In Vitro Studies of Antiglaucomatous Prostaglandin Analogues: Travoprost with and without Benzalkonium Chloride and Preserved Latanoprost

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Purpose. With use of the Wong-Kilbourne derivative Chang conjunctival cell line, this study compared in vitro the ocular toxicity of three topical intraocular pressure (IOP)–lowering agents: travoprost 0.004% containing 0.015% benzalkonium chloride (BAK), travoprost Z 0.004%, a new formulation without BAK, and latanoprost 0.005% containing 0.02% BAK.

Methods. Neutral red, Alamar blue, YOPRO-1, and annexin V/7-AAD assays were used to evaluate the effects of the IOP-lowering agents and BAK on cellular viability, membrane integrity, and apoptosis in the conjunctival cell line using microtitration fluorometric analysis and flow cytometry. All assessments were performed in a masked manner.

Results. Assessment of cell viability and membrane integrity revealed a significant effect by latanoprost with BAK or BAK alone but no effect by travoprost Z without BAK or buffer alone ($P < 0.0001$). Latanoprost with BAK, travoprost with BAK, and BAK alone were cytotoxic in Chang conjunctival cells, whereas no cytotoxicity was observed in cells exposed to travoprost Z without BAK or in cells treated with buffer ($P < 0.0001$). No increase in apoptosis or necrosis was observed in cells treated with control or travoprost Z without BAK compared with BAK, travoprost with BAK, and latanoprost with BAK ($P < 0.0001$).

Conclusions. Latanoprost with BAK, travoprost with BAK, and BAK alone have significant cytotoxic effects on human conjunctiva-derived cells and are associated with apoptosis. These effects likely result from BAK used as a preservative. IOP-lowering agents with alternative preservatives instead of BAK will most likely have fewer ocular surface adverse effects than agents containing BAK. (*Invest Ophthalmol Vis Sci.* 2007;48:4123–4128) DOI:10.1167/iovs.07-0266

Management of glaucoma typically involves pharmacotherapy with topical ocular agents, such as the prostaglandin analogues latanoprost and travoprost.[1] Long-term use of topical ocular drugs is often associated with ocular inflammation, allergy, dry eye syndrome, and failure of filtration surgery.[2–8] Several lines of evidence indicate that these agents are associated with decreased tear turnover and tear film break-up time, infiltration of the conjunctiva by inflammatory cells and fibroblasts, and overexpression of inflammatory markers such as HLA-DR, intercellular adhesion molecule (ICAM-1), FAS antigen, or the apoptotic marker Apo 2.7.[9–14]

The preservative benzalkonium chloride (BAK), the most commonly used ocular preservative, is largely responsible for the ocular toxicities and inflammation associated with the chronic use of many ophthalmic solutions.[9,15–17] BAK is retained in tissues and can be found 168 hours after a single 30-μL drop of 0.01% BAK in rabbits.[18] The half-life of BAK from corneal epithelium and conjunctival tissues is 20 hours.[18] Prolonged exposure to BAK causes indirect and direct toxic effects to the ocular surface, including infiltration by inflammatory cells, reduced cellular viability, and compromised epithelial barrier function through increased corneal epithelial cell permeability.[9,10,19–21] The toxic effects of BAK are dose dependent, with progressive, slow cell growth arrest occurring at concentrations as low as 0.0001%, more rapid apoptosis at 0.01%, and immediate epithelial cell necrosis at higher concentrations of 0.05%.[22]

The development of ocular preservatives that provide a nontoxic alternative to BAK will likely become useful in the management of chronic diseases such as glaucoma. Using a human conjunctiva–derived cell line, we specifically investigated in vitro the first commercially available BAK-free prostaglandin solution, travoprost Z 0.004%, preserved without BAK, and compared the ocular toxicity of this topical solution with that of standard travoprost 0.004% containing 0.015% BAK, latanoprost 0.005% containing 0.02% BAK, and BAK alone. We evaluated the effects of commercial formulations of these IOP-lowering agents and BAK on cellular viability, membrane integrity, and apoptosis through microtitration fluorometric assays and flow cytometry.

Several types of assays were chosen to explore various mechanisms of cell damage caused by the preservative BAK on conjunctiva-derived cells. The neutral red cytotoxicity assay is a cell survival/viability assay that measures the integrity of the cell membrane.[17] Alamar blue assay measures cell proliferation and cytotoxicity.[23] YOPRO-1, a cell-impermeable nuclear dye that stains only cells that have lost membrane integrity, serves as an indication of apoptosis.[24] The double-staining annexin V/7-AAD assay discriminates cells that are undergoing early apoptosis, late apoptosis, and necrosis. These assays were used to provide a broader understanding of the cytotoxic effects of preservatives on the conjunctiva.

