Corneal Neurotoxicity Due to Topical Benzalkonium Chloride

Joy Sarkar, Shweta Chaudhary, Abed Namavari, Okan Ozturk, Jin-Hong Chang, Lisette Yco, Snehal Sonawane, Vishakha Khanolkar, Joelle Hallak, and Sandeep Jain

PURPOSE. The aim of this study was to determine and characterize the effect of topical application of benzalkonium chloride (BAK) on corneal nerves in vivo and in vitro.

METHODS. *Thy1*-YFP+ neurofluorescent mouse eyes were treated topically with vehicle or BAK (0.01% or 0.1%). Wide-field stereofluorescence microscopy was performed to sequentially image the treated corneas in vivo every week for 4 weeks, and changes in stromal nerve fiber density (NFD) and aqueous tear production were determined. Whole-mount immunofluorescence staining of corneas was performed with antibodies to axonopathy marker SMI-32. Western immunoblot analyses were performed on trigeminal ganglion and corneal lysates to determine abundance of proteins associated with neurotoxicity and regeneration. Compartmental culture of trigeminal ganglion neurons was performed in Campenot devices to determine whether BAK affects neurite outgrowth.

RESULTS. BAK-treated corneas exhibited significantly reduced NFD and aqueous tear production, and increased inflammatory cell infiltration and fluorescein staining at 1 week ($P < 0.05$). These changes were most significant after 0.1% BAK treatment. The extent of inflammatory cell infiltration in the cornea showed a significant negative correlation with NFD. Sequential in vivo imaging of corneas showed two forms of BAK-induced neurotoxicity: reversible neurotoxicity characterized by axonopathy and recovery, and irreversible neurotoxicity characterized by nerve degeneration and regeneration. Increased abundance of beta III tubulin in corneal lysates confirmed regeneration. A dose-related significant reduction in neurites occurred after BAK addition to compartmental cultures of dissociated trigeminal ganglion cells. Although both BAK doses (0.0001% and 0.001%) reduced nerve fiber length, the reduction was significantly more with the higher dose ($P < 0.001$).

CONCLUSION. Topical application of BAK to the eye causes corneal neurotoxicity, inflammation, and reduced aqueous tear production. (*Invest Ophthalmol Vis Sci.* 2012;53:1792-1802) DOI:10.1167/iops.11-8775

From the Corneal Neurobiology Laboratory, Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, College of Medicine, Chicago, Illinois.

Supported by National Eye Institute (NEI) Grant EY018874 (SJ), NEI core grant EY001792 and Research to Prevent Blindness.

Submitted for publication October 10, 2011; revised February 15, 2012; accepted February 19, 2012.

Disclosure: J. Sarkar, None; S. Chaudhary, None; A. Namavari, None; O. Ozturk, None; J.-H. Chang, None; L. Yco, None; S. Sonawane, None; V. Khanolkar, None; J. Hallak, None; S. Jain, None

Corresponding author: Sandeep Jain, Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, 1855 W. Taylor Street, Chicago, IL 60612; jains@uic.edu.

Several published studies provide evidence of benzalkonium chloride (BAK)-induced neurotoxicity in extraocular tissues.¹⁻³ BAK has been applied to the gastrointestinal tract and bladder to produce chemical denervation for functional studies. BAK selectively and irreversibly destroys nerve cell membranes without harming muscular tissue. For bladder denervation in rats, 0.3% BAK was applied for 30 minutes.¹ For denervation of the myenteric plexus in mouse colon, 0.05% BAK was applied for 30 minutes.² Subsequent nerve regeneration was reported in the denervated area.³ Despite evidence that topical application of BAK produces chemical denervation in several extraocular tissues, and the knowledge that corneal nerves are essential for maintaining healthy ocular surfaces, the effect of BAK on corneal nerves remains largely unknown.

BAK is the most commonly used bactericidal preservative in ophthalmic preparations (reviewed by Baudouin et al.⁴). It is a quaternary ammonium cationic surface-acting agent that dissolves the bacterial walls and membranes by detergent action. It is well-documented that BAK causes dose-dependent conjunctival and corneal epithelial cell toxicity in vivo^{5,6} and in vitro,^{7,8} tear film instability, and corneal epithelial barrier dysfunction.⁹ Martone et al.¹⁰ have reported that patients treated with BAK-preserved glaucoma eye drops have reduced subbasal nerve density, lower corneal sensitivity, and reduced tear secretion. BAK may enhance transcorneal permeability of ophthalmic drugs¹¹ or increase epithelial permeability.¹² The permeation enhancing actions of BAK have recently been used to perform collagen crosslinking without epithelial debridement.¹³

In *Thy1*-YFP+ mice, corneal nerves are fluorescent, making in vivo visualization of nerves feasible.¹⁴ We have recently described nerve regeneration events in these mice.^{15,16} In the investigations reported here, we applied BAK to eyes of these mice and sequentially visualized the corneal nerves to characterize BAK-induced neurotoxicity. Furthermore, we confirmed BAK-induced neurotoxicity by molecular analyses of markers for nerve degeneration and regeneration and in cultured trigeminal ganglion cells.

METHODS

Animals

All animal experiments were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animal protocol was approved by Animal Care Committee (ACC) of the University of Illinois at Chicago. Neurofluorescent homozygous adult mice (6-8 weeks old) from the *thy1*-YFP line were purchased from Jackson Laboratories (Bar Harbor, ME). For in vivo experiments, mice were anesthetized with intraperitoneal injections of a combination of ketamine (20 mg/kg; Phoenix Scientific, St. Joseph, MO) and xylazine (6 mg/kg; Phoenix Scientific). For terminal experiments, mice were sacrificed according to animal committee protocols.

Animal Treatments

For neurotoxicity experiments, mice ($n = 5/\text{group}$) were treated with BAK (Sigma-Aldrich, St. Louis, MO), either 0.1% or 0.01% dissolved in balanced salt solution (BSS), once a day for 1 week. Mice were gently restrained, and 10 μL BAK solution was applied by micropipette into the inferior conjunctival sac of the eye. Eyes were held open for 30 seconds to allow for adequate ocular surface contact of the drug, and to prevent aggressive blinking during application of the drug, which may cause variability in the ocular surface contact time of the drug. Untreated mouse eyes and eyes treated with vehicle (BSS) were controls. Stereofluorescence imaging was performed and aqueous tear production determined as in the following section. Corneal fluorescein staining was performed in age-matched C57BL/6 mice because *thy1*-YFP mouse corneas show fluorescent properties from the YFP+ inflammatory cells that infiltrate the cornea after BAK treatment and the fluorescence from fluorescein-stained superficial punctate keratitis is indistinguishable. For recovery of neurotoxicity experiments, mice eyes ($n = 7$) were treated with 0.1% BAK once a day for 1 week. After BAK treatment cessation, mice were followed sequentially for another 3 weeks and recovery of nerves was visualized. At 1- and 4-week time points, mice were sacrificed, and corneas were excised and processed for hematoxylin-eosin staining, whole mount confocal microscopy, and Western immunoblot analysis as described in following sections.

