Effects of topical flurbiprofen sodium, diclofenac sodium, ketorolac tromethamine and benzalkonium chloride on corneal sensitivity in normal dogs

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Abstract

To evaluate corneal sensitivity by using the Cochet-Bonnet® esthesiometer in normal canine eyes at different time points following instillation of three different topical non-steroidal anti-inflammatory drugs (flurbiprofen sodium 0.03%, diclofenac sodium 0.1% and ketorolac tromethamine 0.5%) and benzalkonium chloride 0.01%. Six healthy mixed breed dogs from the same litter were used in two different stages. First, one drop of flurbiprofen sodium 0.03% and diclofenac sodium 0.1% in each eye; second, one drop of ketorolac tromethamine 0.5% and benzalkonium chloride 0.01% in each eye. Baseline esthesiometry was obtained before eye drop application and every 15 minutes thereafter until a total of 105 minutes of evaluation time. A one-week interval was allowed between the two treatment phases. Statistical analysis was used to compare means according to time of evaluation and drug used. Diclofenac sodium 0.1% decreased corneal sensitivity at 75 and 90 minutes (P > 0.015) with possible interference on neuronal nociceptive activity and analgesic effect while ketorolac tromethamine 0.5% did not show any variation for esthesiometry means along the evaluation. Flurbiprofen sodium 0.03% resulted in increased esthesiometry values 30 minutes after instillation (P > 0.013), increasing corneal sensitivity and possibly producing a greater irritant corneal effect over its analgesic properties. Benzalkonium chloride 0.01% significantly increased corneal sensitivity at 15 minutes of evaluation (P > 0.001), most likely resulting from its irritating effect. Esthesiometry did not allow a definite conclusion over the analgesic effect of the NSAIDs tested; however it was effective in detecting fluctuations in corneal sensitivity.

Keywords: Cochet-Bonnet, Cornea, Nociceptors, NSAID.

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Introduction

Corneal and bulbar conjunctival innervation is supplied by a relatively small number of primary sensorial nerves originating from the ipsilateral trigeminal ganglion (about 1.5% of the neurons from ganglion´s total number) (De Felipe *et al*., 1999). However, the small dimension of the corneal surface with the vast branching of peripheral nerve axons results in the cornea being the most densely innerved structure of the body (De Felipe *et al*., 1999). Additionally, 70% of these sensorial fibers are classified as polymodal nociceptors (Belmonte *et al*., 2004).

Non-steroidal anti-inflammatory drugs (NSAIDS) are a group comprised of several drugs that are able control inflammation, promote analgesia and reduce hyperthermia by inhibiting cyclooxygenases activity, thereby reducing eicosanoid synthesis created through the arachinodic acid cascade (Bloom, 1996; Insel, 1996; Aragona *et al*., 2000). Prostaglandins are one of the primary eicosanoids released upon insult to the canine eye (Maggs, 2008). After biosynthesis, prostaglandins bind to receptors called pain facilitators (Tranquilli and Thurman, 1999; Marfurt *et al*. 2001), which promote variations in the resting potential of neuronal membranes. These variations in membrane resting potentials result in pain amplification (Bloom, 1996; Tasaka, 1999; Acosta *et al*., 2005; Tranquilli and Thurman, 1999).

NSAIDS are commonly used in ophthalmology to control pain caused by tissue injury and reduce localized inflammation and after surgical procedures (Chen *et al*., 1997; Aragona *et al*., 2000; Weaver and Terrell, 2003; Giuliano, 2004; Acosta *et al*., 2007; Kim *et al*., 2015). Topical NSAIDs such as flurbiprofen, diclofenac and ketorolac tromethamine are commonly prescribed in human and veterinary medicine due to their availability and relatively low cost. Topical NSAIDS are primarily used for inflammatory ocular conditions such as the suppression of uveitis that may be present before and after intraocular surgery

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(Giuliano, 2004; Sigle and Nasisse, 2006; Kim *et al.*, 2010; Maca *et al.*, 2010; Klein *et al.*, 2011).

