

**Development of a Sustained-Release Voriconazole-Containing Thermogel for  
Subconjunctival Injection in Horses**

by

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## Abstract

Subconjunctival administration of a slow-release, voriconazole-containing thermogel may allow for sustained delivery of voriconazole to the cornea and anterior chamber.

Thermogel development consisted of four experiments. **i) *In vitro*** voriconazole release. Liquid (4°C) poly (DL-lactide-co-glycolide-b-ethylene glycol-b-DL-lactide-co-glycolide) (PLGA-PEG-PLGA) (+0mg, 1mg and 5mg voriconazole) was placed in glass vials at 34.5°C to induce gelation. Physiological buffered saline (PBS) was added and samples collected for 28 days. **ii) *In vitro*** permeation. Permeation of voriconazole (1.5% solution, 0.3% and 1.5% thermogel) through equine corneas and sclerae was determined using a Franz Cell diffusion chamber. PBS was collected for 24 hours and voriconazole concentrations measured via high performance liquid chromatography (HPLC). **iii) Characterization of thermogel subconjunctival space (SCS) injection.** Ten normothermic *ex vivo* equine eyes were injected with liquid thermogel (4°C). **iv) *In vivo*** ocular toxicity. SCS thermogel injections were performed in a horse 1 week and 2 hours pre-euthanasia. Toxicity was evaluated via ocular inflammatory scores and histopathology.

Voriconazole was released from the PLGA-PEG-PLGA thermogel *in vitro* and diffused through the cornea and sclera in effective concentrations. The thermogel formed a discrete gel deposit in the dorsal bulbar SCS and did not induce adverse reactions *in vivo*. Voriconazole-containing thermogels have potential application in treatment of equine keratomycosis.

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## List of Abbreviations

AH	aqueous humor
ANOVA	analysis of variance
AUC	area under the curve
AULATH	Auburn University Large Animal Teaching Hospital
HPLC	high performance liquid chromatography
MIC	minimum inhibitory concentration
MW	molecular weight
NaF	sodium fluorescein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	poly(ethylene glycol)
PLGA	poly(lactic-co-glycolic acid)
PLGA-PEG-PLGA	poly(DL-lactide-co-glycolide-b-ethylene glycol-b-DL-lactide-co-glycolide)
SCS	subconjunctival space
SD	standard deviation
$t_{1/2}$	half-life
UBM	ultrasound biomicroscopy
w/w	weight for weight
$W_a$	wet weight
$W_b$	dry weight

## Chapter 1 – Literature Review

### **Section 1: Pathogenesis of Equine Keratomycosis**

Keratomycosis (fungal infection of the cornea) is a vision-threatening disease well described in horses and humans worldwide.<sup>1-3</sup> Fungal keratitis is an emerging cause of corneal disease in horses, with the reported incidence in North America rising from 13% of keratitis cases in the 40 years preceding 2006 to 25% in 2013.<sup>4, 5</sup> Keratomycosis is particularly prevalent in tropical or subtropical environments, such as the southern United States,<sup>2, 6</sup> and consequently accounted for 40% of the equine corneal disease caseload presenting to the Auburn University Large Animal Teaching Hospital (AULATH) during the 2012-13 academic year.

Horses are thought to be prone to fungal ocular infections due to their large globe size, unique orbital shape and resultant globe exposure, suspected tear film instability and living environment.<sup>7, 8</sup> Fungi are ubiquitous in the equine environment (e.g. in hay, soil and bedding) and are also part of the normal ocular surface microbiota of the horse.<sup>1, 4</sup> 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*.<sup>1, 5, 9</sup>

Development of keratomycosis requires disruption of the normal defense mechanisms of the equine cornea and conjunctiva such as the intact corneal epithelium, normal bacterial microbiota, flushing of commensal organisms through tear flow and eyelid movement, and tear film macrophages, lysozymes and immunoglobulins.<sup>7, 10</sup> Following disruption of one or more of these defense mechanisms, commensal or introduced (environmental) fungi are able to adhere to the cornea, proliferate and invade the corneal stroma.<sup>1, 6, 8</sup> 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. This may ultimately result in fungal migration into the anterior chamber, lens and iris, with subsequent development of severe endophthalmitis.<sup>6</sup>

Several clinical manifestations of equine keratomycosis exist. These include corneal ulceration (+/- keratomalacia), corneal microerosions, superficial punctate keratitis, stromal abscessation, stromal plaque formation and corneal perforation with iris prolapse.<sup>1, 8, 11, 12</sup> Of these, stromal ulcerative keratomycosis is the most common manifestation, comprising 50-80% of reported cases of equine keratomycosis.<sup>6</sup>

