2020

An eye on the dog as a translational model for ocular pharmacology

Lionel Sebbag

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DEDICATION

This dissertation is lovingly dedicated to my parents, Monique and Michel

For their endless love, support, and encouragement

Je vous aime très fort, Terbah!!
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‘You are not a reasonable man’ said my major professor during my PhD defense. I agree, there is nothing rational in pursuing a graduate degree while working as an assistant professor and raising a family. I owe this accomplishment to the overwhelming support I received over the last 4 years, for which I will be forever grateful.

To my beautiful wife, Alison, for your patience in surviving the cold winters of Iowa and letting me escape to my office all too often to get work done. I love you deeply, you are an incredible mother to Jacob and Netta and a wonderful person with a kind heart.

To my major professor, Dr. Jonathan Mochel, for pushing me to get out of my comfort zone, both on a professional and personal level. “If I have seen further it is by standing on the shoulders of giants.” — Isaac Newton

To other members of my graduate committee: Dr. Al Jergens, Dr. Heather Greenlee, Dr. Josh Beck and Dr. Ann Perera, I am truly honored to have completed this journey by your side. Thank you for guiding me on the right path.

To my previous mentors and resident-mates at UC Davis, now close friends and colleagues, for believing in me, and for shaping the clinician-scientist career I am pursuing today with passion and dedication.
To the Ophthalmology service at Iowa State University, for providing me the flexibility to juggle between the student and the faculty hat, and for the fantastic times working together in such a great environment.

To the other co-workers and friends at Iowa State University, for the fun and memorable experiences we shared.

To my parents Monique and Michel, my brothers Jérôme and David, mamie Jeanette, Hillel, and all my family scattered throughout the world. Family is everything. You are my inspiration and my strength in life.

To my four-legged companions, Winston, Holly, Morgan and Dexter z”l, for your love that is mostly unconditional (as long as chicken is provided every now and then).

I realize the list of acknowledgements can only capture a small fraction of the people who supported me. I send my gratitude to all.
ABSTRACT

Today’s high failure rate in ophthalmic clinical trials can be largely explained by two major shortcomings: (i) the animals routinely studied (rabbits, mice, rats) are not representative of the affected population due to apparent anatomical and physiological differences with humans; and (ii) studies conducted in healthy eyes do not account for physiological disturbances in ocular homeostasis present in diseased eyes. Unlike traditional laboratory animals, diseases in dogs better reflect the complex genetic, environmental, and physiological variation present in humans; however, the translational potential of canine research is currently limited by scarce information on normative data specific to dogs, and the limited means to mimic ocular disease in a reliable and non-invasive manner in this species.

The work conducted in the dissertation provides a deeper understanding of the canine ocular surface in health and disease states, investigating laboratory Beagle dogs and canine patients of varied breeds and cephalic conformations. Tear fluid was collected from canine eyes in successive experiments – primarily via Schirmer tear strips but also capillary glass tubes and absorbent sponges – and subsequent bioanalytical tools included fluorophotometry (tear film fluorescence), infrared spectroscopy (total protein content), immunoassays (serum albumin, cytokines, chemokines) and liquid chromatography-mass spectrometry (corticosteroids). Data analysis combined conventional statistical tests with nonlinear mixed-effects mathematical modeling to improve the robustness of the predictions.

The main research outcomes of the dissertation work are the following: (i) Normative data were established for canine tear film dynamics, including tear volume (65.3 µL), basal tear turnover rate (12.2%/min) and reflex tear turnover rate (50%/min). In both clinical and research settings, successive lacrimal tests should be spaced by ≥ 10 min in dogs to provide sufficient
time for the tear film to replenish. (ii) The volumetric capacity of the canine palpebral fissure was 31.3 µL, approximating the volume of a single eyedrop. Kinetic studies confirmed that a single drop is sufficient for topical administration in dogs, any excess being lost predominantly by blinking and spillage over the periocular skin. (iii) Topical histamine solutions of 1, 10, and 375 mg/mL induced mild, moderate, and severe conjunctivitis in dogs, respectively. The resulting disruption of the blood-tear barrier promoted leakage of plasma compounds (e.g., albumin) into the tear film, a finding confirmed in dogs with naturally acquired ocular diseases. This ‘large animal’ model was robust, non-invasive, and self-resolving, providing a unique opportunity to investigate the ocular surface in health and disease. (iv) Acute conjunctivitis increased tear quantity and decreases tear stability, although ocular surface homeostasis was rapidly restored. (v) Corticosteroid levels in the tear film did not change significantly between healthy vs. diseased eyes following oral prednisone administration, although findings may differ for drugs with other physicochemical properties. (vi) Albumin in tears lowered the ocular bioavailability of topically administered drugs, as shown for tropicamide and (to a lesser extent) latanoprost in dogs.

The thesis concludes with a comprehensive review of key ocular parameters in humans, dogs, and traditional laboratory species (rabbits, mice, rats), detailing species differences in ocular surface anatomy, physiology, tear film dynamics and tear film composition, and highlighting the benefits of integrating dogs into preclinical studies given striking resemblances between the canine and human eyes (One Health approach).
CHAPTER 1. GENERAL INTRODUCTION

Companion animals such as the dog constitute an underutilized resource for translational research in biomedical sciences.\textsuperscript{1} Unlike traditional laboratory animals (eg., rabbits, mice, rats), naturally-occurring diseases in dogs better reflect the complex genetic, environmental, and physiological background present in humans; therefore, integrating canine subjects into preclinical studies can accelerate and improve the framework in which research is translated to the human clinic, and ultimately generate discoveries that will benefit the health of humans and animals. This ‘One Health’ approach is rapidly growing in several medical fields (eg., oncology, neurology, stem cells),\textsuperscript{2-4} yet the literature in comparative ophthalmology is very limited to date.

Dogs are routinely examined by veterinarians for the diagnosis and management of diverse ocular pathologies, exploiting the expertise of general practitioners and veterinary specialists across the world. Importantly, numerous canine ocular disorders share striking phenotypical resemblances with their human clinical analogues – as exemplified by \textit{keratoconjunctivitis sicca} (‘dry eye’)\textsuperscript{5} – and could therefore serve as spontaneous animal models of ocular diseases. To date, the paucity of canine research for translational purposes is generally explained by perceived limitations such as ethical and economic considerations, lack of transgenic dogs, and limited molecular tools compared to laboratory species.\textsuperscript{1,6,7} In the authors’ opinion, more scientifically relevant hindrances to the use of canines in ophthalmology research include limited data on the physiology of the ocular surface in dogs, and the lack of non-invasive experimental model to mimic disease pathophysiology in this species. Unfortunately, direct extrapolation from other animals is not possible given notable species differences in ocular anatomy and physiology.\textsuperscript{8,9}
The first two chapters of this dissertation describe fundamental work to better understand the physiology of the canine ocular surface, establishing normative data for key parameters such as tear volume, tear turnover rate, volumetric capacity of the palpebral fissure, and tear film kinetics following topical eyedrop administration. The next two chapters describe the development of a robust in vivo model of conjunctivitis in dogs, a translational large animal model that provides a unique opportunity for scientists to investigate the ocular surface in health and disease states. Practical applications of the model are reported in the last two chapters of the thesis, highlighting the clinical significance of blood-tear barrier breakdown on ocular pharmacology and drug bioavailability. Finally, the last chapter of this manuscript provides a comprehensive review of the ocular surface in humans and selected animals, describing major pitfalls that tremendously limit the translational potential of conventional laboratory animals (rabbits, mice, rats) in ophthalmic research. This chapter further highlights the benefits of leveraging information from canine pharmacokinetic, efficacy and safety preclinical studies given striking resemblances between the canine and human eyes.

References


CHAPTER 2. FLUOROPHOTOMETRIC ASSESSMENT OF TEAR VOLUME AND TURNOVER RATE IN HEALTHY DOGS AND CATS

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Modified from a manuscript published in the Journal of Ocular Pharmacology and Therapeutics

Abstract

Purpose: The study establishes normative data of tear volume (TV) and tear turnover rate (TTR) in healthy dogs and cats, two species commonly used for translational research in ophthalmology. Methods: Thirty six dogs and 24 cats were enrolled, encompassing a variety of breeds with diverse skull conformations (brachycephalic, mesocephalic, dolichocephalic). Two μL of 10% fluorescein were instilled onto the upper bulbar conjunctiva of both eyes, followed by tear collection with 2-μL capillary tubes at 0, 2, 4, 6, 10, 15 and 20 minutes. Fluorescein concentrations were measured with a computerized scanning ocular fluorophotometer. The TV and TTR were estimated based upon nonlinear mixed-effects analysis of fluorescein decay curves. Results: In dogs, median (interquartile range) TV, basal TTR and reflex TTR were 65.3 μL (42.3-87.9), 12.2%/min (3.7-22.1) and 50.0%/min (25.9-172.3), respectively. In cats, median (interquartile range) TV, basal TTR and reflex TTR were 32.1 μL (29.5-39.9), 10.9%/min (3.0-23.7) and 50.0%/min (28.4-89.4), respectively. Body weight (r=0.44) and age (r=0.30) were
positively correlated ($P \leq 0.019$) with TV in dogs. Age was negatively correlated ($P \leq 0.018$) with TTR in dogs ($r = -0.33$) and cats ($r = -0.24$). However, TV and TTR were not associated with skull conformation in either species. **Conclusions:** Dogs have greater TV than cats but similar basal and reflex TTR. Tear parameters were impacted by body weight and age, but not skull conformation. In both clinical and research settings, successive lacrimal tests should be spaced by $\geq 10\text{min}$ to provide sufficient time for the tear film to replenish, as basal TTR is approximately 11-12%/min in both species.

**Introduction**

Tear fluid dynamics, or the balance between tear secretion, distribution, absorption, evaporation and drainage, are critical for the maintenance of ocular surface health.\(^1\) Tear volume (TV) and tear turnover rate (TTR) are parameters that provide insight into these complex dynamics, and as such are valuable for numerous clinical and research applications: (i) TTR helps differentiate between aqueous-deficient dry eye and evaporative dry eye in human patients;\(^2\) (ii) TTR impacts the quantity of various tear components such as electrolytes and proteins;\(^3\)\(^-\)\(^5\) and (iii) determination of TV in horses highlights a large dilution effect of tear fluid on exogenously applied drugs, whereby over half of the drug concentration is diluted immediately upon topical instillation onto equine eyes.\(^6\)

Dogs and cats, in addition to being the most common companion animals worldwide, are commonly used as animal models for translational ocular surface research, as exemplified by canine keratoconjunctivitis sicca\(^7\) and feline epithelial wound healing.\(^7\)\(^,\)\(^8\) However, information about tear fluid dynamics is lacking in these species, despite numerous reports in humans\(^1\)\(^,\)\(^2\)\(^,\)\(^9\) and various animals such as rabbits,\(^10\) horses\(^6\) and cows.\(^11\) Evaluation of tear dynamics in dogs and cats is likely confounded by their diversity in facial conformations. Indeed, brachycephaly
(foreshortening of the facial skeleton) can impact tear drainage in dogs and in cats, and the associated lagophthalmos of some brachycephalic animals can impact tear distribution and evaporation.

The present study establishes normative data of TV and TTR in dogs and cats using fluorophotometry, a method considered to be the gold standard in assessing tear dynamics. A secondary objective is to determine the impact of cephalic conformation and other variables (age, body weight, Schirmer values) on canine and feline tear dynamics.

**Materials and Methods**

**Animals**

Thirty-six dogs ($n = 72$ eyes) and 24 cats ($n = 48$ eyes) were enrolled in the study. Prior to study participation, a consent form was signed by owners and each subject was confirmed to be ophthalmoscopically healthy by slit-lamp examination, indirect ophthalmoscopy, rebound tonometry (TonoVet, Icare Finland Oy, Espoo, Finland) and normal Schirmer test values (≥ 15 mm/min in dogs, ≥ 9 mm/min in cats). A soft measuring tape was used to measure the skull width (widest interzygomatic distance), skull length (dorsal tip of the nose to occipital protuberance) and muzzle length (dorsal tip of the nose to the stop). These values were used to calculate the cephalic index (CI = skull width / skull length) and the craniofacial ratio (CFR = muzzle length / skull length) in each animal. The CI was used to characterize canine subjects as brachycephalic ($n = 10$), mesocephalic ($n = 16$) or dolichocephalic ($n = 10$), as described by Evans and De Lahunta. Since similar numerical features are lacking in cats, the CI was still utilized for data analysis but feline subjects were classified as brachycephalic ($n = 9$; e.g. Persian, Himalayan, Exotic Shorthair) or non-brachycephalic ($n = 15$; e.g. Domestic Shorthair, Bengal) based on previous studies. The study was approved by the Institutional Animal Care and Use
Committee of Iowa State University, and was conducted in accordance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

**Fluorophotometry**

Fluorophotometry was performed as previously described with minor modifications. Briefly, 2µL of 10% sodium fluorescein (Akorn Inc., Buffalo Grove, IL) was instilled onto the dorsal bulbar conjunctiva of each eye using a pipette, with care not to touch the ocular surface. Immediately following 3 manual eyelid blinks (0 min), a 2-µL capillary glass tube (Drummond Scientific Co., Broomhall, PA) was placed in contact with the inferior tear lake for ≤ 2 seconds to collect a tear sample. Eyes were then allowed to blink naturally, and tear samples were collected in a similar fashion at 2, 4, 6, 10, 15 and 20 minutes. A maximum of 2 seconds was selected for tear collection as a compromise to obtain sufficient tear fluid for analysis while minimizing the risk of inadvertently touching the ocular surface and causing reflex tearing. This duration, however, was often insufficient to completely fill the 2-µL capillary tubes with tears. Thus, following each collection, the length of fluid contained within the capillary tube was measured to the nearest mm using a ruler, a value extrapolated to the volume of tears collected given that the 2-µL tube is 32 mm in length. The content of each capillary tube was expelled into a 2-mL Eppendorf tube pre-filled with 1mL of phosphate-buffered saline (Gibco® PBS, pH 7.2, Thermo Fisher Scientific, Rockford, IL). An additional 1mL of PBS was added to each tube, followed by vortex mixing for 30 seconds and transfer to a glass cuvette. Fluorescein concentration was measured in each sample with a computerized scanning ocular fluorophotometer (Fluorotron Master™, Coherent Radiation, Palo Alto, CA). All tear sample collection and experiments were performed in the morning (from 8 am to 12 pm) to reduce the
potential impact of circadian rhythm on TTR. All subjects were confirmed to be free of corneal epithelial defects using a cobalt blue light evaluation at completion of the last tear collection. Of note, both eyes of each animal received the same amount of fluorescein at baseline, except for six beagle dogs in whom one random eye received 2 µL of 10% fluorescein while the other eye received 1 µL of 1% fluorescein - an experiment conducted to assess the effect of fluorescein dosing on tear dynamics.

**Data analysis**

Since the fluorophotometer output is nonlinear at high fluorescein concentrations, a calibration curve was established for the Fluorotron Master™ by analyzing a dilution series of known fluorescein concentrations in triplicate (1 to 10,000 ng/mL). Fluorescein concentrations in tear samples were corrected based on the resulting calibration equation (y = -4E-05x^2 + 0.9567x + 20.581).

Fluorescein data of each animal were inputted to Monolix® version 2018R2 (Lixoft, Orsay, France). Selected data points were censored in Monolix® when a peak of fluorescence could not be identified on the fluorophotometer reading, or if the tear fluorescein concentration did not make physiologic sense (e.g. higher fluorescein at 2 min compared to baseline). Overall, <10% (93/944) of all data points were left-censored.

Mathematical models of fluorescein disposition time-course were written as non-linear mixed effects (NLME) models as previously described and detailed in Appendix A. Data collection from the left and the right eye practically constitutes a repeat sample from the same individual. To account for this repeat sampling procedure, biological data collected from the right and left eye of each study subject were modeled using a within-dog variability term in the statistical model structure. Inclusion of covariate relationships with the model parameters TV and TTR
(age, body weight, sex, skull type, cephalic index, craniofacial ratio, STT value, and amount of fluorescein instilled onto the ocular surface) was assessed for statistical significance using a Pearson correlation test (for continuous variables) or Fisher’s exact test (for categorical variables) at a \( P < 0.05 \) threshold. Data modeling best fitted a biphasic decay curve, as previously reported,\(^1\),\(^19\),\(^26\),\(^27\) allowing for the calculation of the following parameters (Figure 1): (i) Tear volume (TV), calculated from the monophasic decay of fluorescence in the first regression line after instillation of fluorescein;\(^1\),\(^26\) (ii) Reflex TTR (rTTR), calculated from the slope of the first regression line;\(^27\) (iii) Basal TTR (bTTR), calculated from the slope of the second regression line.\(^1\),\(^19\),\(^26\),\(^27\) Basal TTR represents tear drainage during non-stimulated physiologic conditions, while reflex TTR represents the faster drainage that occurs as a response to noxious stimulation or irritative conditions (e.g. foreign body, corneal abrasion, eyedrop administration). Data supporting the validity and robustness of the model are shown in Figure 2 and Appendix A. Lastly, theoretical TV was calculated based on tear film thickness measured in a subset of dogs (Appendix B).\(^6\),\(^28\),\(^29\)

**Results**

All eyes (\( n = 72 \) in dogs, \( n = 48 \) in cats) were deemed healthy on ophthalmic examination and were utilized for data analysis. Using the model, TV and TTR for the general canine and feline population were calculated, and results are presented as median and interquartile range (25\(^{th}\)-75\(^{th}\) percentile) in Table 1. Several correlations were found between individual characteristics and tear film parameters (Table 2). In particular, body weight (\( r = 0.44, P < 0.001; \) Figure 3) and age (\( r = 0.30, P = 0.019 \)) were positively correlated with TV in dogs, and a negative correlation was found between age and TTR in dogs (\( r = -0.33, P = 0.007 \)) and cats (\( r = -0.24, P = 0.018 \)). Further, a positive correlation was detected between the dose (amount of
fluorescein instilled onto the ocular surface) and the calculated TV in dogs ($P < 0.001$). Of note, a post-hoc sample size calculation (SigmaPlot version 14.0, Systat Software, Point Richmond, CA) showed that $n = 360$ dogs and $n = 130$ cats would be required to detect statistical differences in TV and TTR among animals with diverse skull conformations, assuming a power of 80% and an alpha of 0.05.

**Discussion**

The present study establishes the tear film dynamics in healthy dogs and cats, accounting for the diversity of skull conformation occurring in these companion animals. Dogs and cats have a few advantages over current preclinical models of ocular surface disease: their ocular anatomy better resembles humans than rabbits or laboratory rodents, and both species develop spontaneous diseases that share strong similarities with human pathologies (e.g. dry eye disease, herpes keratitis). In pharmacology, for instance, extrapolation of findings from rabbits to humans is compromised by significant differences in precorneal residence time of drugs between these two species. In fact, the tear flow in rabbits is much slower than in humans, a difference likely explained by variability in tear film stability, mucin composition, and blink rate. Such differences would be minimized when working with companion animals, since it takes approximately the same amount of time for the tear fluid to replenish in humans as in dogs and cats (5-10 min); indeed, basal TTR in dogs (12.2%/min) and cats (10.9%/min) better mimics the human’s ocular surface physiology (10-20%/min). On the other hand, the TV is different among these species: TV in dogs (65.3 µL) and cats (32.1 µL) is larger than in humans (7.0-12.4 µL), a finding that could explain why Schirmer testing is recommended for 1 min in companion animals vs. 5 min in people. Compared to humans, dogs and cats have a larger corneal surface to lubricate (average corneal diameter in humans is 11.7 mm vs. 16.7 mm and
16.5 mm for dogs and cats, respectively), and they possess an additional secretory tissue (gland of the third eyelid) to supplement the main lacrimal gland with aqueous tear production. Differences in tear film thickness (3.4 µm in humans vs. 15.1 µm in dogs [Appendix B]) could also explain the larger canine TV. Such differences in lacrimal volume have important practical implications. When compared to humans, dogs and cats have a greater initial dilution of a drug administered onto their ocular surface. Conversely, they offer easier collection of sufficient tear fluid for analytical purposes; in fact, up to 106 µL and 43 µL can be easily collected within 1 min using ophthalmic sponges in dogs and cats, respectively.

Fluorophotometry is often considered superior to other tear assessment methods, such as fluorescein clearance test or lacrimal scintigraphy, to study tear film dynamics. However, one must be cognizant of the initial amount of fluorescein used in fluorophotometry studies as it can impact the calculated tear dynamics parameters, a finding verified in the present study. Here, we combined this analytical method with detailed modeling of the data to improve the robustness of our findings. Unlike previous studies in which t = 5 min is empirically selected as the transition between reflex and basal tearing, mathematical modeling appreciates the nuances of fluorescein decay between eyes and subjects, while incorporating individual characteristics (such as age and body weight) into the final analysis. Another value of the NLME approach is the ability to model both eyes simultaneously, thereby taking into account within-subject (between eyes) variability (WSV) in the tear fluid dynamics. This is particularly relevant as this variability was estimated to be fairly high, such that modeling of the data without factoring in WSV could lead to significant model misfits.

Further, we purposely enrolled animals with a variety of cephalic conformations to be representative of the diverse breeds examined by veterinarians and researchers. Brachycephalic
animals could theoretically have reduced TTR due to functional punctal occlusion caused by medial entropion and/or altered anatomy of the nasolacrimal duct associated with the skull conformation. The lack of statistical impact of cephalic conformation on TTR is likely due to strict inclusion criteria (normal subjects without obvious pathological changes) and an overall relatively low sample size. Recruiting brachycephalic animals with healthy ocular surface was indeed rather challenging, particularly in cats. A few significant correlations were detected between individual characteristics and tear dynamics parameters. Notably, lacrimal volume gets larger with increasing body weight in dogs (Figure 3), a finding previously documented in juvenile, but not adult dogs. Moreover, TTR decreases with age in both dogs and cats. In humans, aging reduces eyelid kinematics (blinking amplitude and peak velocity), a physiologic change that could result in a less efficient pump mechanism to drain the tear fluid through the nasolacrimal duct; the same may be true in companion animals.

In both clinical and research settings, we recommend waiting 10 min between successive lacrimal tests. Indeed, 10 min would be required to fully replenish the canine or feline tear film if the initial lacrimal test did not cause ocular irritation (e.g. strip meniscometry), as the basal TTR is approximately 11-12%/min in both species. However, 5 minutes may be sufficient if the diagnostic test is causing reflex tearing (e.g. Schirmer test) given that the initial TTR is very fast (rTTR = 50%/min). From a pharmacological standpoint, the concentration of drug instilled onto the ocular surface is immediately diluted by 3-fold in dogs and 2-fold in cats upon mixing with the tear film, assuming an average drop size of 35 µL. The precorneal residence time of this drug is expected to be < 10 min as TTR following eye drop administration is presumably faster than under physiologic conditions. Further, fluorophotometry data from healthy animals can be compared to clinical cases with ocular surface disease, helping to differentiate between
symptomatic and asymptomatic patients (TTR is significantly lower in symptomatic patients)\textsuperscript{43} – particularly in cats for whom clinical signs of aqueous tear deficiency are not as overt as in dogs\textsuperscript{44} – and between aqueous-deficient and evaporative dry eye (TTR is significantly lower in aqueous deficiency),\textsuperscript{45} a distinction well established in human patients but poorly characterized in veterinary medicine.

The main limitation of our study is the relatively low sample size, which could explain the lack of significant effect of the skull type (brachycephalic, mesocephalic, dolichocephalic) on tear film dynamics. Further, \textit{in situ} assessment of tear fluorescence — as described in most investigations on human subjects\textsuperscript{9,19,21,27} — was not possible in our study, as dogs and cats would not tolerate the 20-min protocol without heavy sedation or general anesthesia, which in turn would affect tear film dynamics. Thus, tear fluid had to be collected with capillary tubes prior to analysis and this could have added another source of variability in the fluorophotometry measurements. The volume of fluid collected was calculated in each sample, but the exact duration of tear collection was not standardized among subjects. Since collection duration did not exceed 2 seconds to avoid reflex tearing, the potential impact of sampling duration on fluorophotometry data is deemed negligible in the present study; however, this parameter should be recorded in future studies should longer collection duration be necessary (i.e. higher risk of inadvertent reflex tearing).

In conclusion, the normative data established in the present study has several implications for both clinicians and researchers. In particular, successive lacrimal tests should be spaced by $\geq 10$ min to provide sufficient time for the tear film to replenish, as basal TTR is approximately 11-12 \%/min in both species. In both species, tear film parameters were impacted by body weight and age, but not skull conformation.
References


Tables and Figures

Table 1. Normative data of tear volume and tear turnover rate in dogs and cats, presented as median and interquartile range (25th-75th percentile). The relative standard errors (RSE) of parameters estimates from the model are listed for each species. For comparison, the human tear film parameters are presented in the right column.

<table>
<thead>
<tr>
<th></th>
<th>Dog  (n = 72 eyes)</th>
<th>RSE (%)</th>
<th>Cat  (n = 48 eyes)</th>
<th>RSE (%)</th>
<th>Human1,9,27 (n = 31-74 eyes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tear volume (µL)</td>
<td>65.3 (42.3-87.9)</td>
<td>7.5</td>
<td>32.1 (29.5-39.9)</td>
<td>20.3</td>
<td>7.0-12.4</td>
</tr>
<tr>
<td>Basal tear turnover rate (%/min)</td>
<td>12.2 (3.7-22.1)</td>
<td>5.4</td>
<td>10.9 (3.0-23.7)</td>
<td>13.7</td>
<td>10-20</td>
</tr>
<tr>
<td>Reflex tear turnover rate (%/min)</td>
<td>50.0 (25.9-172.3)</td>
<td>5.4</td>
<td>50.0 (28.4-89.4)</td>
<td>13.7</td>
<td>31.5-100</td>
</tr>
</tbody>
</table>

Table 2. Correlations between tear parameters and co-variates, using the Pearson test for continuous variables (age, body weight, STT, CI, CFR) and the Fisher’s exact test for categorical variables (gender, dose, skull type). STT = Schirmer tear test; CI = Cephalic index; CFR = Craniofacial ratio. Pearson’s correlation coefficients (r) are noted for continuous variables. N/A = Not assessed; — No correlation found.

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Body weight</th>
<th>Gender</th>
<th>STT</th>
<th>Dose</th>
<th>Skull type</th>
<th>CI</th>
<th>CFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>TV</td>
<td>r = 0.30</td>
<td>r = 0.44</td>
<td>—</td>
<td>—</td>
<td>P &lt; 0.001</td>
<td>—</td>
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<tr>
<td></td>
<td></td>
<td>(P = 0.019)</td>
<td>(P &lt; 0.001)</td>
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</tr>
<tr>
<td></td>
<td>TTR</td>
<td>r = -0.33</td>
<td></td>
<td>—</td>
<td>—</td>
<td>P = 0.005</td>
<td>—</td>
<td>—</td>
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<tr>
<td></td>
<td></td>
<td>(P = 0.007)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Cat</td>
<td>TV</td>
<td></td>
<td></td>
<td>—</td>
<td>r = 0.72</td>
<td>N/A</td>
<td></td>
<td>—</td>
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<td></td>
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<td>—</td>
<td></td>
<td></td>
<td>(P &lt; 0.001)</td>
<td></td>
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<tr>
<td></td>
<td>TTR</td>
<td>r = -0.24</td>
<td></td>
<td>—</td>
<td>—</td>
<td>N/A</td>
<td></td>
<td>—</td>
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<td></td>
<td></td>
<td>(P = 0.018)</td>
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</table>
Figure 1. Representative fluorescein decay curve in tear fluid of a dog or a cat, allowing for calculation of tear volume and tear turnover rate (reflex and basal) using parameters calculated with non-linear mixed effects model. $C_0$ represents the fluorescein concentration in tear fluid at $t = 0$ min, extrapolated from the fluorescein decay curve. NaFl = sodium fluorescein; TTR = tear turnover rate.
Figure 2. Comparison of predicted tear fluorescence over time (purple curve) to observed data (blue points) for a random sample of dogs (A) and cats (B). Censored data are shown as vertical red bars.
Figure 3. A positive association was found between canine body weight and tear volume (Pearson correlation test). Estimated tear volumes are described in the table for body weights ranging from 1 to 65 kg.
CHAPTER 3. KINETICS OF FLUORESCEIN IN TEAR FILM AFTER EYE DROP INSTILLATION IN BEAGLE DOGS: DOES SIZE REALLY MATTER?

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Modified from a manuscript published in Frontiers in Veterinary Sciences

Abstract

The study aimed to determine the impact of drop size on tear film pharmacokinetics, and assess important physiological parameters associated with ocular drug delivery in dogs. Two separate experiments were conducted in 8 healthy Beagle dogs: (i) Instillation of 1 drop (35 µL) or 2 drops (70 µL) of 1% fluorescein solution in each eye, followed by tear collections with capillary tubes from 0 to 180 min; (ii) Instillation of 10 to 100 µL of 0.1% fluorescein in each eye, followed by external photography with blue excitation filter (to capture periocular spillage of fluorescein) and tear collections from 1 to 20 min (to capture tear turnover rate; TTR). Fluorescein concentrations were measured in tear samples with a fluorophotometer. The TTR was estimated based upon nonlinear mixed-effects analysis of fluorescein decay curves. Tear film pharmacokinetics were not superior with instillation of 2 drops vs. 1 drop based on tear film concentrations, residual tear fluorescence, and area under the fluorescein-time curves ($P \geq 0.163$). Reflex TTR varied from 20.2-30.5 %/min and did not differ significantly ($P = 0.935$) among volumes instilled (10-100 µL). The volumetric capacity of the canine palpebral fissure (31.3 ± 8.9 µL) was positively correlated with the palpebral fissure length ($P = 0.023$). Excess
solution was spilled over the periocular skin in a volume-dependent manner, predominantly in the lower eyelid, medial canthus and lateral canthus. In sum, a single drop is sufficient for topical administration in dogs. Any excess is lost predominantly by spillage over the periocular skin, as well as accelerated nasolacrimal drainage.

**Introduction**

Topical administration is the route of choice for treating diseases that affect the anterior segment of the eye. This route is simple, convenient, noninvasive, and allows for the use of relatively high drug concentrations at the target tissue while minimizing systemic exposure. One of the main challenges associated with topical administration, however, remains the poor bioavailability of therapeutic drugs to the inner tissues of the eye given rapid precorneal loss from reflex blinking and efficient nasolacrimal drainage. Optimization of eyedrop delivery can enhance therapeutic benefits for the patient, regardless of the underlying pathology (e.g. dry eye, infectious keratitis, glaucoma), yet little consensus exists on fundamental concepts such as the number of eyedrops to apply. The label of ophthalmic products often recommends to ‘apply one to two drops’ (e.g. Optixcare®, Lotemax®), while diverse publications in veterinary and human ophthalmology describe the use of either ‘1 drop’, ‘1 to 2 drops’, or ‘2 drops’.  

