

Effects of Ophthalmic Medications on Conjunctival Goblet Cell Density in New Zealand White Rabbits

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Keywords

Benzalkonium chloride · Conjunctival goblet cell density · New Zealand white rabbits · Ocular surface

Abstract

Introduction: The conjunctiva contains numerous specialized cells called conjunctival goblet cells (CGCs). The topical application of specific eye drops to the ocular surface has conclusively been linked to cause a reduction in CGCs, a condition which has been associated with dry eye and other ocular surface disorders. The purpose of this study was to assess if the use of benzalkonium chloride (BAK) as a preservative in common ophthalmic medications affects CGCs' density in New Zealand white (NZW) rabbit conjunctivas. **Methods:** Data from seven preclinical studies conducted between March 2016 and April 2021 were analyzed, involving 146 male NZW rabbits aged 2 to 3 months. Prior to study participation, rabbits underwent a 7-day quarantine period in individual housing, during which their general health was monitored. Rabbits had ad libitum access to water and food, with intake data recorded. Comprehensive ophthalmological examinations were performed on all eyes prior to and throughout the studies. The density of corneal endothelial cells was specifically assessed using AB/PAS staining and

quantified with a high-power (40X) field objective (ocular 18 × 22), expressed as a percentage relative to epithelial cells. **Results:** No statistically significant differences were found between the with-BAK group and without-BAK group. The mean density in the 30-day group presented a statistically significant higher density than the >30-day group ($p = 0.005$). Analysis of the treatment revealed that antibiotic/steroid combination group had a higher average number of CGCs compared to both the antihistaminic group ($p = 0.004$) and hypotensive agent group ($p = 0.047$). **Conclusion:** Exposure to BAK-preserved medications for 30 days results in a higher density of CGCs compared to prolonged exposure to BAK-preserved medications exceeding 30 days. The pharmacological effects and associated cellular damage induced by BAK vary depending on the specific medication with which it is combined.

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Introduction

Conjunctival goblet cells (CGCs) are defined as specialized epithelial cells, primarily found in the apical surface of the conjunctiva and which are arranged within narrow layers of stratified epithelium [1, 2]. CGCs'

primary function is to secrete mucin, which allows them to retain water, hence facilitating the maintenance of a hydrated mucus gel that covers the ocular surface. A detriment in CGCs' density usually leads to a decreased mucin secretion and therefore a susceptible tear film [3, 4]. These alterations can cause symptoms such as foreign body sensation, itching, burning, dryness, decreased visual acuity, and can even lead to ocular surface disease [5, 6]. CGCs' loss has been associated with dry eye disease and other inflammatory ocular surface conditions such as Sjögren's syndrome, allergic conjunctivitis, and Steven-Johnson syndrome [4, 7].

The topical installation of specific eye drops to the ocular surface can cause a reduction of CGCs. Several studies have demonstrated that preservatives like benzalkonium chloride (BAK) and other excipients play a significant role in ocular inflammation by inducing a cytotoxic response that leads to tear film instability [6, 8]. In experimental laboratory rabbits, the use of eye drops containing BAK twice a day for 14 days reduced the number of CGCs [9].

BAK is commonly employed in glaucoma medication, and its long-term administration has been demonstrated to induce structural modifications in the ocular surface in a dose-dependent manner and simultaneously decrease the density of CGCs compared to non-preservative medications [3, 5, 10]. Hence, preservative-free ophthalmic medications have been developed for long-term treatments; however, it has been observed that there is no significant improvement in CGCs' density with the use of preservative-free topical agents [9–12]. Based on this evidence, the purpose of this study was to assess if the use of BAK as a preservative in common ophthalmic medications influences CGCs' density in New Zealand white (NZW) rabbit conjunctivas.