## **Section 2: Prognosis and Outcome of Equine Keratomycosis**

Equine keratomycosis is a source of severe morbidity in horses.<sup>4-6, 10</sup> Fungal organisms and infiltrating immune cells are capable of initiating both vigorous inflammatory responses and protease overproduction in the cornea; this in turn may lead to the development of acute complications in affected eyes including rapid keratomalacia (melting of the cornea), unresolving

ulceration, abscess formation, uncontrolled anterior uveitis, endophthalmitis and corneal perforation.<sup>5, 6, 13</sup> Long term sequelae may include corneal scarring, cataract or synechiae formation, phthisis bulbi and vision loss.

Seven retrospective case series have been published that report outcomes in horses affected with equine keratomycosis.<sup>10, 12-18</sup> Success rates (as defined by restoration of functional vision and/or ocular survival) in these different equine populations varied from 50% to greater than 90%, with the majority of case series reporting retention of functional vision in <70% of cases.<sup>10, 12-18</sup> In light of the severity of potential sequelae of keratomycosis described above, these success rates are undesirable. The variation in treatment success reported between case series may reflect improvement in treatment protocols over time or variations in case selection e.g. corneal ulcers only vs. all manifestations of keratomycosis.<sup>10, 16</sup> The largest case series of 65 horses reported a requirement for surgical intervention to control the infection in 65% of cases, with 49% of these surgical patients maintaining globe integrity and 15% ultimately requiring enucleation.<sup>13</sup> In this study genus of fungus cultured (*Aspergillus* vs *Fusarium*), time of year, bacterial co-infection and previous steroid or antifungal use were not associated with outcome.<sup>13</sup>

Treatment success in cases of keratomycosis depends both on the stage of the corneal disease and subsequent uveitis present at the time of diagnosis, and the destructive capability of the fungal organism involved.<sup>5, 13</sup> Equine keratomycosis treatment is challenging, prolonged and expensive and requires compliance from both horses and their handlers.<sup>8</sup> The average duration of treatment varies from 48-72 days, with up to 192 days of treatment described.<sup>6, 12</sup> The average



time in hospital varies from 15-21 days.<sup>8,12</sup> Extended hospitalization represents an investment of thousands of dollars and a source of considerable stress for the horse and has been defined as a risk factor for development of colic.<sup>19</sup>

### **Section 3: Diagnosis and Treatment of Equine Keratomycosis**

Accurate and timely diagnosis, and therefore rapid institution of appropriate antimicrobial therapy, can significantly affect outcome in the face of infectious disease.<sup>1,5</sup> Definitive diagnosis of keratomycosis requires visualization of the organism within corneal tissue in concert with prompt growth of the visualized fungal organism in culture. Timely diagnosis of keratomycosis poses a clinical challenge due to the propensity of fungal organisms to invade the deepest layers of the cornea, thus evading cytologic sampling, their fastidious nature and therefore potential to die during handling or shipping of samples, and slow growth in culture (1-3 weeks). Diagnosis of equine keratomycosis has therefore traditionally been based on identification of fungal organisms on cytologic examination, fungal culture or histopathologic examination of corneal samples, in conjunction with identification of clinical signs supportive of keratomycosis and/or response to therapy.<sup>5, 10, 11</sup> Additional techniques which have been recently reported for diagnosis of keratomycosis in horses include polymerase chain reaction (PCR) and *in vivo* confocal microscopy.<sup>5,20</sup> Nested and quantitative PCR performed on 43 corneal samples from horses with ocular disease were able to identify fungal organisms in both fungal culture positive and negative eyes within 2-5 days of sampling.<sup>5</sup> While the nature of fungal organisms identified (i.e. pathogen, commensal or contaminant) cannot be confirmed by this method, PCR may have a role to play by identifying the fungal population in horses' eyes at presentation, thus providing a basis for

empirical therapy while awaiting other results, and furthermore may provide a rapid method of assessing the efficacy of anti-fungal therapy in horses with keratomycosis. *In vivo* confocal microscopy is a technique whereby high-resolution magnification of tissues allows for non-invasive, real-time, spatial sectioning of the cornea at the cellular level.<sup>20</sup> *In vivo* confocal microscopy of horses with naturally occurring keratomycosis and *ex vivo* equine corneas infected with fungal organisms provided a rapid, non-invasive diagnosis of keratomycosis through visualization of the fungal organisms themselves and accompanying corneal pathology in horses affected *in vivo*, including leukocyte infiltrates, activated keratocytes, anterior stromal dendritic cell infiltrates and vascularization.<sup>20</sup>

