

Original Research

In Vitro Corneal and Conjunctival Wound-Healing Assays as a Tool for Antiglaucoma Prostaglandin Formulation Characterization

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Abstract

Background: Benzalkonium chloride (BAK)-containing antiglaucoma therapies alter the ocular surface over the long term. We used an *in vitro* scraping model to compare the effects of preserved and unpreserved topical commercial prostaglandins (PGs) in a wound-healing model. **Methods:** Standardized mechanical scraping was performed in confluent immortalized human corneal/conjunctival epithelial cell layers. Cytotoxicity, cell migration and proliferation, as well as the percentage of closure, were analyzed 2 h and 1/2/3/6 days after a 30-min exposure to 1/10 dilutions in phosphate buffered saline (PBS) used also as control, BAK solutions at concentrations ranging from 0.0001% to 0.1%, latanoprost-0.02%BAK, travoprost-0.015%BAK, bimatoprost-0.005%BAK, BAK-free Tafluprost, latanoprost in cationic emulsion, and travoprost (Polyquad® and SofZia®). **Results:** PG eyedrop preparations with BAK preservative delayed corneal healing, which is primarily related to the presence of BAK, in a dose-dependent manner, especially at day 1, as evidenced through actin disorganization and decreased Ki-67-positive cell numbers. The PGs (BAK-free tafluprost, latanoprost in cationic emulsion, travoprost (Polyquad® and SofZia®)) maintained a normal healing process with results similar to those of control. Conjunctiva-derived cell layers healed more slowly than corneal cell layers and were more sensitive in *in vitro* cytotoxicity tests. **Conclusions:** This novel *in vitro* scraping model mimics the damaged ocular surface epithelia observed in glaucoma patients affected by ocular surface disease, such as toxic-induced dry eye (TIDE) and offers a tool to assess the potential cytotoxic effects of PG formulations with or without BAK.

Keywords: cornea; conjunctiva; wound-healing; antiglaucoma prostaglandin

1. Introduction

In addition to efficacy, safety and tolerance are the major criteria for evaluating new eye drops in preclinical and clinical trials, especially for antiglaucoma chronic treatments due to their repeated instillations over the long term [1,2]. Classically, experimental toxicological models of tolerance and safety are carried out on a healthy ocular surface (OS). However, in real life, long-term use of BAK-preserved glaucoma therapies is associated with or may induce ocular surface disease (OSD). The overall incidence of glaucoma in patients with OSD was reported to be of 20%, with a prevalence of glaucoma of 66% in patients with severe OSD, as defined by Tsai *et al.* [3] such as limbal stem cell deficiency resulting the conjunctivalization of the corneal surface. OSD is more frequent in older, polymedicated patients presenting severe, hard-to-treat glaucoma [3–5]. The frequency of topical treatment changes consecutive to treatment intolerance and the degree of glaucoma severity were found in a recent observational survey to be positively correlated with the severity of OSD [6]. Therefore, there is a real need to develop

safe and effective antiglaucoma therapies in order to avoid triggering or worsening OSD on the one hand and to alter glaucoma outcome on the other hand. Ideally, such therapy should also offer OS protective properties so that eventual pre-existing OSDs can be cured at the same time. Little knowledge is available on the influence of the antiglaucoma eyedrop formulations on an abnormal or already impaired OS. One *in vivo* study has reported the better effect on rat corneal healing of travoprost preserved with SofZia® compared to travoprost preserved with benzalkonium chloride (BAK) [7]. No *in vitro* corneal wound-healing model has been established for testing the impact of antiglaucoma ophthalmic solutions. Developing an *in vitro* model could be interesting to rapidly screen active antiglaucoma compounds and assess the impact of their formulations while respecting animal care ethics guidelines.

Wound healing is a complex dynamic process widely described in previous studies. Zhang *et al.* [8,9], found using *in vitro* and *in vivo* models, a prolonged and strengthened inflammation and a raised reactive oxygen species production after benzalkonium bromide administra-



tion contributing to delayed skin wound healing. On the OS, in the cornea, limbus and conjunctiva, many mediators through complex pathways coordinate cell migration, proliferation and differentiation in order to repair the epithelial defect [10–12]. Understanding the corneal healing process is essential for refractive surgery, such as photorefractive keratectomy (PRK) or laser in-situ keratomileusis (LASIK). Conjunctival healing is also mandatory for glaucoma filtration surgery: it is the major factor contributing to its long-term efficacy. An insufficiently healed postoperative conjunctival wound is significantly correlated with thin-walled filtration blebs, wound leakage and infection reduces the surgical success rate [13,14]. Furthermore, an excessive scarring process is also a major cause for filtration site fibrosis and surgical failure [15].

BAK, the most widely used preservative in ophthalmic aqueous solutions, induces pro-inflammatory and pro-apoptotic effects proportional to its concentration. BAK severely influences the wound-healing process: in 1985, Kossendrup *et al.* [16] showed that 0.01%BAK was able to delay corneal epithelial wound healing in rabbit and pig eyes. In rats, the cytotoxicity of BAK worsened a debrided corneal epithelium with a matrix metalloproteinase (MMP) increase [7,17]. In a previous study, with *in vivo* and *in vitro* scraping models designed to investigate the negative impacts of BAK-containing solutions on damaged OS, we demonstrated that the two scraping models correlated well [18]. In addition, the *in vitro* model offered the advantages of simplicity and rapidity and limits the unnecessary use of animal models. In that regard, we used this *in vitro* model as a validated toxicological model to test the effect of cyclosporine (CsA) in different formulations, which perfectly matched the known cytotoxicity of certain solutions in established rabbit toxicity models [19].

Following two previous *in vivo* and *in vitro* studies describing the impact of ophthalmic solutions on an altered OS, we aimed to evaluate in this *in vitro* wound-healing model the cytotoxicity of seven commercial antiglaucoma prostaglandin (PG) formulations, with or without preservative (i.e., BAK), or with alternative preservatives. This study is also the first to investigate the impacts of such a large number of PGs in two different OS cell lines, namely human corneal epithelial cell (HCE) and human conjunctiva-derived epithelial cell (HCjE) lines.

2. Materials and Methods

2.1 Human Corneal Epithelium (HCE) Cell Culture

Under standard culture conditions (humidified atmosphere of 5% CO₂ at 37 °C), the human corneal epithelial cell line (HCE, RCB 1384; Riken Cell Bank, Tsukuba, Japan) was cultured using DMEM/F-12 medium (Gibco, Invitrogen, France; L-glutamine, HEPES) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 50 IU/mL penicillin, and 50 IU/mL streptomycin. The cell passages (P) were from P10 to P15. The cells were seeded

in six-well culture dishes or on chamber slides (Lab-Tek™; Nalge Nunc International, Rochester, NY, USA) for the immunological analysis at a density of 100,000 cells per well and kept at 37 °C for 24 h. Cells were grown to reach a 90%–95% confluence in the center area of the well for the scraping assays before all the experiments [19].

