

Sensitive and Real-Time Method for Evaluating Corneal Barrier Considering Tear Flow

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We developed a new electrophysiological method mimicking tear flow to evaluate the epithelial tight junction of rabbit cornea quantitatively. We investigated the effect of tear flow on the corneal damage induced by ophthalmic preservatives using this method. An Ussing chamber system with Ag/AgCl electrodes was used in the electrophysiological experiment. The excised rabbit cornea was mounted in the Ussing chamber and the pre-corneal solution in the chamber was perfused with a peristaltic pump at the rate of human tear flow. Corneal transepithelial electrical resistance (TEER) was monitored as corneal barrier ability. In the electrophysiological method mimicking tear flow, we observed stable TEER, which rapidly decreased with benzalkonium chloride (BAC), an eye drop preservative. Using this system, we first found that 0.004% BAC decreased corneal TEER reversibly. A high concentration of BAC showed strong irreversible damage to the tight junction. The influence of BAC on corneal TEER was not only concentration-dependent but also tear flow rate-dependent. The electrophysiological method mimicking tear flow was useful to evaluate the corneal barrier quantitatively. Using this method, we clarified that the tear flow was important to protect the corneal damage induced by preservatives.

Key words electrophysiological method; mimicking tear flow; corneal barrier; corneal transepithelial electrical resistance; Ussing chamber; benzalkonium chloride

In pharmacotherapy for ophthalmology, long-term use of eye drops has been shown to induce toxic histopathological changes to the ocular surface.¹⁾ Most barrier properties of the corneal epithelium apparently reside at the level of the outer membrane and an apical tight junction between surface epithelial cells produces the greatest resistance to diffusion across the paracellular pathway. The tight junction of the cornea is considered to be influenced by various ophthalmic ingredients,^{2,3)} and alteration of the corneal epithelium must damage the ocular surface.

In the previous study, we developed an electrophysiological method to quantitatively monitor the paracellular pathway of the corneal epithelium in real-time.⁴⁾ Transepithelial electrical resistance (TEER) was the most sensitive electrophysiological parameter for changes occurring with the exposure of eye drops to the corneal epithelium. We found two significant correlations between the cytotoxicity of preservatives and corneal paracellular permeability of fluorescein isothiocyanate dextran (FD-4, average molecular weight of 4400) and between the cytotoxicity of preservatives and conductance (reciprocal value of TEER).⁴⁾

Benzalkonium chloride (BAC) is a preservative often used for eye drops. Animal studies, *in vitro* studies and *in vivo* experiments have demonstrated various adverse effects of BAC.^{2,5-7)} BAC concentrations ranging from 0.01 to 0.04% caused some superficial punctate keratitis, increased superficial cell desquamation, and inhibited the corneal epithelial healing rate.^{2,5)} Clinical studies have also shown an increased incidence of adverse events with BAC. Often underrecognized are the significant cytotoxic effects of preservatives associated with long-term therapy and especially use of multiple preserved drugs.⁸⁻¹⁰⁾ On the other hand, Baudouin sug-

gests that dry eye and impaired tear film reduce the resistance of the cornea to the presence of toxic compounds.¹⁰⁾ However, there have been few reports to clarify the effect of tear flow on the corneal damage induced by BAC.

Therefore, we developed a new electrophysiological method mimicking tear flow in which precorneal solution was exchanged for buffer with a peristaltic pump at the rate of human tear flow. Using this new system, we investigated the effect of tear flow on the corneal damage induced by BAC.

MATERIALS AND METHODS

Animals Male Nippon albino rabbits (KBT: JW; KBT Oriental Co., Ltd., Tosu, Japan), 2.0–2.5 kg, were individually housed in cages in an air-conditioned room and maintained on a standard laboratory diet (ORC4; Oriental Yeast Co., Ltd., Tokyo, Japan). The rabbits were starved for 24 h before use but had free access to water. All experiments conformed to the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23), the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Research, and the Declaration of Helsinki. Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval from the Institutional Animal Care and Use Committee.

Materials FD-4 was obtained from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.). Benzalkonium chloride (BAC) and oxidized glutathione were commercially obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

