

Impact of benzalkonium chloride-preserved and preservative-free latanoprost eye drops on cultured human conjunctival goblet cells upon acute exposure and differences in physicochemical properties of the eye drops

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To cite: Müllertz O, Hedengran A, Mouhammad ZA, *et al*. Impact of benzalkonium chloride-preserved and preservative-free latanoprost eye drops on cultured human conjunctival goblet cells upon acute exposure and differences in physicochemical properties of the eye drops. *BMJ Open Ophthalmology* 2021;**6**:e000892. doi:10.1136/bmjophth-2021-000892

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Received 31 August 2021
Accepted 30 November 2021



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ABSTRACT

Objective To investigate the short-term impact on human conjunctival goblet cell (GC) survival and mucin release of acute exposure to benzalkonium chloride (BAK) preserved and preservative-free (PF) 0.005% (w/v) latanoprost (LT) eye drops, and to compare the eye drops' physicochemical properties.

Methods and analysis Primary GC cultures were established from human conjunctival donor tissue. The impact of eye drops on GC survival was assessed using a lactate dehydrogenase assay. Mucin release was evaluated through mucin-specific immunostaining. pH value, osmolality, drop mass and surface tension for all LT eye drops were measured.

Results After application with PF-LT for 30 min (min), the GC survival was maintained compared with control ($p=0.9941$), while all BAK-LT eye drops reduced survival with approximately 30% ($p<0.02$). Following application with PF-LT for 30 min, mucin was found around the GC nucleus, as seen in the vehicle control, indicating no secretion. In contrast, BAK-LT caused diffuse staining of mucin, similar to the secretagogue histamine, indicating stimulation of secretion. The pH value of the BAK-LT and PF-LT eye drops were 6.0–6.9 and 6.8, respectively. The osmolality was 258–288 mOsm/kg for the BAK-LT eye drops and 276 for PF-LT eye drops. The mean drop mass was 26–31 mg for the BAK-LT eye drops and 30 mg for PF-LT. The surface tension was lower for all BAK-LT eye drops (31.1–32.1 mN/m) compared with PF-LT (42 mN/m).

Conclusion PF-LT compared with various branded and generic LT preparations containing BAK are less cytotoxic when applied to cultured GCs.

INTRODUCTION

Glaucoma is the leading cause of irreversible blindness worldwide. With an ever-increasing

Key messages

What is already known about this subject?

- ▶ Benzalkonium chloride (BAK) has worse tolerability than preservative-free eye drops which affects compliance and quality of life.

What are the new findings?

- ▶ BAK-preserved latanoprost eye drops cause more cell death than preservative-free latanoprost when examined on cultured human conjunctival goblet cells.

How might these results change the focus of research or clinical practice?

- ▶ The findings identify a harmful effect of BAK-preserved latanoprost eye drops on goblet cells and support the use of preservative-free treatment for better compliance and disease control.

number of glaucoma patients, the need for effective and well-tolerated long-term treatments is crucial.^{1,2} The main goal of glaucoma treatment is to control and reduce the intraocular pressure (IOP) to slow disease worsening, as increased IOP is a major risk factor for glaucoma progression.³ Treatments with IOP-lowering drugs consist primarily of application with eye drops, and the treatment is lifelong for the vast majority of patients. Due to their effectiveness in lowering the IOP along with minimal systemic side effects, treatment with prostaglandin analogues (PG) is the first choice for treating glaucoma.⁴ PGs decrease the IOP by increasing the uveoscleral

outflow.⁵ Latanoprost (LT) eye drops make up the majority of prescribed PGs worldwide.⁶ Although generally well tolerated, lifelong use of preserved LT eye drops can cause ocular side effects and affect patients' quality of life. This may lead to reduced compliance, can have serious consequences for the therapeutic effect and thus worsen the disease.^{7,8} Ocular side effects from preserved anti-glaucomatous eye drops have been associated with tear film instability, a common phenomenon found in dry eye disease (DED).⁹ Mucin, which is produced by the conjunctival goblet cells (GCs), is an important component of the tear film. Loss of GCs leads to decreased mucin production, increasing the instability of the tear film, which ultimately causes DED. As GCs are highly involved in the maintenance of a healthy ocular surface, it is important to obtain knowledge of the potential cytotoxic effect of preserved LT eye drops on conjunctival GCs to characterise the tolerance of the ocular surface to these eye drops.