Methods

The data utilized were derived from seven preclinical studies conducted between the months of March 2016 and April 2021, conducted at Laboratorios Sophia S.A. de C.V. (Zapopan, Jalisco, Mexico). All of them were performed to determine the toxicity of several ophthalmic drugs belonging to different treatment groups. For more information, see Supplementary Material (for all online suppl. material, see <https://doi.org/10.1159/000544102>), which provides comprehensive details regarding the preclinical studies, encompassing protocol number approval, active ingredients, number of subjects, CGCs' density % (mean $\pm$ standard deviation

[SD]), study duration, and drug of therapeutic use. For more detailed information, see Supplementary Material (online suppl. Table S1).

All animal studies were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee of Laboratorios Sophia S.A. de C.V. (CICUALS, Approval No.: BEPOVSBEPSTSEP16V2, BEPOVSBEPSTJUN18, PROPVSPROPSTJUL17V2, PAPRVSGAPRSTJUL17V2, TRAVVSTRAVSTFEB18V2, NEDIVSNEDESTJUL18, and PREC185-0221/ST). A total of 146 male (NZW) rabbits aged 2 to 3 months and weighing between 2.0 and 3.0 kg obtained from Productora CORADE (Zapopan, Jalisco, Mexico) were analyzed in this study. Prior to their participation in these protocols, all subjects were subjected to a mandatory quarantine period of no less than 7 days, in individual housing, during which a comprehensive health assessment was conducted. During their stay, NZW rabbits were provided ad libitum access to water and food, and both intakes were registered (LabDiet[®] 5321, Richmond, IN, USA). This feed adheres strictly to nutritional standards established by the Mexican Official Norm NOM-062-ZOO-1999, technical specifications for the production, care, and use of laboratory animals. Before eligibility criteria, topical anesthesia (tetracaine hydrochloride 0.5%; Ponti[®] Ofteno, Laboratorios Sophia S.A. de C.V., Zapopan, Jalisco, Mexico) was administered to prevent any potential discomfort.

An ophthalmic eligibility criteria screening with slit lamp examination (Luxvision[®], Class I Type B, Doral FL, USA, and S4OPTIK, Model SL-H3-LED, Badia A Settimo, Italy) and fluorescein staining (BioGlo, HUB Pharmaceuticals LLC) were performed to ensure no exclusion criteria were met, such as secretion, conjunctival hyperemia, corneal or conjunctival lacerations, scarring, neovascularization, corneal degeneration, cataract, or any pathological findings in the indirect funduscopy performed with a 78 diopters lens (Ocular Instruments, Bellevue, WA, USA). Elimination criteria included any serious adverse event that required the administration of complementary ophthalmic or systemic treatment, including any situation that entailed a compromise to animals' well-being.

The NZW rabbits were housed under controlled 12-h light-dark cycles with free access to food and water. Examinations were performed on all eyes before the studies began and during evaluation days as determined by each individual protocol. Ophthalmological medications were used according to the established duration in each of the studies, such as vasoconstrictors (30 days),

hypotensive agents (30 and 93 days), antihistaminic (60 and 80 days), antibiotic/steroid combination (30 days), and preservative-free lubricants (93 days).

The dosing regimen for each treatment group per protocol was as follows: four times per day for the vasoconstrictor group, once a day for the hypotensive agents' group, two times per day for the antihistaminic group, and four times per day for the antibiotic/steroid combination group. During examinations, an ophthalmologist evaluated the anterior and posterior segment as well as ocular tonometry.

Once the protocols were finalized, the laboratory animals were euthanized with carbon dioxide released into a closed chamber at a minimum concentration of 70%. Subsequently, the eyes of interest were removed and sent to histological analysis.

The enucleation of a total of 258 eyes from 146 NZW rabbits was performed. The enucleated eyes were fixed in a 4% paraformaldehyde solution between 24 and 48 h. Paraffin sections of 4.0 μm were prepared, stained with hematoxylin, eosin, and alcian blue, followed by the periodic acid-Shiff reagent for identification of goblet cells and for general morphology observation with a light microscope [13].

