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PREPARATION AND EVALUATION OF A SUSPENSION OF HUMAN CORNEAL ENDOTHELIAL CELLS ISOLATED FROM THE EYES OF CADAVERIC DONORS FOR TRANSPLANTATION IN AN EX VIVO EXPERIMENT

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Background. According to the World Health Organization, corneal diseases are one of the major causes of blindness globally. Endothelial dystrophy is one of the etiological factors leading to corneal diseases. The corneal endothelium is a monolayer of cells with virtually no mitotic activity. When the density of corneal endothelial cells falls below a critical threshold, the endothelium loses its ability to regulate corneal stromal hydration. This leads to corneal clouding and, consequently, to reduced visual acuity and quality of life of the patient. In this regard, various keratoplasty methods are widely used in clinical practice. Today, it is technically possible to transplant all corneal layers via penetrating keratoplasty, and to transplant the posterior epithelium via layer-by-layer keratoplasty. These surgical approaches are now widely used in everyday practice, but they require the use of scarce material – cadaveric donor corneas, from which grafts for the above-mentioned operations are formed in the conditions of an eye bank. In this regard, protocols for obtaining human corneal endothelial cell (HCEC) culture for subsequent transplantation have been proposed in recent years. However, the use of such approaches in Russia is limited by the law. **The aim** of this study was to experimentally justify the possibility of transplanting uncultured endothelial cells, isolated from cadaveric human corneas. **Materials and methods.** The first stage of the work consisted of obtaining a suspension of endothelial cells from cadaveric donor corneas and studying it; at the second stage, the transplantation effectiveness of the resulting cell suspension was assessed in an *ex vivo* experiment. **Results.** The cell phenotype after transplantation by the proposed method had high viability and preservation. **Conclusions.** The presented results suggest that phenotype and adhesion ability are preserved, and that the cell suspension has a high level of viability under adequate loss of endothelial cells during transplantation in the *ex vivo* experiment.

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

INTRODUCTIONS

The cornea is the most important optical medium of the eye, accounting for most of the eye's refractive power. This is due to the unique histological structure of this tissue. The cornea is one of the parts of the fibrous tunic of the eye and it is in direct contact with the environment. For this reason, many different etiological factors can lead to corneal injuries.

According to the World Health Organization, corneal diseases are one of the major causes of blindness globally and are the 4th leading cause of visual disability [1, 2]. In the Russian Federation, about 18% of patients have corneal opacities leading to partial or complete loss of vision. Endothelial dystrophy is one of the causes of corneal opacities.

The posterior epithelium (endothelium) of the human cornea is a monolayer of hexagonal cells located on the inner surface of the cornea. It plays a crucial role in maintaining corneal hydration balance, regulating the inflow of watery moisture to the stroma, thus ensuring corneal transparency. The posterior corneal epithelial cells are practically incapable of mitotic division, and when one of the cells dies, the neighboring cells migrate to the defect site and/or increase in size to close the monolayer defect. A loss of approximately 0.6% of cells per year of life is considered the physiological norm. When the density of CECs falls below a critical threshold of approximately 500 cells per mm^2, the endothelium loses its ability to regulate stromal hydration, which leads to corneal opacity and, consequently, to decreased visual acuity [3].

Keratoplasty is the main treatment for endothelial dystrophies. Today, there has been significant progress in transplantation methods, which is manifested in transition from end-to-end keratoplasty, implying replacement of all corneal layers, but accompanied by a high risk of complications, to direct transplantation of Descemet's

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membrane with endothelial cells. This has significantly reduced the risk of complications [4]. However, due to severe shortage of donor material all over the world, there are ongoing activities aimed at obtaining human endothelial cell culture for subsequent transplantation.

In 2018, a group of Japanese scientists led by Prof. S. Kinoshita for the first time successfully transplanted cultured CECs with the help of a rho-associated protein kinase inhibitor in patients diagnosed with bullous keratopathy. A prerequisite was that after the procedure, the patient had to stay in a prone position for 3 hours. This technique showed high clinical efficacy in the long-term follow-up period. Five years after surgery, normal corneal endothelial function was restored in 10 out of 11 operated eyes, endothelial cell density (ECD) of the central part of the cornea was 1257 ± 467 cells/mm^2, visual acuity increased statistically significantly in 10 eyes [5].