Fungal keratitis can be treated with either medical therapy alone or a combination of medical and surgical therapy.<sup>6</sup> Whilst surgical procedures are immediately indicated in patients with acute corneal perforation or in those with imminent or pre-existing rupture of a stromal abscess into the anterior chamber, medical treatment with antifungal agents still constitutes the major therapeutic option in most cases, whereby success depends on the agent's ability to penetrate the corneal epithelium and achieve therapeutic concentrations in the anterior segment of the eye.<sup>21</sup> In order to reach therapeutic concentrations in the cornea and anterior chamber, treatment typically involves application of topical medication every 1-2 hours in acute disease, decreasing ultimately to every 6-12 hours, with potential concurrent administration of oral antifungals, anti-inflammatories and antibiotics.<sup>6,22</sup>

Two main classes of antifungal medication are used in the treatment of keratomycosis: the azoles, which include fluconazole, voriconazole, miconazole, ketoconazole and itraconazole, and the polyenes which include amphotericin B and natamycin.<sup>9</sup> These agents target ergosterol, the main lipid present in fungal cell membranes. The polyenes bind to ergosterol in fungal cell membranes, altering membrane permeability and therefore allowing loss of protein, carbohydrate and cations from the cell and ultimately causing cell death. Natamycin is the only antifungal agent approved for ophthalmic use by the United States Food and Drug Administration. It has poor epithelial penetration and therefore may not reach therapeutic concentrations in the corneal tissue if the epithelium is intact.<sup>3</sup> Amphotericin B is potentially epitheliotoxic and has poor penetration through the intact epithelium, therefore is rarely used topically to treat equine keratomycosis.<sup>6,23</sup> The azoles decrease ergosterol synthesis in fungal cell membranes and therefore ultimately result in their destruction. In addition, all azoles with the exception of fluconazole, decrease the function of immune system cells and may therefore lessen the inflammatory response in cases of keratomycosis.<sup>23</sup> Itraconazole displays limited penetration of the intact corneal epithelium, miconazole displays moderately good penetration and voriconazole displays excellent corneal penetration.<sup>23,24</sup>

Antifungal medications are generally applied topically to treat keratomycosis, however isolated case reports of resolution of keratomycosis exist following subconjunctival (amphotericin B, voriconazole) and intrastromal (voriconazole) antifungal administration in horses and following intracameral and subconjunctival administration in humans.<sup>22,25-28</sup> Fluconazole, in addition, can be administered orally in some cases particularly where a good blood supply is present to the site

of infection, enabling delivery of drugs administered systemically to the cornea, or in cases with severe anterior uveitis as it has been shown to achieve therapeutic concentrations in the anterior chamber following oral administration.<sup>29</sup>

#### **Section 4: Horses as a Natural Model of Human Keratomycosis**

The epidemiology, presentation, treatment and prognosis of human keratomycosis is very similar to that of equine keratomycosis.<sup>3, 30</sup> A retrospective study of keratomycosis in the USA revealed a higher incidence in warmer, humid areas than those with cooler climates, which supported previous reports in which keratomycosis accounted for 35% of keratitis cases in Florida, as opposed to 1-5% in the northern states.<sup>2, 21</sup> The most common fungi implicated in cases of human keratomycosis are *Fusarium*, *Aspergillus*, *Curvularia* and *Candida*, and voriconazole, which demonstrated 100% activity against 541 human fungal keratitis isolates, is a recommended treatment.<sup>3, 21, 22, 28</sup> Horses are the only domestic animal that serve as a naturally occurring comparative model of human keratomycosis, and therefore investigation into new treatment modalities in horses may influence development of parallel human therapies and benefit people who contract this disease.<sup>30</sup>

#### **Section 5: Voriconazole Use in Treatment of Keratomycosis**

Voriconazole is a second generation triazole antifungal drug. It acts by inhibiting the P450-dependant enzyme C-14 $\alpha$  demethylase and therefore causes depletion of ergosterol and accumulation of 14-methyl sterols in fungal cell membranes.<sup>4, 21</sup> This in turn disrupts cell membrane synthesis and repair and thus the integrity and function of the fungal organism itself.<sup>4,</sup>

