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Cultivation and characterization of a bovine *in vitro* model of the cornea

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The aim of this study was to develop an *in vitro* model of the cornea of bovine cells, to characterise the model by histochemical methods and to investigate permeation of ophthalmic drugs through the model. As in the *in vivo* situation, an *in vitro* model of the cornea should consist of all three different types of cells. In the current study, the construction of the *in vitro* cornea was performed using cells prepared from primary cultures. To investigate the state of the cells in the cultures, growth curves were established. Immunocytochemical determination of keratin and vimentin was performed for all three isolated and sub-cultivated cell types of the bovine cornea. To further simulate the *in vivo* conditions, corneal epithelial cells were seeded onto the collagen-gel base containing the stromal cells with an underlying sheet of endothelium. Permeation experiments were performed with pilocarpine hydrochloride and timolol hydrogen maleate as model drugs and excised bovine cornea and the *in vitro* cornea as permeation barriers. The immunohistochemical investigations show that excised bovine cornea and the *in vitro* model of the cornea are comparable with respect to the expression of keratin K3, indicating that the primarily isolated cells correspond to the different cell types of the cornea. Culturing of the epithelial cells on the complex basis has led to the formation of a corneal epithelium with several layers, closely resembling the morphology of the *in vivo* epithelium. Although the permeation rates of the drug through the *in vitro* cornea were always higher, the sequence in which the drugs permeate through the two types of barriers was the same. The drug permeation through the *in vitro* cornea may therefore be a useful predictive tool to estimate the permeability coefficients of drugs through excised cornea.

1. Introduction

There is a growing criticism about the use of animals for the purpose of scientific experimentation. From a public viewpoint mainly ethical reasons are given as arguments to look for alternatives to animal experiments. From a scientific viewpoint, the most important question is to what extent results from animal experiments can be transferred to comparable situations in humans. Furthermore, animal experiments are expensive due to the labour of intensive housing of the animals.

The objective of this study was to develop an *in vitro* alternative to the use of animals or excised cornea for ocular permeation studies. Besides the above mentioned problems, these latter procedures have the disadvantage that interindividual differences between the corneas of the animals may increase the variability of the results. The specific aims of the study were to develop an *in vitro* model of the cornea of bovine cells, to characterise the model by histochemical methods and to investigate the permeation of ophthalmic drugs through the *in vitro* model, and thus its barrier function.

As in the *in vivo* situation, an *in vitro* model of the cornea should consist of three different types of cells, endothelial, stromal and epithelial cells. In the current study, the construction of the *in vitro* cornea was performed using cells prepared from primary cultures.

2. Investigations, results and discussion

2.1. Establishment of primary cell cultures

To develop an organotypical cell culture model of the cornea, it is a prerequisite to obtain the different cell types of the bovine cornea. The establishment of a homogeneous population of human endothelial cells of the cornea has been described as problematic (Engelmann et al. 1988). The outgrow of the human endothelial cells form tissue fragments leads to non-homogenous cultures (Hyldahl 1984). In the current study, contamination of bovine endothelial cells however, could be completely suppressed by a modified and improved technique based on the method by Stocker et al. (1958) who established cell cultures of rabbit endothelial cells. Stabilisation of the cornea in a stainless steel cup maintained the natural curvature of the cornea and thus restricted the contact of the enzyme solution with other cell layers, essential for the establishment of non-contaminated endothelial cell cultures.

A total of eight replicates was performed for the isolation of endothelial cells of the cornea. In all but one, adher-

Table 1: Morphological appearance, passage time and yield after first passage (mean ± standard deviation, n = 3) of primary cultures of the different corneal cell types

	Endothelial corneal cells	Epithelial corneal cells	Stromal corneal cells
Morphological appearance	Hexagonal shape	Polygonal shape	Elongated spindle shape
First passage	After 10 days	After 6–8 days	After 10 days
Yield after first passage	$4.8 \pm 1.2 \cdot 10^4$/ml	$5.8 \pm 1.8 \cdot 10^4$/ml	$6.9 \pm 3.1 \cdot 10^4$/ml

ence of the endothelial cells could be observed microscopically within a few hours. After the first passage (ten days) the cell number was determined. For the endothelial cells the cell yield was lower than for epithelial and stromal cells (Table 1).

Isolation of corneal epithelial cells was performed by outgrowth from tissue fragments. After four days of culture the tissue fragment was removed and the adhered cells were allowed to grow for another two to four days, before the first passage, as the cells were confluent after six to eight days. Eight replicates were made out of which seven could be further cultured.

Isolation of corneal stromal cells led to homogenous cell cultures. Again, eight replicates were made out of which seven could be further cultured. The yield after the first passage (10 days) was very high, due to the high proliferation rate of the cells. The results are summarised in Table 1.

2.2. Cryoconservation of corneal cells

To establish a stock of corneal cells and to minimise genetic drift, cells were frozen in liquid nitrogen (−196 °C) with DMSO added as cryoprotectant. The survival rate of the cells was determined to investigate the influence of the freezing process on the different cell types. The results are summarised in Table 2, and indicate that the method is suitable for cryoconservation of the isolated cells. According to Morgan and Darling (1994) the conservation method should lead to a survival rate of at least 50%.

2.3. Growth of corneal cells

To investigate the state of the cells in the cultures, growth curves were established. From these curves the doubling time for the cell numbers was determined. Additionally CPD and CPD/(d) were calculated. Growth curves were determined in DMEM and Minimum Essential Medium/D-Val (MEM/D-Val), in which L-valine of DMEM is replaced by D-valine.

