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Influence of Alkyl Chain Length of Benzalkonium Chloride on Acute Corneal Epithelial Toxicity
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ABSTRACT
Purpose: To evaluate acute corneal epithelial toxicity induced by benzalkonium chloride (BAC) homologs with different alkyl chain lengths using an in vivo electrophysiological method.
Methods: BAC homologs with C12, C14, and C16 alkyl chain lengths were used at concentrations of 0.0025%, 0.005%, and 0.01%, respectively. Cytotoxicity of BAC homologs on the normal rabbit corneal epithelial cells was examined by using a WST-1 assay. Corneal transepithelial electrical resistance (TER) was measured in living Japanese white rabbits by 2 Ag/AgCl electrodes placed in the anterior aqueous chamber and on the cornea. TER changes were then evaluated after a 60-second exposure to these BAC homologs. Morphological changes in corneal epithelium after exposure to the BAC homologs were examined using scanning electron microscopy. The antimicrobial activity of BAC homologs against Escherichia coli was also assessed.
Results: All BAC homologs caused cytotoxicity and corneal barrier dysfunction in a concentration-dependent manner. However, the degree of corneal toxicity differed among the BAC homologs. Based on cytotoxicity and TER measurement, C14-BAC caused the greatest corneal impairment followed in order of severity by mixed BAC/C16-BAC and C12-BAC. Scanning electron microscopy images indicated an intact corneal epithelium after exposure to 0.005% C12-BAC, whereas 0.005% C14-BAC damaged the epithelium. There were no remarkable differences noted in the antimicrobial activity among the BAC homologs.
Conclusions: Acute corneal epithelial toxicity induced by BAC homologs depends on the alkyl chain length. Thus, the use of C12-BAC instead of commercially available BAC is potentially safer for patients undergoing ophthalmological pharmacotherapy.

Key Words: benzalkonium chloride; alkyl chain; eyedrop; cornea; barrier function
The most commonly used antimicrobial preservative in topical eyedrops is the quaternary ammonium cationic surfactant, benzalkonium chloride (BAC), which is a homologous mixture of N-alkyldimethylbenzylammonium chlorides with N-alkyl chain lengths ranging from C8 to C18. In ophthalmology, commonly administered pharmacotherapeutic agents contain commercially produced BAC, which consists of 3 homologs that have different N-alkyl chain lengths (C12, C14, and C16). On the other hand, numerous studies have revealed that there are deleterious corneal effects associated with BAC that include destabilization of the tear film, death of corneal and conjunctival epithelial cells, morphological changes in the corneal epithelial cells, and the reduction of the corneal epithelial barrier function.

It has been reported in previous studies that the carbon chain lengths of phosphatidylcholines or nonionic surfactants can influence the degree of hemolysis of red blood cells. Furthermore, BAC homologs with different alkyl chain lengths have been shown to influence the neuronal ablative activity in the rat jejunum and the mucosal toxicity in slugs when used during alternative tests for ocular irritation. These reports seem to suggest that BAC homologs can result in different degrees of ocular toxicity. However, presently there are few reports that have examined the BAC homolog-induced ocular surface impairment.

We have previously developed an in vivo method that can be used to measure the transepithelial electrical resistance (TER) of live rabbit corneas. This novel approach for examining the clinical instillation of eyedrops has revealed that exposure to BAC concentrations between 0.005% and 0.05% immediately causes acute corneal barrier dysfunction. In the present study, we used this in vivo corneal TER measurement system to evaluate the acute corneal epithelial toxicity induced by BAC homologs that contained C12, C14, and C16 alkyl chain lengths and their mixture. Furthermore, the toxicity was confirmed using a cytotoxicity assay in addition to scanning electron microscopy (SEM). We also assessed the antimicrobial activity of the different BAC homologs.
MATERIALS AND METHODS

Chemicals

Benzyldimethyldecylammonium chloride (C12-BAC), benzyldimethyltetradecylammonium chloride (C14-BAC), and benzyldimethylhexadecylammonium chloride (C16-BAC) were purchased from Sigma-Aldrich, Inc, (St Louis, MO). BAC 10% solution (mixed BAC) was obtained from Wako Pure Chemical, Co, (Osaka, Japan) and was composed of 3 BAC homologs that included approximately 67% C12-BAC, 28% C14-BAC, and 6% C16-BAC. Dojindo Laboratories, Inc, (Kumamoto, Japan) supplied the 2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium, monosodium salt (WST-1) and the 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS), whereas the rabbit corneal epithelial cell growth medium (RCGM) and the culture reagents were purchased from Kurabo, Co, Ltd, (Osaka, Japan). Hank balanced salt Ca2+ and Mg2+ free solution (HBSS) was obtained from Invitrogen, Corp, (Carlsbad, CA). BAC homolog test solutions were prepared in RCGM or HBSS. The concentrations for the BAC homologs were set at 0.0025%, 0.005%, and 0.01%.

