



Impact of short-term exposure of commercial eyedrops preserved with benzalkonium chloride on precorneal mucin

So-Hyang Chung,¹ Su Kyung Lee,¹ Stephen M. Cristol,² Eun Suk Lee,³ Dong Wook Lee,⁴ Kyoung Yul Seo,¹ Eung Kweon Kim^{1,5}

¹Institute of Vision Research, Department of Ophthalmology and ³Brain Korea 21, Division of Medical Sciences, Yonsei University College of Medicine, Seoul, Korea; ²Department of Ophthalmology, Emory Eye Center, Emory University, Atlanta, GA; ³Department of Ophthalmology, Inha University College of Medicine, Incheon, Korea; ⁴Mokdong Yonsei Eye Clinic, Seoul, Korea

Purpose: The aim of this study is to investigate the short-term effects of benzalkonium chloride (BAC), a preservative used in many ophthalmic topical solutions, on precorneal mucin in humans.

Methods: Immortalized human corneal-limbal epithelial (HCLE) cells were exposed to eyedrops containing BAC solutions at 0.0025% and 0.01% concentrations for a period of 15 min. Human corneal epithelium was acquired with consent, as a byproduct of elective excimer photorefractive keratectomy procedures after application of Ocuflax® eyedrops (0.3% ofloxacin with 0.0025% BAC) for 1 week before surgery. The relative expression of the MUC1 and MUC16 mucin gene was determined by conventional and real-time reverse transcription-polymerase chain reaction (RT-PCR). Monoclonal antibodies for MUC1 (HMFG-1) and MUC16 (OC125) were used in western blot analysis to detect MUC1 and MUC16. Human corneas exposed to 0.01% BAC solutions were examined by transmission electron microscopy.

Results: The expression of *MUC1* and *MUC16* gene transcripts was not changed after exposure to BAC in HCLE cells and human corneal epithelium. However, MUC1 and MUC16 were reduced after exposure to BAC in HCLE cells and human corneal epithelium. Transmission electron microscopy of the anterior corneal surface revealed fixation of the mucous layer after exposure to 0.01% BAC for 5 or 15 min; prolonged exposure (60 min) to 0.01% BAC destroys the mucous layer.

Conclusions: This study demonstrates that short-term exposure to BAC can alter the precorneal mucin.

Benzalkonium chloride (BAC), the most commonly used preservative in ophthalmic preparations, is most often used at a concentration of 0.01% (range 0.004%-0.02%) in topical multidose solutions [1]. BAC is a quaternary ammonium compound [2] that has been demonstrated to adversely affect both the cornea and conjunctiva [3-5]. Several studies have suggested that use of preserved drops results in a significant reduction of tear film breakup time, tear turnover, and an increase of corneal epithelial permeability [6-8]. Although the cytotoxic effect of BAC on corneal epithelium has been studied extensively [9-11], little is known about the effects of BAC on precorneal mucin. Moreover, to our knowledge, there have been no reports on the effect of BAC on mucin in the cornea.

Mucins are large, elongated molecules with a protein backbone to which oligosaccharides are attached [12]. Mucins are characterized by their protein structure as secreted or membrane associated. Mucin genes in humans are named *MUC* and suffixed with a number indicating their order of identification. The stratified epithelia of the human cornea produce the membrane associated mucins MUC1 [13], MUC4 [14], and MUC16 [15] which form a dense barrier in the glycocalyx

at the corneal epithelial tear film interface [16,17]. MUC1 and MUC16 are concentrated along the apical membrane of apical and subapical cells in human corneal epithelia, whereas *MUC4* mRNA is mostly found in the conjunctival epithelia with relatively little in the central corneal epithelium [13-15]. This study examined the short term effects of BAC on precorneal MUC1 and MUC16 in human corneal epithelium.

METHODS

Tissue and cell lines: Immortalized human corneal-limbal epithelial (HCLE) cell cultures (kindly provided by Dr. I. K. Gipson, Schepens Eye Research Institute, Harvard Medical School, Boston, MA) were briefly grown in keratinocyte-serum free medium (K-SFM; Gibco-Invitrogen, Carlsbad, CA), a medium optimized for keratinocyte proliferation, and were switched at approximately half confluence to a 1:1 mixture of K-SFM and low calcium DMEM/F12 (Gibco-Invitrogen) to achieve confluence (about 24 h). After reaching confluence, cells were switched to DMEM supplemented with 10% fetal calf serum and 10 ng/ml epidermal growth factor (R&D Systems, Minneapolis, MN) for 7 days to promote stratification and differentiation of the HCLE cells [18].

Human corneal epithelium was acquired, with consent, as a byproduct of elective excimer photorefractive keratectomy procedures. The three patients in the "BAC" group were treated with Ocuflax® eyedrops (details of experimental solutions are listed in Table 1) four times a day for 1 week be-

Correspondence to: Eung Kweon Kim, MD, PhD, Institute of Vision Research, Department of Ophthalmology and Brain Korea 21 Project for Medical Sciences, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, CPO Box 8044, Seoul, Korea, 120-752; Phone: 822-2228-3577; FAX: 822-312-0541; email: eungkkim@yumc.yonsei.ac.kr

fore surgery. The three patients in the control group received no preoperative antibiotic. Anesthesia was achieved with 3 drops of proparacaine solution over a 10 min interval (every 5 min). The corneal epithelium was mechanically scraped with a sterile Bard-Parker #15 blade. Each specimen of collected cells was divided in two for use in PCR and western blotting.