Table 2: Survival rate of different corneal cell types after freezing (mean ± standard deviation, n = 3)

	Endothelial corneal cells	Epithelial corneal cells	Stromal corneal cells
Survival rate (%)	77 ± 9	79 ± 13	71 ± 7

This medium was chosen for the isolation of epithelial cells, because the growth of stromal cells is strongly restricted in this medium, as these cells lack the ability to convert D- to L-valine (Sundar-Raj et al. 1980), thus strongly reducing the risk of contamination of the epithelial cells by stromal cells.

2.3.1. Corneal epithelial cells

The growth curve of the corneal epithelial cells with DMEM as growth medium under given cultivation conditions is shown in Fig. 1a. After a lag time of more than two days growth changes into an exponential phase (log phase). The population doubling time is about one day. In comparison, Fig. 1b shows the growth curve of corneal epithelial cells with MEM/D Val as growth medium, indicating a lag time of almost three days and a population doubling time of approximately one day. These two parameters are thus hardly influenced by the change of the growth medium. It is therefore possible to use MEM/D Val for the establishment of primary cultures of corneal epithelial cells.

Generation number (CPD) and growth rate per day (CPD/d) are shown in Table 3. The generation number indicates, how many cell divisions have taken place during the experimental period. These two characteristic numbers were on similarly high level for both media, again indicating that for bovine corneal epithelial cells D-valine can be used in the growth medium, as their growth rate is not significantly lowered by the exchange of L-valine with D-valine.

2.3.2. Corneal stromal cells

Fig. 1c shows the growth curve of the corneal stromal cells under the described environmental conditions with DMEM as growth medium. These corneal cells are not only morphologically similar to the fibroblasts of the skin, but are likewise characterised by an explosive growth in culture. The lag phase of the stromal cells (0.6 days) is substantially shorter than of the corneal epithelial cells. The population doubling time is also decreased compared to that of epithelial cells (0.7 days).

Fig. 1d shows the growth behaviour of the stromal cells in MEM/D Val. A clear difference in the growth curves can be detected. This difference is also reflected in generation number and the CPD/(d) (Table 3): Generation number and growth rate were 1.6 times higher if the cells were

Table 3: Generation number (CPD) and growth rate per day (CPD/d) of different corneal cell types cultured in DMEM or MEM/D-Val, (mean ± standard deviation, n = 3)

	DMEM		MEM/D-Val	
	CPD	CPD/d	CPD	CPD/d
Epithelial corneal cells	2.93 ± 0.06	0.266 ± 0.005	2.88 ± 0.02	0.26 ± 0.002
Stromal corneal cells	4.05 ± 0.19	0.45 ± 0.02	2.53 ± 0.12	0.281 ± 0.013
Endothelial corneal cells	2.70 ± 0.11	0.246 ± 0.01	–	–