In the same year, a group of scientists from Japan and India presented HCEC transplantation results on three patients diagnosed with bullous keratopathy, who did not respond positively to drug therapy. Transplantation of cultured CECs into the anterior chamber was performed using nanocomposite gel to reduce cell migration into the structures of the eye's anterior chamber. A prerequisite was that after the procedure, the patient had to stay in a prone position for 24 hours. Cells from cadaver eyes were cultured for 26 days, then the resulting cell culture was transferred to the gel carrier, where co-culture lasted for one week. The resulting gel carriers were transplanted into 3 patients via a 23G cannula. Endothelial defect in the patients closed after 11 days. The gel carrier was then removed from the patients' anterior chamber. In two out of three cases, a significant increase in visual acuity was achieved at a follow-up period of up to 18 months.

One of the key issues of endothelial cell transplantation is the study of their native phenotype and its preservation in the process of isolation and cultivation. To study the phenotype of endothelial cells, the immunocytochemical method of staining for the following markers, found in almost all scientific reports, is most often used: positive Na^+/K^+-ATPase; ZO-1, Ki67 and negative vimentin and a-SMA. Many researchers take this set of markers as a basic characteristic of HCECs [6].

However, the presented methods have several limitations for implementation in the Russian Federation due to the Federal Law "On Biomedical Cellular Products", which prohibits the direct use of cultured cells in the hospital. The aim of this study was formulated to create an original endothelial cell transplantation technique that would not contradict the above Federal Law.

Aim: experimental validation of the possibility of transplanting uncultured endothelial cells isolated from cadaveric human corneas.

MATERIALS AND METHODS

Stage 1

Obtaining viable and phenotypically intact endothelial cells from cadaveric human corneas

To perform this stage, nine corneas with viable endothelium (but non-transplantable corneas due to the presence of stromal lesions) were obtained from the Eye Tissue Bank. Studies using biomaterials isolated from human cadaveric eyes were conducted in accordance with officially accepted procedures and special authorization under the laws of the Russian Federation. Donor mean age was 53 ± 4 years, the male/female ratio was 5/4, respectively. The average time from death to entry into the experiment was 20 ± 3 hours. According to the viability classification proposed by S.A. Borzenk, the corneas obtained were of grade 1A (non-transplantable corneas).

Isolation of endothelial cell suspension

Endothelial cell suspension was isolated from cadaveric donor corneas according to the following protocol. First, Descemet's membrane with endothelial cells was surgically isolated. Then the Descemet's membrane and endothelial cells were transferred into a tube for enzymatic treatment with chemically stable trypsin TrypLE (Thermo Fisher Scientific, USA), after which the tubes were transferred into a TS-100 thermo-shaker (BioSan SIA, Latvia) using the following device settings: constant heating temperature 37 °C, 800 rpm, 40 minutes. At the end of this procedure, the tubes were centrifuged, the supernatant was removed, and a culture medium of the following composition was added: DMEM/F12; 5% fetal bovine serum; 2 mM L-glutamine; L ascorbic acid; epidermal growth factor (EGF); insulin; Rho-associated protein kinase (ROCK) inhibitor Y-27632; antibiotic and antimycotic solution in a 1 mL volume, after which cell counting was performed on an automated cell counter Luna II (Logos, South Korea).

Determination of viability

The viability of the resulting suspension was determined by fluorescent staining using a commercial kit "Live and Dead" (Abcam plc., UK). This kit represents fluorescent dyes that stain dead cells in red color and live cells in green color. For analysis, 5 mL aliquots were taken from the resulting suspension and mixed with 5X dye solution for subsequent confocal microscopy. Evaluation was performed using a confocal laser scanning biological microscope FluoView FV10i (Olympus Corporation, Japan).

To determine viability and apoptosis, 7AAD and Annexin V dyes were used; for this purpose, 50 μL of suspension was taken and mixed with dyes. The analysis was performed on a CytoFLEX flow cytometer (Beckman Coulter, USA).

Flow cytometry

To confirm the conservation of endothelial cell suspension phenotype, flow cytometry was performed according to the protocol on a CD panel proposed by Kinoshito. Protocol description: 3 tubes of 1×10^5 cells each were formed, isotopic controls were added to 1 tube, CD166 APC 750, CD44 PC7, CD24 PE were added to 2 tubes, CD105 APC, CD26 PC7, CD133 FITC were added to 3 tubes. The analysis was performed on a CytoFLEX flow cytometer (Beckman Coulter, USA).