The volume of solution instilled through topical administration is known to influence the precorneal residence time, a key parameter in ocular pharmacology. A prolonged contact time between the solution and the ocular surface is often desired, as it enhances drug bioavailability and permits longer intervals between instillations. In humans, best practices for ocular delivery often recommend a single drop of commercial preparations (~ 35 µL) per dosing session, as the maximum volume that the human palpebral fissure can hold without overflowing is estimated at 25-30 µL. Any excess is rapidly lost via nasolacrimal drainage and spillage over the
eyelashes and periocular skin;\textsuperscript{6,15} therefore, a second drop does not provide any therapeutic advantage in humans and may in fact be counterproductive by increasing systemic absorption and the risk of associated adverse effects. In rabbits, a single drop is also sufficient as the lacrimal drainage rate is proportional to the volume of solution instilled (up to 50 µL), hence tear film drug concentrations decrease less rapidly with lower instilled volumes.\textsuperscript{13,16} In fact, the smaller the instilled volume, the greater the fraction of applied dose that is absorbed inside the rabbit’s eye.\textsuperscript{13,16} Similar findings may be true in dogs, albeit direct extrapolation between species is not possible given important differences in ocular anatomy and physiology. In particular, the canine tear volume (65.3 µL)\textsuperscript{17} is nearly 9-fold larger than humans (7.0 µL)\textsuperscript{14} and rabbits (7.5 µL),\textsuperscript{13} while the canine tear turnover rate is comparable to humans (12.2 %/min vs. 10-20 %/min, respectively)\textsuperscript{17,18} but faster than rabbits (7.1 %/min).\textsuperscript{13}

The primary objective of this study was to determine the influence of volume instilled via topical administration (i.e. 1 vs. 2 drops) on tear film kinetics of fluorescein in dogs. Given the aforementioned species differences in tear film dynamics, we originally hypothesized that the kinetic profile would be superior following instillation of 2 drops in canine eyes, a hypothesis that was proven to be wrong. Hence, to explain why a single eyedrop is deemed sufficient in dogs, a secondary objective was to determine the maximal volume that the canine palpebral fissure can hold, as well as the drainage rate relative to diverse volumes (10 to 100 µL) instilled onto the canine ocular surface. The present work focuses on canine-specific ocular physiology, providing valuable information to veterinary practitioners, pet owners, and scientists working with dogs as a translational animal model for ocular surface diseases.
Materials and Methods

Animals

Eight Beagle dogs (4 neutered male, 4 spayed female) were included in the study, all confirmed to be healthy based on physical and ophthalmic examinations performed by a board-certified veterinary ophthalmologist (LS), including Schirmer tear test-1 (Eye Care Product Manufacturing, LLC, Tucson, AZ, USA), rebound tonometry (TonoVet, Icare Finland Oy, Espoo, Finland), slit-lamp biomicroscopy (SL-17; Kowa Company, Ltd., Tokyo, Japan) and indirect ophthalmoscopy (Keeler Vantage; Keeler Instruments, Inc., Broomall, PA, USA). All dogs were 3.0 – 3.5 years old and weighed 7.5 – 10 kg. The study was approved by the Institutional Animal Care and Use Committee of Iowa State University (IACUC #18-398), and was conducted in accordance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

Tear film fluorescein following instillation of 1 vs. 2 drops

A 1% fluorescein concentration was obtained by mixing 10% fluorescein solution (Akorn Inc., Lake Forest, IL, USA) with 1.4% polyvinyl alcohol lubricating eye drops (Artificial Tears, Rugby, Rockville Center, NY, USA). On Day 1, one eye in each dog was randomly selected (Excel software) to receive 35 µL (1 drop) of 1% fluorescein solution while the contralateral eye received 70 µL (2 drops) of the same solution, using a pipette (Eppendorf Reference® 2, 10-100 µL) for accuracy. On Day 2 (24 hours later), the order of eyes was reversed and the experiment was repeated. Of note, the volume chosen for a single drop (35 µL) approximates the average drop size of commercial ophthalmic preparations used in veterinary and human medicine (35-39 µL),¹⁹,²⁰ and is routinely described in previous scientific publications.⁴,⁸,²¹,²² Following topical instillation, tear fluid was collected in each eye with a 2-µL capillary glass tube (Drummond
Scientific Co., Broomhall, PA, USA) at the following time points: 0 min (i.e. immediately after instillation and spontaneous blinking), 1 min, 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 90 min, 120 min, and 180 min. The capillary tube was placed against the inferior tear lake for ≤ 2 seconds, a duration sufficient to collect tear fluid by capillary action while minimizing the risk of inadvertent ocular irritation and reflex tearing. Given the rapid collection time (< 2 sec), the lack of blinking during collection (eyelids manually opened) and the relatively large tear volume in dogs (~65 µL)(Sebbag et al., 2019), the authors believe that it is unlikely for reflex tearing, if any, to affect tear fluorescein concentrations in a significant manner. The length of fluid contained within each capillary tube was measured to the nearest millimeter using a ruler, a value used to calculate the volume of fluid collected (as 32 mm equate to 2 µL). The collected fluid was then expelled into a 2-mL Eppendorf tube that contained 500 µL of phosphate buffered saline (Gibco® PBS, pH 7.2, Thermo Fisher Scientific, Rockford, IL, USA), vortexed for 30 seconds, and transferred to a cuvette for analysis. Fluorescein concentrations were measured (in ng/mL) with a computerized scanning ocular fluorophotometer (Fluorotron Master™, Coherent Radiation, Palo Alto, CA, USA) as previously described,17 with the exception that tear fluid was diluted with 0.5 mL of PBS herein (instead of 2 mL) to improve the sensitivity of fluorescein detection (data not shown); the cuvette was slightly raised in the device’s cuvette holder to account for the lower total volume.

**Volumetric capacity of the palpebral fissure**

A 0.1% fluorescein concentration was obtained by mixing 10% fluorescein solution with 1.4% polyvinyl alcohol lubricating eye drops. Each eye received the following volumes of 0.1% fluorescein solution via pipette administration, the order being selected at random (Excel software, Microsoft Corp., Redmond, WA, USA) in each dog: 10 µL, 20 µL, 30 µL, 40 µL, 50
µL, 60 µL, 70 µL, 80 µL, 90 µL, and 100 µL. To minimize any carry-over effect from one session to another, the eyes and periocular skin were thoroughly rinsed with eye wash (Ocusoft® Eye Wash, OcuSOFT Inc., Richmond, TX, USA) at completion of each experiment, and a 1-hour break was provided between repeated instillations in each eye to allow ample time for the physiological tear film dynamics to be restored. At each session, within 10 seconds of topical instillation and spontaneous blinking, an external photograph was taken with a Nikon D90 camera to capture each eye and associated periocular skin. To enhance detection of fluorescence, the camera was equipped with a screw-on Tiffen Wratten 15 deep yellow filter (Tiffen Manufacturing, Hauppauge, NY, USA) as well as an external flash (Nikon Speedlight SB-700) covered with a blue excitation filter (SJ-4 blue color). Of note, this photographic method better highlighted 0.1% than 1% fluorescein, hence the choice of 0.1% solution for this experiment.

**Tear turnover rate at various instilled volumes**

In the experiment described above, following external photography (taken ~10 seconds after 0.1% fluorescein instillation), tear fluid was collected with 2-µL capillary glass tubes at the following time points in each eye: 1 min, 2 min, 4 min, 6 min, 10 min, 15 min, and 20 min. Tear film fluorescein concentrations were measured in all samples (see above for details) and recorded in ng/mL.

**Data analysis**

**Fluorophotometry** – First, a fluorescein calibration curve was established by analyzing a dilution series of known fluorescein concentrations in triplicate (1 to 10,000 ng/mL). Fluorescein concentrations in tear samples were corrected based on the resulting calibration equation \( y = 19.3 + 0.9 x - 3E-05 x^2 \). Fluorescein data of each animal were inputted to Monolix® version
2019R1 (Lixoft, Orsay, France), and tear turnover rate (TTR) was derived from a non-linear mixed effects model as previously described. Selected data points were censored in Monolix when a peak of fluorescence could not be identified on the fluorophotometer reading, or if the tear fluorescein concentration did not make physiologic sense (e.g. higher fluorescein at 2 min compared with baseline). Overall, 11/640 (1.7%) of all data points were left censored.

**External photography** – The volumetric capacity of the palpebral fissure was calculated in each eye as the average between the lowest instilled volume that led to periocular spillage of fluorescein solution, and the highest instilled volume for which all fluorescence remained on the ocular surface. For instance, a volumetric capacity of 35 µL was calculated for an eye that had spillage first noted at 40 µL of instilled solution, but no spillage noted at 30 µL (Figure 1). When present, the location of spillage was recorded (i.e. lower eyelid, upper eyelid, medial canthus, lateral canthus), and the area of fluorescence that extended beyond the eyelids margins was delineated with the ‘freehand selection’ tool in ImageJ 1.52a software (National Institutes of Health, Bethesda, MD, USA). The area of fluorescein spillage was recorded in mm² (Figure 2) using a length bar specific to each eye (i.e. palpebral fissure length measured in mm with calipers).

**Statistical analysis** – Normality of data was assessed with the Shapiro-Wilk test. A mixed model for repeated measures (MMRM) was fitted to the data using the R software version 3.6.0. In the model, fluorescein concentration was the response variable, the group (1 or 2 drops), time (0 to 180 min) and group-by-time interaction were treated as fixed effects, and the animal and animal-by-group interaction were treated as random effects, using animal as block. After the model was fit, the fixed effects were tested, and comparisons between 1 vs. 2 drops were made for the following outcomes: (i) fluorescein concentration in tears at each time point, and (ii) percent of
fluorescein remaining at each time point, using the baseline data of 1 drop for both groups in order to account for the different volumes instilled in both eyes. The R software was also used to calculate the area under the concentration-time curve (AUC), a parameter that was compared between groups (1 vs. 2 drops) using the paired t-test. Differences among volume instilled in fluorescein periocular spillage and tear turnover rate were assessed with a one-way ANOVA, while the relationship between the volumetric capacity and the palpebral fissure length was assessed with the Pearson’s correlation test. Statistical analyses were performed with SigmaPlot 14.0 (Systat software, Point Richmond, CA), and P values < 0.05 were considered significant.

Results
Data were normally distributed (P > 0.05), therefore results are presented as mean ± standard deviation (range).

Volumetric capacity of the canine palpebral fissure
Mean ± SD (range) volumetric capacity of the canine palpebral fissure was 31.3 ± 8.9 µL (15-45 µL). A moderate positive correlation (r = 0.57, P = 0.023) was found between the length (in mm) and the volumetric capacity (in µL) of the palpebral fissure (Figure 3). Further, mean palpebral fissure length and volumetric capacity were slightly larger in male dogs (22.5 mm and 35 µL) compared to female dogs (22 mm and 27.5 µL), although these differences were not statistically significant (P ≥ 0.090).

Periocular spillage of instilled solution
The lower eyelid represented the most common location (92%, Figure 1B, Figure 2, and Figure 4A) covered by fluorescein spillage from the ocular surface, followed by the medial
canthus (73%, Figure 4B), the lateral canthus (68%, Figure 4C) and the upper eyelid (32%, Figure 4E-F). Instillation of large volumes often resulted in excessive periocular spillage that covered multiple skin locations, although the amount and distribution of spillage varied within and between dogs; for instance, instillation of 100 µL of fluorescein onto the left eye of 3 different dogs resulted in either mild (Figure 4D) or pronounced (Figure 4E-F) periocular spillage. Overall, the area of periocular spillage increased as the volume of instilled solution increased (Figure 5), with statistical differences noted between 90-100 µL vs. 50-60 µL ($P = 0.002$), 90-100 µL vs. 30-40 µL ($P < 0.001$), and 70-80 µL vs. 30-40 µL ($P = 0.003$).

**Tear turnover rate**

Parameters estimation was performed using the stochastic approximation expectation maximization algorithm for nonlinear mixed-effects models implemented in the Monolix Suite, as previously described for analysis of canine pharmacokinetic data.\(^{23,24}\) Standard goodness-of-fit diagnostics were used to assess the validity of the model, including visual predictive checks, individual predictions vs. observations, individual weighted residuals plotted against tear fluorescein concentrations, and simulations of fluorescein vs. time disposition from 500 Monte Carlo simulations (Appendix C). Using the final mathematical model, a visual inspection of individual fluorescein decay curves showed a tendency for a ‘steeper’ initial slope (i.e. a faster tear drainage) with increasing volumes of instilled fluorescein, as seen in a representative animal that received 10-100 µL of topical solution (Figure 6). However, the average rTTR (20.2-30.5 %/min) did not vary significantly among groups ($P = 0.935$), nor did the bTTR (1.1-1.4 %/min, $P = 0.988$) observed a few minutes following fluorescein instillation (Table 1).
Tear film fluorescein concentrations following 1 vs. 2 drops

Tear film fluorescein concentrations in eyes receiving 1 vs. 2 drops are depicted in Figure 7. Immediately following instillation of fluorescein (t = 0 min), tear film concentrations were significantly higher ($P = 0.046$) in eyes receiving 2 drops ($2345 \pm 237 \mu g/mL$) compared to 1 drop ($2104 \pm 403 \mu g/mL$). However, no statistical differences in fluorescein concentrations were noted at $t = 1$ min ($P = 0.163$) or any subsequent time points ($P \geq 0.293$). In fact, the overall exposure of the ocular surface to fluorescein (AUC of fluorescein concentration-time curve) was slightly higher in eyes receiving 1 drop ($30,513 \pm 21530 \mu g*min/mL$) compared to 2 drops ($28,975 \pm 17,410 \mu g*min/mL$). However, this difference was not statistically significant ($P = 0.742$), and the overall effect of volume instilled on tear film fluorescein was non-significant ($P = 0.619$) when taking ‘time’ into account in the model.

The percentage of solution retained on the ocular surface following 1 vs. 2 drops is summarized in Figure 8. At $t = 1$ min, the percent retained was higher in eyes receiving 2 drops ($90.6 \pm 16.7 \%$) compared to 1 drop ($81.8 \pm 8.2 \%$), a difference that approached statistical significance ($P = 0.071$). However, no significant differences were noted for any other time point ($P \geq 0.220$), or in the overall effect of volume instilled on the percentage of solution retained ($P = 0.731$) when the variable ‘time’ was taken into account.

Discussion

The present study supports the use of a single eyedrop in Beagle dogs, whether used therapeutically in canine patients with ocular disease, or experimentally in canine models of translational research.25,26 A second drop achieved higher tear film concentrations immediately after topical administration ($t = 0$ min), a finding that is partly explained by a lower dilution effect for 2 drops (1.9-fold) than 1 drop (2.9-fold) from the tear fluid present on the canine ocular
surface (~65 µL). However, the benefit of instilling two drops was short-lived (<1 min) and unlikely to be clinically important, although the present study focuses on fluorescein and cannot be directly extrapolated to ophthalmic drugs such as antibiotics, corticosteroids or anti-glaucoma medications. A second drop is wasted from an economic perspective, and can potentially exacerbate local and/or systemic adverse effects by overflow on the periocular skin and drainage through the nasolacrimal duct, respectively. The latter was not evaluated herein as fluorescein is non-biologically active, albeit previous studies have shown that overwhelming the lacrimal system can increase the amount of drug that reaches the blood via the naso-buccal mucosa. In sum, the kinetic profile of fluorescein in tears was not superior with 2 drops vs. 1 drop, a finding that is often explained by an accelerated lacrimal drainage with increasing volume in both rabbits and humans. However, this explanation is not valid in dogs as the rate of lacrimal drainage did not change significantly in our canine subjects despite a 10-fold increase in instilled volume (10 to 100 µL); rather, the present study shows that excessive periocular spillage is the main culprit limiting the benefit of using 2 topical drops in canine ophthalmology. The amount of solution that overflowed on the periocular skin was greater with larger volumes of instilled solution, and primarily affected the lower eyelid, medial canthus and lateral canthus. Such spillage can participate to local adverse effects, such as Malassezia sp. overgrowth in dogs receiving topical medications, or skin hyperpigmentation and lengthening of eyelashes in humans receiving topical prostaglandin analogues.

The volumetric capacity of the canine palpebral fissure is 31.3 µL. This value is somewhat similar to the volumetric capacity in humans (25-30 µL), and approximates the average volume of a single eyedrop of commercial preparations (~35 µL). As such, a single eyedrop is deemed sufficient in dogs and humans because their ocular surface is unable to
accommodate volumes larger than ~ 30 µL, yet therapy with a single drop is relatively inefficient in both species given the short precorneal residence time and low ocular bioavailability. Several strategies can be implemented to enhance the benefits of eyedrop administration, including: (i) Eyelid closure and/or nasolacrimal punctal occlusion for several minutes following topical instillation; (ii) Higher drug concentration – Walters et al. showed that topical 1.5% levofoxacin in humans achieved tear concentrations that were 3-10 times higher than those seen with 0.3% ofloxacin at multiple time points over 24 hours; (iii) Higher solution viscosity and/or use of mucoadhesive polymers; (iv) Administration of a second drop ≥ 1 min apart from the first drop – Herring et al. showed that administration of 2 drops (1-min apart) of 0.5% proparacaine in dogs achieved significantly greater and longer anesthetic effect compared to eyes that received a single drop; and (v) Use of volumes smaller than the average commercial drop size.

Strategies to improve ocular drug delivery should ideally be investigated in each species separately, as direct extrapolation between species is hindered by differences in ocular anatomy and physiological parameters such as blink and tear turnover rates. Rabbits, for instance, have a much slower blink rate (3-6 blinks/h) and a slower tear drainage (7 %/min) compared to humans (17 blinks/min and 10-20 %/min, respectively), as well as a different expression of mucins on the ocular surface that could affect the retention of mucoadhesive polymers. These differences explain why an instilled eyedrop is partially lost (20-30%) due to reflex blinking and periocular spillage in humans, but not in rabbits, or why a solution’s viscosity has a great impact on precorneal retention and drug ocular bioavailability in humans, but not in rabbits. In a study from over 4 decades ago, it was recognized that “considerable reservations may be felt about comparing results from rabbits with those from humans because
of the differences between the physiology of tear flow and mixing and general anatomy”, yet “the rabbit is the principal experimental animal in ophthalmology, so comparisons are needed”.42 Since then, rabbits continued to be the ‘species of choice’ for ophthalmic studies given their availability and easy handling, yet the present study shows that dogs likely represent a more relevant model for translational research. Indeed, dogs and humans share many similarities that are relevant to ophthalmic drug delivery, although important differences exist (e.g. tear volume)17 that should be accounted for in comparative studies. The similarities include the blink rate (14.2 vs. 17 blinks/min),39,43 basal TTR (12.2 %/min vs. 10-20 %/min),17,18 reflex TTR following eyedrop instillation (20-30 %/min vs. 30 %/min, respectively),44 volumetric capacity of the palpebral fissure (31.3 µL vs. 25-30 µL),1,14 and periocular spillage of excess solution. The aforementioned similarities justify the use of dogs as a translational model in ophthalmic research, especially given the presence of spontaneous canine diseases that closely resemble human conditions including keratoconjunctivitis sicca,45,46 herpetic keratitis47 and neurotrophic keratopathy.48

The main limitation of the study is the use of dogs from a single breed (Beagles), all being ophthalmoscopically healthy and relatively young (3-3.5 years). The tear film pharmacokinetics of 2 drops may be different in a larger canine breed, presumably due to differences in volumetric capacity and/or tear drainage, as shown in German Shepherd dogs using the fluorescein clearance test.20 Similarly, the ocular surface of older dogs may accommodate a larger volume due to laxity in the eyelids, and the instilled solution may be retained for longer durations due to reductions in tear volume, reflex tearing, and tear turnover rate.49,50 In addition, the present findings do not fully represent the physiology of eyes with ocular surface disease, in which chemosis can reduce the volumetric capacity of the palpebral
fissure, inflammation can affect tear drainage and ocular absorption, and excessive tearing from ocular irritation can further dilute the administered solution. In particular, patients with inflamed nasolacrimal duct (dacryocystitis) may actually benefit from instillation of a second drop, as greater nasolacrimal drainage would theoretically be beneficial in such cases. A second limitation of the study is related to the use of sodium fluorescein as a marker for tear film kinetics. Fluorescein was shown to overestimate tear turnover in human subjects, as a portion of instilled fluorescein can be lost by conjunctival permeation and not nasolacrimal drainage. However, a common alternative described by other investigators (i.e. gamma scintigraphy) is not applicable to dogs, in whom the general anesthesia required to hold still for the procedure would negatively impact the tear film dynamics.

The present study on drop size and tear film pharmacokinetics can be summarized as follows. Instillation of 2 drops provided tear film fluorescein concentrations that were higher than 1 drop at baseline, due to lower dilution effect from tears, although the benefits were short-lived (<1 min) and not clinically important. The kinetic profile of fluorescein in tear film was not superior in eyes receiving 2 drops vs. 1 drop, as determined by the residual tear fluorescence at various time points, and the overall exposure of the ocular surface (AUC) to the solution instilled. Therefore, a single standard size drop is sufficient for topical administration in dogs, a finding supported by the volumetric capacity of the canine palpebral fissure (31.3 µL). Any excess is lost predominantly by spillage over the periocular skin, as well as accelerated nasolacrimal drainage.
References


32. Flach AJ. The importance of eyelid closure and nasolacrimal occlusion following the ocular instillation of topical glaucoma medications, and the need for the universal inclusion of one of these techniques in all patient treatments and clinical studies. Trans Am Ophthalmol Soc 2008;106:138-145; discussion 145-138.


Tables and Figures

**Table 1.** Mean ± standard deviation of reflex tear turnover rate (rTTR) and basal tear turnover rate (bTTR) in 8 Beagle dogs following topical instillation of 10-100 µL of 0.1% fluorescein solution in each eye. *P* values depict the results of one-way ANOVA testing.

<table>
<thead>
<tr>
<th>Volume (µL)</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
<th>100</th>
<th><em>P</em>-value</th>
</tr>
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<tbody>
<tr>
<td>rTTR (%/min)</td>
<td>20.6 ± 14.5</td>
<td>20.2 ± 11.4</td>
<td>22.7 ± 17.1</td>
<td>24.3 ± 12.0</td>
<td>30.3 ± 23.3</td>
<td>22.3 ± 13.0</td>
<td>24.6 ± 13.2</td>
<td>24.6 ± 18.4</td>
<td>30.5 ± 22.1</td>
<td>22.2 ± 11.6</td>
<td>0.935</td>
</tr>
<tr>
<td>bTTR (%/min)</td>
<td>1.4 ± 0.4</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.5</td>
<td>1.1 ± 0.5</td>
<td>1.2 ± 0.6</td>
<td>1.2 ± 0.6</td>
<td>1.1 ± 0.5</td>
<td>1.2 ± 0.6</td>
<td>1.1 ± 0.6</td>
<td>1.2 ± 0.6</td>
<td>0.988</td>
</tr>
</tbody>
</table>

**Figure 1.** Photographs of the right eye and eyelids in a representative Beagle dog. The volumetric capacity of the palpebral fissure was calculated as 35 µL in this eye, based on the lack or presence of periocular spillage of 0.1% fluorescein with instillation of either 30 µL (A) or 40 µL (B), respectively.
Figure 2. Photograph of the right eye and eyelids following topical instillation of 90 µL of 0.1% fluorescein in a representative Beagle dog. The area of periocular spillage was delineated with ImageJ software (version 1.52a, National Institute of Health), and recorded in mm² based on a length bar (10 mm) specific to each eye.

Figure 3. A positive association was found between the length and the volumetric capacity of the palpebral fissure (Pearson’s correlation test).
Figure 4. Representative ocular images following instillation of 60 µL (A), 80 µL (B), 40 µL (C), or 100 µL (D-F) of 0.1% fluorescein solution in different Beagle dogs. Notice the periocular spillage that predominantly affects the lower eyelid (A), medial canthus (B), lateral canthus (C), or multiple locations including the upper eyelid (D-F).

Figure 5. Bar chart depicting the mean area (+SD) of periocular spillage of 0.1% fluorescein solution, instilled at various volumes (10-100 µL) in 8 Beagle dogs (n = 16 eyes).
Figure 6. Comparison of predicted tear fluorescence over time (purple curve) with observed data (blue points) following topical instillation of 0.1% fluorescein solution (10 to 100 µL) in a representative Beagle dog. Censored data are shown as vertical red bars.

Figure 7. Scatter plot depicting the mean ± SD of tear film fluorescence over time in canine eyes receiving either 1 drop (35 µL; red circles) or 2 drops (70 µL; blue triangles) of 1% fluorescein solution. Differences in tear fluorescence were noted at t = 0 min ($P = 0.046$) but no other time points ($P \geq 0.163$).
Figure 8. Bar chart depicting the mean ± SD of residual tear film fluorescence at each time point in canine eyes receiving either 1 drop (35 µL; plain red bars) or 2 drops (70 µL; hatched white bars) of 1% fluorescein solution. For standardization, the residual fluorescence in both groups was compared to the tear fluorescence obtained at t = 0 min in eyes receiving a single drop of fluorescein. No statistical differences were noted between both groups at any time point (P ≥ 0.220).
CHAPTER 4. HISTAMINE-INDUCED CONJUNCTIVITIS AND BREAKDOWN OF BLOOD-TEAR BARRIER IN DOGS: A MODEL FOR OCULAR PHARMACOLOGY AND THERAPEUTICS

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Modified from a manuscript published in Frontiers in Pharmacology

Abstract

Conjunctival inflammation disturbs the blood–tear barrier and thus affects the tear film stability and composition. We aimed to develop a non-invasive and reliable method to induce conjunctivitis in dogs, a large animal model for translational work on ocular surface disease in humans. Six beagle dogs underwent a randomized, vehicle-controlled, balanced crossover trial—on six separate days, one eye received topical artificial tears (vehicle), while the other eye received one of six concentrations of histamine solution (0.005–500 mg/ml). At sequential times after eyedrop administration, a conjunctivitis score was given to each eye based on the degree of palpebral and bulbar conjunctival hyperemia and chemosis, ocular pruritus, and discharge. Total protein content (TPC) and serum albumin were quantified in tear fluid at baseline and 20 min. Additionally, 13 dogs presenting for various ophthalmic diseases with associated conjunctivitis were examined. Experimentally induced conjunctivitis developed rapidly (<1 min) following topical histamine administration and lasted for 1–3 h (four lowest doses) to 6–8 h (two highest...
doses). The severity of conjunctivitis was dose-dependent. Histamine was overall well tolerated, although transient blepharitis, aqueous flare, and ocular hypertension occurred in a few dogs receiving histamine ≥375 mg/ml. TPC and serum albumin levels increased in tears of eyes receiving histamine ≥1.0 mg/ml, being significantly higher than vehicle and baseline in eyes receiving histamine ≥375 mg/ml. Lacrimal albumin levels were also increased in 13 dogs with naturally acquired conjunctivitis, up 2.7–14.9 fold compared to contralateral healthy eyes. Histamine-induced conjunctivitis represents a robust model for translational work on the ocular surface given the low cost, non-invasiveness, self-resolving nature, ability to adjust the duration and severity of the disease, and shared features with naturally occurring ocular diseases. Histamine solutions of 1, 10, and 375 mg/ml induce mild, moderate, and severe conjunctivitis in dogs, respectively. Leakage of serum albumin in tear fluid of eyes with conjunctivitis suggests a breakdown of the blood–tear barrier.

**Introduction**

Conjunctivitis, or inflammation of the vascularized mucous membrane lining the inside of the eyelids, anterior sclera, and (when present) the nictitating membrane, is a common ocular surface disease in both humans and veterinary patients.\(^1\,^{2}\) In addition to well-recognized etiologies (e.g., infectious, allergic, toxic/irritative, immune-mediated), conjunctivitis frequently develops as a bystander to most adnexal and ocular diseases, such as blepharitis, keratitis, uveitis, and glaucoma.\(^1\,^{2}\) Regardless of the cause, conjunctivitis is debilitating to patients due to ocular discomfort, redness, and discharge, as well as the potential development of conjunctival scarring, fornix foreshortening, or symblepharon in severe or untreated cases. Further, conjunctivitis compromises the tear film homeostasis and thereby contributes indirectly to ocular surface damage. Changes in tear composition result from a loss of secretory function and
numbers of mucin secreting goblet cells, but can also be linked to the disruption of the blood–
tear barrier — a critical yet poorly understood mechanism. Indeed, conjunctivitis increases
vascular permeability and results in leakage of plasma compounds into the tear film, as
exemplified by human patients with conjunctivitis and dry eye who were noted to have
significantly greater serum albumin in tears compared to healthy controls, and dogs with
spontaneous keratoconjunctivitis sicca for whom the clinical signs of conjunctivitis were
positively correlated with tear levels of serum proteins.

Despite the prevalence of conjunctivitis, there is limited knowledge about the disease
impact on the ocular surface in clinical patients. Are tear film composition and quality affected
by conjunctivitis-induced breakdown of the blood–tear barrier? Is there an impact on the
pharmacokinetic profile of drugs on the ocular surface? Do medications administered
systemically reach the tear film compartment at higher concentrations? Such knowledge can be
gained from experimentally induced models of conjunctivitis in animals, such as intraperitoneal
injection of ovalbumin in guinea pigs and topical administration of dust mite allergens in
dogs. However, a delayed response occurs with ovalbumin (6 h from antigen exposure to
conjunctival pathology), and dust mite allergens only cause conjunctivitis in individuals already
sensitized to this antigen. In contrast, the use of topical histamine is promising as it causes
conjunctivitis in a nonspecific and rapid manner and the compound is a potent inflammatory
mediator that is associated with various disorders such as allergy, inflammation, autoimmune
conditions, and possibly cancer. Takahashi and colleagues used topical histamine in guinea
pigs and quantified the extravasation of Evans Blue to demonstrate vascular permeability in the
conjunctiva. The authors focused on a single dose of histamine and did not assess the safety of
the drug, disease severity, or disease duration. In the present study, the model of histamine-
induced conjunctivitis was fine-tuned and perfected: we investigated a diverse range of histamine concentrations, performed serial ophthalmic and physical examinations to assess safety, and described in detail the clinical and biochemical changes observed at each dose. The dog is a preclinical species of choice for modeling human ocular diseases as the canine ocular anatomy is more similar to humans than routine laboratory species; dogs share similar environmental stressors with people, and spontaneously occurring ocular surface diseases are common in this species. We are confident that this translatable in vivo model of conjunctivitis will guide future studies to gain a deeper understanding of the disease, elucidating the impact of conjunctivitis on drug pharmacokinetics, tear film dynamics, metabolomics, and proteomics, among others.

Materials and Methods

Experimentally Induced Conjunctivitis in Dogs

Animals

Six beagle dogs (1.5–2.0 years, 7.7–10.1 kg) were used in the study. The gender and neuter status was the same for all subjects (female spayed), as sex hormones are known to be key regulators of vascular tone in various organs, including the eye. All dogs were confirmed to be healthy based on complete physical and ophthalmic examinations, including slit-lamp biomicroscopy (SL-17; Kowa Company, Ltd., Tokyo, Japan), indirect funduscopy (Keeler Vantage; Keeler Instruments, Inc., Broomall, PA, USA), rebound tonometry (TonoVet; Icare Finland Oy, Espoo, Finland), Schirmer tear test-1 (STT-1; Eye Care Product Manufacturing LLC, Tucson, AZ, USA), and fluorescein staining (Flu-Glo, Akorn, Inc., Buffalo Grove, IL, USA). The dogs were group-housed in kennels with ambient temperature maintained at 18–24°C and lights automatically turned on/off at 06:00/18:00. The study was approved by the Institutional Animal Care and Use Committee of Iowa State University (log # 2-18-8704-K) and
adhered to the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research.

**Topical Histamine and Vehicle Solutions**

Histamine ophthalmic solutions were formulated by mixing histamine powder (Histamine dihydrochloride, FCC grade, Arcos® organics, Geel, Belgium) in 1.4% polyvinyl alcohol lubricating eye drops (Artificial tears solution, Rugby, Rockville Center, NY, USA) using a sterile manner under a laminar flow hood. The pH of each solution was tested with a pH meter (B-212 Twin compact pH, Horiba, Kyoto, Japan) and adjusted to 6.5 by adding 1% sodium hydroxide (prepared from granules mixed with sterile water), one drop at a time until the target pH was reached. The following 12 concentrations of histamine solution were compounded into 15-ml sterile eyedropper bottles (Steri-dropper®, Medi-Dose®, Ivyland, PA, USA) by the pharmacist at Iowa State University’s Lloyd Veterinary Medical Center: 0.001, 0.005, 0.01, 0.0375, 0.1, 0.5, 1.0, 10, 100, 375, 500, and 1,000 mg/ml. The vehicle solution consisted of artificial tears solution adjusted to pH of 6.5 with a minute amount of 1% sodium hydroxide (< 5 drops in 15-ml bottle). Histamine and vehicle solutions were used within 24 h of preparation and kept in the dark at room temperature (18–24°C) before use and in between experiments.

**Experimental Design**

*Pilot Study to Assess Tolerability and Appropriate Histamine Concentrations*

A pilot study was conducted to assess the tolerability of histamine solutions in dogs (local and systemic) and the most appropriate concentrations to select for the crossover trial. Each of the 12 eyes (n = 6 dogs) was randomly allocated to one of the 12 histamine concentrations (Excel, Microsoft, Redmond, WA, USA). A single drop of histamine solution was instilled onto
the ocular surface, followed by slit-lamp biomicroscopy and conjunctivitis scoring at 1, 3, 5, 7, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120, 180, 240, 300, 360, and 420 min. Additionally, the following ocular and physical parameters were recorded at 1, 10, 20, 30, 60, 120, and 240 min post-histamine administration: intraocular pressure, aqueous flare grading, body temperature, heart rate, respiratory rate, respiratory efforts, capillary refill time, and indirect blood pressure (Doppler model 811-B, Parks Medical Electronics, Las Vegas, NV, USA).

