A Rabbit Dry Eye Model Induced by Topical Medication of a Preservative Benzalkonium Chloride

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PURPOSE. To establish a rabbit dry eye model with topical medication of the ocular preparation preservative benzalkonium chloride (BAC).

METHODS. Sixteen white rabbits were used. One eye of each rabbit was chosen randomly for topical administration of 0.1% BAC twice daily for 14 days. The other untreated eyes served as controls. Schirmer test, fluorescein, and rose bengal staining were performed before and after BAC treatment on days 3, 5, 7, and 14. Conjunctiva impression cytology specimens were collected on days 0, 7, and 14. The rabbits were killed after day 14. Immunofluorescence staining was performed to detect mucin-5 subtype AC (MUC5AC) on conjunctival cryosections. Corneal and conjunctiva structures were evaluated by light and electron microscopy.

RESULTS. Compared with untreated controls, BAC-treated eyes showed significant decreases in Schirmer scores (P = 0.001) and increases in fluorescein scores (P < 0.001) on days 5, 7, and 14. A significant increase in rose bengal scores was noticed as early as day 5 (P = 0.001). Decreased goblet cell density occurred on days 7 and 14 (P = 0.001). Decreased MUC5AC and histopathologic and ultrastructural disorders of the cornea and conjunctiva were also observed in the BAC group.

CONCLUSIONS. These findings demonstrated that an ophthalmic preservative, benzalkonium chloride, induced a dry eye syndrome in rabbits with damage to the cornea and conjunctiva, decreased aqueous tear basal secretion, goblet cell loss, and MUC5AC deficiency. This rabbit model was consistent with the human dry eye syndrome in both aqueous tear and mucin deficiency or excessive tear evaporation. This condition has been designed to show progressive lacrimal gland inflammation resembling the development of Sjögren syndrome in certain strains of mice,10–12 androgen deficiency after orchiectomy in rats,13 pharmacologic blockade of cholinergic muscarinic receptors in the lacrimal glands,14 and the effect of desiccating environments in mice.15 However, none of these models seems to mirror precisely the complexity and chronicity of this frequent disease.16

Preservative is an important component of ophthalmic preparations, providing antimicrobial activity and preventing decomposition of the active drug. However, significant cytotoxic effects of preservatives are associated with long-term use. The most common preservative in ophthalmic preparations for glaucoma and ocular surface disease is benzalkonium chloride (BAC), which is most often used at a concentration of 0.01% (range, 0.004%–0.02%) in topical multidose solutions.17 BAC is a quaternary ammonium compound that has been shown to hasten the drying of tear film,18,19 worsen preexisting dry eye,20 and affect both the cornea and the conjunctiva.21 Epidemiologic data also show that symptoms and signs of dry eye are more prevalent when BAC-preserved drops are used.22

Based on the side effect of BAC, this study was conducted to evaluate its effects on the ocular surface of normal rabbits and to develop a rabbit dry eye model with topical administration of this preservative. In this manner, the present study sought to determine the clinical subtype of the preservative-induced dry eye syndrome.

MATERIALS AND METHODS

Animals

Sixteen female white New Zealand rabbits (purchased from Guangdong Medical Laboratory Animal Center, Guangdong, China) weighing between 2 and 2.5 kg were used for this study. The rabbits were quarantined and acclimatized a week before the experiments in the Ophthalmic Animal Laboratory of Zhongshan Ophthalmic Center at
Experiments were performed on the rabbits under standard conditions throughout the study as follows: room temperature 23°C ± 2°C, relative humidity 60% ± 10%, and alternating 12-hour light-dark cycles (8 AM to 8 PM). All procedures were performed with adherence with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Experimental Procedure**

One eye of each rabbit was chosen randomly for twice-daily topical administration of 0.1% BAC drops (Sigma, St. Louis, MO) as a BAC-treated group (total, 16 treated eyes). The ophthalmic preparation was adjusted to iso-osmotic by the pharmaceutical preparation section of Zhongshan Ophthalmic Center. This concentration and frequency of medication were carefully selected based on the data of preliminary experiments (see Results). The other untreated eyes of 16 rabbits that were exposed to no solution were used as a control group.

