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Decrease in Corneal Damage due to Benzalkonium Chloride by the Addition of Mannitol into Timolol Maleate Eye Drops

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Abstract: We investigated the protective effects of mannitol on corneal damage caused by benzalkonium chloride (BAC), which is used as a preservative in commercially available timolol maleate eye drops, using rat debrided corneal epithelium and a human cornea epithelial cell line (HCE-T). Corneal wounds were monitored using a fundus camera TRC-50X equipped with a digital camera; eye drops were instilled into rat eyes five times a day after corneal epithelial abrasion. The viability of HCE-T cells was calculated by TetraColor One; and *Escherichia coli* (ATCC 8739) were used to measure antimicrobial activity. The reducing effects on transcorneal penetration and intraocular pressure (IOP) of the eye drops were determined using rabbits. The corneal wound healing rate and rate constant (k_H), as well as cell viability, were higher following treatment with 0.005% BAC solution containing 0.5% mannitol than in the case BAC solution alone; the antimicrobial activity was approximately the same for BAC solutions with and without mannitol. In addition, the k_H for rat eyes instilled with commercially available timolol maleate eye drops containing 0.5% mannitol was significantly higher than that for eyes instilled with timolol maleate eye drops without mannitol, and the addition of mannitol did not affect the corneal penetration or IOP reducing effect of the timolol maleate eye drops. A preservative system comprising BAC and mannitol may provide effective therapy for glaucoma patients requiring long-term treatment with anti-glaucoma agents.

Key words: mannitol, benzalkonium chloride, cornea, timolol maleate, preservative

1 INTRODUCTION

The most common preservative added to ophthalmic preparations used to treat glaucoma and ocular surface diseases is benzalkonium chloride (BAC), most often used at a concentration of 0.005% (range, 0.005-0.02%) in topical multi-dose solutions[1]. BAC, which is surface-active agents, is known to have a strong preservative effect, and its surface-active effects increase the corneal penetration of the main component. Therefore, BAC has been seen as an effective preservative and indispensable in the preparation of eye drops. However, BAC has been shown to be highly toxic both *in vitro* and *in vivo* due to a stimulatory effect on epithelial cell death[2, 3]. BAC is a quaternary ammonium compound that has been shown to hasten drying of the tear film[4, 5], worsen preexisting dry eye[6], and affect both the cornea and conjunctiva[7]. In addition, it is known that BAC changes the ionic resistance of the cornea by intercalating into cellular membranes, resulting in membrane disruption via oxidative stress[8-10]. Therefore, BAC is a pro-inflammatory or proapoptotic mediator[11] because it induces oxidative stress[8-10] or significantly alters precorneal mucins[12]. Clinically, these iatrogenic effects are found most frequently for eye drops used to treat long-term pathologies such as glaucoma. The side effects of BAC seem to be both dose- and time-dependent, increasing with larger amounts used for longer periods. Recently, a new preservative system without BAC has been in development. Travatan Z® (Alcon, TX, U.S.A.) is an anti-glaucoma eye drop formulation preserved with a non-BAC system (sofzia) patented by Alcon. The sofzia preservative system of Travatan Z® consists of boric acid and zinc chloride, which are less damaging than BAC to the ocular surface of glaucoma patients receiving long-term eye drop therapy. However, for reasons of versatility, this potent preservative

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system has not yet been introduced because the sofzia preservative system is not applicable to other eye drops (only Travatan Z®). Taken together, improvements to the BAC preservative system that do not cause corneal epithelial cell damage remain a high priority.

D-mannitol is a nonreducing sugar alcohol that is used as an osmotic agent[13], and has wide applications in the food, pharmaceutical, and chemical industries. In particular, D-mannitol has been used to reduce osmotic pressure in the ophthalmic field. Recently, it was reported that D-mannitol showed an antioxidative role by caused to the up-regulating the level of catalase, which is decreased by hydrogen peroxide[14]. Therefore, it is possible that the use of mannitol in combination with BAC may lessen the epithelial cell death caused by BAC alone.