2.2 Human Conjunctival Epithelium (HCjE) Cell Culture

The Wong-Kilbourne derivative of Chang conjunctival cells (clone 1 to 5c-41 American Type Culture Collection [ATCC, Manassas, VA]-certified cell line [CCL], 20.2) were cultured under standard conditions (humidified atmosphere of 5% CO₂ at 37 °C) in a mixture of Dulbecco's modified Eagle medium (Gibco, DMEM (1X), liquid, with 1000 mg/L D-glucose, sodium pyruvate with L-glutamine, 1000 mg/L D-glucose, sodium pyruvate) supplemented with 10% FBS, 50 IU/mL penicillin, and 50 IU/mL streptomycin. The conjunctival cells used in the subsequent assay were at a passage ranging from P10 to P15. Under our culture conditions, the cell division cycle for both HCE and HCjE cells was similar: 2–3 days.

2.3 In Vitro Wound-Healing Assay with EGF and the Different EyeDrops in Corneal Cell Culture

At day zero (D0) a standardized wound was created by scraping an HCE cell monolayer with a sterile micropipette tip under an inverted microscope (Leica DMIRB; Heidelberg, Germany) equipped with a digital camera (Coolpix 5000, Nikon, Tokyo, Japan). The cells were then incubated for 30 min in 1:10 dilutions (in their corresponding cell culture medium) of the different eyedrop formulations and control as previously published [20]: phosphate buffered saline (PBS), BAK solutions from 0.0001% to 0.1%, 0.005% latanoprost-0.02%BAK (Xalatan®; Pfizer, New York, NY, USA), 0.004% travoprost-0.015%BAK (Travatan®; Alcon, Fort Worth, TX, USA), 0.03% bimatoprost-0.005%BAK (Lumigan®; Allergan, Irvine, CA, USA), BAK-free 0.0015% tafluprost (Santen SAS, Evry, France), 0.005% latanoprost in cationic emulsion (Catioprost®, Santen SAS), 0.004% travoprost-0.001% polyquaternium-1 (Polyquad®), and 0.004% travoprost-SofZia® (ion-buffered solution with borate, zinc and sorbitol (TravatanZ®, Alcon).

For better understanding, only these initial concentrations will be kept for the presentation of the results. Day zero (D0) was set at the beginning of the incubation period. After 30 min, the incubation medium containing the different test items was discarded, the cell culture plates washed to remove any cells in suspension and replaced with fresh cell culture medium. Cytotoxicity, cell migration and proliferation were analyzed 2 h (H) after the end of the incubation and on day (D) 1, 2, 3, 4, 5 and 6. Since epidermal growth factor (EGF) is known to be activated during the corneal epithelial wound-healing process and induces a strong stimulus on corneal epithelial cell proliferation and

migration [21–23], EGF at 100 ng/mL was used as a positive control in this *in vitro* wound-healing model.

2.4 *In Vitro* Wound-Healing Assay and Eyedrop Incubation in Conjunctival Cell Culture

The scraping model in HCjE cells was performed using the same protocol as for HCE cells. Only a subset of the different PG eye drops was selected and evaluated in HCjE cells: PBS, PG formulated with the highest BAK concentration (latanoprost-0.02%BAK) with its corresponding 0.02%BAK control solution, and latanoprost in cationic emulsion. To objectively compare the corneal and conjunctival lines, the same operator analyzed the same cell passages from P10 to P15 for both cell lines.

2.5 Wound-Healing Analyses: Wound Distance and Percentage of Wound Closure

At the different time points, a picture of each well was taken (at $\times 200$ magnification) with an inverted microscope (Leica DMIRB) for further wound distance and closure analysis. As previously published, wound distance (WD) was measured with a framing graticule providing precise length measurements in the superior, middle, and inferior regions alongside the scraping length. The final calculated WD was the mean value of these three measurements. The WD was measured between the edges of the cell aggregates in case of substantial cytotoxicity. The WD at D0, before the incubation with the eye drop dilutions, was used as the reference to calculate the percentage of closure over time. All experiments were repeated six times, and the final WD was the mean of six experiments.

2.6 Immunohistology

For standard immunofluorescence staining, cells were cultured in chamber slides (Lab-Tek™) and were subjected to the aforementioned protocol: the cells were incubated for 30 min with the different test items diluted 1/10, washed with PBS and culture for an additional 24 h in fresh cell culture medium. The cells were then fixed in 4% paraformaldehyde (PFA). The images were analyzed with a confocal fluorescence microscope (E800, PCM 2000; Nikon, Tokyo, Japan) for proliferation and morphological patterns determination with Ki-67 and Alexa 488-conjugated phalloidin antibodies.

2.7 Cell Proliferation (Ki-67)

Only the cells treated with the different PG formulations were used for cell proliferation evaluation given that data with the 0.02%BAK solution were already published using this model [18]. Proliferating cells were identified by indirect immunohistology with Ki-67 (Nuclear Antigen Ki-67 Immunotech, Marseilles, France) as the primary antibody, and Alexa 488-conjugated goat anti-mouse (Molecular Probes, Invitrogen, Montluçon, France; in green) as the secondary antibody. Final staining with 4',6-diamidino-2-

phenylindole (DAPI) allowed for the visualization of the scraped area under the microscope. Ki-67-positive cells at D1 were counted in the superior, middle and inferior areas near the scraping edges and compared in the different conditions.

2.8 Cell F-Actin Cytoskeleton (Phalloidin)

To evidence cytoskeleton alterations we used four different concentrations of BAK (0.0001, 0.001, 0.005 and 0.01%BAK). The other BAK concentrations and PG formulations showed similar or intermediate patterns. The morphological pattern was analyzed using a confocal fluorescence microscope (E800, PCM 2000; Nikon, Tokyo, Japan). Propidium iodide (PI) at the concentration of 1:10,000 in 1X PBS was added just before mounting to localize cell nuclei.

2.9 Statistical Analysis

Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by the Fisher test (Software ID). Statistical significance was set at $p < 0.05$.

3. Results

3.1 Dose-Dependent BAK Inhibitory Effects in HCE Scraping Model

In PBS-incubated HCE cells (Fig. 1A), at D1 and D3, the percentage of closure reached 53.0% and 89.0%, respectively. From D3 to D6, the scraped area was almost covered with cells following PBS treatment, as for EGF treatment; however, the latter presented a better healing rate than with PBS: $>20\%$ at H2, 85.0% at D1 and nearly 100% from D2. The lowest BAK concentration 0.0001% (Fig. 1B at D0 and Fig. 1C at D3) did not inhibit the closure process, showing the same healing rates as for PBS at all time points. BAK at the 0.01% (Fig. 1D at D0 and Fig. 1E at D3) or 0.02% concentrations did not present any sign of wound closure at H2, but worsened for both conditions. Only at H4 and D1 was a slight recovery observed in the 0.01%BAK treatment group that was not confirmed at D3. For the 0.02%BAK group, no recovery was observed at any time points. At D6, no edges were found in either the 0.01% or the 0.02%BAK group. The 0.1%BAK solution induced cell death almost immediately with numerous detached cells floating in the culture medium at H2, and no clear edges could be measured afterwards. At D6, the culture medium became blurred for all conditions, including the lowest concentrations of BAK (from 0.0001% to 0.1%), and the experiment was halted.

