, there is insufficient evidence to determine whether the transplanted CE cells themselves are the reason for functional recovery and not the medium or ROCK inhibitors that the cells are cultured in, or the use of carrier substrates and composite gels used to deliver the donor CE cells. The use of a substrate to deliver the cells could have the benefit of delivering the cultured endothelium in required density to the posterior surface of the cornea. However substrate attachment, maintenance of transparency and biocompatibility requires careful attention prior to translation to clinical trials that are lacking in evidence at present. In comparison, injection of cell suspension to the anterior chamber has the advantage of being an easy technique that will have to overcome the problem of extended dispersion of cells to unwanted targets surfaces such as the iris or anterior chamber angle. A recent study had shown endothelial regenerative effect with the sole use of topical ROCK inhibitor (Moloney, et al., 2017). This approach even circumvents the need for cell therapy and utilises the proliferative reserve of native peripheral corneal endothelial cells to recover function in bullous keratopathy. Therefore, further research is needed into delivery methods, tracking cell fate and grafting sites of the transplanted cells. This would disentangle the effect of cell culture mediums and other adjunct materials from the effect of the transplanted CE cells.

**5.4 Methods for inducing CE dysfunction**

The methodology used in animal *in vivo* models to create endothelial decompensation, such as the use of manual removal or cryo-injury adds limitation and bias to the studies. Although Bullous keratopathy was maintained for 2 months after cryo-injury (Mimura, et al., 2005b) it does not ensure complete removal of native endothelial cells in these models. Likewise, other CE removal techniques, such as excision of the central DM, corneal button trephination or CE scraping, leave the peripheral endothelium intact (Mimura, et al., 2004b; Hsiue, et al., 2006; Sumide, et al, 2006; Koizumi, et al., 2007). Therefore, the influence of the residual native peripheral endothelial cells in the recovery of corneal function following cell therapy using *in vitro* generated cells needs to be taken into account in the interpretation of the clinical results.

In order to overcome this limitation in our previous study (Rolev, et al., 2018) using a corneal *ex vivo* model we removed the entire Descemet’s membrane/Endothelium complex all the way from the Schlemm’s canal of the sclerocorneal junction and demonstrated by histology the complete removal of the native CE. However, this might be more challenging in an *in vivo* study due to a more restricted access to the peripheral posterior cornea.

**5.5 Tracking the cell fate of transplanted cells and setting up appropriate controls in future clinical studies**

Both clinical trials by Parikumar, et al., (2018) and Kinoshita, et al., (2018) are uncontrolled, single-group studies. In the case of Parikumar, et al., (2018) the entire native CE was left intact prior to cell transplantation. In the case of Kinoshita, et al., (2018) the central 8.0 mm of the CE alone were removed. This means that the peripheral CE was intact in all cases, with an intact central DM devoid of CE in other patients. Kimoto, et al., (2014) has pointed out that monkeys with bullous keratopathy often show spontaneous recovery of corneal transparency by regeneration of their own CECs if Descemet’s membrane is intact, which could also be the case in humans. This adds a limitation to the interpretation of the results of both clinical studies because in either case it is not possible to determine if the newly transplanted cells attached to the recipient corneas and restored their normal CE function, or the residual native CE was able to regenerate itself due to being stimulated by factors released by the transplanted cells, or by the ROCK inhibitor as indicated by Kinoshita, et al., (2018), or by spontaneous CE regeneration due to the presence of a DM. Therefore, setting up appropriate control groups and tracking of the cells post-transplantation in order to demonstrate their engraftment and to differentiate between the transplanted hCECs and the host endothelial cells are crucial.

Another limitation in both clinical trials is the lack of tracking of engrafted and ectopic cells. Injected cells in the anterior chamber can enter the circulation through the Schlemm’s canal and migrate to different regions of the eye or of the body. Ectopic tumorigenic cells could pose a significant health risk to the recipients and, therefore, render cell therapy impractical. Although normal human corneal endothelial cells have not been shown to possess tumorigenic capacity, it is yet unknown whether spontaneous transformation might occur after transplantation and cause the cells to acquire tumorigenic properties. Additionally, if iPS-derived hCECs become a new avenue for generation of CE for transplantation it will be of great importance to establish methods for tracking ectopic cells in order to ensure the safety of the procedure since iPS cells might be tumorigenic. Some techniques exist for cell labelling and tracking after transplantation and should be considered for future clinical trial design: such as fluorescence imaging, magnetic resonance imaging, photoacoustic imaging, positron emission tomography (PET) and computed tomography (CT) (Wang, et al., 2013; Takayama, et al., 2019).

