Purpose. In a previous study, it was demonstrated that in vitro in a human conjunctiva-derived cell line, latanoprost in its commercial presentation appeared to be less toxic than the benzalkonium chloride (BAC) it contains as a preservative. Through a microplate cytometry technique, the investigation was furthered by study of whether the three commercially available antiglaucoma prostaglandin analogs could protect the same cell line in vitro against BAC toxicity and whether an antioxidative mechanism could be involved in such prostaglandin effects.

Methods. Human conjunctiva-derived epithelial cells from the Chang cell line were exposed to three prostaglandins in their commercial presentation (latanoprost, travoprost, and bimatoprost) and to three concentrations of BAC (0.02%, 0.015%, and 0.005%), corresponding to the concentrations contained in the three prostaglandin eye drops. Each solution was diluted to 1/10 and was applied for 30 minutes. Cellular membrane integrity, cytotoxic H2O2, cytotoxic O2•−, and apoptosis were evaluated using neutral red, H2DCF-DA, hydroethidine, and Yopro-1 probes, respectively.

Results. Cellular viability decreased as BAC concentration increased, but it was accompanied by concentration-dependent toxicity. Toxicity of latanoprost and travoprost commercial solutions was statistically significantly lower than their respective BAC concentrations (P < 0.01), whereas bimatoprost induced no significant effects. There was a statistically significant decrease in H2O2 detection with cells exposed to latanoprost (P < 0.01) and travoprost (P < 0.01) and a lower detection of O2•− with cells exposed to latanoprost (P < 0.01) compared with the corresponding BAC concentration alone. The Yopro-1 test showed a BAC-induced apoptotic effect that increased with its concentration. Latanoprost and travoprost produced proapoptotic effects compared with control (P < 0.01), but these were lower than their respective preservative concentrations (statistically significant difference; P < 0.01).

Conclusions. Latanoprost and travoprost were responsible for significant protective effects against BAC toxicity on conjunctiva-derived epithelial cells in vitro, probably related to their antioxidative properties. The low toxicity of the bimatoprost solution did not reveal a possible antioxidative effect. Reduced reactive oxygen species production could be the main mechanism by which prostaglandin analogs protect epithelial cells from the proapoptotic effects of BAC. Further studies will be useful to confirm this hypothesis. (Invest Ophthalmol Vis Sci. 2005;46:4594–4599) DOI:10.1167/iovs.05-0776

Prostaglandin analogs are now the most efficient topical treatment used for ocular hypertension and have widely been shown to have a good safety profile. In a previous in vitro study, we showed that latanoprost, travoprost, and bimatoprost toxicity profiles were mild and mostly related to their concentrations in benzalkonium chloride (BAC), which is used as a preservative, and that prostaglandins did not induce an overexpression of various inflammatory markers, such as intercellular adhesion molecule-1, platelet endothelial cell adhesion molecule-1, or human leukocyte antigen DR (HLA DR).1 Pisella et al.2 showed similar results in vivo, with lower HLA DR expression induced by latanoprost eye drops than with topical β-blockers, despite their higher BAC concentrations, and with lower toxicity of the latanoprost commercial preparation in vitro than with BAC solution at the same concentration. These results led us to hypothesize that latanoprost exerts a protective effect against BAC toxicity on conjunctival cells by means that have yet to be investigated.

BAC toxicity is well studied, and we know that it is responsible for proapoptotic and necrotic effects3,4 involving, as shown in vitro, reactive oxygen species (ROS) and, therefore, oxidative stress.5,6 Thus, if the prostaglandin analogs protect conjunctival cells, an antioxidative mechanism could be proposed as a hypothesis and would deserve further attention.

