

The Effect of Preservatives and Antiglaucoma Treatments on the Ocular Surface of Mice With Dry Eye

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Submitted: April 8, 2014

Accepted: August 31, 2014

Citation: Barabino S, Antonelli S, Cimbolini N, Mauro V, Bouzin M. The effect of preservatives and antiglaucoma treatments on the ocular surface of mice with dry eye. *Invest Ophthalmol Vis Sci.* 2014;55:6499–6504. DOI:10.1167/iovs.14-14548

PURPOSE. To test the hypothesis that benzalkonium chloride (BAK) alters the ocular surface in normal and dry eye mice and that a BAK-free commercially available antiglaucoma treatment does not induce the same effects.

METHODS. Eight- to 12-week-old female C57BL/6 mice were used under normal environmental conditions and in a controlled environment chamber (CEC) which induces dry eye. Study and control groups included treatment with BAK, bimatoprost, BAK-free travoprost, and 0.9% NaCl and nontreated mice exposed and nonexposed to the CEC, respectively. Treatments were instilled 4 times a day in the right eye for 7 days. Aqueous tear production was measured by cotton thread test, corneal fluorescein staining (score 0–15), corneal thickness, goblet cell density, and CD45⁺ cell expression in superior, inferior, and fornix conjunctiva by a masked observer.

RESULTS. After 7 days of treatment with BAK, mice showed significant increase of corneal staining, reduction of goblet cells, and increase of inflammation under normal and CEC conditions. The commercial preparations of bimatoprost containing BAK and travoprost did not show the same effects. Travoprost showed a significant corneal thickening under CEC conditions compared to that in all other groups.

CONCLUSIONS. This study indicated that use of BAK has negative effects on the ocular surface under normal and dry eye conditions, even if the association with bimatoprost does not confirm the same results. A BAK-free travoprost preparation showed positive effects on tear secretion and corneal protection.

Keywords: glaucoma medications, ocular surface, tear film

The ocular surface system is a functional unit consisting of the tear film, lacrimal gland, corneal and conjunctival epithelia, and Meibomian glands, which work together to provide an efficient system necessary for the health and normal function of the eye and visual system. Nervous connections, systemic hormones, and immunological mechanisms are well-known factors that maintain the homeostasis of the ocular surface. They control responses to internal and external stimuli and avoid possible negative consequences on the eye's components due to acute response or chronic activation.¹

One of the possible factors responsible for changes of the ocular surface system is topical drugs containing preservatives. Artificial tears and antiglaucoma medications are used frequently and for prolonged periods of time and sometimes a life time, and if multidose preparations require the inclusion of an antimicrobial preservative, the quaternary ammonium benzalkonium chloride (BAK) would be most frequently used. It has now been clearly demonstrated that this compound can induce ocular discomfort, dry eye, and changes to the ocular surface: tear film instability, apoptosis of corneal and conjunctival cells, decrease in the number of goblet cells; increase in macrophage, mast cell, and fibroblast counts in the conjunctiva; and increased expression of markers of inflammation on conjunctival epithelial cells.²

One of the limitations of studies investigating the effect of BAK is that they were performed in an otherwise normal ocular

surface system. The cell lines and animal models used so far to test the toxicity of BAK have not been based on ocular surface diseases such as dry eye. Clinical trials performed to test antiglaucoma treatments exclude patients with dry eye, allergy, and blepharitis in order to avoid selection bias. Therefore, so far we do not have any information regarding the possible effect of BAK in ocular surface diseases such as dry eye syndrome, which is a well-known, highly prevalent disease.³

Use of a controlled environment chamber (CEC) that allows for control and monitoring of temperature, airflow, and humidity and regulation of these parameters have been used in a manner that leads to a dry eye state in mice, characterized by corneal fluorescein staining, loss of conjunctival goblet cells, and diminished lacrimation, with features that mimic human keratoconjunctivitis sicca.⁴ This mouse model of dry eye has now been widely used to study dry eye pathogenesis and to test new treatments for this frequent disease.^{5–7}

Study of the effects of the combination of preservatives and stressful environment on the ocular surface could provide important information about tear secretion and ocular surface response, including changes in corneal and conjunctival epithelia. In particular, we wanted to test the hypothesis that topical use of BAK induces ocular surface damage and decreases tear secretion in mice exposed to the CEC. Furthermore, the secondary objective of our study was to demonstrate that the use of a BAK-free commercially available

antiglaucoma treatment might reduce these adverse effects on the ocular surface under normal and CEC conditions.

