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# Development of a serum-free human cornea construct for in vitro drug absorption studies: The influence of varying cultivation parameters on barrier characteristics

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ABSTRACT

The increased use of ophthalmic products in recent years has led to an increased demand for in vitro and in vivo transcorneal drug absorption studies. Cell-culture models of the human cornea can avoid several of the disadvantages of widely used animal experimental models, including ethical concerns and poor standardisation. This study describes the development of a serum-free cultivated, three-dimensional human cornea model (Hemicornea, HC) for drug absorption experiments. The impact of varying cultivation conditions on the corneal barrier function was analysed and compared with excised rabbit and porcine corneas. The HC was cultivated on permeable polycarbonate filters using immortalised human keratocytes and a corneal epithelial cell line. The equivalence to native tissue was investigated through absorption studies using model substances with a wide range of molecular characteristics, including hydrophilic sodium fluorescein, lipophilic rhodamine B and fluorescein isothiocyanate (FITC)-labelled macromolecule dextran. To study the intra-laboratory repeatability and construct cultivation, the permeation studies were performed independently by different researchers. The HC exhibited a permeation barrier in the same range as excised animal corneas, high reproducibility and a lower standard deviation. Therefore, the HC could be a promising in vitro alternative to ex vivo corneal tissues in preclinical permeation studies.



## 1. Introduction

The increased use of ophthalmic drugs in recent years has led to an increased demand for absorption studies to develop and characterise new substances and new or modified ophthalmic formulations. The permeation of topically applied drugs mainly occurs via the cornea; absorption via the sclera and conjunctiva contributes only marginally to this process (Barar et al., 2008; Huang et al., 1989; Järvinen et al., 1995). Hence, absorption studies are commonly performed using excised rabbit corneas mounted in diffusion chambers. Furthermore in vivo animal experiments are widely used despite numerous disadvantages, including ethical concerns, high costs, poor standardisation and questionable transferability of results to human cases. Several cell-culture-based models of varying complexity have been developed to overcome the problems of animal experimental models and to establish a viable in vitro test system (Hornof et al., 2005; Reichl et al., 2011; Xiang et al., 2009).

The human cornea is composed of three major cell layers: the epithelium, the stroma, and the endothelium. Each layer performs a different task in maintaining vision and has distinct characteristics in the context of drug absorption. The 5-6-layered epithelium is the outermost portion of the cornea. It protects the eye against the environment and, due to the zona occludens formed by superficial epithelial cells that are interconnected by desmosomes and sealed by tight junctions (Chen et al., 2007; Sosnova-Netukova et al., 2007), is the main barrier to the absorption of hydrophilic xenobiotics into the anterior chamber (Klyce and Crosson, 1985). Thereby it represents a considerably stronger barrier for the transport of hydrophilic substances than other epithelia, such as lung, nasal or intestinal mucosa (Rojanasakul et al., 1992). The passive absorption of hydrophilic molecules through the cornea occurs via the paracellular pathway, in which the regulatory mechanisms of the tight junctions that are responsible for the selectivity and magnitude of diffusion are distinct and not completely clear (Steed et al., 2010). In contrast to the epithelium, the stroma only presents a barrier to lipophilic substances, which move along the transcellular route through the epithelium. This effect is a result of the stroma's hydrophilic nature, as it consists of 75% water in a

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collagenous extracellular matrix. The stroma makes up 90% of the corneal thickness. Finally, the endothelium is a leaky monolayer that is easily permeated and participates only marginally in the corneal barrier function (Huang et al., 1989; Prausnitz and Noonan, 1998).

As the epithelium represents the main barrier, many cell-culture models are based exclusively on epithelial cells (Becker et al., 2005; Kawazu et al., 1998; Scholz et al., 2002). Pure corneal epithelial models with adequate TEER values seem to be sufficient for testing the permeability of hydrophilic and moderately lipophilic drugs. In the case of highly lipophilic drugs (such as steroids), however, an in vitro model that includes the stroma offers a benefit with regard to the realistic absorption of this drugs. Furthermore, as shown by Orwin and Hubel (2000), co-cultivation with stromal keratocytes impacts epithelial cells as a result of cell–cell interactions that reflect in vivo properties more accurately. Several corneal models have been developed using primary or immortalised cells of bovine (Minami et al., 1993; Tegtmeyer et al., 2004), porcine (Reichl and Müller-Goymann, 2001, 2003; Schneider et al., 1997), human (Becker et al., 2007, 2008; Ranta et al., 2003; Reichl et al., 2004, 2005; Toropainen et al., 2001, 2003) and in particular rabbit (Becker et al., 2008; Chang et al., 2000; Goskonda et al., 1999, 2000; Kawazu et al., 1998; Sakanaka et al., 2006; Tak et al., 2001) origin. Although primary cells may reflect in vivo conditions more accurately, immortalised cells have advantages over primary cells, including an extended lifespan, unaltered characteristics for a larger number of passages and easier cultivation. Immortalised cell lines are beneficial for completely utilising the advantages of an in vitro model, which means having a highly defined and standardised system available at any time. Moreover, human cell lines have the advantage that the differences between species (and, therefore, the problematic transferability of animal model results to humans) are not a concern. While several corneal epithelial models have been investigated, only the HCE-T cell line and a commercially available corneal epithelial model (Clonetics®) have demonstrated tight epithelial tissue barriers (Becker et al., 2008; Reichl et al., 2011; Seeber et al., 2008; Toropainen et al., 2001; Xiang et al., 2009; Zhang et al., 2008).

However, the cultivation conditions of the in vitro model have a strong influence on the expression of the zona occludens and, therefore, on the characteristics of the corneal barrier function. The type of growth substrate, use of an air–liquid interface, co-cultivation of keratocytes and epithelial cells and cell-culture medium and its supplements (especially serum supplementation) (Chang et al., 2000; Toropainen et al., 2001) must be considered to achieve a barrier that is comparable to in vivo tissue. Supplementation of the medium with animal-derived serum is particularly problematic due to its ill-defined composition and varying quality. The use of serum during cultivation is undesirable, given the aim of obtaining a drug testing system that is reproducible and standardised, so that many investigators consider cultivation in a standardised serum-free medium to be required. Hence, one focus of this study was to reduce or avoid serum supplementation in the culture medium. In addition, the effects of other relevant cultivation parameters on the barrier function were investigated. Their impact on the corneal model was analysed by means of transepithelial electrical resistance (TEER) and absorption experiments. For the absorption studies, the passive diffusions of three model drugs with varying molecular attributes (hydrophilic sodium fluorescein, lipophilic rhodamine B and macromolecular fluorescein isothiocyanate (FITC)–dextran) were examined in the in vitro model and compared with the permeation data for excised rabbit and porcine corneas.

Finally, standard operating procedures (SOP) were developed to permit reproducible cultivation and to provide a basis for later potential prevalidation and validation procedures. The open-source availability of these SOP and the corneal model to all interested institutions could contribute to the advancement of ophthalmic research and to the decreased use of animal experimentation in this field.

## 2. Materials and methods

### 2.1. Materials

Tissue culture flasks were purchased from Sarstedt (Nümbrecht, Germany), and Transwell® inserts were obtained from Corning Costar (Acton, Massachusetts, US). The phosphate-buffered saline (PBS), foetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), Ham's F12, M 199 Earls's medium, 10× Minimum Essential Medium (MEM), insulin, epidermal growth factor (EGF) and L-glutamine were purchased from Biochrom (Berlin, Germany). The $NaHCO_3$, KCl and $MgSO_4 \times 7H_2O$ were purchased from Acros Organics (Geel, Belgium), and the EDTA disodium salt solution was obtained from MP Biomedicals (Solon, Ohio, US). The trypsin-EDTA, penicillin G sodium salt, streptomycin sulfate, amphotericin B and MycoTrace kit were purchased from PAA (Linz, Austria). The trypsin inhibitor, Advanced DMEM culture medium and Defined Keratinocyte serum-free medium (DefK) were acquired from Invitrogen (Karlsruhe, Germany). The dimethyl sulfoxide (DMSO), cholera toxin, retinoic acid, hydrocortisone, sodium bicarbonate, collagen, $CaCl_2 \times 2H_2O$, sodium fluorescein, rhodamine B and FITC–dextran (FD-4) were obtained from Sigma (Deisenhofen, Germany). The Keratinocyte Basal Medium (KBM), which was referred to as the Keratinocyte Growth Medium (KGM) after the addition of the provided SingleQuots, was acquired from Lonza (Rockland, Maine, US), as was the Corneal Epithelial Model Differentiation Medium (CEMDM). The Panserin 401 culture medium and Panexin serum substitute were purchased from PAN Biotech (Aidenbach, Germany). The Keratinocyte Medium (K-SFM) was obtained from ScienCell (San Diego, California, US). The acetic acid, NaCl, HEPES, D-glucose monohydrate and ascorbic acid were purchased from Roth (Karlsruhe, Germany). The Technovit 7100 was acquired from Heraeus-Kulzer (Hanau, Germany). The $NaH_2PO_4 \times H_2O$ was obtained from Merck (Darmstadt, Germany).

The Krebs-Ringer buffer (KRB), which was used for absorption studies, contained the following substances (in 1000 mL of double-distilled water): 6.8 g NaCl, 0.4 g KCl, 0.14 g $NaH_2PO_4 \times H_2O$, 2.1 g $NaHCO_3$, 3.575 g HEPES, 1.1 g D-glucose monohydrate, 0.2 g $MgSO_4 \times 7H_2O$ and 0.26 g $CaCl_2 \times 2H_2O$.

