Rapid Communication

Effect of Cyclodextrins on Morphology and Barrier Characteristics of Isolated Rabbit Corneas

Goutham R. Adelli,[1] Sai Prachetan Balguri,[1] and Soumyajit Majumdar[1,2,3]

Abstract. The objective of the present study is to investigate the confounding effects, if any, of beta-cyclodextrins (βCDs) on corneal permeability coefficients obtained from *in vitro* transmembrane diffusion studies. Transcorneal permeability studies were carried out with 2-hydroxypropyl-beta-cyclodextrin (HPβCD) and randomly methylated-beta-cyclodextrin (RMβCD) at 5 and 2.5%w/v in isotonic phosphate-buffered solution (IPBS) (pH 7.4). Rabbit corneas received from Pel-Freez Biologicals® were used for these studies. Propranolol hydrochloride (PHCl) (1 mg/mL) was used as the paracellular permeability marker. A series of permeation studies were carried out with IPBS as the control, with CDs on the donor side only, CDs on the receiver side only, and CDs on both the donor and receiver sides. At the end of 1 or 3 h, corneas were collected and fixed using a solution containing 2%v/v glutaraldehyde+2%w/v paraformaldehyde+IPBS and histological examinations were performed (Excalibur Pathology, Inc). The order of transcorneal permeability of PHCl was found to be CDs on the receiver side > control (no CDs) ≈ CDs on both the receiver and donor sides > CDs on the donor side. Histology studies revealed that the corneal epithelial and endothelial layers remained intact in the control sets. Damage to the cornea was observed in the order of CDs on the receiver side > CDs on the donor side > CDs on both sides > control. The use of CDs in solutions for *in vitro* permeation experiments with rabbit corneas needs to be carefully considered to avoid confounding effects in the data obtained.

KEY WORDS: corneal histology; cyclodextrins; *in vitro* transcorneal permeability; morphology; propranolol hydrochloride.

INTRODUCTION

Cyclodextrins (CDs) are a group of cyclic oligosaccharides with a hydrophobic inner core and a hydrophilic outer surface. Based on the number of glucopyranose units in the structure, they are classified into alpha (α), beta (β), and gamma (γ) CDs (6, 7, and 8 units, respectively). Over the last few decades, CDs have emerged as an important pharmaceutical excipient for solubility enhancement of lipophilic drugs and permeability improvement across biological membranes (1). CDs act as penetration enhancers by increasing the availability of drug molecules at the surface of the biological membrane barrier. Because of their aqueous solubility improving characteristics, suitable cavity size, and drug complexation efficiency (1), beta-cyclodextrins (βCDs; Fig. 1a) such as 2-hydroxypropyl-beta-cyclodextrin (HPβCD; Fig. 1b) and

randomly methylated-beta-cyclodextrin (RMβCD; Fig. 1c) are widely used in the field of formulation and drug delivery.

It is only recently that the mechanism of permeability enhancement across biological membranes, such as the skin, buccal mucosa, and cornea, have been discussed. CDs interact with cholesterol present in the phospholipid monolayers of the cell membranes, thus exchanging them with the drugs/drug candidates held in the hydrophobic CD core (Fig. 2). In addition to their utility as transcorneal permeability enhancers, by virtue of their ability to extract lipophilic components like cholesterol and phospholipids from the corneal membrane (2), CDs are often added to the receiver solutions to maintain sink conditions during *in vitro* transcorneal permeability studies of lipophilic molecules (3). Unknowingly damaging the cornea (epithelium, stroma lamellae, or endothelium), however, may lead to altered permeability of the drug/drug candidates as a result of the receiver solution characteristics rather than formulation or drug/prodrug candidate properties. This can lead to misinterpretation of the results and overestimation of the permeability enhancement of the formulation/drug candidate if adequate controls were not included in the experiment design. Thus, an understanding of the effect of CDs on the corneal permeability characteristics, when added as a solubilizer in the receiver solution, is very important and has not been analyzed as yet. The aim of the present study is to evaluate the effect of CD

