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International Journal of Pharmaceutics 250 (2003) 191–201
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

The use of a porcine organotypic cornea construct for permeation studies from formulations containing befunolol hydrochloride

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Received 22 April 2002; received in revised form 20 September 2002; accepted 24 September 2002

Abstract

The purpose of this study was to develop an organotypic cornea equivalent consisting of three different cell types (epithelial, stromal and endothelial cells) and to investigate its usefulness as in vitro model for permeation studies. The different cell types of a porcine cornea were selectively isolated and a multilayer tissue construct was created step-by-step in Transwell® cell culture insert. Histology, basement membrane components (laminin, fibronectin) and surfaces of cornea construct were investigated to evaluate the degree of comparability to porcine cornea from slaughtered animals. The cornea construct exhibited similarities to the original cornea. Ocular permeation of befunolol hydrochloride from different formulations across the cornea construct was tested using modified Franz cells and compared with data obtained from excised cornea. The cornea construct showed a similar permeation behavior for befunolol hydrochloride from different formulations compared with excised porcine cornea. However, permeation coefficients K_p obtained with the construct were about three to fourfold higher for aqueous formulations and same for the w/o-emulsion. The reconstructed cornea could be an alternative to excised animal tissue for drug permeation studies in vitro.

Keywords: Cornea construct; In vitro model; Drug permeation studies; Befunolol hydrochloride

1. Introduction

In development of novel ocular drugs or formulations intended for the application to the eye, the investigation of drug absorption into the eye is required. In general, the cornea is the main route of absorption for topically applied ocular drugs (Doane et al., 1978; Burstein and Anderson, 1985; Lee and Robinson, 1986). In vitro studies of transcorneal permeation behavior of drugs and formulations as well as toxicity of substances are usually performed using either the whole eye or excised cornea of animals (Camber, 1985; Igarashi, 1987; Muir, 1987; Richman and Tang-Liu, 1990; Zhu et al., 1996). Recently in vitro models based on cell culture techniques have been created to avoid disadvantages of animal experiments e.g.

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0378-5173/02/$ - see front matter
PII: S0378-5173(02)00541-0

high costs for laboratory animals and interindividual differences in permeability (Kruszewski et al., 1997; Schneider et al., 1997; Kawazu et al., 1998; Offord et al., 1999; Tegtmeyer et al., 2001).

The cornea is a multilayered tissue composed of three cellular layers, the epithelium, the stroma and the endothelium. In previous in vitro permeation studies cell culture models were described using epithelial (Goskonda et al., 1999; Chang et al., 2000) or epithelial and stromal cells (Toropainen et al., 2001). In contrast to previously published studies the aim of the present contribution was (i) to produce a complete organotypic cornea construct including all corneal cell types, in this case porcine epithelial, stromal (fibroblasts) and endothelial cells, (ii) to test drug permeability using befunolol hydrochloride as a model ocular drug in three different formulations, i.e. an aqueous solution, hydrogel and w/o-emulsion, and (iii) to investigate the influence of vehicles on permeation data obtained from cornea construct compared with those from excised cornea. These formulations were chosen due to the fact that they are frequently used ophthalmic preparations. Befunolol hydrochloride as a beta adrenergic antagonist is used in treatment of glaucoma disease.

First all three different cell types were isolated in primary culture. Cornea was reconstructed with epithelial cells cultivated on stromal cells embedded in a collagen matrix with an underlying layer of endothelial cells (Zieske et al., 1994; Tegtmeyer et al., 2001). The construct was investigated regarding typical corneal features. Permeation data obtained with cornea construct were compared with those from excised porcine cornea.

2. Materials and methods

2.1. Materials

Glauconex® 0.5% is a commercial aqueous eye drop solution containing befunolol hydrochloride from Alcon-Pharma (Freiburg, Germany). BefuAG 1% as a hydrogel contains 3.5% polyacrylic acid Carbopol® 940 (Caelo, Hilden, Germany) and 1% befunolol hydrochloride. BefuAS 2% is a w/o-emulsion based on Unguentum Ophthalmicum Emulsificans Ph.Helv.8-1997 with 2% befunolol hydrochloride.