### 2.2. Cell culture

#### 2.2.1. HCE-T cells

The human corneal epithelial cells (HCE-T) were derived from a 49-year-old woman and were immortalised by transfection with a recombinant SV40-adenovirus vector. These cells, which were established and characterised by Araki-Sasaki in 1995 (Araki-Sasaki et al., 1995), form a multilayered epithelium when cultivated at the air–liquid interface (ALI) (Fig. 1). They were obtained from the RIKEN cell bank (Tsukuba, Japan).

#### 2.2.2. HCK cells

The HCK cell line was a gift from Dr. Zorn-Kruppa, who established the line by transfecting human corneal keratocytes with a SV40-adenovirus vector (Zorn-Kruppa et al., 2005). The immortalised keratocytes showed an in vivo-like flattened morphology and conserved their proliferation and differentiation characteristics to a large extent (Manzer et al., 2009).

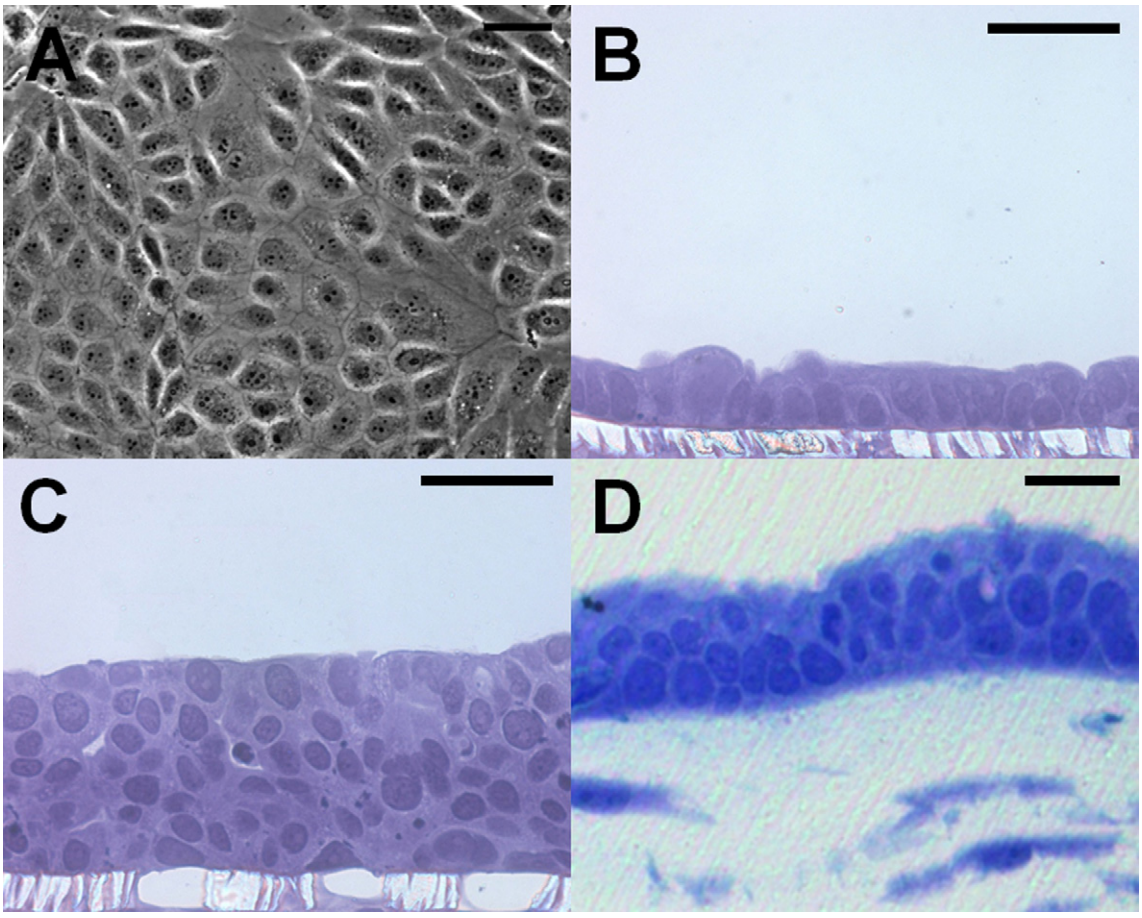


**Fig. 1.** (A) A phase-contrast micrograph of the confluent HCE-T monolayer; (B–D) a cross section and toluidine blue staining of the HCE-T cell epithelial model on a polycarbonate filter exhibiting a monolayer after seven days of submerged cultivation (B) and forming a multilayer after five additional days of cultivation at the ALI (C); the Hemicornea construct with multilayered epithelium on the collagenous stroma matrix, including embedded keratocytes (D); scale bar = 50 μm.

*2.2.3. Cultivation*

By default, the HCE-T and HCK cells were cultivated using DMEM/F12 and 5% FBS in 25-$cm^2$ tissue culture flasks at 37 °C in a humidified atmosphere containing 5% $CO_2$ (Table 1). The growth medium was replaced three times per week. If the type of medium or medium supplementation was changed, the cells were stepwise adapted to the new medium for at least four weeks. The growth media and their variations are listed in Table 1. The cell counting was performed using a Z2 Coulter Counter (Beckman Coulter, Krefeld, Germany), and the cultures were tested routinely for the absence of mycoplasma infection using the MycoTrace PCR detection kit.

## 2.3. Reconstructed corneal tissue

*2.3.1. Corneal epithelium*

The cultivation of the epithelial model, which was composed exclusively of epithelial cells, was performed on permeable, 3.0-μm pore size, polycarbonate filter inserts with a diameter of 12 mm (Transwell®). Before cultivation, an ethanolic solution of type I rat tail collagen (1.5 mg/mL) that was acidulated with acetic acid was cast in the Transwell®. After evaporation of the solvent, an aqueous solution of fibronectin (10 μg/mL) was flowed onto the collagen-coated filter. After removing the fibronectin solution, the HCE-T cells suspended in the culture medium were seeded onto the filter, and the culture medium was added. After the HCE-T cells became confluent, the cell differentiation and multilayered growth of the epithelium was induced by lifting the construct to the air–liquid interface (ALI) after a varying period of submerged cultivation. A metal plate was then placed under the Transwell®, which increased the volume at the basolateral side from 1.5 mL to 2.0 mL, and the apical side of the model came into contact with the surrounding atmosphere.

*2.3.2. Hemicornea construct*

The three-dimensional Hemicornea model (HC) was cultivated in the Transwell® inserts. Initially, the human keratocytes were suspended in an aqueous solution of 10× MEM, L-glutamine (12.9 mM) and $NaHCO_3$ (16.1 mg/mL). This cell suspension was mixed with a solution of type I rat tail collagen in acetic acid (1.7 mg/mL) and cast in the Transwell®; the collagen gelled within 1 h, forming the stromal equivalent of the HC. Subsequently, 1.5 mL of culture medium was added under the filter, and the HCE-T cells that were suspended in the culture medium were seeded onto the collagen matrix. The Hemicornea was initially cultivated submerged for some days and then cultivated at the ALI before the permeation experiment was performed.

## 2.4. Variations in the cultivation conditions

The epithelial model was cultivated in two types of serum-containing growth media: a modified DMEM/Ham's F12 mixture (DMEM/F12), and F99 (Table 1). To analyse the influence of FBS on the barrier function, the serum concentration in the DMEM/F12 was varied from 0.2% to 20%. Furthermore, several serum-free base media were tested, including Advanced DMEM, CEMDM, DefK,

