DOI: 10.15825/1995-1191-2024-2-135-144

CHALLENGES OF OBTAINING CULTURED CORNEAL ENDOTHELIAL CELLS FOR REGENERATIVE PURPOSES

D.S. Ostrovskiy[1], S.A. Borzenok[1, 2], B.E. Malyugin[1, 2], O.P. Antonova[1], M.Kh. Khubetsova[1], T.Z. Kerimov[1, 2]

[1] Fyodorov Eye Microsurgery Complex, Moscow, Russian Federation
[2] Moscow State University of Medicine and Dentistry, Moscow, Russian Federation

Human posterior corneal epithelium (corneal endothelium) has limited proliferative activity both *in vivo* and *in vitro*. Disease or dysfunction in these cells leads to impaired corneal transparency of varying degrees of severity, up to blindness. Currently, the only effective standard treatment for corneal endothelial dysfunction is transplantation of donor cornea that contains a pool of healthy and functionally active cells. However, there is a global shortage of donor corneas, which has led to an unmet clinical need and the fact that only 1 patient out of 10 in need receives surgical treatment. Therefore, creation of cellular constructs and artificial human corneas containing healthy endothelium is a very urgent challenge facing modern ophthalmic transplantology. This review presents the current state of affairs, challenges and prospects for obtaining cultured corneal endothelial cells (CECs) *in vitro* for transplantation purposes.

Keywords: cornea, endothelium, posterior epithelium, transplantation, cells.

INTRODUCTION

The human cornea is a transparent, avascular tissue that is nourished primarily through the anterior chamber moisture from corneal posterior epithelial cells (otherwise known as the endothelium). The corneal endothelium is a monolayer of hexagonal cells, lying on a specialized basement membrane called Descemet's membrane. Its main function is to maintain the cornea in a transparent, relatively dehydrated state by an important layer of metabolic pumps mediated through Na/K-ATPase [1], as well as barrier function through ZO-1 tight contact proteins [2].

It is known that human endothelial cell density is approximately 6000 cells/mm^2 during the first month of life, but gradually decreases with age, with an annual loss of approximately 0.6% of the total cell population per year [3]. In healthy individuals, this natural decrease in density does not result in any clinically significant impairment of corneal structure and function. In case of more active endothelial cell loss, for example, due to surgical interventions or ocular trauma, there may be partial restoration of the functional integrity of the endothelial layer due to cell migration and increased area of healthy cells [3].

When the CEC density falls below the critical threshold of approximately 500 cells per mm^2, the endothelium loses its ability to regulate corneal stromal hydration, which leads to clouding and, consequently, to reduced visual acuity [3]. According to the World Health Organization, corneal diseases in 2020 were the cause of reduced vision in 7% of the world's population, and the third leading cause of blindness and low vision [4]. Penetrating keratoplasty was the gold standard for treating corneal endothelial diseases for a long time. However, taking the first place today are selective methods of lamellar keratoplasty, namely posterior lamellar keratoplasty (PLK), in which the donor graft includes a stromal layer in addition to the endothelium and Descemet's membrane, and Descemet's membrane endothelial keratoplasty (DMEK), in which only the endothelium is transplanted with the underlying Descemet's membrane [5–7]. These techniques today give good clinical and functional outcomes, while surgical methods of obtaining grafts for lamellar keratoplasty continue to improve [8, 9]. However, organizational and medical and legal problems related to donation are still an issue in many countries around the world, which explains the shortage of donor material. On the other hand, postoperative endothelial cell loss after PLK and DMEK reaches 35% or more per year [10] and that leads to the need for repeat keratoplasty [11]. Repeat keratoplasty is the second leading indication for corneal transplantation in some developed countries of the world [12].

Thus, there is a real need to study alternative therapeutic pathways that would help to reduce dependence on donor material in the treatment of corneal endothelial diseases, as well as increase the viability of transplanted endothelial cells, both donor and recipient.

Corresponding author: Dmitriy Ostrovskiy. Address: 59a, Beskudnikovsky Bul'var, Moscow, 127486, Russian Federation. Phone: (499) 488-85-58. E-mail: dmitriy.ostrovskiy@gmail.com

PREPARATION AND *IN VITRO* CULTIVATION OF CORNEAL ENDOTHELIAL CELLS

One of the ways to solve the problem of corneal endothelial dysfunction is the use of cell culture [13]. However, these cells proliferate weakly, due to their origin from neural crest progenitor cells and contact inhibition and expression of TGF-b factor in the anterior chamber moisture, endothelial cells are overwhelmingly in the G1 phase of the cell cycle [14].

Despite this, there is ongoing global effort to obtain cultured CECs. Mannagh J. et al. published the first report on a successful endothelium culture in 1965. They suggested immersing isolated corneoscleral discs in 0.06% pronase, incubating them at 37 °C for 2 hours, and then scraping the endothelium cells. Cultivation was carried out in an Eagle medium with addition of 6 g/L glucose and 20% fetal bovine serum under standard conditions. Corneas of donors aged 28 and 70 years were used in the experiment. The authors visualized attached rounded conglomerates of cells after 48 hours, which took on a characteristic epithelial morphology after 72 hours. However, on day 10, the cells acquired a mesenchyme-like morphology, which indicated an epithelial-mesenchymal transition (EMT) [15].