Three corneoscleral buttons were obtained from the Yonsei Eye Bank (Seoul, Korea) from human donor eyes. The cause of death for the tissue donors was unknown making them unsuitable for transplantation; the tissue appeared healthy microscopically.

The study was conducted in compliance with the provisions of the Declaration of Helsinki.

RNA isolation and reverse transcription: Total RNA was isolated from HCLE cells and human corneal epithelium using TRIzol as the extraction reagent (Gibco-Invitrogen), according to the manufacturer's protocol. HCLE cells and human corneal epithelium were homogenized by adding 1 ml reagent per well. After passing the cell lysate through a pipette several times, samples were incubated at room temperature for 5 min to permit the complete dissociation of nucleoprotein complexes. A volume of 0.2 ml chloroform was added to the reagent solution. Tubes were vigorously shaken by hand and centrifuged for 15 s. The colorless upper aqueous phase containing the RNA was recovered. Samples were then incubated with 0.5 ml isopropanol and centrifuged. After removing the supernatant, the RNA pellet was washed once with 75% ethanol. The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water and incubated for 10 min at 60 °C. Residual genomic DNA from the samples was elimi-

nated by DNase I digestion of the RNA preparation.

cDNA synthesis (SuperScript III Reverse Transcriptase; Gibco-Invitrogen), as described in the manufacturer's protocol, used 1 µg total RNA. Oligo d(T) primers were added to each sample then the tubes were heated to 70 °C for 10 min. After a brief centrifugation, a mixture containing PCR buffer (Gibco-Invitrogen), MgCl₂, dNTPs, and dithiothreitol (DTT) was added to the samples and incubated for 5 min at room temperature. The reverse transcription was performed by adding 1 µl reverse transcriptase to the mixture and incubating the tubes at 25 °C for 10 min, 42 °C for 50 min, and 70 °C for 15 min. Samples were placed on ice for 1 min, 1 µl RNase H was added to each tube, and they were incubated at 37 °C for 20 min. Reverse-transcription products were then ready for use in conventional and real-time PCR reactions. Of the 20 µl of total reverse transcription volume, 1 µl was used for each PCR.

Conventional PCR and real-time PCR: Conventional polymerase chain reaction (PCR) was performed using a Perkin Elmer RNA-PCR core kit (Roche Molecular System, Inc., Branchburg, NJ) in a Perkin Elmer Gene Amp PCR system 2400 (Hoffmann-La Roche Inc., Norwalk, CT). The *MUC1* primers [19] and *MUC16* primers [15] were designed to produce 368 bp and 114 bp PCR products, respectively. The PCR conditions for *MUC1* were an initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, extension at 72 °C for 1 min, and post-elongation at 72 °C for 10 min. The PCR conditions for *MUC16* were an initial denaturation at 95 °C for 10 min, 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min,

TABLE 1. EXPERIMENTAL SOLUTIONS

Solution	BAC	Other	Source
Control (for PCR, WB)	0%	SFM	
BAC media	0.01%	SFM	
Control (for TEM)	0%	DW	
BAC solution	0.01%	DW	
Ocuflox® eyedrops	0.0025%	0.3% ofloxacin, WFI	Samil Pharmaceutical, Seoul, Korea
Ofloxacin solution	0%	0.3% ofloxacin, WFI	Sigma-Aldrich
Alcaine® eyedrops	0.01%	0.5% proparacaine, WFI	S. A. Alcon-Couvreur Co., Puurs, Belgium
Proparacaine solution	0%	0.5% proparacaine, WFI	Sigma-Aldrich

For each of the experimental solutions used in these experiments, the table lists the BAC content, other components, and the source (if purchased). Control and BAC (Sigma-Aldrich) solutions were prepared from tertiary distilled water (DW) or serum free media (SFM; Gibco-Invitrogen). Purchased solutions were all in sterile water for injection (WFI).

extension at 72 °C for 2 min, and post-elongation at 72 °C for 7 min. The 10 µl of PCR products were electrophoresed on 2% agarose gel containing ethidium bromide. β2 microglobulin (β2-m) was used as an endogenous reference to determine the integrity of the mRNA in each sample.

Real-time PCR amplification was performed in the presence of double-labeled fluorogenic probes (*TaqMan* probes; PE-Applied Biosystems, Foster City, CA) that allow the relative quantification of gene expression in real time. Amplification was performed in triplicate with 1 µl of cDNA in a total volume of 50 µl (*TaqMan* chemistry; PE-Applied Biosystems).