Xalatan was the first LT eye-drop product, as well as the first PG, to be marketed. Xalatan eye-drop solution contains 0.005% (w/v) LT and 0.02% (w/v) of the preservative benzalkonium chloride (BAK). In 2013, the first preservative-free (PF) formulation of LT became available in Europe (Monoprost/Monopost, Laboratoires Théa France). Originally, BAK was thought to be an absorption enhancer for drugs. This, however, has been disproven as Monoprost does not have inferior efficacy compared with BAK-preserved eye drops.^{10–12} Furthermore, PF Monoprost has shown much better tolerability and significantly less conjunctival hyperaemia.^{11–13} In a systematic review and meta-analyses, no significant differences in tolerability between BAK-preserved and PF IOP-lowering eye drops could be confirmed.¹⁴ This is likely due to short trial durations and diverse reporting on safety measures, as BAK has in multiple studies shown to be damaging to the ocular surface. In patients treated with BAK-preserved eye drops, GC density was decreased compared with PF eye drops.^{15,16} No difference in GC morphology was identified, but BAK caused worse Ocular Surface Index scores and tear film function.¹⁶ Stevens *et al* identified increased intraocular reaction and Martinez-de-la-Casa *et al* increased cytokine levels in tear film from patients treated with BAK-preserved eye drops compared with PF eye drops.^{17,18} BAK-preserved eye drops increased matrix metalloproteinases nine in tear film, caused longer tear break-up time and more conjunctival and corneal fluorescein staining.¹⁹ Other preservatives such as polyquaternium-1 and sofzia are available, and clinical and cellular studies suggest that these alternatives are less damaging than BAK.^{20–22}

Since Xalatan went off patent, many different BAK-preserved LT generics were introduced to the market. This posed another problem in treating glaucoma. The European Medicines Agency states that generics must have the same concentration of the active substance, indication and bioequivalence, with the latter being of little relevance when addressing eye drops.²³ Variations in

content of excipients as well as minor formulation differences can have an influence on the efficacy and safety of the product. In this context, concerns have been raised and, despite few studies in the field, several significant differences in physical and chemical properties of the LT eye drops have been reported.^{24–26} In the 2020 guidelines from the European Glaucoma Society, closer monitoring of patients is advised when switching between eye drops.⁴

In this study, all preserved and PF-LT products available in Denmark as well as Latanelb, were examined. Latanelb, although not available in Denmark, is a BAK-preserved LT product widely used in Europe. In total, seven different BAK-preserved LT eye drops, and one PF-LT formulation were analysed in terms of acute effect on viability and mucin release on single exposure to GCs. The physical and chemical properties for all eye drops were examined. To our knowledge, no previous studies have examined the effect of LT eye drops on human GC viability and mucin release and compared the eye drops' properties.

MATERIALS AND METHODS

Eye drops

The 0.005% (w/v) LT products: Monoprost (Laboratoires Théa, Clermont-Ferrand, France), Xalatan (Pfizer, New York, New York, USA), latanoprost Mylan (Viatris, Canonsburg, Pennsylvania, USA), latanoprost Pfizer (Pfizer), latanoprost Sandoz (Sandoz AG, Holzkirchen Germany), latanoprost Stada (STADA Arzneimittel AG, Bad Vilbel, Germany) and latanoprost Teva (Teva Pharmaceutical Industries, Petah Tikva, Israel) were kindly provided by the Department of Ophthalmology, Copenhagen University Hospital, purchased from RegionH Pharmacy (Copenhagen, Denmark). Latanelb (axunio Pharma, Hamburg, Deutschland) Latanelb was directly ordered from Deutsch pharmacy.