METHODS

Animals

Eight- to 12-week-old female C57BL/6 mice (Centre d'élevage Janvier, Le Genest Saint Isle, France) were used in these experiments. The protocol was approved by the Iris Pharma Institute Animal Care and Use Committee, and all animals were treated according to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental Procedure

This was a prospective, double-masked, placebo-controlled study. Mice were exposed to the CEC for 7 days (temperature: $22.8 \pm 0.5^\circ\text{C}$; relative humidity: $20.5 \pm 4.5\%$; airflow: 15 L/min), as previously described.⁴ Animals demonstrated normal behavior, similar to that of littermates in standard cages.

Study and control groups were organized as follow: BAK-treated mice (0.2 mg/mL) exposed ($n = 15$) and nonexposed ($n = 15$) to the CEC; saline (0.9% NaCl) solution-treated mice exposed ($n = 15$) and nonexposed ($n = 5$) to the CEC; bimatoprost-treated mice (0.1 mg/mL, BAK 0.2 mg/mL, Lumigan; Allergan, Inc., Irvine, CA, USA) exposed ($n = 15$) and nonexposed ($n = 15$) to the CEC; travoprost-treated mice (Travatan; Alcon, Aliso Viejo, CA, USA) exposed ($n = 15$) and nonexposed ($n = 15$) to the CEC; and mice without any eye drop treatment exposed ($n = 15$) and nonexposed ($n = 15$) to the CEC. Five microliters of BAK and bimatoprost were instilled 4 times a day in the right eye. The same posology was used for the 0.9% NaCl- and travoprost BAK-free treated mice. BAK was prepared using sterile distilled water.

Measurements of tear secretion and corneal fluorescein staining were performed at the beginning and end (day 7) of the study 2 hours after the last eye drop was administered by a masked observer. Corneal thickness measurement, goblet cells density, and conjunctival immunohistochemistry with CD45 detection were studied at day 7.

Measurement of Tear Production. Tear production was measured with phenol red thread test (Zone-Quick; Lacrimedics, Eastsound, WA, USA). The validity of this test in mice has previously been described.⁵ Under a magnifying fluorescent lamp, the threads were held with jeweler's forceps and placed in the lateral cantus of the conjunctival fornix of the right eye for 30 seconds. The length of the wet thread in millimeters was measured under a microscope (S4 model; Zeiss, Munich, Germany), using a hemocytometer.

Corneal Fluorescein Staining. Corneal fluorescein staining was performed by applying 0.5 μL 5% fluorescein to the inferior conjunctival sac of the right eye by using a micropipette. The cornea was examined with slit lamp biomicroscopy using cobalt blue light 3 minutes after the fluorescein instillation. Punctuate staining was recorded in a standard fashion, using a standardized (National Eye Institute) grading system of 0 to 3 for each of the 5 areas in which the corneal surface has been divided.⁸

Histologic Analysis. After 7 days of treatment and exposure to normal and CEC conditions, mice were euthanized. Eyeballs with eyelids were orientated, embedded in OCT compound (Tissue-Tek; Sakura Finetek USA, Inc., Torrance, CA, USA), and frozen in isopentane cooled in liquid nitrogen. Immunostaining procedure was performed with 6- μm frozen nasal, central, and temporal sagittal sections fixed in acetone