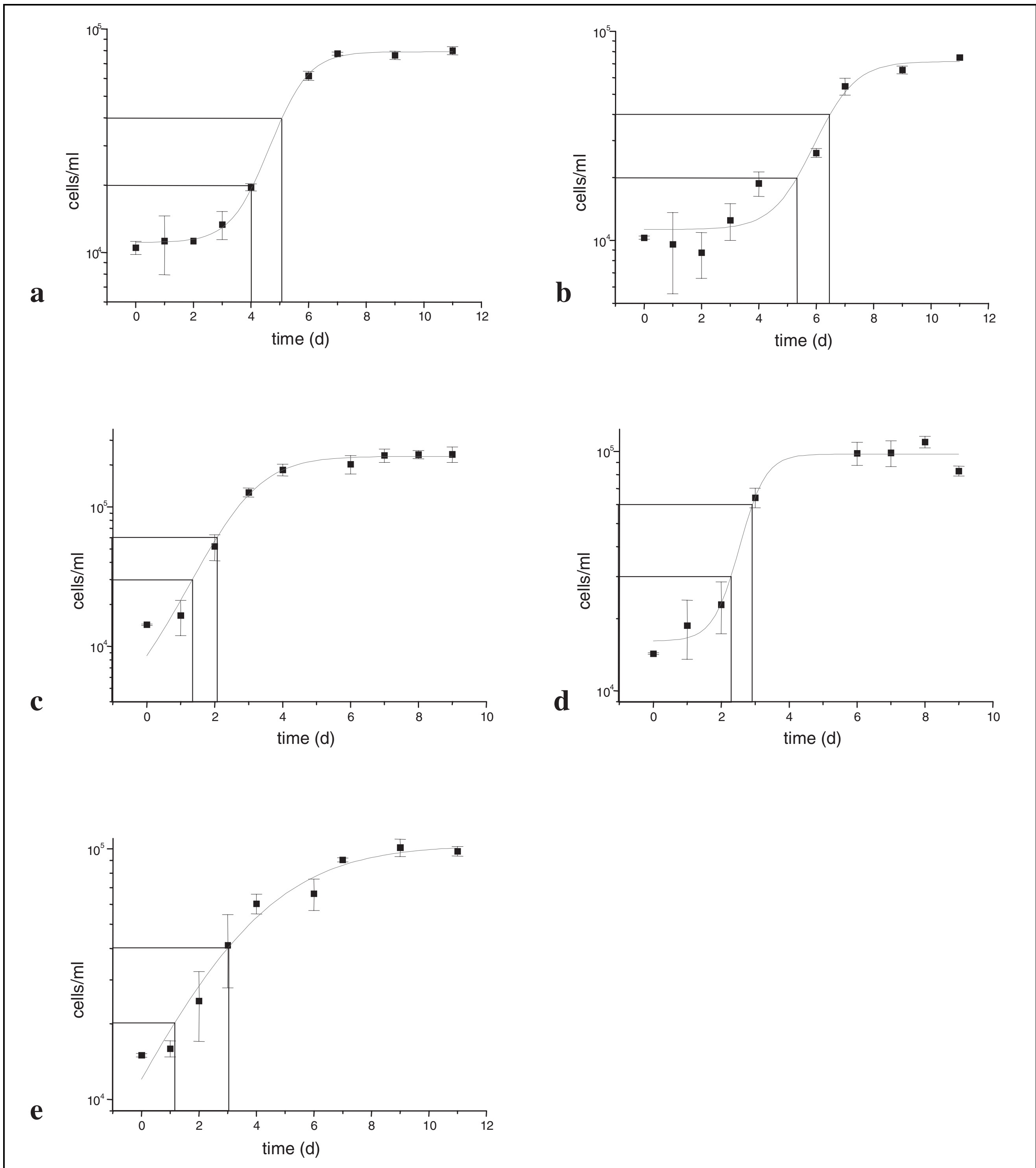


Fig. 1: Growth curves of corneal epithelial cells (a, b) corneal stromal cells (c, d) and corneal endothelial cells (e) cultured in DMEM (a, c, e) or MEM/D-Val (b, d), (n = 3)

cultivated in DMEM, indicating that for the cultivation of stromal cells DMEM is the more suitable medium. Growth in MEM/D Val is probably not as strongly reduced as was expected. Thus, during primary isolation of epithelial cells the contamination by stromal cells may not have been completely prevented using MEM/D Val.

2.3.3. *Corneal endothelial cells*

Fig. 1e shows the growth curve of the corneal endothelial cells with DMEM as cultivation medium. After a lag-phase of half a day the curve changes immediately into the exponential growth phase. From the curve a population doubling time of 1.9 days can be inferred.

Since the method for isolation of the corneal endothelial cells prevents a contamination with stromal cells no growth curve of the cells in MEM/D Val was established. The growth rate in DMEM (Table 3) is somewhat lower than that of epithelial cells, however, it still indicates a healthy status of the culture.

The comparison of the growth curves of the different cell types in the two cultivation media demonstrates that

DMEM is a suitable medium for all three cell types. In order to prevent contamination of epithelial cells with stromal cells, the exchange of DMEM with MEM/D Val does not appear to be sufficient, as this only lowers the growth rate of stromal cells but does not completely prevent their growth.

2.4. Immunocytochemical investigations

Further characterisation of the corneal cell types was carried out by different immunocytochemical investigations. The aim of these investigations was to identify the cell-specific intermediate filaments keratin and vimentin.
Keratin filaments are the tissue specific intermediate filaments of epithelial cells (Sun and Green 1978; Chan and Haschke 1982). Due to the presence of keratin in the primarily isolated cells of the corneal epithelium, the possibility arises that keratin could also be expressed in organotypical cultures.
Vimentin is a protein with a molecular weight of 55.000 Da, which is mainly expressed in non-epithelial cells (Alberts 1995). In 1985, Hayashi et al. immunocytochemically established that vimentin is present in endothelial corneal cells. Vimentin can thus be used as a marker to differentiate epithelial from endothelial cells. Stromal corneal cells also express vimentin (Alberts 1995), but due to their very characteristic morphology they can easily be differentiated from corneal endothelial cells.
The immunocytochemical determination of keratin and vimentin was performed for all three isolated and sub-cultivated cell types of the bovine cornea. The results are summarised in Table 4.
These investigations show that the primarily isolated cells correspond to the different cell types of the cornea. This becomes apparent on the one hand by the morphological characteristics of the cells (see Table 1), and on the other hand by the the presence of the respective intermediate filaments in the cell cultures.

2.5. Development of the in vitro cornea

The aim of the development of a three-dimensional organotypical cornea-equivalent (*in vitro* cornea) is to mimic the structure of the *in vivo* cornea as closely as possible. Cell culture models are used as substitution of the original organ, and it is thus assumed that morphological similarity strongly corresponds to a similar functionality of the organ.
In the current study the particular focus is on the permeability of the *in vitro* cornea. The permeability of the cornea is determined mainly by the epithelium and it is therefore crucial in the development of the *in vitro* cornea to adapt the morphology of the epithelium as closely as possible to the *in vivo* situation, by modifying the culture conditions. This is only possible by the recombination of the three cell types, because only the presence of all three cell types allows the formation of an organotypical epithelium.
An adjustment to the *in vivo* conditions is the dispersion of stromal corneal cells in a collagen gel. Dispersion of these cells leads to a contraction of the gel. This contraction is the stronger, the more stromal cells are present in the gel. The degree of contraction is inversely proportional to the collagen concentration, in agreement with findings in the development of *in vitro* dermal equivalents (Bell et al. 1979; Specht et al. 1998). The contraction of the gel indicates that the stromal cells stay vital following the dispersion in the gel (a gel without incorporated stromal cells does not contract). The mechanism of this phenomenon however, is not understood.
In Fig. 2 epithelial corneal cells are shown, which were cultivated on a matrix of collagen with embedded stromal cells for 14 days at the matrix-air interface. Clearly, a layered structure of the epithelium can be detected. However, the presence of basal cells is not immediately apparent. The cells in the upper layers are flattened. Under natural conditions these cells would have been detached from the epithelium due to the mechanical impact of the eyelid and due to tear flow.
To further simulate *in vivo* conditions, corneal endothelial cells were seeded onto the collagen-gel base. After confluence of the endothelial cells was achieved, the collagen gel containing the stromal cells was applied onto the endothelial cell monolayer. Culturing of the epithelial cells was performed on this complex basis. Again, these cells were cultured for 2 weeks at the air-liquid interface. The medium was adjusted to be exactly level with the surface of the culture. Culturing under these conditions led to the formation of a corneal epithelium with several layers, which closely resembles the morphology of the *in vivo* epithelium. Fig. 3 shows a cross-section of the *in vitro* corneal epithelium, indicating the presence of basal cell layer, together with two layers of suprabasal cells and a layer of stratified, flattened cells at the top.
Due to the addition of endothelial cells in the model, the *in vitro* cornea thus attains a structuring of the epithelium, which could not be attained before. It may therefore be concluded that transmitters from endothelial cells are of considerable importance for the structure and the differentiation of the epithelium.

