

BOVINE CORNEAL EPITHELIAL PRIMARY CULTURES AS AN *IN VITRO* MODEL FOR OPHTHALMIC DRUGS STUDIES

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Abstract: In the recent years a significant development of investigations with regard to bioavailability of ocular drugs has been noticed. The corneal epithelial barrier is the main pathway for ocular penetration of topically applied ophthalmic drugs into the anterior chamber. To work out an *in vitro* model of bovine corneal epithelial primary cultures and exercise it for permeability research with lipophilic and hydrophilic markers, permeability coefficients estimation of the 6-carboxyfluorescein and rhodamin B was made. The corneal epithelial cultures of the 3rd or 4th passage were chosen for layered culture with inserts based on the liquid-liquid interface (for the first week) and the air-liquid interface (for the two following weeks). On the 7th, 12th, 18th, 21st experiment day TER values, and on the 21st day drug permeability coefficients, were determined. The mean TER values of the 7th, 12th, 18th, 21st day of corneal epithelial culture were: 122.14, 155.14, 198.43 and; 247.43 Wcm², respectively. The mean values of permeability coefficients on the 21st day of culture for 6-carboxyfluorescein and rhodamin B were $3.87 \pm 0.10 \times 10^{-6}$ cm/s and $3.65 \pm 0.06 \times 10^{-6}$ cm/s, respectively. We state that the *in vitro* bovine corneal epithelial primary culture model is useful for ocular studies.

Keywords: *in vitro* cell culture model, transepithelial electrical resistance (TER), corneal drug permeability, 6-carboxyfluorescein, rhodamin B

Over the recent years we can observe an intensive development of cell and tissue cultures which enables their wide applications in numerous branches of science, particularly in transplantology (1). Much research efforts leading to discovery and selection of new ophthalmic drugs are focused on penetration of the healing substances carried on an *in vitro* model with the use of an appropriate media of eye drug. One of the determining factors influencing eye drug absorption is its penetration through the barrier of epithelium of cornea, being the main way of drug penetration into the eyeball's interior (2).

Many years ago, the most commonly used experimental model was the dissected cornea of lifeless rabbits (3). However, the isolated corneas were showing signs of life only for 6 h and interspecies differences were deteriorating the predictive value of the results, in the context of drug absorption in humans. Those models were quickly substituted with cell cultures. The most commonly used cell line was the SIRC line (4). Hutak et al. (5) proved that if suspension of those cells is cultured on a poly-

carbonic membrane and is a subject to culturing, then the cells begin to multiply, creating many levels of epithelial cells. A serious flaw of this model is the fact that the SIRC line has the morphology similar to fibroblasts (6). Therefore, it does not provide either species or tissue specificity for the assessment of penetration of eye drug in humans.

There have been many attempts to create a model based on primary cultures of the cells of human cornea epithelium (7). Both isolation and culturing of human cells create numerous problems (7). The age of the donors constitutes one of the major problems. Most often the donors are advanced in years. A serious problem is also the availability of human corneas, whose main source is the Tissues Bank (but those used for experimental research usually have long storage life counted from the moment of their collection). There are other limitations as well. Cells in primary cultures cease to divide and proliferate after 3 or 4 passages and freezing them in liquid nitrogen does not guarantee their full vitality. Consequently, this does not allow for the conduction of all indispensable experiments on the same popu-

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lation of cells, which, in turn, hinders the obtainment of repetitive results.

In recent years, there have been many attempts aimed at obtaining “immortal” cells. Kahn et al. (7) documented obtainment of long living cell line (HCE) from human epithelium of cornea. Primary cultures, as well as lines with prolonged life cycle cultured on the collagen membrane in a liquid/liquid phase and, subsequently, in a liquid/air phase formed closely packed stratified epithelium, resembling human epithelium of cornea (8–11). Thus created model may serve as an imitation of stratified membrane of cornea and provokes specific electrical resistance (TER – transepithelial electrical resistance). Such a model may be used to assess the penetration of various drugs and chemical substances located in media systems (12–15).

The experimental model of the eye drug penetration based on the prepared cell line was devised by Toropainen et al. (16). Similar research that served to TER examination, morphology of gained stratified epithelium and penetration of mannitol, carboxyfluorescein and rhodamin B was conducted by Kahn et al. (7) and Araki-Sasaki et al. (17). The effects of further research conducted by Toropainen et al. (18) was the assessment of transmembrane penetration of 9 lipophilic β -blockers and intercellular penetration of 16 oligomers of polyethylene glycol.

