Aqueous humor concentration and prostaglandin E2 suppression efficacy of topically applied ophthalmic ketorolac 0.5% and diclofenac 0.1% solutions in dogs with cataract

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Aqueous humor concentration and prostaglandin E2 suppression efficacy of topically applied ophthalmic ketorolac 0.5% and diclofenac 0.1% solutions in dogs with cataract

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ABSTRACT

Background: Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for their analgesic, anti-pyretic and anti-inflammatory properties in both human and veterinary patients. Topical ophthalmic NSAIDs are commonly employed in the management of intraocular inflammation (uveitis), corneoconjunctival inflammatory disease and pre-operatively to prevent intraoperative miosis during cataract surgery. Despite their routine application in these clinical scenarios, little is known regarding the corneal penetration and relative anti-inflammatory efficacy of the available topical ophthalmic NSAIDs in the dog. Decisions regarding which of these agents to employ are therefore based upon factors such as cost and ease of acquisition as opposed to established efficacy.

Objectives: To investigate the relative intraocular penetration and anti-inflammatory efficacy of two commonly utilized topical ophthalmic NSAIDs in dogs, diclofenac 0.1% and ketorolac 0.5%.

Animals: Twenty-two client owned dogs (22 operated eyes) presenting to the VTH ophthalmology service for routine cataract surgery for mature or hypermature cataract.

Methods: Subjects were randomized to be treated with either topical ketorolac 0.5% or topical diclofenac 0.1% ophthalmic solutions at specified times in the 24-hour period pre-operatively. Aqueous humor samples were obtained intra-operatively and stored for subsequent evaluation of drug concentrations and prostaglandin E2 (PGE2) concentrations via ultra performance liquid chromatography-mass spectrometry (UPLC-MS/MS) and enzyme-linked immunoassay (ELISA) analysis, respectively.

Results: Median aqueous humor drug concentrations were significantly higher in dogs treated with ketorolac 0.5% (1311.6 ng/mL) compared to those treated with diclofenac 0.1% (284.9 ng/mL). There was no significant difference in aqueous humor PGE2 concentrations between the two treatment groups. No significant association was determined between aqueous humor drug concentration and PGE2 concentration. There was no significant association between diabetic status and aqueous humor drug concentration or PGE2 concentration in either group.

Conclusions and clinical importance: This study suggests that topical ketorolac 0.5% and diclofenac 0.1% are efficacious in decreasing aqueous humor PGE2 concentrations and are equally suitable for use based on their comparable anti-inflammatory profiles. The results of these assays provide clinically relevant information regarding intraocular penetration and anti-inflammatory efficacy of these medications in dogs with cataract.
Aqueous humor concentration and prostaglandin E2 suppression efficacy of topically applied ophthalmic ketorolac 0.5% and diclofenac 0.1% solutions in dogs with cataract

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GENERAL AUDIENCE ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for their analgesic, anti-pyretic and anti-inflammatory properties in both human and veterinary patients. Topical ophthalmic NSAIDs are commonly employed in the management of intraocular inflammation (uveitis), corneoconjunctival inflammatory disease and pre-operatively to prevent intraoperative miosis during cataract surgery. Despite their routine application in these clinical scenarios, little is known regarding the intraocular penetration and relative anti-inflammatory efficacy of the available topical ophthalmic NSAIDs in the dog. Decisions regarding which of these agents to employ are therefore based upon factors such as cost and ease of acquisition as opposed to established efficacy.

Efficacy of topical anti-inflammatory medications in controlling intraocular inflammation is primarily related to the ability of the medication to penetrate the cornea and its efficacy at suppressing inflammatory mediators. The purpose of this study, therefore, is to investigate the relative intraocular penetration and anti-inflammatory efficacy of two commonly utilized topical ophthalmic NSAIDs in dogs, diclofenac 0.1% and ketorolac 0.5%.

Twenty-two dogs presenting to the VTH ophthalmology service for routine cataract surgery with the presence of a mature or hypermature cataract were enrolled in a prospective, randomized clinical trial. Subjects were treated with either topical ketorolac 0.5% or topical diclofenac 0.1% ophthalmic solutions at specified times in the 24-hour period pre-operatively. Aqueous humor samples were obtained intra-operatively and stored for subsequent evaluation of drug concentrations (n=22) and prostaglandin E2 (PGE2) concentrations (n=19) via ultra performance liquid chromatography (UPLC) and enzyme-linked immunoassay (ELISA) analysis, respectively.

Treatment with topical ketorolac 0.5% resulted in higher median aqueous humor drug concentrations when compared to treatment with diclofenac 0.1% (1311.6 ng/mL vs. 284.9 ng/mL). However, there was no significant difference in anti-inflammatory efficacy when comparing PGE2 concentrations between the two groups. Furthermore, no significant association was determined when drug concentration was directly compared with PGE2 concentration. The results of these assays suggest that topical ketorolac 0.5% and diclofenac 0.1% are equally suitable for use based on their comparable anti-inflammatory profiles, and provides clinically relevant information regarding intraocular penetration and anti-inflammatory efficacy of these medications in dogs with cataract.
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<tr>
<td>ACAID</td>
<td>Antigen chamber associated immune deviation</td>
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<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AH</td>
<td>Aqueous humor</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IOP</td>
<td>Intraocular pressure</td>
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<tr>
<td>IS</td>
<td>Internal standard</td>
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<tr>
<td>LIU</td>
<td>Lens induced uveitis</td>
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<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear cell</td>
</tr>
<tr>
<td>PGs</td>
<td>Prostaglandins</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>UPLC-MS/MS</td>
<td>Ultra performance liquid chromatography with tandem mass spectrometry</td>
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A. Uveitis: Pathogenesis

Uveitis, or intraocular inflammation, refers to inflammation of the uveal tissue, which is comprised of the iris, ciliary body, and choroid. The anterior uvea is comprised of the iris and ciliary body and is also the site of the blood-aqueous barrier. This barrier consists of tight junctions between non-pigmented ciliary body epithelial cells, tight junctions and gap junctions in the iris vascular endothelium and nonfenestrated, impermeable capillaries in the iris. As such, the blood-aqueous barrier normally prevents large, high-molecular weight proteins from entering the aqueous humor.\(^1,2\) Differences in the stability of the blood-aqueous barrier vary among species with primates, for example, having a very stable barrier and rabbits having a highly sensitive barrier.\(^3\) The stability of the dog’s blood-aqueous barrier lies somewhere between these two extremes.

The rich blood supply, close proximity to other structures, and immunosensitivity of the anterior uvea make it the source of many inflammatory responses in the eye.\(^1\) Uveitis is incited by tissue injury and occurs secondary to a variety of conditions including lens, corneal and scleral disease, immune-mediated disease, infectious disease, trauma (including surgical insult) and neoplasia, among others.\(^1,4\) When tissue injury occurs, a cascade of events occur including increased blood supply, enhanced vessel permeability and white blood cell migration to the site of injury. Various chemical mediators are also released in response to injury including histamine, serotonin, kinins, plasmin, complement, prostaglandins, and peptide growth factors. These chemical mediators increase vascular permeability by causing the intercellular tight junctions in the vascular endothelial cells to open thereby allowing fluid to leak into the tissues.\(^1\) In
addition, plasma proteins, namely albumin and globulin, leak through the vessel walls. Reported mean values for aqueous protein in the non-inflamed canine eye range from 21 ± 1.2 mg/dL to 37.4 ± 7.9 mg/dL.\textsuperscript{5-8} In cases of uveitis, aqueous protein values range from 1200 mg/dL to 6600 mg/dL.

\textit{Mediators of ocular inflammation}

Ocular inflammation is mediated by several compounds including: prostaglandins, leukotrienes, platelet activating factor, neuropeptides like substance P and bradykinin, and cytokines such as tumor necrosis factor alpha and interleukins.\textsuperscript{9}

Prostaglandins (PGs) are the most important and widely studied mediators of ocular inflammation. They are produced in almost all ocular tissues and have been demonstrated to be synthesized in the irides of dogs and other species.\textsuperscript{1,4,10} Prostaglandin receptors have also been detected in the iris and ciliary body of several mammals.\textsuperscript{11-13} Notable pathologic ocular effects of PGs, particularly PGE\textsubscript{2}, include miosis, hyperemia, changes in vascular permeability, and alterations in intraocular pressure (IOP).\textsuperscript{1,4,14,15} PG mediated disruption of the blood-aqueous barrier results in exudation of plasma proteins and cellular components into the anterior chamber, which is detected clinically as aqueous flare, a hallmark of uveitis.\textsuperscript{1,4} PGs also have normal physiologic functions but are present in excessive quantities during episodes of uveitis. The eye has limited amounts of PG 15-dehydrogenase which is responsible for the inactivation of PGs and, as such, PGs must be removed by active transport through the ciliary body. When uveitis is present, these active transport mechanisms are diminished.\textsuperscript{16-17}

PGs (PGE\textsubscript{2}, PGD\textsubscript{2}, PGF\textsubscript{2α}, and PGI\textsubscript{2}) are end products of the arachidonic acid cascade, in which arachidonic acid is mobilized from damaged cellular membranes by the
enzymatic action of phospholipase $A_2^{18}$ and enters one of multiple pathways, including the cyclooxygenase (COX) pathway, which results in PG production.$^{1,9,19}$ The COX enzyme exists in two prominent isoforms: COX-1 (constitutive) and COX-2 (inducible). COX-1 enzymes are expressed on the endoplasmic reticulum of all cells, including platelets, gastrointestinal mucosa, vascular endothelium, renal medullary collecting ducts, interstitium and pulmonary, hepatic and splenic sites.$^{20}$ As such, COX-1 produces PGs responsible for homeostatic functions including gastrointestinal mucosal integrity, platelet aggregation, and regulation of renal perfusion. COX-2 is synthesized by macrophages and inflammatory cells that have been stimulated by cytokines and other inflammatory mediators in response to cellular insult or injury.$^{14,21}$ However, COX-2 can also be found in low amounts in physiologically normal tissues. Constitutive COX-2 expression has also been demonstrated in the kidney, central nervous system, vascular endothelium and gastrointestinal tract.$^{22-25}$

![Figure 1: COX isoenzyme properties and functions.](image-url)
Other arachidonic acid derivatives play a key role in ocular inflammation. Arachidonic acid can enter one of three metabolic pathways once it is released from damaged cellular membranes including the cyclooxygenase, lipoxygenase or oxidation pathway. Each of these pathways has been identified in the eyes of various species but the relative contribution of each in the role of uveitis is poorly defined. The cyclooxygenase pathway produces PGs, as described above, along with thromboxane, and the lipoxygenase pathways produces leukotrienes, hydroperoxy and hydroxeicosa-tetraenoic acids. Leukotrienes are potent vasoactive substances and chemoattractants that are synthesized in several ocular tissues. Levels of leukotriene B4 were found to be increased during early inflammation in a canine model of lens induced uveitis (LIU). In a paracentesis model of uveitis in dogs, it was demonstrated that leukotrienes are not important mediators of blood-aqueous barrier disruption in dogs suggesting that leukotriene inhibitors may exacerbate uveitis through shunting of arachidonic metabolites to alternate pathways. Substance P is thought to be associated with uveitis secondary to corneal irritation. Substance P release from the ciliary body and iris is thought to be mediated by the trigeminal nerve, which results in vascular dilation and altered permeability as well as PMN chemotaxis. These effects are likely transient and do not result in permanent damage. Intraocular administration of bradykinin has also been shown to cause miosis and breakdown of the blood aqueous barrier with a rise of aqueous humor protein levels. It has been suggested that bradykinin has its effect through the release of neuronal substance P. The role of these neuropeptides however appears to be minimal in canine uveitis. Platelet activating factor (PAF) also plays a role in the inflammatory response in uveitis, as it activates the release of arachidonic acid and the
subsequent production of prostaglandins.\textsuperscript{33-34}

Figure 2: Arachidonic acid cascade and prostaglandin mediated ocular effects.