Cytotoxicity Assay

The normal rabbit corneal epithelial (NRCE) cells at the second passage were obtained from Kurabo, Co, Ltd. The NRCE cells were maintained in RCGM, which was supplemented with 5 µg/mL of insulin, 10 ng/mL of epidermal growth factor, 0.5 µg/mL of hydrocortisone, 50 µg/mL of gentamicin, 50 ng/mL of amphotericin B, and 0.4% bovine pituitary extractives reagent. The cells were grown at 37°C in a humidified atmosphere with 5% CO2. Cytotoxicity tests on NRCE cells were carried out using a WST-1 commercially available cell proliferation reagent. The assay was based on the cleavage of the tetrazolium salt WST-1 by active mitochondria, which led to the production of a soluble colored formazan salt. Because this conversion can only occur in the presence of viable cells, results are directly correlated to the cell number. The stock solutions of WST-1 (5.5 mM) and 1-methoxy PMS (2 mM) were prepared in sterilized phosphate-buffered saline. Just before each of the experiments, the mixture was prepared by combining 0.9 mL of WST-1 solution and 0.1 mL of 1-methoxy PMS solution. NRCE cells were plated at 3 × 10^3 cells per well in 96-well microtiter plates (Becton-Dickinson, Franklin, NJ). At 6 days after plating, the growth medium was replaced with 100 µL of the test solutions for 60 seconds. The cells were washed with fresh growth medium, with 100 µL of fresh growth medium and 10 µL of WST-1 mixture
solution, and then added to each well. After incubation of the cells for 3 hours at 37°C, a microplate reader (Thermo Fisher Scientific, Inc, Waltham, MA) was used to measure the absorbance at a wavelength of 450 nm, with a reference wavelength of 630 nm. The results are presented as a percentage of the untreated cells. The procedure for the cytotoxicity assay with the short-time exposure was performed in accordance with a previously published method.20

Experimental Animals

Japanese white male rabbits (KBT Oriental, Tosu, Japan) weighing 2.5-3.0 kg were individually housed in cages under a controlled temperature (21°C) and humidity (50% ± 5%) and a 12:12-hour light/dark cycle at the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine. Initiation of the study occurred once the rabbits had reached weights of 3.0-4.0 kg, which is the point where corneal diameters were of a suitable size for experimentation. The rabbits had free access to food and water. All experiments in the present study confirmed to the guiding principles in the care and use of animals (DHEW Publication, National Institutes of Health 80-23), the Association for Research in Vision and Ophthalmology Resolution for the use of animals in ophthalmic research, and the Declaration of Helsinki.

Corneal TER Measurements In Vivo

The rabbits were anesthetized with an intramuscular injection of 30·mg/kg ketamine (Ketalar, Sankyo, Tokyo, Japan) and 5·mg/kg xylazine (Celactal; Bayer Health Care, Osaka, Japan). The experimental procedure was started within 10 minutes of the induction of anesthesia. After a slit-lamp examination of the eyes to confirm that the cornea was intact, adhesive tape was applied so that one eye was kept open, whereas the other was kept closed.

After a small incision was made with an 18-gauge sharp needle (Terumo, Tokyo, Japan) in the peripheral cornea, a 1.0-mm diameter custom-made Ag/AgCl electrode (Physiotech, Tokyo, Japan) was inserted into the anterior chamber. A 6.0-mm internal diameter (0.28-cm² inner area) nitrile rubber O-ring (Union Packing; SAN-EI, Osaka, Japan) was fixed on the cornea using biomedical adhesive (Alon-Alpha A; Sankyo, Tokyo, Japan). Subsequently, 80 µL of HBSS was placed inside the ring, with the second electrode then placed in HBSS on the cornea. This initial procedure was carefully conducted to avoid damaging the center of the cornea.