Table 4: Immunocytochemical determination of the intermediate filaments keratin and vimentin in different corneal cell types, according to Tegtmeyer et al. (2001)

	Keratin	Vimentin
Epithelial corneal cells	+	−
Stromal corneal cells	−	+
Endothelial corneal cells	−	+

2.6. Immunohistochemical investigations

For the development of a three-dimensional model of the cornea it is not sufficient just to obtain morphological similarity.
For the application in permeation studies the mechanical stability is of particular importance, for which in turn, the presence of structural proteins is crucial. The structuring intermediate filaments of the corneal epithelium are the keratins. Keratins are initially formed in the basal cells and are present in the outer cell layers as molecular proteins.
In the corneal epithelium a basic 64-KDa-protein (K3) and an acidic 55-KDa-protein can be found. Both these keratins can be regarded as markers for fully differentiated corneal epithelial cells (Kurpakus et al. 1992).
In Fig. 4 a freeze section through excised bovine cornea is shown. The complete corneal epithelium shows a bluish colour, indicating the presence of K3. Likewise, the presence of K3 in the *in vitro* cornea was investigated in unfixed freeze-sections. The colour of the corneal epithelium in this case, appears brownish, since no cobalt chloride was added. Again, the whole epithelium is stained, indi-

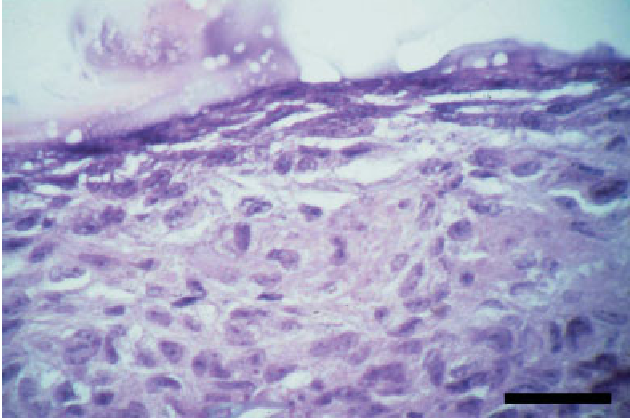


Fig. 2: Cross section through a culture of corneal epithelial cells, cultivated for 14 days at the air liquid interface on a matrix of collagen with embedded corneal stromal cells, H & E staining, Bar: 30 μm

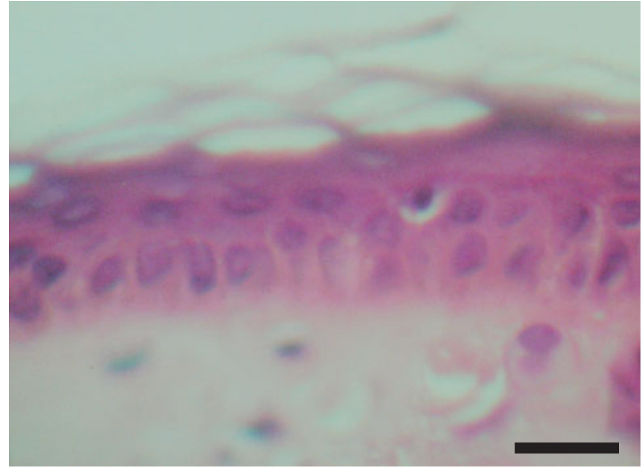


Fig. 3: Cross section through a culture of corneal epithelial cells, cultivated for 14 days at the air liquid interface on a matrix of collagen with embedded corneal stromal cells and an underlying endothelial cell layer, H & E staining, Bar: 25 μm

cating that K3 is expressed in all layers of the cornea (Fig. 4b).

The immunohistochemical investigations show that excised bovine cornea and the organotypical model of the cornea are comparable with respect to the expression of K3. The finding that in the *in vitro* cornea the distribution of cells that express K3 is similar to the bovine cornea, indicates that the *in vitro* model may be regarded as a suitable substitution for excised cornea in further studies.

2.7. Permeation investigations

One of the main aims of the current study was to develop an *in vitro* cornea in order to use it for permeation studies. The *in vitro* cornea was thus compared to excised bovine cornea with regards to its suitability as a permeation barrier.

An important advantage of a cell culture model for permeation studies is the independence of the availability of bovine eyes and cornea respectively. A further advantage is that interindividual differences in the quality of the cornea could be minimised using *in vitro* models.

The development of an *in vitro* model on the basis of human cells would further increase the merit of such a model, as human cornea is not available in quantities necessary to perform permeation studies.