2.2. Primary cultures, cultivation and characterization of cell types

Eyes from 6 month old swine were obtained from the slaughterhouse of Institute for animal science and animal husbandry (Braunschweig, Germany). The cornea was excised with an attached 1–2 mm wide scleral ring and was rinsed three times with phosphate-buffered saline (PBS) (ICN, Eschwege, Germany). To obtain endothelial cells the inner side of the cornea was treated with trypsin 0.5 g/l-EDTA 0.2 g/l-solution (Gibco BRL Life Technologies, Karlsruhe, Germany) and incubated for 10 min at 37 °C. The cells were detached with a rubber spatula and cultivated in 25 cm^2 tissue culture flasks (Costar, Fernwald, Germany). Dulbecco's modified Eagle's medium (DMEM) (ICN) supplemented with 10% new born calf serum (NBCS) (Gibco BRL), 4 mM L-glutamine and 1% antibiotic solution (Gibco BRL) was used as standard growth medium for all three cell types. The medium was changed three times per week. To obtain corneal fibroblasts, both endothelial and epithelial sheets were dissected off, the stroma was cut into small pieces and 3 mm explants were attached to a 100 mm diameter plastic dish (Costar). Fibroblasts showed outgrowth after 6–7 days. For isolation of porcine epithelial cells the cornea was treated as described above. The posterior half of the stroma and endothelium were removed and the anterior portion of the cornea was incubated in 1.8 units/ml Dispase II (Roche Diagnostics, Mannheim, Germany) at 37 °C for 150 min. Next the cornea was washed twice with PBS, single cells and small clumps of epithelium were lifted from the stroma and seeded on 25 cm^2 tissue culture flasks (Costar) coated with rat tail acid-extracted collagen of type I.

Cells could have been cultivated until passage six without a change of their typical morphology became visible.

To examine purity of isolated primary cultures, cells were in addition characterized regarding

specific cytoskeletal components. Cytokeratin is a specific marker for epithelial and vimentin for stromal and endothelial cells. Mouse monoclonal anti-cytokeratin pan (Biotrend, Köln, Germany) diluted 1:100 and anti-vimentin (Sigma, Deisenhofen, Germany) diluted 1:40 were used as primary antibodies. As secondary antibodies, horseradish peroxidase conjugated anti-mouse IgG antibody (Sigma) diluted 1:50 was used. Cells were grown on Lab Tek® chamber slides (Nunc, Wiesbaden, Germany), incubated with every antibody each time for 30 min at 37 °C and washed with PBS. Subsequently cells were treated with 1.6 mg/ml urea hydrogen peroxide and 0.7 mg/ml diaminobenzidine (DAB) (Sigma). Positive response led to a brown precipitate and was examined by light microscopy.

2.3. Cornea construct

Cells from passages two to six were used for reconstruction of the cornea equivalent. Cornea construct was constructed step-by-step in Transwell® (Costar) cell culture inserts. The cultures were maintained in a humidified incubator at 37 °C with 5% CO_2 in DMEM-10% NBCS and medium was replaced three times per week too. 3×10^5 endothelial cells were seeded onto a polycarbonate filter (surface area, 4.7 cm^2; pore size, 3.0 μm) covered with a layer of type I collagen and grown to confluence within 7 days. A type I collagen gel matrix containing 2×10^5 stromal fibroblasts was then cast atop the confluent endothelial cell layer. Endothelium-stroma equivalent was cultivated approximately 5–7 days submerged. 2×10^5 epithelial cells were seeded onto the contracted collagen lattice (Hibino et al., 1998) and grown for additional 7 days submerged to confluence. After epithelium became confluent tissue construct was lifted to the air liquid interface for additional 2 weeks and cultivated with a 3:1 mixing of DMEM (ICN) and Nutrient Mixture Ham's F-12 supplemented with 2% NBCS, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B, 4 mM L-glutamin (Gibco BRL), 5 μg/ml insulin, 0.4 μg/ml hydrocortisone, 20 pM triiodthyronine, 5 μg/ml transferrin, 24.3 μg/ml adenine (Sigma), 6.1 μg/ml ethanolamine, 14.1 μg/ml phosphoethanolamine, 6.8 ng/ml selenious acid (Biochrom, Berlin, Germany). Within 2 weeks a multilayered epithelium was performed.

