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Clinical and Histologic Comparison of Conjunctival Changes Induced by Antiglaucoma Beta Blockers, Carbonic Anhydrase Inhibitors, Alfa Agonists and Fixed Combinations with and without Preservatives

Heloisa Helena Russ[1*], Amalia Turner-Gianicco[2], Fabiano Montiani-Ferreira[3] and Leandro Lima[2]

[1]*Federal University of Parana. Glaucoma Service. Centro da Visão/Hospital de Clinicas da Universidade Federal do Paraná, Brazil*

[2]*Veterinary Ophthalmolgy, Federal University of Parana, Brazil*

[3]*Veterinary Ophthalmolgy, Graduate School of Veterinary Sciences, SCA, Federal University of Parana, Brazil*

[*]**Corresponding author:** Heloisa Helena Russ, Centro da Visão/ Hospital de Clinicas da Universidade Federal do Paraná, Rua Paster, 26- Batel. Curitiba, Paraná, CEP 80250080, Brazil, Tel: +55413523-0086; E-mail: heloisaruss@onda.com.br, heloisa@glaucomacuritiba.com.br

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Abstract

Background: The objective of this research was to evaluate and compare clinical tests, histological and immunohistochemical changes induced by antiglaucoma beta blockers, carbonic anhydrase inhibitors, alfa agonists and fixed combinations containing benzalkonium chloride (BAK) and without preservative (BAKFREE) in the conjunctiva of rabbits. A total of 60 rabbits (120 eyes), were divided into six groups, and treated during 30 days with: dorzolamide 2%+timolol maleate 0.5% BAK, dorzolamide 2%+timolol maleate 0.5%^{BAKFREE}, brinzolamide 1% +timolol maleate 0.5% BAK, brimonidine 0.2%+timolol maleate 0.5%^{BAK}, timolol maleate 0.5%^{BAK} and control solution BAK. Right eyes served as controls and received no medication. Corneal touch threshold (CTT), Schirmer tear test (STT) and intraocular pressure (IOP) were measured during pre and post treatment periods. Conjunctival goblet cells density and vascular endothelium thickness (VET) were evaluated. Immunohistochemistry was used to detect reactive macrophages (RAM11), vascular endothelial inflammation (VCAM-1), and reactive T-lymphocytes (CD45RO).

Results: No differences were observed concerning CTT and STT. IOP was reduced in all drugs after treatment, except control solution BAK. No variation was noted in goblet cells density and VET after treatment in all groups. An increased macrophages response was observed after treatment with all BAK groups. Conjunctival reactive lymphocytes were increased after treatment only in dorzolamide 2%+timolol maleate 0.5%^{BAK}.

Conclusion: Antiglaucoma beta blockers, carbonic anhydrase inhibitors and fixed combinations appear to have a small influence in the clinical ophthalmic tests, but with alteration in macrophage inflammatory response. The reactive macrophage stimulation was associated with the presence of preservative BAK that may induce changes in rabbit's healthy conjunctiva, trends to increase an inflammatory response. Lymphocytic inflammatory response was observed only in animals treated with dorzolamide 2%+timolol maleate 0.5%^{BAK}, suggesting some toxic effect of this association, during 30 days treatment.

Keywords: Glaucoma therapy; Conjunctival inflammation; Benzalkonium chloride; Histomorphometric; Immunohistochemistry

Background

According to the World Health Organization, glaucoma is the second leading cause of blindness worldwide [1,2]. Medical therapy usually constitutes the first line treatment for glaucomatous patients. Patients often use topical therapy for many years, and may experience the occurrence of signs and symptoms of inflammation of the ocular surface [3,4].

Ocular surface reacts specifically to a wide range of external insults, such as environmental changes, air pollution, infectious agents, allergens and topical eye drop treatments [4-7].

Side effects of chronic use of eye drops could be due to active component as well as to preservatives. The most commonly used is benzalkonium chloride (BAK), which exerts an antimicrobial effect by its powerful detergent action on bacterial walls and membranes [8]. These toxic effects are well documented in the ophthalmological and biomedical literature, and encompass a large variety of mechanisms involving the immune system, conjunctival and corneal epithelia, tear film and most likely corneal nerve sensitivity [9-11]. This detergent effect of BAK in combination with a partial destruction of mucous goblet cells is responsible for the induced instability of the lacrimal film, and an immunologic reaction with an increased presence of lymphocytes, macrophages and Langerhans cells during chronic therapy [12,13].

Several classes of drugs are currently available for treating glaucoma, including cholinergic agents, beta blockers, alpha adrenergic agonists, carbonic anhydrase inhibitors, prostaglandins analogues (PGAs) and the fixed combinations of alpha adrenergic agonists, carbonic anhydrase inhibitors or prostaglandin analogues associated with timolol maleate [14-16], most of them were associated with BAK. Trying to reduce BAK adverse effects, other studies using different preservatives or preservative-free (BAKFREE) have arisen. Some

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studies reported lower prevalence of ocular symptoms and signs in preservative-free eye drops during long-term therapy [14,17].