**Balanced Crossover Vehicle-Controlled Trial**

Six histamine solutions were selected for the crossover trial based on good tolerability and diversity of conjunctivitis scoring (pilot study, data not shown). The six histamine solutions are listed as follows: $H_1 = 0.005\, \text{mg/ml}$, $H_2 = 0.1\, \text{mg/ml}$, $H_3 = 1.0\, \text{mg/ml}$, $H_4 = 10\, \text{mg/ml}$, $H_5 = 375\, \text{mg/ml}$, and $H_6 = 500\, \text{mg/ml}$. For each dog, one eye received histamine solution (random selection by coin-toss) while the other eye received vehicle solution, and this order was kept consistent throughout the study. Each dog received all six histamine solutions over six consecutive days, using one solution per day (once in the morning) in a balanced crossover trial (Appendix D, Figure 1). In each dog, ocular and physical parameters were recorded at selected times (similar to pilot study), while tear collection and conjunctivitis scoring were performed as outlined below:

- **Tear collection:** At baseline and at 20 min post-eyedrop administration, tear fluid was collected from both eyes and total protein content (TPC) was analyzed as previously described. Briefly, a standardized Schirmer strip was inserted into the ventrolateral conjunctival fornix until the 20-mm mark was reached. Each wetted Schirmer strip was placed into a 0.2-ml tube (pre-punctured at its bottom with an 18-gauge needle), secured into a 2-ml tube with adhesive tape, and centrifuged at $3,884 \times g$ for 2 min (Mini Centrifuge, VWR International, Radnor, PA, USA). After estimating the volume of extracted tear fluid with a pipette, each tear sample was diluted.
1:3 with phosphate-buffered saline (PBS 1X, Gibco™, Thermo Fisher Scientific, Inc., Waltham, WA, USA). TPC was calculated with Direct Detect™ infrared spectrometer and expressed in mg/ml after adjusting for the threefold dilution of each sample. Subsequently, the residual tear fluid was diluted fourfold with diluent provided with the albumin ELISA kit (Serum albumin ELISA kit, Life Span Biosciences, Inc., Seattle, WA, USA). Serum albumin and various cytokines/chemokines/growth factors (Canine Procarta Plex™ 11-plex immunoassay, Cat No EPX11A-50511-901, Thermo Fischer Scientific, Waltham, MA, USA) were quantified in each tear sample following the manufacturer’s protocol.

• **Conjunctivitis score:** At baseline and at selected times post-eyedrop administration (similar to pilot study), one examiner (LS) who was masked to which eye received histamine or vehicle solution performed slit-lamp biomicroscopy of both eyes to determine a conjunctivitis score at each time point. The conjunctivitis score was calculated as the sum of the following categories, each graded on a three- to four-point scale (Appendix D, Table 1): hyperemia, chemosis and follicles of the palpebral and bulbar conjunctiva, conjunctival discharge, and ocular pruritus.17,18

**Naturally Acquired Conjunctivitis in Dogs**

Thirteen dogs presenting to Iowa State University’s Lloyd Veterinary Medical Center (ISU-LVMC) for clinical ophthalmic disease with associated conjunctivitis were enrolled. A verbal informed consent was obtained from all owners, which was sufficient for the hospital’s ethics committee given that Schirmer tear test is part of routine clinical care. Dogs were examined by a board-certified veterinary ophthalmologist (LS or RA) for diagnosis and treatment of various ocular complaints including corneal ulceration, uveitis, and glaucoma. Details of the dogs (breed, sex, age) and their clinical diagnosis are presented in Table 1. Tear collection and albumin analysis were performed in both eyes of each dog as described above.
Data Analysis

Normality of the data was assessed with the Shapiro-Wilk test. The one-way ANOVA and Tukey post hoc test was used to compare the six histamine doses for i) duration of conjunctivitis, determined as the time for conjunctivitis score to return to zero, and ii) severity of conjunctivitis, determined by the area under the curve of conjunctivitis score from 0 to 180 min. Within each histamine dose, the one-way repeated measures ANOVA and Bonferroni post hoc test was used to assess differences in conjunctivitis scores between time points. The Student t-test was used to evaluate differences between vehicle and histamine-treated eyes for TPC and albumin levels, both at baseline and at 20 min following eyedrop administration. Further, a ratio was calculated for TPC and albumin levels for each histamine dose as follows: (Concentration histamine 20 min/histamine baseline)/(Concentration vehicle 20 min/vehicle baseline). This ratio describes the percentage change in protein levels between baseline and 20 min post-induction of conjunctivitis, taking into account the variability inherent to the tear collection method itself by adding the vehicle-treated eyes as the denominator.16 The one-way ANOVA and Tukey post hoc test was used to assess differences in protein ratios and levels of cytokines/chemokines/growth factors in tears of different groups. In dogs with naturally acquired conjunctivitis, differences in lacrimal concentrations of albumin between affected and unaffected eyes were analyzed with the Mann–Whitney test. Statistical analysis was performed using SigmaPlot version 14.0 (Systat Software, Point Richmond, CA), and values $P < 0.05$ were considered statistically significant.
Results

Experimentally Induced Conjunctivitis in Dogs

Clinical Features of Conjunctivitis

All eyes receiving the vehicle solution were scored at zero for all time points. In eyes receiving histamine, the ocular surface was examined from different angles to evaluate the canine conjunctiva in a comprehensive manner, including a view from the front (Figure 1A), side (Figure 1B), top (Figure 1C), and globe retropulsion with lower lid retraction (Figure 1D), which facilitated assessment of the palpebral conjunctiva and nictitating membrane. Figure 2 shows representative photographs and conjunctivitis scoring of a canine eye at 30 min following H₄ administration (10 mg/ml histamine solution), while Figure 3 demonstrates the development and progression of conjunctivitis from 0 to 420 min in a canine eye receiving H₅ (375 mg/ml histamine solution). Data were normally distributed such that results are presented as mean ± standard deviation (range). Conjunctivitis developed rapidly (<1 min) for all doses (Figure 4A). Of note, not a single eye developed conjunctival follicles. Details of conjunctivitis scoring for each subsection (palpebral chemosis, bulbar hyperemia, etc.) is described in Appendix D (Table 2).

The duration of conjunctivitis was statistically different among histamine doses ($P < 0.001$), ranging from $61 ± 37$ min (25–120 min) for H₁, $110 ± 36$ min (90–180 min) for H₂, $115 ± 52$ min (60–180 min) for H₃, $190 ± 45$ min (120–240 min) for H₄, $390 ± 33$ min (360–420 min) for H₅, and $400 ± 31$ min (360–420 min) for H₆. All pairwise comparisons were statistically significant ($P ≤ 0.029$) except for H₁–H₃ ($P = 0.201$) and H₅–H₆ ($P = 0.998$).

The severity of conjunctivitis was significantly different among histamine doses ($P < 0.001$), with an AUC₀–180min (in score x min) ranging from $59.2 ± 36.8$ (21.5–119) for H₁, $200.2 ± 53.9$ (107.5–254.5) for H₂, $277.2 ± 128.3$ (144.5–495.5) for H₃, $522.3 ± 131.8$ (358–735)
for H4, 1,168.5 ± 266.4 (892.5–1,585) for H5, and 1,186.5 ± 242.4 (914–1,443.5) for H6. All pairwise comparisons were statistically significant ($P \leq 0.037$) except for H5–H6 ($P = 1.000$) and H2–H3 ($P = 0.794$; Figure 4B).

A post hoc power analysis showed that $n = 5$ dogs were sufficient to detect a difference in total clinical scores of 2.2 (as observed clinically in eyes with mild vs. moderate vs. severe conjunctivitis), a standard deviation of 0.9, a power of 80%, and an $\alpha$ value of 0.05.

**Tolerance**

Locally, histamine administration was very well tolerated in dogs except for transient adverse effects noted with H5 and H6: i) Blepharitis, manifested by mild blepharedema and erythema (Figure 5A), was noted in 1/6 dogs receiving H5 and 2/6 dogs receiving H6, developing within 10–30 min of histamine administration and self-resolving within 3 h. ii) Aqueous flare, subtle in intensity (trace to 1+), was noted in 2/6 dogs receiving H5 and 6/6 dogs receiving H6, developing within 25–90 min and self-resolving within 1–3 h. Aqueous flare was commonly accompanied by miosis (Figure 5B). iii) Ocular hypertension, defined as IOP > 25 mmHg, was noted in 1/6 dogs receiving H5 at 30 min (IOP = 32 mmHg), although it was not statistically greater than baseline IOP ($P = 0.529$) and it self-resolved within 10 min. Ocular hypertension was also noted in 3/6 dogs receiving H6 in which IOP was significantly higher at 30 min (23.8 ± 5.5 mmHg; $P = 0.011$) and 60 min (23.2 ± 2.6 mmHg; $P = 0.029$) compared to baseline (16.8 ± 2.6 mmHg). Importantly, no fluorescein stain uptake or corneal changes were noted for any histamine dose.

Systemically, all the vital parameters monitored were stable and not a single dog receiving H1 to H6 developed systolic hypotension (<90 mmHg). However, the systolic blood pressure dropped from 130 to 88 mmHg in a single dog receiving 1,000 mg/ml histamine during
the pilot phase, and the dog exhibited transient depression and weakness until blood pressure
returned to baseline 10 min later.

**Total Protein Content and Serum Albumin Levels in Tears**

At baseline, lacrimal TPC varied from 3.0 to 29.0 mg/ml (8.8 ± 4.0 mg/ml) and no
differences were noted between vehicle and histamine-treated eyes for any dose \( (P \geq 0.365) \).
Twenty minutes following eyedrop administration, lacrimal TPC varied from 2.3 to 36.3 mg/ml
(9.9 ± 5.6 mg/ml) and was statistically greater in histamine vs. vehicle-treated eyes for H5 \( (P =
0.009) \) and H6 \( (P = 0.021) \) but no other doses \( (P \geq 0.310, \text{ Figure 6A}) \). Mean ± SD (range)
changes in TPC were 171 ± 112% (6–271%) and 170 ± 181% (13–499%) for H5 and H6,
respectively, values that were significantly greater than H1 and H2 \( (P \leq 0.036, \text{ Figure 6B}) \).

At baseline, albumin levels in tears varied from 0.015 to 1.431 mg/ml (0.413 ± 0.455
mg/ml) and no differences were noted between vehicle and histamine eyes for any dose \( (P \geq
0.394) \). Twenty minutes following eyedrop administration, albumin levels varied from 0.019 to
9.595 mg/ml (1.158 ± 2.098 mg/ml) and were statistically greater in histamine vs. vehicle-treated
eyes for H5 \( (P = 0.021) \) and H6 \( (P = 0.029) \) but no other doses \( (P \geq 0.106; \text{ Figure 6C}) \). Mean ±
SD (range) changes in albumin levels were 2,348 ± 1,454% (314–4,348%) and 4,031 ± 4,679%
(583–12,689%) for H5 and H6, respectively, values that were significantly greater than H1 and H2
\( (P \leq 0.016; \text{ Figure 6D}) \).

**Canine Chemokines/Cytokines/Growth Factors in Tears**

The levels of interferon-gamma (IFNγ), interleukin-2 (IL-2), and beta nerve growth
factor (NGF-β) were below limits of quantification in all tear samples. Tear concentrations of
other chemokines/cytokines/growth factors, described as mean ± standard deviation (range),
were as follows (Figure 7): 4.5 ± 19.4 pg/ml (0–147.6 pg/ml) for interleukin-6 (IL-6), 2,647.8 ± 5,680.4 pg/ml (0–25,640.2 pg/ml) for interleukin-8 (IL-8), 17.8 ± 16.3 pg/ml (0–86.8 pg/ml) for interleukin-10 (IL-10), 30.6 ± 104.0 pg/ml (0–807.3 pg/ml) for interleukin-12 (IL-12), 532.5 ± 510.6 pg/ml (0–2,650.8 pg/ml) for vascular endothelial growth factor A (VEGF A), 0.80 ± 2.9 pg/ml (0–20.9 pg/ml) for tumor necrosis factor alpha (TNFα), 1.3 ± 4.3 pg/ml (0–24.8 pg/ml) for stem cell factor (SCF), and 5.4 ± 13.1 pg/ml (0–80.2 pg/ml) for chemokine monocyte chemoattractant protein-1 (MCP-1). Statistical differences among groups (vehicle histamine solutions) were noted for IL-8 (Figure 7B), IL-10 (Figure 7C), IL-12 (Figure 7D), and VEGF A (Figure 7E).

Naturally Acquired Conjunctivitis in Dogs

Ocular disease was unilateral in all dogs, and spontaneous conjunctivitis was noted upon examination of all affected eyes (Figures 8–10). Lacrimal concentrations of albumin ranged from 1.1 to 17.95 mg/ml in affected eyes, representing a significant change by 2.67–14.86 fold ($P < 0.001$) compared to lacrimal concentrations noted in contralateral unaffected eyes (0.13–3.06 mg/ml). Re-examination of two dogs following therapy of the underlying ocular disease (cases #12–13; Table 1, Figure 10) showed reduction in lacrimal albumin concentrations concomitant with a reduction in the conjunctivitis score (Table 1).

Discussion

The present study establishes a robust in vivo model of conjunctivitis in dogs, a translational large animal model that provides a framework for ocular surface studies in clinically relevant subjects, investigating the impact on conjunctivitis on tear film dynamics, pharmacokinetics, metabolomics, and other relevant fields. Dogs are an ideal large animal
species for such translational model: not only does the canine ocular anatomy better resemble humans than small laboratory animals do, but dogs also share similar environmental stressors to humans and conjunctivitis is a common and naturally occurring disease in this species. As a proof of concept, our study examined 13 dogs with naturally acquired conjunctivitis and confirmed the presence of elevated albumin levels in tears of affected eyes. Similar to histamine-induced conjunctivitis, eyes with naturally occurring disease exhibited a breakdown of the blood–tear barrier regardless of the underlying etiology (e.g., corneal ulceration, uveitis, glaucoma, and orbital cellulitis). Of note, the “blood–tear barrier” is not as well defined as other ocular barriers (e.g., blood–aqueous and blood–retinal barriers) and would benefit from future anatomical and physiological studies to confirm the terminology used in the present study and in previous work.

Histamine-induced conjunctivitis is not novel. The ocular use of histamine has been described in humans, guinea pigs, and rabbits as the compound is inexpensive and triggers local inflammation in a non-specific manner. In dogs, we showed that histamine-induced conjunctivitis is non-invasive, self-resolving, and dose-dependent, allowing investigators to modulate the severity and duration of conjunctival inflammation by adjusting the dose of histamine solution administered. In fact, both parameters increased with histamine concentration until saturation in dose response observed at 375 mg/ml. Thus, we propose doses of 1, 10, and 375 mg/ml to induce a mild, moderate, or severe conjunctivitis in dogs, respectively (Figure 11). Of note, histamine is not only associated with ocular allergies but also implicated in general inflammation, autoimmune conditions, and possibly cancer. Ocular allergy was not the scope of the present study, as many models and detailed descriptions of the condition already exist.
The model presented herein provides a unique opportunity for scientists to investigate the ocular surface in health and disease. First, since topical histamine had no impact on the contralateral eye, the model is applicable for studies that compare efficacy between drug and placebo, allowing for precise measurements of a drug action given the rapid (1 min) and sustained (120–420 min) development of conjunctivitis. Second, the changes noted in tear fluid levels of TPC and albumin strongly suggest a breakdown of the blood–tear barrier. In particular, albumin represents a marker of vascular permeability and plasma leakage given it is not produced by the lacrimal gland or corneo-conjunctival tissue. Our findings are consistent with previous reports of conjunctivitis, whether experimentally induced or naturally occurring from dry eye, corneal ulcers, allergies, or others. The mechanism of histamine-induced disruption of the blood–tear barrier is unknown. Increased vascular permeability likely plays a role, combined with a disruption of tight junctions between conjunctival epithelial cells due to increased contractility of actin linked to these adhesion complexes. The breakdown of the blood–tear barrier could be exploited for assessing the impact of conjunctivitis on drug pharmacokinetics, tear film metabolomics, and other fields. In the field of pharmacology, for instance, the vast majority of studies to date are limited to healthy subjects with intact blood–tear barriers, in whom the lacrimal drug concentrations likely under-estimate the ones noted in actual clinical patients with ocular surface inflammation. Such discrepancies likely result in inappropriate dosing, thus affecting the drug efficacy and increasing the risk of toxicity or antimicrobial resistance.

In addition to protein quantification, the present study investigated a panel of cytokines/chemokines/growth factors in tear samples of dogs. Similar to human subjects with ocular surface disease such as dry eye or vernal keratoconjunctivitis, an increase in VEGF A,
pro-inflammatory cytokines (IL-8, IL-12), and anti-inflammatory cytokine (IL-10) was detected in tears of dogs with experimentally induced conjunctivitis. Analysis of other biomarkers such as acidic mammalian chitinase would be beneficial in future studies, but the paucity of tear fluid collected in each dog limited the number of compounds that could be analyzed.

The relatively low number of dogs enrolled in the histamine experiment is a clear limitation of our work, although a post hoc power analysis showed that $n = 5$ dogs were sufficient to detect significant differences between histamine doses for the main study outcome (clinical severity of conjunctivitis). Furthermore, although the subjectivity of our clinical scoring may be perceived as a drawback, given that photograph-based methods are mainstream in human studies, the method described herein is purposely adapted to working with dogs. Indeed, i) a handheld slit-lamp greatly facilitates examination of animals for whom the skull conformation and behavior traits are poorly suited for table-mounted devices, and ii) the presence of the nictitating membrane and the minimal bulbar conjunctival exposure in dogs require a “dynamic” examination of the ocular surface that is not conducive to photographic scales. The ocular adverse effects noted with high doses of histamine represent another potential limitation of the proposed model. There was minimal to absent local irritation from all doses, likely favored by adjusting the test solutions to more physiologic pH values, but histamine concentrations $\geq 375$ mg/ml resulted in meaningful side effects in selected cases. A transient breakdown of the blood–ocular barrier likely explains the anterior uveitis, while ocular hypertension maybe linked to the aforementioned acute uveitis and/or episcleral venous compression from the overlying swollen conjunctiva, causing an increased resistance to aqueous outflow. Future studies are required to characterize these adverse effects in detail. Lastly, the study does not explain the mechanistic reason of breakdown of the blood–tear barrier, as we mainly focused on documenting the safety
and clinical features of the model in dogs. Our group is now working on characterizing the biochemical and histological changes resulting from histamine-induced conjunctivitis in dogs. In summary, histamine-induced conjunctivitis in dogs represents a robust and reliable model for translational research on the ocular surface. The model is particularly appealing given the low cost, non-invasiveness, self-resolving nature, ability to adjust the duration and severity of the disease, and shared features with naturally occurring diseases in human and veterinary medicine. The model could be used to assess new therapeutics and to better understand the impact of conjunctivitis on drug pharmacokinetics–pharmacodynamics, tear film dynamics, and tear film composition, among many other applications.

References


**Tables and Figures**

**Table 1.** Patient information and tear albumin concentrations in the affected and unaffected eyes of dogs presented to the Ophthalmology service at Iowa State University’s Lloyd Veterinary Medical Center with various ocular diseases. * Cases 12 and 13 were examined twice: findings at the initial visit are shown in the 1st row while findings following treatment of the ocular disease are depicted in the 2nd row. yo = year old; FS = female spayed; MC = male castrated; FI = female intact.

<table>
<thead>
<tr>
<th>Case #</th>
<th>Patient information</th>
<th>Ocular disease</th>
<th>Conjunctivitis score (affected eye / unaffected eye)</th>
<th>Tear albumin concentration in affected eye (mg/mL)</th>
<th>Tear albumin concentration in unaffected eye (mg/mL)</th>
<th>Ratio affected vs. unaffected eye</th>
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<tbody>
<tr>
<td>1</td>
<td>9 yo FS Yorkshire terrier</td>
<td>Keratoconjunctivitis sicca</td>
<td>4 / 0</td>
<td>3.50</td>
<td>1.22</td>
<td>2.87</td>
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<tr>
<td>2</td>
<td>9 yo MC French Bulldog</td>
<td>Corneal ulcer (superficial), Eyelid mass</td>
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<td>2.44</td>
<td>0.80</td>
<td>3.05</td>
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<td>4 yo MC Shih Tzu</td>
<td>Corneal ulcer (superficial), Distichia</td>
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<td>1.10</td>
<td>0.38</td>
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<td>6 yo MC English Bulldog</td>
<td>Spontaneous chronic corneal epithelial defect</td>
<td>8 / 0</td>
<td>9.98</td>
<td>1.67</td>
<td>5.99</td>
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<tr>
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<td>12 yo FS Shih Tzu</td>
<td>Corneal ulcer (stromal)</td>
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<td>4.18</td>
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<td>Spontaneous chronic corneal epithelial defect</td>
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<tr>
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Figure 1. A comprehensive evaluation of the canine conjunctiva is facilitated by examination of the ocular surface at different angles, including afront view (A), side view (B), top view (C), and globe retropulsion with lower lid retraction (D). The latter permits visualization of the palpebral conjunctiva and nictitating membrane.

Figure 2. Photographs of the left eye in a dog, 30 min following topical administration of 10 mg/ml histamine solution. The overall conjunctivitis score was 6, based on the absence/presence and degree of follicles, hyperemia, and chemosis of both palpebral and bulbar conjunctiva, conjunctival discharge, and ocular pruritus.
Figure 3. Development and progression of conjunctivitis in a representative canine eye receiving topical 375 mg/ml histamine solution. Conjunctivitis developed rapidly (< 1 min), with progression of conjunctival hyperemia and chemosis for 20–30 min, and subsequent slow improvement and self-resolution by 420 min.
Figure 4. (A) Graphs depicting the mean + SD of conjunctivitis score over time in dogs receiving H₁ (0.005 mg/ml; white circles), H₂ (0.1 mg/ml; black circles), H₃ (1.0 mg/ml; white triangles), H₄ (10 mg/ml; black triangles), H₅ (375 mg/ml; white squares), and H₆ (500 mg/ml; black squares). Within the same dose, a blue asterisk (*) depicts statistical significance of conjunctivitis scoring compared to baseline (for readability, only the first and last significant time points are depicted). (B) Box-and-whiskers plots depicting the area under the curve of conjunctivitis score from 0 to 180 min in dogs receiving topical histamine of various concentrations. Mean and median values are shown by horizontal dotted and solid lines, respectively. First and third quartiles (25th and 75th percentiles) are represented by the lower and upper limits of the box, respectively, while the 2.5th and the 97.5th percentiles are shown as the lower and upper whiskers, respectively. The conjunctivitis severity of histamine doses with different letters differ significantly ($P < 0.05$).
Figure 5. Blepharitis (A) and miosis (B) in a dog that received high dose of histamine ophthalmic solution (500 mg/ml).

Figure 6. Bar charts depicting the total protein content (A, B) and serum albumin levels (C, D) in tears of six beagle dogs receiving vehicle or histamine eyedrops. An asterisk (*) indicates statistical significance (P < 0.05) between vehicle and histamine (A, C) or among histamine doses (B, D).
**Figure 7.** Bar charts depicting mean + SD of various chemokines, cytokines and growth factors quantified in tears of six beagle dogs receiving vehicle (vehicle) or histamine solutions: interleukin-6 (IL-6; A), interleukin-8 (IL-8; B), interleukin-10 (IL-10; C), interleukin-12 (IL-12; D), vascular endothelial growth factor A (VEGF A; E), tumor necrosis factor alpha (TNFα; F), stem cell factor (SCF; 1.3 ± 4.3 pg/ml; G), and chemokine monocyte chemoattractant protein-1 (MCP-1; H). Differences among groups are depicted by an asterisk (*) if statistically significant ($P < 0.05$).
Figure 8. Clinical photographs of canine eyes from patients presented to the Ophthalmology Service at Iowa State University’s Lloyd Veterinary Medical Center. Conjunctivitis is present in all eyes, a condition noted concurrently to various ocular diseases. Patient case numbers are listed in the top left corner of each photograph (see Table 1 for additional patient information).
Figure 9. Clinical photographs of a 7-year-old female intact Coonhound dog (A) diagnosed with end-stage glaucoma in the right eye (case #11). Tear concentrations of albumin were much higher in the affected right eye (B) compared to the contralateral healthy left eye (C).

Figure 10. Clinical photographs of a 5-year-old male castrated Pitbull (A, B) diagnosed with a spontaneous chronic corneal epithelial defect (case #12) and a 4-year-old female spayed Beagle (C, D) diagnosed with uveitis secondary to blastomycosis (case #13). Following treatment of each ocular disease (B, D), the severity of conjunctivitis was reduced and the concentration of albumin in tears was lower compared to the initial visit (A, C).
Figure 11. Representative clinical pictures of mild conjunctivitis (A; score = 3), moderate conjunctivitis (B; score = 6), and severe conjunctivitis (C; score = 9) following topical administration of histamine in dogs.
CHAPTER 5. IMPACT OF ACUTE CONJUNCTIVITIS ON OCULAR SURFACE HOMEOSTASIS IN DOGS

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Modified from a manuscript under review in Veterinary Ophthalmology

Abstract

Objective: To investigate the effects of acute conjunctivitis on tear film characteristics and corneal sensitivity in dogs. Animals studied: Eight female spayed Beagle dogs (1.5-2 years old, 7.5-10 kg). Procedures: On two consecutive days, one randomly selected eye in each dog received 1 or 375 mg/mL histamine solution to induce mild or severe conjunctivitis, while the contralateral eye served as control. Diagnostic tests were performed in the following order: fluorescein instillation and repeated tear collection over 20 minutes (to determine tear volume [TV] and turnover rate [TTR] by fluorophotometry), Schirmer tear test-1 (STT-1), tear ferning, corneal esthesiometry and tear film breakup time (TFBUT). Results: Results are presented as median values for severe conjunctivitis, mild conjunctivitis, and control eyes. Eyes with severe conjunctivitis had significantly higher STT-1 (24, 19.5, 17.5 mm/min) and significantly lower TFBUT (10.5, 13.5, 15.5 sec), but no changes were noted in corneal tactile sensation (2, 2.5, 2.5 cm) or tear ferning (grades 2, 2, 2.5). Severe conjunctivitis significantly increased TV by nearly 10-fold (631, 97, 65 µL) initially (reflex tearing), although basal TV returned rapidly (<5 minutes) in all eyes (46, 58, 48 µL). Finally, there was a non-significant trend for higher reflex TTR in the conjunctivitis vs. control eyes (68, 58, 43 %/min). Conclusions: Acute conjunctivitis
increases tear quantity and decreases tear quality in dogs, but has no impact on corneal sensitivity. Changes in tear film dynamics could affect ocular pharmacology (e.g. precorneal retention time), although homeostasis of lacrimal volume and drainage is rapidly restored.

**Introduction**

A thin layer of tears covers the ocular surface and serves vital functions such as lubrication, nutrition, removal of debris, and defense against microbes. Thus, ocular surface health is compromised when tear film homeostasis is disturbed, as noted with changes in tear quantity, tear quality, and tear film dynamic.\(^1\) Quantitative and qualitative tear film deficiencies are well recognized in humans and veterinary species,\(^3,4\) but changes in tear film dynamics (i.e. production/distribution/drainage) could also play an important role in the pathophysiology of ocular surface diseases. For instance, excess lacrimal production dilutes the levels of endogenous (e.g. IgA) and exogenous (e.g. medication) compounds in tears,\(^5\) while faster drainage reduces their contact time with the ocular surface.\(^6\) Equally important, diseases of the ocular surface such as blepharitis and conjunctivitis often result in, or exacerbate tear film instability.\(^3,7,8\)

Conjunctivitis is a common ocular surface disease and is known to lower tear film stability in many species, including humans,\(^9\) dogs\(^10,11\) and cats.\(^12,13\) Primarily, the loss of tear film homeostasis results from altered mucin secretion onto the ocular surface by the conjunctival goblet cells.\(^14\) However, the relationship between conjunctivitis and the tear film is more complex and also involves neurosensory stimulation from ocular irritation,\(^15,16\) changes in tear volume, and variations in tear clearance.\(^9,17,18\)

In the present study, conjunctivitis was experimentally induced in dogs and a series of diagnostic tests were conducted to assess tear quantity, tear quality, tear dynamics, and corneal sensitivity. A deeper understanding of the interaction between conjunctivitis and tear film will
help clinicians break the vicious cycle whereby ocular surface disease leads to tear film instability, which subsequently exacerbates inflammation, and so forth.³

**Materials and Methods**

**Animals**

Eight Beagle dogs were enrolled in the study. All dogs were female spayed, aged 1.5-2.0 years, weighed 7.5-10 kg, and confirmed to be healthy based on a complete physical and ophthalmic examination, including Schirmer tear test-1 (STT-1; Eye Care Product Manufacturing LLC, Tucson, AZ, USA), tonometry (TonoVet, Icare Finland Oy, Espoo, Finland), slit-lamp biomicroscopy (SL-17; Kowa Company, Ltd., Tokyo, Japan) and indirect ophthalmoscopy (Keeler Vantage; Keeler Instruments, Inc., Broomall, PA, USA). The study was approved by the Institutional Animal Care and Use Committee of Iowa State University.

**Procedures**

A series of procedures were conducted in both eyes of each dog in a specific order, as described in Figure 1. All measurements were completed in the morning (to reduce diurnal variability), in the same examination room under controlled temperature (70-72°F) and ambient humidity (25-30%).

- **Induction of conjunctivitis:** Histamine powder (Histamine dihydrochloride, FCC grade, Arcos® organics, Geel, Belgium) was sterily mixed with 1.4% polyvinyl alcohol lubricating eye drops (Artificial tears solution, Rugby, Rockville Center, NY, USA) to compound 1 mg/mL and 375 mg/mL ophthalmic solutions.¹⁹ A single drop (35 µL)²⁰ of histamine solution was applied onto one randomly selected eye of each dog (Excel software, Microsoft Corp., Redmond, WA), while the other eye received artificial tears and served as control.
Both mild (1 mg/mL) and severe (375 mg/mL) conjunctivitis were induced in the selected eye, one day apart, the order of which was randomized for each dog (Excel software).

- **Fluorophotometry:** Tear film fluorophotometry was performed in each eye as previously described. Briefly, 2 µL of 10% fluorescein (Akorn Inc., Buffalo Grove, IL, USA) was instilled onto the dorsal bulbar conjunctiva using a pipette, followed by 3 manual blinks (0 minutes) and tear collection with 2-µL capillary tubes (Drummond Scientific Co., Broomhall, PA) at times 0, 2, 4, 6, 10, 15 and 20 minutes. Fluorescein concentrations were measured in each sample with a computerized scanning ocular fluorophotometer (Fluorotron Master™, Coherent Radiation, Palo Alto, CA), and tear fluorescence was recorded in ng/mL.

- **Schirmer tear test-1 (STT-1):** A Schirmer strip (Eye Care Product Manufacturing LLC, Tucson, AZ, USA) was placed in the ventrolateral conjunctival fornix of each eye, and tear quantity was recorded at 1 minute (mm/min) using a stopwatch. Strips were left until the ≥ 20-mm mark was reached, followed by centrifugation for 2 minutes at 3,884 g in a punctured 0.2-mL tube (secured to 2-mL tube) to extract tear fluid for the ferning test.

- **Tear ferning test:** For each sample, 2 µL of tear fluid was deposited on a glass slide inside a 2-mm circular area delineated with a marking pen. Following air drying at room temperature for 10 minutes, each sample was examined with light microscopy (10 X magnification) and classified as grade 1, grade 2, grade 3, or grade 4 according to specific criteria described by Oria and colleagues.

- **Corneal sensitivity:** A nylon filament (0.12 mm, Cochet-Bonnet esthesiometer, Luneau Ophtalmologie, Chartres, France) was extended to 6 cm and advanced until it contacted the central cornea, creating a slight deflection in the filament. The filament length was shortened
by 0.5-cm increments until a blink response was consistently noted in at least 3 out of 5 attempts, recorded in cm as the corneal tactile sensation (CTS).\textsuperscript{25}

- **Tear film breakup time (TFBUT):** Fluorescein 10% solution was diluted 1:5 with eyewash (OCuSOFT\textsuperscript{®} eye wash, OCuSOFT Inc., Richmond, TX), and 5-µL of the resulting 2% fluorescein solution was instilled onto the dorsal bulbar conjunctiva of each eye.\textsuperscript{26} After 3 manual blinks, the eyelids were kept open and the dorsotemporal corneal surface was observed at 16X magnification with a cobalt blue filter (SL-17). The TFBUT was recorded with a stopwatch (to the nearest tenth of a second) as the time from eyelid opening to the appearance of \( \geq 1 \) dark spot(s) within the fluorescent green tear film. The average of 2 measurements per eye was used for data analysis.