In both the BAC-treated and the control groups, Schirmer test, fluorescein and rose bengal staining, and conjunctiva impression cytology (CIC) were performed before and after BAC treatment. At day 14, all rabbits were killed with overdoses of phenobarbital sodium. The conjunctival MUC5AC was detected with immunofluorescence staining on cryosections. The structures of the cornea and conjunctiva in the same area of the two groups, BAC treated and control, were observed by light microscopy, scanning electron microscopy, and transmission electron microscopy.

**Measurement of Aqueous Tear Production**

Tear production was measured with a modified Schirmer test using Whatman 41 filter paper strip (Tianjin Jingming New Technology Development Co., Ltd., Tianjin, China) on days 0, 3, 5, 7, and 14. Intramuscular injection of a mixture of 50 mg ketamine and 25 mg chloropromazine was administered to keep the rabbits immobile. After topical application of proparacaine (Alcaine; Alcon, Fort Worth, TX), the Schirmer paper strip was inserted into the conjunctival sac location around the junction of the middle and outer thirds of the lower lid. The wetted length (mm) of the paper strip was read after 5 minutes.

**Fluorescein and Rose Bengal Staining on Ocular Surface**

Corneal fluorescein staining was performed on days 0, 3, 5, 7, and 14. Two minutes after 2 μL of 1% fluorescein sodium was dropped into the conjunctival sac, the ocular surface was examined and graded under a slit lamp microscope (Topcon). Using the Van Bijsterveld grading system, the scores were graded after 15 seconds.

Rose bengal was applied on days 0, 3, 5, 7, and 14. After instillation of 2 μL 1% rose bengal into conjunctival sac, the ocular surface was

**Immunofluorescence Staining for MUC5AC**

Immunodetection of MUC5AC was performed by immunofluorescence staining on cryosections of the nasal and temporal bulbar conjunctiva from the BAC-treated and control groups on day 14. Corneal specimens were used as one of the negative controls. All specimens were fixed in acetone at 4°C for 10 minutes. After 3 washes in phosphate-buffer saline (PBS), the specimens were blocked with 10% goat serum for 30 minutes at room temperature and incubated for 12 hours at 4°C with a 1:150 dilution of mouse anti-rabbit MUC5AC antibody (Abcam, Cambridge, UK). After PBS washes, the specimens were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary goat anti-mouse IgG (Cell Signaling Technology, Inc., Danvers, MA) for 45 minutes at room temperature, followed by three washes in PBS and nuclei counterstaining with 0.5 μg/mL Hoechst 33342 dye (Sigma). The specimens were observed under a confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany).

**Conjunctival Impression Cytology**

CIC specimens were collected on days 0, 7, and 14. Intramuscular injection of 50 mg ketamine and 25 mg chlorpromazine was given to immobilize the rabbit. Two 3.5 mm × 3.5 mm circular disks of nitrocellulose filter paper (Pall Corporation, New York, NY) were placed separately on the nasal and temporal bulbar conjunctiva with the filter paper dull-side down. After the filter paper was pressed for 10 seconds with constant pressure, it was gently lifted and fixed with 95% alcohol. Hematoxylin and periodic acid-Schiff (PAS) reagents were used to stain the specimens. After staining, the number of goblet cells was counted under a microscope (Olympus, Tokyo, Japan) with a 40× objective, and the morphology of the conjunctival epithelium was graded according to the Nelson system. Three different sections of each specimen were selected randomly for counting, and an average was calculated (cells/high-power [HP] visual field with 400×).

**Light Microscopy**

Corneas and conjunctivas were fixed in 10% formalin. After dehydration, the specimens were embedded in paraffin, cross-sectioned, and stained with hematoxylin and eosin or with the PAS reagent.
Scanning Electron Microscope

Corneas were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and postfixed with osmium tetroxide in 0.1 M phosphate buffer. The specimens were dehydrated in a graded series of ethanol, critical-point dried, gold-coated with platinum, and examined under a scanning electronic microscope (JSM-6330F; JEOL, Ltd., Tokyo, Japan).