(-20°C , 10 minutes). Slides were then incubated with 2% bovine serum albumin (BSA) to block nonspecific binding. Inflammatory cells were analyzed with specific antibody against CD45 (1:50 dilution; monoclonal rat anti-mouse antibody, clone 30-F11; BD Pharmingen, San Diego, CA, USA). The secondary antibody used was donkey anti-rat immunoglobulin G labeled with Alexa Fluor 594 (1:500 dilution; Molecular Probes, Inc., Eugene, OR, USA). Controls were performed by incubating slides with nonimmune serum in phosphate-buffered saline (PBS) 2% BSA replacing primary antisera, or with PBS replacing conjugated antibodies. For each sample, the extent of immunohistochemical reactivity was evaluated by the number of cells positive for CD45 expression per millimeter of epithelium. Periodic acid-Schiff (PAS) staining was performed with 6- μm nasal, central, and temporal sagittal sections fixed in Bouin-Holland liquid. Slides were then stained successively with PAS and hematoxylin. Goblet cells were then evaluated by number of positive cells per millimeter of epithelium.

Tarsal, fornix, and bulbar conjunctiva were considered in superior and inferior parts of the eyeball. Cell counting was performed manually using ImageJ software and Cell Counter plugin (National Institutes of Health, Bethesda, MD, USA).

Corneal Thickness Measurement. The evaluation of corneal thickness was performed in vivo by using the Heidelberg retina tomograph (HRT) II and its corneal module, Rostock Cornea Module (RCM; Heidelberg Engineering GmbH, Heidelberg, Germany). Anesthetized animals were laid on a support, and the right eye was placed so that the central cornea was in the axis of the camera. The contact between cornea and camera was allowed by a gel tear substitute. Images were then taken from the external layers of the epithelium to the endothelium, and the software provided thickness of the cornea in micrometers.

Statistical Analysis

Statistical analysis was performed using SPSS version 20.0 software (SPSS, Chicago, IL, USA). A mixed between-within subject analysis of variance (two-way ANOVA for repeated measures) was used to analyze the impact of treatment and environmental conditions (exposure or not to the CEC) on tear production and corneal fluorescein staining over time. A two-way between-group analysis of variance was performed to study the impact of treatment and CEC on conjunctival goblet cells, corneal thickness, and CD45⁺ cells in cornea and conjunctiva. If necessary, post hoc comparisons were assessed by using a Tukey honest significant difference (HSD) test. All *P* values were 2-tailed, with statistical significance set at 0.05. Representative results are means \pm SD.

RESULTS

Tear Production

Statistical analysis revealed large significant interactions among the time at which the tear production was measured, treatment, and environmental condition (partial $\eta^2 = 0.150$, $P < 0.001$). The interaction between time and environment was not significant, whereas a moderately significant effect was revealed for the interaction between time and treatment (partial $\eta^2 = 0.097$, $P = 0.010$). In particular, travoprost and bimatoprost showed a significant increase in tear production over time compared to that with BAK and no treatment and also to 0.9% NaCl in the case of bimatoprost (Fig. 1).