[1] Department of Pharmaceutics and Drug Delivery, School of Pharmacy, The University of Mississippi, 111 Faser Hall, University, Mississippi 38677, USA.
[2] Research Institute of Pharmaceutical Sciences, The University of Mississippi, University, Mississippi 38677, USA.
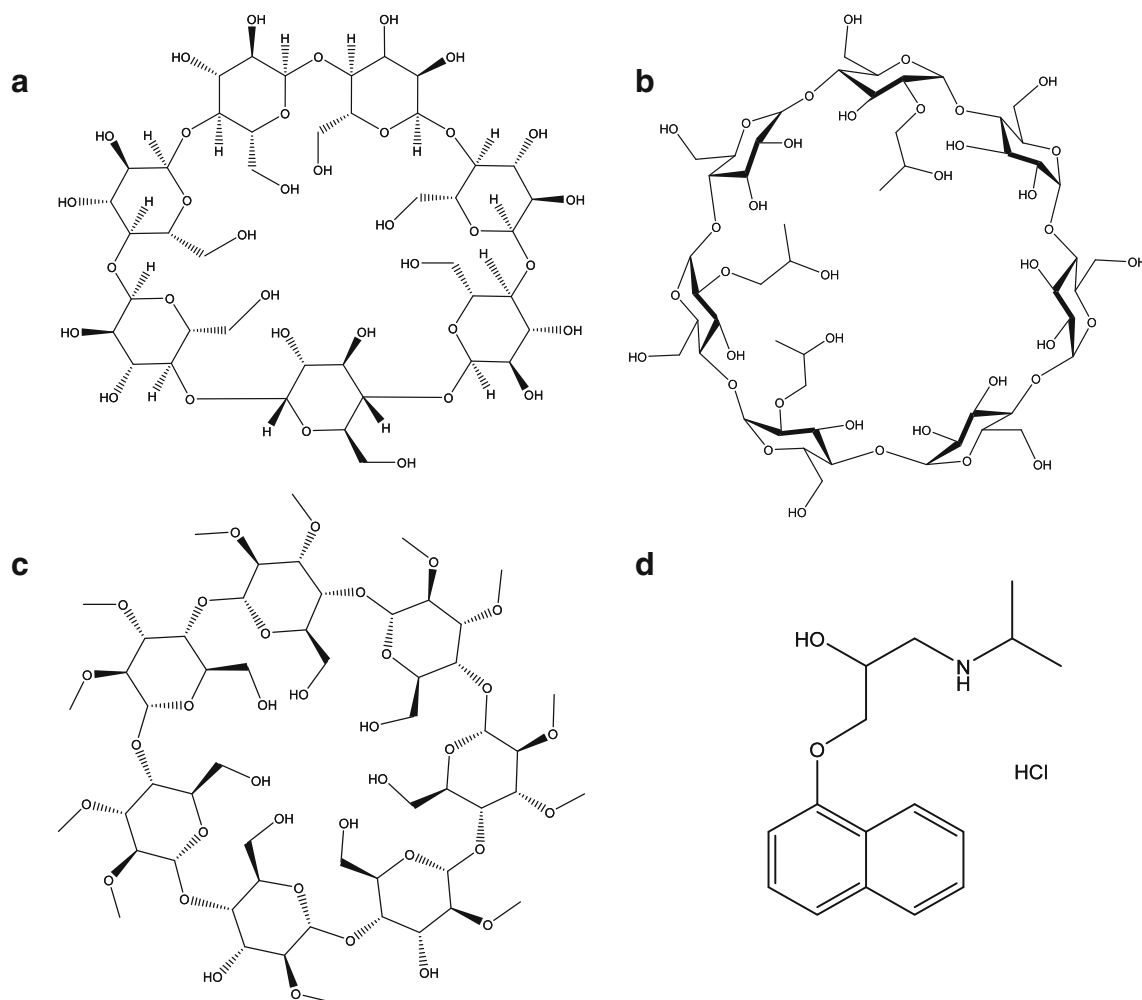[3] To whom correspondence should be addressed. (e-mail: majumso@olemiss.edu)