Chen *et al.* (1997) showed that the application of topical NSAID ophthalmic solutions effectively reduced corneal sensitivity as well as the sensitivity of polymodal nociceptors. These findings support that analgesia and ocular comfort reported by human beings may, in part, be due to decreased PGs excitatory activity on nervous fibers (Martini *et al.*, 1984; Yamada *et al.*, 2002).

The aim of this study is to evaluate variations in the corneal sensitivity of dogs after topical application of three different NSAIDS and one preservative agent using a Cochet-Bonnet esthesiometer, which measures the corneal surface sensitivity threshold. In dogs, the accepted criteria for measurement of corneal sensitivity threshold is the degree of corneal stimulation that is required to elicit a blink reflex (Barrett *et al.*, 1991; Stiles *et al.*, 2001; Good *et al.*, 2003; Blocker *et al.*, 2007; Venturi *et al.*, 2016; Dorbandt *et al.*, 2017).

Materials and Methods

This study followed ARVO´s Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Ethics Committee of the Agrarian Sciences Sector, Federal University of Parana.

Six nine-month-old nonbrachycephalic mixed breed dogs, all littermates, were used in the study. Three males and three females were used, and all dogs were previously evaluated, vaccinated and free from signs of systemic or ophthalmic diseases. Prior to enrollment in the study, a complete ophthalmic examination was performed including a Schirmer tear test I (STT-I) (Drogavet, Curitiba, Brazil), fluorescein stain (fluorescein sodium; Drogavet, Curitiba, Brazil), tonometry (Tono-Vet; Icare Finland, Espoo, Finland), slit-lamp biomicroscopy (Hawk Eye; Dioptrix, L'Union, France), and indirect ophthalmoscopy (Heine Omega 180 Headworn Binocular Indirect Ophthalmoscope, Dove, USA). Additionally, a complete blood count, serum biochemical analysis, and urinalysis were obtained.

A masked prospective study design was used to determine the immediate effects of three commonly available ophthalmic NSAIDS and one common ophthalmic preservative on corneal sensitivity in normal canine eyes. The ophthalmic NSAIDS evaluated were flurbiprofen sodium 0.03% (ALLERGAN; Syntex Inc., USA), diclofenac sodium 0.1% (ALLERGAN; Syntex Inc., USA) and ketorolac tromethamine 0.5% (ALLERGAN; Syntex Inc., USA). The ophthalmic preservative evaluated was benzalkonium chloride 0.01%, which is the preservative agent found in ketorolac tromethamine. Benzalkonium chloride eye drops were specially prepared in a compounding pharmacy (OPHTHALMOS, São Paulo, Brazil).

Corneal sensitivity data were acquired using a Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, Chartres Cedex, France) following a previously described protocol (Barrett *et al.*, 1991; Good *et al.*, 2003). Data collection began approximately two weeks after the screening examination. The handheld esthesiometer possesses a thin, retractable, nylon monofilament that extends up to 6 cm in length. Since normal baseline corneal sensitivity threshold range of the normal dog varies from 1.0 to 3.5 cm (Stiles *et al.* 2001; Blocker *et al.*, 2007; Kobashigawa *et al.*, 2015; Venturi *et al.*, 2016), measurements started with a nylon monofilament length of 4 cm, which is a usual initial set point for corneal sensitivity evaluation in dogs (Venturi *et al.*, 2016).

The esthesiometer was gently advanced perpendicularly toward the center of corneal surface until a slight deflection of the monofilament was noted after contact. If a blink reflex was not observed on at least three attempts, the length of the monofilament was decreased 0.25 cm, and the procedure was then repeated. The length of the filament is directly proportional to corneal sensitivity such that a longer nylon monofilament length required to stimulate a blinking reflex equates to a more sensitive cornea. The experiment was conducted in two different stages, and for each stage, a repetition was made in the same group of animals.