Stage 2

Assessment of transplantation efficiency for the resulting cell suspension in an ex vivo experiment

For the second stage, cadaveric donor corneas with a viability score of 1A, n = 4. Transplantation of the cell suspension onto pre-prepared cadaveric donor corneas with a standard defect zone was carried out under sterile conditions at the rate of 1,000 cells/mm^2, followed by cultivation for 1 week in complete cell culture medium of the following composition: DMEM/F12; 5% bovine calf serum; 2 mM L-glutamine; L ascorbic acid; EGF; insulin; ROCK Y-27632; antimycotic and antimycotic solution. For transplantation, we used a syringe with a glass nozzle with a 0.2 ml volume and <2 mL nozzle diameter, which allows optimizing microsurgical manipulations with the cell suspension.

The main stages of endothelial cell suspension transplantation included the mechanical removal of native endothelium using a sterile swab, detection of defect site by trypan blue stain, transfer of prepared endothelial cell suspension using a glass nozzle to the cornea at 1,000 cells/mm^2 per 100 μL of complete cell culture medium. Next, the corneas were cultured in 7 mL of complete cell growth medium in a 6-well plate. Cultivation lasted for 7 days.

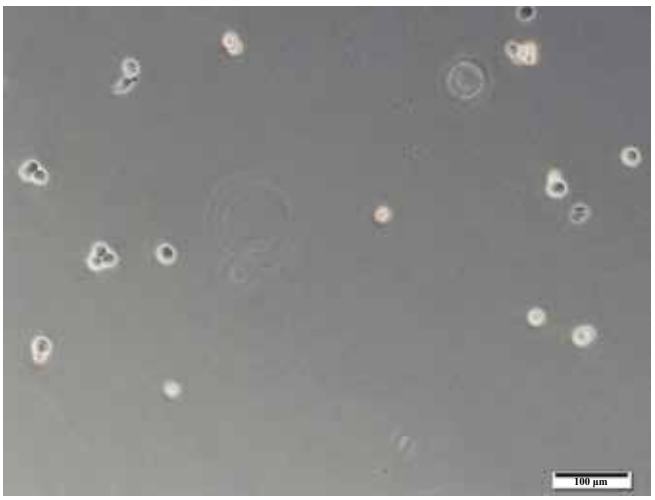

Fig. 1. Endothelial cell suspension. Light phase-contrast microscopy; 100× magnification

Immunohistochemistry

At the end of cultivation, to verify the functional activity of the transplanted posterior corneal epithelial cells, immunohistochemistry (IHC) was performed for the following markers: tight intercellular junction protein (ZO-1), functional proteins Na^+/K^+-ATPase, Lumican, Vimentin and proliferative activity protein Ki67. The corneas were washed once with sterile phosphate-buffered saline (PBS), then fixed in 10% formalin for 24 hours. The protocol of the IHC study included the following main steps: Permobilization with 0.1% Triton X100, blocking of non-specific binding with 0.3% Tween 20 and 1% albumin solution, incubation with primary and secondary antibodies, nuclei were counterstained with Hoechst #33258 nuclear dye. Analysis was performed using a FluoView FV10i confocal laser scanning biological microscope (Olympus Corporation, Japan).

Electron scanning microscopy

Corneal electron microscopy was also performed to determine the quality of closure of the formed defect by transplanted endothelial cells at the end of culturing. The obtained samples were washed once with sterile PBS, then fixed in 10% formalin for a day. The specimens were dehydrated in an ascending battery of acetone, followed by critical point drying, gold sputtering, and analysis on a Jeol 6000 plus electron-scanning microscope (Jeol, Japan).

RESULTS

Stage 1

At this stage, a suspension of endothelial cells was isolated, viability was assessed and the number of cells in apoptosis was determined. It was shown that with the enzymatic method of isolation, the suspension consisted of single cells with single small clusters of 5–10 cells (Fig. 1). The number of viable cells in the resulting suspension was 98.07 ± 1.21% (Fig. 2). The number of cells in a state of apoptosis was 0.1 ± 0.012%, dead cells 1.53 ± 0.61% (Fig. 3).

Flow cytometry showed an expression of more than 98% of CD166, low expression of CD105 and CD133, with no expression of CD24, CD26, and CD44, which is consistent with the results obtained by Kinoshito et al. (Figs. 4, 5).

Stage 2

For transplantation of endothelial cell suspension, it was proposed to use a glass nozzle, which is used in surgery. The main advantages of this nozzle include weak adhesion of cells to the glass walls of the nozzle, internal volume of the nozzle, which allows the cell suspension not to contact with the syringe piston, and small nozzle diameter, which allows to optimize surgical manipulations. Previously, we found that loss of endothelial cells in the glass nozzle is 10% ± 2.5%, and with this method

of transplantation is 40% ± 5.5%. The author's method of transplanting the prepared endothelial cell suspension is presented in Fig. 6.