Investigations of the permeation properties of the cornea are usually performed on the rabbit eye. In these experiments usually the drug concentration in the aqueous humour is determined after certain time intervals. For this purpose, the animals are sacrificed and their eyes are taken out. This procedure has some disadvantages:

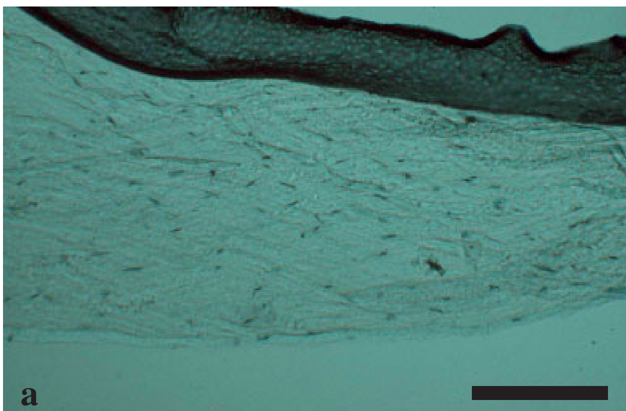
Firstly, housing of laboratory animals is expensive and labour intensive and secondly, there is a growing criticism by the public about the use of animals for scientific experimentation, which becomes more relevant if useful alternatives are available. Secondly, the question arises, to what extent data found in the rabbit eye model can be transferred to the conditions in the human eye, especially as other species than rabbits are hardly used for such investigations.

For the permeation investigations on the organotypical cell culture model in the current study drugs were used that are frequently employed in glaucoma therapy (pilocarpine hydrochloride and timolol hydrogen maleate).

Furthermore, the influence of the vehicles on the extent and rate of permeation should be determined. For this purpose, permeation investigations were performed with the same drugs in different formulations.

In Table 5 and 6 the results of these investigations are summarised. Permeation of the drugs from various formulations through the *in vitro* model followed the same rank order than through excised bovine cornea in terms of the permeation coefficient. The permeation coefficient however, was 2–4 fold higher with the *in vitro* cornea construct than with the excised cornea.

Thus, in all experiments, the *in vitro* model of the cornea has shown a higher permeability for the drugs, compared



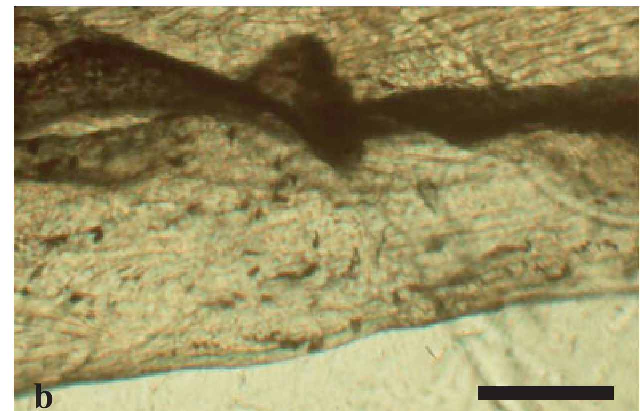


Fig. 4: Freeze section through excised bovine cornea (a) and *in vitro* model of the cornea (b). Immunohistological staining of Keratin K3, Bar: 60 μm

Table 5: Permeation coefficient (P) of pilocarpine hydrochloride in various formulation through *in vitro* model of the cornea and excised bovine cornea

		In vitro model of the cornea		Excised bovine cornea	
		P (cm/s)	s_{rel} (%)	P (cm/s)	s_{rel} (%)
Borocarpin® AT	×	$8.59 \cdot 10^{-6} \pm 1.38 \cdot 10^{-6}$	16.1	$2.29 \cdot 10^{-6} \pm 0.73 \cdot 10^{-6}$	31.9
Vistacarpin® AT		$2.55 \cdot 10^{-6} \pm 0.26 \cdot 10^{-6}$	10.2	$1.89 \cdot 10^{-6} \pm 0.25 \cdot 10^{-6}$	13.2
Pilocarpol® AT	×	$2.51 \cdot 10^{-6} \pm 0.39 \cdot 10^{-6}$	15.5	$1.14 \cdot 10^{-6} \pm 0.17 \cdot 10^{-6}$	14.9
Spersacarpin® AT	×	$13.4 \cdot 10^{-7} \pm 0.74 \cdot 10^{-7}$	5.5	$7.77 \cdot 10^{-7} \pm 1.57 \cdot 10^{-7}$	20.2
RMS	×	$2.54 \cdot 10^{-7} \pm 0.39 \cdot 10^{-7}$	15.4	$6.11 \cdot 10^{-8} \pm 1.15 \cdot 10^{-8}$	18.8

Values represents mean +/− standard deviation and relative standard deviation (s_{rel}), (n = 3–8). Marked data (×) according to Tegtmeyer et al. (2001)

Table 6: Permeation coefficient of timolol hydrogen maleate in various formulation through *in vitro* model of the cornea and excised bovine cornea

	In vitro model of the cornea		Excised bovine cornea	
	P (cm/s)	sd (%)	P (cm/s)	sd (%)
Timomann® AT	$8.23 \cdot 10^{-6} \pm 1.26 \cdot 10^{-6}$	15.3	$3.76 \cdot 10^{-6} \pm 0.84 \cdot 10^{-6}$	22.3
HPMC-formulation	$1.92 \cdot 10^{-6} \pm 0.69 \cdot 10^{-6}$	35.9	$9.75 \cdot 10^{-7} \pm 1.81 \cdot 10^{-7}$	18.6
RMS	$6.17 \cdot 10^{-8} \pm 1.89 \cdot 10^{-8}$	30.6	$5.25 \cdot 10^{-8} \pm 2.85 \cdot 10^{-8}$	54.3

