Sustained-Release Voriconazole-Thermogel for Subconjunctival Injection in Horses: Ocular Toxicity and *in-vivo* Studies

By

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Abstract

Subconjunctival (SC) injection of a thermosensitive voriconazole-hydrogel poly (DL-lactide-co-glycolide-b-ethylene glycol-b-DL-lactide-co-glycolide) (PLGA-PEG-PLGA) may allow for sustained delivery of voriconazole to the anterior segment of the eye. Equine corneas were exposed to voriconazole thermogel (1 and 5 mg), plain thermogel and phosphate buffered solution (PBS) using a Franz Cell diffusion chamber, and analyzed by histology. At 2 hours, no difference was found between treatments for corneal epithelial and stromal thickness. Artifactss at longer time points precluded analysis. Six horses received 1% topical voriconazole or 1.7% subconjunctival (SC) voriconazole-thermogel. Using a Hackett-McDonald scoring system for inflammation, there were changes in conjunctival swelling and congestion following injection, but no signs of ocular pain. On day 2, drug concentration in tears was not different between groups. For the thermogel group, voriconazole was not detected in the aqueous humor (AH). Three horses received a 1.7% SC voriconazole-thermogel injection 48 and 2 hours prior euthanasia; voriconazole concentrations were above the target minimum inhibitory concentration (MIC) in the tissues of the anterior segment of the eye. Voriconazole-containing thermogel was easy and safe to administer in horses with no adverse effects. The thermogel provided sustained release of voriconazole, and the high lipophilicity and volume of distribution of the drug enhanced the distribution to the ocular tissues, thus making this drug delivery system of potential importance for the treatment of equine keratomycosis.

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List of Abbreviations

AH   Aqueous humor

ATP   Adenosine triphosphate

CBC   Complete blood count

DLEK   Deep lamellar endothelial keratoplasty

FL   Fluorescein leakage

HE   Hematoxylin and eosin

HPLC   High performance liquid chromatography

MIC   Minimum inhibitory concentration

MW   Molecular weight

PBS   Phosphate buffered solution

PCR   Polymerase chain reaction

PEG   Poly(ethylene glycol)

PLGA   Poly(lactic-co-glycolic acid)

PLK   Posterior lamellar keratoplasty

SBA   Serum biochemical analysis

SC   Subconjunctival

SEM   Standard error of the mean

SPL   Subpalpebral lavage system

TG   Thermogel

USP   United States Pharmacopeia

VOR1   1 mg voriconazole thermogel

VOR5   5 mg voriconazole thermogel

Vori-Top   Topical 1% voriconazole solution

Vori-Gel   1.7% voriconazole thermogel SC injection

Chapter 1 – Literature review

Section 1: Definition and Pathogenesis of Equine Keratomycosis

Keratomycosis (corneal fungal infection), also known as fungal keratitis, is a relatively common, sight threatening condition in horses with several clinical presentations, including ulceration with or without keratomalacia, fungal plaque, microerosions, and stromal abscess. [1] This condition occurs with higher frequency in horses compared with other mammals, particularly in warm and humid climates such as the southeastern United States. Areas of the mid-Atlantic United States have higher incidence of this condition during the summer months. [2] The incidence of fungal ocular infections has been increasing in America, from 13% of keratitis cases in the 40 years preceding 2006 to 25% in 2013. [3,4] Several factors contribute for the high incidence of fungal keratitis in horses, including the exposure to vegetative material, presence of fungal organism in the normal ocular surface, [5] large and prominent eyes, and their unpredicted behavior. [6,1]

Any disruption of the natural defense mechanisms of the cornea, such as the intact corneal epithelium, normal bacterial flora, eyelid movement, or tear film macrophages, lysozymes and immunoglobulins, with subsequent exposure of the stroma, is the starting point for the development of keratomycosis. [7-9] More than 30 genera of fungi have been implicated in equine keratomycosis, with the most common

being the filamentous fungi *Aspergillus*, *Fusarium* and *Penicillium*, and the yeast *Candida*. [3,6,9,10]

Fungal agents are present in the equine environment, and are also isolated from normal equine cornea and conjunctiva. [5,9] Fungal organisms, damaged keratocytes and inflammatory cells attracted to the area by the fungi then release proteases which facilitate vertical movement of fungi through the corneal stroma and Descemet's membrane. Final migration into the anterior chamber, lens, and iris causes severe endophthalmitis. [11] From the different clinical presentations of fungal keratitis in horses, stromal keratomycosis is the most common, comprising 50-80% of reported cases. [11] If left unattended, this condition could lead to severe ocular complications such as melting process or keratomalacia, stromal abscess formation, severe anterior uveitis, endophthalmitis, or corneal perforation. [1,3,11] Diagnosis is based on cytology, fungal culture, histopathology, polymerase chain reaction (PCR) and *in vivo* confocal microscopy. [3,8,12,13]

A guarded prognosis for vision is advisable for equine patients with keratomycosis due to the risk of extensive corneal scarring, uncontrollable uveitis, keratomalacia and secondary corneal perforation which can occur despite aggressive treatment. [14] Moreover, if treatment is discontinued prematurely, recurrence is not uncommon, necessitating longer and more frequent medication. [12] Due to treatment failure, some cases may progress to deep ulceration that often times requires surgical intervention, thus increasing cost and hospitalization time. [2,12] If clinical situation is worsening

despite the use of intensive treatment, surgical therapy may be used including conjunctival grafts, lamellar keratectomy, posterior lamellar (PLK) or deep lamellar endothelial keratoplasty (DLEK) and penetrating keratoplasty combined with the use of grafts with natural (autologous cornea, amnion) or synthetic/biosynthetic (Vet BioSISt™, ACell™) tissues. [15] In the worst case scenario, ocular perforation may occur, necessitating enucleation. [16] In a report of cases of equine keratomycosis in a hospital, ocular survival was achieved in 70% of the eyes treated. [16] Among various reports, visual outcome ranges from 53% to 90%, and will depend on the stage of the corneal lesion, the degree of uveitis and destructive capability of the fungal organism. [8,16-18] However, a recent work showed that genus of fungus cultured was not significantly associated with the need for surgical intervention nor was it significantly associated with the necessity of globe-sparing surgery versus enucleation. [19] Cases that present with severe melting ulcers and intense neovascularization had worse prognosis than superficial ulcers or fungal plaques. [16] Following recovery from corneal disease, it is common some degree of corneal opacification, which may affect vision and client compliance. [12] Generally, better response and outcome to treatment is obtained when aggressive treatment is begun in the early stages of the disease. [8,16]

Mycotic keratitis is an often severe disease in which diagnosis can be challenging, response to medical therapy slow, and outcome poor with severe ocular morbidity and vision loss, [20] being a major concern in human and veterinary ophthalmology.[6,21-23] In addition to benefits to veterinary medicine, investigation into new treatment

modalities in horses may influence development of parallel human therapies and benefit people with this disease. Current treatment protocols for equine keratomycosis are lengthy, expensive, and labor intensive. [4] Keratomycosis can cause impaired vision and lead to total blindness if not diagnosed and treated effectively. [6,8,21,22]

Section 2: Diagnosis and Treatment of Equine Keratomycosis

Consequences of mycotic keratitis in horses are potentially severe leading to progressive ulceration and perforation of the cornea. Thus, prompt identification of fungal etiology followed by appropriate therapy is desirable. Cytological samples are easily collected during examination, and even though they don't provide a specific diagnosis, if active ocular disease is present, the presence of fungal hyphae is the most unequivocal evidence for the role of that agent in the pathogenesis. [3,1,21] A positive fungal culture is considered the gold standard for diagnosis of equine fungal keratitis. The use of quantitative polymerase chain reaction (PCR) for the most common fungi implicated in equine keratomycosis has been described in addition to other diagnostic tests as well as *in vivo* confocal microscopy or histopathology if corneal specimen is available. [3,13]

Treatment success in cases of keratomycosis greatly depends on the stage of the corneal disease, amount or presence of uveitis, and the use of an appropriate antifungal agent. [2,6,8] Keratomycosis treatment is often prolonged and expensive, requiring compliance from horses and their handlers. [24] Even if the condition has been

controlled, many horses stay in the hospital due to inability to be treated at home, which adds a considerable stress to the animal, and has been established as a risk factor for development of colic. [2,25] The treatment of keratomycosis in horses also has an economic impact, since many animals are not allowed to perform while on therapy, especially if a subpalpebral lavage system (SPL) is in place. [2]

The ultimate goal in the treatment of fungal keratitis is to preserve vision and conserve the eye. Fungal keratitis can be treated with either medical therapy, surgical intervention or a combination of both. [11,26] While surgical procedures are more indicated in patients with acute corneal perforations, ruptured stromal abscesses or deep corneal defects, antifungal agents are still the major medical therapeutic option. Effectiveness of antifungal therapy depends on the agent's ability to penetrate the cornea and aqueous humor and achieve therapeutic concentrations. [27] The use of eye drops instilled into the conjunctival *cul-de-sac* results in extensive drug loss due to tear nasolacrimal drainage, tearing reflex and blinking movement, consequently, very small amount (1–6%) actually reaches in intraocular tissues. [23] This resultant in low ocular bioavailability following topical eye drops is influenced by the ocular barriers, which if intact, can further reduce the amount of drug available for effective ocular absorption. [28,29] Ocular barriers can be static including the tight epithelial junction of the cornea and conjunctiva, nasolacrimal drainage; or dynamic, such as tear turnover and systemic absorption through the conjunctival blood flow and lymphatic clearance mechanism. [29,30] To overcome this, frequent instillations are required typically every 1-2 hours for acute disease in horses, with potential concurrent administration of oral

antifungals, SC and/or corneal intrastromal injections. [11,20,31-33] Regardless of the initial therapeutic protocol, treatment for keratomycosis is frequently necessary for weeks to months. [15] Two main classes of antifungal medications are used in the treatment of keratomycosis: the azoles and the polyenes. [34] Fungicidal activity of most antifungal drugs is concentration dependent. However, the high fungicidal concentrations of these drugs in the horse cornea are difficult to reliably achieve in practice, and many agents, are generally considered to exhibit fungistatic activity only. [15,35]

Polyene antibiotics (natamycin and amphotericin B) are antifungal drugs with broad spectrum activity, which acts by disruption of the membrane barrier. [35] The fungi cells bind polyenes to ergosterol in the plasma membrane, resulting in altered membrane permeability and inhibition of cytochrome P-450 and the electron transport chain. [35] Polyenes have affinity to cholesterol in mammalian cells, thus they are considered to have toxic effects especially when given systemically, being nephrotoxicity the most common complication. To avoid this, diluted amphotericin B is often used by SC injection, however local irritation, and reversible iritis and cloudiness of the lens have been reported. [35] Azoles are the most widely used antifungal agents in horses, and are divided into imidazole's (miconazole and ketoconazole) and triazoles (fluconazole, itraconazole and voriconazole); they are fungistatic by inhibition of ergosterol biosynthesis and the disturbance of lipid organization in cell membranes. [11,35] Azoles inhibit the fungal cytochrome P450 3A enzyme lanosterol 14-α-demethylase. [35,36] The prevention of conversion of lanosterol

to ergosterol causes disruption of fungal cell membranes, thus increasing permeability. Azoles have a broad spectrum of activity against yeasts and filamentous fungi, and side effects are less common than with other antifungal groups. [35] Voriconazole is a recent antifungal in the azole group, and has shown to effectively penetrate the equine eye after topical administration, as well as to have good *in vitro* activity against *Aspergillus* and *Fusarium* spp. [11,36]

Section 3: Voriconazole Use in Treatment of Keratomycosis

Based on *in vitro* studies, voriconazole, a second generation triazole antifungal drug appears to be the most effective antifungal for initial treatment of keratomycosis in the midwestern and southern United States. The effectiveness is due to its wide spectrum of antifungal activity, low MIC against common pathogens, and excellent corneal penetration. [11,27,36,37]

Voriconazole has a large volume of distribution and is highly lipid-soluble with excellent tissue penetration. [38] The use of voriconazole at 4 mg/Kg orally once daily achieved concentrations above 0.5 μg/mL in body fluids including the AH. [38] In the ranges of MICs for filamentous and yeast organisms, most isolates are < 0.5 μg/mL. [11] Occasional isolates, particularly for *Fusarium* spp., can yield MIC values > 8 μg/mL. [39]

Voriconazole has a broad spectrum of antifungal activity against most filamentous fungi, including some isolates of *Aspergillus* spp. [11,39] In a recent study of *Aspergillus*

spp. and *Fusarium* spp. isolates from equine keratomycosis cases in the Midwestern and southern United States, all isolates were significantly more susceptible to voriconazole than to natamycin, itraconazole, fluconazole and ketoconazole. [36] These *in vitro* findings are supported in the literature, with voriconazole now recommended as a first line agent for treatment of keratomycosis. [32,36,37,40]

For topical ocular administration of 1% voriconazole for the treatment of keratomycosis in horses, the intravenous lyophilized formulation is used (extralabel). This formulation is solubilized by hydroxypropyl-B cyclodextrin sodium, forming a homogeneous solution, which has been proven to be stable for over 30 days. [39,41] However, due to the lack of preservatives, caution must be taken to prepare this formulation under aseptic technique. Voriconazole in horses is often used topically either by direct instillation or by the use of a SPL system, and in many occasions depending on the severity of the disease, frequent administrations are required. Application of 1% voriconazole topically every 4 hours in horses achieves therapeutic concentrations in AH with no adverse effects; [11] and is a common protocol used to manage clinical cases of keratomycosis in horses.

Alternate routes of administration of voriconazole in horses include intrastromal and subconjunctival injections. [31,32] Additionally in humans, ophthalmic medications are also applied intracamerally directly in the anterior chamber of the eye. [42] Intrastromal injections of voriconazole are the current treatment of choice for fungal stromal abscesses that failed to respond to traditional medical therapy or as part of initial

treatment to increase drug levels within the cornea. Volumes of 0.2 mL of 1% or 5% voriconazole solution, in 3 different sites have been injected in the cornea of horses with no significant complications reported. [31,32] In refractory cases to the conventional therapy of topical voriconazole, treatment success was documented using concomitantly intrastromal and SC injections of 1% voriconazole solutions. [32] Intracorneal injections of voriconazole for the treatment of equine keratomycosis proposed several benefits that include achieving higher concentration of drug within the cornea, mechanical disruption and thinning of purulent debris, and stimulation of the corneal immune defenses through disruption of corneal architecture, however, the efficacy and safety of this route has not been assessed objectively. [15,31]

The SC route of drug administration provides transient high and prolonged drug concentration to the anterior segment of the eye, including the corneal stroma and AH compared with topical administration. [32,43] Subconjunctival injection of a water-soluble drug can provide therapeutic levels for 8 to 12 hours. [40]

The systemic use of voriconazole for treatment of fungal keratitis in horses has its limitations. Due to the horse's size, the intravenous use is often cost prohibited, and bot the intravenous and oral route, have to potential for drug accumulation, thus chronic use could be a potential for adverse effects as described in humans. [39] Novel routes of administration and drug delivery systems to the eye warrant investigation for the treatment of equine keratomycosis, with the objective of achieving therapeutic voriconazole levels in the eye with minimal systemic adverse effects.

