Original article

Experimental allogenic transplantation of cornea endothelial cells in cats

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Abstract

Background: The aim of the present study was assessing the possibility of experimental allogenic transplantation of cat cornea endothelial cells, multiplied in vitro, into the anterior chamber of the eyeball in recipient cats. The reason for undertaking the research is the need to develop a method that would help in the cornea treatment in animals with corneal opacification following cataract surgery, as well as lens dislocation, injuries and endothelium degeneration.

Methods: Cats aged 10-12 months were used in the experiment. Cornea fragments consisting of the posterior limiting membrane and posterior epithelium were placed in Iscove;s medium with addition of 10% foetal calf serum. Multiplied in vitro cells were injected into the anterior chamber of recipient cats. The cornea was subject to histological, histometric and SEM examination on the 3rd, 7th, 20th and 30th day after the surgery.

Results: Micromorphological examination of the cornea showed full restitution of its endothelium 30 days after transplantation. Complete regeneration of structures indispensable for normal functioning of the posterior epithelium occurred as a result of implantation.

Conclusions: In this study the results show that implantation of the cells of posterior corneal epithelium of donor cats, multiplied into vitro and injected into the anterior chamber of recipient cats. The cornea regained its full function, the layer of the posterior epithelium was regenerated and the stroma stabilized, presenting the image of full and proper corneal translucency.

Key words: cornea, cat, endothelium, transplantation.

Abbreviations: DSAEK/DSEK, Descemet's stripping automated endothelial keratoplasty; DLEK, Descemet's Lamellar Endothelial keratoplasty; IOL, implantation of intraocular lens; PBK, pseudophakic bulbous keratopathy; ABK, aphakic bulbous keratopathy; FCS, foetal calf serum; SEM, scanning electron microscope.

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Introduction

First attempts at culture of cells of the corneal endothelium, also called the posterior epithelium, were made in the 1970s, however selection of adequate culture media posed a problem (Jumblatt et al. 1978, Baum et al. 1979, Insler and Lopez 1986). Cells of the corneal endothelium in man, ape, dog and cat are incapable of regeneration, while the rabbit cells regenerate following damage (Nishida 2005). Research was aimed at estimating of the possibility of xenogenic transplantation of endothelial cells from cattle to rabbit (Gospodarowicz et al. 1979 a,b). Good results were obtained with allogenic transplantations where endothelial cells of the donor rabbit, incubated on the posterior limiting membrane, were implanted on a trepanation lobule into recipient rabbit (Fei et al. 1994). Cornea of mice with experimentally induced mucopolisaccharidosis was transplanted into rabbits (Aguirre et al. 1992). Fluorocarbon polymer implants were implanted into the posterior surface of rabbit cornea (Renard et al. 1996). The most recent research on rabbits used hydroxyethyl methacrylate polymer as carrier of endothelial cells in penetrating transplantations (Shimmura et al. 2005). Experimental studies included also xenogenic transplantations of endothelial cell cultures from cattle to cats (Bahn et al. 1982).

The most modern technique of cornea transplantation (Descemet's stripping automated endothelial keratoplasty – DSAEK/DSEK), applied to humans in the USA, makes it possible for the patient to regain vision one month after surgery. In traditional penetrating keratoplasty, the patient regains vision in 12 months, in Descemet's Lamellar Endothelial keratoplasty (DLEK) – in 6 months (Price and Price 2006).

Performing a penetrating or lamellar cornea transplantation in animals is difficult because of the cost and numerous complications during convalescence which lasts about a year (Wilkie and Whittaker 1997, Pena Gimenez and Morales Farina 1998, Gelatt 1999, Gelatt 2000). Stitches are removed 9-12 months after penetrating keratoplasty. In the past, experimental attempts were made at anterior lamellar transplantation in cattle, mule and goat (Mashira and Reddy 1991, Sarma et al. 1991, Hoque et al. 1993).

At present, the most frequent reason for cornea opacity and thus recommendation for penetrating transplantation in humans and animals is corneal oedema associated with decompensation of endothelial cells following implantation of intraocular lens (IOL) after cataract surgery – the condition called pseudophakic bulbous keratopathy (PBK), or aphakic bulbous keratopathy (ABK). Following IOL implantation, decrement in the corneal endothelium may range from 10% to 40% (Peiffer 1983, Waring 1989, Glover and Constantinescu 1997, Spalton et al. 2007).

The reason for our research was the need to develop a method that would help in the cornea treatment in animals with corneal opacification following cataract surgery, as well as with lens dislocation, injuries and endothelium degeneration. Besides, following cataract surgery or other surgery necessitating opening of the anterior chamber of the eyeball, cells of the corneal endothelium undergo damage. Following microsurgery within the anterior pole of the eyeball, the cornea may loose its translucency. The most frequent complication is the damage of corneal structure and disturbance of the function of the damaged corneal endothelium in the form of bulbous keratopathy in post-operational aphakia, or pseudophakia. The lack of endothelium regeneration capacity in cats and dogs results in permanent loss of cornea translucency, not receding after pharmacological treatment.