Human conjunctival GC culture

Tissue was stored at 5°C in CorneaMax (CMXSTO01F, Eurobio, Les Ulis, France) until cultivation. No data of donors was obtained as each donor served as its own control in the cytotoxicity assays. No exclusions were made. Pure donor cultures were cultivated from one conjunctival sample per donor. Controls were included for all donors in all assays. The cultivation method was based on the work of Shatos *et al*.²⁷ Tissue pieces were incubated for 14 days at 37°C and 5% CO₂ in Roswell Park Memorial Institute medium medium 1640 1x (32404–014; Gibco, Life technologies, Massachusetts, USA), 10% (v/v) fetal bovine serum (10270–106; Gibco, Life technologies), 1% (v/v) Penicillin/Streptomycin (15140–122; Gibco, Life technologies), 1% (v/v) Non-Essential-Amino-Acid solution (M7145; Sigma-Aldrich, Missouri, USA), 1% (v/v) 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (15630–080; Gibco, Life technologies), 1% (v/v) L-glutamine (25030–024; Gibco, Life technologies) and 1% (v/v) sodium pyruvate (11360–039; Gibco, Life technologies). Culture medium

was added on day 1, 2 and 3 and then changed every other day. The cultures were examined before medium change by light microscopy and non-GCs were scraped away to obtain as pure cultures of GCs as possible.

Cytotoxicity assay

Cytotoxicity was determined using a lactate dehydrogenase assay (LDH). After 14 days, cultures were trypsinised using 1M EDTA (E5134; Sigma-Aldrich) in phosphate-buffered saline (PBS), 0.48mM Versene (15040-033; Gibco, Life technologies) and 0.25% (w/v) trypsin (T4799; Sigma-Aldrich) and replated to a density of 25,000 cells/cm² for the LDH assays. These first passage GCs were cultured for 5–7 days at 37°C and 5% CO₂ before analyses. At the time of analyses the GCs were 21–23 days old. The LT products were diluted 1:7 (v/v) in the culture medium to mimic the dilution in the tear film.

An LDH kit (MK401 from Takara BIO, Shiga, Japan) was used to measure LDH release from cells which indicates cell death. Culture medium was removed, and the GCs were incubated for 30 min with eye drops diluted 1:7 (v/v) in culture medium under static conditions at 37°C and 5% CO₂. The diluted eye drops were removed, fresh medium was added, and the GCs were incubated for an additional 20 hours. After spinning supernatant was moved to a well in a new plate and 0.1% (v/v) Triton X-100 (1001325622; Sigma-Aldrich) in PBS was added to the old well to permeate the membrane. The plate was incubated at room temperature (RT) for 10 min. After spinning the supernatant was moved to the new plate. LDH solution was prepared and added as instructed by the manufacturer. The GCs were incubated in the dark at RT until enough colouring (up to 15 min) before adding stopping solution, 10% (v/v) 1M hydrogen chloride.

The absorbance was measured at 490 nm using SpectraMax i3X multimode microplate reader (Molecular devices, California, USA). A minimum of three batches of human conjunctival GCs from different donors were analysed for each eye-drop. Cultures from 11 donors were included. A control was added for each donor in all assays with culture medium applied instead of eye drops. All analyses were performed in triplicates. Cytotoxicity was calculated as LDH release before membrane permeation divided by total LDH release. The mean percent relative to the control was then calculated.