Matrix metalloproteinases (MMPs) are a group of enzymes with the ability to degrade extracellular matrix and have also been associated with ocular inflammatory disease. MMPs can regulate chemokines, cytokines, growth factors and cell surface receptors which allow them to modify the course of inflammatory processes. Specifically, high MMP-2 and MMP-9 levels have been associated with intraocular inflammatory disease.\textsuperscript{35} A multitude of cytokines, including IL-1\textbeta, IL-6, IFN-\gamma, and TNF-\alpha, have been
detected in cases of acute uveitis as well as in experimentally induced cases of uveitis in both animals and humans.\textsuperscript{36} The mechanism of action for this process includes the initial triggering of IL-1 and TNF-\(\alpha\) and subsequent prostaglandin production and induction of chemokines which activate inflammatory cells.\textsuperscript{37} The production of nitric oxide (NO) and its associated derivatives is also induced by immunologic and inflammatory stimuli and has been implicated in ocular inflammatory states in endotoxin induced uveitis.\textsuperscript{37-39}

\textit{Inflammatory immune response}

Three phases of inflammation have been identified including acute, subacute and chronic stages. In the acute stages of inflammation, polymorphonuclear cells (PMN) predominate and death of these cells causes additional tissue destruction leading to increased inflammation.\textsuperscript{40} During the subacute stage, immunologic reactions are initiated and healing occurs, or there is necrosis, recurrence or chronicity.\textsuperscript{40} Chronic uveitis occurs when there are permanent alterations in uveal vascular structure or permeability due to inability to control the inflammatory event or eliminate the underlying cause.

Inflammation plays a critical role in host defenses and immune responses. The innate immune response is initiated during times of acute inflammation and local innate immune cells such as macrophages and natural killer (NK) cells are activated. Vascular adhesion molecules are expressed and chemoattractant cytokines are released. Leukocyte migration from the blood to the site of injury is also stimulated secondary to vasodilation, increased vascular permeability and the expression of specific adhesion molecules.\textsuperscript{41} Inflammation is also necessary in activating antigen presenting cells (APCs) and initiating antigen specific immune responses. Failure to get rid of the underlying cause will lead to stimulation of an antigen-specific immune response and, as such, long
standing inflammation is typically the result of an active adaptive immune response. The eye is considered an immune privileged site characterized by the absence of certain effector mechanisms and by the enhanced generation of tolerance to the antigen called antigen chamber associated immune deviation (ACAID). This unique immune response serves as a protective mechanism to preserve the function of the eye as it has limited tolerance for tissue damage before significant loss of function develops. As such, control of inflammation is critical to preserve ocular function and vision.

**B. Uveitis: Clinical Signs and Diagnosis**

*Clinical signs*

Anterior uveitis manifests many ocular clinical signs including conjunctival hyperemia, corneal edema, excessive lacrimation, blepharospasm, visual deficits, aqueous flare, miosis, fibrin formation, keratic precipitates, hyphema, hypopyon, ciliary flush, synechia formation, iris color change, iris swelling, decreased intraocular pressure, deep corneal neovascularization, rubeosis iridis, pre-iridal fibrovascular membrane formation, cataract, lens instability, secondary glaucoma, iris bombé, ectropion uvea, and phthisis bulbi. Clinical signs of posterior uveitis include retinal detachment, retinal hemorrhage, choroidal effusion, optic neuritis, chorioretinal granulomas, and vitreal opacities. These clinical signs vary in the acute versus chronic stages of intraocular inflammation and may vary in severity correlating to the severity of the disease.

Pupillary constriction, or miosis, is a common sign of anterior uveitis and occurs in response to PGF2α acting on the iris sphincter. Inflammatory mediators also cause spasm of the ciliary body musculature which can be painful and has been described to cause a “brow ache” in humans. Aqueous flare occurs as proteins and cellular
components accumulate within the aqueous humor after disruption of the blood-aqueous barrier. Aqueous flare is visualized when light scattering from particles suspended in the anterior chamber causes a continuous beam effect called the Tyndall phenomenon.\textsuperscript{44} The presence of aqueous flare is pathognomonic for anterior uveitis with increasing degrees of flare correlating to an increasing severity of uveitis. Decreased intraocular pressure is one of the earliest and most subtle clinical signs of uveitis. Proposed mechanisms for decreased IOP include decreased aqueous humor production with breakdown of the blood-aqueous barrier and increased uveoscleral outflow mediated in part by PGs.\textsuperscript{9,45,46} Intraocular pressure will vary with chronicity and severity of uveitis. In acute uveitis, IOP is typically decreased whereas in chronic uveitis, fibrosis or atrophy of the ciliary body may contribute to decreased secretory functions with resulting ocular hypotony.\textsuperscript{1} Marked and continued decreased IOP may lead to phthisis bulbi which describes a shrunken, non-functional eye. Glaucoma may result secondary to obstruction of the iridocorneal angle by inflammatory debris, pre-iridal fibrovascular membrane formation, or iris bombé development as a result of extensive posterior synechia formation.

\textit{Diagnosis}

A diagnosis of uveitis in veterinary medicine is typically made via slit lamp biomicroscopy, ophthalmoscopy, and tonometry. It is also imperative to perform a complete physical examination as uveitis can commonly present as a manifestation of underlying systemic disease. Slit lamp biomicroscopy allows magnified, three-dimensional evaluation of the adnexa, cornea, anterior chamber, lens and vitreous to evaluate for the presence of the clinical signs outlined above. Although not practical clinically, laser flaremetry and slit lamp fluorophotometry have been applied in the dog in
experimental settings as more objective quantitative measures of uveitis severity compared to clinical slit lamp examination.\textsuperscript{47-52} Ophthalmoscopy, using both direct and indirect techniques, allows for visualization of the posterior aspect of the eye including the retina and optic nerve to assess for changes such as retinal detachment, retinal hemorrhages and optic neuritis. Tonometry allows for measurement of intraocular pressure (IOP) to confirm the presence of low intraocular pressure or to evaluate for the presence of secondary glaucoma. An IOP of less than 10mmHg is consistent with uveitis and a difference of more than 5-8mmHg between eyes should be considered significant even if those values are in the normal range.\textsuperscript{53} The normal IOP for most animals is between 15-25mHg.

A complete physical examination in addition to a complete ophthalmic examination is indicated when a diagnosis of uveitis has been made as other signs of systemic disease may be revealed. A complete blood count, serum biochemistry profile and urinalysis, with serologic tests for various infectious diseases should be performed, as dictated by a number of factors. Knowledge of common endemic agents can be useful in assisting with selection of specific serologic tests. Thoracic radiographs and abdominal ultrasound is helpful in determining a diagnosis of neoplastic or fungal disease. Aqueocentesis and cytological examination of the aqueous humor may also be helpful in the diagnosis of lymphoma, specifically.\textsuperscript{54} Ocular ultrasound is useful if corneal or lens opacifications preclude visualization of the intraocular structures and posterior segment.

C. Uveitis: Etiology

Many etiologies for uveitis exist in all animal species and most causes can be divided into endogenous and exogenous causes. Endogenous causes originate from
within the eye or spread to the eye hematogenously. Endogenous causes include infectious, neoplastic, toxic, metabolic and immune-mediated diseases. Exogenous causes arise from outside of the eye and include trauma, surgical trauma, chemical injury and radiation exposure. Etiologies for uveitis can also be categorized as infectious or non-infectious. Infectious diseases include viral, bacterial, protozoal, rickettsial, fungal, algal, and parasitic diseases. Non-infectious causes include corneal and scleral disease, primary intraocular and metastatic intraocular neoplasia, trauma, toxin exposure, lens-induced uveitis, metabolic disease, idiopathic, and immune mediated diseases. In canine cases of anterior and panuveitis, idiopathic/immune mediated uveitis is most commonly diagnosed.

Infectious etiologies

Examples of viral diseases that can cause uveitis in dogs include infectious canine hepatitis and canine distemper. Bacterial causes include *Brucella sp.*, *Leptospira sp.*, *Bartonella sp.*, and *Borrelia burgdorferi* among others. Protozoal diseases include toxoplasmosis, leishmaniasis, and neosporosis. Common systemic fungal diseases include blastomycosis, coccidiomycosis, cryptococcosis, histoplasmosis, and less commonly aspergillosis and candidiasis. Prototheca is an algal organism known to cause uveitis. Common parasitic diseases include heartworm disease, ocular larval migans caused by *Toxocara canis*, and onchocerciasis. Rickettsial diseases include ehrlichiosis and Rocky Mountain spotted fever caused by *Rickettsia rickettsia*.

Non-infectious etiologies

Anterior uveitis can occur secondary to corneal ulceration, putatively through an axonal reflex causing substance P release. Traumatic uveitis may result from
penetrating and blunt trauma, with or without associated intraocular foreign bodies and lens rupture. The two most common primary intraocular neoplasms to result in uveitis in dogs include melanocytic tumors and iridociliary epithelial tumors, respectively. Lymphoma is the most common secondary neoplasm to metastasize to the canine eye. Uveodermatologic syndrome is an immune mediated disease directed against melanocytes which causes anterior uveitis in addition to dermatologic signs in dogs. Metabolic conditions such as diabetes mellitus, hyperadrenocorticism and hypothyroidism may result in hypertriglyceridemia from elevated cholesterol or triglyceride levels which can result in lipid-laden aqueous humor termed lipemic uveitis.

**Lens-induced uveitis**

Lens-induced uveitis (LIU) is a common complication of cataract in the dog due to overwhelming of T-cell tolerance and induction of a cell mediated and/or humoral response. Two types of lens-induced uveitis are recognized in the dog including phacolytic and phacoclastic uveitis. Phacolytic uveitis occurs more commonly in dogs with rapidly developing or hypermature cataracts in which soluble lens proteins leak through an intact lens capsule. It has been confirmed that aqueous humor PGE2 concentrations in dogs with mature (378.40 +/- 140.50 pg/mL) and hypermature (442.50 +/- 213.00 pg/mL) cataract are significantly elevated compared to dogs without cataract (5.98 +/- 1.41 pg/mL), although PGE2 concentrations in dogs with these two stages of cataract are not significantly different to each other. The prevalence of phacolytic uveitis has been reported to be as high as 71% of dogs screened for cataract surgery and may lower surgical success rates. Phacoclastic uveitis results from lens capsular rupture which causes sudden and rapid exposure of intact lens proteins, overwhelming
the normal low-dose T-cell tolerance to lens proteins. This type of LIU most commonly occurs in dogs with rapidly developing cataracts (diabetic or otherwise) and in cases of traumatic lens rupture.

Surgically induced trauma may also exacerbate or cause uveitis. For example, a 72-fold increase in anterior chamber protein has been demonstrated 24 hours after cataract surgery, remaining significantly elevated for up to 15 days postoperatively. The antioxidant capacity and ascorbic acid concentrations of the aqueous humor have also been shown to be decreased for 7-15 days following cataract surgery, both indirect reflections of ongoing inflammation. A retrospective study documented a 16.2% incidence of long term uveitis, categorized as three weeks or longer, after cataract surgery in dogs.

**D. Uveitis: Current Treatments**

The primary treatment goals for uveitis are halting inflammation and preventing or controlling complications caused by inflammation, which include pain and vision loss. Identification and adequate treatment of the underlying cause is necessary to achieve the best outcome. However, in many situations, an underlying cause cannot be determined and symptomatic treatment must be pursued. A variety of topical and systemic anti-inflammatory agents, both steroidal and nonsteroidal anti-inflammatory drugs, are utilized in treating uveitis.