The TER was measured in real time using a volt-ohm meter (EVOMX; World Precision Instruments, Sarasota, FL) that generated a ±20 µA AC square wave current.
at 12.5 Hz. Data were recorded using a thermal array recorder (WR300-8; Graphtec, Tokyo, Japan). In a period of just a few seconds, 1 mL of the test solutions were gently poured into the ring, with all of the overflow aspirated, followed by exposure for 60 seconds. The results were then calculated as a percentage of the preexposure TER value (100%). This specific methodology and photographs of the in vivo corneal TER measurement system have been previously published.19,20

**SEM Observation**

The rabbits were anesthetized with an intramuscular injection of 30-mg/kg ketamine and 5-mg/kg xylazine. Corneas were evenly soaked in the BAC homologs solution for 60 seconds. After the corneal washing, the rabbits were immediately killed using a lethal dose of intravenous sodium pentobarbital (Nembutal; Dainippon Pharmaceutical, Japan). The corneas were carefully excised, fixed in 4% glutaraldehyde in 0.05 M of cacodylate buffer for 1 hour, and then postfixed in 1% osmium tetroxide in veronal acetate buffer containing 0.22 M of sucrose. The fixed materials were dehydrated through a series of ethanol washes. Corneas were placed in t-butyl alcohol, treated in a freeze-drying apparatus (EIKO ID-2; EIKO, Japan), and spatter coated with gold using an auto fine coater (JEOL JFC-1600; JEOL, Japan). After processing, the surface of the corneal epithelium was observed by a scanning electron microscope (Hitachi S2360; Hitachi, Japan).

**Minimum Inhibitory Concentration Determination**

Minimum inhibitory concentrations (MICs) of the antimicrobial agents were determined by a microdilution method that used a cation-adjusted Luria-Bertani broth in accordance with the recommendations of the National Committee for Clinical Laboratory Standards. BAC homologs were diluted 2-fold serially, ranging from 6.25 to 100 µg/mL (0.000625%-0.01%), with 20 mL of the media then dispensed into the 100-mm diameter dishes (Corning Glass Works, NY). All of the media containing each of the BAC homologs were inoculated with 1 × 10^6 colony-forming units of Escherichia coli strain XL-1 blue that was suspended in phosphate-buffered saline, with the dishes then incubated for 24 hours at 37°C. The lowest concentration in which the colony of E. coli was not observed was defined as the MIC.

**Statistical Analysis**

The results of the cytotoxicity assay and TER measurement were expressed as the mean ± standard error of at least 3 experiments. The results of the MIC determination
were expressed as the median of 3 experiments. Statistical comparisons were performed by analysis of variance followed by Scheffe test for the cytotoxicity assay and TER measurement. Values of P < 0.05 were considered to indicate statistical significance.
RESULTS

Cytotoxicity Assay

Cytotoxicity of BAC homologs on the NRCE cells was confirmed through the use of a WST-1 assay (Fig. 1). All of BAC homologs showed a concentration-dependent cytotoxicity as compared with the untreated cells (control). Cell viability after C12-BAC exposure was 67.3%, 48.9%, and 31.5% for 0.0025%, 0.005%, and 0.01% of the solution, respectively. Cytotoxicity of C12-BAC was milder at all of the concentrations employed when compared with the other BAC homologs. On the other hand, cell viability after C14-BAC exposure was 41.8%, 21.6%, and 7.4% for 0.0025%, 0.005%, and 0.01% of the solution, respectively. C14-BAC induced the highest cytotoxicity for all of the concentrations used. For C16-BAC and mixed BAC, cytotoxicities were found to be between those of C12-BAC and C14-BAC.

FIGURE 1. Cytotoxicity assay of BAC homologs (●, C12-BAC; ■, C14-BAC; ▲, C16-BAC; □, mixed BAC) to NRCE cells. NRCE cells were exposed for 60 seconds. Data represent the percentage of the untreated cells (control). Each value is the mean ± standard error. (n = 6). *P < 0.05, **P < 0.01 as compared with untreated cells.
**TER Change**

Corneal TER changes after exposure to BAC homologs for 60 seconds are shown in Figure 2, whereas Table 1 lists the TER values after a 60-second exposure. The corneal exposure to HBSS, which served as the control, did not cause any changes in the TER. After exposure to 0.0025% BAC homologs for 60 seconds, C12-BAC and C16-BAC did not induce any significant changes in the TER, whereas mixed BAC and C14-BAC caused corneal TER to decrease by 83.6% and 61.5%, respectively (Fig. 2A). Although exposure to 0.005% BAC homologs resulted in no significant TER changes for C12-BAC, there were decreases of 71.5% and 61.1% for mixed BAC and C16-BAC, respectively. Furthermore, the lowest corneal TER value (34.1%) was noted for C14-BAC (Fig. 2B). Similarly, after exposure to the 0.01% BAC homologs, TER decreases were observed for C12-BAC, C16-BAC, mixed BAC, and C14-BAC, with values of 65.6%, 43.7%, 42.9%, and 19.5%, respectively (Fig. 2C). A concentration-dependent corneal barrier dysfunction was noted for all of the BAC homologs. Among these, C12-BAC exhibited the lowest corneal impairment, whereas C14-BAC induced the most severe corneal barrier dysfunction (Table 1).