**Data analysis**

Tear volume (TV) and tear turnover rate (TTR) were estimated based upon nonlinear mixed-effect analysis of fluorescein decay curves in tear samples (Monolix\textsuperscript{®} version 2018R2; Lixoft, Orsay, France).\textsuperscript{21,27,28} The biphasic decay of fluorescein allowed for calculation of reflex (first phase) and basal (second phase) values for both tear parameters. Normality of data was assessed with the Shapiro-Wilk test. For all parameters, differences among control vs. mild conjunctivitis vs. severe conjunctivitis eyes were assessed with the Kruskal-Wallis test and post hoc Dunn’s. Statistical analysis was performed using SigmaPlot 14.0 (Systat Software Inc., San Jose, CA, USA), and \( P \) values < 0.05 were considered statistically significant.

**Results**

Results are presented below as median (interquartile range) as the data from tear film diagnostic tests and fluorophotometry were not normally distributed.
No statistical differences \((P = 0.582)\) were noted in baseline values of STT-1 between eyes selected for control \([19 (4.8) \text{ mm/min}]\), mild conjunctivitis \([20 (8.8) \text{ mm/min}]\) and severe conjunctivitis \([20 (2) \text{ mm/min}]\). However, when STT-1 was repeated 20 min after eyedrop administration (Fig. 1), tear values were higher in eyes with conjunctivitis \([\text{mild} = 19.5 (5.5) \text{ mm/min}; \text{severe} = 24 (3.3) \text{ mm/min}]\) compared to control eyes \([15.5 (9.8) \text{ mm/min}]\), with statistical differences noted between control and severe conjunctivitis groups \((P = 0.002)\) (Fig. 2A).

Differences in tear production were also noted with fluorophotometry data (Table 1). Compared to control, median reflex TV was 1.5-fold and 9.7-fold higher in eyes with mild and moderate conjunctivitis, respectively, a difference that was statistically significant for the severe conjunctivitis group \((P = 0.015)\). However, no statistical differences were noted among groups for basal TV \((P = 0.965)\), basal TTR \((P = 0.402)\) or reflex TTR \((P = 0.201)\).

While tear quantity increased with conjunctivitis, the tear quality was reduced (Fig. 2B). Median (interquartile range) TFBUT was lower in eyes with conjunctivitis \([\text{mild} = 13.5 (2.6) \text{ seconds}; \text{severe} = 10.5 (2.3) \text{ seconds}]\) compared to control eyes \([15.5 (5.1) \text{ seconds}]\), with statistical differences noted between control and severe conjunctivitis groups \((P = 0.002)\). Lastly, tear ferning (Fig. 2C) and corneal sensitivity (Fig. 2D) were not affected by conjunctivitis \((P \geq 0.322)\).

**Discussion**

The present study demonstrates that acute conjunctivitis increases tear quantity and decreases tear quality in dogs, but has no significant impact on corneal sensitivity or tear ferning. These results provide useful information for clinicians who manage canine patients with an acute injury to the ocular surface (e.g. corneal ulcer, foreign body or chemical burn with secondary
conjunctivitis), and for basic scientists who utilize histamine-induced conjunctivitis as a model to investigate ocular pharmacology and therapeutics.\textsuperscript{19}

It is well established that corneal irritation causes reflex tearing across species,\textsuperscript{29-32} with greater corneal nociceptive stimulation leading to greater tear production.\textsuperscript{32} In contrast, little is known about the impact of conjunctival irritation on lacrimal secretion. Here, we showed that tear production was increased in the presence of conjunctivitis in dogs – irrespective of corneal stimulation (no changes noted in corneal tactile sensation) – as demonstrated by STT-1 values and TV calculation (fluorophotometry). In fact, the quantity of tears increased with the severity of conjunctivitis, a finding likely explained by increasing noxious stimulation to the conjunctival nerves that contribute to the afferent pathway of lacrimation, activating the efferent parasympathetic and sympathetic nerves to the lacrimal gland.\textsuperscript{33} Similar findings were noted in humans with acute catarrhal conjunctivitis\textsuperscript{9} and in cats with experimentally induced herpetic conjunctivitis, in whom STT-1 doubled (from 8 to 16 mm/min) concurrently with conjunctival disease scoring.\textsuperscript{29} Further, the amplitude of STT-1 increase with conjunctivitis (15.5 to 19.5-24 mm/min) is similar to changes noted in the presence of corneal ulceration; Williams and Burg showed that dogs with a unilateral corneal ulcer had significantly greater STT-1 values in the affected eye compared to the non-ulcerated fellow eye (20.2 vs. 16.7 mm/min, respectively).\textsuperscript{30}

Importantly, the present study demonstrates the tremendous capacity of the canine ocular surface to restore tear homeostasis in a rapid manner. Indeed, the volume of tears quickly stabilized (<5 min) after induction of conjunctivitis, despite ongoing conjunctival inflammation (lasting \( \geq 1 \) h with topical histamine),\textsuperscript{19} and no significant differences were noted in basal TV among control, mild conjunctivitis or severe conjunctivitis eyes.
This finding could be explained by rapid increase in the drainage of tears through the nasolacrimal duct, as shown in rabbit eyes that were instilled various volumes of eyedrops. However, tear drainage (assessed with TTR) did not differ significantly in dogs with or without conjunctivitis (present study), nor did it change in a recent report of dogs receiving artificial tears at a volume of 10 to 100 µL. Discrepancies between rabbits and dogs are likely due to notable differences in blinking rates (3-6 blinks/h vs. 14.2 blinks/min, respectively), an important physiologic response that promotes spillage of excessive tearing onto the periocular skin.

The quality of tears, or tear film stability, was reduced in canine eyes with acute conjunctivitis. Shortened TFBUT is an established feature of dogs, cats and humans with chronic conjunctivitis, as chronicity of inflammation reduces the density of goblet cells that secrete pre-ocular mucins, although this finding is not well documented in eyes with acute conjunctivitis. The authors hypothesize that excessive aqueous secretion onto the ocular surface (reflex tearing) changes the relative abundance of lipids and mucins in the tear film, resulting in faster tear evaporation and shorter TFBUT, although rapid loss of conjunctival goblet cells could also be an explanation; future studies assessing tear composition and conjunctival impression cytology are therefore needed to support that hypothesis. Regardless of the underlying cause, a short TFBUT is important to recognize in practice as it can participate to ocular discomfort experienced by the patient, necessitating mucinomimetic supplementation to relieve irritation and prevent exacerbation of conjunctival inflammation; in fact, rapid TFBUT results in the same degree of ocular discomfort than patients with aqueous tear deficiency, likely due to development of neuropathic pain.

Evaluation of tear ferning can provide valuable information in patients with ocular surface disease. In fact, a recent study showed that all dogs with keratoconjunctivitis sicca had
abnormal ferning patterns (grades 3-4) while the majority of healthy canine eyes had a normal ferning pattern. In contrast, our study did not find significant differences in tear ferning between control eyes and eyes with acute conjunctivitis. This finding may be physiologically true, although it could also be skewed by the low sample size, possible technical issues (e.g. inconsistent drying times among samples), relative inexperience of the investigators with tear ferning, or lack of standardization of grading. The latter was addressed in a recent equine study by using a computerized stereology tool to characterize the tear crystallization in an objective manner.

The present study is limited to the short-term effects of conjunctivitis on ocular surface homeostasis, in a relatively small population of dogs of a single breed. Topical histamine provides a robust method to induce conjunctivitis in a rapid and non-invasive manner in dogs, however the duration of conjunctivitis is limited in time as the disease is self-resolving (average duration of 115 and 390 min with 1 mg/mL and 375 mg/mL solutions, respectively). One could consider repeating administration of topical histamine to prolong the duration of conjunctivitis, as recently performed in a pharmacokinetic study of prednisone in dogs, although the safety profile of consistent extended dosing with histamine is currently unknown. Nevertheless, the present study shows that conjunctivitis alone is enough to activate the lacrimal functional unit and cause reflex tearing, irrespective of noxious stimulation to the cornea, and that the homeostasis of the ocular surface is rapidly restored. This is important because tear film disturbances noted with acute conjunctivitis likely affect the comfort level of canine patients, and might justify the use of hyaluronic acid-based lacrimomimetics to improve tear stability and reduce discomfort; further, the kinetics of solutions delivered to the ocular surface might be
affected in the short-term (e.g. dilution factor, precorneal retention time), influencing the bioavailability of topical drugs.

References


Tables and Figures

Table 1. Median (interquartile range) of basal and reflex tear film dynamics in healthy eyes (control) and eyes with acute conjunctivitis. TV = Tear volume; TTR = Tear turnover rate. † Kruskal-Wallis test. * Significant difference between control and severe conjunctivitis eyes (post hoc Dunn’s test).

<table>
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<tr>
<th></th>
<th>Control</th>
<th>Mild conjunctivitis</th>
<th>Severe conjunctivitis</th>
<th>P value †</th>
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<td>Basal TV (µL)</td>
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<td>Reflex TV (µL)</td>
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<td>630.8 (891.3)*</td>
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<td>Basal TTR (%/min)</td>
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<tr>
<td>Reflex TTR (%/min)</td>
<td>43.2 (46.4)</td>
<td>57.7 (32.9)</td>
<td>65.7 (30.8)</td>
<td>0.201</td>
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</tbody>
</table>

Figure 1. Flow chart of the ophthalmic procedures performed in 8 healthy Beagle dogs, before and after experimental induction of conjunctivitis with topical histamine. STT-1 = Schirmer tear test-1; TFBUT = Tear film breakup time.
Figure 2. Box-and-whiskers plots depicting test results of Schirmer tear test-1 (A), tear film breakup time (B), tear ferning (C) and corneal tactile sensation (D) in 8 healthy Beagle dogs receiving artificial tears (control, white), 1 mg/mL ophthalmic histamine (mild conjunctivitis, light gray) or 375 mg/mL ophthalmic histamine (severe conjunctivitis, dark gray). Each plot represents the mean (dashed line), median (solid line), 2.5th percentile (lower whisker), 25th percentile (lower limit of box), 75th percentile (upper limit of box), and 97.5th percentile (upper whisker).
CHAPTER 6. TEAR FLUID PHARMACOKINETICS FOLLOWING ORAL PREDNISONE ADMINISTRATION IN DOGS WITH OR WITHOUT CONJUNCTIVITIS

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Modified from a manuscript published in the Journal of Ocular Pharmacology and Therapeutics

Abstract

Purpose: To describe the pharmacokinetics of prednisone and prednisolone in tear fluid of dogs receiving oral prednisone at anti-inflammatory to immunosuppressive doses, and to assess the impact of induced conjunctivitis on lacrimal drug levels. Methods: Six healthy Beagle dogs were administered 4 courses of prednisone at 0.5, 1.0, 2.0 and 4.0 mg/kg given orally once a day for 5 days. At steady state, topical histamine was applied to induce mild (1 mg/mL) or severe (375 mg/mL) conjunctivitis in one eye of each dog and tear samples were collected from both eyes at selected times. Prednisone and prednisolone were quantified in tears by liquid chromatography-mass spectrometry. Results: Lacrimal prednisone and prednisolone
concentrations ranged from 2-523 ng/mL and 5-191 ng/mL, respectively. Drug concentrations were overall greater in dogs receiving higher doses of prednisone, but were not correlated with tear flow rate. Eyes with conjunctivitis often had larger amounts of prednisone and prednisolone in tear fluid compared to control eyes (up to +64%), but differences were not statistically significant. Significantly greater, but clinically insignificant, levels of prednisolone were found in eyes with severe vs. mild conjunctivitis for oral prednisone doses ≥ 1.0 mg/kg. **Conclusions:** Disruption of the blood-tear barrier with conjunctivitis did not significantly affect drug levels in tears. Based on drug pharmacokinetics in tears, oral prednisone is likely safe for the management of reflex uveitis and ocular surface diseases. However, further prospective trials using systemic corticotherapy in diseased animals are warranted to confirm findings from this preclinical study.

**Introduction**

Prednisone is a corticosteroid with a wide range of pharmacological indications that is commonly used for the treatment of inflammatory and immune-mediated diseases in human and veterinary medicine. In ophthalmology, corticosteroids can alleviate ocular inflammation and help prevent devastating sequelae that could be painful or vision threatening. Of the various routes of administration, systemic therapy is generally recommended when the target tissue cannot be reached with topical ophthalmic corticosteroids (e.g. eyelids, posterior segment, orbit), or as a complement to topical medications in cases of anterior uveitis.

Systemically administered medications readily distribute to the vascular tissues of the eye, but can also affect the ocular surface if the drug reaches the tear compartment. For instance, oral doxycycline can be used as adjunctive therapy for keratomalacia in dogs and horses, while oral famciclovir is highly effective in managing herpetic keratoconjunctivitis in cats. For oral prednisone, detection of steroid levels in the tear film could support the use of systemic
corticotherapy for adjunctive treatment of inflammatory diseases such as chronic superficial keratitis, immune-mediated keratitis, and keratoconjunctivitis sicca. Conversely, lacrimal levels of corticosteroids could inhibit corneal wound healing and potentiate infection.\textsuperscript{7,8} This therapeutic dilemma is exemplified in patients with ulcerative keratitis and concurrent reflex uveitis: systemic steroids are superior to nonsteroidal anti-inflammatory medications for controlling severe uveitis and preventing devastating sequelae,\textsuperscript{9} yet ulceration could worsen and result in corneal perforation if wound healing is compromised and infection is potentiated.

The main goal of the study was to describe the PK of prednisone and its active metabolite prednisolone in tear fluid of dogs following oral administration at doses ranging from anti-inflammatory to immunosuppressive use (0.5 to 4 mg/kg/d). We hypothesized that prednisone and prednisolone would be quantifiable in canine tear fluid and concentrations would be greater with increasing oral dosing. In an effort to make the findings of this study more clinically relevant, a secondary objective was to determine the impact of conjunctivitis on drug concentrations in tears. Indeed, conjunctivitis, a common bystander of most ocular diseases, increases conjunctival vascular permeability and therefore enhances vascular leakage of plasma compounds onto the ocular surface.\textsuperscript{10,11} We hypothesized that tear concentrations would be greater in eyes with conjunctivitis (\textit{i.e.} compromised blood-tear barrier) compared to healthy eyes.

**Materials and Methods**

**Animals**

Six Beagle dogs were included in the study. All were spayed females of 1.5-2 years old and weighing 7.5-10 kg. Prior to study inclusion, dogs were confirmed to be healthy based on physical and ophthalmic examination, complete blood count, serum chemistry and urinalysis.
The study was approved by the Institutional Animal Care and Use Committee of Iowa State University, and adhered to the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research.

**Procedures**

Over a period of 2 months, all dogs received 4 successive dosing regimens of oral prednisone (Cadista™ predniSONE tablets, Jubilant Cadista Pharmaceuticals Inc., Salisbury, MD, USA), characterized by 5 days of drug administration interrupted by 9 days washout period, which included: (i) 0.5 mg/kg once daily for 5 days; (ii) 1.0 mg/kg once daily for 5 days; (iii) 2.0 mg/kg once daily for 5 days; and (iv) 4.0 mg/kg once daily for 5 days. The following procedures were performed on Day 4 of each dosing regimen, a time chosen to ensure steady state drug levels were reached:12

- **Induction of conjunctivitis in one eye:** Histamine ophthalmic solutions were formulated by mixing histamine powder (Histamine dihydrochloride, FCC grade, Arcos® organics, Geel, Belgium) with 1.4% polyvinyl alcohol lubricating eye drops (Artificial tears solution, Rugby, Rockville Center, NY, USA) in a sterile manner under a laminar flow hood. Twenty minutes prior to prednisone administration, a single drop of histamine solution was applied to one randomly selected eye in each dog, while the other eye received artificial tears (Control). This ocular selection was kept constant throughout the study. Histamine rapidly induced conjunctivitis (<1 min) that was either mild (n = 3 dogs, 1.0 mg/mL histamine solution; Figure 1A) or severe (n = 3 dogs, 375 mg/mL histamine solution; Figure 1B), as previously described.11 Conjunctivitis was maintained throughout the 12 h collection period by repeating topical histamine administration every 1-4 h as needed to maintain effect. Topical 0.035% ketotifen fumarate (Zaditor®, Novartis Pharmaceuticals Corporation, East Hanover, NJ,
USA) was instilled onto each eye at the end of the day to control any residual conjunctival swelling.

- **Tear collection:** Tear fluid was sampled simultaneously in both eyes before prednisone administration (t = 0 min) and at 15, 30, 60, 90, 120, 240, 480, and 720 min following drug administration. The bent tip of a Schirmer tear strip (Eye Care Product Manufacturing, LLC, Tucson, AZ, USA) was placed in the ventrolateral conjunctival fornix of each eye. While recording test duration with a stopwatch, each Schirmer strip was removed and transferred into a 2-mL Eppendorf tube when the 20-mm mark of wetness was reached, as to standardize the volume of tears collected in each sample. The distal portion of each strip (25-35 mm marks, not wetted with tears) was spiked with 5 μL internal standard (prednisone-d7, Toronto Research Chemicals, North York, Canada) prepared as 10 ng/μL solution in 1:1 acetonitrile:water, and samples were stored at -80°C until analysis.

**Preparation of tear samples for analysis**

The details of tear fluid extraction are described in Appendix E. Briefly, both centrifugation and elution in solvent were used as complementary methods to extract the drug from Schirmer strips. Wetted strips containing tear fluid and internal standard were first centrifuged to retrieve the majority of absorbed tear fluid, followed by cutting and shredding the strips into small pieces and eluting them in methyl tert-butyl ether (MTBE). Of note, this particular solvent was chosen based on its superior ability to extract prednisone from Schirmer strips as compared to methanol, acetonitrile and water (small pilot study, data not shown).
Liquid chromatography – mass spectrometry

Prior to study initiation, blank tears were collected from the same Beagle dogs using polyvinyl ophthalmic sponges as previously described. Eight standard curve solutions were prepared by spiking blank canine tears with stock solutions of prednisone/prednisolone (Cerriliant, Round Rock, Texas, USA) to obtain the following concentrations: 1, 2, 5, 10, 20, 50, 100 and 200 ng/mL. Calibration curve samples were processed in a similar fashion to biological tear samples, which involved wetting Schirmer strips with standard solutions until the 20-mm mark was reached, spiking prednisone-d7 internal standard onto the distal (dry) portion of the strips, centrifugation and elution in MTBE, etc. (see Appendix D for details). Concentrations of prednisolone and prednisone in canine tears were determined using high-pressure liquid chromatography (Agilent 1100 Pump, Column Compartment, and Autosampler, Santa Clara, CA, USA) with ion trap mass spectrometry detection (LTQ, Thermo Scientific, San Jose, CA, USA). The injection volume was set to 20 μL. The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile at a flow rate of 0.25 mL/min. The mobile phase began at 20% B with a linear gradient to 95% B in 5.0 minutes, which was maintained for 2 minutes at 0.325 mL/min, followed by re-equilibration to 20% B at 0.325 mL/min for 3.5 min. Separation was achieved with an ACE Ultracore C18 column, 100 mm x 2.1 mm, 2.5 μm particles (Mac-Mod Analytical, Chadds Cord, PA, USA) maintained at 40°C. The chromatographic peaks for the internal standard, prednisone and prednisolone (each eluted at 4.69± 0.05 min) were integrated using Xcalibur software (Thermo Scientific, San Jose, CA, USA). Drug quantitation was based on linear regression analysis of calibration curves (weighted 1/X) using the analyte to internal standard area ratio. Calibration curves exhibited a correlation coefficient ($r^2$) exceeding 0.995 across the concentration range. The limits of quantitation for
prednisone and prednisolone were 2 ng/mL and 5 ng/mL, respectively, while the limits of detection for prednisolone and prednisolone were 0.5 ng/mL and 1 ng/mL, respectively.

**Data analysis**

Noncompartmental analysis of prednisone and prednisolone pharmacokinetics was conducted with Phoenix software (WinNonlin, version 8.0, Pharsight Corporation, CA, USA) to determine the maximum concentration (C\text{max}), time to maximum concentration (T\text{max}), and area under the curve from time zero to time of last measurable concentration (AUC\text{last}).

The Shapiro-Wilk test was used to assess data for normality. Non-normally distributed data were expressed as median and 95% central range (2.5-97.5th percentiles) and were analyzed with nonparametric statistics. Normally distributed data were expressed as mean ± standard deviation (95% central range), and were analyzed with parametric statistics. Associations between tear flow rate and lacrimal concentrations of prednisone or prednisolone were assessed with the Spearman’s correlation test. The Student t-test was used to assess differences in AUC\text{last} between eyes with mild or severe conjunctivitis. For each oral dose and for each PK parameter (AUC\text{last}, C\text{max}, T\text{max}), differences between control and conjunctivitis eyes (mild + severe) were assessed with the Student t test or Mann-Whitney test. For each PK parameter, differences among oral doses (0.5, 1.0, 2.0 and 4.0 mg/kg) were assessed with the one-way ANOVA or Kruskal Wallis test. Statistical analysis was performed using SigmaPlot 14.0 (Systat Software Inc., San Jose, CA, USA), and values P < 0.05 were considered statistically significant.

**Results**

Following oral administration of prednisone, both prednisone and prednisolone were quantifiable in tear fluid, with concentrations ranging from 2-523 ng/mL and 5-191 ng/mL,
respectively. Lacrimal concentrations were not correlated with tear flow rate for either prednisone ($P \geq 0.412$) or prednisolone ($P \geq 0.388$). However, higher doses of oral prednisone resulted in higher tear concentrations of both steroids, as depicted by the individual concentration-time curves in Figure 2. In fact, the overall drug exposure in tears (depicted by AUC$_{\text{last}}$) was statistically different among the 4 doses for both prednisone ($P \leq 0.002$; Figure 3A) and prednisolone ($P \leq 0.001$; Figure 3B). Similarly, statistical differences were detected among oral doses for prednisone $C_{\text{max}}$ ($P = 0.008$) and for prednisolone $C_{\text{max}}$ ($P = 0.003$) and $T_{\text{max}}$ ($P = 0.039$; Table 1). In general, eyes with conjunctivitis showed a trend for larger concentrations of prednisone and prednisolone in tear fluid compared to control eyes (Figures 2 and 3), with differences in average concentrations ranging from +5% to +64%. However, differences in AUC$_{\text{last}}$ between control and conjunctivitis eyes were not statistically significant for either prednisone ($P \geq 0.095$; Figure 3A) or prednisolone ($P \geq 0.485$; Figure 3B). The severity of conjunctivitis did have an impact on lacrimal concentrations, as significantly greater levels of prednisolone were found in eyes with severe vs. mild conjunctivitis for oral doses 2-4 mg/kg/d ($P \leq 0.042$; Figure 4), although these changes were not considered to be large enough to be clinically relevant.

**Discussion**

The present study describes, for the first time, the tear film pharmacokinetics of prednisone and prednisolone in veterinary medicine. Tear film concentrations of prednisone and prednisolone varied from 2-523 ng/mL and 5-191 ng/mL respectively, with higher doses of oral prednisone leading to higher lacrimal levels of both steroids. The question remains whether concentrations of the active metabolite (prednisolone) are relevant in clinical patients, that is potentially beneficial or detrimental to managing ocular surface diseases. Overall, tear film
prednisolone levels were $\geq 10^{-9}$M (i.e. 0.4 ng/mL) in all dogs throughout the 12-hour sampling time, a concentration shown to decrease the expression of deleterious cytokines (TNF-α, IL-6) and matrix metalloproteinases in a rat model of keratitis.\textsuperscript{15} Therefore, oral prednisone might have therapeutic benefits in managing corneal inflammation, assuming similar exposure-response between species. In fact, although the use of corticosteroid in infectious keratitis remains controversial,\textsuperscript{16} some authors believe that a judicious use of corticosteroids (combined with the appropriate antimicrobial) could improve the outcome of keratitis as it reduces damage caused by the host’s inflammatory response, decreases corneal scarring, and inhibits neovascularization.\textsuperscript{17,18} From a safety viewpoint, the use of corticosteroids is known to potentially delay corneal wound healing and exacerbate signs of ocular infection. \textit{In vitro}, inhibition of corneal wound healing in dogs is only reported for prednisolone concentrations that are much higher ($\geq 620$ µg/mL)\textsuperscript{19} than the ones reported herein. \textit{In vivo}, topical corticosteroid use can be detrimental in patients with ulcerative keratitis\textsuperscript{2} although tear film concentrations following topical 1% prednisolone acetate are unknown to date in any species. Data extrapolation from pharmacokinetics of 0.3% ciprofloxacin in dogs\textsuperscript{20} shows that (i) topical 1% prednisolone acetate could reach concentrations as high as 909 µg/mL, which is 1,000 to 10,000-fold greater than drug levels noted in the present study; and (ii) topical 1% prednisolone acetate (applied every 6h) could result in drug exposure over 12h that is 14,000-27,000 fold and 3,300-5,300 fold greater than oral prednisone given at anti-inflammatory dose (0.5-1 mg/kg/d) or immuno-suppressive dose (2-4 mg/kg/d), respectively. Of note, drug exposure over time is more relevant than single lacrimal concentrations (e.g. $C_{\text{max}}$) given differences in pharmacological disposition between topical and oral routes. While the ocular bioavailability of topical administration is $<10$-20% given efficient washout by tears,\textsuperscript{21} oral administration could be
considered as a form of sustained-release at the ocular surface through lacrimal gland diffusion and conjunctival leakage. As for the negative impact on the immune system, there is no consensus on what concentration is considered harmful. In one study, prednisolone levels as low as 0.005 µg/mL were shown to reduce the phagocytosis function of human leucocytes, while prednisolone concentrations as high as 4.32 µg/mL did not impact leucocyte phagocytosis or bactericidal activity in another study.

Conjunctivitis is a common disorder in dogs that develops concurrently to most ocular diseases, whether affecting the adnexa (e.g. blepharitis), ocular surface (e.g. corneal ulcer) or intraocular tissues (e.g. uveitis). With conjunctivitis, plasma constituents tend to ‘leak’ into the tear compartment as the permeability of conjunctival vessels is typically increased. This breakdown of the blood-tear barrier explains the large quantities of albumin in tears of diseased eyes, regardless of the underlying etiology of conjunctivitis (e.g. dry eye, corneal ulcer, allergies). Thus, to make the present PK findings more clinically relevant, conjunctivitis was experimentally induced in selected canine eyes using a recently described model. Lacrimal levels of prednisone and prednisolone were overall higher in conjunctivitis vs. control eyes, with greater disease severity leading to generally greater drug levels in tears, especially for prednisolone. However, differences between control vs. conjunctivitis eyes were not statistically significant, and were fairly minimal (up to 64% increase) when compared to plasma albumin (up to 12,000%). Unlike albumin, a very large molecule (66,500 Da) that does not permeate through intact conjunctival tissue, we suspect that smaller molecules like prednisone (358 Da) and prednisolone (360 Da) readily cross the blood-tear barrier under normal conditions, and are therefore not significantly impacted by conjunctival inflammation. However, we cannot exclude that larger amounts of corticosteroids actually reach the lacrimal fluid in eyes with conjunctivitis;
although the concurrent leakage of albumin binding to free prednisolone would probably reduce
its bioavailability at the ocular surface.26 Further, physicochemical properties other than
molecular weight may explain differences in lacrimal distribution between prednisone,
prednisolone, and other drugs reported in the veterinary literature3, 5, 13 – namely protein binding,
lipophilicity and degree of ionization.27 Here, the competitive nature of plasma protein binding
between prednisone and prednisolone could justify the slightly higher lacrimal concentrations of
prednisone in canine tears.28

The present study has a few limitations. First, the sample size of our experiment was
relatively small, and only females from a single dog breed were evaluated. Tear film PK could
theoretically differ in male vs. female dogs,29 or breeds other than Beagle, especially in
brachycephalic dogs in whom the lacrimal lipid layer is thin and corneal exposure is large.30 Yet,
previous studies did not find significant differences between mesocephalic and brachycephalic
dogs in regards to tear film dynamics (tear volume, tear turnover rate)31 or tear film drug
concentrations.20 Second, it is possible that we did not find statistical differences in tear film
concentrations between healthy vs. conjunctivitis eyes because of the conjunctivitis model itself.
Experimental induction of conjunctivitis, although rapid and non-invasive,11 could have falsely
lowered lacrimal concentrations by causing reflex tearing and accelerated tear turnover. We
minimized this risk by inducing conjunctivitis \( \geq 20 \) min before drug administration and sample
collection, but a small degree of ocular irritation could have lingered. Regardless, increased
tearing should not have affected drug levels in a notable manner, as we did not find a significant
correlation between tear flow rate and prednisone/prednisolone levels in tears. Last, the present
study used Schirmer strips to collect tears in dogs, and although this method yielded sufficient
tear fluid for analysis, the method has several disadvantages that could partly explain the large
variability in tear concentrations noted among subjects. Not only do Schirmer strips absorb tear fluid but they also retain a certain amount of tear components (adsorption), the degree of which can vary depending on the concentration. Here, we minimized the impact of adsorption by incorporating two important steps in our sample preparation: (i) the internal standard was spiked onto the distal end of Schirmer strips before tear extraction, and (ii) standard curves were processed in a similar fashion to biological samples. We also maximized the amount of drug extracted from Schirmer strips by combining centrifugation with solvent elution. Such combination may improve the assay sensitivity (i.e. able to detect lower concentrations), but the process is very labor intensive and may not be necessary for all drugs. Future studies should consider a pilot experiment to assess the extraction efficacy of the combination method vs. centrifugation or solvent elution alone.

In conclusion, our data indicate that oral prednisone might be safe and beneficial as adjunctive therapy for reflex uveitis, ulcerative keratitis or other ocular surface disease in dogs. However, these preliminary pharmacokinetic findings need to be complemented with prospective controlled studies using systemic corticotherapy in diseased animals. Similarly, future anatomical and physiological studies are needed to better understand the role of conjunctivitis in diffusion of systemically administered drugs into the tear film.

References


Tables and Figures

**Table 1.** Mean ± standard deviation of the maximal concentration ($C_{\text{max}}$) and time to reach $C_{\text{max}}$ ($T_{\text{max}}$) for prednisone and prednisolone in control and conjunctivitis eyes. Within the same dose, comparisons between control and conjunctivitis eyes are described with $P$ values above the plots and an asterisk (*) if statistically significant ($P < 0.05$). Within the same group (control or conjunctivitis), differences among doses are depicted with symbols to demonstrate statistically greater values compared to dose 1 (#), dose 2 (†) and dose 3 (‡).