Because of the similarity of the structure and reaction of endothelial cells in man and cat, the cat was chosen as the biological model for the study. Pertinent literature includes few papers on transplantation of cell cultures of the cat corneal endothelium multiplied in vitro and implanted into the anterior chamber of eyeball of recipient cats, thus it was decided to study the possibility of such implantation. Besides, we aimed at assessing the functional state and structure of the posterior limiting membrane and substantia propria, adjoining the implanted cultured cells, and their effect on the cornea translucency following the surgery.

Materials and Methods

Twenty five cats aged 10-12 months, body mass ca. 2.5-4.0 kg, were used in the experiment. The animals were pharmacologically prepared for surgery by intramuscular administration of 2% xylasin (1mg/kg body weight) and anaesthesised with ketamin hydrochloride (10 mg/kg body weight i.m.). The cornea wall was separated under surgical microscope. This consisted in removal with a knife of the anterior epithelium with the substantia propria to the posterior limiting membrane. Then the anterior chamber was opened with a knife at the corneoscleral junction and a circle of a part of its wall, composed of the posterior limiting membrane and endothelium, was cut out with scissors.

Isolation and in vitro multiplication of corneal endothelium cells were performed in the laboratory. The material was placed in the Iscove's medium with addition of 10% foetal calf serum (FCS) on a glass Petri dish and cut into small fragments with scissors. All the procedures were performed in high sterility conditions under laminar extractor with flow of sterile air. The

medium was then removed, the cornea fragments were transferred to a plastic Petri dish and covered with 2 cm^3 of 1% collagenase I solution in PBS. The dish was placed in a thermostat with regulated CO_2 concentration and incubated during 1 hour at 37°C in 5% CO_2 atmosphere. The incubation with collagenase was to facilitate the release of endothelial cells from the collagen posterior limiting membrane. Following the incubation, the collagenase solution was removed with a pipette and the cornea fragments were rinsed in PBS.

Then the Iscove's medium with 20% FCS was added and the material was transferred to a 24-well culture plate, previously coated with collagen, dividing the mixture into 15 wells. The wells were filled with the medium to the volume of 1 ml, and the cell culture was conducted at 37°C in 5% CO_2 atmosphere. After 5-6 days, the cells occupied half of the well. After ca. 10 days they covered the whole bottom of the well and were ready for further use. One well yielded the average of 200 000 live cells of the corneal endothelium.

The cells showed a strong adherence to the substratum and were collected from the plate using trypsin in the following way. After removal of the medium, the cell layer was rinsed with 0.5 cm^3 of 0.25% trypsin solution, and incubated with 1 ml of trypsin solution at 37°C for 3 minutes. Following the incubation, the suspension of detached cells was stirred with Pasteur pipette and transferred to a tube with 5 ml of medium with 10% foetal serum, where the excess of protein stopped the trypsin reaction. 50 μl of suspension served to count the cells, and the rest of suspension was centrifuged at 1200 revolutions per minute during 6 minutes. Quantitative analysis of the cells was performed under the microscope on the Burker chamber grid. 50 μl of cell suspension were mixed with 50 μl of 0.2% tripane blue solution. Dead cells stained blue, live cells were yellow. Counting blue and yellow cells made it possible to calculate the percentage of dead cells i.e. the suspension viability. Suspension with the proportion of dead cells not exceeding 10% was regarded as viable. In the endothelium cell cultures the viability was 2-3% dead cells. The cells from the suspension were precipitated through cenrifugation during 6 minutes, at 1200 revolutions per minute, and used in the experiments.

The corneal endothelium of recipient cats was removed through kryodestruction. Kryoextractor pre-cooled in a container with liquid nitrogen, with the tip in the form of cylinder of 10 mm in a diameter and of a temperature of about -70°C, was applied to the cornea surface for 20 seconds. This caused destruction of the monolayer cuboid corneal endothelium and uncovering of the posterior limiting membrane of an area 10 mm in a diameter.

Following such preparation of the anaesthesised recipient cat, paracenthesis was made in the corneoscleral junction at the angle of 30° with injection needle 25G; 0.2 cm^3 of aqueous humor were removed from the anterior chamber with the syringe and replaced with the same quantity of liquid with corneal endothelium cells multiplied *in vitro*.