**Corticosteroids**

Topical corticosteroids are potent anti-inflammatory medications commonly employed in the management of ocular inflammation as they inhibit phospholipase and the release of arachidonic acid, thereby preventing the subsequent formation of both
prostaglandins and leukotrienes. They work to decrease inflammation by decreasing cellular and fibrinous exudation and tissue infiltration, inhibiting fibroblastic and collagen-forming activity, diminishing post-inflammatory neovascularization, and decreasing vascular permeability.\textsuperscript{73} Topical 1% prednisolone acetate and 0.1% dexamethasone sodium phosphate solutions are commonly prescribed corticosteroids in cases of uveitis. Topical 1% prednisolone acetate has been shown to be more effective than 0.1% dexamethasone sodium phosphate in stabilizing the blood-aqueous barrier\textsuperscript{51} and is generally considered the most effective topical anti-inflammatory agent for anterior segment inflammation.\textsuperscript{74} Frequency of application of topical corticosteroids is determined by the severity of the uveitis. Subconjunctival corticosteroid injections with triamcinolone acetonide and betamethasone have also been used for their long-acting benefit; however, this route of administration holds risks including trauma to the globe, granuloma formation, and the inability to reverse the medication’s effects after administration.\textsuperscript{75} Intravitreal injections of corticosteroids have also been utilized in human patients with uveitis, although they are not commonly used in veterinary medicine.\textsuperscript{76} In addition to their desired and potent anti-inflammatory effects, topical corticosteroids possess local effects that may be detrimental to the eye, including corneal lipid and mineral deposition, delayed corneal healing and potentiation of corneal collagenase activity, decreased epithelial healing rates, and reduction of neutrophil and macrophage migration, thereby increasing the risk of infection.\textsuperscript{14, 19, 20, 77} As such, topical corticosteroids are contraindicated in cases of corneal ulceration and other ocular infections. Corticosteroids have also been documented to cause an increase in IOP and have been associated with cataract formation in humans and cats.\textsuperscript{78-80}
Systemic corticosteroids may be used to treat uveitis in conjunction with topical corticosteroids. In addition, inflammatory conditions of the posterior segment and optic nerve typically require systemic administration of corticosteroids. However, systemic corticosteroid therapy should not be implemented until a complete workup is performed as systemic corticosteroids can potentiate the severity of some systemic infectious diseases and can mask the presence of systemic neoplasms, namely lymphoma. The lowest effective dose should be used when administering oral steroids to minimize the occurrence of adverse systemic side effects. Systemic corticosteroid use may also be contraindicated by concurrent disease and their associated systemic side effects may be detrimental even in otherwise healthy animals.14

Nonsteroidal anti-inflammatory drugs

Topical nonsteroidal anti-inflammatory drugs (NSAIDs) are particularly useful in the treatment of uveitis when the use of corticosteroids is contraindicated. NSAIDs may also be used in combination with topical steroids to reduce intraocular inflammation through an additive effect and may allow for less frequent administration of topical steroids. Many ophthalmic NSAIDs are available in the United States including 0.4% and 0.5% ketorolac, 0.1% diclofenac, 0.03% flurbiprofen, 0.1% nepafenac and 0.09% bromfenac. Currently, topical formulations of indomethacin are only commercially available outside of the United States. Efficacy of topically applied NSAIDs in the control of uveitis is determined by two primary factors: effective entry into the anterior chamber and effective COX suppression. Most studies to date evaluate the effectiveness of topical NSAIDs in preventing blood-aqueous barrier disruption in experimental uveitis. In one study evaluating blood-aqueous barrier stabilizing effects of topical
NSAIDs in dogs, diclofenac was found to be superior to flurbiprofen and suprofen. In a rabbit model, ketorolac and bromfenac have been shown to effectively suppress inflammation and ketorolac demonstrated greater anti-inflammatory effects than diclofenac. Few studies have been performed which evaluate the efficacy of NSAIDs in the situation of pre-existing anterior uveitis. In the case of topical ophthalmic NSAIDs in dogs, objective comparative studies are few and, for some medications, non-existent. In contrast to topical steroids, significant side effects of topical ophthalmic NSAIDs are uncommon, although ocular irritation with epiphora, blepharospasm, and conjunctival hyperemia may be noted on occasion. Topical NSAIDs may also delay wound healing and have been reported to increase IOP in veterinary species, possibly secondary to decreased aqueous outflow. In human patients, superficial punctate keratitis, corneal infiltrates and epithelial defects have been reported as well as keratomalacia. Systemic effects associated with topical NSAIDs are rare, however, exacerbation of bronchial asthma secondary to administration has been reported in humans. Overall, these complications are uncommon in humans and rarely reported in veterinary patients where topical NSAIDs are generally considered a much safer alternative to topical corticosteroids.

Many different systemic NSAIDs are available for use in the treatment of uveitis including carprofen, flunixin meglumine, phenylbutazone, piroxicam, meloxicam, ibuprofen, acetaminophen, naproxen, deracoxib and firocoxib. Systemic NSAIDs are utilized most commonly, in conjunction with topical anti-inflammatory therapy, to maximize the inhibition of inflammation and when corticosteroid use is contraindicated such as in infectious diseases and in diabetic patients. Several studies have been
performed that demonstrate the ocular anti-inflammatory effects of systemic NSAIDs.\textsuperscript{27, 49, 51, 92-95} Specifically, oral carprofen has been demonstrated to reduce the inflammatory response by 68\% in experimentally induced uveitis in dogs.\textsuperscript{49} Although their use may be beneficial in the treatment of uveitis, systemic NSAIDs are associated with adverse effects including gastrointestinal ulceration and hemorrhage, hepatotoxicity, platelet dysfunction, and decreased renal perfusion and glomerular filtration rate.\textsuperscript{96-99} Systemic NSAID administration in cats has been associated with an increased risk of adverse effects, such as bone marrow suppression, GI upset, and acute renal failure due to a reduced glucuronide metabolism and slow drug clearance.\textsuperscript{100-102} These side effects are particularly of concern in high risk patients such as those with pre-existing renal disease and under general anesthesia and as such these medications should be used judiciously.

\textit{Immunosuppressive therapy}

Immunosuppressive medications are sometimes employed in cases of immune-mediated uveitis that are unresponsive to other therapies. Immunosuppression can be achieved through high doses of orally administered steroids; however, side effects make long term use undesirable. Other immunosuppressive agents are utilized more frequently for long term therapy, including azathioprine, mycophenolate and systemic cyclosporine. The topical use of cyclosporine is not indicated in cases of uveitis due to its hydrophobic nature thus preventing its ability to penetrate into the eye. Suprachoroidal cyclosporine implants, however, have been utilized successfully in the treatment of equine recurrent uveitis in horses.\textsuperscript{1, 41} Frequent monitoring of bloodwork to evaluate blood and platelet counts, as well as liver values, is recommended due to the potentially hepatotoxic and myelosuppressive effects associated with these therapies.\textsuperscript{103-105}
Parasympatholytic agents

Parasympatholytic agents are used in the treatment of uveitis to relieve associated pain and secondary complications. These agents paralyze the iris and ciliary body musculature to alleviate ciliary spasm and its associated discomfort. Dilating the pupil also minimizes the risk of posterior synechia development and subsequent secondary glaucoma. Atropine is the most commonly used ophthalmic parasympatholytic agent due to its potent mydriatic and cycloplegic effects.\(^1\) In addition, atropine is long acting and has been demonstrated to stabilize the blood-aqueous barrier.\(^{106}\) Use of atropine is contraindicated in cases of elevated IOP and lens instability and therefore continued monitoring of IOP during administration is recommended. Atropine has also been associated with decreased secretions and as such should be used cautiously in patients with low or borderline tear production.\(^{103}\)

E. Uveitis: Prognosis and Sequelae

In general, regardless of the underlying cause, uncontrolled uveitis has a poor prognosis for vision. However, long term prognosis ultimately relies on the severity and duration of the inflammation, underlying cause, development of secondary complications and timeliness of appropriate treatment. Unfortunately, despite our best treatment efforts, vision loss and secondary ocular complications may ensue, particularly in cases of recurrence or uncontrolled inflammatory states. Such complications include cataract formation, glaucoma and retinal detachment.\(^{14,19}\) Chronic uveitic states can lead to cataract formation as inflammatory mediators in the aqueous humor interfere with normal lens metabolism.\(^1\) Chronic inflammatory states can also lead to glaucoma development as inflammatory debris obstructs the iridocorneal angle. Formation of pre-iridal...
fibrovascular membranes can also lead to glaucoma and inflammation causes adhesions of the iris to the lens capsule termed posterior synechia. Severe posterior synechia can cause obstruction of the pupil resulting in disruption of normal aqueous humor outflow paths. Phthisis bulbi, a shrunken, non-functional globe, may also occur as chronic inflammation destroys the ability of the ciliary body to produce aqueous humor. Prompt and appropriate treatment of uveitis is therefore critical to relieving associated ocular discomfort and preventing potential vision loss. Improving our understanding regarding the efficacy of the various commercially available anti-inflammatory therapies will allow for the most effective and judicious treatment planning when treating uveitis in our canine patients.
CHAPTER 2: TOPICAL NSAID LITERATURE REVIEW WITH A PRIMARY FOCUS ON KETOROLAC AND DICLOFENAC

A. NSAIDs: Mechanism of Action

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit PG-mediated inflammation by inhibiting the COX enzyme, which converts arachidonic acid to prostaglandins and thromboxane A$_2$. Additional anti-inflammatory properties of NSAIDs include decreasing polymorphonuclear leukocyte migration and chemotaxis, decreasing cytokine expression and mast cell degranulation and acting as free-radical scavengers. Being organic acids, NSAIDs also accumulate at sites of inflammation thereby increasing their overall anti-inflammatory effect. As mentioned previously, the COX enzyme exists in two prominent isoforms: COX-1 (constitutive) which is responsible for production of prostaglandins and required for normal tissue homeostasis and COX-2 (inducible) which produces prostaglandins at sites of inflammation. NSAIDs inhibit both isoforms of the COX enzyme and are often classified according to their ability to preferentially select for either COX-1 or COX-2 isoforms. The molecular action of the drug is based upon competitively blocking the enzyme active site in a reversible or irreversible manner. Selective inhibition of the inducible COX-2 isoform is believed to lead to fewer deleterious side effects, as protective functions mediated by COX-1 are spared. Although currently available NSAIDs in veterinary ophthalmology vary in their COX selectivity, most inhibit both isoenzymes. It is important to note that COX enzyme localization and expression, and subsequent PG expression and PG receptor sensitivities vary among species which affects pharmacologic response and efficacy of intervention. Efficacy of topically applied NSAIDs in the control of uveitis is determined by two primary factors,
including effective entry to the anterior chamber and effective COX suppression. The ideal ocular NSAID in a given species would exhibit high corneal penetration and demonstrate effective suppression of prostaglandin-mediated inflammation via COX suppression in that species.