IHC was performed after 1 week of culturing the cell suspension on the corneas. As a result of the study, expression of the characteristic markers of endothelial cells was detected in the corneal samples: ZO-1 protein (marker of dense intercellular junctions), Na^+/K^+-ATPase (marker of intact pumping function), Ki67 protein (marker of proliferative activity), with the presence of single cells expressing vimentin (protein of connective tissue cell cytoskeleton) and Lumican, which plays an important role in cell migration and proliferation during tissue repair and is also responsible for regulation of the assembly of the collagen matrix in the cornea. The images of endothelial cells obtained during IHC are presented in Figs. 7–9.

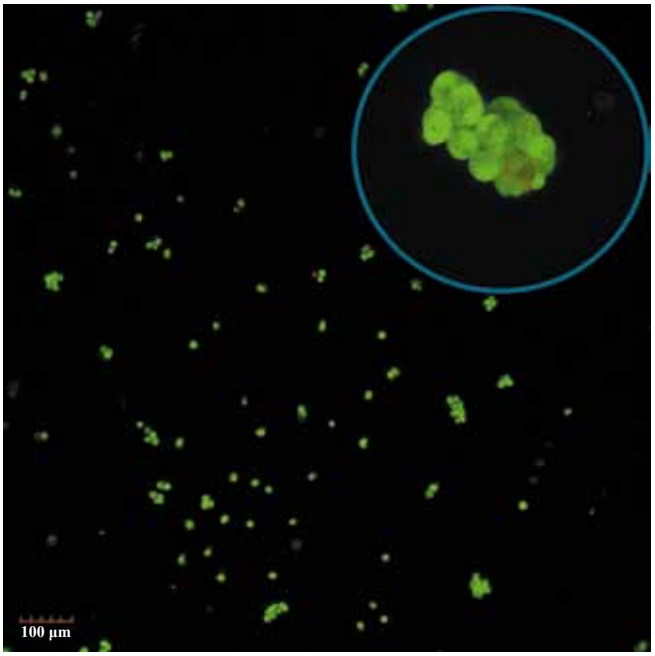

Fig. 2. Determination of endothelial cell suspension viability: live cells are stained in green, dead cells in red. Immunohistochemical staining, confocal laser scanning microscope; 100× magnification

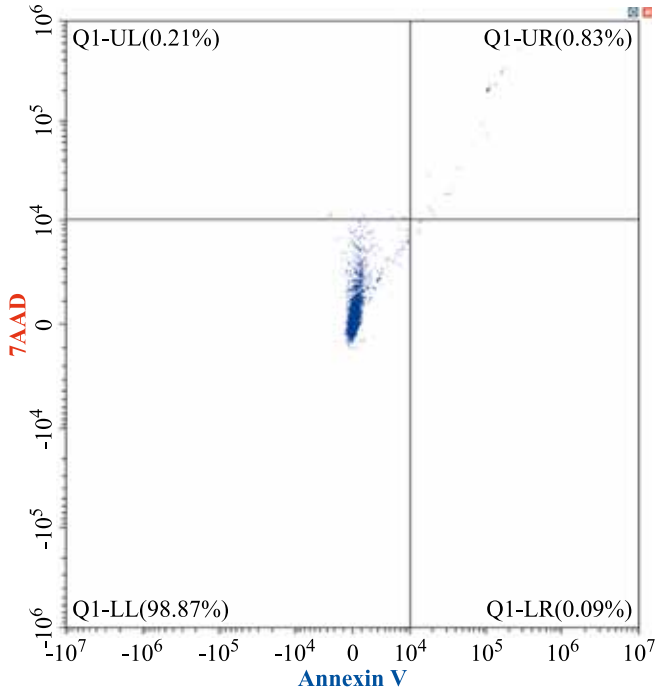

Fig. 3. Determination of viability and apoptosis of endothelial cell suspension. Flow cytometry

Scanning electron microscopy was performed to assess the ability of the resulting cell suspension to adhere to Descemet's membrane and create a monolayer subsequently. The results showed that the endothelial cell suspension formed a monolayer and closed the defect area. The images obtained during scanning electron microscopy are presented in Fig. 10. The red dotted lines indicate cell boundaries.

DISCUSSION

The current Federal Law of the Russian Federation "On Biomedical Cellular Products" regulates the use of cellular products in Russia. According to the adopted provisions, transplantation of in vitro cultured CECs is practically impossible today. For this reason, today it is not possible to use published and successfully proven algorithms for transplantation of human corneal posterior epithelial cells [5, 6]. In this regard, a proprietary endothelial cell transplantation method, which does not contradict the current Law, was used in the present study, since uncultured posterior epithelial cells of cadaveric donor corneas are used for transplantation.

