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Excised Porcine Cornea Integrity Evaluation in an in vitro Model of Iontophoretic Ocular Research

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Key Words
Ocular diffusion cell · Excised porcine cornea · Iontophoresis · Corneal integrity · Electron transmission microscopy

Abstract
Background/Aims: It is a challenge to adapt traditional in vitro diffusion experiments to ocular tissue. Thus, the aim of this work was to present experimental evidence on the integrity of the porcine cornea, barrier function and maintenance of electrical properties for 6 h of experiment when the tissue is mounted on an inexpensive and easy-to-use in vitro model for ocular iontophoresis. ***Methods:*** A modified Franz diffusion cell containing two ports for the insertion of the electrodes and a receiving compartment that does not need gassing with carbogen was used in the studies. Corneal electron transmission microscopy images were obtained, and diffusion experiments with fluorescent markers were performed to examine the integrity of the barrier function. The preservation of the negatively charged corneal epithelium was verified by the determination of the electro-osmotic flow of a hydrophilic and non-ionized molecule. ***Results:*** The diffusion cell was able to maintain the temperature, homogenization, porcine epithelial corneal structure integrity, barrier function and electrical characteristics throughout the 6 h of permeation experiment, without requiring CO_2 gassing when the receiving chamber was filled with 25 mM of HEPES buffer solution. ***Conclusion:*** The system described here is inexpensive, easy to handle and reliable as an in vitro model for iontophoretic ocular delivery studies.

Introduction

Iontophoresis is a non-invasive physical method that involves the application of a low electrical potential gradient across tissues to enhance molecular transport. In general, the drug is applied with an electrode carrying the same charge as the drug, and the ground electrode, which is of the opposite charge, is placed elsewhere on the body to complete the circuit [1]. The ease of application, the minimization of systemic side effects, and the increased drug penetration directly into the target region resulted in the extensive clinical use of iontophoresis, mainly in the transdermal field [2–4].

However, experimental conditions, such as formulations of pH values, total electric current, drug concentration and competitive ions, among others, strongly interfere with the efficiency of iontophoresis. For that reason, in vitro experiments are undoubtedly an important step

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during drug delivery research for screening of formulations and experimental conditions before in vivo tests are performed.

Despite this need, in vitro ocular iontophoresis is very poorly evaluated compared to the work that has been done in the transdermal iontophoretic field. Ocular drug absorption is usually studied in vivo using rabbits as the animal model [5, 6]. Due to the low volume of aqueous humour in rabbits (less than 200 μl) and analytical method limitations, at least 3 animals must be killed per time point to generate a drug concentration profile [7, 8]. Furthermore, the general lack of a strong theoretical foundation for the practice of ocular iontophoresis has led to many useless animal experiments with no beneficial results due to drug or current unsuitability [1]. Consequently, the choice of predictive in vitro penetration models is highly important.

Eye diseases are mostly treated by topical administration of ophthalmic drugs, normally to the cul de sac of the eye. The applied drug can penetrate into the eye via the corneal and conjunctival-scleral pathways. The latter is highly efficient for intra-ocular drug delivery; however, only a few high-molecular-weight substances are delivered by this route [9]. Most substances penetrate the eye via the transcorneal route [10], which represents the direct pathway into the eye [9]. In this way, corneal-penetration experiments play an essential role in the selection of drugs, formulations and conditions for the application of physical methods for the treatment of eye diseases.

Because of the poor availability of human donor corneas for experimental purposes, in vitro studies are largely performed using excised corneal tissues from laboratory animals [11]. However, the sacrifice of animals for in vitro experimental purposes has been deemed inappropriate. Because of this, several corneal cell culture models have been established [12–14]. Nevertheless, these models, at least up to now, have been expensive, time-consuming to develop and, most importantly, have not been fully proven to be equivalent in barrier properties to excised corneas. In several recent studies, corneal epithelium models and 3-dimensional cornea equivalents were evaluated for their usefulness as in vitro models for permeation studies. The corneal models investigated showed clear differences in epithelial barrier function when compared to excised bovine cornea. Only one in vitro model that was developed seems to be appropriate to replace excised animal corneas for assessing corneal permeability, but it is not yet commercially available [9].

As long as reliable alternative culture models for permeation experiments remain unavailable, a simple alternative is the use of excised animal corneas obtained in abattoir houses. There are no ethical concerns in their use since the animal would already be sacrificed for provisioning. The disadvantages of these membranes are the decreasing viability of the isolated cornea and the species differences compared to human tissue. Nevertheless, the porcine cornea has been used in permeation experiments because of its similar permeability properties compared to the human cornea [15].

