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BASIC SCIENCE

A novel ophthalmic latanoprost 0.005% nanoemulsion: a cytotoxicity study

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Abstract

Background Benzalkonium chloride (BAK), the most commonly used preservative in anti-glaucoma eye drops, inflicts damage to the ocular surface. A novel anti-glaucoma formulation that avoids the use of BAK has been developed. The aim of this study was to evaluate the cytotoxicity of this formulation and to compare it with an ophthalmic solution containing BAK.

Methods Two different latanoprost eye drops were used: one ophthalmic solution (LSc) containing BAK 0.02% and one ophthalmic nanoemulsion (LNe) with a soft preservative (potassium sorbate 0.18%). Human epithelial conjunctival cells were incubated for 15, 30, and 60 min with either LSc or LNe. The cytotoxicity was determined by MTT assay. Cell death was measured by flow cytometry using annexin V–FITC and propidium iodide.

Results The values of cell viability and proliferation obtained from cells exposed to LNe were between 80 and 90% relative to the control group, whereas values obtained from cells exposed to LSc were around 30% at all study times ($p < 0.05$ at 15 and 30 min; $p < 0.01$ at 60 min). The percentage of viable cells decreased significantly when cells were incubated with LSc compared with cells incubated with LNe at all the study times, while the percentage of cells in late apoptosis/necrosis increased significantly in cells exposed to LSc compared to LNe.

Conclusions The new latanoprost nanoemulsion is significantly less cytotoxic on human conjunctival cells than LSc. These results suggest that the new formulation might be gentler on the eye surface than currently available BAK-preserved latanoprost solutions.

Keywords Glaucoma · Latanoprost · Ocular surface · Toxicity · Nanoemulsion

Key messages

- BAK is widely known due to its toxic effects on ocular surface, especially harmful in long-term treatments.
- Nanotechnology allows to address new pharmaceutical forms that enable to formulate BAK-free latanoprost eye drops.
- Latanoprost BAK-free nanoemulsion presents much better toxicity profile in comparison to latanoprost with BAK solution.
- Latanoprost BAK-free nanoemulsion presents really high values of viability, comparable to control group.

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Introduction

The term "glaucoma" comprises a group of optic neuropathies with diverse and complex etiologies, with primary open angle glaucoma (POAG) being the most common type and is one of the leading causes of blindness worldwide [1]. One of the major risk factors for the development and

progression of glaucoma is elevated intraocular pressure (IOP), and currently, IOP reduction is the only therapy to prevent visual field damage progression [2]. This is a chronic eye disease which needs long-term treatment (usually lifelong) and follow up [3]. The treatment is principally administered topically, by eye-drops, and if IOP is not controlled, different surgical procedures can be performed [3, 4].

Benzalkonium chloride (BAK) is the most commonly used preservative in eye drops, although there is a considerable amount of evidence of its deleterious effects on the ocular surface, especially when used over an extended period of time [5–9]. The reported effects, both in in vitro and in vivo models, include dose-dependent and time-dependent toxicity to the corneal epithelium, the conjunctival epithelium, the stroma, and tear film constituents. BAK may also reduce epithelial cell integrity, impair healing, induce cytokine secretion, cause elevated production of conjunctival inflammatory cells, and reduce goblet cell number [5, 6, 9–13]. Furthermore, BAK may impair tear function and reduce tear film integrity through its effects on the integrity of the meibomian layer and disruption of the lipid film continuous multilayer structure, thus decreasing tear film break-up time [6, 13–15]. At the cellular level, BAK not only induces growth arrest, but also increases epithelial cell apoptosis and cytotoxicity [5, 9–11].

The development of ocular surface disease (OSD) triggered by BAK can compromise treatment compliance since a strong link between treatment tolerability and compliance has been established [16–18]. Currently, there is an unmet medical need for the development of new multidose BAK-free formulations, that preserve the integrity of the ocular surface, especially when dealing with conditions that require long-term treatment [19, 20].