The CGCs' density was evaluated specifically using alcian blue/periodic acid-Shiff staining. Goblet cells were counted using a high-power field (40X) objective (ocular 18 $\times$ 22), in percentage, in relation to epithelial cells. A minimum of one hundred cells were counted in the same anatomical portion of the conjunctiva at the corneal margin, and whichever presented the higher value was reported. This technique has been used previously with conclusive results. As a matter of importance, samples were excluded if they could not be evaluated due to tangential sectioning conjunctival epithelium and if inflammation were severe [14].

Statistical analysis was performed with a specialized statistical language, R statistical software (The R Foundation for Statistical Computing; <http://www.R-project.org>). All numerical data reported are expressed as means $\pm$ SD. The Kolmogorov-Smirnov test was applied to the values obtained from the goblet cells to determine their distribution ($p > 0.05$). To compare the effects of BAK on goblet cells, two groups were formed: BAK-exposed (with BAK) and non-BAK-exposed (without BAK) not-treated eyes and lubricants.

To assess the effects of BAK based on exposure duration, subjects were stratified into two groups: 30 days (30 d) and more than 30 days (>30 d). The analysis between groups to compare means was performed using a Student's *t* test for independent groups. To compare the mean of CGCs by treatment type, lubricants were excluded, and treatments preserved with BAK were analyzed: antihistaminic, hypotensive agents, vasoconstrictors, and antibiotic/steroid

combination. A one-factor ANOVA was performed, followed by a TukeyHSD post hoc test. The 95% confidence interval (CI) of these differences was computed. For all analyses, a value of $p < 0.05$ was considered statistically significant. It is important to note that eyes reported as unavailable and zero values of goblet cells were excluded from consideration in the statistical analysis.

Results

A total of 258 eyes (from 146 NZW rabbits) were studied. A light microscopy evaluation was performed in all included eyes. No apparent signs of toxicity or structural damage were observed in any eyes as shown in Figure 1.

In the with-BAK group ($n = 114$), the mean percentage of goblet cells $\pm$ SD was $22.07 \pm 4.83\%$ (ranged 12 to 38%), while in the without-BAK group ($n = 144$) it was $22.70 \pm 5.20\%$ (ranged 9 to 40%), with no difference between groups ($p = 0.322$, 95% CI $[-0.62$ to $1.86]$), shown in Figure 2. For average density of CGCs according to BAK exposure duration, the 30-day group ($n = 59$) had an average density of $23.28 \pm 4.46\%$ CGCs (ranged 14 to 36%), which was statistically higher than the $20.78 \pm 4.90\%$ (ranged 12 to 38%) in the >30-day group ($n = 55$) ($p = 0.005$, 95% CI $[-4.25$ to $-0.75]$), shown in Figure 3.

ANOVA and Tukey's post hoc test were performed to compare the average density among treatments. In the group treated with the antibiotic/steroid combination ($n = 31$), the mean was $24.58 \pm 4.62\%$ (ranged 18 to 36%), which was higher than the antihistaminic group ($n = 45$) mean of $20.86 \pm 5.31\%$ (12 to 38%) ($p = 0.004$, 95% CI $[-6.53$ to $-0.89]$) and hypotensive agents' group ($n = 22$) mean of $21.18 \pm 4.26\%$ (ranged 14 to 30%) ($p = 0.047$, 95% CI $[-6.76$ to $-0.03]$). There were no statistically significant differences among the other groups (vasoconstrictor vs. hypotensive agents; antihistaminic vs. vasoconstrictors; hypotensive agents vs. antihistaminic), shown in Figure 4.