The animals were divided into 5 experimental groups, 5 cats each, with cornea samples for histological studies taken as follows:
– group I – control
– group II – after 3 days
– group III – after 7 days
– group IV – after 20 days
– group V – after 30 days

The removed cat corneas were fixed in AFA fluid (Culling 1974). Each fixed dome-shaped cornea was cut through the middle. The material was dehydrated in a graded ethanol series and embedded in paraffin. Serial sections 5-6 μm thick from the medial profile of the cornea were stained with hematoxylin and eosin, and alcian blue (Lev and Spicer 1964). The right cornea was experimental, the left one served as control. Two series, each including 100 slides, were made from each cornea. Cells located within the zone of the posterior epithelium (endothelial cells, macrophages, fibroblasts) were analyzed on slides stained with hematoxylin and eosin. Slides stained with alcian blue served as the basis of histometric analysis of the endothelium. Endothelial cells, as well as macrophages and fibroblasts were counted.

Counts included 1 mm sections and were then converted to the surface of 1 mm^2. Three fields were analyzed for each cornea. As a result, for each animal counts were made from the total of 90 fields in the right and left cornea.

Material for scanning electron microscope (SEM) analysis was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2-7.4 with addition of 7.5% sucrose during 48 hours (Millonig 1961). It was then rinsed in the same buffer for 24 hours and post-fixed in 1% osmium tetroxide during 2 hours. Dried material was gold-coated.

The material was analyzed in a scanning electron microscope Stereoscan – 180 (Cambridge Instruments).

Eyes of the cats after allogenic transplantation of endothelial cells into the anterior chamber were clinically examined every day, using slit lamp, ophthalmoscope, surgical microscope and tensiometer.

Results

Histological and histometric analysis of the cat cornea showed a complete restitution of the corneal endothelium 30 days after transplantation (Table 1: 2874.03 ± 264.56 towards 3412.19 ± 305.78 in the control cornea). Some degenerated cells were still present

Table 1. Histometric analysis of the transplanted endothelium of the cornea in cats.

Cells undergoing histometric analysis		Particular days of experiment									
		Day 1		Day 3		Day 7		Day 20		Day30	
		control	experiment	control	experiment	control	experiment	control	experiment	control	experiment
1	2	3	4	5	6	7	8	9	10	11	12
Endothelium cells unchanged	x	3224.62	956.16	3538.61	1051.49	3141.87	1372.18	3641.93	2481.61	3412.19	2874.03
	SD	±269.62	319.16	244.69	324.32	232.14	170.41	269.59	238.14	305.78	364.56
Degenerated and degenerating cells	x	14.52	1124.24	20.26	907.15	12.96	854.06	21.36	518.94	37.54	284.01
	SD	±2.65	296.31	2.33	281.63	1.19	67.91	2.79	48.07	3.67	61.18
Macrohages	x	5.31	96.48	7.39	84.69	9.81	79.45	6.15	34.98	8.46	17.62
	SD	±1.78	±8.51	±1.54	±7.42	±0.68	±8.56	±0.82	±3.17	±1.21	±2.37
Fibroblasts	x	4.66	49.37	5.68	38.06	6.77	36.94	4.98	16.54	4.28	10.14
	SD	±1.38	±6.12	±1.15	±4.81	±1.42	±5.29	±0.84	±2.77	±0.95	±1.93

x – measurements were made on the surface of 1.00 mm^2
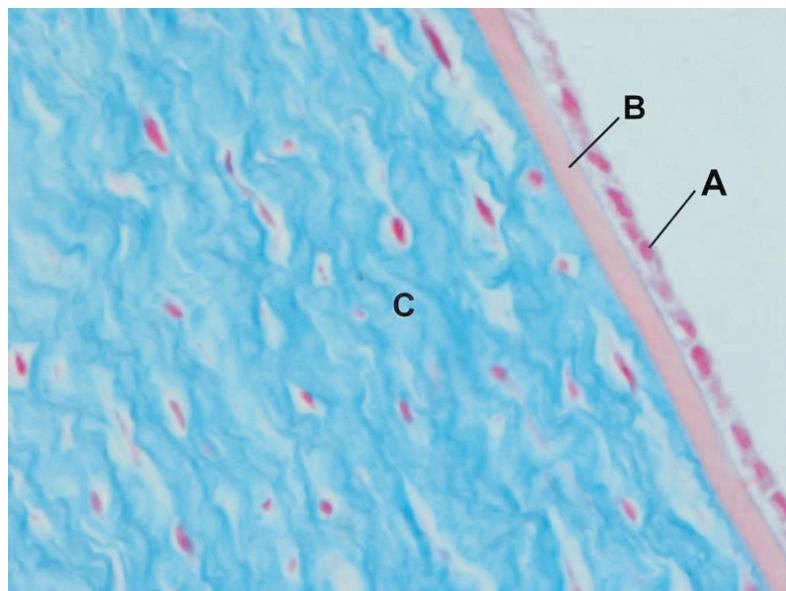

Fig. 1. Histological image of the corneal endothelium (A) on the basal membrane (B), adhering to the stroma (C) in the control group. Lev and Spicer's staining, 400x.

