Effects of the pharmaceutical cosolvent hydroxypropyl-β-cyclodextrin on porcine corneal endothelium

Abstract  ● Background: The influence of the pharmaceutical cosolvent hydroxypropyl-β-cyclodextrin (HPBCD) on porcine corneal endothelium was investigated. The purpose was to find out if this substance causes severe damage to the cornea.
● Methods: One hundred and ninety-five pig corneas were preserved in Eagle’s minimal essential medium with dextran and HPBCD. They were stained with trypan blue, examined using a light microscope and then reincubated. Changes in cell density and cell morphology as a function of the duration of preservation and the HPBCD concentration were evaluated. We developed a morphological classification combining the morphological aspects observed using the light microscope and the scanning electron microscope. The vitality of the endothelium was analyzed by cell separation and monolayer cultivation.
● Results: The cell density stayed stable without significant alterations in 0.1% HPBCD, 1% HPBCD and control solutions. In 10% HPBCD, however, the endothelium showed significant loss of cells. The morphological classification revealed high-grade endothelial damage in 10% HPBCD and low-grade damage in 1% HPBCD. The changes observed in 0.1% HPBCD and control medium were comparable. The degree of alteration conformed to the results of monolayer cultivation: endothelial cells of damaged corneoscleral buttons were limited in their ability to proliferate.
● Conclusion: Severe endothelial destruction in 10% HPBCD and changes in membrane integrity at lower concentrations limit the use of HPBCD in ophthalmic solutions.

Introduction

Pharmaceutical cosolvents are useful for helping drugs to penetrate pharmacological barriers. Substances for topical application are often limited in their solubility and stability [18]. Cyclodextrins (CDs) have a positive influence on these parameters [12, 14]. Additionally, much of the drug instilled into the conjunctival sac is washed out by tears or drained by conjunctival vessels [24]. Penetration through the cornea can be enhanced by the cosolvent hydroxypropyl-β-cyclodextrin (HPBCD) [30]. As there are still problems concerning the copyright of HPBCD the substance has not yet been used in ophthalmic formulations. In view of the intensive research into CDs their use in eye drops is probable in future [30].

HPBCD is an enzymatically modified starch. It consists of seven glucopyranose monomers (Fig. 1). The ring molecule is apolar in its interior parts and polar in its exterior parts [37]. Drugs can be integrated into the inner cavity [41]. Previous investigations showed HPBCD to have good pharmacokinetics and good bioavailability [37].

On the one hand, CDs enhance the concentration of drugs, such as steroids, in the anterior chamber. On the other hand, there are substances which have to be applied systemically, for example cyclosporin A. If penetration of topically applied drugs could be improved, for example in corneal graft rejection, this would be a step forward [19].
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β-CDs are stable in aqueous solutions; they are not metabolized [37]. CDs are not integrated into cell membranes [27]. However, they interfere with membranous structures [12] and the skin [42]. On the other hand, CD accumulates in kidney cells [13]. To our knowledge there is no evidence of intracellular accumulation in the eye.

We chose the endothelium as an indicator of toxicity for two reasons. First, the endothelial function is essential in order to maintain the cornea’s vitality [6]. Second, it has only a limited ability to regenerate [39]. To our knowledge no exact data on the endothelial cell toxicity of HPBCD have been published.

In this study, the effects of various concentrations of HPBCD on porcine corneoscleral buttons were examined during organ culture and cell culture.

