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Research paper

Postoperative Use of Cyclosporine, Prednisolone or a Cyclosporine/Prednisolone Combination to Treat Inflammation of the Conjunctiva Following Trabeculectomy in Rabbits

ABSTRACT

OBJECTIVES: The objective of this investigation was to compare different topical treatment protocols for controlling inflammation in rabbits during the postoperative period following trabeculectomy. **METHODS:** Thirty rabbits had their right eyes treated with a daily eye drop of 0.03% bimatoprost for 30 days. They then underwent trabeculectomy and were divided into three groups (G1, G2 and G3). Rabbits from G1 received prednisolone acetate 1.0% four times a day, G2 received cyclosporine 0.05% twice a day and G3 received both prednisolone acetate 1.0% four times a day and cyclosporine 0.05% twice a day. Seven days after the surgery all the rabbits were euthanized and their eyes enucleated. Histomorphometry was performed in HE-stained sections to evaluate epithelial thickness, blood vessel diameter and vascular area. Immunohistochemical staining using monoclonal antibodies against smooth muscle actin, factor VIII and IL-6 was performed to evaluate fibrosis, neovascularization and inflammatory infiltrate, respectively. **RESULTS:** In group G3 there was a reduction in the thickness and diameter of the conjunctival epithelium and the diameter and number of blood vessels and less inflammatory infiltrate and fibrosis. **CONCLUSION:** This study suggests that a combination of prednisolone and cyclosporine is more efficient than these drugs on their own in controlling inflammation of the conjunctiva in rabbits in the postoperative period following trabeculectomy.

Keywords: conjunctiva, glaucoma, drug therapy, histology, histomorphometry, immunohistochemistry.

29 INTRODUCTION

30

31 Glaucoma is a multifactorial progressive optical neuropathy characterized by structural
32 changes in the optic disc and frequently accompanied by changes in the field of vision that requires
33 treatment over a long period with topical hypotensive drugs (1,2). Various classes of drugs are
34 available to treat this disease, including cholinergics, beta blockers, alpha-adrenergic agonists, carbonic
35 anhydrase inhibitors and, more recently, prostaglandin analogs (3).

36 In patients treated with topical drugs, the conjunctiva acts as a semipermeable membrane,
37 allowing these active ingredients to be absorbed and exert their action within the eye. However, when
38 the topical treatment is chronic, the conjunctiva responds with the formation of scar tissue,
39 keratinization and neovascularization, which can change the shape of the membrane and the way it
40 operates (4). The intensity of these effects depends on the length of treatment as well as the
41 concentration and amount of drugs used (5).

42 There is solid evidence that these changes on the surface of the conjunctiva can increase the
43 risk of surgical failure in glaucoma patients who undergo filtering surgery (5,6,7).

44 Broadway et al. (5) identified subclinical conjunctival inflammation due to prior topical
45 therapy as a risk factor for unsuccessful trabeculectomy. The reduction in the success rate for glaucoma
46 filtering procedures can probably be attributed to the activation of fibroblasts in the conjunctiva,
47 Tenon's capsule and episclera, leading to scar tissue formation around the scleral flap (8,9,10). In the
48 present study, a preoperative protocol consisting of topical bimatoprost (0.03%) was used to simulate
49 conditions that adversely affect filtering surgery.

50 Trabeculectomy is the most frequently used procedure for glaucoma. In the first step of the
51 technique a flap is cut in the conjunctiva and Tenon's capsule. The base of this flap can be at the
52 corneal limbus or conjunctival fornix. After the conjunctiva is dissected, the scleral bed is exposed. A
53 limbus-based scleral flap is then made and a portion of the trabecular meshwork and edge of the cornea
54 is removed to allow greater outflow of aqueous humor after surgery. Next, the scleral flap is sutured
55 with nonabsorbable thread in a simple interrupted pattern, and the conjunctiva and Tenon's capsule are
56 sutured with absorbable thread in a continuous pattern (11).