(284.01 ± 61.18). The number of macrophages decreased distinctly (17.62 ± 2.37) as well as that of fibroblasts (10.44 ± 1.93). The results of histometric analysis are presented in Table 1.

Restitution of the endothelium with all its consequences started on the 7th day after transplantation. The number of regenerating cells increased significantly (1372.18 ± 17.41 compared to the first day after surgery). A considerable number of mitoses was an expression of the progressive restitution. The number of degenerated cells decreased (854.06 ± 67.91 towards 1124.24 ± 296.31). The decreased number of degenerated and degenerating cells was accompanied by corresponding changes in the number of macro-phages (79.45 ± 8.56 towards 9.81 ± 0.68 in the control cornea). Progressive restitution was observed on the 20th day post-surgery and it reached its maximum on the 30th day.

The corneal endothelium in the control animals had the form of a monolayer of cuboid cells resting on the distinctly developed basal membrane underlained by a network of collagen fibres, forming the proper layer of the cornea (Fig. 1).

The presented morphological image of the endothelium indicates also ongoing processes of exchange of intra-tissue fluid. The cells had the form of monolayer cuboid, and in places flat, epithelium. Some cells were distinctly flat, other cuboid, which
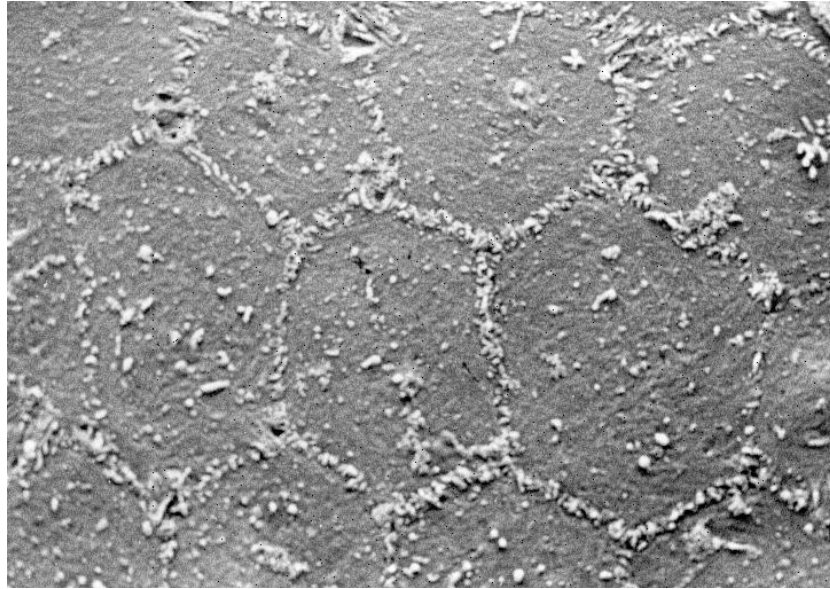
Fig. 2. Ultrastructural image of cells of the corneal endothelium. Visible borders between the endothelial cells and uneven surface of the cells. SEM, 3,000x.
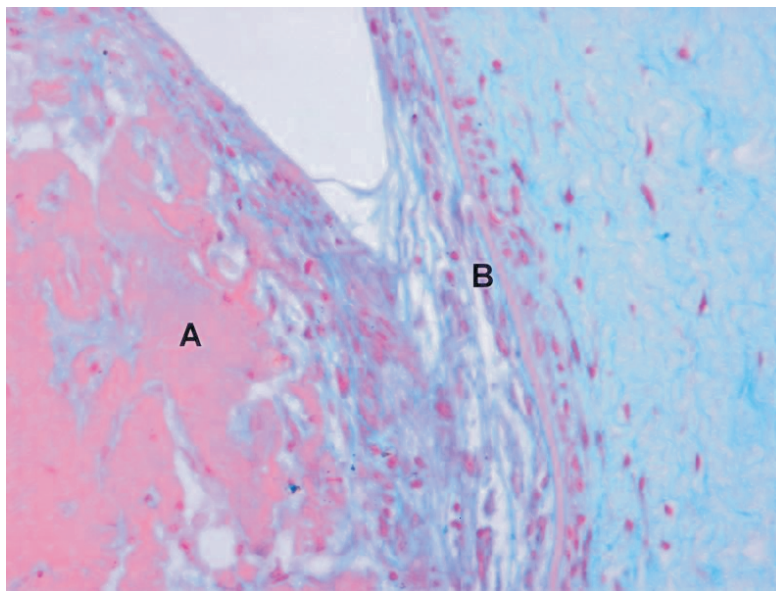

Fig. 3. A cluster of multiplied cells in the anterior chamber, creating matrix (A). Allograft of cells 7 days after transplantation remaining in the anterior chamber, close to the corneal endothelium (B). Lev and Spicer's staining, 300x.

might result from different behaviour of the free zone of cells delimiting the anterior chamber. Free surfaces of the epithelium, especially in case of high cuboid cells, were provided with microvilli forming a "border ridge".