In studies to evaluate the effects of BAC on corneal wound healing, the selection of the experimental animal is very important. The rat debrided corneal epithelium model has been used in studies aimed at the development of corneal healing drugs[15-17], and the mechanism of corneal wound healing in this model is similar to that in humans. In this study, we investigated the protective effects of mannitol on BAC-induced corneal damage using the rat debrided corneal epithelium and a human cornea epithelial cell line (HCE-T). In addition, we demonstrate the usefulness of a new preservative system for timolol maleate (TM) eye drops consisting of BAC and mannitol.

2 EXPERIMENTAL

2.1 Animals and reagents

Male Wistar rats and rabbits were housed under standard conditions (12 h/d fluorescent light (07:00-19:00), 25℃ room temperature), and allowed free access to a commercial diet (CE-2 or CR-3, Clea Japan Inc., Tokyo, Japan) and water. All procedures were performed in accordance with the Kinki University School of Pharmacy Committee Guidelines for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. D-mannitol (mannitol) was provided by Wako Pure Chemical Industries Ltd. (Osaka, Japan). Commercially available 0.5% TM eye drops (Timoptol®) and 0.4% Benoxil were obtained from Santen Pharmaceutical Co., Ltd. (Osaka, Japan). BAC was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). *Escherichia coli* (*E. coli*, ATCC 8739) was provided by the National Institute of Technology and Evaluation (Tokyo, Japan). All other chemicals used were of the highest purity commercially available.

2.2 Preparation of BAC solution and commercially available TM eye drops with or without mannitol

Mannitol and BAC were each dissolved in saline and used to prepare BAC solutions with or without mannitol, which were then filtered through a Minisart CE (pore size of 0.20 μm, Costar, MA, USA). The changes in the mannitol or BAC concentration was not observed with or without filtration [the mannitol and BAC concentration was analyzed by D-mannitol colorimetric assay kit, HPLC method (mobile phase, 20 mM sodium perchlorate/acetonitrile (2/8, v/v); wavelength, 265 nm), respectively]. BAC eye drops with or without mannitol were adjusted to pH 7.0 by the addition of 1N NaOH solution. For the preparation of commercially available TM eye drops with or without 0.5% mannitol, 0.04 mL of filtered saline or 5% mannitol solution was added to 1.96 mL of commercially available TM eye drops. In this study, the BAC (0.005%-0.02%) and mannitol (0.1%-1%) concentrations used were determined according to the generally used range in many ophthalmic solution products.

2.3 Instillation of eye drops in rats

Ten microliters of saline, BAC or commercially available TM eye drops were instilled into the eyes of rats subjected to corneal abrasion (next section) five times per day (9:00, 12:00, 15:00, 18:00 and 21:00). The eyes were kept open for approximately 1 min following instillation to prevent the eye drops from washing out.

2.4 Image analysis of corneal wound healing in rats

Seven-week-old rats were anesthetized with isoflurane, and a patch of corneal epithelium removed with a BD Micro-Sharp™ (blade 3.5 mm, 30°, Becton Dickinson, Fukushima, Japan) as described previously[15, 16, 18]. The areas of debrided corneal epithelium were as follows: saline, 10.38 ± 0.56 mm^2; mannitol, 11.01 ± 0.69 mm^2; 0.005% BAC with or without mannitol, 10.57 ± 0.68 mm^2, 10.82 ± 0.78 mm^2; 0.02% BAC with or without mannitol, 10.85 ± 0.84 mm^2, 10.63 ± 0.74 mm^2; commercially available TM eye drops with or without mannitol, 10.91 ± 0.84 mm^2, 11.06 ± 0.87 mm^2 (mean ± S.E. for 5-10 independent rat corneas). The debrided corneal epithelium was dyed by instilling a solution containing 1% fluorescein (Alcon Japan, Tokyo, Japan) and 0.4% Benoxil (Santen Pharmaceutical Co., Ltd., Osaka, Japan). Changes in the corneal wounds were monitored under a TRC-50X fundus camera (Topcon, Tokyo, Japan) equipped with a digital camera (EOS Kiss Digital N, Canon Inc., Tokyo, Japan)[15, 16, 18], and the images obtained were analyzed with Image J[19]. The amounts of corneal wound healing (%) were calculated according to equation 1:

$$\text{Corneal wound healing}(\%) = (\text{wound area}_{0h} - \text{wound area}_{12\text{-}36h})/\text{wound area}_{0h} \times 100 \quad (1)$$