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<th></th>
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<td>$C_{\text{max}}$ (ng/mL)</td>
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<td>(4.0 mg/kg)</td>
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<td>Control</td>
<td>116 ± 67.2 #</td>
<td>127.5 ± 93.8</td>
<td>123.6 ± 47.8 #†</td>
<td>160.0 ± 92.3</td>
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<tr>
<td>Conjunctivitis</td>
<td>204.3 ± 173.5</td>
<td>120.0 ± 180.7</td>
<td>120.0 ± 38.1 #††</td>
<td>160.0 ± 92.3</td>
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Figure 1. Topical histamine rapidly induced conjunctivitis (<1 min) that was either mild [(A); 1.0 mg/mL solution] or severe [(B); 375 mg/mL solution]. Color images are available online.
Figure 2. Tear film concentrations (mean + standard deviation) of prednisone (A, B) and prednisolone (C, D) in dogs receiving oral prednisone at 0.5 mg/kg once daily (black line, circles), 1.0 mg/kg once daily (red lines, down triangles), 2.0 mg/kg once daily (green lines, squares), and 4.0 mg/kg once daily (blue lines, up triangles). The concentrations are depicted for control eyes (A, C) and eyes with experimentally induced conjunctivitis (B, D). Of note, the conjunctivitis group includes eyes with mild and severe conjunctivitis.
Figure 3. Box-and-whiskers plots depicting the area under the tear concentration–time curve from time zero to time of last measurable concentration (AUClast). Each plot depicts the mean (dotted line), median (solid line), 2.5th percentile (lower whisker), 25th percentile (lower limit of box), 75th percentile (upper limit of box), and 97.5th percentile (upper whisker). Data for prednisone (A) and prednisolone (B) are shown for all 4 oral doses of prednisone (0.5–4.0 mg/kg) in both control eyes (white boxes) and eyes with experimentally induced conjunctivitis (dark gray). Of note, the conjunctivitis group includes eyes with mild and severe conjunctivitis. Within the same drug dose, comparisons between control and conjunctivitis eyes (t test) are described with P values above the plots. Within the same ocular group (control or conjunctivitis), differences among doses (one-way analysis of variance) are depicted with symbols to demonstrate statistically greater AUClast compared to dose 1 (#), dose 2 (†) and dose 3 (‡).
Figure 4. Bar charts depicting mean ± standard deviation of area under the tear concentration-time curve from time zero to time of last measurable concentration (AUClast). Data for tear concentrations of prednisone (A) and prednisolone (B) are shown for all 4 oral doses of prednisone (0.5–4.0 mg/kg) in eyes with experimentally induced mild conjunctivitis (light gray) or severe conjunctivitis (dark gray). Within the same dose, statistical comparisons between mild and severe conjunctivitis (t test) are described with $P$ values above the plots, and statistical significance ($P < 0.05$) is depicted with an asterisk (*).
CHAPTER 7. ALBUMIN LEVELS IN TEAR FILM MODULATE THE BIOAVAILABILITY OF MEDICALLY-RELEVANT TOPICAL DRUGS

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Modified from a manuscript published in Frontiers in Pharmacology

Abstract

The breakdown of blood-tear barrier that occurs with ocular pathology allows for large amounts of albumin to leak into the tear fluid. This process likely represents an important restriction to drug absorption in ophthalmology, as only the unbound drug is transported across the ocular tissue barriers to exert its pharmacologic effect. We aimed to investigate the effects of albumin levels in tears on the bioavailability of two commonly used ophthalmic drugs: tropicamide, an antimuscarinic that produces mydriasis and cycloplegia, and latanoprost, a PGF2α analog used for the treatment of glaucoma. Eight female beagle dogs underwent a randomized, vehicle-controlled crossover trial. For each dog, one eye received 30 µL of artificial tears (control) or canine albumin (0.4 or 1.5%) at random, immediately followed by 30 µL of 1% tropicamide (2 days, 24 h washout) or 0.005% latanoprost (2 days, 72 h washout) in both eyes. Pupil diameter (digital caliper) and intraocular pressure (IOP; rebound tonometry) were recorded at various times following drug administration (0 to 480 min) and compared between both groups with a mixed model for repeated measures. Albumin in tears had a significant impact on pupillary diameter for both tropicamide ($P \leq 0.001$) and latanoprost ($P \leq 0.047$), with no differences noted between 0.4% and 1.5% concentrations. Reduction in the maximal effect (pupil...
size) and overall drug exposure (area under the effect-time curve of pupil size over time) were significant for tropicamide (6.2-8.5% on average, $P \leq 0.006$) but not for latanoprost ($P \geq 0.663$). The IOP, only measured in eyes receiving latanoprost, was not significantly impacted by the addition of either 0.4% ($P = 0.242$) or 1.5% albumin ($P = 0.879$). Albumin in tear film, previously shown to leak from the conjunctival vasculature in diseased eyes, may bind to topically administered drugs and reduces their intraocular penetration and bioavailability. Further investigations in clinical patients and other commonly used ophthalmic medications are warranted.

**Introduction**

Topical instillation is the most common route of drug administration in ophthalmology, especially for the treatment of anterior segment diseases.\(^1\) This mode of administration is convenient and non-invasive, although drug bioavailability is generally poor (typically < 10%) due to physiological, structural, and biochemical barriers to drug penetration into the eye.\(^1\)\(^-\)\(^3\) Upon instillation, an eyedrop is immediately diluted in the tear film and a large portion of the drug is lost through reflex tearing, nasolacrimal drainage, and systemic absorption. Residual drug has to cross ocular tissue barriers (i.e. cornea, sclera and conjunctiva) to reach targets within the globe.\(^1\)\(^,\)\(^3\)\(^,\)\(^4\) In general, small lipophilic drugs permeate through the cornea while larger or hydrophilic compounds permeate through the conjunctiva and sclera.\(^4\) Protein binding in tear film represents another important restriction to drug absorption, as only the unbound drug is transported across the tissue barriers.\(^5\) In fact, the presence of albumin in tears can dramatically reduce the bioavailability of topical drugs via protein-drug interactions, as previously shown for pilocarpine in rabbit eyes.\(^5\)
Albumin is a relatively large (66 kDa) and negatively charged protein that is widely distributed in the body. Given the protein’s remarkable capacity for binding ligands, albumin serves as a reservoir and transporter for drugs and other molecules such as hormones, metabolites, and nutrients. At the level of the eye, plasma-derived albumin leaks onto the ocular surface from conjunctival vessels and mixes with the tear film. Albumin concentration in tears is generally low in healthy state but increases substantially in diseased eyes. In fact, albumin is often considered a biomarker of ocular insult or inflammation as the breakdown of blood-tear barrier noted with ocular pathology allows for large amounts of albumin to leak into the lacrimal fluid. A recent study by Sebbag et al. showed that canine eyes with diverse ocular diseases (e.g. corneal ulcer, uveitis, glaucoma) had lacrimal albumin levels that were up to 14.9-fold greater than contralateral healthy eyes.

The impact of albumin binding on the drug’s pharmacological activity is extensively studied in blood, yet little is known about the physiology and function of albumin in tears or other biological fluids. In the present study, we examined the bioavailability of topically delivered drugs in the presence of clinically relevant levels of albumin in tears. We hypothesized that the drugs’ intraocular effect will be reduced by lacrimal albumin given the inability of protein-bound drugs to permeate through ocular tissue barriers. Two ophthalmic medications were investigated as a proof-of-concept experiment: 0.005% latanoprost and 1% tropicamide. These drugs are commonly used in human and veterinary practice, and possess different physicochemical properties (e.g. solution pH, drug concentration) that could influence protein-drug interactions. Latanoprost, a PGF2α analog, is used for the treatment of glaucoma and ocular hypertension in human and veterinary patients. Tropicamide, an antimuscarinic drug, is used to achieve short-acting mydriasis for enhanced visualization of the lens, vitreous
body and fundus, as well as cycloplegia to control accommodation during the assessment of refractive error.\textsuperscript{14} Pupil response to tropicamide was also suggested as a noninvasive neurobiological test for Alzheimer’s disease and other neurodegenerative disorders,\textsuperscript{15,16} although this diagnostic test fell out of favor given large inter- and intra-individual variations and subsequent poor test specificity.\textsuperscript{17} Drug binding to proteins in tear fluid could partly explain the aforementioned variability in pupil size, a phenomenon our group investigated in the present study to help guide future diagnostic and therapeutic applications in ophthalmology. The present work was conducted in dogs, a species that represents an excellent large animal model for translational research in humans given similarities in ocular anatomy\textsuperscript{18} and physiologic parameters pertinent to topical route of drug administration,\textsuperscript{19} as well as spontaneous disease development such as glaucoma,\textsuperscript{20} dry eye\textsuperscript{21} and conjunctivitis.\textsuperscript{10}

\textbf{Materials and Methods}

\textbf{Animals}

Eight female spayed Beagle dogs (1.5-2.0 years, 7.5-10 kg) were recruited. Prior to study enrollment, dogs were part of a teaching colony and underwent weekly physical and ophthalmic examinations, including tonometry (TonoVet, Icare Finland Oy, Espoo, Finland). At study inclusion, dogs were confirmed to be healthy based on a complete physical and ophthalmic examination, including tonometry (TonoVet), Schirmer tear test-1 (STT-1; Eye Care Product Manufacturing LLC, Tucson, AZ, USA), slit-lamp biomicroscopy (SL-17; Kowa Company, Ltd., Tokyo, Japan) and indirect ophthalmoscopy (Keeler Vantage; Keeler Instruments, Inc., Broomall, PA, USA). The study was approved by the Institutional Animal Care and Use Committee of Iowa State University (protocol # 19-049), and conducted in accordance with the Association for Research in Vision and Ophthalmology guidelines for animal use.
**Experiment**

Two canine albumin ophthalmic solutions (0.4% and 1.5%) were formulated by mixing canine albumin lyophilized powder (Animal Blood Resource International, Stockbridge, MI) with lubricating eye drops (Artificial tears solution, Rugby, Rockville Center, NY, USA) in a sterile manner under a laminar flow hood. Of note, albumin concentrations selected herein (0.4% and 1.5%) aimed to achieve tear film albumin levels of ~ 1 to 5 mg/mL (i.e., after dilution of the instilled drop with the canine tear film, accounting for ~ 3-fold dilution)\(^{19}\) thus representing a spectrum of albumin levels in tears of dogs with spontaneous or experimentally-induced conjunctivitis.\(^{10}\) Artificial tears solution without albumin (vehicle only) was used as control for the experiment. Albumin and vehicle solutions were kept in the refrigerator (4 °C) and used within 7 days of preparation.

For each dog, one eye was randomly selected to receive albumin solutions while the contralateral eye served as control (vehicle solution); this choice was kept constant throughout the study. Tropicamide 1% (Sandoz Inc., Princeton, New Jersey, USA) and latanoprost 0.005% (Sandoz Inc., Princeton, New Jersey, USA) were each investigated over two separate days. To allow pupil size to return to baseline and avoid a carry-over effect, the washout between experimental days was 24h for tropicamide\(^{22}\) and 72h for latanoprost.\(^{23}\) For each drug, the eye allocated to albumin was randomly assigned to receive either 4 mg/mL or 15 mg/mL albumin solution on the first experimental day, and vice versa on the second day.

The experiments took place in a quiet and uniformly illuminated room (500 lux) under controlled temperature (70-72°F) and ambient humidity (25-30%). Measurements of pupil diameter (PD) were obtained with a digital Vernier caliper (± 0.01 mm, Ultratech No. 1433, General Tools & Instruments, Secaucus, NJ) held adjacent to the cornea, while measurements of
intraocular pressure (IOP) were obtained with rebound tonometry (TonoVet, Icare Finland Oy, Espoo, Finland). Baseline PD and IOP were recorded in both eyes of each dog at the beginning of each study day.

Using a pipette, 30 µL of experimental solution were delivered topically: albumin in one eye, vehicle in the other. This was immediately followed (<10 sec) by topical instillation of 30 µL of the drug (tropicamide or latanoprost) in both eyes. Then, PD (tropicamide and latanoprost) and IOP (latanoprost only) were recorded in both eyes at the following time points: 2, 4, 6, 8, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, and 480 min.

Data analysis

Normality of data was assessed with the Shapiro-Wilk test. Differences in pupil diameter (tropicamide, latanoprost) and IOP (latanoprost) between eyes receiving saline (control) or albumin (0.4% or 1.5%) were assessed with a mixed model for repeated measures (MMRM)²⁴ using the R software version 3.6.0. In the model, PD or IOP were the response variable, the group (control or albumin), time (0 to 480 min) and group-by-time interaction were treated as fixed effects, and the animal and animal-by-group interaction were treated as random effects, using animal as block. After the model was fit, the fixed effects were tested, and comparisons between control and albumin eyes at baseline and each time point were made. The R software was also used to calculate the area under the effect-time curve (AUETC) and the maximal effect on pupil diameter (maximal dilation for tropicamide, maximal constriction for latanoprost). Paired t-tests were conducted with SigmaPlot 14.0 (Systat Software Inc., San Jose, CA, USA) to assess the following parameters: (i) AUETC for tropicamide and latanoprost, (ii) PD\text{max} for tropicamide, and (iii) PD\text{min} for latanoprost. P values < 0.05 were considered statistically significant.
Results

Results from the Shapiro-Wilk test confirmed that the experimental data were normally distributed. Results are therefore presented as mean ± standard deviation (SD).

Pupil dilation from tropicamide

Taking the variable ‘time’ into account, albumin had a significant effect on pupil diameter post- tropicamide administration for both 0.4% ($P = 0.001$) and 1.5% concentrations ($P = 0.001$). Compared to the contralateral eye (control), pupillary dilation was significantly reduced in eyes receiving 0.4% albumin as early as 8 min ($P = 0.043$) and as late as 240 min ($P = 0.009$) (Figure 1A), and in eyes receiving 1.5% albumin as early as 8 min ($P = 0.027$) and as late as 240 min ($P = 0.021$) (Figure 1B) following instillation of 1% tropicamide. A representative clinical image is depicted in Figure 2, showing a lower degree of mydriasis in the dog’s left eye (0.4% albumin and 1% tropicamide) compared to the right eye (artificial tears and 1% tropicamide) at 45 min following eyedrop administration. Further, the cumulative effect of tropicamide on pupillary dilation (from 0 to 480 min) was significantly reduced with the addition of 0.4% or 1.5% albumin ($P < 0.001$), representing an average reduction in biological response of 7.1% and 7.2% compared to controls, respectively (Figure 3); however, no differences were noted in AUETC (pupil size over time) between both albumin concentrations ($P \geq 0.625$). Last, mean ± SD maximal pupillary dilation in eyes receiving tropicamide and 0.4% albumin (11.9 ± 0.7 mm) or 1.5% albumin (11.7 ± 1.3 mm) was significantly lower ($P \leq 0.006$) compared to contralateral controls (12.7 ± 0.8 mm, and 12.8 ± 1.1 mm, respectively), representing a reduction in biological response of 6.2% and 8.5%, respectively (Figure 4).
Pupil constriction from latanoprost

Taking the variable ‘time’ into account, albumin had a significant effect on pupil diameter post-latanoprost administration for both 0.4% \((P = 0.016)\) and 1.5% concentrations \((P = 0.047)\). Compared to the contralateral eye (control), pupillary constriction was overall reduced in eyes receiving 0.4% albumin (Figure 5A) or 1.5% albumin (Figure 5B), although differences in pupil diameter were not statistically significant at any time point following instillation of 0.005% latanoprost \((P \geq 0.158\) and \(P \geq 0.416\), respectively). No differences were noted in AUETC (pupil size over time) between groups \((P \geq 0.663)\), nor in maximal pupillary constriction \((P = 1.000)\) obtained with 0.4% albumin \((1.5 \pm 0.1\) mm), 1.5% albumin \((1.5 \pm 0.2\) mm) and their respective contralateral controls \((1.5 \pm 0.1\) mm and \(1.5 \pm 0.2\) mm).

IOP changes from latanoprost

Compared to the contralateral eye (control), IOP was significantly lower in eyes receiving 0.4% albumin at 10 min \((P = 0.010)\), 20 min \((P = 0.010)\), 45 min \((P = 0.010)\) and 240 min \((P = 0.010)\) following instillation of 0.005% latanoprost (Figure 6A), while no significant changes were noted at any time point \((P \geq 0.262)\) for the 1.5% albumin group (Figure 6B). However, after taking ‘time’ into account in the mixed effects model, it is important to note that the impact of albumin on IOP was not statistically significant for either 0.4% \((P = 0.242)\) or 1.5% concentration \((P = 0.879)\). Further, no differences were noted in AUETC (IOP over time) between control and albumin groups \((P \geq 0.351)\) or between both albumin groups \((P = 0.979)\).

Discussion

The bioavailability of ophthalmic drugs can be reduced by the presence of albumin in tears, a protein that leaks onto the ocular surface in large amounts in the diseased eye. A deeper
understanding of albumin-drug interactions in tears is critical to basic researchers in pharmacology and vision science, but also physicians and veterinarians. Indeed, drug binding to albumin may partly explain the challenge of treating certain diseases in ophthalmology. For instance, a poor response of uveitis to topical corticosteroid may be due to high affinity of the drug to albumin in tears, while a poor response of infectious keratitis to topical antibiotics may be explained by the fact that only the unbound portion of an antimicrobial is microbiologically active. Here, we showed a differential impact of albumin in tears on the ocular response of tropicamide and latanoprost, two common ophthalmic drugs in human and veterinary patients, and the same could be investigated in the canine model for other relevant drug classes. Dogs are particularly suited for translational research in ocular pharmacology as – unlike small laboratory animals – dogs share similar anatomical and physiological features to humans, similar environmental stressors and genetic variation, and a range of naturally occurring ophthalmic diseases that resemble the ones diagnosed in human patients.

**Ocular response of tropicamide and latanoprost in the presence of albumin**

The biological effect of tropicamide (*i.e.* mydriasis) was significantly reduced in canine eyes that received concurrent topical administration of serum albumin, regardless of the protein’s concentration. Albeit minimal (6.2-8.5 %), the impact of albumin on tropicamide-induced pupillary dilation is likely underestimated compared to clinical patients given limitations inherent to the study design (described below). Interestingly, this lower degree of tropicamide-induced mydriasis was also noted in canine eyes covered with a soft contact lens, a physical barrier to drug penetration. Similar findings were noted by Mikkelson et al. in rabbit eyes receiving pilocarpine, although the magnitude of drug-response reduction was much greater in
rabbits (75-100 fold) compared to the present study in dogs (6-8%). Such discrepancy is likely explained by two important differences in study designs. In the present experiment, the concentrations of albumin (0.4% and 1.5%) were specifically chosen based on clinically-relevant albumin levels detected in canine patients with diverse ocular diseases,\textsuperscript{10} taking into account the 3-fold dilution effect from resident tears.\textsuperscript{19} to better account for the true binding constant seen in dialysis experiments.\textsuperscript{27} In contrast, the concentrations of albumin used in rabbits were higher (1% and 3%) and may not reflect the range of biological concentrations of albumin at the ocular surface. Another key difference is the way albumin was delivered to the ocular surface. While albumin was pre-mixed with pilocarpine solution in the rabbit study, allowing for protein-drug binding to occur \textit{ex situ} (\textit{i.e.} away from the ocular surface) over an extended duration, albumin and tropicamide were delivered separately in the present study (albumin first, tropicamide within <10 seconds) so that protein-drug interactions would occur \textit{in situ} (\textit{i.e.} in the tear film) over a short duration. From a physiological standpoint, the latter method is more appropriate as the interaction time of a topical drug to albumin in tears is generally short, limited by the rapid tear turnover rate that occurs following eyedrop administration in dogs\textsuperscript{19} or other species.\textsuperscript{28,29} Of note, tonometry was not performed in dogs receiving tropicamide (with or without albumin) in the present study given the lack of effect of tropicamide on IOP in healthy canine eyes,\textsuperscript{30} but this parameter could be considered in future studies as IOP can vary from tropicamide in dogs receiving sedation\textsuperscript{30} or dogs with glaucomatous eyes.

The pharmacological activity of latanoprost (\textit{i.e.} miosis) was also reduced in the presence of albumin in tears, although not to the same extent as for tropicamide. Indeed, differences in pupil size between albumin and control eyes were limited in duration (up to 30 min, compared to 240 min for tropicamide) and somewhat limited in magnitude (1% non-significant change in
AUETC). These findings are likely explained by the high sensitivity of the iris sphincter muscle to the drug. The minimum amount of PGF2α required to generate contraction of the iris sphincter in dogs is $10^{-10}$ M, while the concentration of latanoprost applied topically is approximately $10^6$ higher (0.005% ~ $10^{-4}$ M). The amount of drug lost to albumin in tears is therefore insignificant, as only a small fraction of intraocular drug penetration is sufficient to cause miosis. Furthermore, once latanoprost reaches the anterior chamber, the drug acts directly on the iris sphincter muscle but also indirectly through the release of endogenous prostaglandins. Endogenous PGF2α, which further acts on the prostanoid FP receptors and contributes to the sphincter muscle’s tone in dogs, is released inside the anterior chamber and is thereby not affected by albumin levels in the tear film.

Following latanoprost administration, the overall effect of lacrimal albumin on IOP values was non-significant for either albumin concentration. Compared to control eyes, a significantly lower IOP was noted at selected times (10, 20, 45 and 240 min) in eyes receiving 0.4% albumin concurrently to latanoprost, although it is important to note that IOP readings displayed a large variability within-and between-subjects in all groups. To reduce IOP variability, a recent study in healthy Beagle dogs recommended a minimum of 5 training days immediately prior to the start of the study, and collecting IOP readings in triplicate at each time. In absence of such precautions, the IOP results of the present study are likely confounded by a large measurement noise.

**Factors affecting the impact of albumin on drug bioavailability**

The present findings provide evidence that albumin levels in tears does not affect all drugs in a uniform manner. Rather, the mechanism of action of a drug and/or potency for its
biological target can modulate the impact of lacrimal albumin on the drug’s pharmacological activity. The dose-response relationship, a cornerstone of pharmacology/toxicology, defines the role of a dose for a chemical (e.g. drug, toxic agent) in evoking biological response. Figure 7A depicts two drugs (A & B) with different dose-response profiles. In this scenario, if lacrimal albumin reduces the amount of free drug available inside the eye by 50%, the response observed (e.g. pupillary dilation) will be greatly reduced for drug B but minimally affected for drug A. Along the same line, the amount of drug applied topically is an important factor to take into consideration. For a given drug, if the dose administered topically falls in the ‘far right’ of the drug’s dose-response curve, a reduction in drug available after albumin binding would only minimally affect the observed response (dose X, Figure 7B); in contrast, if the dose delivered produces an effect that falls within the steep portion of the dose-response curve, the impact of dose reduction from albumin would be more pronounced (dose Y, Figure 7B). Mikkelson and colleagues showed that the entire biological response (pilocarpine-induced miosis) could be suppressed when low drug concentrations were used in the presence of serum albumin. Future studies leveraging this work should assess the ocular response obtained from different concentrations of the same drug (e.g. tropicamide 0.05%, 0.5%, 1%, 2%) in the presence of albumin in tears.

A number of other factors can influence the drug-protein interactions on the ocular surface, including albumin concentrations in tears and physicochemical properties of the individual drug. In plasma, higher levels of albumin can further reduce the biological response of a drug, as exemplified by lower antimicrobial activity of fluoroquinolones with increasing levels of serum albumin. In tears, however, the present study did not find statistical differences between 0.4% and 1.5% albumin for either tropicamide or latanoprost. It is possible that the
The magnitude of albumin levels is not as critical in tears as it is in plasma, as lacrimal concentrations of albumin are relatively small (<2%)\textsuperscript{10} in comparison to blood (≥4%).\textsuperscript{36} Similarly, the influence of molecular weight on drug-albumin affinity may be minimal in tears, as most ophthalmic drugs have a relatively small and narrow range of molecular weights (e.g. 284 Da for tropicamide, 432 Da for latanoprost). In contrast, the pH of ophthalmic drugs is likely more impactful: pH varies from one ophthalmic solution to another, and albumin is known to change its binding affinity and conformation when exposed to changes in solution pH.\textsuperscript{37} Here, tropicamide solution is slightly more acidic (pH 5.8) than latanoprost solution (pH 6.7), although both solutions are near physiologic pH for the ocular surface and may have minimal impact on albumin affinity compared to other ophthalmic drugs such as dorzolamide (pH 4.5). Future studies should investigate the importance (or lack thereof) of other relevant biological factors on albumin-drug interactions in tear fluid, such as drug lipophilicity, viscosity, temperature at the ocular surface, fatty acids levels in tears, and drug-drug interactions.\textsuperscript{38}

The study may underestimate drug-proteins interactions and their impact on bioavailability

The present work likely underestimates the true impact of proteins on ocular bioavailability of drugs, as the study design has two noteworthy limitations. First, although the lag time between albumin and drug instillation was short (<10 s), it may be sufficient for a portion of the administered albumin to be washed out of the ocular surface by the time the drug mixes with the tear film, as most of an administered eyedrop is lost to drainage in the first 15 to 30 seconds.\textsuperscript{28,29} Second, although albumin is a major actor of drug-protein binding on the ocular surface, other proteins play a critical role too. Using equilibrium dialysis, Chrai and Robinson showed that sulfisoxazole primarily binds to albumin in tears, but also α-globulin and (to a lesser
extent) γ-globulin and lysozyme, all of which are normal components of tears in dogs and other species. Ultimately, the authors recommend that future investigations be conducted with experimental models of blood-tear barrier breakdown, such as histamine-induced conjunctivitis in dogs. With such models, albumin and other key proteins are already present on the ocular surface when the drug is administered topically, although individual protein concentrations may vary from one eye to another, and this variability should be accounted for in the interpretation of study results. Furthermore, such models would account for other important changes that occur with ocular surface inflammation and that could affect drug-protein interactions, such as altered tear volume and turnover rate, tear film instability, and variations in mucin composition.

**Strategies to minimize drug-proteins interactions and enhance ocular bioavailability**

A few strategies can be used to minimize the effects of drug-protein interactions on the ocular surface, and thereby maximize the drug pharmacological action by enhancing intraocular bioavailability. First, a higher drug concentration should be considered, especially if available commercially (e.g. tropicamide 1% instead of 0.5%), as the resulting concentration gradient of unbound drug will be higher (Fick’s first law of diffusion). Second, the amount of protein leakage into the tear film can be reduced by stabilizing the blood-tear barrier in the diseased eye, a process achieved by treating the underlying ocular disease and/or using vasoprotective drugs such as calcium dobesilate. Last, drug-protein interactions can be reduced by using competitive inhibitors of protein binding; for instance, Mikkelson and colleagues showed that the biological activity of pilocarpine (a miotic agent) increased 10-fold in the presence of the competitive inhibitor cetylpyridinium chloride. However, the use of competitive inhibition of albumin binding is discouraged until the importance of albumin on the ocular surface is fully elucidated.
In fact, albumin in tear film may serve as a double-edged sword, being detrimental to the ocular bioavailability of topically administered medications, but also beneficial for symptomatic relief of dry eye,\textsuperscript{42,43} corneal wound healing,\textsuperscript{42} and anti-oxidative and anti-inflammatory activities.\textsuperscript{44}

**Conclusion**

Albumin in tears modulate the ocular bioavailability of topically administered drugs, as observed in a ‘large animal’ model that shares similar anatomical and physiological features with humans. The effect of albumin depends on the medication (\textit{e.g.} drug concentration and inherent physicochemical properties) and was overall mild (<10\%) in the present work on healthy canine eyes, albeit likely underestimated given the rapid tear turnover rate following eyedrop administration. Models of ocular surface inflammation could enable future pharmacological studies to gain a deeper understanding of protein-drug interactions, accounting for albumin leakage in tears as well as other relevant factors that affect ocular surface homeostasis.

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**Figure 1.** Mean ± SD pupil diameter from 0 to 480 min in dogs receiving 1% tropicamide in both eyes, immediately preceded by topical instillation of artificial tears (control, white circles) in one randomly selected eye, and either 0.4% albumin (A, black triangles) or 1.5% albumin (B, black triangles). Statistical differences ($P < 0.05$) obtained with mixed model for repeated measures are depicted by gray asterisks (*).

**Figure 2.** Clinical image of a Beagle dog at 45 min following topical instillation of 1% tropicamide in both eyes, immediately preceded by topical artificial tears (right eye, control) and 0.4% albumin (left eye). Note the lower degree of mydriasis in the left eye.
Figure 3. Box-and-whisker plots depicting the area under the effect-time curve (AUETC) of pupil diameter over time (0 to 480 min) in dogs receiving 1% tropicamide in both eyes, immediately preceded by topical instillation of artificial tears (control, white boxes) in one randomly selected eye, and either 0.4% albumin (light gray box) or 1.5% albumin (dark gray box) in the other eye. Mean and median values are shown by horizontal dotted and solid lines, respectively. First and third quartiles (25th and 75th percentiles) are represented by the lower and upper limits of the box, respectively, while the 2.5th and the 97.5th percentiles are shown as the lower and upper whiskers, respectively.

Figure 4. Box-and-whisker plots depicting the maximal pupillary diameter in dogs receiving 1% tropicamide in both eyes, immediately preceded by topical instillation of artificial tears (control, white boxes) in one randomly selected eye, and either 0.4% albumin (light gray box) or 1.5% albumin (dark gray box) in the other eye. Mean and median values are shown by horizontal dotted and solid lines, respectively. First and third quartiles (25th and 75th percentiles) are represented by the lower and upper limits of the box, respectively, while the 2.5th and the 97.5th percentiles are shown as the lower and upper whiskers, respectively.
**Figure 5.** Mean + SD pupil diameter from 0 to 480 min in dogs receiving 0.005% latanoprost in both eyes, immediately preceded by topical instillation of artificial tears (control, white circles) in one randomly selected eye, and either 0.4% albumin (A, black triangles) or 1.5% albumin (B, black triangles). No statistical differences were noted between groups at any time point (mixed model for repeated measures, $P \geq 0.05$).

**Figure 6.** Mean + SD intraocular pressure from 0 to 480 min in dogs receiving 0.005% latanoprost in both eyes, immediately preceded by topical instillation of artificial tears (control, white circles) in one randomly selected eye, and either 0.4% albumin (A, black triangles) or 1.5% albumin (B, black triangles). Statistical differences ($P < 0.05$) obtained with mixed model for repeated measures are depicted by gray asterisks (*).
Figure 7. Hypothetical scenarios highlighting the importance of dose-response relationship in understanding the impact of albumin in tears on the biological activity of an ophthalmic drug. 

(A) A 50% reduction in the amount of drug that can penetrate inside the eye will have a minimal effect on the biological effect of drug A (dotted line), but a profound effect on drug B (solid line). 

(B) For the same drug, a 50% reduction in the amount of drug that can penetrate inside the eye will have a minimal effect on the biological response if the initial drug concentration was high (X), but a profound effect if the initial drug concentration was relatively low (Y).
CHAPTER 8. GENERAL CONCLUSION - AN EYE ON THE DOG AS THE SCIENTIST'S BEST FRIEND FOR TRANSLATIONAL RESEARCH IN OPHTHALMOLOGY: FOCUS ON THE OCULAR SURFACE

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Modified from a manuscript under review in Medicinal Research Reviews

Abstract

Preclinical animal studies provide valuable opportunities to better understand human diseases and contribute to major advances in medicine. This review provides a comprehensive overview of ocular parameters in humans and selected animals, with a focus on the ocular surface, detailing species differences in ocular surface anatomy, physiology, tear film dynamics and tear film composition. We describe major pitfalls that tremendously limit the translational potential of traditional laboratory animals (i.e., rabbits, mice and rats) in ophthalmic research, and highlight the benefits of integrating companion dogs with clinical analogues to human diseases into preclinical pharmacology studies.

This One Health approach can help accelerate and improve the framework in which ophthalmic research is translated to the human clinic. Studies can be conducted in canine subjects with naturally occurring or non-invasively induced ocular surface disorders (e.g., dry eye disease, conjunctivitis), reviewed herein, and tear fluid can be easily retrieved from canine eyes.
for various bioanalytical purposes. In this review, we discuss common tear collection methods, including capillary tubes and Schirmer tear strips, and provide guidelines for tear sampling and extraction to improve the reliability of analyte quantification (drugs, proteins, others).

**Introduction**

Preclinical animal models provide critical information to better understand human diseases’ characteristics, identify biomarkers, develop diagnostic tools and novel therapeutics. Rabbits and laboratory rodents (mice, rats) are widely used for ophthalmic research as they are economical and easy to handle;\(^1\) however, serious drawbacks limit the translational usefulness of data obtained in these species, notably due to the need to artificially induce pathology in these animals (e.g., through genetic manipulation or experimental surgery), as well as apparent differences in ocular anatomy and physiology compared to humans. For instance, precorneal residence time of topically applied solutions is much prolonged in rabbits owing to their low blink rate, resulting in 3-fold overestimation of ocular drug exposure if findings were directly extrapolated from rabbits to humans.\(^2\) Another example is topical nepafenac, a potent nonsteroidal anti-inflammatory drug (NSAID) that reaches therapeutic levels in the posterior segment of mice (owing to their thin cornea and small globe size), inhibiting choroidal neovascularization by decreasing production of VEGF\(^3\) – in contrast, humans require intravitreal injections of anti-VEGF compounds to achieve the same outcome. Multiple other examples exist in the scientific literature, together participating to the unacceptably low success rate of ophthalmic clinical trials to date, and resulting in substantial economic loss and burden for scientists, consumers, and society overall.\(^4\) In fact, the main cause for clinical trial failure is
either lack of safety or efficacy, two components that are supposedly ‘validated’ in initial preclinical animal studies.

Under the umbrella of the One Health Initiative, a growing number of investigations have integrated companion animals into preclinical studies to complement and expand the knowledge gained from studies in other animal models, accelerate and improve the framework in which research is translated to the human clinic, and ultimately generate discoveries that will benefit the health of humans and animals. Over the last few years, several review articles have highlighted the benefits of using dogs for translational research in oncology, neurology and other biomedical fields, yet such information is not available in ophthalmology.

The present review provides a comprehensive comparison of key ocular parameters in humans, dogs and traditional laboratory animals (ie., rabbits, mice, rats), highlighting selected strengths and important pitfalls that must be addressed when ocular research is conducted in animal models. This review is focusing on the ocular surface, a critical element of vision that includes the secreted tear film, lacrimal gland(s), eyelids, meibomian glands, cornea, conjunctiva, sclera, and nasolacrimal drainage apparatus. The ocular surface dictates the bioavailability of medications administered topically to the eye, and is a common site of pathology in both human and veterinary medicine. Methods of tear fluid collection for bioanalytical purposes are also being discussed, with special consideration on the safety and efficiency of the collection technique at hand. Lastly, this review highlights on spontaneous and experimental ocular surface disorders in dogs, providing a tool for researchers to better model disease pathophysiology in clinical patients suffering from ocular surface disorders.
Comparative anatomy and physiology of the ocular surface

Anatomy

The anatomy of the ocular surface is depicted in Figure 1 for dogs, and its parameters are being summarized in Table 1 for all species discussed in this review (i.e., humans, dogs, rabbits, mice, rats).