It is important to note that there has never been a single protocol for isolation and cultivation, nor a standard formula for the nutrient medium and required additives. This is because the criteria for choosing donor material have been controversial since the first culture of endothelial cells was obtained. In this review, we present protocols that made it easier to produce CEC cultures that did not undergo EMT at least up to passages 2.

DONOR CHARACTERISTICS

Joyce and Zhu conducted a comparative analysis of the possibility of obtaining cultured CECs. They were able to present the donor selection criteria (Table) [16].

In addition, exclusion criteria included the presence of diabetes mellitus, glaucoma, generalized infection (sepsis), and chemotherapy in the donor [17]. However, according to several Russian authors, diabetes and cancer (without the intoxication stage) are the preferred choice of donor as they can drive endothelial cells to mitosis [18, 19].

Table

Donor selection criteria for corneal endothelial cell culture

Donor age	2–79 years
Endothelial cell density	1,800–3,891 cells/mm^2
Average time from death to corneal preservation	≤12 hours
Average time from death to introduction into experiment	≤7 days

Parekh and Ahmad also showed that endothelial cell culture could be obtained from donors with mean ages greater than 75 years and mean endothelial cell densities of 1943.75 ± 222.02 kl/mm^2. In order to force cell conglomerates to adhere to one another during cultivation, the scientists employed Rho-associated kinase (ROCK) inhibitors and viscoelastic [17].

CELL ISOLATION METHODS

All currently available corneal endothelium isolation methods can be divided into 4 groups: mechanical, mechanical with the use of enzymes; enzymatic and based on organ culturing.

In the first of these isolation methods, the endothelium is mechanically separated from Descemet's membrane and then transferred to the culture surface. Obtaining a uniform CEC culture is this method's undeniable advantage. However, the culture obtained by this technique has a very low cellular activity, and most of the obtained cells express markers of early apoptosis [20].

The enzymatic isolation method involves incubation of the corneoscleral disc with collagenase, dispase, ethylenediaminetetraacetic acid (EDTA) or trypsin solution, which inevitably leads to a heterogeneous cell culture containing corneal stromal fibroblast admixtures. Subsequent application of selective culture media does not yield the expected homogeneous CEC culture [21–23].

Combined use of mechanical and enzymatic methods provides a gentler isolation of CECs, but the use of enzymes requires a long incubation time, which leads to increased cellular injury [21–24].

Obtaining a heterogeneous cell culture due to the presence of underlying stromal layers is one of the drawbacks of the method of organ culture of Descemet's membrane with an adjacent endothelial layer. Furthermore, this technique yields a culture with incredibly low mitotic activity [21].

COMPOSITION OF NUTRIENT MEDIUM

A comparative analysis of basic nutrient media for obtaining cultured CECs was carried out by Peh G. et al. [25]. Such nutrient media as DMEM [26], OptiMEM [27], DMEM/F12 [28], and Ham's F12/M199 [29] were investigated. It was shown that when DMEM and DMEM/F12 were used, the cell culture lost mitotic activity after the second passage, the cells became larger and underwent apoptosis. In turn, the use of OptiMEM and Ham's F12/M199 promoted culture maintenance up to the third passage with preservation of mitotic activity and expression of specific epithelial markers, such as ZO-1 and Na/K ATP-ase. However, despite the presence of a typical immunocytochemical pattern, the cells lost their characteristic morphologic shape. According to Zhu et al., these changes are caused by the presence of the main fibroblast growth factor in the composition of the nutrient media, which helps stimulate EMT [30].

Today, the most effective CEC culture scheme involves the step-by-step use of different nutrient media, the so-called "two media method". The first one is a medium containing the basic serum-free endothelial medium Human Endothelial SFM, which is necessary for stabilization of the cell culture and maintenance of the characteristic phenotype. To activate the proliferative activity of cells, the culture is placed in the second medium, Ham's F12/M199. The use of this approach allows obtaining a more homogeneous cell culture with a characteristic morphology and preventing the occurrence of EMT [31]. Despite the advantages of this method, currently available preclinical and clinical trials are based on the use of DMEM/F12 [32] and OptiMEM [33] media.

CULTURE ADDITIVES

In addition to the choice of the basic nutrient medium, selection of factors that support CEC culture is highly relevant. Factors such as bFGF [35], LIF [36], EGF [36], NGF [37], endothelial cell growth supplement [38] and L-Ascorbic acid 2-phosphate [39] have been reported to promote endothelial growth. The use of LIF is thought to delay contact inhibition and, together with bFGF in a serum-free medium, promote endothelial cell proliferation with preservation of the characteristic phenotype [35].

In scholarly publications, there are reports on the production of a non-transformable CEC cell line brought to passage 224 [39]. In this study, the cells were maintained in a DMEM/F12-based nutrient medium supplemented with 20% fetal bovine serum, antibiotics, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), N-acetylglucosamine hydrochloride, glucosamine hydrochloride, chondroitin sulfate, oxidation and degradation products of chondroitin sulfate, carboxymethyl chitosan, bovine eye extract and culture supernatant of human corneal stromal cells in logarithmic phase. The presented result, as stated by the authors, was achieved due to the use of conditioned medium. Zhu et al. reported that the use of conditioned keratocyte medium in the logarithmic growth phase stimulates CEC proliferation better than the use of bone marrow-derived mesenchymal stem cells [40]. The presented data possibly show the potential of using conditioned media to stimulate CEC proliferation.