There are many reported side effects of BAK in glaucomatous drugs that may result in changes of some ophthalmic clinical tests, and can cause conjunctival inflammation affecting the normal cellular and immunological function [17-20]. Some of these side effects were studied in clinical trials, some in cellular culture and others in animal models. Until now, there are small reports that evaluate in the same study the effects of preservative and preservative-free prostaglandin analogs on conjunctiva cellular morphology, inflammatory response and changes in some specific ophthalmic tests.

Thus the aim of this study was to examine and compare the effects of different prostaglandin analogs therapy, with and without preservative, on selected ophthalmic tests, histomorphometry and immunohistochemistry in rabbit's conjunctiva, before and after 30 days of topical therapy.

Methods

Animals

All procedures using live rabbits were conducted in accordance with Federal University of Paraná Animal Use Committee (Curitiba City, Paraná State, Brazil) and with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Sixty New Zealand white rabbits (*Oryctolagus cuniculus*) were selected randomly from a commercial breeder collection. All animals (27 males and 33 females) have similar mean weight (2.5 kg) and age on average five months old.

Physical and ophthalmic examinations were performed ten days prior to initiation of treatment to exclude animals with indications of systemic and ocular diseases. Procedures and tests necessary to produce this work were performed one day prior to start treatment by the same investigator, to avoid discrepancies related to inter-observer repeatability. After 30 days of continuous treatment, the animals were reevaluated.

The drugs chosen in this research were commercial antiglaucoma eye drops beta blockers, carbonic anhydrase inhibitors, alfa agonists and fixed combinations containing benzalkonium chloride (BAK) and without preservative (BAKFREE).

Drugs	N	Label name
Dorzolamide 2%+timolol maleate 0.5%$^{BAK\ 0.0075\%}$	10	Cosopt*1
Dorzolamide 2%+timolol maleate 0.5%^{BAKFREE}	10	Cosopt*2
Brinzolamide 1%+timolol maleate 0.5% $^{BAK\ 0.005\%}$	10	Azorga[3]
Brimonidine 0.2%+timolol maleate 0.5%$^{BAK\ 0.005\%}$	10	Combigan[4]
timolol maleate 0.5%BAK 0.005%	10	Timoptol[5]
Control solutionBAK 0.01%	10	BAK**6

* There was no difference between Label from Cosopt® with BAK and Cosopt® (BAKFREE). **BAK = benzalkonium chloride.

[1]Cosopt® (MSD - Merck Sharp & Dohme Ltda., Guarulhos, SP, Brasil).

[2]Cosopt® (MSD - Merck Sharp & Dohme France, Paris, France).

[3]Azorga® (Alcon Laboratórios do Brasil Ltda., São Paulo, SP, Brasil).

[4]Combigan® (Allergan Indústria Farmacêutica Ltda., Guarulhos, SP, Brasil).

[5]Timoptol® (MSD - Merck Sharp & Dohme Ltda., Guarulhos, SP, Brasil).

[6]Benzalkonium chloride (Ophthalmos Farmácia Oftalmológica de Manipulação, São Paulo, SP, Brasil).

Table 1: Ophthalmic drugs used to treat left eye of the New Zealand white rabbits, according to the respective number of animals (N) and label name.

Rabbits were divided into six groups containing 10 animals, and were treated with dorzolamide 2%+timolol maleate 0.5%$^{BAK\ 0,0075\%}$ (Cosopt®-MSD-Merck Sharp & Dohme Ltda., Guarulhos, SP, Brasil), dorzolamide 2%+timolol maleate 0.5%^{BAKFREE} (Cosopt®-MSD-Merck Sharp & Dohme France, Paris, France), brinzolamide 1%+timolol maleate 0.5%$^{BAK\ 0,005\%}$ (Azorga®-Alcon Laboratórios do Brasil Ltda., São Paulo, SP, Brasil), brimonidine 0.2%+timolol maleate 0.5%$^{BAK\ 0,005\%}$ (Combigan® Allergan Indústria Farmacêutica Ltda., Guarulhos, SP, Brasil), timolol maleate 0.5%$^{BAK\ 0,005\%}$ (Timoptol®-MSD-Merck Sharp & Dohme Ltda., Guarulhos, SP, Brasil), and control solutionBAK 0.01% (phosphate-buffered saline with benzalkonium chloride 0.01%, Ophthalmos Farmácia Oftalmológica de Manipulação, São Paulo, SP, Brasil). All groups were treated onto the left eye with one daily drop of the selected substance. Right eyes served as controls and received no medication. Treatments were performed daily in fixed hour at 8:00 AM. All drugs can be seen in Table 1.

Ophthalmic tests

A total of 120 eyes, from 60 healthy rabbits were evaluated. The anterior ocular structures were evaluated using a Finoff transilluminator (Welch Allyn, Skaneateles Falls, NY, USA) as a source of focal light and a slit lamp biomicroscope (Hawk Eye; Dioptrix, L'Union, France). Clinical tests were performed while rabbits were manually restrained by an experienced handler, taking care to keep the animal comfortable. When the head was manually stabilized for taking measurements special attention was given to avoid applying pressure to the neck region, to prevent iatrogenic alterations in intraocular pressure (IOP). The sequence of procedures performed in this study was: (1) ocular inspection, (2) corneal touch threshold, (3) Schirmer tear test (STT) and (4) IOP tonometry. To avoid interobserver discrepancies, the same investigator (LL) performed all ophthalmic tests. Humidity and temperature were monitored during tests. Humidity varied from 70 to 73% and temperature from 21 to 23°C.

