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Induced Corneal Ulcers in Cats - Effects of 2% Dorzolamide on Epithelization Time and on the Expression of Matrix Metalloproteinase-9

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ABSTRACT

***Background*:** Topically administered 2% dorzolamide is among the most commonly used agents to lower IOP. As a complication of glaucoma, blind patients may develop corneal ulcers secondary to trauma. Nonetheless, in patients with a hypertensive or glaucomatous eye, in which the cornea has also been ulcerated, medical hypotensive therapy should not be discontinued. Therefore, the present study aimed to determine whether the instillation of a benzalkonium chloride (BAK)-preserved 2% dorzolamide alters corneal wound healing time and the levels of matrix metalloproteinases (MMP-9) in the tears of cats with experimentally induced corneal ulcers.

***Materials, Methods & Results*:** Sixteen cats (8/group) were randomly assigned to receive 40 µL of 2% dorzolamide (TG) or saline (CG) 3 times daily until corneal re-epithelialization. Experimental keratectomies were performed under general and topical anesthesia using an operating microscope. For this purpose, a millimitred trephine was calibrated and used to create a temporal paraxial corneal ulcer with a diameter of 6 mm and a depth of 200 µm. After corneal wounding, the ulcerated area, the healing time, blepharospasm, conjunctival hyperemia, and aqueous flare were compared between groups. Tears were collected at baseline and 24 and 48 h after keratectomy, and the total MMP-9 was quantified by ELISA. Data were assessed statistically using unpaired Student's *t* test, one-way, and two-way ANOVA followed by a Bonferroni post hoc test. Statistical significance was set at $P < 0.05$ for all analyses. The average time to achieve corneal wound healing did not differ between groups ($P = 0.36$) and was 65.50 ± 3.62 h in the CG and 71.00 ± 4.58 h in the TG. Twenty-four h after keratectomy, the ulcerated area in the CG was 3.34 mm^2 larger than that observed in the TG ($P = 0.04$); the rest of the comparisons did not reach statistical significance at any time point between groups ($P > 0.05$). Higher blepharospasm scores were observed in cats of TG ($P = 0.04$). When compared with baseline of both groups, the levels of MMP-9 increased significantly at 24 and 48 h post-keratectomy ($P < 0.001$), but differences between groups were not observed at 24 and 48 h post-keratectomy ($P > 0.05$).

***Discussion*:** In cats, 9 mm axial corneal ulcers created by superficial debridement re-epithelize approximately 48 h post-wounding. In the present study, re-epithelialization post keratectomy occurred within an average time of 68.25 h in most cats and in a delayed manner in one cat of the TG after 96 h. In the current study, the lesions in both groups healed without corneal scarring, pigmentation, or vascularization. Although BAC was present in all topical medications used in the present study, the authors attribute the higher scores of blepharospasm in the TG to the rheological characteristics and the pH of the dorzolamide ophthalmic solution. Indeed, the pH value of dorzolamide (5.58) may cause signs of irritation, as the tear film has an approximate pH of 7.6. Previous studies showed that ulcerated corneas presented significantly higher levels of MMP-9 in tears at the early stages (8 to 36 h) post-wounding. In the current study, the levels of this enzyme after wounding did not change significantly in the tears of cats treated with 2% dorzolamide when compared to the eyes in the control group. This study showed that the instillation of a BAC-preserved 2% dorzolamide ophthalmic solution did not impair the corneal wound healing time or the early expression of MMP-9 in the tears of cats with experimentally induced corneal ulcers. However, our results warrant further investigation in patients with ocular hypertension or glaucoma presenting concomitant naturally occurring corneal ulcers to certify our findings.

Keywords: carbonic anhydrase inhibitor, benzalkonium chloride, ulcerative keratitis, corneal healing, glaucoma.