Stereofluorescence Imaging

Sequential in vivo photography was performed using a fluorescence stereoscope (StereoLumar V.12, Carl Zeiss, GmbH, Hamburg, Germany) equipped with a digital camera (AxioCam MRm) and Axiovision 4.0 software. An anesthetized *thy1*-YFP mouse was placed on the stereoscope stage. Seven microliters of proparacaine (0.5%, Bausch & Lomb, Tampa, FL) was applied for 3 minutes, and the pupil was constricted with 0.01% carbachol (Miostat, Alcon, Fort Worth, TX) for 5 minutes. Z-stack images were obtained at 5- μm intervals and compacted into one maximum intensity projection image after alignment using Zeiss Axiovision software. Nerve fibers were traced manually using Neurolucida software (MBF Bioscience, Williston, VT). Corresponding points on the images from different time points were selected to make a contour, and nerve tracing was performed within the contour area. Only stromal nerves were included in the analysis. Subbasal hairpin nerves were excluded. Neuroexplorer software (Nex Technologies, Littleton, MA) was used to measure the total length of stromal nerves and the area of the contour in which nerves had been traced. Corneal nerve fiber density (NFD) was calculated by dividing the total length of nerve fibers (mm) by the area of the contour (mm^2) as described by Al-Aqaba et al.¹⁷

Measurement of Aqueous Tear Production

Tear production was measured with the phenol red thread test (Zone-Quick; Lacrimedics, Eastsound, WA). Under a stereo-fluorescent microscope, the threads were held with jeweler forceps and placed in the lateral canthus of the conjunctival fornix of the eye for 30 seconds. The thread was imaged and the tear distance (in millimeters) was determined using Axiovision software.

Corneal Fluorescein Staining

Corneal fluorescein staining was performed by applying 0.5 μL of 0.1% fluorescein by micropipette into the inferior conjunctival sac of the eye as reported previously.¹⁸ The cornea was imaged using the stereo-fluorescent microscope using blue filter 3 minutes after fluorescein instillation. The images were analyzed with a standardized (National Eye Institute) grading system.¹⁹

Hematoxylin-Eosin Staining

Excised eyes were fixed in 4% paraformaldehyde (PFA) for 48 hours, embedded in paraffin, and processed for sectioning by the UIC Research Resources Center Pathology Core facility. Sections (5- to 8- μm -thick sagittal slices) were cut, mounted onto glass slides (R 7200, Mercedes Medical, Sarasota, FL), stained with hematoxylin-eosin, and viewed using an upright Axioscope 100 microscope (Carl Zeiss Meditec GmbH). Pictures were loaded through a color camera into Axiovision software and analyzed.

Corneal Whole-Mount Immunostaining and Confocal Microscopy

Excised corneas were directly fixed in 4% PFA for 1 hour at room temperature, and washed four times with PBS (15 minutes each). The epithelium and endothelium were removed using a rotating brush to increase penetration of the reagents. Corneas were then permeabilized and blocked for 1 hour at room temperature in 1% Triton X-100, 1% bovine serum albumin, and 10% normal donkey serum in PBS. Corneas were incubated in primary antibody diluted in the blocking solution (1:100) for 24 hours at 4°C, washed four times in PBS (15 minutes each), and incubated with secondary antibody diluted in the blocking solution (1:400) overnight at 4°C. Corneas were further washed and mounted in mounting medium on glass slides. Primary antibodies used were Neurofilament H Non-Phosphorylated Monoclonal Antibody (SMI-32; catalog no. SMI-32R, Covance Inc., Princeton, NJ; antibody specificity for axonopathy validated by Bannerman and Hahn²⁰ and Irvine and Blakemore²¹), rat anti-mouse F4/80 and CD11b (F4/80; catalog no. MCA 497R and CD11b; catalog no. MCA711 both from AbD Serotec, Raleigh, NC). The F4/80 antibody specificity for macrophages has been previously validated by Brissette-Storkus et al.²² and Kuffová et al.,²³ whereas the CD11b antibody specificity for monocytes, granulocytes, and agranulocytes has been validated by Bannerman and Hahn²⁰ and Irvine and Blakemore.²¹ Secondary antibodies used were Dylight 594-conjugated AffiniPure donkey anti-mouse and anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). Dylight 594 was chosen to ensure non-overlap with the yellow fluorescent protein (YFP) wavelength and to minimize false positive staining. Primary antibody was omitted for negative control. Z-stack images of corneal whole-mounts were obtained using a LSM 510 META confocal microscope (Carl Zeiss, GmbH, Hamburg, Germany).

Isolation and Immunostaining of Bone Marrow Resident Cells

Thy1-YFP mice were sacrificed, and their femurs were carefully cleaned from adherent soft tissue. The tip of each bone was removed, and the marrow was harvested by inserting a syringe needle (25-gauge) into one end of the bone and flushing with Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Invitrogen, Carlsbad, CA). The bone marrow cells were filtered through a 70- μm nylon mesh filter (BD Biosciences, Durham, NC). Cells were plated into six-well plastic cell culture plate and immediately imaged using wide-field fluorescent microscope to determine the presence of YFP fluorescent cells. Bone marrow cells were cultured in DMEM/F12 containing 10% fetal bovine serum (Sigma), 2 mM L-glutamine, 100 U/mL penicillin (Sigma), and 100 U/mL streptomycin (Sigma). At day 2 of culture, bone marrow cells were processed for immunostaining with antibody specific for leukocyte common antigen (rat anti-mouse CD45, catalog no. 550,539, BD Pharmingen, San Diego, CA).

