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Corneal Penetration of Polyhexamethylene Biguanide and Chlorhexidine Digluconate

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Abstract

Objective: Cationic antiseptics, such as polyhexamethylene biguanide (PHMB) and chlorhexidine digluconate (CHG), are widely used for the topical treatment of *Acanthamoeba keratitis*. The aim of this study was to investigate the corneal penetration of PHMB and CHG topically administered as eye drops and to study the effect of PHMB and CHG on the epithelial barrier function.

Methods: The penetration was evaluated in vitro in rabbit corneas clamped in artificial perfusion chambers. Two different preparations of PHMB 0.02% (Cosmocil and Lavasept) and CHG 0.02% eye drops were administered twice each hour for up to 8 hours to the rabbit corneas with and without epithelium. The amount of drug penetrating into the anterior chamber was measured using capillary electrophoresis with contactless conductivity detection. The integrity of the epithelial barrier function was evaluated by adding fluorescein to the PHMB or CHG eye drops. The fluorescence of the anterior chamber perfusate was measured continuously throughout the experiment. Corneas treated with fluorescein alone (in either NaCl 0.9% or benzalkoniumchloride (BAC) eye drops) served as controls.

Results: Neither PHMB nor CHG were detectable at any time in the anterior chamber perfusate of either the corneas with or without epithelium. PHMB and CHG treatment resulted in a minimal increase of fluorescein penetration as compared to the controls treated with 0.9% NaCl/0.05% fluorescein eye drops indicating a slight disruption of the epithelial barrier function caused by the biguanides. In contrast fluorescein penetration was much further enhanced when BAC 0.01% control eye drops were administered.

Conclusion: This study showed that neither PHMB nor CHG readily penetrated through the cornea to the anterior chamber, which may explain why treatment of *Acanthamoeba keratitis* requires many months of sustained topical drug administration. PHMB and CHG had little effect on the epithelial barrier function compared to BAC, which is widely used as a preservative in eye drops.

Keywords: Chlorhexidine digluconate; Polyhexamethylene biguanide *Acanthamoeba keratitis*; Corneal penetration; Artificial anterior chamber; *In vitro* model; Fluorescein; Benzalkonium chloride

Introduction

Acanthamoeba keratitis is a rare but severe infection of the cornea that causes corneal ulceration resulting in scarring and severe visual impairment. Treating *Acanthamoeba keratitis* is difficult. When *Acanthamoebae* reach the ocular surface, the trophozoites penetrate the corneal epithelium by secreting a variety of proteases that invade the corneal stroma [1], from which they are difficult to eradicate. In the stroma, *Acanthamoeba* transform into a thick-walled cyst that shelters against adverse environmental effects and drugs [2,3]. Long-term treatment is mandatory, as encystment and recurrent infection occur when the drug concentration decreases before the vital cysts are completely eradicated. The introduction of the two cationic antiseptics polyhexamethylene biguanide (PHMB) [4] and chlorhexidine digluconate (CHG) [5], which are administered topically as eye drops, represented a great progress in the treatment of *Acanthamoeba keratitis*. In vitro, both substances show excellent efficacy against trophozoites and cysts, but CHG is slightly more effective than PHMB [6,7]. In clinical settings, when PHMB or CHG is used as monotherapy, the success rate is approximately 80% [8]. Topical treatment is usually maintained for many months, but despite intensive treatment and proven drug susceptibility of the clinical isolates, there are still many therapeutic failures [4,9,10]. A possible explanation for the high incidence of failure may be low drug bioavailability in the deep corneal stroma because of low drug penetration. Polymeric biguanides and CHG are used as off-label antimicrobial agents; thus, little is known about their pharmacokinetic properties when they are used as eye drops.

The aim of this study was to investigate the corneal drug penetration as well as the effect on the epithelial barrier function of three different cationic antiseptics that are commonly used to treat *Acanthamoeba keratitis*. In particular, we investigated the two PHMB preparations: a) Cosmocil, which features a uniform length of the

PHMB molecular chains, and b) Lavasept, which claims to produce a better "wetting" interaction with the non-hydrophilic surface due to the addition of macrogolum. The third anti-amoebic substance tested was CHG, which is approved as a preservative for eye drops. Since UV-absorption and fluorescence are unsuitable to assess PHMB and CHG concentrations we used capillary electrophoresis with contactless conductivity detection [11] to investigate the penetration of PHMB and CHG into the anterior chamber of intact rabbit corneas.