2.4. Immunohistochemistry

Rabbit antibodies against basement membrane components, anti-fibronectin (Dako, Hamburg, Germany) diluted 1:50 and anti-laminin (Sigma) diluted 1:20 were utilized. Frozen sections (14 μm) of a cornea construct were fixed with acetone at −20 °C, washed in PBS and then incubated in a moist chamber with primary antibodies for 3 h. Slides were rinsed and treated for 1.5 h with fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG (Dako) diluted 1:20 in case of fibronectin detection or horseradish peroxidase conjugated anti-rabbit IgG (Sigma) diluted 1:20 to identify laminin. The sections were examined with a fluorescence microscope Axioskop 2 plus (Zeiss, Jena, Germany) to detect fibronectin or stained with DAB (Sigma) and examined as described above to determine laminin.

2.5. Light microscopy

Tissue was fixed in a 75 mM phosphate buffer pH 7 containing 3.5% formaldehyde, dehydrated in isopropyl alcohol and embedded in paraffin. Four micrometer cross sections were cut, stained in hematoxylin eosin and examined with a photomicroscope (Zeiss, Oberkochen, Germany).

2.6. Scanning electron microscopy

Samples were fixed with 1.5% paraformaldehyde, 3% glutaraldehyde, 1.5% acrolein in 0.2 M cacodylate buffer (pH 7.2) for 24 h at 20 °C, treated with 2% glycine, sodium glutamate, sucrose (pH 6.2) for 12 h at 20 °C, then with 2% tannic acid pH 4 for 12 h at 20 °C, rinsed in H_2O and postfixed with 2% osmium tetroxide for 12 h at 20 °C (Fujita et al., 1986). Tissue equivalent was dehydrated in a graded series of isopropyl alcohol and sputtered with gold particles before examination in a Stereoscan 250 (Cambridge Instruments, Cambridge, UK) SEM.

2.7. Permeation studies

Diffusion experiments for the evaluation of transcorneal drug permeability from three formulations containing befunolol hydrochloride were performed for 420 min using modified Franz diffusion cells at 37 °C. To compare the barrier function both excised porcine cornea and organotypic cornea construct were used. The donors were Glauconex® 0.5% as an aqueous solution, BefuAG 1% as an aqueous hydrogel and BefuAS 2% which is a w/o-emulsion described above. The receiver solution contained phosphate buffered saline pH 7.4 and was stirred with a magnetic stirrer (Janke&Kunkel, Staufen, Germany) at 400 rpm during the experiment. Samples were taken from the receiver chamber at fixed time intervals (every 60 min) and analyzed using a Waters 515, 717 plus, 486 HPLC system (Waters, Eschborn, Germany) and a column of Gromsil® 120 ODS-3 CP 5 μm, 125×4 mm (Grom, Herrenberg, Germany) at ambient temperature. The mobile phase was a mixture of methanol/50 mM NaH_2PO_4 in the ratio 45:55 (V/V) with a flow rate of 1.0 ml/min. Peaks were detected at $\lambda = 292$ nm with retention time of approximately 2.3 min. Data analysis and calculation were performed by Waters Millenium 32 Chromatography Manager software (Waters). Correlation coefficient of calibration was 0.9999 within a concentration range from 0.25 to 50.0 μg/ml. The permeation coefficient K_p was calculated as flux/drug concentration from the linear ascent of a permeation curve.

3. Results

3.1. Corneal cells

Both enzymatic and outgrowth techniques were carried out to selectively submit primary cultivation of pure corneal epithelial, stromal and endothelial cells from pig cornea. The cells were investigated regarding their typical morphology, rate of proliferation and cytoskeletal components to be sure of purity of specific cell types. The epithelial and endothelial cells show a polygonal shape and are easily differentiated from the elongated corneal stroma cells with their characteristic spindle shape (Fig. 1). With immunocytochemical analysis of cytoskeletal components, cytokeratin was detected in epithelial cells but not present in fibroblasts and endothelial cultures whereas vimentin was found in isolated stromal and endothelial cells but not in epithelial cultures (Table 1). The morphological and immunocytochemical characterization together with the selective isolation methods certify pure cultures of the three different cell types.