Western Immunoblot Analysis

Excised corneas were snap-frozen in liquid nitrogen and homogenized using a Biopulverizer (Biospec Products Inc., Bartlesville, OK) in a modified RIPA cell lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% IGEPAL, 2.5 mM sodium pyrophosphate,

1 mM β -glycerophosphate, pH 7.4) supplemented with a complete protease inhibitor and a phosphatase inhibitor Cocktail I and II (Sigma Chemical Co., St. Louis, MO). Samples were then centrifuged at 10,000g for 15 minutes at 4°C, and the supernatant (cell lysate) was collected. Total protein was determined using a modified Lowry method (BioRad DC Protein assay, BioRad Laboratories, Hercules, CA). For Western blot analysis, 50 μ g total protein was electrophoretically run on 4% to 12% Tris-glycine SDS polyacrylamide gel (XCell SureLock Mini-Cell Electrophoresis System, Invitrogen). Samples were transferred to 0.2- μ m nitrocellulose membranes (Whatman Inc., Florham Park, NJ) by electro-elution. Membranes were blocked in Li-Cor blocking buffer (Li-Cor Biosciences, Lincoln, NE), followed by incubation overnight at 4°C with either mouse anti-IL6 (1:500; Abcam, Cambridge, MA), mouse anti-CD3e antibody (1:500; BD Pharmingen), mouse anti-GAP-43 antibody (1:1000; Millipore, Billerica, MA), or anti-chicken beta III tubulin antibody (TUBB3, 1:1000; Abcam, Cambridge, MA) diluted in blocking buffer. Rabbit polyclonal anti- β -actin (1:2000; Cell Signaling, Danvers, MA) was used as a loading control. After three 10-minute washes in PBS containing 0.1% Tween-20, the blots were incubated for 2 hours at room temperature in the fluorescently labeled secondary antibody mixture (Rockland Immunoresearch, Gilbertsville, PA) of goat anti-mouse (IRDye 800CW, 1:15,000) and goat anti-rabbit (IRDye700DX, 1:10,000) antibodies diluted in blocking buffer. Membranes were then imaged using LiCor Odyssey Infrared imager (Li-Cor Biosciences). The relative intensity of each band was determined with the LiCor Odyssey application software (LiCor Biosciences). Quantification was performed by subtracting background readings from the relative intensity for each sample band and normalizing it with that of β -actin. Data are expressed as fold-increase in protein expression of the 0.1% BAK-treated groups versus respective vehicle (BSS)-treated groups.

In Vitro Experiments

Trigeminal ganglia neurons were isolated from 10-day-old *thy1*-YFP pups and compartmental culture performed as previously described.^{16,24–26} On day 1, trigeminal neuronal cells (1×10^3) were plated in the central compartment. The side compartments were filled with a mixture that was identical except that it also contained cytosine arabinoside (AraC, 0.3 μ M; Sigma).²⁶

BAK Treatment of Dissociated Trigeminal Ganglion Neuronal Cell Cultures

Cultures were treated with BAK (0.001% and 0.0001%) or vehicle (BSS) for 4 hours on day 5. The central compartment was filled with F12 media containing 10% fetal calf serum, penicillin-streptomycin, NGF, and AraC (0.3 μ M), while the side compartments were filled with a mixture that was identical except that it lacked AraC and contained BAK (0.001% and 0.0001%) or vehicle (BSS). After 4 hours, media in all the side compartments was replaced with F12 supplemented with NGF, and cells were cultured for an additional 48 hours. Neurites were then exposed to BAK or vehicle for 4 hours. The total time of neurite growth after intervention was 48 hours. The BAK concentrations were selected based on studies by De Saint Jean et al.²⁷ Images of neurite outgrowth along tracks were acquired on day 5 and 48 hours after treatments (on day 7). All images were analyzed using Neurolucida software.

Analysis of Neurite Outgrowth in Trigeminal Ganglion Cultures

Nerve fiber length (NFL) was measured in each track using Auto-neuron software. NFL was calculated on day 5 (before vehicle or BAK treatment) and on day 7 (after vehicle or BAK treatment). The increase in NFL between days 5 and 7 was determined. In vitro experiments were terminated on day 7.

Statistical Analysis

Mean values and their standard errors were computed for untreated and treated groups at each time point. Paired two-sample t-tests and Pearson's correlation coefficient (*r*) were used for analysis. Microsoft Excel office and GraphPad Prism 4 statistics software packages were used for analysis and graphs. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

BAK Neurotoxicity In Vivo

A BAK-containing eye drop (0.01% or 0.1%) was applied to the eye once a day for 1 week. Untreated eyes and BSS-treated eyes served as controls. At baseline prior to treatment, the stromal NFD and corneal fluorescein staining were similar for all groups. Stromal NFD and corneal fluorescein staining in untreated eyes and BSS-treated eyes did not change after 1 week (Fig. 1). NFD in eyes treated with 0.01% BAK was reduced significantly (*P* = 0.02) from 8.4 ± 0.7 mm/mm² at baseline to 6.10 ± 1.4 mm/mm² at 1 week (mean $\pm$ SEM). NFD in eyes treated with 0.1% BAK also was reduced significantly (*P* = 0.001) from 8.5 ± 0.8 mm/mm² at baseline to 3.4 ± 0.8 mm/mm² at 1 week (Fig. 2H). With 0.1% BAK, mean NFD was reduced by 60% at 1 week as compared to baseline, whereas with 0.01% BAK the mean NFD reduction was 31%; showing a significant dose-related effect (*P* = 0.03).

The number of YFP+ inflammatory cells prior to initiation of BAK application was similar in 0.01% BAK group (7.4 ± 2.2) and 0.1% BAK group (6.6 ± 0.7). At day 7 of BAK application, the number of YFP+ cells had increased significantly in 0.01% BAK group (335 ± 73.8 , *P* = 0.008) and 0.1% BAK group (1064 ± 102.3 , *P* = 0.0003). The number of cells in the 0.1% BAK group was significantly more than in the 0.01% BAK group (Fig. 2I). The extent of YFP+ cell infiltration in the cornea showed a significant negative correlation with NFD (*r* = -0.78 , *P* = 0.007).

Aqueous tear production changes mirrored NFD changes (Fig. 2J). Tear production in untreated eyes and BSS-treated eyes did not change at 1 week (*P* = 0.3 and *P* = 0.07, respectively). Tear production in eyes treated with 0.01% BAK was reduced significantly (*P* = 0.05) from 2.9 ± 0.3 mm at baseline to 2.3 ± 0.4 mm at 1 week. Tear production in eyes treated with 0.1% BAK also was reduced significantly (*P* = 0.002) from 3.5 ± 0.5 mm at baseline to 1.2 ± 0.2 mm at 1 week. The reduction in tear production with 0.1% BAK (2.4 ± 0.53 mm) was significantly greater (*P* = 0.01) than the reduction in tear production with 0.01% BAK (0.6 ± 0.35 mm), showing a dose-related effect.