Corneal touch threshold

To evaluate corneal sensitivity, all rabbits were manually restrained, and a Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, Chartres Cedex, France) was used. This instrument contains an adjustable nylon filament with a defined diameter, which is applied in different lengths to the center of the cornea. A stimulus produced by the instrument's nylon monofilament that reaches the corneal touch threshold induces a corneal reflex, consisting of prompt eyelid closure. In this study only the center of the cornea was analyzed for corneal touch threshold (CTT), which was repeated five times using the same length of the nylon filament. The length of the nylon filament was then decreased at 0.5 cm increments until each rabbit responded with a corneal blink reflex. The CTT was then quantified in cm length of the filament necessary to cause a blink reflex.

Schirmer tear test

Sterile standardized Schirmer tear test (STT) strips (Schering Plough Animal Health, Union, NJ, USA) were used to perform the STT type I, which measures the basal plus a portion of the reflex tear secretion in all rabbits eyes.

Intraocular pressure

Intraocular pressure (IOP) was measured by a rebound tonometer (Tonovet, Lumic International, Baltimore, MD, USA) to assess IOP of both eyes. No topical anesthetic was used and the tonometer was set for an undefined species. Tonovet uses an electromagnetic probe that is propelled off the central cornea six times and an average is obtained to provide an estimate of the IOP.

Euthanasia, sample collection and histological processing

On the final day of drop instillation, after 30 days of treatment, rabbits were anesthetized and euthanized with an overdose bolus of pentobarbital (200 mg/kg) intravenously by ear vein. After death, the entire eyes and ocular annexes (eyeballs and eyelids) were collected. The samples were placed in 10% buffered formaldehyde for 24 hours. After that, eyes were sliced at longitudinal axis followed by routine paraffin embedding [16]. Tissue blocks were sectioned at 5 µm thicknesses and mounted on charged glass slides (Starfrost adhesive slide, Waldemar Knittel GmbH, Hamburg, Germany). Slides were stained with HE (hematoxylin-eosin) and PAS (periodic acid-Schiff) to the histomorphometric quantitative analyses, and prepared with three immunohistochemical (IHC) markers to detect: (1) reactive macrophages, with rabbit anti-macrophage RAM11 (DakoCytomation, CA, USA,in dilution 1:800); (2) reactive T-linfocites, with an anti-CD45RO (BD Biosciences Pharmingen, USA, in dilution 1:800) and (3) reactive vascular cellular adhesion molecule, with VCAM-1 (Novocastra Laboratories Ltd, UK, in dilution 1:200).

Histomorphometric quantitative analyses were performed with the software Image Pro-Plus version 4 (Media Cybernetics, Silver Spring, MD). Digital images were acquired under 200x magnification and stored using the same software. Subsequently, two segments of 200 µm of length and with 5 µm of thickness of conjunctival tissue were selected from each examined field of all slides. These same-sized linear segments allowed measurement of the following parameters: vascular endothelial thickness and number of goblet cells.

Goblet cells were manually counted using a 200 µm virtual ruler. Optical qualitative microscopy evaluation was made in linear conjunctival segments of 200 µm using a virtual ruler on digital images. The researcher responsible for measuring all histologic parameters was masked to the medication group of the rabbit.

IHC analysis were performed with the same software in order to detect by the sum of tissue areas positively labelled to activated macrophages (RAM11), reactive lymphocytes (CD45RO), and endothelial cells reactive against inflammation (VCAM-1), all of which are present in a chronic inflammatory response.

Statistical analyses

Results were expressed as mean ± standard deviation (SD). The ophthalmic tests were evaluated in each drug comparing pre and post-treatment values (mean ± standard deviation) to the left (treated) and right eye (control) using t-tests with a significance level of 5%. Histomorphometric and IHC values were compared only at post-treatment. One-way ANOVA with a significance level of 5% was used to compare continuous variables. If any statistically significant difference was found, the data were further analyzed using post hoc comparisons with Tukey-Kramer test (Statview V; SAS Institute Inc., Cary, NC, USA). Differences were deemed statistically significant when P<0.05.

Results

Ophthalmic tests

Corneal touch threshold: Comparison of corneal touch threshold (CTT) before and after treatment did not show significant differences for all drugs (P>0.05). All CTT (mean ± SD) values with respective P values can be seen in Table 2.

Drugs	Pre CTT Mean ± SD (cm)	Post CTT Mean ± SD (cm)	P Value
Dorzolamide 2% + timolol maleate 0.5%$^{BAK\ 0.0075\%}$	2.0 ± 0.6	2.1 ± 0.7	0.7313
Dorzolamide 2% + timolol maleate 0.5%^{BAKFREE}	1.7 ± 0.5	1.8 ± 0.5	0.6490
Brinzolamide 1% + timolol maleate 0.5% $^{BAK\ 0.005\%}$	2.2 ± 0.8	2.8 ± 0.5	0.0544
Brimonidine 0.2% + timolol maleate 0.5%$^{BAK\ 0.005\%}$	2.3 ± 0.5	2.4 ± 0.4	0.8621
timolol maleate 0.5%$^{BAK\ 0.005\%}$	2.3 ± 0.4	2.1 ± 0.6	0.2163
Control solution$^{BAK\ 0.01\%}$	2.2 ± 0.5	2.4 ±0.4	0.3466

*P values greater than 0.05 were not considered significant.

