ORIGINAL ARTICLE

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Effects of benzalkonium chloride and cyclosporine applied topically to rabbit conjunctiva: a histomorphometric study

Efeitos do uso tópico do conservante cloreto de benzalcônio e ciclosporina na conjuntiva de coelhos: estudo histomorfométrico

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ABSTRACT | Purpose: Chronic instillation of benzalkonium chloride, a preservative, has inflammatory effects on the ocular surface. However, addition of the anti-inflammatory agent cyclosporine to a therapeutic protocol may mitigate these effects. This study compared the toxic effects of a 0.1% benzalkonium chloride solution and the possible protective effect of 0.05% cyclosporine when applied topically to the rabbit conjunctiva. **Methods:** Fifteen age- and weight-matched, female New Zealand white rabbits were categorized into three groups and treated for 30 consecutive days. Group 1, 2, and 3 - benzalkonium chloride received 0.1% every 24 h, 0.05% cyclosporine every 6 h, and both treatments, respectively. In each rabbit, the left eye was subjected to treatment and the right eye was a control. The rabbits were euthanized at after the experiment. Goblet cells and blood vessels were then enumerated in conjunctival tissues stained with periodic acid-Schiff and hematoxylin-eosin, respectively. Differences between treated and untreated eyes and between groups were compared using the *t*-test and analysis of variance. **Results:** Benzalkonium chloride treatment, with and without cyclosporine, significantly reduced ($p \leq 0.05$) in the number of goblet cells in treatment eyes compared with that in respective control eyes. Alternatively, adding cyclosporine to benzalkonium chloride did not prevent the loss of conjunctival goblet cells, and a significant reduction in the number of goblet cells was noted. Benzalkonium chloride-induced significant increase in the number of new blood vessels was mitigated significantly by the addition of cyclosporine. **Conclusion:** This study demonstrated the magnitude of conjunctival injury caused by chronic instillation of benzalkonium chloride. Although cyclosporine did not mitigate the effects on goblet cells, its addition minimized inflammatory angiogenesis induced by benzalkonium chloride.

Keywords: Benzalkonium chloride; Cyclosporine; Goblet cells; Blood vessels; Conjunctiva

RESUMO | Objetivo: A instilação crônica de cloreto de benzalcônio, um conservante, tem efeitos inflamatórios na superfície ocular. No entanto, a adição do agente anti-inflamatório ciclosporina a um protocolo terapêutico pode atenuar esses efeitos. Este estudo comparou os efeitos tóxicos de uma solução de cloreto de benzalcônio a 0,1% e o possível efeito protetor de ciclosporina a 0,05% quando aplicado topicamente à conjuntiva de coelho. **Métodos:** Quinze coelhos fêmeas brancos da raça Nova Zelândia, pareados por idade e peso, foram categorizados em três grupos e tratados por 30 dias consecutivos. Os grupos 1, 2 e 3 - receberam cloreto de benzalcônio 0,1% a cada 24h, ciclosporina a 0,005% a cada 6h e ambos os tratamentos, respectivamente. Em cada coelho, o olho esquerdo foi submetido a tratamento e o olho direito foi controle. Os coelhos foram submetidos à eutanásia após o experimento. Células caliciformes e vasos sanguíneos foram então enumerados em tecidos conjuntivais corados com ácido periódico-Schiff e hematoxilina-eosina, respectivamente. As diferenças entre os olhos tratados e não tratados e entre os grupos foram comparadas usando o teste t e análise de variância. **Resultados:** O tratamento com cloreto de benzalcônio, com e sem ciclosporina, reduziu significativamente ($p \leq 0,05$) o número de células caliciformes nos olhos tratados em comparação com os olhos controle correspondentes. Alternativamente, a adição de ciclosporina ao cloreto de benzalcônio não impediu a perda

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de células caliciformes conjuntivais, e foi observada uma redução significativa no número de células caliciformes. O aumento significativo induzido pelo cloreto de benzalcônio no número de novos vasos sanguíneos foi significativamente mitigado pela adição da ciclosporina. **Conclusão:** Este estudo demonstrou a magnitude da lesão conjuntival resultante da instilação crônica de cloreto de benzalcônio. Embora a ciclosporina não tenha atenuado os efeitos nas células caliciformes, sua adição minimizou a angiogênese inflamatória induzida pelo cloreto de benzalcônio.

