Benzalkonium Chloride-Preserved Anti-Glaucomatous Eye Drops and Their Effect on Human Conjunctival Goblet Cells in vitro

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Keywords
Goblet cells · Glaucoma · Benzalkonium chloride · Anti-glaucomatous treatment · In vitro study

Abstract

Introduction: Most intraocular pressure (IOP)-lowering eye drops are preserved with benzalkonium chloride (BAK). This can increase side effects and decrease adherence. Particularly, damage to the mucin-producing conjunctival goblet cells may be an issue due to instability of the tear film. We aimed to investigate the effect of IOP-lowering eye drops preserved with BAK on cultured human conjunctival goblet cells. Methods: Eye drops Brimonidine Tartrate Teva (BT) with 0.005% BAK, Dorzolamide Stada (DS) with 0.0075% BAK, Optimol\textsuperscript{®} (OP) with 0.01% BAK, and Latanoprost Teva (LT) with 0.02% BAK were included. Human primary cultured goblet cell survival was evaluated using a lactate dehydrogenase assay on human goblet cells after treatment for 30 min and 6 h with the different anti-glaucoma drug formulations. Results: All eye drops examined, except BT, reduced goblet cell survival. The impact of eye drops on goblet cell viability was correlated with the time of exposure as well as to the concentration of BAK. After 30 min of exposure, cell viability was 93% for BT (0.005% BAK; \( p = 0.93 \)), 71% for DS (0.0075% BAK; \( p = 0.067 \)), 70% for OP (0.01% BAK; \( p = 0.054 \)), and 69% for LT (0.02% BAK; \( p = 0.022 \)), and exposure for 6 h reduced cell survival to 74% for BT (\( p = 0.217 \)), 52% for DS (\( p = 0.011 \)), 34% for OP (\( p = 0.017 \)), and 31% for LT (\( p = 0.0007 \)). Conclusion: LT, OP, and DS reduced human goblet cell survival in a time-dependent manner. BT did not affect goblet cell survival. Cell survival was correlated with the BAK concentration in the eye drops making 0.02% BAK-preserved LT most toxic and 0.005% BAK-preserved BT least toxic.
Primary open-angle glaucoma (POAG) affects approximately 3% of the world’s population aged 40–80 years [1]. POAG is an optic neuropathy and is characterized by a progressive degeneration of the innermost retinal neurons, the retinal ganglion cells. The etiology of POAG is not known, but an elevated intraocular pressure (IOP) is a proven risk factor [2]. IOP can be lowered with eye drops, laser treatment, or surgery, with the far most common treatment being eye drops. Eye drops lower IOP by decreasing aqueous humor production and/or by increasing outflow. Eye drops available at Danish pharmacies that decrease aqueous humor production include beta-adrenergic receptor blockers (BB), carbonic anhydrase inhibitors (CAI), and alpha-2-receptor agonists (AA), with AA also increasing uveoscleral outflow. Prostaglandin analogs (PG) increase the outflow. In addition to the eye drops listed, the muscarinic eye drop, pilocarpine, increases outflow by inducing miosis. Due to the incurable nature of POAG, most patients require lifelong treatment. In Denmark, 47% of glaucoma patients use PG monotherapy making PG the most frequently used glaucoma treatment [3], followed by BB, CAI, and AA. While most glaucoma patients are treated with monotherapy, 23% of Danish glaucoma patients are prescribed 2 or more preparations [3].

The available IOP-lowering eye drops cause substantial side effects. The most common side effects are burning, itching, redness, tearing, and blurred vision [4]. The same symptoms can be seen in patients with ocular surface disorders (OSD). The prevalence of OSD in an average population is reported to be approximately 15% [5, 6], while the prevalence of OSD among glaucoma patients has been reported to be around 60% [6].