Fig. 1. Chemical structure of **a** general structure of beta cyclodextrin (*β-CD*), **b** hydroxypropyl-βeta-cyclodextrin (*HPβCD*), **c** randomly methylated-βeta-cyclodextrin (*RMβCD*), and **d** propranolol (*PHCl*)

concentration, duration, and type on the morphological characteristics and barrier properties, using propranolol hydrochloride (PHCl; Fig. 1d) as a paracellular diffusion marker, of isolated rabbit cornea *in vitro*.
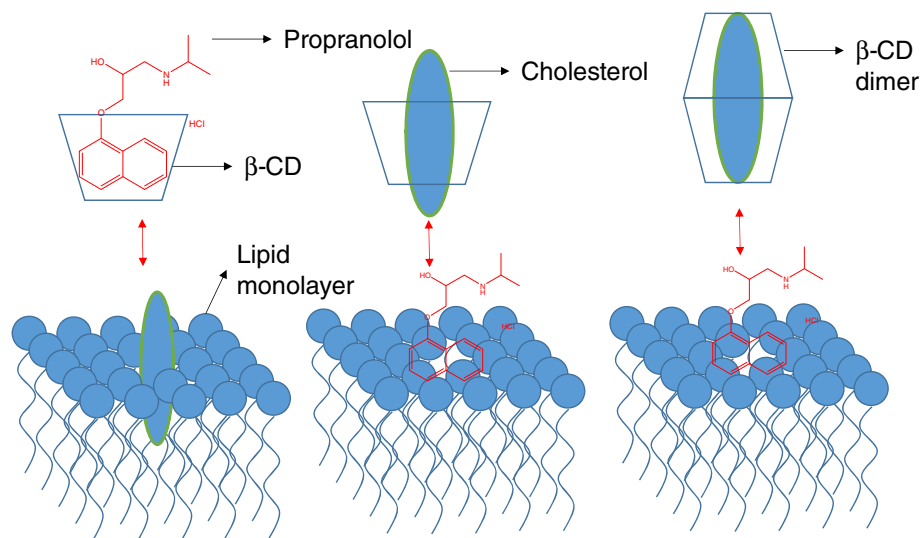

Fig. 2. Extraction of cholesterol and the exchange of active moiety by cyclodextrins from the phospholipid cell membrane

MATERIALS AND METHODS

Chemicals

Propranolol hydrochloride (PHCl), 2-hydroxypropyl-beta-cyclodextrin (HPβCD), randomly methylated-beta-cyclodextrin (RMβCD), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Animal Tissues

Whole eye globes of New Zealand albino rabbits were purchased from Pel-Freez Biologicals® (Rogers, AK), shipped overnight in hanks balanced salt solution (HBSS) over wet ice. Corneas excised from the whole eye globes were used immediately on the day of receipt.

Phase Solubility Studies

Complexation of PHCl with HPβCD and RMβCD was evaluated using phase solubility studies according to the method of Higuchi and Connors (4). Excess amount of PHCl was added to 1 mL IPBS, containing increasing concentrations of CDs. The concentrations ranged from 2.5–10%*w/v*. The resulting suspensions were shaken at 25°C for 24 h in a reciprocating water bath. Following equilibration, the suspensions were centrifuged at 13,000 rpm for 20 min at 25°C and the supernatant thus obtained was analyzed using an HPLC-UV method.

Phase solubility profile was obtained by plotting the solubility of PHCl against the concentration of CDs used. The binding constants ($K_{1:1}$) and complexation efficiencies (CE) for the PHCl-CD complex were calculated from the linear region of the solubility curves using Eq. 1 and 2:

$$K_{1:1} = \frac{\text{Slope}}{S_o(1-\text{Slope})} \tag{1}$$

$$CE = K_{1:1} \times S_o = \frac{\text{Slope}}{S_o(1-\text{Slope})} \tag{2}$$

where $K_{1:1}$ is the binding constant, S_o is the saturation concentration of PHCl in pure water, and slope denotes the slope of the straight line.

In vitro Transcorneal Permeability

A series of *in vitro* transcorneal permeability studies were carried out using a 9-mm side-by-side diffusion apparatus (PermeGear Inc., Hellertown, PA). Freshly excised rabbit whole eye globes received from Pel-Freez Biologicals® were used for these studies. Briefly, corneas were excised by making a brief incision, about 2 mm from the corneal-scleral junction and cutting radially along the sclera. The excised corneas were immediately mounted between the diffusion cells. The half-cell facing the epithelial layer was termed as the donor compartment, and the other half towards the endothelium was termed as the receiver chamber. The nomenclature is based on the addition of PHCl to the epithelial side half-cell. A circulating water bath was used to maintain the temperature at 34°C during the transport studies.