In SEM images, the free surface of these cells showed also differentiated structure associated with microvilli and the covering secretion. Contacting endothelial cells formed adherence junctions. The arrangement of epithelial cells was hexagonal (Fig. 2).

In some places in the free zone of the epithelium, hollows and variations were observed, indicating degradation processes in the cells. The varied height of the epithelium could result from various functional states. The epithelium forms a limiting barrier and the fact has many implications. It delimits the anterior chamber and is also a zone participating in the transport of intra-tissue fluid.

Remains of degraded epithelial cells located directly on the basal membrane were observed on the
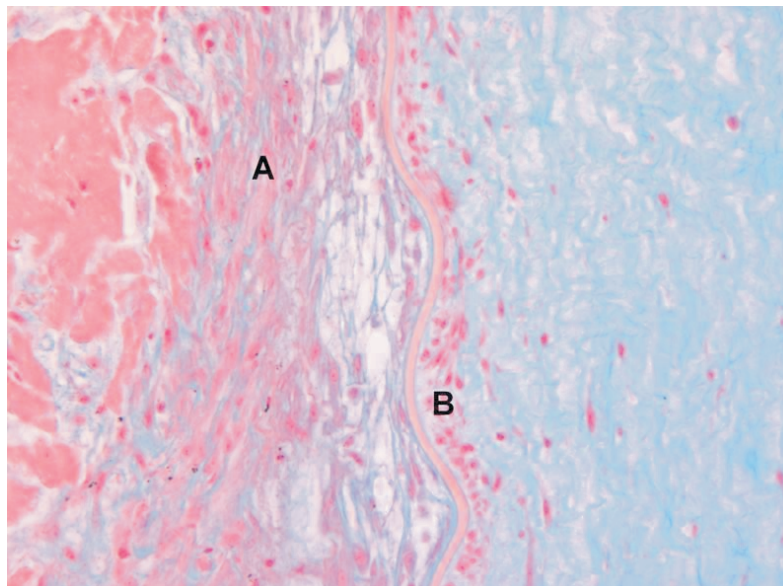
Fig. 4. A cluster of allograft cells 7 days post surgery adherent to the damaged corneal endothelium (A). In the stroma adjoining the basal membrane a clear proliferation of fibroblastic cells (B). Lev and Spicer's staining, 300x.

Fig. 5. 7 days after transplantation. Visible implantation of the multiplied cells. In their close vicinity, the corneal endothelium is preserved. SEM, 1,000x.

7th day of the experiment. Numerous cultured cells adhered to the uncovered basal membrane. Some of them were flattened, creeping onto the basal membrane. In other places, macrophages were observed removing remains of the degraded endothelial cells. Within the zone of the corneal stroma adjoining the basal membrane, a considerable proliferation of cells of fibroblastic lineage was observed (Figs 3, 4).

Endothelial cells were represented by cytoplasm remains and degenerating, fragmenting nuclei. The basal membrane was adjoined by a mantle of cul-

ture-originating cells. This was accompanied by a reaction of the connective tissue which adhered to and integrated the above-mentioned tissue with the epithelium (Fig. 5).

Fibroblasts and, in smaller numbers, also fibrocytes forming a delicate network were visible, as well as macrophages participating in phagocytosis of degenerated endothelial cells (Fig. 6).