**Table 1**
The cell-culture media and its compositions.

| Abbreviation | Base medium | Supplements |
|---|---|---|
| DMEM/F12 | DMEM/Ham's F12 1:1 mixture | 0.2–20% foetal bovine serum (FBS)<br>2 mM glutamine<br>5 μg/mL insulin<br>0.5% dimethyl sulfoxide (DMSO)<br>0.1% cholera toxin<br>10 ng/mL epidermal growth factor (EGF)<br>100 U/mL penicillin G sodium salt<br>100 μg/mL streptomycin sulfate<br>0.25 μg/mL amphotericin B |
| DMEM/F12 wPX | DMEM/Ham's F12 1:1 mixture | 3.0–12.5% Panexin<br>2 mM glutamine<br>5 μg/mL insulin<br>0.5% dimethyl sulfoxide (DMSO)<br>0.1% cholera toxin<br>10 ng/mL epidermal growth factor (EGF)<br>100 U/mL penicillin G sodium salt<br>100 μg/mL streptomycin sulfate<br>0.25 μg/mL amphotericin B |
| F99 | F99 (M 199 Earle's medium/Ham's F12 1:1 Mixture) | 5% foetal bovine serum (FBS)<br>100 U/mL penicillin G sodium salt<br>100 μg/mL streptomycin sulfate<br>0.25 μg/mL amphotericin B |
| Advanced DMEM | Advanced DMEM | 0–1.0% foetal bovine serum (FBS)<br>2 mM glutamine<br>20 U/mL penicillin G sodium salt<br>20 μg/mL streptomycin sulfate<br>0.5 μg/mL amphotericin B |
| Advanced DMEM+ | Advanced DMEM | 0–1.0% foetal bovine serum (FBS)<br>2 mM glutamine<br>5 μg/mL insulin<br>0.5% dimethyl sulfoxide (DMSO)<br>0.1% cholera toxin<br>10 ng/mL epidermal growth factor (EGF)<br>20 U/mL penicillin G sodium salt<br>20 μg/mL streptomycin sulfate<br>0.5 μg/mL amphotericin B |
| CEMDM | Corneal epithelial model differentiation medium | From the SingleQuot Kit:<br>Recombinant human epidermal growth factor (rhegf)<br>Insulin<br>Bovine pituitary extract (BPE)<br>Hydrocortisone<br>Ascorbic acid<br>Retinoic acid<br>Calcium chloride<br>Gentamicin<br>Amphotericin B |
| DefK | Defined keratinocyte-SFM | 20 U/mL penicillin G sodium salt<br>20 μg/mL streptomycin sulfate<br>0.5 μg/mL amphotericin B |
| KGM | Keratinocyte growth medium | 30 μg/mL bovine pituitary extract (BPE)<br>0.1 ng/mL human epidermal growth factor (hEGF)<br>0.5 μg/mL hydrocortisone<br>5 μg/mL insulin<br>0.5 mM calcium chloride<br>15 ng/mL amphotericin B<br>30 μg/mL gentamicin sulfate |
| KGM special | Keratinocyte growth medium | 30 μg/mL bovine pituitary extract (BPE)<br>0.1 ng/mL human epidermal growth factor (hEGF)<br>1.0 μg/mL hydrocortisone<br>5 μg/mL insulin<br>0.5 mM calcium chloride<br>50 μg/mL ascorbic acid<br>1.5 ng/mL retinoic acid<br>15 ng/mL amphotericin B<br>30 μg/mL gentamicin sulfate |
| K-SFM | Keratinocyte Medium | 10 μg/mL bovine serum albumin (BSA)<br>50 μg/mL bovine pituitary extract (BPE)<br>2.5 μg/mL insulin<br>25 ng fibroblast growth factor (FGF)<br>500 ng/mL epinephrine<br>0.5 μg/mL hydrocortisone |

Table 1 (*Continued*)

| Abbreviation | Base medium | Supplements |
|---|---|---|
| | | $10^{-8}$ M prostaglandin $E_2$<br>30 nM triiodothyronine ($T_3$) |
| Pan 401 | Panserin 401 | 50 U/mL penicillin G sodium salt<br>50 μg/mL streptomycin sulfate<br>0.125 μg/mL amphotericin B |
| Pan+ | Panserin 401 | 5 μg/mL insulin<br>0.5% dimethyl sulfoxide (DMSO)<br>0.1% cholera toxin<br>10 ng/mL epidermal growth factor (EGF)<br>50 U/mL penicillin G sodium salt<br>50 μg/mL streptomycin sulfate<br>0.125 μg/mL amphotericin B |
| Pan 401 wPX | Panserin 401 | 5.0–10.0% Panexin<br>50 U/mL penicillin G sodium salt<br>50 μg/mL streptomycin sulfate<br>0.125 μg/mL amphotericin B |
| Pan+ wPX | Panserin 401 | 5.0–10.0% Panexin<br>5 μg/mL insulin<br>0.5% dimethyl sulfoxide (DMSO)<br>0.1% cholera toxin<br>10 ng/mL epidermal growth factor (EGF)<br>50 U/mL penicillin G sodium salt<br>50 μg/mL streptomycin sulfate<br>0.125 μg/mL amphotericin B |

The DMEM/F12 medium containing 5% FBS was the standard medium, as recommended by the provider of the HCE-T cells. Starting with this medium, modifications were undertaken to improve the epithelial barrier characteristics. The abbreviation "wPX" means the medium containing the serum replacement Panexin, and "+" stands for the medium containing the same supplements as the DMEM/F12 standard medium.

KGM, K-SFM and Pan 401, with varying supplementation. The most promising serum-free media, KGM and Pan+, were subsequently used for cultivating the Hemicornea construct. In addition to the type of medium, the influence of the medium replacement frequency after the air-lift was investigated by replacing the medium at the basolateral side either once daily or every other day, starting at the date of the air-lift (the replacement frequency during the submerged cultivation period remained the same).

The influence of the initial cell count on the epithelial barrier was investigated within the range of 60,000 to 140,000 HCE-T cells per Transwell® using the epithelial model. To analyse the influence of the air-lift date, the corneal tissue was cultivated submerged for 5 to 18 days before it was lifted to the ALI to induce multilayered growth.

Moreover, the cultivation of the Hemicornea construct after the air-lift was varied by either supplementing the medium with 0.5% DMSO or leaving a lachrymal fluid equivalent (50 μL KGM) on the surface. Thus, the epithelium was slightly moistened by a lachrymal-fluid equivalent even under air-lifted conditions.

In addition to the cultivation parameters impacting the epithelial layer that have been noted so far, the effect of differing stromal thicknesses was investigated by varying the volume of the collagen gel from 200 μL to 500 μL per Hemicornea.

### 2.5. Light microscopy

The morphological appearances of the HCE-T and HCK cultures were observed using an Olympus IX50 photomicroscope (Olympus, Hamburg, Germany). For the histological characterisation of the epithelial model and Hemicornea, the tissues were fixed in an aqueous solution of 4% formaldehyde for 24 h. After an ethanol dehydration series, the tissue was embedded in plastic resin on a base of hydroxyethylmethylacrylate (Technovit 7100). Cross sections of these samples were cut to a 3.5-μm thickness, stained with toluidine blue and examined by microscopy.

### 2.6. Transepithelial electrical resistance (TEER) measurements

To evaluate the barrier function of the reconstructed corneal tissue during cultivation and to investigate the influence of variations in the cultivation conditions on tissue integrity, TEER measurements, which reflect the resistance across epithelial tight junctions, were taken using the EVOM® resistance meter and Endohm® chamber (World Precision Instruments, Sarasota, Florida, US).

### 2.7. Permeation studies

The transcorneal permeability of the Hemicornea construct was evaluated using three different marker substances with a wide range of molecular attributes. The Hemicornea construct and excised rabbit and porcine tissues were used to compare the barrier characteristics. The animal corneas were obtained from a local slaughterhouse and from local breeders, removed within 30 min of death, transported in Krebs-Ringer buffer (KRB) to maintain the tissues' physiological properties and used immediately for the absorption studies.

The marker compounds sodium fluorescein, rhodamine B and FD-4 were dissolved in KRB (pH 7.4), which also served as the acceptor solution. The particular concentrations of the donor solution, donor volume, acceptor volume and dimension of the permeation surface are specified in Table 2. The permeation experiments, including the experiments on the HC, were performed at 37 °C directly in the Transwell®. Before the donor solution was applied to the epithelial side, the HC was rinsed with KRB and incubated for 30 min with tempered KRB. Samples from the acceptor compartment were taken at 30, 90, 150, 210, 270 and 330 min after adding the donor solution; the acceptor solution was replaced by the same volume of tempered KRB. During the experiment, the donor and acceptor solutions were agitated continuously with an orbital shaker. The excised corneas were treated in the same manner and permeated using a vertical Ussing diffusion chamber system (Harvard Apparatus, Holliston, Massachusetts, US). The

**Table 2**
The experimental parameters of the permeation studies of the Hemicornea model and the excised rabbit and porcine corneas.

| | | Hemicornea | Excised cornea |
|---|---|---|---|
| Concentration of donor solution | Sodium fluorescein | 250 μg/mL | 500 μg/mL |
| | Rhodamine B | 50 μg/mL | 250 μg/mL |
| | FD-4 | 2500 μg/mL | 5000 μg/mL |
| Donor volume | | 400 μL | 4000 μL |
| Acceptor volume | | 2000 μL | 4000 μL |
| Permeation area | | 1.12 $cm^2$ | 0.126 $cm^2$ |

donor and acceptor solutions were mixed with carbogen gas during the experiments.

The levels of the marker compounds in the samples taken from the acceptor compartment were quantified using a fluorescence plate reader (Tecan, Männedorf, Switzerland) with 485-nm excitation and 535-nm emission filters for sodium fluorescein and FD-4 as well as 535-nm excitation and 590-nm emission filters for rhodamine B. The permeation coefficients ($P_{app}$ [cm/s]) of the three markers were determined by plotting the amount of the compound that permeated through the HC or the excised tissue with respect to time. By the means of the steady-state flux $J$ [μg/($cm^2$ s)] estimated from the linear ascent of the curve:

$$J = \frac{dQ}{dt \cdot A}$$

The permeation coefficient $P_{app}$ was calculated as:

$$P_{app} = \frac{J}{c_0}$$

where $Q$ indicates the quantity of the substance crossing the HC or excised cornea, $A$ indicates the corneal area exposed and $t$ indicates the exposure time. The term $c_0$ represents the initial marker concentration in the donor at $t = 0$.

### 2.8. Statistical analysis

The experiments were performed at least in triplicate; the exact numbers of replicates are stated for each data set. All the results are presented as the mean ±SD, and the Student's $T$-test was used to analyse statistical significance. $P$-values less than 0.05 were considered statistically significant.

The intra-laboratory repeatability of the HC cultivation and permeability testing was analysed by the equivalence test. Therefore, 95% confidence intervals for the permeation coefficients of each HC batch were generated using MS Excel 2007. The confidence intervals had to match the predefined acceptance criteria to confirm equivalence. The limits of the acceptance interval were defined using the permeation data of the excised rabbit cornea, which served as a reference due to its widespread use in such studies. As the batches of rabbit corneas had to meet our defined criteria for equivalence, the rabbit-derived $P_{app}$ values in our laboratory showing the greatest variability (i.e., the permeation values of FD-4) were used to specify the acceptance limits.