The aim of research presented in this paper was the discussion of optimal conditions of culturing stratified cells of bovine cornea epithelium whose functionality resembles cornea epithelium of an *in vitro* environment. Moreover, the paper strives to examine the penetration of substances with hydro- and lipophilic features through this cornea epithelium. In the experiments conducted, the hydrophilic chemical compound was imitated by 6-carboxyfluorescein, whereas the lipophilic one by rhodamin B. We hypothesized that this system may be useful as a predictive kinetic model *in vitro*, because of cultured corneal epithelium exhibits morphologic characteristics and permeability similar to those observed in the intact cornea. There was a search for experimental data confirming this hypothesis.

MATERIALS AND METHODS

Materials

In culturing of anterior epithelium of cornea, animal corneas were used. They derived from 30-month-old cows from the butchery near Radom, Poland. Bovine eyeballs were transported from the butchery to the laboratory on ice. The isolation of

cornea from eyeballs was conducted within 2 h after the slaughter.

The age of the donors brings about one of the major problems in culturing of cornea epithelium. As results from our experience, it is much easier to produce primary culture of bovine cornea epithelium, got from calves than captured from older animals. Therefore, for the purpose of these experiments, the corneas of 30-month-old cows were used. The age of donors have an impact on the culture success. It was observed that the younger organism, from which the corneas derived, the better their ability to proliferate in the culture (19).

Methods

Culturing was conducted in incubators with constant (5%) access to CO₂, constant humidity (90%) and temperature 37°C. The best epithelium cell growth in the initial stage of culturing was achieved by using the following culturing media: 50% DMEM (Dulbecco Minimal Essential Medium) and 50% Ham's F-12 medium (Gibco) without serum, 10 μ L/mL of ITS-X (Gibco) solution consisting of insulin, transferrin and selenium, 10 ng/mL of epidermic growth factor (EGF – Gibco), 30 μ g/mL of pituitary gland extract (Gibco), 0.5 μ g/mL of hydrocortisone and antibiotics (100 IU/mL of penicilin, 100 μ g/mL of streptomycin, 25 μ g/mL of amphotericin and 50 μ g/mL of gentamycin). The literature data (7, 20) suggest resignation from serum in the initial days of culturing with strips. Lack of serum prevents the migration of fibroblasts from explants and enables the growth of epithelium cells (7). Only after the removal of fragments of tissue culturing was conducted in the medium with 20% of fetal bovine serum (FBS). FBS provides many compounds which condition the appropriate adherence and cell proliferation. Such a method of culturing is supported by the data from the literature (21, 22). In our case, the culture medium was changed 2–3 times a week. Such a frequency of medium change is also suggested by the data from the literature (7, 21–24).

During culturing, the cells proliferated covering the bottom of the vessel with even layer and within 2 weeks the cells achieved the stage of confluence. After achieving about 80% of confluence, the cells were passaged. According to the data from the literature (7), it is the best moment to conduct the passage because the cells are still in the phase of logarithmic growth. The cultures of cornea epithelium cells of the 3rd or 4th passage were destined to layer culturing – on inserts (Transwell Costar) (16, 18) initially in the liquid/liquid structure (first

week), and for another 14 days in the liquid/air structure. For this purpose, both the exterior vessel and the insert with epithelium cell culture inside it, were filled with culture medium. Subsequently, culture medium was removed from the insert and for another 14 days culturing was conducted in the liquid/air environment. The cells, being in the liquid/air structure, had contact with atmospheric air on their exterior surface, whereas at the bottom, they had constant contact with culture medium. The method of maintaining epithelium cells in the liquid/air condition for about 2 weeks is supported by the literature (7, 16, 25–27).

During culturing (on the 7th, 12th, 18th and 21st day), transepithelial electrical resistance (TER) was measured with the use of Millicer-ERS (Millicer Electrical Resistance System) (Millipore) (16, 18).