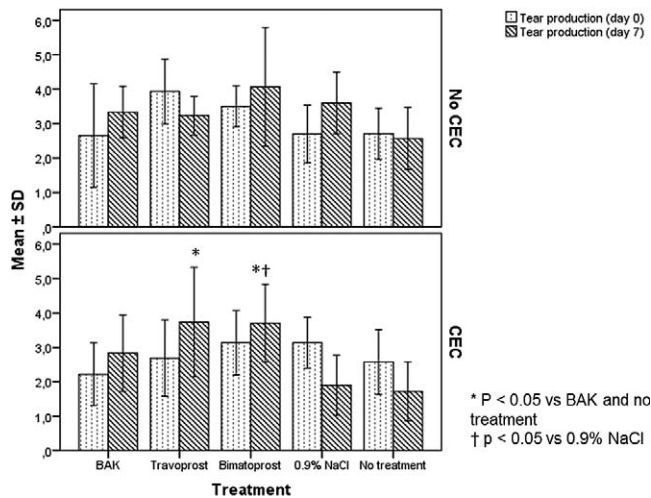


FIGURE 1. Two-way ANOVA representation of tear secretion (in millimeters of length of wet thread) under normal and CEC conditions after 7 days of instillation of BAK, travoprost, bimatoprost, 0.9% NaCl and in the nontreated group. The Tukey HSD test shows a significant increase in tear production over time with travoprost and bimatoprost compared to BAK and no treatment ($^*P < 0.05$) and also compared to 0.9% NaCl with bimatoprost ($†P < 0.05$). BAK, benzalkonium chloride; CEC, controlled environment chamber. Results are means \pm SD. Number of eyes, 15 for all groups; 0.9% NaCl no CEC, $n = 5$.

Corneal Fluorescein Staining

Corneal fluorescein staining was used to evaluate corneal epitheliopathy. Minimal punctuate corneal fluorescein staining was recorded at baseline in all the studied groups, without significant differences.

A moderate interaction was found between treatment and exposure to the CEC over time (partial $\eta^2 = 0.093$, $P = 0.012$). A significantly large interaction was revealed by considering both

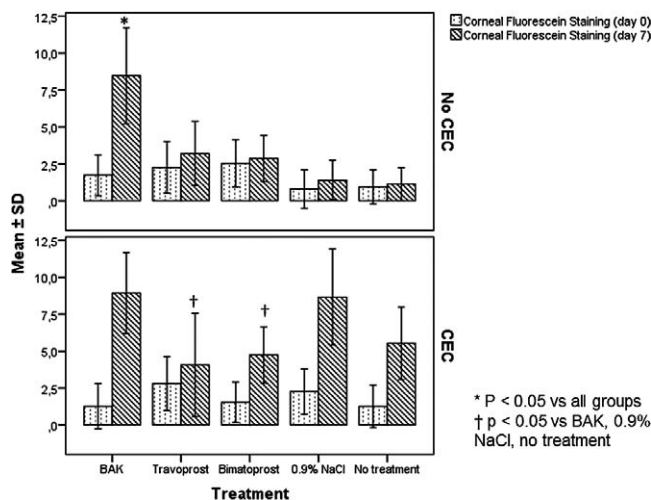


FIGURE 2. Two-way ANOVA representation of corneal fluorescein staining (grades 0-15) under normal and CEC conditions after 7 days of instillation of BAK, travoprost, bimatoprost, and 0.9% NaCl and in the nontreated group. Significant differences between corneal staining with BAK and staining with all other treatments over time is shown ($^*P < 0.05$). Travoprost- and bimatoprost-treated groups showed significantly less corneal damage than BAK and 0.9% NaCl ($†P < 0.05$, Tukey HSD test). BAK, benzalkonium chloride; CEC, controlled environment chamber. Results are means \pm SD. Number of eyes = 15 for all groups; 0.9% NaCl no CEC, $n = 5$.

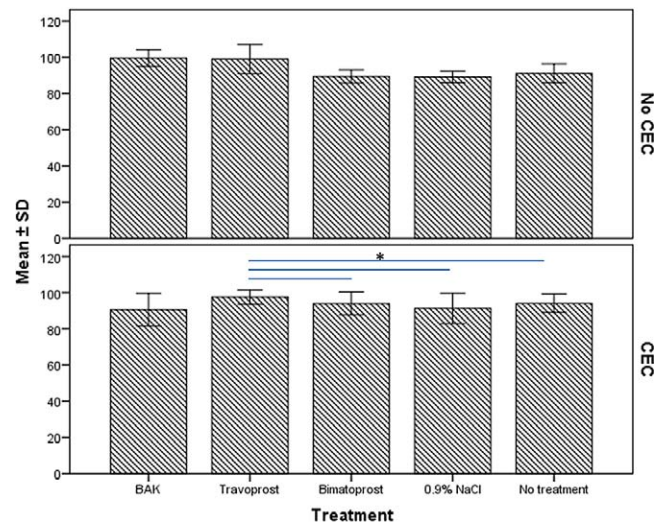


FIGURE 3. Corneal thickness. The travoprost group shows a statistically higher corneal thickness ($^*P < 0.05$) than all other groups, except for the BAK group, after 7 days of treatment. BAK-treated animals do not show any significant differences compared to the other groups. Number of eyes, 15 for all groups; 0.9% NaCl no CEC $n = 5$; 0.9% NaCl CEC $n = 13$. BAK, benzalkonium chloride; CEC, controlled environment chamber. Results are means \pm SD.