3.2 Organization of the Actin Cytoskeleton in the HCE Scraping Model

Pictures in Fig. 2 of the phalloidin staining showed F-actin fibers near the scraped area. At D1 for the 0.0001%BAK-treated cells (Fig. 2A), cells near the edges of the scraped area (identified as “scraping” on the images)

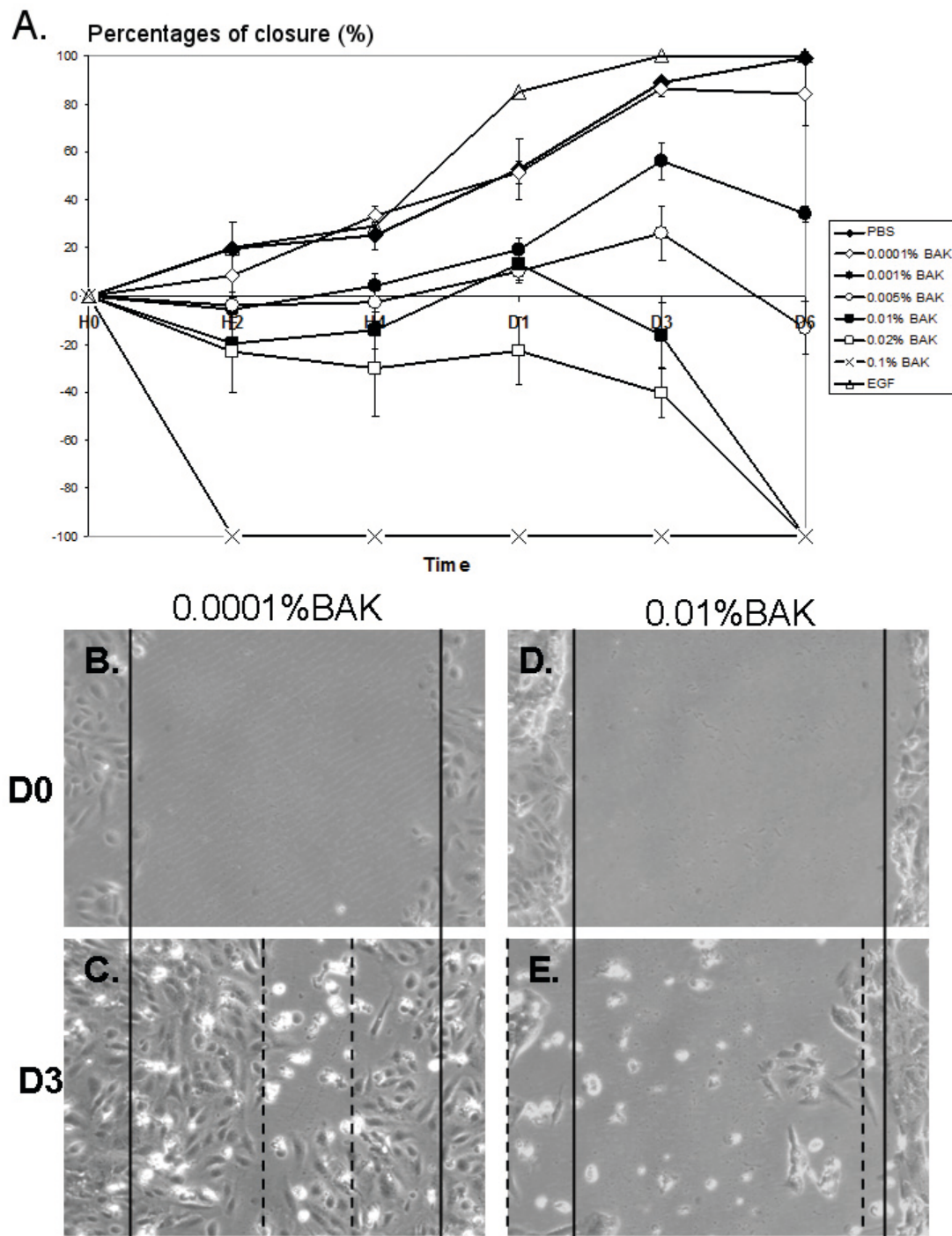


Fig. 1. Effects of different concentrations of BAK in the corneal wound-healing model. Percentage of closure over 6 days (D) with different concentrations of BAK ranging from 0.0001% to 0.1%, and EGF (100 ng/mL) as positive control (A). BAK at 0.1% caused cell desquamation and no closure can be measured (red asterisk). Pictures of scraped HCE cells at D0 (B and D) and D3 after treatment with 0.0001%BAK (C) and 0.01%BAK (E). Note: no clear healing edges could be observed after incubation with 0.01%BAK. $p < 0.01$ when PBS or EGF compared to all other groups from hour (H) 2 to D6, $p < 0.01$ when EGF compared to PBS from D1 to D3; $p < 0.01$ when 0.0001%BAK compared to other BAK groups from H4; $p < 0.01$ when 0.001%BAK compared to other BAK groups from D1; $p < 0.0001$ when 0.0051%BAK compared to other BAK groups from D3. The solid lines measure the initial wound width; the dotted lines evaluate the healing (magnification $\times 200$).

presented a normal and regular actin cytoskeleton showing extension of actin fibers (arrowhead) towards the scraped

area. BAK at the 0.001% (Fig. 2B) and 0.005% concentrations (Fig. 2C) presented moderate anisocytosis with short-

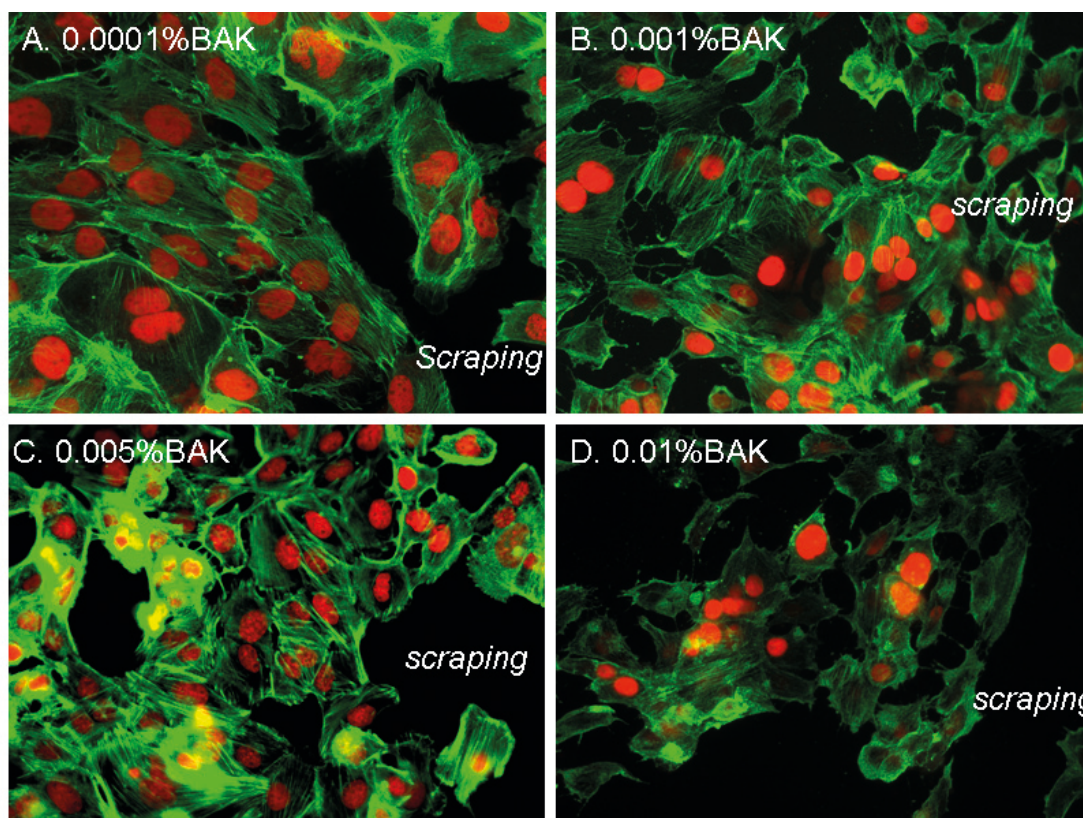


Fig. 2. F-actin fiber organization near the scraped area following treatments with BAK solutions. Phalloidin immunohistology staining (in green) at D1 after treatment with 0.0001%BAK (A), 0.001%BAK (B), 0.005%BAK (C) and 0.01%BAK (D). “Scraping” localizes the area where the cellular monolayer was scraped. Nuclei are stained in red with propidium iodide (magnification $\times 400$).