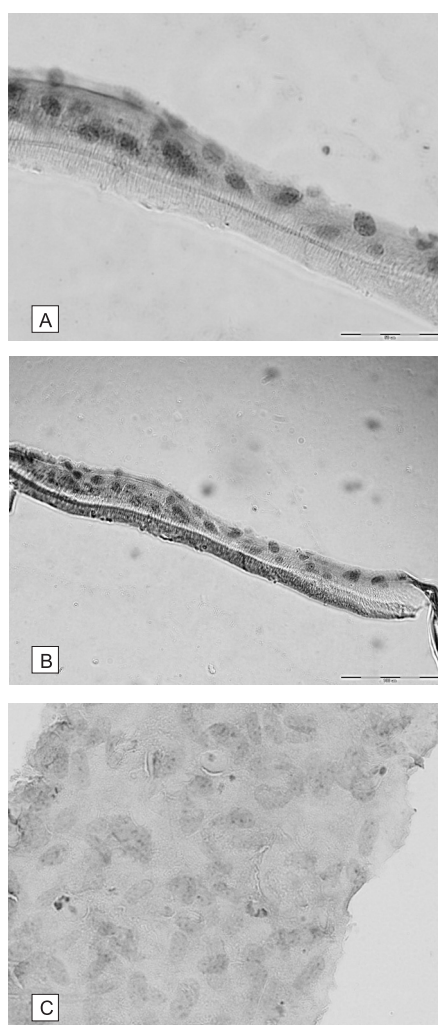


Figure 1. Transverse cross-sections (A, B) and beforehand sight (C) in cultured epithelium of bovine cornea

In the last phase of experiments (on the 21st day), the penetration of rhodamin B (lipophilic substance) (Sigma) and 6-carboxyfluorescein (hydrophilic substance) (Sigma) through stratified epithelium of bovine cornea was examined in appropriate time periods with the use of fluorescence microplate reader (Synergy 'HT BIO-TEK). The experiments were performed after cell growth in different conditions at 37°C. The penetration study with 6-carboxyfluorescein was initiated by using 2.5 mL Hank's balanced salt solution (containing balanced quantity of ions, 5.6 mM glucose and 10 mM HEPES to maintain pH 7.4) to the basolateral side (acceptor part) and 2.0 mL Hank's balanced salt solution containing 6-carboxyfluorescein (10 µM) to the apical side (donor part). The salt solution was used because the red color of the medium could disturb the fluorescence measurements. At 30, 60, 90, 120, 150, 180, 240 and 300 min, aliquots of 200 µL were withdrawn from the acceptor chamber and replaced with an equal volume of blank salt solution. The concentrations of 6-carboxyfluorescein were determined using a fluorescence plate reader with 485 nm excitation and 530 nm emission filters. The transport was studied also with lipophilic marker – rhodamine B as a probe, in a manner similar to the 6-carboxyfluorescein experiments. The donor concentration of rhodamine B was 10 mM, and samples were taken at 10, 20, 30, 45, 60, 75, 90, 105 and 120 min. The concentrations of rhodamine B were determined by the fluorescence plate reader with 530 nm excitation and 590 nm emission filters.

The values of the factors of permeability through the cultured epithelium were calculated on the basis of the formula derived from the work of Toropainen et al. (16). The factor of permeability (P_{tot} in cm/s) of the cultured epithelium and membrane was calculated based on our results using the formula:

$$P_{\text{tot}} = V(dc/dt)AC_0 \quad (1)$$

where: P_{tot} is the apparent permeability of the filter and cells together, dc/dt is the drug flux across the membrane (µM/min), V (2.6 cm³) is the volume of the medium in the acceptor part, A is the surface area of the filter (4.6 cm²) and C_0 (10 µM) the initial concentration of the examined substance added to the insert with cells (donor compartment).

The cultured corneal epithelium and the support (filter, extracellular matrix) caused serial resistance against drug permeation. The resistance for drug permeability was provided both by the barrier of the support (R_{sup}) and cell layer (R_{cell}). Total resistance of the system (R_{tot}) was defined as:

$$R_{\text{tot}} = R_{\text{cell}} + R_{\text{mem}} \quad (2)$$

Permeability is inversely proportional to resistance: $R = 1/P$.

Thus

$$1/P = 1/P_{\text{cell}} + 1/P_{\text{mem}} \quad (3)$$

Permeability of the cultured epithelium without the influence of the membrane was calculated through conversion of the above formula:

$$P_{\text{cell}} = P_{\text{tot}} \times P_{\text{mem}} / (P_{\text{mem}} - P_{\text{tot}}) \quad (4)$$

where P_{tot} is the apparent permeability of the filter and the cells together (obtained experimentally using equation 1), P_{sup} is the apparent permeability of the filter and possible coatings without the cells (obtained experimentally), and P_{cell} is the apparent permeability coefficient of the cell layer calculated by equation 4.