The drug bottles were labeled as 'A', 'B,' 'C', 'D', and the investigators responsible for application of the study drug and esthesiometry measurements were masked to the drug being used. The key was maintained by the principal investigator and revealed to the study investigators at the conclusion of the study. In the first stage, one drop of flurbiprofen sodium (drug A) was administered onto the right eye, and one drop of diclofenac sodium (drug B) was administered onto the left eye of each animal. In the second stage, after a washout period of 7 days, one drop of ketorolac tromethamine (drug C) was administered onto the right eye and one drop of benzalkonium chloride was administered onto the left eye (drug D).

For each stage, the length of the nylon monofilament able to stimulate the dogs' blink reflex was recorded. Baseline esthesiometry (before the administration of any drug) was represented as time 0. Once each eye drop was applied onto the ocular surface, subsequent esthesiometry data were collected every 15 minutes after initial administration until one hour and 45 minutes after the last drop (105 minutes).

Each stage was performed with same room conditions, controlling external variables such as weather, noise, and people and animal traffic inside and outside the room. Only two study investigators were present during the data collection, one responsible for esthesionometry and the other responsible for animal restraint. The same

study investigator was responsible for each function throughout the experiment. Temperature and humidity were evaluated during each stage of the investigation using a wireless thermos hygrometer (TM005X-M Meade Instruments, Irvine, CA).

Statistical analyses were performed to compare the esthesiometry data obtained in relation to time (in minutes) and each drug used. To check whether or not the distribution of the data errors followed a Gaussian distribution, a Shapiro-Wilk test was applied. The test revealed that the data did not follow a non-Gaussian distribution. Therefore the non-parametric Friedman´s test (similar to the parametric repeated measures ANOVA) was used to detect differences in treatments across multiple test attempts on the same group of animals (paired data). Descriptive and inferential statistical analysis were performed using the JMP v7 (SAS Institute, Cary, NC, EUA), statistical package for computers.

Results

In the first stage, the mean environmental conditions during the time in which the experiment was conducted was 21°C and 71% humidity. During the second stage, temperature was 18ºC with 77% humidity.

Table 1 shows the esthesiometry results (medians) by each evaluation time for each drug tested. No significant difference was found comparing baseline results (time 0). An overall (considering all drugs together) significant difference for higher corneal sensitivity values within the initial 15 (P = 0.001) and 30 minutes (P = 0.03) after topical administration of all eye drops tested was observed compared to baseline values. This initial increase in corneal sensitivity was followed by a gradual and significant decrease in mean values (P≤ 0.025) compared to baseline values. Considering the results for the individual drugs, after the topical use of flurbiprofen sodium 0.03%, an increase in corneal sensitivity at 30 minutes was observed (P = 0.013) when compared with baseline values. Subsequently, a tendency towards a decrease in mean corneal sensitivity values was observed until the last evaluation time. However these lower mean corneal sensitivity values were not significantly different than baseline values. A slight increase of esthesiometry means also was observed 30 minutes after topical administration of diclofenac sodium 0.1%; however, this slight increase was not statistically significant (P=0.75).

Subsequently, however, significant decreases (P ≤ 0.034) in corneal sensitivity were observed between 75 and 90 minutes post-application of diclofenac sodium 0.1% when compared to baseline values. Nonetheless, at 105 minutes after initial topical administration of diclofenac, the difference was no longer significant. Corneal sensitivity data obtained after topical application of ketorolac tromethamine 0.5% showed no

significant differences between esthesiometry data means and baseline means at 15 minutes (P = 0.1), 30 minutes (P = 0.8) or at any other time point after topical administration. After topical application of benzalkonium chloride 0.01%, a significant increase (P = 0.001) in corneal sensitivity values compared with baseline values was observed at 15 minutes.