<table>
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<th>Relative TER Value (%)</th>
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<tr>
<td><strong>HBSS</strong></td>
<td>100.6 ± 2.0</td>
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<td><strong>0.0025%</strong></td>
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<tr>
<td>C12-BAC</td>
<td>97.7 ± 1.2</td>
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<tr>
<td>Mixed BAC</td>
<td>83.6 ± 1.9*</td>
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<tr>
<td>C16-BAC</td>
<td>94.8 ± 1.5</td>
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<td><strong>0.005%</strong></td>
<td></td>
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<tr>
<td>C12-BAC</td>
<td>92.2 ± 5.5</td>
</tr>
<tr>
<td>Mixed BAC</td>
<td>71.5 ± 2.5**†</td>
</tr>
<tr>
<td>C16-BAC</td>
<td>61.1 ± 1.2***††</td>
</tr>
<tr>
<td>C14-BAC</td>
<td>61.5 ± 5.5**††§§§§</td>
</tr>
<tr>
<td><strong>0.01%</strong></td>
<td></td>
</tr>
<tr>
<td>C12-BAC</td>
<td>65.6 ± 7.0*</td>
</tr>
<tr>
<td>Mixed BAC</td>
<td>42.9 ± 6.2**</td>
</tr>
<tr>
<td>C16-BAC</td>
<td>43.7 ± 6.2**</td>
</tr>
<tr>
<td>C14-BAC</td>
<td>19.5 ± 1.2**†</td>
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Each value is the mean ± SE (n = 3-4). *P < 0.05, **P < 0.01 as compared with HBSS. †P < 0.05, ††P < 0.01 as compared with C12-BAC. §P < 0.05, §§P < 0.01 as compared with mixed BAC. §§§P < 0.01 as compared with C16-BAC.

**TABLE 1. Corneal TER Value After a 60-Second Exposure to BAC Homologs**
FIGURE 2. Corneal TER changes after a 60-second exposure to HBSS (white circle) and BAC homologs (●, C12-BAC; ■, C14-BAC; ▲, C16-BAC; □, mixed BAC). BAC homolog concentrations were 0.0025% (A), 0.005% (B), and 0.01% (C). Data represent the percentage of the preexposure TER value (100%). Each value is the mean ± standard error. (n = 3-4).
SEM Observation

Figures 3 and 4 show the SEM images of the corneal epithelium after 60-second exposures to 0.005% C12-BAC and C14-BAC, respectively. Although the corneal epithelium exposed to 0.005% C12-BAC exhibited a regular appearance of the superficial cells with a high density of microvilli (Fig. 3), there were detaching superficial cells and degenerated microvilli observed in the corneal epithelium after exposure to 0.005% C14-BAC (Fig. 4).

FIGURE 3. SEM images of the corneal epithelium after a 60-second corneal exposure to 0.005% C12-BAC. Magnification: ×2000 (A), and ×12,000 (B).

FIGURE 4. SEM images of the corneal epithelium after a 60-second corneal exposure to 0.005% C14-BAC. Magnification: ×2000 (A), and ×12,000 (B).

MIC Determination

The antimicrobial activities of the BAC homologs against E. coli were determined using a microdilution method that employed a serial 2-fold dilution. Equivalent MICs of 25 µg/mL (0.0025%) were noted for all of the BAC homologs.
DISCUSSION

The corneal epithelium, which may be in direct contact with topically instilled drugs, is recognized as the primary source of the corneal barrier function. Therefore, evaluations of corneal epithelial barrier function have been performed to assess the corneal toxicity of ophthalmic agents, including BAC. Tear flow also plays an important role in the protective mechanism that eliminates instilled drugs from the precorneal area. The turnover of tears rapidly dilutes the instilled BAC to 26% of its original concentration in 1 minute and to 9% in 5 minutes. This fact led us to realize that corneal toxicity assays performed over long periods of time do not sufficiently reflect actual clinical conditions, whereas evaluations of BAC-induced corneal toxicity over short periods of time could provide valuable data. In the current study, we used a corneal exposure period of 60 seconds to evaluate the acute corneal epithelial effect induced by the BAC homologs.