Therefore, the aim of this work was to present experimental evidence on the porcine cornea integrity, barrier and electrical properties maintenance throughout 6 h of permeation using a modified Franz diffusion cell type and to present this modified system as a reliable, inexpensive and easy-to-handle in vitro model for future ocular passive and iontophoretic research.

Materials and Methods

Chemicals

Fluorescein, fluorescein diacetate, Ag wire (99.99%, diameter = 1.5 mm), AgCl (99.99%) and Pt wire were all purchased from Sigma Aldrich (Steinheim, Germany). Fluconazole (FLU) was purchased from Galena (Campinas, Brazil), and N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) was purchased from J.T. Baker (Phillipsburg, N.J., USA). All other reagents were BDH or HPLC reagents. Deionized water (Milli-Q Millipore Simplicity 185, Bedford, Mass., USA) was used to prepare all solutions.

Cornea

Corneas used in the experiments were obtained from pig eyes that were collected immediately after the slaughter of the animals (Frigorífico Pontal Ltda, Pontal, Brazil). The eyes had not been heat treated in the abattoir in any way. They were then kept at 4°C while transported to the laboratory and used within 1 h of enucleation. Any eye with a collapsed anterior chamber was discarded. Corneoscleral buttons were dissected using standard eye bank techniques, and care was taken to minimize tissue distortion [16]. Immediately after corneal preparation, the tissue was mounted in the diffusion cell and kept in contact with the receiving compartment (HEPES) at 35°C.

Apparatus and Experimental Procedure

The vertical diffusion cells that were developed consisted of a receiving and a donor chamber fixed together with a clamp (fig. 1). The receiving chamber contained two openings: one for the placement of the cornea and another for manual sampling of the receiving solution. In order to fit the concave cornea appropriately in the cell, the opening intended for the cornea placement had its edges raised by 2 mm. The area of the cornea exposed to drug diffusion was 0.6 cm^2, and the receiving compartment volume was 35 ml. Magnetic stirrers (15 mm) were put at the bottom of the receiving compartment to adequately homogenize the contents of these chambers at 600 rpm. The cells were placed in an outer bath-

ing compartment for temperature maintenance at 35°C. The receiving medium consisted of 25 mM HEPES buffer containing 133 mM NaCl, pH 7.4.

Validation of the Mechanical Elements of the Diffusion Cell

Volume of the Receptor Chamber for Each Cell

All volumes were measured by gravimetric methods. The cells with empty receptor chamber were weighed and then filled with Milli-Q water. The difference in the weight after the water addition (milligrams) was then assumed to be the volume of each cell (millilitres), assuming that the water density was 1 g/ml [17]. All the determinations were made in triplicate for each cell.

Diffusional Area

The diffusional area of each cell was calculated by measuring the diameter of the main hole in the receptor chamber.

Temperature Control in the Different Chambers

Ten cells were mounted with a laboratory film (Parafim™) between the donor and the receiving compartments. The receiving chambers were filled with HEPES buffer solution (pH 7.4 ± 0.1) containing 133 mM NaCl, and 1 ml of this solution was added to the donor chambers. The cells were placed into an outer bath filled with distilled water maintained at 35°C with a thermostat. The temperature of each cell chamber was measured after 10, 15, 20 and 30 min using a calibrated thermometer.

Stirring Efficiency

Magnetic stir bars (15 mm) were placed in the interior of the receiving chamber and maintained at 600 rpm. A solution of methylene blue (25 μl) was added to the receiving compartment. The time needed for complete homogeneous distribution of the dye was measured by visual observation.

Integrity of the Cornea

Electron Transmission Microscopy

Porcine corneas were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C at the following times: (a) immediately after the slaughter of the animal, (b) after transportation from the abattoir to the laboratory (30 min), (c) after montage of all corneas in the perfusion chambers, (d) after 2 h of the perfusion experiment, (e) after 4 h of the perfusion experiment and (f) after 6 h of perfusion experiment. They remained in the glutaraldehyde solution for 24 h. After this period the tissues were maintained in 0.1 M sodium cacodylate buffer (pH 7.4) until the routine process for transmission electron microscopy, when corneas were divided into quarters and refixed in 1% OsO_4 and 0.1 M phosphate buffer at 4°C for 2–3 h. The samples were then washed and dehydrated in a graded series of acetone solutions and embedded in a mixture of araldite and pure acetone (1:1) for 24 h at room temperature. They were then sectioned, stained with uranyl acetate and lead citrate, and examined on a transmission electron microscope (Phillips EM208, Eindhoven, the Netherlands) with final images from ×3,200 to ×30,000 or higher for the ultrastructural analysis of the corneal epithelium.