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INTRODUCTION

Topical dorzolamide significantly decreases IOP in healthy and glaucomatous individuals [1,5,9,10,13,17,18,20,23-26]. As a complication of glaucoma, blind patients may develop corneal ulcers secondary to trauma, and medical hypotensive therapy should not be discontinued [13,20]. It has been reported the occurrence of corneal ulcers in the early postoperative period in nearly 27% of cats subjected to phacoemulsification [2]. In this same study, the authors described that medical hypotensive therapy was maintained until corneal re-epithelialization in cats who presented corneal ulcers associated with post-operative ocular hypertension [2].

In humans, ocular adverse reactions reported with the use of benzalkonium chloride (BAC)-preserved dorzolamide include a higher oxidative stress index of the tear film, superficial punctate keratitis, corneal endothelium and epithelium decompensation, and increased central corneal thickness [1,5,9,10,23,26]. Systemic adverse effects have also been reported in humans and cats receiving topical 2% dorzolamide [7,14,26]. An *in vitro* study showed that the combination of BAC-preserved dorzolamide/0.5% timolol decreases the viability of human corneal epithelial cells in culture after 30 min of exposure [4]. Thus, we hypothesize that BAC-preserved 2% dorzolamide could impair corneal wound healing time as a possible short-term adverse effect. Therefore, the aims of the current study were to determine whether the instillation of 2% dorzolamide 3 times daily alters corneal wound healing time and the early expression of MMP-9 in the tears of cats with experimentally induced corneal ulcers.

MATERIALS AND METHODS

Animals

A total of 16 adult domestic shorthair cats were enrolled in this study, with the following averages: weight = 3.27 kg (2.35 - 4.04 kg) and age = 4.0 years old (1.5 - 4.10 years). Healthy animals were selected if no abnormalities were detected after a full clinical, ophthalmic, and hematological exam. Selected cats were kept in individual cages in a room for 2 consecutive days and acclimated to the procedures and staff. Furthermore, they were exposed to a 12 h light/dark cycle, fed dry cat pellets twice daily, and provided water *ad libitum*.

Procedures and study design

Cats were premedicated with a mixture of ketamine[1] [Cetamin® - 8 mg/kg, intramuscular route (IM)], midazolam2 [Dormire® - 0.2 mg/kg, IM], and tramadol[2] [Tramal® - 2 mg/kg, IM]. General anesthesia was induced with intravenous propofol[2] [Propovan® - 5 mg/kg, IV], and anesthesia was maintained with inhaled isoflurane[2] [Isoforine® - inhalant route] in 100% oxygen.

The procedure was performed in the right eye using an operating microscope[3] [SM Novus®] by a single surgeon. All eyes were rinsed with 1:40 diluted betadine solution, and 1 drop of proxymetacaine[4] [Anestalcon®] was instilled into the ocular surface. A millimitred trephine[5] was calibrated and used to create a temporal paraxial corneal ulcer with a diameter of 6 mm and a depth of 200 µm. Subsequently, the corneal lamella was removed with the aid of 69 mini Beaver scalpel[6] and corneal scissors. The ulcerate area measured in pixels was converted to mm^2. The conversion of the wound size from pixels to mm^2 was calculated using the following formula: $Dh \times 6mm^2/D0$, where *D0* is the dimension of the ulcerated wound at time point 0, and *Dh* is the dimension of the wound area at the next time points (hours) until complete epithelialization.

At the end of keratectomy and after 24, 36, and 48 h with evaluations every 4 h thereafter, fluorescein was instilled in the corneas and examined with a slit lamp and cobalt filter[7] [SL-15®] by the same blinded examiner. Images were taken with a cellular phone camera[8] [Iphone 6S®] at a fixed distance of 18 cm until no fluorescein uptake by the cornea was observed. The dimensions of the ulcerated areas were measured using image analysis software[9] [ImageJ], by another observer blinded to the treatment used in that eye.

Clinical signs were evaluated in all cats at the same time point that tears were collected by grading blepharospasm, conjunctival hyperemia, and aqueous flare as absent (0), mild (1), moderate (2), or severe (3) [Table 1]. To facilitate the interpretation, only an average score was reported. When the wounds showed no fluorescein uptake, the appearance of the corneal scar tissue was evaluated clinically to check for the occurrence of scarring, pigmentation, and vascularization. After healing, the occurrence of corneal scarring, vascularization, and pigmentation were interpreted as absent (0), mild (1), moderate (2), or severe (3) [Table 1].