Section 4: Ocular anatomy and physiology: Drug Diffusion to the Eye

An understanding of the ocular anatomy and physiology is key to design of effective drug delivery systems. The equine cornea is composed of four anatomically distinct layers, with a total central corneal thickness measured using confocal microscopy, of 835 µm (725–920 µm). [7,15] The epithelium, with its respective basement membrane, is the most outer layer of the eye, which comprises a non-keratinized stratified squamous epithelium that forms a relatively impermeable layer, mainly because of its lipoidal nature. [32,44] In contrast to hydrophilic drug, in average, the corneal epithelium accounts for only 10 percent of the resistance to lipophilic drug permeation since these drugs can penetrate the epithelium easily via the transcellular (intracellular) pathway. [45] The stroma is the thicker layer of the cornea and is made up of a parallel lamellar array of type I collagen fibrils within an intensely hydrophilic proteoglycan substance. The stroma is 75-80% water in horses, making it a strong barrier to lipophilic molecules. [44] Underneath the stroma, there is a limiting membrane called the Descemet's membrane, composed mainly of type IV collagen fibrils; it is elastic and resistant to enzymatic and chemical hydrolysis. Finally, the endothelium is a permeable monolayer of hexagonal cells, which is responsible for preservation of normal stromal hydration. [15] Knowing these characteristics, the ideal drug to penetrate the cornea to the anterior chamber should have both lipophilic and hydrophilic properties. The scleral stroma has a greater degree of fibrillary interweave than the corneal stroma which offers less resistance to permeability of drugs and makes the sclera more permeable to hydrophilic drugs. [46,47] Compared to cornea, transconjunctival route allows passive passage of larger molecules or more

hydrophilic than the transcorneal route because of its diffusely scattered goblet cell population, which contains leakier tight junctions however, once of the limitation of the transconjunctival route is the presence of conjunctival blood flow and lymphatics, which can cause significant drug loss into the systemic circulation. Like the corneal stroma, the main transportation route through the sclera is by passive diffusion through the interfibrillar spaces. [45]

In addition, drugs absorbed via the arterial network of the conjunctiva and sclera enter the ciliary body and uvea directly, resulting in the active substances reaching their site of action with a higher concentration than transcorneal absorption; thus the conjunctiva and sclera could be promising drug absorption targets. [44] From the AH or through the scleral tissue, the drug does have easy access to the anterior uvea (iris and ciliary body). Here the drug may bind to melanin, which may form a reservoir source that gradually releases the drug to the surrounding cells, thereby prolonging the drug activity. [45] The vascular layer or uvea is composed of iris and ciliary body in the anterior segment and choroid for the posterior segment. The blood ocular barriers in the anterior segment of the eye (blood-aqueous barrier) consist of the non-pigmented epithelium of the ciliary body and the iris endothelium. In the posterior segment, the barrier (blood-retinal barrier) will be composed of the retinal pigmented epithelium and vascular endothelium of the retinal vessels. [48]

The ophthalmic artery is a branch of the internal carotid artery. It enters the orbit alongside the optic nerve, and its branches supply the eyeball and extraocular

muscles, primarily the posterior segment. [49] The anterior ciliary arteries will provide blood supply to the anterior segment of the eye, initiating at the conjunctiva and sclera before reaching the uveal vessels, iris and ciliary body. [45] Aqueous humor provides nutrition to the cornea and lens, and is formed from diffusion, ultrafiltration and active secretion by the ciliary body. [45]

Section 5: Ocular drug delivery systems

In ocular drug delivery, the goal is to attain adequate concentrations of drugs at the target tissue, maintenance of these therapeutic levels for a prolonged period of time, and ease and safety of delivery with minimal intervention. [50] When using the topical route, in order to maintain MIC, the agent needs to be frequently dosed resulting in poor patient and owner compliance, as well as substantial fluctuations in drug levels. [51] Placement of a SPL assists with medication delivery, however these systems are expensive and often difficult to maintain in horses. Though the topical and systemic routes are convenient for the anterior and posterior segments respectively, due to lack of adequate bioavailability and failure to deliver therapeutic amounts of drugs, alternative routes of administration or delivery formulations are needed. [46]

As described previously, ocular drug delivery remains a major challenge due to multiple barriers within the eye. [28,30] The use of systemic drugs for the treatment of ophthalmic disease is limited by the ocular barriers or toxic effects since higher doses might be needed to reach therapeutic concentrations. [52,53]

To overcome the challenges of topical eye medications, other routes and formulations for ocular administration of medications have been investigated for horses and used in the clinical setting. The advantage of using other alternatives for drug delivery include higher concentrations in the targeted region and less frequency of treatment. Other routes of administration of ocular drugs include intravitreal, intracameral, suprachoroidal, episcleral, SC, subtenon, intrastromal and retrobulbar. [51] To target the posterior segment of the eye, medications such as anti-inflammatories, anti-angiogenics, antifungals and antibiotics are often used in human and veterinary medicine as intravitreal or suprachoroidal injections. [53,54] In humans, periocular injections (SC, subtenon, episcleral, retrobulbar) are associated with fairly high patient compliance as compared to intravitreal injections.[46] When higher drug concentrations are needed in the anterior segment of the eye, other routes of administration, such as periocular injections and implants, used alone or in conjunction with the topical drops are gaining popularity. Episcleral cyclosporine-loaded silicone implants have been used in horses diagnosed with immune-mediated keratitis to provide a sustained release of the drug to the cornea, avoiding constant topical treatment for prolonged periods of time. [55]

In horses, most specifically for the medical treatment of fungal keratitis, intrastromal corneal injections of 1-5% voriconazole have been used with reported positive outcomes and no significant complications due to the procedure.[31,56] The SC route of administration is often use for delivering antifungal agents like voriconazole and amphotericin B to the anterior segment of the eye, and the use of the former drug

by this route has been proposed to be useful in cases refractory to the conventional topical therapy. [32] Visualization of a hydrophilic contrast agent after subconjunctival injection in live rabbits demonstrated significant penetration into the ciliary body before reaching the anterior chamber. [57] After SC injection in live rabbit different mechanism of drug transport have been proposed including transconjunctival permeation followed by corneal absorption, reflux out of the injection site, and direct penetration through the sclera. [58] Subconjunctival injections can be applied on either the dorsal or ventral bulbar conjunctiva, depending on the ease of access. Although these routes (intrastromal and SC) produce the highest peak concentrations of drugs in the cornea or anterior segment, the major disadvantage is that the peak is often followed by low and persistent trough concentrations, unless given repeatedly and frequently, however, sustained concentrations of drug are definitely needed for full biological effect on the cornea or anterior segment. [58,59]

Depending on the degree of lipophilicity of the drug, there is limited capacity to penetrate corneas with intact epithelium using the topical route, however SC injections, because of direct deposit onto the sclera, offer less resistance to diffusion, resulting in better penetration of the corneal stroma despite not having neovascularization. [59] In human patients, SC administration is well tolerated and considered to be safe. [60] A study in humans showed that SC injection of dexamethasone was the most effective method of delivering of the drug into both the anterior and posterior segments of the eye when compared to the systemic and retrobulbar routes, achieving higher concentrations in the aqueous humor and

vitreous. [61] Challenges of the transscleral route include high choroidal blood flow that can affect drug clearance, and only drugs with molecular weight less than 70 kDa are able to enter the sclera. [62] The diffusion of drugs by the SC route will be limited by the anterior blood-ocular barrier and part of the drug will be lost into the systemic circulation through the conjunctival vascular bed. [58]

In addition to route of administration, new drug formulations that could offer properties such as sustained release are desirable. Continuous drug release would reduce the need for daily administration which could improve client compliance and treatment outcome. Sustained release systems could also be more economical than application of eye drops since smaller amounts of drugs may achieve the same effect since less would be lost. [60]

Implantable and injectable devices can extend the release time up to several weeks or even months. [63] Silicone episcleral implants as a sustained release ocular drug delivery devices have been developed over the past years. To direct drug to the cornea, the implants are placed in the episcleral space and are efficacious in delivering drugs such as cyclosporine for the control of immune mediated keratitis and keratoconjunctivitis sicca in horses. [55,64] These implants are not biodegradable and require surgical intervention for either replacement or removal. A biodegradable gel-containing drug for delivery of medication to the anterior chamber will be easier to implant with no need for removal.

Biomaterials for sustained drug delivery include micro- and nanospheres developed from biodegradable synthetic polymers, which can encapsulate both hydro- and lipophilic molecules. These micro- and nanospheres can be produced from the copolymer poly(D,L-lactide-co-glycolide) (PLGA). [62] In order to increase the low encapsulation efficiency of the micro- and nanospheres, PLGA block copolymers such as poly(D,L-lactide-co-glycolide)-poly(ethylene glycol)-poly(D,L-lactide-co-glycolide) (PLGA-PEG-PLGA) were developed. Hydrogels are triblock PLGA copolymers, and have been developed mainly for biomedical applications. The three dimensional network protect the encapsulated drug from degradation, thus preserving their activity. [62,65] They also have chemical characteristics that allow for the control of their phasic properties at different temperatures, and it is an attractive candidate for drug delivery into small spaces within the body, specifically, the ocular structures. [44,62] In vitro analysis of the release properties of thermogels for voriconazole using the horse as a model are promising, raising interest in the investigation of this sustained delivery system for the treatment of fungal keratitis. [4]

Section 6: Thermogels as Ocular Sustained-Drug Delivery Vehicles

Hydrogels are polymeric "smart" materials that can respond to stimuli such as pH, temperature, ionic strength, electric or magnetic fields, chemical and biological stimuli and consequently have a wide range of applications, in medicine, gene delivery and tissue engineering. [66] These gels have the ability to swell in a solvent if given enough time and space. Specifically, thermoresponsive polymers exhibit a change in their degree of swelling in response to temperature. [66] These

biocompatible, biodegradable polymers have been investigated for their potential as drug delivery carriers to improve ocular drug bioavailability. [67] Drugs are loaded into the thermogel at room temperature, and once heating to body temperature, the gel shrinks and entrap the drug molecules limiting their release. [66]

The desired hydrophobic therapeutic agent can be added to the aqueous polymer solution to be physically entrapped in the PLGA segments which form crosslinks as the temperature of the solution increases. [68] The more hydrophobic the drug, the more it will partition into the PLGA micellar core in the hydrogel, and consequently have a sustained drug release profile. The higher the initial polymer solution concentration, the slower the drug release rate, and this release mainly comes from the degradation, except during the initial stage when there is some degree of diffusion. [69] Such polymers are capable of maintaining a sustained release of drug at the site of administration over weeks to months, thus increasing the local bioavailability of the medication, decreasing systemic side effects, and improving client compliance. [67]

Once in the organism, the polymers from the thermogel safely biodegrades away to lactic and glycolic acids, as well as water soluble poly ethylene glycol, which are safely eliminated from the body by the kidneys. [68] Since biodegradable polymers have the advantage of disappearing from the site of action after releasing the drug, they can be developed as an alternative to the implants prepared from non-biodegradable polymers, which may need surgical removal after a certain period. [46,70]

The mild preparation conditions and high water content of hydrogels are beneficial in preserving the activity of biopharmaceuticals. [71] Applications of this technology in human medicine has gone beyond the ocular use, and hydrogels have been studied and used for sustained drug delivery of chemotherapeutic agents like docetaxel, applied directly to tumors therefore reducing side effects and increasing efficacy. [72] Thermo-sensitive hydrogels have shown promise for treatment and management of ocular conditions, where a topical dexamethasone hydrogel has been studied for treatment of uveitis [73], and a ganciclovir-containing hydrogel for intravitreal injection in cases of cytomegalovirus retinitis. [74] Thermosensitive hydrogels can be injected in a liquid form to the vitreous cavity, intracamerally or into the SC space through a small gauge needle. [75] When it is exposed to body temperature, the solution rapidly becomes a solid gel that releases the encapsulated protein. [70] In an *in vitro* study using equine corneas in a transcorneal permeation model, a 1.5% voriconazole containing thermogel (PLGA-PEG-PLGA) effectively released the drug for more than 28 days above the target MIC of 0.5 μg/ml. [4] Moreover, the same study demonstrated in an *ex vivo* model that SC injection of the voriconazole thermogel in equine eyes was feasible, making this sustained drug release method of potential clinical importance. [4]

There are some limitations of the hydrogel-based drug delivery systems, and the clinical use has been limited, due to difficulties in sterilization and unpredictable drug delivery. The degradation rate may depend on the underlying diseases and may vary among patients, thus it might be difficult to predict the release rate of the

incorporated drug. [71] Heat sterilization or radiation may induce degradation or side reactions to the polymers, particularly the extended release type, affecting the gel properties. [76,77] In order to avoid this, in pharmacological studies for human use of thermogels, 0.22 μm needle filtration is commonly used as the sterilization method. [78] Needle filtration was also used when a voriconazole thermogel was administered by SC injection of a horse in a pilot study, however there was drug remaining in the filter. [4]

Section 7: Safety of Thermogel Use in Other Species

The use of thermogels for the treatment of ocular and other conditions has been studied in animals as a model for humans. The PLGA-PEG-PLGA thermogel was used in mice for a model of treatment of ovarian cancer, where the hydrogel was loaded with a chemotherapeutic agent and delivered by intraperitoneal injection, also showing sustained release of the medication and less tumoral growth, with no systemic or local adverse effects. [65] An injectable formulation of a pentablock copolymer was administered intracamerally to rabbits for sustained delivery of drug to the anterior segment of the eye. The injection was well tolerated, with no ocular inflammation recorded, and a well-defined gel was observed in the anterior chamber for nearly 28 days post injection. [75] The antifungal fluconazole showed better corneal penetration and a sustained release in combination with a thermogel applied over rabbit corneas both in ex vivo and *in vivo* models. [52] Thermogels have also been used in combination with antioxidants in rabbits to study corneal wound healing, showing a potential application of the drug delivery system to treat chemical ocular injury in

humans and other species. [79] The thermogel PLGA-PEG-PLGA has been used in rabbits for slow release of dexamethasone after intravitreal injection, showing significant concentration of the drug for up to 1 week. The morphology of the ocular tissues, including the retina and cornea was not altered. [78] *In situ* dexamethasone thermogel also appeared to be safe over rabbit eyes, achieving a sustained drug release. [73] Voriconazole loaded thermogels have been studied for treatment of conditions of the skin using mice, where they showed better penetration of the drug and a sustained release. [80]

There are currently no temperature sensitive hydrogels approved for human or veterinary use. [71] Regarding the SC injection of the thermogel, there are limited number of studies, however this route has shown to deliver substances to the anterior chamber in a sustained manner, is easy to perform, and considered to be safe, with no evidence of inflammation, infection, or irritation to the eyes. [50]

The safety of ocular drugs or routes of administration can be evaluated by the use of a modified Hackett-McDonald scoring system. The Hackett-McDonald scoring system is a modification of the McDonald-Shadduck scoring system developed for recording the findings of slit lamp examinations for toxicology studies evaluating topical ophthalmic medications. [81] This system has been widely used in research animals, with modifications according to the segment of the eye evaluated. [82,83] This scoring system grades the inflammation in different sections of the eye during the ophthalmic examination. It evaluates conjunctival congestion (grades 0 to 3), swelling (grades 0

to 4) and discharge (grades 0 to 3), as well as corneal cloudiness (grades 0 to 4), pannus (grades 0 to 2), fluorescein staining intensity (grades 0 to 4), aqueous flare (grades 0 to 3), iris congestion (grades 0 to 4), and appearance of lens and fundus. [81]

Section 8: Justification of the Study

Keratomycosis is a significant disease of horses worldwide, with higher frequency in tropical and subtropical climates. [22] Treatment of fungal keratitis could be challenging, lengthy and costly. Drug delivery to the eye is difficult in horses due to their size and behavior, and ocular barriers can decrease drugs from reaching the affected areas within the globe. For the treatment of keratomycosis in horses, voriconazole is the first choice of antifungal due to its high lipophilicity, facilitating corneal penetration, as well as its low MIC for the most common fungi implicated in this disease, such as *Aspergillus* spp. and *Fusarium* spp. [27,36]

Alternate routes and vehicles for drug delivery to the eye have the potential to increase the drug concentration to the anterior segment of the eye, as well as to decrease treatment frequency. Subconjunctival injections in horses have been performed using 1% voriconazole sterile solution. Improved treatment success has been seen in cases refractory to the conventional use of topical voriconazole. [32] The SC route for drug delivery to the anterior segment of the eye could be used to deposit a voriconazole thermogel for sustained release, decreasing the treatment frequency

and increasing therapy success. A continuous lower dose delivered could improve healing time as verses intermittent or sporadic topical therapy. [11] This has been demonstrated by Cuming *et al*., where a voriconazole thermogel was easily injected into the dorsal SC space of equine eyes, forming a well-defined gel deposit upon reaching the horse's SC space temperature of 34.5°C. [4] Furthermore this study showed sustained release of 1.5% voriconazole from a PLGA-PEG-PLGA thermogel for more than 28 days in vitro. [4] The voriconazole concentrations were above the MIC of 0.5 μg/mL for 28 days, and antifungal activity was maintained. The use of this voriconazole thermogel did not show clinical adverse reactions in a live horse for 7 days following injections and no lesions were found in the histopathological exam of the eye. [4] PLGA-PEG-PLGA is a biocompatible, biodegradable thermogel that has also been investigated as a drug delivery vehicle for human chemotherapeutic and ocular medications. [4,38,72,74,84-87]

As is common in hydrogel preparations, different molecular weights and formulations of gels may be combined to give initial efficacy followed by slower release efficacy. Cuming *et al*. reported an initial burst release of voriconazole from the shorter portion of PLGA-PEG-PLGA thermogel as this was a combination of 2 hydrogels (short and extended release) *in vitro*, with peak concentration at 6 hours. [4] *In vivo* conditions are expected to behave different since the ocular barriers and blood vessels will clear part of the drug molecules before they enter the ocular tissues. [49] However the topical use of voriconazole every 4 hours on concentrations above 3% showed mild transient ocular irritation, thus toxic effects of high voriconazole concentrations in the cornea

warrant further investigation. [11] A real justification for smaller dosages delivered over continuous extended times made possible by hydrogel formulations.

There is a need for a controlled evaluation of the previous *in vitro* voriconazole thermogel technology in equine ophthalmology using live animals. In a previous work, Abarca *et al*., [88] presented a simple Franz diffusion cell-type modification for use in *ex vivo* ocular drug delivery investigations in the equine eye using a fluorescein leakage (FL) assay. Fluorescent concentrations detected in the receiving compartment of experiments maintained for 2 and 6 hours were not significantly different than those obtained from the negative control showing that the diffusion cell was able to maintain the integrity of equine epithelial corneal barrier function throughout the 6 hours of the permeation experiment. [88]

The use of a voriconazole-containing thermogel by SC injection in horses has the potential to deliver and maintain therapeutic concentrations of the drug in the anterior segment of the eye, especially the cornea; this is of clinical interest as a novel therapy for ocular fungal infections. At the time of this study, there were no reports determining the voriconazole concentrations in the AH and ocular tissues of horses following SC injection of voriconazole thermogel. Based on the high voriconazole release reported in an *in vitro* study, [4] determination of adverse effects to the cornea using *ex vivo* corneal models from horses euthanized for other reasons, will help in determining safety of this technique. Safety of the SC injection and

thermogel formulation can also be supported by the use of ocular inflammatory scores in live horses for a longer period following treatment than previously reported.[4]

Future positive results from the use of the voriconazole thermogel will be of interest for the pharmacology industry, and could be the first step to the commercialization of an efficacious, safer and more economic drug, that can be dosed by general veterinary practitioners.