Transmission Electron Microscope

Corneas were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed with osmium tetroxide in 0.1 M phosphate buffer, embedded in resin, and cut in 60-nm sections. The specimens were observed and photographed under a transmission electron microscope (H600; Hitachi, Ltd., Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed with special software (SPSS, ver. 13.0; SPSS, Inc., IL). Student's paired $t$-test for aqueous tear production and conjunctival goblet cell density and Wilcoxon signed-rank test for fluorescein and rose bengal scores were used to compare the differences between the BAC-treated and control groups. All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Effects of BAC on Rabbit Ocular Surface

The effects on the rabbit ocular surface of different concentrations of BAC (0.01%, 0.05%, 0.1%, 0.2%, 0.3%, 0.5%, and 1.0%), different frequencies of topical application (2–4 times daily), and different lengths of treatment (1–4 weeks) were evaluated with the Schirmer test, ocular surface staining, CIC, and microscopy. Twice-daily topical administration of 0.1% BAC drops for 2 weeks was chosen as our optimal procedure to induce dry eye syndrome in white New Zealand rabbits. The use of lower BAC concentrations was found to be insufficient to stimulate tear deficiency over a 2-week period. Higher concentrations and multiple applications damaged the ocular surface seriously and even caused corneal ulcer, vascularization, and scarring, similar to what occurs with chemical burn.

Aqueous Tear Production

No statistical differences were recorded at baseline (day 0) between the BAC-treated and untreated control groups. Significant differences were found on days 5, 7, and 14 ($P = 0.01$, 0.01, and 0.01, respectively; Fig. 1). Compared with baseline, statistically significant decreases were observed on days 5, 7, and 14 in the BAC group ($P = 0.01$, 0.01, and 0.01, respectively). There were no statistically significant differences between baseline and each time point in the control group ($P > 0.05$).

Fluorescein and Rose Bengal Staining on Ocular Surface

There was no significant differences in minimal corneal punctate staining between the BAC and control groups at baseline. There were significant differences on days 5, 7, and 14 ($P = 0.001$, all time points) between the two groups (Figs. 2, 3). In
the BAC group, fluorescein scores were significantly increased on days 5, 7, and 14 compared with baseline ($P < 0.001$, all time points). There were no apparent changes in control group scores throughout the entire study period.

In the BAC group, significant increases in the rose bengal scores were also observed on days 3, 5, 7, and 14 (Figs. 4, 5) compared with baseline at day 0 (all $P < 0.001$). In the control group, no apparent changes in scores were observed throughout the study.

**Goblet Cell Density in Conjunctival Impression Cytology**

There was no difference in goblet cell number between the 2 groups at day 0 as a baseline. The number of goblet cells in the BAC group was significantly lower than that in the control group at days 7 and 14 (both $P = 0.001$; Fig. 6). In the BAC group, the number of goblet cells decreased significantly on days 7 and 14 compared with baseline (both $P = 0.001$). Grading scores increased from grade 0 to grade 1 on day 7 and up to grade 2 on day 14 (Figs. 7A–C). No apparent changes in scores were observed on days 7 and 14 in the control group.

**MUC5AC Staining on Conjunctiva**

Immunofluorescence staining for MUC5AC using anti-MUC5AC antibody showed an obvious decrease of MUC5AC in the BAC group, fluorescein scores were significantly increased on days 5, 7, and 14 compared with baseline ($P < 0.001$, all time points). There were no apparent changes in control group scores throughout the entire study period.

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were unremarkable between these 2 groups (Figs. 9A, 9B). Differences in the Descemet membrane and the endothelium and more eosinophilic than in healthy controls. In addition, corneal epithelium was thinner, and the stroma was edemic rabbit corneas of the control group. In the BAC group, the five layers of epithelial cells and collagen fibrils of the stroma in As could be seen under a light microscope, there were three to five layers of epithelial cells and collagen fibrils of the stroma in rabbit corneas of the control group. In the BAC group, the corneal epithelium was thinner, and the stroma was edemic and more eosinophilic than in healthy controls. In addition, differences in the Descemet membrane and the endothelium were unremarkable between these 2 groups (Figs. 9A, 9B).