Discussion

It is well known that corneal sensitivity exhibits a great variability and it is influenced by several factors. For instance, corneal sensitivity is significantly reduced in brachycephalic and corneal region evaluated, with the central part being more sensitive that the peripheral cornea in dogs (Barrett *et al.*, 1991) and cats (Blocker and Van Der Woerdt, 2001). Humans over the age of 40 have a significantly reduced corneal sensitivity (Millodot, 1972). Interestingly, the influence of age was not observed in dogs (Barrett *et al.*, 1991) or cats (Blocker and Van Der Woerdt, 2001). These variables were controlled in the present study since a repeated measure statistical design was used with the same test-subjects (paired data), including the initial control. Data were collected in a longitudinal fashion in which change over time was assessed and time 0 is the control of each subject. This method reduces the variance of estimates of treatment-effects, allowing statistical inference to be made with fewer subjects (Gueorguieva and Krystal, 2004). Additionally, to reduce the influence of different examiners on data variability, the same study investigator was responsible for all corneal sensitivity evaluations.

Topical application of diclofenac sodium 0.1% was the only study drug that resulted in a decrease in corneal sensitivity, and this occurred at 75 and 90 minutes post-application. Paradoxically, topical application of flurbiprofen sodium 0.03% demonstrated a significant increase in corneal sensitivity 30 minutes post-application while a nonsignificant trend for an increase in corneal sensitivity occurred for all other study drugs during the initial 15 to 30 minutes. For all treatment protocols, this initial increase in corneal sensitivity was followed by a steady decrease (Table 1).The magnitude and significance of the initial increase and subsequent decrease is different between drugs, but this trend is consistently observed for each specific anti-inflammatory drug evaluated.The control of corneal pain has limited options, as topical sodium channel blockers, such as proparacaine and tetracaine, are not acceptable due to their limited duration of activity and epitheliotoxic effects (Grant and Acosta, 1994; Herring *et al.*, 2005; Venturi *et al.*, 2016). Topical morphine has been shown to provide acceptable analgesia without a delay of corneal epithelialization (Peyman *et al.*, 1994; Stiles *et al.*, 2003; Clark *et al.*, 2011); however, the corneal analgesic effectiveness in dogs has recently come into question (Thomson *et al.*, 2013).

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Table 1. Esthesiometry values (median in cm) in relation with each time point evaluated after topical use of sodium flurbiprofen 0.03%, sodium diclofenac 0.01%, ketorolac tromethamine 0.5% and benzalkonium chloride 0.01% on corneal surface in dogs.

Drugs	Time (minutes)							
	0	15	30	45	60	75	90	105
Flurbiprofen Sodium 0.03%	0.875	1.125	1.375*	0.875	1.000	1.000	0.875	0.875
Diclofenac Sodium 0.1%	1.250	1.125	1.250	1.000	1.00	0.750*	0.750*	0.875
Ketorolac Tromethamine 0.5%	1.250	1.375	1.250	1.250	1.250	1.000	1.000	1.000
Benzalkonium Chloride 0.01%	1.000	1.5*	1.375	1.125	1.000	1.000	1.250	1.000

*Means that presented significant differences compared with the baseline esthesiometry, $P < 0.05$.

Therefore, it is paramount to identify alternative options for corneal analgesia. In humans, the use of topical NSAIDS has been shown to decrease corneal pain (Weaver and Terrell, 2003; Kim *et al*., 2015). To the authors' knowledge, there are no studies evaluating the antinociceptive effect of topical NSAIDS on dogs with corneal ulcers. The current study provides evidence that diclofenac decreases corneal sensitivity, and this may correlate with improved comfort when used in the presence of a corneal ulceration; however, controlled studies should be performed to evaluate its efficacy. This decrease in corneal sensitivity after topical application of diclofenac has also been reported in humans (Szerenyi *et al*., 1994; Seitz *et al*., 1996).

In contrast to the effect of diclofenac, topical application of flurbiprofen demonstrated a possible increase of nociceptor excitatory activity after 30 minutes. However, between 45 to 105 minutes after application, corneal sensitivity decreased, and nociceptor activity conceivably started to decline as well. During this period of decreasing sensitivity, mean corneal sensitivity values still remained higher than baseline. Our data support that flurbiprofen may result in a more evident irritant action than an analgesic one. The authors hypothesize that the initial increase reflects a common time pattern for the irritant action on the canine corneal surface after topical administration of all drugs tested.