Discussion

Exposure to BAK

Our study assessed the effects of a long-term exposure of ophthalmic medications preserved with BAK on the ocular surface of NZW rabbits. The use of ophthalmic medications with BAK as a preservative for more than 30 days significantly decreased goblet cell density, compared to treatments of 30 days or less, whether with or without preservatives. Upon analyzing treatment groups in days (30 days vs. >30

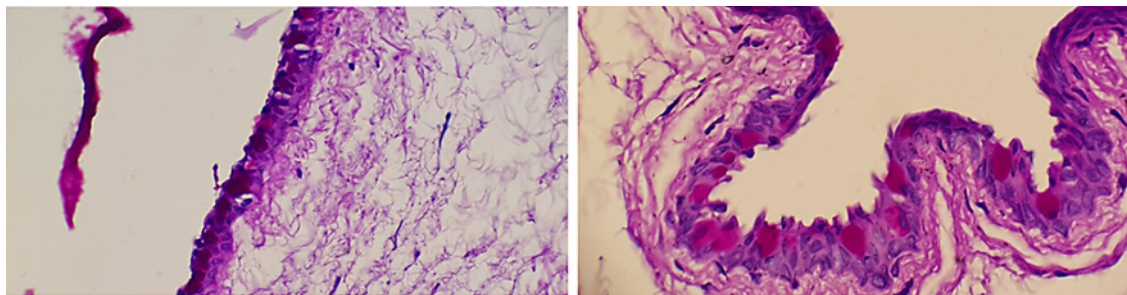


Fig. 1. Representative images of CGC histopathology, each H&E and with AB, followed by the PAS in NZW rabbits' eyes treated with ophthalmic medications containing BAK as a preservative. Magnification x40. AB, alcian blue; PAS, periodic acid-Schiff; H&E, hematoxylin and eosin.

Fig. 2. Comparison of the effect of ophthalmic medications with BAK versus without BAK on CGCs' density. No significant differences were observed between treatments with and without BAK. The black dot on the graph represents the mean.

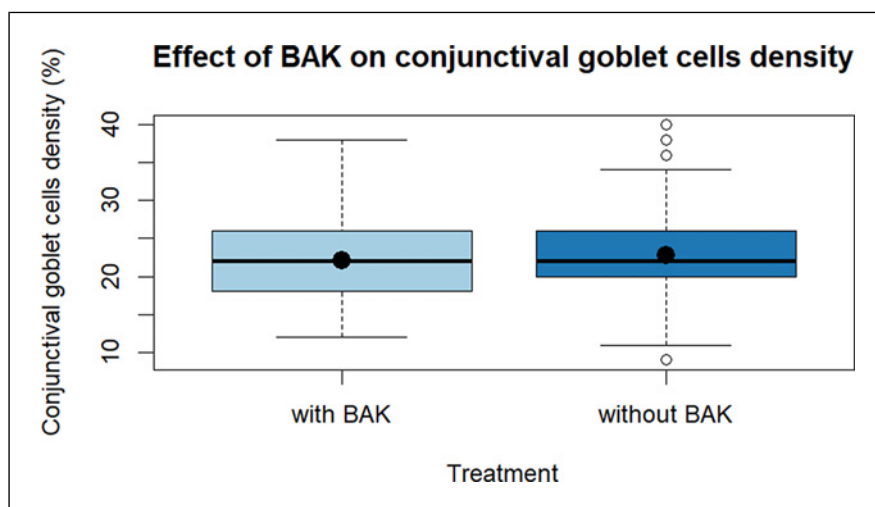
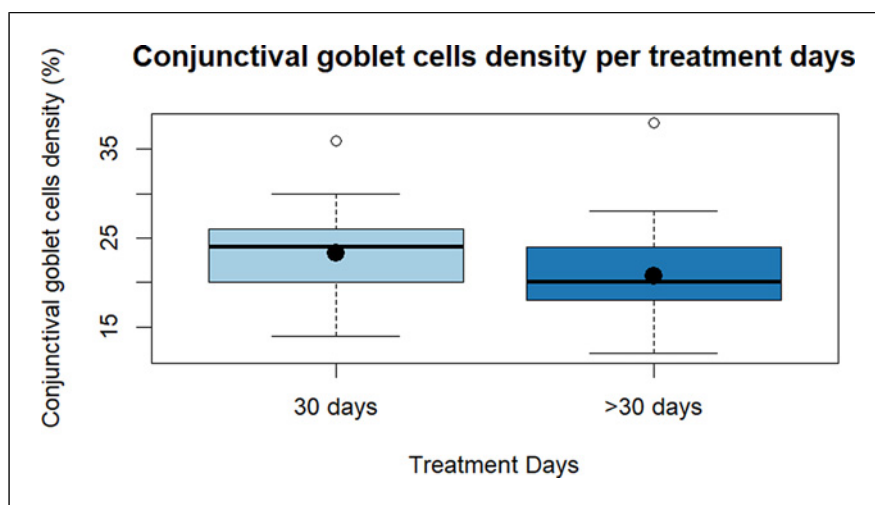