time and treatment (partial $\eta^2 = 0.389$, $P < 0.001$) and time and CEC (partial $\eta^2 = 0.182$, $P < 0.001$). Significant differences in corneal staining were found between BAK and all the other treatments over time, as shown in Figure 2. Travoprost- and bimatoprost-treated groups showed significantly lower corneal damage than those treated with BAK and 0.9% NaCl.

Corneal Thickness

As shown in Figure 3, after 7 days of treatment, travoprost showed a significant increase of corneal thickness compared to that in all other groups, except for BAK. We did not record any changes in corneal thickness due to the instillation of BAK. Overall, there was a statistically large main effect of the treatment (partial $\eta^2 = 0.176$, $P < 0.001$), whereas exposure to the CEC did not have an impact on corneal thickness.

Conjunctival Goblet Cells

The interaction effect of treatment and CEC on the number of goblet cells was not statistically significant. There was a significantly large effect of treatment (partial $\eta^2 = 0.267$, $P < 0.001$) but not of the CEC. Figure 4 shows changes in the number of goblet cells. BAK treatment demonstrated a significantly lower number of goblet cells than that in all other groups. Interestingly, we recorded a higher number of goblet cells in the bimatoprost group than in nontreated animals.

Cd45⁺ Cells in Cornea and Conjunctiva

The numbers of CD45⁺ cells in the cornea after 7 days of treatment did not show any statistical differences. The effect of treatment showed a significantly moderate effect (partial $\eta^2 = 0.117$, $P < 0.05$) on the number of CD45⁺ cells in the inferior bulbar and fornix conjunctiva only, whereas the effect of the CEC was not evident. In particular, as shown in Figure 5, BAK induced a significant increase of CD45⁺ cells in the bulbar conjunctiva compared to bimatoprost, and compared to bimatoprost and nontreated mice in the fornix conjunctiva (Fig. 6).

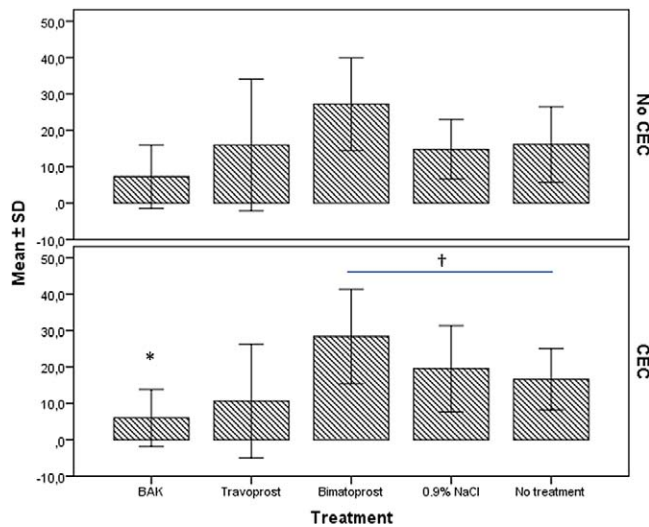


FIGURE 4. Number of goblet cells per millimeter of epithelium is shown in *superior* and *inferior* conjunctiva after 7 days of treatment. BAK-treated eyes show a significantly lower number of cells than those in all other groups ($*P < 0.05$), whereas bimatoprost-treated eyes show a significantly higher number than those in nontreated animals ($\dagger P < 0.05$, Tukey HSD test). Number of eyes, 15 for all groups; 0.9% NaCl no CEC $n = 5$; and no treatment $n = 10$. BAK, benzalkonium chloride; CEC, controlled environment chamber. Results are means \pm SD.