Descritores: Cloreto de benzalcônio; Ciclosporina; Células caliciformes; Vasos sanguíneos; Conjuntiva

INTRODUCTION

Glaucoma is a progressive, multifactorial form of optic neuropathy characterized by structural changes to the optic disc, often followed by visual field changes that require long-term treatment with topical ocular hypotensive substances[1]. Several classes of drugs including cholinergic agents, β-blockers, α-adrenergic agonists, carbonic anhydrase inhibitors, and more recently, prostaglandin analogs are available for treating glaucoma[2]. Controlling intraocular pressure (IOP) is a main objective of glaucoma treatment and is most frequently attempted using instilling topical eye drops over the ocular surface.

The majority of eye drops have preservatives that serve a twofold purpose: maintaining sterility by inhibiting the growth of opportunistic bacteria and yeast and improving drug penetration, enhancing its effects. However, certain preservatives are toxic to the conjunctiva, especially when continuously used (e.g., in glaucoma treatment). Benzalkonium chloride (BAK), a quaternary ammonium used as a detergent, antiseptic, disinfectant, fungicide, and bactericide, is the most commonly used preservative in eye drops and improves the corneal penetration capability of some drugs. However, BAK has pro-apoptotic and -inflammatory actions, and its chronic application to the ocular surface can significantly impair the tear film, resulting in ocular surface epithelial dysfunction[3,4]. The resulting aesthetic effects, such as conjunctival hyperemia and ocular discharge, can cause discomfort and even public embarrassment. Therefore, attempts to reduce these effects will additionally help patient adherence to treatment and improve patient general well-being and self-esteem.

Cyclosporine is an immunomodulatory drug with antimetabolite properties and has been shown to control cell proliferation both *in vitro* and *in vivo*[5]. Ophthalmology studies of cyclosporine have proven its efficacy in the treatment of autoimmune keratoconjunctivitis sicca, which was first discovered in 1989 by a veterinary ophthalmologist named Renee Kaswan in 1989. The existing literature suggests that the addition of cyclosporine to therapeutic eye drops containing BAK may reduce the toxic effects of BAK and reduce inflammatory activity in the ocular tissues, including the conjunctiva. Ophthalmologic studies assessing these changes in the conjunctiva are extremely important when evaluating the effectiveness and safety of the various treatment protocols for glaucoma, as most involve the use of multiple preservative-containing drops.

Therefore, this investigation aimed to compare different treatment protocols mimicking chronic (i.e., 30 days) topical exposure to BAK at a concentration of 0.1% (approximately 13 times greater than the average concentration in eye drops) in the presence or absence of 0.05% cyclosporine (Restasis®; Allergan, Irvine, CA, USA). We assessed the inflammatory effects of these protocols in the conjunctiva of rabbits by evaluating the histological changes through a histomorphometric study.

METHODS

Fifteen healthy New Zealand female white rabbits (age, ~5 months; body weight, ~3 kg) were randomly divided into three groups of five animals each. During a 30-day period, these groups were treated with the following therapeutic protocols to mimic chronic use: Group 1, BAK 0.1% every 24 h; Group 2: cyclosporine 0.05% every 6 h; and Group 3: BAK 0.1% every 24 h and 0.05% cyclosporine every 6 h. For all groups, one drop of each protocol eye drop was instilled into the left eye (OS) of each animal at each indicated time point and the right eye (OD) served as a control.

Thirty days after the experiment, the animals were euthanized. The eye globes and adnexa were removed and immediately fixed in 10% buffered formaldehyde solution for 24 h. Subsequently, the tissues were cut longitudinally and submitted to routine paraffin embedding and staining with hematoxylin-eosin (HE)[6] or periodic acid-Schiff (PAS).

Sixty slides were prepared for the histomorphometric study. Four slides were prepared per rabbit: two (left eye and right eye) each were stained with HE and PAS. Blood vessels were counted in the 30 slides (15 left eyes and 15 right eyes) stained with HE, while goblet cells were counted in the 30 corresponding slides stained with PAS. Histological samples of the eye globes were then analyzed

using light microscopy to evaluate and compare possible conjunctival tissue changes between the treated and control eyes after the experimental treatment. Goblet cells and blood vessels were counted in the upper and lower palpebral conjunctiva. Twelve high-power fields (HPFs) were analyzed in the conjunctival tissue of each eyelid (upper and lower). Six immediately adjacent HPFs starting from the bulbar conjunctiva (immediately after the limbus) and six HPFs starting at the conjunctival fornix were analyzed toward the palpebral conjunctiva.