Fluorescein staining of cornea was greater with 0.1% BAK treatment than with 0.01% BAK treatment. Hematoxylin-eosin staining of corneal sections showed a mixed inflammatory infiltrate and edema of corneas treated with 0.1% BAK (Figs. 2A, 2B). The inflammatory cells were identified as neutrophils (Fig. 2B). The YFP+ cells were bone marrow-derived inflammatory cells (Figs. 2D–F). Immunostaining with leukocyte common antigen (CD45) antibody confirmed their hematopoietic lineage (Fig. 2G).

Recovery after BAK Neurotoxicity

A BAK-containing eye drop (0.1%) was applied to the eye once a day for 1 week. Sequential stereofluorescence imaging was performed, and NFD and tear production were determined for 3 weeks after cessation of BAK treatment. We visualized two forms of stromal nerve neurotoxicity (Fig. 3). Reversible neurotoxicity or axonopathy (Fig. 3, top panel A1–D1) was

FIGURE 1. In vivo maximum intensity projection image of fluorescent nerves in the *thy1*-YFP mouse cornea (A1–D1 are baseline and A3–D3 are 1 week later) and the corresponding corneal fluorescein staining images (A2–D2 and A4–D4). Untreated corneas (A1–A4) and BSS-treated corneas (B1–B4) showed no change in corneal nerves or corneal fluorescein staining. Corneas treated with 0.01% BAK (C1–C4) and 0.1% BAK (D1–D4) once a day for 1 week had reduced corneal nerve density (C3 and D3) and increased fluorescein staining (C4 and D4) at 1 week. The changes in corneal nerves and fluorescein staining were most prominent after 0.1% BAK treatment.

characterized by initial disappearance of nerve fluorescence and its subsequent reappearance, replicating precisely the same nerve pattern as baseline. Irreversible neurotoxicity or degeneration (Fig. 3, top panel A2–D2) was characterized by disappearance of nerve fluorescence. In areas of nerve degeneration, subsequent appearance of fluorescence in a different pattern and location as compared to baseline signified regeneration.

During the 0.1% BAK treatment period, NFD decreased significantly ($P = 0.002$) from 8.3 ± 0.7 mm/mm² at baseline to 3.6 ± 0.8 mm/mm² at 1 week (Fig. 4A). One week after cessation of BAK (2-week time point), NFD had recovered to 5.9 ± 0.8 mm/mm². The increase in NFD between week 1 and week 2 was significant ($P = 0.005$). NFD continued to significantly increase between weeks 2 and 3 ($P = 0.003$). At week 4, NFD (8.0 ± 0.7 mm/mm²) was similar to baseline NFD ($P = 0.3$).

Aqueous tear production recovery mirrored NFD changes (Fig. 4B). During the BAK treatment period, tear production was significantly reduced ($P = 0.001$) from 3.6 ± 0.4 mm at baseline to 1.1 ± 0.2 mm at 1 week. One week after cessation of BAK (2-week time point), tear production recovered to 2.2 ± 0.2 mm. The increase in tear production between week 1 and week 2 was significant ($P = 0.006$). Tear production continued to significantly increase between weeks 2 and 3 ($P = 0.03$). At week 4, tear production (3.4 ± 0.3 mm) was similar to baseline ($P = 0.4$).

Molecular Changes in Cornea after BAK Neurotoxicity

After 0.1% BAK treatment for 1 week, Western immunoblot analyses of corneal lysates at 1 week and 4 weeks were

FIGURE 2. Corneal inflammation, NFD, and aqueous tear production after 1 week of treatment with BSS or BAK. (A) Hematoxylin–eosin staining of corneas treated with BSS are compact with no inflammation. (B) Corneas treated with 0.1% BAK have corneal edema and inflammatory cells (arrowhead points to neutrophils). (C) Whole mount confocal immunostaining with CD11b and F4/80 antibody for inflammatory cells show normal nerves and the absence of inflammatory cells in BSS-treated corneas. (D and E). In 0.1% BAK-treated corneas, nerve fluorescence is lost and inflammation is present. The YFP+ inflammatory cells in D and E (green), CD11b antibody (D, red), and F4/80 antibody (E, red) colocalize (D and E, arrowheads). (F) The YFP+ cells are present in the bone marrow as yellow fluorescent spheres. (G) Immunostaining with leukocyte common antigen (CD45) antibody (G, red) confirmed that YFP+ cells are of hematopoietic lineage. (H) Graph shows that corneal NFD was significantly less after 1 week of 0.01% BAK treatment and 0.1% BAK, but there was no change in NFD with BSS treatment. (I) Graph shows that YFP+ inflammatory cells were significantly increased after 1 week of 0.01% BAK treatment and 0.1% BAK. The inflammatory cells in normal and BSS-treated corneas were less than 10 cells/cornea, therefore the bars are not visible in the graph. (J) Graph shows that aqueous tear production was significantly less after 1 week of 0.01% and 0.1% BAK, but there was no change in tear production with BSS treatment. * $P < 0.05$, White scale bar for C, D, and E = 20 μ m, F = 50 μ m, and G = 10 μ m.

performed to determine fold changes in abundance of proteins in BAK-treated corneas relative to BSS-treated corneas (Figs. 4C and 4D). Inflammatory markers, interleukin-6 (IL6, 1.7 ± 0.06) and CD3 (1.3 ± 0.01), were increased at 1 week, but at 4 weeks the levels of these proteins were similar to those in BSS-treated corneas. These results suggest resolution of inflammation after 4 weeks. The abundance of GAP43, a regeneration-associated protein, was reduced at 1 week (0.6 ± 0.02) but was increased at 4 weeks (1.4 ± 0.15). The abundance of TUBB3, a nerve structural protein, was reduced at 1 week (0.7 ± 0.01) but at 4 weeks, the abundance was similar to that in BSS-treated corneas (0.9 ± 0.02). Of the proteins analyzed, only GAP43 abundance was

increased at 4 weeks, suggesting ongoing nerve regenerative activity in the cornea.