B. Topical NSAIDs: Indications for Use

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for their analgesic, anti-pyretic and anti-inflammatory properties in both human and veterinary patients. In humans, topical NSAIDs are used for the management of postoperative ocular inflammation and the prevention of cystoid macular edema after cataract surgery.\textsuperscript{14, 83, 90, 113} They are also commonly utilized to prevent intraoperative miosis, control postoperative pain and inflammation after intraocular surgery, control symptoms of allergic conjunctivitis and alleviate signs of uveitis.\textsuperscript{1, 14, 83, 89, 111, 113} Several studies have also demonstrated the analgesic effects of topical NSAIDs in humans through a decrease in corneal sensitivity.\textsuperscript{114-120} In veterinary medicine, topical NSAIDs are used most commonly to decrease intraocular inflammation and to prevent intraoperative miosis during cataract surgery.\textsuperscript{14, 83, 121} Studies have been performed to evaluate the effects of corneal sensitivity and analgesia of topical NSAIDs with mixed results. In dogs, diclofenac and flurbiprofen were shown to be ineffective in reducing corneal sensitivity\textsuperscript{122} whereas a study performed in cats showed decreased responsiveness of corneal nerves after treatment with diclofenac and flurbiprofen.\textsuperscript{123} As such topical NSAIDs are not used commonly to treat or reduce corneal pain in veterinary medicine. When utilizing topical NSAIDs to manage intraocular inflammation, substituting topical NSAIDs for steroids or combining them with topical steroid use may decrease or negate the need for topical
steroids, thereby reducing their associated local deleterious effects.\textsuperscript{1, 14, 83} Topical nonsteroidal anti-inflammatory medications also have the benefit of having minimal and uncommon side effects as compared to systemic NSAIDs and their corticosteroid counterparts. Compared to systemic NSAIDs, topical ophthalmic NSAIDs also provide the advantage of good ocular bioavailability.\textsuperscript{83} Generally, topical NSAIDs are considered a much safer alternative to topical corticosteroids and as such are commonly utilized in managing anterior uveitis and inflammatory corneconjunctival disease and to prevent intraoperative miosis and inflammation associated with cataract surgery.\textsuperscript{14, 83, 124}

C. Ocular Drug Penetration

Topical therapy for ocular disease has the benefit of direct application to the desired site with minimal systemic side effects. However, efficacy of local administration is, to varying degrees, limited by ocular anatomical and physiological barriers, including blinking, the pre-corneal tear film, the lacrimal drainage system and the corneal layers.\textsuperscript{125} The primary barrier to topically applied medication entering the anterior chamber is the cornea, particularly the corneal epithelium.\textsuperscript{125-126} The cornea is comprised of three main layers: the epithelium, stroma and endothelium which differ in their lipophilicity. The epithelium and endothelium are hydrophobic whereas the stroma is hydrophilic. As such, transfer through the epithelium is the rate-limiting step for absorption of hydrophilic compounds whereas transfer through the stroma is the rate-limiting step for lipophilic compounds. For successful penetration through the cornea, it is necessary for an ophthalmic drug to possess intermediate solubility characteristics.\textsuperscript{127} Passage of drugs through the corneal epithelium can occur through transcellular (across cells) and paracellular (between cells) routes; however, the paracellular route is typically blocked
by tight junctions in the cornea. In addition to these tight junctions, mechanisms exist to regulate the entry and exit of substances and contribute to the barrier properties of ocular membranes. Membrane-bound proteins called transporters have been found in various ocular tissues including the conjunctiva, cornea, and retina and have been reported to exert a role in drug delivery. Efflux transporters pose significant barriers to the entry of drug molecules through effluxing molecules out of cell membranes and cytoplasms. The conjunctiva has also been shown to contribute to the intraocular penetration of certain topical drugs; however, the cornea is generally considered the main route of passage to the anterior chamber.

Ocular drug penetration is also influenced by the pH of the drug and of the tear film. The degree of ionization of a drug and its ability to diffuse across cellular barriers is determined by its dissociation constant (pKa) and the solvent’s pH. As such, the pH can be adjusted to increase the proportion of unionized drug and facilitate its transport through the corneal epithelium. The size of the molecule, concentration of the drug and the drug’s ability to reduce surface tension also affect corneal penetration. The factors that influence the rate of drug elimination from the ocular surface include the size of the drop delivered, blinking frequency, and tear flow dynamics. When applied to the ocular surface, ophthalmic solutions typically exhibit a fast drug delivery with an initial high concentration that rapidly declines as the medication is cleared through the nasolacrimal system and washed away by tear turnover. It is generally considered that less than 1% to no more than 10% of a topical dose enters the eye. The drop volume delivered by many ophthalmic dropper bottles is ~40 µL and the palpebral fissure is only able to hold ~25-30 µL, so complete drop retention rarely occurs.
NSAIDs are weak acids with pKas typically between 3.5 and 4.5 and are poorly soluble in the water. Aqueous ophthalmic solutions of NSAIDs have been made using sodium, potassium, tromethamine and lysine salts. Because most NSAIDs are weakly acidic, they exist in their ionized forms in the tear film and as such poorly penetrate the cornea. Reducing the pH of topical formulations increases the intraocular penetration however it also increases irritant effects after administration. The anionic nature of NSAIDs also favors the formation of insoluble complexes with preservatives such as benzalkonium chloride. Benzalkonium chloride alters biological membranes and as such is capable of enhancing drug penetration. It has been shown to enlarge intercellular spaces in the superficial layers of the cornea facilitating increased corneal drug penetration.

D. Ketorolac Ophthalmic Solution

Ketorolac, available as 0.4%, 0.45% and 0.5% solutions, is approved for use in reducing ocular pain and inflammation after corneal refractive surgery and cataract surgery and is an effective treatment for post-operative cystoid macular edema caused by PGE$_2$ as well as ocular surface inflammatory conditions in people. The 0.45% solution was developed to reduce adverse effects on the corneal epithelium as it is formulated without the preservative present in the 0.4% and 0.5% solutions and is also formulated with carboxymethylcellulose, which may increase epithelial wound healing. Ketorolac 0.4% was produced for the treatment of ocular pain, burning, and stinging following refractive surgery. With 20% less active ingredient, 0.4% ketorolac was demonstrated to be equivalent in potency to the 0.5% ketorolac in animal and human studies. Ketorolac tromethamine ophthalmic solution 0.5% is the most commonly
used formulation in veterinary patients. It is classified as an aryl acetic acid derivative and is a member of the pyrrolo-pyrrole group of NSAIDs with a pH of 7.4 as a solution. In a study evaluating systemic absorption after topical installation, only 5/26 subjects had a detectable amount of ketorolac in their plasma. In studies in humans and rabbits, ketorolac has been shown to inhibit both COX-1 and COX-2 but to have potent, selective COX-1 inhibition with peak concentrations in aqueous humor at approximately 60 minutes following topical application. Studies comparing the relative efficacy of ketorolac with other topical NSAIDs performed in humans undergoing cataract surgery generally show evidence of superior corneal penetration and superior PGE2 inhibition by ketorolac compared to other topical NSAIDs. Anti-inflammatory activity of topical ophthalmic ketorolac has not been studied in the dog, but in a study comparing anti-inflammatory activities of ketorolac and diclofenac in a uveitis induced rabbit model, ketorolac was found to have greater anti-inflammatory effects than diclofenac. The lack of information regarding the efficacy of topical ophthalmic ketorolac in canine patients confirms that further investigation is warranted.

**E. Diclofenac 0.1% Ophthalmic Solution**

Diclofenac sodium 0.1% solution is a topical ophthalmic NSAID approved for use in humans for achieving mydriasis for cataract surgery and to reduce ocular pain following corneal refractive surgery. Diclofenac is classified as a non-selective COX inhibitor as it inhibits both COX-1 and COX-2 isoenzymes. In addition to inhibition of the COX enzyme, diclofenac has been suggested to have inhibitory effects on the lipoygenase pathway by decreasing the amount of free arachidonic acid available for metabolism. Diclofenac sodium is an aryl acetic acid derivative with a pH of 7.2 as a
solution. Plasma levels of diclofenac following ocular installation were below the limit of quantification over a 34 hours period suggesting limited, if any, systemic absorption.\textsuperscript{149} In humans, diclofenac has demonstrated good intraocular penetration within 2.5 hours after installation with detectable levels in the aqueous humor for 24 hours.\textsuperscript{150} Following topical administration, diclofenac was also found in higher concentrations subretinally when compared with ketorolac in human patients.\textsuperscript{151} Diclofenac and its ability to control blood-aqueous barrier breakdown in dogs has been studied with mixed effects. In one study utilizing the centesis model and fluorophotometric assessment of inflammation, diclofenac was found to be superior to flurbiprofen and suprofen.\textsuperscript{21, 47} However, in another study utilizing a pilocarpine-induced inflammatory model with laser flaremetry assessment of inflammation, diclofenac was found to be inferior to flurbiprofen.\textsuperscript{21, 86}

**F. Detecting PGE\textsubscript{2}: The Enzyme-Linked Immunosorbent Assay**

Clinical measures of inflammation used to judge severity of uveitis are subjective and semi-quantitative at best. The most common of these is scoring of severity of aqueous flare as determined by slit lamp examination, wherein elevated aqueous humor protein concentrations related to severity of inflammation result in progressively more severe aqueous flare as demonstrated by the Tyndall effect.\textsuperscript{44} Although not practical clinically, laser flaremetry and slit lamp fluorophotometry have been applied in the dog in experimental settings as more objective quantitative measures of uveitis severity compared to clinical slit lamp examination.\textsuperscript{47-52} Because PGE\textsubscript{2} is a known mediator of ocular inflammation, its levels are commonly assayed to quantify intraocular inflammation and evaluate the effectiveness of medications at suppressing such inflammation.\textsuperscript{81-82, 147, 152-153} Several studies in the dog have successfully utilized aqueous
PG concentrations to document effectiveness of various systemically administered NSAIDs, utilizing an aqueous centesis-induced model of inflammation in experimental animals. PGE2 levels can be detected utilizing enzyme-linked immunosorbent assay specific for PGE2 detection.

ELISAs are utilized to detect and quantify specific substances in a given sample wherein enzyme-labeled antibodies and antigens are used to detect a specific biological molecule. First, sample is placed into the well where antigens are previously coated to the well surface. The sample is then incubated with a specific primary antibody which binds to a targeted molecule followed by a specific enzyme-linked antibody (tracer) which binds to the primary antibody. The plate is washed and a reagent is added to the well. This addition initiates an enzymatic reaction and produces a color change that can be quantified. This specific assay is based on the competition between PGE2 and a PGE2-acetylcholinesterase (AChE) conjugate (PGE2 tracer) for a limited amount of antibody. The amount of tracer is held constant while the concentration of PGE2 varies. As such, the amount of tracer that binds to the antibody is inversely proportional to the concentration of PGE2 in the well.

G. Conclusion and Research Justification

Uveitis occurs commonly in dogs secondary to many ocular and systemic diseases. Prompt and effective control of inflammation is mandatory in order to prevent secondary complications that may otherwise lead to vision loss. While several avenues of topical and systemic treatment are available and often appropriate, certain conditions may preclude the use of systemic anti-inflammatories or topical steroids. In particular, long-term management of chronic inflammation with such medications carries a high risk of
significant adverse effects. Due to their ocular and systemic safety profile, topical NSAIDs are particularly useful agents to employ in the management of ocular inflammation by general practitioners and veterinary ophthalmologists alike. Despite their routine clinical application, little is known regarding the corneal penetration and relative anti-inflammatory efficacy of the available topical ophthalmic NSAIDs in the dog. Decisions regarding which of these agents to employ are therefore based upon factors such as cost and ease of acquisition as opposed to established efficacy.