We have previously reported that the cytotoxicity assay is a useful method for estimating the corneal epithelial barrier function. Thus, we used this assay to initially confirm the corneal change that is induced by the BAC homologs on NRCE cells. The concentrations for each of the homologs were set at 0.0025%-0.01%, values that correspond to those found in the clinically used ophthalmic solutions. Results of the cytotoxicity assay demonstrated that all of the BAC homologs caused a concentration-dependent cytotoxicity. The results also indicated that there was a tendency for C12-BAC to induce lower toxicity, whereas C-14 BAC was responsible for inducing a much more severe cytotoxicity. These results suggest that the BAC carbon alkyl length can influence the degree of the cytotoxicity. Based on our initial findings, we performed further in vivo experiments designed to electrophysiologically examine the influence of BAC homologs on live rabbit cornea.

We have previously developed an in vivo corneal TER measurement system that makes it possible to measure corneal changes both continuously and quantitatively within only a few seconds. In general, the TER reflects the barrier function of the epithelium. The lower corneal TER values are indicative of the penetration of greater amounts of electrical current through the damaged superficial cells and tight junctions that exists in the epithelium. By using this method, we were able to determine that the TER of the cornea of normal live rabbits was $821.8 \pm 36.6 \text{[OMEGA] cm}^2$ (43 eyes). This value is similar to our previous data and to that reported for previous studies that have used the conventional Ussing chamber system. Among BAC homologs, lower concentrations of C12-BAC did not cause any changes in the TER,
whereas C-14 BAC exhibited the lowest TER for all the concentrations tested. Overall, our results suggest that acute corneal barrier dysfunction is the greatest for C14-BAC, followed by mixed BAC/C16-BAC, and then C12-BAC. The decrease in TER that was caused by the mixed BAC is attributable to the synergic activity of the BAC homologs that were of various concentrations within the mixed BAC solution. The study results suggest that the toxicity may primarily be attributable to C14-BAC, which makes up approximately 28% of the solution.

We were able to confirm the BAC homolog-caused corneal impairment by performing a histological analysis using SEM. The SEM-based histological analyses of the corneas treated with 0.005% C12-BAC indicated that the appearance of the superficial layer was almost intact. However, there was an apparent damage of the corneal epithelium in corneas exposed to 0.005% C14-BAC.

A previous study has also demonstrated that the alkyl chain length influences the physical property of BAC.30 It is known that the longer the hydrocarbon chain, the greater the tendency to interact with the cell membrane via stronger interfacial activity. It has also been shown that aqueous solubility is an important factor with regard to BAC activity and that longer alkyl chain length causes a decrease in the aqueous solubility. Therefore, the magnitude of the BAC surface activity is dependent upon a balance between these 2 physical properties. Thus, the more severe corneal toxicity that was seen for C14-BAC may be because of this combination. However, all of the concentrations of the BAC homologs used in this study were soluble in water. Further studies will need to be undertaken to elucidate the mechanism of BAC’s toxicity. Another previous study has confirmed the influence of the BAC homologs on the neuronal ablative activity in the rat jejunum.17 In this particular study, parabolic structure activity was observed among the BAC homologs that had alkyl chain lengths ranging from C6 to C18, whereas C14-BAC mostly caused ablated neurons of the myenteric plexus and produced thickening of intestinal smooth muscle. These results are consistent with our present study and agree with the above-mentioned theory.

As a final step, we investigated whether or not the BAC homologs have an influence on antimicrobial activity. E. coli was chosen as the representative susceptible species, and we made sure that the MICs of the BAC homologs were of the same value. Thus, we can assume that there were no remarkable differences in the antimicrobial activity among the BAC homologs in the present study. In several studies that previously measured the antimicrobial activity of the BAC homologs, results indicated that the antimicrobial activity of BAC homologs differed in accordance with the bacterial species employed.17,31 To definitively elucidate the details of the antimicrobial activity of the
BAC homologs, further studies that use several different bacterial species will be necessary.

From the present study, we can conclude that the BAC homolog-induced acute corneal epithelial toxicity is dependent upon the alkyl chain length. Among the BAC homologs, C12-BAC exhibited the lowest corneal impairment, whereas C14-BAC induced the most severe impairment. Therefore, the current data suggest that use of C12-BAC instead of the commercially available BAC might provide greater security and safety for patients during ophthalmological pharmacotherapy.

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