57 In the postoperative period following trabeculectomy, tissue repair processes take place, as in
58 any surgical procedure. These are modulated by inflammatory mediators (histamine, serotonin,

59 prostaglandin, leukotrienes and other kinins) that cause cells such as neutrophils, monocytes,
60 macrophages and fibroblasts to migrate to the healing area. While this process is desirable in other
61 surgical procedures, in glaucoma filtering surgery the fistula should be kept clear to allow the aqueous
62 humor to drain and, consequently, the intraocular pressure (IOP) to fall (12).

63 To avoid the proliferation of fibroblasts and subconjunctival fibrosis, which cause the fistula
64 to close in filtering surgery, antimetabolites are used to reduce the rate of cell mitosis (13,14).
65 Mitomycin C (MMC), an antibiotic produced from fermentation of the fungus *Streptomyces*
66 *caespitosus*, which acts in all stages of the cell cycle, and 5-fluorouracil (5-FU), an analog of
67 pyrimidine that is incorporated into the DNA chain and prevents synthesis only in the S phase of the
68 cell cycle, are the most commonly used drugs (15,16).

69 The use of these drugs increases the chances of continued patency of the fistula but also
70 increases the likelihood of complications related to hypotonia and endophthalmitis (11,17,18).

71 Cyclosporine is an immunomodulatory drug that also has antimetabolic properties. It can
72 control in vitro cell proliferation and has been studied in vivo in the postoperative period following
73 trabeculectomy for some years (19,20).

74 Corticosteroids act by suppressing synthesis of inflammatory mediators and, consequently,
75 reducing the number of leukocytes and fibroblasts at the inflammation site (13). To date, the effect of
76 these drugs in trabeculectomy has not been fully elucidated, but studies have shown that their topical
77 use preoperatively and postoperatively prolongs the reduction in IOP and increases surgical success
78 rates (13,15,21).

79
80 This study sought to compare three different post-trabeculectomy protocols based on
81 cyclosporine and a corticosteroid (prednisolone acetate 1%, cyclosporine 0.05% and a combination of
82 prednisolone and cyclosporine at these concentrations) by evaluating the subsequent inflammatory
83 reaction.

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89 MATERIAL AND METHODS

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91 Thirty female New Zealand rabbits with similar ages (5 months) and weights (1.7kg) were
92 treated with bimatoprost (0.03%) eye drop for thirty days. One drop of the eye drop was instilled in the
93 right cornea of each animal, and the left eye was used as a control. The eye drop used contains
94 benzalkonium chloride (0.005%) as a preservative. On the thirtieth day trabeculectomy was performed
95 without antimetabolites on all the animals by the same surgeon. The rabbits were then divided
96 randomly into three groups of ten animals (G1, G2 and G3).

97 The three postoperative protocols were as follows: prednisolone acetate 1% four times a day
98 (G 1); cyclosporine 0.05% twice a day (G 2); and a combination of prednisolone acetate 1% four times
99 a day and cyclosporine 0.05% twice a day (G 3). All the groups were given antibiotic therapy with
100 gatifloxacin (0.03%) eye drops four times a day. After one week, the animals were euthanized. The
101 eyeballs were immediately fixed in 10% formaldehyde for 24 hours. After fixation, the eyes were
102 sectioned, and the sections were dehydrated in a graded alcohol series (up to absolute alcohol), cleared
103 with xylol and embedded in paraffin (Bacha Jr. and Wood, 1991). Sections cut from the paraffin block
104 with a microtome were then stained with HE (hematoxylin-eosin) and assessed by
105 immunohistochemistry using monoclonal antibodies against the following markers: factor VIII, smooth
106 muscle actin and IL-6.

107 Histomorphometric analysis was performed with Image Pro-Plus version 4.5 software (Media
108 Cybernetics®, Silver Spring, MD). Digital images were captured at 400x magnification and stored
109 using the same program. Two points corresponding to the bulbar conjunctiva closest to the corneal
110 limbus were selected on each slide. After the images had been captured, two segments of the
111 conjunctival epithelium 200 µm long and 5 µm thick were selected at random from each photograph.
112 By measuring linear segments of the same size, the diameter of the blood vessels and the thickness of
113 the conjunctival epithelium could be determined with the aid of virtual tools included in the program.