Fig. 3. Effect of exposure time to the combination of BAK with other active components on the CGCs' density. The group exposed for 30 days has a higher cell density than the group exposed for more than 30 days. The black dot on the graph represents the mean.



days), there were no significant differences between the two groups. Our findings align with those of Faria et al. [15], who reported that the prolonged eye drop application (exposure

of 30 days) was associated with CGCs' density loss, regardless of whether they have preservatives or not. While their study was a short-term simulation of chronic exposure to BAK, it

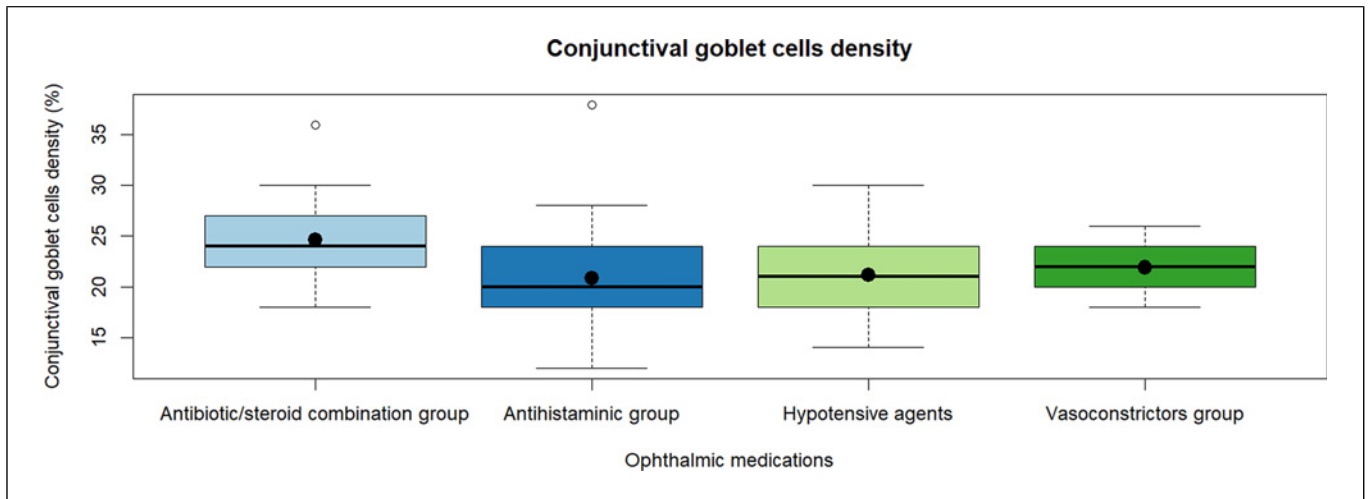


Fig. 4. Effect of different ophthalmic medications containing BAK on CGCs' density. The steroid-antibiotic combination group presented a higher average of CGCs than the one compared to the rest of the drugs. The black dot on the graph represents the mean.