Materials and Methods

Materials

The Wong-Kilbourne derivative of Chang conjunctiva, clone 1-5c-4, was obtained from American Type Culture Collection (CCL-20.2; Manassas, VA). Kanamycin and phosphate-buffered saline (PBS) without

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calcium and magnesium (PBS) were purchased from Eurobio, amoxicillin from GlaxoSmithKline (London, UK), EDTA from Sigma-Aldrich (Saint Quentin Fallavier, France), and fetal calf serum (FCS) from Dominique Dutscher (Brumath, France). Dulbecco minimum essential medium (DMEM 1×) with L-glutamine substitute (GlutaMAX-I), Alamar blue, and YOPRO-1 were obtained from Invitrogen (Cergy-Pontoise, France); annexin V/7-aminoactinomycin D (7-AAD) was obtained from Immunotech (Luminy, France); and neutral red was obtained from Fluka (Buchs, Switzerland). The cytotoxic effects of travoprost Z 0.004% preserved without BAK and travoprost 0.004% containing 0.015% BAK (Travatan Z and Travatan, respectively; Alcon, Fort Worth, TX), and latanoprost 0.005% containing 0.02% BAK (Xalatan; Pfizer, New York, NY) were compared with those of the culture medium, using PBS as a negative control, and with those of BAK (Sigma-Aldrich) dissolved in PBS to obtain the concentration of 0.02% as a positive control of cytotoxicity.

Methods

Conjunctival Cell Culture. Chang conjunctiva-derived cells were cultured under standard conditions (moist atmosphere of 5% CO_2 at 37°C) in 75-cm^2 bottles in DMEM with L-glutamine substitute (GlutaMAX-I; Invitrogen) containing 10% FCS, 4.5 g/L glucose, 1% kanamycin, and 50 mg/mL amoxicillin. For each assay, culture dishes were seeded with 10^5 cells/mL and were treated when they reached 80% confluence. For tests using microtitration fluorometric assays, cells were cultured in 96-well microplates. Cells used for flow cytometry assays were cultured in six-well plates. At the end of the incubation in the presence of the drugs tested, they were collected after 5 minute-incubation in 2 mL of 1 mM EDTA. These cells had been used previously to assess the toxicity of various prostaglandin analogues and served as a basis for toxicological uses with similar assessment of cell viability and apoptosis.[9,17]

Assessment of Cell Viability and Membrane Integrity with Neutral Red. Neutral red is a fluorescent molecule that enters the lysosomes of living cells. Thus, coloration depends on cell viability.[9,17] Cells were incubated for 30 minutes at 37°C with 50 μL undiluted test solution and then were washed with 200 μL PBS. A 200-μL volume of medium containing 50 μg/mL neutral red was added to each well and was incubated for 3 hours at 37°C with an atmosphere containing 5% CO_2. After another washing, 200-μL of ethanol–acetic acid was added, and the coloration was homogenized by agitation for 15 minutes. The fluorescence of neutral red taken up by viable cells was measured with a Safire microplate reader (Tecan Instruments, Trappes, France), with excitation at 535 nm and emission at 600 nm.

Assessment of Cytotoxicity with Alamar Blue. Alamar blue is made of resazurin, which is blue and nonfluorescent and can be reduced in the cells through enzyme activity to resorufin, which is pink and highly fluorescent.[25] Fifty microliters of the undiluted test solutions was added to each well of a microtiter plate containing conjunctival cells and was incubated for 30 minutes at 37°C. The cells were then rinsed with 200 μL PBS. Two hundred microliters of resazurin at a concentration of 50 μg/mL was added to the cells and incubated for 6 hours at 37°C in an atmosphere containing 5% CO_2. The conversion of resazurin to resorufin by the cells was measured by fluorescence with an excitation wavelength at 535 nm and an emission wavelength at 600 nm.