Lacrimal glands - Four types of lacrimal glands can be distinguished in mammals: (i) the orbital lacrimal gland (glandulae lacrimales superior), located in the dorsolateral orbit just caudal to the orbital rim, with secretory ducts that open into the upper conjunctival fornix (humans, dogs, rabbits); (ii) the gland of the third eyelid, located in the ventromedial orbit at the base of the nictitating membrane with secretory ducts than open into the nictitans’ bulbar conjunctiva (dogs); (iii) the infraorbital gland (glandulae lacrimales inferior), located either intraorbital and ventromedial to the globe (rabbits) or extraorbital and caudal to the globe (rodents), with a single secretory duct that opens into the lower conjunctival fornix; and (iv) the Harderian gland, or Harder’s gland, extending from the base of the third eyelid into the caudal orbit, with secretory ducts opening at the nictitating membrane (rabbits, rodents).10,11 The histomorphology of lacrimal glands varies with age and sex of the individual.12 In dogs, an orbital lacrimal gland and gland of the third eyelid contribute to 60-70% and 30-40% of the overall tear secretion, respectively.13 The morphological and histological features of the canine glands resemble the human lacrimal gland including distinct lobules and acini that provide serous and mucous secretions, as well as intralobular ducts that drain into small excretory tubules.14,15 Likewise, an Harderian gland is not present in the canine or human orbit.14-16 However, two notable differences exist between species: (i) the combined volume of the two canine glands is smaller than the main lacrimal gland in humans (0.24 vs. 0.60 cm²)17,18; and (ii)
the accessory lacrimal glands of Krause and Wolfring are absent in dogs (or not yet reported), presumably being consolidated through evolution into the single gland of the third eyelid. In rabbits, the histoarchitecture of the main lacrimal gland is comparable to humans with loosely packed acini and round/oval lumen; in contrast, mice and rats have densely packed acini with small pleiomorphic lumen and numerous intercellular tight junctions. Like humans, rabbits also possess accessory lacrimal glands of Wolfring in the tarsal portion of the palpebral conjunctiva. However, the Harderian gland present in rabbits and rodents is a unique anatomical feature that has important repercussions for comparative studies; in fact, the gland’s lipid secretions in the tear film have profound effects on the ocular surface physiology (e.g., tear composition, tear film dynamics, blink rate) and pharmacology of topically applied medications (see sections 2.2 and 2.3).

**Nasolacrimal apparatus** - The morphology of the canine lacrimal drainage system is remarkably similar to that of humans, except for a longer nasolacrimal duct (notably in long-nosed dogs), and the presence of accessory duct openings into the nasal cavity. In both species, tear drainage begins with the lower and upper nasolacrimal puncta and canaliculi in the medial canthus, joining into a lacrimal sac in the bony lacrimal fossa, and extending into the nasolacrimal duct that runs through an osseous channel towards the nasal cavity. Species similarities are also evident on a microscopic level, including an epithelial lining with microvilli and mucin-secreting goblet cells, sub-epithelial seromucous glands, and mucosal-associated lymphoid tissue. In contrast, the nasolacrimal apparatus of rabbits has distinct differences compared to humans. Rabbits only have a single nasolacrimal punctum/canaliculus (medial lower eyelid) and the nasolacrimal duct has two very distinct flexures due to the ventral
deflection of the snout, a unique feature that results in a convoluted path for tear drainage.\textsuperscript{25,26} The fetal development of the rabbit’s nasolacrimal apparatus is also unique in mammals, more closely resembling reptiles vs. humans.\textsuperscript{27} At an ultrastructural level, the epithelium lining the duct is double-layered (similar to humans) but there are no goblet cells or subepithelial seromucous glands.\textsuperscript{28} Nonetheless, the use of the rabbit is still recommended as a practical model to characterize the nasolacrimal apparatus,\textsuperscript{29} albeit this choice is described as ‘less than ideal’ by the authors. Mice and rats have a well-developed nasolacrimal apparatus that shares similar ontogenetic origin to humans,\textsuperscript{27} although the histological features are different. The duct lining is covered by a multi-layered stratified squamous epithelium with goblet cells but without subepithelial seromucous glands.\textsuperscript{25}

**Third eyelid** - The nictitating membrane (third eyelid) is a large fold of the conjunctiva that protrudes from the medial canthus over the anterior surface of the globe in many animals, including dogs, rabbits and rodents. The counterpart in humans is the plica semilunaris, a vestigial remnant in the form of a crescent-like conjunctival fold in the medial canthus.\textsuperscript{11,30} Despite gross differences, both structures have important physio-morphological similarities such as the presence of goblet cells and lymphoid follicles, contributing to the lubrication and immune protection of the ocular surface.\textsuperscript{30} Nonetheless, the presence of a third eyelid should be considered in comparative studies as it could impact ocular examinations (eg., third eyelid protrusion from ocular irritation) or ocular drug delivery (eg., altered retention time of a contact lens),\textsuperscript{31} among others. If required for ease of experimentation, a simple fixation of the nictitating membrane can be performed\textsuperscript{31} as an alternative to complete surgical removal,\textsuperscript{13,32,33} as the latter negatively impacts ocular surface homeostasis.\textsuperscript{13,34}
**Eyelids** - Similar to humans, the canine upper and lower eyelids are comprised of an outer dermis, tarsus, orbicularis oculi muscle, palpebral conjunctiva and secretory tissues including meibomian glands (20-40 per eyelid), glands of Zeis and Moll.\textsuperscript{35,36} The main anatomical difference is the tarsal plate, which is comprised of dense fibrous tissue and cartilage-specific components in humans\textsuperscript{37} – providing a rigid internal support to the eyelids – compared to a much thinner and poorly-developed fibrous tissue in dogs.\textsuperscript{38} Also, the interpalpebral fissure area is approximately 20% larger in dogs (2.2 vs. 1.8 cm\textsuperscript{2})\textsuperscript{39,40}, although the measurements of the palpebral fissure width depend on the dog’s size and body weight.\textsuperscript{40}

The palpebral opening in the rabbit is relatively small (10-16 mm),\textsuperscript{38,41,42} albeit much larger than mice (3.7-5 mm)\textsuperscript{43} and rats (6-9 mm)\textsuperscript{44,45}, with a shorter and thicker upper eyelid compared to the inferior palpebrae; consequently, the interpalpebral fissure area is 20% smaller in rabbits than in man (1.44 vs. 1.8 cm\textsuperscript{2}).\textsuperscript{39} The meibomian gland ducts and acini are also larger in rabbits than mice and rats,\textsuperscript{46} but the overall volume and distribution of meibomian glands is different than in humans: the total meibomian gland volume in the human (39.5 mm\textsuperscript{3}) is twice that of the rabbit (18.8 mm\textsuperscript{3}), with a larger volume in the upper eyelid (man) compared to similar volumes in the upper and lower eyelids (rabbit).\textsuperscript{39}

**Conjunctiva** - The conjunctiva is a thin mucous membrane that serves important roles on the ocular surface including mucin secretion and immune surveillance. The anatomical subdivision of the conjunctiva is the same in humans, dogs, and common laboratory species (rabbits, rodents): the palpebral conjunctiva – lining the inside of the eyelids – reflects back at the level of the conjunctival fornix to form the bulbar conjunctiva, a region that covers the anterior portion of the sclera and attaches to the corneoscleral limbus.\textsuperscript{16} However, the amount of bulbar conjunctiva exposed (‘scleral show’) is notably larger in humans compared to animals
given differences in eyelid opening and/or corneal diameter. Another important species difference is the presence of a nictitating membrane in animals (but not man), as the third eyelid is covered by conjunctiva on its anterior and posterior surfaces. As such, animals have two conjunctival fornices in the inferonasal region – one on each side of the third eyelid – and the overall conjunctival surface is generally larger in animals compared to humans. In dogs, the conjunctival area is supposedly larger than in humans given the depth of the canine conjunctival fornices and the amount of conjunctiva covering the canine nictitating membrane, although no objective data exist to date. In rabbits, the upper conjunctival fornix depth (20.36 mm) is larger than in humans (15 mm), while the conjunctival area is reportedly comparable (13.34-18.48 vs. 17.65 cm², respectively), although the measurements did not include the rabbit’s third eyelid (surgically removed by investigators).

Conjunctival goblet cells are distributed individually in humans, dogs and rabbits, in contrast to clustered organization in mice and rats. The distribution of goblet cells is overall similar in dogs and humans, with high density in the canine third eyelid and human plica semilunaris, relatively high density in the conjunctival fornices and palpebral conjunctiva, and lower density in the bulbar conjunctiva. In rabbits, the highest density is noted at the lid margin of both upper and lower palpebral conjunctivae, while the density in the bulbar conjunctiva is generally higher than in humans (399-1576 cells/mm² vs. 7-979 cells/mm²). In addition to mucin-secreting goblet cells, the conjunctiva also contains an organized immune network termed conjunctiva-associated lymphoid tissue (CALT), a structure that plays a key role in protecting the ocular surface by initiating and regulating immune responses. The presence of lymphoid follicles was confirmed in the conjunctiva of most mammals studied by Chodosh and colleagues – including humans, dogs, rabbits – with the exception of mice and rats, although a
later report detected lymphoid tissue in the nictitating membrane of BALB/c mice. At an ultrastructural level, specialized M cells are present in the epithelium overlying the conjunctival follicles in dogs and rabbits, similar to humans.

**Cornea** - The anatomy of the cornea is unique to each species with important differences in corneal dimensions and ultrastructural features (e.g., thickness, collagen arrangement, nerve supply). First, the cornea is generally larger in dogs and rabbits compared to humans, while the dimensions are much smaller in mice and rats. As such, the relative amount of cornea and conjunctiva exposed on the ocular surface varies among species, an anatomical fact that has important implications in ocular pharmacology and other research fields; for instance, the surface area ratio of conjunctiva to cornea is two times smaller in rabbits (8.6-8.9) than humans (17.1), a finding that could largely explain species differences in drug penetration into the anterior chamber. Second, the corneal thickness varies among mammals and is generally correlated to the size of the animal. From highest to lowest, the mean central corneal thickness is 497-594 µm in dogs, 505-563 µm in humans, 354-407 µm in rabbits, 159-170 µm in rats, and 90-137 µm in mice. The average canine cornea is only slightly thicker than in humans. In contrast, the thinner cornea in rabbits and rodents can limit the use of these laboratory species for selected experiments; for instance, cross-linking is discouraged in corneas thinner than 400 µm due to potential damage to the corneal endothelium or intraocular tissues.

On a structural level, the main layers of the cornea are the same in humans and animals (epithelium, stroma, Descemet membrane, endothelium) with the notable exception of the Bowman’s membrane. Bowman’s membrane is present in nearly all primates (including humans) and selected animals (e.g., sheep, deer, giraffe), but is absent in dogs and common laboratory species. The number of layers and overall thickness of the corneal epithelium
vary among species: humans (5-7 layers, 44-55 µm),79,83 dogs (6-9 layers, 52-64 µm),70,71,78 rabbits (5-7 layers, 45-49 µm),38,79,83 rats (10-14 layers, 26-33 µm),84,85 and mice (13 layers, 37-46 µm).74 The corneal stroma, comprising nearly 90% of the total corneal thickness in most mammals, is primarily composed of collagen fibrils arranged in lamellae. While extensive collagen intertwining is noted in the majority of the corneal stroma in humans, it is only present in the anterior most-aspect of the cornea in dogs and rabbits.86,87 Differences in collagen intertwining, along with the absence of Bowman’s membrane in laboratory species, explain the vast disparity in stiffness of the anterior stroma (16.2, 1.3 and 1.1 kPa) and posterior stroma (2.5, 0.5 and 0.4 kPa) in humans, dogs and rabbits, respectively.86,87 The elastic modulus of the cornea is reportedly higher in rodents, although the methodology used was different.88,89 Corneal rigidity should be considered in comparative studies in which the biophysical attributes of the cornea are important (eg., wound healing, keratoprosthesis). The corneal endothelium shares a similar morphological blueprint among species (single cell layer, honey-comb pattern), while the cellular density varies from 3233 cells/mm² in rabbits, 2875 cells/mm² in mice, 2818 cells/mm² in dogs, 2732 cells/mm² in humans, and 2242 cells/mm² in rats.68,90

The mammal cornea is the most densely innervated tissue in the body. Corneal nerves play important roles to maintain ocular surface health and homeostasis, including sensory functions (touch, pain, temperature), release of trophic neuropeptides, maintenance of the limbal stem cell niche, and activation of brainstem circuits to promote reflex blinking and lacrimation. From highest to lowest, the sensitivity of the cornea to mechanical stimulus is as follows: humans (0.2-1.0 g/mm²), rats (0.42-0.47 g/mm²), mice (0.59 g/mm²), dogs (2.16-2.9 g/mm²), and rabbits (6.21-10 g/mm²).2,91-95 The murine model is the most extensively studied of all laboratory species given gross similarities between mice and humans in corneal sensitivity and nerve
architecture. The canine model is also studied in detail given shared features with humans in several spontaneous diseases such as diabetes mellitus, herpetic keratitis, and non-healing corneal ulcers; importantly, investigators should account for the canine breed selected for the experiment as corneal sensitivity depends on the dog’s cephalic conformation. In regard to rabbits, two striking species differences exist: (i) Corneal sensitivity in rabbits is much lower than in humans, dogs and rodents; and (ii) Morphology of the rabbit subbasal plexus is unique, with nerve fibers sweeping horizontally across the corneal surface in a temporal-to-nasal direction compared to a typical whorl-like or spiraling pattern in other species.

**Sclera** - Humans have a widely exposed white sclera, a feature that is unique when compared to other primate species (Kobayashi, 1997). In contrast, the scleral exposure is minimal in dogs and routine laboratory species. The thickness of the sclera also differ among species: at the ocular surface (limbal sclera), recorded measurements vary from 0.8 mm in dogs, 0.5 mm in humans, 0.29 mm in rabbits, 0.1 mm or less in rats, and 0.05-0.06 mm in mice.

**Tear film dynamics**

Effective tear dynamics, combined with well-balanced composition of the tear film (discussed in the next section), are critical for the maintenance of ocular surface homestasis and physiology. Tear fluid dynamics – or the balance between tear secretion, distribution, absorption, evaporation, and drainage – are closely regulated by the lacrimal functional unit. The lacrimal functional unit is unique to each species (see aforementioned anatomical differences), comprised of secreting glands (orbital, accessory, third eyelid, Harder’s, meibomian), eyelids, conjunctival goblet cells, corneo-conjunctival surface, and their interconnecting innervation.
Key physiological parameters provide insight into the complex tear dynamics – highlighted in Figure 2 and Table 2 – and are therefore important to account for in translational studies that involve the ocular surface:

- **Basal tear turnover rate:** Tear turnover rate is considered a global measure of the tear dynamics and integrity of the lacrimal functional unit.$^{107,108}$ The basal tear turnover rate is reportedly 13.1-17.5 %/min in humans,$^{109,110}$ 12.1 %/min in dogs,$^{111}$ 6.2-7.1 %/min in rabbits$^{112}$ and 5.2 %/min in mice;$^{113}$ no information was available in rats. In other words, it takes approximately the same time for the tear film to replenish in dogs and humans (~6-8 min) but the duration is longer in rabbits (~14-16 min) and mice (~20 min). The slow tear turnover of rabbits and rodents has important repercussions in translational research, including a longer precorneal retention time of instilled eyedrops (see next subsection), or exaggeration of ocular surface disease due to delayed clearance of inflammatory mediators from the tear film.$^{114}$

- **Tear volume:** The volume of tears on the ocular surface is highest in dogs (65.3 µL)$^{111}$ followed by humans (7-12.4 µL)$^{109,115}$ rabbits (1.9-7.5 µL)$^{112,116}$ rats (4.6 µL)$^{117}$ and mice (0.06-0.2 µL)$^{113,118}$ Canine tear volume depends on the subject’s body weight but not the dog’s cephalic conformation.$^{111}$ Differences in study methodology notwithstanding, the canine tear volume is approximately 5 to 9-fold larger than in humans. This discrepancy can be partly explained by the additional secretory tissue in dogs (third eyelid gland) and the larger corneal surface to lubricate in dogs (1.2-2.1 cm$^2$ vs. 1.04-1.3 cm$^2$)$^{50,71,77}$ The canine tear film may also be thicker than in humans (15.1 µm vs. 2.3-11.5 µm), although measurements of tear thickness were only obtained in 6 dogs$^{111}$ and the calculation of tear thickness is reportedly highly variable within and between species.$^{119}$
**Spontaneous blink rate:** The blink action distributes fresh tears on the ocular surface in a uniform layer, promotes secretion of tears from the accessory tear glands, and pumps excess tears (or instilled drop) into the nasolacrimal drainage system. Spontaneous blinking is triggered by higher centers in response to corneo-conjunctival nerve stimulation, presumably due to changes in ocular surface temperature that result from thinning and evaporation of unstable tear film. The spontaneous blink rate is very similar between dogs (14.2 blinks/min) and humans (8.5-17.6 blinks/min), although it is lower in rodents (< 5.3 blinks/min) and much lower in rabbits (0.05-0.19 blinks/min). In other words, *humans and dogs blink approximately every 4-7 seconds*, while *mice/rats blink every 11 seconds* (or more) and *rabbits only blink every 313-1200 seconds*. This large disparity in mammals’ blink rate can be explained by species differences in (i) ocular surface sensitivity, (ii) tear composition, and (iii) the inherent stability of the animal’s tear fluid. In fact, (i) the corneo-conjunctival sensitivity is higher in humans > rodents > dogs >> rabbits, a key parameter that is linked to spontaneous blinking as well as reflex secretion of tear components from the lacrimal glands, conjunctival goblet cells and meibomian glands; (ii) tear composition is unique to each species (see next section), for instance large discrepancies exist in the tear lipidomic profile of rabbits vs. man; and (iii) tear film stability is strongly associated with the maximum blink interval, as recently shown in humans. Tear stability is often measured with the tear film breakup time (TFBUT), defined as the interval between the last complete blink and the first appearance of a dry spot in the tear film. Results of TFBUT and other tear film diagnostics are summarized in **Table 2**, with care given to discard or highlight values obtained in anesthetized or sedated animals (e.g., TFBUT of 29.8 min in...
sedated rabbits)\textsuperscript{131} as chemical intervention negatively impacts ocular surface homeostasis (\textit{ie.}, abolished blinking, reduced tear secretion).

Importantly, investigators should account for additional parameters (and their species differences) in any study that involves topical drug administration. In fact, an eyedrop can be considered as a transient ocular irritant – especially if the solution’s pH or osmolarity is different than the tear film – thereby stimulating reflex blinking and lacrimation upon contact with the ocular surface.\textsuperscript{132}

- \textbf{Reflex blinking} (or lack thereof): In dogs, a blink occurs immediately after eyedrop administration and is responsible for removal of any excess solution onto the periocular skin and nasolacrimal drainage system.\textsuperscript{40} The same is true in humans, in whom an instilled eyedrop is partially lost (20-30\%) due to reflex blinking and spillage onto the eyelids and eyelashes.\textsuperscript{133} Blinking in response to eyedrop instillation is also reported in mice\textsuperscript{134} and rats.\textsuperscript{135} In contrast, rabbits rarely blink following eyedrop administration, or do so infrequently. In one study, rabbits did not blink for 20-30 minutes after instillation of an eyedrop, and this alone could result in overestimating ocular drug exposure by 3-fold if findings were to be extrapolated to humans.\textsuperscript{2}

- \textbf{Reflex tear turnover rate}: Eyedrop administration abruptly increases the volume of fluid in the conjunctival sac and ocular surface. The sudden disruption in homeostasis promotes a faster nasolacrimal drainage until baseline conditions return. This physiologic response is prominent in dogs (50 \%/min)\textsuperscript{111} and humans,\textsuperscript{109,115} but is minimal in rabbits (6.1-6.9 \%/min).\textsuperscript{112} In fact, the tear turnover rate in rabbits is mostly unchanged whether a small (1-5 µL) or large volume (25-50 µL) of eyedrop is instilled on the ocular surface,\textsuperscript{112} a finding
likely related to the poor corneal sensitivity and inexistent/minimal reflex blinking in this species. No available report in mice or rats can be found in the literature.

- **Volumetric capacity of the palpebral fissure**: The surface of the canine eye can ‘hold’ on average 31.3 µL of fluid, nearly identical to the volumetric capacity of the human eye (25-30 µL) and the volume of a single ophthalmic drop (35 µL). Of note, the volumetric capacity of the canine eye is positively correlated with the length of the palpebral fissure, and may be larger in breeds larger than Beagles (e.g., German Shepherd dogs). The exact volumetric capacity of the eye is not reported in laboratory species, but is presumably around 10-25 µL in rabbits (based on drug quantification in tears at various instilled volumes), ≤ 5 µL in mice and ≤ 20 µL in rats.

**Tear film composition**

The tear film is a complex biological fluid containing thousands of compounds of diverse structures and functions, including proteins, lipids and mucins, as well as minor constituents such as electrolytes, vitamins, and growth factors. The integrated interactions of these constituents are responsible for the promotion of a stable tear film and, ultimately, the homeostasis of the ocular surface. Species differences in tear film components are summarized in Table 3.

*Proteins* – The total protein content is generally similar in dogs (5.2-14.6 mg/mL) and humans (6.0-11.0 mg/mL), although qualitative and quantitative differences exist. Specifically, the three major constituents of the human tear proteome (lactoferrin, lysozyme, lipocalin) are only detected at low levels in dogs, although the relative abundance of other common proteins (e.g., lacritin, secretory IgA, serum albumin) is generally similar between
the two species. Importantly, homologous proteins have been described in canine tears and may play similar functions to their human counterparts – for instance, *transferrin* is an iron-binding protein with similarities to lactoferrin, while *major canine allergen* is an abundant protein in canine tears with similarities to lipocalin.\textsuperscript{148-150} From a qualitative aspect, a recent in-depth proteomic study showed that 25 out of 125 proteins detected in canine tears were common to humans.\textsuperscript{149} In rabbits, Wei et al. found that the total protein content was two-fold higher in rabbits compared with humans (20.6 mg/mL vs. 9.4 mg/mL), although the number of different proteins detected in tear samples was lower in rabbits.\textsuperscript{151} Other differences in tear proteins among species are summarized in Table 3.

*Mucins* – Ocular mucins are large glycoproteins expressed by conjunctival goblet cells, the corneal epithelium and the lacrimal gland(s), playing important roles on the ocular surface in lubrication, wettability and barrier function.\textsuperscript{152} The main secretory mucin, MUC5AC, is described at large levels on the ocular surface of humans and animals.\textsuperscript{11,55,152} The expression of membrane-associated mucins, however, differs among species. In a recent study by Leonard et al., dogs were found to have a very similar pattern of mucin expression to that of humans and rhesus macaques, with MUC16 being the most abundant mucin transcript.\textsuperscript{153} In contrast, the rabbit had a unique mucin expression pattern with all mucin transcripts expressed at relatively similar levels; as such, the authors concluded that the predictive value of the rabbit as a model in ocular surface studies should be called into question.\textsuperscript{153} In another study, the majority of ocular mucins detected in dogs and rabbits were neutral fucosylated glycans, while the ones in humans were mainly negatively charged sialylated glycans;\textsuperscript{154} however, the experiment lysed the ocular surface epithelium and could not discriminate between mucins of differing origin.
**Lipids** – In a comprehensive lipidomic study comparing the meibum collected in several species, Butovich et al. found that the highest degree of biochemical similarity with humans was observed in mice, closely followed by the dog. An earlier study by Butovich et al. also reported the close resemblance of the tear lipid composition between dogs and humans. In these 3 species (humans, dogs, mice), the major lipid classes included wax esters, cholesterol esters, and ω-acyl-ω-hydroxy fatty acids (OAHFA). In contrast, the major lipid classes in rabbit tears were DiHL esters (24,25-dihydro--lanosterol esters), diacylated diols, and OAHFA, with low to trace amounts of wax and cholesterol esters. Such discrepancy between rabbits and humans was confirmed in a separate study by Wei et al, who noted significant differences in the tear film concentrations of triglycerides (higher in rabbits), free cholesterol (lower in rabbits), phosphatidylcholine (higher in rabbits) and phosphatidylethanolamine (higher in rabbits). Taken together, the authors of these two studies argued that the rabbit is too different to serve as a valid animal model for humans, at least from a biochemical standpoint.

**Tear collection for bioanalytical purposes**

The tear film, a complex body fluid uniquely exposed to both internal and external environments, contains numerous endogenous and exogenous molecules (e.g., proteins, lipids, mucins, xenobiotic) that can be assayed for clinical or research purposes. Topically and systemically administered drugs can be quantified in tear fluid to determine the clinical efficacy and dosing frequency from fitting of kinetic data. Multiple ‘omics’ approaches can also be utilized for analysis of the tear fluid including proteomics, lipidomics and metabolomics, providing valuable information for the development of novel diagnostics and therapeutics in ophthalmology, as well as biomarkers identification for various ocular and
systemic diseases. However, collecting tears and obtaining reproducible analytical results in ophthalmology is challenging; in particular, the volume of tear fluid is limited (unlike other biological fluids, such as blood or urine), and the biochemical profile of a tear sample is intimately affected by the collection, storage, extraction, handling, and analytical methods used by the investigator.

In this section, we review the main sampling methods reported in the scientific literature and discuss their respective advantages and limitations. Further, based on the authors’ experience with dogs in clinical and research settings (board-certified veterinary ophthalmologist [LS] and pharmacologist [JPM]), the section provides recommendations specific to canine subjects and their use in translational research (Figure 3).

**Direct tear sampling**

A microcapillary glass tube (1-10 µL) placed in contact with the inferior lacrimal lake is the most commonly reported technique to collect tear fluid. This method directly samples tear fluid by capillary action and is extensively described in humans, dogs, rabbits, mice, and rats. Other direct techniques (seldom reported) involve micropipettes, polypropylene tubing or polytetrafluoroethylene tubing. With capillary glass tubes, it is possible to obtain unaltered tear samples by avoiding reflex tearing from ocular irritation, especially if the collection is performed by an experienced operator on a cooperative patient. The minimal binding of tear compounds to glass is another reported advantage of capillary tubes. However, the main limitation of microcapillary collection is the long collection time, generally ≥ 5 min – this is particularly true in small laboratory animals, with up to 15-30 minutes and 15-60 min required to collect sufficient tear
fluid in rabbits and rodents (mice and rats), respectively. Another critical limitation of direct sampling is the low volume of tear fluid retrieved, generally ≤ 5 µL – as such, the small sample collected may be grossly insufficient in some individuals, may require excessive dilution that renders the target analyte undetectable, and does not take into account possible losses (e.g., transfer, storage) or the need to repeat certain assays in duplicates.

Several strategies can be used to overcome current obstacles with the volume of the tear volume; however, each come with its own set of drawbacks (listed in parentheses): (i) Sedate or anesthetize the animal to extend collection duration and obtain a larger volume (altered lacrimal functional unit and ocular surface homeostasis); (ii) Pool tear samples from several subjects (reduced statistical power and loss of information regarding inter-individual variability); (iii) Induce reflex tearing with a stimulant – either physical (e.g., irritation to nasal mucosa or cornea), chemical (e.g., parenteral pilocarpine or ammonium fumes) or physiological (e.g., yawn or sneeze reflex) – thereby accelerating tear flow and shortening collection time (diluted tear sample, unable to control flow rates); (iv) Instill fluid (e.g., saline) on the ocular surface immediately prior to tear collection, a process called ‘flush’ or ‘washout’ that yields a larger tear sample in a shorter amount of time (diluted tear sample, non-standardized instilled volume, non-homogenous mixing of fluid with tears).

In particular, the diluting effect of reflex tearing or flush methods may drop the concentration of low-abundant compounds below the analytical limit of quantification, and potentially mask differences between groups due to reduced variance in tear composition. A third limitation of microcapillary tubes is the technical difficulty associated with the collection method. In fact, it is nearly impossible (or very challenging) to avoid reflex lacrimation in a consistent manner,
even with cooperative patients and experienced personnel,\textsuperscript{175,176,180,197,200} for instance, capillary tear collection by Markoulli et al. resulted in tear secretion that was approximately 4-fold faster than basal tear flow in humans (4.6 vs. 1.2 µL/min, respectively).\textsuperscript{109,180} Of note, sampling itself may act as a stimulant due to environmental factors (air movement, light)\textsuperscript{200} and the stress/anxiety experienced by patients when capillary tubes are used.\textsuperscript{173,176,179,197} Importantly, the technical challenge of capillary tubes is amplified in animals given their uncooperative nature, and in any patient with aqueous tear deficiency given the low tear volume; tear sampling can be extra slow in these cases, possibly impeded/interrupted if an air bubble or mucinous material enters the capillary lumen.\textsuperscript{185}

Taken together, although direct tear collection remains the preferred method of some investigators given the ‘undisturbed’ tear sample retrieved,\textsuperscript{199} the serious drawbacks listed above have prompted a growing number of clinicians and researchers to consider indirect tear sampling in humans as suitable alternatives.\textsuperscript{149,176} It is the authors’ opinion that indirect tear sampling is also preferred in dogs, cats and laboratory animals. Ultimately, the patient’s safety and comfort during tear collection is paramount and, as suggested by Berta, ‘it is better to use well-controlled methods than to try to cause as little irritation as possible’.\textsuperscript{200}

**Indirect tear sampling**

Indirect techniques involve tear fluid absorption with either Schirmer tear strips or absorbent sponges, followed by extraction of tear compounds by centrifugation and/or solvent elution.

**Schirmer tear strips** are routinely used to measure tear volume for clinical assessment of dry eye disease in humans and veterinary species.\textsuperscript{165,201-206} The strips are made of Whatman no.
cellulose filter paper and possess specific characteristics to promote tolerance (5mm width x 35mm length, 0.22 mm thick, 20-25 µm porosity, foldable extremity for ease of insertion). In addition to their conventional use for aqueous tear assessment, Schirmer strips can retrieve tear fluid for bioanalytical purposes in humans, dogs, and small laboratory species. For instance, Schirmer strips were used for in-depth characterization of proteomics in human and canine tears, and can also successfully recover specific analytes such as cytokines, clusterin, and xenobiotics.

**Absorbent sponges** exist in different material types such as cellulose, polyvinyl acetal, polyester, and polyurethane. A material with hydrophilic and hydrophobic properties (e.g., polyvinyl acetal, polyurethane) is generally preferred in order to optimize the amount of fluid absorbed and the amount of fluid retrieved from the sponge. For tear fluid collection, the sponge is held against the lacrimal lake by the operator (to minimize reflex tearing), or placed beneath the lower eyelid for a given period of time. Tear fluid recovered from absorbent sponges can be assayed for selected tear compounds, similar to Schirmer strips.

Indirect tear collection is superior to direct capillary sampling in many aspects, namely: (i) Improved tolerance and acceptability by patients; (ii) Ease of use and operator safety, especially for Schirmer strips, allowing non-specialists to perform the procedure with minimal training; and (iii) Larger volume of tears collected in a shorter duration.

Absorbent materials collect tears but can also pick up cellular and extracellular ‘debris’, an attribute considered beneficial by some as the sample obtained is more representative of the dynamic microenvironment at the ocular surface, but also perceived as a limitation by others as the fluid retrieved is not ‘pure’ tears. On this note, the main limitation
of indirect sampling is the ‘invasiveness’ of the technique, at risk of promoting reflex tearing and altering the composition of the tear fluid; indeed, several studies showed variable tear composition between directly- and indirectly-collected samples, with notable differences in the qualitative and quantitative profiles obtained for tear lipids\textsuperscript{167} and tear proteins.\textsuperscript{161,166,182,220} Another important drawback is related to the adsorptive properties of Schirmer strips or absorbent sponges, \textit{i.e.} incomplete release of tear compounds following extraction;\textsuperscript{143,159,207,208,216} however, the authors believe this limitation can be minimized/controlled with adequate precautions (see section 3.3).