Values represents mean +/− standard deviation and relative standard deviation (s_{rel}), (n = 3–6)

with the excised bovine cornea. There could be several reasons for this finding:
Firstly, the epithelium of the *in vivo* cornea has five to six layers, whereas the *in vitro* cornea has an epithelium with three to four layers only. As the epithelium is determining the permeability of the cornea, this difference may explain the difference in permeability. Secondly, in the *in vitro* model the density of collagen fibres in the stroma may not be as high as in the *in vivo* cornea (Schneider et al. 1997). This hypothesis is supported by the lack of transparency of the artificial cornea. The specific density and lamellar layering of the collagen fibres in the native cornea, together with a specific water concentration, leads to a complete transparency of the *in vivo* cornea (Schneider et al. 1997). Thirdly, differentiation of the cells in the *in vitro* model may not happen in exactly the same way as for the cells *in vivo*.
Such a phenomenon (higher permeability) has also been observed with cell culture models of the skin. Investigations of the keratin pattern of those cell cultures indicated that the artificial skin is more comparable to skin in the status of wound healing or psoriatic skin than to normal skin, with a manifold higher permeability than excised stratum corneum (Specht et al. 1998).
Nevertheless from all permeation experiments with pilocarpine hydrochloride and timolol hydrogen maleate as drug model and excised bovine cornea and the *in vitro* cornea as permeation barriers, it becomes apparent that although the permeation rates of the drug through the *in vitro* cornea are always higher, the rank order in which the drugs permeate through the two types of barriers is the same, and therefore drug permeation through the *in vitro* cornea may be a predictive tool to estimate the permeability coefficients of drugs through excised cornea.
Of particular interest however, would be an estimation of the permeation conditions *in vivo*, especially if organotypical cell culture models from human corneal cells could be established.

3. Experimental

3.1. Material

3.1.1. Biological materials

Eyes from freshly slaughtered cattle were obtained from a local slaughterhouse. The corneas were excised and used as source for the primary cell cultures.

3.1.2. Ophthalmic formulations

Several ophthalmic formulations with identical drug concentrations were chosen for permeation studies through excised bovine cornea and through the *in vitro* model of the cornea. These formulations varied in their contents of excipients. The compositions of the various formulations are listed in Table 7. In addition, self-prepared ophthalmic formulations were used such as an aqueous solution with 0,45% (w/w) hydroxypropyl methylcellulose as an additive as well as a reverse micellar solution (RMS), according to Tegtmeyer et al. (2001).

Table 7: Composition of formulations for permeation studies (Rote Liste 1999)

Formulation	Active ingredient	Additives
Borocarpin® S 2%	20 mg/g Pilocarpine hydrochloride	0.1 mg/g Benzalkonium chloride
Spersacarpin® 2%	20 mg/g Pilocarpine hydrochloride	0.1 mg/g Benzalkonium chloride 4.5 mg/g Hydroxypropyl methylcellulose
Vistacarpin® N 2%	20 mg/g Pilocarpine hydrochloride	0.04 mg/g Benzalkonium chloride 14.0 mg/g Polyvinylalcohol
Pilocarpol® 2%	20 mg/g Pilocarpine	0.002 mg/g Cetalkonium chloride
Timomann® 0.25%	3.42 mg/g Timolol hydrogen maleate	0.1 mg/g Benzalkonium chloride

3.2. Methods

3.2.1. Isolation of the cornea and establishment of primary cell cultures

Eye bulbs from freshly slaughtered cattle were used to obtain the corneas. After rinsing of the bulbs with water the attached tissue was removed with a surgical knife. Using a sterile scalpel the cornea was then excised along the limbus leaving a 1–2 mm scleral edge. The cornea was cleaned with tap water and stored for short periods in phosphate buffered saline (PBS), pH 7.4, containing 2% of a Gibco antibiotic and antimycotic mixture (Gibco, D-München).

For the isolation of endothelial cells the cornea was placed in a sterilised stainless steel cup of the same shape as the cornea with the endothelial cell layer side up. The endothelium was carefully wetted with Trypsin-EDTA solution (Gibco, D-München), avoiding contact of the solution with the epithelium or stroma. After incubation at 37 °C for 7–8 min in an atmosphere containing 5% CO_2, the endothelial cells could be removed from the Descemet's membrane with a silicone scraper.

In order to obtain corneal epithelial cells, these cells were allowed to grow out of an isolated piece of tissue. First the endothelial monolayer was removed from the cornea by scraping. Then circular pieces (6 mm diameter) were cut out of the cornea and placed in separate wells of a six-well plate. Enough medium was added so that the tissue fragments were covered with liquid. The epithelial cells started to grow after a lag period of 24 h. After 4 days of culture the tissue pieces were removed in order to avoid contamination with stromal cells.

The isolation method for the corneal stromal cells was similar to that used for epithelial cells. Not only the endothelium was removed mechanically, but the epithelium was also eliminated from the surface of the cornea by scraping. Cells were allowed to grow for one week before the tissue fragments were removed.