ened phalloidin-stained cytoskeleton marks. HCE cells treated with 0.01%BAK (Fig. 2D) showed cell loss with considerable anisocytosis. The phalloidin staining for F-actin fibers was less intense with no extension towards the scraped area. Cells also showed significant anisocytosis and the PI-stained nuclei were much smaller, with many nuclear bodies.

3.3 Effects of BAK-Containing and PGs in the HCE Scraping Model

Following the characterization of the various BAK concentrations in the HCE scraping model, we assayed all the commercially available anti-glaucoma PGs over a 3-day time period.

The percentage of closure in the different conditions is presented in Fig. 3A. At D1, no migration to a very slight migration was observed for the all BAK-containing PG formulations. The other PG formulations (BAK-free Tafluprost/Catioprost®/travoprost (Polyquad® and SofZia®) already presented significant wound closure at D1. At D2, we found -4.3% for latanoprost-0.02%BAK, while the percentage of closure ranged from 5.0 to 20.0% for the other two BAK-containing PG formulations. In contrast, between D1 and D2 the percentage of wound closure increased steadily for the BAK-

free Tafluprost/Catioprost®/travoprost (Polyquad® and SofZia® with a percentage of closure ranging from 45.0 to 56.0%. At D3, this closure ranging from 74.0 to 82.0% was observed, which was similar to the closure observed in the PBS group. Interestingly, BAK-containing PGs showed a better closure rate than the corresponding concentrations of BAK alone (Fig. 3B). Latanoprost-0.02%BAK presented a higher closure percentage than that of 0.02%BAK alone; and bimatoprost-0.005%BAK also had a better rate than 0.005%BAK alone.

3.4 Proliferating, Ki-67-Positive Cells in the HCE Scraping Model

Ki-67 was used to detect the proliferating cells at the edges of the scraped areas at D1, as shown in Fig. 4A–H. Following PBS treatment, 666 ± 13 Ki-67-positive HCE cells/mm² (Fig. 4H) were present. While all the BAK-containing formulations significantly reduced the number of proliferating cells in a BAK concentration-dependent manner: 300 ± 100 cells/mm² for latanoprost-0.02%BAK ($p < 0.02$ compared to all other groups), 500 ± 57 cells/mm² for travoprost-0.015%BAK, and Bimatoprost-0.005%BAK was able to maintain a proliferating cell density of 633 cells/mm², close to the PBS density. BAK-free Tafluprost and latanoprost in cationic emulsion ($>783 \pm 14$

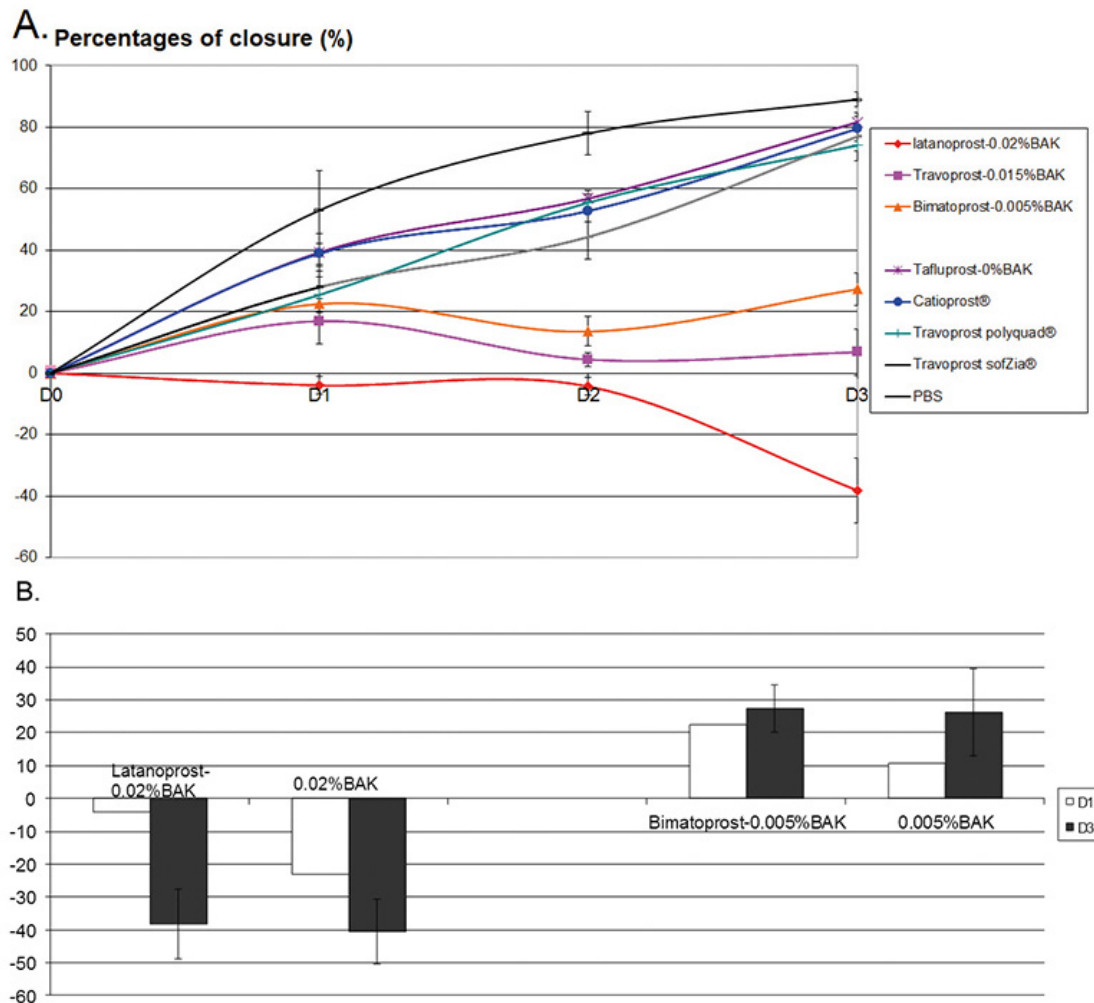


Fig. 3. Effects of PG formulations on corneal wound-healing model. (A) Percentage of closure over time (up to D3) following the treatment with the different PG formulations. (B) Comparison of two BAK-containing PG formulations with their corresponding BAK concentrations at D1 and D3.

cells/mm²) presented levels of Ki-67-positive cells higher than the PBS level. The delay observed in wound closure with BAK-containing PG formulations may be the consequence of a reduction in the number of proliferating cells.