We performed immunolocalization of a molecular marker of axonopathy (SMI-32) in whole-mount corneas to confirm neurotoxicity (Fig. 5). In BAK-treated corneas, few nerves in the corneal periphery stained with SMI-32 antibody (Fig. 5B). Corneal nerves in the corresponding location in normal cornea did not show the SMI-32 staining (Fig. 4A). We were unable to determine abundance of SMI-32 in the cornea, perhaps because of low abundance. Therefore, we determined the fold increase in SMI-32 abundance in the trigeminal ganglion after BAK treatment as compared to after BSS treatment (Figs. 5C, 5D). The 180-kDa band of SMI-32 was increased in the

FIGURE 3. Sequential in vivo imaging of cornea showing BAK-induced neurotoxicity. The eye was treated with 0.1% BAK once a day for 1 week. The cornea was imaged at baseline (A) and 1 week (B), 2 weeks (C), and 4 weeks (D) after cessation of BAK treatment. Panels A1–D1 and A2–D2 show magnified images of two areas of the same cornea. Corneal neurotoxicity manifested in two forms. Top panel (A1–D1) shows reversible neurotoxicity (axonopathy and recovery). The corneal nerve (A1, arrow) at baseline completely lost fluorescence after 1 week of BAK treatment (B1), followed by incomplete recovery of fluorescence at 2 weeks (C1) and finally complete recovery at 4 weeks (D1, arrow). The fluorescent dots in B1 are YFP+ inflammatory cells. Bottom panel (A2–D2) shows irreversible neurotoxicity (degeneration and regeneration). The corneal nerve (A2, arrowhead) at baseline completely lost fluorescence at 1 week (B2), and the fluorescence did not recover subsequently, signifying degeneration of this nerve. In the same area, new fluorescent nerves were seen at 4 weeks that were not present at baseline (D2, arrow), signifying nerve regeneration. Areas with reversible neurotoxicity (B1) had less YFP+ cells as compared to areas with nerve degeneration (B2). Black scale bar = 500 μ m. White scale bar = 100 μ m.

trigeminal ganglion (3.5 ± 0.52 fold) in BAK-treated corneas but had similar abundance as in BSS-treated corneas at 4 weeks (0.9 ± 0.04 fold). Analysis of the 200-kDa band mirrored the 180-kDa band abundance pattern.

BAK Neurotoxicity In Vitro

After day 3 of culture, neurites extended from cell bodies in the central compartment and crossed the Teflon divider to reach the tracks in the side compartment. On day 5, BAK (0.0001% or 0.001%) was added to compartmental culture of trigeminal ganglion cells in the side (neurite) compartment for 4 hours, and the effect on neurite length per track determined (Figs. 6B–D). Vehicle addition served as control (Fig. 6A). At baseline (day 5), prior to BAK or vehicle treatment, nerve fiber length/track (NFL/track in mm) was similar in all groups. On day 7, NFL in the BAK-treated group was significantly reduced

compared to that at baseline. With 0.0001% BAK, NFL was reduced from 2.7 ± 0.28 mm to 0.89 ± 0.12 mm ($P \leq 0.001$). With 0.001% BAK, NFL was reduced from 2.9 ± 0.30 mm to 0.28 ± 0.02 mm ($P \leq 0.001$). In contrast, NFL in the vehicle-treated group was significantly increased ($P \leq 0.001$) at day 7 (5.2 ± 0.27 mm) compared to that at baseline (3.1 ± 0.33 mm). BAK treatment led to a dose-related reduction in NFL. Although NFL was reduced with both BAK doses (0.0001% and 0.001%), the reduction was significantly greater with the higher dose ($P \leq 0.001$).

DISCUSSION

The ocular surface cytotoxicity of BAK, particularly the toxic effects on conjunctival and corneal epithelial cells, precorneal tear film stability, and corneal epithelial barrier function, are

FIGURE 4. In vivo corneal neurotoxicity caused by BAK. (A1–A4) Sequential stereo-fluorescent images of the cornea were obtained, stromal nerves were traced with NeuroLucida software as shown, and NFD was calculated. (A) Bar diagram shows significant reduction of stromal NFD after BAK treatment with recovery by 4 weeks. (B) Bar diagram shows that aqueous tear production, determined by phenol red thread test, was reduced significantly after 1 week of BAK treatment with recovery by 4 weeks. (C and D) Western blot analyses of corneal lysate showed an increase in abundance of proteins associated with inflammation (IL6 and CD3) at 1 week and reduced abundance at 4 weeks. The abundance of GAP43 (prototype regeneration-associated protein) and TUBB3 (nerve structural protein) was increased at 4 weeks, signifying nerve regeneration activity. (* $P < 0.05$)

well described.^{4,28} In this study, we performed in vivo and in vitro experiments to characterize BAK-induced neurotoxicity. The key finding of our study is that topical application of BAK to the eye causes neurotoxicity. We also found that corneal inflammation and reduction in aqueous tear production accompany neurotoxicity. Cessation of BAK treatment leads to resolution of inflammation, normalization of tear production, and recovery of stromal nerve density.

In this study, we determined in vivo neurotoxicity by measuring stromal nerves only and not hairpin-like subbasal nerves. We have previously reported that changes in subbasal nerve pattern may occur with sequential stereofluorescence microscope imaging in *thy1*-YFP mice corneas, even without any intervention.¹⁵ The deeper stromal nerve pattern and density remained constant. Therefore, we reasoned that

because stromal nerves are less prone to variability in sequential measurements, changes in their pattern and density will be indicative of true neurotoxicity. Furthermore, because subbasal nerves arise from stromal nerves, degeneration of stromal nerves should lead to simultaneous degeneration of subbasal nerves. *Thy1*-YFP mice have been used previously to demonstrate that the disappearance of YFP fluorescence in axotomized neurons signifies atrophy, whereas the reappearance of YFP fluorescence fibers signifies nerve regeneration.²⁹ We, as well as others, have reported that regenerating nerves in the cornea do not follow pre-existing tracks; therefore, the appearance of new fluorescent nerve fibers in a new pattern signifies nerve regeneration.^{14–16}

We visualized two forms of stromal nerve neurotoxicity. Reversible neurotoxicity (axonopathy) was characterized by