Assessment of Apoptosis with YOPRO-1. YOPRO-1 is a cell-impermeable nuclear dye that stains cells when they have lost their membrane integrity; it is a useful probe to assess apoptosis.[24] The opening of specific membrane pores that appear during apoptosis induces cationic movements and reactive oxygen species (ROS) delivery from mitochondria. The fluorescence of YOPRO-1 is related to the generation of ROS when apoptosis occurs. Fifty microliters of undiluted test solution was added to each well of a microtiter plate containing conjunctival cells, incubated for 30 minutes at 37°C, and then rinsed with 200 μL PBS. YOPRO-1, at a concentration of 2 μM in PBS, was added to the conjunctival cells, and the degree of apoptosis was measured by fluorescence with excitation at 491 nm and emission at 509 nm. Results of the YOPRO-1 assay were expressed as a ratio to the results of the neutral red assay to correlate apoptosis with cellular viability.

Assessment of Apoptosis and Necrosis with Annexin V/7-AAD. Annexin V binds to the membrane phosphatidyl-serine, which becomes exposed from the inner part of the cell membrane to the outer part during apoptosis and is an indicator of the first step of cell membrane alteration that occurs during the early phase of apoptosis. By reacting like propidium iodide, 7-AAD binds to DNA when the cell membrane is disrupted in the late phase of apoptosis and in necrosis. Conjunctiva-derived cells, cultured in six-well plates, were incubated for 30 minutes with 50-μL of undiluted test solution and were collected after incubation for 5 minutes in 2 mL of 1 mM EDTA. Cells were suspended in binding buffer at a concentration of 100,000 cells/mL and 50-μL of cell suspension were combined with 5 μL annexin V as a marker of early apoptosis and with 10 μL 7-AAD as a marker of necrosis, and were incubated for 15 minutes on ice. After the incubation period, 500 μL binding buffer was added, and the samples were analyzed by flow cytometry (Beckman Coulter XL-MCL, Miami, FL) with discrimination of annexin V and 7-AAD on a biparametric histogram giving four cell populations, cells negative to both markers (normal viable cells), cells positive only to annexin V (early apoptotic), cells positive to both annexin V and 7-AAD (late apoptotic), and cells positive only to 7-AAD (necrotic).

Statistical Analysis

All analyses were performed in a masked manner toward the drug tested and were repeated in independent assays. Results were expressed as percentages of the untreated cell control and were the means of 18 wells (six wells in three different assays). Mean values for each concentration were analyzed with a one-way ANOVA test followed by Bonferroni test using statistics software (Statview IV for Windows; Abacus, Berkeley, CA); the level of significance was fixed at 0.05.

Results

Assessment of Cell Viability and Membrane Integrity with Neutral Red

There was no decrease in the viability of cells exposed to PBS or travoprost Z 0.004% preserved without BAK to incorporate neutral red compared with cells exposed to culture medium (Fig. 1). By comparison, latanoprost 0.005% containing 0.02% BAK, travoprost 0.004% with 0.015% BAK, and 0.02% BAK alone had significant effects on cell viability, as assessed by neutral red uptake.

Assessment of Cytotoxicity with Alamar Blue

Travoprost Z preserved without BAK had no cytotoxic effects on Chang conjunctival cells compared with cells treated with PBS (Fig. 2). There was a significant cytotoxic effect of latanoprost with BAK and 0.02% BAK alone. Travoprost with 0.015% BAK evoked an intermediate response that was significantly different from that in the latanoprost group. The reduction of resazurin to resorufin in the BAK- or latanoprost with 0.02% BAK-treated cells was less than 10%.

Assessment of Apoptosis by YOPRO-1

There was no increase in apoptosis, measured by fluorescence of YOPRO-1, in cells treated with travoprost Z without BAK compared with control medium. In contrast, apoptosis increased in cells treated with latanoprost with BAK, travoprost with BAK, and BAK alone ($P < 0.0001$ compared with PBS and travoprost Z without BAK). The degree of apoptosis for each treatment was adjusted for cell viability by the ratio of