## 3. Results

### 3.1. Cultivation of the corneal epithelium

First, studies were undertaken to evaluate the optimal cultivation conditions for forming an in vivo-like epithelium that represented multilayered tissue with a pronounced zona occludens, as determined by the TEER measurements. Fig. 1B and C show the confluent monolayer of the HCE-T cells grown on the polycarbonate filter before being lifted to the ALI and after five days of cultivation under ALI conditions, resulting in a multilayered corneal epithelium. The general histology of the model was not significantly altered when certain cultivation conditions, such as the number of cells seeded or the medium components, were modified. However, some modifications strongly influenced the formation and function of the tight junctions and, consequently, the barrier properties of the epithelium.

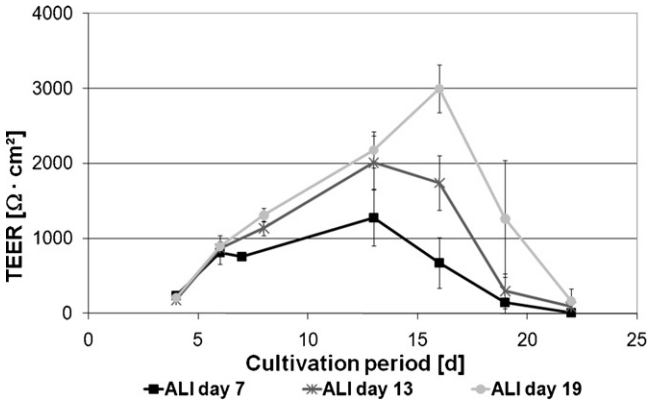


**Fig. 2.** The mean ±SD of the TEER levels resulting from submerged cultivation in the standard DMEM/F12 medium containing 5% FBS and subsequent lifting to the ALI at cultivation Day 7, 13 or 19 ($n = 6$).

The initial number of epithelial cells seeded per Transwell® affected the TEER values. The lowest and highest cell numbers (60,000 and 140,000 cells, respectively) produced significantly lower TEER values than did the mid-range cell counts (90,000 and 110,000 cells), both of which led to nearly identical TEER values (a mean of 2,900 Ω $cm^2$). For the subsequent experiments, therefore, 100,000 cells were used per Transwell®. Variations in the cultivation conditions, such as the time the model was lifted to the ALI and modifications to the DMEM/F12 cultivation medium by variations from the standard 5% FBS concentration or by DMSO/cholera toxin supplementation, had major influences on the TEER level. Fig. 2 shows the TEER behaviour of the epithelial model cultivated in the standard DMEM/F12 medium containing 5% FBS with respect to cultivation time. When the lift date was varied from Day 7 to Day 19, the extended submerged cultivation periods of 13 to 19 days led to higher peak TEER values, but these values were not maintained under the air-lifted conditions. The TEER values decreased rapidly immediately after the air-lift, indicating a decrease in the epithelial barrier. This effect was more pronounced when the FBS level in the growth medium was decreased to 0.2% (Fig. 3). However, the TEER level decreased slightly (Fig. 3) or continued to increase (Fig. 2) when the epithelial model was lifted at Day 7. Due to this

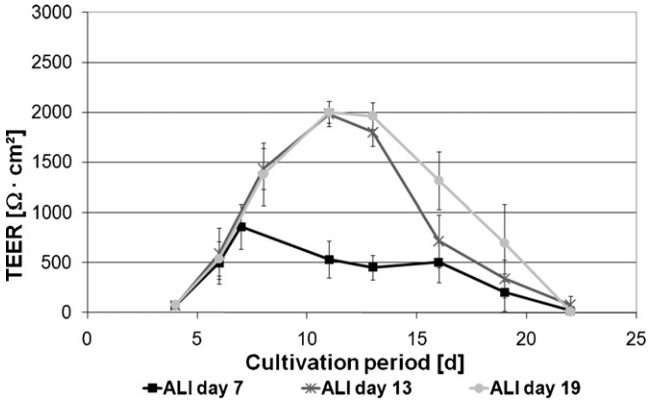


**Fig. 3.** The mean ±SD of the TEER levels during submerged cultivation in DMEM/F12 containing 0.2% FBS and subsequent lifting to the ALI at cultivation Day 7, 13 or 19 ($n = 6$).

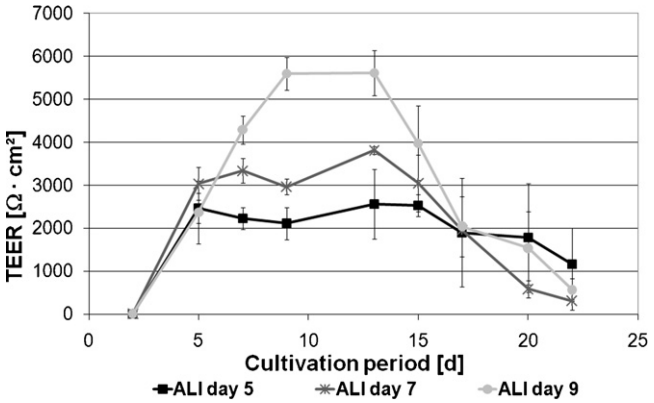


**Fig. 4.** The mean ±SD of the TEER levels during submerged cultivation in DMEM/F12 containing 15% FBS and subsequent lifting to the ALI at cultivation Day 5, 7 or 9 ($n = 6$).

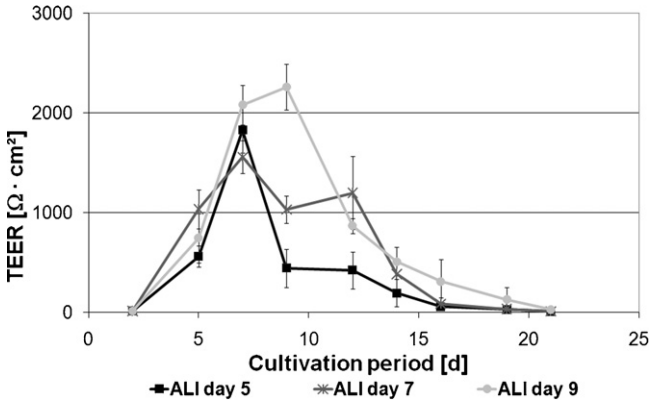


**Fig. 5.** The mean ±SD of the TEER levels during submerged cultivation in serum-free KGM and subsequent lifting to the ALI at cultivation Day 5, 7 or 9 ($n = 6$).

early air-lift, the TEER level plateaued. When the possible air-lift dates were narrowed to cultivation Days 5–9, lifting the model at Day 7 resulted in an optimal balance between high TEER values and a stable TEER level for an adequate time period. This finding was more pronounced for increased serum concentrations, with an optimum at a 15% FBS concentration (Fig. 4). By reducing the FBS concentration in the DMEM/F12 gradually from 5% to 0.2%, the TEER values remained at comparable levels in the submerged cultures (Figs. 2 and 3); a further serum reduction to 0% led to significantly lower values (data not shown). However, when the model was lifted to the ALI, FBS concentrations of up to 15% led to higher and more-stable TEER values (Fig. 4) and permitted a further increase in epithelial tightness after the air-lift.

The absence of cholera toxin, which was included in the standard DMEM/F12 medium by default (Araki-Sasaki et al., 1995), had no significant effect on the epithelial barrier function. By contrast, the lack of DMSO supplementation led to decreasing TEER values, which were at least 40% lower than those from the DMSO-containing DMEM/F12. Cultivation in the F99 medium, a different base medium (Table 1), containing 5% FBS did not result in the formation of a tighter barrier independently of the air-lift date (TEER <800 Ω cm$^2$).

### 3.1.1. Serum-free cultivation of corneal epithelium

The analysis of several serum-free culture media did not generally result in satisfactory barrier functions in the epithelial model. When the FBS in the DMEM/F12 standard medium was replaced with the chemically defined serum substitute Panexin (at 3–12.5% concentrations), a maximum TEER value of 300 Ω cm$^2$ was obtained. The models that were cultivated in CEMDM, DefK and K-SFM exhibited barriers of less than 100 Ω cm$^2$, which are identical to those found for the epithelium that was cultivated using serum-free Advanced DMEM and Advanced DMEM+. Only by supplementing Advanced DMEM and Advanced DMEM+ with 1% FBS were TEER levels of approximately 200 Ω cm$^2$ achieved. Cultivation in Pan 401 resulted in a slightly higher barrier function, and supplementation of Pan 401 with components of the standard DMEM/F12 medium (referred to as Pan+) resulted in increased TEER values of up to 900 Ω cm$^2$. Additional supplementation of Pan 401 and Pan+ with 5–10% Panexin produced slightly higher TEER levels (approximately 600 Ω cm$^2$ (Pan 401 wPX) and 1000 Ω cm$^2$ (Pan+ wPX)), but these higher values were accompanied by greater day-to-day variability. When the corneal epithelium was cultivated in KGM, a tighter barrier was achieved, and TEER values of more than 2000 Ω cm$^2$ were observed. However, further supplementation of KGM with ascorbic acid, retinoic acid and hydrocortisone (referred to as KGM special) was inefficient and resulted in a decrease in the TEER values to approximately 200 Ω cm$^2$. Adding 0.5% DMSO to KGM, by contrast, led to an increase in the TEER level, but the tight barrier was not maintained for as long as with normal KGM.