Chapter 2 – Statement of Hypotheses and Objectives

Specific Aim 1: Ex-vivo corneal model to determine corneal toxicity

A. Hypothesis Aim 1: 1 mg voriconazole-PLGA-PEG-PLGA hydrogel (referred as the "0.3% thermogel") will result in no microstructural changes in the cornea, while application of voriconazole hydrogel at a higher concentration 5 mg voriconazole ("1.7% thermogel") will result in loss of corneal tissue integrity observed under histological analysis of HE stained cornea sections.

B. Objectives Aim 2:

   a. Evaluate toxicity to the equine cornea of a 0.3% thermogel versus a 1.7% thermogel in an *ex vivo* model at multiple time points using standard histopathological analysis.

   b. Determine the ideal composition of the thermogel (0.3% versus 1.7% thermogel) for use *in vivo.*

Specific Aim 2: Ocular concentrations of voriconazole after subconjunctival injection of voriconazole-PLGA-PEG-PLGA thermogel in horses.

A. Hypotheses Aim 2:

   a. Therapeutic concentrations of voriconazole in the AH will be maintained for at least 7 days following a single SC injection of 1.7% voriconazole-thermogel; concentrations of voriconazole in AH and tears will be

b. significantly higher using the thermogel versus standard 1% topical voriconazole when compared at day 2.

c. Voriconazole concentrations in the target tissues of the ocular anterior segment will be above the target MIC (0.5 μg/ml) for at least 2 days following a SC injection of 1.7% voriconazole thermogel; drug concentrations will be significantly higher in the dorsal segment of the eye due to proximity to the site of injection.

B. Objectives Aim 2:

a. To describe clinical effects of SC injection of voriconazole-thermogel in horses using an ocular inflammatory scoring system.

b. Determine concentrations of voriconazole in AH and tears on days 2, 7, 14 and 23 post SC injection of voriconazole-thermogel in horses.

c. Compare concentrations of voriconazole in AH and tears on day 2 between eyes treated with the thermogel and the standard topical treatment.

d. Determine the voriconazole concentrations in the ocular tissues after 2 and 48 hours of a SC injection of voriconazole-thermogel and compare the effect of location (anterior and dorsal segments) in drug distribution.

Chapter 3 – *Ex vivo* corneal toxicity to determine ocular safety

Introduction

The safety of voriconazole has been demonstrated in equine, leporine and human eyes, and in human cultured corneal endothelial cells. [11,27,89,90] Voriconazole solutions applied topically in human corneas, at concentrations of 1% and 2% have been reported to be safe and non-irritating. [91-93] This drug was not associated with adverse effects detected by clinical examination following topical administration in humans and horses, or intracameral injection in rabbits, when administered at concentrations ≤1%. [11,15,27,90] In horses the most common concentration used on the cornea, without any adverse reaction reported is 1%. [11,12,33]

Alternate routes of voriconazole administration in horses, such as intracorneal injections for treatment of fungal stromal abscesses showed no adverse effects at concentrations of 5%. [31] Subconjunctival injections of 1% voriconazole solution have been used in conjunction with the conventional topical therapy for treating refractory cases, improving outcome with no clinical evidence of ocular toxicity. [32,40] From the injection procedure, mild transient conjunctival hyperemia and hemorrhage were observed. [32]

In toxicology studies of the ocular use of voriconazole, a variation in cellular tolerance at different concentrations has been reported. Voriconazole 0.01% showed safety on

human corneas intended for use in transplants when added to the preservation medium; however, on cultured human corneal endothelial cells, there is evidence of dose dependent cytotoxic effects, seeing by activation of the proapoptotic pathway. [89,93] Mild transitory blepharospasm and epiphora with no residual adverse effects have been described in equine eyes with the use of 3% voriconazole solution applied topically every 4 hours. [11] Systemic side effects of ocular administration of voriconazole are unlikely. [11,27] Considering the information from other species, dose dependent topical voriconazole applied to the equine cornea has the potential to cause cell damage with potential increased risk of cutaneous malignancies, primarily squamous cell carcinoma. [94] *In vitro* testing of a 5 mg voriconazole thermogel formulation for SC injection in horses showed significantly high drug release during the first 24 hours, with a peak at soon as 6 hours, therefore there is a potential for cornea toxicity if used in live animals. [4]

Ex vivo models are used in pharmacological studies to investigate permeation of drugs as well as toxic effects of the drug formulation in tissues. [23] Transmembrane permeation using a Franz cell diffusion chamber is a common model used in ophthalmologic studies to determine drug diffusion through the cornea [4,23,88] We have used it previously to evaluate if epithelial function was maintained in the *ex vivo* equine corneas for 6 hours; [88] however the effect of the drugs on the tissue, when in contact with the solutions in the Franz cell device, has not been studied. Assessment of toxic changes to the cornea by histological analysis in *ex vivo* models using whole corneas immersed in the solution of interest, [23] or the whole ocular globe after a

treatment are acceptable models to determine microstructural changes to the corneas. Formalin fixed corneas prepared with HE stain may show structural changes such as endotheliallayer integrity, epithelial thickness and organization, keratocyte density, and stromal bed thickness and appearance. [95-98]

Using whole ocular globes in equine ophthalmic toxicology studies, where a large sample size is needed, is difficult due to the availability and the cost to maintain horses on a therapy while awaiting for euthanasia. Thus, *ex vivo* models represent an important research tool to reduce the necessity of euthanizing animals and costs related to research. The use of cornea sections instead of whole ocular globes will further reduce the number of animal needed in ocular toxicology studies.

To our knowledge, this study is the first one that uses the technique of transmembrane permeation to assess the corneal integrity by histological analysis. The purpose of this study was to evaluate potential toxic effects of two different concentrations of voriconazole thermogel using an *ex vivo* model of equine cornea sections maintained in a Franz cell diffusion chamber followed by histological analysis of the tissues at different timepoints. Results from this study will determine the safety of this sustained drug release method in treating equine keratomycosis.

Materials and Methods:

Preparation of the equine corneas

Use of animals in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and was approved and monitored by the Auburn University Institutional Animal Care and Use Committee.

Twelve eyes were obtained from horses euthanized for reasons not related to this project. The eyes were enucleated following euthanasia with intravenous lethal dose of pentobarbital. Enucleation was performed using a transpalpebral technique to preserve the integrity of the cornea. The whole ocular globe was transported in a 0.9% sodium chloride solution (Hospira Inc., Lake Forest, IL) and the corneas were harvested within one hour of enucleation. The corneas were carefully excised along with 1 mm of surrounding scleral tissues and washed with cold saline solution.

Thermogel preparation

The thermogel was selected based on an *in vitro* study by Cuming *et al*.[4], where a combination of the thermogels PEG-PLGA-PEG AK19 (MW 1500-1500-1500, 1:1 lactide:glycolide) and AK24 (MW 1100-1000-1100, 3:1 lactide:glycolide) Akina Inc, West Lafayette, IN) in a 1:1 ratio was used in order to reach a gelation temperature between 33.3°C - 35.3°C (temperature of the SC space in horses). [4]

Following the manufacturer's instructions, in order to create a 20% solution of the thermogel, the gel was reconstituted with 10 mL of sodium chloride 0.9% sterile

injection, USP (Hospira Inc., Lake Forest, IL). The thermogel solution was kept at 4°C for at least 48 hours, in which period it was vortexed every 8 hours to promote the contact of the solidified gel with the sodium chloride. The thermogel was stored at 4°C thereafter. The day prior utilization of the thermogels, both the AK19 and AK24 were mixed in a single container.

Voriconazole powder (U.S. Pharmacopeia, Rockville, MD) was aseptically added to the thermogels while in a liquid state to create a suspension. Two different concentrations were prepared and four groups defined: TG (PLGA-PEG-PLGA thermogel), PBS (phosphate buffered solution), and VOR1 (1 mg voriconazole thermogel), VOR5 (5 mg voriconazole thermogel). Gelation of both voriconazole-laden and control thermogel was tested by immersing tubes containing the solutions into a water bath set at 34°C. The thermogels were confirmed to go from liquid to gel once at 34°C and back to liquid when stored at 4°C. The thermogels were discarded after 4 weeks of reconstituted based on the manufacturer's recommendations.

Ex vivo maintenance of corneas

Each cornea was divided in 4 equal sections, obtained by cutting a whole cornea in quarters with a corneal knife. The sections were then placed in a Franz cell diffusion chamber of 5 mm diameter that consisted of an upper and lower compartment (Figure 1). The upper compartment served as a donor chamber in which 0.3 mL of the sample solution (TG, PBS, VOR1, and VOR5) was placed (Figure 2). The corneal epithelial

surface faced the donor compartment. The receiving compartment was filled with phosphate buffered saline (PBS) until it was in contact with the cornea. The PBS in the receiving compartment was stirred using a small magnetic bar and maintained at 34°C. Aliquots of VOR1 and VOR5 were placed on the donor chamber in contact with the epithelial surface of the corneas, with both groups studied in triplicate. Two controls were used to compare with VOR1 and VOR2, consisting of TG and PBS. The procedure was repeated in triplicate, and the corneas were exposed to the solutions for different time points: 2, 4, 6, and 8 hours.
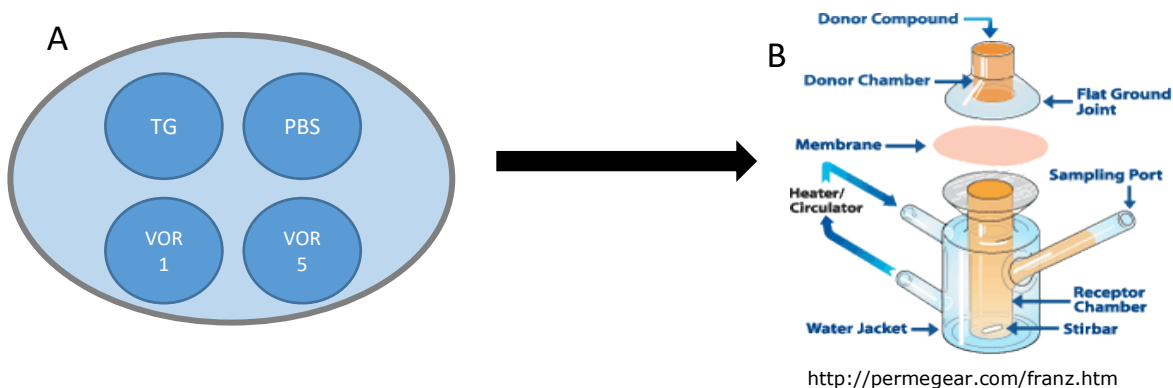
Figure 1. A. Diagram of the cornea with the corneal sections designated for each group. TG (thermogel); PBS (phosphate buffer solution); VOR1 (1 mg voriconazole-thermogel); VOR5 (5 mg voriconazole-thermogel). **B**. Diagram of the Franz cell diffusion chamber, where membrane corresponds to the cornea section.

Figure 2. Franz cell diffusion chamber setting. The arrows show the voriconazole-thermogel in the solid state in the donor chamber in contact with the corneal epithelium once reaching 34°C for both the 1 mg and 5 mg voriconazole-thermogels.

Preparation and analysis of histology samples

The corneal sections were removed from the chamber at each timepoint (2, 4, 6, 8 hours) after exposure to each treatment and analyzed histologically. The corneas were placed in between 2 slices of cucumber of about 0.5 mm thickness to preserve the architecture as previously described [99] prior to being placed in a tissue cassette and immersed in 10% neutral buffered formalin for at least 24 hours (Figure 3). Subsequent to fixation, corneal sections were routinely processed and stained with HE.

The histology slides were evaluated by a board certified specialist in veterinary pathology (S. Shrader). Histological evaluation parameters included direct measurements of corneal epithelial and stromal thickness and presence or absence of endothelium. When possible, for the epithelium and stroma, 5 measurements were performed at different locations and a mean with standard deviation was calculated and then used for subsequent statistical analysis.
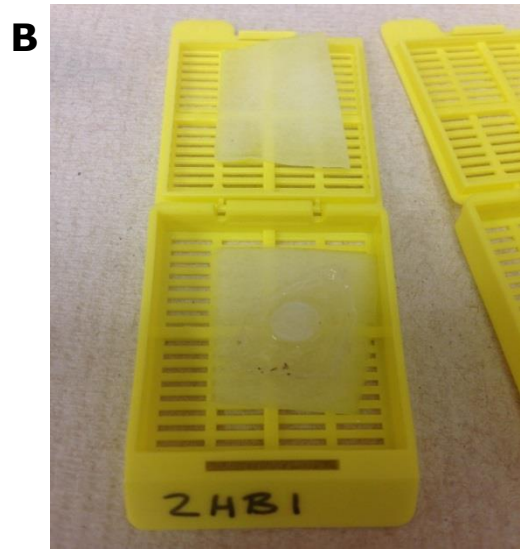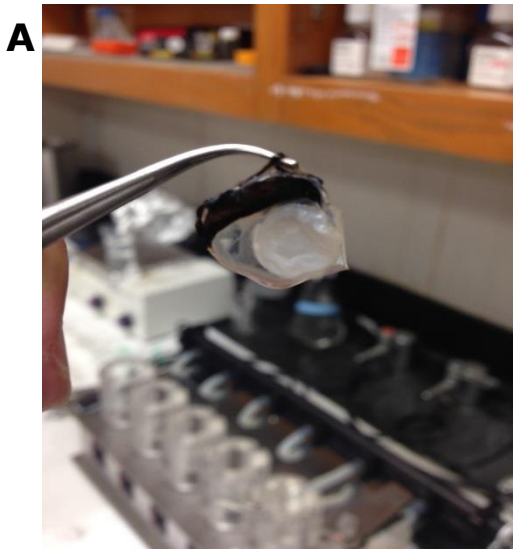
Figure 3. **A.** Corneal section removed from the Franz cell diffusion chamber after being exposed to the samples for 4 hours. Note the degree of macroscopic damage secondary to fluid absorption by the stroma. **B.** Corneal segment over a thin slice of fresh cucumber before fixation in 10% formalin for histological analysis.

Data analysis

Continuous data are presented as mean and standard error of the mean (SEM). Presence and absence of endothelium were analyzed descriptively. For corneal epithelial thickness and stromal thickness, for each corneal section, 5 measurements were performed at different locations and a mean with standard deviation was calculated and then used for subsequent statistical analysis. Treatments (VOR1, VOR5, TG and PBS) were compared at each timepoint using one way analysis of variance with Tukey's multiple comparison test. Significance was set at a p value ≤0.05.

Results

Comparisons of the 4 different groups were done for the corneal epithelial and stromal thicknesses after 2 hours of exposure to the solutions in the Franz cell diffusion chamber, with no significant difference observed between treatments. As the time of exposure to the solutions increased, the more significant artifacts were observed. Samples at the times 4, 6 and 8 hours presented significant artifacts that affected the morphological measurements and less samples were analyzed per group (Figure 4).
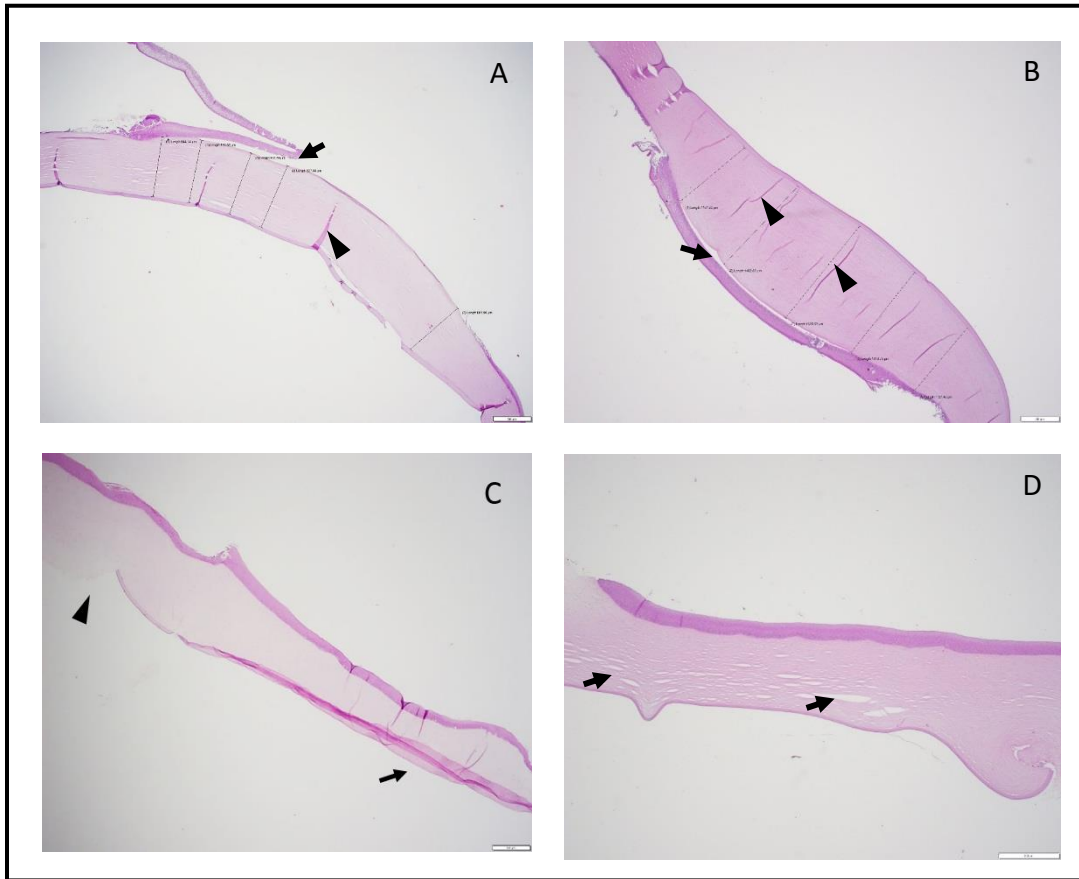
Figure 4. Example of artifacts present in the corneal samples when analyzed under light microscopy at 10X and stained with H&E. **A** and **B**. Epithelial clefting (arrows) and wrinkles in the stroma (arrow heads) of a sample from the TG group after 8 hours of exposure (A) and from the VOR1 group after 2 hours (B). **C**. Folding (arrow) and loss of endothelium (arrow head) in a sample exposed to PBS for 4 hours. **D**. Stromal edema (arrows) of a sample from the PBS group at 2 hours.