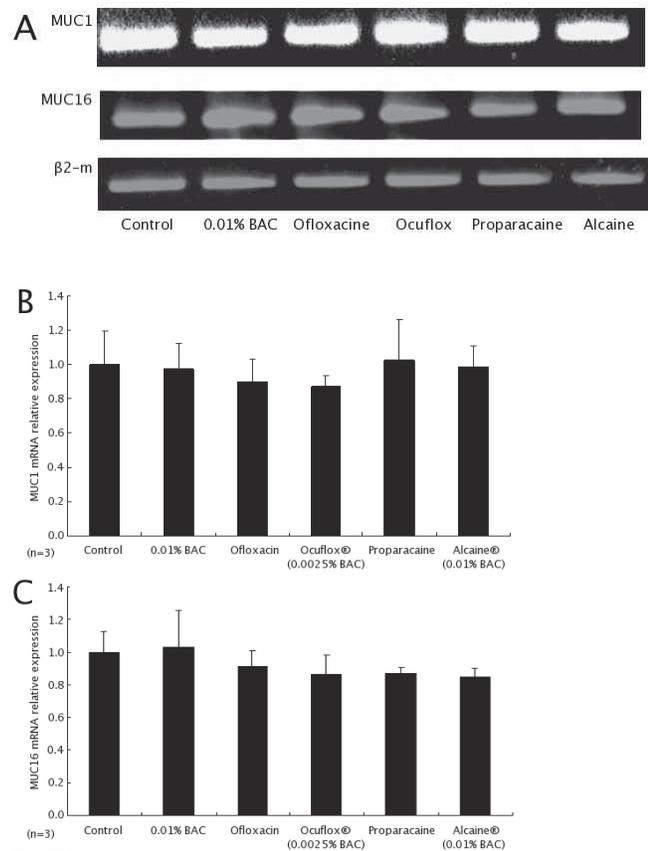


Figure 1. *MUC1* and *MUC16* gene expression in human corneal limbal epithelial (HCLE) cells. **A:** No differences were detected qualitatively in the expression of *MUC1* and *MUC16* mRNA among groups in conventional RT-PCR. β2 microglobulin (β2-m) was a housekeeping gene for the conventional RT-PCR. **B:** Real time RT-PCR demonstrated that there was no significant difference in the amount of *MUC1* transcripts between the control group and the BAC media treatment group ($p=0.827$), between the Ocuflox® eyedrops and ofloxacin solution treatment groups ($p=0.275$), and between the Alcaine® eyedrops and proparacaine solution treatment groups ($p=0.400$). **C:** Real time RT-PCR demonstrated that there was no significant difference in the amount of *MUC16* transcripts between the control group and the BAC media treatment group ($p=0.187$), between the Ocuflox® eyedrops and ofloxacin solution treatment groups ($p=0.275$), and between the Alcaine® eyedrops and proparacaine solution treatment groups ($p=0.377$). The graphs show the mean values of three independent experiments; the error bars represent the standard deviation.

Assays were performed using an ABI Prism 7700 Sequence Detection System (PE-Applied Biosystems). The average threshold cycle (CT) values for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were used as an internal calibrator to correct for differences in the integrity and amount of total RNA added to each reaction [20]. To validate the relative quantification, the efficiency of the target gene amplification was compared with the efficiency of the GAPDH amplification, as described in the manufacturer’s protocol (PE-Applied Biosystems). A nontemplate control was included in all the experiments performed with real-time PCR to evaluate DNA contamination of the reagents used for amplification. None of the experiments resulted in a positive signal from the nontemplate control, indicating that the RNA used in the assays was not contaminated by DNA.

Western blot analysis: HCLE cells were incubated for 15 min under six different conditions: serum free media (SFM; control group), BAC media, ofloxacin solution, Ocuflox® eyedrops, proparacaine solution, and Alcaine® eyedrops.

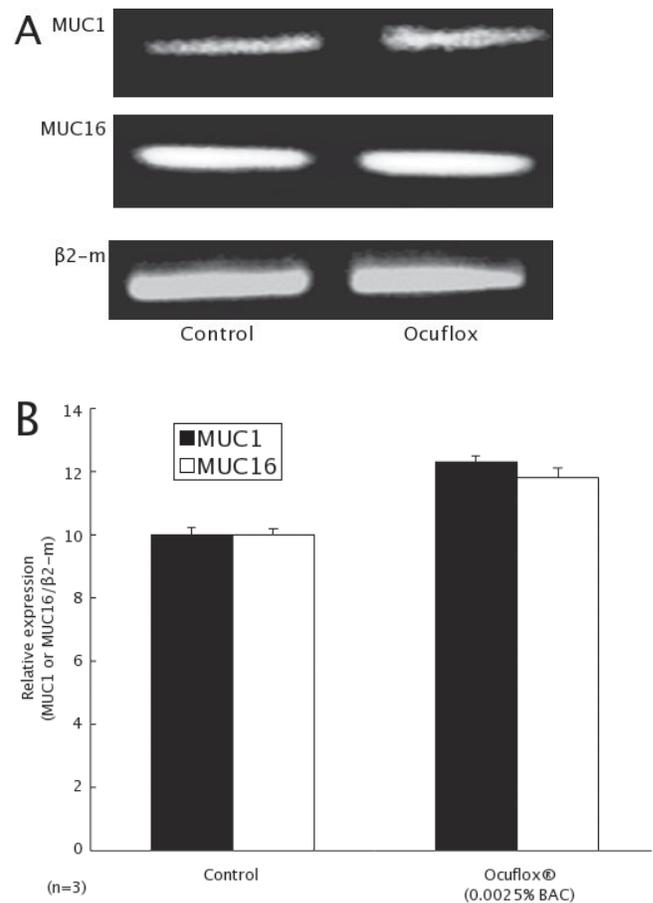


Figure 2. *MUC1* and *MUC16* gene expression in human corneal epithelium. **A:** Conventional RT-PCR demonstrated no differences in *MUC1* and *MUC16* mRNA expression between the Ocuflox® eyedrops treatment and control group. Corneal epithelia were treated with Ocuflox® eyedrops or with nothing (control). **B:** The graphs show the mean values of three independent experiments; the error bars represent the standard deviation. Values were normalized to the density of the respective band of β2 microglobulin (β2-m).