Table 1. Description of the scales used for assessing the extent of clinical signs.

	Absent (0)	Mild (1)	Moderate (2)	Severe (3)
Blepharospasm	Eyelids held completely open and no tear impregnation in the eyelids	Eyelid is partially closed so that the palpebral opening is decreased by 1/3	Eyelid is partially closed so that the palpebral opening is decreased by 2/3	Eyelid is completely closed
Aqueous flare	Clear aqueous humor. Normal anterior chamber (AC)	The intensity of the light beam in the AC is less than the intensity of the slit beam as it passes through the lens.	The slit lamp beam in the AC is equally opaque compared with the beam passing through the normal lens.	The slit beam in the AC is more opaque than that passing through the lens.
Conjunctival hyperemia	No ingurgitation of conjunctival vessels	A flushed, reddish color predominantly confined to the palpebral conjunctiva with some perilimbal injection	The palpebral conjunctiva appears bright red with accompanying perilimbal injection covering at least 75% of the circumference of the perilimbal region	Both the bulbar and palpebral conjunctiva exhibit a dark, beefy red color with pronounced perilimbal injection.
Corneal scar	Clear and transparent	Slight cloudiness allowing the visualization of the AC	Slight white plaque decreasing the visualization of structures in the AC	Completely withe plaque preventing the visualization of structures in the AC
Corneal vascularization	Clear and transparent	One or two branches of vessels migrating into the axial corneal region	Three or five branches of vessels migrating into the axial corneal region	Several branches of vessels migrating into the axial corneal region
Corneal pigmentation	Clear and transparent	Slight melanocyte dispersion allowing the visualization of the AC	Slight dark cluster decreasing the visualization of structures in the AC	Completely dark spot preventing the visualization of structures in the AC

Treatments

At the end of keratectomy, the wounded eyes received one drop of 0.3% tobramycin[4] [Tobrex® - QUID] until the corneas became fluorescein negative. One drop of 1% atropine sulfate[10] [Atropina 1% - SID] was also administered during the first 48 h. The animals were randomly allocated to 2 different groups (8 cats per group). The eyes of cats in the treatment group (TG), received 2% dorzolamide eye drops[11] [Cloridrato de dorzolamida 2%® - 40 µL, TID], while the eyes of cats in the control group (CG) received saline at the same dose regimen and time points adopted for TG. These treatments were applied 5 min after the administration of the antibiotic and mydriatic medications listed above. For pain management, individuals in both groups received meloxicam[12] [Maxicam® - 0.1 mg/kg, subcutaneous route, SID], for 3 consecutive days.

Tear collection

In both groups, tears were collected before and 24 and 48 h after keratectomy. Sampling occurred between 9 am and 10 am to avoid diurnal changes in tear volume. To collect the tears from the cats, a Schirmer tear test strip[13] [Teste de Schirmer®] was placed in the ventral fornix until moistening reached the 20 mm mark of the strip [22]. This procedure was repeated in 2 separate sessions with a 10 min interval between them. For the extraction of tears, the Schirmer strips were placed in a 0.2 mL microtube with a fenestrated bottom. The fenestrated tube was then placed within another tube (2 mL), which collected the residual fluid. The tubes were centrifuged at 3881 *g* for 1 min, and the samples were stored at -80°C until further analysis.

Quantification of MMP-9 in tears

Tear samples were thawed at room temperature, and a commercial ELISA kit[14] [Matrix Cat®]

was used to quantify the total MMP-9 (latent and active) according to the manufacturers' protocols. Tear samples were measured in duplicate with no dilution. The absorbance was read at 450 nm, and the values were converted to ng/mL by 4-parameter logistic extrapolation. The lower and higher detection limits of the assay were 6.25 and 200.00 ng/mL of total MMP-9, respectively.