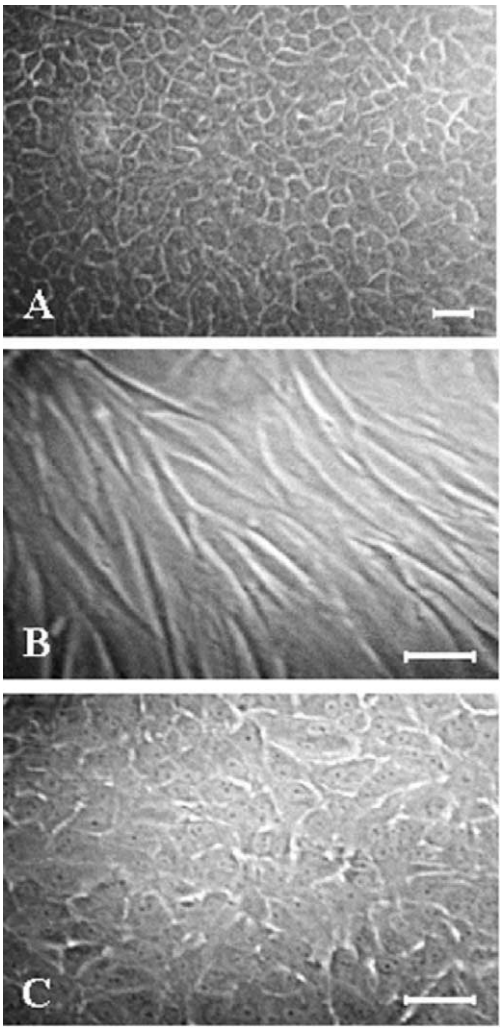

Fig. 1. Primary cultures of the three different cornea cell types. Phase-contrast micrographs of epithelial (A), stromal (B) and endothelial (C) cells, Scale bar 25 μm.

Table 1
Detection of typical cytoskeletal components for the different corneal cell types

	Epithelial cells	Stromal cells	Endothelial cells
Cytokeratin	+	–	–
Vimentin	–	+	+

3.2. Corneal tissue construct

A multilayered tissue construct was formed step-by-step during the cultivation period of about 5 weeks as described in Section 2. The construct was characterized in terms of typical corneal features to examine comparability with original porcine cornea. Fig. 2A shows a cross section of epithelium with underlying stroma from excised porcine cornea. Epithelium can be differentiated in basal, wing and superficial cells. Fig. 2B shows a cross section of the superior part of an organotypic cornea construct. The micrograph represents cultured corneal epithelial cells grown on stroma equivalent consisting of a collagen matrix with incorporated stromal cells. An epithelium of 4–5 layers is detectable similar to original, but obviously differences in appearance occur in comparison with Fig. 2A: cells are rather flattened. The construct epithelium does not show full thickness either, as the original does. These findings are in agreement with other reconstructed corneal epithelium (Minami et al., 1993; Germain et al., 1999; Tegtmeyer et al., 2001) and may be affected by the absence of lacrimal fluid and eyelid blinking in construct cultivation.

Scanning electron microscopy (SEM) analysis was performed to look for similarities of surface structures between original and cornea construct. A confluent monolayer was formed by seeding porcine endothelial cells onto a collagen gel. Endothelial monolayer exhibits a tight cobblestone-like pattern similar to normal endothelium. However, a change in appearance is visible, in terms of a hexagonal shape of endothelial cells in vivo altering to a polygonal shape in construct (Fig. 3A). The same phenomenon also occurs in