This study investigates the corneal penetration and anti-inflammatory efficacy of two commonly used, commercially available topical NSAIDs, ketorolac 0.5% and diclofenac 0.1% in dogs with mature or hypermature cataracts presenting for cataract surgery. It has recently been reported that aqueous humor PGE2 concentrations in dogs with mature and hypermature cataract are significantly elevated compared to dogs without cataract, although PGE2 concentrations in dogs with these stages of cataract are not significantly different to each other. Thus, dogs presenting for cataract surgery (who exhibit mature or hypermature cataract) present an ideal, naturally-occurring inflammatory condition against which the efficacy of anti-inflammatory medications can be evaluated, without resorting to the use of experimental animals. We hypothesized that aqueous humor drug concentrations and PGE2 levels would significantly differ between these study medications. Improving our understanding of the efficacy of the various commercially available topical NSAIDs will allow for the most effective and judicious treatment planning when utilizing these medications in our canine patients. The model proposed for this study is a novel one and, if effective, will be suitable to future studies evaluating other ophthalmic anti-inflammatory agents.
CHAPTER 3: AQUEOUS HUMOR CONCENTRATION AND PROSTAGLANDIN E2 SUPPRESSION EFFICACY OF TOPICALLY APPLIED OPHTHALMIC KETOROLAC 0.5% AND DICLOFENAC 0.1% SOLUTIONS IN DOGS WITH CATARACT

A. Introduction

Nonsteroidal anti-inflammatory drugs are used commonly in both human and veterinary medicine in the management of intraocular inflammation (uveitis), to prevent intraoperative miosis, and to treat corneoconjunctival inflammatory disease. Uncontrolled uveitis can lead to a host of complications including cataract development, glaucoma and retinal detachment, all of which may be blinding.\textsuperscript{14, 19} As such, prompt and appropriate control of intraocular inflammation is critical to relieving associated ocular discomfort and reducing the odds of vision loss. While several avenues of topical and systemic anti-inflammatory treatment are available, certain conditions may preclude the use of systemic anti-inflammatories or topical steroids. Although topical steroids are potent inhibitors of inflammation, they also possess local effects that may be detrimental to the eye.\textsuperscript{14, 19, 20, 77} Substituting topical NSAIDs for steroids or combining them with topical steroid use may decrease or negate the need for topical steroids, thereby reducing their associated local deleterious effects.\textsuperscript{1, 14, 83} Topical NSAIDs also have the benefit of increased ocular bioavailability and decreased systemic side effects. Due to their ocular and systemic safety profile, topical NSAIDs are particularly useful agents to employ in the management of ocular inflammation by general practitioners and veterinary ophthalmologists alike.

Efficacy of topical anti-inflammatory medications in controlling intraocular
inflammation is primarily related to the ability of the medication to penetrate the cornea and its efficacy at suppressing inflammatory mediators. Despite the routine application of topical NSAIDs, little is known regarding their corneal penetration and relative anti-inflammatory efficacy in the dog. Decisions regarding which of these agents to employ are therefore based upon factors such as cost and ease of acquisition, as opposed to established efficacy. There are also a host of species differences, both anatomical and physiological, that must be considered when extrapolating pharmacologic information from one species to another. Therefore, while information driving therapeutic decision making can be, and often is, extrapolated from other species, species-specific information is ideal when considering the use of any medication.

Ketorolac 0.5% and diclofenac 0.1% ophthalmic solutions are commonly utilized topical NSAIDs in dogs. However, there are few studies in the dog evaluating the relative efficacy of these medications in a natural disease model. In this study, we investigated the relative corneal penetration and anti-inflammatory efficacy of ketorolac 0.5% and diclofenac 0.1% in dogs presenting for cataract surgery with mature or hypermature cataracts acting as a natural model of intraocular inflammation. We hypothesized that aqueous humor concentrations as well as prostaglandin E₂ levels would differ significantly between these study medications.

**B. Materials and Methods**

*Pilot study*

A pilot study was performed prior to execution of the final study described below to refine and determine any necessary changes in the established protocol. Thirteen dogs
with a mature or hypermature cataract presenting to the Virginia-Maryland College of Veterinary Medicine Teaching Hospital for cataract surgery between March and August 2018 were included in this pilot study and received treatment with either ketorolac or diclofenac in the immediate pre-operative period. All dogs received the following treatment in the operated eye(s): prednisolone acetate 1% suspension every 6 hours the night prior to surgery, neomycin-polymyxin-gramicidin solution every 6 hours the night prior to surgery and Cosopt (dorzolamide 2%/timolol 0.5%) 12 hours and 2 hours prior to surgery. A standardized topical ophthalmic pre-operative medication regimen was initiated two hours prior to the time of induction as listed in table 1 below.

<table>
<thead>
<tr>
<th>Time (hr:min pre-induction)</th>
<th>Treatments at 5 minute intervals (1 drop of each drug in the prescribed order)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:00</td>
<td>A. Neomycin-Polymyxin-Gramicidin</td>
</tr>
<tr>
<td></td>
<td>B. Prednisolone acetate 1%</td>
</tr>
<tr>
<td></td>
<td>C. Tropicamide1%</td>
</tr>
<tr>
<td></td>
<td>D. Diclofenac 0.1% or ketorolac 0.5% (based upon treatment group)</td>
</tr>
<tr>
<td>1:30</td>
<td>A-D</td>
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<tr>
<td>1:00</td>
<td>A-D</td>
</tr>
<tr>
<td>0:30</td>
<td>A-D and</td>
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<tr>
<td></td>
<td>Phenylephrine 10% ophthalmic</td>
</tr>
<tr>
<td>0:00</td>
<td>A-D</td>
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</tbody>
</table>

Table 1: Pilot study pre-operative standardized topical ophthalmic medication regimen.

Overall, there were 2 dogs in the diclofenac group and 11 dogs in the ketorolac group with 2 dogs having the presence of a hypermature cataract and 11 having the presence of a mature cataract. There was no standardized, controlled approach to the treatment in the days to weeks leading up to surgery in these patients, as the aim of this pilot study was to gain familiarization with the UPLC-MS/MS and ELISA methods and to detect any necessary adjustments in method development. As such, some patients received a topical NSAID or steroid in the days to weeks leading up to sample collection.
Two patients included also had the presence of lens-induced uveitis as detected on slit-lamp biomicroscopic evaluation. Aqueous humor samples were collected from the first operated eye following routine surgical entry to the anterior chamber. Aqueous humor (0.2mL) was collected and divided into two microcentrifuge tubes which were stored at -80°C until analysis for drug or PGE2 concentration.

Concentrations of ketorolac and diclofenac in aqueous humor samples as well as PGE2 concentrations were quantified as outlined below. For PGE2 determination, 50 μL aqueous samples were used. Four samples were variably diluted with buffer solution to reach a final volume of 50 μL as there was insufficient volume to use 50 μL of sample. Three samples, which were originally not diluted, were assayed again on the same plate at a 1:1 dilution (25 μL sample, 25 μL buffer) to determine if dilution of the samples was necessary. The samples were assayed in duplicate. Three samples were unable to be run in duplicate due to insufficient samples volume. Generation of a standard curve as outlined by the protocol booklet was performed (see Figure 3 below). In addition, a standard curve was generated using blank aqueous collected from recently euthanized dogs without cataract to determine if this was an appropriate method for use in the final analysis (see Figure 4 below).

Ultimately it was determined that dilution was not necessary, that an increase in sample volume was necessary to run samples in duplicate, and that a higher level of protein was detected in the blank aqueous samples than expected. As such, it was decided to generate a standard curve without the use of blank aqueous humor for the final assays and to increase the volume of aqueous humor collected to 0.5mL. Although dilution was
determined to be unnecessary, the final samples were diluted 1:1 to maximize the volume of sample available. In addition, it was decided to completely eliminate topical steroids in the immediate pre-operative treatment period to eliminate any associated influence on PGE$_2$ concentrations. Dogs with the presence of lens-induced uveitis as detected by slit-lamp biomicroscopic evaluation were also excluded from the final study. Gaining a better understanding of the variability in the timing between the last dose of NSAID and the time of fluid collection was also obtained from the pilot study, which aided in minimizing this variability in final sample collection.

Figure 3: Standard curve generated from pilot study.

Figure 4: Standard curve generated using blank aqueous humor from pilot study.
**Main study**

**Animals and study design**

Canine patients with mature or hypermature cataracts presenting to the Virginia-Maryland College of Veterinary Medicine Teaching Hospital between March and December 2019 for pre-operative evaluation for cataract surgery were potential candidates for study enrollment. Patients that received systemic or topical NSAIDs or corticosteroid therapy within two weeks prior to surgery were excluded from the study. Additional exclusion criteria included dogs with the presence of active lens-induced uveitis (as determined by detection of aqueous flare on slit lamp examination), those with patient-specific historical, systemic or surgical complications preventing adherence to the study protocol, and those who were not tolerant of topical treatments. Historical data collected included signalment, diabetic status, eye(s) affected, cataract stage and current topical and systemic medication. All patients received a complete ophthalmic examination performed by 1 of 3 clinical faculty ophthalmologists (IPH, RGR, or RVR), of whom 2 were board certified (IPH, RGR), or 1 of 2 ophthalmology residents in training (KAW and AME). In all cases, this examination included a neuro-ophthalmic examination to document menace response, dazzle reflex and pupillary light reflex, along with anterior segment examination via slit-lamp biomicroscopy and indirect ophthalmoscopy where possible. A cataract was classified as mature if the cataract comprised approximately 100% of the lens and subsequently obscured all fundic reflection. A cataract was classified as hypermature if there was evidence of one or more of the following: lens resorption and subsequent loss of lens volume, dystrophic mineralization resulting in retractile foci within the lens or capsule, or lens capsule
wrinkling. Schirmer tear test I, intraocular pressure measurement via rebound tonometry (TonoVet) and fluorescein staining were also performed. Gonioscopic examination of the iridocorneal angle, ocular ultrasonography and electroretinography were performed in most cases, per clinical discretion. All dogs underwent routine physical examination. Pre-operative laboratory evaluation was determined at clinical discretion upon perceived clinical need ranging from minimal (packed cell volume, serum total solids, Azostix®, blood glucose and urine specific gravity) to more comprehensive (complete blood count, serum chemistry, urinalysis and urine bacterial culture) as indicated by patient care needs. The following data was recorded for study purposes and statistical evaluation: treatment group, signalment, eye to be operated, diabetic status, stage of cataract, and time of aqueous humor collection relative to last NSAID treatment. After ophthalmic examination and owner consent were obtained, patients were scheduled for cataract surgery on a prescribed date. A separate and complete ophthalmic examination was performed on dogs at the time of the drop off appointment. Enrollees were assigned a number and randomized to be in either the ketorolac 0.5% or diclofenac 0.1% treatment groups.

Study treatment and Pre-operative protocol

Dogs were hospitalized the night prior to surgery and received 1 drop of the assigned study medication to the operated eye(s) every 6 hours starting the night prior to surgery equating to a total of 4 doses the night prior (starting no earlier than 12pm and initiated no later than 7pm with the last dose administered at 7am). Two dogs received only 3 doses of the assigned NSAID the night prior as the treatment protocol was initiated starting at 7pm. Study dogs also received 1 drop of the assigned study
medication every 30 minutes beginning 2 hours prior to surgical induction equating to a total of 4 doses the morning of (starting at 7:30am or after). However, two dogs received only 3 doses the morning of and 4 dogs received 5 doses. In addition to NSAID treatment as dictated by study group enrollment, all dogs received standardized pre-operative medical treatments to include the following treatment in the operated eye(s): neomycin-polymyxin-gramicidin solution every 6 hours the night prior to surgery and Cosopt (dorzolamide 2%/timolol 0.5%) 12 hours and 2 hours prior to surgery. Lastly, a standardized topical ophthalmic pre-operative medication regimen was initiated two hours prior to the time of induction as follows in table 2 below. The anesthetic protocols were not standardized for all subjects and were based upon the discretion of the attending anesthesiologist. All dogs received IV cefazolin at a dose of 22 mg/kg immediately following anesthetic induction and every 90 minutes intraoperatively. Routine surgical aseptic preparation was performed on all operated eyes and neuromuscular blockade was achieved with either atracurium or rocuronium intravenously prior to surgical entry to the anterior chamber. No oral or systemic anti-inflammatories were administered until after completion of the surgery.