Electrophysiological Experiment The Ussing chamber

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system (World Precision Instruments, FL, U.S.A.) was used in the electrophysiological experiment. Ag/AgCl half-cells were screwed into short tubes, which plugged firmly into the chamber luer ports. Rabbits were sacrificed by injecting an overdose of sodium pentobarbital into a marginal ear vein. The cornea was dissected and washed with warm glutathione-bicarbonated Ringer's solution (GBR).¹¹ GBR is a pH 7.4 isotonic solution containing 106 mM NaCl, 4.8 mM KCl, 0.66 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 29.2 mM NaHCO_3 , 0.78 mM CaCl_2 , 0.78 mM MgCl_2 , 5.0 mM D-glucose, and 0.15 mM oxidized glutathione. The excised cornea was immediately mounted on the Ussing chamber CHM 1 (World Precision Instruments) using rubber supporters and O-rings. Figures 1A, B and C show photographs and the scheme of the electrophysiological experiment mimicking tear flow. The chambers were filled with GBR, and aeration and circulation in the tissue bath were provided by bubbling with a mixture of 95% O_2 and 5% CO_2 . Corneas with high membrane resistance (mean \pm S.E.: $1122.3 \pm 61.3 \text{ ohm} \times \text{cm}^2$) were used in this study. All experiments were conducted at 37°C using a constant-temperature bath connected to the jacket of the Ussing chambers. A 0.53-cm^2 area of tissue was exposed to donor and receiver compartments, with volumes of 6 ml and 7 ml, respectively.

The electrical output of the Ag/AgCl electrodes was fed to an automatic voltage-clamp unit (CEZ-9100; Nihon Koden, Tokyo, Japan) and spontaneous potential difference (PD) was measured with two matched Ag/AgCl electrodes. A direct current was sent across the tissue with a pair of matched Ag/AgCl electrodes whose tips were positioned away from the tissue surfaces at the far end of the two reservoirs. Current flow in the bath-tissue-bath circuit under short-circuit conditions was monitored and the short-circuit current (Isc) was

measured as the current passing through the cornea under zero voltage clamp conditions. At 60-sec intervals, a 10-mV voltage pulse was imposed for 1 sec across the short-circuited tissue to estimate corneal TEER.

After 80-min preincubation, isotonic pH 7.4 ophthalmic solution (GBR containing BAC 0.004, 0.02 or 0.1%) was applied to the precorneal side and preparations displaced half of the buffer on the precorneal side. After the ophthalmic solution was added, the precorneal side was immediately perfused with a peristaltic pump at a rate of 0.96 ml/min (16%/min) with fresh GBR for 160 min. In another experiment, the precorneal side was perfused with a peristaltic pump at a rate of 0.48 ml/min (8%/min) as a poor tear flow (dry-eyes) and the precorneal side was not perfused as the static tear system. TEER was determined in the electrophysiological experiment at 10-min intervals for 160 min. In order to confirm tear flow, FD-4 was added to the precorneal side and determined fluorometrically.

Figure 1D shows an equivalent circuit model for corneal epithelium and electrophysiological parameters (PD, Isc and TEER). Transepithelial electrical resistance was described as follows: $\text{TEER} = R_L \times (R_a + R_b) / (R_L + R_a + R_b)$. As the electrical resistance of apical and basolateral cell membranes (R_a and R_b) was much higher than that of tight junctional resistance ($R_a + R_b \gg R_L$), TEER reflects tight junctional conductance (reciprocal value of TEER); therefore, TEER, in general, is sensitive to changes occurring in the paracellular permeability of epithelial tissues.¹²

Drug Determination Samples for FD-4 were determined with a spectrofluorophotometer (FP-770; Jasco, Tokyo, Japan) at an excitation wavelength of 489 nm and an emission wavelength of 515 nm.