In vitro transcorneal permeability studies were carried out with 2.5 and 5%*w/v* HPβCD and RMβCD in IPBS (pH 7.4) for 1 or 3 h at 34°C. PHCl (1 mg/mL) was used as the paracellular marker (PHCl; pKa=9.4). PHCl solution was always added on the donor side. Permeation studies were performed with IPBS as control; with CDs on the donor side only, CDs on the receiver side only, and CDs on both the donor and receiver sides as represented in Table I. Aliquots, 600 μL, were withdrawn at predetermined time points for 1 or 3 h and replaced with an equal volume of the receiver solution. Samples were analyzed by HPLC-UV system.

Corneal Histology

Histological evaluation was carried out at Excalibur Pathology Inc. (Oklahoma City, OK). At the end of each study, corneas were fixed in 2%*w/v* paraformaldehyde and 2%*v/v* gluteraldehyde in IPBS. Corneas embedded in paraffin were sliced into 5-μm cross sections using a microtome (American Optical® 820 Rotary Microtome). These sections were mounted on a slide and dried overnight in an oven. The slide was washed with xylene to remove the paraffin and washed with alcohol and water to hydrate the tissue. This was then stained with nuclear dye Gill III hematoxylin (StatLab medical) for 10 min and rinsed, and then counterstained with eosin. These slides were then washed in reverse manner (running water, alcohol, and xylene), cover slipped, and examined under microscope (Chromavision ACIS II).

Analytical Method for Measurement of PHCl

Waters HPLC system with 600E pump controller, 717 plus autosampler, and 2487 UV detector was used. Data handling was carried out using an Agilent 3395 integrator. Mobile phase consists of 160 mL of water, 180 mL of methanol, 70 mL of acetonitrile, 2.5 mL of acetic acid, and 125 μL of

Table I. *In vitro* Transcorneal Permeability Coefficients of PHCl at Different Concentrations of CDs. (I) IPBS without CDs (Control; 1), (II) CDs in the Donor Solution (3 and 6), (III) CDs in the Receiver Solution (2 and 5), and (IV) CDs in Both the Donor and Receiver Solutions (4 and 7). Experiments were Carried out for 1 or 3 h

S. No	Donor 3 ml	Receiver 3.2 ml	Permeability×10^6 cm/s	
			5%*w/v* CDs	2.5%*w/v* CDs
1	IPBS	IPBS	27.0±1.3	21.3±1.0
2	IPBS	HPβCD	40.8±5.7	32.2±4.5
3	HPβCD	IPBS	8.1±1.2	6.4±1.0
4*	HPβCD	HPβCD	17.6±1.1	13.8±0.9
5	IPBS	RMβCD	52.1±8.4	41.3±6.6
6	RMβCD	IPBS	14.5±5.2	11.4±4.1
7[a]	RMβCD	RMβCD	24.5±3.4	19.2±2.7

CDs cyclodextrins, *IPBS* isotonic phosphate-buffered solution, *HPβCD* 2-hydroxypropyl-beta-cyclodextrin, *RMβCD* randomly methylated-beta-cyclodextrin
[a] Experiments were carried out for 1 and 3 h. Permeability coefficients were calculated only for a 1-h experiment.

triethylamine (v/v). The pH of the whole mixture was adjusted to 3.4. Phenomenex *Luna®* 5 μm C_{18} 100 Å, 250×4.6 mm column was used at a flow rate of 0.5 mL/min and detection wavelength of 291 nm.

RESULTS

Phase Solubility Studies

Binding constants of PHCl with HPβCD and RMβCD were very low (278.1 and 326.4 μM^{-1}, respectively). Complexation efficiency of PHCl with HPβCD and RMβCD were 0.94 and 1.1, respectively. RMβCD was observed to have slightly higher CE with PHCl than HPβCD. Low-binding constant values demonstrate that only a low fraction of PHCl was bound to either of the CDs and that there was more free concentrations of CDs and PHCl in the solution.