In the control group, the conjunctiva contained a cuboidal basal epithelial layer and three or four overlying columnar layers with interspersed goblet cells. In the BAC-treated group, the conjunctiva became thinner and lost the cuboidal layer. PAS-positive goblet cells were significantly decreased compared with the control group (Figs. 9C, 9D).

Under a scanning electron microscope, many bright cells could be seen among the corneal epithelium, and high-density microvilli and microplacae covered the surfaces of cells in the control group. In the BAC group, a large area was covered with dark cells, and the corneal epithelial microvilli were destroyed (Fig. 10).

With the transmission electron microscope, it could be seen that corneal epithelial cells displayed a microvillar structure on the surfaces of their outer membranes in the control eyes. High magnification revealed that many desmosomes anchored cell-cell junctions and many Golgi apparatus, mitochondria, and rough endoplasmic reticulum (Fig. 11A). In the BAC group, reduction of number and size of microvilli, swelling of mitochondria, and rough endoplasmic reticulum were observed (Figs. 11B, 11C).

Similar changes in conjunctiva were observed. Under the scanning electron microscope, the conjunctival epithelium in the control group showed many microvilli. Goblet cells were interposed between the epithelial cells with many secretory granules, which play an important role in the synthesis of MUC5AC (Fig. 12A). In epithelial cells of the BAC group, reductions of microvilli and nuclear ultrastructural changes, consistent with apoptosis (as chromatin condensation and peripheral migration), were observed (Fig. 12B). There were fewer secretory granules in goblet cells in the BAC group than in the control group.

**DISCUSSION**

A good animal model that simulates dry eye problems would be a useful tool, not only for investigation of the pathophysiology and pathogenesis of dry eye but also for the development of new therapeutic treatments. Developing a new animal model would be important, though several animal models have been reported. Dry eye is a complicated condition caused by different etiologies and affecting different tear film levels, and it manifests as various clinical subtypes. In this study, we introduced a rabbit model of dry eye developed by a novel treatment with topical application of an ophthalmic preservative, BAC. To avoid errors caused by individual ocular variations and measurement protocols, we used the contralateral eyes as controls. We strictly applied the same defined procedures to both eyes in our study. Our findings demonstrated that the abnormal ocular surface manifestations induced by topical medication of 0.1% BAC were consistent with human dry eye syndrome, including tear deficiency, lower conjunctival goblet cell density, and decreased mucin secretion.

The Schirmer test is the classic method for measuring the production of the aqueous component in tears. In this study, Schirmer test scores decreased significantly in the BAC-treated group, consistent with human tear-deficient dry eye. We found that the Schirmer test, performed with standard filter paper for 5 minutes with topical anesthesia, could maximize reproducibility and minimize confounding; however, the type of anesthesia and the removal of excess tears may be why the average wet length was shorter than in previous reports. Fluorescein staining and rose bengal staining are effective methods for ocular surface evaluation. Fluorescein staining is the result of uptake caused by the disruption of corneal epi-

**FIGURE 10.** Representative images of scanning electron microscopy for the corneal epithelium. (A) In the control group (15,000×), cells were covered with high-density microvilli and microplacae. (B) In the BAC group (15,000×), cells had flat surfaces with destroyed microvilli.

**FIGURE 11.** Representative images of transmission electron microscopy showing the corneal epithelial ultrastructure. (A) In the control group, there were many microvilli (Mv), desmosomes (De), Golgi apparatus (Go), and rough endoplasmic reticulum (RER) in the corneal epithelial cells (15,000×). (B) In the BAC-treated group, corneal cells exhibited disruption of the Mv and widening of the perinuclear cisterna (PN). (C) In the BAC group, the corneal cells showed swelling of mitochondria (Mi), Go, and RER (15,000×).
Tseng,30 rose bengal can stain live and dead cells if they are not protected by the tears or tears substitute.29 According to Feenstra and Tseng,30 rose bengal can stain live and dead cells if they are not protected by an intact mucin layer. As shown in Figures 3 to 5, the BAC-treated group had diffuse punctuate epitheliopathy. Rose bengal staining was apparent earlier than fluorescein staining, which is consistent with clinical human dry eye syndrome. The limitation of these methods when used in rabbits is that the evaluation schemes used in humans do not fit rabbits very well. These evaluations will have to be further standardized.