Assessment of the Corneal Penetration Barrier

The cornea was placed between the donor and the receiving compartments of the diffusion cell. The donor compartment was filled with 1 ml of a solution containing 10 μM fluorescein. Samples (1 ml) were withdrawn from the receiving solution (HEPES buffer solution, pH 7.4) each hour throughout the 6 h of the experiment and replaced with fresh receiving fluid. The fluorescence intensities of the receiving solution samples were monitored at 490/512 nm (excitation/emission) using a Hitachi F4500 spectrofluorometer (Hitachi, Tokyo, Japan).

The positive control consisted of 1 ml of a solution containing 10 μM of fluorescein diacetate added to the donor compartment. Experiments without fluorescein in the donor solution were also performed as negative controls. All the experiments were performed in quadruplicates.

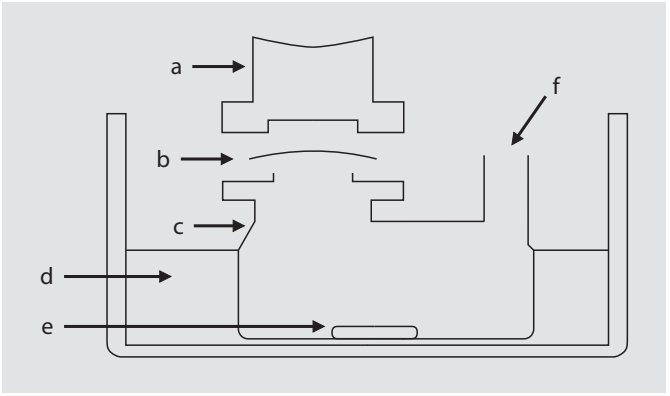

Fig. 1. Diagrammatic representation of the diffusion cell design. Cell components as follows: a = donor compartment, b = membrane, c = receptor compartment, d = water bath, e = magnetic stir bar and f = sampling port.

In vitro Ocular Iontophoresis

Experiments were performed in vitro using the aforementioned diffusion cell, and Ag/AgCl electrodes were prepared as previously described [18]. The cornea was placed between the donor and the receiving compartments of the diffusion cell. The area of cornea that was exposed was 0.64 cm^2. The donor compartment was filled with 1 ml of a saline solution containing FLU (0.2 mg/ml), used as a model drug. The positive electrode was placed in contact with the drug solution in the donor compartment, and the ground electrode was placed in contact with the receiving solution (HEPES, pH 7.4, 133 mM NaCl) to complete the circuit. A constant electrical current of 0.5 mA/cm^2 generated by a Kepco APH 500DM apparatus (Kepco Power Supply, USA) was applied. The voltage of the complete circuit and of each cell was measured hourly with a voltmeter (Freak, MY-63). Samples of 1 ml were withdrawn from the receiving solution each hour for 6 h and replaced by fresh receiving fluid. The amount of FLU that permeated across the cornea, i.e. the amount of the drug in the receiving solution, was analysed by HPLC. C_{18} reversed-phase columns of 125 × 4 mm (5 μm) were used, with water:acetonitrile:methanol (80:15:5 vol/vol) as the mobile phase at a flow rate of 1.0 ml/min, detection at 210 nm and an injection volume of 50 μl.

'Passive' experiments were also performed with donor formulations containing 0.2 mg/ml FLU. In these experiments, all conditions were identical to those described above except that no current was applied.

Results

Validation of the Mechanical Elements of the Diffusion Cell

The volumes of the receiving compartment (34.63 ± 0.86 ml) and the diffusion area (0.64 ± 0.03 cm^2) of the cells were found to be homogeneous in the 10 units analysed.

The time necessary for the receiving chamber solution at the initial temperature of 27°C to equilibrate with the outer bathing solution temperature of 35°C was 15 min. Only a difference of 1°C was seen between the donor and receiving solutions.

It was visually observed that when a dye was added to the receiving solution under stirring with a magnetic bar of 15 mm at 600 rpm, it was almost instantaneously homogeneously distributed in a volume of 35 ml, indicating that stirring at 600 rpm is adequate.

Integrity of the Cornea

Electron Transmission Microscopy

The corneal epithelium structure was analysed by electron transmission microscopy immediately after the animal had been sacrificed (fig. 2), after the transportation period, and during the 6 h of the diffusion experiments (fig. 3).