Materials and methods

Preservation and media

195 porcine bulbi from pigs approximately 6 months old were obtained from the local slaughterhouse. The eyes were transported in a thermos container at 4°C to the laboratory. There, corneoscleral rims were dissected and preserved at 37°C in a 5% CO2 atmosphere. The culture medium was changed every 3 days. One liter of standard culture medium (Serva, Heidelberg, Germany) comprised: Eagles minimal essential medium (MEM) with Earles salts (100 ml), MEM (1 M, 12.47 ml), sodium bicarbonate (2.2 g), l-glutamine (200 M, 10 ml), dextran T500 (6%), fetal calf serum (FCS; 2%), penicillin (100 IU), streptomycin (100 mg), amphotericin B (2.5 mg) and distilled water ad 1000 ml. This medium included 0.1%, 1% and 10% HPBCD. We used HPBCD (Amaizo, Hammond, Indiana, USA) with a mean degree of substitution of 0.546, a molecular weight of 1356.7 and remaining humidity of 6.75%. In the control group for the 0.1% and 1% concentration the standard medium was used (control 1). In order to avoid a significant increase in osmolarity in 10% HPBCD, a solution of 7 parts standard medium, 2 parts distilled water and 1 part HPBCD was used. In this case a modified control medium (control 2) was used. It consisted of 7 parts standard medium, 3 parts distilled water and sodium chloride. The osmolarity of all media was in the range between 322 and 335 mosmol/kg. Before cultivation the corneoscleral buttons were stained with 0.3% trypan blue solution and examined using a light microscope. The endothelial cell density and the endothelial morphology were surveyed. After different periods of incubation the corneoscleral buttons were examined and then reincubated. During short-term cultivation the corneas were examined after 0, 6, 18, 30, 42, 54, 66, 78 and 90 h. In another experiment they were studied after 0, 1, 3, 6, 18, 30 and 42 h. During long-term cultivation the corneas were investigated after 0, 5, 10, 15, 20, 25 and 30 days.

Quantitative analysis

Each experiment consisted of 10 corneas examined after different periods of preservation. The central cell densities of each cornea after each incubation time were counted. Since the density varies locally [11, 12], we counted the cells in selected sample areas of the central corneal parts eight times – twice in each quadrant – and calculated the mean value. In this way we were able to minimize the error caused by the counting method. Interindividual differences were reduced by calculating the average of the means of 10 corneas per medium and incubation time and by expressing the cell densities in percent of the initial cell density. How well the data fit a gaussian curve was tested with the Schapiro-Wilks method (BMDF New System Version 1.0). Significant changes in cell density were detected by block variance analysis. The influence of the culture medium and of different concentrations of HPBCD on the cell density at different points of time was tested with analysis of variance (ANOVA) and the t-test.

Scanning electron microscopy

In another experiment we examined the surface of endothelial cell membranes and the integrity of the cell layer of 17 corneoscleral rims using a scanning electron microscope (Stereoscan S 4–10, Cambridge, UK). The corneas were cut with a 7-mm trephine. The tissue was fixed in 3% glutaraldehyde (pH 7.4, buffered with phosphate buffer) and then washed in phosphate buffer three times for 30 min each time. Then it was stored in 30, 40, 50, 60, 70, 80, 90, 95, and finally 100% ethanol, twice for 30 min per ethanol concentration. After critical point drying (Balzers CPD 030, Liechtenstein) and sputtering with gold (ION Tech, Teddington, UK) the endothelium was examined at magnifications between 22- and 10800-fold.

Cell cultures

The endothelial cells differed in their vitality. Sporadically we investigated their ability to regenerate in cell cultures. We removed corneoscleral rims from culture in 10% HPBCD after 18 h and in 1% HPBCD after 90 h and compared them with freshly dissected corneas. For this purpose the endothelial side of the corneoscleral rims was covered with trypsin-EDTA solution (0.05%; Gibco, Grand Island, N.Y., USA) for 15 min. Endothelial cells were washed away with a jet of 5 ml standard culture medium and 10% FCS. For each experiment we collected the medium from three corneas and obtained standardized cell suspensions with 10^6 cells/cm^2 in standard culture medium with 10% FCS. The standardization was checked by cell counting in a Neubauer chamber. Contact of trypsin-EDTA solution and culture medium with the epithelial surface or the edge of the rims was studiously avoided in order to prevent contamination with keratocytes or epithelial cells. Indeed, these cells could not be found with the light microscope during the subsequent cell culture. The hollows of a microtiter culture dish (Becton