FIGURE 5. Corneal axonopathy caused by BAK. Ex vivo widefield fluorescent image of a quadrant of cornea that received BSS eye drops (A) or 0.1% BAK eye drops (B). There was loss of nerve fluorescence in the cornea after BAK treatment and influx of YFP+ inflammatory cells. These quadrants were processed for confocal immunofluorescence microscopy using antibodies specific for SMI-32, a marker of axonopathy. Corneal stromal confocal images from within the area demarcated by a white box in A and B are shown. Top panel (A1–A3) confocal images from BSS-treated cornea show a strongly fluorescent YFP+ nerve (A1, green) that did not stain with SMI32 antibody (A2, absence of red). Bottom panel (B1–B3) confocal images from BAK-treated cornea show a weakly fluorescent YFP+ nerve (B1, green) that stained with SMI32 antibody (B2, red). B3 shows a merged image overlay with DAPI staining. Western blot analyses of cell lysate of ophthalmic division of trigeminal ganglion (C and D) shows that SMI-32 abundance increased after BAK treatment at 1 week and reduced thereafter at 4 weeks. A, B scale bar = 500 μ m. A1–A3, B1–B3 scale bar = 20 μ m.

initial disappearance of nerve fluorescence and its subsequent reappearance in precisely the same nerve pattern as baseline. Irreversible neurotoxicity (degeneration) was characterized by disappearance of nerve fluorescence and subsequent reappearance in a different pattern and location as compared to baseline. Neurotoxicity was also observed with the clinically relevant lower dose of BAK (0.01% BAK in vivo and 0.0001% BAK in vitro). The finding of reversible nerve toxicity suggests that discontinuing BAK exposure to the cornea may allow some nerves with disrupted axonal function to recover structurally. Therefore, it is possible that discontinuation of BAK-preserved eye drops improves corneal function (surface disease, sensitivity, aqueous tear production). Our data agree with data reported by Shriver and Dittell,³⁰ which suggests that YFP loss correlates with a disruption in axonal function that can be detected at the very early stages of acute experimental autoimmune encephalomyelitis in *thy1-YFP* mice and that there is a reversal of the axonal dysfunction when inflammation is resolved and the mice recover clinically. Irreversible neurotoxicity (nerve degeneration) recovers by regeneration. The pattern and density of the regenerated nerves differs markedly from normal innervation, and it is unknown whether they are functionally normal.

We performed immunostaining of corneas and Western analyses of trigeminal ganglion lysates with antibody specific to SMI-32 to confirm axonopathy. The monoclonal antibody SMI-32, which recognizes hypophosphorylated neurofilament-H

(hypoP-NF-H), is an established marker of central nervous system axonopathy.²⁰ Neurofilament phosphorylation states are dynamically regulated by dysmyelination and intrinsic axonal pathologic changes. Neuronal inflammation, as occurs in multiple sclerosis, causes decreased neurofilament phosphorylation and an increase in nonphosphorylated neurofilament epitopes, thus increasing SMI-32 immunoreactivity.³¹ We observed SMI-32 immunoreactivity only in peripheral corneal nerves in BAK-treated corneas. This is to be expected because myelinated nerves are present only in the periphery of normal cornea.³² Nerve degeneration and regeneration activity were confirmed by Western immunoblot analyses of corneal lysates with antibody specific to TUBB3, a nerve structural protein, and GAP43, a prototype nerve regeneration-associated protein.

Our study also revealed that BAK neurotoxicity was dose related. In topical applications, 0.1% BAK applied to the eye once a day for 1 week caused greater toxicity than the same application of 0.01% BAK. Several published studies have also reported dose-related toxicity of BAK (reviewed by Baudouin et al.⁴). We chose the higher BAK dose (0.1%) for the sequential in vivo study to allow for easier characterization of neurotoxicity. Additionally, the use of 0.1% BAK has been reported in the past in rabbit models to produce dry eye, allowing us to benefit by comparing our findings with published data.³³ Our findings of corneal inflammation and reduced aqueous tear production with topical application of

FIGURE 6. Effect of BAK on neurite outgrowth in vitro in compartmental cultures of dissociated trigeminal ganglion cells. (A1–C2) Widefield fluorescent image showing neurites in side compartment. The cell bodies were isolated in the central compartment, and the neuritis (shown here) extended into the side compartment. Neuritis was imaged at 5 days (A1–C1) and at 7 days (A2–C2). Neurites in the side compartment were exposed either to vehicle (A1, A2), 0.0001% BAK (B1, B2), or 0.001% BAK (C1, C2) on day 5 for 24 hours. The cell bodies in the central compartment received normal culture media and were not exposed to vehicle or BAK. Neurite length was assessed on days 5 and 7 to calculate neurite outgrowth (D). The bars show the NFL per track before (day 5) and after (day 7) vehicle or BAK treatment. A dose-related significant reduction in neurites occurred after BAK treatment, signifying neurotoxicity. * $P < 0.05$. Scale bar = 2 mm.

BAK are in agreement with previous findings.^{18,33} In our study, the frequency of BAK application (0.1% once a day for 1 week) was lower than in the study by Lin et al.¹⁸ in mice (0.2% twice a day for 1 week). Therefore, less frequent administration of the same dose of BAK produced similar ocular surface toxicity. BAK can accumulate and remain in ocular tissues for relatively lengthy periods of time; therefore, even infrequent applications of BAK are cytotoxic (reviewed by Yee²⁸).

The mechanism of BAK-induced neurotoxicity is well established. BAK induces neurotoxicity directly due to its detergent action and indirectly due to infiltrating inflammatory cells. Sato et al.³⁴ postulated that the neuronal damage of BAK was due to its positive charge. In contrast, Fox et al.³⁵ found that neurotoxicity can be attributed to the generalized property of cationic surfactants rather than to charge specificity. BAK destroys nerve cell membranes by solubilizing the lipid barrier, leading to irreversible damage to the neurons.³⁶ Parr and Sharkey³⁷ found enormous numbers of T lymphocytes on immunohistochemical analysis of whole-mounts 3 days after BAK treatment, pointing to a T cell-mediated immune response during the degeneration induced by BAK. Immunosuppression with cyclosporine delayed a significant proportion of neuronal loss elicited by BAK treatment, supporting the idea that some neuronal death is not directly due to detergent-mediated lysis and that the immune system has an active role in the neurotoxic effects of BAK. Our results are also along the same lines. We have also found evidence of neurotoxicity as well as inflammatory cell infiltration after topical BAK application to cornea.