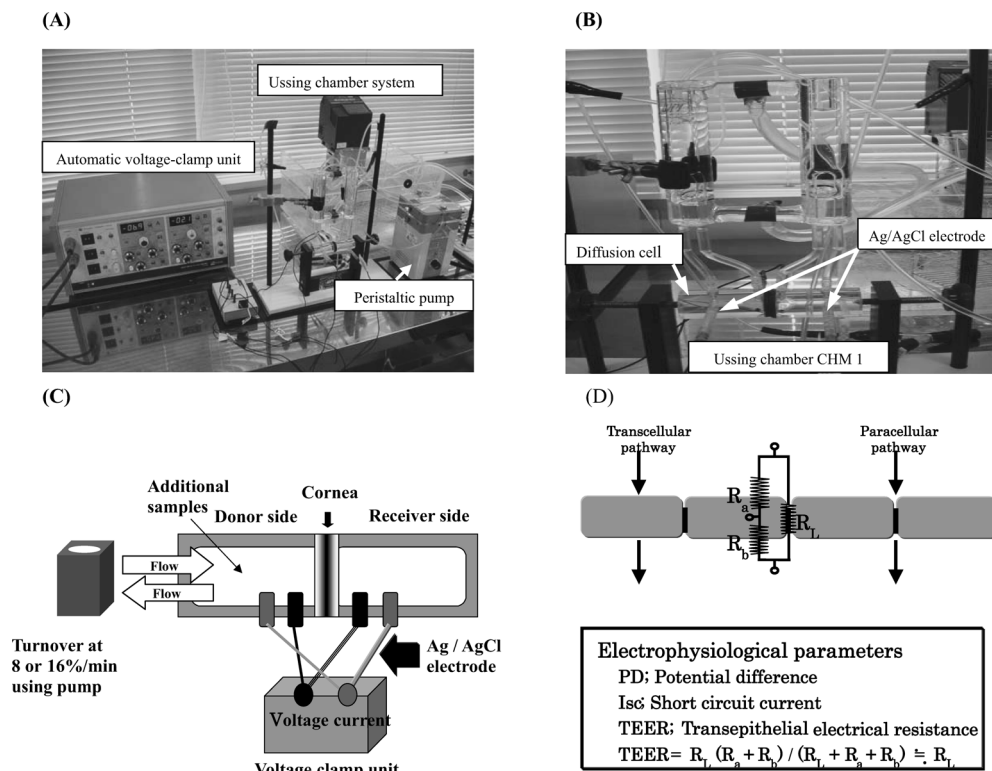


Fig. 1. Photographs (A, B) and Schematic Illustration (C) of Electrophysiological Device, and Equivalent Circuit Model for Corneal Epithelium (D)

RESULTS

Electrophysiological Method with Tear Flow Figure 2 shows the elimination profile of FD-4 from the precorneal side mimicking tear flow. Concentration of FD-4 (Y) was eliminated with time (X) at a first-order rate constant (Ke) of 0.0027/sec or 0.16/min from the donor phase. Corneal TEER was approximately 1000 ohm×cm² at steady state after 80-min incubation. Penetration of FD-4 from the donor side was not observed on the endothelial side.

Effect of BAC on Corneal Epithelial Barrier Figure 3

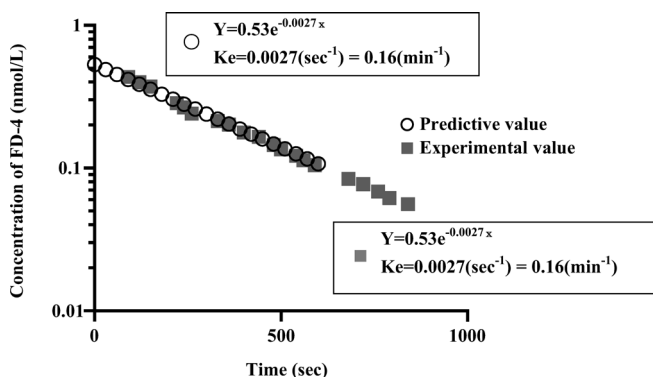


Fig. 2. Elimination Profile of Fluorescein Isothiocyanate Dextran (FD-4) from Donor Phase When Mimicking Tear Flow

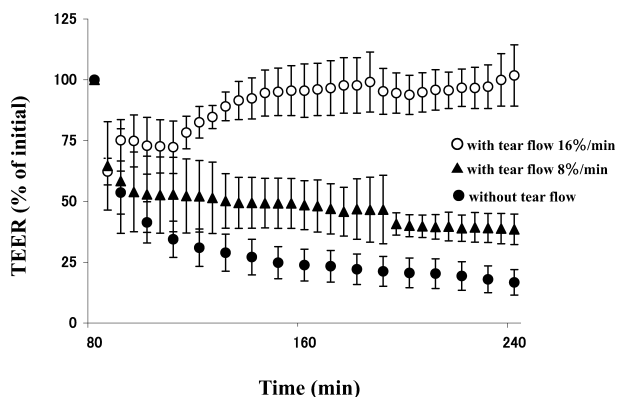


Fig. 3. Effect of 0.004% Benzalkonium Chloride (BAC) on Corneal Transepithelial Electrical Resistance (TEER) in the System with or without Tear Flow

Tear flow is prepared in 16%/min and 8%/min turnover. Each value represents the mean±S.E. of three experiments.

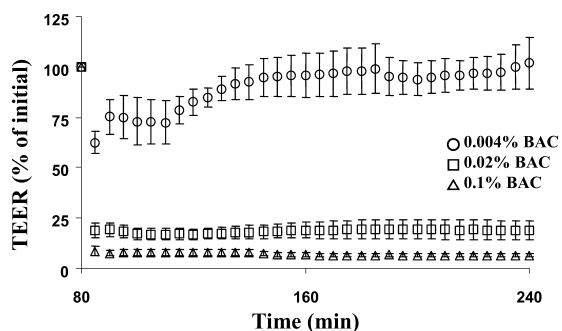


Fig. 4. Effect of Benzalkonium Chloride (BAC) Concentrations on Corneal Transepithelial Electrical Resistance (TEER) in the Tear Flow Mimicking System

Each value represents the mean±S.E. of three experiments.

shows the effect of 0.004% BAC on corneal TEER in 8%/min or 16%/min tear flow, and the effect was compared with that in the static tear system. The 0.004% BAC rapidly decreased corneal TEER for 160 min in the static tear system. In 16%/min tear flow, however, corneal TEER decreased slightly by 0.004% BAC and recovered after 40 min. On the other hand, in 8%/min tear flow, corneal TEER decreased slightly and didn't recover.