HCLE cells and human corneal epithelium were lysed in buffer (25 mM HEPES pH 7.5, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.05% Triton X-100, 0.5 mM DL-Dithiothreitol, 0.4 mM PMSF, 2 µg/ml leupeptin, and 1 µg/ml pepstatin A). Proteins at each purification step were subjected to SDS polyacrylamide gel electrophoresis [21] using a 6% separating, 4% stacking gel followed by electroblotting on Immobilon™-P polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). The membranes were washed with Tris buffered saline and treated with 0.1 U/ml of Type V neuraminidase (Sigma-Aldrich, St. Louis, MO) in a 50 mmol sodium acetate and 10 mmol calcium acetate buffer (pH 6.5) at 37 °C

for 1 h. The blots were then exposed to monoclonal antibodies recognizing MUC1 (HMFG-1, 1:1000; Biotools, Saco, ME) [13] or MUC16 (OC125, 1:1000; Dako Corp., Carpinteria, CA) [15] overnight at 4 °C. After removing the antibodies and washing the reactive proteins, the membrane was treated with peroxidase conjugated antimouse IgG (1:1000 dilution; Amersham Pharmacia Biotech, Buckinghamshire, England). Detection was carried out using enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) on Hyperfilm ECL (Amersham Pharmacia Biotech [USA]). β-actin was used as an endogenous reference to determine the integrity of the protein in each sample.

Preparation of corneal tissue for transmission electron microscopy: Three human corneas were dissected from corneoscleral buttons. Each human cornea was divided into six pieces; a negative control was fixed overnight in a 5% glutaraldehyde (containing 0.1 M sodium cacodylate-HCl and 1% sucrose at pH 7.4). A positive control was fixed overnight in 5% glutaraldehyde (containing 0.1 M sodium cacodylate-HCl and 1% sucrose at pH 7.4) with 0.5% cetylpyridinium chloride (CPC). Four pieces were treated with 0.01% BAC solution diluted in tertiary distilled water for 1, 5, 15, or 60 min at 4 °C. Treated corneas were fixed overnight in a 5% glutaraldehyde (containing 0.1 M sodium cacodylate-HCl and 1% sucrose, pH 7.4). After fixation, all specimens were washed in 0.15 M sodium cacodylate-HCl, pH 7.3, and fixed again for 1 h at room temperature in 1% glutaraldehyde in 0.1 M sodium cacodylate-HCl with 1% tannic acid. They were washed again in 0.1 M sodium cacodylate-HCl and were postfixed in Palade's buffer containing 2% osmium tetroxide for 1 h at 4 °C. Finally, corneas were dehydrated in ethanol, and embedded in Epon [21,22].

Transmission electron microscopy: Thin sections were cut with a diamond knife using a LEICA ultracut microtome, stained with aqueous uranyl acetate and Reynolds lead citrate (or with the latter alone), and examined in a Philips CM-10 microscope operating at 80 KV with a 60 µm objective aperture.