The authors believe that blocking prostaglandin action in peripheral nociceptors is able to promote a decrease of neuronal excitatory activity even in non-inflamed eyes, which is supported by previous studies (Chen, *et al*., 1997; Aragona *et al*., 2000). Esthesiometry data obtained after topical application of diclofenac in the current study suggests the peak analgesic action of this drug to occur 75 to 90 minutes after topical application. After this period, however, corneal sensitivity returned to baseline values. Topical application of ketorolac did not produce significant changes in corneal sensitivity. Data obtained after topical application of benzalkonium chloride demonstrates a significant increase in corneal sensitivity at 15 minutes compared with baseline values. Additionally, higher mean sensitivity values were obtained when compared to other study treatments during the same time point, which may represent a potentially higher corneal irritant effect. This finding was interesting, as a change in corneal sensitivity was not expected because benzalkonium chloride is a preservative devoid of anti-inflammatory properties and is instead used in ophthalmic solutions to increase drug penetration through the lipid cell membrane (Madhu *et al*., 1996; Malhotra and Majumdar, 2006). It is also an ingredient present in ketorolac ophthalmic solution. Importantly, benzalkonium chloride has also been shown to promote eicosanoid synthesis and, consequently, ocular irritation suggests that topical application of this preservative, even in concentrations as low as 0.0001%, decreases proliferative cellular activity and promotes apoptosis (Madhu *et al*., 1996; De Saint Jean *et al*., 1999).

Although the present study demonstrated an increase in corneal sensitivity after application of benzalkoniun chloride, the same effect was not observed with ketorolac, despite containing benzalkonium chloride as a preservative. It is possible that the anti-inflammatory action of ketorolac may reduce the irritant effect of benzalkonium chloride on corneal nociceptor fibers.

The results of flurbiprofen and diclofenac identified in the current study are somewhat different than the results obtained in a recent study (Dorbandt *et al*., 2017), and this may be explained, in part, by variations in study design as well as differences in ambient humidity between the two studies. The current study evaluated corneal sensitivity with decreasing increments of 0.25 cm, and this may have allowed the investigators to assess for smaller changes in corneal sensitivity. The current study also evaluated corneal sensitivity up to 105 minutes after application of the specified study drug. Specifically, for diclofenac, a decrease in corneal sensitivity was observed at 75 and 90 minutes after application (Table 1). One of the most important differences between the two studies is the ambient humidity. In the current study, ambient humidity values during data collection were 71% to 77%, and it has been shown that humidity may have profound effects on corneal esthesiometry measurements (Dorbandt *et al*., 2017). In the recent comparable study, ambient humidity ranged from 19% to 44%, which may explain the higher sensitivity values, as the same study found that a lower humidity

results in a higher corneal sensitivity measurement (increased filament length) (Dorbandt et al., 2017). With these results of humidity kept in mind, it can explain why corneal sensitivity values (filament length) obtained in the present study are lower than those obtained in previous studies (Barrett et al., 1991; Good et al., 2003; Dorbandt et al., 2017).

Although some previous studies in humans and dogs demonstrated the analgesic action of flurbiprofen and ketorolac (Sigle and Nasisse, 2006; Maca et al., 2010), the present study suggests that the ophthalmic application of these two drugs do not promote analgesic activity on corneal nociceptor activity in healthy canine eyes. Another recent study (Dorbandt et al., 2017) also demonstrated a lack of analgesic activity after topical application of flurbiprofen to canine eyes. Nevertheless, Acosta et al. (2007) found that topical ketorolac 0.4% resulted in analgesic efficacy in healthy feline eyes and that this drug was capable of decreasing peripheral nociceptor activity. Although the study by Acosta et al. (2007) also uses the Cochet-Bonnet esthesiometer as mechanical stimuli, data collection was performed using the evaluation of the evoked potential of neuronal fibers. This methodology, previously developed by Chen et al. (1997), comprises a more quantifiable parameter for the evaluation of corneal nociceptors. In this way, the single use of a blinking reflex in the current investigation as an indirect observational parameter of corneal sensitivity promoted by Cochet-Bonnet esthesiometry may have resulted in insufficient data due to inherent variability in the blink response.