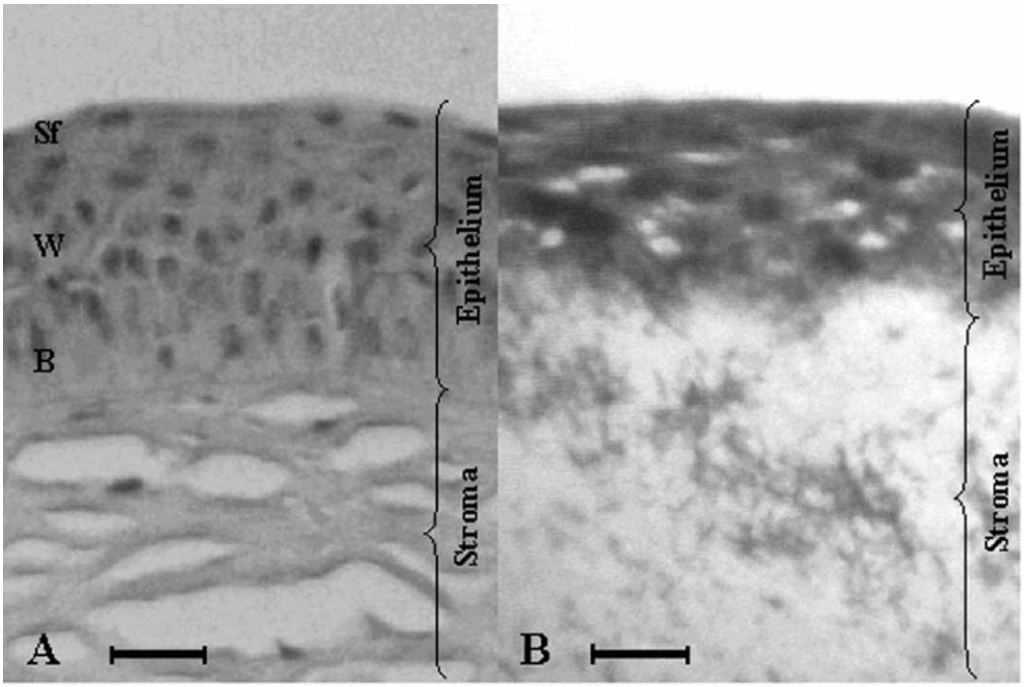

Fig. 2. Histologic cross sections of original porcine corneal epithelium (A) and cultured epithelial cells grown on a stroma equivalent (B) stained with hematoxylin-eosin (Sf-superficial cell layers, W-wing cell layers, B-basal cell layer), Scale bar 20 μm.

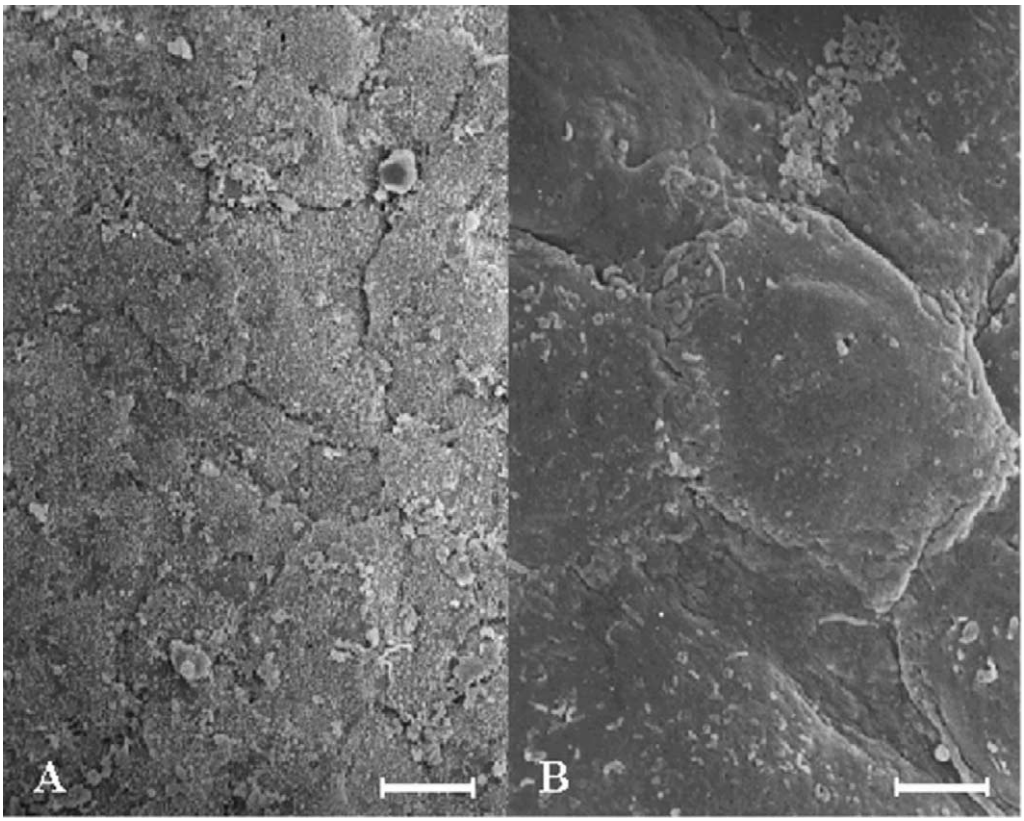

Fig. 3. Scanning electron micrographs of cultured endothelial cells confluent grown on a type I collagen gel (A) Scale bar 20 μm, and of epithelial surface of cornea construct (B) Scale bar 5 μm.

standard cultivation of endothelial cells (Stocker et al., 1958; Easty et al., 1986; Engelmann et al., 1988). Epithelial cells also formed a tight surface whereas strong interlocked superficial cells were detected. Compared to original, cultured epithelium shows fewer microvilli or microplicae (Fig. 3B), probably due to absence of lacrimal fluid too.