Statistical analysis

Statistical analysis was performed using Statistical software[15] [Prism version 7.04®]. Data were confirmed to be normally distributed using the Shapiro-Wilk test. Unpaired Student's t test was used to assess whether there were differences between groups in the time for complete corneal wound healing, in the re-epithelialization rate, and in the parameters related to comfort. The dimensions of the ulcerated area were compared between groups using a two-way analysis of variance followed by a Bonferroni post hoc test. The levels of MMP-9 in tears collected at baseline and at 24 and 48 h were compared within the same group using an analysis of variance (ANOVA) for repeated measures, followed by a Bonferroni post hoc test. To compare the levels of MMP-9 between groups, a regular ANOVA followed by a Bonferroni post hoc test was used. Statistical significance was set at $P < 0.05$ for all analysis. The results are shown as the mean ± standard error of the mean (SEM).

RESULTS

Dimensions of the ulcerated areas and clinical signs

Regardless of therapy, healing occurred in 2 distinct phases: an initial rapid phase with an average duration of 52 to 56 h and a second, slower phase with a variable average duration of 60 to 96 h (Figure 1). The only significant difference with regard to the dimensions of the ulcerated areas was observed in the first 24 h of evaluation, with the area of the CG being 3.34 mm^2 larger than that observed in the TG (P = 0.04); the rest of the comparisons did not differ at any time point between groups (P > 0.05) [Figure 1].

The corneal re-epithelialization rate did not differ between groups (P = 0.99; 95% confidence interval: -0.3062 to 0.3078), being 0.48 ± 0.13 mm^2/h in the CG (ranging from 0.21 to 1.38 mm^2/h) and 0.48 ± 0.05 mm^2/h in the TG (ranging from 0.33 to 0.77 mm^2/h). Similarly, the average time to achieve corneal wound healing did not differ between groups (P = 0.36; 95% confidence interval: -7.027 to 18.03) and was 65.50 ± 3.62 h in the CG and 71.00 ± 4.58 h in the TG (Figure 2).

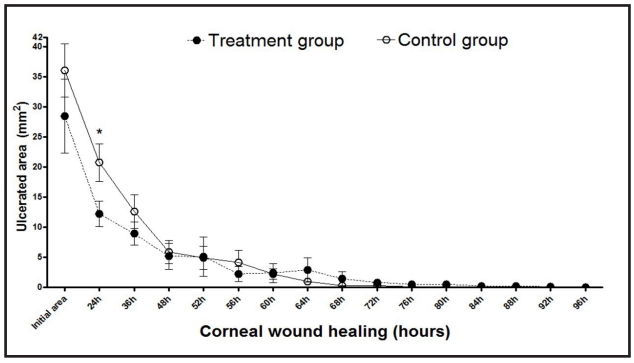

Figure 1. Mean ± SEM* of relative ulcerated area size after fluorescein instillation in control and treatment groups. *Bonferroni's test (P = 0.04).

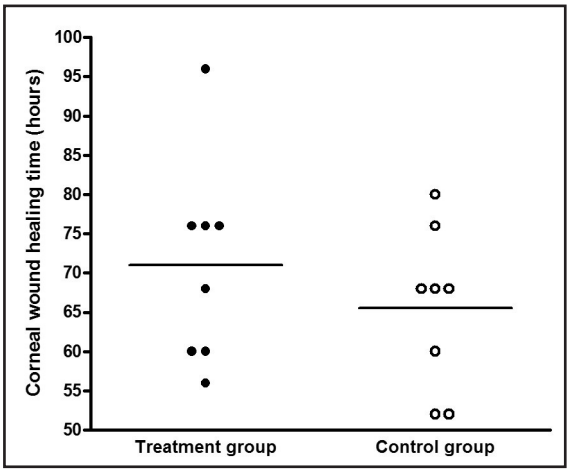

Figure 2. Time to achieve corneal re-epithelialization in the control and treatment groups. The line represents the mean, and the circles refer to the individual values. *Unpaired t-test (P = 0.36).