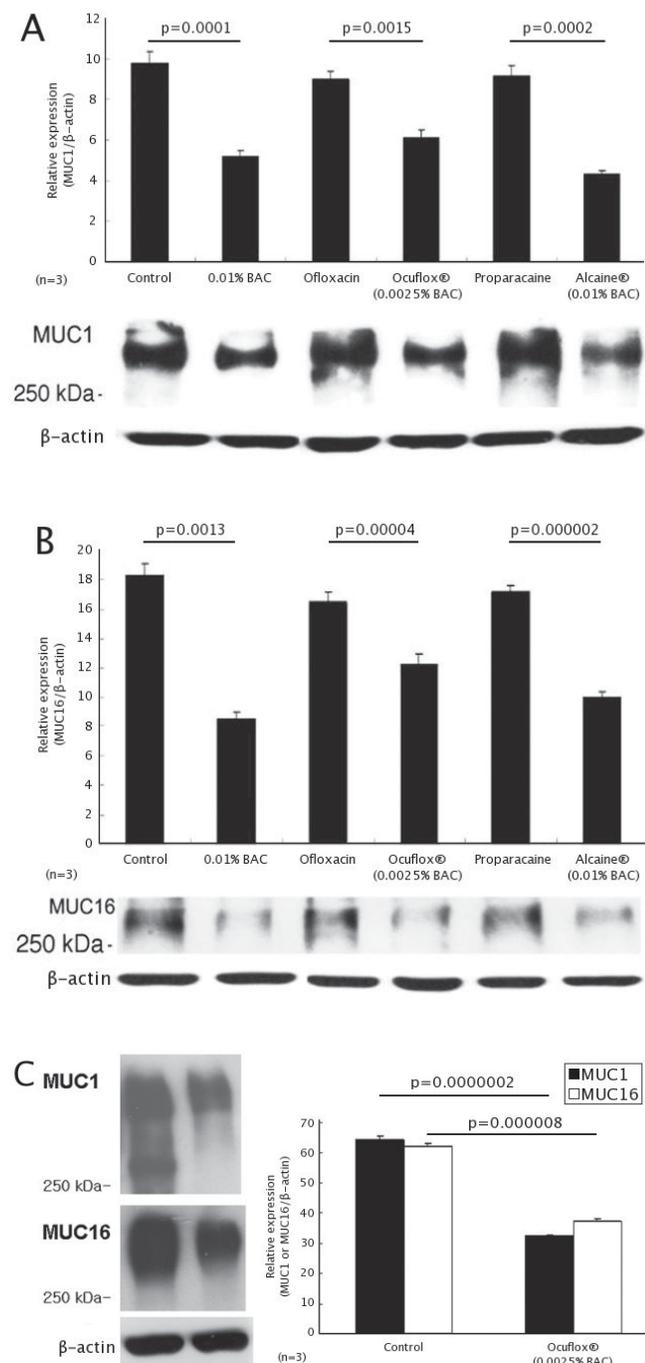


Figure 3. Western blot and densitometric analyses. Western blotting was used to measure MUC1 and MUC16 in both human corneal-epithelial (HCLE) cells and human corneal epithelium exposed to different concentrations of benzalkonium chloride (BAC). HCLE cells (A,B) exposed to BAC had decreased MUC1 and MUC16 protein levels (p<0.05). There is the suggestion of a concentration response in the data, but the data set is not suitable for a statistical test of trend. The human corneal epithelium (C) of patients treated with Ocuflor® eyedrops also had a decrease in MUC1 and MUC16 protein levels (p<0.05). A: The bar graph shows the quantity of MUC1 (relative to β-actin) in the six groups: control, BAC media, ofloxacin solution, Ocuflor® eyedrops, proparacaine solution, and Alcaine® eyedrops. B: The bar graph shows the quantity of MUC16 (relative to β-actin) in the same six groups. C: The bar graph shows the quantities of MUC1 and MUC16 (relative to β-actin) in the control and Ocuflor® eyedrops groups. The values in these graphs represents the mean of three experiments; the error bars represent the standard deviation. Values were normalized to the density of the respective band of β-actin.

Statistics: Statistical differences between measured mRNA and protein levels were assessed using parametric testing with SPSS (version 12). An α level of 0.05 was chosen for all tests.

RESULTS

MUC1 and MUC16 mRNA expression in HCLE cells and human corneal epithelia: The expression of *MUC1* and *MUC16* mucin gene transcripts in corneal epithelia was initially determined by conventional RT-PCR (Figure 1A). Amplification of cDNA products from HCLE cells treated with serum free media (control), HCLE cells treated with BAC media, HCLE cells treated with ofloxacin solution, HCLE cells treated with Ocuflor® eyedrops, HCLE cells treated with proparacaine solution, and HCLE cells treated with Alcaine®

eyedrops produced bands corresponding to the *MUC1* and *MUC16* sizes that qualitatively had the same density (Figure 1A). The housekeeping gene β 2-m was used to normalize the results.

The use of real-time PCR demonstrated that no change was detected in the expression of the *MUC1* and *MUC16* mRNA by the treatment with BAC in HCLE cells (Figure 1B,C). The amount of *MUC1* and *MUC16* transcripts was not significantly different between the control group and the BAC media treatment group (Figure 1B,C). Levels of the *MUC1* and *MUC16* mRNA remained essentially unaltered after treatment with Ocuflor® eyedrops and Alcaine® eyedrops compared to ofloxacin solution and proparacaine solution treatment groups, respectively (Figure 1B,C).