Immunohistological staining of laminin and fibronectin between epithelium and stroma equivalent of cornea construct (Fig. 4) indicates the formation of basement membrane components in the construct. This is similar to the situation under in vivo conditions.

3.3. Permeation studies

Furthermore it was of interest whether histological similarities between construct and original cornea lead to a similar barrier function. Therefore, the permeation of different model formulations of befunolol hydrochloride was investigated. Resultant permeation coefficients K_p of befunolol hydrochloride from various formulations are shown in Table 2. Fig. 5 shows the permeation profiles across excised porcine cornea, as expected with the highest K_p for the aqueous solution (Glauconex®) followed by the aqueous gel (BefuAG) and with the lowest K_p for the w/o-emulsion of befunolol hydrochloride (BefuAS). Permeation of the same formulations across the cornea construct indicates the same rank order of the different formulations in terms of K_p (Fig. 6, Table 2). However for the aqueous formulations K_p is about three to fourfold higher with construct than with excised cornea. The w/o-emulsion shows the same K_p for both permeation barriers.

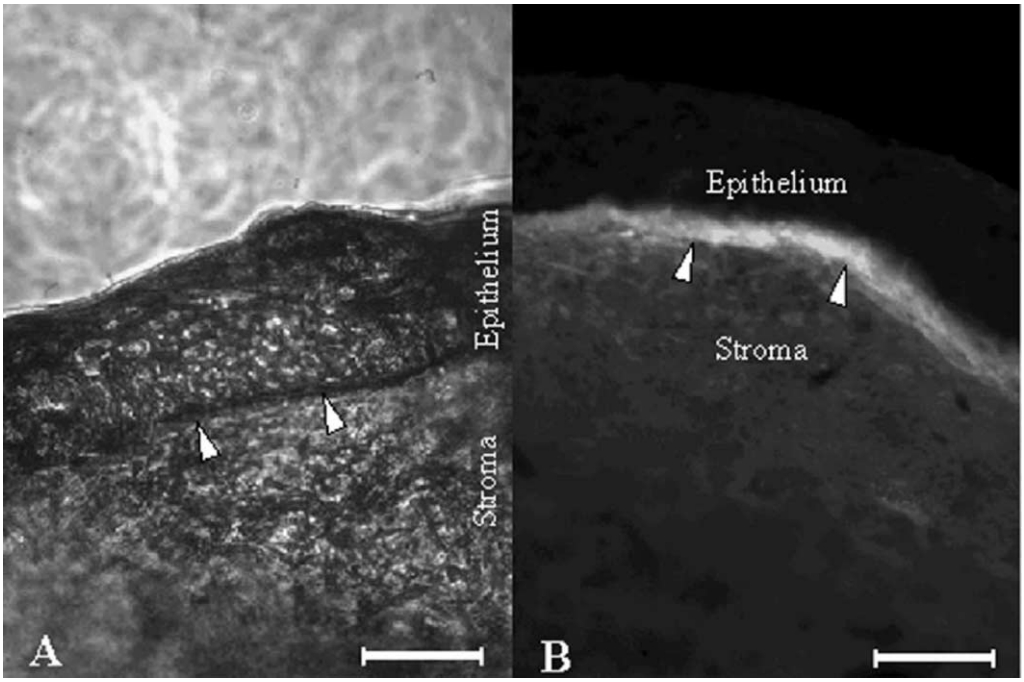

Fig. 4. Immunohistochemical detection of laminin (A) positive DAB-staining and fibronectin (B) positive FITC-detection in basement membrane of cultured cornea construct between epithelium and stroma, Scale bar 50 μm, (arrowheads).

4. Discussion

To create a functional corneal in vitro model it is important to prepare pure cultures of different cell types with their typical properties. Enzymatic and outgrowth techniques can be used for selective isolation and standard cultivation of cells (Stocker et al., 1958; Chan and Haschke, 1982; Easty et al., 1986; Engelmann et al., 1988; Hackworth et al., 1990). Furthermore reconstruction of corneal tissue has already been described. However, cultivated cornea models are used predominantly for histological and toxicological studies (Minami et al., 1993; Zieske et al., 1994; Parnigotto et al., 1998; Germain et al., 1999; Griffith et al., 1999; Schneider et al., 1999). Analogously to utilized dermal and epidermal cell culture systems (Specht et al., 1998; Asbill et al., 2000) or corneal epithelial models (Kawazu et al., 1998; Chang et al., 2000; Toropainen et al., 2001), we report on the usefulness of a complete reconstructed cornea for permeation studies.