The newly developed latanoprost BAK-free nanoemulsion may meet this present need for alternative formulations that are gentle to the ocular surface and compatible for use in multidose BAK-free prostaglandin analogue formulations. The aim of this study was to compare the cytotoxicity of two different latanoprost eye drops: one ophthalmic solution containing BAK, and the newly developed ophthalmic nanoemulsion with a soft preservative.

Methods

Design, settings, and ophthalmic preparations

An experimental study was performed in the "Laboratory of Ocular Research," of the Pathology Department, Faculty of Medicine, University of Buenos Aires, Argentina. Two different latanoprost eye drops were used: one ophthalmic solution (LSc) containing BAK (latanoprost 0.005%, BAK 0.02%, Xalatan®, Pfizer) and one ophthalmic nanoemulsion (LNe) with a soft preservative (latanoprost 0.005%, potassium sorbate 0.18%, Louten® Emulsión, POEN). Both eye drops were purchased from commercial sources and were used within their labeled expiration dates.

Cell culture

The normal human epithelial cell line (IOBA-NHC) was kindly provided by Yolanda Diebold, Ph.D. (University Institute of Applied Ophthalmobiology, University of Valladolid, Valladolid, Spain) [21]. Cells were subcultured once a week in order to maintain the cell line. The medium consisted of Dulbecco's modified Eagle's medium–Nutrient Mixture F-12 (DMEM-F12) supplemented with 10% fetal bovine serum (Internegocios, Mercedes, Buenos Aires, Argentina), 2 ng/mL EGF, 5 µg/mL hydrocortisone (Sigma, St. Louis, MO), 1 µg/mL bovine pancreas insulin (Sigma), 50 U/mL penicillin, 50 µg/mL streptomycin, and 2 mg/mL amphotericin B. Cells were incubated at 37 °C in a humidified 5% CO_2 incubator.

Cell treatment with eye drops

A 1:6 dilution of the eye drops in culture medium (either LSc or LNe) was added to cell culture for 15, 30, and 60 min. Cells incubated with culture medium at the same time periods were used as control group.

Cytotoxicity evaluation

Cytotoxicity of eye drops on IOBA-NHC cells was examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay which measures viability and proliferation[22]. MTT is a yellow aqueous solution, which is reduced by dehydrogenases and reducing agents present in metabolically active cells, to insoluble violet-blue formazan crystals. IOBA-NHC cells were plated at 3×10^4 cells per well in a 24-well culture plate for 24 h prior to treatment. Culture medium was removed and eye drop treatment was performed. After this, test solutions and control medium were removed, and each well was washed twice with 1 × PBS, $pH = 7.4$. Culture medium with 0.4 mg/mL MTT (Sigma, St. Louis, MO) was added to each well (final volume = 500 µL). Plates were incubated for 3 h in a humid atmosphere of 37 °C with 5% CO_2. After the incubation time, supernatants were discarded, and a solution of isopropyl alcohol was added to dissolve formazan crystals, and incubation was carried out for 30 min in the dark. The absorbance of each supernatant was recorded at 570 nm using a plate reader (iMark Microplate Absorbance Reader; Bio-Rad, Hercules, CA). Results are expressed as percentage

of cell viability and proliferation, where 100% represents mean value of the absorbance of the control group.

Cell viability and cell death

The number of dead cells was determined by annexin V–FITC and propidium iodide (PI) kit for flow cytometry (BD Biosciences, Franklin Lakes, NJ). Annexin V binds to negatively charged phospholipid surfaces with a higher specificity for phosphatidylserine (PS). PS is an internal plasma membrane phospholipid that in the early apoptotic cascade is exposed on the outer layer of the plasma membrane, before the cells become permeable to PI. Therefore, early apoptotic cells are annexin V–FITC positive and PI negative, and cells that are in late apoptosis or already dead (necrosis) are both annexin V–FITC and PI positive. Viable cells are annexin V–FITC and PI negative.