114 Immunohistochemical analysis was carried out with images captured at 400x magnification
115 using the same software. A virtual grid 150 X 200 µm was applied to each image and included the
116 epithelium and lamina propria. The three subfields closest to the epithelium were then examined to
117 allow a more specific analysis of the subepithelial region of the conjunctiva rather than the conjunctival
118 lamina propria on each slide. The anti-actin antibody revealed the degree of inflammatory fibrosis

(fibroblasts marked by the antibody). This parameter was evaluated using qualitative categorical variables, and numbers were assigned according to the staining intensity of the marker (1: minimum; 2: mild; 3: moderate; and 4: strong) in the histological section for each 400x field. This analysis was carried out twice for each slide. Factor VIII is a marker of vascular endothelial cells and therefore of vessel walls in the conjunctival tissue. The degree of neovascularization was determined by counting the number of marked blood vessels in each 400x field. Two counts were taken for each slide. The degree of inflammatory infiltrate was determined by counting the areas stained with anti-interleukin-6 antibody in the histological section. This was also performed for each 400x field and carried out twice for each slide. The person responsible for reading the slides was blinded to the identities of the groups. The parameters were measured twice by the same investigator, and the coefficients of variability between the two sets of readings were low.

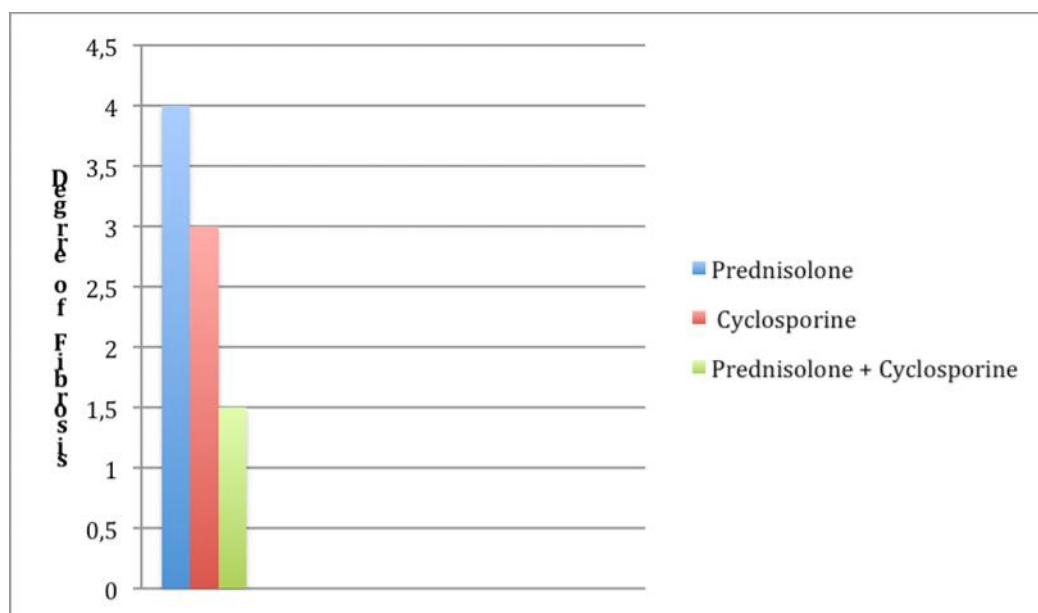
The categorical variables were analyzed with Fisher's exact test and presented as medians. One-way ANOVA with a 5% significance level was used to compare the continuous variables and was followed by Fisher's post-test (for comparisons between up to three groups) or the Tukey-Kramer test (for comparisons between more than three groups). Statistical significance was defined as $p < 0.05$. Continual variables were presented as means $\pm$ standard deviations. The experimental procedures followed the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the study was approved by the Commissions for Animal Welfare at São Paulo University and Hospital Evangélico, Curitiba, (CAPESQ no. 161/05).