demonstrated statistically significant BAK dose-dependent effects such as tear film destabilization, irritation, and dryness occur. In an NZW rabbit model of dry eye disease, it was observed that the eye exposed to 0.1% BAK compared to the control eye exhibited a decrease in Schirmer's score, an increase in fluorescein scores, as well as a decrease in CGCs' density at 7 (≈ 40 cells per field) and 14 days (≈ 15 cells per field) relative to baseline (≈ 80 cells per field) and control (≈ 85 cells per field) [16]. However, the concentration of BAK as a preservative in ophthalmic medications is set to be within the range of 0.003 to 0.02%. Kim et al. [17] evaluated the effects of BAK on the ocular surface of rabbits that were exposed to BAK for 14 days at two concentrations (0.01% and 0.1%) or to PBS (control group). Rabbits exposed to BAK exhibited a decrease in CGCs' density, along with more prominent histopathologic changes. These changes were more noticeable in the 0.1% group than in the 0.01% group. Exposure to BAK has also been assessed longitudinally. Yu et al. [18] investigated the ocular surface toxicity by prolonged use of BAK-preserved brimonidine in New Zealand rabbits exposed for 61 days to brimonidine, compared to control (9.43 ± 0.57 mm), showing significant decrease in CGCs' density (6.61 ± 0.38 mm). Comparable outcomes were noted for white rabbits exposed to latanoprost with BAK, which compared to control had a decrease in goblet cell density at days 31 (≈ 75 cells per field) and 61 (≈ 50 cells per field) of treatment ($p < 0.005$), demonstrating the toxicity of latanoprost with BAK (0.02%) in a time-dependent manner, in addition to increased inflammatory cell infiltration and increased apoptosis rate [19].

Effect of Drugs Containing BAK

Our study analyzed the instillation of BAK in conjunction with diverse types of ophthalmic medications, and it was determined that the antibiotic/steroid combination group had a higher CGCs' density as compared to the antihistaminic and hypotensive agents' groups. Hedengran et al. [20] evaluated the changes of four different hypotensive drugs in combination with different concentrations of BAK (brimonidine with 0.005% BAK, dorzolamide at 0.0075% BAK, timolol with 0.01% BAK, and latanoprost with 0.02% BAK) in primary human goblet cell cultures. They noted a reduction in CGCs' survival at 30 min only in those cultures exposed to the highest concentrations of BAK. Meanwhile, after 6 h of exposure the 0.02%, 0.01%, and 0.0075% concentrations showed decreased cell survival of 31, 35, and 52%, respectively. The lowest concentration of BAK (0.005%) did not significantly affect cell survival [20]. It has been proved that the association between BAK and prostaglandins (hypotensors) in mice does not carry the same effects as BAK alone. BAK decreased goblet cell density in the conjunctiva when administered alone, but when administered in combination with travoprost it showed a significant increase [11]. The effect of the combination of BAK and prostaglandin analogues has also been evaluated in rabbits that were exposed to BAK in combination with prostaglandin analogues (travoprost with 0.015% BAK and tafluprost with 0.001% BAK). It was observed that rabbits treated with the combination of travoprost with 0.015% BAK showed a decrease in CGCs' density (8.2 ± 3.8 cells per field). The same changes (although less severe) were