The rates of corneal wound healing, represented by the corneal wound healing rate constant (k_H, h^{-1}), over the period 0-36 h after corneal epithelial abrasion were deter-

mined according to equation 2:

$$H_t = H_\infty \cdot (1 - e^{-k_H \cdot t}) \quad (2)$$

where t is time (0-36 h) after corneal abrasion, and H_∞ and H_t are the percentages of corneal wound healing (%) at time ∞ and t, respectively.

2.5 Cell culture and treatment

The immortalized human corneal epithelial cell line (HCE-T) developed by Araki-Sasaki *et al.*[20] was used in this study. HCE-T cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 (GIBCO, Tokyo, Japan) containing 5% (v/v) heat-inactivated fetal bovine serum and 0.1 mg/mL streptomycin and 1000 IU/mL penicillin (GIBCO, Tokyo, Japan). For experiments, HCE-T cells (1×10^4 cells) were seeded in 96-well microplates (IWAKI, Chiba, Japan). Saline or 0.02% BAC solution with or without 0.1%-1% mannitol was added to the cell cultures one day after seeding, and the cells were stimulated for 0-120 sec. Following stimulation, culture medium containing TetraColor One (SEIKAGAKU Co. Tokyo, Japan) was added, and the absorbance (Abs) at 490 nm was measured. Cell viability was calculated according to the manufacturer's instructions as represented by equation 3:

$$\text{Cell viability}(\%) = \text{Abs}_{\text{treatment}}/\text{Abs}_{\text{non-treatment}} \times 100 \quad (3)$$

2.6 Hemolysis of rabbit red blood cells (RBC) by the treatment of of BAC with or without mannitol

The blood was removed from the marginal ear vein of adult Japanese albino rabbits weighing 2.5 to 3.0 kg, and the 1 mL blood was mixed with 100 mL heparin (10 mg/mL). The mixture was centrifuged at 3,000 rpm for 5 min at 37℃, and the pellets was washed by phosphate buffered saline (pH 7.4). The resulting pellets was used as the red blood cells (RBC) in this study. The 40 μL RBC was incubated with isotonic saline containing 0.005% BAC or 0.5% mannitol for 20 min. After the incubation, the solution containing RBC was centrifuged at 2,300 rpm for 5 min at 37℃, and absorbance at 576 nm was measured. The rate of hemolysis was calculated according to the manufacturer's instructions as represented by equation 4:

$$\text{Rate of hemolysis}(\%) = \text{Abs}_{\text{treatment}}/\text{Abs}_{\text{non-treatment}} \times 100 \quad (4)$$

2.7 Antimicrobial activity of BAC with or without mannitol

BAC solutions with or without 0.5% mannitol were tested for antimicrobial activity against *E. coli* (ATCC 8739), which was selected based on Japanese Pharmacopoeia (JP) test protocols[21]. According to the standard methodology, the bulk dilution was split into 10 mL aliquots, each of which was inoculated with between 10^5 and 10^6 colony-forming units (CFU)/mL of *E. coli* (ATCC 8739) (1 organism per aliquot) and incubated in the presence of saline, mannitol or BAC solution with or without 0.5% mannitol at 20℃ to 25℃. Sampling and enumeration of the inoculated samples were done at 2, 7, 14 and 28 days. One mL aliquots were serially diluted in phosphate buffer, plated in duplicate on soybean-casein digest agar (Casein soya bean digest agar for JP general test, Wako, Osaka, Japan), and incubated at 31℃ for 3 days. Raw data counts were converted to log (CFU) values. Since the samples were diluted at least 1:10 at the time of testing, 10 CFU reduction is the lowest sensitivity allowed by the test.