<table>
<thead>
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<th>Time (hr:min pre-induction)</th>
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| 2:00                        | A. Neomycin-Polymyxin-Gramicidin  
B. Tropicamide 1%  
C. Diclofenac 0.1% or ketorolac 0.5% (based upon treatment group) |
| 1:30                        | A-C |
| 1:00                        | A-C |
| 0:30                        | A-C and Phenylephrine 10% ophthalmic |
| 0:00                        | A-C |

Table 2: Main study pre-operative standardized topical ophthalmic medication regimen.
Sample collection and storage

Samples were obtained from the eye of study dogs undergoing unilateral surgery and in cases of bilateral surgery, from the first operated eye in the following manner. Immediately following routine surgical entry to the anterior chamber, a 0.5mL aqueous humor sample was collected by aspiration utilizing a 27-gauge cannula on a 1mL syringe through the surgical incision. The time of collection was recorded and each sample was immediately divided into two microcentrifuge tubes and stored at -80°C until analysis for drug or PGE2 concentration. Following aqueous humor collection, data collection for the study ended and medical and surgical care decisions for the patient were determined by the attending surgeon/clinician based upon patient needs.

Aqueous humor NSAID concentration evaluation

Concentrations of ketorolac and diclofenac in aqueous humor samples were quantified using ultra performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) in the VetMed Analytical Laboratory. Samples were subjected to a simple protein precipitation method with acetonitrile prior to injection onto the machine. Diclofenac and ketorolac reference standards were purchase from Toronto Research Chemicals and Cayman Chemical, respectively. Diclofenac-d4 (Dd4) and ketorolac-d5 (Kd5) were used as internal standards (IS) and were also purchased from Toronto Research Chemicals. Stock solutions of all compounds were initially made up in ACN and then separately diluted in ACN to their final standard concentrations.

Aqueous humor (AH) samples were prepared by combining 100 µL of AH with 300 µL of the internal standard addition solution (1.6 µg/mL Dd4 and 150 ng/mL Kd5 in
ACN) in 0.6 mL microcentrifuge tubes. The samples then were briefly shaken and then vortexed for 30 seconds to extract before being centrifuged (Eppendorf Microcentrifuge Model 5415R) at 16,100 g for 5 minutes. The resulting supernatant solutions were diluted 1:5 by adding 200 µL of the supernatant to 800 µL of 10/90/0.1 ACN/ 1 mM aqueous ammonium acetate / acetic acid in a 2 mL amber autosampler vial. These diluted extracts were then briefly shaken and vortexed to mix before being placed in the refrigerated autosampler of the UPLC-MS/MS for analysis.

Sample extracts were subjected to chromatographic separation performed on a Waters H-Class UPLC system with a Phenyl column (Waters Acquity UPLC BEH Phenyl, 100 mm length x 2.1 mm ID x 1.7 µm) and matching guard column (Waters Acquity UPLC BEH Phenyl VanGuard Pre-Column, 5 mm length x 2.1 mm ID x 1.7 µm) maintained at 40°C. Five microliters of sample was injected onto the column using a refrigerated autosampler maintained at 8 °C. Mobile phase A consisted of 1 mM aqueous ammonium acetate (NH₄Ac) + 0.1% acetic acid (HAc), and mobile phase B was ACN. The mobile phase was delivered to the UPLC column at a flow rate of 0.4 mL per min. The gradient elution program is shown below in Table 3.

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>%A (1mM NH₄Ac+0.1%HAc)</th>
<th>%B (ACN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>0.25</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>2.75</td>
<td>2</td>
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</tr>
<tr>
<td>3.00</td>
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<tr>
<td>3.01</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>5.00</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 3: UPLC gradient method used for the chromatographic separation of diclofenac and ketorolac.
In order to keep the MS clean, the divert valve was used to transfer the column effluent to the MS from 1.0 to 3.75 minutes. From 0 to 1.0 and 3.75 to 5.0 minutes, all the column effluent was transferred to waste. The retention times of ketorolac and diclofenac were approximately 1.79 and 2.95 minutes, respectively. The UPLC column effluent was pumped directly without any split into a triple-quadrupole mass spectrometer (Waters Xevo TQD) equipped with a Zspray ionization source which was operated in positive-ion electrospray mode (ESI+) for ketorolac from 1 to 2.50 minutes and negative-ion electrospray mode (ESI-) for diclofenac from 2.51 to 3.75 minutes with both using multiple reaction monitoring (MRM). The parent and product ion transitions for the compounds of interest are shown in Table 4.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Parent Ion (amu)</th>
<th>Product Ion (amu)</th>
<th>Cone Energy (V)</th>
<th>Collision Energy (eV)</th>
<th>Quant/Qual Transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diclofenac</td>
<td>254.0 [M-H]^-</td>
<td>250.0</td>
<td>22</td>
<td>10</td>
<td>Quantifier</td>
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<td></td>
<td>214.0 [M-H]^-</td>
<td></td>
<td>22</td>
<td>18</td>
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</tr>
<tr>
<td>Ketorolac</td>
<td>105.0 [M+H]^+</td>
<td></td>
<td>32</td>
<td>20</td>
<td>Quantifier</td>
</tr>
<tr>
<td></td>
<td>77.0 [M+H]^+</td>
<td></td>
<td>32</td>
<td>36</td>
<td>Qualifier 1</td>
</tr>
<tr>
<td></td>
<td>51.1 [M+H]^+</td>
<td></td>
<td>32</td>
<td>56</td>
<td>Qualifier 2</td>
</tr>
<tr>
<td>Diclofenac-d4 (IS)</td>
<td>254.0 [M-H]^-</td>
<td></td>
<td>20</td>
<td>10</td>
<td>Quantifier</td>
</tr>
<tr>
<td>Diclofenac-d5 (IS)</td>
<td>217.1 [M-H]^-</td>
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<td>20</td>
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<tr>
<td>Ketorolac-d5 (IS)</td>
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<tr>
<td></td>
<td>82.1 [M+H]^+</td>
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<tr>
<td></td>
<td>106.0 [M+H]^+</td>
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<td>34</td>
<td>12</td>
<td>Qualifier 2</td>
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</table>

Table 4: MRM transitions and specific mass spectrometry tuning parameters for the quantification of ketorolac and diclofenac.
Commercial software (MassLynx) was used to analyze the data. Tuning was performed on each analyte by direct infusion of standard solution (0.1 ng/µL) at a rate of 10 µL per min. Mass spectrometer parameters used for the detection of ketorolac and diclofenac are shown in Table 5 below.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
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<tr>
<td>Cone (V)</td>
<td>39 / 21</td>
</tr>
<tr>
<td>RF (V)</td>
<td>2.50</td>
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<tr>
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<tr>
<td>Source Temperature (°C)</td>
<td>150</td>
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<tr>
<td>Desolvation Temperature (°C)</td>
<td>600</td>
</tr>
<tr>
<td>Cone Gas Flow (L/Hr)</td>
<td>10</td>
</tr>
<tr>
<td>Desolvation Gas Flow (L/Hr)</td>
<td>1000</td>
</tr>
</tbody>
</table>

Table 5: Mass spectrometer tuning parameters for the detection of ketorolac and diclofenac. The first number is the ESI+ value followed by the ESI- value. A single value indicates the value was the same for both ESI+-/ modes.

A six-point calibration curve made up in blank AH was prepared in the same manner as the samples but was spiked with a range of approximately 20 to 1,960 ng/mL AH for both ketorolac and diclofenac. A linear calibration curve was constructed for both compounds of interest using the MassLynx software to determine analyte concentration in samples based on the sample / IS ratio. The coefficient of determination ($R^2$) for all curves was >0.999, and all standard values were within ±10% of the expected range. The system had a limit of detection (LOD) of approximately 6 ng diclofenac/mL AH and approximately 0.05 ng ketorolac/mL AH, as determined by the signal-to-noise ratio (S/N = 3), and the limit of quantification (LOQ), determined by the lowest concentration on a linear regression line of the calibration curve, was 20 ng/mL AH for both analytes. The calibration curves used in the diclofenac and ketorolac analyses are shown in Figures 1 and 2 of the appendix, respectively.
Aqueous humor PGE2 concentration evaluation

Aqueous PGE2 concentrations were determined according to the protocol of a commercially available competitive enzyme-linked immunoassay kit (Cayman Chemical #514010) using 25 µl aqueous samples and 25 µL buffer at a dilution of 1:1 for a final volume of 50 µL. The samples were assayed in duplicate. One sample was unable to be assayed in duplicate due to insufficient sample volume. A standard curve was generated with the following concentrations: 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 pg/mL. The plate was incubated for 18 hours at 4°C and then washed 5 times and developed over a period of 80 minutes. The plate was read at an absorbance wavelength of 412 nm. The minimum detectable concentration of PGE2 for this test is reported by the vendor as 15 pg/mL, with a standard range of 7.8-1000 pg/mL. Data analysis and calculations were performed according to the assay booklet protocol recommendations. Dilution of the samples (1:1) was accounted for when calculating final PGE2 concentrations.

Figure 5: Standard curve generated for main study.
**Statistical analysis**

Normal probability plots showed that the primary outcomes (drug concentrations and PGE2 concentrations) were skewed. Accordingly, data were summarized as medians (minimum, maximum). Outcomes (one at a time) were compared between the two treatment groups (ketorolac vs. diclofenac) using the Wilcoxon rank sum test.

Outcomes were also compared between dogs with diabetes and dogs without diabetes using the Wilcoxon rank sum test. Associations between PGE2 concentration and drug concentrations were assessed using scatter plots and analysis of covariance. The linear model specified PGE2 concentration as the outcome and drug concentration, treatment group, and the interaction between drug concentration and treatment as predictors. Associations between PGE2 concentrations and time of sample collection and between drug concentration and time of sample collection were assessed using scatter plots and Spearman’s correlation coefficient. Associations between PGE2 concentrations and number of doses and between drug concentration and number of doses were assessed using the Kruskall-Wallis test (for number of tests with 3 levels) or the Wilcoxon rank sum test (for number of tests with 2 levels). Statistical significance was set to \( p < 0.05 \).

All analyses were performed using SAS version 9.4 (Cary, NC, USA).

**C. Results**

During the study period, a total of 25 dogs presenting to the VTH Ophthalmology service for routine cataract evaluation were deemed candidates for the study. Ultimately, a total of 22 samples were collected from 22 dogs (eyes) and were included for data analysis. The 3 remaining eyes were excluded from the study due to accidental alternations in the pre-operative treatment protocol as follows: treatment with topical
steroids the morning of surgery, treatment with topical steroids the night prior to surgery and a combination thereof. The final study population of 22 dogs included 1 intact female, 9 spayed females, 1 intact male, and 11 castrated males. The mean ± SD age was 9.68 ± 3.51 years (range 4-17 years). Breeds included mixed (n=10), Boston Terrier (n=2), Shih Tzu (n=1), Labrador Retriever (n=1), Miniature Pinscher (n=1), Maltese (n=1), Dachshund (n=1), Weimaraner (n=1), Siberian Husky (n=1), Australian Cattle Dog (n=1), Yorkshire Terrier (n=1), and Saint Bernard (n=1). Cataracts were most commonly categorized as mature (82% or 18/22 eyes) followed by hypermature (18% or 4/22 eyes). Diabetes mellitus was present in 59% of dogs in the study population (13 diabetic and 9 non-diabetic dogs). Of the dogs with diabetes mellitus, two dogs had the concurrent presence of hyperadrenocorticism. Samples were collected from 14 right eyes (OD) and 8 left eyes (OS). No dogs had evidence of lens-induced uveitis upon biomicroscopic evaluation at either the time of the initial evaluation or the drop off appointment for surgery. The median time from last NSAID treatment administration to aqueous humor collection was 50 minutes (range 5-80 minutes). Of the 22 dogs enrolled, 12 dogs were enrolled in the diclofenac treatment group and 10 dogs were enrolled in the ketorolac treatment group. Out of the 12 dogs in the diclofenac treatment group, 8 dogs were diabetics and 4 were non-diabetics. Eight dogs were classified as having mature cataracts and 4 dogs were classified as hypermature cataracts. Out of the 10 dogs enrolled in the ketorolac treatment group, 5 dogs were diabetics and 5 were non-diabetics. All 10 dogs in the ketorolac treatment group were classified as having mature cataracts.
Drug Concentrations

22 samples (12 diclofenac and 10 ketorolac) were analyzed for aqueous humor drug concentration. The median aqueous humor concentration for the diclofenac group was 284.9 ng/mL with a range of 144.3-1202.2 ng/mL. The median aqueous humor concentration for the ketorolac group was 1311.6 ng/mL with a range of 466.0-4176.5. A significant difference was detected between aqueous humor drug concentrations between the two treatment groups (p=0.0017) with the ketorolac group having significantly higher aqueous humor drug concentrations (4.6 times higher) compared to the diclofenac treatment group.