observed in rabbits treated with tafluprost with 0.001% BAK (17.6 ± 5.8 cells per field) [21]. However, it has been observed that tafluprost combined with BAK causes more damage to conjunctival epithelial cells compared to BAK administration alone. Travoprost with 0.015% BAK exhibits lower toxicity than BAK alone at an equivalent concentration [22, 23]. Sezgin Akcay et al. [24] observed that subjects exposed to travoprost-BAK and subjects exposed to travoprost-PQ (polyquaternium-1 0.001% [Polyquad] as preservative) had an increase in impression cytology grading according to Nelson classification at 1- and 6-month follow-up from their baseline. Among both groups, BAK administration presented the highest grades (values equal or greater than 2) on the Nelson scale, in which goblet cells have decreased markedly [24]. The variances identified in our study, as well as potentially in other studies, may be explained by variations in the concentrations of BAK and the associated drug to which it binds, since it has been suggested that BAK toxicity depends on the prostaglandin analogue or compound to which it is bound [21]. It has been observed that when BAK is combined with an immunomodulatory drug, such as cyclosporine, to mitigate the negative effects of BAK and prevent the loss of CGCs, it does not have a positive outcome [15]. While antibiotics, such as netilmicin, have been shown to be more toxic than BAK [25], however, these data are not conclusive since it has been observed that netilmicin, used at the concentration of 3 mg/mL and even at higher concentrations, has a moderate or even no toxic effect on the cellular integrity or epithelial cells [26, 27]. Therefore, our work would help clarify the effect of antibiotics on the corneal surface during longer exposure and with BAK-preserved antibiotics.

Another significant factor contributing to the observed statistical differences between the groups of drugs is the dosage scheme used. For the antibiotic/steroid combination, it was set to be four times a day, while dosage of the hypotensive agents was once a day and for the antihistaminic group it was twice a day. The duration of treatment was 30 days for the antibiotic/steroid combination group, while the duration of treatment was more than 30 days for the other groups. Since the groups had an identical exposure time duration, the absence of significant differences suggests that the duration of treatment may play a more pivotal role than the type of treatment in reducing CGCs' density.

Limitations

A significant limitation of this study was the inability to independently assess and compare the effect of BAK on goblet cell density alone. This would have allowed for

a more comprehensive understanding of how BAK interacts with other drugs and its potential role in altering goblet cell density in combination with other drugs. Another limitation was not being able to evaluate by the concentration of BAK contained in each drug; since the concentration in variable BAK contained in drugs has been evaluated, most of these studies have been performed in *in vitro* models and the conditions under which the exposures are performed vary, from diluting the drops in a medium to apply to the culture, to the concentration being stable, to having a different pH, to having a different osmolarity [20]. Further animal studies comparing the effects of diverse types of BAK-preserved drugs at different concentrations are needed to help clarify what determines the extent of damage to the ocular surface caused using these drugs.

Although these results cannot be directly extrapolated to humans, they do provide information and confirm that BAK can cause damage depending on the compound to which it is binding. Conducting clinical studies with a longer follow-up could help better identify the effects of BAK-preserved drugs at different concentrations. It would also help evaluate interactions with drugs used for other conditions, since, in the real clinic, some ocular conditions are derived from other diseases.

Conclusion

Our findings show a statistically significant increase in the average CGCs' density on NZW rabbits after exposed to BAK-preserved ophthalmic medications preserved with BAK for a period of 30 days was compared to those exposed for longer duration over 30 days. Furthermore, the antibiotic/steroid combination has a higher density of CGCs, suggesting that the effects of BAK-preserved drugs are dependent on the particular drug formulation used.

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Statement of Ethics

All animal studies were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision

Research and approved by the Institutional Animal Care and Use Committee of Laboratorios Sophia SA de C.V. (CICUALS, Approval No.: BEPOVSBEPOSTSEP16V2, BEPOVSBEPOSTJUN18, PROPVSPROPSTJUL17V2, PAPRVSGAPRSTJUL17V2, TRAV-VSTRAVSTFEB18V2, NEDIVSNEDESTJUL18, and PREC185-0221/ST).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

S.C.G.M: methodology, investigation, writing original draft preparation, review, and editing. R.O.J.F: methodology, investigation, and writing original draft preparation. J.M.T.A: formal analysis, writing original draft preparation, and review and editing. E.Y.C.G and J.M.M.E: investigation, writing, review, and editing. A.S.R and O.O.M: conceptualization, review, and editing.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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