Materials and Methods

Corneas and perfusion chambers

Corneas were obtained from 3-month-old albino rabbits that were bred and killed at the local abattoir for human consumption. The eyes were transported to the laboratory at 4°C in 0.9% NaCl and were used within 2-3 hours after enucleation. The study was approved by the Animal Welfare Committee of the Canton of Zurich. The corneas were dissected using standard eye banking techniques and were mounted in a corneal perfusion chamber, as previously described [12], but a commercially available artificial anterior chamber (Barron artificial anterior chamber, Katena Products, Inc., Denville, New Jersey, USA) was used rather than a custom-made clamping system. The inflow tube of the perfusion chamber was connected to a peristaltic pump with a flow rate of 0.3 ml/min. The outflow tube from the perfusion chamber was connected to a small reservoir that was elevated by 20 cm to mimic the intraocular pressure of 15 mmHg. The total fluid volume recirculating through the perfusion chamber, reservoir and tubes was 3 ml. To mimic a physiologic corneal environment, the perfusion chamber, tubes and reservoirs were placed into a covered, partially filled, temperature-controlled water bath. The corneal temperature was regularly measured with an infrared based optical thermometer device (ThermoScan IRT4520, Braun, Kronberg, Germany), aiming for a surface temperature of 36°C. The chambers were perfused with freshly prepared BSS Plus solution (Alcon, Hünenberg, Switzerland), and the corneas were allowed to equilibrate for at least 30 minutes prior to starting the penetration experiments. Central corneal thickness was measured with a handheld ultrasound probe (Medical Pachymeter Echograph Model Pocket, Quantel Medical, Cournon d'Auvergne, France) and was used to control for endothelial viability during the course of the experiment. Experiments in which the corneas swelled by more than 10 % of the baseline pachymetry values (after the 30-minute equilibration time) were discarded.

Chemicals

CHG was provided by Sigma (Buchs, Switzerland) as a 20% solution. Two different preparations of PHMB were purchased. The first PHMB preparation, Cosmocil CQ, was obtained from ArchChemicals GmbH (Ratingen, Germany) as a 20% stock solution. The second PHMB preparation, Lavasept, was purchased from Fresenius Kabi (Stans, Switzerland) as an aqueous solution containing 20% PHMB and 1% macrogolum, a polyethylene glycol agent that is

thought to improve the contact between aqueous fluids and lipophilic surfaces. CHG and PHMB were each diluted with 0.9% NaCl to obtain 0.02% eye drop solutions. Fluorescein 10% solution was purchased from Novartis (Basel, Switzerland). Benzalkonium chloride (BAC) was purchased from Sigma in a powdered form. Eye drops containing 0.9% NaCl and 0.05% fluorescein, with or without 0.01% BAC, were used to compare the effect of CHG and PHMB on the barrier function of the corneal epithelium. BSS Plus, a balanced salt solution with high similarity to aqueous humor (Alcon, Hünenberg, Switzerland), was freshly prepared at the start of each experiment and then used to perfuse the chamber system.

Experimental protocol

During the penetration experiments, one to two eye drops were used to moisten the entire corneal surface every 30 minutes. To prevent the surface from drying, the corneal surface was moisturized with BSS Plus drops every 15 minutes. Aqueous samples from the posterior side of the cornea were obtained by collecting 200 µl samples from the circulating fluid out of the little reservoir; the fluid was immediately replaced by the same amount of BSS Plus. The samples were transferred to Eppendorf tubes and immediately frozen for storage at -20°C until the electrophoretic analysis. PHMB and CHG were measured using capillary electrophoresis combined with contactless conductivity detection, allowing a minimal drug concentration of 0.4 µg/ml for PHMB and 4 µg/ml for CHG [11].

Fluorescein was added to the eye drops (0.05%) to control for the integrity of the epithelial penetration barrier. Fluorescein penetration was measured with a 538 nm UV light meter (Fluostar, BMG Labtech, Ortenberg, Germany) using an excitation spectrum of 485 nm. At the end of each experiment, the corneal buttons were stored in 40% formalin for the histological analysis.