3.2.2. Subculture of corneal cells

Cells were subcultured after they had reached confluence. The medium was removed and the cells were washed twice with PBS. Cells were then incubated for 10 min in 0.25 mmol/l EDTA solution. After removal of the EDTA solution the cells were incubated for up to 15 min in a solution containing 0.1% trypsin and 0.02% EDTA (ICN, D-Eschwege), and carefully pipetted into new culture plates.

3.2.3. Cryoconservation of corneal cells

A stock of the three corneal cell types was established by freezing. A cell suspension of 10^6 cells in 1 ml freezing medium was prepared. The freezing medium consisted of DMEM supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma, D-Deisenhofen). Aliquots were transferred into cryo vials, placed into the gas phase above liquid nitrogen for two hours, and then lowered into liquid nitrogen for long term storage.

The cells were thawed, using warm, running tap water until all ice crystals were molten. Cells were then rapidly diluted with 24 ml culture medium in a culture plate. After a period of no longer than 24 h the medium was changed in order to remove traces of DMSO.

3.2.4. Growth curves

In order to plot growth curves, the cells were seeded in 24-well plates. For every cell count three wells were treated with a solution of 0.1% trypsin and 0.02% EDTA and the 24-well plate placed in an incubator for 15 min. The cell number was determined until the stationary phase of cell growth was reached.

To estimate the cumulative population doublings (CPD), the following formula was used:

$$CPD = (\ln N - \ln N_0)/\ln 2\,, \qquad (1)$$

where N = harvest cell count and N_0 = inoculum cell count. To calculate the rate of proliferation per day CPD(d), the following equation was used:

$$CPD(d) = CPD/t_2 - t_1\,, \qquad (2)$$

where t_2 = seeding day and t_1 = harvesting day.

3.2.5. Construction of the in vitro model of the cornea

For the bottom layer of the *in vitro* model corneal endothelial cells were seeded onto a Transwell membrane insert (Costar, D-Fernwald). Confluent endothelial cell cultures between the fourth and eighth passage were used. The cell number was adjusted to $3–4 \times 10^4$/ml, and 100 µl of cell suspension were added to each gel. Cells were cultured for 7 days.

For the next layer of the *in vitro* model a monolayer of stromal cells was required. These cells were used between the fourth and tenth passage. Cells were trypsinized, and the cell number was adjusted to 5×10^4/ml. For the preparation of six gels, a mixture of 1.83 ml 10 × MEM, 170 µl L-glutamine, 580 µl $NaHCO_3$ solution (71.2 mg/ml), 2.08 ml NBCS, 15.42 ml collagen solution with a collagen content of 1–2 mg/ml, and 1.67 ml of the described cell suspension were required. From this mixture, 3 ml were added to the bottom layer of the *in vitro* model described above. The gels were incubated for an additional week.

For the top layer corneal epithelial cells were seeded onto the gel. Confluent cells between passage 3 and 8 were used. The cell number was adjusted to $2–3 \times 10^5$/ml. 50 µl of this suspension were added to each contracted gel. Five ml medium, a mixture of three parts of glucose-free DMEM (ICN, D-Eschwege) and one part of HAM's F12 (25%), were added. The medium was supplemented with 20 µl/ml NBCS (Life Technologies, D-Eggenstein), 5 µg/ml insulin (Sigma, D-Deisenhofen), 20 pM triiodothyronine (Sigma), 5 µg/ml transferrin (Sigma), 24.3 µg/ml adenine (Sigma), 6.1 µg/ml ethanolamine (Biochrom, D-Berlin), 14.1 µg/ml phosphoethanolamine (Biochrom), 6.8 ng/ml selenious acid (Biochrom), 100 U/ml penicillin G sodium (Gibco, D-München), 100 µg/ml streptomycin sulfate (Gibco), and 0.25 µg/ml amphotericin B (Gibco).

The *in vitro* model was cultured submersed in medium for one week. The medium was changed three times per week. Then the medium level was lowered to lift the constructs to the air-liquid interface and the constructs were cultured for additional two weeks.

3.2.6. Histological sections

The *in vitro* model was fixed in 7% formaldehyde for 24 h and then placed into 2% agar solution (Merck, D-Darmstadt) for mechanical stabilisation. After the fixation, the constructs were dehydrated in acetone and embedded in molten paraffin. Sections suitable for light microscopy (4 µm) were cut with a microtome.

3.2.7. Histochemical staining

Before staining with haematoxylin-eosin, the histological sections were incubated in xylene for 10 min to remove the paraffin and washed in a series of ethanol-water solutions with descending ethanol concentrations for 5 min.

3.2.8. Immunocytochemical studies

For the immunocytochemical investigations of the monolayers, cells were cultured in Lab Tek® Chamber Slides (Nunc, D-Wiesbaden). All three corneal cell types were examined for the presence of vimentin and keratin after passage 2 and 10. The monoclonal antibody anti-vimentin clone V9 (Sigma) was used to detect vimentin in endothelial and stromal cells. Keratin in the corneal epithelial cells was labelled with a specific monoclonal antibody against keratin K3, anti-epithelial keratin AE5. As secondary antibody a peroxidase-conjugated anti-mouse IgG anti-body was used, which recognises the entire mouse IgG molecule as antigen (Sigma). The primary antibodies were diluted 1 : 100 in a dispersion of 5% milk powder (Heirler, D-Radolfzell) and 1% triton X-100 (ICN, D-Eschwege) in PBS. Conjugated peroxidase catalysed the reaction of diaminobenzidine to a coloured brown product.