The experimental phase of the study was conducted in the vivarium of the Comparative Ophthalmology Lab (LABOCO) of the Veterinary Hospital, Federal University of Paraná (UFPR), following approval by the Animal Use Ethics Committee of the Agrarian Sciences Sector. The histomorphometric analysis was also conducted at LABOCO.

Statistical analysis

After compiling the results in a spreadsheet, differences between the groups were compared using an analysis of variance (ANOVA) to investigate numerical differences between continuous variables with normal distribution. Comparisons within the same animal (right eye and left eye) were performed using paired t-tests, while comparisons among all groups were performed using an ANOVA and the Tukey-Kramer post *hoc* test. *P*-values of ≤0.05 were considered significant.

RESULTS

After a 30-day treatment period, clinical signs of inflammation were detected in the left eyes of animals treated with BAK and BAK+cyclosporine. Varied amounts of ocular discharge, conjunctival hyperemia, chemosis, and corneal neovascularization were observed.

In the PAS-stained slides, the left eyes of animals treated with BAK had a significantly lower mean number of goblet cells per field, whereas eyes treated with cyclosporine contained a significantly higher mean number of these cells (Figure 1). However, there was no significant difference in the number of goblet cells between eyes treated with BAK only and those treated with BAK+cyclosporine (Figure 2). The main results of the statistical group comparisons are shown in Figure 2.

Angiogenic effects

The eye globes treated with BAK alone (Group 1) contained a larger number of blood vessels per field. By contrast, globes treated with cyclosporine alone (Group 2) or BAK+cyclosporine (Group 3) had a significantly lower number of blood vessels per field relative to Group 1 (Figure 3). Notably, Group 3 also contained a significantly larger number of blood vessels, compared with Group 2. These results confirmed the clinical observations in each group before euthanasia. Among the treated eyes, conjunctival hyperemia was more evident in Group 1 relative to Groups 2 and 3 and in Group 3 relative to Group 2 (Figure 4).

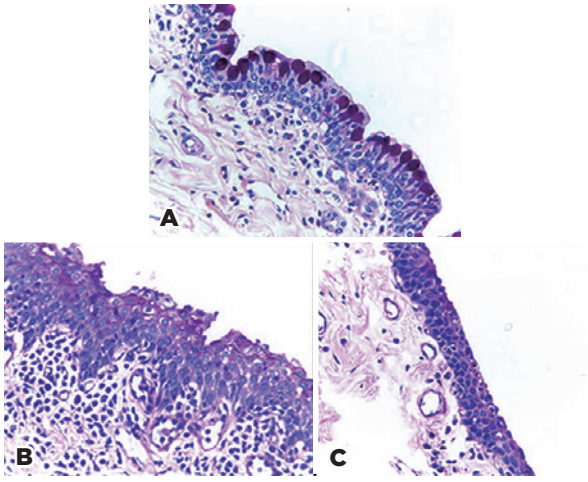

Figure 1. Photomicrographs (40× magnification) of representative conjunctival tissues from rabbits subjected to 30 days of treatment. Tissues were stained with periodic acid-Schiff to highlight goblet cells (red arrows). A, highlighted goblet cells from an eye in Group 2 (cyclosporine 0.05%); B, highlighted goblet cells from an eye in Group 1 (benzalkonium chloride [BAK] 0.1%); and C, highlighted goblet cells from an eye in Group 3 (cyclosporine 0.1%+BAK 0.05%). Note the paucity of goblet cells after treatment with BAK in B and C. All conjunctival tissue samples were collected from the same ocular location (one high-power field from the fornix toward the palpebral conjunctiva).

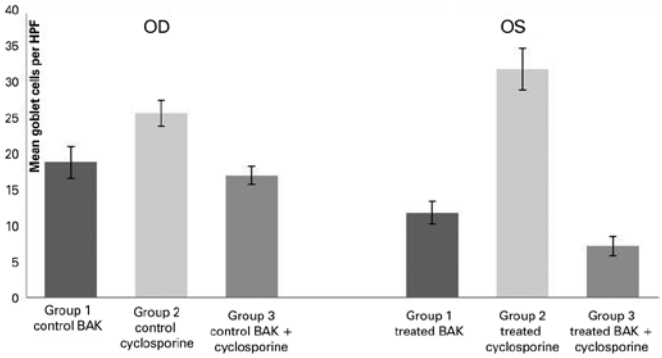

Figure 2. Mean number of goblet cells per high-power field (HPF) in different groups [benzalkonium chloride (BAK) OD/OS, cyclosporine OD/OS, and BAK+cyclosporine OD/OS]. Note the similar mean values between Groups 1 (BAK) and 3 (BAK+cyclosporine) for both control and treated eyes, suggesting that cyclosporine had no significant protective effect on goblet cells.

