

REVIEW ARTICLE

Research progress of corneal endothelial cell regeneration and replacement

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Abstract

Corneal endothelial cells (CECs) are crucial for the maintenance of corneal transparency and normal visual function. Corneal endothelial dysfunction can lead to corneal edema, opacity, and even blindness. Due to the limited proliferative capacity of human CECs and the global shortage of donor cornea, corneal endothelial regeneration and replacement always represent the most challenge in the basic research and clinical treatment of corneal diseases. Although there is a potential existence of corneal endothelial progenitor cells, the efficiency of Descemet stripping without endothelial keratoplasty remains controversial. In recent years, significant advancements have been made in the field of cultured endothelial cell regeneration and artificial material replacement. Here, we reviewed the current research and clinical progress of corneal endothelial cell regeneration and replacement, including the in vitro cultivation of primary human CECs, in vitro differentiation of stem cell-derived CECs, tissue-engineered corneal endothelium, and fabrication of artificial corneal endothelium. We also discussed the remaining questions regarding innovating clinical preventive and therapeutic strategies for corneal endothelial dysfunction.

KEYWORDS

artificial corneal endothelium, cell therapy, corneal endothelial dysfunction, regeneration

INTRODUCTION

The corneal endothelium, the innermost layer of the cornea, plays a vital role in maintaining corneal transparency and thickness, both of which are indispensable for normal visual function. However, adult human corneal endothelial cells (CECs) possess very limited proliferative capacity, relying on the neighbor cell enlargement and migration for wound healing [1]. The age-related loss of human CECs occurs at a rate of 0.6% per year, and corneal endothelial dysfunction emerges when the cell density drops below a critical threshold of 400–500 cells/mm². Despite various corneal transplantation, including Descemet's membrane endothelial keratoplasty (DMEK)

and Descemet's stripping endothelial automated keratoplasty (DSEAK), were specifically designed and performed as the major clinical therapeutic approach for corneal endothelial dysfunction, their applications are severely limited by the global shortage of human donor corneas [2].

To solve the limitation of human CEC proliferation and the shortage of donor cornea, many research groups are focusing on the corneal endothelial regeneration and replacement to develop alternative therapeutic approaches. Here, we reviewed the major background and current progress of basic research and clinical treatment, including the characteristics of corneal endothelial repair, the intracameral injection of CECs, the challenges of CEC culture, the

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alterations of human embryonic stem cell/induced pluripotent stem cell (hESC/iPSC)-derived CECs, tissue-engineered corneal endothelium, and artificial corneal endothelial replacement.

CHARACTERISTICS OF CORNEAL ENDOTHELIUM

Corneal endothelial function

The human cornea is composed of six distinct layers, including the epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium [3]. The corneal endothelium comprises a monolayer of hexagonal cells that functioned as the physiological barrier separating the corneal stroma from the aqueous humor [4]. Corneal endothelium plays a vital role in maintaining the physiological hydration, thickness, and transparency of the corneal stroma through its barrier and fluid pumping capabilities [5–7]. Hence, the normal corneal endothelium is critical for the preservation of the cornea's normal visual function [8]. During embryonic development, CECs originate from mesenchymal cells derived from the periocular neural crest and subsequently migrate into the space located between the lens and corneal epithelium [9, 10]. Upon birth, these cells undergo arrest in the G1 phase of the cell cycle, resulting in the limited proliferative capacity. Subsequently, the cell density of corneal endothelium undergoes an annual decline of 0.6%. Corneal endothelial dysfunction manifests when the density falls below the range of 400–500 cells/mm² [11].

Corneal endothelial damage

Because of the limited proliferative capacity, the repairing of human CECs after damage depends on the enlargement and migratory behavior of adjacent CECs. Severe injury can impair normal corneal endothelial function, thereby triggering corneal edema, vesicular keratopathy, and subsequent visual impairment. Corneal endothelial damage can be caused by multiple factors, such as systemic influences, primary corneal pathologies, refractive anomalies, glaucoma, ocular inflammation, and eye trauma [12–14]. The systemic factors include diabetes mellitus, hypertension, metabolic syndrome, and chronic kidney failure [15–18]. The primary pathologies include Fuchs' corneal endothelial dystrophy, Peters' anomaly, and irido-corneal endothelial syndrome [2, 14, 19–22]. In addition, intraocular surgeries, such as cataract surgery, corneal transplantation, and glaucoma surgery, have the potential to induce corneal endothelial damage [23, 24].