CDs on the Receiver Side

With 5%w/v HPβCD and RMβCD on the receiver side, permeability coefficient of PHCl at the end of 1 h was found to be 40.8±5.7×10^{-6} and 52.1±8.4×10^{-6} cm/s, respectively (1.5-fold and 1.9-fold higher than the control) (Table I). Similar results were encountered with the use of 2.5%w/v HPβCD and RMβCD on the receiver side at the end of 1 h (32.2±4.9×10^{-6} and 41.3±6.6×10^{-6} cm/s, respectively).

Histological studies at the end of 1 h revealed damage to both the endothelium and epithelium in the case of 5%w/v HPβCD on the receiver side. Stroma lamellae were found to be disrupted at several points on the cornea. When 5%w/v RMβCD was used on the receiver side, less damage was caused to the epithelium, and little or no damage to the endothelium was observed. HPβCD at 2.5%w/v caused significant rupture of the corneal epithelium, but the endothelium was found to be intact. At the same concentration, RMβCD showed little damage to the epithelium and none to the endothelium. These studies revealed that more damage was caused when HPβCD is used in the receiver solution in comparison to RMβCD (both 5%w/v and 2.5%w/v) (Figs. 3 and 4).

CDs on the Donor Side

The permeability coefficients of PHCl at the end of 1 h with 5%w/v HPβCD and RMβCD were found to be 8.1 ±1.2×10^{-6} and 14.5±5.2×10^{-6} cm/s. Permeability coefficient of PHCl in IPBS (control) was found to be 27.02±1.2×10^{-6} cm/s. Permeability coefficients of PHCl were also observed to be lower than the control in case of 2.5%w/v HPβCD and RMβCD (Table I).

Histological studies revealed rupture of the corneal epithelium with the use of 5%w/v and 2.5%w/v HPβCD in the donor solution. With 5%w/v and 2.5%w/v RMβCD on the donor side, little or no damage was caused to the corneal epithelium. In the case of the control group, corneal morphology was found to be intact. In all the studies, HPβCD and RMβCD in the donor chamber, at the concentrations used, did not cause any damage to the endothelium (Figs. 3 and 4).

CDs in Both the Donor and Receiver Chamber

Surprisingly, use of 5%w/v and 2.5%w/v RMβCD in both the donor and receiver chambers simultaneously did not show significant difference compared to the control. Permeability coefficients of PHCl in the control, 5%w/v HPβCD and 5%w/v RMβCD solutions on the donor and the receiver sides were observed to be 27.0±1.3×10^{-6}, 17.6 ±1.1×10^{-6}, and 24.5±3.4×10^{-6} cm/s, respectively. Permeability coefficients of PHCl in the control, 2.5%w/v HPβCD and 2.5%w/v RMβCD solutions on the donor and the receiver sides were observed to be 21.3 ±1.03×10^{-6}, 13.8±0.9×10^{-6}, and 19.2±2.7×10^{-6} cm/s, respectively. On an average, a 20% drop in the permeability was observed as a function of 50% drop in CD concentrations.

No damage was observed to the corneal epithelia with 5%w/v and 2.5%w/v RMβCD. At the end of 1 h, when 5%w/v and 2.5%w/v HPβCD solution was used in both the donor and receiver chambers, the corneal epithelia were found to be damaged. In all cases, endothelia were found to be intact without any significant damage compared to the control (Figs. 3 and 4). When corneas were exposed to 2.5%w/v HPβCD on both sides for 3 h, complete erosion of the epithelium was observed, exposing the stroma. But no significant damage to the endothelium was noted (Fig. 5).

DISCUSSION

CDs have become important pharmaceutical excipients due to their ability to improve solubility of lipophilic molecules and to enhance permeability across biological membranes. It is considered that CDs increase permeability across biological membranes through their surface activity, membrane partitioning, and cholesterol extraction characteristics (5,6).