Results

Administered twice per hour, CHG and PHMB caused only minimal damage to the epithelial penetration barrier, which was evident in the slightly increased penetration of fluorescein compared to unpreserved 0.9% NaCl control eye drops (Figure 1). This effect was clearly enhanced when eye drops containing 0.01% BAC administered twice per hour were used instead. The histological findings of the epithelial surfaces of the corneas exposed for 8 hours to PHMB or CHG eye drops did not differ from the control corneas treated only with NaCl or BSS Plus eye drops (Figure 2). In corneas with intact epithelium, penetration of CHG or PHBM into the artificial anterior chamber was not detected within the 8-hour exposure time (n=10).

A second set of experiments measured the penetration of CHG and PHMB through corneas after mechanical epithelial debridement. While the penetration of fluorescein, which was used as a control substance, increased dramatically, no corneal penetration of CHG (n=3) or PHMB (n=3 for Lavasept and n=3 for Cosmocil) was detected even after 8 hours of drug exposure.
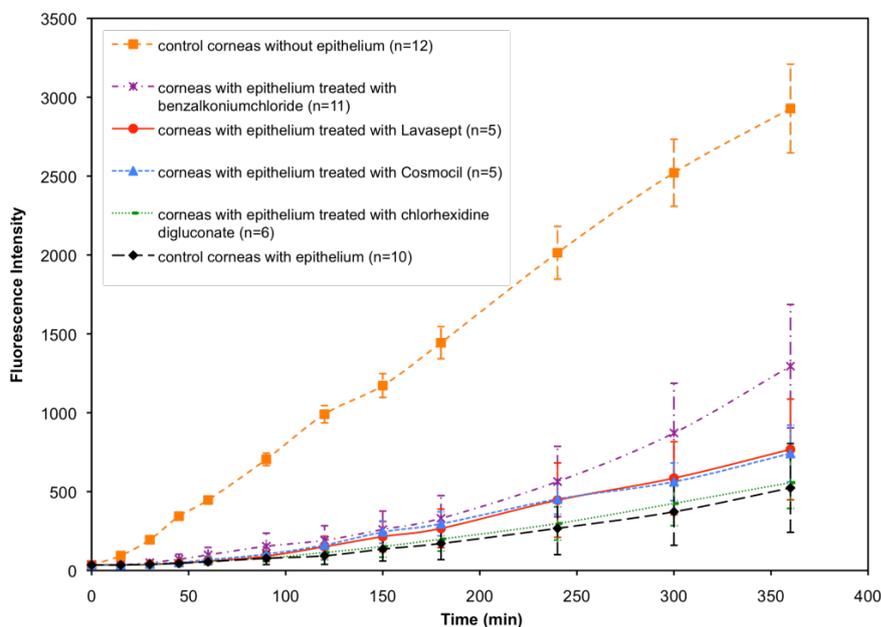
Figure 1: Effects of the various drugs on the corneal epithelial barrier function. Fluorescein penetration was used as a marker for the viability of the epithelial barrier during the experiments. The corneas were exposed to eye drops containing 0.9% NaCl; 0.05% fluorescein; and 0.02% Lavasept, 0.02% Cosmocil, 0.02% chlorhexidine digluconate, or 0.01% benzalkonium chloride for 6 hours. The control corneas with intact epithelium or with mechanically removed epithelium were treated with eye drops containing 0.9% NaCl and 0.05% fluorescein.
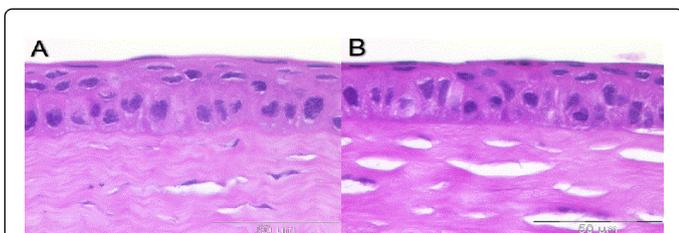

Figure 2: Histological findings of representative examples of corneas after an 8 hour penetration experiment in which the corneas were exposed to (A) BSS Plus eye drops only or (B) 0.02% Lavasept.