When investigating the effect of the air-lift date on the serum-free cultures, analogous results to those obtained from the serum-containing cultures were observed. As was observed for the DMEM/F12, lifting the KGM-cultivated epithelium at Day 7 resulted in the best balance between height and stability of the TEER level (Fig. 5), and a barrier comparable to that obtained with DMEM/F12 containing 5% FBS was achieved. Nevertheless, the plateau-like period was slightly shortened compared to the FBS-supplemented media.

### 3.2. Cultivation of the Hemicornea model

The most promising media used to cultivate the epithelium in Pan+ and KGM were investigated for the serum-free cultivation of the Hemicornea model. Fig. 1D shows the histological feature of the corneal tissue equivalent, which consists of the collagenous stroma-biomatrix containing keratocytes (HCK) covered by the multilayer corneal epithelium (HCE-T). Using the standard cultivation conditions (100,000 HCE-T cells per HC, a stromal volume of 500 μL collagen gel, seven days of submerged cultivation, and additional three days on the ALI and medium replacement every two days), the HC cultivated in Pan+ exhibited a TEER value of approximately 220 Ω cm$^2$, and the KGM-cultivated HC possessed higher values (approximately 350 Ω cm$^2$). Fig. 6 shows the HC permeation coefficients for the hydrophilic marker sodium fluorescein, compared with those for the excised

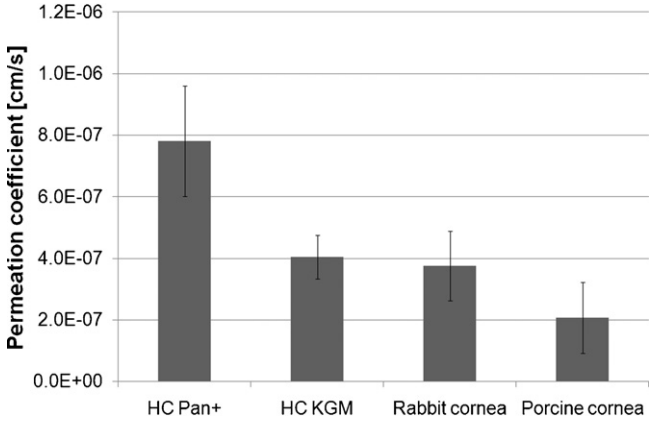


**Fig. 6.** A comparison between the sodium fluorescein permeation coefficients obtained from the HC cultivated in Pan+ and KGM and those of the excised rabbit and porcine corneas (mean ±SD, $n = 12$).

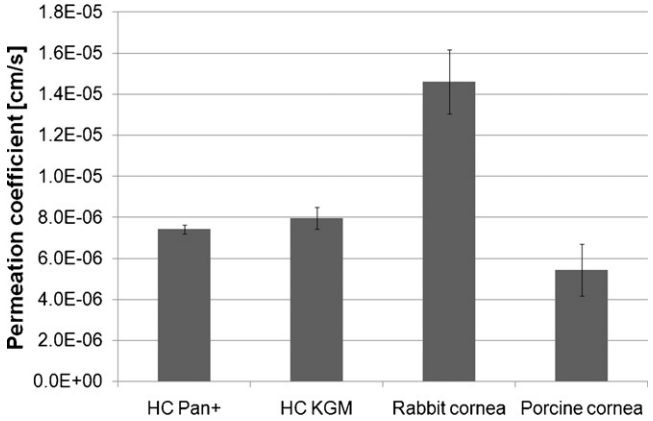


**Fig. 7.** A comparison between the rhodamine B permeation coefficients obtained from the HC cultivated in Pan+ and KGM and those of the excised rabbit and porcine corneas (mean ±SD, $n = 12$).

porcine and rabbit corneas. The $P_{app}$ of the Pan+-cultivated HC ($7.81 \pm 1.79 \times 10^{-7}$ cm/s) was significantly higher than that of the KGM-cultivated HC ($4.04 \pm 0.70 \times 10^{-7}$ cm/s), which was in the same range as the rabbit cornea and slightly higher than the porcine cornea. By contrast, the permeation experiments with the lipophilic marker rhodamine B demonstrated no significant discrepancy between the HCs cultivated in Pan+ or KGM (Fig. 7). The HC permeability was distinctly lower than that measured for the rabbit cornea and slightly higher than that measured for the porcine cornea. This finding can be explained by the different stromal thicknesses of the corneal samples. While the rabbit cornea had a thinner stromal layer, the HC stroma-biomatrix was similar to that of the porcine corneal stroma, which is considerably thicker than the rabbit stroma and consequently forms a stronger diffusion barrier for lipophilic molecules.

Modifications to the HC stromal thickness by varying the volume of collagen gel from 200 μL to 500 μL resulted in changing permeation coefficients for the lipophilic rhodamine B and the hydrophilic sodium fluorescein (Fig. 8). When the stromal thickness was diminished gradually, the permeation coefficient of the hydrophilic sodium fluorescein decreased from $4.00 \pm 0.98 \times 10^{-7}$ cm/s to $2.79 \pm 0.29 \times 10^{-7}$ cm/s, whereas the permeation coefficient of lipophilic rhodamine B increased from $7.96 \pm 0.53 \times 10^{-6}$ cm/s to $21.15 \pm 3.12 \times 10^{-6}$ cm/s when the stromal thickness was reduced. The influence of further modifications to the cultivation procedure on the epithelial barrier properties, as determined by

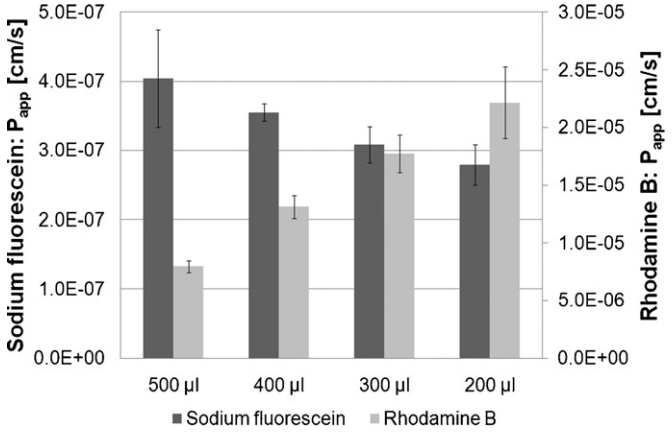


**Fig. 8.** The influence of varying volumes of HC collagenous stromal equivalent, from 200 to 500 μL, on the sodium fluorescein and rhodamine B permeation coefficients (mean ±SD, $n = 6$).

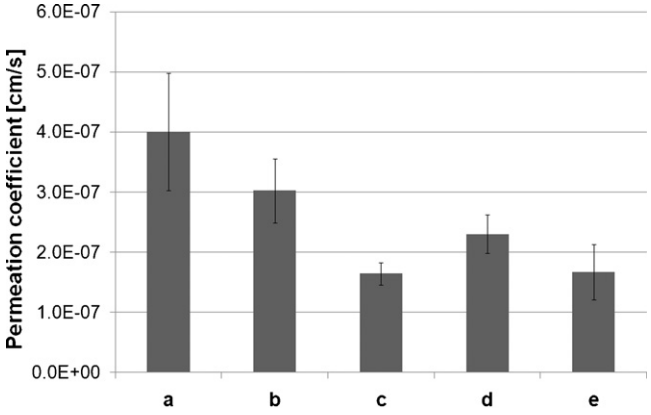


**Fig. 9.** The influence of different cultivation parameters on the sodium fluorescein permeation coefficients obtained from the Hemicornea model with a homogeneous 500 μL stroma volume (mean ±SD, $n = 6$). a: Every two days medium replacement; b: daily medium replacement; c: lachrymal fluid equivalent and daily medium replacement; d: DMSO supplementation and every two days medium replacement; e: DMSO supplementation and daily medium replacement.

the permeation coefficient of sodium fluorescein, is presented in Fig. 9. A change in the medium replacement frequency from every other day (standard) to once daily after the Hemicornea construct was lifted to the ALI resulted in a $P_{app}$ value that decreased from $4.04 \pm 0.70 \times 10^{-7}$ cm/s to $3.02 \pm 0.53 \times 10^{-7}$ cm/s. When the daily medium exchange was maintained and a lachrymal fluid equivalent of 50 μL KGM remained on the epithelial surface after the air-lift, a further reduction in the permeation coefficient, to $1.64 \pm 0.19 \times 10^{-7}$ cm/s, occurred. Moreover, supplementation of the KGM culture medium with 0.5% dimethyl sulfoxide (DMSO) beginning on the date of the air-lift led to an increase in the epithelial barrier properties. In this way, a permeation coefficient of $2.30 \pm 0.32 \times 10^{-7}$ cm/s was achieved when the DMSO-supplemented medium was replaced every two days, and a value of $1.68 \pm 0.46 \times 10^{-7}$ cm/s was achieved with daily medium replacement. In contrast, the DMSO had a negative effect on the barrier function when it was added at day 0 (i.e., at the beginning of the cultivation period).