Corneal samples were analyzed by triplicate for each of the treatments. At each timepoint, a total of 12 cornea sections (3 per treatment) were analyzed. The mean measurements for 5 sites for corneal epithelium and stroma was obtained. Corneal endothelium was analyzed descriptively.

As previously reported [4], the combination of the hydrogels AK19 and AK24 showed a reversible liquid-gel transition, with a phase transition temperature of 34°C, which depended on the concentration, molecular weight and poly(ethylene oxide):poly(propylene oxide) ratio. [4] A well-defined gel was formed in the donor chamber for the entire period of exposure.

Corneal epithelial thickness

Corneal epithelium thickness between groups at 2 hours was not significantly different (p =0.3098) (Figure 5). The mean (+/- SEM) measurements for corneal epithelial thickness for each group at 2 hours are presented in table 1.

At the 2-hour timepoint, from the 12 samples analyzed, measurements were performed in 91.67% of the samples. Epithelial thickness was measured in 9 out of 12 samples (75%) for the 4-hour timepoint. At 6 hours, the epithelium was measured in 7 out of 12 samples (58.33%). For the 8-hour timepoint, epithelium was measured in 4 out of 12 samples (33.33%).

Only the groups that were exposed to the different treatments for the 2-hour period did not present significant artifacts, thus it was possible to perform measurements at 5 different sites (Figure 6). Artifacts to the corneal epithelium included edema, folding, clefting (epithelial and subepithelial), tearing and partial loss.
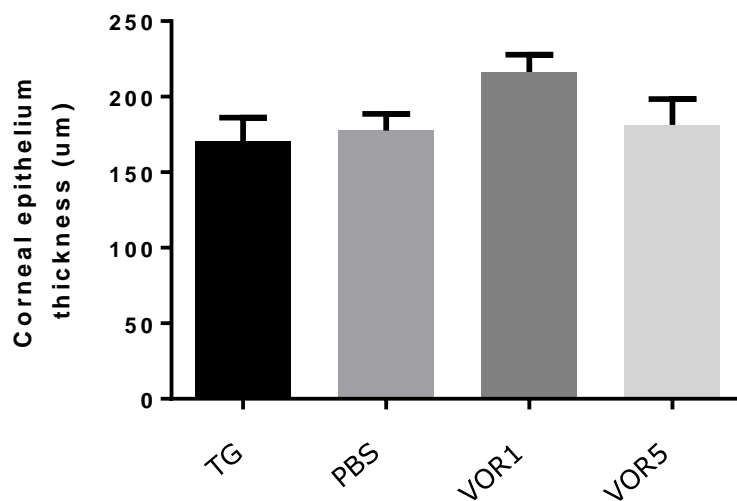
Figure 5. Corneal epithelium thickness between groups at 2 hours. No statistical difference was observed for epithelial thickness. Data are mean +/- SEM. p =0.3098.

Group	Mean (μm)	SEM
TG	136.3	15.8
PBS	177.62	11
VOR 1	216.42	11.34
VOR 5	181.32	17.22

Table 1. Mean corneal epithelium measurements (+/- SEM) in μm for the 4 different groups at the 2-hour timepoint.

Figure 6. Corneal section stained with HE after 2 hours of exposure to TG in the Franz cell diffusion chamber. Five measurements of the epithelial thickness are represented at different sites. There is presence of edema of the epithelium (arrow). Sample analyzed by light microscopy at 20X.

Corneal stromal thickness

Corneal stroma thickness between groups at 2 hours was not significantly different (p =0.1931) (Figure 7). The mean (+/- SEM) measurements for corneal stromal thickness for each group at the different timepoints are presented in table 2.

The number of measurements performed depended on the integrity of the cornea (Figure 8A). When measured at 2 hours after exposure to the solutions, 10 out of the 12 samples were analyzed (93.33%). At 4 hours, 8 out of the 12 corneal sections (66.67%) were used for measurements. No measurements could be performed for the corneas in the TG group due to the presence of significant artifacts. Artifacts for the corneas exposed for 4 hours also precluded the analysis by triplicate. Analysis was performed in 8 out of 12 samples (66.67%) for the 6-hour timepoint. At 8 hours, it was possible to perform morphological measurements in 9 out of 12 samples (75%).

Artifacts to the stroma (Figure 8B) included edema, folding, and vacuolation. There was also partial loss of the stroma.
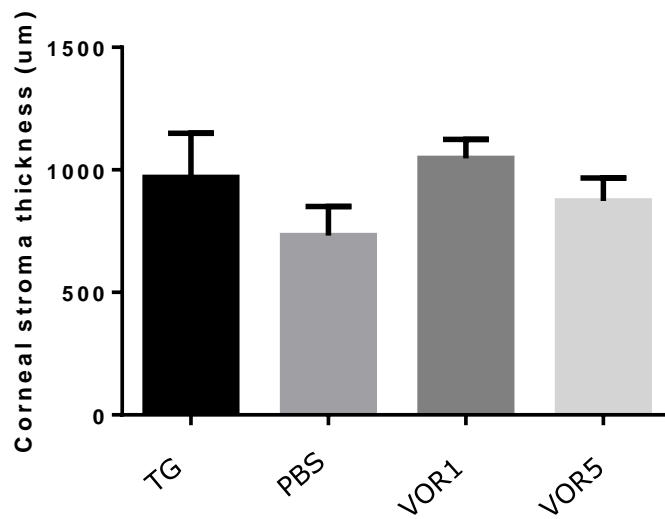
Figure 7. Corneal stroma thickness between groups at 2 hours. No statistical difference was observed for epithelial thickness. Data are mean +/- SEM. p = 0.1931.

Group	Mean (μm)	SEM
TG	969.76	90.06
PBS	731.37	84.71
VOR 1	1046.99	54.48
VOR 5	873.41	53.63

Table 2. Mean corneal stromal thickness (+/- SEM) in μm for the 4 different groups at the 2-hour timepoint.
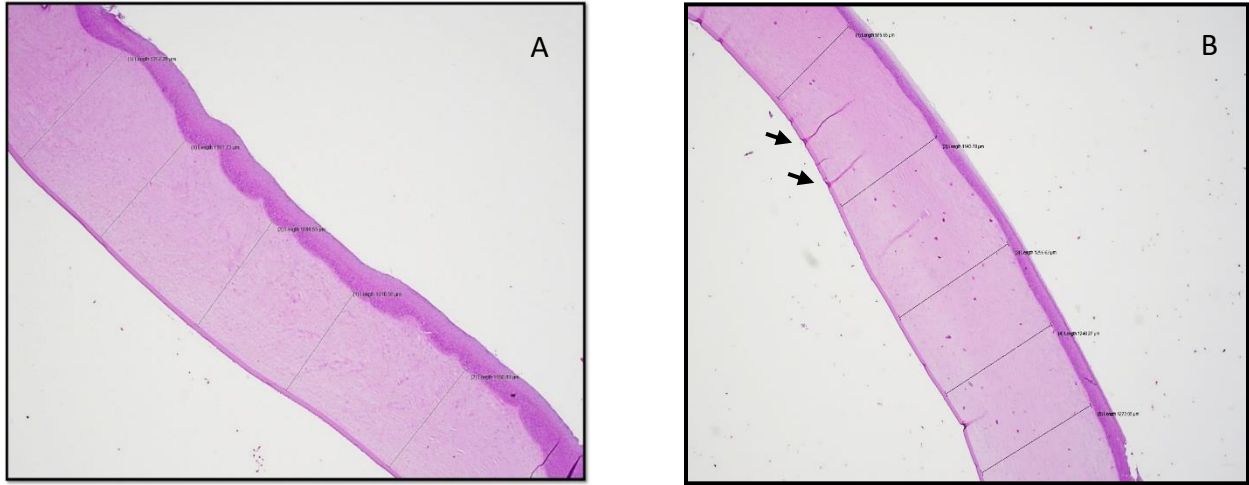
Figure 8. Corneal sections stained with HE. **A**. Corneal section after 2 hours of exposure to TG in the Franz cell diffusion chamber. **B**. Corneal section of the VOR1 group after 2 hours of exposure in the Franz cell diffusion chamber with measurements of the corneal stroma at 5 different sites; the arrows show a site with increase in wrinkles with subsequent decrease in the stromal depth. Samples analyzed by light microscopy at 10X.

<u>Corneal endothelium</u>

None of the samples analyzed, at any time points or group had complete presence of endothelium. At 2 hours, 75% of the samples lacked of an endothelial layer, and a 100% of the samples at 4 and 8 hours. At 6 hours there was partial presence of endothelium in 58% of the samples analyzed.

Discussion

In an attempt to deliver effective drug concentrations to the ocular tissues without adverse systemic effects, novel drug delivery systems have been developed. Hydrogels like PEG-PLGA-PEG, which respond to temperature can be administered by minimally invasive methods and have shown many benefits for treatment of ocular conditions. [71,75,78] For this phase of the study, the previously described [4] voriconazole-thermogel was evaluated for potential damage to the corneal integrity in an *ex vivo* model using equine cornea sections.

The potential toxicity of voriconazole has been tested by evaluation of the corneal thickness and endothelial integrity in rabbit eyes, where concentrations up to 1% did not result in microstructural damage. [90] Voriconazole in horses showed mild transient adverse effects when used topically at 3% concentration. The side effects included epiphora and blepharospasm. [11] On the other hand, reports of intrastromal use of voriconazole have not cause visible adverse effects in the clinical setting. [31] In this study we evaluated the histological structure of normal corneas after exposure to a

voriconazole thermogel, since reports of higher concentrations used are focused on ophthalmic evaluations rather than potential structural damage. Evaluation using this model was successful up to 2 hours, but longer timepoints resulted in damage to the corneas in the Franz cell diffusion chamber regardless of treatment. These artifacts precluded the correct morphological analysis of the tissues, hence a potential adverse effect of the voriconazole thermogel could not be determined (Figure 3A). Based on the results from this phase of the study, the technique used with the equine corneal sections in the Franz cell diffusion chamber to evaluate corneal toxicity for longer timepoints than 2 hours is not recommended since it yielded inconclusive results. Further studies are warranted to improve the corneal preservation after harvesting, such as by the use of other culture corneal storage media, or *ex vivo* techniques using the isolated cornea, perfused ocular anterior segment, cell cultures or whole globe.[100-102]

Handling of the corneas for *in vitro* or *ex vivo* studies is challenging, which is the reason studies using histopathologic analysis often fix the whole globe right after enucleation. [96,103] Corneal tissue is very elastic and has a strong tendency of roll or fold on itself, therefore tangentially or obliquely cutting tissue can introduce errors in interpretation, making proper histological analysis very difficult. [99,104] The use of fresh or frozen cucumber has been described to preserve the shape of the ocular tissues prior to fixation in formalin. [99] This technique was applied in our study, by using fresh cucumber to prevent the corneas from folding prior fixation in formalin.

Severe damage was still present in the corneas despite the use of cucumber in all the samples. Since major damage was seen with time in the Franz cell diffusion chamber, it was speculated that failure of corneal preservation, mechanical damage from the clamps in the chamber, and exposure of the tissues to the fluid were responsible for the artifacts seen in histology rather than the fixation technique.

Comparison among the 4 different groups was done for the corneal epithelial and stromal thickness after 2 hours of exposure to the solutions in the Franz cell diffusion chamber, with no significant differences observed between treatment groups which is encouraging for clinical use of the voriconazole thermogel. Results from this phase of the study for the 4, 6, and 8 hour time periods were inconclusive, thus potential adverse effects of the thermogel itself or the voriconazole-loaded thermogel at different concentrations could not be determined. This *ex vivo* model failed in preserve the equine cornea for accurate histological analysis, based on appearance of artifacts that compromised the integrity of all the corneal layers. With longer time of exposure to the solutions all the corneal samples showed more evidence of damage, and a fewer number of samples were analyzed as the time points increased. The corneal stroma is mostly composed of Type I collagen fibrils within a hydrophilic polysulphated proteoglycan ground substance, and integrity of the corneal epithelium and endothelium are essential in preserving a relative state of dehydration to the stroma to maintain transparency. [105] Damage to the corneal epithelium by desiccation, handling, or continuous exposure to the substances studied will allow the

stroma to attract great amounts of fluid, leading to corneal edema, resulting in morphologic alterations and increase of thickness with time of exposure. Stromal edema might have led to disruption and clefting of the epithelium and endothelium by stretching of these layers. The corneal endothelium is a permeable monolayer of cells that also works with a Na-K ATP-ase-dependent electrolyte pump which counters the natural influx of water intro the stroma. There is the possibility that with *ex vivo* corneas, with longer time of energy deficits the ATP-ase pump will lack efficacy and more water will enter the stroma, causing severe edema and damage to the entire cornea. [105] Equine corneas are significantly thicker in the dorsal and ventral portions compared to the central, lateral or medial. [106] Cornea sections instead of a whole cornea were analyzed in this study, and the location of the segment (i.e. central versus dorsal) could have given different measurements. To reduce this factor, 5 different areas were measured within the segment, and each quarter of the cornea was cut as equal as the others as possible, containing portions of both the central and peripheral regions. Moreover, all the 4 samples for each of the groups were obtained from the same cornea to minimize individual variability.

Different studies describe the preservation of corneas using buffered saline solution, and then continuous infusion with either PBS or artificial tear fluid after placement of the corneas into the Franz cell chamber.[4,23] *Ex vivo* models for corneal toxicity have been also used with whole ocular globes from pigs, where the vitreous cavity was infused with tissue-culture medium for preservation of the organs; however in this

study no histological analysis was performed. [100] In our study, using whole globes was not suitable due to the large number of horses needed to study the different voriconazole concentrations at the different time points. *Ex vivo* corneal toxicity has been studied by immersion of whole goat corneas into the solutions to be studied for a maximum of 30 minutes before fixation in 10% formalin. By this technique the corneas were well preserved and suitable for analysis. [23] This is similar to our study since the cornea was in direct contact with the solution studied, however the exposure times were different, and one can conclude that exposure for just 30 minutes may produce less tissue damage compared with the exposure times used in our study. In contrast using the same settings than our study, histological evaluation of whole porcine corneas maintained in a Franz cell chamber preserved the histological integrity; [107] thus cutting the corneas, and consequently exposing more of the stroma, might be the cause of the increase in edema of our samples.

One limitation of the study is the difficulty of obtaining enough equine corneas to study the different drug concentrations at multiple timepoints, therefore the corneas had to be divided in quadrants. By cutting the corneas, there is more manipulation involved, which inevitably will increase the risk for tissue damage. Another limitation is the use of the Franz cell diffusion chamber, which is designed for permeation studies, rather than preservation of tissues; when using corneal sections the clamps can easily damage the samples, thus larger samples may be required.