As a result of transplantation of endothelial cell suspension from cadaveric donor corneas, it was found that the cells can successfully adhere to the cornea. The success of endothelial cell transplantation was evaluated by IHC and scanning electron microscopy. The phenotype of the obtained cell cultures was studied by several markers such as Na^+/K^+-ATPase and ZO-1. ZO-1 is a characteristic protein of tight intercellular junctions for epithelial cells. Expression of this tight intercellular junction marker is an indication of preservation of the epithelial phenotype of the studied cells after transplantation by the method presented in this work. The pumping function of the transplanted posterior epithelium was assessed by evaluating the expression levels of Na^+/K^+-ATPase. IHC revealed high levels of Na^+/K^+-ATPase enzyme expression. It was therefore concluded that the pumping function of endothelial cells, which is the key in maintaining corneal homeostasis, is preserved after transplantation according to the specified technique. Also, insignificant levels of expression of the vimentin protein show the absence of epithelial-mesenchymal plasticity of cells. The choice of vimentin is justified by the known role of this protein as a marker of mesenchymal cell transformation. All protein markers used in this study are widely used

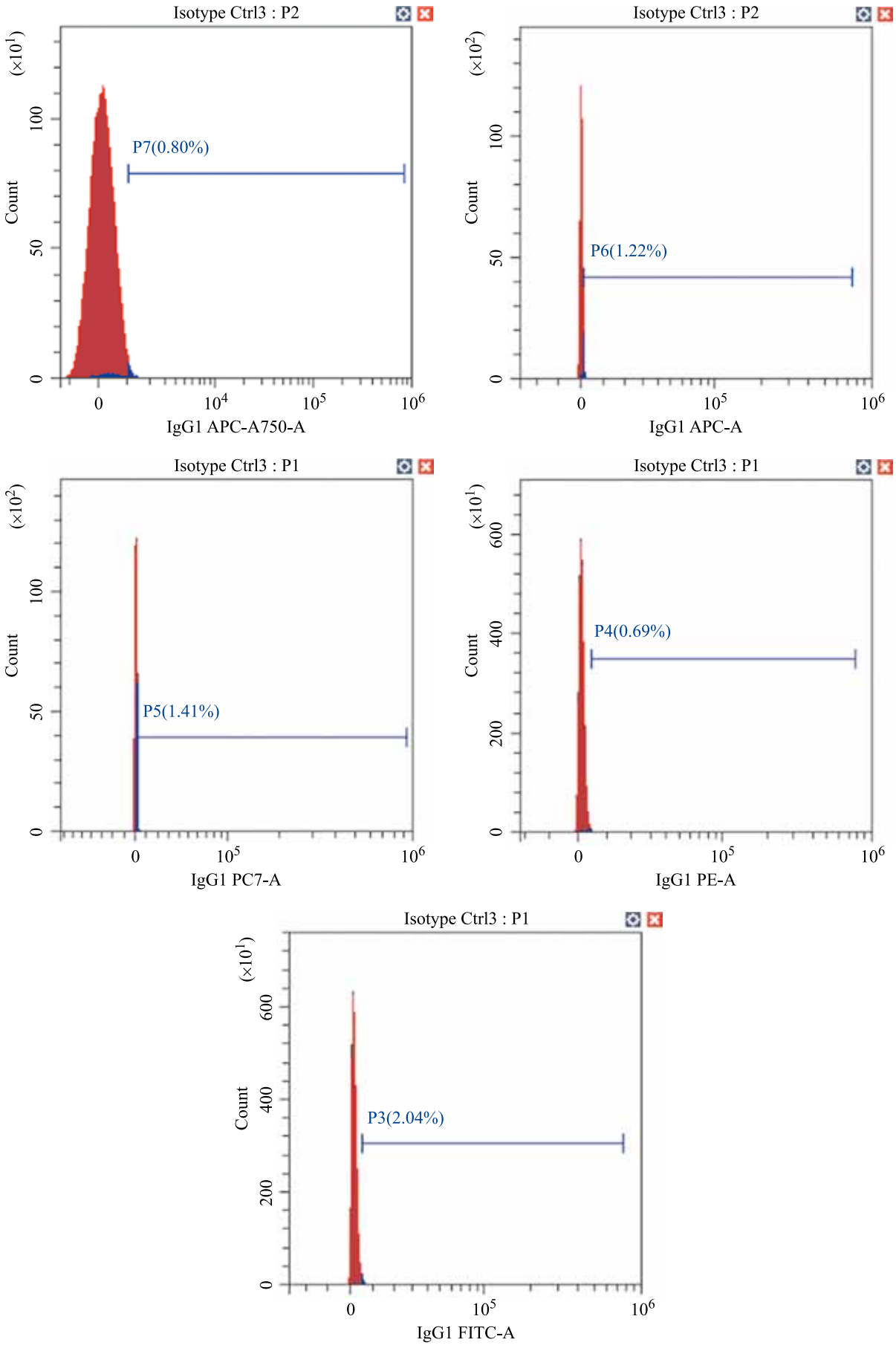

Fig. 4. Isotype control. Flow cytometry

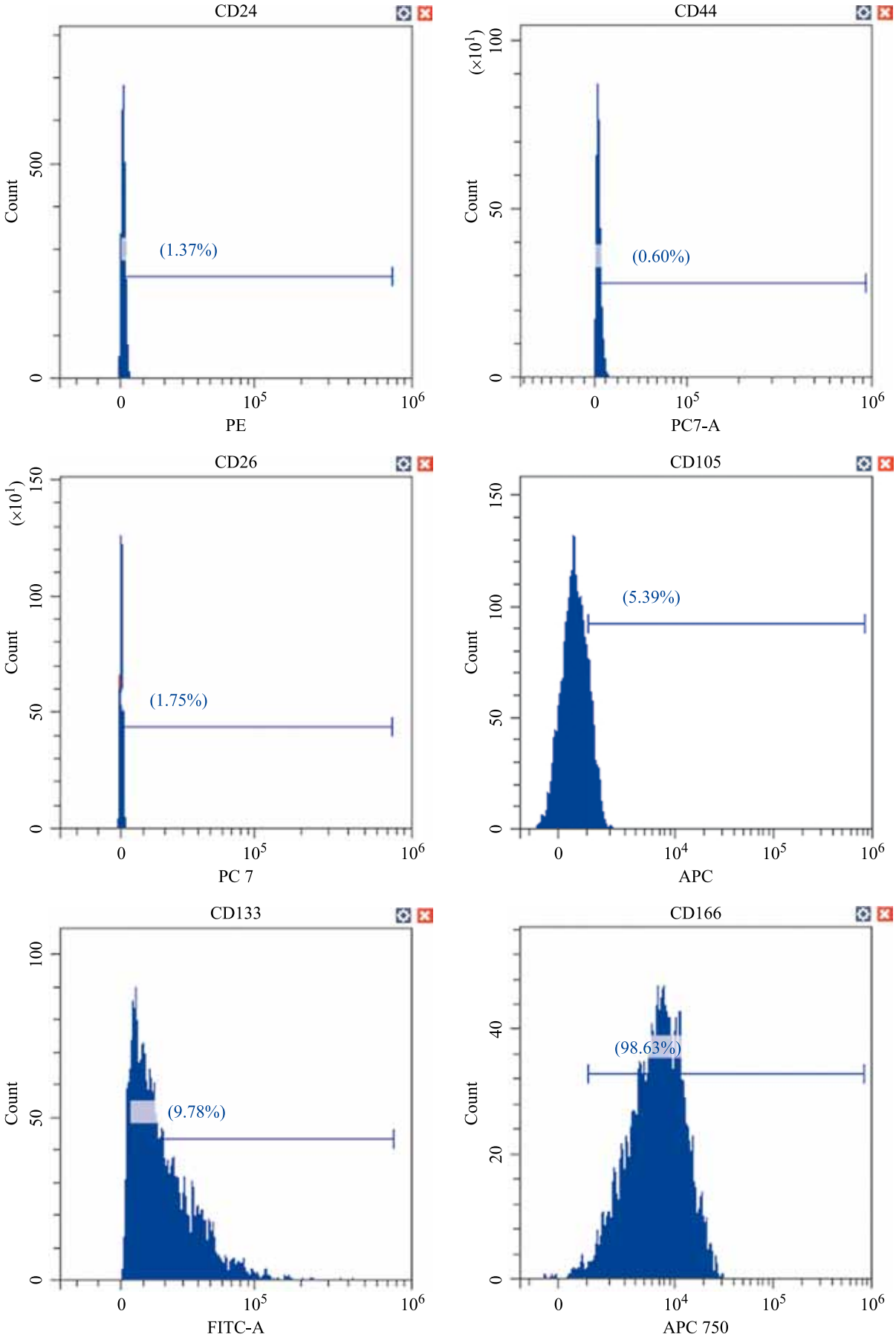

Fig. 5. Flow cytometry of a corneal endothelial cell suspension

in the study of corneal endothelium by various groups of scientists [7, 8].