### 3.3. Comparative absorption studies and intra-laboratory repeatability

Based on the results of the previously analysed cultivation parameters, the Hemicornea constructs were cultivated according to the optimised parameters shown in Table 3; TEER values of approximately 600 Ω cm$^2$ were obtained. The Hemicornea constructs were used in comparative studies to evaluate the utility of HC for replacing excised cornea in drug absorption studies and to determine the degree of intra-laboratory repeatability of such experiments. The HC cultivation and permeation experiments using sodium fluorescein and rhodamine B as hydrophilic and

**Table 3**
The optimised cultivation parameters of the Hemicornea model for obtaining an in vivo-like barrier function.

| Parameter | Optimum |
|---|---|
| Culture medium | Keratinocyte Growth Medium (KGM) |
| Number of epithelial cells per Transwell® | 100,000 cells |
| Number of keratocytes per Transwell® | 80,000 cells |
| Volume of stromal equivalent | 400 μL |
| Date of lifting HC to air–liquid interface | cultivation Day 7 |
| Date of permeation study | cultivation Day 10 |
| Medium replacement frequency | daily from cultivation Day 6 until Day 10 |

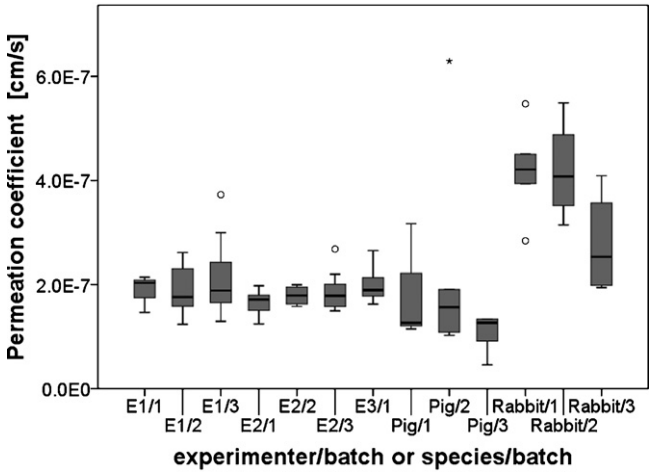


**Fig. 10.** The sodium fluorescein permeation coefficients for different HC batches (cultivated according to the optimised SOP) and the excised porcine and rabbit corneas. The HC results were obtained by three different researchers (E1–E3) in the same laboratory. Each HC batch includes $n = 12$ independent constructs, and the permeation experiments with the excised corneas were performed with $n = 6$.

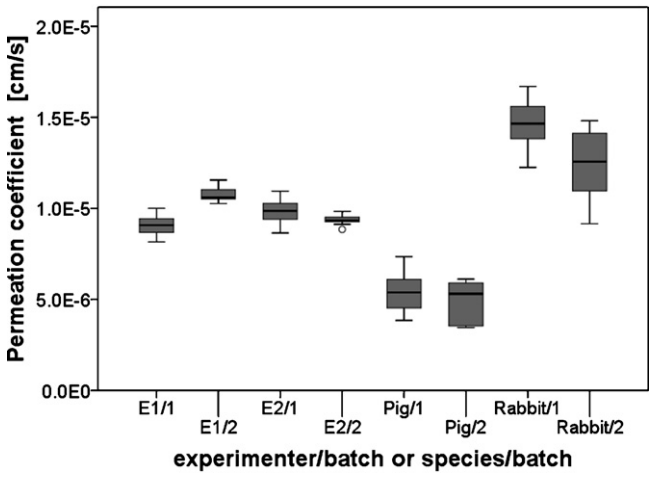


**Fig. 11.** The rhodamine B permeation coefficients for different HC batches (cultivated according to the optimised SOP) and the excised porcine and rabbit corneas. The HC results were obtained by two different researchers (E1 and E2) in the same laboratory. Each HC batch includes $n = 12$ independent constructs, and the permeation experiments with the excised corneas were performed with $n = 6$.

lipophilic markers and FD-4 as a high-molecular-weight compound were performed independently by two or three researchers. The results for each experimental batch are illustrated as box plots in Figs. 10–12 and are summarised as the means ±SD in Table 4. The permeation coefficients obtained from the different HC batches exhibited extensive uniformity compared to the excised animal corneas for all three of the permeation markers. In addition, the HC batches possessed considerably lower variability than the isolated rabbit or porcine corneas and demonstrated extensive equivalence with the excised tissue for sodium fluorescein, rhodamine B and FD–4. More precisely, the HC had a greater permeation barrier

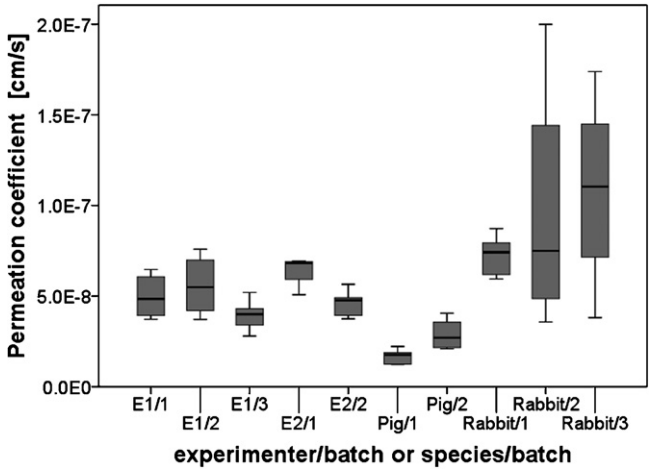


**Fig. 12.** The FD-4 permeation coefficients for different HC batches (cultivated according to the optimised SOP) and the excised porcine and rabbit corneas. The HC results were obtained by two different researchers (E1 and E2) in the same laboratory. Each HC batch includes $n = 12$ independent constructs, and the permeation experiments with the excised corneas were performed with $n = 6$.

than the excised rabbit cornea but a lower permeation barrier than porcine cornea in all cases.

All 17 tested batches of the Hemicornea constructs fulfilled the acceptance criteria for equivalence when the experiments were performed by up to three different researchers in the same laboratory. Thus, the equivalence test indicated that different researchers using the optimised SOP were able to cultivate several batches of HC having same level of permeability. Moreover, while the extreme values of the confidence intervals varied from 20.7% to 84.2% for the rabbit corneas and from 24.4% to 129.9% for the porcine corneas, the HC batches exhibited a variability of only 6.4% to 52.2%.

## 4. Discussion

Investigating the transcorneal absorption of new drug substances and the impact of novel ophthalmic formulations is essential before they can be applied to humans. Experiments are usually performed with excised animal corneas mounted in a diffusion chamber setup. As was mentioned earlier, however, in vivo and ex vivo animal experiments have many disadvantages; therefore, extensive research has been conducted on alternative methods, such as tissue engineering techniques, to develop an in vitro model of the corneal barrier (Hornof et al., 2005; Reichl and Becker, 2008). The exceptional epithelial barrier due to the zona occludens and the multilayered structure of the native corneal tissue (epithelium, stroma, and endothelium) make the development of a realistic barrier a demanding task, whereby the optimisation of the cultivation conditions is especially important for achieving intact intercellular junctions. Supplementation of the culture medium with FBS has been shown to have a positive impact on the formation of the barrier function (Toropainen et al., 2001), independent of its potentially negative effects on cell differentiation (Kruse and Tseng, 1993) and (especially) on process standardisation. In the present study, the benefit of FBS for the tightness and stability of the epithelial barrier was confirmed. However, the TEER values observed in our study

**Table 4**
The mean ±SD of the permeation coefficients [cm/s] of the three permeation markers through the Hemicornea and rabbit and porcine corneas ($n = 10$–52).

| Marker substance | Hemicornea | Porcine cornea | Rabbit cornea |
|---|---|---|---|
| Sodium fluorescein | $1.98 \pm 0.45 \times 10^{-7}$ | $1.75 \pm 1.51 \times 10^{-7}$ | $3.76 \pm 1.12 \times 10^{-7}$ |
| Rhodamine B | $9.80 \pm 0.72 \times 10^{-6}$ | $5.43 \pm 1.26 \times 10^{-6}$ | $13.49 \pm 2.11 \times 10^{-6}$ |
| FD-4 | $4.96 \pm 1.46 \times 10^{-8}$ | $1.67 \pm 0.43 \times 10^{-8}$ | $9.08 \pm 4.88 \times 10^{-8}$ |

for the HCE-T model cultivated in a serum-containing medium, which reached a maximum of 5500 Ω cm$^2$ (in DMEM/F12 with 15% FBS and ALI lifting on Day 9), were considerably higher than those described in previous studies using the same cell line, which have ranged between 200 Ω cm$^2$ and 1200 Ω cm$^2$ (Becker et al., 2008; Ko et al., 2009; Nagai et al., 2008; Reichl, 2008; Seeber et al., 2008; Toropainen et al., 2001; Wang et al., 2008). The elimination of some additional supplements from the standard medium (Araki-Sasaki et al., 1995) to simplify it for improved standardisation had no effect on the TEER (in the case of cholera toxin), whereas the removal of DMSO resulted in decreased TEER values. The optimal date of lifting the model to the ALI after reaching a confluent epithelial monolayer is an important parameter for achieving high, stable TEER values over an extended period of time, which is considered to be essential for achieving reproducible permeation data; this optimal date was found to be the seventh day of cultivation. Bringing the epithelium to the ALI later (Day 13 or 19) led to rapidly decreasing TEER values that resulted in insufficient barrier properties and strongly increased permeability. Due to the extended cultivation time, an increase in the cell count and the number of epithelial layers results that consequently requires greater amounts of culture medium and medium supplements to maintain the TEER. A possible explanation for the rapidly decreasing TEER values with delayed lifting to the ALI may be a decreased number of superficial cells that receive essential nutrients through the elongated diffusion distances.