3.5 Comparison of the Two Scraping Models in HCE and HCjE Cells

HCjE cells were scraped according to the protocol used for the HCE scraping model (Fig. 5) and treated with the PBS control to characterize the difference in response, if any, between HCE and HCjE cells. At D0 following the scraping and PBS treatment, both cell types presented similar patterns (Fig. 5A,B). However, a delay in the closure process was observed with HCjE cells. At D5, while HCE cells reached completed closure (>99.0%, Fig. 5C,E), HCjE cell wound closure reached only 64.6% closure (Fig. 5E). In conjunctival cells at D5 (Fig. 5D), the cells at the edges seemed to extend their cytoplasmic expansions toward the center of the scraped area without be-

ing able to complete the closure. We did not observe closure in HCjE cells until D10, with all cells floating due to toxic effects induced by an excessively long culture (data not shown).

3.6 Effects of Antiglaucoma PG in the HCjE Scraping Model

HCjE cells were scraped and treated with different BAK-containing and PG formulations, as in the HCE scraping model. The percentage of closure in Fig. 6. At D1 all the HCjE cells were floating with no identifiable edges and no measurable distance, clearly suggesting that the 0.02%BAK and latanoprost-0.02%BAK were more toxic for HCjE cells than for HCE cells. Interestingly, the closure process was much slower in HCjE cells: at D1, the percentage of closure was only 30.6% for PBS, 40.7% for latanoprost in cationic emulsion, without any significant difference between them. At D3, this percentage of closure was 36.1% for PBS and 49.3% for latanoprost in cationic emulsion, with no signifi-

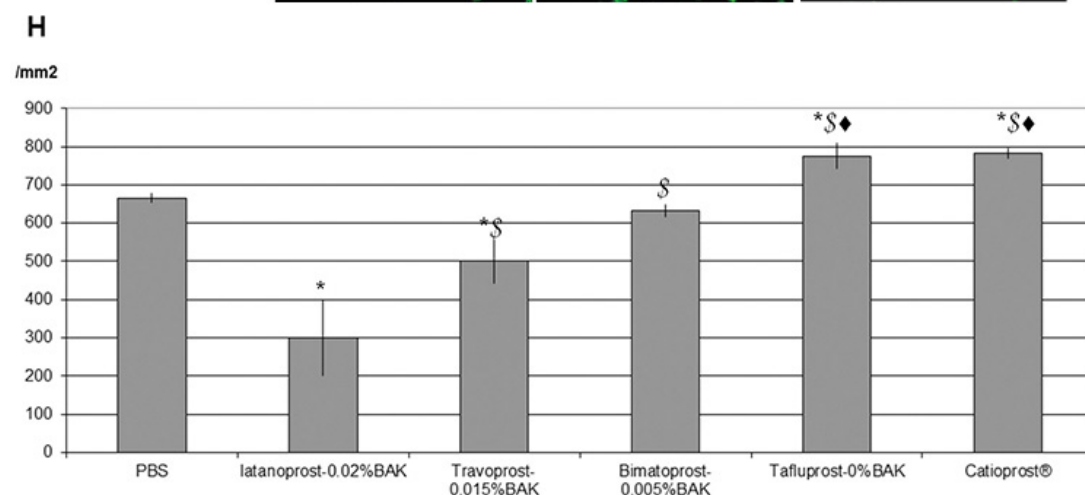
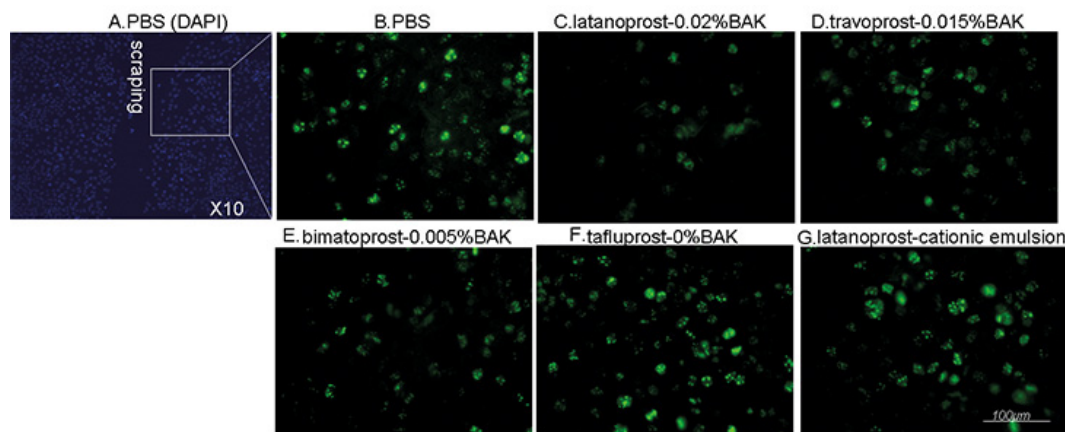


Fig. 4. Immunohistology of Ki-67 proliferating cells and cell counts at D1. Immunohistology for the proliferation marker Ki-67 (in green) near the scraped area (A) at day 1 (D1) after treatments with PBS (B), latanoprost-0.02%BAK (C), travoprost-0.015%BAK (D), bimatoprost-0.005%BAK (E), BAK-free Tafluprost (F), latanoprost in cationic emulsion (G) (bar = 100 μ m). Note the decrease in proliferating cells following treatments with BAK-containing solutions. Quantification of Ki-67-positive cells per mm² at D1 (H). * $p < 0.04$ – 0.004 compared to PBS; \$ $p < 0.02$ compared to latanoprost-0.02%BAK; ♦ $p < 0.0005$ compared to travoprost-0.015%BAK.

cant differences between them. From D4, the solutions became too toxic for the cultured cells, and no clear edges could be measured (data not shown).

4. Discussion

In this study we used an established *in vitro* wound-healing assay as a dynamic and relatively simple *in vitro* model to test eye drop PG formulations, not only their safety/cytotoxic profile but also their impact on the epithelial cell's proliferative capabilities. In this study, we compared commercially available antiglaucoma PGs using the same model. Our results demonstrated that in the HCE cells: (1) BAK-containing PGs inhibited the healing process in a BAK concentration-related manner; (2) none of the BAK-free Tafluoprost/Catioprost®/travoprost (Polyquad® or SofZia®) altered the normal healing process without altering the capacity of two OS cells to multiply despite using different preservative systems; (3) the BAK inhibition and cytotoxic effects were associated with an alteration of

F-actin fiber organization and the decrease or loss of proliferating Ki-67-positive cells; (4) BAK-containing PG formulations progressed faster than their corresponding concentration of BAK, suggesting a positive, protective effect of the PGs and/or formulation. These toxic effects of BAK seem to affect both cell survival and cell motility, as a possible consequence of a more general metabolic toxicity. BAK is known to inhibit the cornea's healing due to its pro-apoptotic cytotoxicity, which has been largely demonstrated in the literature [1,24–28]. Both conjunctival and corneal epithelial DNA synthesis was inhibited in a dose-dependent manner by a 15-min exposure to 0.001%, 0.01% or 0.1%BAK solutions [29]. In a 3D-reconstituted corneal epithelial model, expression of Ki-67 decreased in a BAK concentration-dependent manner, in accordance with the decreased cell viability observed by MTT tests and the increased apoptosis evidenced with the TUNEL assay [27,28]. The BAK inhibited the cornea epithelium wound healing in a concentration-dependent manner. This inhi-