RESULTS

In the histomorphometric assessment, the group treated with prednisolone had a thicker conjunctival epithelium ($P=0.03$); the means for the prednisolone, cyclosporine and prednisolone+cyclosporine groups were $29.3 \pm 10.27 \mu\text{m}$; $25.1 \pm 5.16 \mu\text{m}$; and 24.4 ± 3.81 , respectively. The diameters of the blood vessels were significantly larger in the animals treated with prednisolone ($30.3 \pm 11.28 \mu\text{m}$) or cyclosporine ($27.7 \pm 10.90 \mu\text{m}$) ($P=0.0011$ and $P<0.0001$, respectively) than in the group treated with a combination of the two drugs ($19.8 \pm 6.9 \mu\text{m}$) (Table 1 and Figure 1).

148 Fibrosis was also less intense in the group that received the combination treatment (median 1.5)
149 than in the groups treated with prednisolone (median 4.0) or cyclosporine (median 3.0) on their own
150 ($P<0.001$) (Graph 1).

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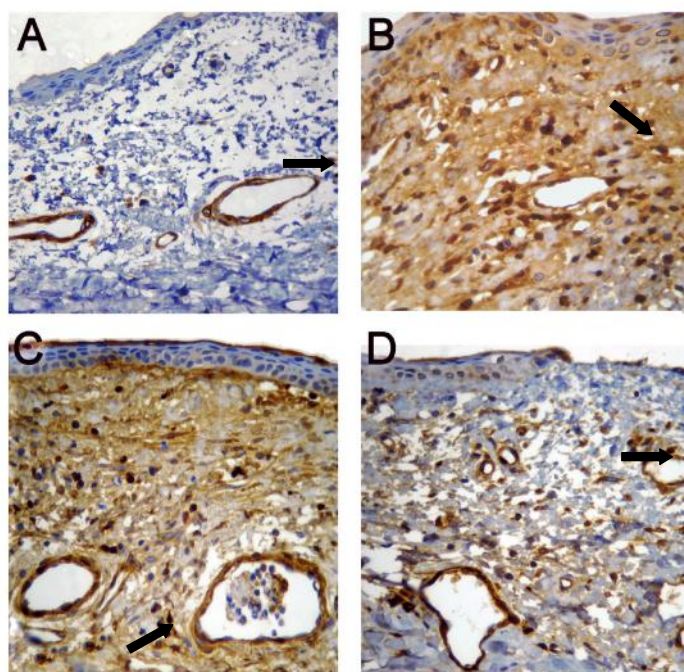
154 Graph 1 - Comparison of the degree of conjunctival fibrosis in the three treatment groups:
155 prednisolone 1.0% (G1), cyclosporine 0.05% (G2) and prednisolone 1.0% with cyclosporine 0.05%
156 (G3). Note the less intense fibrosis in the group that received the combination treatment.
157 Immunohistochemical assessment using anti-actin antibody and discrete numeric values (1: minimal; 2:
158 mild; 3: moderate; and 4: intense).

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 164 Figure 1 – Photomicrographs of conjunctivae (400x magnification) stained with anti-actin antibody.
 165 Subepithelial fibroblasts can be seen for the control group (A) and groups G1 (B), G2 (C) and G3 (D).
 166 Note the smaller number of fibroblasts in the eye treated with cyclosporin+prednisolone (D) than in the
 167 eye treated with prednisolone alone (B).

168
 169 Factor VIII revealed less vascularization in response to the inflammatory process in G3 (8.07)
 170 than in G1 (17.50) or G2 (11.86) ($P<0.001$) (Table 1 and Figure 2).