Table 2: Pre and post treatment values for corneal touch threshold (CTT) in New Zealand white rabbits, according to the drugs, expressed in mean ± SD values.

Schirmer tear test: Pre and post treatment Schirmer tear test (STT) comparisons did not show significant difference for all drugs (P>0.05). All STT (mean ± SD) values with the respective P values can be seen in Table 3.

Drugs	Pre STT Mean ± SD (mm)	Post STT Mean ± SD (mm)	P Value
Dorzolamide 2%+timolol maleate 0.5%$^{BAK\ 0.0075\%}$	4.8 ± 0.9	4.4 ± 0.7	0.2878
Dorzolamide 2% + timolol maleate 0.5%^{BAKFREE}	6.3 ± 1.3	6.1 ± 1.4	0.7287
Brinzolamide 1% + timolol maleate 0.5%$^{BAK\ 0.005\%}$	4.5 ± 0.7	5.2 ± 0.8	0.0511
Brimonidine 0.2% + timolol maleate 0.5%$^{BAK\ 0.005\%}$	5.6 ± 1.6	5.3 ± 1.1	0.6795
timolol maleate 0.5%$^{BAK\ 0.005\%}$	5.1 ± 1.4	5.7 ± 1.3	0.3349
Control solution$^{BAK\ 0.01\%}$	6.4 ± 1.1	6.6 ± 1.0	0.5716

*P values greater than 0.05 were not considered significant.

Table 3: Pre and post treatment values for Schirmer tear test (STT) in New Zealand white rabbits, according to the drugs, expressed in mean ± SD millimeters values, during one minute.

Intraocular pressure

A comparison of the intraocular pressure (IOP) pre and post treatment show a statistical difference in all drugs, and except to the control solution with only BAK. Values of IOP (mean ± SD) with the respective P values of all groups can be seen in Table 4.

Drugs	Pre IOP Mean ± SD (mmHg)	Post IOP Mean ± SD (mmHg)	P Value
Dorzolamide 2% + timolol maleate 0.5%$^{BAK\ 0.0075\%}$	13.3 ± 1.4	11.4 ± 2.0	0.0229*
Dorzolamide 2% + timolol maleate 0.5%^{BAKFREE}	12.3 ± 1.6	10.7 ± 1.5	0.0312*
Brinzolamide 1% + timolol maleate 0.5% $^{BAK\ 0.005\%}$	13.9 ± 1.0	12.2 ± 0.9	0.0009*
Brimonidine 0.2% + timolol maleate 0.5%$^{BAK\ 0.005\%}$	13.1 ± 0.9	11.4 ± 1.2	0.0034*
timolol maleate 0.5%$^{BAK\ 0.005\%}$	12.7 ± 1.9	11.1 ± 1.2	0.0363*
Control solution BAK 0.01%	13.2 ± 0.4	13.0 ± 1.9	0.8220
*P values lower than 0.05 were considered significant.			

Table 4: Pre and post treatment values for intraocular pressure (IOP) in New Zealand white rabbits, according to the drugs, expressed in mean ± SD values.

Histomorphometric and immunohistochemical analysis

Goblet cells density: A comparison of number of conjunctival goblet cells between left (treated) and right (control) eyes, after 30 treatment days shows no significant changes in goblet cells in all drugs. Goblet cells count (mean ± SD) with the respective P values is shown on Table 5.

Drugs	OD	OS	P Value
Dorzolamide 2% + timolol maleate 0.5%$^{BAK\ 0.0075\%}$	11.6 ± 1.265	12.3 ± 1.567	0.2862
Dorzolamide 2% + timolol maleate 0.5%^{BAKFREE}	12.3 ± 2.710	13.0 ± 3.367	0.6147
Brinzolamide 1% + timolol maleate 0.5% $^{BAK\ 0.005\%}$	10.5 ± 2.838	10.7 ± 2.058	0.8588
Brimonidine 0.2% + timolol maleate 0.5%$^{BAK\ 0.005\%}$	10.0 ± 2.667	11.4 ± 3.134	0.2962
timolol maleate 0.5%$^{BAK\ 0.005\%}$	12.1 ± 2.514	11.6 ± 2.633	0.6692
Control solution$^{BAK\ 0.01\%}$	11.2 ± 2.168	13.8 ± 5.119	0.3262
*P values greater than 0.05 were not considered significant.			

Table 5: A comparison of conjunctival goblet cells counts (mean ± SD) between left (OS) and right (OD) eyes, after 30 days' treatment of the New Zealand white rabbits, according to the drugs.