2.8 *In vitro* transcorneal penetration of TM from commercially available TM eye drops with or without mannitol

The *in vitro* transcorneal penetration of commercially available TM eye drops with or without 0.5% mannitol was examined using the method of Iwata *et al.*[22]. Adult Japanese albino rabbits weighing 2.5 to 3.0 kg were killed by injecting a lethal dose of pentobarbital into the marginal ear vein. The eyes were removed and the corneas were carefully separated from other ocular tissues. The individual corneas were placed on a methacrylate cell designed for transcorneal penetration studies. The side of the chamber (donor chamber) exposed to the exterior surface of the cornea was filled with commercially available TM eye drops with or without 0.5% mannitol. The other side of the chamber (reservoir chamber) was filled with 10 mM HEPES buffer (pH 7.4) containing 136.2 mM NaCl, 5.3 mM KCl, 1.0 mM K_2HPO_4, 1.7 mM $CaCl_2$ and 5.5 mM glucose. The experiments were performed at 35℃ for 6 h. Fifty microliters of sample solution was withdrawn from the reservoir chamber at the indicated time intervals and replaced with the same volume of buffer. The TM concentrations of the samples were determined by the following HPLC method. Fifty microliters of filtrate was added to 50 μL methanol containing 10 μg propyl p-hydroxybenzoate (internal standard), and the mixture solution was filtered through a Chromatodisk 4A (pore size 0.45 μm, Kurabo Industries Ltd., Osaka, Japan). The solution (10 μL) was injected onto an Inertsil® ODS-3 (3 μm, column size: 2.1 mm × 50 mm) column (GL Science Co., Inc., Tokyo, Japan) on a Shimadzu LC-20AT system equipped with a column oven CTO-20A (Shimadzu Corp., Kyoto, Japan). The mobile phase consisted of 25 mM phosphate buffer (pH 7) containing 30% methanol and 10% acetonitrile at a flow rate of 0.2 mL/min, the column temperature was 35℃, and the wavelength for detection was 294 nm[16]. Corneal viability was monitored by measuring thickness (0.0625 cm, average for 5 rabbits; no significant changes in thickness were observed over the 6 h period). The obtained data were analyzed by following equations:

$$J_c = \frac{K_m \cdot D \cdot C_{TM}}{\delta} = K_p \cdot C_{TM} \quad (5)$$

$$\tau = \frac{\delta^2}{6D} \quad (6)$$

$$Q_t = J_c \cdot A \cdot (t - \tau) \quad (7)$$

where J_c is the TM penetration rate, K_m is the cornea/preparation partition coefficient, D is the diffusion constant within the cornea, C_{TM} is the TM content in the ophthalmic preparation, τ is the lag time, δ is thickness of the cornea (0.0625 cm, average of 4 rabbits), Q_t is the total amount of TM appearing in the reservoir solution at time t, and A is the effective area of the cornea (0.78 cm^2). J_c and τ were estimated by fitting each penetration profile to Eq. 7. The penetration coefficient through the cornea, K_p, is given by J_c/C_{TM}. A nonlinear least-squares computer program was employed for the calculation[16].

2.9 Measurement of intraocular pressure in rabbits

The enhancement of intraocular pressure (IOP) was induced in rabbits by keeping them in a dark room for 5 h (11:00–16:00)[23]. A calibrated tonometer TonoPen XL (Medtronic SOLAN, FL, USA) was used to monitor IOP after surface anesthesia (0.4% Benoxil ophthalmic solution) instilled into the rabbit eyes. IOP was monitored at intervals of 5-15 min for 90 min after the instillation of 40 μL of commercially available TM eye drops with or without mannitol. Differences in the IOPs (ΔIOP, mmHg) among rabbits receiving different preparations of drugs and saline were measured. The area under the curve for ΔIOP ($AUC_{\Delta IOP}$, mmHg·min) was calculated according to equation 8:

$$AUC_{\Delta IOP} = \int_0^t \Delta IOP dt \quad (8)$$

Briefly, $AUC_{\Delta IOP}$ was determined according to the trapezoidal rule up to the last IOP measurement point.

2.10 Statistical analysis

All values are presented as mean ± standard error of the mean (S.E.). Unpaired Student's t-test was used to evaluate statistical differences, and multiple groups were evaluated by one-way analysis of variance followed by Dunnett's multiple comparison. P values less than 0.05 were considered significant.