Several types of prostaglandins have been described. Various studies show that the prostaglandin E2 (PGE2) type is responsible for neuronal toxicity during lateral amyotrophic sclerosis through the release of ROS and glutamate.7 Another class of prostaglandins has also been studied—PGJ2 or, more precisely, 15-deoxy-Δ12,14-prostaglandin J2 (15dPGJ2), an agonist of the peroxisome proliferator-activated receptors (PPARs)—that may present an antioxidative effect by reducing mitochondrial membrane depolarization and the release of ROS and an anti-apoptotic effect on retinal pigment epithelium cells in vitro.8,9 To our knowledge, no antioxidative effect of prostaglandins of the F type (PGF) has been shown to date. The PGFs, known as proinflammatory molecules, are instead regarded as mediators of oxidative processes; several studies show that ROS is involved in the activation of phospholipase A2, which leads to PGF formation.10,11

Moreover, oxidative stress has recently been suggested as a possible player in glaucoma pathophysiology. Overexpression of oxidative stress markers was found in the aqueous humor and plasma of patients with glaucoma compared with healthy controls.12,13 Another study found that levels of 8-oxo-deox-
yguanosine, a major marker of the oxidized DNA, increased in the trabecular meshwork of patients with glaucoma compared with that of controls.\textsuperscript{14}

As a continuation of our previous study on toxicologic profiles of prostaglandin analogs used in glaucoma treatments, the present study compares the three commercially available prostaglandin analogs—latanoprost, travoprost, and bimatoprost—in vitro with regard to their protective effects against BAC toxicity on conjunctiva-derived epithelial cells and attempts to determine whether the antioxidative properties of these prostaglandins could explain this protection.

**Materials and Methods**

**Conjunctival Cell Line**

As previously described,\textsuperscript{1,5,6} Wong-Kilbourne–derived human conjunctival epithelial cells, an established cell line (Wong-Kilbourne derivative of Chang conjunctiva, clone 1 to 5c-4l American Type Culture Collection [ATCC, Manassas, VA]—certified cell line [CCL], 20.2), were cultured under standard conditions (humidified atmosphere with 5% CO\textsubscript{2} at 37°C) in Dulbecco’s minimum essential medium (DMEM; Eurobio, Les Ulis, France) supplemented with 10% fetal calf serum, 1% glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin (Eurobio). Cells from passages 7 through 20 were used in all experiments. Normal culture development was assessed daily by phase-contrast microscopy (Leica, DMRBB, Wetzlar, Germany).

Confluent cultures were removed by 5-minute trypsin incubation, and cells were counted. They were then seeded onto 96-well culture plates (5000 cells per well; Corning BV, Schiphoul-Rijk, The Netherlands) for microtitation analyses and onto six-well culture plates (40,000 cells per well) for flow cytometry analyses. Cultures were kept at 37°C for 24 hours. After subconfluence (culture surface covering nearly 70%) was attained, cells were exposed to the different formulations. Because this cell line spontaneously undergoes apoptosis at 100% confluence (data not shown), 70% confluence was chosen to avoid any artifact in membrane integrity assays.

**Microplate Cold Light Fluorometry**

Microplate cold light fluorometry provides fluorometric detection (280–870 nm) with high sensitivity (picograms to femtograms per microliter) and specificity. Fluorometry was performed using a microplate cytofluorimeter (Fluorolite 1000; Dynex; Cergy Pontoise, France). Four different tests were used according to previously validated methods.\textsuperscript{6,15}

Membrane integrity, closely correlated with cellular viability, was evaluated with neutral red (Fluka, Ronkonkoma, NY) using fluorometric detection (excitation, 535 nm; emission, 600 nm), used at 50 μg/ml. In accordance with the validated protocol of Borenfreund and Puerner,\textsuperscript{16} 200 μl/well medium containing neutral red was added to living cells, and the microplates were incubated for 3 hours at 37°C in atmosphere with 5% CO\textsubscript{2}. The neutral red fluorescence was measured as previously described.