Drugs	OD Mean ± SD (μm)	OS Mean ± SD (μm)	P Value
Dorzolamide 2% + timolol maleate 0.5%BAK 0.0075%	4.279 ± 0.847	4.228 ± 0.932	0.8997
Dorzolamide 2% + timolol maleate 0.5%BAKFREE	4.021 ± 0.815	4.598 ± 1.069	0.1913
Brinzolamide 1% + timolol maleate 0.5% BAK 0.005%	4.406 ± 0.758	4.427 ± 0.828	0.9525
Brimonidine 0.2% + timolol maleate 0.5%BAK 0.005%	4.643 ± 0.721	4.557 ± 0.755	0.7974
timolol maleate 0.5%BAK 0.005%	4.353 ± 1.006	4.169 ± 0.928	0.6762
Control solutionBAK 0.01%	4.804 ± 1.232	4.554 ± 1.184	0.7522

*P values greater than 0.05 were not considered significant.

Table 6: Conjunctival vascular wall thickness (VWT) comparison between left (OS) and right (OD) eyes, after 30 days' treatment in New Zealand white rabbits, according each drug. Values (mean ± SD) are expressed in μm.

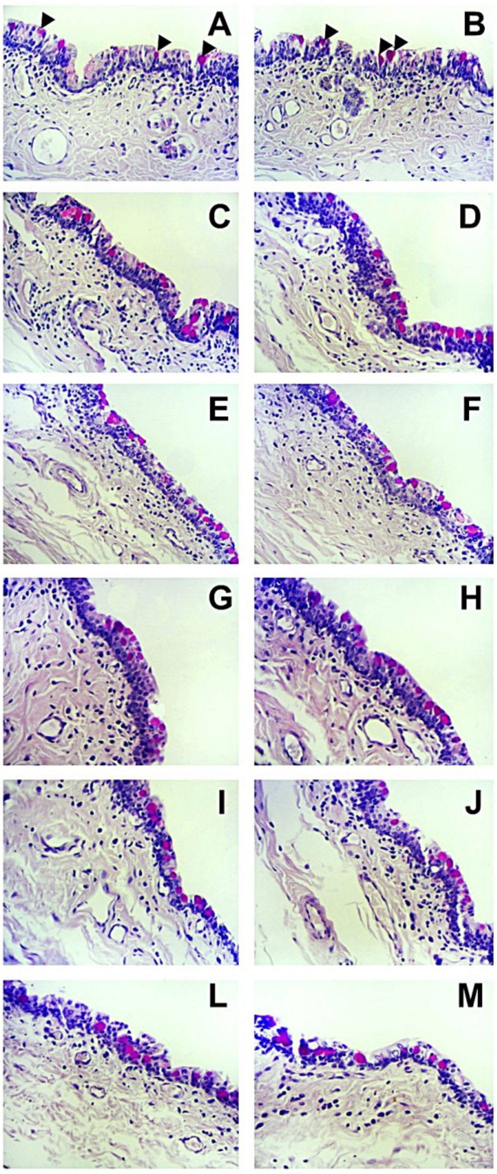

Figure 1: Photomicrographs of New Zealand white rabbit's conjunctiva (200x) stained with PAS. Density of goblet cells (black arrow head) in treated left eyes (B,D,F,H,J,M) were similar than non-treated right eyes (A,C,E,G,I,L). A and B refers to dorzolamide 2% + timolol maleate 0.5%BAK. C and D to dorzolamide 2% + timolol maleate 0.5%^{BAKFREE}. E and F to brinzolamide 1% + timolol maleate 0.5% ^{BAK}. G and H to brimonidine 0,2% + timolol maleate 0.5%^{BAK}. I and J to Control solution^{BAK}.

Representative photomicrographs demonstrating the histological differences between conjunctival goblet cell numbers of treated and non-treated eyes can be seen in Figure 1.

Vascular wall thickness

Vascular wall thickness (VWT) of the conjunctival tissue was compared between left (treated) and right (control) eyes, after 30 days' treatment. There were no significant changes observed in all tested drugs. All VWT (mean ± SD) values with the respective P values of all groups can be seen in Table 6.

Anti-RAM11 was compared between left (treated) and right (control) eyes, after 30 days treatment. Treated eyes show a higher RAM11 response to all drugs, except to dorzolamide 2% + timolol maleate 0.5%^{BAKFREE} (Table 7). The RAM11 response was represented in the Figure 2.

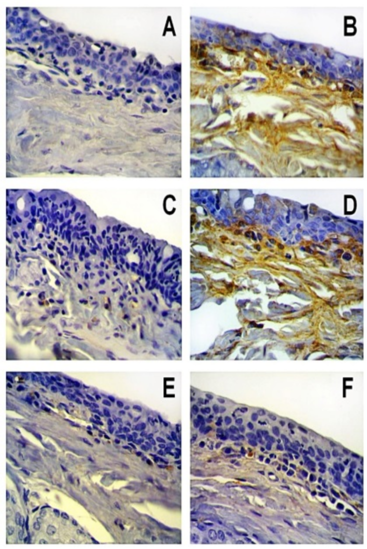

Figure 2: Photomicrographs of New Zealand white rabbit's conjunctiva (400x) after 30 days treatment stained with RAM11 IHC antibody. A and B refers to dorzolamide 2% + timolol maleate 0.5%^{BAK}, C and D to control solution^{BAK}, and E and F to dorzolamide 2% + timolol maleate 0.5%^{BAKFREE}. Observe that the density of immune response (brown areas) were higher in left (B,D) than right (A,C) eyes, in contrast with background eosin stain (blue areas), and has no difference in (E,F).