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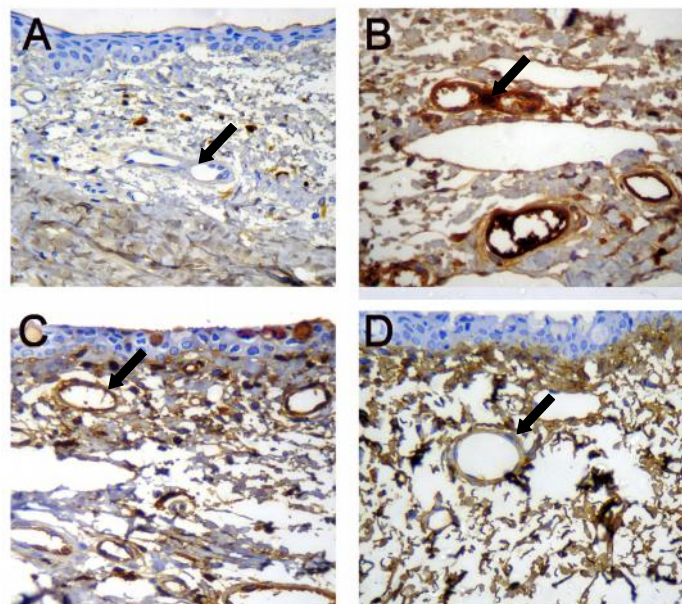


Figure 2 – Photomicrographs of conjunctivae (400x magnification) stained with anti-factor VIII antibody. The endothelial cells in the blood vessels formed in response to the inflammatory process can be seen for the control group (A) and groups G1 (B), G2 (C) and G3 (D). Note the greater number of vessels in the eye treated with prednisolone alone (B) than in the eye treated with prednisolone+cyclosporine (D).

In the immunohistochemical assessment using anti-interleukin 6, less inflammatory infiltrate was observed in G3 (6.60) than in G1 (27.37) ($P=0.0005$) or G2 (13.20) ($P<0.001$) (Table 1 and Figure 3).

	Thickness of the conjunctiv al epithelium µm	P	Diameter of the blood vessels µm	P	Vascula rization (no. of vessels)	P	Anti- interleuki n 6	P
Prednisolon e (G1)	29.3 ± 10.27	P<0.03	30.3 ± 11.28	P<0.0011	17.50	P<0.001	27.37	P=0.0005
Cyclosporin e (G2)	25.1 ± 5.16		27.7 ± 10.90	P<0.0001	11.86	P<0.001	13.20	P<0.001
Prednisolon e + Cyclosporin e (G3)	24.4 ± 3.81		19.8 ± 6.9		8.07		6.60	

192

193 Table 1- Comparison of the parameters measured for the three treatment groups: prednisolone 1%
194 (G1), cyclosporine 0.05% (G2) and prednisolone 1.0% with cyclosporine 0.05% (G3).

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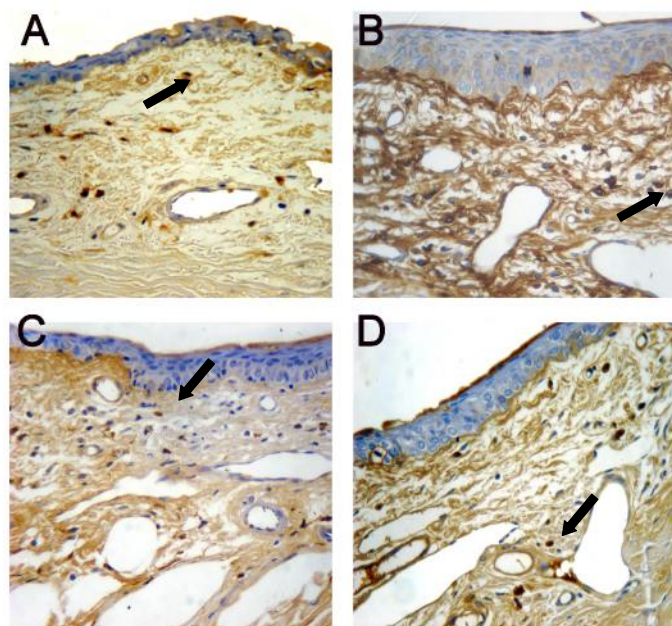


Figure 3 – Photomicrographs of conjunctivae (400x magnification) stained with anti-interleukin-6 antibody. Inflammatory subepithelial cells can be seen for the control group (A) and groups G1 (B), G2 (C) and G3 (D). Note the smaller number of inflammatory cells in the eye treated with cyclosporin+prednisolone (D) than in the eye treated with prednisolone alone (B).