Conclusion

With the advance in laboratory techniques, the use of live animals for testing toxicological effects of ocular drugs is decreasing, and both *in vitro* and *ex vivo* techniques are gaining popularity. This study showed that using a transmembrane diffusion model with a Franz cell chamber for equine corneas is an acceptable method to morphologically assess structural changes for at least 2 hours of exposure to a voriconazole thermogel. Further investigation in preservation of corneal segments is warranted in order to use this technique for longer exposure times. *Ex vivo* models are appropriate techniques to assess the impact of ophthalmic drugs, thus limiting the need for experiments with live animals. [100]

Chapter 4 – Ocular Concentrations of Voriconazole Following Subconjunctival Injection of Voriconazole-PLGA-PEG-PLGA Thermogel in Horses

Introduction

Horses are prone to corneal trauma and infections due to their large globe size, orbital shape, and living environment. [9,24] Disruption of the defense mechanisms of the cornea, such as the epithelium, with subsequent exposure of the stroma, will be the starting point for the development of keratomycosis. [7-9] Filamentous fungi including *Aspergillus*, *Fusarium* and *Penicillium*, are the most common fungi implicated in equine keratomycosis. [3,6,9] Fungal keratitis have several clinical presentations, including superficial and deep ulceration with or without corneal melting process, plaque formation, non-ulcerative stromal abscess, and corneal perforation with iris prolapse. [1,12,16,108] Treatment is prolonged and expensive and requires compliance from horses and their handlers. [24]

Effectiveness of antifungal therapy depends on the agent's ability to enter the aqueous humor and achieve therapeutic concentrations. [27] Voriconazole is a second generation triazole antifungal drug, which inhibits the P450-dependant enzyme C-14α demethylase and causes depletion of ergosterol and accumulation of 14-methyl sterols in fungal cell membranes. [11,27] Voriconazole has become the first line antifungal for treatment of keratomycosis in horses. The effectiveness is due to its

wide spectrum of antifungal activity, low MIC against common pathogens, and excellent corneal penetration. [11,27,36,37] Based on MICs for filamentous and yeast organisms, MICs between 0.5 to 4 μg/mL [11] for voriconazole are required to inhibit fungal growth. Some isolates of *Fusarium* spp. can yield MIC values > 8 μg/mL. [39]

The poor bioavailability of drugs is mainly related to the topical administration, due to protective mechanisms of the eye and presence of ocular barriers. [46,52] To overcome the challenges of topical eye medications, other routes for administration have been used in horses. The advantage of using other alternatives for drug delivery include higher concentrations in the targeted region, and less frequency of treatment. The SC route of drug administration provides transient high and prolonged drug concentration to the anterior segment of the eye compared with topical administration, [32,43] and can maintain therapeutic levels for 8 to 12 hours for a water soluble drugs. [40] Subconjunctival injections, because of being deposited onto the sclera, offer less resistance to diffusion, resulting on better penetration of the corneal stroma. [59]

Continuous drug release will reduce the need for daily administration, which could improve client compliance and treatment outcome. [60] Thermosensitive biodegradable gels, such as PLGA-PEG-PLGA are administered as a liquid but convert to form a gel deposit upon reaching body temperature. [67,84] Thermogelation allows for sustained drug delivery at the site of administration over weeks to months, thus increasing the local bioavailability, decreasing systemic and ocular side effects, and improving

therapeutic outcome. [67] The properties of a voriconazole thermogel have been previously studied, where sustained release for up to 28 days of the drug from a 5 mg voriconazole thermogel over the target MIC of 0.5 μg/ml was demonstrated. [4] The technique for injection has been previously described using an *ex vivo* equine eye model. [4]

The voriconazole thermogel has the potential to release significantly high and lower steady state concentrations of the drug [4], therefore investigation of adverse ocular effects by the use of a modified Hackett-McDonald scoring system is important for validation of this novel technique in the clinical use. [81]

The purpose of this study was to describe the ocular toxicity based on clinical inflammatory effects of a voriconazole-thermogel in healthy horses, and to document if concentrations above the target MIC were maintained over time in the AH and the tissues of the anterior segment of the eye.

Materials and Methods

The study was divided in two phases. The first one consisted of determination of tear and aqueous humor voriconazole concentrations and ocular toxicity based on clinical scores after a SC injection of voriconazole thermogel in an *in vivo* model at different timepoints. The second phase further studied the distribution of the voriconazole released from a thermogel after SC injection in the different ocular tissues with

special focus in the anterior chamber of the eye.

Animal Selection

The animals used for the study were part of the Auburn University teaching and research herd. Use of animals in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and was approved and monitored by the Auburn University Institutional Animal Care and Use Committee.

For both phases, horses were determined to be healthy for inclusion in the study based on a normal physical examination and results from complete blood count (CBC) and serum biochemical analysis (SBA). All the horses underwent a complete ophthalmic examination performed by a board certified specialist in veterinary ophthalmology (E. Abarca) and were determined to be free of ocular abnormalities. None of the horses received any systemic or ophthalmic medication for at least 60 days prior to the start of the study. Complete ophthalmic examination included Schirmer tear test (STT, Schering-Plough, Charlotte, NC), biomicroscopy examination (Kowa SL-14, Tokyo, Japan), Tonovet tonometry (iCare, Finland), fluorescein test (Akorn Inc., Buffalo Grove, IL). After pupillary dilation with tropicamide 1% (Akorn Inc., Buffalo Grove, IL), the ocular fundus was photographed (Optibrand Clearview, Optibrand Ltd., Fort Collins CO). The horses were examined twice daily and monitored closely for signs of inflammation, reaction or pain in the treated eyes.

<u>Preparation of voriconazole thermogel</u>

The thermogel was selected based on a study in vitro by Cuming et al.[4] A combination of the thermogels PEG-PLGA-PEG AK19 (MW 1500-1500-1500, 1:1 lactide:glycolide) and AK24 (MW 1100-1000-1100, 3:1 lactide:glycolide) (Akina Inc, West Lafayette, IN) in a 1:1 ratio was used in order to reach a gelation temperature between 33.3°C-35.3°C (temperature of the SC space in horses). [4] Following the manufacturer's instructions, a 20% solution was prepared by adding 10 mL of sodium chloride 0.9% injection USP (Hospira Inc., Lake Forest, IL) to each of the thermogel vials. The reconstituted thermogel was kept at 4°C for at least 48 hours, in which period it was vortexed every 8 hours to promote the contact of the solidified gel with the sodium chloride. The thermogel was stored at 4°C thereafter. The day prior utilization of the thermogels, both the AK19 and AK24 were mixed in a single container.

Voriconazole powder USP (U.S. Pharmacopeia, Rockville, MD) was added to the thermogels while in a liquid state to create a suspension. The 5 mg of voriconazole powder was weighed and added to the thermogel in liquid state in an amount necessary to create a 1.7% suspension. Preparation of the voriconazole thermogel was compounded under a sterile products laminar flow hood in a USP 797 sterile products compounding room. Prior to using the voriconazole loaded thermogel, gelation was tested by immersing tubes containing the suspension into a water bath set at 34°C. The thermogel was confirmed to go from liquid to gel once at 34°C and back to liquid when stored at 4°C. The thermogel was not used for longer than 4 weeks after reconstitution based on the manufacturer's recommendations.

For the first phase of the study, both the voriconazole and the thermogel were sterilized prior to reconstitution by the use of gamma-rays (cobalt-60) with doses of 8, 13 and 25 kGy, with a rate dose between 5 and 6 kGy/h. Preliminary studies were performed *in vitro* in similar manner to Cuming *et al*. [4] to determine release kinetics from irradiated voriconazole thermogel. These results are presented in appendix 1. For the second phase of the study (tissue concentrations), the thermogel was prepared using sterile gloves in a biosafety cabinet.

Determination of voriconazole concentrations in tears, aqueous humor, and tissues

Phosphate-buffered saline samples were analyzed by reverse phase high performance liquid chromatography (HPLC) as previously described. [38] The HPLC system (Agilent 1200 series) consisted of pumps, an autosampler, UV/visible light absorption detector, column (Thermo BetaBasic-18, 4.6 mm x 15 cm, 5μ; Bellefonte, PA, USA), and computer interface. The flow rate opf the mobile phase was 35% 0.1 M N, N, N', N'-tetramethylenediamine (Fisher Scientific, Inc., Waltham, MA, USA) and methanol (Fisher Scientific, Inc.). An injection volume of 100 μL was selected. UV detection was at 254 nm with voriconazole and the internal standard, ketoconazole, the internal standards, showing retention times of 3.7 and 13.5 min respectively. Calibration standards for voriconazole concentrations ranged from 0.001 to 10 μg/mL and were prepared in dissolution media selected fluids. Plasma and tissue samples collected from horses that had not received voriconazole were used as the blank controls. Tissues were minced and extracted with 4 mL of ethyl acetate. The lower limit of detection and quantification for voriconazole was 0.001 μg/mL and 0.005

μg/mL respectively. Values of 0.96% and 5.01% were noted for intra- and inter-day variations respectively. Extraction efficiency appeared greater than 89% for drug and internal standard. [109]

Phase 1: *In vivo* tear and aqueous humor voriconazole concentrations and ocular toxicity

Animals

Six horses (5 mares and 1 gelding) from the Auburn University teaching herd were included in the first phase of the study. The ages ranged from 8 to 14 years (mean of 11 years), and they weighed between 1,012 and 1,230 pounds. Breeds included 4 American Quarter Horses and 2 Tennessee Walking Horses.

Treatment groups and overall design

For this phase of the study, a cross-over design was used. Two groups of 6 horses each were defined; a group receiving topical 1% voriconazole (Vori-Top), and a group that received a SC injection of 1.7% voriconazole thermogel. The Vori-Top group received 1% voriconazole solution on the right eye every 4 hours for 2 days (48 hours). Voriconazole for intravenous use in humans (Sandoz, Princeton, NJ) was used for topical ophthalmic application. A 1% solution was prepared by reconstitution of the powder by adding 20 mL of sterile water. The compounding was performed by the Auburn University Veterinary Pharmacy under US Pharmacopeal Convention (USP) 797 sterile compendium guidelines. The 1% voriconazole was prepared the day prior utilization, and was stored in the refrigerator at 4°C for the 48 hours

duration of the treatment.

One hour after the last dose, AH and tears were collected for determination of voriconazole concentrations using HPLC. This treatment was considered a positive (standard treatment in clinical field) control to validate AH concentrations of voriconazole in this group of horses and to compare to previously published results.[11] After a 45 day washout period, the same 6 horses formed the voriconazole thermogel group (Vori-Gel), which received a SC injection of 1.7% voriconazole thermogel in the dorsal bulbar conjunctiva of the left eye.

Samples of AH and tears were collected on days 0, 2, 7, 14 and 23 after the SC injection for determination of voriconazole concentrations. Following the SC injection, the horses were stalled for 24 hours, and then transferred to a pasture. They were monitored twice daily for evidence of systemic or ocular disease. While a complete ophthalmic examination was performed on day 2, 7, 14 and 23.

Procedures on animals

Horses allocated in the Vori-Top group received 1% voriconazole solution in the right eye. The drug was administered by direct instillation of 0.2 mL of voriconazole using a 25 gauge needle previously broken to the hub. The needle was attached to a 1 ml syringe containing the drug.

Horses in the Vori-Gel group received a sterile, previously irradiated voriconazole

thermogel injection in the dorsal bulbar conjunctiva of the left eye. A total of 0.3 mL of thermogel containing 5 mg of voriconazole (1.7%) were drawn from a plain sample collection tube that was previously vortexed for homogeneous distribution of the voriconazole particles. The preloaded syringe was stored in ice to maintain the liquid state of the thermogel, and the voriconazole thermogel suspension was mixed in the syringe just prior to injection. The injection was performed using a 30 gauge, ½ inch needle, and a well-defined gel deposit was observed in the SC space (Figure 9).

For all thermogel injections and for tear and AH collections, the horses were sedated with 5 mg of detomidine hydrochloride (Dormosedan®, Zoetis Kalamazoo, MI) intravenously. Auriculo-palpebral and supraorbital nerve blocks were performed by subcutaneous injection of 1 mL of 2% lidocaine hydrochloride (Hospira, Inc., Lake Forest, IL). Local anesthesia using proparacaine hydrochloride 0.5% (Akorn, Lake Forest, IL) was applied at the site of injection in the dorsal bulbar conjunctiva.

Evaluation of ocular inflammation

The horses in the Vori-Gel group underwent complete ophthalmic examination prior to the injection and at each day of tears and aqueous humor collection (days 0, 2, 7, 14 and 23 post injection). Ocular inflammatory changes were giving a score following a Hackett-McDonald scoring system on days 0, 2, 7, 14 and 23 of treatment. [81] Horses were also observed twice daily for signs of ocular pain (blepharospasm, blepharedema, epiphora) for the duration of the study. The Hackett-McDonald scoring system is a modification of the McDonald-Shadduck scoring system developed for

recording the findings of slit lamp examinations for toxicology studies evaluating topical ophthalmic medications. [81] When assigning a score to the ocular inflammation in this study, the scale was adjusted for equine eyes, thus varying from the original version created for white rabbits, therefore being more accurate for evaluating inflammation for this model.

The Hackett-McDonald scoring system used is shown in (Figure 10). It evaluates conjunctival congestion (grades 0 to 3), swelling (grades 0 to 4) and discharge (grades 0 to 3), as well as corneal cloudiness (grades 0 to 4), pannus (grades 0 to 2), fluorescein staining intensity (grades 0 to 4), aqueous flare (grades 0 to 3), iris congestion (grades 0 to 4), and appearance of lens, anterior vitreous involvement and funduscopy. [81]

Figure 9. Gel deposit formed in the SC space following injection of the voriconazole thermogel.

| Study day: | Conjunctival | | | Aqueous | Iris | Corneal | Pannus | Fluorescein | Lens | Anterior | Funduscopy |
Animal	Congestion 0 - 3	Swelling 0 - 4	Discharge 0 - 3	flare 0 - 3	congestion 0 - 4	cloudiness 0 - 4	0 - 2	exam 0 - 4		vitreous	
1											
2											
3											
4											
5											
6											

SC injection
Left eye

Figure 10. Example of the modified Hackett-McDonald scoring table used for assessing ocular inflammation in horses.

Adapted from Hackett and McDonald, 1996. [110]

Sample Collection

Under standing sedation, first tears were collected from the lacrimal lake (cul-de-sac) using disposable plain clean glass microcapillary tubes (Fischer Scientific, Pittsburgh, PA), where the fluid flowed inside the tube by direct contact (Figure 11). During the collection, contact between microcapillary tube and conjunctiva was avoided to reduce lacrimal stimulation and extra sample dilution. The tear fluid was placed in Eppendorf Tubes® and stored at -80°C for voriconazole concentration analysis. Aqueous humor samples were collected under standing sedation and periocular blocks as described above. The sampling area was also irrigated with 0.5% povidone-iodine solution. The bulbar conjunctiva was then grasped using a 0.3 colibri forceps and a 30 gauge needle advanced through the limbal cornea into the anterior chamber and 0.3-0.5 mL of AH aspirated (Figure 12). Immediately following sample collection, a drop of topical moxifloxacin 0.5% (Vigamox®, Alcon, Fort Worth, TX) was applied topically to the eye. Following the procedure each horse received a single dose of flunixin meglumine (Banamine®, Merck, Germany) at a dose of 1.1 mg/kg. Samples were stored at -80°C until determination of voriconazole concentrations.

Figure 11. Tear collection in the tear lake (*cul-de-sac*) using a microcapillary tube.

Figure 12. Extraction of aqueous humor for determination of voriconazole concentration.

Phase 2: Ocular tissue voriconazole concentrations

Animals

Four horses (2 geldings and 2 mares, American Quarter Horses) were included in the phase 2 of the study; the ages ranged from 8 to 17 years (mean of 14 years).

Treatments and overall design:

Horses included in the second phase received a SC injection of 1.7% voriconazole thermogel in the dorsal bulbar conjunctiva of the left eye 48 hours prior to euthanasia as described above. Two hours prior euthanasia the horses received the same SC injection in the dorsal bulbar conjunctiva of the right eye.

Horses were euthanized using a lethal dose of pentobarbital sodium. Immediately after euthanasia both eyes were enucleated and snap frozen in liquid nitrogen, followed by storage at -80°C until dissection was performed.

Tissue collection and processing

Prior snap freezing of the eyes for voriconazole tissue concentration determination, the periocular tissues surrounding the sclera were removed. The frozen eyes were cut in half along the horizontal axis into dorsal and ventral segments using a microtome blade (Accu-Edge®) (Figure 13). The frozen ocular globes were dissected over a cooled ceramic tile placed over a cooler filled with liquid nitrogen, in order to prevent rapid thawing of the tissues and drug diffusion. [53] Once cut in halves,

dissection was started on the dorsal segment and the ventral segment was stored in a -20°C freezer until dissection of the other half was finished. The different tissues were individually dissected over the cooled tile in the following order: AH, cornea, iris-ciliary body, vitreous, retina, choroid, and sclera (Figure 14). Each tissue was weighed and placed in a plastic container. The tissues were immediately stored at -80°C for voriconazole concentration determination.