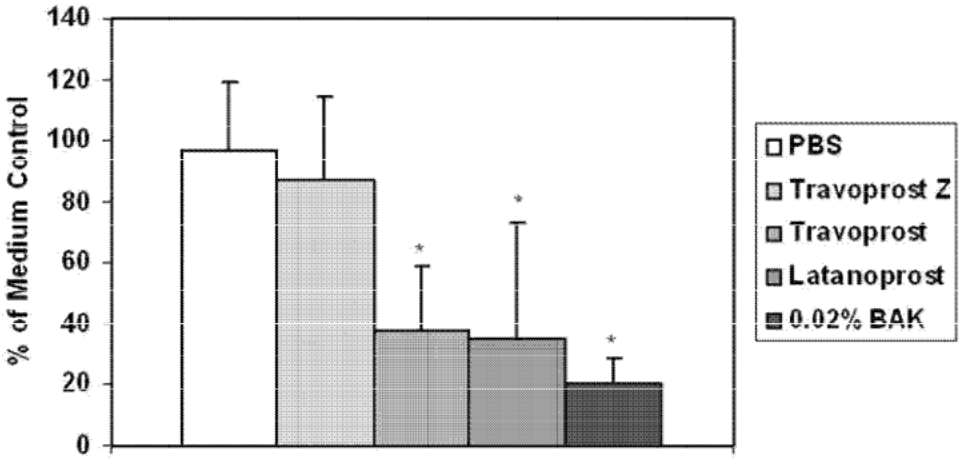

FIGURE 1. Cellular viability and membrane integrity were preserved in cells exposed to PBS and travoprost Z without BAK compared with medium, as assessed by the neutral red assay. Latanoprost and travoprost containing BAK and BAK alone showed significantly decreased cellular viability. $^*P < 0.0001$ compared with medium (referred to as 100%), PBS, and travoprost Z.

YOPRO-1 and neutral red (Fig. 3). The ratio was not significantly different in cells treated with travoprost Z and culture medium, but it was increased approximately 38-fold in cells exposed to either latanoprost or BAK ($P < 0.001$ compared with medium, PBS, and travoprost Z). Travoprost with 0.015% BAK showed an intermediate response that was significantly different from the response in the latanoprost with 0.02% BAK and BAK groups.

Assessment of Apoptosis and Necrosis with Annexin V/7-AAD

In flow cytometry, the double-staining annexin V/7-AAD discriminates on a biparametric histogram cells that are undergoing early apoptosis (annexin positive, 7-AAD negative), late apoptosis (annexin positive, 7-AAD positive), and necrosis (annexin negative, 7-AAD positive). No significant apoptosis or necrosis occurred in cells treated with PBS or with travoprost Z preserved without BAK (Fig. 4). Cells treated with latanoprost with 0.02% BAK, travoprost with 0.015% BAK, or 0.02% BAK alone had significant late apoptosis and necrosis compared with cells treated with medium or travoprost Z ($P < 0.0001$). Significant early apoptosis occurred in cells treated with latanoprost with 0.02% BAK that was not observed with other treatments. The total toxic effect describes the damage to conjunctival cells caused by apoptosis and necrosis. Latanoprost with 0.02% BAK, travoprost with 0.015% BAK, and 0.02% BAK alone had similar total toxic effects on conjunctiva-derived cells (Fig. 5). There were no significant differences between cells treated with PBS representing spontaneous apoptosis in the cell line and travoprost Z.

DISCUSSION

Glaucoma, a chronic disease characterized by increased intraocular pressure that commonly leads to blindness,[25] is often treated with topical prostaglandins or β-blockers. Unfortunately, the chronic use of most IOP-lowering medications is associated with some toxicity, such as allergic reactions, ocular pseudopemphigoid, allergic contact dermatitis, punctate corneal staining, and failure of filtration surgery.[2,26] This toxicity has often been associated not with the active component of the medication but with the preservative BAK, which damages corneal epithelial cells, even at concentrations as low as 0.005%.[15] Samples et al.[27] demonstrated that BAK caused a significant inhibition of the growth of trabecular meshwork cells at extremely low concentrations. Sherwood et al.[3] reported in eyes chronically exposed to preservatives an increase in macrophages, lymphocytes, and fibroblasts in the conjunctiva and Tenon capsule and a decrease in the number of conjunctival goblet cells. Although there are subtle differences with primary cultures of human conjunctival cells, the Wong-Kilbourne derivative of the Chang conjunctiva-derived cell line has been used largely to determine the effects of toxic preservatives and IOP-lowering agents and is a well-recognized model.[17,28] The present study used this conjunctival cell line to assess the direct cytotoxicity of travoprost Z, the first commercially available BAK-free prostaglandin analogue (which contains 0.004% travoprost and a preservative system [SofZia; Alcon Laboratories, Fort Worth, TX]), travoprost 0.004% with 0.015% BAK, and latanoprost 0.005% with 0.02% BAK and to assess the development of apoptosis after exposure to these