Drugs	OD Mean ± SD (μm^2)	OS Mean ± SD (μm^2)	P Value
Dorzolamide 2% + timolol maleate 0.5%$^{BAK\ 0.0075\%}$	633.981 ± 229.532	1207.866 ± 385.829	0.0008*
Dorzolamide 2% + timolol maleate 0.5%^{BAKFREE}	263.966 ± 173.984	642.484 ± 601.322	0.0719
Brinzolamide 1% + timolol maleate 0.5% $^{BAK\ 0.005\%}$	618.203 ± 262.670	1460.454 ± 725.052	0.0028*
Brimonidine 0.2% + timolol maleate 0.5%$^{BAK\ 0.005\%}$	624.797 ± 243.333	1418.755 ± 467.904	0.0002*
timolol maleate 0.5%$^{BAK\ 0.005\%}$	1564.251 ± 508.218	2517.6 ± 1028.104	0.0170*
Control solution$^{BAK\ 0.01\%}$	1250.832 ± 297.694	7460.292 ± 2253.231	0.0003*

Table 7: Comparison between anti-RAM11 IHC-reactive areas between left (OS) and right (OD) eyes, after 30 days' treatment in New Zealand white rabbits, according to each drugs. Values are expressed in sum of areas (mean ± SD) in μm^2.

Conjunctival anti-CD45RO response was compared between left (treated) and right (control) tissues, after 30 days treatment. Eyes treated with dorzolamide 2% + timolol maleate$^{BAK0,0075\%}$ show a higher CD45RO response than the respective control eyes. There were no significant differences between left and right eyes other drugs and in control solution^{BAK} (Table 8). The CD45RO response was represented in the Figure 3.

Drugs	**OD Mean ± SD (μm^2)**	**OS Mean ± SD (μm^2)**	**P Value**
Dorzolamide 2% + timolol maleate 0.5%$^{BAK\ 0.0075\%}$	351.594 ± 237.079	654.758 ± 337.314	0.0320*
Dorzolamide 2% + timolol maleate 0.5%^{BAKFREE}	370.094 ± 347.154	598.324 ± 272.577	0.1194
Brinzolamide 1% + timolol maleate 0.5% $^{BAK\ 0.005\%}$	34.738 ± 19.066	48.35 ± 25.278	0.1908
Brimonidine 0.2% + timolol maleate 0.5%$^{BAK\ 0.005\%}$	306.179 ± 181.713	486.926 ± 276.919	0.1015
timolol maleate 0.5%$^{BAK\ 0.005\%}$	316.198 ± 206.693	418.958 ± 198.714	0.2719
Control solution$^{BAK\ 0.01\%}$	102.088 ± 63.088	125.239 ± 65.27	0.5842
*P values lower than 0.05 were considered significant.			

Table 8: Comparison of the CD45RO IHC response between left (OS) and right (OD) eyes, after 30 days treatment of the New Zealand white rabbits, according to the drugs. Values are expressed in sum of areas (mean ± SD) in μm^2.

Drugs	OD Mean ± SD (μm^2)	OS Mean ± SD (μm^2)	P Value
Dorzolamide 2% + timolol maleate 0.5%$^{BAK\ 0.0075\%}$	347.451 ± 246.418	545.816 ± 265.014	0.1001
Dorzolamide 2% + timolol maleate 0.5%^{BAKFREE}	246.686 ± 183.916	450.35 ± 160.442	0.2482
Brinzolamide 1% + timolol maleate 0.5% $^{BAK\ 0.005\%}$	175.444 ± 127.699	261.106 ± 180.328	0.2360
Brimonidine 0.2% + timolol maleate 0.5%$^{BAK\ 0.005\%}$	619.007 ± 463.714	933.467 ± 818.428	0.3044
timolol maleate 0.5%$^{BAK\ 0.005\%}$	242.631 ± 158.681	255.527 ± 151.796	0.8548
Control solution$^{BAK\ 0.01\%}$	393.866 ± 231.270	467.414 ± 244.763	0.6384
*P values greater than 0.05 were not considered significant.			

Table 9: Comparison of anti-VCAM reactive areas between left (OS) and right (OD) eyes, at post treatment of the New Zealand with rabbits, according to each drug. Values are expressed in sum of areas (mean ± SD) in μm^2.