3 RESULTS

3.1 Preventive Effects of Mannitol on Corneal Damage Caused by BAC

Figure 1 shows the percent of corneal wound healing following the instillation of BAC solutions with or without mannitol or saline as a control. The levels of corneal wound healing in rat eyes instilled with saline were approximately

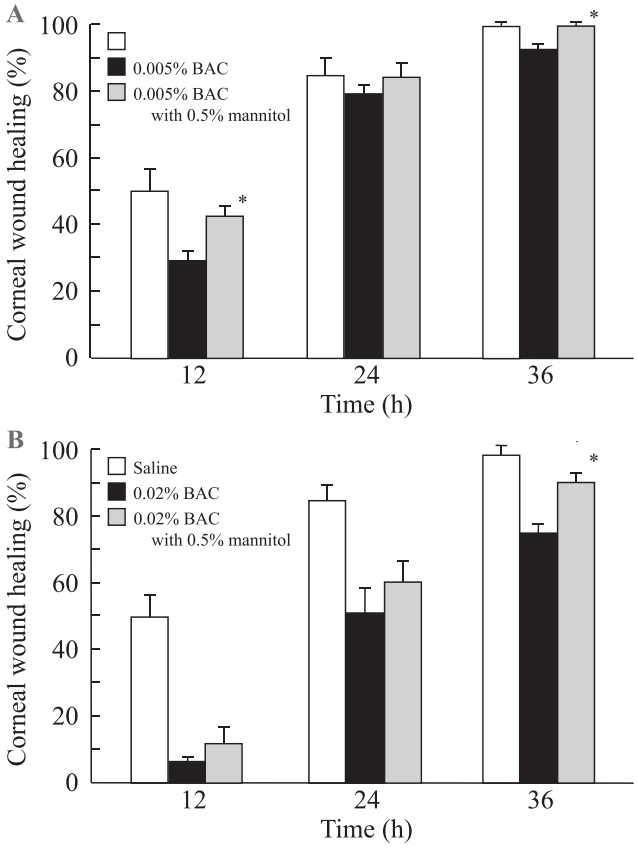

Fig. 1 Corneal Wound Healing in Rat Eyes Treated by the Instillation of 0.005% (A) or 0.02% (B) BAC solution containing 0.5% Mannitol.
The degree of corneal wound healing (%) was calculated according to equation 1 in EXPERIMENTAL. 0.005% (A) and 0.02% (B) BAC solutions with or without 0.5% mannitol were instilled into rat eyes five times per day. The data are presented as means ± S.E. of 5-10 independent rat corneas. *$p < 0.05$, vs. 0.005% or 0.02% BAC-instilled rat eyes.

51.4% at 12 h, and 86.2% at 24 h after corneal epithelial abrasion. The corneal wounds in rats instilled with saline were almost entirely healed at 36 h after corneal epithelial abrasion (99.6%). The corneal wounds of rat eyes instilled with 0.02% BAC solution were 75.0% healed while those instilled with 0.02% BAC solution containing 0.5% mannitol were 90.1% healed at 36 h after corneal epithelial abrasion. The rate constants (k_H) for rat eyes instilled with BAC solution containing mannitol was significantly higher than for eyes instilled with BAC solution without mannitol (saline, 5.33 ± 1.10; 0.5% mannitol, 5.40 ± 1.18; 0.005% BAC with or without 0.5% mannitol, 4.13 ± 0.85, 3.17 ± 0.77; 0.02% BAC with or without 0.5% mannitol, 1.76 ± 0.84*, 0.38 ± 0.10, $\times 10^{-2}$/h, means ± S.E., n = 5-10 independent rat corneas, *$p < 0.05$, vs. BAC solution without