RESULTS

Fourteen independent measurements of transepithelial electrical resistance of cornea epithelium cells were conducted on the 7th day of culturing (in liquid/liquid conditions) and on the following days (on the 12th, 18th, 21st day) of culturing in liquid/air conditions (Fig. 2). The average value of TER on the 7th day of culturing in the liquid/liquid environment was $122.14 \pm 25.29 \Omega\text{cm}^2$. On the 12th day of layer culturing (liquid/air conditions) the average value of TER was $155.14 \pm 26.59 \Omega\text{cm}^2$. Then, on the 18th day of layer culturing (still in liquid/air conditions) all TER values were around $200 \Omega\text{cm}^2$, surpassing TER values observed in former measurements. The average TER value conducted on the 18th day of layer culturing was $198.43 \pm 25.35 \Omega\text{cm}^2$. The last TER measurements of cornea epithe-

lium cells were conducted on the 21st day of layer culturing and the obtained results surpassed the value of $200 \Omega\text{cm}^2$, resulting in higher TER than on the 18th day of culturing. The average TER value conducted on the 21st day of layer culturing was $247.43 \pm 33.93 \Omega\text{cm}^2$. Figure 2 presents the dynamics of changes in TER measurements of cornea epithelium cells on the 7th, 12th, 18th and 21st day of layer culturing. TER values were increasing systematically in the subsequent days of culturing achieving maximum values on the 21st day of culturing, which proves the growth of further layers of cornea epithelium cells.

In this work, 7 independent experiments (Fig. 3) were conducted for 6-carboxyfluorescein. In each experiment the dynamics of penetration of the examined compound through cultured anterior epithelium of bovine cornea was monitored. After placing 6-carboxyfluorescein into the insert with layer culture of epithelium (the donor part) it was assessed how many substances penetrated the epithelium to the exterior vessel (the acceptor part) after 30, 60, 90, 120, 150, 180, 240 and 300 min. In 30th min, the concentration range of 6-carboxyfluorescein was $0.05\text{--}0.09 \mu\text{M}$, whereas in 300th min it was $0.62\text{--}0.82 \mu\text{M}$. The values of concentrations measured in the subsequent time points of the experiment were growing up systematically. The obtained results were used to calculate the permeability factor (P) of cultured epithelium for 6-carboxyfluorescein (Fig. 3). In each experiment very similar P values were obtained. The average value of permeability factors for 6-carboxyfluorescein was: $3.87 \pm 0.10 \times 10^{-6} \text{ cm/s}$, with small CV at only 2.57%.

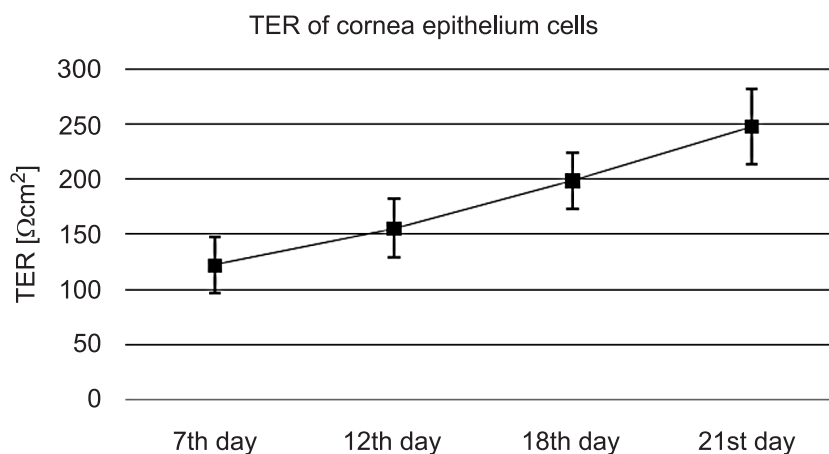


Figure 2. The dynamics of changes in TER values during corneal epithelial culture. Each point represents the mean \pm SD from 14 independent experiments