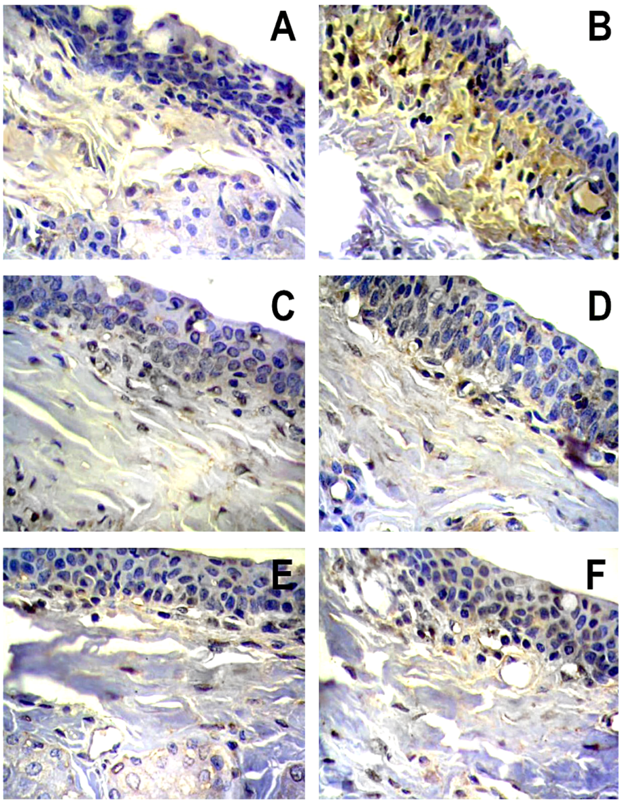

Figure 3: Photomicrographs of New Zealand white rabbit's conjunctiva (400x) stained with anti-CD45RO IHC. A and B refers to dorzolamide 2% + timolol maleate 0.5%BAK, C and D to dorzolamide 2% + timolol maleate 0.5%^{BAKFREE}, E and F to Control solution BAK. Observe that the density of immune response (dark brown areas) was higher in left (B) than right (A) eye, in contrast with background eosin stain (blue areas), and has no difference in (C e D) e (E e F).

Anti-VCAM-1 response was compared between left (treated) and right (control) eyes, after 30 days treatment. There were no significant differences between left and right eyes in all tested drugs (Table 9). The VCAM-1 response was represented in the Figure 4.

Discussion

In this investigation, we showed that topical beta blockers, carbonic anhydrase inhibitors, alfa agonists and fixed combinations containing benzalkonium chloride (BAK) and without preservative (BAKFREE) in the conjunctiva of rabbits' therapies, commonly used in glaucoma patients may induce ocular surface changes in normal rabbit´s eyes by cellular modifications, showing no variation in corneal touch threshold (CCT) nether in Schirmer´s tear test (STT) analysis. Apparently, preservatives used to avoid contamination of topical ophthalmic compounds or to enhance their permeability still remain the main suspects of detrimental effects [21,22]. However, in recent years plenty of evidence has shown that they are not the only players of the inflammatory cascade triggered in the ocular surface challenged by chronic topical therapies. Medical compounds themselves may induce ocular damage in predisposed patients in a cumulative effect related to dosage and duration of therapy or preexisting diseases [21,23]. An example of this, was the discovered that timolol maleate 0.5% has mechanisms that could decrease hemangiomas in infancy [24].

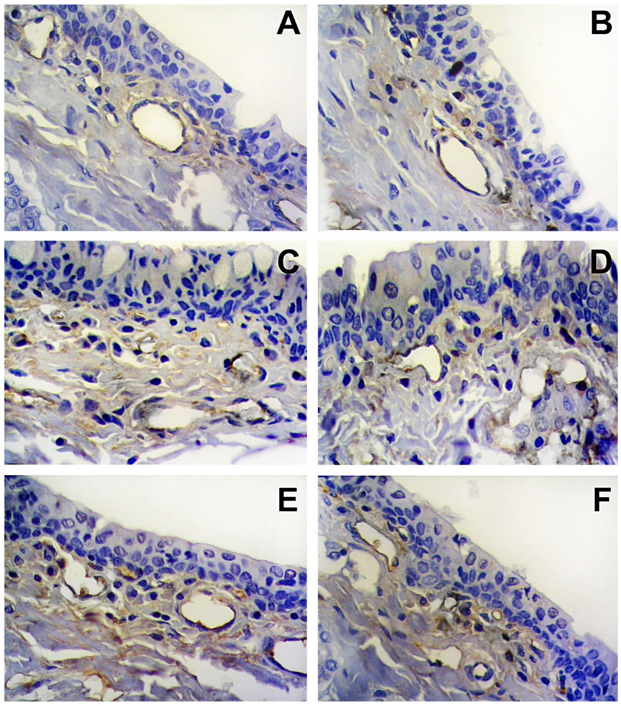

Figure 4: Photomicrographs of New Zealand white rabbit's conjunctiva (400x) stained with VCAM-1 IHC antibodies. A and B refers to dorzolamide 2% + timolol maleate 0.5%^{BAK}, C and D to brinzolamide 1% + timolol maleate 0.5% ^{BAK}, and E and F Control solution BAK. Observe that the density of immune response (brown areas) showed no difference between left (B, D and F) than right (A, C and E) eyes comparisons.

The literature suggests that the use of timolol maleate can result in a decrease of CTT in elderly people treated with such therapy [25], and the presence of preservative BAK could be the most important factor involved in the modification of corneal neurophysiological response of rabbits [26].

The ophthalmic tests evaluation did not change CTT or STT during treatment in all tested drugs. One limitation of CTT analysis is that the measurement technique used in our study resulted in considerable inter- and intraindividual variability, which could have a different interpretation by other researchers [27]. The STT alteretions are frequentely associated in other studys that evaluate the effect of preservatives like BAK [2,27-29] during long term treatment. None of the drugs, with or witout BAK, has changed STT, and this abserse of change could be related with the short period of treatment. Frezzotti et al. [30] describes significant superfitial ocular changes in patients treated with timolol maleate^{BAK} compared with timolol maleate^{BAKFREE}, showing reduction in tear production, during one year of treatment.