Prostaglandin E2 Concentrations

Due to insufficient volume necessary for proper analysis in 3 samples, only 19 samples (11 diclofenac and 8 ketorolac) were analyzed for aqueous humor PGE2 concentration. The median PGE2 concentration for the diclofenac group was 73.5 pg/mL with a range of 36.8-119.1 pg/mL. The median PGE2 concentration for the ketorolac group was 55.8 pg/mL with a range of 36.6-104.5 pg/mL. There was no significant difference between PGE2 concentrations in the ketorolac and diclofenac treatment groups (p=0.4196).

Associations between PGE2 and drug concentrations

A comparison between PGE2 concentration and drug concentration showed no significant association between PGE2 and drug concentration for the diclofenac group (p=0.0776) or the ketorolac group (p=0.0525).
Diabetic dogs vs. non-diabetic dogs

Associations between diabetic status and PGE2 and aqueous humor drug concentrations were also evaluated overall and between the two treatment groups. Out of the 22 dogs included in determining aqueous humor drug concentrations, 13 dogs were diabetic and 9 were non-diabetic. Out of the 19 dogs included in determining PGE2 concentrations, 11 dogs were diabetic and 8 were non-diabetic. When divided by treatment group, there were 4 diabetic and 4 non-diabetic dogs in the ketorolac treatment group and 7 diabetic and 4 non-diabetic dogs in the diclofenac treatment group. Overall, there was no significant difference between diabetic dogs and non-diabetic dogs with regard to PGE2 concentrations (p=0.5171) or aqueous humor drug concentrations (p=0.645). When evaluated by treatment group, there was no significant difference between diabetic dogs and non-diabetic dogs in the ketorolac group for PGE2 (p=0.4939) or aqueous humor drug concentrations (p=0.425). For the diclofenac group, no significant difference was detected between diabetic and non-diabetic dogs for PGE2 (p=0.9266) or aqueous humor drug concentrations (p=0.4608).

Associations between drug concentration and time of sample collection

Overall, there was no significant difference between drug concentration and time of sample collection (p=0.6825). When evaluated by treatment group, there was no significant difference between drug concentration and time of sample collection for either the ketorolac (p=0.6374) or diclofenac (p=0.7837) treatment groups.

Associations between PGE2 concentration and time of sample collection
Overall, there was no significant difference between PGE2 concentration and time of sample collection (p=0.6322). When evaluated by treatment group, there was no significant difference between PGE2 concentration and time of sample collection for either the ketorolac (p=0.3032) or diclofenac (p=0.8668) treatment groups.

**Associations between drug concentration and number of doses**

Overall, there was no significant difference between drug concentration and total number of doses administered (p=0.2422). When evaluated by treatment group, there was no significant difference between drug concentration and total number of doses administered for the ketorolac (p=0.3857) or diclofenac (p=0.5233) treatment groups.

**Associations between PGE2 concentration and number of doses**

Overall, there was no significant difference between PGE2 concentration and total number of doses administered (p=0.0855). When evaluated by treatment group, there was no significant difference between PGE2 concentration and total number of doses administered for the ketorolac (p=0.1773) or diclofenac (p=0.5113) treatment groups.

**D. Discussion**

This study is the first to investigate the efficacy profile of topical NSAIDs in a naturally occurring model of intraocular inflammation. It has been previously reported that aqueous humor PGE2 concentrations in dogs with mature and hypermature cataracts were significantly elevated compared to dogs without cataract, although PGE2 concentrations in these cataract stages were not significantly different from each other.64 As such, dogs presenting for cataract surgery with the presence of mature or hypermature cataracts were utilized in this study as a naturally occurring model of inflammation.
against which the efficacy of topical NSAIDs could be evaluated without resorting to the use of experimental animals or conditions. In addition, cataracts in the dog are a common clinical condition requiring anti-inflammatory treatment in order to prevent clinical complications of lens-induced uveitis as well as to control pre- and post-operative inflammation related to cataract surgery. Overall, topical ketorolac and diclofenac were determined to have comparable anti-inflammatory efficacy, while ketorolac had increased aqueous humor drug concentrations when compared to diclofenac. Although significantly higher aqueous humor drug concentrations were determined in the ketorolac treatment group in this study, differences in commercial drug concentrations between the two drugs must be taken into consideration. The ketorolac solution utilized in this study was the 0.5% concentration, whereas the diclofenac used was a 0.1% concentration. As such, the 4.6 fold increase in aqueous humor drug concentration seen with ketorolac treatment is expected given the known factors that influence ocular drug penetration including drug concentration, pH, size of the molecule, and the drug’s ability to reduce surface tension. It can also be concluded, as evidenced by the difference in aqueous humor concentrations between the two drugs, that drug penetration across the cornea did not reach the point of saturation at the concentrations used in this study. In addition, the drug concentration values obtained in this study differ from previously reported values, which are widely variable. For example, studies evaluating human patients in which cataract surgery was performed found a mean aqueous humor concentration of 130.5 +/- 37.8 ng/mL and 95 ng/mL (range 40 to 170 ng/mL) for ketorolac 0.4% and ketorolac 0.5% respectively which is substantially lower compared to the results of this study (range 466.0-4176.5 ng/mL). Another study performed in rabbits with experimentally
induced uveitis, determined a mean peak aqueous humor concentration for ketorolac 0.45% of 737.8 +/- 64.7 ng/mL and trough concentrations of 127.0 +/- 18.9 ng/mL. The peak concentrations of this study are more consistent with the findings of the current study. When comparing diclofenac, one study found a peak aqueous humor concentration of 82 ng/mL with concentrations remaining greater than 20 ng/mL for over 4 hours.

Another study directly compared the penetration of diclofenac and ketorolac into the aqueous humor in human patients with cataract and found a mean diclofenac concentration of 4.98 ng/mL and a mean ketorolac concentration of 20.17 ng/mL. Both of these studies demonstrate substantially lower aqueous humor diclofenac concentrations compared to the results of this study (range 144.3-1202.2 ng/mL).

Differences in drug kinetics, especially considering species variation, differences in dosing protocols, and/or variation in sample collection times relative to the last NSAID dose likely influence the variation in these concentrations. Determining the influences of these factors can be better investigated by developing a better understanding of drug kinetics for the medication in use for a particular species and by standardizing NSAID treatment times and doses relative to collection times.

In the current study, ketorolac and diclofenac were demonstrated to have comparable anti-inflammatory efficacy as measured by aqueous humor PGE2 concentrations via ELISA. Median aqueous humor PGE2 was determined to be 73.5 pg/mL with a range of 36.8-119.1 pg/mL for diclofenac and 55.8 pg/mL with a range of 36.6-105.4 pg/mL for ketorolac. Based on the previously mentioned study, which established and compared aqueous humor PGE2 concentrations in dogs with mature (378.40 +/- 140.50 pg/mL) and hypermature (442.50 +/- 213.00 pg/mL) cataract to dogs
without cataract (5.98 +/- 1.41 pg/mL), our results indicate that ketorolac and diclofenac work to substantially minimize intraocular inflammation, but do not completely eliminate it. However, there are limitations in applying a historical control as a basis for comparison in the current study. For example, a relatively small sample size was utilized in the aforementioned study, and as such, it is possible that PGE2 concentrations vary from the reported values. In addition, the above study only included dogs ranging from 7-10 years of age, did not include diabetic patients, and included dogs described as having mild signs of lens-induced uveitis, which may have impacted the results of their study. In our study, aqueous humor samples were not collected as a comparative baseline prior to initiating treatment, as it would violate patient care standards. In addition, while this would have allowed for a comparison of pre-treatment aqueous humor PGE2 concentrations to the post-treatment PGE2 concentrations, anterior chamber paracentesis is known to induce blood aqueous barrier breakdown and uveitis, which would confound subsequent evaluation of treatment response.

Alternatively, laser flaremetry is a noninvasive method which would have allowed for objective quantification of inflammation both before and after sample collection. This technique quantitates the level of aqueous humor protein by measuring photon counts of scattered light, which is proportional to the amount of protein in the anterior chamber. Unfortunately due to equipment limitations, this technique was not an option in this study. Aqueous humor samples were also not collected post-operatively or in the early post-operative period and as such NSAID effect was measured only on basal PGE2 levels. One study performed in an experimentally induced model of inflammation in rabbits found that both ketorolac and bromfenac effectively suppressed inflammation at
peak but that only ketorolac effectively suppressed inflammation at trough. It was proposed that the difference in inflammation suppression found could potentially be due to differences in tissue concentrations or greater COX-1 suppression by ketorolac as ketorolac is known to have potent, selective COX-1 inhibition, whereas diclofenac is classified as a non-selective COX inhibitor. In the future, more information could perhaps be gathered by evaluating peak and trough anti-inflammatory concentrations to allow for the most efficacious use of these medications.

Previously performed studies in other species have compared the relative efficacy of ketorolac with other topical NSAIDs and have found evidence of superior PGE2 inhibition by ketorolac compared to others. One study in human patients undergoing cataract surgery found ketorolac to have better anti-inflammatory efficacy as evidenced by lower PGE2 levels compared to treatment with nepafenac. Another study found a twenty-fold higher aqueous humor concentration and greater PGE2 inhibition in human cataract surgery patients treated with ketorolac compared to another potent topical NSAID, bromfenac. Few studies have directly compared the anti-inflammatory effects of ketorolac and diclofenac as in the current study. However, one study found ketorolac to have greater anti-inflammatory effects than diclofenac when using rabbits as an animal model of ocular inflammation. In contrast, a study performed in human patients found ketorolac and diclofenac to be equally effective for the control of postoperative inflammation after uncomplicated cataract surgery. However, evaluation of inflammatory mediators such as PGE2 was not performed in this study and only objective and subjective measurements of inflammation were made using laser flaremetry and slit lamp biomicroscopic evaluation. It should be noted that these aforementioned studies
were performed in human patients and in experimental rabbit models and that drawing accurate comparisons regarding pharmacologic profiles cannot necessarily be made across species, as anatomic and physiologic differences must be considered. This is particularly true regarding corneal epithelial thickness, surface area, and molecule transporters. COX expression is also variable across species under normal and pathologic conditions. For example, expression of COX-1 in the anterior uvea is variable among species in the following descending order: cat, rabbit, dog, human and cow. When COX-2 is expressed, it too is variable among different species with COX-2 expression being present in the iris and ciliary body of normal rabbits but not in the cornea or anterior chamber of normal dogs. In humans, COX-2 expression is largely confined to the nonpigmented epithelium of the ciliary body, but in normal dogs, only minimal COX-2 expression is noted in the ciliary epithelium. Additionally, species-specific efficacy of COX suppression of a given NSAID must be taken into consideration. As such, further studies of topical NSAID efficacy are necessary in the dog as species-specific information is sparse or non-existent.