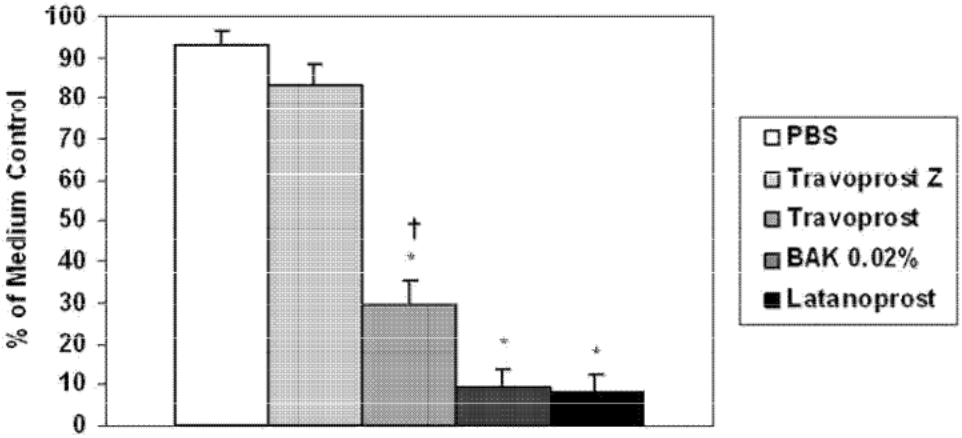

FIGURE 2. Cytotoxicity assessment using the Alamar blue assay. Latanoprost with BAK and BAK alone were cytotoxic in Chang conjunctiva-derived cells and, to a lesser extent, in BAK-containing travoprost, whereas no cytotoxicity was observed in cells exposed to travoprost Z without BAK or cells treated with PBS. $^*P < 0.0001$ compared with medium (referred to as 100%) and travoprost Z. $^\dagger P < 0.0001$ compared with latanoprost with BAK and BAK alone.

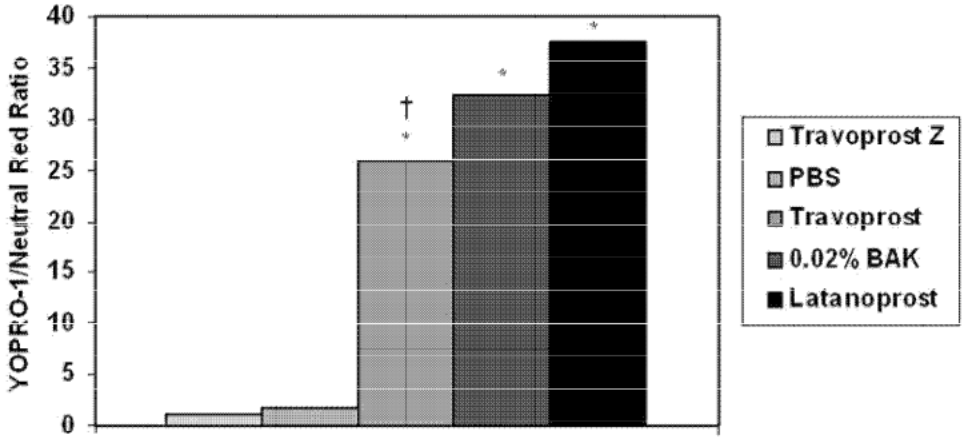

FIGURE 3. Apoptosis measured by the ratio of YOPRO-1 and neutral red. No increase was observed in cells treated with PBS or travoprost Z without BAK compared with PBS, whereas significant apoptosis was observed with BAK and latanoprost with BAK and, to a lesser extent, with BAK-containing travoprost. *$P < 0.0001$ compared with PBS and travoprost Z without BAK. †$P < 0.0001$ compared with latanoprost.

agents. These agents are all prostaglandin analogues, but they differ in the concentration and the type of preservative used. The neutral red assay measures the ability of viable cells to incorporate dye in their lysosomes, and the Alamar blue assay assesses intracellular reduction of resazurin by intact enzymatic systems in viable cells. Both assays assess cell viability by different mechanisms and, when they are used in association, improve the analysis of cytotoxic effects. In both assays, latanoprost with 0.02% BAK, travoprost with 0.015% BAK, and BAK alone demonstrated deleterious effects on the viability of the Chang conjunctiva-derived cells, with travoprost 0.015% BAK the least toxic of the three, which is consistent with the well-demonstrated dose-dependent toxicity of BAK.[22] Indeed previous studies comparing the three commercially available prostaglandin analogues found differences in their toxic profiles corresponding to their concentration in BAK (the least concentrated, the least toxic).[17]