\textbf{Proposed strategy for lachrymal determinations in dogs}

\textit{Schirmer strips vs. absorbent sponges} – Sponges can rapidly absorb up to 106 µL of tear fluid in dogs,\textsuperscript{214} while the maximum absorptive capacity of Schirmer strips is \sim 31 µL (\textit{i.e.}, 35 mm wetness).\textsuperscript{221} With sponges, however, the operator can only control the duration of tear collection and not the volume of tears soaked up in each individual. The resulting variability in tear volume absorbed often translates into large intra- and inter-subject variability in the concentration of the compound(s) of interest, as shown for protein content\textsuperscript{143} and various drugs such as doxycycline,\textsuperscript{159} minocycline,\textsuperscript{222} voriconazole,\textsuperscript{223} and ofloxacin.\textsuperscript{191} On the other hand, the ability to control the volume of tears absorbed with Schirmer strips (\textit{i.e.}, same mm mark) generally improves the reproducibility of the results.\textsuperscript{143,159,160,224}

As such, the authors prefer (\textit{i}) absorbent sponges for collecting large volumes of tears in canine subjects – \textit{i.e.}, for further use as blank tears in bioanalytical assays for example – and (\textit{ii}) Schirmer strips for collecting known amounts of tears in any scenario where reproducibility of the data is important (\textit{i.e.}, for group comparisons, or follow-up of the same individual over time).
Schirmer strips for protein quantification – For consistency purposes, the authors recommend the use of dye-free Schirmer tear strips, being consistent with the manufacturer and lot number (given the reported variability in absorptive and adsorptive properties among Schirmer strips), as well as the time of collection (e.g., morning), because of known diurnal variability in lacrimal protein composition in humans and dogs. The distal end of the Schirmer strips should remain in position (ventrolateral conjunctival fornix) until 20 mm, 25 mm or 30 mm wetness is reached. Strip wetness < 20 mm is discouraged given the potential 'concentrating effect' of the absorbent filter with low tear volumes, while complete wetness of the strip (35 mm) should be avoided as the total protein content is significantly greater with 35 mm compared to 20-30 mm mark, likely due to vascular fragility and excessive irritation ensued by the prolonged test duration. Importantly, investigators should be consistent with the selected mm-mark (strip wetness) within and between patients in order to standardize the volume of tears collected among subjects. This strategy provides a lower coefficient of variability in tear protein content compared to ophthalmic sponges and capillary glass tubes in dogs, thereby improving the reliability and reproducibility of the data. Tear extraction and protein analysis can be done directly after tear collection, or can be postponed to a future date as long as Schirmer strips are stored immediately at -80°C and the stability of the compound(s) of interest is verified. Following tear extraction with centrifugation, elution in solvent, or a combination of both, total protein content (TPC) should be quantified in order to standardize the amount of sample used for subsequent analyses. The authors’ preferred method is infrared spectroscopy with Direct Detect™ (EMD Millipore, Danvers, MA) as the technique utilizes merely 2 µL of tear sample, without
any of the drawbacks of colorimetric protein assays (eg., Bradford, Lowry), including variability with specific protein composition and potential contamination from the absorbent material.179,228

*Schirmer strips for drug quantification* – The following steps should be considered to optimize drug quantification in pharmacological studies:

- **Study design:** In studies that assess tear film pharmacokinetics following topical drug administration, one must consider a potential limitation associated with Schirmer strips which remove most of the tear fluid in early collection times, thereby negatively impacting the ‘true’ tear concentration at later time points.197 For this reason, the authors recommend to conduct pharmacokinetic studies in tears over several days (eg., 10 days for 10 collection time points), repeating topical administration each day with a standardized volume and limiting the collection to a single time point per day. An alternative is to use a larger sample size and randomly allocate each time point to a subset of individuals or eyes (eg., 40 eyes with n = 5 eyes for 8 separate time points),156 although this method should account for differences between subjects such as greater tear volumes in dogs of larger body weight.111 Another aspect to consider in the study design is the assessment of drug kinetics in diseased eyes, rendering the study results more clinically applicable (see section 4.2); in fact, tear film concentrations and ocular bioavailability are likely to differ in healthy vs. diseased eyes (eg., excessive lacrimation, increased absorption into congested conjunctival vessels, albumin binding),160,229 yet the majority of ocular studies to date are conducted in healthy individuals which is a clear limitation for translation of research findings from bench to bedside.

- **Tear collection with Schirmer strips:** It is important to homogenize the volume collected within and between subjects by standardizing the extent of strip wetness (≥ 20 mm mark) – this approach limits the variability in tear concentrations related to the collection method
The amount of wetness is then converted to a volume (µL) in order to calculate actual tear film concentrations; data reporting is otherwise limited to µg/g of strip. In dogs, the median volume absorbed by Schirmer strips is 18 µL (20 mm), 22 µL (25 mm), 26 µL (30 mm) and 31 µL (35 mm), information obtained from hundreds of in vivo collections with pre- and post- weighing of Schirmer strips. This method is preferred over in vitro use of phosphate buffered saline given differences in fluid viscosities and the inability of an in vitro experiment to mimic the complex dynamics of tear absorption noted in vivo (eg., rapid initial uptake, tear evaporation). In parallel, investigators should record the duration of tear collection (eg., 50 seconds to reach 20 mm) in order to calculate a flow rate (µL/min) for each sample obtained. In one of our experiments with doxycycline in dogs, flow rate did not influence tear concentrations, but this finding might not be generalizable to other drugs and/or other species of interest.

**Extraction protocol optimization:** A drug can be extracted from Schirmer strips via centrifugation, solvent elution, or a combination of both methods. However, a single extraction protocol cannot be generalized to all pharmacological studies as the specific physicochemical properties of each drug (eg., molecular weight, lipophilicity) can affect the extraction efficiency from the filter papers. As such, investigators should consider conducting a preliminary experiment to determine the optimal extraction protocol for the drug studied, and report specific recovery rates (mean ± standard, range). For instance, the recovery of prednisone and prednisolone was maximized with a combination of centrifugation and elution in methyl tert-butyl ether, a solvent chosen over methanol and acetonitrile based on superior drug extraction from Schirmer strips (Figure 4). Of note, a comprehensive review of all reported protocols is beyond the scope of the present work, and
Further research is warranted to assess the potential benefits (or lack thereof) of extraction steps reported in the literature, such as cutting Schirmer strips into small pieces\textsuperscript{162,165,213} or using ultrasonic agitation.\textsuperscript{160,165,197} Ultimately, an optimized extraction protocol is important as it enhances the reliability of the data at hand, improving the sensitivity of the bioassay, and providing drug concentrations closer to ‘true’ biological levels in the tear film.

- **Bioanalytical method optimization:** First, internal standard should be applied directly onto the dry portion of the Schirmer strip (Figure 4),\textsuperscript{160} i.e. before tear extraction instead of post-elution with solvent as routinely described;\textsuperscript{156,213} this step allows for drug quantification to account for potential variability in extraction efficiency between samples. Second, the standard calibration curve solutions should be constructed by spiking known drug concentrations and internal standard onto Schirmer strips, followed by the same extraction protocol as for biological samples; this step is equivalent to ‘spike and recover’ experiments recommended for other analytes such as cytokines\textsuperscript{216} and proteins.\textsuperscript{207} Third, actual tear fluid should be used whenever possible as the selected matrix for standard calibration curve and quality control solutions,\textsuperscript{159,160,223} as the reported surrogates (e.g., artificial tear solution)\textsuperscript{230} do not account for chemical interferences and matrix effects that typically occur with a complex biological fluid (e.g., ionization suppression).\textsuperscript{223} Blank tears can be collected with absorbent materials prior to study initiation, retrieving up to 84 µL in 1 min with ophthalmic sponges in dogs\textsuperscript{214} and 132 µL in 12 min with successive substitutions of polyurethane mini-sponges in humans.\textsuperscript{181}

**Spontaneous and experimental models of ocular surface disorders in dogs**
Spontaneous ocular surface diseases in dogs with translational applications to humans

Spontaneous ocular surface disorders are common in dogs and represent one of the major causes for referral visits to veterinary practitioners.\textsuperscript{231} In contrast, naturally-acquired ocular surface pathology is much less common in rabbits\textsuperscript{232,233} and is rare in mice and rats.\textsuperscript{234-23}

Keratoconjunctivitis sicca

Keratoconjunctivitis sicca (KCS), or ‘dry eye’, represents one of the most common ocular diseases in humans with an estimated prevalence ranging from 5 to 50\% in different regions worldwide.\textsuperscript{237} The disease is also very common in dogs (prevalence 1.5 to 35\%),\textsuperscript{231} although not a single report of spontaneous KCS case exists in laboratory animals such as rabbits, mice and rats.

The pathogenesis of KCS is very complex, involving diverse physio-anatomical factors such as lacrimal gland integrity, meibomian glands function, hormonal balance and neuronal input.\textsuperscript{237} Numerous models of dry eye have been established in animals over the years,\textsuperscript{238-240} helping to elucidate complex pathological mechanisms involved in KCS and develop novel therapeutics for humans. However, the major drawbacks of most animal models are the acute nature of the induced pathology (vs. chronic disease in humans) and the focus on a single component of the lacrimal functional unit, such as surgically removing the lacrimal gland in mice to reduce tear secretion,\textsuperscript{32} cauterizing the lid margin in rats to induce meibomian gland dysfunction,\textsuperscript{241} or instilling topical 1\% atropine in rabbits to disrupt the efferent neural input.\textsuperscript{242} These experimental models can be generally improved by increasing the number of interventions in the study animals, for instance combining lacrimal glands removal with chemical destruction of the conjunctiva in rabbits,\textsuperscript{243} or combining scopolamine administration with desiccating
environmental stress in mice; yet, these complex models remain suboptimal at best given the acute nature and the inability to fully encompass the complexity of KCS pathophysiology.\textsuperscript{244} Dogs, on the other hand, develop KCS in a spontaneous manner and do not require invasive procedures to disrupt the lacrimal functional unit.\textsuperscript{201,205} Most importantly, the disease is clinically and immunopathologically similar to dry eye in humans, and possesses several attributes that are beneficial for translational research:

- Canine KCS is typically bilateral, develops in middle-aged animals, is more common in female dogs and in certain breeds (\textit{eg.}, American Cocker spaniel, English Bulldog), mimicking the diversity of dry eye in humans related to sex and race.\textsuperscript{201,237}
- Immune-mediated dacryoadenitis is the most common etiology of KCS in dogs – similar to human patients with Sjögren’s syndrome – in which progressive lymphocytic infiltration of the lacrimal gland(s) damages the secretory tissues and reduces aqueous tear production.\textsuperscript{201}
- Meibomian gland dysfunction is recognized in many canine patients with ocular surface disorders, affecting tear film stability in a similar manner than evaporative dry eye in human patients.\textsuperscript{245}
- Spontaneous symptoms of ocular irritation, conjunctival hyperemia and corneal scarring correlate directly with aqueous tear production, a parameter that is easily measured/quantified using a standard Schirmer tear test strip.
- Multiple diagnostic tools used in humans can easily be applied in dogs (but not rodents) given the large size of the canine globe,\textsuperscript{205,239} including tear osmometry, vital staining, strip meniscometry test, infrared meibography and comeo-conjunctival impression cytology.
- Dogs and humans display a similar responses to common therapeutics for dry eye disease; in fact, the two FDA approved anti-inflammatory drugs for dry eye disease in humans
(cyclosporine, lifitegrast) were first developed in canine patients with spontaneous KCS.\textsuperscript{201,246}

The main limitation to consider in dogs is the tendency for clinical signs to be more pronounced in that species vs. humans (\textit{e.g.}, tenacious mucoid discharge, corneal melanosis, neovascularization), in part because canine KCS is often diagnosed at a later stage when owners fail to recognize more subtle clinical signs early on.

\textbf{Allergic conjunctivitis}

Allergic conjunctivitis is a common disorder in humans with an approximate prevalence of 40\% in the North American population.\textsuperscript{247} The disease is characterized by an immunopathological reaction of the ocular surface to the external environment, resulting in clinical symptoms that range from mild conjunctivitis (seasonal or perennial) to the more severe, vision-threatenng vernal keratoconjunctivitis and atopic keratoconjunctivitis.\textsuperscript{247} Over the past few decades, extensive research on small laboratory species (mice, rats, guinea pigs) has helped elucidate some of the complex molecular and cellular processes involved in the pathogenesis of ocular allergies.\textsuperscript{248,249} However, these experiments primarily relied on a relatively small selection of allergens (\textit{e.g.}, ovalbumin, compound 48/80, ragweed pollen), using an experimental design that merely mimics acute forms of the disease – not chronic allergen exposure over months to years – therefore limiting the long-term clinical significance of these findings. On the other hand, dogs possess notable benefits for the comparative study of allergic conjunctivitis, especially when considering companion animals rather than laboratory Beagles: \textit{(i)} these animals share the same environment (and related allergens) as their human owners, unlike commonly used species who are housed in a laboratory setting; \textit{(ii)} companion dogs are outbred, providing a genetic
diversity background that better reflects the human population than inbred laboratory species; and (iii) dogs develop a spontaneous form of allergic conjunctivitis. Spontaneous allergic conjunctivitis is relatively common in dogs, often associated with other allergic disorders such as canine atopic dermatitis. Similar to humans, the clinical signs of allergic conjunctivitis involve conjunctival hyperemia, chemosis, pruritus and ocular discharge, the disease can be diagnosed with high sensitivity and specificity using the conjunctival provocation test, and similar therapeutics are used in both species including topical antihistamines, mast-cell stabilizers, NSAIDs and immunomodulators.

Microbial keratitis

It is well recognized that a natural host is best suited for studying infection, as several species-specific factors (e.g., anatomical, physiological, genetic, immune) closely influence the host-pathogen interactions and subsequent clinical response. These factors likely explain why rabbits and rodents – traditionally used to model ocular surface infections in humans – cannot fully recapitulate the disease presentation and progression that occur in human patients. As such, there is an emerging appreciation for the translational advantage of studying spontaneous (and not experimental) ocular infections in dogs:

- Herpetic keratitis: Recent work has highlighted the robustness and reproducibility of the canine model to study ocular herpesvirus infections and disease, showing striking similarities in the pathogenesis of canine herpesvirus-1 and herpes simplex virus-1, both members of the alphaherpesvirinae subfamily with a seroprevalence of 21-98% in dogs (CHV-1) and 67-90% in humans (HSV-1).
• **Bacterial keratitis**: The most common bacterial genera isolated from canine patients overlap with the ones recognized in human patients (Staphylococcus, Streptococcus, Pseudomonas).\(^{255,256}\) In fact, the major culprit in canine bacterial keratitis (Staphylococcus pseudintermedius) is now considered an emerging zoonosis in humans.\(^{257}\)

**Others**

Dogs can serve as models for other ocular surface diseases such as corneal endothelial dystrophy (analogous to Fuch’s dystrophy in humans),\(^{258}\) limbal stem cell deficiency,\(^{259}\) ocular surface squamous neoplasia\(^{260}\) and neurotrophic keratopathy,\(^{261}\) among others.

**Breakdown of the blood-tear barrier in dogs: A model for ocular pharmacology and therapeutics**

**Histamine-induced conjunctivitis**

To date, the vast majority of preclinical ocular studies for evaluation of candidate drug efficacy and safety are conducted in healthy eyes – in part for simplicity, but at a higher risk of treatment failure rate when translating these findings to clinical studies. Indeed, healthy eyes do not account for the disruption of ocular homeostasis that occurs with inflammatory diseases, including (but not limited to) changes in tear film dynamics, tear composition and permeability of ocular tissues. To address this shortcoming, the authors have recently established a robust *in vivo* model of conjunctivitis in dogs, a translational large animal model that provides a unique opportunity for scientists to investigate the ocular surface in health and disease states.\(^{212}\) The model specifically focused on conjunctivitis as this condition is frequently encountered in humans and dogs,\(^{262,263}\) developing either as a primary condition (*eg.*, bacterial, viral), or as a bystander to common ophthalmic diseases such as blepharitis, keratitis, uveitis and glaucoma.
This model is particularly appealing given the low cost, non-invasiveness, self-resolving nature, ability to adjust the duration and severity of the disease, and shared features with naturally occurring diseases in human and veterinary medicine. The main highlights of the translational ‘large animal’ model are as follows:

- The selected compound (histamine) is inexpensive and triggers local inflammation in a non-specific manner.
- Disease severity is dose-dependent, allowing investigators to induce mild (1 mg/ml), moderate (10 mg/ml) or severe (375 mg/ml) conjunctivitis (Figure 5).
- Disease duration is dose-dependent, self-resolving within an average of 115 min (1 mg/ml), 190 min (10 mg/ml) or 390 min (375 mg/ml). The duration of conjunctivitis can be lengthened by repeating topical histamine administration at set intervals.160
- Topical histamine is safe and generally well-tolerated, although selected eyes receiving the highest dose of histamine (375 mg/ml) can develop mild ocular irritation (lasting < 1 min), blepharitis or miosis.
- Tear film composition changes in eyes with experimentally-induced conjunctivitis (eg., higher levels of serum albumin and inflammatory cytokines), mimicking clinical patients with ocular surface inflammation.
- A transient increase in tear quantity and decrease in tear quality occur, although tear film homeostasis is rapidly restored in ≤ 5 min.264

   Levels of serum albumin are increased in tear film of canine eyes with experimentally-induced or naturally-acquired conjunctivitis,212,224 a physiological variation caused by the breakdown of the blood-tear barrier (Figure 6). Disruption of the blood-tear barrier is also described in human patients with spontaneous ocular surface disorders (eg., dry eye, allergic
conjunctivitis)\textsuperscript{182,265-267} and other animal species (rabbits, horses, guinea pigs).\textsuperscript{268-270} Increased vascular permeability and disruption of tight junctions between conjunctival epithelial cells likely play a role (Figure 6),\textsuperscript{271,272} although the exact etiopathogenesis is unknown and require further investigation. A few noteworthy limitations are listed here: \textit{(i)} the model is not adequate to study ocular allergy given the lack of characteristic features noted in canine patients with allergies (eg., follicular conjunctivitis); \textit{(ii)} pro-inflammatory mediators other than histamine are also responsible for triggering conjunctival inflammation in clinical patients (eg., leukotrienes, cytokines); \textit{(iii)} conjunctival inflammation is relatively short-lived (115-390 min) and cannot mimic the physiological changes noted in patients with chronic conjunctivitis (eg., reduced goblet cell density).

\textbf{Clinical relevance of serum albumin leakage in tear film}

Elevated serum albumin levels in the tear film represents a biomarker for ocular insult or inflammation in humans, dogs and other species.\textsuperscript{182,212,265,270} In brief, plasma-derived albumin leaks onto the ocular surface from congested conjunctival vessels and mixes with the tear film; as such, albumin concentration in tears is generally low in healthy state but increases substantially in diseased eyes.\textsuperscript{182} For instance, a recent study showed that canine eyes with diverse ocular diseases (eg., corneal ulcer, uveitis, glaucoma) had lacrimal albumin levels that were up to 14.9-fold greater than contralateral healthy eyes.\textsuperscript{212} Albumin is a relatively large protein that has a remarkable capacity for binding ligands.\textsuperscript{273} At the level of the eye, protein binding represents an important restriction to drug absorption as only the unbound fraction of the drug diffuses across the ocular tissue barriers.\textsuperscript{268} Combined with the rapid drainage of tears following eyedrop administration (in humans/dogs, not true in rabbits), any portion of drug that
binds to albumin in tear film can be considered as ‘lost’ from a pharmacological standpoint. Broader implications of the blood-tear barrier breakdown on ocular drug pharmacokinetics are listed below:

- **Reduced bioavailability for intraocular targets**: The inability of bound therapeutic drugs to penetrate the cornea lowers the amount of drug available inside the eye to exert its pharmacological action. The physiological effects of increased albumin levels in tears was recently demonstrated with tropicamide (and to a lesser extent latanoprost) in dogs, as well as pilocarpine in rabbits. Of note, the impact of lacrimal albumin on the pharmacological activity of a given drug is likely modulated by various factors, the concentration of the formulation, the mechanism of action and the potency of the drug for its biological target.

- **Reduced bioavailability for ocular surface targets**: Drug-albumin interactions in the tear film could also be detrimental for management of ocular surface disorders, for instance reducing the efficacy of therapeutics for bacterial keratitis as only the unbound portion of an antibiotic is microbiologically active. Preliminary experiments conducted by the authors showed that the presence of albumin results in higher minimal inhibitory concentrations (i.e., reduced susceptibility) for various antibiotics against common bacterial isolates in dogs (in-house unpublished data).

- **Tear film concentrations of systemically administered drugs**: Drug in the plasma compartment can access the tear film by active secretion from the lacrimal gland, or passive diffusion through the conjunctival vessels. The latter is theoretically enhanced when the blood-tear barrier is disrupted. In humans, this physiological feature could explain why the concentration-time profiles of cetirizine were similar in serum and tears in patients with allergic conjunctivitis. In dogs, tear film corticosteroid levels were generally higher in
conjunctivitis vs. control eyes following oral prednisone administration (up to +64%), although differences were not statistically significant.\textsuperscript{160} The degree of conjunctival permeation is likely to vary among therapeutic drugs given differences in their physico-chemical properties.\textsuperscript{275,276}

These findings highlight the importance of conducting pharmacological studies in clinically relevant preclinical species that are able to recapitulate leaky conjunctival vessels and elevated albumin levels in the tear film of clinical patients with ocular diseases. For topical drug administration, the authors recommend using an experimental model of blood-tear barrier breakdown (\textit{eg.}, histamine-induced conjunctivitis or alkali burn models)\textsuperscript{212,277} so that albumin and other relevant proteins are already present on the ocular surface at the time the drug mixes with the tear film.\textsuperscript{229} For systemic drug administration, the authors suggest conducting a preliminary experiment to assess whether conjunctival inflammation affects tear film concentrations to a significant extent. If not, pharmacological assessment in healthy eyes should be sufficient. Incidentally, the physico-chemical properties of some drugs (\textit{eg.}, size, lipophilicity, polar surface area)\textsuperscript{275,276} may allow for high conjunctival permeation under normal conditions, thereby rendering differences between healthy vs. diseased eyes insignificant.

**Corneal injury in dogs: \textit{in vivo} and \textit{ex vivo} models**

Corneal injury is common in human and veterinary patients – whether due to trauma, surgery, or other causes – and the resulting corneal scar remains one of the leading causes of blindness in animals and people worldwide.\textsuperscript{278} Although small laboratory animal species are commonly used in corneal scarring research,\textsuperscript{279} results derived from these models have several limitations. The corneal thickness is much smaller in rabbits and rodents compared to humans.
In addition to thin corneas, mice and rats have corneas that are much smaller in diameter compared to people; consequently, it is often difficult to isolate the central cornea when performing the experimental procedure (e.g., chemical burn) and the damage caused to surrounding limbal stem cells negatively impacts the wound healing process. Using the dog as an animal model is therefore more appropriate, not only due to closer resemblances in ocular surface anatomy and physiology with humans, but also the relatively high prevalence of naturally-acquired corneal pathology in the canine species. In that regard, Gronkiewicz et al. recently developed a novel in vivo corneal fibrosis model in canines; the authors induced corneal scarring with an alkali burn and investigated the ability of suberanilohydroxamic acid (SAHA) to inhibit fibrosis using this large animal model. The availability of such a model presents a clear opportunity for translational research (i.e., intact innervation, tear film, blood supply), although experimentally-induced corneal wounding (at risk of secondary infection) and subsequent corneal scar in dogs represent potential ethical challenges. As an alternative, other authors have established ex vivo canine corneal cultures that can be used to model wound healing and assess anti-fibrotic compounds, or better understand the pathophysiology of herpesvirus in a virus-natural-host environment; in that study, the authors established an air-liquid canine corneal organ culture model to study acute herpetic keratitis, showing important similarities in the response to CHV-1 to what has been described for HSV-1.

**Conclusions**

“Considerable reservations may be felt about comparing results from rabbits with those from humans because of the differences between the physiology of tear flow and mixing and general anatomy. Nevertheless, the rabbit is the principal experimental animal in ophthalmology,
so comparisons are needed”.284 Sadly, this quote published over 45 years ago is still representative of today’s state of ophthalmic research. Rabbits and small laboratory rodents continue to be used primarily (if not exclusively) in most areas of ophthalmic research,1 a concerning fact given the vast anatomical and physiological differences that exist with humans. Of note, such differences should not be regarded as merely ‘weaknesses’ for translational research, but rather evolutionary adaptations optimally suited to the environment and behavior of each species; for instance, rabbits likely developed a very stable tear film to limit intermittent blindness that occurs with each blink,285 thereby reducing the risk of predation. Noteworthily, recent innovations have helped mitigate some of the drawbacks of traditional laboratory species – for instance providing manual blinking and supplementary tear flow in anesthetized rabbits,286 or reverse engineering the ocular surface using human cells in vitro287 – however the authors believe the complexity of the ocular surface and integrated lacrimal functional unit cannot be fully recreated without in vivo conditions in awake subjects.

The comparative work presented throughout this review provides evidence that dogs are best suited for translational research in ophthalmology. Unlike small laboratory animals, dogs share similar anatomical and physiological features to humans, similar environmental stressors and genetic variation, and a range of naturally occurring ophthalmic diseases that closely resemble clinical phenotypes in human patients. The resemblance between dogs and humans is particularly relevant in the field of ocular pharmacology, with notable similarities in blink rate, tear turnover rate (basal, reflex), volumetric capacity of the palpebral fissure, and other factors pertinent to drug diffusion (eg., globe volume, corneal thickness); nonetheless, a few differences should be accounted for in comparative studies, such as the presence of a nictitating membrane, greater tear volume, larger corneal size and lower corneal elastic modulus in dogs. Similar to
other fields of medicine, preclinical studies in ophthalmology could involve canine patients with spontaneous ocular diseases – many of which share striking resemblances with their human counterparts – integrating the expertise of veterinarians, physicians and basic science researchers under the umbrella of the One Health Initiative.\textsuperscript{6,288} Alternatively, or complementarily, preclinical animal work could be performed in laboratory dogs in whom ocular disease is experimentally-induced, making sure to account for the blood-tear barrier breakdown (noted in clinical patients with ‘red eyes’). In all cases, tear fluid can be easily collected from canine eyes for various bioanalytical purposes, favoring Schirmer tear strips over other collection methods given the excellent safety profile and enhanced reliability in analyte quantification (eg., proteins, drugs) provided by this method.

References


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**Tables and Figures**

**Table 1.** Comparative anatomy of the ocular surface and globe between humans, dogs and common laboratory species used in preclinical ophthalmic research.

<table>
<thead>
<tr>
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<th>Rabbit</th>
<th>Mouse</th>
<th>Rat</th>
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<td><strong>Lacrimal glands and</strong></td>
<td><strong>Lacrimal glands</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>nasolacrimal apparatus</strong></td>
<td>Lacrimal gland Accessory glands of Wolfring and Krause 20</td>
<td>Lacrimal gland, Third eyelid gland 11,14</td>
<td>Lacrimal gland, Third eyelid gland, Infraorbital (infraorbital), Accessory glands of Wolfring 10,11,22,23</td>
<td>Infraorbital (extraorbital) 10,11,22</td>
<td>Infraorbital (extraorbital) 10,11,22</td>
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<td><strong>Lacrimal glands</strong></td>
<td>0.59-0.61 cm³ † 18</td>
<td>0.14/ 0.1 cm³ 17</td>
<td>– / 1.5 cm³ 289</td>
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<td><strong>Harderian gland</strong></td>
<td>Absent 22</td>
<td>Absent 11,16</td>
<td>Present 11,22</td>
<td>Present 11,22</td>
<td>Present 11,22</td>
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<td><strong>Nasolacrimal drainage apparatus</strong></td>
<td>Two puncta/canalici, no flexure 25,27</td>
<td>Two puncta/canalici, 1 dorsal flexure 16,25,27</td>
<td>Single punctum/canalicus, two pronounced flexures 27,38,41</td>
<td>Two puncta/canalici 25</td>
<td>Two puncta/canalici 25</td>
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<td><strong>Eyelids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Third eyelid</strong></td>
<td>Absent 66</td>
<td>Present 16,66</td>
<td>Present 66</td>
<td>Present 66</td>
<td>Present 66</td>
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<tr>
<td><strong>Palpebral fissure width (mm)</strong></td>
<td>21.3-34.5 290</td>
<td>18.9-34.1 40,291</td>
<td>10-16 38,41,42</td>
<td>3.7-5 † 43</td>
<td>6-9 45</td>
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<tr>
<td><strong>Interpalpebral fissure area (cm²)</strong></td>
<td>1.8 39</td>
<td>2.2 ‡ 290</td>
<td>1.4 39</td>
<td>0.13 ‡ 43</td>
<td>0.5 ‡ 45</td>
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<td><strong>Meibomian glands (eyelid)</strong></td>
<td>20-40 39,292</td>
<td>20-40 16</td>
<td>30-50 38,39,41</td>
<td>20 293</td>
<td>20-30 # 241</td>
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<td><strong>Dog</strong></td>
<td><strong>Rabbit</strong></td>
<td><strong>Mouse</strong></td>
<td><strong>Rat</strong></td>
</tr>
<tr>
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<tr>
<td><strong>Conjunctiva</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Conjunctival fornix depth (mm) ††</td>
<td>15</td>
<td>49</td>
<td>–</td>
<td>20.36</td>
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<tr>
<td>Conjunctival surface (cm²)</td>
<td>17.65</td>
<td>50</td>
<td>13.3-18.48 ††</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Conjunctiva/Cornea surface ratio</td>
<td>17.17</td>
<td>50</td>
<td>8.62-8.94</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Goblet cell spatial configuration</td>
<td>Individual</td>
<td>51</td>
<td>Individual</td>
<td>51</td>
<td>Clusters 51</td>
</tr>
<tr>
<td>Goblet cell distribution</td>
<td>Highest in plica semilunaris and lower nasal fornix Low in bulbar conjunctiva 30,53,54,56</td>
<td>Highest in third eyelid and lower nasal fornix Low in bulbar conjunctiva 52,55</td>
<td>Highest in palpebral conjunctiva Relatively dense in bulbar conjunctiva 57-59</td>
<td>–</td>
<td>Highest in fornix Low in bulbar conjunctiva Kim 2019</td>
</tr>
<tr>
<td>Conjunctiva-associated lymphoid tissue</td>
<td>Present</td>
<td>61</td>
<td>Present</td>
<td>16</td>
<td>61</td>
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<tr>
<td><strong>Cornea and Sclera</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Corneal diameter, horizontal/vertical (mm)</td>
<td>11.8 / 11.2</td>
<td>75,77</td>
<td>13-17 / 12-16</td>
<td>71,77</td>
<td>13.4-15 / 13-14</td>
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<tr>
<td>Corneal surface (cm²)</td>
<td>1.04-1.3</td>
<td>75,77</td>
<td>1.2-2.1</td>
<td>71,77</td>
<td>1.55-2.03</td>
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<tr>
<td>Corneal thickness (µm)</td>
<td>505-563</td>
<td>79</td>
<td>497-594</td>
<td>68,78</td>
<td>354-407</td>
</tr>
<tr>
<td>Corneal epithelial thickness (µm)</td>
<td>44-55</td>
<td>79</td>
<td>52-64</td>
<td>71,78</td>
<td>45-49</td>
</tr>
<tr>
<td>Endothelial cell density (cells/mm²)</td>
<td>2732</td>
<td>90</td>
<td>2818</td>
<td>68</td>
<td>3233</td>
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<td></td>
<td>Human</td>
<td>Dog</td>
<td>Rabbit</td>
<td>Mouse</td>
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<tr>
<td><strong>Subbasal nerve plexus pattern</strong></td>
<td>Whorl-like 67</td>
<td>Whorl-like 67</td>
<td>Horizontal 67</td>
<td>Whorl-like 67</td>
<td>Whorl-like 67</td>
</tr>
<tr>
<td>Corneal sensitivity (g/mm²)</td>
<td>0.2-1.0</td>
<td>2.16-2.9</td>
<td>6.21-10</td>
<td>0.59</td>
<td>0.42-0.47</td>
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<tr>
<td></td>
<td>2</td>
<td>92.95</td>
<td>2.92</td>
<td>93</td>
<td>91.94</td>
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<td>Stiffness/elastic modulus (kPa) ‼</td>
<td>16.2-33.1</td>
<td>1.3</td>
<td>1.1</td>
<td>25-40</td>
<td>6.2</td>
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<tr>
<td></td>
<td>87.294</td>
<td>87</td>
<td>86</td>
<td>89</td>
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<td>Scleral thickness at the limbus (mm)</td>
<td>0.50</td>
<td>0.80</td>
<td>0.29</td>
<td>0.05-0.06</td>
<td>&lt; 0.1</td>
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<td></td>
<td>102</td>
<td>101</td>
<td>103</td>
<td>105</td>
<td>104</td>
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<tr>
<td><strong>Globe</strong></td>
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<td></td>
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<tr>
<td>Globe volume (mL)</td>
<td>5.7-6.0</td>
<td>5.0-5.8</td>
<td>2.3-2.9</td>
<td>0.014 1</td>
<td>0.13 11</td>
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<td></td>
<td>295</td>
<td>296</td>
<td>296</td>
<td>297</td>
<td>72</td>
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<td>Anterior chamber (mL)</td>
<td>0.17-0.31</td>
<td>0.77</td>
<td>0.28-0.30</td>
<td>0.0044-0.007</td>
<td>0.0036-0.015</td>
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<tr>
<td></td>
<td>66.77</td>
<td>101</td>
<td>41.66,77</td>
<td>11.66,77</td>
<td>11.66,77</td>
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<tr>
<td>Vitreous chamber (mL)</td>
<td>3.5-5.4</td>
<td>1.7-3.0</td>
<td>1.1-1.8</td>
<td>0.0053</td>
<td>0.013-0.054</td>
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<tr>
<td></td>
<td>65</td>
<td>101,296</td>
<td>66,296</td>
<td>11.65</td>
<td>11.65</td>
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</tbody>
</table>

- Information not available or not found
- † Lacrimal gland (human), Lacrimal gland / TEL gland (dog), Lacrimal gland / Harderian gland (rabbit)
- ‡ Estimated based on clinical images43
- § Estimated based on clinical images40
- ¶ Calculated based on average palpebral fissure length45
- # Estimated based on clinical images241
- †† Central upper conjunctival sac (from fornix to lid margin)
- ‡‡ Does not account for the nictitating membrane, surgically removed prior to the experiment
- §§ Estimated from corneal radius, assuming a circular shape for the cornea in rodents72
- ¶¶ Whole cornea in rats, epithelium and anterior stroma in other species
- ## Estimated from axial length, assuming a spherical shape for the globe
Table 2. Comparative physiology and characteristics of the ocular surface and tear film between humans, dogs and common laboratory species used in preclinical ophthalmic research.