DISCUSSION

The study of the influence of antiglaucoma treatments on the ocular surface is a critical element to optimize the management of patients undergoing long-term therapy. In this study, we confirmed the negative effects of BAK on normal ocular surface conditions as previously shown in published reports, and for the first time, we demonstrated that BAK also has negative effects in dry eye. Furthermore, we demonstrated that the association between BAK and prostaglandin does not demonstrate the same effects as BAK alone and that a BAK-free

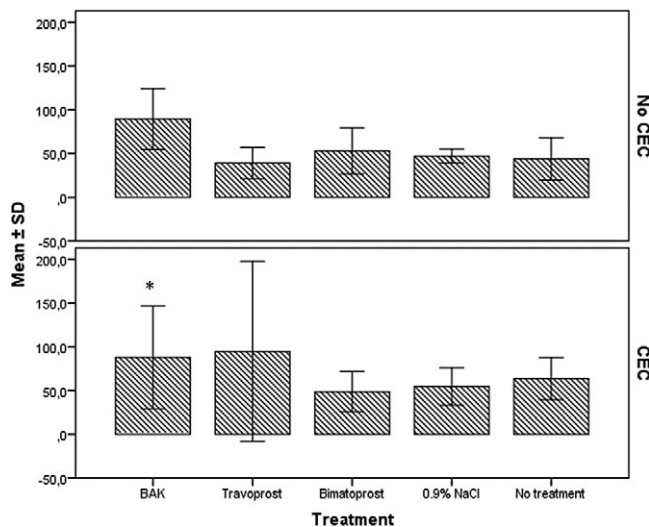


FIGURE 5. Number of CD45⁺ cells is shown at the level of bulbar inferior conjunctiva after 7 days of treatment. BAK induced a significant increase of CD45⁺ cells in the bulbar conjunctiva compared to bimatoprost ($*P < 0.05$, Tukey HSD test). Number of eyes (n) for each group: BAK, 10; travoprost, 7; bimatoprost, 13; 0.9% NaCl, no CEC, 5; and no treatment, 10. BAK, benzalkonium chloride. Results are means \pm SD.

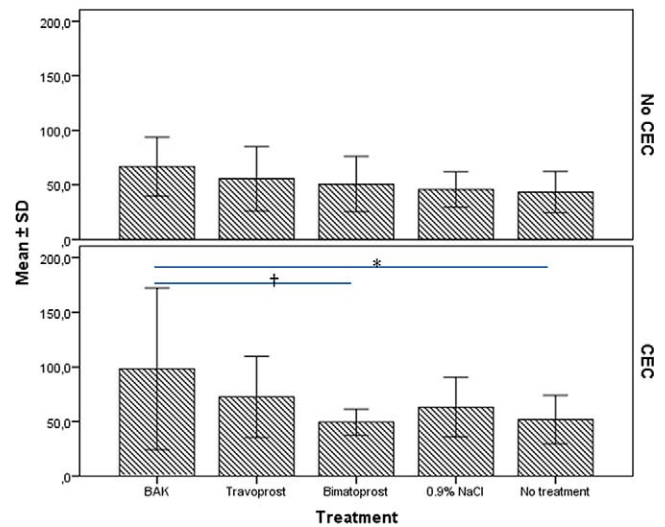


FIGURE 6. Number of CD45⁺ cells is shown at the level of inferior fornix conjunctiva after 7 days of treatment. BAK induced a significant increase of CD45⁺ cells compared to bimatoprost ($\dagger P < 0.05$) and nontreated mice ($*P < 0.05$, Tukey HSD test). Number of eyes (n) for each group: BAK, 10; travoprost, 7; bimatoprost, 13; 0.9% NaCl, no CEC, 5; no treatment, 10. BAK, benzalkonium chloride; CEC, controlled environment chamber. Results are means \pm SD.

travoprost preparation may have positive effects on tear secretion and corneal protection.