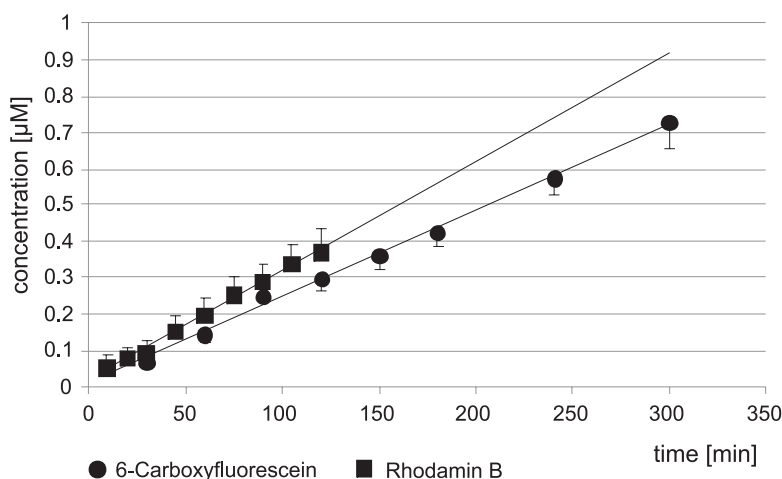


Figure 3. Permeability across cornea epithelium cells at the 21st day of culture of 6-carboxyfluorescein and rhodamin B. Each point is the mean \pm SD; n = 7. Permeability coefficients for 6-carboxyfluorescein: $3.784 \pm 0.108 \times 10^{-6}$ cm/s, CV 2.57% and rhodamin B: $3.653 \pm 0.068 \times 10^{-6}$ cm/s, CV 1.74%

In this work, 7 independent experiments (Fig. 3) with the use of layer culture of bovine cornea epithelium were also conducted for rhodamine B. After placing rhodamin B into the insert with culture (the donor part) measurements of concentration of the examined compound in the exterior vessel (the acceptor part) after 10, 20, 30, 45, 60, 75, 90, 105, 120 min were conducted. In the subsequent measurement time points the increasing concentration of rhodamin B was observed. In the 10th min of the measurement the concentration of rhodamin B was between 0.02 to 0.11 μ M, whereas in the 120th min it was already at 0.32–0.49 μ M. The values of permeability factors (P) for rhodamine B (Fig. 3) oscillate around the average of: $3.65 \pm 0.06 \times 10^{-6}$ cm/s, which confirms CV equal to 1.74%.

DISCUSSION

Transplantations of cornea are becoming an indispensable operation in the recovery of eyesight which was impaired due to various dystrophies and dysfunctions of cornea along with various types of injuries. Moreover, it is commonly known that cornea comprises a tissue barrier which plays a crucial role in drug penetration into the eyeball (2, 28, 29).

The mitotic activity of cornea epithelium cells is very high, their exchange in the organism occurs more or less on a weekly basis. The growth and differentiation of cells in the organism is influenced by various factors, coming from e.g., blood, intercellu-

lar substance and adjacent cells. Applying accurately enriched media an *in vitro* cultures, the cells are provided with supplements indispensable for their survival. The layer cultures of cornea epithelium cells were conducted on inserts. The bottom of such an insert is a half-permeable membrane covered with extracellular matrix (ECM). ECM is a frame for cells and enables their movement and adherence to surface; it also stimulates their proliferation and differentiation (30, 31). It should be emphasized that in culturing some of the ECM constituents may come from their own matrix produced by the cells themselves (11, 19). In inserts that were later used in this research, the main ECM constituent is type I collagen. Collagen facilitates the cells' adherence to the culture surface, as well as stimulates their growth and differentiation (32, 33). Various literature data (7, 16, 34, 35) inform about the advantages of using inserts covered with collagen in culturing of the cornea epithelium cells.

The analysis of obtained results of the TER of cornea epithelium cells measured on the 7th, 12th, 18th and 21st day of layer culturing presents systematic increase in resistance values in the following days of culturing for each of the 14 experiments (Fig. 2). Increasing values of resistance indicate the emergence of further layers of cornea epithelium. Consequently, the 14 independent experiments of layer culturing on inserts presented in this paper were successful and in each case several layers of bovine cornea epithelium cells were obtained (Fig. 1).