The images showed no differences between the control group at time point zero and the corneas fixed at the end of the 6-hour diffusion experiment. The corneal epithelium was well preserved, consisting of a basal layer of columnar cells, approximately 3 layers of wing cells, and 1 or 2 outermost layers of squamous, polygonally shaped superficial cells. As expected, intercellular tight junctions (zonula occludens) completely surrounded the most superficial cells, but the intercellular spaces were wider between basal cells. The superficial cells were normal and well integrated after 6 h of experiment. Microvilli observed at the surface of those cells were also preserved. Wing cells showed sinusoidal limits, which securely contributes to cell adhesion. They also presented abundant desmosomes between cells. Basal cells were normal, with a pleated basal surface abutting the stroma. The basement membrane remained intact with abundant hemidesmosomes.

Assessment of the Corneal Penetration Barrier

After 6 h of permeation experiments containing fluorescein as a donor solution, the fluorescence values detected in the receiving compartment in 3 h of experiment were similar to those found for the negative control experiment (without fluorescein in the donor solution). After 3 h they were a little bigger, but not statistically different ($p > 0.05$), than those found in the control.

The transcorneal permeation profile of fluorescein diacetate, used as a positive control, is shown in figure 4.

In vitro Ocular Iontophoresis

The iontophoretic permeation profile of FLU in aqueous solution, compared to its passive permeation profile, is presented in figure 5. Iontophoresis significantly ($p < 0.05$) improved the drug permeation when compared to the passive drug delivery. A linear relationship ($r > 0.99$) was obtained when the amount of FLU in the receptor phase was plotted against time. The steady-state flux obtained was 22.67 and 47.55 $\mu g/cm^2/h$ for passive and iontophoretic transport, respectively.

Discussion

Most of the work published that investigates the iontophoresis potential on ocular delivery is done only in vivo using animal models [19, 20]. In many cases the high number of animals employed, i.e. 50 [21] to 60 [22] rabbits per study, could be severely diminished using in vitro screening before the in vivo studies. The use of the Franz-type diffusion cells to perform in vitro experiments was quickly established as a common method, but there are some challenges in adapting these diffusion cells to corneal tissue because of the natural curvature and small area of this membrane [17]. The difficulties on adapting these systems especially increase for iontophoretic experiments, in which two electrodes need to be inserted in different compartments. Mostly described iontophoretic diffusion cell models are vertical, comprised of three chambers in the donor compartment for introducing the two electrodes separated by a spacer, both over the membrane [23]. This model, largely used in transdermal iontophoretic research [2, 4, 24, 25], comprises a large diffusion area (approximately 1.5 cm^2) that is not suitable for the placement of the cornea, which is only about 1.0 cm^2.

The horizontal diffusion cell model could also be used for iontophoretic studies through the cornea. In this model the membrane is placed vertically between the donor and receiving compartments which have two ports for introducing the iontophoretic electrodes on each side of the cornea [26]. Nonetheless, it is more difficult to place semisolid formulations, as a gel, in contact with the membrane in horizontal cells than in the vertical ones.