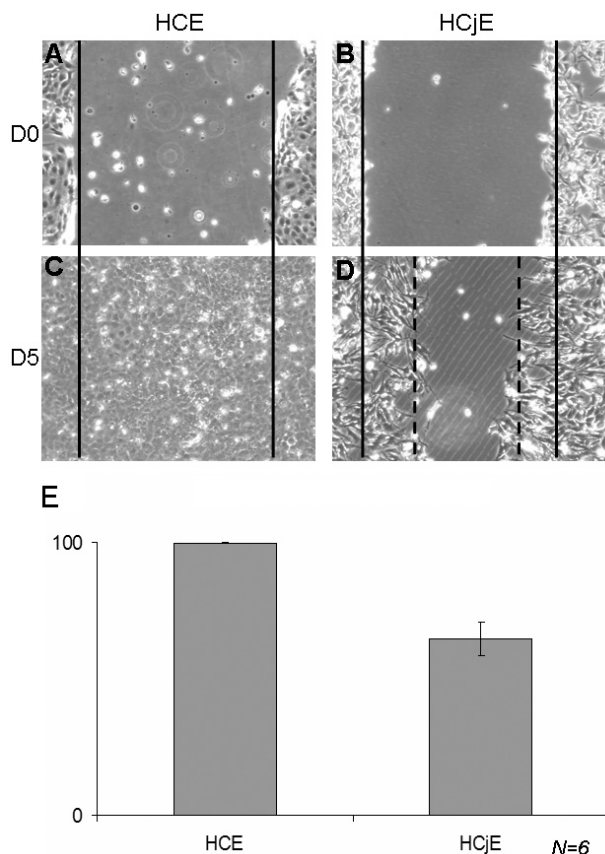


Fig. 5. Comparison of the scraping model with HCE and HCjE cells. Comparison of the normal healing process in human corneal epithelial cells (HCE) and human conjunctiva-derived epithelial cells (HCjE) at D0 (A for HCE, B for HCjE) and D5 (C for HCE, D for HCjE). While the HCE healing process was complete at D5 (C with 99% wound closure in E), HCjE cells reached only 64.60% closure at D5 ($n = 6$).

bition started at H4, and increased after one day. Indeed, BAK is known to induce adverse effects on the ocular surface and is, at the same time, considered useful in promoting the permeation of active molecules. But, it is interesting to note, that, the formulation of prostaglandin analogs as an ester prodrug, with a high lipophilicity, further hydrolyzed by cellular esterases into the acid form of prostaglandin, allows a better absorption by the cornea [30]. Additionally, several studies comparing prostaglandin analogs, with or without BAK showed similar efficacy especially on lowering IOP [31–33] suggesting that a penetration enhancer might not be helpful.

BAK induces cell apoptosis, necrosis and inhibition of proliferation, but the PGs seem to have a positive effect on the proliferation process. PGF2alpha agonists have been shown to induce a significant stimulation of bovine corneal endothelial cell (BCEC) proliferation [34]. Latanoprost and brimonidine increased the number of proliferating subconjunctival fibroblasts [35]. In latanoprost-incubated conjunctival specimens from glaucoma patients, the upregu-

lation of matrix MMPs, MMP-1 and MMP-3 reduced extracellular matrix accumulation in the conjunctival stroma [36]. Daily treatments with exogenous PGF2alpha agonist ($\leq 10^{-6}$ M) significantly increased epithelial cell proliferation [37]. In our experimental conditions, the beneficial effects induced by PGs could partially make up for the negative effects of BAK. All BAK-containing PG formulations (mixed micelles of BAK with solubilized PGs) showed a slightly better healing rate than the aqueous solutions containing the corresponding concentrations of BAK (BAK micelles). We also observed that treatments with latanoprost in cationic emulsion and BAK-free tafluprost resulted in the presence of slightly more Ki-67-positive cells than following PBS treatment.

Two different epithelial cell types of the OS, namely corneal and conjunctiva-derived epithelial cells, were used to evaluate the effects of the PG analog formulations on their healing capacity.

Even though there is some discussion that HCjE was originally derived from Hela cells, HCjE remains an acceptable and simple tool for testing toxicity without biopsy or primary culture. Indeed, two conjunctival cell lines ‘IOBA-NHC’ and ‘Chang cells’ showed similar toxicological profiles in *in vitro* studies [38]. In the future, primary cultures of conjunctival epithelial cells could be used in this model. The conjunctival cells seemed to heal more slowly than corneal cells and are apparently more sensitive to BAK, with inhibitory effects seen at lower concentrations. Lin *et al.* [39] have shown that corneal epithelium cells expressed EGFR, while only minimal to no staining for EGFR was observed in the superficial conjunctival and limbal epithelia. This reduced EGFR expression may explain the differences observed in the healing capacity between the two cell lines.

The safety and ocular tolerance of any eye drops are major criteria that are usually investigated on healthy ocular tissues via the Draize test [40] in animal models or during clinical trials that exclude severely afflicted patients, i.e., the patients whose disease severity is susceptible to leading to drug intolerance or to an increased patient dropout rate. Therefore, diseased preclinical models (*in vitro* and *in vivo*) might be of help [18,41,42]. The biological difference observed in the corneal/conjunctival wound-healing response results most probably from a difference in cytokine, growth factor, and chemokine expression, which would warrant further exploration and the development of appropriate models.

It is becoming increasingly evident that it will no longer be acceptable to lower elevated intraocular pressure (IOP) in glaucoma patients at the detriment of the OS, or without protecting the OS, while at the same time controlling elevated IOP. In that regard, cationic emulsion vehicles seemed to be the appropriate answer, because they have been shown to offer OS protective properties improving signs and symptoms of dry eye in clinical trials [43].

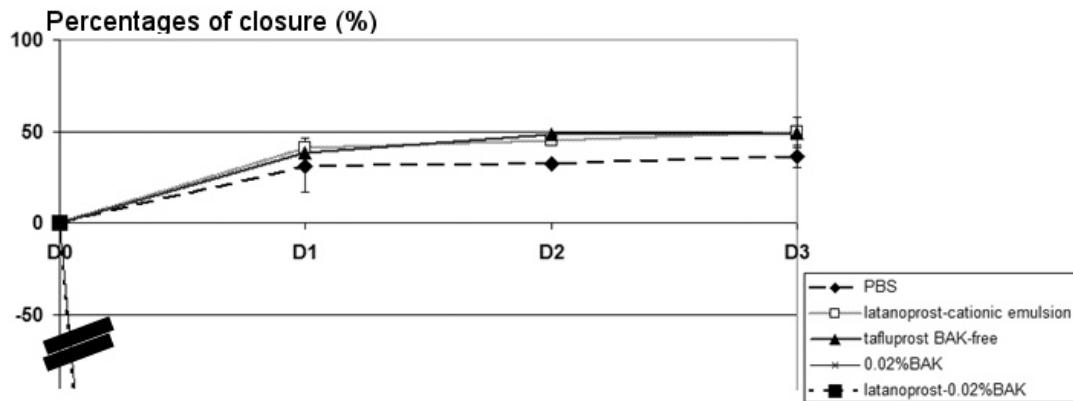


Fig. 6. Effects of PG formulations in the HCjE wound-healing model.