Toropainen et al. (16) conducted similar research, including those on human cornea epithelium cells. Conducting (16) culturing in liquid/air conditions in the period of 2–3 weeks they obtained TER values higher than ours (from 200 to 800 Ωcm^2), which was connected with the presence of even 5–8 layers of human cornea epithelium cells. When layer culturing was conducted only in liquid/liquid conditions, merely 2–3 layers of cells were obtained (16). Dohring et al. (26), using the method of layer culturing of human cornea epithelium cells in liquid/air conditions, obtained only 4–5 layers.

After completion of the first and most fundamental assumption of this paper, further experiments using the cultured ox cornea epitheliums were initiated (Fig. 1). In the second stage of the conducted research the assessment of permeability factor of anterior epithelium of cornea for hydro- and lipophilic healing substances was performed.

There are three major ways of eye drug application: surface (external), general, subconjunctival and through the eyeball. In the case of surface application, healing substances penetrate the eyeball through cornea (2, 28, 29). Therefore, cornea is one of the barriers that regulate drug penetration into the eyeball (cornea-ventricular barrier). Each layer of cornea shows different permeability, depending on the character of the healing substance. An important role in the process of substance penetration through cornea is played by: epithelium, proper substance and endothelium (28). This paper was concerned with permeability of the cornea epithelium itself.

The transfer of a drug depends on its characteristics, such as: size of compound particles, level of ionization and solubility in lipids and body fluids. The paper judges the ability of healing substances' penetration through anterior epithelium of cornea applied to conjunctival sac, depending on their solubility in fats. For this purpose 6-carboxyfluorescein and rhodamin B were used.

6-Carboxyfluorescein imitated a hydrophilic healing substance which penetrates cornea epithelium by transcellular means. Such compounds generally have difficulties in penetrating the barrier of a lipid membrane. Their penetration through the structure of the membrane usually occurs *via* active transfer i.e., with the use of appropriate media and energy obtained of disintegration of ATP. On the other hand, rhodamin B penetrates the cornea epithelium only through intercellular spaces. Its application was used in the assessment of permeability of anterior epithelium of cornea for lipophilic healing substances. Well-soluble in fats compounds

usually have no difficulties in penetrating the biological membrane. However, it must be emphasized that it also depends on other compound characteristics which were discussed above. Transfer of lipophilic substance through membranes occurs by means of passive diffusion. On the basis of measured values of concentration for 6-carboxyfluorescein and for rhodamin B, further measurements of permeability factors of those substances were conducted, achieving the result of the average value of the permeability factor for 6-carboxyfluorescein: $3.87 \pm 0.10 \times 10^{-6} \text{cm/s}$, and for rhodamin B: $3.65 \pm 0.06 \times 10^{-6} \text{cm/s}$ (Fig. 3).

The obtained permeability factors for both substances were marked by high repetitiveness (Fig. 3). Statistical assessment showed that the differences between the arithmetic means of permeability factors for 6-carboxyfluorescein and rhodamin B through cornea epithelium cells are statistically relevant ($p < 0.05$). It suggests that the proposed experimental model *in vitro* may be useful for the evaluation of penetration of healing substances applied to conjunctival sac.

The use of cell culturing for research on drugs is important because in recent years there have been attempts to limit the usage of laboratory animals in experiments. Nevertheless, *in vitro* tissue models are not, contrary to expectations, sufficiently accurate to fully present the processes in a living organism. Thus, the method of *in vitro* culturing is constantly being improved. Such improvements can be exemplified by culturing of the "immortals". Kahn et al. (7) obtained long living cell lines (HCE) subjecting the cells obtained from human cornea epithelium to virus Ad12-SV40 infection or transfection of the plasmid RSV-T. Cell lines obtained in this way are of similar character and retain many phenotypical features characteristic for human cornea epithelium. The immortal cell line was used by other authors (17, 24, 36). Toropainen et al. (16) with the use of cell lines devised a model to examine the penetration of drugs, based on similar parameters which were used in the preparation of the experimental model in this paper: TER, morphology of epithelium and penetration of 6-carboxyfluorescein and rhodamin B.

CONCLUSION

In this study the methodology of bovine cornea epithelium cells culturing was established and permeability of hydro- and lipophilic substances were assessed. A few layers of epithelium have been obtained after three weeks of carrying out the strati-

fied culture (Fig. 1). The evidence of that were increasing TER values observed gradually day-by-day over the period of experiment.

On the basis of conducted research, the following conclusion can be drawn: the devised experimental *in vitro* model of bovine cornea epithelium cells may prove useful in assessment of penetration of eye healing substances.

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