The *thy1*-YFP transgenic mice used in our study were generated by Feng et al.³⁸ In these transgenic mice YFP is

stably expressed in neurons. Longitudinal studies with repeated imaging performed by Feng et al.³⁸ over a period of 9 months showed stable expression of YFP in neurons with negligible toxicity and structural changes. We have performed repeated imaging of corneal nerves over a 12-month period and have not noticed changes in YFP fluorescence over time. These data suggest that imaging of corneal nerves to measure NFD for long-term follow-up is feasible in *thy1*-YFP mice. With our imaging technique, it should be possible to assess the overall drug-induced corneal neurotoxicity. However, this imaging technique will not be able to differentiate the mechanism of neurotoxicity, whether direct or indirect. If drug permeation does not occur then direct neurotoxicity is likely to be negligible; however, the drug may indirectly cause neurotoxicity by inducing inflammation or by perturbing normal physiological processes. Our imaging technique may still be used to visualize corneal neurotoxicity, if drug permeation does not occur. However, additional cellular and molecular investigations will be needed to elucidate neurotoxicity mechanisms for such drugs.

The BAK doses used in our in vitro experiments were 0.001% and 0.0001%. The in vitro experiments were performed to complement the in vivo experiments and confirm neurotoxicity. It is generally accepted that to be complementary, the in vitro dose should be 1/100 of the in vivo dose.³⁹ In these doses, in vitro addition of BAK to trigeminal ganglion neurites caused dose-related neurite loss that was greater with the 0.001% dose (complementary to a 0.1% topical dose). Our data regarding dose-related toxicity of BAK agree with published data showing significant in vitro dose-related toxicity of BAK at doses of 0.001% or more.⁴⁰ We used compartmental

cultures of trigeminal ganglion cells so that only the neurites (axonal and dendritic processes) were exposed to BAK and not the cell bodies. This was done to simulate normal physiological conditions, because the trigeminal axons are resident in the cornea, not the cell bodies.

The most direct practical application of our finding of BAK-induced neurotoxicity lies in avoiding the chronic application of BAK-preserved eye drops in patients who have neurotrophic corneas and in patients who have dry eyes due to reduced aqueous tear production. One example of a clinical situation necessitating chronic administration of BAK-preserved eye drops is the treatment of patients with glaucoma, especially those who have co-existent dry eyes. Skalicky et al.⁴¹ recently reported that higher daily exposure to BAK-preserved eye drops is associated with presence of ocular surface disease and poorer glaucoma-related quality of life. The association of BAK-preserved antiglaucoma eye drop use and a decrease in the number and density of corneal subbasal nerve fiber bundles has also been reported.^{10,42}

One limitation of our study is that we were unable to confirm whether the reduced aqueous tear production after BAK application is a consequence of neurotoxicity-induced changes in nerve function or whether tear production decrease was secondary to inflammation-induced reduction in lacrimal gland function. This unanswered question is important because if BAK-induced neurotoxicity, in and of itself, is sufficient to cause a reduction in aqueous tear production, then some cases of dry eye disease may be iatrogenic, due to chronic use of BAK-preserved eye drops. Furthermore, BAK-induced neurotoxic dry eye may not be treatable by reducing ocular surface inflammation. Although Martone et al.¹⁰ also reported an association between the use of BAK-containing eye drops, reduced number of corneal nerves, corneal hypoesthesia, and reduced tear secretion, the differential contribution of ocular surface inflammation and neurotoxicity in reducing tear production remains unknown. We were unable to reliably measure corneal sensitivity in mice using Cochet-Bonnet aesthesiometry due to several confounding variables such as restraining method and inadvertent whisker touch (Jain S, 2011, unpublished data). Therefore, we were unable to probe associations between neurotoxicity, corneal hypoesthesia, and reduced aqueous tear production.

In conclusion, using in vivo and in vitro methodologies, we describe corneal neurotoxicity due to topical application of BAK to the eye. The neurotoxicity may reverse or be irreversible on cessation of BAK application. If irreversible, regenerating nerves reinnervate the affected area.

Acknowledgments

We thank Ke Ma, Ph.D., for help with confocal imaging and Eryn Gangi-Simon, Ph.D., for help with isolation of cells from bone marrow.

References

1. Kaya M, Baba F, Deniz M, Baykara S, Yucesan S. Effects of benzalkonium chloride application on the rat bladder. A functional and histopathological study. *Urol Int*. 2005;74:74-78.
2. Faussone-Pellegrini MS, Vannucchi MG, Ledder O, Huang TY, Hanani M. Plasticity of interstitial cells of Cajal: a study of mouse colon. *Cell Tissue Res*. 2006;325:211-217.
3. Hanani M, Ledder O, Yutkin V, et al. Regeneration of myenteric plexus in the mouse colon after experimental denervation with benzalkonium chloride. *J Comp Neurol*. 2003;462:315-327.
4. Baudouin C, Labbé A, Liang H, Pauly A, Brignole-Baudouin F. Preservatives in eyedrops: the good, the bad and the ugly. *Prog Retin Eye Res*. 2010;29:312-334.
5. Labbé A, Pauly A, Liang H, et al. Comparison of toxicological profiles of benzalkonium chloride and polyquaternium-1: an experimental study. *J Ocul Pharmacol Ther*. 2006;22:267-278.
6. Uematsu M, Kumagami T, Shimoda K, et al. Influence of alkyl chain length of benzalkonium chloride on acute corneal epithelial toxicity. *Cornea*. 2010;29:1296-1301.
7. Buron N, Micheau O, Cathelin S, Lafontaine PO, Creuzot-Garcher C, Solary E. Differential mechanisms of conjunctival cell death induction by ultraviolet irradiation and benzalkonium chloride. *Invest Ophthalmol Vis Sci*. 2006;47:4221-4230.
8. Zhou Y, Liu Q, Zhou T, et al. Modulation of the canonical Wnt pathway by benzalkonium chloride in corneal epithelium. *Exp Eye Res*. 2011;Apr 23. [Epub ahead of print].
9. Georgiev GA, Yokoi N, Koev K, et al. Surface chemistry study of the interactions of benzalkonium chloride with films of meibum, corneal cells lipids, and whole tears. *Invest Ophthalmol Vis Sci*. 2011;52:4645-4654.
10. Martone G, Frezzotti P, Tosi GM, et al. An in vivo confocal microscopy analysis of effects of topical antiglaucoma therapy with preservative on corneal innervation and morphology. *Am J Ophthalmol*. 2009;147:725-735.
11. Majumdar S, Hippalgaonkar K, Repka MA. Effect of chitosan, benzalkonium chloride and ethylenediamine tetraacetic acid on permeation of acyclovir across isolated rabbit cornea. *Int J Pharm*. 2008;348:175-178.
12. McCarey B, Edelhauser H. In vivo corneal epithelial permeability following treatment with prostaglandin analogs [correction of analoges] with or without benzalkonium chloride. *J Ocul Pharmacol Ther*. 2007;23:445-451.
13. Wollensak G, Iomdina E. Biomechanical and histological changes after corneal crosslinking with and without epithelial debridement. *J Cataract Refract Surg*. 2009;35:540-546.
14. Yu CQ, Rosenblatt MI. Transgenic corneal neurofluorescence in mice: a new model for in vivo investigation of nerve structure and regeneration. *Invest Ophthalmol Vis Sci*. 2007;48:1535-1542.
15. Namavari A, Chaudhary S, Sarkar J, et al. In vivo serial imaging of regenerating corneal nerves following surgical transection in transgenic Thy1-YFP mice. *Invest Ophthalmol Vis Sci*. 2011;52:8025-8032.
16. Namavari A, Chaudhary S, Chang JH, et al. Cyclosporine immunomodulation retards regeneration of surgically transected corneal nerves. *Invest Ophthalmol Vis Sci*. 2012;53:732-740.
17. Al-Aqaba MA, Alomar T, Miri A, Fares U, Otri AM, Dua HS. Ex vivo confocal microscopy of human corneal nerves. *Br J Ophthalmol*. 2010;94:1251-1257.
18. Lin Z, Liu X, Zhou T, et al. A mouse dry eye model induced by topical administration of benzalkonium chloride. *Mol Vis*. 2011;Jan 25;17:257-264.
19. Lemp MA. Report of the National Eye Institute/Industry workshop on clinical trials in dry eyes. *CLAO J*. 1995;21:221-232.
20. Bannerman PG, Hahn A. Enhanced visualization of axonopathy in EAE using thy1-YFP transgenic mice. *J Neurol Sci*. 2007;260:23-32.
21. Irvine KA, Blakemore WF. Age increases axon loss associated with primary demyelination in cuprizone-induced demyelination in C57BL/6 mice. *J Neuroimmunol*. 2006;175:69-76.
22. Brissette-Storkus CS, Reynolds SM, Lepisto AJ, Hendricks RL. Identification of a novel macrophage population in the normal mouse corneal stroma. *Invest Ophthalmol Vis Sci*. 2002;43:2264-2271.