In the present study, we used rabbit corneas because of their similarities to human corneal ultra-structure (7) and common use in *in vitro* studies evaluating corneal drug penetration. We selected HPβCD and RMβCD because of their widespread use in the ocular formulations and permeability studies. Permeability coefficients of PHCl were in the order of CDs on the receiver side > control > CDs on both sides > CDs on the donor side. Corneal histology revealed the damage to the membranes in the order of CDs on the receiver side > CDs on the donor side > CDs on both sides > control. CDs after forming inclusion complexes are known to improve permeability of compounds across the biological membranes. Thus, we observed higher permeability coefficients with the RMβCD-PHCl complex which could be because of the higher CE of PHCl with RMβCD compared to HPβCD.

RMβCD produced less corneal damage than HPβCD. This is in agreement with previous literature reports. Savolainen *et al.* studied toxicity/irritation caused by CDs on human corneal epithelial cell lines and observed that the cytotoxic effect was in the order of αCD > dimethyl-
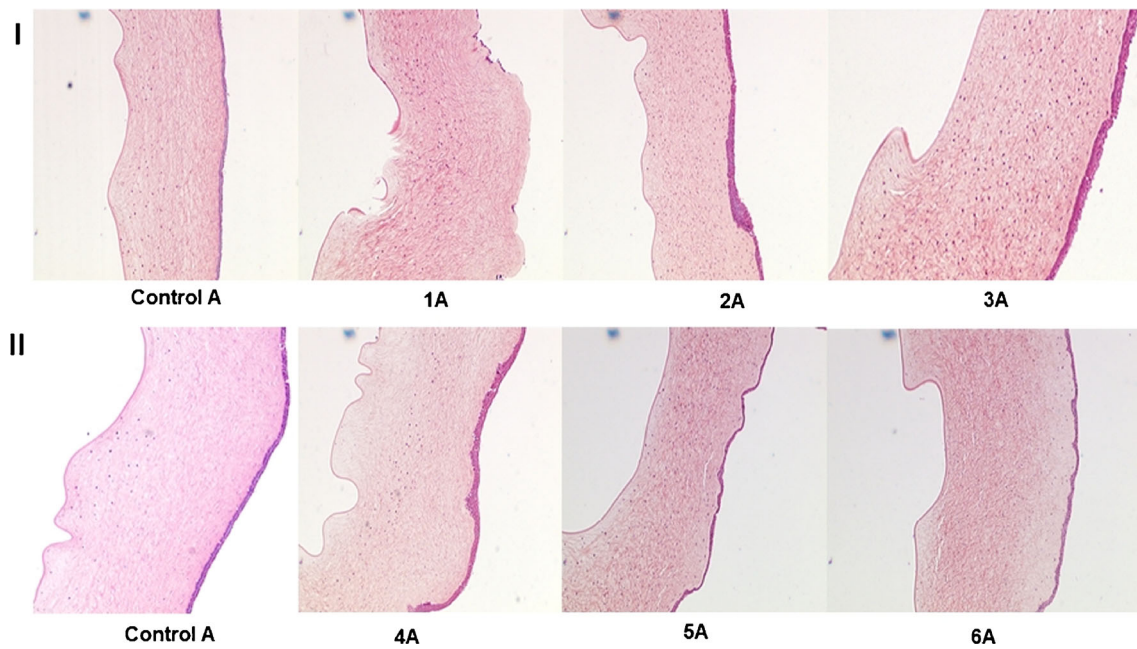
Fig. 3. Hematoxylin-eosin-stained corneal cross-sections 1 h postexposure to 1 IPBS: control A, *1A* 5%*w/v* HPβCD in the receiver solution, *2A* 5%*w/v* HPβCD in the donor solution, *3A* 5%*w/v* HPβCD in the receiver and donor solutions, *4A* 5%*w/v* RMβCD in the receiver solution, *5A* 5%*w/v* RMβCD in the donor solution, and *6A* 5%*w/v* RMβCD in the receiver and donor solutions