This fibroblasts formed a network providing the basis for adaptation of culture-originated cells. Multiplying fibroblasts were observed under the basal
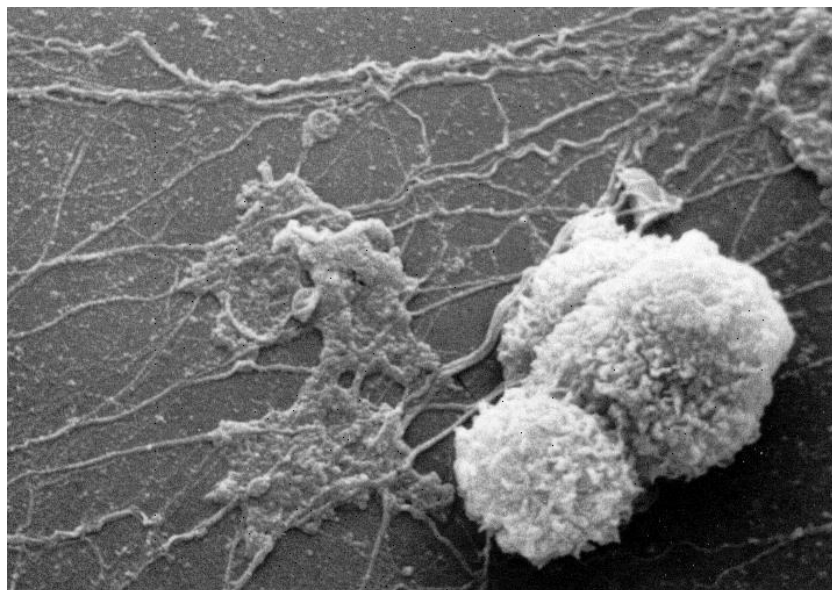
Fig. 6. Macrophages removing the remains of the degenerated endothelial cells. In their vicinity implanting multiplied endothelium cells. 7th day after surgery. SEM, 6,000x.
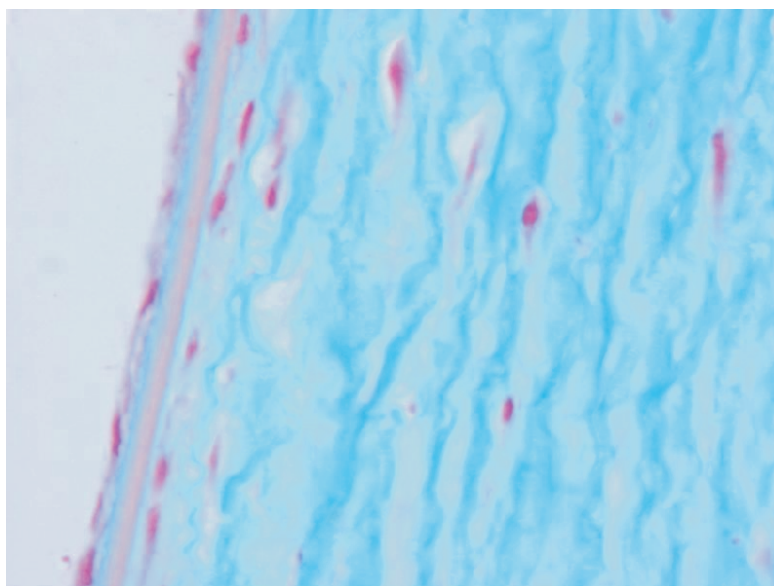

Fig. 7. Changes in the arrangement of collagen fibres in the corneal stroma on the 7th day after transplantation. Area of implanting multiplied cells visible. Lev and Spicer's staining, 400x.

membrane. In the zone of adhesion of cells to the basal membrane in the proper zone of cornea, bundles of collagen fibres changed their orientation. Their arrangement was wavy, and some were transverse (Fig. 7).

On the 20th day of experiment, flat cells distinctly adhering to the basal membrane were observed. The mantle of culture-originated cells in places formed a wide zone. Individual cells adhering the basal membrane were at different stages of implantation. Numerous fibroblasts were visible under the basal membrane in zones of implanting cells.

Where the basal membrane was damaged, the epithelium was repaired. Culture-originating cells adopted a compact arrangement filling the zone of the damaged membrane. Multiplying fibroblasts were visible in the layer of the corneal stroma adjoining the damage zone (Fig. 8).

The fibroblast population became sparser and fibroblasts were less numerous under the basal membrane. In the proper layer of cornea they formed an insular arrangement characteristic of a healthy, unchanged cornea.
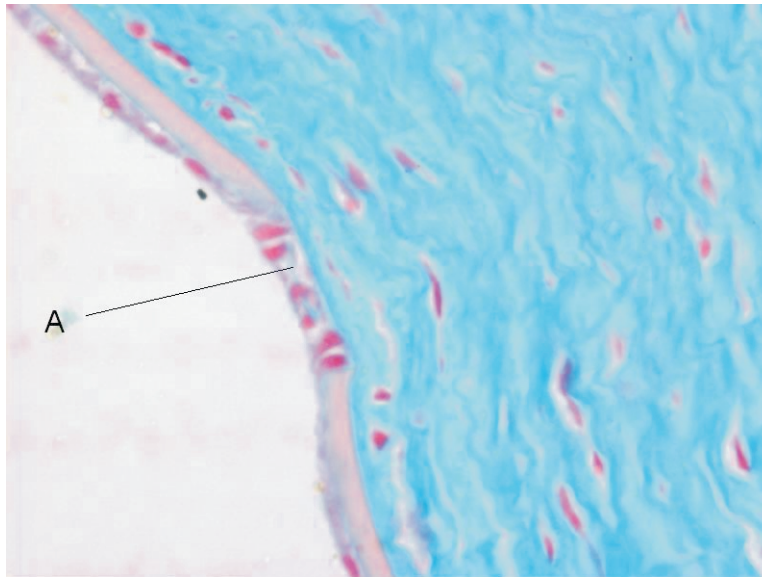
On the 30th day of the experiment, the en-