Immunocytochemistry

GC cultures cultivated on coverslips were incubated for 30 min with Xalatan, latanoprost Pfizer or Monoprost diluted 1:7 (v/v) in culture medium. GCs were incubated with culture medium as negative control and 10^{-3.5} M histamine as positive control. Cultures were fixed using 4% (v/v) paraformaldehyde and stored at 4°C until immunostaining. The cell membrane was permeated using 0.1% (v/v) Triton X-100 in PBS and non-specific binding was blocked using 3% (w/v) bovine serum albumin (ab181831; Sigma-Aldrich) in PBS. Coverslips

were incubated with antibodies to specific markers for cytokeratin-7 (anti-cytokeratin7, 1:500 v/v) (ab181831; Abcam, Cambridge, England) and mucin5AC (anti-mucin, 1:200 v/v) (M5293; Sigma-Aldrich), washed with PBS and incubated with fluorescent secondary antibodies Alexa488 (anti-rabbit, 1:500 v/v) (A11034; Gibco, Life Technologies) and Texas red (anti-mouse, 1:200 v/v) (T862; Gibco, Life Technologies). Nuclei were stained using DAPI (0.3 μM) (D3571; Invitrogen, Massachusetts, USA). A minimum of cultures from three different donors were analysed. Imaging was performed using Axioskop 2 (Zeiss; Göttingen, Germany) with an AxioCam MRm camera (Zeiss; Göttingen, Germany) and HXP 120 lighting unit (Zeiss; Göttingen, Germany). Fiji ImageJ 1.49 was applied for picture optimising and merging.

Physical and chemical characterisation of LT products

Three withdrawn samples from three preparations of different batches for each LT product were analysed in triplicates with respect to pH value, osmolality and surface tension. Experiments were conducted at RT. pH value was measured using a calibrated 744 pH-metre (Metrohm; Nordic ApS, Herisau, Switzerland). Osmolality was determined by measuring the freezing point depression (Osmomat 3000; Gonotec, Berlin, Germany). Surface tension was measured using Wilhelmy method with platinum rod probe (PL03) and a K100c Force tensiometer and Laboratory Desktop, software version 3.2.2.3068 from KRÜSS (Hamburg, Germany). Drop mass was assessed by manually weighing all drops from three preparations of different batches for each LT eye-drop by releasing one drop and returning the bottle upright between each measure using a XS105 Dual Range analytical balance (Mettler Toledo International, Ohio, USA). Drop mass was measured by one person.

Statistics

Graphics and statistical analysis were performed using GraphPad Prism V.8. Results were expressed as mean unless otherwise described. Comparative one-way analysis of variance (ANOVA) was performed on all data sets after normal distribution was confirmed through QQ-plots. Dunnett's test was used for multiple comparison when comparing cell survival with control. Tukey's multiple comparison test was used to compare LT products with respect to cell survival and physical and chemical characteristics. Mixed-effects analysis was applied when comparing cell survival. A $p \leq 0.05$ was considered statistically significant.

RESULTS

Cell viability assays

The acute effect of the LT eye drops on GC viability was analysed by release of the intracellular enzyme LDH. GC survival was not altered after 30 min instillation with Monoprost compared with control, while all the preserved LT drops caused significant cell death ($p < 0.02$) (figure 1).