No significant association was found between drug concentration and PGE2 levels in the current study. Therefore, higher concentrations of drug found in the aqueous humor may not necessarily correlate with better anti-inflammatory effect, contrary to previous studies indicating superior corneal penetration and anti-inflammatory effect of ketorolac compared to other topical NSAIDs such as bromfenac and nepafenac. However, it is possible that higher concentrations of the drug may have better sustained efficacy of reduced prostaglandin levels compared with those of lower concentrations. Although a statistically significant association was not determined between drug and
PGE2 concentrations, p values reached near significance, particularly regarding the ketorolac treatment group (p=0.0525). Based on scatter plot analysis, ketorolac showed a trend towards higher concentrations being associated with increased levels of PGE2. This is an unexpected finding and is contrary to what would generally be expected regarding higher concentrations of a drug and its association with greater anti-inflammatory effect. Intraocular inflammation may affect ocular pharmacokinetics of topical medications, however, and it is possible that more inflamed eyes may be associated with increased corneal permeability. However, it has been previously determined that levels of diclofenac in the aqueous humor of uveitis induced rabbits were lower compared to controls which refutes this theory.166 Alternatively, higher amounts of drug in the eye may produce increased inflammation. To the authors’ knowledge, there are no studies in which ketorolac was directly compared to diclofenac to evaluate these factors.

In addition, no significant association was found in diabetic dogs when evaluating drug and PGE2 concentrations. The relatively small sample size in this study population may have influenced these results. The results of this study suggest that underlying systemic diseases such as diabetes mellitus do not influence aqueous humor drug penetration or anti-inflammatory effects of topical ketorolac or diclofenac. Although not specifically evaluated in this study, two of the enrolled dogs were concurrently affected with hyperadrenocorticism. Hyperadrenocorticism results in an overproduction of cortisol, which can result in the reduction of vasodilatory prostaglandins.167 As such it is plausible to question whether these animals had reduced levels of baseline intraocular inflammation compared to the other study candidates which could have influenced our results. Of the two dogs in the study with diagnosed hyperadrenocorticism, one dog was
in the ketorolac treatment group while the other was in the diclofenac treatment group. The aqueous humor PGE2 concentrations of these two dogs were 36.6 pg/mL and 97.0 pg/mL respectively. Interestingly, the dog in the ketorolac group had the lowest PGE2 concentration when compared to the remainder of the dogs in that treatment group (range 36.6-104.5 pg/mL). However, the dog in the diclofenac group demonstrated the third highest PGE2 level when compared to the remainder of dogs in the diclofenac treatment group (range 36.8-119.1 pg/mL). The aqueous humor drug concentrations in these dogs were 759.02 ng/mL and 191.92 ng/mL for the ketorolac and diclofenac treatment groups respectively. The dog in the ketorolac group (range 466.0-4176.5 ng/mL) had the fourth lowest drug concentration and the dog in the diclofenac group (range 144.3-1202.2 ng/mL) had the third lowest concentration when compared to the remainder of the group. Due to the very small sample size of dogs with hyperadrenocortism in this population of dogs, no conclusions based on these findings can be made. Patients with diabetes mellitus have been reported to be at an increased risk of comorbidities and in one study, 23% of dogs with diabetes (n=211) had concurrent hyperadrenocorticism. As such, it is also possible that additional dogs in this study may have been concurrently affected with undiagnosed hyperadrenocorticism due to the similar clinical and laboratory findings that occur with hyperadrenocorticism and diabetes mellitus.

Several study limitations must be addressed. A primary limitation of this study design is the inability to utilize a completely untreated control group. However, patient care standards mandating the use of topical anti-inflammatories in the immediate pre-surgical treatment period precluded the inclusion of untreated controls. Topical steroids are routinely utilized in conjunction with topical NSAIDs in the immediate pre-operative
period in dogs undergoing cataract surgery. Their use, however, was eliminated from the current study to eliminate any associated influence on PGE2 concentrations. As such, if topical NSAIDs were eliminated in the pre-operative period to utilize a control group, topical steroids would be required as a substitute, which considering their effects on PG synthesis would render the comparative data unstable.

The variability between the time of the last dose of NSAID and the time of aqueous humor sample collection is also a limitation of this study. Ideally, this timing would be perfectly standardized, but due to variation in anesthetic and pre-operative care demands, this would require removing fluid from the eye prior to surgical entry, which is not ideal from a patient care perspective and as such was not performed. Peak concentration in the aqueous humor has been reported to occur 0.5 to 3 hours after instillation of a topically applied medication in the dog. In humans and rabbits, peak aqueous humor concentrations of ketorolac have been reported 1 hour after administration with detectable levels remaining at least 6-11 hours after administration respectively. Peak aqueous humor concentrations of diclofenac have been reported approximately 2.5 hours after administration remaining detectable for 24 hours after application. Given this, it is possible that aqueous humor drug concentrations were affected for those dogs who received the last NSAID dose outside of this time range, although most dogs received multiple doses in the two hour window prior to sample collection making this an unlikely influence. Aqueous humor drug kinetics also likely influenced these findings. Once in the anterior chamber, ocular medications are eliminated mostly by aqueous humor turnover, which is estimated to be approximately 5µL/min in dogs and 1.5-5µL/min in humans. Therefore, differences in aqueous humor
turnover such as increased outflow in uveitic states may affect drug concentrations due to more rapid clearance of the drug. In contrast, topical NSAIDs have also been associated with the ability to decrease aqueous humor outflow facility, which could perhaps lead to longer drug retention. The dogs included in this study also received treatment with dorzolamide 2%/timolol 0.5% prior to sample collection which may have also influenced aqueous humor kinetics and subsequent drug retention due to the ability of these medications to alter and slow aqueous humor outflow. When evaluating the effect of time between the last NSAID dose and sample collection in this study, no statistically significant difference was determined for either drug concentration or PGE2 concentration.

The variability in the number of preoperative doses of the assigned NSAID may have influenced results as prolonged and more frequent administration of NSAIDs have been demonstrated to lead to higher aqueous humor levels. When evaluating the effect of drug concentration and PGE2 concentration and the number of pre-operative doses in the current study, no statistical significance was determined. Ideally, it would have been standardized for all dogs to receive the same number of treatments, however, due to variations in pre-operative and anesthetic demands, this was difficult to perform. Although no statistically significant difference was determined when evaluating this factor, this may have been attributed to the small sample size in this study and future studies should aim to standardize treatments to eliminate this variable.

Variability in baseline ocular inflammation is also a limitation as it was unable to be objectively measured in this study. However, measures were taken to prevent and eliminate extraneous factors from the study population that could have influences on
levels of inflammation. For example, dogs that were treated with topical or systemic
NSAIDs or steroids at the time of evaluation were excluded from the study. In addition,
all dogs included in the study were confirmed to have the absence of lens-induced uveitis
as evidenced by the absence of flare, cell and keratic precipitates; however, these are all
subjective assessments rendered via slit-lamp biomicroscopy. As such, more objective
measurements of uveitis severity such as laser flaremetry and slit lamp fluorophotometry
would have provided a more quantitative and accurate assessment of the intraocular
inflammation. Ultimately, objective, pre-operative assessment of baseline intraocular
inflammation was not possible due to the inability to obtain an aqueous humor sample
prior to treatment, which would violate patient care standards, as well as due to
equipment limitations and availability at this particular institution. It has been previously
reported in rabbits that PGE2 levels do not demonstrate diurnal variation unlike other
prostaglandins and as such, assuming a similar phenomenon occurs in dogs, samples
collected at various time points throughout the day should not have an effect on baseline
PGE2 concentrations. We suspect that any variation in baseline ocular inflammation
was unlikely to affect our results or conclusions as this variation is likely randomly
distributed between treatment groups. Sample size is one of the greatest limitations in this
study. The relatively small sample size in this study may account for the fact that no
significant differences were determined PGE2 concentrations between the two treatment
groups. Unfortunately, due to the rising frequency and popularity of pre-surgical
treatment with topical NSAIDs by the referring veterinary community prior to referral for
cataract surgery, many patients presenting to the VTH for routine cataract evaluation
were currently receiving topical NSAIDs at the time of evaluation and as such were
excluded from enrollment. Only 19 samples were evaluated for PGE2 concentrations compared to 22 samples that were evaluated for aqueous humor drug concentrations due to insufficient aqueous humor volume available in 3 samples, which contributed to a smaller sample size when evaluating anti-inflammatory effect. Additionally, one sample was unable to be run in duplicate when performing analysis via ELISA. When performing ELISA, it is recommended to test each sample in replicates to minimize the variability within the assay and so it is possible that the accuracy of this particular sample is skewed. However, it is unlikely for that single sample’s results to significantly affect the overall conclusions and results of this study. In addition, when evaluating aqueous humor drug concentrations in the diclofenac treatment group, an outlier was identified that demonstrated substantially higher drug concentration when compared to the remainder of the diclofenac values. Despite the significantly higher drug concentration, the corresponding aqueous humor PGE2 level in this sample was the second highest out of all the dogs in the study (118.76 pg/mL). In addition, visible precipitation following ACN extraction during UPLC/MS-MS evaluation was identified, likely indicating higher protein or the presence of more severe inflammation in this sample. This finding further supports the notion that higher concentrations of drug found in the aqueous humor may not necessarily correlate with better anti-inflammatory effect. Because a significant difference was ultimately determined in aqueous humor drug concentration between the two treatment groups, it is not likely for this outlier to influence the results and conclusions of this study.
CHAPTER 4: CONCLUSION AND FURTHER RESEARCH

Treatment with topical ketorolac 0.5% and diclofenac 0.1% ophthalmic solutions both demonstrated similar anti-inflammatory efficacy, although treatment with ketorolac resulted in increased aqueous humor drug concentrations. Furthermore, no significant association was determined between drug concentration and PGE2 concentration. As current decisions regarding topical anti-inflammatory use is oftentimes based upon factors such as cost and ease of acquisition, this study indicates that topical ketorolac and diclofenac are equally suitable for use in veterinary medicine based on their comparable anti-inflammatory profiles. The results of the assays in this study provide clinically relevant information regarding relative intraocular penetration and anti-inflammatory efficacy of these medications in a naturally occurring model of uveitis in dogs with cataract. This model is a novel one and may be suitable to future studies evaluating other ophthalmic anti-inflammatory agents.

Despite our results, larger studies and further investigation in other domestic species is warranted, as comparisons cannot be made across species due to the host of species differences that must be considered when extrapolating pharmacologic information from one to another. Future research might also evaluate anti-inflammatory efficacy on the basis of alternative mediators of inflammation as well as in models using techniques to objectively measure baseline intraocular inflammation as opposed to subjective methods. Due to the few comparative studies of topical NSAIDs in veterinary medicine, particularly in the dog, future studies are warranted to evaluate and compare other commonly utilized topical NSAIDs to allow for the most effective and judicious treatment planning for our patients.
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APPENDIX: FIGURES

Figure 6. Linear calibration curve used in the analysis of diclofenac in aqueous humor. It should be noted that the concentration range on the x-axis is in ng diclofenac / mL solution. This is easily converted to AH concentrations by multiplying by the 5x dilution, the final extract volume (0.4 mL) and then dividing by the initial AH sample volume (0.1 mL).
Figure 7. Linear calibration curve used in the analysis of ketorolac in aqueous humor. It should be noted that the concentration range on the x-axis is in ng diclofenac / mL solution. This is easily converted to AH concentrations by multiplying by the 5x dilution, the final extract volume (0.4 mL) and then dividing by the initial AH sample volume (0.1 mL).
Figure 8: Median and range of aqueous humor drug concentrations (ng/mL) of diclofenac and ketorolac.
Figure 9. Median and range of aqueous humor PGE2 concentrations (pg/mL) between diclofenac and ketorolac.
Figure 10: Association between PGE2 concentrations (pg/mL) and drug concentrations (ng/mL) for diclofenac and ketorolac.