23. Kuffová L, Netuková M, Duncan L, Porter A, Stockinger B, Forrester JV. Cross presentation of antigen on MHC class II via the draining lymph node after corneal transplantation in mice. *J Immunol*. 2008;180:1353–1361.
24. Malin SA, Davis BM, Molliver DC. Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. *Nat Protoc*. 2007;2:152–160.
25. Campenot RB, Lund K, Mok SA. Production of compartmented cultures of rat sympathetic neurons. *Nat Protoc*. 2009;4:1869–1887.
26. Pazyra-Murphy MF, Segal RA. Preparation and maintenance of dorsal root ganglia neurons in compartmented cultures. *J Vis Exp*. 2008;Oct 17;(20). pii: 951. doi: 10.3791/951.
27. De Saint Jean M, Brignole F, Bringuier AF, Bauchet A, Feldmann G, Baudouin C. Effects of benzalkonium chloride on growth and survival of Chang conjunctival cells. *Invest Ophthalmol Vis Sci*. 1999;40:619–630.
28. Yee RW. The effect of drop vehicle on the efficacy and side effects of topical glaucoma therapy: a review. *Curr Opin Ophthalmol*. 2007;18:134–139.
29. Greer JE, McGinn MJ, Povlishock JT. Diffuse traumatic axonal injury in the mouse induces atrophy, c-Jun activation, and axonal outgrowth in the axotomized neuronal population. *J Neurosci*. 2011;31:5089–5105.
30. Shriver LP, Dittel BN. T-cell-mediated disruption of the neuronal microtubule network: correlation with early reversible axonal dysfunction in acute experimental autoimmune encephalomyelitis. *Am J Pathol*. 2006;169:999–1011.
31. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mörk S, Bö L. Axonal transection in the lesions of multiple sclerosis. *N Engl J Med*. 1998;338:278–285.
32. Müller LJ, Pels L, Vrensen GE. Ultrastructural organization of human corneal nerves. *Invest Ophthalmol Vis Sci*. 1996;37:476–488.
33. Xiong C, Chen D, Liu J, et al. A rabbit dry eye model induced by topical medication of a preservative benzalkonium chloride. *Invest Ophthalmol Vis Sci*. 2008;49:1850–1856.
34. Sato A, Yamamoto M, Imamura K, Kashiki Y, Kunieda T, Sakata K. Pathophysiology of aganglionic colon and anorectum: an experimental study on aganglionosis produced by a new method in the rat. *J Pediatr Surg*. 1978;Aug;13(4):399–435.
35. Fox DA, Epstein ML, Bass P. Surfactants selectively ablate enteric neurons of the rat jejunum. *J Pharmacol Exp Ther*. 1983;Nov;227(2):538–544.
36. Hanani M, Ledder O, Yutkin V, et al. Regeneration of myenteric plexus in the mouse colon after experimental denervation with benzalkonium chloride. *J Comp Neurol*. 2003;462:315–327.
37. Parr EJ, Sharkey KA. Multiple mechanisms contribute to myenteric plexus ablation induced by benzalkonium chloride in the guinea-pig ileum. *Cell Tissue Res*. 1997;289:253–264.
38. Feng G, Mellor RH, Bernstein M, et al. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron*. 2000;28:41–51.
39. Hamard P, Blondin C, Debbasch C, Warnet JM, Baudouin C, Brignole F. In vitro effects of preserved and unpreserved antiglaucoma drugs on apoptotic marker expression by human trabecular cells. *Graefes Arch Clin Exp Ophthalmol*. 2003;241:1037–1043.
40. Davis D, Cyriac M, Ge D, You Z, Savoie FH. In vitro cytotoxic effects of benzalkonium chloride in corticosteroid injection suspension. *J Bone Joint Surg Am*. 2010;92:129–137.
41. Skaliky SE, Goldberg I, McCluskey P. Ocular Surface Disease and Quality of Life in Patients With Glaucoma. *Am J Ophthalmol*. 2011;Aug 25. [Epub ahead of print].
42. Baratz KH, Nau CB, Winter EJ, et al. Effects of glaucoma medications on corneal endothelium, keratocytes, and sub-basal nerves among participants in the ocular hypertension treatment study. *Cornea*. 2006;25:1046–1052.