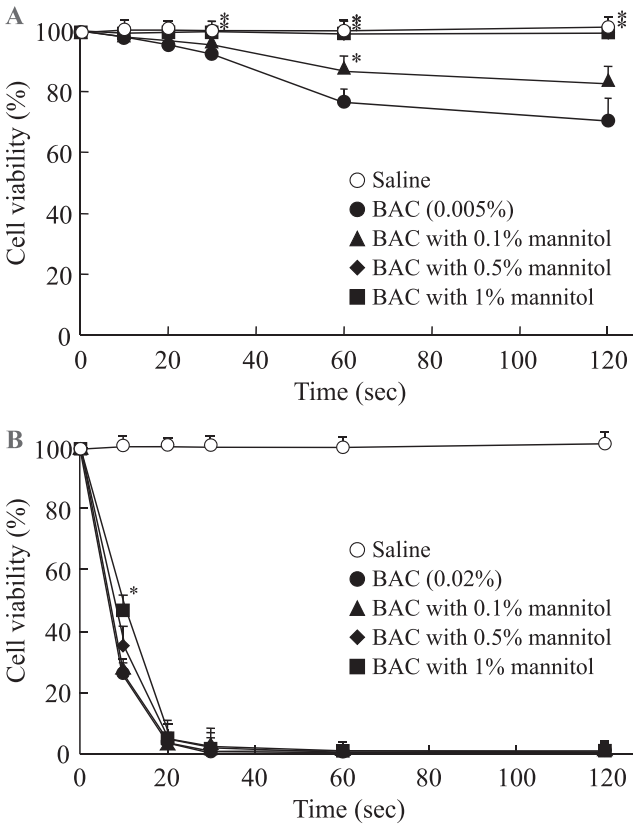

Fig. 2 Effect of BAC Solutions with or without Mannitol on the Viability of HCE-T Cells.
HCE-T cells in 96-well microplates were treated with 0.005% (A) and 0.02% (B) BAC solutions with or without 0.1 - 1% mannitol for 0 - 120 sec. Cell viability was calculated using TetraColor One according to equation 3 in EXPERIMENTAL. The data are presented as means ± S.E. of 8-15 experiments. *$p < 0.05$, vs. 0.005% or 0.02% BAC-treated HCE-T cells.

mannitol for each category). Figure 2 shows the changes in the viability of HCE-T cells following treatment with BAC solutions containing 0.1%-1% mannitol. The cell toxicity was not observed by the treatment of 1% mannitol (cell viability in 120 sec, 98.6 ± 3.4%, n = 6). The viability of HCE-T cells treated with 0.02% BAC solutions were lower than that with 0.005% BAC, and the viabilities of HCE-T cells treated with 0.02% BAC solution for 10 or 20 sec were approximately 26.9% or 4.1%, respectively. On the other hand, in the both groups treated with 0.005% and 0.02% BAC, the viability of HCE-T cells treated with the BAC solutions containing mannitol were higher, and the viability increased with increasing mannitol concentration. In this study, we investigated the effect of 0.005% BAC solutions containing 0.5% mannitol on rate of hemolysis of RBC, a model of cell injury. RBC membranes are broken by treatment with 0.005% BAC for 20 min with a hemolysis rate of 98.9 ± 1.6 (%, n = 6). However, the hemolysis rate when RBC are treated with 0.005% BAC containing 0.5% mannitol for 20 min is 40.1 ± 1.8 (%, n = 6). In addition, we also demonstrated that the antimicrobial activity of 0.005% BAC solution with or without 0.5% mannitol. No antimicrobial activity was observed by the addition of saline containing 0.5% mannitol. The addition of a 0.005% BAC solution containing 0.5% mannitol showed high antimicrobial activity approximately equal to that of BAC solution without mannitol.

3.2 Effects of Mannitol on Stimulation, Transcorneal Penetration and IOP of TM Eye Drops

Table 1 summarizes the pharmacokinetic parameters calculated from the data for corneal healing (Fig. 3), *in vitro* transcorneal penetration and changes in IOP. Figure 3 shows corneal wound healing following the instillation of commercially available TM eye drops with or without mannitol. The levels of corneal wound healing of rat eyes instilled with TM eye drops with and without mannitol were approximately 90.3% and 75.4%, respectively, 24 h after corneal epithelial abrasion. The k_H for rat eyes instilled with TM eye drops containing mannitol was also significantly higher than that of eyes instilled with TM eye drops without mannitol (Table 1). In the *in vitro* transcorneal penetration experiments, the amount of penetrated TM increased linearly for 6 h after the addition of TM eye drops with or without 0.5% mannitol into the donor chambers, and there were no significant differences in the amount of penetration between the two eye drop formulations (with or without 0.5% mannitol, Table 1). The IOP of rabbits kept in the dark for 5 h was found to rise by 7.1-9.6 mmHg as compared with untreated rabbits (16.5 mmHg). When the rabbits were treated with TM eye drops containing 0.5% mannitol before being placed in the dark, the increase in IOP was less, and the IOP reducing effects of TM eye drops both with and without mannitol were similar (Table 1).