Due to the disadvantages of serum supplementation, a major goal of our study was to find an appropriate serum-free alternative to the gold-standard FBS-containing medium. Unfortunately, few studies have described the cultivation of HCE-T cells under serum-free conditions (Hahne and Reichl, 2010; Seeber et al., 2008). Therefore, six different serum-free base media and several variations of these media created by the addition of different supplements or by the replacement of serum with Panexin were investigated. Two variants, KGM and Pan+, were identified as appropriate for cultivating corneal epithelium. They resulted in TEER values between 1000 Ω cm$^2$ and 2000 Ω cm$^2$, which indicated barrier properties in the same range as those often described for excised rabbit corneas (Nakamura et al., 2007; Rojanasakul et al., 1992).

A previous study has shown the impact on achieving equivalence with native corneal tissue in the permeation of high lipophilic drugs from an additional stromal layer in corneal cell-culture models (Reichl, 2008). To keep the in vitro model simple, in particular in view of a sufficient co-culturing of different cell types in the same serum-free medium, we developed a two-layered Hemicornea model consisting of an epithelium growing on a stromal equivalent of keratocytes embedded in a collagen matrix that exhibited the same barrier properties to hydrophilic, lipophilic and macromolecular compounds as excised animal corneas. The avoidance of serum supplementation showed in this case, apart from defined cultivation conditions, the additional advantage that the serum-dependent contraction of the stromal matrix due to transformation of keratocytes in myofibroblasts did not occur (Beales et al., 1999; Borderie et al., 1999; Jester and Ho-Chang, 2003; Manzer et al., 2009). The unaltered shape of the stromal equivalent provided the opportunity to perform the absorption studies directly in the Transwell® inserts, which eliminated possible sources of errors, such as tissue transfer from the Transwell® to an appropriate diffusion setup and mechanical damage of the model during clamping in diffusion chambers. This new experimental setup was a distinct improvement over previously described corneal constructs for drug absorption studies (Reichl et al., 2004; Tegtmeyer et al., 2001) and consequently led to higher reproducibility.

The best HC cultivation results were achieved using the KGM medium. The standard procedure led to an in vivo-like corneal histology and permeability to hydrophilic sodium fluorescein that was equal to that of a rabbit cornea. Changes in the volume of the collagen gel when constructing the HC, which resulted in different stromal layer thicknesses, yielded different permeation coefficients for both rhodamine B and sodium fluorescein. The permeability of the lipophilic rhodamine B was reduced with increasing volumes of collagen gel due to the increase in the rhodamine B diffusion distance in the hydrophilic stromal layer. The same effect was observed when comparing rabbit and porcine corneas. The porcine cornea, with its distinctly thicker stromal tissue, revealed a lower permeability for rhodamine B than did the rabbit cornea. An increased thickness in the HC stromal layer resulted in a slight increase in permeability to the hydrophilic sodium fluorescein. This finding could be explained by the restriction of nutrient diffusion as a consequence of elongating the diffusion distance, resulting in a decrease in the TEER value. However, certain modifications of the cultivation procedure were studied. It was found that daily medium replacement after lifting to the ALI in combination with DMSO supplementation of medium can further strengthen the barrier properties of HC, resulting in decreased permeability to the hydrophilic sodium fluorescein. This effect could be further improved by adapting the cultivation conditions to more closely mimic the in vivo situation through leaving a lachrymal fluid equivalent (50 μL KGM) on the HC surface after lifting to the ALI, resulting in a further decrease in the sodium fluorescein permeability.

Based on these results, standard operating procedures (SOP) for Hemicornea construction and drug absorption studies were developed. In this context, the optimised parameters for HC cultivation were chosen (Table 3). These parameters led to permeability properties that were assumed to be equivalent to those of the human cornea in vivo. Unfortunately, due to the poor availability of human corneal tissue for such experiments, only a few studies of drug permeation through the human cornea have been described (Van Der Bijl et al., 2002; Van Eyk et al., 2009). Thus, a comparison between the permeabilities of human and excised animal corneas with respect to the three marker compounds is not available. However, the biology of the porcine eye is close to that of the human eye in many respects (Pond and Houpt, 1978). Furthermore, the histology of the porcine cornea more closely resembles that of the human cornea than does the histology of the rabbit cornea. Hence, we expect that the actual permeability of the human cornea is between that of rabbit and porcine corneas, although closer to the permeability of the porcine cornea. Comparative permeation studies of numerous HC batches with sodium fluorescein, rhodamine B and FD-4 showed permeability values that were, in all cases, between those measured for the rabbit and porcine corneas. Furthermore, the permeation data obtained from the HCs possessed distinctly less variability within each batch and between batches. In addition, the statistical analysis of the permeation coefficients obtained from different researchers cultivating and permeating the HCs according to the SOPs revealed intra-laboratory equivalence and repeatability between the batches.

## 5. Conclusions

The human corneal epithelial cell line HCE-T, cultivated on permeable filters using either culture medium containing serum or serum-free medium, can form multilayered epithelial tissue that exhibits the same barrier properties as corneal epithelium in vivo. Based on this cell line, SOPs were established for the serum-free cultivation of a more organotypical HC model that demonstrates extensive equivalence with the barrier properties of isolated rabbit and porcine tissues for three marker substances with a wide range of molecular attributes. A distinct advantage over excised animal corneas is the lower variability and excellent intra-laboratory repeatability of the HC. Thus, our study describes the next step toward a valid, open-source corneal cell-culture model by intro-

ducing SOPs for the standardised, serum-free cultivation of a tissue equivalent that may be a promising in vitro alternative for replacing animal ex vivo tissue when studying corneal drug absorption. To move further toward universal open-source use, the method must be transferred to other laboratories for a prevalidation that includes more studies of ophthalmically relevant drugs. Doing so will prove the robustness and inter-laboratory repeatability of the HC model. Furthermore, studies to compare the expression levels of drug transporter proteins and metabolic enzymes in the HC to those of human corneas in vivo are needed.

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## References

Araki-Sasaki, K., Ohashi, Y., Sasabe, T., Hayashi, K., Watanabe, H., Tano, Y., Handa, H., 1995. An SV40-immortalized human corneal epithelial cell line and its characterization. Invest. Ophthalmol. Vis. Sci. 36, 614–621.

Barar, J., Javadzadeh, A.R., Omidi, Y., 2008. Ocular novel drug delivery: impacts of membranes and barriers. Expert Opin. Drug Deliv. 5, 567–581.

Beales, M.P., Funderburgh, J.L., Jester, J.V., Hassell, J.R., 1999. Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: maintenance of the keratocyte phenotype in culture. Invest. Ophthalmol. Vis. Sci. 40, 1658–1663.

Becker, U., Ehrhardt, C., Schaefer, U.F., Gukasyan, H.J., Kim, K.J., Lee, V.H., Lehr, C.M., 2005. Tissue distribution of moxaverine-hydrochloride in the rabbit eye and plasma. J. Ocul. Pharmacol. Ther. 21, 210–216.

Becker, U., Ehrhardt, C., Daum, N., Baldes, C., Schaefer, U.F., Ruprecht, K.W., Kim, K.J., Lehr, C.M., 2007. Expression of ABC-transporters in human corneal tissue and the transformed cell line, HCE-T. J. Ocul. Pharmacol. Ther. 23, 172–181.

Becker, U., Ehrhardt, C., Schneider, M., Muys, L., Gross, D., Eschmann, K., Schaefer, U.F., Lehr, C.M., 2008. A comparative evaluation of corneal epithelial cell cultures for assessing ocular permeability. Altern. Lab. Anim. 36, 33–44.

Borderie, V.M., Mourra, N., Laroche, L., 1999. Influence of fetal calf serum, fibroblast growth factors, and hepatocyte growth factor on three-dimensional cultures of human keratocytes in collagen gel matrix. Graefes Arch. Clin. Exp. Ophthalmol. 237, 861–869.

Chang, J.E., Basu, S.K., Lee, V.H., 2000. Air-interface condition promotes the formation of tight corneal epithelial cell layers for drug transport studies. Pharm. Res. 17, 670–676.

Chen, L., Ebihara, N., Fujiki, K., Murakami, A., 2007. Expression and distribution of junctional adhesion molecule-1 in the human cornea. Jpn. J. Ophthalmol. 51, 405–411.

Goskonda, V.R., Khan, M.A., Hutak, C.M., Reddy, I.K., 1999. Permeability characteristics of novel mydriatic agents using an in vitro cell culture model that utilizes SIRC rabbit corneal cells. J. Pharm. Sci. 88, 180–184.

Goskonda, V.R., Hill, R.A., Khan, M.A., Reddy, I.K., 2000. Permeability of chemical delivery systems across rabbit corneal (SIRC) cell line and isolated corneas: a comparative study. Pharm. Dev. Technol. 5, 409–416.

Hahne, M., Reichl, S., 2010. Simulation of corneal epithelial injuries by mechanical and corrosive damage: Influence of fetal bovine serum and dexpanthenol on epithelial regeneration in a cell culture model. Ophthalmologe 107, 529–532, 534-526.

Hornof, M., Toropainen, E., Urtti, A., 2005. Cell culture models of the ocular barriers. Eur. J. Pharm. Biopharm. 60, 207–225.

Huang, A.J., Tseng, S.C., Kenyon, K.R., 1989. Paracellular permeability of corneal and conjunctival epithelia. Invest. Ophthalmol. Vis. Sci. 30, 684–689.