Corneal endothelial transplantation

Corneal transplantation plays a pivotal role in the management of corneal endothelial dysfunction. The field of corneal transplantation surgery has witnessed significant advancements, leading to the

emergence of diverse surgical techniques ranging from penetrating keratoplasty to lamellar keratoplasty, deep lamellar keratoplasty, posterior lamellar keratoplasty, and corneal endothelial transplantation [3, 25–28]. At present, Descemet's stripping endothelial keratoplasty (DSEK) and Descemet's membrane endothelial keratoplasty (DMEK) represent the predominant surgeries for the treatment of corneal endothelial dysfunction [29–31]. However, the broader promotion of corneal transplantation is hindered by the scarcity of high-quality donor corneas and the compromised viability of cryopreserved donor CECs. Consequently, the mechanical exploration of corneal endothelial regeneration and the development of innovative strategies to prevent and treat corneal endothelial dysfunction remains the unsolved key problem in the field of corneal research.

ENDOGENOUS REGENERATION OF CORNEAL ENDOTHELIUM

Despite the inherently limited proliferative capacity of human CECs, extensive research has been conducted to investigate strategies for corneal endothelial regeneration. The major investigations include the comparative regenerative analysis of different species-derived CECs, the exploration of corneal endothelial progenitor, and the pharmacologic promotion of corneal endothelial regenerative capacity.

Different regenerative capacity of different species

The rabbit model was first used to investigate the regenerative mechanism of CECs, revealing that damaged areas of rabbit corneal endothelium were repaired through cellular division [32]. Subsequent investigations employing murine and bovine models reported similar findings, suggesting that CECs in these species possessed proliferative potential [33, 34]. However, it was observed that the proliferative capacity of CECs is species-specific. In 1972, an assessment of the regenerative ability of human corneal endothelium revealed the absence of significant proliferation of human CECs during the repair process [35]. Instead, the endothelial repair predominantly occurred through cellular expansion and migration, accompanied with the fibrotic and multinucleated characteristics. Similar repair capacity and patterns were further identified in CECs of primates and felines [36, 37]. The mechanical investigations of different regenerative capacity of different species may provide the clues of promoting human corneal endothelial cell proliferation.

Limited regeneration of human corneal endothelium

Previous investigations have revealed the variations of cell densities across different regions of human corneal endothelium, where the peripheral region possesses the highest cell density [38]. Peripheral

CECs express the stem/progenitor markers LGR5 and PITX2, suggesting the potential existence of corneal endothelial progenitor within the peripheral region of corneal endothelium [39, 40]. Accordingly, several research groups are focusing on exploring the clinical evaluation of Descemet stripping without endothelial keratoplasty (DWEK) for the treatment of corneal endothelial dysfunction [41]. However, comprehensive analyses have demonstrated a direct correlation between the success rate of DWEK and the extent of Descemet membrane removal. Specifically, when the removal diameter is ≤ 4 mm, the surgical success rate is significantly higher, resulting in the restoration of corneal transparency and normal thickness. These findings imply that neighboring CECs potentially contribute to the repair of damaged regions through cellular migration and enlargement. However, if the extent of the removal area surpasses a certain threshold, the migrated and enlarged cells may be unable to adequately restore the impaired corneal endothelial function. Despite these observations, there remains a lack of definitive consensus regarding the DWEK surgery.

Pharmacologic promotion of corneal endothelial regeneration

Pharmacologic treatment entails the local administration of pharmacological agents to promote cell survival, proliferation, and migration of CECs in corneal endothelial disease, potentially mitigating the onset of surgical complications (Figure 1). Previous studies have investigated various growth factors to promote the migration and proliferation of human CECs, such as epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor [42–44]. Currently, the ROCK inhibitors represent the most promising drugs

for the treatment of corneal endothelial diseases, including Y-27632 and Ripasudil, which promote cell proliferation, migration, and adhesion through the Rho/ROCK signaling pathway, thereby facilitating corneal endothelial regeneration [45]. ROCK inhibitors have shown the effectiveness in several small clinical trials. However, multiple-center randomized controlled clinical trials are still needed to optimize the dosage, duration, and indications of corneal endothelial dysfunctions.