This innovative technology was used to develop a new latanoprost formulation: 0.005% latanoprost in cationic emulsion (Catioprost®) [44,45]. These cationic emulsions used 0.005% cetalkonium chloride (CKC), a C16 alkyl derivative of BAK, for its cationic property and its binding capacity to the oil nanodroplets rather than for any preservative effect [18,19,46]. For antiglaucoma treatments, it is recommended to reduce the use of BAK in eye drops as much as possible or even avoid it, and avoid use of any other excipients which may be deleterious to the ocular surface on the long term [27,41]. Therefore, removing BAK will allow for the exploration of other therapeutic strategies, such as goblet cell activation and growth factor stimulation, evidencing the possible beneficial effects of the PG analogs for the OS.

5. Conclusions

Using an *in vitro* scraping model aimed at mimicking a damaged OS and using two OS epithelial cell lines, we demonstrated that BAK-containing formulations delayed the wound-healing processes while the antiglaucoma active compounds maintain the healing capacity of the epithelial cells, and as such may offer a therapeutic interest for glaucoma patients already suffering from OS disorders. Outcomes of this *in vitro* study are in line with similar observations described in other preclinical models (*in vivo*, *ex vivo*). 2D and 3D reconstructed corneal epithelial models exposed to BAK solutions or BAK-containing prostaglandin formulations confirmed the toxicity through a delayed corneal healing [18,27,41,42]. Furthermore, a BAK-free travoprost preparation showed positive effects on tear secretion and corneal protection [42]. The next steps for antiglaucoma treatments might be to target elevated IOP, OSD, and its associated inflammation, the latter of which, if left untreated, may lead to trabecular meshwork complications and antiglaucoma treatment failure. Therefore, for the greater benefit of glaucoma patients, the concomitant treatment of ocular inflammation and elevated IOP might be of great value.

Author Contributions

HL—Design, experiments and main manuscript writing; CB, FBB—Design and manuscript correction; PD, JSJ—Figures and manuscript correction.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

References

- [1] Baudouin C, Labbé A, Liang H, Pauly A, Brignole-Baudouin F. Preservatives in eyedrops: the good, the bad and the ugly. *Progress in Retinal and Eye Research*. 2010; 29: 312–334.
- [2] Rouland J, Traverso CE, Stalmans I, Fekih LE, Delval L, Renault D, *et al*. Efficacy and safety of preservative-free latanoprost eye-drops, compared with BAK-preserved latanoprost in patients with ocular hypertension or glaucoma. *The British Journal of Ophthalmology*. 2013; 97: 196–200.
- [3] Tsai JH, Derby E, Holland EJ, Khatana AK. Incidence and prevalence of glaucoma in severe ocular surface disease. *Cornea*. 2006; 25: 530–532.
- [4] Leung EW, Medeiros FA, Weinreb RN. Prevalence of Ocular Surface Disease in Glaucoma Patients. *Journal of Glaucoma*. 2008; 17: 350–355.
- [5] Fechtner RD, Godfrey DG, Budenz D, Stewart JA, Stewart WC, Jasek MC. Prevalence of ocular surface complaints in patients with glaucoma using topical intraocular pressure-lowering medications. *Cornea*. 2010; 29: 618–621.
- [6] Baudouin C, Renard J, Nordmann J, Denis P, Lachkar Y, Sellem E, *et al*. Prevalence and risk factors for ocular surface disease

among patients treated over the long term for glaucoma or ocular hypertension. *European Journal of Ophthalmology*. 2012; 0.