Most side effects on the ocular surface can be related to a reduced quality of the tear film. The tear film consists of 3 layers: an inner layer of lubricating mucin; a middle layer of proteins, electrolytes, and water; and an outer layer of lipids [7]. Mucins are secreted from the conjunctival goblet cells and stabilize the tear film as well as protect the ocular surface from dryness. The goblet cells are present in the entire conjunctiva in clusters or individually. In the human conjunctiva, the highest density of goblet cells is found nasally, and the cells reach partway down the apical surface of the stratified epithelium [8, 9]. Goblet cells are also present in the gastrointestinal, urogenital, and respiratory tracts. The development of goblet cells and hence the production of mucins has been shown to depend on the epithelial transcription factor SPDEF [9]. In SPDEF null mice, increased fluorescein staining of the cornea, inflammatory cells in the conjunctival epithelium, tear volume, and accumulation of debris have been found [7]. These findings substantiate the important role of goblet cells and their mucins in protecting the health of the ocular surface.

The current study compares the effect of AA, CAI, BB, and PG eye drops on human conjunctival goblet cells. The eye drops included represent 4 multidose eye drops available in a glaucoma clinic in Denmark, and each contains different percentages of BAK. The advantage of this study is that it was performed on human goblet cells making it more clinically relevant than studies on animal models. We have successfully cultured goblet cells from donor tissue and developed purified cultures due to fibroblast removal.

Materials and Methods

Treatments

Anti-glaucomatous treatments included the AA Brimonidine Tartrate Teva (BT) (brimonidine tartrate 2 mg/mL and 0.005% BAK; Teva Pharmaceutical Industries Ltd., Petah Tikva, Israel), the CAI Dorzolamide Stada (DS) (dorzolamide 20 mg/mL and 0.0075% BAK; STADA Arzneimittel AG, Bad Vilbel, Germany), the BB Lumigan 0.02% Bausch & Lomb (Lumigan 2%, 0.02% BAK; Bausch & Lomb, Rochester, NY), and the PG Alphagan 0.5% (Pilocarpine HCl 0.5% and 0.02% BAK; Allergan, Inc., Santa Ana, CA) and Lumigan 0.2% (Pilocarpine HCl 0.2% and 0.005% BAK; Allergan, Inc., Santa Ana, CA). A pure saline solution (PSS) served as a control.

Treatments

Before treatment, the prepared goblet cells were incubated in PSS for 24 hours to ensure that no BAK was present on the cells. After 24 hours, the cells were treated with the different concentrations of BAK for 24 hours. The BAK concentrations were 0%, 0.05%, 0.025%, 0.01%, 0.0075%, and 0.005% BAK. The control group was treated with only PSS.

Slides were prepared for each group and each concentration of BAK. The slides were stained with periodic acid-Schiff (PAS) and stained with hematoxylin and eosin (HE) to visualize the goblet cells. The mean percent of PAS-positive goblet cells was calculated in each group for each concentration of BAK.

Results

The results showed that the BAK concentrations of 0.05%, 0.025%, and 0.01% BAK significantly decreased the number of goblet cells. The BAK concentrations of 0.0075% and 0.005% BAK did not significantly decrease the number of goblet cells.

Discussion

The results of this study show that BAK has a significant effect on goblet cell populations. The BAK concentrations of 0.05%, 0.025%, and 0.01% BAK significantly decreased the number of goblet cells. The BAK concentrations of 0.0075% and 0.005% BAK did not significantly decrease the number of goblet cells.

Conclusion

The results of this study show that BAK has a significant effect on goblet cell populations. The BAK concentrations of 0.05%, 0.025%, and 0.01% BAK significantly decreased the number of goblet cells. The BAK concentrations of 0.0075% and 0.005% BAK did not significantly decrease the number of goblet cells.
Effect of Anti-Glaucomatous Drugs on Goblet Cells