IOBA-NHC cells were plated at 3×10^5 cells per well in a 24-well culture plate for 24 h prior to treatment. After treatment with test solutions, cell monolayers were washed twice with 1 mL of PBS and trypsinized (250 µL per well for 2–5 min, 37 °C, 5% CO2). Trypsin was neutralized with culture media and cells were centrifuged at 1000 g for 5 min. A total of 2.5×10^5 cells were placed in cytometer tubes, washed twice with cold 1 × PBS, and resuspended in 100 µL 1 × annexin V binding buffer. Then, 10 µL annexin V–FITC and 5 µL IP were added to each tube. The tubes were gently vortexed and incubated for 15 min in the dark. Finally, 150 µL 1 × annexin V binding buffer was added to each tube, and the flow cytometric analysis was performed (FACSCalibur; BD Biosciences,Argon laser, 488 nm). The values obtained were analyzed by Cyflogic 1.2.1 (CyFlo Ltd., Turku, Finland).

Statistical analysis

Data is available at Laboratory of Ocular Research. Results presented correspond to three independent experiments performed in triplicate. Data are presented as mean ± SD. Statistical analysis was performed using SPSS 17 (WinWrap Basic, Copyright 1993–2007 Polar Engineering and Consulting), the percentage of absorbance and fluorescence were the outcome variables evaluated. Student's *t*-test for two independent samples was used to calculate differences between groups. Statistical significance was set at $p < 0.05$.

Results

Cytotoxicity was significantly higher in cells exposed to ophthalmic solution (LSc) compared with ophthalmic nanoemulsion (LNe) at all the study times (Fig. 1; $p < 0.05$ for 15 and 30 min and $p < 0.001$ for 60 min). The values of viability and proliferation obtained from cells exposed to LNe were between 80 and 90%, whereas the values obtained from cells exposed to LSc were around 30% at all study times.

Cell viability, apoptosis, and necrosis were assessed by flow cytometry using the annexin V and propidium iodide (PI) kit in IOBA-NHC cells exposed to eye drops. The percentage of viable cells (negative for annexin V and PI) decreased significantly in cells incubated with LSc compared with cells incubated with LNe at all the study times (Fig. 2A; $p < 0.01$ for15 and 30 min; $p < 0.05$ for 60 min). It is interesting to note that the values of viable cells obtained from cells exposed to LNe were higher than 90% at all study times, whereas the values obtained from cells exposed to LSc were timely decreased. Non-significant differences were found for early apoptotic cells (positive for annexin V and negative for PI) at all study times (Fig. 2B).

In concordance with the results obtained for viable cells, a significant increase of cells in late apoptosis and necrosis (positive for annexin V and PI) was observed at all study times for cells incubated with LSc (Fig. 2C, $p < 0.01$ for 15 min and $p < 0.05$ for 30 and 60 min). It is important to point out that the values obtained from cells exposed to LNe were around 5% at all study times, whereas the values obtained from cells exposed to LSc were timely increased.

Finally, Table 1 shows the viability values obtained from both methods used. Despite the differences in absolute values obtained from each assay, the differences in viability between eye drops formulation are clearly visible by both methods.

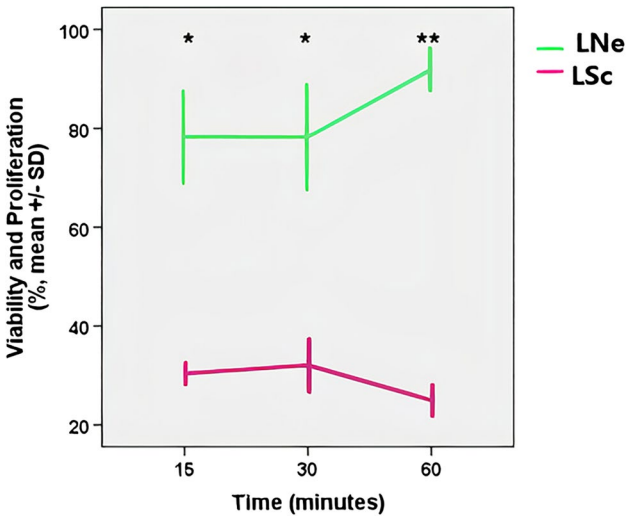

Fig. 1 Cytotoxicity evaluation by MTT assay. Percentage of cell viability and proliferation; *$p < 0.05$ **$p < 0.01$