REGENERATION STRATEGIES FOR CORNEAL ENDOTHELIAL DYSFUNCTION

The global shortage of donor corneas represents the major limitation of corneal transplantation for the treatment of endothelial dysfunction. Therefore, more groups are focusing on the alternative regeneration strategies of CECs. The major progress includes tissue-engineered corneal endothelium and intracameral cell injection of cultured human CECs and hESC/iPSC-derived CECs, as well as other cell sources (Figure 1).

Tissue-engineered corneal endothelium

Tissue-engineered corneal endothelium is originally developed approach that involves the cultivation of in vitro expanded or differentiated CECs onto appropriate substrates for the treatment of corneal endothelial dysfunction. The substrates must fulfill with the favorable biocompatibility, transparency, mechanical strength, thickness, and curvature, as well as permeability and cellular adhesion capabilities [46]. Natural carriers, such as amniotic membrane

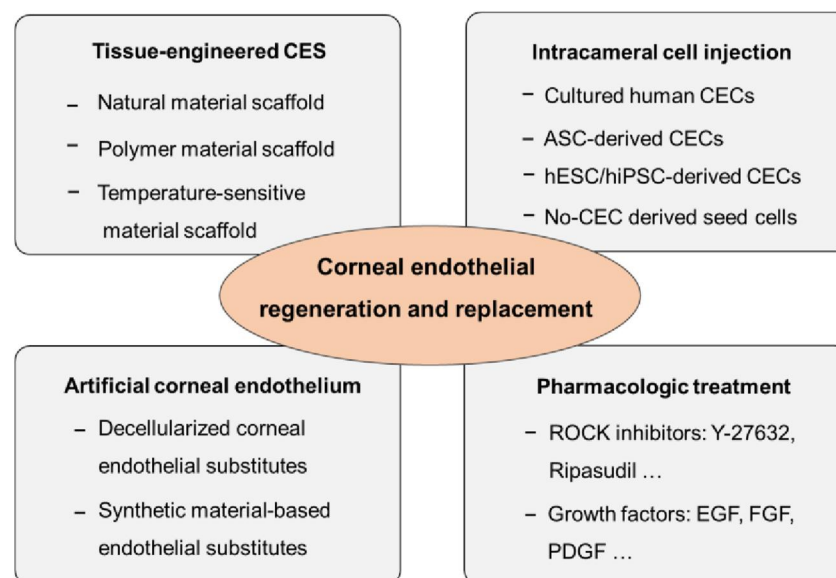


FIGURE 1 Multiple strategies of corneal endothelial regeneration and replacement. ASC, adult stem cells; CECs, corneal endothelial cells; CES, corneal endothelial sheet; EGF, epidermal growth factor; FGF, fibroblast growth factor; hESC, human embryonic stem cells; hiPSC, human induced pluripotent stem cells; PDGF, platelet-derived growth factor; ROCK, Rho-associated kinase.

and decellularized corneal stroma, exhibit favorable biocompatibility characteristics, but these carriers are constrained by the limited sources and donor quality variations. Moreover, other endothelial carriers contain polycaprolactone, chitin, and temperature-sensitive material poly (N-isopropyl acrylamide). They exhibit the benefits of uniformity, fast production, and the absence of donor restrictions. However, synthetic materials may assume the inadequate adhesion to corneal stroma and the risk of causing stromal fibrosis [46–49]. Zhang et al. employed the ultrathin decellularized porcine corneal stroma as a carrier for culturing rabbit CECs and generating tissue-engineered corneal endothelium. Upon transplantation in rabbits, the tissue-engineered corneal endothelium facilitated the restoration of normal corneal thickness [47]. Zhao et al. employed the cross-linked amniotic membrane in combination with human CECs to construct tissue-engineered corneal endothelium, affirming the efficacy in feline and monkey models [48]. Therefore, significant advantages of tissue-engineered corneal endothelium include the reduced requirement of transplanted cell number, the increased cellular adhesion on the carrier, and the improved systemic safety, in comparison to intracameral injection of single cell suspensions. Furthermore, the transplantation procedure resembles the traditional procedures of corneal endothelial transplantation, rendering it easily adoptable. However, this technique requires the extensive consideration of both seed cell function and favorable carrier quality, which contribute to the difficulties in construction and assessment.