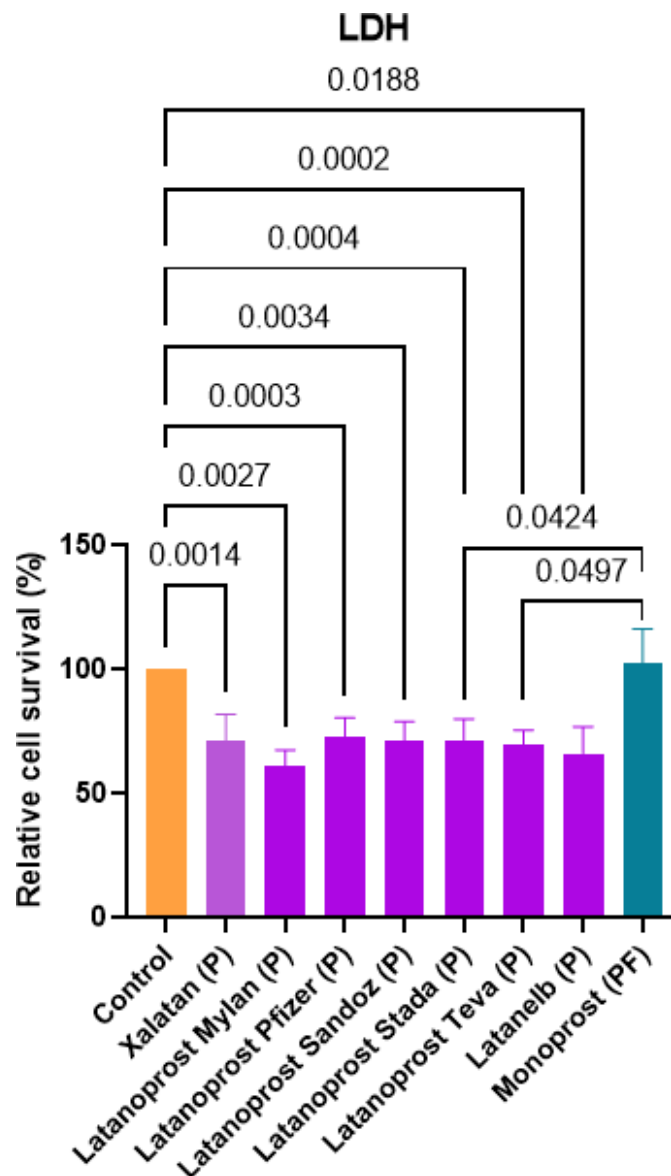


Figure 1 Mean relative survival of the human goblet cell (%) \pm SD obtained by the lactat dehydrogenase (LDH) assay after 30 minutes of application compared with 100% survival for medium applied goblet cells. Only $p < 0.05$ are shown. P, preserved; PF, preservative-free.

No significant differences were identified between the preserved eye drops. Latanoprost Stada and Latanoprost Teva caused more cell death than Monoprost ($p < 0.05$).

Immunocytochemistry

Mucin release was evaluated through immunohistochemical stainings (figure 2). The negative control incubated with medium showed unreleased vesicles seen as red dots located around the nuclei suggesting limited secretion (figure 2, row A). The positive control incubated with histamine showed a diffuse red staining in the cytoplasm suggesting secretion from vesicles (figure 2, row B). The cultures to which Monoprost was applied revealed mucin in vesicles around the nuclei as seen in the negative control (figure 2, row C). Application with Xalatan and

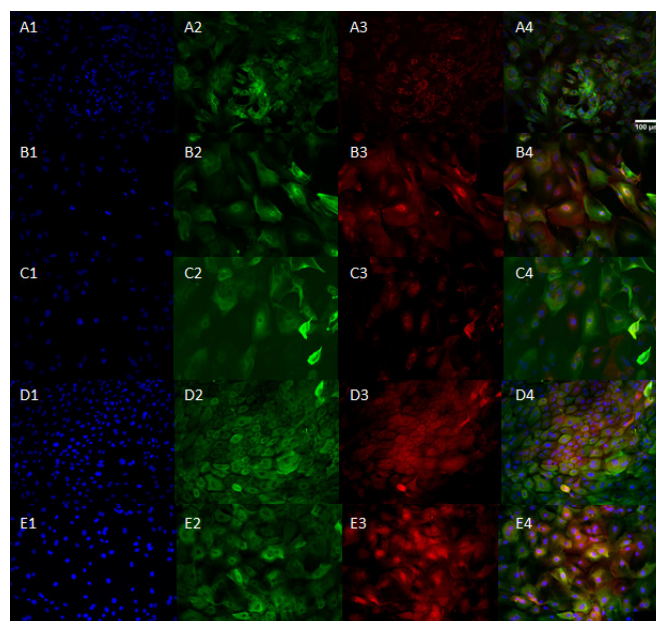


Figure 2 Immunocytochemistry staining specific for; the nucleus colored blue (column 1), the cytoskeleton colored green (column 2) and the mucin colored red with (column 3). Finally, the three stainings were merged (column 4). The cultures have been incubated with cell medium (row A), histamine (row B), Monoprost (row C), Xalatan (row D) and latanoprost Pfizer (row E). Scalebar was made using Fiji ImageJ 1.49.