Discussion

Eye drops given for the treatment and management of chronic conditions should avoid induction of unwanted side effects that may compromise the integrity of the ocular surface [6, 23]. This is especially true for glaucoma treatment and BAK, the most frequently used preservative in anti-glaucoma eye drops, has been clearly established as a risk factor for the development of dry eye disease [5, 8–10, 15–17]. This has led to considerable effort to develop new formulations that avoid the use of BAK, and preserve the integrity of the ocular surface, thereby improving the quality of life of glaucoma patients, as well as treatment compliance.

In this study, we evaluated the cytotoxicity induced by two different latanoprost eye drops: one solution preserved with BAK (LSc), and one newly developed ophthalmic nanoemulsion with a soft preservative (LNe) on a monolayer of human conjunctival epithelial cells.

The original pharmacological formulation used in this study contains potassium sorbate, described as a "soft preservative" given its advantages over BAK for the ocular surface health, previously confirmed in experimental and clinical studies [24, 25].

Nanoemulsions are excellent formulations to deliver lipophilic drug substances to the eye, as they provide enhanced ocular penetration [26]. The implementation of nanotechnology allows not only to formulate lipophilic drug products without the need to add BAK as a solubilizing agent, but also to encapsulate the active principle inside the nanoparticles, isolating it from contact with the ocular surface on its way to the site of action, avoiding the interaction with the prostanoid receptor EP, responsible for the characteristic hyperemia observed with the use of prostaglandin analogues in clinical practice [27, 28].

Latanoprost nanoemulsion also presents some technical advantages such as improvements in conservation conditions because nanoemulsions are formulations with adequate thermodynamic stability, which allows them to be stored at room temperature.

The product used in this study was approved by different regulatory offices in many Latin American countries, and is commercially available since 2017. Recently a clinical study has been published where this formulation in nanoemulsion was evaluated for its efficacy and safety. This study confirms that it maintains hypotensive efficacy with respect to the solution preserved with BAK and that improves dry eye disease signs and symptoms [28].