Intracameral injection of cultured CECs

The intracameral injection approach involving the administration of in vitro cultured human CECs through single-cell suspension into the anterior chamber to restore corneal endothelial function [50]. Prof. Kinoshita's group has successfully cultivated CECs from both human and nonhuman primate sources in vitro [51–54]. They used the ROCK inhibitor Y-27632 to elevate the viability and promote rapid adhesion and proliferation of CECs. Notably, animal experiments involving rabbits and monkeys demonstrated the efficacy of combining intracameral injection of human CECs with Y-27632 for corneal endothelial function recovery [55, 56]. The group subsequently reported the outcomes of clinical trials focusing on cultivated human CEC transplantation in 2018 [51]. This approach involves the initial utilization of a specifically designed needle to remove CECs and aberrant extracellular matrix from the central corneal lesion. Subsequently, a single cell suspension containing Y-27632 is directly injected into the anterior chamber. Following surgery, patients are positioned in a prone posture for 3 h, capitalizing on the combined effects of gravity and Y-27632 to facilitate the adhesion of the transplanted cells. They conducted a comprehensive 5-year clinical follow-up of 11 patients diagnosed with corneal endothelial dysfunction. The findings revealed that all patients displayed restored corneal transparency, among which 10 patients with corneal thickness below 630 μm and 9 patients with the improvement of at least two lines in the best corrected visual acuity (BCVA). Recently, they reported the 5-year

results after surgery, 10 patients retained the restored corneal transparency, with an average central CEC density of 1257 ± 467 cells/ mm^2 . Visual sensitivity improved from preoperative 0.876 logarithm to postoperative 0.046. No systemic or ocular abnormalities were found postoperatively. Notably, guttae structures were observed again on the endothelium in 7 patients with the restored corneal transparency [51, 57].

The intracameral injection strategy possesses specific attributes, including the absence of a carrier requirement, uncomplicated surgical procedures, and minimal tissue damage. However, a series of issues still need to be clarified and resolved. In terms of long-term safety and its mechanism, due to the significantly higher number of transplanted cells than the amount needed to reconstruct corneal endothelium, it is unclear whether the transplanted cells will adhere to the lens, iris, ciliary body, or even enter the bloodstream through the trabecular meshwork. Additionally, this strategy still relies on healthy donor corneas to provide a high-quality source of CECs and has high requirements on the ex vivo culture techniques of human CECs, such as the standardized cell production, identification of cellular purity, and functional cell subpopulation.

Corneal endothelial cell culture

In vitro culture strategy

The corneal endothelium, originating from the neural crest, exhibits cell cycle arrest predominantly in the G0/G1 phase. Therefore, overcoming cell cycle arrest, achieving extensive in vitro expansion, and maintaining a homogeneous and functional population of CECs represent the most challenges in the expansion of adult human CECs [58–61]. During the expansion and cultivation process, multiple factors, including cell cycle arrest, tight junction disruption, and alterations in cell polarity can lead to cellular senescence and endothelial mesenchymal transition (EnMT) [60, 62]. These changes are characterized by increased cell volume, fibroblastic transformation, diminished expression of tight junction-associated protein ZO-1, and upregulation of mesenchymal-related protein α -SMA, among others. To address the challenges of cellular senescence and EnMT during the in vitro expansion of human CECs, most researchers have made efforts in exploring various cell culture media (e.g., M199, DMEM, and Opti-MEM), growth factors (e.g., epidermal growth factor and basic fibroblast growth factor), and other additives (e.g., pituitary extract, ascorbic acid, and Rho kinase inhibitor) [63–67]. Through optimization, these culture systems have demonstrated the ability to support CEC expansion while preserving their original phenotype and functionality. Currently, two predominant methods are utilized for CEC expansion and cultivation. The first method involves a dual culture system, where a proliferation medium is initially employed to promote CEC expansion and monolayer formation. Subsequently, the differentiation medium is introduced to restore the phenotype and function of CECs [68, 69]. The other method entails the addition of ROCK inhibitor Y-27632, which mitigates apoptosis and EnMT by antagonizing

the Rho/ROCK signaling pathway that is activated during cell isolation and culture, thus facilitating endothelial cell expansion [70].