DISCUSSION

The results of this study confirm the existing evidence that a wide variety of medications that are topically administered for treating glaucoma can affect the structure and integrity of the ocular surface[7-9]. These drugs have potential side effects such as conjunctival hyperemia, foreign body sensations, and symptoms of dry eye disease, which are caused not only by the active ingredients but also, and perhaps more importantly, by the addition of preservatives and excipients such as BAK[4,10].

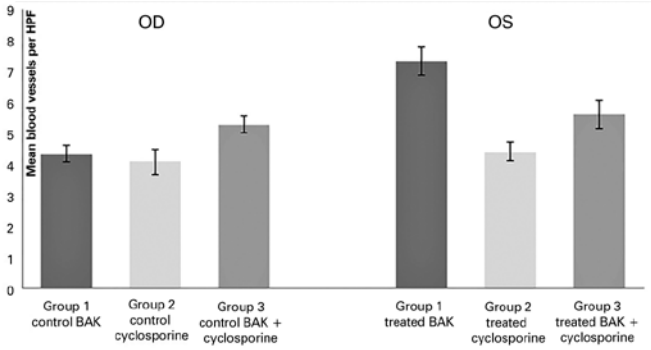

Figure 3. Mean number of blood vessels in different groups [benzalkonium chloride (BAK) OD/OS, cyclosporine OD/OS, and BAK+cyclosporine OD/OS]. Note the significant reductions in the mean number of treated eyes in Groups 2 and 3 compared with that of those in group 1.

Our histomorphometric analysis revealed that the topical administration of BAK effectively simulated chronic exposure. In our study, BAK induced rapid and severe morphological changes in the ocular surfaces of healthy rabbits that have only been observed with chronic exposure to this chemical. To mimic the effects of chronic exposure within 30 days, we selected a concentration of BAK higher than those found in commercially available eye drops (0.005%-0.02%) but lower than those used in that used in previous *in vitro* experiments (0.06%-0.09%)[11-13] and a previous rabbit study (0.2%)[14]. We emphasize that our short-term simulation of chronic exposure to a higher BAK concentration does not necessarily replicate prolonged usage at lower concentrations. However, shorter studies demonstrating the effects of exposure to higher concentrations of this preservative are an alternative to the challenges associated with longer-term *in vivo* studies of real conjunctival changes induced by standard concentrations of BAK.

Cyclosporine 0.05% (Restasis®), the drug of choice in our study, was approved by both the United States Food and Drug Administration and ANVISA for inducing increased tear production in patients with ocular inflammation associated with dry keratitis. Cyclosporine is also known as a potent immunosuppressive agent