DISCUSSION

Trabeculectomy has been used to control glaucoma for over thirty years. It has a relatively high success rate and is still the most commonly used incisional technique to treat the disease (5,18).

Many clinical and experimental studies have reported strong evidence that chronic use of topical glaucoma drugs can induce changes in the conjunctival surface, causing discomfort during instillation, conjunctival inflammation, tear film instability, subconjunctival fibrosis, apoptosis of conjunctival epithelial cells and changes in the corneal surface (22,23,24,25). Some researchers suggest that these changes result in a worse prognosis for trabeculectomy in patients who had previously been receiving topical glaucoma therapy (5,10,26,27,28). In clinical, laboratory and experimental studies, many of these conjunctival changes have been associated not only with glaucoma drugs themselves but also with benzalkonium chloride (BAK), the most commonly used preservative in topical ophthalmic preparations (6,19).

218 In the present study, previous treatment with bimatoprost, in which BAK is used as the
219 preservative, resulted in side effects in the conjunctiva. These have already been reported by other
220 authors and include changes in epithelial thickness, conjunctival inflammatory infiltrate,
221 vascularization and fibrosis. In another study by the same group of authors these changes were more
222 apparent in the bimatoprost group than in groups using other ophthalmic preparations (25,29). The
223 magnitude of these changes was modulated at different levels by the action of cyclosporine and
224 prednisolone.

225 The most frequent cause of failure in trabeculectomy is the formation of episcleral
226 fibrovascular tissue around the surgical flap (30). The inflammation that can cause this tissue to adhere
227 to the flap is prevented with the aid of antimetabolites, which act by inhibiting DNA synthesis and
228 reducing fibrosis, thus ensuring surgical patency and controlled IOP (13,21).

229 The use of antimetabolites, however, can also cause side effects, such as infection (blebitis
230 and endophthalmitis) and leakage from the bleb, leading to an excessive drop in IOP and loss of visual
231 acuity (18,31). Some studies suggest that these complications are less severe in patients using 5-
232 fluorouracil but that there is still a drop in IOP (17). MMC and 5-FU are the most frequently studied
233 drugs, but cyclosporine has also been the subject of research. Cristofanilli et al. (20) showed that
234 cyclosporine was able to control cell proliferation by inducing in vitro apoptosis, suggesting that it
235 could be of benefit in post-trabeculectomy antifibrosis therapy.

236 Cyclosporine acts by inhibiting genetic transcription of some interleukins (IL-2, IL-3, IL-4
237 and IL-6) and interferon- γ , thus modulating the immune response. It also has an effect on non-immune
238 cells, such as endothelial cells, keratinocytes, fibroblasts and mast cells. Recent studies have shown
239 that cyclosporine induces a reduction in fibroblast proliferation in the conjunctival mucosa in rabbits
240 (32,33). This inhibition of the proliferation of fibroblasts, endothelial cells and inflammatory cells such
241 as mast cells explains the smaller degree of fibrosis, vascularization and inflammatory infiltrate
242 observed both in the group that received cyclosporine on its own and in the group that received the
243 combination of cyclosporine and prednisolone.