βCD > SBEβCD = HPβCD > γCD (9). In another study, Lopez *et al.* performed a computational simulation of CDs interacting with cholesterol (8). It was reported that within 10 ns of the initial interaction, CDs orient in a
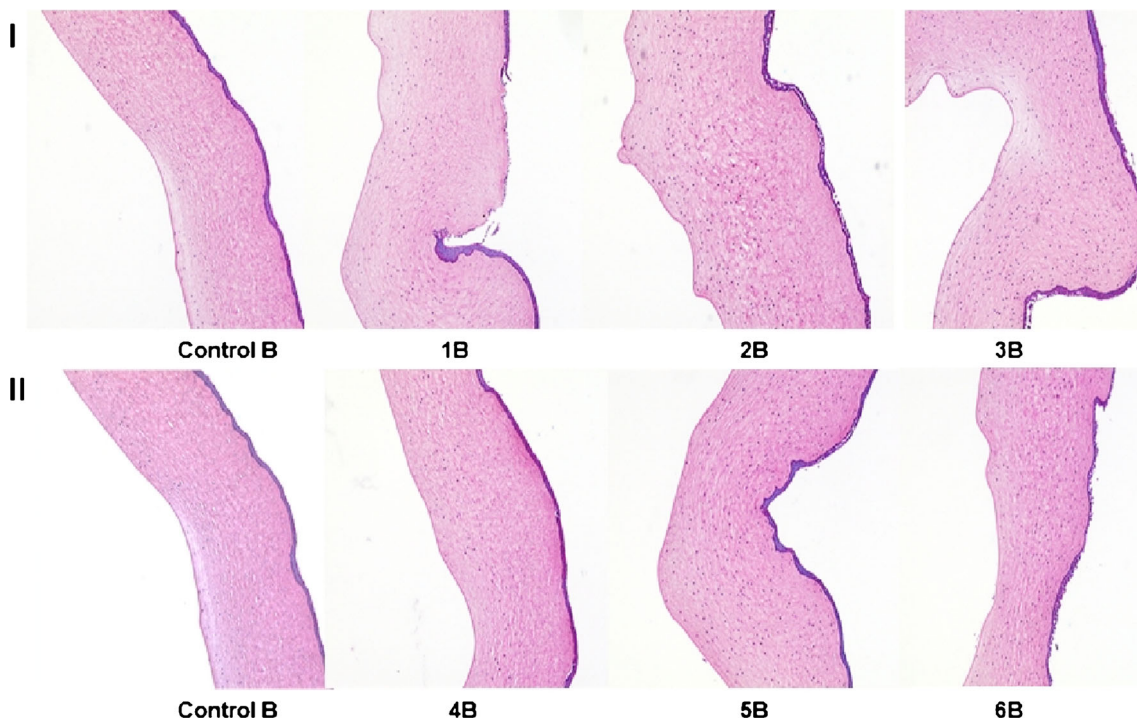

Fig. 4. Hematoxylin-eosin-stained corneal cross-sections 1 h postexposure to 1 IPBS: Control B, *1B* 2.5%*w/v* HPβCD in the receiver solution, *2B* 2.5%*w/v* HPβCD in the donor solution, *3B* 2.5%*w/v* HPβCD in the receiver and donor solutions, *4B* 2.5%*w/v* RMβCD in the receiver solution, *5B* 2.5%*w/v* RMβCD in the donor solution, and *6B* 2.5%*w/v* RMβCD in the receiver and donor solutions
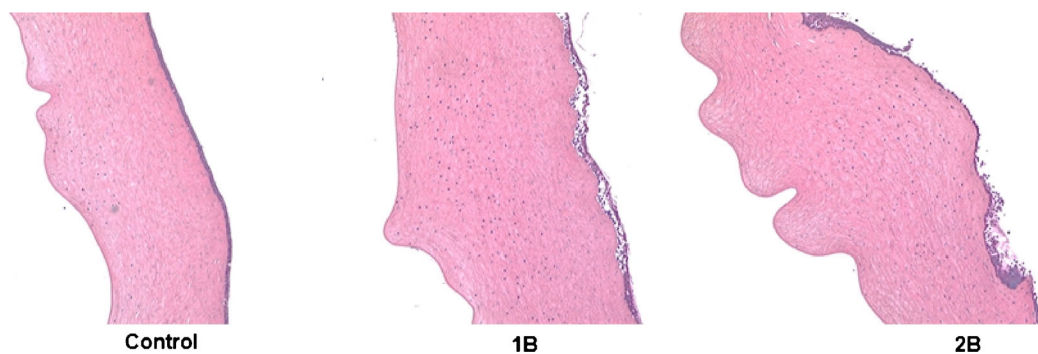
Fig. 5. Hematoxylin-eosin-stained corneal cross-sections 3 h postexposure to 1 IPBS: control, *1B* 2.5%*w/v* HPβCD in the receiver and donor solutions, and *2B* 2.5%*w/v* RMβCD in the receiver and donor solutions