3.2.9. Immunohistological studies

Immunohistological investigations were carried out on cryosections of the cultures and of the excised bovine cornea. Keratin K3, a differentiation marker specific for corneal epithelial cells, was used. The keratin K3 was labelled with the monoclonal primary antibody anti-epithelial keratin AE 5 (ICN, D-Eschwege). As secondary antibody a peroxidase-conjugated anti-mouse IgG was used as described above. The sections were analysed by light-microscopy.

3.2.10. Microscopy

A photo microscope type III (Zeiss, D-Oberkochen) was used for light microscopical investigations of the stained histological preparations and the stained monolayer cultures.

3.2.11. Permeation studies

Permeation studies with pilocarpine hydrochloride and timolol hydrogen maleate as ophthalmic model drugs from various ophthalmic formulations were performed with a modified Franz cell (Franz 1975) at 32 °C. In this experimental set-up the *in vitro* model of the cornea was left on the polycarbonate filters, on which they had grown. The acceptor media were PBS without calcium and magnesium and phosphate buffer pH 7.0 (DAB 1996) for timolol hydrogen maleate and pilocarpine hydrochloride respectively. The samples were taken hourly over a period of six to eight hours. The sample volume (250 µl) was immediately replaced with fresh buffer solution. The drug concentrations in the samples were determined by HPLC. The permeation parameters of model drugs were calculated by plotting the amounts (µg/cm^2) of drug permeated through the excised cornea or cornea construct versus the time (min). The permeation coefficient P was calculated as flux/drug concentration from the linear ascent of a permeation curve.

3.2.12. HPLC analysis

The quantitative determination of the permeated drugs was carried out by HPLC.

Pilocarpine hydrochloride: The HPLC system consisted of an isocratic pump (Spectroflow 400) and an UV detector (Spectroflow 757, Kratos, D-Weiterstadt). The peak identification and analysis was performed using a Beckman System Gold Chromatography Software Version 6.01 (Beckman, D-Munich). A reversed phase column (125.4 mm) filled with Gromsil ODS 5 µm (Grom, D-Herrenberg) was used for the chromatographic separation. The mobile phase consisted of acetonitrile (23%) and buffer (6.8 g/l KH2PO4, 1.7 g/l KOH, pH 7.0) (77%). The calibration was carried out over a concentration range of 0.2–25 µg/ml. The correlation coefficient was 0.999.

Timolol hydrogen maleate: The HPLC system consisted of an isocratic Waters 515 HPLC pump and a Waters 486 Tunable Absorbance UV detector (Waters, D-Eschborn). Samples were injected with an automatic Waters 717 plus autosampler. Millenium 32 Chromatography Manager software (Waters) was used for peak identification and analysis. For separation of the substances a Symmetry Shield Reversed Phase (RP 8) column (150, 3.9 mm, Waters) was used. The mobile phase consisted of acetonitrile (12.5%) and a solution of 0.1% triethylamine in water (87.5%). The pH of the water phase was adjusted to 2.9 with glacial acetic acid. The calibration was carried out within the range of 100 ng to 20 µg. The correlation coefficient was 0.999.

References

Alberts B (1995) Molekularbiologie der Zelle, ed 3, Weinheim, VCH Verlagsgesellschaft.

Bell E, Ivarsson B, Merril C (1979) Production of tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc Natl Acad Sci USA 76: 1274–1278.

Chan KY, Haschke RH (1982) Isolation and culture of corneal cells and their interactions with dissociated trigeminal neurons. Exp Eye Res 35: 137–156.

DAB 1997 Deutsches Arzneibuch (1996) Frankfurt am Main, Deutscher Apotheker Verlag, Stuttgart & Govi Verlag GmbH.

Engelmann KJ, Böhnke M, Friedl P (1988) Isolation and long-term cultivation of human corneal endothelial cells. Invest Ophthalmol Vis Sci 29: 1656–1662.

Franz TJ (1975) Percutaneous absorption. On the relevance of in vitro data. J Invest Derm 64: 190–195.

Hayashi K, Sueishi K, Tanaka K, Inomata H (1986) Immunohistochemical evidence of the origin of human corneal endothelial cells and keratocytes. Graefes Arch Clin Exp Ophthalmol 224: 452–456.

Hyldahl L (1984) Primary cell cultures from human embryonic corneas. Cell Sci 66: 343–351.

Kurpakus MA, Stock EL, Jones JCR (1992) The role of the basement membrane in differential expression of keratin proteins in epithelial cells. Developm Biol 150: 243–255.

Morgan SJ, Darling DC (1994) Kultur tierischer Zellen, Heidelberg, Spektrum, Akademischer Verlag.

Schneider AI, Maier-Reif K, Graeve T (1997) The use of an in vitro cornea for predicting ocular toxicity. In vitro Toxicology 10: 309–318.

Specht C, Stoye I, Müller-Goymann CC (1998) Comparative investigations to evaluate the use of organotypic cultures of transformed and native dermal and epidermal cells for permeation studies. Eur J Pharm Biopharm 46: 273–278.

Stocker FW, Eiring A, Georgiade R, Georgiade N (1958) A tissue culture technique for growing corneal epithelial, stromal and endothelial tissues separately. Am J Ophthalmol 46: 294–298.

Sun TT, Green H (1978) Immunofluorescent staining of keratin fibers in cultured cells. Cell 14: 469–476.

Sundar-Raj CV, Freeman IL, Brown SI (1980) Selective growth of rabbit corneal epithelial cells in culture and basement membrane collagen synthesis. Invest Ophthalmol Vis Sci 10: 1222–1230.

Tegtmeyer S, Papantoniou I, Müller-Goymann CC (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: 119–225.