Selective isolation of pure porcine corneal cells and their standard cultivation for 5–6 passages

Table 2
The permeation coefficients K_p (cm/s) of befunolol hydrochloride from Glauconex® 0.5%, BefuAG 1%, BefuAS 2% across excised porcine cornea and organotypic cornea construct, (mean ± SD)

	Formulation	Excised porcine cornea	Cornea construct
BefuAS 2%	w/o-emulsion	$0.30 \times 10^{-7} \pm 0.07 \times 10^{-7}$	$0.28 \times 10^{-7} \pm 0.03 \times 10^{-7}$
BefuAG 1%	Aqueous polyacrylate gel	$1.51 \times 10^{-6} \pm 0.43 \times 10^{-6}$	$4.08 \times 10^{-6} \pm 1.22 \times 10^{-6}$
Glauconex® 0.5%	Aqueous solution	$5.95 \times 10^{-6} \pm 0.52 \times 10^{-6}$	$24.8 \times 10^{-6} \pm 4.79 \times 10^{-6}$

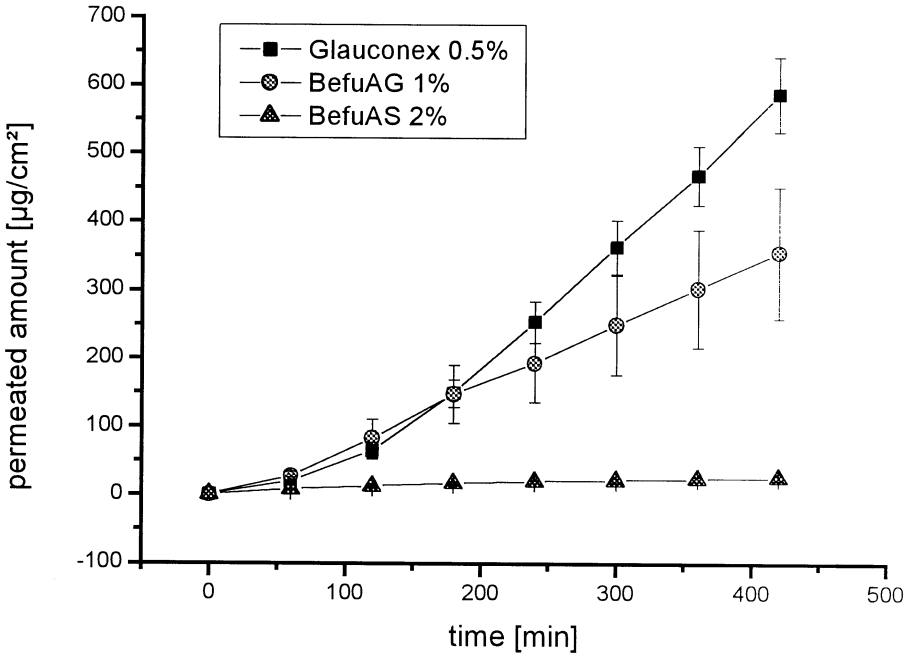

Fig. 5. Permeation graph of befunolol hydrochloride from Glauconex® 0.5% ($n = 5$), BefuAG 1% ($n = 4$) and BefuAS 2% (n = 4) across excised porcine cornea. The values represent the mean ± SD.