The average blepharospasm score was less than mild in both groups. However, significantly higher scores were observed in cats of TG (0.82 ± 0.17) in comparison with the cats of CG (0.27 ± 0.18) [P = 0.04, 95% confidence interval: 0.003882 to 1.096]. Such an event was observed immediately after the instillation of dorzolamide. Conjunctival hyperemia was absent in the cats with GC and less than mild (0.5 ± 0.75) in the cats with TG (P = 0.08; 95% confidence interval: -1.061 to 0.08640). In those evaluations, clinical signs lasted only until corneal re-epithelialization and cessation of

dorzolamide and the other topical medications. In both groups, corneas healed without developing scarring, pigmentation or vascularization.

MMP-9 in tears

The baseline values of MMP-9 in the eyes of the CG and TG were 18.09 ± 0.92 and 19.09 ± 0.96, respectively (P = 0.61; 95% confidence interval: -9.160 to 10.16). When compared with baseline of both groups, the levels of MMP-9 increased significantly at 24 and 48 h post-keratectomy ($P < 0.001$; 95% confidence interval: -54.03 to -34.86) [Table 2]. At 24 h post-keratectomy, the levels of MMP-9 were 6.09 ± 2.62 ng/mL higher in the CG than in the TG at the same time point. At 48 h, the levels of MMP-9 decreased by 6.40 ng/mL in the tears of cats with GC, but statistical significance was not observed at any of these time points ($P > 0.05$; 95% confidence interval:). In the TG, a slight increase in the levels of this enzyme was observed from 24 to 48 h, but statistical significance was not reached within and between groups at this time point ($P > 0.05$) [Table 2].

Table 2. Tear MMP-9 (ng/mL) obtained before, 24 and 48 h post-keratectomy in cats of the control and treatment groups.

Groups	Before	24 h	48 h	*P**
Control				
Men ± SEM	18.09 ± 0.92*	69.55 ± 1.43	63.11 ± 3.46	< 0.001
(Range)		(65.63 - 75.74)	(47.68 - 74.84)	
Treatment				
Men ± SEM	19.09 ± 0.96*	63.46 ± 6.20	65.26 ± 2.87	< 0.001
(Range)		(56.95 - 75.26)	(54.59 - 78.28)	
P between groups	0.61	0.31	0.12	

*Before and post-keratectomy time points.

DISCUSSION

In cats, 9 mm axial corneal ulcers created by superficial debridement re-epithelize approximately 48 h post-wounding [16]. In the present study, re-epithelialization post keratectomy occurred within an average time of 68.25 h in most cats and in a delayed manner in 1 cat of the TG after 96 h. In the current study, the lesions in both groups healed without corneal scarring, pigmentation, or vascularization. From our results, we observed that thrice-daily instillation of 2% dorzolamide did not decrease the corneal wound healing time in cats. In Brazil, a BAC-preservative free 2% dorzolamide ophthalmic solution is not available on the market. This was responsible for one of the limitations of the present study, which did not allow us to compare BAC-preserved dorzolamide with a BAC-preservative free dorzolamide ophthalmic solution. Notwithstanding, one can assume that cats from the control group also received BAC-preserved medications once the ophthalmic solutions of 0.3% tobramycin and 1% atropine sulfate were also supplemented with such preservative. This may in part explain the similar corneal re-epithelialization rates observed in both groups. In this regard, tobramycin was chosen once no deleterious effects of this antibiotic have been described in the corneal epithelium of humans and dogs [6,12]. Although in cats, the instillation of topical atropine for 7 consecutive days may induce a reduction in the number of corneal endothelial cells, no studies describing the effects of such a mydriatic over the corneal healing time are found in the literature [28].