The effect of BAK has so far been studied in animal models such as rabbits and rats to assess potential acute irritation and corneal changes, using confocal microscopy. Despite the small size of the mice, we used a mouse model because of the advanced murine immunogenetics and extensive availability of reagents and a standardized model which mimics dry eye syndrome. As we demonstrated in a previous study,⁴ the model is based on the use of a controlled environment chamber that induces dry eye in mice by decreasing humidity and by a constant controlled air flow. The reason for choosing 7 days as endpoint in this study was related to the maximum effect in terms of reduced tear secretion and corneal damage reached by the C57BL/6 mice at this time point.⁹ The reasons for choosing female C57BL/6 mice were that this mouse strain develops a predominantly Th-1 response, based on interferon- γ and tumor necrosis factor- α responses, and that it has been demonstrated to develop more significant signs of dry eye than in BALB/c mice.⁹ In our study, BAK did not induce any changes in tear secretion under normal conditions, similarly to what has been demonstrated in rabbits¹⁰ and humans.¹¹ Interestingly, after 7 days of exposure to CEC, BAK did not induce a significant decrease of tear production, in contrast to what could be expected considering the well-known effect of an adverse environment,^{4,12} whereas travoprost and bimatoprost were able to increase tear production. We do not have any definitive explanation for the mechanisms underlying these two different effects. Because the ocular surface is a functional unit, we should probably study possible changes at the level of the lacrimal gland, even if a previous report has not demonstrated any tissue modifications induced by BAK¹³ and/or changes in corneal nerves and accessory lacrimal glands induced by the use of BAK and antiglaucoma treatments.

Precisely evaluating the condition of the ocular surface is a critical aspect of identifying dry eye syndrome signs in humans and animals alike. Use of fluorescein represents an important diagnostic tool in our hands, to study the corneal epithelium. We adopted the use of a determined quantity and concentration of fluorescein and its delivery by means of a micropipette,

allowing for a 3-minute time interval between dye instillation and observation, and use of a standardized grading scheme specific to the cornea in order to assess ocular surface epitheliopathy in mice. Corneal epithelial damage is a well-known effect of BAK. In 1944, Swan¹⁴ found that BAK causes punctate corneal epithelial damage at a concentration of 0.04% and edema and cellular desquamation with corneal epithelial lesions at a concentration of 0.1%. Similar results were obtained in animal studies and human clinical trials.¹⁵ We confirmed that BAK induces corneal damage under normal conditions when administered alone, and similar levels of damage were recorded after exposure to CEC, which means that BAK maintains the toxic effect compared to other groups even under dry eye conditions. The effect of CEC was significant in all treatments and demonstrated that travoprost protects ocular surface epithelia, inducing a corneal damage significantly lower than environmental conditions with and without 0.9% NaCl treatment. Also, a similar effect was shown by the combination of bimatoprost and BAK. These data seem to be very important, because for the first time, they show that prostaglandins are not toxic to the ocular surface under normal and dry eye conditions.