latanoprost Pfizer caused the mucin vesicles to release and the mucin was spread to the cytoplasm, as seen for the positive control (figure 2, row D and E). This indicates that Monoprost does not have a secretagogue effect, while Xalatan and latanoprost Pfizer do.

Physicochemical properties

pH value, osmolality, surface tension and drop mass were measured for Xalatan, the generics hereof, Latanelb and Monoprost, and significant differences were observed (figure 3). The most notable pH value was that of 6.0 for Xalatan which was significantly lower than the pH values for the generics ranging from 6.7 to 6.9 as well as the pH value for Latanelb and Monoprost of 6.7 and 6.8, respectively ($p < 0.0001$). Mean osmolality was lowest for Xalatan measuring 258 mOsm/kg. The osmolality of the generics varied from 264 to 278 mOsm/kg which was significantly higher compared with Xalatan for all but latanoprost Stada ($p \leq 0.025$). Latanelb had the highest osmolality of 288 mOsm/kg and Monoprost measured 276 mOsm/kg. The surface tension of 42.4 mN/m for Monoprost was significantly higher than for all the preserved eye drops measuring 31.1–32.1 mN/m ($p < 0.0001$). Mean drop mass was significantly different between most eye drops but varied with only 5 mg. Xalatan and the generics hereof varied from 28 to 31 mg and Monoprost weighed 30 mg. Latanelb measured the smallest drop of 26 mg which was significantly smaller than the remaining eye drops ($p \leq 0.001$). Generally, substantial differences were identified when comparing Xalatan with the generics

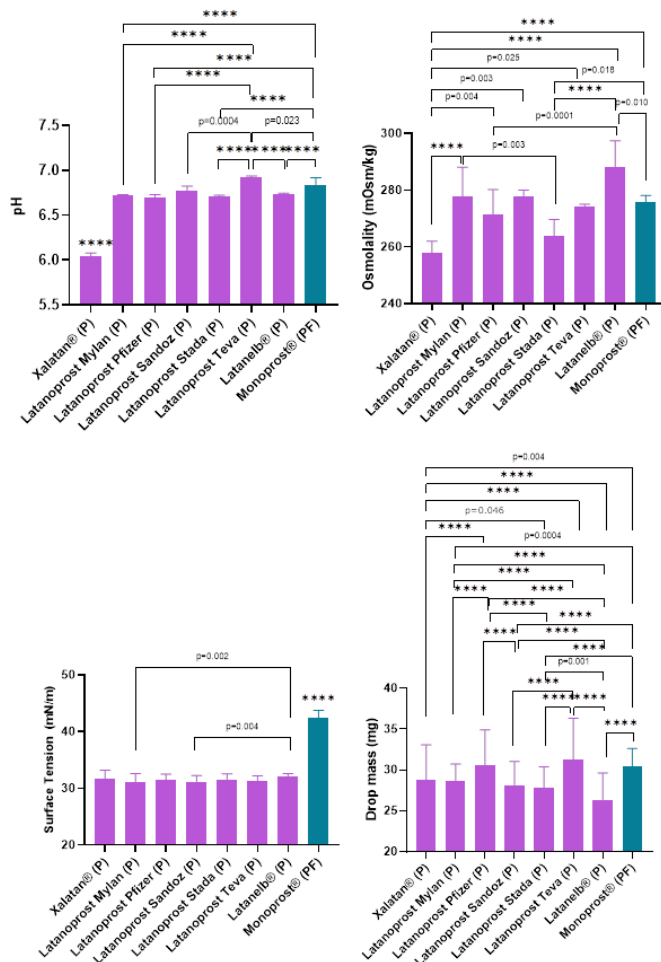


Figure 3 Physicochemical properties of latanoprost eye drops; pH value, osmolality (mOsm/kg), surface tension (mN/m) and drop mass (mg). Values are mean \pm SD. **** $P < 0.0001$. Only $p < 0.05$ are shown. P, preserved; PF, preservative-free.

regarding pH value and osmolality. Furthermore, the surface tension was notably higher for Monoprost compared with the preserved LT drops.