The expression of *MUC1* and *MUC16* mucin gene tran-

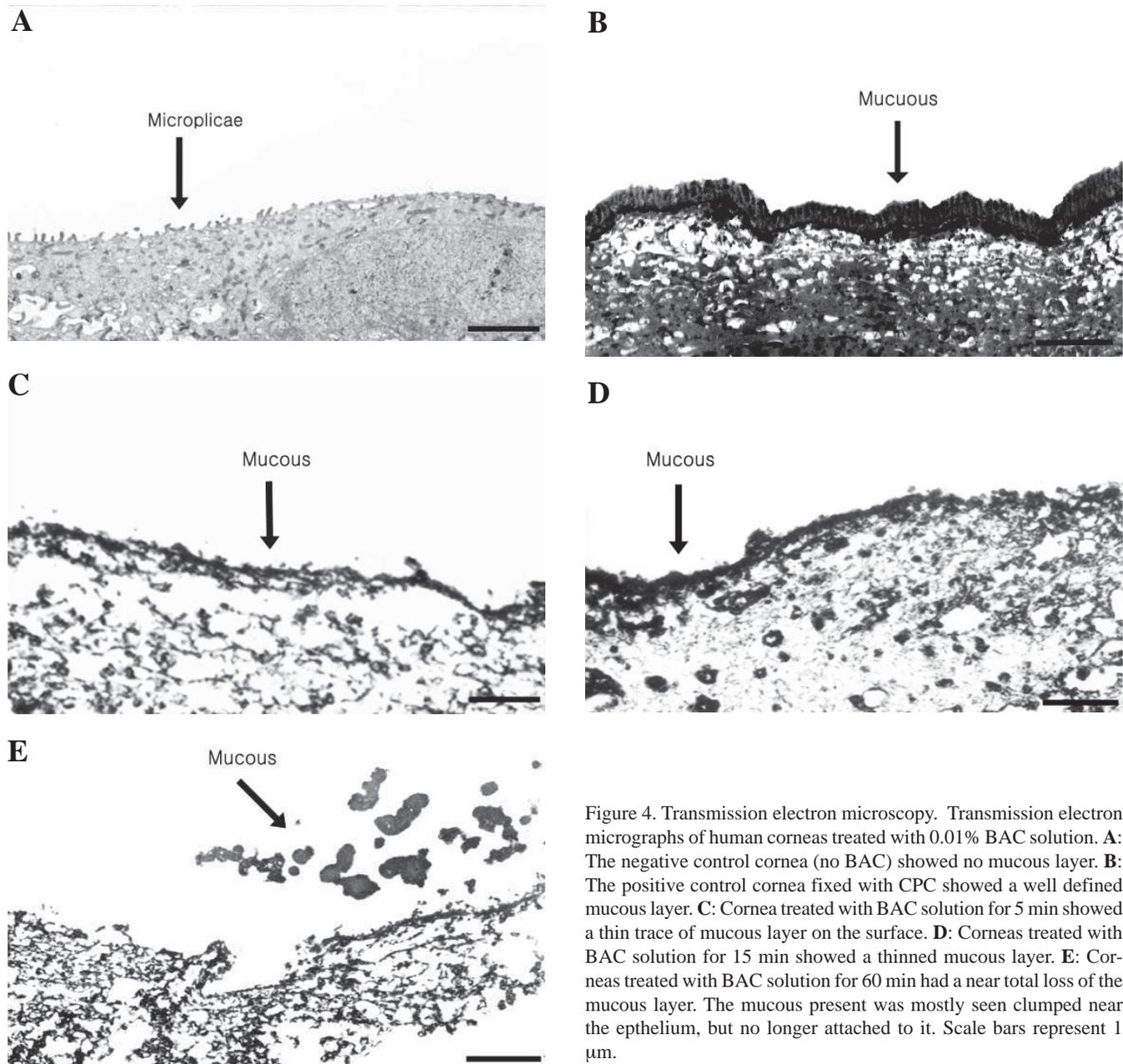


Figure 4. Transmission electron microscopy. Transmission electron micrographs of human corneas treated with 0.01% BAC solution. **A:** The negative control cornea (no BAC) showed no mucous layer. **B:** The positive control cornea fixed with CPC showed a well defined mucous layer. **C:** Cornea treated with BAC solution for 5 min showed a thin trace of mucous layer on the surface. **D:** Corneas treated with BAC solution for 15 min showed a thinned mucous layer. **E:** Corneas treated with BAC solution for 60 min had a near total loss of the mucous layer. The mucous present was mostly seen clumped near the epithelium, but no longer attached to it. Scale bars represent 1 μ m.

scripts in human corneal epithelium was determined by conventional RT-PCR. The steady-state levels of the two mucins were not decreased in human corneal epithelium that had been treated with Ocuflor® eyedrops for a week (Figure 2).

MUC1 and MUC16 in HCLE cells and human corneal epithelia: Western blot analysis was performed to determine the effect of BAC on MUC1 and MUC16. Figure 3A showed a reduction of MUC1 in the group treated with BAC media compared to the control group. A reduction of MUC1 was also observed in the Ocuflor® eyedrops and Alcaïne® eyedrops treatment groups (Figure 3A). The group treated with 0.0025% BAC (Ocuflor® eyedrops) has a tendency to show less reduction of MUC1 than groups treated with 0.01% BAC (Alcaïne® eyedrops, BAC media). Figure 2B shows the western blot analysis of MUC 16; as with MUC1, the MUC16 was also decreased in groups treated with BAC. Also, the higher concentration of BAC resulted in a greater reduction of MUC16 (Figure 3B). Experiments using three human corneal epithelia demonstrated that the groups that received Ocuflor® eyedrops for 1 week before surgery had a lower level of MUC1 and MUC16 than the control group (Figure 3C).

Transmission electron microscopy of human corneas exposed to BAC: The mucous layer was absent in negative control corneas (Figure 4A) and the group treated with 0.01% BAC solution for 1 min (data not shown). The positive control corneas fixed with CPC demonstrated a well-defined mucous layer 0.55 μm thick (standard deviation 0.116 μm ; Figure 4B). The corneas treated with BAC solution for 5 min had a thin mucous layer on the surface (Figure 4C). A definite mucous layer was observed in the corneas treated with BAC solution for 15 min (Figure 4D). When treated with BAC solution for 60 min, corneas had diffuse damage to the epithelial cell layer; the mucous layer was essentially lost and what mucous remained was no longer adherent to the epithelium (Figure 4E).