There is a known pharmacological approach to endothelial repair using ROCK inhibitor Y-27632 [41]. The use of ROCK in culturing helps to regulate cell shape and movement by affecting the cytoskeleton [42]. ROCK inhibitor Y-27632 has been shown to promote better cell adhesion, inhibit apoptosis, is non-toxic and does not alter the morphology of human corneal endothelium [43, 44]. ROCK has been proposed as an active agent for restoring endothelial cell loss *in vivo* in animal models [8].

According to a number of publications, EMT can be inhibited using low molecular weight inhibitors RhoA and ROCK inhibitor Y-27632. EMT may be triggered by destruction of intercellular junctions during culture and culture passing [40].

Consequently, further research into the peculiarities of production and long-term cultivation of endothelial cells are needed in order to optimize and standardize conditions for their cultivation with confirmation of functional properties and to further translate these studies into clinical practice.

SEEDING DENSITY OF ENDOTHELIAL CELLS

A very important factor is the initial seeding density of cells required to preserve their hexagonal morphology and expression of all markers. It has been shown in studies that such a density is 1×10^4 cells per cm^2 [45].

SUBSTRATE FOR CORNEAL ENDOTHELIAL CELLS CULTURE

In addition to selecting the proper composition of the nutrient medium, another unresolved issue is the choice of an optimal culture surface [45]. Many research teams use coatings such as type I collagen [46] and fibronectin [16], type IV collagen [48], chondroitin sulfate and laminin [49], matrigel [50], and FNC coating mix [27] to obtain 2D cultured CECs. It is worth noting that the use of type IV collagen as well as laminin-511 E8 [51], as components of the corneal Descemet's membrane, seems to be the best solution to obtain homogeneous CEC culture [40]. Silk fibroin is also well established. It has been shown that its use can support CEC growth with characteristic morphology and expression of specific markers [52].

3D CULTURE OF CORNEAL ENDOTHELIAL CELLS

Several authors use 3D cultured CECs because of its undeniable advantage in terms of introducing cell culture into the anterior chamber of the eye. The use of 3D spheroids has been shown to preserve expression of ZO-1 and Na/K ATPase. This culturing option helps to overcome EMT [53, 54].

CRYOPRESERVATION

It should be noted that cultured CECs are unique, and their production is an expensive procedure. Therefore, it is important to prevent the loss of valuable cell cultures by cryopreservation. The possibility of using the following media for CEC cryopreservation has been shown: Opti-MEM + 10% DMSO + 10% FBS; Cellbanker 2; Bambanker; KM Banker; Stem-Cellbanker; Bambanker hRM; ReproCryo DMSO Free RM. Among them, only the use of Bambanker hRM, which does not contain xeno additives, complies with the requirements of good manufacturing practice (GMP) [55].

CELL CHARACTERIZATION

Cell characterization is one of the main points of culturing. It involves both determination of the functional

properties of the culture and confirmation of its authenticity. Studying morphology is the simplest and most obvious method of cell identification.

Peh et al. [45] proposed the use of the cell circularity index to evaluate the shape of CECs in order to distinguish them from elongated fibroblasts.

$$\text{Cell circularity} = \frac{4\pi \cdot \text{Area}}{\text{Perimeter}^2}$$

where a value approaching 1.0 indicates a circular profile.

Currently, the main standard procedures for cell culture identification are techniques for studying their genotype and the markers specific to the cells under study.

GENOTYPE OF CORNEAL ENDOTHELIAL CELLS

To assess the genotype of CECs obtained *in vitro*, reverse transcription-polymerase chain reaction (RT-PCR) is used to study mRNA expression. It should be noted that at present, there is no clear set of investigated genes necessary to confirm endothelial cell species-specificity. The most frequently investigated genes in primary human CEC culture are Na+/K+ ATPase (ATPA1), ZO-1 (TJP1), collagen type 8 (COL8) and transporter family (SLC4). The study of the following genes is also sporadically reported: Vimentin, N-cadherin, CD166, Nestin, OCT 3/4, Snail, p27, a-SMA, and Laminin [56–58].

PHENOTYPE OF CORNEAL ENDOTHELIAL CELLS

To study the phenotype of CECs obtained *in vitro*, the immunocytochemical method of staining for the following markers – Na+/K+ ATP-ase, ZO-1, Ki67 – found in almost all scientific reports, is mainly used. These markers are accepted by many researchers as basic and characteristic for human CECs [59]. In addition, N- and E-cadherin, Actin, CD 166, CD44, CD77, nestin, vimentin, type IV and VIII collagen, cytokeratin 3, a-SMA, and GFAP are also determined.

CLINICAL MARKERS FOR TESTING THE HOMOGENEITY OF CULTURED CORNEAL ENDOTHELIAL CELLS

The first clinical panel of CD markers was proposed by Kinoshita et al[33]. In their study, it was shown that endothelial cells expressing CD166+ and not expressing CD44- CD133- CD105- CD24- CD26- have unaltered genotype and phenotype and can be used for cell therapy. The absence of CD44, CD24, and CD26 indicates the exclusion of aneuploid cells, thus linking phenotypic analysis to cellular karyotype [60, 61].

It is worth noting a number of studies that report that functioning endothelial cells express CD166, CD200, GPC4, HLA-ABC and PD-L1 [57, 62–64] and do not express a-SMA, CD9, CD24, CD24, CD26, CD44, CD73, CD90, CD105, CD133, SNAIL, ZEB1 and vimentin [64–66].