**6. Conclusion and future areas of research**

All of the research reports discussed above support the notion that cell therapy is indeed a viable treatment method for corneal blindness. However, culture protocols for *in vitro* culture of human corneal endothelial cells need to be further optimized in order to enable generation of sufficient cell quantities with normal morphology to be used routinely in the clinic. Furthermore, there is a clear need for the development of experimental protocols which enable the fate mapping of transplanted corneal cells to allow the experimental therapies to be traced. Simple transplantation of CE cells onto the posterior cornea or injecting them into the anterior chamber does not allow researchers to calculate the percentage of cells which are incorporated into the regenerating endothelial layer and thus there is no clear protocol available to measure the efficiency or efficacy of transplanted CE cells. Additionally, the methodology used to measure corneal thickness varies between studies. The CCT in many studies is measured by ultrasound at a single point. Only four *in vivo* animal studies have reported using OCT for measuring corneal thickness (Vázquez, et al., 2016 and 2017; Peh, et al., 2017; Shen, et al., 2017), which might be a more accurate way of measurement since it allows the imaging of an entire cross section of the cornea rather than just a single point of measurement. Therefore, the interpretation of the results could be biased. For example, corneas might defer slightly in thickness at different points, which are very close to each other, and this might affect the end result depending on where the single-point measurement was taken before and after cell therapy treatment. Moreover, no objective measurement of corneal clarity has been reported in clinical and non-clinical studies. Researchers tend to use Slit-Lamp Microscopic Images to assess corneal clarity but this method is subjective and interpretation of the results may vary from person to person.

Therefore, some technical issues remain unaddressed, such as:

* Lack of means to culture cells without the use of xenogenic products and in cost-effective, good manufacturing practice (GMP)-compliant standard.
* Lack of method to trace the cell fate of transplanted CE cells.
* Lack of methods to trace donor cell location, continued mitosis, migration and sustenance of transplanted CE cells.
* Lack of standardized reports on functional assessment on corneal transparency and stromal thickness in the measurement of outcomes.

The use of induced pluripotent stem cells is an avenue of research that has yet to be applied to the generation of corneal endothelial cells. There has been recent progress in this approach through the work of Pellegrini et al., (2014), who successfully generated corneal epithelial cells from cultured limbal cells. This approach has yet to be applied in the generation of corneal endothelium and could be an option in the future to provide a large number of donor cells from individual patients.

Currently, many experimental models of hCEC and animal derived CE cell lines use animal serum in the cell culture and injection medium of cell therapies. In the two clinical studies examined in this review, either serum-free medium (Optimem) or a nanocomposite scaffold is used to deliver the CE to the patient (Table 4). Kinoshita, et al., (2018) used animal-derived products such as 8% FBS and trypsin to culture the hCECs and also for cell detachment and passaging prior transplantation. The use of xenogenic products increases the risk of infection from an animal-derived product and, therefore adds a limitation to the translational value of cell therapy to clinical practice. To the best of our knowledge no hCEC culture protocols currently exist with xenogenic-free and fully chemically defined confirmation that could be used for long-term hCEC *in vitro* culture. A cost-effective solution to this is required in order to diminish the risk of cross infection.

Regarding the use of serum, further research is necessary to assess whether or not these experiments can achieve the same functional recovery results with the use of serum-free media. In addition to the use of serum, many of the models of CE therapy analysed in this review use various kinds of carriers, such as nanocomposite (D25-NC) or collagen gel sheets, injections or cell suspensions to deliver CE to the damaged cornea. There is, therefore, a need to establish a validated and consistent delivery system in order to reduce confounding factors in the assessment of functional recovery. This way the observed functional recovery in damaged corneas could be convincingly attributed to the use of transplanted corneal endothelium cells and not to the use of supporting scaffolds and concurrent use of serum medias, growth factors, or the use of ROCK inhibitors.

Considering the future of cell therapy in the context of the political environment of the UK, it is worth noting the potential impact that the exit of the UK from the European Union may have on the regulations of stem cell transplant research. At the time of writing, CE transplants are regulated by the European Tissue and Cells Directive (EUTCD). It remains to be seen what exactly this drift from the EU regulatory bodies may mean in terms of consequences for the clinical trial operations in the UK.

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