DISCUSSION

In this study, the potentially cytotoxic effect on human GCs and mucin release was investigated for seven 0.02% (w/v) BAK-preserved LT eye drops and one PF-LT eye-drop, Monoprost. Furthermore, the physicochemical properties of the eye drops were compared. Monoprost was superior to the BAK-preserved LT eye drops in GCs survival and mucin release. Physicochemical properties varied significantly between eye drops with pH value and osmolality being notably low for Xalatan, and surface tension being low for all preserved eye drops.

In the current study, we evaluated the acute effect of LT eye drops on cultured GC. We assessed cell survival using an LDH assay. To mimic the dilution in tear film on ocular administration, we diluted the eye drops 1:7 (v/v) in cell medium. After 30 min incubation Monoprost proved to be non-cytotoxic while all the BAK-preserved eye drops caused significant GC death.

Our current study and other studies confirm the toxicity of BAK-preserved eye drops. Previously, we found the GC toxicity of BAK-preserved eye drops to be associated with the concentration of BAK in IOP-lowering eye drops with different active ingredients. The higher the BAK concentration the higher the toxicity.²⁸ In a study on conjunctival cell line cultures BAK-preserved eye drops caused significant cell death while PF eye drops did not.¹⁵ BAK has also been associated with toxicity of deeper ocular structures such as the trabecular meshwork (TM). Baudouin *et al* identified BAK in the TM of rabbits treated with BAK-preserved eye drops and found that BAK caused TM cell death in TM cell cultures.²⁹ This could cause TM degeneration and increased outflow resistance, which again could cause an increase in IOP. Impairment of mitochondrial function has been suggested as a potential mechanism for BAK's cytotoxic effect.³⁰

The main function of GCs is to secrete mucin and create a protective lubricating layer at the cornea.³¹ We evaluated mucin release using immunohistochemical stainings. Incubation with Xalatan and latanoprost Pfizer appeared to cause mucin release similar to the known GC agonist histamine, which was not seen for Monoprost.³² This could indicate an irritant effect of preserved eye drops. The effect of PG and BAK on mucin secretion has not been rigorously investigated, and studies on the mechanism of BAK's secretagogue effect would be of interest. Of note, the current results are qualitative and quantitative measurements are needed to confirm these results. Furthermore, GCs may act differently when cultured compared with in vivo. The current setting is static, while in in vivo the eye-drop concentration would continuously decrease and not be constant for 30 min.

The investigated BAK-preserved eye drops appeared identical according to the SmPCs. However, our detailed investigations on pH value, osmolality, surface tension and drop mass of the eye drops have shown that there are significant differences. These differences may affect tolerability as well as efficacy.

A pH value below or above the tear film value of 7.4 can be irritating to the eye.³³ In this study, the pH value of Xalatan was notably low (6) compared with the generic eye drops as well as Latanelb and Monoprost as previously reported.²⁵ Leitritz *et al* found that, compared with 23 preserved LT eye drops, Monoprost had a significantly higher pH value of 7.2 and was closest to the pH value of the tear film.³⁴ Angmo and coworkers identified a pH value of 7.1 for Xalatan.²⁴ In 2013, the declared pH value of Xalatan available in the United Kingdom (UK), was changed from 6.7 to 6.0, to allow long-term RT storage.³⁵ Shortly after, more Xalatan-induced adverse events were reported, suggesting that a deviation of pH value from the physiological conditions causes a higher incidence of side effects.³⁵ Noteworthy, the formulation was not changed in the USA.³⁶ The pH value of Xalatan eye drops is

not declared in the SmPC in the UK nor in Denmark, which makes it difficult for the patients and physicians to be aware of its significance.^{37,38} Angmo and coworker may have used the Xalatan formulation, available in the USA. Our results show that one cannot assume that the branded Xalatan and generics hereof are similar with regard to pH value. The generics drops as well as Monoprost and Lataneolb had pH values closer to the tear film compared with Xalatan and may, therefore, have better tolerability.