RUSSIAN INNOVATIONS IN OBTAINING ENDOTHELIAL CELLS

Today the process of procurement and transplantation of posterior corneal epithelial cells in the Russian Federation is limited by Federal Law No. 180 “On Biomedical Cell Products”. However, the use of suspension of uncultured CECs is a legitimate method of using these cells and does not conflict with the above Law. According to some reports, corneal endothelial transplantation can be considered as a variant of selective endothelial keratoplasty [24].

Up until now, a comparison of different methods of endothelial cell isolation in an *ex vivo* experiment has been carried out in Russia. It has been found that the modified enzymatic approach of isolating endothelial cells is more efficient and desirable than the modified mechanical isolation method: a statistically significant difference ($p < 0.05$) in the number of isolated cells and particle size was found via analysis of flow cytometry data. At the same time, there were no statistically significant differences in the viability of endothelial cells isolated using the modified enzymatic method compared to the modified mechanical method. Development of endothelial cell isolation protocols has fundamental and clinical significance, as demonstrated by the authors of this study [24].

APPLICATIONS OF IPS CELLS

In 2006, a group of scientists from Kyoto University led by Takahashi and Yamanaka first obtained induced pluripotent stem (iPS) cells, which were isolated from fibroblasts by epigenetic reprogramming [67].

Currently, development of protocols for obtaining corneal epithelial cells from iPS cells is still ongoing. For instance, Martínez et al. investigated the optimal time to start cultivating so that iPS cells may start differentiating into the phenotypes of corneal epithelial precursors – limbal epithelial stem cells [68]. Several methods for producing CECs from iPS cells have been published so far [69].

There are ongoing studies aimed at deriving keratocytes from iPS cells. For instance, well-known protocols outline a step-by-step keratocyte production scheme, where it is suggested to synthesize keratocytes through the stage of obtaining neural crest cells to prevent iPS cells from transitioning into fibroblast-like cells [70].

It is established that iPS techniques can be used to derive CECs from embryonic stem cells [21, 66, 71–73] and umbilical cord blood stem cells [74].

ANIMAL TESTING MODELS

When modeling cultured CEC transplantation, the choice of an animal model must be carefully considered.

It is not possible to use animals belonging to the rodent or hare groups since their corneal endothelium is capable of regeneration and proliferation in contrast to human CECs [75]. For effective evaluation of endothelial cell transplantation, it is possible to use other animal models, such as cats [76], pigs or primates [77], as there are similar morphofunctional features between the endothelial cells of these animals and humans. However, it should be noted that these animals are expensive to maintain and care for, obtaining all permits and ethical approvals is not easy either.

CLINICAL APPLICATIONS

Today, there are two known studies on the clinical application of cultured human CECs [32, 33]. It is worth noting that in both studies, patients with bullous keratopathy were treated. These works were confirmed by preclinical studies *in vivo*, as well as on cadaveric eye in an *ex vivo* model. In the work of Kinoshita et al., cells were administered by injection in combination with ROCK inhibitor at a 1×10^6 kL/mL concentration; Parikumar et al. [32] used a nanocomposite gel in the form of a sheet with cultured cells on its surface at 5×10^5 cl/mL.

However, the researchers did not evaluate the migration of the injected cells, which led to much debate about the efficacy and precise role of cell culture in favorable outcomes. It is also worth noting that neither group reported side effects in patients. Kinoshita et al. stated the theoretical possibility of the cell culture entering the trabecular meshwork and the development of glaucoma, but no patient was found to have this pathology during a two-year follow-up [33].

Kinoshita et al. also published the results of a five-year follow-up of 11 patients. Normal corneal transparency was restored in 10 out of 11 patients. In the study of the final endothelial cell density after 5 years, 1000 cells per mm^2 and 2000 cells per mm^2 were obtained in 8 and 2 patients, respectively. Central corneal thickness returned to normal in 10 of 11 patients (<630 μm) within 5 years [78].

CONCLUSION

Transplanted cultured CEC has the potential to quickly restore vision and lessen the requirement for donor tissue, which could drastically alter how corneal endothelial diseases are treated. Results from different research groups show that CEC transplantation can be used in the treatment of corneal endothelial disorders. However, there are a few challenges that must be resolved before this technology can be used in a clinical setting. One of these issues is optimizing the CEC culture isolation and cultivation protocols. In addition, it is necessary to develop experimental methods that would allow tracking and mapping transplanted cells to assess the success of cell therapy. There is also a need to establish objective methods for assessing corneal transparency. Currently, macrophotography using a slit lamp is accepted, but interpretation of resulting images is subjective and varies significantly from author to author.

The only work published to date on human endothelial cell transplantation is by Kinoshita et al. (2018). It was carried out with 8% fetal bovine serum used in endothelial cell culturing. The existence of xenogeneic products, however, poses a serious obstacle to the continued mass use of cell products. It is important to note that, as of right now, no data on the development of a protocol for obtaining and culturing endothelial cells without the use of xenogenic products have been published.

In our view, it is best to take into account progressive techniques of using cell cultures, namely 3D spheroids, since the introduction of cell suspension makes it difficult to trace the point cell engraftment and to assess its efficiency. Because biocompatible substrate carriers require a somewhat larger surgical incision than that required for injecting a cell culture, they may technically complicate surgery in the hospital due to the increased traumatic nature of the intervention. In addition, using various matrices may need their removal later on, which could lead to side effects and complications. Thus, in the aspect of the aforementioned, the complex of issues surrounding endothelial keratoplasty is highly relevant, although it has not been well investigated and calls for more investigation.