DISCUSSION

In our clinical experience, even a single eyedrop containing BAC can cause conjunctival injection and ocular irritation. Ishibashi et al. [6] have shown that an eyedrop containing 0.005% BAC significantly reduces the tear film breakup time for 30 min after instillation. This indicates that BAC destabilizes the tear film [23]. The current model of the tear film suggests that there is a superficial lipid layer above an aqueous layer that contains secreted mucins and membrane associated mucins that have been shed [24]. The glycocalyx is made up of the extracellular domains of the membrane associated mucins (MUC1, MUC4, and MUC16) on the apical corneal epithelial cells with the aqueous phase of the tear film in contact with the glycocalyx [25]. The hydrophilic nature of mucins is important in maintaining the tear film because it keeps water on the corneal surface [26]. We report alterations in the precorneal mucins after short term exposure to BAC.

By using a combination of conventional and real-time RT-PCR, we demonstrated that BAC exposure did not alter the expression of *MUC1* and *MUC16* mRNA in a cell culture

model (Figure 1). Nor is the expression of *MUC1* and *MUC16* affected in patients using Alcaïne® eyedrops (Figure 2) in conventional RT-PCR. These suggest that any reduction of MUC1 and MUC16 following BAC exposure is not a result of gene regulation in our cell culture model or in patients' corneal epithelia.

The western blot analysis assessed the effect of BAC on the amount of MUC1 and MUC16 in a cell culture model. We found a significant reduction of MUC1 and MUC16 after exposure to BAC for only 15 min (Figure 3). Experiments using patient corneal epithelia demonstrated that the groups that received Ocuflor® eyedrops for 1 week before surgery had a lower level of mucins than the control group (Figure 3).

Our TEM results demonstrated a thin mucous layer after treatment with 0.01% BAC solution for as little as 5 min. With longer exposures to BAC solution, the observable mucous layer was noticeably thicker. However, after exposures to BAC solution for about an hour, the mucous layer is disrupted and there is diffuse damage to superficial epithelial cells across the cornea. Why does this layer appear with exposure to BAC solution? Conventional electron microscopy techniques do not preserve the mucous layer. Nichols et al. [21] demonstrated the mucous layer on the corneal epithelium by using quaternary ammonium compounds (e.g., CPC) to fix the mucous for TEM processing. Quaternary ammonium compounds fix soluble acid mucopolysaccharides (e.g., mucin) by forming highly insoluble complexes [27]. We theorize that exposure to BAC (another quaternary ammonium compound [2]), fixes the mucin within the mucous layer leading to the visualization of the mucous layer under TEM (Figure 4). As our BAC solution was not part of an optimized fixation protocol like the CPC used in the positive control (Figure 4B), we believe that it was an inefficient fixative and that prolonged exposure to it ultimately did not preserve the layer for electron microscopic staining. Thus, it is possible the loss of mucins in the western blot analysis corresponds to the fixation of mucin with the alteration of its structure to the point that antibodies do not bind to it.

The decrease in the amount of functional mucin on the corneal surface may affect the stability of the tear film. Mucins contribute to the formation of highly hydrated gel structures, mainly through the hydrophilic O-linked oligosaccharide side chains. A mucus gel with 0.5-5% mucins can contain 90% water [28]. Loss of the mucous layer may lead to decreased water retention and consequently to the ocular irritation symptoms commonly associated with the use of eyedrops containing BAC. It may itself contribute directly to epithelial damage. In a TEM study of rabbit cornea, damaged corneal epithelial cells were seen to slough off after a 1.5 h exposure to BAC solution [29].

In conclusion, BAC can alter the precorneal mucin and seems likely to cause tear film dysfunction. As BAC remains in wide use, it is worthwhile to continuously re-evaluate its use in patients with ocular surface disorders. Eliminating BAC may be desirable to protect the tear film, but must be weighed against the potential dangers of unpreserved eyedrops.

ACKNOWLEDGEMENTS

This study was supported by a grant of the MYUNG-SUN KIM memorial foundation. The authors thank Dr. Gipson for providing us with two human corneal-limbal epithelial (HCLE) cell lines. This study was presented in part at the 2005 Association for Research in Vision and Ophthalmology meeting on May 4, 2005 in Fort Lauderdale, FL.