Data analysis

Data were evaluated for normality using a Shapiro-Wilk test and non-parametric analysis used when appropriate. For phase 1, scores from the modified Hackett-McDonald scoring system were compared among days and treatments with Friedman's test and Dunn's post hoc test. Non-continuous score data were presented as median and range. Voriconazole concentrations in tears at day 2 between the Vori-Top and Vori-Gel groups were compared using a Wilcoxon Rank Sums test. For phase 2, voriconazole concentrations were compared among the different tissues, the 2 time points and between dorsal and ventral segments using a Kruskal Wallis test and Dunn's post hoc test. Both tissue concentrations from the anterior segment (cornea, AH, iris-ciliary body, sclera and lens) and posterior segment (vitreous, retina, choroid) were pooled separately to compare for location and time points. Significance was set at $p \leq 0.05$. Data were analyzed using commercial software (GraphPad Prism v.6).
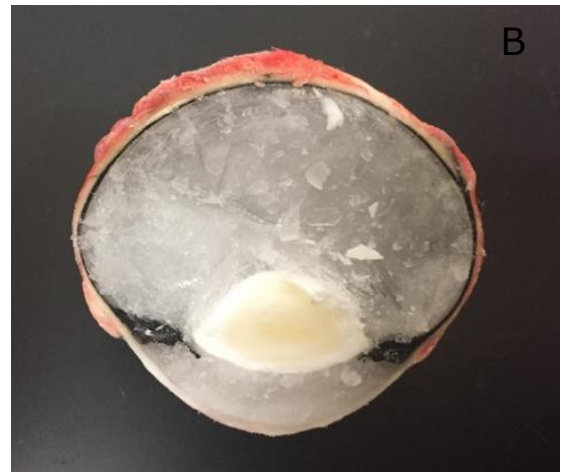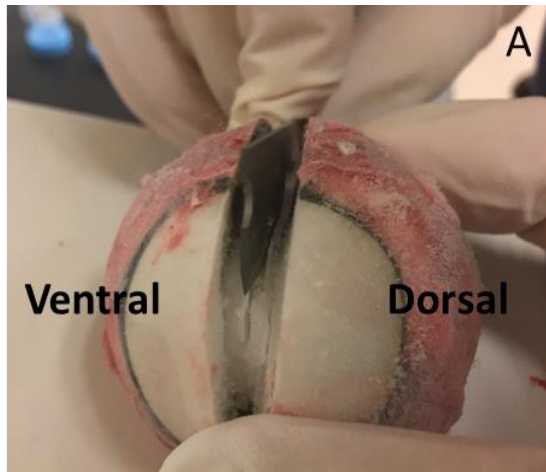
Figure 13. Initial process of eye dissection following snap freezing. **A**. The eye globe is divided into ventral and dorsal segments; each half, represented in image **B**, is then separately dissected in all of the tissues for measurement of voriconazole concentrations.
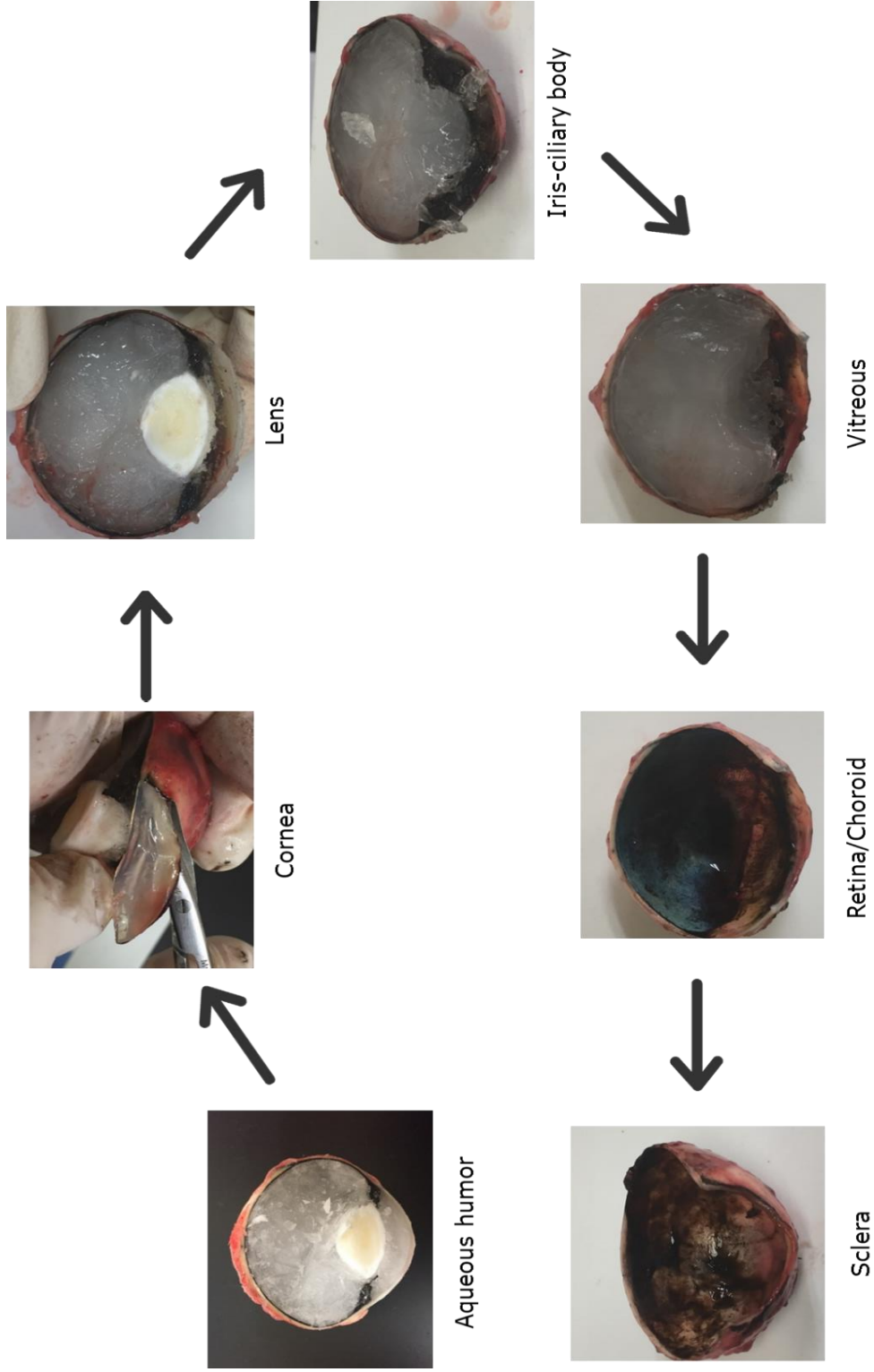
Figure 14. Sequence of dissection of the ocular tissues. Each legend indicates the tissue to be dissected for the correspondent image.

Results

<u>Phase 1: *In vivo* tear and aqueous humor voriconazole concentrations and ocular</u>

<u>toxicity</u>

Clinical findings and ocular inflammation assessment

Results from the evaluation of conjunctival congestion, swelling and discharge are presented in table 3. There was significantly more swelling on day 14 when compared to day 0 ($p=0.0021$) (Figure 15). Conjunctival congestion on days 2 and 7 post treatment with the voriconazole-thermogel were significantly higher than day 0 ($p=0.0053$) (Figure 16). There were no significant differences between days for conjunctival discharge ($p=0.2311$).

Scores for corneal cloudiness, pannus, fluorescein staining intensity, aqueous flare, iris congestion, anterior vitreous involvement were 0 for all the horses at all evaluation times. No changes were observed on evaluation of the lens and fundus on any animal throughout the study. Ophthalmic examinations revealed mild changes in the conjunctiva (Figure 17). Physical examinations remained normal for all the horses throughout the study.

	Conjunctival Inflammatory score		
Day	**Congestion**	**Swelling**	**Discharge**
0	0	0	0.17 (0-1)
2	2.5 (1-3)	2 (1-2)	0.17 (0-1)
7	2 (1-3)	2 (1-2)	0
14	1 (1-2)	2	0
23	1 (0-3)	1.5 (1-3)	0.33 (0-1)

Table 3. Means and ranges of the conjunctival inflammatory scores for each of the timepoints, n=6
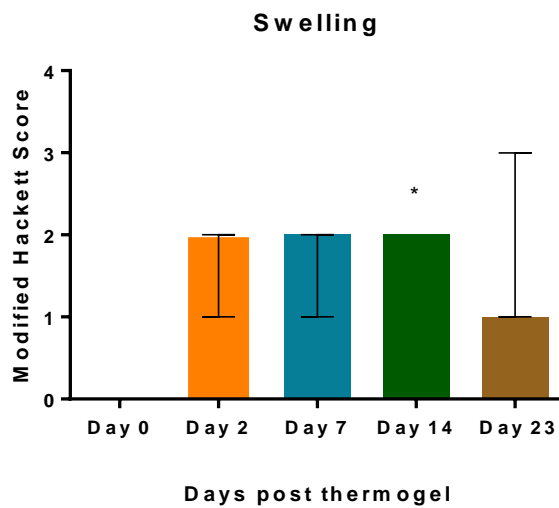
Figure 15. Conjunctival swelling scores compared between days; * Day 14 is significantly different from day 0 (p = 0.0021). Modified Hackett-McDonald score for conjunctival inflammation; data are presented as median and range.
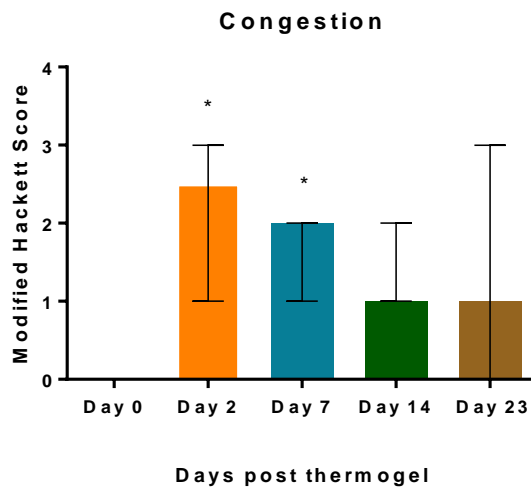
Figure 16. Conjunctival congestion scores compared between days; * Days 2 and 7 are significantly different from day 0 (p = 0.0053). Modified Hackett-McDonald score for conjunctival inflammation; data are presented as median and range.
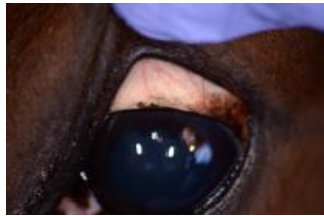
Baseline   Day 2   Day 7

Day 14   Day 23

Figure 17. Assessment of conjunctival inflammation at different timepoints for one of the horses. Note after day 2 the raise in the conjunctiva due to the gel deposit in the SC space, which persisted for the remaining of the study.

Voriconazole concentrations in aqueous humor and tears

The voriconazole concentrations in tears on day 2 between Vori-Top and Vori-Gel were not significantly different (p=0.0625). Results from the voriconazole concentrations in AH and tears for the Vori-Top group are presented in table 4.

For the animals in the Vori-Gel group, results of voriconazole concentration in tears and AH are presented in table 5. Following the SC injection, voriconazole concentrations in tears were not detectable on days 7, 14 and 23. In AH, on days 2 and 7 post injection, voriconazole was detectable but non-quantifiable (minimum concentration quantifiable of 0.001 μg/mL). Concentrations in AH were not detectable on days 14 and 23 post-injection.

Sample	Horse						Mean (μg/mL)	SD
	1	2	3	4	5	6		
	Voriconazole concentration (μg/mL)							
Tears	3.058	8.945	0.961	1.491	3.036	---	3.498	3.184
Aqueous Humor	2.074	1.355	1.479	1.647	1.254	1.282	1.515	0.31

Table 4. Voriconazole concentration in tears and AH (Vori-Top group) on day 2. Mean and standard deviations (μg/mL), n=6.

Sample	Day	Horse						Mean (μg/mL)	SD
		1	2	3	4	5	6		
		Voriconazole concentration (μg/mL)							
Tears	2	0.05	0.052	0.188	0.071	0.018	0.015	0.149	0.207
	7	0.015	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0	0
	23	0	0	0	0	0	0	0	0
Aqueous Humor	2	*	*	*	*	*	*	*	*
	7	*	*	*	*	*	*	*	*
	14	0	0	0	0	0	0	0	0
	23	0	0	0	0	0	0	0	0

Table 5. Voriconazole concentration in tears and AH (Vori-Gel group) on days 2, 7, 14, 23. Mean and standard deviations (μg/mL), n=6.

*Voriconazole concentrations in the range 0.001 – 0.005 μg/mL. (Detectable, non-quantifiable).

Phase 2: Ocular tissue voriconazole concentrations

Table 6 shows the means (+/- SEM) for the four horses included in this phase, units μg/g. One horse was excluded from the statistical analysis due to confounding values for the drug concentrations due to potential passive drug diffusion during dissection; table 7 shows the means (+/- SEM) for the horses included. Figure 17 compares voriconazole concentrations between the different tissues and timepoints. At the 2 hour time point, corneal concentrations were significantly higher than lens, vitreous and retina ($p<0.0001$). At the 48 hour time point corneal concentrations were significantly higher than iris-ciliary body and lens ($p<0.0001$). Sclera concentrations of voriconazole were significantly higher than AH, lens, iris-ciliary body, vitreous, retina and choroid 48 hours after the SC injection ($p<0.0001$). There were no significant differences in voriconazole tissue concentrations between timepoints ($p=0.7794$).

When the pooled tissue concentrations between anterior and posterior segments and between timepoints were compared (Figure 18), the anterior segment concentrations at 48 hours were significantly higher than posterior segment at 2 and 48 hours. ($p = 0.0013$). Figure 19 represents the comparison of each of the tissues in the anterior segment between the time points. At the 2 hour time point, corneal concentrations were significantly higher than the lens ($p<0.0001$). At the 48 hour time point, corneal concentrations were significantly higher than iris and lens ($p<0.0001$); sclera

concentrations were significantly higher than AH, lens, and iris (p<0.0001). There were no significant differences in concentrations for the tissues of the anterior segment between time points (p=0.7794). No significant differences were found among the tissues of the posterior segment of the eye for each time point or between time points (Figure 20).

No significant differences were observed between the grouped dorsal and ventral segments of the eye (p=0.4823) when compared at different timepoints, therefore no effect of the injection on location was observed (Figure 21).

		2 hours					48 hours				
		Horse 1	Horse 2	Horse 3	Mean (μg/g)	SEM	Horse 1	Horse 2	Horse 3	Mean (μg/g)	SEM
Cornea	Dorsal	6.82	2.58	3.9	4.43	2.17	3.15	9.45	2.52	5.04	3.83
	Ventral	4.19	1.65	3.19	3.01	1.28	1.2	3.49	1.03	1.91	1.37
Aqueous Humor	Dorsal	0.1	0.15	0.16	0.14	0.03	0.46	0.76	0.61	0.61	0.15
	Ventral	1.12	2.32	1.23	1.56	0.66	0.49	0.73	0.52	0.58	0.13
Lens	Dorsal	0.16	0.37	0.03	0.19	0.17	0.16	0.4	0.34	0.30	0.12
	Ventral	0.16	0.11	0.06	0.11	0.05	0.19	0.18	0.01	0.13	0.10
Iris Ciliary body	Dorsal	2.28	1.18	0.78	1.41	0.78	1.41	3.67	1.02	2.03	1.43
	Ventral	0.29	0.43	0.44	0.39	0.08	0.59	0.95	0.63	0.72	0.20
Vitreous	Dorsal	0.02	0.01	0.06	0.03	0.03	0.01	0.01	0.03	0.02	0.01
	Ventral	0.01	0.01	0.27	0.10	0.15	0.01	0.01	0.03	0.02	0.01
Retina	Dorsal	0.63	0.4	1.63	0.89	0.65	0.26	0.39	0.41	0.35	0.08
	Ventral	0.28	0.22	0.5	0.33	0.15	0.31	0.24	0.12	0.22	0.10
Choroid	Dorsal	0.68	0.23	1.07	0.66	0.42	0.64	1	0.78	0.81	0.18
	Ventral	0.72	0.99	1.11	0.94	0.20	0.73	0.58	0.68	0.66	0.08
Sclera	Dorsal	11.04	3.68	2.35	5.69	4.68	4.88	14.22	2.95	7.35	6.03
	Ventral	0.19	0.06	0.12	0.12	0.07	1.14	3.3	1.04	1.83	1.28

Table 6. Voriconazole tissue concentrations (μg/g). Means and SEM (μg/g).
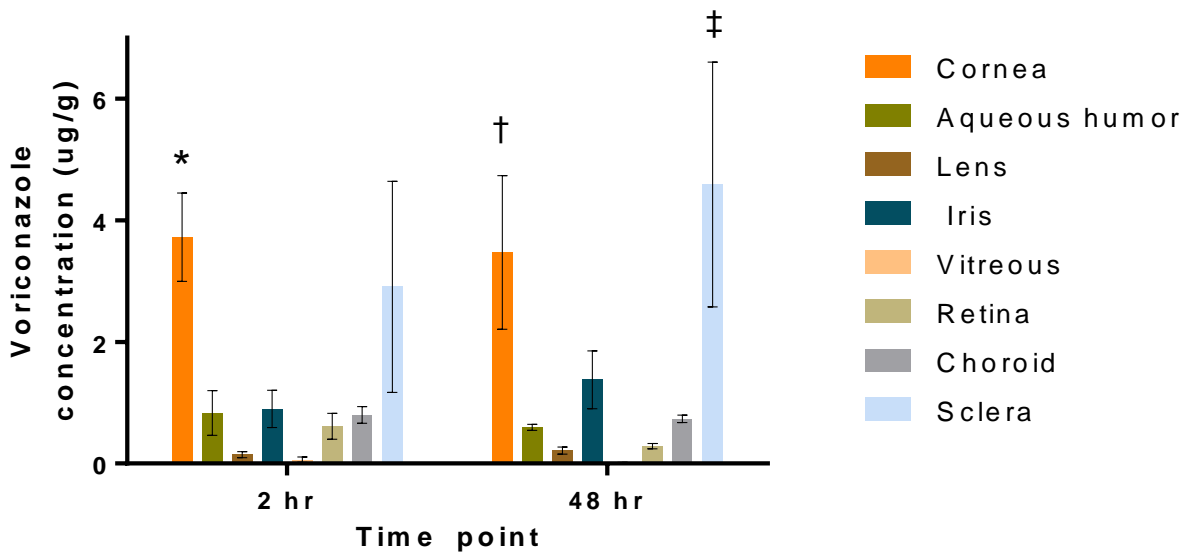
Figure 18. Comparison of the voriconazole concentrations (ug/g) between the different ocular tissues and between timepoints. For each tissue, drug concentrations for the dorsal and ventral segments were combined.