Reactive oxygen species and especially H\textsubscript{2}O\textsubscript{2} were detected with the 2′,7′-dichlorofluorescin diacetate (H\textsubscript{2}DCF-DA; Molecular Probes, Eugene, OR) dye added to live cells for 30 minutes before treatment. This probe is a nonfluorescent cell-permanent compound that is used in fluorescence units and were expressed as a percentage of the control. Each drug was tested in six wells, and each experiment was performed in three independent procedures. H\textsubscript{2}DCF-DA, hydroethidine, and Yopro-1 results were expressed using a ratio of the results of these tests to those of the neutral red test to correlate them to cellular viability. A protective effect induces an increase in the H\textsubscript{2}DCF-DA/neuralred or hydroethidine/neurald ratio (≥1), whereas an antioxidative effect is characterized by a decreased ratio (<1). In the same way, apoptotic cells showed an increase in the Yopro-1/neuralred ratio (>1), whereas necrotic cells were characterized by a decrease in Yopro-1/neurald ratio (<1).

**Prostaglandins and Preservatives**

We used six solutions: the three PGF\textsubscript{2α} analogs latanoprost (Xalatan; Pfizer, New York, NY), travoprost (Travatan; Alcon, Fort Worth, TX), and bimatoprost (Lumigan; Allergan, Irvine, CA) and three different concentrations of benzalkonium chloride (0.02%, 0.015%, and 0.005%) corresponding to the concentrations contained in latanoprost, travoprost, and bimatoprost, respectively. We evaluated cellular viability (neutral red), apoptosis (Yopro-1), and oxidative stress (H\textsubscript{2}DCF-DA and hydroethidine) with each solution diluted to 1/10 and with an exposure time of 30 minutes.

**Statistical Analysis**

Statistical comparisons were performed using analysis of variance (ANOVA) followed by the Tukey-Kramer test with Statview V for Windows (SAS Institute, Cary, NC).

**Results**

**Cellular Viability (Neutral Red Probe)**

All three BAC formulations were responsible for significant reductions in cellular viability. The most important toxicity was obtained with BAC at 0.02% (58% of control; \( P < 0.01 \)), followed by BAC at 0.015% (69% of control; \( P < 0.01 \)) and BAC at 0.005% (85% of control; \( P < 0.01 \)). A statistically significant difference was found between BAC at 0.02% and 0.005% (\( P < 0.01 \)) and between BAC 0.015% and 0.005% (\( P < 0.01 \)), but there was no statistical difference between BAC concentrations of 0.02% and 0.015%. Thus, cell viability decreased in a concentration-dependent manner. The same tendency was found when we compared cellular viability with the three prostaglandins. Indeed, toxicity increased as the concentration of BAC contained in the eyedrop increased: low but significant toxicity was thus found with latanoprost (76% of control, \( P < 0.01 \)) but was not significantly found with travoprost (88% of control; no statistical difference) or bimatoprost (95% of control; no statistical difference). There was a statistically significant difference between latanoprost and travoprost (\( P < 0.05 \)) and between latanoprost and bimatoprost (\( P < 0.01 \)) but not between bimatoprost and travoprost. Comparisons among the three prostaglandins and their respective BAC concentrations showed a tendency to lower toxicity of the eyedrops com-
pared with their respective BAC concentrations, with a statistically significant difference found between travoprost and BAC at 0.015% ($P < 0.01$) and between latanoprost and BAC at 0.02% ($P < 0.01$; Fig. 1) but not between bimatoprost solution and BAC at 0.005%, which was in fact subtoxic.

**Reactive Oxygen Species Detection**

(H$_2$DCF-DA Probe)

Results are expressed using the ratio of the fluorescence obtained with the H$_2$DCF-DA test and the neutral red test (H$_2$DCF-DA/neutral red).

With regard to the cells exposed to BAC, the highest ROS production was obtained with BAC at 0.02% (ratio, 4.45 [$P < 0.01$] compared with control), then with BAC at 0.015% (ratio, 3.5 [($P < 0.01$] compared with control), and finally with BAC at 0.005% (ratio, 2.03 [$P < 0.01$] compared with control). Thus, the amount of ROS detected was correlated with the BAC concentration: there was a statistically significant difference between BAC at 0.02% and 0.015% ($P < 0.01$) and between BAC at 0.02% and 0.005% ($P < 0.01$). Results concerning the three prostaglandin analogs were not perfectly correlated with their BAC concentrations; rather, the lowest ROS production was found with travoprost (ratio, 1 [$P < 0.01$] compared with bimatoprost and latanoprost), followed by bimatoprost (ratio, 1.7) and latanoprost (ratio, 2.26). Furthermore, ROS detection on cells exposed to the prostaglandins was lower than that of their corresponding BAC, but a statistically significant difference was found only between BAC at 0.02% and latanoprost ($P < 0.01$) and between BAC at 0.015% and travoprost ($P < 0.01$; Fig. 2).