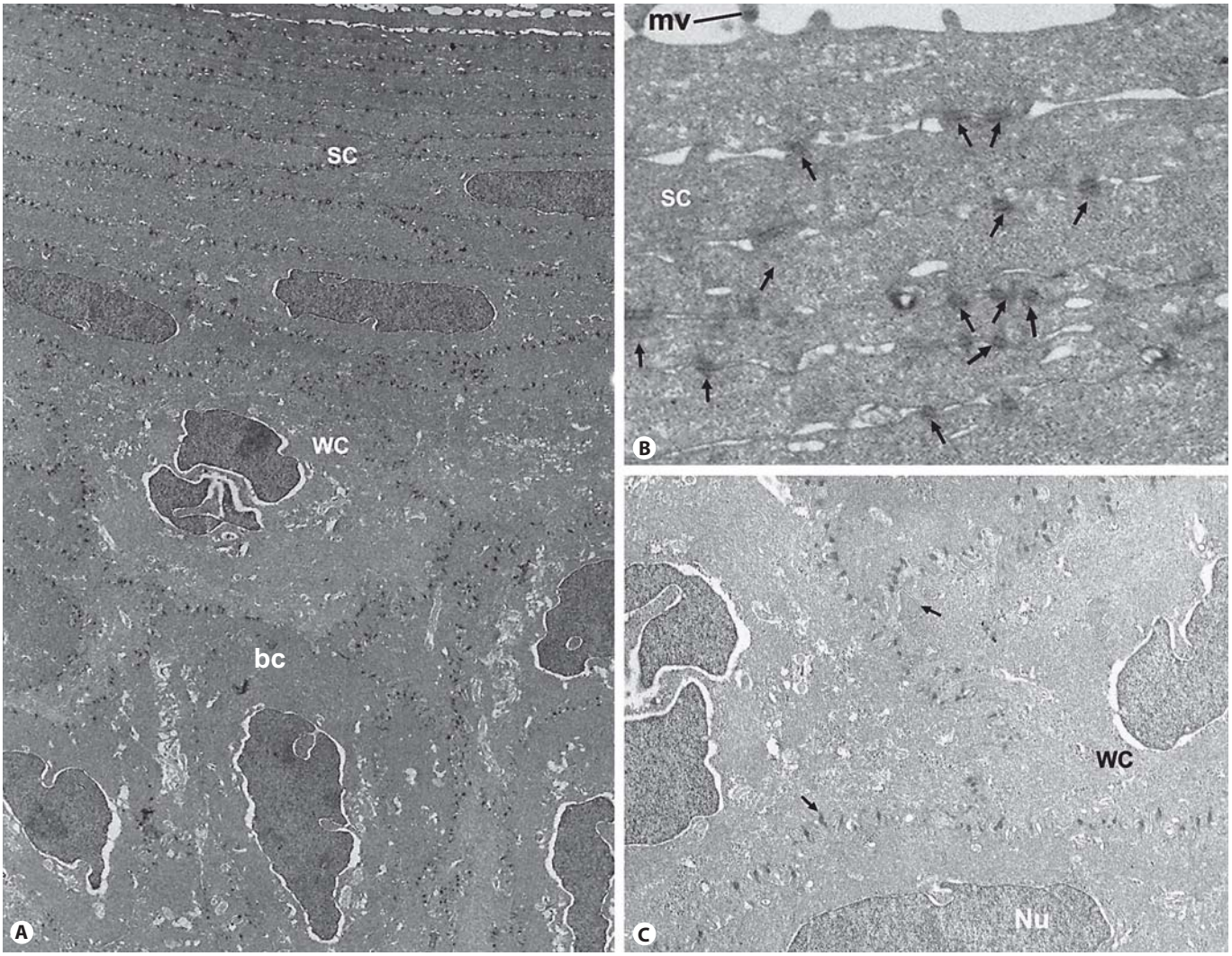

Fig. 2. Transmission electron micrographs of the control corneal epithelium (time zero), uranyl acetate and lead citrate contrasting. **A** A typical corneal epithelium is observed, the corneal epithelium was well preserved, consisting of a basal layer of columnar cells (bc), 2–3 layers of wing cells (wc) and outermost layers of squamous, polygonally shaped superficial cells (sc). As expected, intercellular tight junctions completely surrounded the superficial cells. Original magnification ×2,000. **B** Higher magnification of superficial cells (sc) and the desmosomes (arrows) between cells. A high density of microvilli (mv) is observed on the surface of the superficial cells. Original magnification ×8,000. **C** Wing cells (wc) showed sinusoidal limits with abundant desmosomes between cells (arrows). Nu = Nucleus. Original magnification ×8,000.

Also, to completely embed the electrodes a higher volume of donor solution should be used as, in some cases, when the drug is expensive or the donor solution is comprised of materials derived from synthesis or any material not available in high quantity, this need of higher volumes represents a drawback.

The modified Franz diffusion cell proposed in this work (fig. 1) used the concept of the horizontal model by placing one electrode in contact with the formulation and the ground electrode in the receiving compartment. However, it is still a vertical diffusion cell in which the membrane is placed horizontally between the donor and the receiving compartments. Also, the sampling port of these cells is large enough to make it possible to introduce the ground electrode into the receiving compartment without any difficulty, eliminating the necessity of using special micro-electrodes. It also possesses the capability of applying a small volume of donor solution. So it could