Fig. 8. Corneal endothelium on the 20th day after transplantation. Multiplied cells in various stages of adhesion to the basal membrane. Where the membrane is damaged, the multiplied cells are arranged in a palisade, filling the damaged area of the membrane (A). Lev and Spicer's staining, 400x.
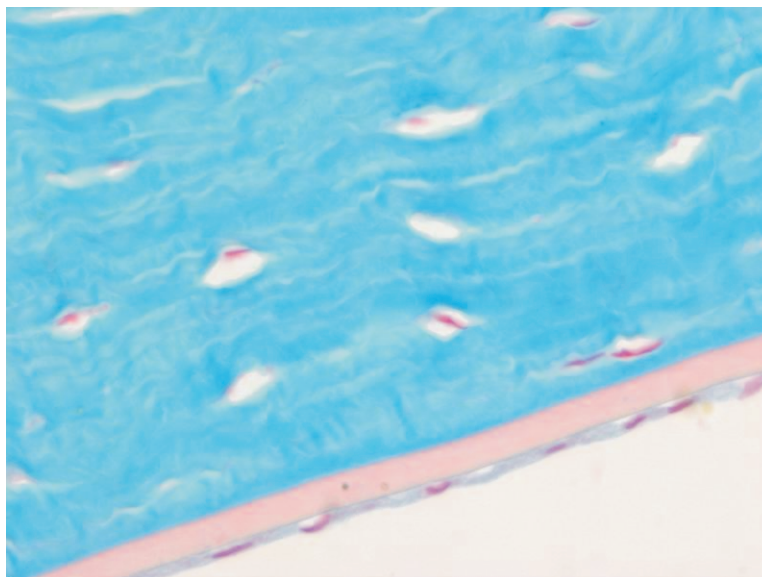

Fig. 9. On the 30th day after transplantation, the endothelium resembles that of the healthy cornea. In the zone below the basal membrane, single fibroblasts are visible. Lev and Spicer's staining, 400x.

dothelium to a large extent resembled that of the normal cornea. It was built mostly of flat and cuboid cells (Fig. 9).

Single macrophages removing remains of degraded cells were visible in some places. Besides, where the basal membrane was damaged, numerous macrophages and multiplying fibroblasts were visible. The remaining endothelium zone represented the normal endothelium (Fig. 10).

Hollows and grooves with implanting endothelium cells were present in some places (Fig. 11).

Discussion

The presented model of the experiment with transplantation of cat endothelial cells, multiplied in vitro and introduced into the anterior chamber shows that the cultured cells implant on the surface of the posterior limiting membrane of the recipient cats.