- [7] Nagai N, Murao N, Okamoto N, Ito Y. Comparison of corneal wound healing rates after instillation of commercially available latanoprost and travoprost in rat debrided corneal epithelium. *Journal of Oleo Science*. 2010; 59: 135–141.
- [8] Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. *Frontiers in Bioscience*. 2004; 9: 283–289.
- [9] Zhang J, Yan Y, Li Y, Shen C, Zhang Y. Topical effect of benzalkonium bromide on wound healing and potential cellular and molecular mechanisms. *International Wound Journal*. 2021; 18: 566–576.
- [10] Iwamoto S, Koga T, Ohba M, Okuno T, Koike M, Murakami A, *et al.* Non-steroidal anti-inflammatory drug delays corneal wound healing by reducing production of 12-hydroxyheptadecatrienoic acid, a ligand for leukotriene B4 receptor 2. *Scientific Reports*. 2017; 7: 13267.
- [11] Wilson SE. Corneal wound healing. *Experimental Eye Research*. 2020; 197: 108089.
- [12] Daniels JT, Dart JK, Tuft SJ, Khaw PT. Corneal stem cells in review. *Wound Repair and Regeneration*. 2001; 9: 483–494.
- [13] Li F, Huang W, Zhang X. Efficacy and safety of different regimens for primary open-angle glaucoma or ocular hypertension: a systematic review and network meta-analysis. *Acta Ophthalmologica*. 2018; 96: e277–e284.
- [14] Narita A, Morizane Y, Miyake T, Seguchi J, Baba T, Shiraga F. Characteristics of early filtering blebs that predict successful trabeculectomy identified via three-dimensional anterior segment optical coherence tomography. *The British Journal of Ophthalmology*. 2018; 102: 796–801.
- [15] Schlunck G, Meyer-ter-Vehn T, Klink T, Grehn F. Conjunctival fibrosis following filtering glaucoma surgery. *Experimental Eye Research*. 2016; 142: 76–82.
- [16] Kössendrup D, Wiederholt M, Hoffmann F. Influence of cyclosporin a, dexamethasone, and benzalkonium chloride (BAK) on corneal epithelial wound healing in the rabbit and guinea pig eye. *Cornea*. 1985; 4: 177–181.
- [17] Sharma C, Velpandian T, Baskar Singh S, Ranjan Biswas N, Bihari Vajpayee R, Ghose S. Effect of fluoroquinolones on the expression of matrix metalloproteinase in debrided cornea of rats. *Toxicology Mechanisms and Methods*. 2011; 21: 6–12.
- [18] Liang H, Baudouin C, Daull P, Garrigue J, Buggage R, Brignole-Baudouin F. *In vitro* and *in vivo* evaluation of a preservative-free cationic emulsion of latanoprost in corneal wound healing models. *Cornea*. 2012; 31: 1319–1329.
- [19] Liang H, Baudouin C, Daull P, Garrigue J, Brignole-Baudouin F. Ocular safety of cationic emulsion of cyclosporine in an *in vitro* corneal wound-healing model and an acute *in vivo* rabbit model. *Molecular Vision*. 2012; 18: 2195–2204.
- [20] Guenoun J, Baudouin C, Rat P, Pauly A, Warnet J, Brignole-Baudouin F. *In vitro* comparison of cytoprotective and antioxidative effects of latanoprost, travoprost, and bimatoprost on conjunctiva-derived epithelial cells. *Investigative Ophthalmology & Visual Science*. 2005; 46: 4594–4599.
- [21] Shu DY, Hutcheon AEK, Zieske JD, Guo X. Epidermal Growth Factor Stimulates Transforming Growth Factor-Beta Receptor Type II Expression in Corneal Epithelial Cells. *Scientific Reports*. 2019; 9: 8079.
- [22] Martínez-Rendón J, Sánchez-Guzmán E, Rueda A, González J, Gullías-Cañizo R, Aquino-Jarquín G, *et al.* TRPV4 Regulates Tight Junctions and Affects Differentiation in a Cell Culture Model of the Corneal Epithelium. *Journal of Cellular Physiology*. 2017; 232: 1794–1807.
- [23] Rush JS, Bingaman DP, Chaney PG, Wax MB, Ceresa BP. Administration of Menadione, Vitamin K3, Ameliorates off-Target Effects on Corneal Epithelial Wound Healing Due to Receptor Tyrosine Kinase Inhibition. *Investigative Ophthalmology & Visual Science*. 2016; 57: 5864–5871.
- [24] De Saint Jean M, Debbasch C, Brignole F, Rat P, Warnet J, Baudouin C. Toxicity of preserved and unpreserved antiglaucoma topical drugs in an *in vitro* model of conjunctival cells. *Current Eye Research*. 2000; 20: 85–94.
- [25] Debbasch C, Pisella PJ, De Saint Jean M, Rat P, Warnet JM, Baudouin C. Mitochondrial activity and glutathione injury in apoptosis induced by unpreserved and preserved beta-blockers on Chang conjunctival cells. *Investigative Ophthalmology & Visual Science*. 2001; 42: 2525–2533.
- [26] Pisella P, Debbasch C, Hamard P, Creuzot-Garcher C, Rat P, Brignole F, *et al.* Conjunctival proinflammatory and proapoptotic effects of latanoprost and preserved and unpreserved timolol: an ex vivo and *in vitro* study. *Investigative Ophthalmology & Visual Science*. 2004; 45: 1360–1368.
- [27] Liang H, Pauly A, Riancho L, Baudouin C, Brignole-Baudouin F. Toxicological evaluation of preservative-containing and preservative-free topical prostaglandin analogues on a three-dimensional-reconstituted corneal epithelium system. *British Journal of Ophthalmology*. 2011; 95: 869–875.
- [28] Pauly A, Meloni M, Brignole-Baudouin F, Warnet J, Baudouin C. Multiple Endpoint Analysis of the 3D-Reconstituted Corneal Epithelium after Treatment with Benzalkonium Chloride: Early Detection of Toxic Damage. *Investigative Ophthalmology & Visual Science*. 2009; 50: 1644–1652.
- [29] Sosne G, Albeiruti A, Hollis B, Siddiqi A, Ellenberg D, Kurpakus-Wheeler M. Thymosin beta4 inhibits benzalkonium chloride-mediated apoptosis in corneal and conjunctival epithelial cells *in vitro*. *Experimental Eye Research*. 2006; 83: 502–507.
- [30] Alm A. Latanoprost in the treatment of glaucoma. *Clinical Ophthalmology*. 2014; 8: 1967–1985.
- [31] Pellinen P, Lökkila J. Corneal penetration into rabbit aqueous humor is comparable between preserved and preservative-free tafluprost. *Ophthalmic Research*. 2009; 41: 118–122.
- [32] Janulevičienė I, Derkač I, Grybauskienė L, Paulauskaitė R, Gromnickaitė R, Kuzmienė L. Effects of preservative-free tafluprost on tear film osmolarity, tolerability, and intraocular pressure in previously treated patients with open-angle glaucoma. *Clinical Ophthalmology*. 2012; 6: 103–109.
- [33] Halder A, Khopade AJ. Pharmacokinetics and Pharmacology of Latanoprost 0.005% without Benzalkonium Chloride Vs Latanoprost 0.005% with Benzalkonium Chloride in New Zealand White Rabbits and Beagles. *Current Eye Research*. 2021; 46: 1031–1037.
- [34] Rieck P, Edelmann B, Schmidt S, Hartmann C. Effect of prostaglandin F2 alpha on proliferation of corneal endothelial cells *in vitro*. *Der Ophthalmologe*. 2001; 98: 1157–1161. (In German)
- [35] Pérez-Roca F, Rodrigo-Morales E, Garzón I, Oliveira A, Martín-Piedra M, Carriel V, *et al.* Effects of Four Formulations of Prostaglandin Analogs on Eye Surface Cells. A Comparative Study. *PLoS ONE*. 2015; 10: e0129419.
- [36] Terai N, Schlötzer-Schrehardt U, Lampel J, Böhm AG, Rummelt C, Schmidt E, *et al.* Effect of latanoprost and timolol on the histopathology of the human conjunctiva. *The British Journal of Ophthalmology*. 2009; 93: 219–224.
- [37] Conconi MT, Spinazzi R, Tommasini M, Limoli A, Parnigotto PP. Prostaglandin F2 alpha can modulate the growth and the differentiation of bovine corneal epithelial cells cultured *in vitro*. *Annals of Anatomy*. 2001; 183: 567–573.
- [38] Brasnu E, Brignole-Baudouin F, Riancho L, Warnet J, Baudouin C. Comparative study on the cytotoxic effects of benzalkonium chloride on the Wong-Kilbourne derivative of Chang conjuncti-

- p>val and IOBA-NHC cell lines.
- Molecular Vision*
- . 2008; 14: 394–402.
- [39] Liu Z, Carvajal M, Carraway CA, Carraway K, Pflugfelder SC. Expression of the receptor tyrosine kinases, epidermal growth factor receptor, ErbB2, and ErbB3, in human ocular surface epithelia. *Cornea*. 2001; 20: 81–85.
- [40] Ciuchta HP, Dodd KT. The determination of the irritancy potential of surfactants using various methods of assessment. *Drug and Chemical Toxicology*. 1978; 1: 305–324.
- [41] Pinheiro R, Panfil C, Schrage N, Dutescu RM. The Impact of Glaucoma Medications on Corneal Wound Healing. *Journal of Glaucoma*. 2016; 25: 122–127.
- [42] Barabino S, Antonelli S, Cimbolini N, Mauro V, Bouzin M. The effect of preservatives and antiglaucoma treatments on the ocular surface of mice with dry eye. *Investigative Ophthalmology & Visual Science*. 2014; 55: 6499–6504.
- [43] Daull P, Buggage R, Lambert G. Novasorb® Cationic Nanoemulsion and Latanoprost: the Ideal Combination for Glaucoma Management? *Glaucoma: Open Access*. 2017; 2: 107.
- [44] Lallemand F, Daull P, Benita S, Buggage R, Garrigue J. Successfully improving ocular drug delivery using the cationic nanoemulsion, novasorb. *Journal of Drug Delivery*. 2012; 2012: 604204.
- [45] Daull P, Buggage R, Lambert G, Faure M, Serle J, Wang R, *et al.* A comparative study of a preservative-free latanoprost cationic emulsion (Catioprost) and a BAK-preserved latanoprost solution in animal models. *Journal of Ocular Pharmacology and Therapeutics*. 2012; 28: 515–523.
- [46] Daull P, Lallemand F, Garrigue J. Benefits of cetalkonium chloride cationic oil-in-water nanoemulsions for topical ophthalmic drug delivery. *Journal of Pharmacy and Pharmacology*. 2014; 66: 531–541.