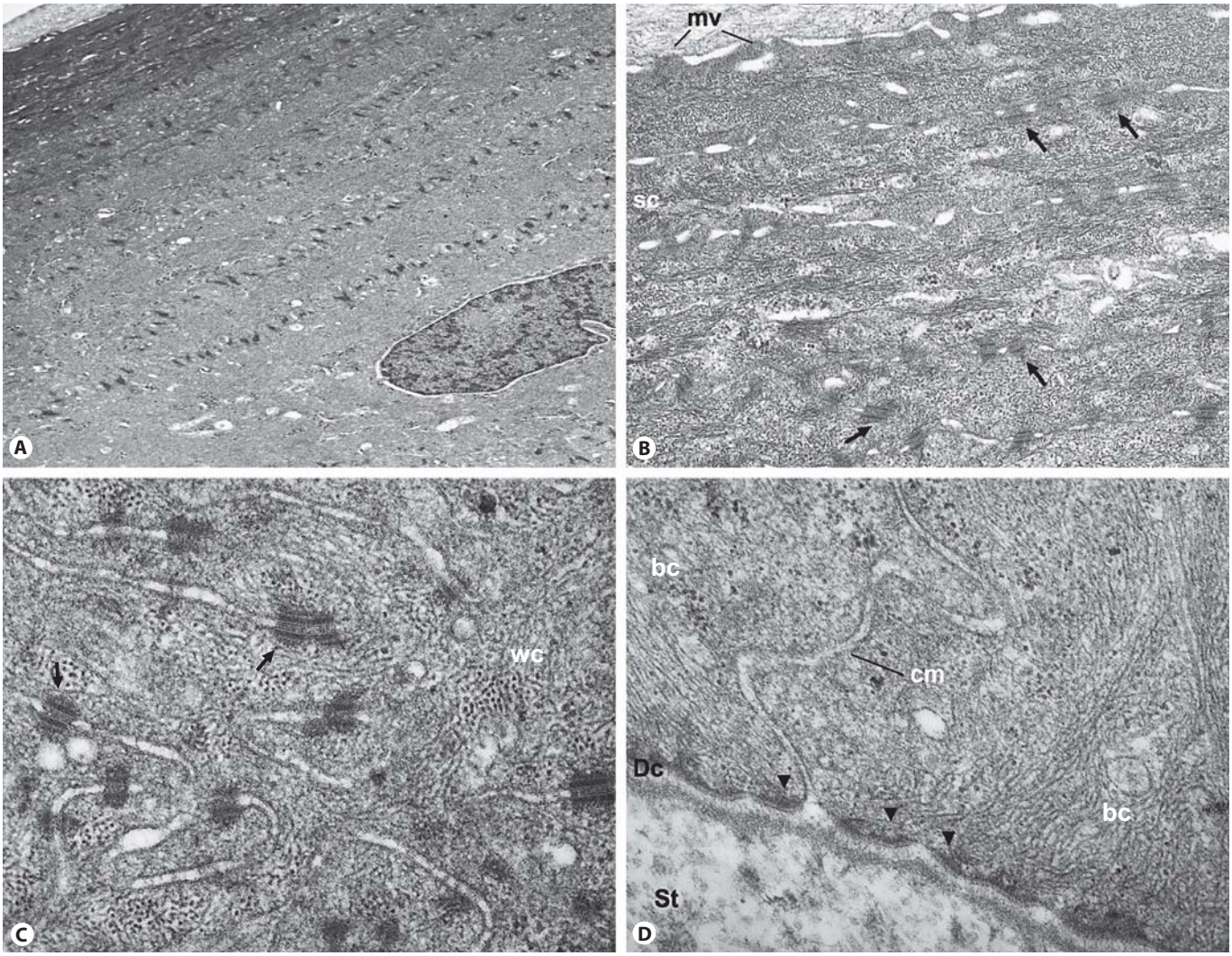

Fig. 3. Transmission electron micrographs of the corneal epithelium after 6 h of the permeation experiment, uranyl acetate and lead citrate contrasting. **A** The corneal epithelium remains well preserved with abundant intercellular tight junctions. Original magnification ×8,000. **B** Higher magnification of superficial cells (sc) and the desmosomes (arrows) between cells. Microvilli (mv) are observed on the surface of the superficial cells. They remain intact. Original magnification ×25,000. **C** Wing cells (wc) maintained their normal appearance with abundant desmosomes between cells (arrows). Original magnification ×40,000. **D** View of two basal cells (bc) with a pleated basal surface abutting the stroma (St) and Descemet membrane (Dc). The basement membrane showed well-preserved hemidesmosomes (arrowheads). cm = Cellular membrane. Original magnification ×32,000.

be easily adapted for all kinds of formulations and electrodes available in most laboratories. This modified diffusion cell was shown to be robust, inexpensive and easy to handle. No leakage from the donor or receiving compartment was observed. It is worth to mention the high volume of the receiving compartment. In general, in vitro permeation experimental conditions are chosen such that sink conditions are maintained [27]. This means that the kinetics of transfer is followed until a maximum of 10% of the total drug dose has accumulated in the receiving chamber. In this way, the larger volume of the diffusion cells developed (35 ml) in comparison to the smaller (6 ml) of the Franz diffusion cell [26] is very useful, especially in studies using peptides or lipophilic compounds.