**Superoxide Anion Detection**

(Hydroethidine Probe)

Results are expressed using the ratio of the fluorescence obtained with the hydroethidine test and the neutral red test (hydroethidine/neutral red).

The detection of superoxide anions (O$_2$.$^-$) on cells exposed to BAC was also correlated with BAC concentration: the ratio was 1.74 for BAC at 0.02% ($P < 0.01$ compared with control), 1.38 for BAC at 0.015% ($P < 0.05$ compared with control), and
1.27 for BAC at 0.005% (not significant), with a significant difference only between BAC at 0.02% and 0.005% ($P < 0.05$).

Results obtained with the three prostaglandins showed that superoxide anion measurements seemed less correlated with their BAC content: the ratio was 1.24 for latanoprost, 1.06 for travoprost, and 1.08 for bimatoprost, and no significant difference was found among the three eyes. The comparison between the prostaglandins and their corresponding BAC showed a statistically significant difference between latanoprost and BAC at 0.02% ($P < 0.01$). On the other hand, there was no significant difference between bimatoprost and BAC at 0.005% or between travoprost and BAC at 0.015%, but there was a tendency to lower ratios for these two prostaglandin analogs (Fig. 3).

**Apoptosis Assay (Yopro-1 Probe)**

Results are expressed using the ratio of the fluorescence obtained with the Yopro-1 test and the neutral red test (Yopro-1/neutral red).

Among cells exposed to BAC, the highest ratio was that obtained with BAC at 0.02% (ratio, 4.6 [$P < 0.01$] compared with the control), whereas cells exposed to BAC at 0.015% showed a ratio of 3.1 ($P < 0.01$ compared with the control). Then the lowest ratio was that obtained with BAC at 0.005% (ratio, 1.32; not significant compared with the control). Thus, the results obtained increased as the concentration of BAC increased: there was a significant difference between BAC at 0.02% and 0.015% ($P < 0.01$), between BAC at 0.015% and 0.005% ($P < 0.01$), or between BAC at 0.02% and 0.005% ($P < 0.01$).

Results obtained with cells exposed to the prostaglandins were comparable with those obtained with BAC alone: latanoprost solution, which presented the highest BAC concentration, induced a ratio of 3.3 ($P < 0.01$ compared with control, travoprost, and bimatoprost), higher than that obtained with travoprost (1.8; not significant) and bimatoprost (1.05; not significant). The comparison between the prostaglandins and their respective BAC showed a significant difference between latanoprost and BAC at 0.02% ($P < 0.01$) and between travoprost and BAC at 0.015% ($P < 0.01$), but we did not find a significant difference between bimatoprost and BAC at 0.005% (Fig. 4), which, again, appeared almost subtoxic.

**Discussion**

Our experiments aimed to test two main hypotheses. The first is that prostaglandin analogs available for the treatment of ocular hypertension have a protective effect against the well-known BAC toxicity, and the second is that an antioxidant effect can explain this property.

Why BAC concentrations are so different is not clear. Preservatives are used in eyedrops because of their antimicrobial functions, but other effects of these molecules, such as increasing the prostaglandin biodisponibility or their solubility, are probably useful though precise information is unavailable. As we had shown in a previous study, cytotoxicity was proportional to the BAC concentration contained in the eyedrops; indeed, bimatoprost, which contains the lowest BAC concentration, seemed to be less toxic than latanoprost and travoprost. However, the most important conclusion from our results obtained with the neutral red test was that we found a statistically significant protective effect of latanoprost and travoprost against BAC toxicity. Indeed, cellular viability was higher for the cells exposed to these two prostaglandins than for each respective BAC concentration alone, which demonstrates that the association of the prostaglandin analogs with BAC is less toxic than it is with BAC alone, as we had already hypothesized in a previous study.