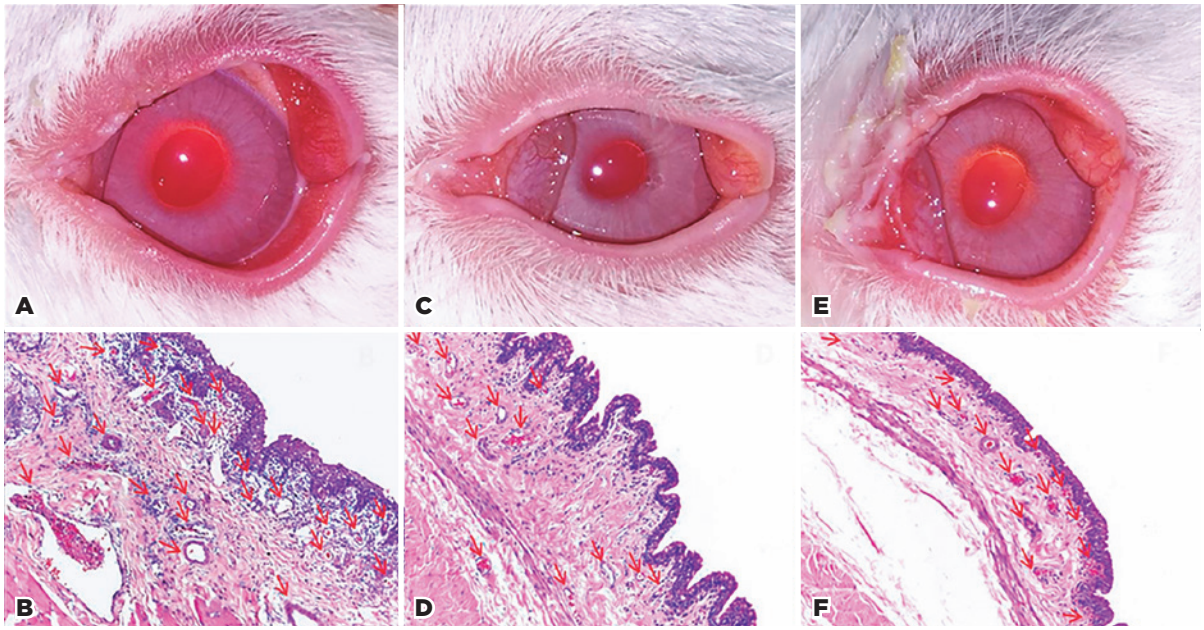

Figure 4. Clinical and histologic comparisons of the left (i.e., treated) eyes in each group after 30 days of treatment. Representative photomicrographs (bottom row) demonstrate the blood vessel counts per field (40× magnification) in hematoxylin-eosin-stained sections. A and B, Group 1 [benzalkonium chloride (BAK)]; C and D, Group 2 (cyclosporine); and E and F, Group 3 (BAK+cyclosporine). All groups (treated and control) were statistically compared with each other. *P*-values ≤0.05 were considered significant.

that inhibits T lymphocyte proliferation and activity by blocking cytokine synthesis and has been shown to attenuate some of the changes associated with dry keratitis. Accordingly, cyclosporine has been used to treat ocular surface diseases, including uveitis, corneal transplant rejection, and keratoconjunctivitis sicca (dry eye)[15,16]. Several clinical studies have demonstrated the efficacy and safety of long-term treatment with Restasis® in patients with moderate to severe dry eye disease. The observed symptom improvement and inflammation control[17,18] corroborated the clinical and histological findings in cyclosporine-treated rabbits in our study, particularly in terms of conjunctival hyperemia and neovascularization.

BAK has been shown to have deleterious effects on the ocular surface both *in vitro* and *in vivo* and in both animal and human studies. The use of this preservative can induce significant and conservative dissolution of the tear film, particularly with long-term exposure. BAK has also been shown to induce cytotoxic and pro-inflammatory effects on the ocular surface by inducing squamous metaplasia of the conjunctival epithelium and reducing the number of goblet cells[19-21]. In our study, Group 1, which was treated with BAK 0.1%, experienced a significant decrease in the number of goblet cells relative to control eyes in the same group and the eyes in Group 2 (cyclosporine alone). Notably, even the eyes in Group 3 (BAK+cyclosporine) exhibited a significant reduction in the number of goblet cells per field, compared to the corresponding controls and eyes in Group 2. A further comparison of the treated eyes in Groups 3 and 1 (BAK+cyclosporine vs. BAK) revealed no significant difference in the number of goblet cells. In other words, cyclosporine had no significant effect on goblet cell preservation in eyes treated with BAK, despite its scientifically proven anti-inflammatory effects.

Our results suggest that cyclosporine has no effect on the cytotoxicity induced by BAK, even at concentrations lower than the one used in this study, and does not prevent cell lysis or apoptosis. BAK exerts its cytotoxic actions directly, as indicated by the cumulative (i.e., dose-dependent) effects of the repeated administration of preservative-containing eye drops. Previous studies found that a BAK concentration of 0.007% induced the lysis of 50% of cultured epithelial cells within 2 minutes and that the prolonged use of topical ocular drugs containing BAK as a preservative may exacerbate the symptoms and signs of surface eye diseases and induce serious adverse effects on the cornea and conjunctiva[22,23]. These effects may include subclinical inflammation, reduced corneal epithelial barrier function, tear film destabilization, and sensations of irritation and dryness[24].