It is also important to consider the integrity of the cornea that is intended to be used as the membrane for in vitro diffusion experiments that will be conducted with

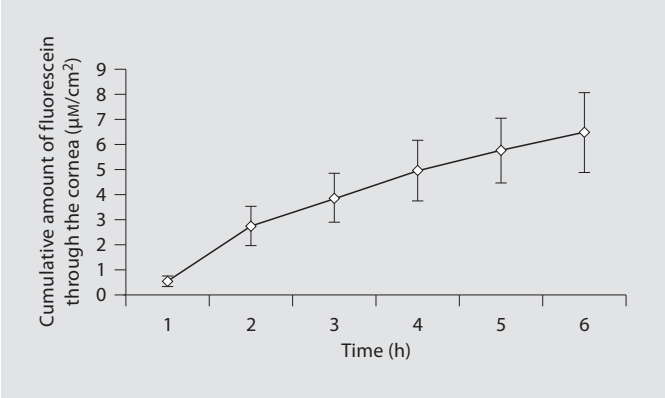

Fig. 4. Cumulative amount of fluorescein measured in the receiving compartment during 6 h of in vitro permeation across porcine cornea from a fluorescein diacetate solution at 10 μM. Data shown are the mean ± SD of 4 replicates.

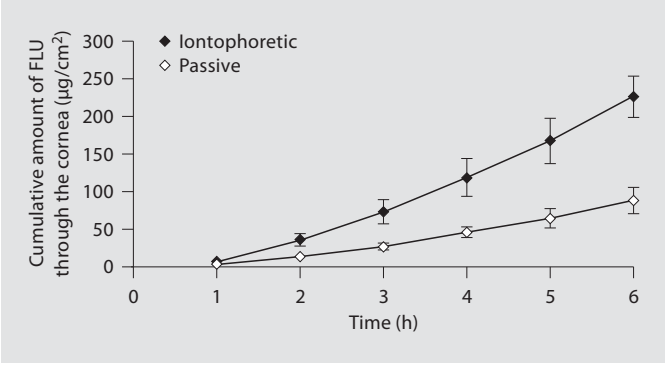

Fig. 5. Permeation profile of FLU through porcine cornea from an aqueous solution (0.2 mg/ml) during 6 h of in vitro passive (no current applied) and iontophoretic (the density of the current applied was 0.5 mA/cm^2) experiments. Data shown are the mean ± SD of 6 replicates.

the developed diffusion cells. This is because, unlike the skin that has the stratum corneum, a dead tissue, as the main layer responsible for its barrier property, the cornea has a live tissue, the most superficial epithelial layers, as mainly responsible for its barrier properties. Drugs penetrate the corneal epithelium via the transcellular or paracellular route. The transcellular route of permeation is limited by the 3 main layers of which the cornea is composed: epithelium, stroma and endothelium, which are lipophilic, hydrophilic and lipophilic, respectively. Conversely, the paracellular pathway is limited by the corneal outermost layer, the epithelium, mainly because of the closely packed arrangement of these cells [28]. Several studies indicate that after epithelial debridement the permeation of molecules is highly increased [29, 30]. In this way, the integrity of the cornea and the maintenance of the epithelial barrier function have to be preserved to adequately interpret the results obtained from the in vitro experiments.

To maintain the corneas, most of the receiving compartments in in vitro diffusion experiments are gassed with carbogen (95:5 vol:vol of air:CO_2), implying the need for either a shaking water bath with regular manual gassing or a rotating air/CO_2 incubator [31, 32]. The isotonic HEPES-based bicarbonate-free buffer that was used in the present study as the receiving solution does not require carbogen gassing, but instead can be exposed to atmospheric levels of about 300 ppm CO_2 in a water bath at 35°C [33, 34], which again facilitates the handling of the perfusion experiments.

The use of HEPES buffer is advantageous for the iontophoretic transport of drugs as well. Buffers and electrolytes used to maintain pH and electrochemical milieu in the donor and receiving solutions furnish ions that are more mobile than the ionized drug molecule and hence compete with the drug for carrying the charge, thereby reducing the efficiency of the drug to be transported iontophoretically [35]. The HEPES buffer, however, has large ions with low mobility through biological membranes (in comparison to other buffer ions, such as phosphate) and consequently does not compete with the drug for the electrical current. For this reason, HEPES has been largely employed as the receiving medium in iontophoretic experiments [2, 25, 35].

To verify the corneal integrity during in vitro diffusion experiments, the corneal epithelium structure was analysed by electron transmission microscopy immediately after the animal had been sacrificed (fig. 2), after the transportation period and during the 6 h of the diffusion experiments. The results showed that the experimental set-up was able to maintain the principal tissue structures, once there were no differences observed between the control (fig. 2) and the cornea after 6 h (fig. 3) of diffusion experiments. In addition to the images from these experiments, the corneal barrier function was evaluated by the determination of the in vitro permeation of sodium fluorescein as a function of time as described below.