4 DISCUSSION

In this study, we investigated the preventive effects of mannitol on corneal damage caused by BAC using rat debrided corneal epithelium and HCE-T cells. In addition, we determined the usefulness of BAC containing mannitol for the preparation of anti-glaucoma eye drops.

The instillation of BAC solution caused a delay in corneal wound healing in the rat debrided corneal epithelium, however, the addition of 0.5% mannitol to the BAC solution resulted in an increase the corneal wound healing rate (Fig. 1). In addition, we evaluated the effects of mannitol on the cell toxicity and antimicrobial activity of BAC using

Table 1 Effect of Commercially Available TM Eye Drops with or without 0.5% Mannitol on Corneal Wound Healing, Transcorneal Penetration and IOP.

	without mannitol	with mannitol
k_H ($\times 10^{-2}$/h)	2.46 ± 0.41	3.40 ± 0.43*
J_c ($\times 10^2$ nmol/cm^2/h)	4.21 ± 0.67	4.23 ± 0.61
k_p ($\times 10^{-4}$ cm^2/h)	2.40 ± 0.59	2.39 ± 0.51
k_m ($\times 10^{-4}$)	1.52 ± 0.34	1.54 ± 0.37
τ ($\times 10^{-1}$ h)	4.05 ± 0.01	4.01 ± 0.02
D ($\times 10^{-3}$ cm^2/h)	1.62 ± 0.01	1.64 ± 0.01
$AUC_{\Delta IOP}$ (mmHg·h)	4.43 ± 0.48	4.49 ± 0.50

These k_H, J_c, k_p, k_m, τ and D were analyzed using rat, and the $AUC_{\Delta IOP}$ was estimated using rabbit. These parameters were calculated according to equations 2, 5-7, and 8 (see EXPERIMENTAL). The data are presented as means ± S.E. of 5-6 independent rats or rabbits. *$p < 0.05$, vs. commercially available TM eye drops without mannitol for each category.

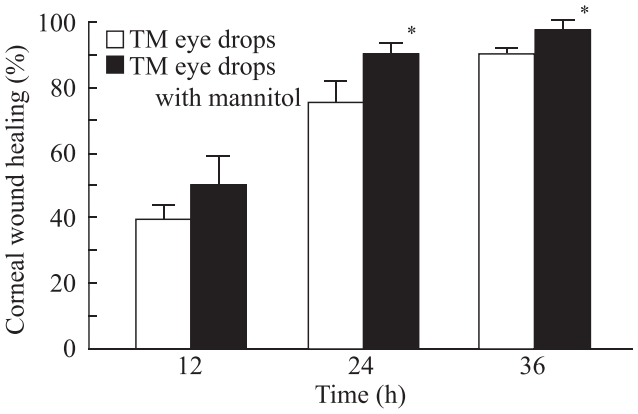

Fig. 3 Corneal Wound Healing of Rat Eyes Treated by the Instillation of Commercially Available TM Eye Drops with or without 0.5% Mannitol
The degree of corneal wound healing (%) was calculated according to equation 1 in EXPERIMENTAL. Commercially available TM eye drops with or without 0.5% mannitol were instilled into rat eyes five times per day. The data are presented as means ± S.E. of 5-6 independent rat corneas. *$p < 0.05$, vs. commercially available TM eye drop-instilled rat eyes.