The micromorphological analysis (histological, histometric, ultrastructural) provides verification for the clinical observations. Cultured cells introduced into the anterior chamber show implantation ten-
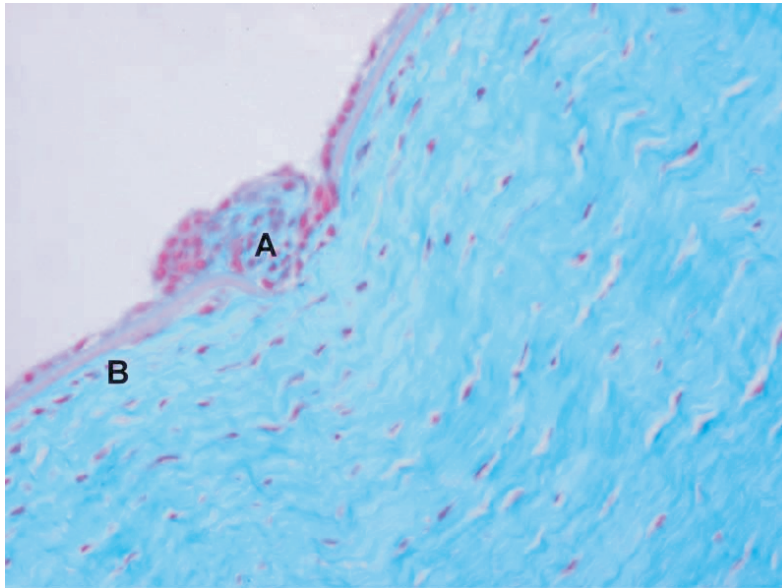
Fig. 10. On the 30th day after transplantation numerous macrophages (A) are present on the damaged basal membrane, whereas in the corneal stroma there are multiplying fibroblasts (B). Lev and Spicer's staining, 300x.
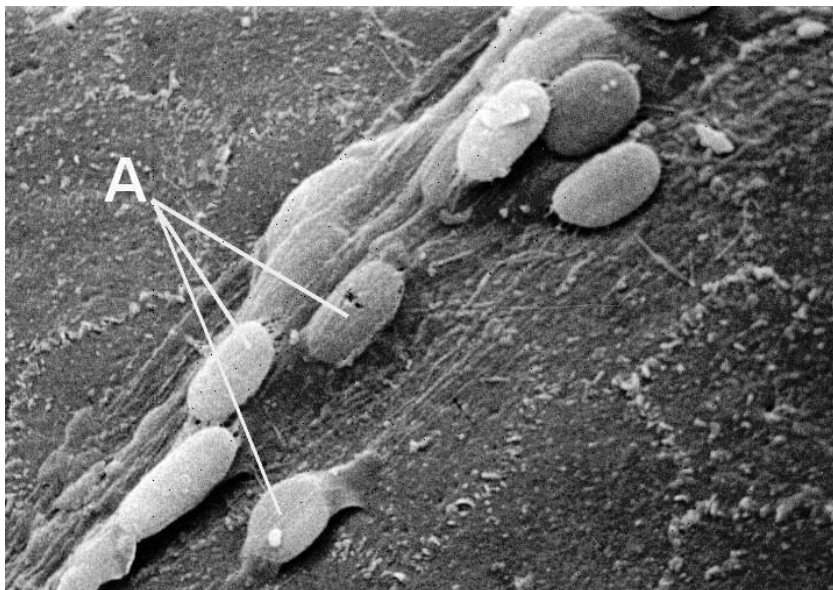

Fig. 11. On the 30th day after transplantation, hollows filled by multiplying cells are observed (A). SEM, 3,000x.

dency already on the 7th day after surgery. These cells adhere to the uncovered posterior limiting membrane whose structure corresponds to the basal membrane. Initially they group in the form of a multi-layered mantle and then migrate onto the basal membrane. Parallelly the basal membrane is cleaned of the remains of endothelial cells, hence numerous macrophages appear. Cells of the fibroblastic lineage show an increased proliferation on the side of the proper layer of the cornea. Multiplying fibroblasts are a manifest of a specific reaction to the changes taking place on the other side of the basal membrane.

It should be pointed out that not all the cultured endothelial cells become implanted. Some of them degenerate which is the reason for the appearance of the great number of macrophages. It can be assumed that a very important factor favouring implantation is the presence of adhesive molecules in the zone of basal membrane. Adhesive molecules in the form of integrins, cadherins and selectines (Kłyszejko-Stefanowicz 1995) are essential for adaptation of the cultured cells. It can be supposed that such molecules determine successful and complete adaptation of such cells. When implantation of the cornea lasts relatively long,

the possibilities of adequate endothelium reconstruction decrease because of the destructive changes in the basal membrane.

On the 20th day post-operation, the implanted cells have the form of a monolayer flat epithelium. The cells of this epithelium adhere to the basal membrane. There are still no complete intercellular junctions. Free surface of such cells is much diversified which indicates that they are resuming their function. Parallel to progressive incorporation of the endothelial cells, the basal membrane is further cleaned of remains of other cells which have undergone degeneration and of remains of cells of damaged endothelium. In the zone of the proper layer of cornea, proliferating fibroblasts produce matrix and collagen fibres. This results in some rearrangement of the fibres. It can be conjectured that after complete implantation and resumption of the endothelium function, a full restitution of the proper layer adjoining the posterior limiting membrane will take place.

On the 30th day post-operation, the cornea endothelium is fully reconstructed. It is the low cuboid epithelium with clearly marked intercellular junctions. The free surface is diversified, with vesicles and secretion granules. This shows that the endothelium is functional, and structurally and functionally stabilised. The zone of the proper layer of cornea adjoining the posterior limiting membrane presents a normal image. The cells of fibroblastic-fibrocytic lineage are scattered, and the collagen fibres are arranged in an orderly fashion. As a result the cornea resumes its full function with reconstructed endothelial layer and stabilised proper layer, being completely and adequately translucent.

It can be stated that the present study revealed a complete restitution of the cat cornea endothelium 30 days after transplantation. The restitution started on the 7th day post-operation, as shown by implantation of the cultured cells. As a result, structures necessary for normal functioning of the endothelium, with preserved translucency of the cornea, are restored. These positive results indicate that transplantation of cultured endothelium cells into the anterior chamber may become an alterative therapy of many eye diseases in humans and animals.

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