Corneal damage could be related to a direct action of BAK or to qualitative changes of the tear film. In vitro studies with a reconstructed three-dimensional model of human corneal epithelial cells showed an increased number of apoptotic cells after exposure to BAK in a dose-dependent manner.¹⁶ Also, the authors demonstrated that BAK induces overexpression of ICAM-1, which facilitates attachment of inflammatory cells that are responsible for cell damage, similar to what happens on conjunctival cells in dry eye patients¹⁷ and after long-term use of antiglaucoma medications containing BAK.¹⁸ Studies using in vivo tandem scanning confocal microscopy and scanning electron microscopy in rabbit corneas showed that immediately after the application of 0.02% and 0.01% BAK, corneal superficial cells were damaged,¹⁹ showing disruption of tight junctions²⁰ and appearance of inflammatory infiltrates in the peripheral cornea.²¹ Similarly, confocal microscopy studies in rat showed a dose-dependent relationship between BAK concentrations and corneal epithelial damage and stromal inflammation.²² Inflammation could be secondary to BAK instillation, because in our study there was a significant increase of CD45⁺ cells at the level of the bulbar conjunctiva which was not exacerbated by CEC conditions. The association of BAK and prostaglandins does not seem to be able to induce the same inflammatory reactions in the conjunctiva, consistent with results of the study of Whitson et al.,²³ who randomized 106 patients from BAK-preserved latanoprost to receive either BAK-preserved latanoprost, bimatoprost, or SofZia-preserved travoprost (Travatan Z; Alcon) for 3 months and found no differences in conjunctival hyperemia. Travoprost preserved with polyquaternium-1 (PQ-1) did not show any significant inflammation under normal conditions and after exposure to CEC. The possible explanation is that PQ-1 is a polycationic preservative that lacks a large hydrophobic domain and therefore does not act as detergent.²⁴

A better understanding of the inflammatory pathway on the ocular surface is a key point in our opinion to better clarify the influence of BAK and antiglaucoma drops. In this study we used markers for CD45⁺ cells only. It would be interesting to further investigate these cells for CD4 and CD11b expression.

Tear film has an important protective role for ocular surface epithelia. Part of this effect is due to the mucin layer produced mainly by the goblet cells in the conjunctiva. Goblet cell density is a critical parameter that reflects the overall health of the ocular surface.²⁵ These cells synthesize, store, and secrete large gel-forming mucins that lubricate and protect the ocular surface.²⁶ Previous studies have demonstrated that decreased

numbers of mucin-containing goblet cells is characteristic of human dry eye.²⁷ Because in our study we demonstrated that BAK can decrease the number of goblet cells in the conjunctiva after exposure to the CEC compared to all other groups, we can speculate that at least part of the corneal damage is due to the lack of mucins on ocular surface epithelia and tear film. Other studies have shown similar results in patients after short exposure to BAK²⁸ or BAK-containing timolol²⁹ and after long-term antiglaucoma treatment in conjunctival biopsy specimens from patients undergoing filtration surgery.³⁰ Interestingly, travoprost, and especially bimatoprost, increased the number of goblet cells after 1 week of treatment compared to that in 0.9% NaCl and nontreated groups. A similar effect was shown in rabbits by Russ et al.,³¹ who observed an increase in the conjunctival goblet cells in animals treated with prostaglandin analogues, and by de Faria et al.³² by using a fixed combination of prostaglandin analogs and timolol. In this case, the increase was significantly higher in the group treated with combined bimatoprost plus timolol than in the group treated with travoprost plus timolol. Because previous studies have shown that β -blockers induce a reduction in the number of goblet cells,³³ we can argue that the increased number of goblet cells is due to the prostaglandin effect. The significance of this change in goblet cells could be interpreted as a positive or a negative response of the ocular surface system, because pollution has been shown to induce goblet-cell hyperplasia in human conjunctival epithelium.³⁴

Alteration of corneal epithelium does not seem to influence corneal structure. In our study, we measured corneal thickness after 7 days of exposure to CEC, and the BAK-treated group did not show significant changes compared to the control group. Travoprost, instead, showed a significant increase compared to all other groups. The importance of this parameter is still debated. Previous studies in humans have reported a reduction in central corneal thickness in Sjögren's and non-Sjögren's dry eye,^{35,36} whereas Fabiani et al.⁶ reported an increased thickness of the epithelium layer in dry eye mice.

In conclusion, further studies are certainly necessary for a better understanding of the effect of preservatives and prostaglandins on the ocular surface, but our results indicate the importance of studying antiglaucoma treatments under normal and ocular surface disease conditions.

Acknowledgments

Supported in part by Alcon.

Disclosure: **S. Barabino**, None; **S. Antonelli**, None; **N. Cimbotini**, None; **V. Mauro**, None; **M. Bouzin**, None

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