HCE-T cells and *E. coli*, respectively (Fig. 2). The viability of HCE-T cells treated with BAC solutions containing mannitol was higher than that of cells treated with BAC solutions without mannitol, and the viability increased with increasing mannitol concentration. A 0.005% BAC solution containing 0.5% mannitol showed high antimicrobial activity approximately equal to that of TM eye drops with or without mannitol. Therefore, the data suggest that the mannitol diminishes some of the side effects of BAC. It is known that BAC changes the ionic resistance of the cornea by intercalating into cellular membranes, which results in membrane disruption via oxidative stress[8-10]. In this study, we investigated the effect of 0.005% BAC solutions containing 0.5% mannitol on rate of hemolysis of RBC, a model of cell injury. Although, RBC membranes are broken by treatment with 0.005% BAC, the hemolysis rate when RBC are treated with 0.005% BAC containing 0.5% mannitol was significantly lower than that in 0.005% BAC. From these results, mannitol may prevent the changes of the ionic resistance in the membrane by BAC stimulation, and protect the membrane disruption.

It is important to confirm the usefulness of BAC containing mannitol in the preparation of anti-glaucoma eye drops. Glaucoma is characterized by nerve degeneration that results in the disappearance of retinal ganglion cells, visual field loss, excavation of the optic disk, and ophthalmopathy[24,25]. The major risk factor for glaucoma is elevated IOP, which leads to apoptosis and a loss of retinal ganglion cells[26]. In treating glaucoma, the focus is on the reduction of IOP and the prevention of retinal and optic nerve damage. Anti-glaucoma eye drops are frequently used in clinical treatment, and treatment with anti-glaucoma eye drops must be continued in glaucoma patients even if they cause corneal damage. Recently, anti-glaucoma combination eye drops, such as Xalacom®(latanoprost/ TM combination eye drops), Duotrav®(travoprost/ TM combination eye drops) and Cosopt®(dorzolamide hydrochloride/ TM combination eye drops), have been developed. Eye drops containing TM are currently the most prescribed glaucoma medications, and the preservative used in eye drops containing TM is BAC. Therefore, we investigated the rate of corneal wound healing following the instillation of commercially available TM eye drops containing saline or mannitol using the rat debrided corneal epithelium model. The rate constants for corneal wound healing (k_H) for rat eyes instilled with TM eye drops containing mannitol were significantly higher than those of eyes instilled with TM eye drops without mannitol (Table 1). These results indicate that TM eye drops without mannitol are much more toxic than TM eye drops with mannitol. It is known that the surface-active effects of BAC increase the corneal penetration of the main component. Therefore, BAC solutions with or without mannitol were examined for their ability to penetrate the cornea and reduce IOP in rabbits. In an *in vitro* transcorneal penetration experiment, the amount of TM penetrating the cornea increased linearly up to 6 h after the addition of TM eye drops with or without 0.5% mannitol into the donor chambers, with no significant differences observed between the presence or absence of mannitol (Table 1). The instillation of TM eye drops containing

0.5% mannitol reduced the enhanced IOP of rabbits kept in the dark, and the IOP reducing effect of TM eye drops containing mannitol was similar to that of TM eye drops alone. These results indicate that the addition of mannitol may provide effective therapy for patients requiring long-term anti-glaucoma eye drops. Further studies are needed to elucidate the usefulness of BAC containing mannitol in anti-glaucoma eye drop preparations. In addition, it is important to clarify the mechanism to diminish some of the side effects of BAC. We also investigated the protective effect of other sugar alcohols (sorbitol and xylitol) on BAC stimulation using the HCE-T cell, and the viabilities of HCE-T cells treated with 0.005% BAC solution containing 1% sorbitol or xylitol for 120 sec were 96.5 ± 3.3%, 83.7 ± 2.9%, respectively (n = 7). Therefore, we are now investigating the relationships between the characterization of these sugar alcohols and protective effect against BAC toxicity.

5 CONCLUSIONS

In the present study, we demonstrate that BAC solutions containing mannitol are tolerated better on the ocular surface of rats than the classic BAC only preservative system. In addition, mannitol does not affect the antimicrobial activity of BAC against *E. coli* or the corneal penetration of TM from commercially available TM eye drops. A BAC plus mannitol preservative system may provide effective therapy for glaucoma patients requiring long-term anti-glaucoma agents.

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