YOPRO-1 is only taken up by membrane pores within cells undergoing apoptosis. Annexin V binds to phosphatidyl serine exposed on cell membranes only during apoptosis. Therefore, test results are positive only in cells undergoing apoptosis. However, the combined assay annexin V/7-AAD discriminates cells undergoing early apoptosis (annexin positive, 7-AAD negative), late apoptosis (annexin positive, 7-AAD positive), and necrosis (annexin negative, 7-AAD positive). As with the cytotoxicity assays, cells exposed to latanoprost with BAK, travoprost containing BAK, and BAK alone reached a significant level of apoptosis compared with media or travoprost Z without BAK. These results are consistent with previous studies in which the Chang cells were exposed to latanoprost with BAK, travoprost with BAK, and bimatoprost with BAK.[17] In these studies, the development of cell cytotoxicity and apoptosis was clearly related to BAK.

As with any other experimental in vitro models, the present study did have some limitations. Evaluations were conducted in only one cell line, but these results are consistent with our previous results and with those previously published in other cell lines. In vitro studies remove the cells from the influence of circulating substances, such as hormones and inflammatory mediators, and from other cells present in the tissue, especially inflammatory cells. However, the present study is valuable for aiding our understanding of the effects of preservatives because the experimental stimulus can be well controlled, and the resultant cellular response can be quantitated. It would be of interest to extend these studies to other clones and to other cell types, such as corneal epithelial and trabecular meshwork cells. Additional studies evaluating the effects of BAK on inflammatory mediators such as prostaglandins and leukotrienes would be of value and interest.

Although the results of the present study cannot be directly extrapolated to humans, the toxicity to BAK in this human cell line was demonstrated to occur after exposure to the test solutions for only 30 minutes The half-life of BAK in the conjunctiva is nearly 12 hours, suggesting that the damage to conjunctival cells observed in vitro may also occur in vivo.[18] Because of its complex structure and heterogeneous cell populations, tissue reaction after repeated contact with a topically administered drug differs from that of a cell monolayer ex-

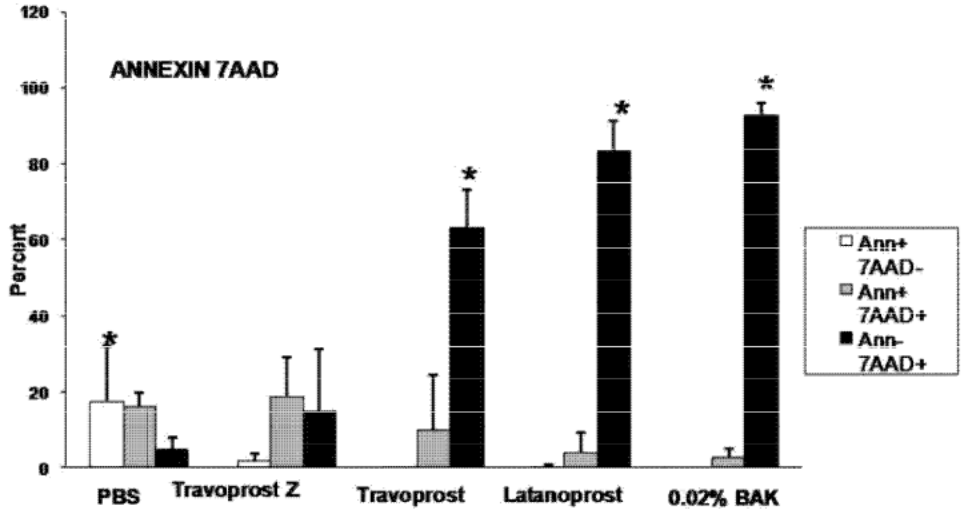

FIGURE 4. No increase in apoptosis and necrosis, measured by annexin V/7-AAD assays, was observed in cells treated with PBS or with travoprost Z without BAK compared with cells treated with travoprost with BAK and latanoprost with BAK or BAK. Annexin +/7-AAD-, PBS compared with all other groups ($P < 0.001$). Annexin +/7-AAD+, no significant differences. Annexin-/7-AAD+, PBS and travoprost Z compared with travoprost with BAK latanoprost with BAK and BAK ($P < 0.0001$). Travoprost with BAK compared with latanoprost with BAK and BAK ($P < 0.001$).