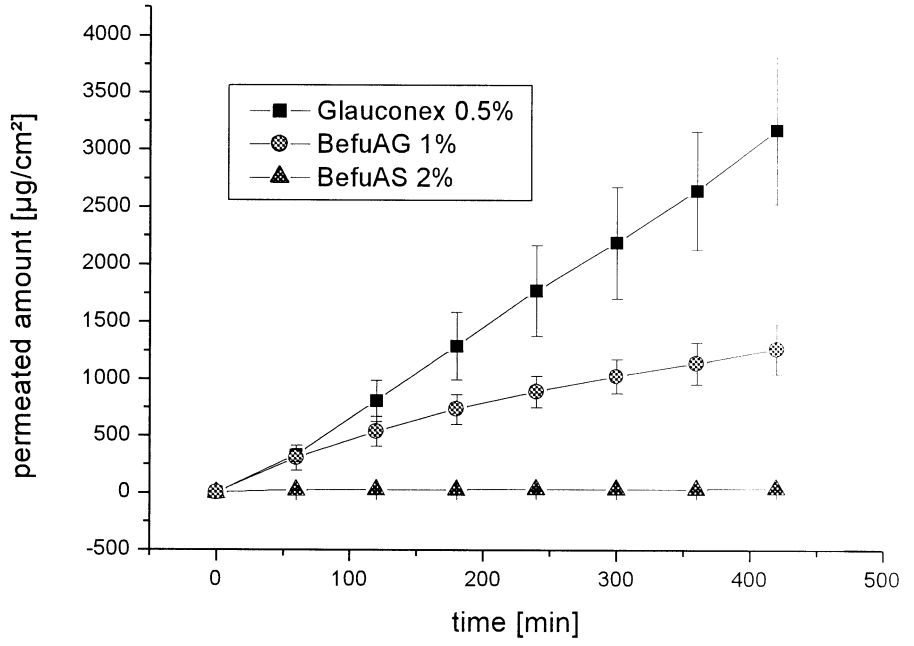

Fig. 6. Permeation graph of befunolol hydrochloride from Glauconex® 0.5% ($n = 5$), BefuAG 1% ($n = 5$) and BefuAS 2% ($n = 8$) across organotypic cornea construct. The values represent the mean ± SD.

allowed reconstruction of a corneal tissue which resembles original in morphological, ultrastructural and histological features. Although there are small structural differences between construct and original, which may be due to the culture conditions such as the medium composition, the absence of lacrimal fluid and eyelid blinking, still major details are very similar. A multilayered epithelium with flattened strong interlocked superficial cells like in original tissue is also formed in vitro. Furthermore a confluent layer of endothelial cells like corneal endothelium in vivo is detectable. Although there are deviations in epithelium thickness (construct 4–5 layers, original 6–8 layers) and structure, basement membrane components such as laminin and fibronectin are expressed in the cornea construct. These findings indicate that despite certain structural differences typical corneal features are formed.

With respect to permeability of befunolol hydrochloride K_p is just three to fourfold higher with cornea construct than with excised cornea for the aqueous formulations. Increased permeability of in vitro models based on cell culture technique is frequently described (Nielsen et al., 1999; Goskonda et al., 2000; Schmook et al., 2001; Zghoul et al., 2001). Structural differences of the construct from the original tissue, especially epithelium thickness, number and tightness of cell junctions, expression of stratum corneum, etc., are probably the main reasons for the increased permeability. However, a three to fourfold increased permeability from aqueous ophthalmic formulations with a cornea construct or reconstructed corneal epithelium is in accordance with previous investigations on different drugs (Tegtmeyer, 2000; Reichl and Müller-Goymann, 2001; Tegtmeyer et al., 2001; Toropainen et al., 2001). For the w/o emulsion K_p is even the same. Equal K_p across construct and excised cornea from w/o emulsion are slightly unexpected in so far as the construct exhibits a higher permeability than the original. A possible explanation is the rate limiting drug release from the w/o emulsion. In the case of a slower diffusion coefficient with regard to K_p, the permeation coefficients measured have to be identical for both the construct and the original. Yet differences between permeation coefficients of the in vitro cornea and the excised cornea are fairly small and constant for porcine and bovine cornea constructs, whereas permeation coefficients in similar permeation experiments across reconstructed rabbit corneal epithelium (Kawazu et al., 1998), rabbit corneal (SIRC) cell line (Goskonda et al., 2000) or other in vitro models as organotypic cultures of human transformed and native dermal and epidermal cells (Specht et al., 1998) reveal higher differences in comparision to data from excised rabbit cornea or human stratum corneum, respectively.

The development of a porcine corneal in vitro model as a useful model for ophthalmic permeation studies has been demonstrated. The good comparability of data from both bovine and porcine cornea construct with data from excised animal cornea gives hope that a corneal model based on human cells could be helpful to predict drug absorption into human eye.

Acknowledgements

We would like to thank Dr E. Parussis, Braunschweig, for preparing the sections of the cultures, Arnold Eilmes from Institute for animal science and animal husbandry, Braunschweig, for the supply of porcine eyes, Rüdiger Hoche from Institute for welding technology, Technical University of Braunschweig, for his support with SEM and Alcon-Pharma for the supply of materials.

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