In our study, we observed some interesting effects of treatment even in the unexposed control eyes. Notably, we observed a significant difference in the number of goblet cells in the contralateral (i.e., control) eyes in Groups 1 and 3 (i.e., BAK-treated), compared with the contralateral eyes in Group 2 (cyclosporine only). In other words, a low level of BAK absorption may have systemic effects, as indicated by the observations in untreated eyes. The systemic absorption of eye drops has been well-described in the literature. When topically administered, these medicines are exposed directly to systemic circulation mainly via the nasolacrimal canal and are absorbed by the nasal mucosa. This route of absorption is more rapid than the conjunctival and corneal paths. The latter lead to absorption via the aqueous humor and entry into circulation via drainage, and the effects are minimal when compared to absorption directly through the nasal mucosa. In reports supporting the systemic absorption of eye drops, some have demonstrated even β-antagonist-induced respiratory failure secondary to the instillation of anti-glaucoma eye drops, as the drug is absorbed more rapidly by the nasal mucosa[25,26].

Our analysis of blood vessels in the eye globes revealed a significant increase in neovascularization in Group 1 (BAK only), when compared to the contralateral control eyes and all other groups. This observation confirms the inflammatory action of BAK and its consequent stimulation of angiogenesis. This preservative acts as a detergent on the lipid layer of the tear film and is known to cause irritation, inflammation, and neovascularization. Patients with a longer exposure to a higher dosage of preservative-containing eye drops exhibit more severe symptoms of ocular superficial disease[27-29]. The treated eyes in Group 3 (BAK+cyclosporine) exhibited a significant increase in the number of new vessels when compared to the control eyes in Groups 1 and 2 and the treated eyes in Group 2, but a significant reduction when compared to the treated eyes in Group 1 (BAK). In other words, cyclosporine effectively prevented the formation of new vessels. However, cyclosporine did not reduce the number of vessels in Group 3, as the treated eyes did not differ significantly from the control eyes or eyes treated with cyclosporine only. In brief, only a comparison of groups treated with BAK (BAK vs BAK+cyclosporine) revealed the effectiveness of cyclosporine because the number of blood vessels remained greater than that in untreated or cyclosporine-treated

eyes although cyclosporine significantly reduced the degree of neovascularization.

In a comparison of control eyes, Group 3 (BAK+ cyclosporine) exhibited a significant increase in the number of new vessels when compared to Group 2. A similar result was observed in a comparison of control eyes in Group 3 to treated eyes in Group 2. Again, this finding suggests the systemic absorption of drugs from eye drops. These absorbed drugs can thus influence conjunctival inflammation even if no direct treatment is applied.

We did not observe any statistical difference in the number of vessels between the cyclosporine-treated eyes in Group 2 and the untreated eyes in any control groups. This suggests that cyclosporine only blocks inflammatory neovascularization but does not reduce the number of pre-existing vessels. Another study reported a positive response to cyclosporine A treatment in corneal epithelial cells in an *in vitro* inflammatory model of keratoconus[30]. In that study, significant short-term decreases in the levels of the inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α and a long-term decrease in the level of matrix-9 metalloproteinase were observed after cyclosporine A treatment. These decreases may have been blocked the progression of disease[30].

Plausibly, the complications associated with the instillation of eye drops could be decreased by reducing the number of instillations. Furthermore, the use of preservative-free eye drops is the ideal option whenever possible. Our study demonstrates a third option, namely that cyclosporine could be used to reduce the side effects of preservatives. In our study, cyclosporine reduced the increased neovascularization associated with BAK, compared to eyes treated with BAK alone. However, cyclosporine had no protective effect on goblet cells. Therefore, cyclosporine should be combined with other anti-inflammatory protocols to control ocular surface changes in chronically treated patients.

This study aimed to simulate the effects of chronic exposure to BAK in a 30-day experimental model and determine the influence of cyclosporine. Notably, longer studies are needed to better demonstrate these effects in the conjunctiva. To accelerate these effects, we have increased the concentration of BAK and frequency of cyclosporine administration. However, this represents a study limitation because a longer study time using BAK concentrations similar to those in commercially available eye drops, as well as a reduced frequency of cyclosporine instillation, would be more representative of real clinical cases. The small sample (15 animals) is another limiting factor; accordingly, our findings may not accurately represent human disease. However, the short-term use of animals according to appropriate standards provides a less expensive and suitable alternative, given the requirements for animal maintenance, eye irritation, and histopathological analysis.

In conclusion, our findings demonstrate the importance of conjunctival injury during glaucoma treatment and suggest that the cyclosporine could be used to minimize this injury.

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