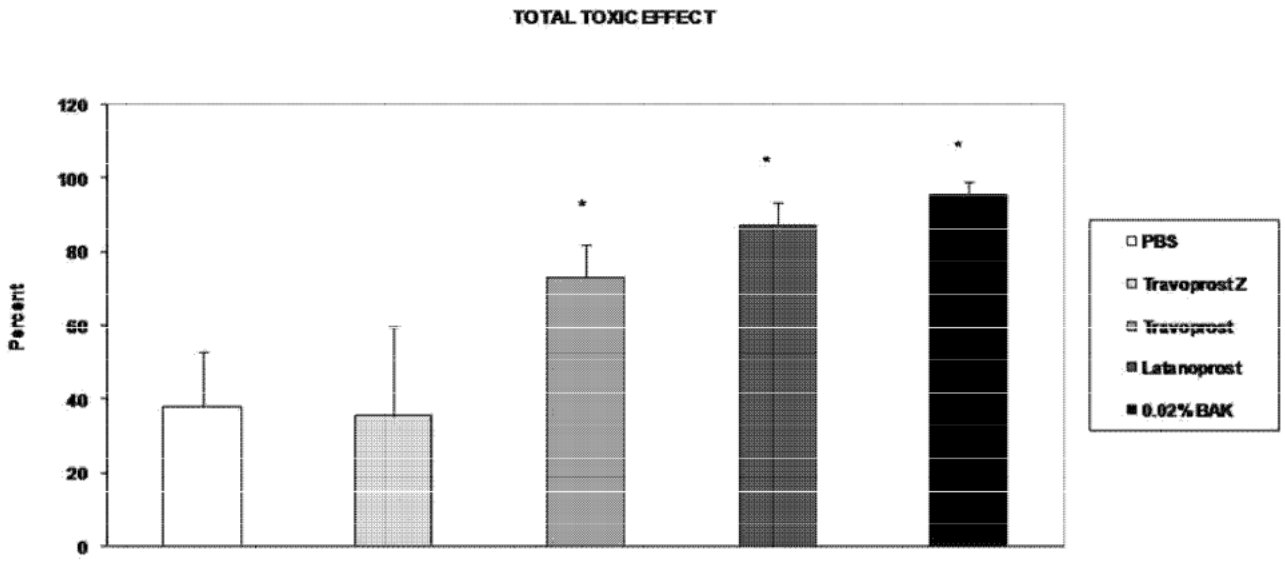

FIGURE 5. No increase in the total toxic effect, measured as either apoptosis or as necrosis using annexin V/7-AAD assays, was observed in cells treated with PBS or with travoprost Z without BAK compared with cells treated with travoprost with BAK, latanoprost with BAK, or BAK alone. PBS compared with travoprost with BAK ($P = 0.0004$), lantanoprost with BAK, and BAK alone ($P < 0.0001$). Travoprost Z compared with travoprost with BAK ($P = 0.0009$), lantanoprost with BAK, and BAK alone ($P < 0.0001$). Travoprost with BAK, latanoprost with BAK, and BAK alone (P = NS).

posed to chemical compounds. Repeated administration over the long term of a weakly toxic drug would most likely cause increased renewal of epithelial structures and tissue stimulation on an inflammatory mode rather than destruction. Indeed, many reports show increased inflammatory responses in the conjunctiva of patients treated over the long term as a reaction to the chronic use of antiglaucoma drugs[5–7,12] and especially to their preservatives; this was not found in patients treated with unpreserved beta blockers.[9] The clinical profile of prostaglandin analogues is usually satisfactory, probably because they are administered once a day. We reported mild inflammatory reactions in conjunctival specimens from patients receiving these treatments.[9] Nevertheless, ocular surface involvement may be pathologically enhanced after years or decades of treatment, after multiple treatments, or when patients have associated ocular surface diseases, such as dry eye disease or meibomian gland dysfunction. In such populations, the use of nontoxic compounds may be of critical importance, especially because the ocular surface has a major influence on compliance and filtering surgery outcome. In a series of in vitro assays, the absence of toxicity consistently observed with travoprost Z without BAK, compared with the high level of toxicity noted with exposure of conjunctival cells to BAK, suggests that use of topical prostaglandin analogues without BAK may reduce the topical ophthalmic toxicity reported with chronic use of these agents. Future studies of these agents in humans will assist in further characterizing the presence or absence of topical ophthalmic toxicity.

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