The normal tear film osmolality is 302 mOsm/kg.³⁹ Osmolality was measured, as osmolality can be determined strictly experimentally and does not require any calculations in contrast to osmolarity. Furthermore, due to the low protein content of the tear film any difference is deemed clinically insignificant.⁴⁰ Hypoosmolality was identified for all LT eye drops, with Xalatan having the lowest osmolality of 258 mOsm/kg. It is, however, hyperosmolality and not hypoosmolality, that is considered a key element in the development of dry eyes. Hyperosmolality is associated with increased tear film evaporation and causes inflammatory responses in epithelial cells and GC loss.⁴¹ While studies identifying ocular discomfort due to hypoosmolality could not be identified, it cannot be ruled out that the hypoosmolalities measured for the LT eye drops may add to the side effect profile. The effect will, however, likely not be as significant as the effect of hyperosmolality.

The surface tension of the air/tear-fluid interface is around 43.6 mN/m.⁴² Various critical surface tensions of the ocular surface are found.⁴³ For instance the surface tension of the cornea needs to be high, while the tension of the aqueous surface needs to be lower. Tiffany *et al* associated dry eyes with a high tear film surface tension (49.6 mN/m). This correlated with a low tear break-up time.⁴² In the current study, Monoprost had a surface tension equivalent to the characteristics of the tear film. As BAK is a surfactant and, therefore, lowers the surface tension, all the preserved eye drops had low surface tensions of around 31 mN/m. While one could theorise that a low surface tension might protect against dry eyes, reduction of ocular discomfort is more likely when using eye drops with a surface tension equivalent to the tear film.

Regarding efficacy, variations in the described physiochemical properties may affect drug absorption. Furthermore, variations in drop mass is of importance. Variations in LT applied to the ocular surface may lead to fluctuations in IOP. We found variations of 5 mg between the LT eye drops. Xalatan and the generics, while significantly different, only varied with 3 mg. While we cannot exclude that a variation of this size will affect tolerability and efficacy, it is a small difference and clinical significance is unlikely.

We conclude that PF-LT eye drops are superior to BAK-preserved eye drops with regard to cultured GC viability and mucin release. Clinical studies are needed to identify whether these findings can be verified in

vivo. Furthermore, we show that the branded LT and the generics hereof cannot be assumed comparable with regard to physiochemical properties.

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Correction notice This article has been corrected since it first published. Author name 'Tor P Utheim' has been updated.

Acknowledgements Special thanks to laboratory technician Charlotte Taul for excellent laboratory assistance.

Contributors OM and AH are cofirst authors on the paper. The study was planned by MK, SH and GP. Concept and design were performed in collaboration with MK, DAD, JB, JJ, SWL and TU. Analyses were conducted by OM, AH, ZAM, JF and RN. Interpretation and analysis of data was performed by OM, AH and MK. The manuscript was written by OM, AH and MK. The manuscript was revised and approved by all authors. MK acted as guarantor.

Funding The work was funded by Laboratoires THEA (France), The Danish Eye Research Foundation and Fight for Sight (Denmark). Hørslev-Fonden granted funds for a tensiometer.

Competing interests The study was funded by Laboratoires THEA (France).

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval The study has been approved by the Danish National Committee on Health Research (H-17007902) and the Norwegian Regional Committees for Medical and Health Research Ethics (REK: 2013/803).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. Datasets are available on reasonable request.

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