Conjunctival tissue was obtained from human donor eyes from the eye bank, Department of Ophthalmology, Oslo University Hospital. The protocol complied with the Declaration of Helsinki and was 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). No data on donors were recorded as a control for each donor was included in all assays. Goblet cell cultivation is based on the work by Shatos et al. [15]. Conjunctival tissue was stored at 5°C in CorneaMax® (CMXSTO01F; Eurobio, Les Ulis, France) until cultivation. For cultivation, the tissue was dissected into 2 x 2 mm pieces and placed into a 6-well culture dish. Immediately after 3–4 drops of culture medium (RPMI medium 1640 1X [32404-014; Gibco, Life Technologies, Waltham, MA, USA], 1% [vol/vol] FBS [10270-106; Gibco, Life Technologies, Waltham, MA, USA], 1% [vol/vol] penicillin/streptomycin [15140-122; Gibco, Life Technologies, Waltham, MA, USA], 1% [vol/vol] nonessential amino acid solution [M7145; Sigma-Aldrich, St. Louis, Missouri, USA], 1% [vol/vol] 1 m Hepes [15630-080; Gibco, Life Technologies, Waltham, MA, USA], 1% [vol/vol] l-glutamine [25030-024; Gibco, Life Technologies, Waltham, MA, USA], and 1% [vol/vol] sodium pyruvate [11360-039; Gibco, Life Technologies, Waltham, MA, USA]) were added per tissue piece, the plates were kept in an incubator at 37°C, 5% CO2. For the following 3 days, 3–4 drops were added per tissue piece. Thereafter, the medium was changed every other day, adding 1 mL of medium. Routinely, the cells were checked employing microscopic visual examination with a light microscope to monitor growth of fibroblasts. In case of fibroblast growth, the fibroblasts were removed by scraping and the well rinsed with culture medium. After 14 days of cultivation, the goblet cells were trypsinized. Trypsinization was implemented with 1 m EDTA (E5134; Sigma-Aldrich, St. Louis, Missouri, USA) in PBS, 0.48 m M versene (15040-033; Gibco, Life Technologies, Waltham, MA, USA), and 0.25% (wt/vol) trypsin (T4799; Sigma-Aldrich, St. Louis, Missouri, USA). After trypsinization, cells were rinsed with medium containing 10% FBS serum, which inactivates trypsin. Finally, cells were pelleted, resuspended in medium, and rinsed with medium containing 10% FBS serum, which inactivates trypsin. As a control, cells were treated with basal culture medium. After the treatment, the medium was changed, and the cells were left in the fresh medium for an additional 20 h before measurement of LDH release. The medium was removed and centrifuged at 500 rpm for 10 min. Each supernatant was transferred to a corresponding well in a new plate. To measure the amount of LDH remaining in the cells, 200 μL of 1% Triton-X (1001325622; Sigma-Aldrich, St. Louis, Missouri, USA) was added to each well in the original plate and incubated for 10 min at room temperature (RT). Plates were centrifuged, and each supernatant was transferred to a corresponding well. LDH solution was added and incubated for 3–15 min at RT; 1 m HCl was added to each well, bubbles were removed, and the plates were read at 490 nm on the SpectraMax i3X multi-mode microplate reader (Molecular Devices, San Jose, CA, USA). Cell survival was assessed as the ratio between LDH release prior to membrane permeation by Triton-X and total LDH. Percentage compared to control was calculated. Cell survival was analyzed on at least 3 cell cultures from different donors.

Measurement of Eye Drop pH and Osmolarity

Measurements were performed in triplicate on 3 containers of each type of eye drop. Measurements were performed promptly to reduce any contact with atmospheric air and thereby prevent any chemical reactions. The pH of each drop was measured with a calibrated standard laboratory 744 pH meter (Metröhm; Nordic ApS, Herisau, Switzerland) at RT. Osmolarity was measured using freezing point depression (Osmomat 3000; Gonotec, Berlin, Germany).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism version 8.0.0. All statistical comparisons with control and in between eye drops were made with ordinary one-way ANOVA multiple comparisons (Tukey’s multiple comparisons test). All values are expressed as mean. Significant outliers were eliminated prior to analyses. A p value <0.05 was considered significant. Normal distribution of data was confirmed through QQ-plots.

Results

Effect of BAK-Containing Eye Drops on Goblet Cell Survival

Goblet cell survival was assessed by LDH assay on cell cultures from at least 3 different donors after exposure to diluted BT, DS, OP, and LT eye drops for 30 min or 6 h. After a 30-min treatment, only LT (0.02% BAK) that contains the highest percentage of BAK had a significant effect on cell survival compared to control with a cell viability of 69% (p = 0.022) (Fig. 1). After 6 h, LT (0.02% BAK), OP (0.01% BAK), and DS (0.0075%) all affected cell survival compared to control with viabilities of 31, 34, and 52%, respectively (p = 0.0007, p = 0.0017, and p = 0.011) (Fig. 1). BT (0.005% BAK), the lowest percentage of BAK, did not significantly affect cell survival after 30 min or 6 h. There was no significant difference in cell survival between eye drops after 30 min, though BT tended to cause less damage compared to LT, OP, and DS with 22–24% greater cell survival. After 6 h, BT caused 43% greater cell survival compared to LT (p = 0.036).
**pH Measurement of BAK-Containing Eye Drops**