Conjunctival goblet cells are the main source of ocular surface mucopolysaccharides that lubricate and protect the ocular surface.31 It is likely that the poorly lubricated ocular surface resulting from reduced mucin production makes the tear film unstable. Thus, resultant ocular surface damage is indicated by fluorescein and rose bengal staining. Goblet cell density is a critical parameter that reflects the overall health of the ocular surface.32 Conjunctival impression cytology is the most useful noninvasive technique24 and can be performed during the entire treatment period for dry eye. Therefore, we used this method to quantify the density of goblet cells and to grade the ocular surface. A striking finding in the BAC group was a marked decrease in the number of conjunctival goblet cells by CIC and paraffin section in this study. The results of immunofluorescence staining confirmed the decrease in MUC5AC. However, the reason for the disappearance of PAS-stained goblet cells in our rabbit was not investigated. Transmission electron micrographs showed a reduction of glycogen inside the goblet cells. It is possible that these cells still exist but cannot be visualized with CIC because of inhibition of mucin production by BAC. It has been reported that long-term instillation of preservative-containing antiglaucoma drugs induces the loss of goblet cells53 and decreases the MUC5AC expression of conjunctival cells by flow cytometry.54 Mucin deficiency was originally proposed as a leading cause of dry eye3; hence, the loss of goblet cells and the decrease of mucin in this model may be an excellent choice for studying the subtype of mucin-deficiency dry eye.

Besides the decrease of goblet cells, CIC also showed that squamous metaplasia of the conjunctiva developed with time by the flattening of conjunctival epithelial cells and the increase of nucleocytoplasmic ratio. Light microscopy supported this finding and showed a decrease of corneal epithelial cells.

The application of preservatives induces the reduction of corneal cell proliferation and viability, impairs corneal healing, and disrupts epithelial barrier function. BAC has been associated with dry eye syndrome.35 In a previous study, BAC exerted direct cell toxicity, damaging cell membranes and cytoplasmic organelles and impeding cellular metabolic function.36 In this study, although the corneal epithelial cells in the BAC group appeared unremarkably different under light microscopy, scanning electron and transmission electron microscopy revealed an obvious decrease of microvilli. These structures are important for the attachment of mucin to the cornea. This cell damage is also the reason for the positive staining in the fluorescein and rose bengal tests in the BAC group.

Most existing dry eye models were introduced by mechanical damage, chemical cauterization, and surgical wounding, including lacrimal gland extirpation or obstruction of the main lacrimal gland opening through trauma, which only damages the main lacrimal gland. In nature, the aqueous component of tear film was secreted mainly by the accessory lacrimal glands in conjunctiva but did not include irritants excreted from the main lacrimal gland. This BAC-induced dry eye model, the decrease in the aqueous component of tear film was caused by damage to the cornea and conjunctiva, in accordance with the natural etiology and pathophysiology as they relate to the effects of stability of the precorneal tear film. It is more important that conjunctival damage lead to the decrease of goblet cell density and alter the production of mucin and the glycosylation of membrane-associated mucins.

In conclusion, our findings demonstrated that an ophthalmic preservative, BAC, induced a dry eye syndrome in rabbits, caused damage to the cornea and conjunctiva, decreased aqueous tear basal secretion, and caused goblet cell loss and MUC5AC deficiency. Phenotypic changes on the ocular surface in this rabbit model were consistent with human dry eye syndrome in both aqueous and mucin deficiency. This new rabbit model would be an appropriate one for studying dry eye syndrome, especially for the subtype of mucin-deficiency dry eye.

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References