Functional subpopulation of cultured human CECs

While recent research has made significant progress in addressing the challenges of *in vitro* expansion and cultivation of human CECs, cell heterogeneity remains. Prof. Kinoshita's group undertook the subpopulation analysis of primary cultured human CECs *in vitro* and successfully identified the surface markers of functional cell subpopulations (CD166+/CD133-/CD105-/CD44-/CD24-/CD26-) [51, 71]. They assessed the transplant outcomes of distinct proportions of functional cell subpopulation. The first group of 11 individuals of 18 patients underwent cell transplantation with a lower proportion of functional cell subpopulation (0.1%–76.3%), while the second group of 7 patients received a higher proportion (>90%). Following a 3-year examination, 10 out of 11 patients in the first group and all 7 patients in the second group exhibited enhanced BCVA. The second group exhibited a substantially elevated CEC density (3083 cells/mm²) in comparison to the first group (1349 cells/mm²). Over the 24-week to 3-year postoperative follow-up, the second group displayed an average density reduction of 3.2%, accompanied with more rapid and stable recovery of corneal thickness. In contrast, the first group experienced 23.6% reduction in CEC density [72]. Notably, variability exists in the proliferative capacity of human CECs derived from different donors, as well as in the ability to cultivate functional CD44- cell subpopulations [73]. Mechanical research has revealed that a higher CD44- cell ratio in cultured CECs leads to reduced expression of inflammatory factors and an elevation in oxidative phosphorylation levels [74]. CD44 is capable of modulating the metabolic flux associated with mitochondrial respiration and stimulating the metabolic conversion toward glycolysis [75, 76]. Consequently, the CD44 may serve as a useful marker of functional population purification of cultured CECs.

Alternative cell sources beyond cornea

Apart from *in vitro* cultivation of human CECs, several groups are investigating the efficacy of utilizing stem cell-derived CECs as an alternative cell source. These include not only adult stem cells, hESCs, hiPSCs, but also non-corneal derived cells.

Adult stem cells

Adult stem cells are regarded as safe due to their easy acquisition, autologous origin, plastic differentiation capability, lack of immune rejection, absence of ethical concerns, and tumorigenic risks [77, 78]. Currently, several groups have reported on the application of adult stem cells derived from bone marrow, adipose tissue, skin, and

other sources for transplantation to treat corneal endothelial dysfunction in animal models [79–81]. Shen et al. induced the differentiation of skin-derived precursor cells into corneal endothelial-like cells and effectively improved the corneal transparency and thickness of rabbit and monkey models through intracameral cell injection [82]. Pan et al. reprogrammed the fibroblasts into neural crest cells using small molecule compounds and further induced differentiation into corneal endothelial-like cells [83]. Cell transplantation via intracameral injection verified to partially recover corneal transparency and normal thickness in rabbit models. Research on CEC transplantation using adult stem cells is primarily conducted in rabbit models, and the efficacy of skin-derived stem cells has been validated in nonhuman primate models. Nonetheless, further research is required to explore the standardization, inductive differentiation efficiency, transplanted cell purity, and their long-term effectiveness in nonhuman primate models.

Pluripotent stem cells

hESCs and hiPSCs possess unlimited expansion capabilities and the potential for multidirectional differentiation, which serve as the optimal cell sources for CECs [84, 85]. At present, the induced differentiation of hESC/hiPSCs into corneal endothelial-like cells mainly recapitulate the developmental process of the embryonic cornea. Initially, hESC/hiPSCs are differentiated into neural crest cells by inhibiting the TGFβ-SMAD signaling pathway and activating the Wnt signaling pathway using small molecule compounds. Subsequently, these cells are further differentiated into corneal endothelial-like cells through various combinations of cytokines, small molecule compounds, or corneal endothelial conditioned medium [86–90]. Morphological and immunofluorescence staining are commonly employed for the *in vitro* characterization of the differentiated cells, while *in vivo* functional verification is frequently conducted using a rabbit corneal endothelial scraping model. Promisingly, hESC/hiPSC-derived corneal endothelial-like cells have demonstrated preliminary evidence of safety and efficacy. Yu et al. employed clinical-grade hESCs to induce the differentiation of corneal endothelial-like cells, which were transplanted into the rabbit model. They found that the differentiated CECs effectively ameliorated corneal edema and restored corneal transparency [91]. Our research team successfully induced hESC/hiPSCs differentiation into corneal endothelial precursors [92]. *In vivo* transplantation revealed that these precursors, combined with the small molecule compound nicotinamide, effectively restored and maintained corneal transparency and thickness in both rabbit and monkey models. Despite considerable progress of corneal endothelial-like cells derived from hESC/hiPSCs, the translation to clinical application remains many unsolved issues, such as the differentiation efficacy, purity of differentiated cells, tumorigenic potential of transplant cells, long-term safety and efficacy, as well as their functional mechanisms in nonhuman primates.