Studies in humans have found that topical application of diclofenac demonstrates comparatively higher corneal analgesic potential (Szerenyi et al., 1994; Seitz et al., 1996) which corroborates the results from Aragona et al. (2000). Studies have also demonstrated evidence for the interaction of diclofenac with β-endorphin (endogenous inhibitory neurotransmitter) and substance P (nociceptors endogenous excitatory neurotransmitter). In these studies, there was an observed increase in plasma β-endorphins (Martini et al., 1984) and a decrease in levels of substance P in tears (Yamada et al., 2002), which may contribute to the potential analgesic action of this drug. Thus, the authors believe that topical diclofenac effectively caused inhibition of the eicosanoid production in the arachidonic acid metabolic pathway in the canine corneas. This mechanism was then responsible for decreasing corneal nociceptor activity of polymodal nociceptors, which are present in the cornea and are responsive to prostaglandins and bradykinin (Belmonte et al., 2004). This decrease in corneal nociception generated a clinically-detectable degree of topical analgesia (Bloom, 1996). This assumption corroborates the findings from other authors (Szerenyi et al., 1994;

Seitz et al., 1996; Chen et al., 1997; Aragona et al., 2000; Yamada et al., 2002; Acosta et al., 2005, 2007). A limitation of the current study is that the low number of individuals evaluated may have a profound effect on significance (Type II statistical error), and it is possible that a significant increase would have been achieved with higher study numbers. Topical administration of all eye drops tested (considered together) show a significant increase in corneal sensitivity values within the initial 15-30 minutes. However, when evaluated individually only flurbiprofen (at 30 minutes) and benzalkonium chloride (at 15 minutes) demonstrated an increase in corneal sensitivity. For diclofenac, although not statistically significant, a trend exists towards an increase in corneal sensitivity values 30 minutes after topical application. Another limitation is that the only preservative evaluated was benzalkonium chloride. Given the result of benzalkonium chloride application alone, the initial increase in corneal sensitivity noted between 15 and 30 minutes may suggest an irritant action caused by different preservative agents present in each of these ophthalmic solutions: thimerosal 0.005% for flurbiprofen; boric acid for diclofenac; benzalkonium chloride 0.01% for ketorolac. For ketorolac, the trend towards an increase in sensitivity after the administration was an expected result given the significant increase in corneal sensitivity after benzalkonium chloride alone was observed. The fact that benzalkonium chloride did not show a significant difference when used in combination with ketorolac may be due to the slightly higher variation of the corneal sensitivity means observed at baseline. This overall effect also might be responsible by the burning sensation reported by several human patients that use these drugs topically (Chen et al., 1997; Aragona et al., 2000; Acosta et al., 2005). However, new investigations are necessary to prove the mechanism of action for these agents on the corneal surface. Although the subjectivity of the blink reflex by observational evaluation may have interfered directly in obtaining esthesiometry results, both baseline and experimental values were always evaluated by the same observer, thus minimizing the possibility of subjectivity on variable measurement.

The results of the current study demonstrate that corneal sensitivity of normal, nonbrachycephalic dogs is decreased by topical diclofenac sodium 0.1% at 75 and 90 minutes after application. However, the topical administration of flurbiprofen sodium 0.03% and benzalkonium chloride 0.01% result in an immediate increase in corneal sensitivity between 15 and 30 minutes after application while ketorolac tromethamine 0.5% has no effect. The results of topical diclofenac demonstrated in the current study mimics those found in humans in that diclofenac was the only topical NSAID observed to cause an immediate, but transient,

decrease in corneal sensitivity. More studies with controlled variables, including the use of new methodologies of corneal nociceptor electrophysiological activity, are necessary to obtain trustworthy data regarding analgesic efficacy of anti-inflammatories tested in this study.

Conflict of interest

The authors declare that they have no competing interests.

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