Figure 4 shows the effect of BAC concentration on corneal TEER in tear flow. A high concentration (0.02, 0.1%) of BAC rapidly decreased corneal TEER and did not recover the initial level of corneal TEER. The decrease of corneal TEER was dependent on the concentration of BAC.

DISCUSSION

The cornea is generally recognized as the major route of ocular penetration for topically instilled drugs. Three primary layers indicate corneal composite structures: the epithelium, stroma and endothelium,¹³⁾ of which the epithelium, being lipoidal in nature, is considered to contribute to the corneal penetration barrier of particularly hydrophilic drugs. An apical tight junction between surface epithelial cells produces the greatest resistance to diffusion across the paracellular pathway. Tight junctions, sealing the paracellular pathway of the epithelial barrier, are elaborate structures composed of integral membrane proteins, linker or adaptor proteins connecting them to the actin cytoskeleton. Some junctional proteins, occluding, claudins and tricellulin, can play an important role in the regulation of epithelial paracellular permeability.^{14,15)}

The paracellular pathway of epithelial tissues is often evaluated using an electrophysiological method. TEER, in general, is sensitive to changes occurring in the paracellular permeability of epithelial tissues.^{12,16)} We found a significant correlation between the cytotoxicity of preservatives and paracellular permeability of FD-4, and between the cytotoxicity of preservatives and conductance (reciprocal value of TEER).⁴⁾ Rojanasakul *et al.*¹⁷⁾ compared TEER of various epithelial tissues and demonstrated that the corneal membrane is very tight compared with other tissues. During preincubation, TEER reached approximately 1000 ohm×cm² within 80 min after reaching the cornea, and the permeability of FD-4 was extremely low. TEER in the present study is consistent with the value in a previous report.⁴⁾

To ensure proper vision, the eye has protective mechanisms to maintain the ocular surface and to eliminate foreign matter.¹⁸⁾ Among these protective mechanisms, tear flow is very important and is reported to be approximately 16%/min in humans.^{19,20)} Most instilled drugs are rapidly eliminated from the precorneal area by tear flow^{19,20)}; therefore, we designed a new electrophysiological method using an Ussing chamber system and considering human tear flow. The donor phase solution was exchanged with GBR solution by a peristaltic pump at a rate of 16%/min as a normal tear flow or 8%/min as a poor tear flow (Figs. 1, 2). This method was very useful to determine the influence of tear flow on damage to the corneal barrier by instilled eye drops.

BAC is a powerful cationic surfactant that destroys bacteria after ionic attraction¹⁷⁾ and is frequently used as an ophthalmic preservative at concentrations between 0.002 and

0.01%.²¹⁾ Green¹³⁾ suggested that clinical concentrations of BAC in commercial eye drops were well-tolerated; however, 0.004% BAC rapidly decreased corneal TEER in the static tear system, suggesting that BAC influenced electrophysiological properties in the corneal epithelium even at clinical concentrations. BAC is incorporated with the lipid bilayer at low concentrations, changes the physical properties of cell membranes, and consequently regulates the tight junction.^{13,22)}

On the other hand, it is worth noting that the effect of 0.004% BAC on corneal TEER was reversible in the 16%/min tear flow (Fig. 3). Tear turnover clearly reduces the topical damage of eye drop preservatives to the ocular surface by dilution. This result indicates that tear flow is a strong protective mechanism of the corneal surface against harmful preservatives. However, a poor tear flow (8%/min) showed the extensive corneal damage induced by BAC. These results suggest that BAC is more harmful in poor tear flow patients. Therefore, we have to carefully use the eye drops containing preservative for dry-eye patients. In population-based studies, the prevalence of dry eye in elderly patients varies between 15 to 34%. Preservative-free eye drops may be beneficial clinically for elderly patients. The repeated treatment of eye drops must increase the corneal damage.

As BAC concentration increased, greater damage to the tight junction was indicated (Fig. 4). Furthermore, a high concentration of BAC caused irreversible damage to the cornea barrier. It was reported that lifting cell borders with desquamation was observed in the cornea exposed to a high concentration of BAC.^{5,6,23)} Irreversible damage to the cornea barrier may be explained by desquamation. These results indicate that the influence of BAC on corneal TEER was not only tear flow rate-dependent but also concentration-dependent. Irreversible damage may be caused by desquamation of epithelial cells.²⁴⁾ These phenomena were found clearly by using the electrophysiological method with tear flow for the first time. Further experiments may be necessary for the mechanism of preservatives on tight junction.

In conclusion, the new electrophysiological method with tear flow was sensitive and quantitative, and was useful for evaluating the corneal barrier in real-time. Using this method, we clarified that the tear flow was important to protect the corneal damage induced by preservatives.

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