* Corneal concentrations significantly higher than lens, vitreous, and retina at 2 hours.

† Corneal concentrations significantly higher than iris or lens at 48 hours.

‡ Sclera concentrations significantly higher than AH, lens, iris, vitreous, retina, and choroid at 48 hour.

$p < 0.0001$ for tissue differences within timepoints.

$p = 0.7794$ for differences between timepoints.
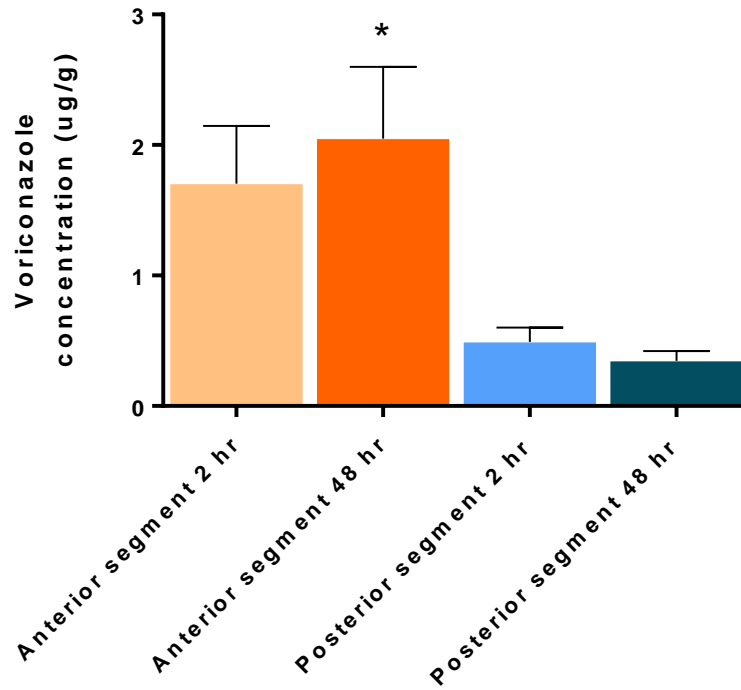
Figure 19. Comparison of the voriconazole concentrations (ug/g) between the anterior and posterior segments at each timepoint.

* Anterior segment concentrations at 48 hours significantly higher than posterior segment at 2 and 48 hours.

p = 0.0013
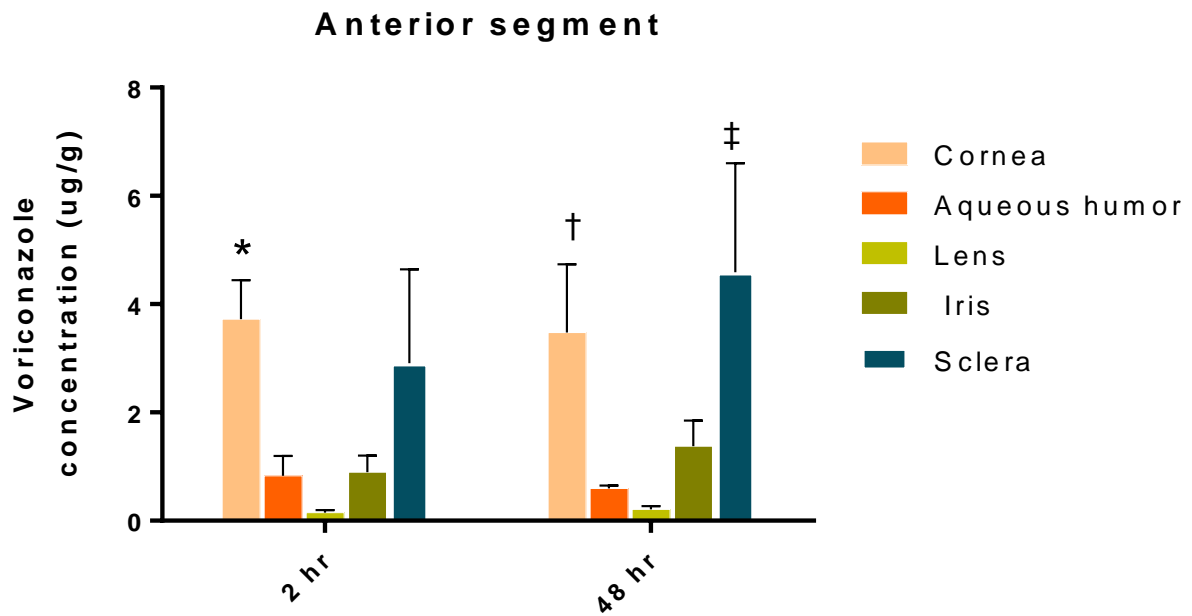
Figure 20. Comparison of the voriconazole anterior segment tissue concentrations (μg/g) for each timepoints and between times.

* Corneal concentrations significantly higher than lens at 2 hours.

† Corneal concentrations significantly higher than iris or lens at 48 hour.

‡ Sclera concentrations significantly higher than AH, lens, and iris at 48 hour.

$p < 0.0001$ for tissue differences within timepoints.

Figure 21. Comparison of the voriconazole concentration (μg/g) posterior segment concentrations for each timepoint and between tissues.

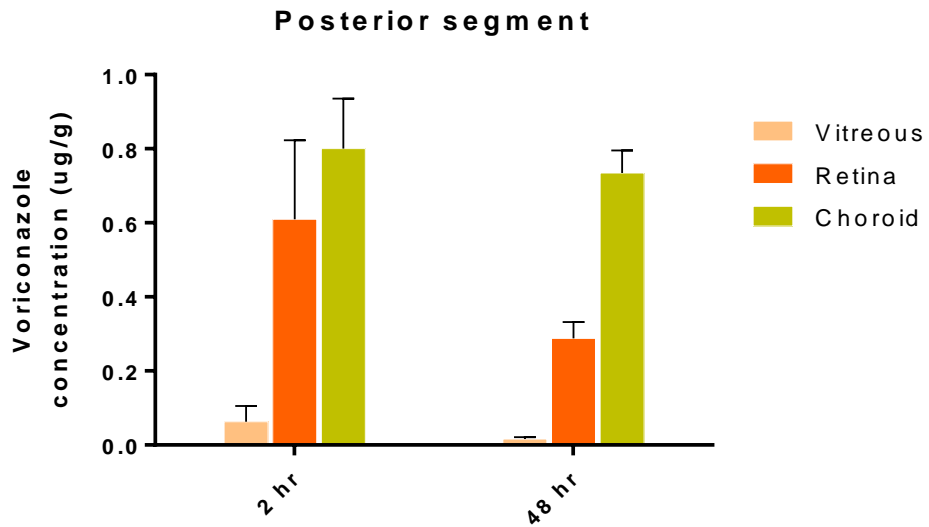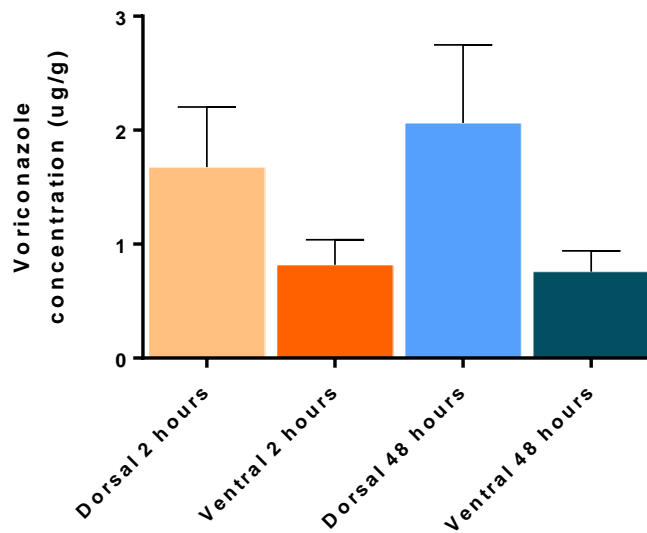**Figure 22**. Comparison of voriconazole concentrations μg/g between the dorsal and ventral segments. p = 0.4823.

Discussion

Mycotic keratitis is an often severe disease in which diagnosis can be challenging, response to medical therapy slow, and outcome poor with severe ocular morbidity and vision loss. [20,27] To overcome treatment challenges, alternatives routes and vehicles for drug delivery may help in achieve higher concentrations in the targeted region, and decrease frequency of treatment. In our study, we demonstrated the sustained release of voriconazole, by measuring the drug concentrations in tears, for 2 days after a SC injection of a 1.7% voriconazole thermogel in live horses, however concentrations were below the target MIC of 0.5 μg/mL. However, no significant difference was found between Vori-Top (standard treatment) and Vori-Gel (p = 0.0625) Results from our study showed the presence of voriconazole in aqueous humor for up to 7 days after the SC injection of the thermogel, however in concentrations that were not quantifiable (0.001 – 0.005 μg/mL), thus behavior of the thermogel *in vivo* differed from the reported *in vitro* release study by Cuming *et al*. where voriconazole loaded thermogel at the same concentrations released the drug for at least 28 days. [4] When drugs are applied in the SC space, they will enter the anterior segment by diverse mechanisms including direct drug diffusion into the cornea and sclera, an important one is through corneal absorption once the drug enters the tear film, from reflux from the puncture site or diffusion across the conjunctiva. Some of the drug will reach the anterior segment from the ciliary arteries, however once the drug molecules reach the blood vessels, most of it will be cleared and not reach the ocular tissues. [58] The *in vivo* phase of this study used horses with no ocular inflammation, therefore the diffusion of drugs by this route will

be limited by the blood-ocular barrier and part of the drug will be lost into the systemic circulation. [45] It is expected that in diseased eyes with presence of inflammation and impairment of the blood-ocular barrier, larger amounts of drug will be reaching the anterior segment of the eye. [59] This was observed in a clinical case of keratomycosis in a horse treated with a SC injection of 1.7% voriconazole thermogel, where significantly higher concentrations of voriconazole were detected in the AH 2 and 5 days and on tears on day 1,2,3,4 and 5 post treatment. The case description is presented in appendix 2.

A possible cause of the lack of detection of voriconazole in the AH after the SC injection of the voriconazole thermogel was the presence of an intact blood-ocular barrier. The high volume of distribution of voriconazole will enhance binding of the drug to the uveal tissues; [38] which was demonstrated in our study based on the voriconazole ocular tissue concentrations obtained, therefore it is expected that as time increases after the administration of the drug, the molecules will stay bound to the uveal tissues instead of the AH. One of the most important mechanisms of drug delivery to the anterior chamber is reflux from the injection site, and this has been demonstrated by the use of drugs in solution after SC injection. [58] For our case, the liquid voriconazole thermogel formed a gel immediately after injection, once in contact with the SC space, therefore it is expected that leakage from the puncture site will be less than previously reported. [58] Even though there was no significant differences in tear concentrations at day 2 between the Vori-Top and Vori-Gel groups,

the low voriconazole concentrations in tears at day 2 for the Vori-Gel group, could be explained by lower leakage from the SC space.

In order to sterilize the voriconazole hydrogel prior to injection in the live horses, both the thermogel and the voriconazole powder were irradiated using gamma-rays. Irradiation can damage the materials resulting in hydrolysis, oxidation, and depolymerization [111,112]; even at low dosages, gamma irradiation can lead to formation of sediments. [111] Gamma irradiation can also enhance the formation of free radicals, propagating polymer chain scission overtime. [113]

Sterilization of thermosensitive polymers is challenging since common methods used in medicine for elimination of microorganisms such as steam autoclaving and irradiation have the potential to alter the physico-chemical properties of the thermogels, thus altering the sustained-release capacity and limiting its availability to the tissues. *In vitro* release of voriconazole from a PLGA-PEG-PLGA thermogel, where both the drug and hydrogel were exposed to gamma-rays, showed release kinetics that mimicked what was previously reported by Cuming *et al*., however it is uncertain if the exposure of irradiated thermogel to the enzymes responsible for degradation in the organism will be different because of a weaker polymeric network. [113] Other methods of sterilization including filter needle and ozone gas should be studied and consider in order to maintain the characteristics of these polymers and avoid loss of efficacy. [114]

The use of the 1% voriconazole solution in the Vori-Top group was based on what is commonly used in our institution when topical voriconazole is used for the treatment of fungal keratitis, as well as results from a previous studies showing the safety and therapeutic concentrations when used at this frequency and concentration. [11] As demonstrated in previous studies, the direct administration of voriconazole to the intact cornea will effectively penetrate the tight corneal epithelium. [11,37] The high lipophilicity and volume of distribution of voriconazole is an important factor in the penetration of the drug through the lipophilic corneal epithelium and endothelium, which are important barriers for many medications. [11] Concentrations of voriconazole in the aqueous humor after 2 days of treatment with topical 1% voriconazole were above the target MIC; which was in agreement with data previously reported by Clode et al. [11]

Concentrations in tears between the Vori-Top and Vori-Gel groups on day 2 were not significantly different, which confirms a sustained release of the voriconazole after the SC injection. The low concentration in tears in the Vori-Gel group could be due to a low diffusion of voriconazole from the injection site to the tear film, either because of low leakage from the puncture site or high blood clearance and low diffusion across the conjunctiva. [58] For both groups the standard deviation was high, reflecting a wide variability among the horses. To prevent this a higher sample might be needed, however with tear samples, dilution has to be taken under consideration, and marked difference in volume was observed while collecting the samples. In normal conditions there will be a basal tear production, however reflex tears in response to stimuli such

as light, wind or irritants can increase the volume of production and therefore dilute the drug concentration. In our case, the tears collecting method (capillary tube) may induce reflex tearing. [45]

In the ranges of MICs for filamentous and yeast organisms, most isolates are < 0.5 μg/mL., [11] therefore antifungal therapies are expected to have MICs above this value to be consider of clinical efficacy. In our study, concentrations of voriconazole in the ocular tissues at both 2 and 48 hours after SC injection of the thermogel were, for most of them, above this target MIC. Even 2 days after the injection, the concentrations in the anterior segment of the eye were significantly higher than the posterior segment. This is of clinical importance since in cases of keratomycosis the goal is to maintain therapeutic concentrations of the voriconazole in the cornea. Within the tissues classified in the anterior segment, sclera achieved higher concentrations than other tissues, therefore it might have contributed to the significance in concentration difference between the anterior and posterior segments. The sclera was included as a tissue of the anterior segment due to the fact that the SC injection was applied over the anterior sclera. Higher drug concentrations were expected in the anterior segment since one important mechanisms of drug diffusion to the eye using the SC route, and especially using a lipophilic drug as voriconazole, is direct penetration through the sclera before reaching the tissues. [58] Due to the location of the injection, the drug could also reach the anterior tissues via the limbal vessels. [58] The previously discussed findings are in correlation with other studies of drug diffusion to the eye, were after SC injection, the drug reaches the anterior

segment of the eye, through mechanisms that will highly depend on the drug characteristics (hydrophilic versus lipophilic). [58,59,62] The lower voriconazole concentrations detected in the ocular fluids, especially the AH, for the second phase of the study, correlates with the findings of the first phase. Voriconazole is a lipophilic drug, and this confirms our initial hypothesis in which the drug is expected to accumulate in the tissues, especially the uvea, instead of hydrophilic areas.

Moreover, as soon as 2 hours after the treatment, mean concentrations of the drug were above the target MIC, which is potentially important in the treatment of a clinical case, where initial therapy with topical 1% voriconazole solution might not be necessary to reach high levels soon after initiation of therapy.

Further analysis of the tissues in the anterior segment of the eye showed significant higher concentrations of voriconazole in the cornea compared to the lens and iris-ciliary body. In the treatment of equine keratomycosis, the cornea is the target tissue of interest, thus the findings are showing that even at 2 days after injection, mean concentrations of almost 4-fold increase from the target MIC of 0.5 μg/g are maintained in the cornea.