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
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<th>Rabbit</th>
<th>Mouse</th>
<th>Rat</th>
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<td><strong>Ocular surface physiology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Blink rate (blinks/min)</td>
<td>8.5-17.6</td>
<td>14.2</td>
<td>0.05-0.19</td>
<td>0-4</td>
<td>2-5.3</td>
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<td></td>
<td>122-124</td>
<td>121</td>
<td>2,123,127</td>
<td>125,126</td>
<td>91,124</td>
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<td>Volumetric capacity of the palpebral fissure (µL)</td>
<td>25-30</td>
<td>31.3</td>
<td>10-25</td>
<td>≤ 5</td>
<td>≤ 20</td>
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<tr>
<td></td>
<td>109,136</td>
<td>40</td>
<td>112,138</td>
<td>139,140</td>
<td>141</td>
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<tr>
<td>Ocular surface temperature (°C)</td>
<td>32.8-37.1</td>
<td>35.2</td>
<td>39.1</td>
<td>37.2</td>
<td>36.5</td>
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<td></td>
<td>298,299</td>
<td>300</td>
<td>298</td>
<td>298</td>
<td>298</td>
</tr>
<tr>
<td><strong>Tear film characteristics</strong></td>
<td></td>
<td></td>
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<tr>
<td>Tear film thickness (µm)</td>
<td>2.3-11.5</td>
<td>15.1</td>
<td>6.5-18.4</td>
<td>7.4-21.1</td>
<td>2-12.6</td>
</tr>
<tr>
<td></td>
<td>119</td>
<td>111</td>
<td>119</td>
<td>119,301</td>
<td>119,302</td>
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<tr>
<td>Lipid layer thickness (nm)</td>
<td>62-78</td>
<td>13-581</td>
<td>&gt; 180</td>
<td>–</td>
<td>12</td>
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<td></td>
<td>123,303</td>
<td>121</td>
<td>123</td>
<td>123</td>
<td>301</td>
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<tr>
<td>Tear volume (µL)</td>
<td>7-12.4</td>
<td>65.3</td>
<td>1.9-7.5</td>
<td>0.06-0.2</td>
<td>4.6</td>
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<tr>
<td></td>
<td>109,115</td>
<td>111</td>
<td>112,116</td>
<td>113,118</td>
<td>117</td>
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<tr>
<td>Basal tear turnover rate (%/min)</td>
<td>13.1-17.5</td>
<td>12.1</td>
<td>6.2-7.1</td>
<td>5.2</td>
<td>–</td>
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<tr>
<td></td>
<td>109,110</td>
<td>111</td>
<td>112</td>
<td>113</td>
<td>–</td>
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<tr>
<td>Reflex tear turnover rate (%/min)</td>
<td>31.5-100</td>
<td>50</td>
<td>6.1-6.9</td>
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<td></td>
<td>109,115</td>
<td>111</td>
<td>112</td>
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<td>Evaporative rate (µm/min)</td>
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<td>0.47</td>
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<td></td>
<td>304</td>
<td>–</td>
<td>127</td>
<td>–</td>
<td>312</td>
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<td><strong>Tear film diagnostics ¶</strong></td>
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<td></td>
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<tr>
<td>Schirmer tear test-1 (mm) ‡</td>
<td>10.0-18.6</td>
<td>18.1-24.3</td>
<td>4.6-7.6</td>
<td>–</td>
<td>5.6-9.4</td>
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<tr>
<td></td>
<td>365,366</td>
<td>13,52,95,205,307,308</td>
<td>42,116,309</td>
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<td>91,241</td>
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<td>Phenol red thread test (mm/15s)</td>
<td>9-20</td>
<td>17.5-39.2</td>
<td>20.9-25.0</td>
<td>2.8-11.2</td>
<td>7.6</td>
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<td>95,205,307,308</td>
<td>42,309</td>
<td>125,244,311</td>
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Table 2 Continued

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<tr>
<td>Tear film breakup time</td>
<td>7.4-13.0</td>
<td>13.9-24.0</td>
<td>2-1788 ††</td>
<td>5-25</td>
<td>5.2-6.0</td>
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<tr>
<td>(sec)</td>
<td>305,306</td>
<td>13,52,205,307</td>
<td>313</td>
<td>125,129</td>
<td>241</td>
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<tr>
<td>Tear osmolarity ‡‡</td>
<td>300.8</td>
<td>337.4-339.0</td>
<td>291.3</td>
<td>346.3-366.8</td>
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<td>(mOsm/L)</td>
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<td>205,314</td>
<td>315</td>
<td>310</td>
<td>312</td>
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<td>Tear pH</td>
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<td>215</td>
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</table>

– Information not available or not found
† Based on greater percent of drug lost at 1 min in rabbit eyes receiving 25 or 50 µL eyedrop vs. 5 or 10 µL eyedrop,138 despite no changes in tear turnover rate for instilled volumes up to 50 µL112
‡ Excludes an outlier measurement of 41-46 µm318
§ Estimated from rabbit eyes receiving a large volume instilled eyedrop (25-50 µL)
¶ Reported values prioritized studies that did not use sedation or general anesthesia
# Values reported in mm/5min (humans) or mm/min (all other species)
†† Large variability in studies’ methodology, most using topical and/or general anesthesia prior to testing, resulting in non-physiologic and highly variable measurements for tear film break up time
‡‡ Measurements obtained with the same device (TearLab™, OcuSense Inc., San Diego, CA)
### Table 3. Comparative composition of the major components in tear film between humans, dogs and common laboratory species used in preclinical ophthalmic research.

<table>
<thead>
<tr>
<th>Proteins</th>
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<th>Rabbit</th>
<th>Mouse</th>
<th>Rat</th>
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</thead>
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<tr>
<td>Lactoferrin †</td>
<td>Abundant</td>
<td>Low 144,147</td>
<td>Low 144,147</td>
<td>Low 319,320</td>
<td>Low 147</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Abundant</td>
<td>Low 144,147</td>
<td>Low 145,146</td>
<td>Low 190,319</td>
<td>Low 147</td>
</tr>
<tr>
<td>Lipocalin ‡</td>
<td>Abundant</td>
<td>Low to moderate 144,147,149,150</td>
<td>Low 147,148,319,321</td>
<td>Low 147,198</td>
<td>Absent 147,148</td>
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<tr>
<td>Lacritin</td>
<td>Moderate</td>
<td>Moderate 322</td>
<td>–</td>
<td>Absent 198</td>
<td>Present 323</td>
</tr>
<tr>
<td>Secretory IgA</td>
<td>Moderate</td>
<td>Moderate 144,324,325</td>
<td>Present 22</td>
<td>Low 11</td>
<td>Moderate 326</td>
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<tr>
<td>Serum albumin</td>
<td>Low 182</td>
<td>Low 146,150,212</td>
<td>Low 190,319</td>
<td>Present 198</td>
<td>–</td>
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<tr>
<td>Peroxidase</td>
<td>Low 327</td>
<td>Low 146,325</td>
<td>Absent 190</td>
<td>–</td>
<td>Abundant 194</td>
</tr>
<tr>
<td>Amylase</td>
<td>Low 327</td>
<td>–</td>
<td>Absent 190</td>
<td>–</td>
<td>Low 194</td>
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</table>

<table>
<thead>
<tr>
<th>Mucins</th>
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<th>Rabbit</th>
<th>Mouse</th>
<th>Rat</th>
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<tbody>
<tr>
<td>MUC5AC</td>
<td>Present 11</td>
<td>Present 11,55</td>
<td>Present 11</td>
<td>Present 11</td>
<td>Present 11</td>
</tr>
<tr>
<td>MUC1, MUC4, MUC16</td>
<td>MUC16 &gt;&gt; MUC1 &gt; MUC4 153</td>
<td>MUC16 &gt;&gt; MUC1 &gt; MUC4 153</td>
<td>MUC1 ~ MUC4 ~ MUC16 153</td>
<td>Present 328</td>
<td>Present 329</td>
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<tr>
<td>Major O-glycans</td>
<td>Sialylated glycans 154</td>
<td>Fucosylated glycans 154</td>
<td>Fucosylated glycans 154</td>
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Table 3 Continued

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Human</th>
<th>Dog</th>
<th>Rabbit</th>
<th>Mouse</th>
<th>Rat</th>
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<tbody>
<tr>
<td>Wax esters</td>
<td>Abundant</td>
<td>Abundant</td>
<td>Low</td>
<td>Abundant</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>129,155</td>
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<td>129</td>
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<td>Cholesterol</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Abundant</td>
</tr>
<tr>
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<tr>
<td>Cholesteryl esters</td>
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<td>Low</td>
<td>Abundant</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>129,155</td>
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<tr>
<td>DiHL</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
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<td>–</td>
</tr>
<tr>
<td></td>
<td>129,155</td>
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<td>129</td>
<td>129</td>
<td>–</td>
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<tr>
<td>DiHL esters</td>
<td>Low</td>
<td>Low</td>
<td>Abundant</td>
<td>Low</td>
<td>–</td>
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</tr>
<tr>
<td>DiAD</td>
<td>Low</td>
<td>Low</td>
<td>Abundant</td>
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<td>–</td>
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<tr>
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<td>129,155</td>
<td>129,155</td>
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</tr>
<tr>
<td>OAHFA</td>
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</tr>
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<td>–</td>
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<tr>
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<tr>
<td>Others §</td>
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<td>–</td>
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<td></td>
<td>129,155</td>
<td>129,155</td>
<td>129</td>
<td>129</td>
<td>–</td>
</tr>
</tbody>
</table>

– Information not available or not found
† Homologous iron-binding protein called transferrin is reported in dogs\textsuperscript{146} and rabbits\textsuperscript{319}
‡ Homologous proteins are reported in dogs (major canine allergen),\textsuperscript{148-150} rabbits (lipophilin)\textsuperscript{151} and rats (VEGr1)\textsuperscript{148}
§ Triacylglycerol, squalene, ceramides, phospholipids and sphingomyelins
Figure 1. Graphical representation of the canine ocular surface and lacrimal functional unit.
Figure 2. Diagram depicting the complexity of tear film dynamics and ocular surface physiology. Secretion of tear components, distribution of tears through blinking, and elimination through nasolacrimal drainage and evaporation must be precisely regulated to maintain homeostasis. Drug kinetics following topical eyedrop administration are impacted by key parameters highlighted in yellow, each being unique in different species. Adapted with permission from “Tsubota K, Tseng SCG, Nordlund ML. Anatomy and Physiology of the Ocular Surface In: Holland EJ, Mannis MJ, eds. Ocular Surface Disease Medical and Surgical Management. New York, NY: Springer New York, 2002;3-15”.
Figure 3. Tear collection in dogs using microcapillary glass tubes (a), Schirmer tear strips (b) or absorbent sponges (c).

Figure 4. Step-by-step protocol to extract tear fluid from Schirmer strips for analytical purposes, using a combination of centrifugation and solvent wash. Centrifugation of wetted Schirmer strip (containing tear sample and internal standard) retrieves tear fluid in a large tube, while subsequent solvent elution washes residual content from the absorbent filter paper.
**Figure 5.** Representative clinical pictures of mild conjunctivitis (a), moderate conjunctivitis (b), and severe conjunctivitis (c) in dogs following topical administration of histamine at concentrations of 1 mg/mL, 10 mg/mL and 375 mg/mL, respectively. Reprinted from “Sebbag L, Allbaugh RA, Weaver A, et al. Histamine-Induced Conjunctivitis and Breakdown of Blood-Tear Barrier in Dogs: A Model for Ocular Pharmacology and Therapeutics. Front Pharmacol 2019;10:752”.
Figure 6. Graphical representation of the blood-tear barrier in the canine eye. The barrier is intact in healthy eyes (left) but is disrupted in diseased eyes (right), enhancing the flow of compounds (eg, albumin, xenobiotics) between the tear film and the blood compartments. Breakdown of the blood-tear barrier can have important clinical implications such as enhanced systemic absorption from greater conjunctival vascular permeability, or reduction in ocular drug bioavailability due to drug-albumin interactions in the tear film.
APPENDIX A.  MATHEMATICAL MODELING USING NONLINEAR MIXED-EFFECTS

Parameters estimation was performed using the stochastic approximation expectation maximization (SAEM) algorithm for nonlinear mixed effects-models as implemented in the Monolix Suite. Competing models were evaluated numerically using Bayesian information criteria (BIC) and precision of parameter estimates – defined as relative standard error of the estimate (RSE). Standard goodness-of-fit diagnostics, including observed vs. predicted fluorescein concentrations, individual fits and weighted residuals time-course were used to graphically assist comparison. SAEM convergence and final model parameterization were assessed graphically by inspection of search stability, distribution of the individual parameters, distribution of the random effects, individual prediction vs. observation, individual fits, and distributions of the weighted residuals. The numerical precision of parameter estimates was assessed using RSE. The numerical normality of individual parameters was assessed using a Shapiro-Wilk test for normality. The normality of the distribution of residuals was assessed using a Shapiro-Wilk test and the centering of the distribution of residuals (i.e. 0) was assessed using a Van Der Waerden test. *P* values < 0.05 were considered as statistically significant.

A suitable mathematical model has the following features (see Figures below): (i) the line of identity is aligned with the regression line while (ii) the residues (differences between observations and predictions) are centered on a mean value of 0, with (iii) a homogeneous dispersion around the mean.
Figure A1. Comparison of predicted tear fluorescence over time (purple curve) to observed data (blue points) for a random sample of dogs (A) and cats (B). Censored data are shown as vertical red bars. The ID of each individual is listed above the individual fit, followed by #1 for the right eye, and #2 for the left eye.

Figure A2. Standard Goodness-Of-Fit diagnostics: Individual predictions vs. Observations (log10) for the fluorophotometry data in dogs (A) and cats (B). The solid black line represents the identity line; the regression line is portrayed in light green color; censored data points (<10%) are represented with red dots.
Figure A3. Individual weighted residuals (IWRES) vs. fluorescein concentrations in tears of dogs (A) and cats (B). Censored data points (<10%) are represented with red dots. The turquoise line represents the spline (loess regression).

Figure A4. Simulations of fluorescein vs. time disposition from 500 Monte Carlo simulations using final parameter estimates from the NLME model. Predictions derived from the 5th to the 95th percentile of the model simulations were able to reproduce the variability in the observed data from the original population of dogs/cats.
APPENDIX B. TEAR FILM THICKNESS AND THEORETICAL TEAR VOLUME

Tear thickness was measured with spectral-domain optical coherence tomography (SD-OCT, Optovue iVue, Fremont, CA) in six beagle dogs, selected based on their homogenous subject characteristics (e.g. similar breed, age, skull type, body weight, etc.). Dogs were manually restrained with their eyelids held open by an assistant. Tear thickness was measured with ImageJ software (National Institutes of Health, Bethesda, MD) from images captured with the SD-OCT. The average tear thickness from all eyes ($d = 0.01512$ mm) was used to calculate the theoretical canine tear volume (TV), assuming a radius of the canine globe ($r$) of 10.45 mm.

$$TV \text{ (µL)} = \frac{1}{2} \left\{ \frac{4}{3} \pi (r + d)^3 \right\} - \frac{1}{2} \left\{ \frac{4}{3} \pi r^3 \right\}$$

The theoretical TV value calculated with this method (10.4 µL) was lower than the one calculated with fluorophotometry (59 µL for a median beagle body weight of 9 kg), a finding likely explained by the unevenness of tear film thickness on the ocular surface. The mathematical equation assumes the tear film thickness to be homogenous when in reality tears are pooling in conjunctival fornices and tear film is much thicker at the lower and upper tear menisci. However, the tear menisci could not be readily imaged in our dogs as we did not use sedation or general anesthesia.
APPENDIX C. MATHEMATICAL MODELING USING NONLINEAR MIXED-EFFECTS

Figure C1. Individual predictions versus observations (log10) for the fluorophotometry data in 8 dogs (16 eyes) receiving 10-100 µL of 0.1% fluorescein solution. The solid black line represents the identity line; the regression line is portrayed in light green color.

Figure C2. IWRES plotted against the tear fluorescein concentrations in 8 dogs (16 eyes) receiving 10-100 µL of 0.1% fluorescein solution. The orange line represents the spline (loess regression). IWRES, individual weighted residuals.
**Figure C3.** Simulations of fluorescein vs. time disposition from 500 Monte Carlo simulations using final parameter estimates from the NLME model. Predictions derived from the 5th to the 95th percentile of the model simulations were able to reproduce the variability in the observed data from the original population dogs. NLME, nonlinear mixed effects.
**APPENDIX D. HISTAMINE-INDUCED CONJUNCTIVITIS**

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 1</td>
<td>H₂</td>
<td>H₃</td>
<td>H₄</td>
<td>H₅</td>
<td>H₆</td>
</tr>
<tr>
<td>Dog 2</td>
<td>H₄</td>
<td>H₅</td>
<td>H₁</td>
<td>H₂</td>
<td>H₃</td>
</tr>
<tr>
<td>Dog 3</td>
<td>H₆</td>
<td>H₁</td>
<td>H₂</td>
<td>H₅</td>
<td>H₄</td>
</tr>
<tr>
<td>Dog 4</td>
<td>H₁</td>
<td>H₅</td>
<td>H₆</td>
<td>H₃</td>
<td>H₂</td>
</tr>
<tr>
<td>Dog 5</td>
<td>H₅</td>
<td>H₂</td>
<td>H₄</td>
<td>H₆</td>
<td>H₅</td>
</tr>
<tr>
<td>Dog 6</td>
<td>H₆</td>
<td>H₄</td>
<td>H₃</td>
<td>H₁</td>
<td>H₆</td>
</tr>
</tbody>
</table>

H₁ = 0.005 mg/mL  
H₂ = 0.1 mg/mL  
H₃ = 1.0 mg/mL  
H₄ = 10 mg/mL  
H₅ = 375 mg/mL  
H₆ = 500 mg/mL

**Figure D1:** Diagram of the study design showing the balanced crossover trial (top) and the timing of the procedures prior to and following topical histamine/placebo administration (bottom). BP = Blood pressure; CS = Conjunctivitis scoring; IOP = Intraocular pressure.
Table D1: A semi-quantitative conjunctivitis scoring system.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Palpebral conjunctiva</strong></td>
<td></td>
</tr>
<tr>
<td>0 (none)</td>
<td>No swelling of the palpebral conjunctival tissue.</td>
</tr>
<tr>
<td>1 (mild)</td>
<td>Diffuse thin swelling with no eversion of the eyelid(s) or change in eyelid margin contour.</td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>Diffuse definite swelling with misalignment of the normal approximation of the lower and upper eyelids.</td>
</tr>
<tr>
<td>3 (severe)</td>
<td>Diffuse marked swelling with partial eversion of the eyelid(s). The eyelid margin(s) may have an irregular, ‘undulating’ contour, but can still be closed completely.</td>
</tr>
<tr>
<td>4 (very severe)</td>
<td>Extremely severe swelling with pronounced eversion of both eyelids. Eyelid closure is incomplete with exposed swollen conjunctiva protruding between the eyelid margins and masking the corneal surface.</td>
</tr>
<tr>
<td><strong>Hyperemia</strong></td>
<td></td>
</tr>
<tr>
<td>0 (none)</td>
<td>Small individual vessels are noted, blanched to pale pink in color. It is normal to observe a few prominent vessels on the palpebral surface of the third eyelid.</td>
</tr>
<tr>
<td>1 (mild)</td>
<td>Dilatation of only a few vessels with minimal branching and/or tortuosity. Pink to light red in color.</td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>Dilatation of the majority of vessels with pronounced branching and/or tortuosity. Bright red to crimson red in color. The conjunctiva between large vessels may have a flushed pink-to-red appearance.</td>
</tr>
<tr>
<td>3 (severe)</td>
<td>Diffuse beefy red appearance to the conjunctiva, difficult to distinguish individual blood vessels.</td>
</tr>
<tr>
<td><strong>Follicles</strong></td>
<td></td>
</tr>
<tr>
<td>0 (none)</td>
<td>No manifestations</td>
</tr>
<tr>
<td>1 (mild)</td>
<td>1-9 follicles</td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>10-19 follicles</td>
</tr>
<tr>
<td>3 (severe)</td>
<td>20 or more follicles</td>
</tr>
<tr>
<td><strong>Bulbar conjunctiva</strong></td>
<td></td>
</tr>
<tr>
<td>0 (none)</td>
<td>No swelling of the bulbar conjunctival tissue.</td>
</tr>
<tr>
<td>1 (mild)</td>
<td>Focal perilimbal and/or diffuse thin swelling. Underlying episcleral tissue is easily observed through the conjunctiva.</td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>Diffuse definite swelling. Underlying episcleral tissue is difficult to observe.</td>
</tr>
<tr>
<td>3 (severe)</td>
<td>Diffuse marked swelling, masking the corneoscleral limbal region.</td>
</tr>
<tr>
<td><strong>Hyperemia</strong></td>
<td></td>
</tr>
<tr>
<td>0 (none)</td>
<td>Small individual vessels are noted, blanched to pale pink in color.</td>
</tr>
<tr>
<td>1 (mild)</td>
<td>Dilatation of only a few vessels with minimal branching and/or tortuosity. Pink-to-reddish in color.</td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>Dilatation of the majority of vessels with pronounced branching and/or tortuosity. Bright red to crimson red in color. The conjunctiva between large vessels may have a flushed pink-to-red appearance.</td>
</tr>
<tr>
<td>3 (severe)</td>
<td>Diffuse beefy red appearance to the conjunctiva, difficult to distinguish individual blood vessels.</td>
</tr>
<tr>
<td><strong>Follicles</strong></td>
<td></td>
</tr>
<tr>
<td>0 (none)</td>
<td>No manifestations</td>
</tr>
<tr>
<td>1 (mild)</td>
<td>1-9 follicles</td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>10-19 follicles</td>
</tr>
<tr>
<td>3 (severe)</td>
<td>20 or more follicles</td>
</tr>
<tr>
<td>Score</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>0 (none)</td>
<td>No discharge, or small amount of clear/mucoid material found in the medial canthus</td>
</tr>
<tr>
<td>1 (mild)</td>
<td>Discharge is above normal and present on the surface of the eye or in the medial canthus, but not on the lids or hairs of the eyelids</td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>Discharge is abundant, easily observed, and has collected on the lids and around the hairs of the eyelids.</td>
</tr>
<tr>
<td>3 (severe)</td>
<td>Discharge has been flowing over the eyelids so as to wet the hairs substantially on the skin around the eyes, past the orbital rim</td>
</tr>
</tbody>
</table>

**Ocular pruritus**

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (none)</td>
<td>No ocular itching</td>
</tr>
<tr>
<td>1 (mild)</td>
<td>Subtle, rapidly resolving itch</td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>Mild persistent itch, resolving within 30 seconds</td>
</tr>
<tr>
<td>3 (severe)</td>
<td>Pronounced itch, not resolving within 30 seconds</td>
</tr>
<tr>
<td>4 (extremely severe)</td>
<td>Incapacitating itch</td>
</tr>
</tbody>
</table>
Table D2: Summary table detailing each subsection of the conjunctivitis score for each histamine dose. The score selected in each individual was the maximal score documented between 0 and 420 min following histamine administration. $T_{\text{max}}$ describes the time (in min) to reach this maximal score in each dog. Results are described as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Palpebral chemosis</th>
<th>Palpebral hyperemia</th>
<th>Palpebral follicles</th>
<th>Bulbar chemosis</th>
<th>Bulbar hyperemia</th>
<th>Bulbar follicles</th>
<th>Conjunctival discharge</th>
<th>Ocular pruritus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H1</strong> (0.005 mg/mL)</td>
<td>Dogs affected</td>
<td>0/6</td>
<td>6/6</td>
<td>0/6</td>
<td>1/6</td>
<td>3/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Score</td>
<td>0</td>
<td>1.2 ± 0.4</td>
<td>0</td>
<td>1</td>
<td>1.0 ± 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$T_{\text{max}}$ (min)</td>
<td>0</td>
<td>4.7 ± 1.5</td>
<td>0</td>
<td>7</td>
<td>15 ± 10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>H2</strong> (0.1 mg/mL)</td>
<td>Dogs affected</td>
<td>0/6</td>
<td>6/6</td>
<td>0/6</td>
<td>2/6</td>
<td>6/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Score</td>
<td>0</td>
<td>1.8 ± 0.4</td>
<td>0</td>
<td>1 ± 0</td>
<td>1.5 ± 0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$T_{\text{max}}$ (min)</td>
<td>0</td>
<td>4.5 ± 3.1</td>
<td>0</td>
<td>6.0 ± 1.4</td>
<td>4.3 ± 2.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>H3</strong> (1.0 mg/mL)</td>
<td>Dogs affected</td>
<td>2/6</td>
<td>6/6</td>
<td>0/6</td>
<td>1/6</td>
<td>6/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Score</td>
<td>1.0 ± 0</td>
<td>2.0 ± 0</td>
<td>0</td>
<td>1</td>
<td>2.0 ± 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$T_{\text{max}}$ (min)</td>
<td>5.0 ± 0</td>
<td>4.0 ± 2.1</td>
<td>0</td>
<td>25</td>
<td>6.8 ± 3.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>H4</strong> (10 mg/mL)</td>
<td>Dogs affected</td>
<td>6/6</td>
<td>6/6</td>
<td>0/6</td>
<td>6/6</td>
<td>5/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Score</td>
<td>1.0 ± 0</td>
<td>2.0 ± 0</td>
<td>0</td>
<td>1.5 ± 0.5</td>
<td>2.0 ± 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$T_{\text{max}}$ (min)</td>
<td>5.2 ± 2.6</td>
<td>2.0 ± 1.1</td>
<td>0</td>
<td>6.5 ± 2.0</td>
<td>3.2 ± 3.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>H5</strong> (375 mg/mL)</td>
<td>Dogs affected</td>
<td>6/6</td>
<td>6/6</td>
<td>0/6</td>
<td>6/6</td>
<td>6/6</td>
<td>0/6</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>Score</td>
<td>2.2 ± 0.8</td>
<td>2.2 ± 0.4</td>
<td>0</td>
<td>2.5 ± 0.5</td>
<td>2.0 ± 0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$T_{\text{max}}$ (min)</td>
<td>6.3 ± 4.5</td>
<td>2.0 ± 2.4</td>
<td>0</td>
<td>10.7 ± 5.8</td>
<td>1.0 ± 0</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>H₆ (500 mg/mL)</td>
<td>Dogs affected</td>
<td>Palpebral chemosis</td>
<td>Palpebral hyperemia</td>
<td>Palpebral follicles</td>
<td>Bulbar chemosis</td>
<td>Bulbar hyperemia</td>
<td>Bulbar follicles</td>
<td>Conjunctival discharge</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
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<td>-------------------</td>
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<tr>
<td>Score</td>
<td>2.7 ± 1.0</td>
<td>2.0 ± 0</td>
<td>0</td>
<td>2.3 ± 0.8</td>
<td>2.0 ± 0</td>
<td>0</td>
<td>0</td>
<td>2.8 ± 0.4</td>
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<td>Tₘₐₓ (min)</td>
<td>9.2 ± 5.4</td>
<td>1.0 ± 0</td>
<td>0</td>
<td>11.2 ± 15.0</td>
<td>1.0 ± 0</td>
<td>0</td>
<td>0</td>
<td>1.0 ± 0</td>
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APPENDIX E. PREPARATION OF SCHIRMER STRIPS FOR LC-MS ANALYSIS

1) Transfer the Schirmer strip (wetted until 20-mm mark) to a 2-mL tube using single-use tweezers.
2) Spike 5 µL of 10 ng/µL prednisone internal standard (10 ng/µL prepared in 1:1 acetonitrile:water) on the dry portion of the strip (i.e. 25 to 35 mm strip end).
3) Place the Schirmer strip into a 0.2-mL tube that was pre-punctured at its bottom with a 18-gauge needle.
4) Secure the 0.2-mL tube into a 2-mL tube using adhesive tape.
5) Centrifuge the combination for 2 minutes at 3884 x g to extract tear fluid out of the Schirmer strip into the 2-mL tube.
6) Transfer the centrifuged strip to another 2-mL tube and cut it into multiple <5mm pieces.
7) Add 600 µL of methyl tert-butyl ether (MTBE) into the tube containing the cut Schirmer strip.
8) Grind for 1 min with a handheld pestle.
9) Store in +4°C fridge for 2 hours.
10) Ultrasonic agitation for 30 min.
11) Centrifuge for 1 min at 3824 g.
12) Transfer the fluid to a new 2-mL tube, leaving the cut/shredded Schirmer strip behind.
13) Dry solvent with nitrogen for 6-8 min at 5-8 psi.
14) Add 75 µL of 25% acetonitrile.
15) Vortex for 1 min.
16) Centrifuge for 1 min at 3824 g.
17) Transfer the fluid to the 2-mL tube containing the centrifuged tear sample (step #5).
18) Vortex for 30 sec.
19) Centrifuge for 30 sec at 3824 g.
20) Transfer into LC-MS vial containing a glass insert and a snap cap.
21) Centrifuge at 1074 x g for 10 min.
22) Transfer the sample from the glass insert to a 2-mL tube.
23) Add 320 µL of iced cold 100% acetonitrile.
24) Centrifuge for 10 min at 10621 g.
25) Transfer to a new 2-mL tube, leaving the precipitated proteins behind
26) Dry solvent with nitrogen for 10-15 min
27) Add 100 µL of 25% acetonitrile
28) Vortex for 5 seconds
29) Transfer the sample to a new glass insert
30) Centrifuge at 998 g for 15 min

*** Steps 23-31 were added after the initial samples were deemed inappropriate for LC-MS analysis. The proteins contained in the centrifuged tear sample were clogging the LC columns, so further precipitation in acetonitrile was required. ***

a. 2 mL cryogenic vial, Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA
b. Evident® disposable plastic tweezer, Evident, LLC, Union Hall, VA, USA
c. Prednisone-d7, Toronto Research Chemicals Inc., North York, Canada
d. Eppendorf® safe-lock micro test tubes, Eppendorf North America Inc., Westbury, NY, USA
e. VWR® mini centrifuge, VWR International, Mississauga, Ontario, Canada
f. Argos® pestle mixer and pestle, Argos Technologies Inc., Elgin, IL, USA
g. Branson® ultrasonic cleaner, Branson Ultrasonics Corporation, Danbury, CT, USA
h. Eppendorf® Centrifuge 5417C, Eppendorf North America, Inc., Hamburg, Germany
i. Biotage® nitrogen evaporator, Biotage, LLC, Charlotte, NC, USA
j. LP vortex mixer, Thermo scientific Inc., Waltham, MA, USA
k. Xpertek® 2-mL12mm*32mm snap seal vial, P.J. Cobert Associates, Inc., St. Louis, MI, USA
l. Sorvall ST40 centrifuge, Thermo scientific Inc., Waltham, MA, USA