Fluorescein has been largely applied to humans for the verification of corneal epithelium integrity due to its high lipophilicity (water solubility = 1.77 μg/ml) and high log p (3.35), which lead to a very low corneal pene-

tration rate [16, 31]. Thiel et al. [16], who also applied this method to verify the corneal integrity in a perfusion chamber developed by their group, found that around 5.0 μg/ml of fluorescein permeate the cornea after 6 h of experiment. These authors compared the amount of fluorescein permeated through normal cornea and cornea without the epithelium (positive control), and concluded that this low permeability of fluorescein in normal conditions indicated that the epithelial barrier was well preserved. Therefore, as the amount of fluorescein found in our experiments after 6 h (0.018 μg/ml; fig. 4) was much smaller than that found by their group, we presumed that the corneal barrier function was preserved in our model. Nevertheless, as the corneal epithelium is known to be metabolically active, composed mainly of esterases, peptidases and proteases, esterase function can be expected [36]. In this way, the transcorneal permeation of the fluorescein diacetate, an essentially non-fluorescent molecule, was also analysed as a positive control. In other words, if preserved and metabolically active, corneal epithelium should have esterases capable of hydrolysing the fluorescein diacetate molecules that penetrate the cornea to the fluorescent fluorescein [31]. As expected, when fluorescein diacetate was placed in the donor compartment, a high dose of fluorescence was observed in the receiving solution (fig. 4) in the first hours, and not only after the third hour, as it was observed with fluorescein. Toropainen et al. [31] also used this method to verify the viability of a cell culture epithelial model compared to rabbit cornea. They found that fluorescein diacetate permeation was 49 and 31 times larger for the cell culture epithelial model and the rabbit cornea, respectively, than the fluorescein permeation. In our experiments it was verified that the permeation of the prodrug was 86 times greater than the fluorescein one. This higher increase observed in our model can be related to esterase activity differences between porcine and rabbit corneas, but also to the total period of time of permeation studies. In the study mentioned above for the cell culture model, the permeation was performed only for 2.5 h and for the rabbit's cornea model for 4 h. In our experiments it was conducted for 6 h. Even so, based only on these experiments, it is not possible to assure cell viability, since these results only indicate that the esterase activity remained in the isolated cornea at least for 6 h. As in vitro iontophoretic experiments are normally performed for this period of time [2, 4, 24, 25], HEPES buffer as a receiving solution can be used to maintain the corneal barrier function integrity and esterase activity in this kind of experiments.

Also important for the in vitro iontophoretic experiments is the membrane charge. The corneal epithelium is negatively charged above its iso-electric point (pI 3.2) [10]. Consequently, when an electrical current is applied, cationic compounds have a prevalent ratio of transport over anionic ones. Therefore, if the negatively charged characteristic of the cornea is maintained in the proposed in vitro model when an electric current is applied, a flux of counter ions from the membrane to the ground electrode is expected and, consequently, a solvent flow in the anode-to-cathode direction occurs. This phenomenon is called electro-osmosis, and it is the main responsible phenomenon for the delivery of neutral molecules by iontophoresis [37]. To verify the existence of the electro-osmosis and, consequently, guarantee that the negative charges were still present in the corneal surface after the dissection process, FLU (MW 306.27 g/mol) was used as a model drug in the iontophoretic experiments. FLU is a hydrophilic, non-ionized molecule at physiological pH; it cannot undergo electrorepulsion when delivered by iontophoresis [38]. It is an antifungal compound largely used in the treatment of fungal keratitis [39]. It is also a stable compound with easy quantification.

Figure 5 shows that iontophoresis of the solution containing FLU improved around 2 times this drug corneal permeation when compared to the passive drug delivery, indicating that the drug has benefited from the electro-osmotic flow. This fact supports the concept that the electric characteristics of the excised cornea are maintained in the proposed in vitro model. Moreover, the linear relationship obtained when the amount of FLU in the receptor phase was plotted against time indicates that both the drug's passive and iontophoretic transport can be described by zero order kinetics. The fact that the drug achieved a steady-state flux when electric current was applied is another indication that the barrier function of the cornea is constantly maintained under the experimental conditions for 6 h.

Conclusion

This work presented a simple glass Franz diffusion cell type modification for the use in passive and iontophoretic in vitro ocular drug delivery investigations. The results are experimental evidence that the diffusion cell was able to maintain the temperature, homogenization, integrity of porcine epithelial corneal structures, barrier function and electrical characteristics throughout the 6 h of permeation experiment, without requiring CO_2

gassing when the receiving chamber was filled with 25 mM of HEPES buffer solution. In this way this system represents a reliable, easy and inexpensive alternative to perform predictive in vitro passive and iontophoretic ocular research.

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