The most important drug delivery mechanism to the anterior chamber of the eye is through corneal diffusion. With the topical route there is continuous passage of voriconazole through the cornea, which is expected due to the high lipophilicity, and has been demonstrated in our study as well as in previous experiments. [11]

Voriconazole concentrations in the aqueous humor from the frozen eyes had values lower than the ones from the Vori-Top group, and one can conclude that it could be due to other mechanism of drug diffusion, rather than transcorneal. The gel will most likely release drug directly onto the sclera that will diffuse to the anterior segment of the eye.

Even though the SC route is used to treat conditions in the anterior segment of the eye, the results showed mean concentrations of voriconazole in the tissues of the posterior segment, when pooled together, above the target MIC; this could be of interest for further investigation in the use of other medications to treat conditions in the posterior segment, especially if intravitreal injections are not suitable to perform. This data suggests that transscleral diffusion is the likely the major route of drug delivery to the choroid/retina after SC, subtenon or retrobulbar injections. [115]

Within the posterior segment, as it was expected, concentrations of voriconazole are higher in the choroid at both time points. This is based on the fact that transscleral diffusion is enhanced with lipophilic drugs, thus reaching the choroid more effectively. [45,62]

When the tissue concentrations on the dorsal segment were pooled together and compared to the concentrations in the ventral segment, no statistical difference was observed, however means were higher in the dorsal aspects, and maintained at least for 2 days following injection. Further studies are warranted to determine whether

the site of injection has an effect in the tissue concentrations, in order to make therapeutic decisions in accordance to the location of the lesion. Abarca *et al*. [53] identified regional differences in drug distribution after suprachoroidal injection of a liphophilic substance, with levels higher on the dosed side, most likely because the drug will remain bound to the choroidal stroma. Nevertheless, it should be considered that in our study, at both 2 and 48 hours after the SC injection, the voriconazole concentrations in the ventral segments remained above the target MIC; thus in cases where the injection can only be administered in either the dorsal or ventral bulbar conjunctiva, therapeutic concentrations will be most likely achieved. This is supported by a previous report using bevacizumab by SC injection in mice with intact cornea, where by immunohistochemical analysis the drug was found along the entire corneal stroma 24 hours after injection and remained unchanged up to 14 days. [59]

There is limited literature describing the use of voriconazole by SC injection in horses, however this route has been proven to provide higher concentrations of drugs to the anterior segment of the eye [59]. Some isolates of fungi, including *Fusarium* spp., have a higher MIC for voriconazole [39], thus higher drug concentrations are desired for treatment of more fastidious microbes. This study reported the use of SC injections of 1.7% voriconazole-thermogel in six horses with no significant adverse reactions observed. All the animals were comfortable with no signs of uveitis. The mild conjunctival swelling and congestion were transient and most likely secondary to the injection procedure itself, the gel deposit and the collection of the aqueous humor. The voriconazole-thermogel used in this study was developed in a previous study for

SC injection in horses. [4] The technique for injection was also previously described and mimicked in the current study. [4] The injection was easy to apply in all the horses by administration of 0.3 mL of the voriconazole thermogel in the dorsal bulbar conjunctiva using a 30 gauge, ½ inch needle attached to a 1 mL syringe following standing sedation and local anesthesia. The use of a small gauge needle caused minimal leakage of the voriconazole thermogel and the horses reacted less to the injection, which was in consistency with previous reports of SC injections in horses. [4,32]

The 1.7% voriconazole-thermogel (5 mg/0.3 mL) was well tolerated *in vivo* for 23 days, did not cause inflammation in the eye globe itself, with no evidence of uveitis. This is consistent with previous reports of the use of thermogels for treatment of ocular conditions applied by routes different from SC, including intracameral, intravitreal or directly over the cornea. [75,78] Subconjunctival injections to treat fungal keratitis in horses using voriconazole 1% solution has been reported with no evidence of post injection blepharospasm, epiphora, chemosis or conjunctival reaction. [40] Subconjunctival injections may cause hemorrhage of the conjunctival tissue, which usually resolves within a few days with no discomfort in the patient. Reports in humans have described adverse effects of medications such as methylprednisolone acetate and triamcinolone acetonide when applied by the SC route, including conjunctival necrosis and persistent inflammation. [116] In horses amphotericin B is an antifungal commonly used by the SC route for the treatment of keratomycosis, and uncomplicated conjunctival hyperemia is often a consequence. [117] There are no

reports of the use of voriconazole subconjunctivally in concentrations above 1%, however intrastromal injections of voriconazole in concentrations of 5% for treatment of fungal stromal abscesses did not report significant complications. [31,32] Conjunctival swelling had scores equal or lower than 2, and there was a significant difference in day 14 compared to baseline. A score of 2 is considered to be mild swelling and confined to a focal area. [81] This difference in conjunctival swelling was considered to be a raising in the conjunctiva due to the gel deposit in the subconjunctival space. The conjunctival swelling was restricted to the area of injection, and a well-defined elevated area in the conjunctiva was observed rather than diffuse edema and inflammation. The median score in swelling for day 23 was 1, which was consistent with the degradation of the gel. On day 2, the congestion was attributed to the injection itself, causing hemorrhage of the scleral vessels. Despite this degree of congestion, none of the horses showed signs of ocular pain including blepharospasm or epiphora. On day 7, the degree of conjunctival congestion was most likely due to the extraction of aqueous humor on day 2 or the injection performed on day 0. The conjunctival congestion was always restricted to a focal area on the dorsal bulbar conjunctiva at the location of the injection and aqueous centesis. The previous reports of the use of voriconazole solution 1% by SC injection did not report complications such as congestion or swelling, so it is unlikely that the voriconazole will cause any adverse reaction to the tissues; however none of this studies used an inflammatory scoring system for a more objective assessment. [40] Some of the horses had ocular discharge scored below grade 1 on days 0, 2 and 23, which is considered to be mild. The discharge was clear and present just on the inner portion of the eye, but not on

the eyelids. Since no significant difference was observed between day 0 and days 2 and 23, the discharge was considered to be unrelated to the injection. Horses can have mild discharge with no signs of ocular pain due to environmental hypersensitivity or irritation by small airborne particles. [118]

There were a number of limitations in this study. The availability of horses for terminal laboratories was a limitation regarding the sample size for the voriconazole tissue concentrations phase, since a larger sample size could have given more conclusions for injection location effect. The horses for the voriconazole tissue phase couldn't be used for the study for more than 2 days, limiting the analysis of tissue concentrations for a longer period of time. There are no ideal techniques for sterilization of thermogels, and this is a limitation for its clinical use. Even though needle filter sterilization preserves the physicochemical characteristics of the polymer, since we used a voriconazole suspension, some of the drug will remain in the filter. The design of the study, where AH samples were taken while evaluating inflammation in the conjunctiva could have been a confounding factor while assessing for congestion, since it was common to have small transient subconjunctival hemorrhages, thus altering the grading.

Conclusions

This study is the first to use the SC route for voriconazole delivery from a biodegradable thermogel in live animals. The results of the injection technique were in agreement with a previous report in *ex vivo* model. [4] The injection was performed

with no clinical side effects, and the drug was released to the ocular tissues for at least 2 days following injection of the thermogel. Moreover, during the 2-day period, voriconazole concentrations were above the target MIC, primarily for tissues of the anterior segment, which is of significance for the future treatment of equine keratomycosis.

This study is an example of the mechanisms of drug delivery to the eye by the transscleral and transcorneal route. It was demonstrated that following the SC route, lipophilic drugs will enter the anterior chamber, most likely through scleral diffusion, and concentrate in greater amounts in the tissues of the anterior segment of the eye. This information can be translated to the clinical setting to increase treatment efficacy and client compliance. Together with the use of the PLGA-PEG-PLGA thermogel as a vehicle for sustained release, this technique is promising for the treatment of equine keratomycosis.

Further studies are warranted to better determine the frequency of injections, and to better assess the effect of the voriconazole thermogel in clinical cases. There is a need to seek for better techniques for sterilization of thermogels, looking forward to improve the quality of the therapy and move forward into using the subconjunctival injections of voriconazole thermogel in the clinic.

Summary

Subconjunctival injections are an alternate route to deliver higher drug concentrations to the anterior segment of the eye. The use of the thermogel PEG-PLGA-PEG as a vehicle for sustained delivery of voriconazole in this study demonstrated concentrations in the anterior segment of the eye above the target MIC for the most common fungi implicated in equine keratomycosis. These high concentrations were reached as soon as 2 hours after a 1.7% voriconazole thermogel injection and maintained for at least 48 hours. Within the tissues of the anterior segment, voriconazole concentrations maintained therapeutic levels in the cornea, which is of special interest in treating this clinical condition. It was demonstrated that following the SC route, voriconazole will enter the anterior chamber, most likely through scleral diffusion, and concentrate in greater amounts in the tissues of the anterior segment of the eye, which could explain the low levels of the drug in hydrophilic areas such as the AH. Sterilization of thermosensitive polymers is challenging and conventional methods such as gamma-irradiation have the potential to alter the physico-chemical properties of the thermogels. Despite the potential affection of the thermogels limiting its release drug capacity, the intact ocular barriers in healthy animals will limit the amount of drug reaching the anterior chamber, which was supported by the high voriconazole concentrations found in a horse with natural acquired keratomycosis

The voriconazole thermogel was easily administered by SC injection, and no clinically significant systemic or ocular adverse effects were observed. Using a modified Hackett-McDonald scoring system for ocular toxicity, differences in conjunctival swelling and congestion were recorded, however they were transient and most likely related to the injection procedure itself, the gel deposit in the SC space, and the collection of the AH. This method of assessing ocular toxicity in life animals was useful for the duration of the study. However the impact of the drug at a cellular level using an *ex vivo* model with equine corneas was not determined for longer than 2 hours after exposure to the voriconazole thermogel. Maintaining corneal sections in the Franz cell diffusion chamber for longer than 2 hours resulted in significant artifacts that precluded the histological analysis of the samples; further investigation in preservation of equine corneas is warranted in order to use this technique for longer exposure times.

Results from this study are of potential interest for the treatment of keratomycosis in horses, with future applications to other animal species. The use of this voriconazole sustained-release technology in a larger number of clinical cases is the next step to better understand the behavior of the drug-thermogel combination in diseased eyes, and to further assess patient outcome, treatment cost and client compliance.

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Voriconazole release from a thermogel (PLGA-PEG-PLGA) previously sterilized with gamma-rays.

Preparation of the thermogel was done as previously described by Cuming *et al*., 2017. Release from 1 mg and 5 mg voriconazole thermogels were measured for 23 days. The thermogel was irradiated before reconstitution using gamma-rays (cobalt-60) with doses of 8, 13 and 25 kGy, with a rate dose between 5 and 6 kGy/h.

Results

	Vori-Gel 1 mg		Vori-Gel 5 mg	
Time (Day)	Voriconazole (µg/mL)	Cumulative release (µg)	Voriconazole (µg/mL)	Cumulative release (µg)
0	0	0.0	0	0.0
0.25	82.85	82.9	137.5	137.5
0.5	100.0	182.9	177.4	314.8
2	117.4	300.3	207.5	522.3
3	110.8	411.1	245.5	767.8
4	97.21	508.3	195.1	962.8
5	82.24	590.5	203.1	1166
6	56.45	647.0	176.8	1343
7	54.96	701.9	90.95	1434
10	9.750	711.7	62.58	1496
13	3.121	714.8	21.19	1517
15	1.190	716.0	9.252	1527
17	0.460	716.4	5.345	1532
19	0.215	716.7	1.698	1534
21	0.0480	716.7	0.701	1534
23	0.0310	716.7	0.323	1535

Table 1. Voriconazole concentration and cumulative release from the thermogel for both 1 mg and 5 mg concentrations.
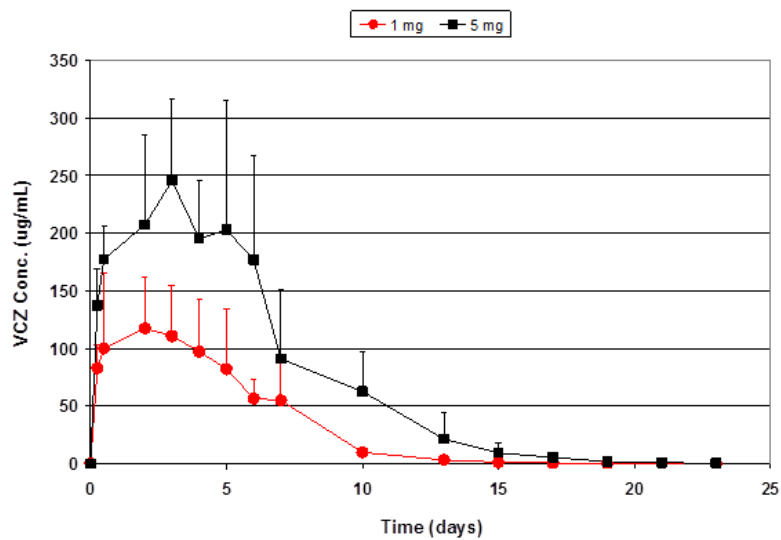
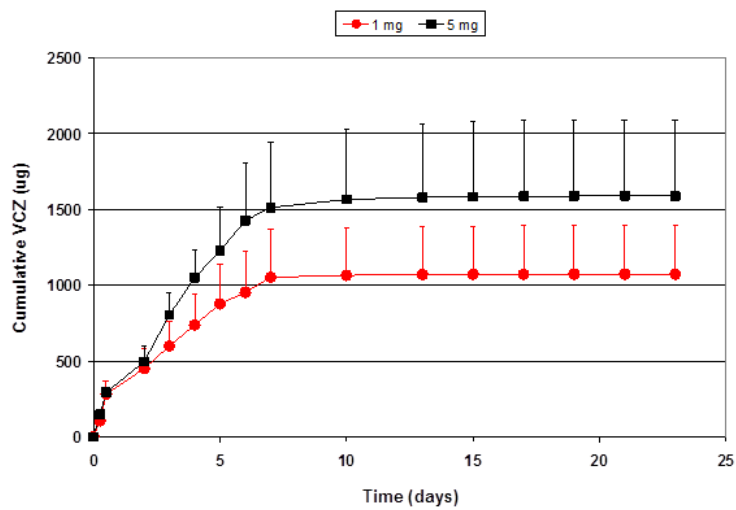**Figure 1.** Release of voriconazole (μg/ml) from the irradiated thermogel for 23 days.

Figure 2. Cumulative release of voriconazole (μg/ml) from the irradiated thermogel.

Appendix 2

Clinical case:

Eighteen-year-old Warmblood gelding, was presented for ophthalmic evaluation of the left eye. The horse had blepharedema, blepharospasm, and epiphora of the left eye. Ophthalmic evaluation revealed ulcerative keratitis with a superficial plaque, which was confirmed to be fungal upon identification of hypae in cytological evaluation of the cornea. There was marked neovascularization, corneal edema and miosis. While the voriconazole thermogel was prepared, the gelding received topical miconazole 1% solution, 0.2 mL every 6 hours, for 3 days. Following this period, a SC injection of 1.7% voriconazole thermogel (0.3 mL) was applied in the dorsal bulbar conjunctiva as previously described (Figure 1). Aqueous humor samples were obtained on days 2 and 5 following injection; additionally, tears were collected daily for 5 days after the injection. Samples of AH and tears were analyzed for voriconazole concentrations using HPLC (Table 1). The horse received a second injection of 1.7% voriconazole thermogel subconjunctivally on day 7 and was released to a paddock. The gelding showed improvement on his condition and remained visual. Additional therapy included superficial keratectomy, and topical ophthalmic atropine and neomycin/polymyxin B/gramicidin.
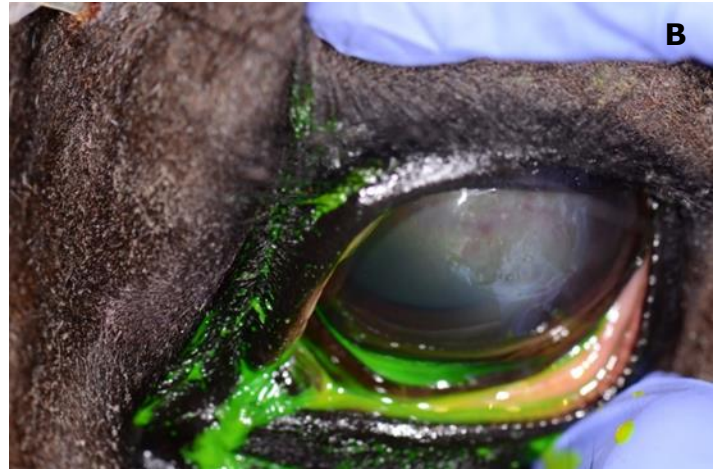
Figure 1. A. Subconjunctival injection of 1.7% voriconazole thermogel in a case of superficial fungal keratitis. **B.** Superficial fungal keratitis in a horse treated with a sustained release voriconazole thermogel.

	Voriconazole (μg/mL)	
Days	**Tears**	**AH**
0	---	
1	4.94	
2	2.76	414.0
3	9.84	
4	1.76	
5	4.95	424.5

Table 1. Voriconazole concentrations (μg/mL) in tears and AH after a SC injection of 1.7% voriconazole thermogel in a clinical case of fungal keratitis.