perpendicular direction to the surface of the phospholipid monolayer and the hydroxyl groups of membrane cholesterol penetrate into the inner core of the CD. On an average, rate of extraction of one cholesterol molecule per CD was found to be 0.5 μs. Based on the stoichiometric ratios, it was observed that one HPβCD is required to extract one molecule of cholesterol or membrane lipid forming a 1:1 soluble complex (9). In some cases, two molecules of CDs interact with one another in a head-head, head-tail, or tail-tail fashion to form a dimer (10). Both head-tail and tail-tail interactions are less stable than head-head interactions due to their spontaneous dissociation in water. Tsamaloukas *et al.* reported that extraction of one molecule of cholesterol/membrane lipid requires two molecules of RMβCD stoichiometrically (cholesterol: RMβCD::1:2), since the formation of 1:1 complex is not a spontaneous process (11). These reports suggest that at a given concentration, the number of cholesterol molecules extracted per RMβCD is less compared to HPβCD. This could probably explain why RMβCD caused less damage to the corneal membranes.

Besides the type of CDs used, the duration of contact and concentration used are other important parameters. Duckner *et al.* studied the effect of HPβCD concentration on porcine corneal endothelium (12). It was observed that use of 10%*w/v* HPβCD caused high grade of endothelial damage followed by minimal damage with 1%*w/v* HPβCD and none with 0.1%*w/v* HPβCD within 3 h of the study. Similar observations were recorded in our studies also. In the current report, use of 5%*w/v* HPβCD caused severe damage to the corneal epithelium and endothelium layers within 3 h of exposure. Disruptions in the stromal lamellae were also observed when CDs were employed in the receiver media. Damage to the corneal layers was observed to be minimum with the use of 2.5%*w/v* CDs. In our study, we observed that 2.5%*w/v* RMβCD causes minimal damage compared to HPβCD, but only after 3 h of exposure.

In the current study, permeability coefficients of PHCl were found to be higher when CDs were taken on the receiver side rather than on the donor side. This was attributed to the fact that the corneal endothelium is more permeable when compared to the epithelium (13). Due to the concentration gradient, CDs incorporated in the receiver side probably cross the endothelium and stroma and interacts with basal cells and tight junctions of the corneal epithelium, thus causing more damage to the epithelia and endothelia. In contrast, CDs on the donor side are unable to pass through the non-keratinized squamous cells of the epithelia; thus causing less damage to the cornea. When CDs were employed on both the donor and receiver side, due to the absence of net concentration gradient, little or no change was observed in the corneal integrity. But prolonged exposure (3 h) to CD solutions leads to the loss of the ZO-1, initiating significant damage and complete erosion of the corneal epithelium. As a result, we observed severe damage of the corneal epithelia after 3-h exposure of the cornea to both HPβCD and RMβCD.

Several studies in the literature, including some from our own laboratory, have used CDs in the receiver solution during *in vitro* transcorneal permeability evaluation (14–20). In view of the findings from this study, it is apparent that use of CDs as solubilizers in the receiver solution could significantly impact transcorneal flux. Thus, it is very important to understand and use proper controls and experimental design during *in vitro* transcorneal permeability studies using CDs.

CONCLUSION

The effect of CDs on the corneal membrane *in vitro* was observed to be dependent on factors such as type of CDs employed, type of inclusion complex formed, CD concentration, and the duration of exposure to the biological membrane. Thus, the use of CDs in *in vitro* transcorneal permeation experiments as solubilizers in the receiving medium, especially when used only on one side of the tissue, needs to be carefully considered to avoid confounding effects in the permeability data obtained due to damage to the corneal ultra-structure.

ACKNOWLEDGEMENTS

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