Discussion

This study indicates that neither CHG nor the two preparations of PHMB readily penetrate the cornea even under ideal laboratory conditions. This finding is surprising because PHMB and CHG have a molecular size that is suitable for penetrating an intact cornea. The molecular weight of a PHMB chain is 219 Daltons (Da). In an aqueous solution, 2 to a maximum of 40 PHMB chains typically polymerize into a larger molecule [13]. CHG has a molecular weight of 897 Da [14] with no tendency for aggregation. In aqueous solution the chlorhexidine (505 Da) [14] separates from the gluconates. Hence, the molecular weight of short PHMB molecules and CHG are similar to other substances that are regularly used for topical administrations,

including fluorescein (332 Da) [15,16], ofloxacin (361 Da) [17,18], latanoprost (432 Da) [19,20] and prednisolone phosphate (440 Da) [21,22], which all rapidly penetrate the intact cornea.

CHG and the two biguanides caused surprisingly little damage to the epithelial penetration barrier compared to eye drops containing 0.01% BAC. Both preparations of PHMB caused slightly more damage to the corneal epithelium than CHG, which is consistent with a previous study that found PHMB to be more harmful to keratinocytes than CHG [23]. In addition, the two different preparations of PHMB (Lavasept and Cosmocil) showed no difference in terms of damage to the penetration barrier. This finding indicate that the two PHMB preparations have similar effects on the ocular surface, independently of macrogolum, which is added to Lavasept to improve wetting and contact to non-hydrophilic surfaces, such as skin or the cornea. These findings closely reflect the clinical experience in which minor punctuated fluorescein staining of the epithelium is present after several weeks of treatment.

In corneas devoid of the epithelium penetration of hydrophilic substances usually increase dramatically, as shown here with fluorescein. In a similar experimental setup, much larger molecules than PHMB or CHG, such as single-chain antibody fragments (28,000 Da) or Dextran 75 (75,000 Da), have been shown to penetrate deepithelialized corneas within 6 to 8 hours [24,25]. Hence, even a maximally polymerized 40-chain PHMB molecule (8760 Da) would have been expected to penetrate deepithelialized corneas within the 8-hour drug exposure that was used in our experiments.

The lack of detectable penetration requires further consideration. The chemical detection assay of capillary electrophoresis with contactless conductivity detection has good detection limits and is well suited when the available sample volumes are limited as in the present study. The minimal detection level is 0.4 µg/ml for CHG and approximately 4 µg/ml for PHMB, which corresponds to a dilution of the eye drop solution by 1:5,000 for CHG and 1:500 for PHMB. Hence the detection limit is well below the expected drug penetration, particularly in an 8-hour experiment using corneas without epithelium. Note, that the contradictory preliminary findings concerning the penetration of the cornea reported by us in [11] could not be reproduced in the subsequent more elaborate investigations reported here. The most likely explanation for the apparent lack of penetration through the cornea is an accumulation of these amphiphilic substances within the corneal stroma. There may be an electrostatic interaction between the positively charged cationic biguanides and the negatively charged corneal proteoglycans that prevents or slows the penetration through the corneal stroma and into the perfusion chamber. Previous studies have shown that the same mechanism causes PHMB and CHG to strongly adsorb on pretreated cellulose (containing anionic carboxylic acid groups) [26,27].

We acknowledge that we performed a technical measurement in a controlled environment and do not fully understand the extent to which the findings presented here translate into clinical practice. We expect that in vivo several additional parameters such as drug dilution by reflex tearing or stromal inflammation of infected corneas may interact with drug penetration. However this study has shown that even under ideal circumstances topically administered PHMB and CHG do not readily penetrate the corneal stroma. Nevertheless we think that three general conclusions for practice can be drawn: 1) initial treatment should consist of very frequent drug dosing to foster drug penetration into deeper stromal layers, 2) an extended treatment period of many weeks or even months may be necessary for PHMB and CHG to reach the deeper stromal layers; and 3) due to its chemical properties PHMB and CHG may accumulate within the cornea, thereby possibly adding to the anti-acanthamoebic effect within the cornea and preventing high and potentially toxic peak drug concentrations that may cause toxic side effects in the anterior chamber structures. This conforms to the clinical observation that despite frequent extensive treatment with these potentially toxic substances, there have been virtually no reports of proven intraocular toxicity.

In summary, this study shows that PHMB and CHG do not readily penetrate through the cornea into the anterior chamber, which may explain why the treatment of deep *Acanthamoeba keratitis* requires many months of sustained topical drug administration.

Funding

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