Given that BAC toxicity has been shown to be related to a pro-oxidative effect, an antioxidant action of the prostaglandins could be suspected. Oxidative stress assays that we used highlighted that the prostaglandins exerted an antioxidative action. This was particularly true for latanoprost, which had H$_2$DCF-DA and hydroethidine probes that detected the presence of ROS, mainly H$_2$O$_2$, and O$_2^-$, respectively, and for travoprost using the H$_2$DCF-DA test. No statistical difference was found on viability and oxidative stress assays with bimatoprost; thus, it may present neither protective nor antioxidant properties, but its very low toxicity may not allow protective or antioxidative effects to appear. Antioxidative activity can result from various mechanisms: direct anti–free radical mechanism, activation of the endogenous antioxidative defenses, or reduction in the overproduction of ROS. A direct anti-free radical effect could not be shown with the tests we carried out. This type of study requires an electronic paramagnetic resonance technique. The increase in the endogenous antioxidative balance (such as glutathione) was not studied, but it could be...
investigated using the same microplate assays and deserves further study. Furthermore, the decrease in ROS we observed could be explained by a reduction in their release. We know that apoptosis induces substantial overproduction of ROS, especially of $O_2^{-}$. Because BAC is known to be responsible for a proapoptotic effect on conjunctival cells in vitro,4–6 we can hypothesize that prostaglandins decrease this BAC-induced proapoptotic effect and involve a reduction in ROS overproduction, especially in $O_2^{-}$ overproduction. Thus we carried out the Yopro-1 test to evaluate apoptosis. The Yopro-1 probe measures membrane permeability, whose modifications allow cationic movements involved in the apoptotic process and in ROS increase.19–21 Our results obtained with the Yopro-1 test showed that BAC was responsible for modified membrane permeability, with a concentration-dependent action. These results are consistent with previous data concerning BAC toxicity (ie, its apoptotic action toward conjunctival cells). Nevertheless, results obtained with latanoprost and travoprost showed a lower proapoptotic effect than observed for their respective preservative concentrations. The two prostaglandin analogs could thus exert an antiapoptotic effect responsible for a reduction in the release of ROS, which explains the lower toxicity observed with these two eyedrops compared with each respective BAC concentration tested alone. Moreover, we observed that latanoprost significantly decreased $O_2^{-}$ production. This radical, mainly of mitochondrial origin, is directly involved in apoptotic phenomena.

We cannot, however, be sure that the cytoprotective and antioxidative effects induced by latanoprost and travoprost result from a specific effect of the prostaglandin analogs rather than from a chemical change in the different solutions. Indeed, because of chemical composition, prostaglandins could create an emulsion with BAC, and we should perhaps not exclude the hypothesis that it decreases the proportion of active BAC in the aqueous phase of this emulsion.22 Thus, the decrease in active BAC proportion could explain the lower cytotoxicity observed and consequently the lower induction of oxidative stress without a real antioxidative effect of prostaglandins. The fact that prostaglandin analogs could present antioxidative properties is only a hypothesis, whereas chemical interactions between prostaglandins and BAC are not well established. Moreover, results obtained in this in vitro model cannot fully be extrapolated to in vivo conditions. Our results must be interpreted with caution. Further studies should be performed in tissues and in vivo animal models to confirm this hypothesis, but all our previous experimental research was in good agreement with data from the literature assessing toxic adverse effects induced in the ocular surface by preservatives and, in some cases, by active compounds.

Nevertheless, latanoprost and travoprost were shown in vitro to protect human conjunctiva-derived epithelial cells against BAC toxicity. This property was not found with bimatoprost because of its already low toxicity, making a protective effect impossible to reveal. This protective effect seems related to an antioxidant action that can be explained, at least in part, by an inhibition of the BAC proapoptotic effects through the oxidative stress pathways. These probable and previously unknown antioxidant and anti-apoptotic activities of the prostaglandin analogs are interesting because oxidative stress is now thought to be involved in the pathophysiology of glaucoma and many other diseases.12–14

References