This work was supported by a grant (No. 22-25-00356) from the Russian Science Foundation.

The authors declare no conflict of interest.

REFERENCES

1. *Clausen MV, Hilbers F, Poulsen H*. The structure and function of the Na,K-ATPase isoforms in health and disease. *Front Physiol*. 2017; 8: 371.
2. *Hutcheon AEK, Zieske JD, Guo X*. 3D *in vitro* model for human corneal endothelial cell maturation. *Exp Eye Res*. 2019; 184: 183–191. doi: 10.1016/j.exer.2019.04.003.
3. *Yamamoto A, Tanaka H, Toda M, Sotozono C, Hamuro J, Kinoshita S et al*. A physical biomarker of the quality of cultured corneal endothelial cells and of the long-term prognosis of corneal restoration in patients. *Nat Biomed Eng*. 2019; 3 (12): 953–960. doi: 10.1038/s41551-019-0429-9.
4. *Robaei D, Watson S*. Corneal blindness: a global problem. *Clin Exp Ophthalmol*. 2014; Apr; 42 (3): 213–214. doi: 10.1111/ceo.12330.
5. *Malyugin BE, Moroz ZI, Borzenok SA, Drozdov IV, Ayba EE, Pashtaev AN*. Pervyy opyt i klinicheskie rezul'taty zadney avtomatizirovannoy posloynoy keratoplastiki (ZAPK) s ispol'zovaniem predvaritel'no vykroennykh konservirovannykh ul'tratonkikh rogovichnykh transplantatov. *Oftal'mokhirurgiya*. 2013; 3: 12–16.
6. *Malyugin BE, Shilova NF, Antonova OP, Anisimova NS, Shormaz IN*. Sravnitel'nyy analiz kliniko-