REFERENCES

- Pisella PJ, Fillacier K, Elena PP, Debbasch C, Baudouin C. Comparison of the effects of preserved and unpreserved formulations of timolol on the ocular surface of albino rabbits. *Ophthalmic Res* 2000; 32:3-8.
- Noecker R. Effects of common ophthalmic preservatives on ocular health. *Adv Ther* 2001; 18:205-15.
- Burstein NL. Preservative cytotoxic threshold for benzalkonium chloride and chlorhexidine digluconate in cat and rabbit corneas. *Invest Ophthalmol Vis Sci* 1980; 19:308-13.
- Mietz H, Niesen U, Kriegelstein GK. The effect of preservatives and antiglaucomatous medication on the histopathology of the conjunctiva. *Graefes Arch Clin Exp Ophthalmol* 1994; 232:561-5.
- De Saint Jean M, Debbasch C, Brignole F, Rat P, Warnet JM, Baudouin C. Toxicity of preserved and unpreserved antiglaucoma topical drugs in an in vitro model of conjunctival cells. *Curr Eye Res* 2000; 20:85-94.
- Ishibashi T, Yokoi N, Kinoshita S. Comparison of the short-term effects on the human corneal surface of topical timolol maleate with and without benzalkonium chloride. *J Glaucoma* 2003; 12:486-90.
- de Jong C, Stolwijk T, Kuppens E, de Keizer R, van Best J. Topical timolol with and without benzalkonium chloride: epithelial permeability and autofluorescence of the cornea in glaucoma. *Graefes Arch Clin Exp Ophthalmol* 1994; 232:221-4.
- Kuppens EV, de Jong CA, Stolwijk TR, de Keizer RJ, van Best JA. Effect of timolol with and without preservative on the basal tear turnover in glaucoma. *Br J Ophthalmol* 1995; 79:339-42.
- Chang SW, Chi RF, Wu CC, Su MJ. Benzalkonium chloride and gentamicin cause a leak in corneal epithelial cell membrane. *Exp Eye Res* 2000; 71:3-10.
- Doughty MJ. Acute effects of chlorobutanol- or benzalkonium chloride-containing artificial tears on the surface features of rabbit corneal epithelial cells. *Optom Vis Sci* 1994; 71:562-72.
- Cha SH, Lee JS, Oum BS, Kim CD. Corneal epithelial cellular dysfunction from benzalkonium chloride (BAC) in vitro. *Clin Experiment Ophthalmol* 2004; 32:180-4.
- Silberberg A, Meyer FA. Structure and functions of mucus. In: Chantler EN, Elder JB, Elstein M, editors. *International symposium on mucus in health and disease. Vol II.* New York: Plenum; 1981. p. 53-74.
- Inatomi T, Spurr-Michaud S, Tisdale AS, Gipson IK. Human corneal and conjunctival epithelia express MUC1 mucin. *Invest Ophthalmol Vis Sci* 1995; 36:1818-27.
- Pflugfelder SC, Liu Z, Monroy D, Li DQ, Carvajal ME, Price-Schiavi SA, Idris N, Solomon A, Perez A, Carraway KL. Detection of sialomucin complex (MUC4) in human ocular surface epithelium and tear fluid. *Invest Ophthalmol Vis Sci* 2000; 41:1316-26.
- Argueso P, Spurr-Michaud S, Russo CL, Tisdale A, Gipson IK. MUC16 mucin is expressed by the human ocular surface epithelia and carries the H185 carbohydrate epitope. *Invest Ophthalmol Vis Sci* 2003; 44:2487-95.
- Gipson IK. Distribution of mucins at the ocular surface. *Exp Eye Res* 2004; 78:379-88.
- Watanabe H. Significance of mucin on the ocular surface. *Cornea* 2002; 21:S17-22.
- Gipson IK, Spurr-Michaud S, Argueso P, Tisdale A, Ng TF, Russo CL. Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. *Invest Ophthalmol Vis Sci* 2003; 44:2496-506.
- Kim SS, Kim KS, Lee JG, Park IY, Koo JS, Yoon JH. Levels of intracellular protein and messenger RNA of mucin and lysozyme in normal human nasal and polyp epithelium. *Laryngoscope* 2000; 110:276-80.
- Argueso P, Balaram M, Spurr-Michaud S, Keutmann HT, Dana MR, Gipson IK. Decreased levels of the goblet cell mucin MUC5AC in tears of patients with Sjogren syndrome. *Invest Ophthalmol Vis Sci* 2002; 43:1004-11.
- Nichols BA, Chiappino ML, Dawson CR. Demonstration of the mucous layer of the tear film by electron microscopy. *Invest Ophthalmol Vis Sci* 1985; 26:464-73.
- Kim EK, Cristol SM, Kim HL, Kang SJ, Park JW, Edelhauser HF. The mucinous layer of corneal endothelial cells. *Yonsei Med J* 2000; 41:651-6.
- Djalilian AR, Hamrah P, Pflugfelder SC. Dry eye. In: Krachmer JH, Mannis MJ, Holland EJ, editors. *Cornea. Vol 1.* Philadelphia: Elsevier Mosby; 2005. p. 521-40.
- Chen HB, Yamabayashi S, Ou B, Tanaka Y, Ohno S, Tsukahara S. Histochemical study on rat tear film and ocular surface epithelial cells. *Curr Eye Res* 1998; 17:642-9.
- Argueso P, Gipson IK. Epithelial mucins of the ocular surface: structure, biosynthesis and function. *Exp Eye Res* 2001; 73:281-9.
- Gipson IK, Argueso P. Role of mucins in the function of the corneal and conjunctival epithelia. *Int Rev Cytol* 2003; 231:1-49.
- Scott JE. Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues. *Methods Biochem Anal* 1960; 8:145-97.
- Carlstedt I, Sheehan JK, Corfield AP, Gallagher JT. Mucous glycoproteins: a gel of a problem. *Essays Biochem* 1985; 20:40-76.
- Ubels JL, McCartney MD, Lantz WK, Beaird J, Dayalan A, Edelhauser HF. Effects of preservative-free artificial tear solutions on corneal epithelial structure and function. *Arch Ophthalmol* 1995; 113:371-8.

The print version of this article was created on 26 Apr 2006. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.