Considering ocular absorption of antiglaucomatous drugs, it was obviously expected that treated eyes would show a reduction in IOP

[31-34]. Indeed in all PGAs studied, and except to control solution^{BAK}, IOP reduction was considered significant, with IOP values near to other described in the literature [20].

Conjunctival goblet cells participate in tear film stability [35], markedly decreased in presence of inflammatory and toxic stimulations, although they tended to regenerate when the irritating stimuli were relieved [36,37]. There are reports of decreasing goblet cell numbers investigations with patients, under short and long-term therapy with BAK-containing antiglaucoma drugs [14,38,39].

Our study showed no changes in goblet cells after 30 days of treatment in all drugs tested groups, with and without preservatives. It may suggest relative safety for use during this time period. A prolonged period of treatment is necessary to conclude if the preservative presence, like timolol maleate^{BAK} and timolol maleate^{BAKFREE} could promote changes, as seen in other studies [23,24,40].

Inflammation of blood vessels frequently changes the cellular morphology of the vascular endothelium [40,41] causing an alteration in vascular endothelium thickness (VET). Our results demonstrated no variation in VET in all tested drugs, making us to believe that the use of this formulations are safe and do not direct contribute to vascular inflammation during the conditions of the research.

During the vascular inflammation, there are an activation vascular cellular adhesion molecule [42], that contributes to enhance vascular permeability and extravasation of lymphocytes, monocytes, basophils and eosinophils. Detection of vascular cellular adhesion molecules (VCAM-1) was not significant to all tested drugs too, corroborating the hypothesis that these ophthalmic drugs did not cause an important initial vascular inflammation. By the way, long period of treatment with preservative BAKFREE drugs show minimal vascular alterations than the other associated with BAK [43]. Russ et al. [15] observed a more evident blood vessels response in rabbit's eyes treated with latanoprost$^{BAK\ 0.02\%}$ however, the mechanism involved in inflammation response is different and specific vascular cell adhesion molecule (VCAM-1) was not investigated.

Histopathology and impression cytology studies of the conjunctiva have demonstrated inflammation with an increase cellular response in eyes treated with antiglaucoma drugs [44-47]. Early phase conjunctival inflammation response is characterized by vasodilatation, increased vascular permeability, itching, and is followed by a late phase reaction that involves infiltration of inflammatory cells, especially macrophages and lymphocytes [48,49].

The increase in rabbit´s conjunctival inflammation mediated by reactive macrophages (RAM11) was observed in eyes treated with drugs associated with BAK and non-observed in association dorzolamide+timolol maleate^{BAKFREE}, suggest that BAK could be the responsible for stimulate inflammatory macrophage reactions. We believe that BAK preservative, present in these formulations, was responsible for causing inflammation in the rabbit's conjunctiva by cytotoxic effect and perhaps by exert a direct effect over macrophages. A recent in vitro study reported that BAK has a direct stimulating effect on macrophages, increasing phagocytosis, cytokine release in conjunctival tissue [50]. They concluded that long-term exposure to low concentrations of BAK should be considered as a stimulating factor responsible for inflammation through macrophage activation [50].

Higher numbers of inflammatory cells, like T-lymphocytes, T-helper lymphocytes, T- cytotoxic lymphocytes, were found in the conjunctiva of the glaucoma patients on long-term medical treatment compared with the normal conjunctiva of the controls [51].

Stimulation of reactive T-lymphocytes (anti-CD45RO response) detected in the conjunctiva of rabbits treated with dorzolamide 2% +timolol maleate 0.5%$^{BAK\ 0.0075\%}$, in contrast with the absence of response of the same formulation without BAK make us to conclude that BAK himself can influence the lymphocyte response. The higher concentration of preservative BAK could be the responsible of this stimulation of T-lymphocytes, but maybe the association of BAK with the fixed combination could have a synergism, enhancing the lymphocytic response, but the mechanisms involved in this process still needs to be better understood. Cho et al. [52], compared the cytotoxic effect of treatment with timolol maleate^{BAK}, dorzolamide^{BAK} alone, with the fixed combination of timolol maleate with dorzolamide^{BAK}, and noted that the association presents higher cytotoxic effect than the use of concomitant individual drugs. However, the study concluded that a long period of treatment with both single solutions in association could have a higher cumulative BAK effect than the fixed combination over the ocular surface, highlighting the cytotoxic effect of the drugs, however with less intensity than the cumulative effect of BAK.

Russ et al. [15] report the presence of moderate inflammatory infiltrate in prostaglandin treated eyes, without great histopathological changes in conjunctiva stroma. Other study observed that the presence of BAK in antiglaucoma eye drops could involve more than the reported toxic effects on the ocular surface epithelium and may affect immune balance of the conjunctiva [53]. However, as a suggestion for future research, a longer experimental drug treatment with and without preservatives could provide more information on the intensity of the lesions evaluated in this study.

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