funktsional'nykh rezul'tatov zadney posloynoy keratoplastiki s ispol'zovaniem femtosekundnogo lazera i mikrokeratoma. *Oftal'mokhirurgiya*. 2019; 1: 20–26.
7. *Maliugin BÉ, Shilova NF, Anisimova NS, Antonova OP*. Transplantation of endothelium and Descemet's membrane. *Vestnik Oftalmologii*. 2019; 135 (1): 98–103. (In Russ.). https://doi.org/10.17116/oftalma201913501198.
8. *Rajan MS*. Surgical strategies to improve visual outcomes in corneal transplantation. [e-journal]. *Eye*. 2014; 28 (2): 196.
9. *Kalinnikov YuYu, Dinh THA, Zolotarevskiy AV, Kalinnikova SYu*. A new surgical approach to pre-Descemet's endothelial keratoplasty. *Vestnik Oftalmologii*. 2023; 139 (1): 55–66. (In Russ., In Engl.). doi: 10.17116/oftalma202313901155.
10. *Baydoun L, Tong CM, Tse WW*. Endothelial cell density after Descemet membrane endothelial keratoplasty: 1 to 5-year follow-up. *Am J Ophthalmol*. 2012; 154: 762–763.
11. *Tarantino-Scherrer JN, Kaufmann C, Bochmann F, Bachmann LM, Thiel MA.* Visual recovery and endothelial cell survival after Descemet stripping automated endothelial keratoplasty for failed penetrating keratoplasty grafts: A cohort study. *Cornea*. 2015; 34: 1024–1029.
12. Eye Bank Assotiation of America (EBAA). Eye banking statistical report. Washington, D.C., 2019. 110 p.
13. *Malyugin BE, Borzenok SA, Komakh YuA, Arbukhanova PM, Zheltonozhko AA, Saburina IN et al.* Modern possibilities of cell technologies in biological equivalent of artificial cornea constructing. Byulleten' SO RAMN. 2014; 34 (5): 43–47.
14. *Joyce NC, Navon SE, Roy S, Zieske JD.* Expression of cell cycle-associated proteins in human and rabbit corneal endothelium *in situ*. [e-journal]. *Investig Ophthalmol Vis Sci*. 1996; 37 (8): 1566.
15. *Mannagh JJ, Irving A*. Human corneal endothelium: Growth in tissue cultures. *Arch Ophthalmol*. 1965; 74: 847–849.
16. *Joyce NC, Zhu CC.* Human corneal endothelial cell proliferation: potential for use in regenerative medicine. *Cornea*. 2004; 23: S8–S19.
17. *Parekh M, Ahmad S, Ruzza A, Ferrari S*. Human Corneal Endothelial Cell Cultivation From Old Donor Corneas With Forced Attachment. *Sci Rep*. 2017; 7 (1): 142. doi: 10.1038/s41598-017-00209-5.
18. *Ronkina TI.* Zakonomernosti vozrastnyh izmenenij endoteliya rogovicy cheloveka v norme i patologii, vozmozhnosti aktivacii proliferacii endoteliya i ih znachenie v oftal'mologii. [Dissertation]. M., 1994; 50.
19. *Yavisheva TM.* Morfo-funkcional'nye osobennosti endoteliya rogovicy cheloveka v norme i patologii i otbor donorskogo materiala dlya keratoplastiki. [Dissertation]. M., 1990; 198.
20. *Bartakova A, Kuzmenko O, Alvarez-Delfin K, Kunzevitzky NJ, Goldberg JL.* A Cell Culture Approach to Optimized Human Corneal Endothelial Cell Function. *Invest Ophthalmol Vis Sci*. 2018; 59 (3): 1617–1629. doi: 10.1167/iovs.17-23637.
21. *Parekh M, Ferrari S, Sheridan C, Kaye S, Ahmad S.* Concise Review: An Update on the Culture of Human Corneal Endothelial Cells for Transplantation. *Stem Cells Transl Med*. 2016; 5 (2): 258–264. doi: 10.5966/sctm.2015-0181.
22. *Kazantsev AD, Ostrovskiy DS, Borzenok SA*. Experience of a new method of isolation and cultivation of human corneal endothelial cells *ex vivo*. *Vestnik Soveta molodykh uchenykh i spetsialistov Chelyabinskoy oblasti.* 2017; 3 (4 (19)): 50–52.
23. *Kazantsev AD, Ostrovskiy DS, Gerasimov MYu, Borzenok SA*. Izuchenie eksperimental'nykh metodov vydeleniya i kul'tivirovaniya kletok endoteliya rogovitsy cheloveka. *Sovremennye tekhnologii v oftal'mologii*. 2017; 4: 105–108.
24. *Malyugin BE, Borzenok SA, Ostrovskiy DS, Antonova OP, Hubetsova MH, Tsikarishvili NR.* Development of a method for obtaining a suspension of human corneal endothelial cells and its subsequent transplantation in an *ex vivo* experiment. *Fyodorov Journal of Ophthalmic Surgery*. 2022; 4: 56–64. doi: 10.25276/0235-4160-2022-4-56-64.
25. *Peh GS, Toh KP, Wu FY, Tan DT, Mehta JS*. Cultivation of human corneal endothelial cells isolated from paired donor corneas. *PLoS One*. 2011; 6 (12): e28310. doi: 10.1371/journal.pone.0028310.
26. *Ishino Y, Sano Y, Nakamura T, Connon CJ, Rigby H, Fullwood NJ, Kinoshita S.* Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. *Invest Ophthalmol Vis Sci*. 2004; 45: 800–806.
27. *Zhu C, Joyce NC*. Proliferative response of corneal endothelial cells from young and older donors. *Invest Ophthalmol Vis Sci*. 2004; 45: 1743–1751.
28. *Li W, Sabater AL, Chen YT, Hayashida Y, Chen SY, He H, Tseng SC.* A novel method of isolation, preservation, and expansion of human corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2007; 48: 614–620.
29. *Engelmann K, Friedl P*. Optimization of culture conditions for human corneal endothelial cells. *In Vitro Cell Dev Biol*. 1989; 25: 1065–1072.
30. *Zhu YT, Chen HC, Chen SY, Tseng SC.* Nuclear p120 catenin unlocks mitotic block of contact-inhibited human corneal endothelial monolayers without disrupting adherent junctions. *J Cell Sci*. 2012; 125 (Pt 15): 3636–3648.
31. *Peh GS, Chng Z, Ang HP, Cheng TY, Adnan K, Seah XY et al.* Propagation of human corneal endothelial cells: a novel dual media approach. *Cell Transplant*. 2015; 24 (2): 287–304. doi: 10.3727/096368913X675719.
32. *Parikumar P, Haraguchi K, Senthilkumar R.* Human corneal endothelial cell transplantation using nanocomposite gel sheet in bullous keratopathy. *Am J Stem Cells*. 2018; 7 (1): 18–24.
33. *Kinoshita S, Koizumi N, Ueno M, Okumura N, Imai K, Tanaka H et al*. Injection of cultured cells with a ROCK inhibitor for bullous keratopathy. *N Engl J Med*. 2018; 995–1003.
34. *Lee JG, Kay EP*. FGF-2-mediated signal transduction during endothelial mesenchymal transformation in corneal endothelial cells. *Exp Eye Res*. 2006; 83 (6): 1309–1316.
35. *Liu X, Tseng SC, Zhang MC, Chen SY, Tighe S, Lu WJ, Zhu YT*. LIF-JAK1-STAT3 signaling de-