244 Because of these properties, cyclosporine could also be used as an antifibrotic drug in
245 trabeculectomy. Turacli et al. (15), in a randomized prospective study, suggested that cyclosporine is
246 both an effective and safe antimetabolite for use in trabeculectomy. The same group of researchers also
247 carried out another study comparing the effects of mitomycin and cyclosporine, in which they assessed

248 86 patients (30 in the mitomycin group, 28 in the cyclosporine group and 28 controls). IOP was
249 controlled in both treatment groups (mitomycin and cyclosporin) with similar success rates (90% and
250 87.5%, respectively), suggesting that cyclosporine can also be used as an antimetabolite in
251 trabeculectomy and leads to fewer complications.

252 Corticosteroids also inhibit the inflammatory response but through different mechanisms, one
253 of the main ones being the inhibition of phospholipase, thus preventing the conversion of membrane
254 phospholipids into arachidonic acid and then into leukotrienes and prostaglandins (chemotactic factors
255 that attract inflammatory cells). This reduction in the amount of inflammatory mediators, together with
256 the decrease in vascular permeability caused by the drug, reduces the concentration of lymphocytes,
257 monocytes and basophils and leads to a fall in macrophage activity at the inflammation site. This
258 cascade of events inhibits mitotic activity, the release of growth factors and the production of fibrin in
259 the lesion, culminating in a reduction in fibroblast activity and, therefore, inflammatory healing.

260 The postoperative use of corticosteroids after eye surgery has been routine for several
261 decades. However, the beneficial effects of using corticosteroids only started to be studied in the '80s.
262 Studies in which patients who had undergone trabeculectomy were followed up for more than ten years
263 showed that better control of IOP and parameters associated with the optic disc and field of vision were
264 achieved in patients who used topical corticosteroids for long periods (13).

265 Subsequent studies began to investigate the effectiveness of corticosteroids in controlling
266 preoperative inflammation of the conjunctiva caused by prior drug therapy. Broadway et al. (5) used
267 topical corticosteroids post-trabeculectomy and concluded that they were ineffective in suppressing
268 subclinical inflammation caused by glaucoma therapy that these patients had already been receiving.
269 Two years later, however, the same group of researchers used a protocol based on topical corticosteroid
270 therapy (fluorometholone 1%) prior to trabeculectomy and, although subclinical inflammation had
271 already developed as a result of the glaucoma therapy, observed a significant reduction in the number
272 of macrophages and lymphocytes in the conjunctival epithelium and the number of macrophages,
273 lymphocytes, mast cells and fibroblasts in the substantia propria of the conjunctiva (34). As the
274 antiproliferative properties of corticosteroids take time to have an effect, some authors recommend that
275 they be administered preoperatively so that they can be more effective in the postoperative period (35).
276 Prednisolone 1.0% is quite effective for this purpose but, like other corticosteroids, can lead to
277 cataracts and infections, particularly keratitis (13).

278 Our study showed that the inflammatory parameters assessed here by histomorphometry
279 (epithelial thickness and diameter of the blood vessels) were less intense for the group treated with
280 cyclosporine alone than for the group treated with prednisolone alone. The same was true of the
281 parameters assessed by immunohistochemistry (degree of inflammatory infiltrate and fibrosis and the
282 number of vessels formed in response to the inflammatory process). All the parameters assessed had
283 lower mean values for treatment with the drugs on their own than for treatment with the
284 cyclosporine+prednisolone combination, suggesting that these drugs act synergistically to reduce the
285 inflammatory response induced by topical therapy in the postoperative period following
286 trabeculectomy.

287

288 **CONCLUSION**

289 The synergistic action of cyclosporine and prednisolone appears to be beneficial and to promote a
290 greater reduction in conjunctival inflammation, fibrosis and vascularization.

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294

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295

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296

c. Contribution to authors: Design of the study (NVF,HHR,FM), Conduction of the study (NVF,PR),

297

analysis and interpretation (NVL,HHR,FM), writing the article (NVF,PR,HHR), critical revision of the

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article (HHR,SC), final approval of the article (NVF,HHR,SC,PAM) Provision of materials and

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resources (NVF,HHR), statistical expertise (FMF), obtaining funds (NVF,HHR),literature search

300

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