The pH value was measured in triplicate on 3 different containers for each eye drop. DS had the most acidic pH of 5.58 and OP the least acidic of 6.84. All eye drops, except LT and OP, differed significantly from each other (p ≤ 0.001) (Fig. 2). All the eye drops differed significantly from the tear film pH of 7.6 (p < 0.001) [16].

**Osmolality Measurement of BAK-Containing Eye Drops**

The osmolality was measured in triplicate on 3 different containers for each eye drop. None of the eye drops differed significantly from one another (shown in Fig. 3). LT had the lowest osmolality of 267.6 mOsm/kg, and BT had the highest osmolality of 293.2 mOsm/kg.
Effect of Anti-Glaucomatous Drugs on Goblet Cells

Discussion/Conclusion

In the present study, we found significant impact on human goblet cell survival in a time-dependent manner after treatment with different BAK-preserved IOP-lowering eye drops. Exposure to LT, OP, and DS eye drops demonstrated cytotoxicity, which increased with exposure duration. BT did not affect cell viability. The concentration of BAK may explain the differences in cytotoxicity. All the eye drops contained BAK. The highest BAK concentration of 0.02% was found in LT, which was also the most toxic, while BT with 0.005% BAK, the lowest concentration, was not cytotoxic. DS eye drops contained 0.0075% BAK, and OP eye drops contained 0.01% BAK. The eye drops, however, had different active components (AA, CAI, PG, and BB), and it is possible that the difference in goblet cell survival was due to the active component and/or the BAK concentration. This cannot be ruled out based on the current study, and further investigation is encouraged. It has, however, previously been demonstrated on cultured corneal and conjunctival epithelial cells that BAK-preserved PG eye drops have similar cytotoxicity as observed in control drops with the corresponding BAK concentrations [14]. These results indicate that the toxicity is due to the preservative and not the active drug. Preservative-free PG even appears to stimulate goblet cell growth [17], and treatment with PG is associated with less treatment failure compared to CAI, miotics, AA, and BB [18].

No previous comparison study has been made on cultured human goblet cells, but comparisons of different preservatives have been performed on human corneal and conjunctival epithelial cells. SofZia and PQ1-preserved PG eye drops have similar cytotoxicity as observed in control drops with the corresponding BAK concentrations [14]. These results indicate that the toxicity is due to the preservative and not the active drug. Preservative-free PG even appears to stimulate goblet cell growth [17], and treatment with PG is associated with less treatment failure compared to CAI, miotics, AA, and BB [18].

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correspond the tear film. The buffers in the eye drops may act differently in the tear film than in the culture medium. The pH and osmolality of the eye drops, when diluted in tear film, may, therefore, be different from what we measured in the drops themselves. Our results cannot be directly transferred to the clinic yet do provide some insight as to which eye drops are cytotoxic.

We conclude that BAK-preserved IOP-lowering eye drops are cytotoxic in a dose-dependent manner making 0.02% BAK-preserved Latanoprost most toxic and 0.005% BAK-preserved Brimonidine Tartrate least toxic. Studies on human goblet cell survival when treating with preserved and preservative-free eye drops of the same treatment group would be of interest.

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Statement of Ethics

The protocol complied with the Declaration of Helsinki and was 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).

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

M.K., S.H., G.P., and B.C. contributed to the outline of the study. A.H., X.B., Z.M., and O.M. performed laboratory analyses. R.V. contributed to the setup of the analyses. G.P. provided tissue for cultivation. J.B. instructed in goblet cell culture. A.H. and X.B. performed statistical analyses and wrote the article manuscript. D.A.D. and M.K. helped in manuscript writing. The manuscript has been approved by all authors.

Data Availability Statement

Data available on request from the authors.
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