lays contact inhibition of human corneal endothelial cells. *Cell Cycle*. 2015; 14 (8): 1197–1206. doi: 10.1080/15384101.2015.1013667.
36. *Zhu YT, Chen HC, Chen SY, Tseng SC.* Nuclear p120 catenin unlocks mitotic block of contact-inhibited human corneal endothelial monolayers without disrupting adherent junctions. *J Cell Sci*. 2012; 125: 3636–3648.
37. *Chen KH, Azar D, Joyce NC.* Transplantation of adult human corneal endothelium *ex vivo*: a morphologic study. *Cornea*. 2001; 20 (7): 731–737.
38. *Liu Y, Sun H, Hu M, Zhu M, Tighe S, Chen S et al*. Human Corneal Endothelial Cells Expanded *In Vitro* Are a Powerful Resource for Tissue Engineering. *Int J Med Sci*. 2017; 14 (2): 128–135. doi: 10.7150/ijms.17624.
39. *Yokoi T, Seko Y, Yokoi T, Makino H, Hatou S, Yamada M et al*. Establishment of functioning human corneal endothelial cell line with high growth potential. *PLoS One*. 2012; 7 (1): e29677. doi: 10.1371/journal.pone.0029677.
40. *Zhu YT, Tighe S, Chen SL, John T, Kao WY, Tseng SC.* Engineering of Human Corneal Endothelial Grafts. *Curr Ophthalmol Rep*. 2015; 3 (3): 207–217.
41. *Koizumi N, Okumura N, Ueno M.* Rho-associated kinase inhibitoreye drop treatment as a possiblemedicaltreatment for Fuchs corneal dystrophy. *Cornea*. 2013; 32 (8): 1167–1170. doi: 10.1097/ico.0b013e318285475d.
42. *Okumura N, Kinoshita S, Koizumi N.* The Role of Rho Kinase Inhibitors in Corneal Endothelial Dysfunction. *Curr Pharm Des*. 2017; 23 (4): 660–666. doi: 10.2174/1381612822666161205110027.
43. *Galvis V, Tello A, Fuquen JP, Rodríguez-Barrientos CA, Grice JM*. ROCK Inhibitor (Ripasudil) as Co-adjuvant After Descemetorhexis Without an Endothelial Graft. *Cornea*. 2017; 36 (12): e38–e40. doi: 10.1097/ICO.0000000000001381.
44. *Bi YL, Zhou Q, Du F, Wu MF, Xu GT, Sui GQ.* Regulation of functional corneal endothelial cells isolated from sphere colonies by Rho associated protein kinase inhibitor. *Exp Ther Med*. 2013; 5: 433–437.
45. *Peh GS, Toh KP, Ang HP, Seah XY, George BL, Mehta JS.* Optimization of human corneal endothelial cell culture: density dependency of successful cultures *in vitro*. *BMC Research Notes.* 2013; 6: 176.
46. *Ostrovskiy DS, Gerasimov MYu, Khubetsova MKh, Kazantsev AD, Borzenok SA.* Kul'tivirovanie kletok zadnego epiteliya rogovitsy cheloveka s ispol'zovaniem biopokrytiya kollagena I tipa. *Geny i kletki*. 2019; 14 (S): 174.
47. *Malyugin BE, Borzenok SA, Saburina IN, Repin VS, Kosheleva NV, Kolokol'tsova TD i dr.* Razrabotka bioinzhenernoy konstruktsii iskusstvennoy rogovitsy na osnove plenochnogo matriksa iz spidroina i kul'tivirovannykh kletok limbal'noy zony glaznogo yabloka. *Oftal'mokhirurgiya*. 2013; 4: 89–97.
48. *Zhu YT, Hayashida Y, Kheirkhah A, He H, Chen SY, Tseng SC.* Characterization and comparison of intercellular adherent junctions expressed by human corneal endothelial cells *in vivo* and *in vitro*. *Invest Ophthalmol Vis Sci*. 2008; 49 (9): 3879–3886.
49. *Engelmann K, Böhnke M, Friedl P.* Isolation and long-term cultivation of human corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 1988; 29 (11): 1656–1662.
50. *Miyata K, Drake J, Osakabe Y, Hosokawa Y, Hwang D, Soya K et al*. Effect of donor age on morphologic variation of cultured human corneal endothelial cells. *Cornea*. 2001; 20 (1): 59–63.
51. *Okumura N, Kakutani K, Numata R, Nakahara M, Schlötzer-Schrehardt U, Kruse F et al*. Laminin-511 and -521 enable efficient *in vitro* expansion of human corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2015; 56 (5): 2933–2942.
52. *Vázquez N, Rodríguez-Barrientos CA, Aznar-Cervantes SD, Chacón M, Cenis JL, Riestra AC et al*. Silk Fibroin Films for Corneal Endothelial Regeneration: Transplant in a Rabbit Descemet Membrane Endothelial Keratoplasty. *Invest Ophthalmol Vis Sci*. 2017; 58 (9): 3357–3365. doi: 10.1167/iovs.17-21797.
53. *Audrey EK, Hutcheon James D, Xiaoqing G.* 3D *In Vitro* Model for Human Corneal Endothelial Cell Maturation. *Exp Eye Res*. 2019; 184: 183–191.
54. *Ostrovskiy DS, Kazantsev AD, Komakh YuA, Malyugin BE, Borzenok SA.* Poluchenie kul'tury kletok zadnego epiteliya rogovitsy cheloveka dlya transplantatsii. *Russian Journal of Transplantology and Artificial Organs*. 2018; 20 (S1): 157.
55. *Okumura N, Kagami T, Watanabe K, Kadoya S, Sato M, Koizumi N.* Feasibility of a cryopreservation of cultured human corneal endothelial cells. *PLoS One*. 2019; 14 (6): e0218431. doi: 10.1371/journal.pone.0218431.
56. *Chen Y, Huang K, Nakatsu MN, Xue Z, Deng SX, Fan G.* Identification of novel molecular markers through transcriptomic analysis in human fetal and adult corneal endothelial cells. *Hum Mol Genet*. 2013; 22: 1271–1279.
57. *Dorfmueller S, Tan HC, Ngoh ZX, Toh KY, Peh G, Ang HP et al*. Isolation of a recombinant antibody specific for a surface marker of the corneal endothelium by phage display. *Nat Publ Gr*. 2016; 6: 1–12.
58. *Bartakova A, Alvarez-Delfin K, Weisman AD, Salero E, Raffa GA, Merkhofer RM et al*. Novel identity and functional markers for human corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2016; 57: 2749–2762.
59. *Kazantsev AD, Ostrovskiy DS, Gerasimov MYu, Borzenok SA*. Razrabotka protokola fenotipirovaniya kletok zadnego epiteliya rogovitsy kadavernykh glaz cheloveka. *Sovremennye tekhnologii v oftal'mologii*. 2018; 4: 158–162.
60. *Hamuro J, Ueno M, Toda M, Sotozono C, Montoya M, Kinoshita S.* Cultured human corneal endothelial cell aneuploidy dependence on the presence of heterogeneous subpopulations with distinct differentiation phenotypes. *Invest Ophthalmol Vis Sci*. 2016; 57: 4385–4392.
61. *Toda M, Ueno M, Hiraga A, Asada K, Montoya M, Sotozono C et al*. Production of homogeneous cultured human corneal endothelial cells indispensable for innovative cell therapy. *Invest Ophthalmol Vis Sci*. 2017; 58: 2011–2020.
62. *Cheong YK, Ngoh ZX, Peh GS, Ang HP, Seah XY, Chng Z et al*. Identification of cell surface markers glypican-4 and CD200 that differentiate human corneal endotheli-