The dust-like expression of the ZO1 marker was noted, which normally has a honeycomb-like nature. Apparently, this is down to the dissociation of endothelial cell monolayer that occurred when obtaining the suspension. Subsequently, as the monolayer and intercellular junctions are restored, we assume that this marker is fully restored.

The proposed method of transplantation of uncultured posterior epithelial cells using a glass nozzle showed high efficiency and safety for cells. The use of a glass nozzle provided weak adhesion of cells to the glass walls of the nozzle and, thus, ensured low loss of endothelial cells at the direct injection stage. Also, sufficient internal volume of the nozzle allows the cell suspension not to contact with the syringe piston, while the small nozzle diameter allows to optimize surgical manipulations. According to the data obtained, due to the above-mentioned features of the glass nozzle, a <10% cell loss was achieved during transplantation. In known publications, authors also emphasize the need to control transplantation speed to minimize trauma and the most delicate surgery [9]. Such control is possible when using a glass nozzle and performing transplantation according to the described technique.

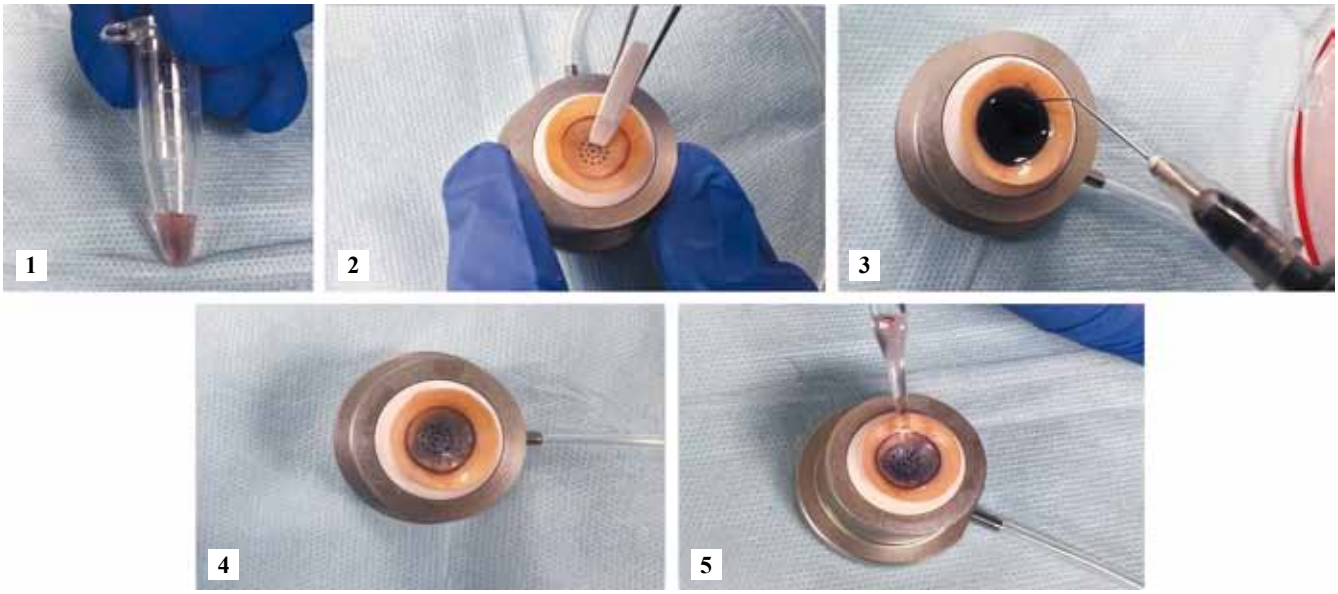

Fig. 6. Method of transplantation of a posterior epithelial endothelial cell suspension, main stages: 1) Preparation of cell suspension; 2) Removal of native endothelium; 3) Staining of defect area with trypan blue, 4) Identification of the defect area, 5) Application of the endothelial cell suspension into the defect area

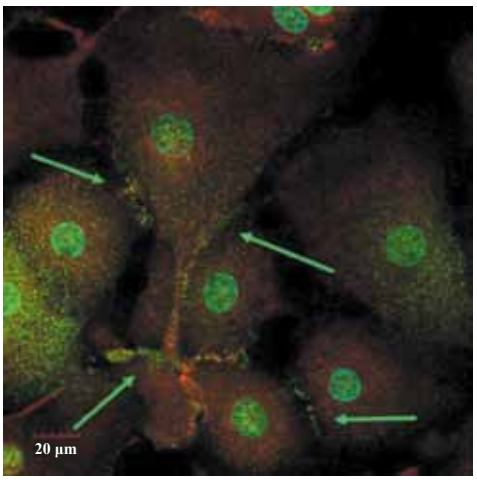

Fig. 7. Immunohistochemical analysis of corneal endothelium. Expression of ZO-1 (green arrows) and Na^+/K^+-ATPase (red stain). Immunohistochemical staining, confocal laser scanning microscope; 600× magnification