Throughout the experiment, signs of discomfort were less than mild in most cats of both groups. However, significantly higher blepharospasm scores were observed in cats with TG. Irritation, pruritus and conjunctival hyperemia are adverse effects described in the package insert of all 2% dorzolamide ophthalmic solutions commertially available. However, such complications were described as being absent in previous studies in which BAC-preserved 2% dorzolamide solution was tested in cats with healthy and glaucomatous

eyes for an average of 15 days [17,18,25]. One study revealed that among 4 hypotensive ophthalmic solutions, dorzolamide had the most acidic pH [5]. Although BAC was present in all topical medications used in the present study, the authors attribute the higher scores of blepharospasm in the TG to the rheological characteristics and the pH of the dorzolamide ophthalmic solution. Indeed, the pH value of dorzolamide (5.58) may cause signs of irritation, as the tear film has an approximate pH of 7.6 [5].

One study revealed that the activity of the enzyme carbonic anhydrase II (ACII) in the corneal epithelium and stroma was not affected by the instillation of dorzolamide [8]. Such findings may suggest one of the reasons why the instillation of dorzolamide did not impair corneal wound healing in the cats in our study [8]. In addition, ocular adverse effects observed in humans under treatment with BAC-preserved CAIs are observed after long-term use of these substances [1,5,9,10,23,26]. Hence, the short-term period in which dorzolamide was used may be another reason why the corneal re-epithelialization time was not impaired in the present study.

MMP-9 acts in the early stages of corneal epithelial wound healing, cleaving the membrane protein β4 (integrin) within the hemidesmosome and facilitating the movement of the corneal epithelial sheet across the basement membrane to close the wound [3]. Previous studies have investigated the presence of total MMP-9 in normal and ulcerated feline corneas [15,16,20]. In one of these studies, immunohistochemistry showed increased MMP-9 expression during epithelial migration and wound closure, returning to the baseline level following full restratification of the corneal epithelium [15]. In this same study, zymograms showed that ulcerated corneas presented significantly higher levels of MMP-9 in tears at the early stages (8 to 36 h) post-wounding [15]. Another study used ELISA to quantify the total MMP-9 in the tears of cats and reported nearly undetectable expression in unwounded corneas, whereas higher amounts of this gelatinase were found during the first 24 h post-wounding [16]. In those 2 experiments conducted in cats, re-epithelization occurred in an average time of 48 h [15,16]. Similarly, experimental studies conducted in rabbits and dogs in which the quantification of MMP-9 was performed by zymography showed that the latent and active forms of this enzyme tended to increase significantly at the early stages (24 to 48 h) post corneal wounding, with a dramatic decrease at the time of re-epithelialization [3,19].

In the current study, the levels of this enzyme did not change significantly in the tears of cats treated with 2% dorzolamide when compared to the eyes in the control group at any time point. Although corneal re-epithelialization occurred before 72 h in most cats in our study, where supposedly higher levels of MMP-9 can be detected [3,15,16,19], the authors acknowledge that further studies should be conducted to check the possible effects of a BAC-preserved 2% dorzolamide on altering the levels of MMP-9 at the time of corneal re-epithelialization.

Carbonic anhydrases (CAs) and MMPs act on different sites but share molecular structure similarities, since both are zinc-containing enzymes [11,21]. The principal difference between the enzymatic mechanisms of CAs and MMPs lies in the fact that the nucleophilic adduct formed after the attack of the substrate by the zinc-bound nucleophile is the reaction product in the case of the CAs (bicarbonate ion), whereas the nucleophilic adduct is only a reaction intermediate in the case of MMPs. This difference is of crucial importance for the interaction of these enzymes with their inhibitors [11,21]. It has been described that sulfonamides may inhibit both MMPs and CAs, but there are no previous studies reporting the direct role of dorzolamide, a nonbacteriostatic sulfonamide derivative, on the expression of MMPs in healthy or damaged corneas [21].

CONCLUSION

Despite its limitations, this study certainly adds to our understanding that the instillation of a BAC-preserved 2% dorzolamide ophthalmic solution did not impair the corneal wound healing time or the early expression of MMP-9 in the tears of cats with experimentally induced corneal ulcers. However, our results warrant further investigation in patients with ocular hypertension or glaucoma presenting concomitant naturally occurring corneal ulcers to certify our findings.

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