um from stromal fibroblasts. *Invest Ophthalmol Vis Sci*. 2013; 54: 4538–4547.
63. *Okumura N, Hirano H, Numata R, Nakahara M, Ueno M, Hamuro J et al*. Cell surface markers of functional phenotypic corneal endothelial cells. *Invest Ophthalmol Vis Sci.* 2014; 55: 7610–7618.
64. *Ueno M, Asada K, Toda M, Hiraga A, Montoya M, Sotozono C et al*. Micro RNA profiles qualify phenotypic features of cultured human corneal endothelial cells. *Invest Opthalmol Vis Sci*. 2016; 57: 5509.
65. *Ueno M, Asada K, Toda M, Nagata K, Sotozono C, Kosaka N et al*. Concomitant evaluation of a panel of exosome proteins and MIRS for qualification of cultured human corneal endothelial cells. *Invest Ophthalmol Vis Sci*. 2016; 57: 4393–4402.
66. *Wei Z, Batagov AO, Carter DRF, Krichevsky AM*. Fetal bovine serum RNA interferes with the cell culture derived extracellular RNA. *Sci Rep*. 2016; 6: 31175.
67. *Takahashi K, Yamanaka S.* Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006 Aug 25; 126 (4): 663–676. doi: 10.1016/j.cell.2006.07.024. Epub 2006 Aug 10. PMID: 16904174.
68. *Martínez García de la Torre RA, Nieto-Nicolau N, Morales-Pastor A, Casaroli-Marano RP*. Determination of the Culture Time Point to Induce Corneal Epithelial Differentiation in Induced Pluripotent Stem Cells. *Transplant Proc*. 2017; 49 (10): 2292–2295.
69. *Chakrabarty K, Shetty R, Ghosh A.* Corneal cell therapy: with iPSCs, it is no more a far-sight. *Stem Cell Res Ther*. 2018 Oct 25; 9 (1): 287.
70. *Naylor RW, McGhee CN, Cowan CA, Davidson AJ, Holm TM, Sherwin T.* Derivation of Corneal Keratocyte-Like Cells from Human Induced Pluripotent Stem Cells. *PLoS One*. 2016 Oct 28; 11 (10): e0165464.
71. *Lee JB, Song JM, Lee JE, Park JH, Kim SJ, Kang SM et al*. Available human feeder cells for the maintenance of human embryonic stem cells. *Reproduction*. 2004; 128 (6): 727–735.
72. *Chen P, Chen JZ, Shao CY, Li CY, Zhang YD, Lu WJ et al*. Treatment with retinoic acid and lens epithelial cell-conditioned medium *in vitro* directed the differentiation of pluripotent stem cells towards corneal endothelial cell-like cells. *Exp Ther Med*. 2015; 9 (2): 351–360.
73. *Zhang K, Pang K, Wu X.* Isolation and transplantation of corneal endothelial cell-like cells derived from in-vitro-differentiated human embryonic stem cells. *Stem Cells Dev*. 2014; 23 (12): 1340–1354.
74. *Joyce NC, Harris DL, Markov V, Zhang Z, Saitta B.* Potential of human umbilical cord blood mesenchymal stem cells to heal damaged corneal endothelium. *Mol Vis*. 2012; 18: 547–564.
75. *Mimura T, Yamagami S, Yokoo S, Araie M, Amano S.* Comparison of rabbit corneal endothelial cell precursors in the central and peripheral cornea. *Invest Ophthalmol Vis Sci*. 2005; 46 (10): 3645–3648. doi: 10.1167/iovs.05-0630.
76. *Van Horn DL, Sendele DD, Seideman S, Buco PJ.* Regenerative capacity of the corneal endothelium in rabbit and cat. *Invest Ophthalmol Vis Sci*. 1977; 16 (7): 597–613.
77. *Van Horn DL, Hyndiuk RA.* Endothelial wound repair in primate cornea. *Exp Eye Res*. 1975; 21 (2): 113–124. doi: 10.1016/0014-4835(75)90076-7.
78. *Numa K, Imai K, Ueno M, Kitazawa K, Tanaka H, Bush JD et al*. Five-Year Follow-up of First 11 Patients Undergoing Injection of Cultured Corneal Endothelial Cells for Corneal Endothelial Failure. *Ophthalmology*. 2021; 128 (4): 504–514. doi: 10.1016/j.ophtha.2020.09.002.

The article was submitted to the journal on 24.11.2023