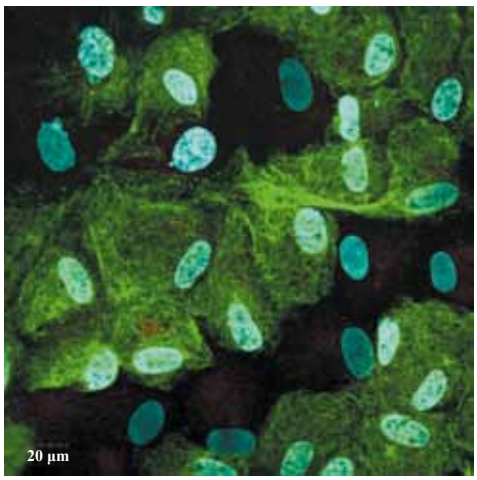

Fig. 8. Immunohistochemical analysis of corneal endothelium. Expression of lumican (green stain) and Na^+/K^+-ATPase (red stain). Immunohistochemical staining, confocal laser scanning microscope; 600× magnification

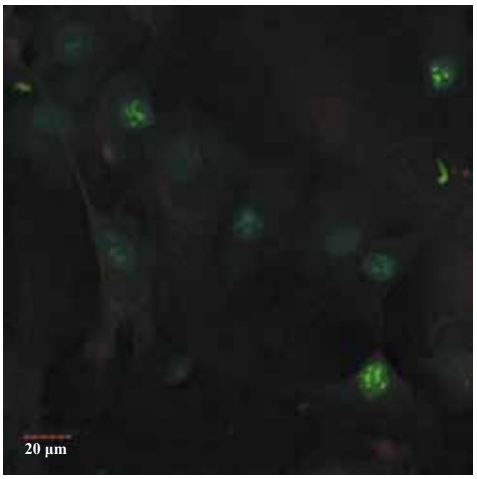

Fig. 9. Immunohistochemical analysis of corneal endothelium. Expression of proliferation marker protein Ki67 (green stain) and Vimentin (red stain). Immunohistochemical staining, confocal laser scanning microscope; 600× magnification

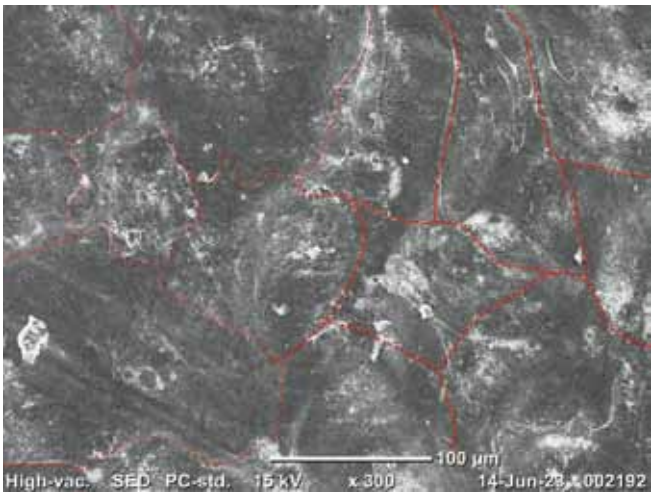

Fig. 10. Endothelial cell layer after culturing on a cadaveric donor cornea. Scanning electron microscopy, 300× magnification

CONCLUSIONS

1. The proposed enzymatic method for obtaining CEC suspension using chemically stable trypsin allows to obtain 98% of viable cells.
2. The resulting endothelial cell suspension expresses 96% CD133, expresses CD105 poorly and does not express CD24, 26, 44. This confirms that the phenotype is preserved after the enzymatic production method.
3. The proposed transplantation method allows minimizing external influence on the endothelial cell suspension and reducing up to 10% cell loss during surgical manipulations.
4. The transplanted suspension of corneal endothelial cells in the experiment showed that these cells can adhere to the Descemet's membrane, forming a monolayer and expressing the characteristic cellular proteins ZO-1, Na^+/K^+-ATPase and Lumican.

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The authors declare no conflict of interest.

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