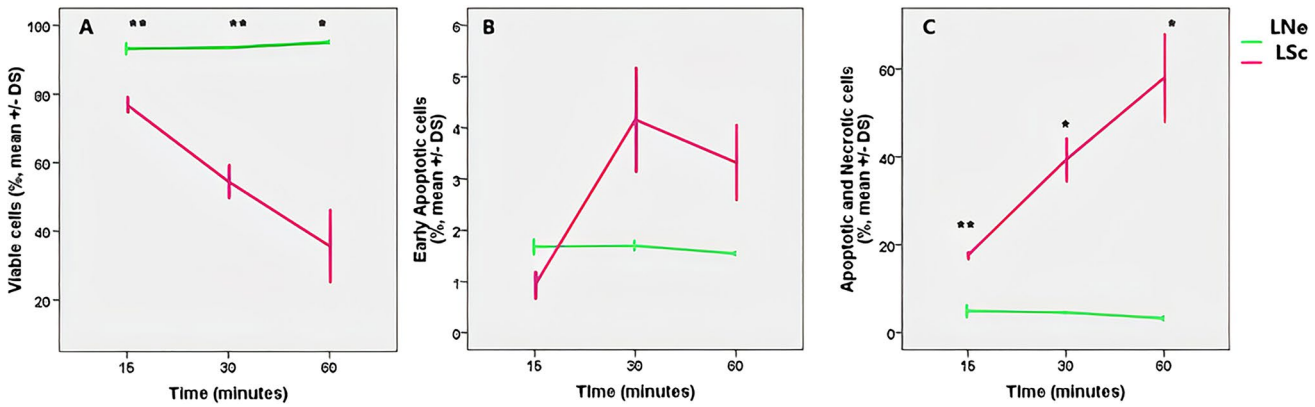

Fig. 2 Cell viability and apoptosis/necrosis evaluation. **A** Percentage of viable cells (Negative to annexin V–FITC and PI). **B** Percentage of early apoptotic cells (Positive to annexin V–FITC and PI negative) **C**. Percentage of Apoptotic and necrotic cells (Both annexin V–FITC and PI positive); $*p<0.05$. $**p<0.01$

Table 1 Cell Viability (%, mean ± SD) by MTT assay and flow cytometry using annexin V and propidium iodide kit

	MTT assay			Annexin V–FITC and PI kit		
Time (min)	LNe	LSc	*p*-value	LNe	LSc	*p*-value
15	78.27 ± 15.89	30.38 ± 3.55	< 0.05	93.23 ± 2.40	76.90 ± 3.55	< 0.05
30	78.24 ± 18.12	32.06 ± 9.03	< 0.01	93.50 ± 0.40	54.53 ± 8.05	< 0.05
60	91.93 ± 7.04	24.96 ± 5.30	< 0.01	95.07 ± 0.60	35.7 ± 18.02	< 0.01

LNe latanoprost nanoemulsion BAK-free, *LSc* latanoprost solution preserved with BAK, *PI* propidium iodide

The advantage of using IOBA-NHC cell lines to evaluate the cytotoxic effect of BAK was previously published by Brasnu E et al. [29]. Later, the same author [30] and others, [31, 32] use the IOBA-NHC cell lines, comparing the effect of preserved versus preservative-free prostaglandin analogues. In the three published studies, it was demonstrated that preservative-free formulations were better for the ocular surface in comparison to the BAK eye drops [30–32]. Nevertheless, this is the first experimental study to evaluate cytotoxicity of a new pharmacological formulation of latanoprost 0.005% BAK-free nanoemulsion, using IOBA-NHC cell line.

The MTT assay showed that cytotoxicity was significantly higher in conjunctival epithelial cells incubated with LSc than in cells exposed to LNe at all the studied times. The percentages of viable cells for those incubated with LSc were below 50% and seem to be independent of exposure time. Furthermore, the results obtained by flow cytometry were in line with the values observed in the MTT assay. Differences in the percentage of cells in late apoptosis/necrosis and viable cells incubated with LSc reached statistical significance at all the studied times compared with the cells incubated with LNe. These differences (an increase in cells in late apoptosis/necrosis and a decrease in viable cells) were time dependent. Also, it is important to point out that the values obtained from cells exposed to LNe were similar to the control group at all the studied times.

There are some concerns of the present experimental study set up to discuss. The tested eye drops were used at 1:6 dilution, with an exposure time up to 30 min, according to previous validated studies [33]. However, modeling of eye drop dilution in the tear film is very complex because of the highly dynamic nature of the process. For future studies, it will be interesting to consider a concentration curve to obtain the physiologically relevant concentration range. In this way, the reduction in viability with the BAK-preserved formulation is very high, which raises questions about the physiological relevance of the in-vitro conditions. However, the differences obtained with the LNe product in the present study are clear. This is an experimental study with the advantage of working under controlled conditions, giving us information about the in vitro behavior of the LNe eye drops in comparison with the Lsc eye drops. The presented study supports evidence for continuous research and in deep evaluation about signal transduction pathways leading to these effects.

Finally, although further studies are needed to confirm our findings in the clinical setting, our results suggest that the new latanoprost nanoemulsion may be gentler on the ocular surface than BAK-preserved eye drops, and it represents a suitable alternative to BAK-preserved eye drops for the treatment of glaucoma.

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Data availability Data is available anytime upon request to the corresponding author. Data is stored by investigators at Laboratory of Ocular Research, Pathology Department, Faculty of Medicine, Buenos Aires University.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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