Järvinen, K., Järvinen, T., Urtti, A., 1995. Ocular absorption following topical delivery. Adv. Drug Deliv. Rev. 16, 3–19.

Jester, J.V., Ho-Chang, J., 2003. Modulation of cultured corneal keratocyte phenotype by growth factors/cytokines control in vitro contractility and extracellular matrix contraction. Exp. Eye Res. 77, 581–592.

Kawazu, K., Shiono, H., Tanioka, H., Ota, A., Ikuse, T., Takashina, H., Kawashima, Y., 1998. Beta adrenergic antagonist permeation across cultured rabbit corneal epithelial cells grown on permeable supports. Curr. Eye Res. 17, 125–131.

Klyce, S.D., Crosson, C.E., 1985. Transport processes across the rabbit corneal epithelium: a review. Curr. Eye Res. 4, 323–331.

Ko, J.A., Yanai, R., Nishida, T., 2009. Up-regulation of ZO-1 expression and barrier function in cultured human corneal epithelial cells by substance P. FEBS Lett. 583, 2148–2153.

Kruse, F.E., Tseng, S.C., 1993. Serum differentially modulates the clonal growth and differentiation of cultured limbal and corneal epithelium. Invest. Ophthalmol. Vis. Sci. 34, 2976–2989.

Manzer, A.K., Lombardi-Borgia, S., Schafer-Korting, M., Seeber, J., Zorn-Kruppa, M., Engelke, M., 2009. SV40-transformed human corneal keratocytes: optimisation of serum-free culture conditions. ALTEX 26, 33–39.

Minami, Y., Sugihara, H., Oono, S., 1993. Reconstruction of cornea in three-dimensional collagen gel matrix culture. Invest. Ophthalmol. Vis. Sci. 34, 2316–2324.

Nagai, N., Inomata, M., Ito, Y., 2008. Contribution of aldehyde dehydrogenase 3A1 to disulfiram penetration through monolayers consisting of cultured human corneal epithelial cells. Biol. Pharm. Bull. 31, 1444–1448.

Nakamura, T., Yamada, M., Teshima, M., Nakashima, M., To, H., Ichikawa, N., Sasaki, H., 2007. Electrophysiological characterization of tight junctional pathway of rabbit cornea treated with ophthalmic ingredients. Biol. Pharm. Bull. 30, 2360–2364.

Orwin, E.J., Hubel, A., 2000. In vitro culture characteristics of corneal epithelial, endothelial, and keratocyte cells in a native collagen matrix. Tissue Eng. 6, 307–319.

Pond, W.G., Houpt, K.A., 1978. The Biology of the Pig. Cornell University Press, Ithaca and London.

Prausnitz, M.R., Noonan, J.S., 1998. Permeability of cornea, sclera, and conjunctiva: a literature analysis for drug delivery to the eye. J. Pharm. Sci. 87, 1479–1488.

Ranta, V.P., Laavola, M., Toropainen, E., Vellonen, K.S., Talvitie, A., Urtti, A., 2003. Ocular pharmacokinetic modeling using corneal absorption and desorption rates from in vitro permeation experiments with cultured corneal epithelial cells. Pharm. Res. 20, 1409–1416.

Reichl, S., 2008. Cell culture models of the human cornea - a comparative evaluation of their usefulness to determine ocular drug absorption in-vitro. J. Pharm. Pharmacol. 60, 299–307.

Reichl, S., Becker, U., 2008. Cell culture models of the corneal epithelium and reconstructed cornea equivalents for in vitro drug absorption studies. In: Ehrhardt, C., Kim, K.J. (Eds.), Drug Absorption Studies—In Situ, In Vitro and In Silico Models. , first ed. Springer, New York, pp. 283–306.

Reichl, S., Müller-Goymann, C.C., 2001. Development of an organotypic corneal construction as an in vitro model for permeability studies. Ophthalmologe 98, 853–858.

Reichl, S., Müller-Goymann, C.C., 2003. The use of a porcine organotypic cornea construct for permeation studies from formulations containing befunolol hydrochloride. Int. J. Pharm. 250, 191–201.

Reichl, S., Bednarz, J., Müller-Goymann, C.C., 2004. Human corneal equivalent as cell culture model for in vitro drug permeation studies. Br. J. Ophthalmol. 88, 560–565.

Reichl, S., Dohring, S., Bednarz, J., Müller-Goymann, C.C., 2005. Human cornea construct HCC—an alternative for in vitro permeation studies? A comparison with human donor corneas. Eur. J. Pharm. Biopharm. 60, 305–308.

Reichl, S., Kölln, C., Hahne, M., Verstraelen, J., 2011. In vitro cell culture models to study the corneal drug absorption. Expert Opin. Drug Metab. Toxicol. 7, 559–578.

Rojanasakul, Y., Wang, L.Y., Bhat, M., Glover, D.D., Malanga, C.J., Ma, J.K., 1992. The transport barrier of epithelia: a comparative study on membrane permeability and charge selectivity in the rabbit. Pharm. Res. 9, 1029–1034.

Sakanaka, K., Kawazu, K., Nishida, K., Nakamura, J., Nakashima, M., Nakamura, T., Oshita, A., Ichikawa, N., Sasaki, H., 2006. Transport of timolol and tilisolol in rabbit corneal epithelium. Biol. Pharm. Bull. 29, 2143–2147.

Schneider, A.I., Maier-Reif, K., Graeve, T., 1997. The use of an in vitro cornea for predicting ocular toxicity. In Vitro Toxicol. 10, 309–318.

Scholz, M., Lin, J.E., Lee, V.H., Keipert, S., 2002. Pilocarpine permeability across ocular tissues and cell cultures: influence of formulation parameters. J. Ocul. Pharmacol. Ther. 18, 455–468.

Seeber, J.W., Zorn-Kruppa, M., Lombardi-Borgia, S., Scholz, H., Manzer, A.K., Rusche, B., Schafer-Korting, M., Engelke, M., 2008. Characterisation of human corneal epithelial cell cultures maintained under serum-free conditions. Altern. Lab. Anim. 36, 569–583.

Sosnova-Netukova, M., Kuchynka, P., Forrester, J.V., 2007. The suprabasal layer of corneal epithelial cells represents the major barrier site to the passive movement of small molecules and trafficking leukocytes. Br. J. Ophthalmol. 91, 372–378.

Steed, E., Balda, M.S., Matter, K., 2010. Dynamics and functions of tight junctions. Trends Cell Biol. 20, 142–149.

Tak, R.V., Pal, D., Gao, H., Dey, S., Mitra, A.K., 2001. Transport of acyclovir ester prodrugs through rabbit cornea and SIRC-rabbit corneal epithelial cell line. J. Pharm. Sci. 90, 1505–1515.

Tegtmeyer, S., Papantoniou, I., Muller-Goymann, C.C., 2001. Reconstruction of an in vitro cornea and its use for drug permeation studies from different formulations containing pilocarpine hydrochloride. Eur. J. Pharm. Biopharm. 51, 119–125.

Tegtmeyer, S., Reichl, S., Muller-Goymann, C.C., 2004. Cultivation and characterization of a bovine in vitro model of the cornea. Pharmazie 59, 464–471.

Toropainen, E., Ranta, V.P., Talvitie, A., Suhonen, P., Urtti, A., 2001. Culture model of human corneal epithelium for prediction of ocular drug absorption. Invest. Ophthalmol. Vis. Sci. 42, 2942–2948.

Toropainen, E., Ranta, V.P., Vellonen, K.S., Palmgren, J., Talvitie, A., Laavola, M., Suhonen, P., Hamalainen, K.M., Auriola, S., Urtti, A., 2003. Paracellular and passive transcellular permeability in immortalized human corneal epithelial cell culture model. Eur. J. Pharm. Sci. 20, 99–106.

Van Der Bijl, P., Engelbrecht, A.H., Van Eyk, A.D., Meyer, D., 2002. Comparative permeability of human and rabbit corneas to cyclosporin and tritiated water. J. Ocul. Pharmacol. Ther. 18, 419–427.

Van Eyk, A.D., Seifart, H.I., Meyer, D., Van der Bijl, P., 2009. In vitro transcorneal diffusion of the antimicrobial macrolides azithromycin and clarithromycin and the impact on microbial keratitis. Cornea 28, 441–446.

Wang, S., Jiang, T., Wang, Z., 2008. Permeability and anticataract effects of a topical ocular drug delivery system of disulfiram, BioMedical engineering and informatics: new development and the future - Proceedings of the 1st International Conference on BioMedical Engineering and Informatics. Vol. 1. BMEI; pp. 596-600.

Xiang, C.D., Batugo, M., Gale, D.C., Zhang, T., Ye, J., Li, C., Zhou, S., Wu, E.Y., Zhang, E.Y., 2009. Characterization of human corneal epithelial cell model as a surrogate for corneal permeability assessment: metabolism and transport. Drug Metab. Dispos. 37, 992–998.

Zhang, T., Xiang, C.D., Gale, D., Carreiro, S., Wu, E.Y., Zhang, E.Y., 2008. Drug transporter and cytochrome P450 mRNA expression in human ocular barriers: implications for ocular drug disposition. Drug Metab. Dispos. 36, 1300–1307.

Zorn-Kruppa, M., Tykhonova, S., Belge, G., Bednarz, J., Diehl, H.A., Engelke, M., 2005. A human corneal equivalent constructed from SV40-immortalised corneal cell lines. Altern. Lab. Anim. 33, 37–45.