Non-corneal derived cells

“Ectopic” cell transplantation represents a promising therapeutic approach in the field of regenerative medicine. One typical example is the use of cultured oral mucosal epithelial cells for the treatment of limbal stem cell deficiency [93–98]. Considering the barrier function of CECs to separate the aqueous humor and corneal stroma, several groups examined the potential of non-corneal derived cells as an alternative for the treatment of corneal endothelial dysfunction. Previous studies have provided evidence supporting the effectiveness of utilizing vascular endothelial cells in tissue-engineered corneal endothelial transplantation, which has shown promising outcomes of partially ameliorating corneal edema [99, 100]. Recently, our group explored the feasibility of replacing corneal endothelium with retinal pigment epithelial (RPE) cells. Analogous to CECs, RPE cells exhibit similar characteristics of regular hexagonal morphology and expressions of ZO1 and Na⁺/K⁺-ATPase. In rabbit models, the transplantation of both primary cultured RPE cells and hESC-derived RPE cells effectively resolved corneal edema and restored normal corneal thickness and transparency [101]. Importantly, previous clinical trials have demonstrated the efficacy and safety of hESC-derived RPE cell transplantation in treating retinal degenerative diseases [102–105]. Therefore, hESC-derived RPE cells may provide a potential non-corneal cell source for the treatment of corneal endothelial diseases.

REPLACEMENT OF CORNEAL ENDOTHELIAL SUBSTITUTES

Cell-free corneal endothelial substitutes, commonly known as artificial corneal endothelium, primarily depend on the barrier function to preserve corneal transparency by preventing aqueous humor infiltration into the corneal stroma. Two distinct substitutes are currently evaluated as decellularized corneal endothelial substitutes and synthetic material-based endothelial substitutes. The absence of CECs eliminates concerns of cellular damage during the transplantation, thereby simplifying the surgical procedure, avoiding the complications of immune rejection and long-term immunosuppressive application (Figure 1).

Decellularized corneal endothelial substitutes

Decellularized corneal endothelial substitutes refer to the decellularized Descemet's membrane and endothelial cells. Mehta et al. reported the first application of decellularized corneal endothelial substitution in rabbit model. The study involved the implantation of decellularized Descemet's membrane, which resulted in increased CEC migration and a faster resolution of corneal edema compared to the control rabbit without the substitute [106]. Ying et al. conducted penetrating keratoplasty using corneal grafts lacking CECs. Among 195 cases, 18 patients experienced corneal graft edema 1–4 months

postoperation, during which no CECs were detected. Subsequently, corneal edema gradually resolved with the finally restored transparency, when the CEC density was 991 cells/mm², suggesting the potential endogenous regeneration of host CECs promoted by the presence of Descemet's membrane [107].

Artificial corneal endothelium

The artificial corneal endothelium is an optically transparent sheet constructed from synthetic materials. Acrylates serve as the predominant choice of synthetic materials due to their favorable intraocular biocompatibility and stability. EyeYon Medical has pioneered the development of EndoArt, an artificial endothelial implant fabricated via hydrophilic acrylates [108]. Preliminary findings of an ongoing multicenter clinical trial (NCT03069521) demonstrate the efficacy of the EndoArt implant in alleviating corneal edema and improving visual acuity in 7 out of 8 patients suffering from chronic corneal edema. However, more additional comprehensive research is necessary to substantiate its safety and efficacy before the clinical application, especially the investigations of the adhesion interface between the artificial endothelium and the posterior corneal stroma, as well as the intricate mechanisms related to nutrient supply and metabolite release within the central corneal stroma.

SUMMARY AND OUTLOOK

Corneal endothelial dysfunction is an important cause of corneal blindness and corneal transplantation worldwide. Consequently, the investigation of corneal endothelial regeneration and replacement approaches has consistently remained among the most challenging in the field of corneal research. Cell injection and substitute replacement are two promising therapeutic strategies, although their widespread clinical application still faces various challenges. Moreover, apart from systemic influences and primary corneal diseases, intraocular surgeries, including cataract surgery, penetrating keratoplasty, and glaucoma surgery, can lead to a rapid decline in CEC density and increase the risk of corneal endothelial dysfunction. Therefore, investigating the mechanisms of endothelial damage and the development of protection strategies may represent another avenue for effective prevention of corneal endothelial dysfunction.

AUTHOR CONTRIBUTIONS

Zongyi Li: Writing – original draft (lead). **Haoyun Duan:** Writing – original draft (supporting). **Qingjun Zhou:** Conceptualization (lead); Writing – review & editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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