<sup>21</sup> Voriconazole is a relatively new antifungal agent in equine and human ophthalmology which, based on *in vitro* evidence, appears to be the most effective antifungal for initial treatment of keratomycosis in the midwestern and southern United States due to its wide spectrum of antifungal activity, low minimum inhibitory concentrations (MIC)s against common pathogens and excellent corneal penetration.<sup>4, 21, 30, 31</sup> Voriconazole has been shown to adequately penetrate the cornea following topical administration in horses and to thus overcome the barrier of poor transcorneal penetration which limits efficacy of alternative antifungal drugs.<sup>4, 31</sup> Topical administration of a 1% voriconazole solution achieved voriconazole levels of 2.35µg/ml in the aqueous humor (AH) of horses 1 hour post-administration; a concentration greater than the reported MIC of most filamentous and yeast fungal keratitis isolates (0.5 µg/ml).<sup>4</sup> Despite the excellent corneal penetration of voriconazole, several additional barriers exist in the eye which must be overcome in order to obtain higher or more sustained therapeutic voriconazole concentrations in the cornea and anterior chamber following topical delivery.

### **Section 6: Challenges in ocular drug delivery**

The principal challenge in advancing therapeutics for treating diseases of the front, as well as back, of the eye is attainment of effective drug concentrations in the target tissue for prolonged periods of time, whilst minimizing any side effects.<sup>32</sup> Upon topical administration, pre-corneal factors and anatomical barriers negatively affect the bioavailability of topical formulations.<sup>32, 33</sup> Anatomical drug transport barriers include corneal and conjunctival epithelial tight junctions and clearance from the vasculature in the conjunctiva.<sup>34, 35</sup> The barriers associated with poor client/patient compliance are intensified in horses due to their size, the strength of their eyelid

musculature and their lack of tolerance for repeated application of topical ocular medications, especially to painful eyes. Placement of a subpalpebral lavage system assists with medication delivery, but medications still need to be given frequently, and these systems are expensive and easily broken by horses. Pre-corneal factors in both people and animals include normal tear turnover, gravity induced tear flow, blinking and induced lacrimation.<sup>36</sup> The cumulative effect of these pre-corneal factors is to decrease contact time between the medication and the absorptive membranes, and this is considered to be the primary reason for less than 5% of the applied dose reaching the intraocular tissues.<sup>36</sup>

Inherent drug properties can be exploited and alternative drug formulations created to improve delivery of topical medications to the anterior segment of the eye.<sup>32,33</sup> Voriconazole, for example, is a highly lipophilic, small molecular weight drug, and as such has excellent corneal penetration, as is evidenced by the fact that therapeutic levels are reached in the anterior chamber following application to people and horses with intact corneas.<sup>4,37</sup> Despite effective corneal penetration, sustained drug delivery is still severely limited by ocular barriers and this effect is short lived, with studies in rabbits demonstrating the need for administration of voriconazole every 30 minutes in order to achieve a sustained high level of voriconazole in the anterior chamber.<sup>38</sup> Research has been conducted into liposomal, nanoparticle-encapsulated and microemulsion formulations of voriconazole to try and increase the contact time with the cornea, and therefore provide more sustained therapeutic drug levels, following administration.<sup>39-41</sup> Further work is needed in this area before these products are used in a clinical setting.

## **Section 7: Sustained Release Drug Delivery in Veterinary Medicine**

Sustained release drug delivery technology is a medical field that has expanded exponentially over the past twenty years. In order for sustained release drug delivery to be successful, an effective medication must be selected which will reach high levels in the tissue of interest, and a vehicle must be selected which can be combined with this drug and will release it slowly over time. Applications of such technology currently employed in veterinary medicine include implantation of antibiotic-impregnated polymethylmethacrylate beads or collagen sponges into joints for treatment of joint sepsis, subcutaneous deslorelin implants for timing of ovulation in mares and male contraception in companion animals, and trace mineral rumen boluses for mineral deficiency in cattle and sheep.<sup>42-46</sup> In the last decade, surgical implantation of cyclosporine-containing suprachoroidal implants has become a standard treatment option for management of equine recurrent uveitis.<sup>47</sup> More recently, successful use of cyclosporine-containing silicone episcleral implants to treat immune-mediated keratitis and keratoconjunctivitis sicca in horses has been described.<sup>48, 49</sup> The reported clinical success of these implants demonstrates the potential advantage of sustained drug delivery devices in treatment of equine ocular diseases.

## **Section 8: Hydrogels as Ocular Drug Delivery Vehicles**

Over the past decade, a variety of biocompatible, biodegradable polymers have been investigated for their potential as drug delivery carriers in an attempt to overcome the aforementioned limitations of topical ocular drug delivery.<sup>50</sup> Applications of this technology in human medicine which have shown promise include a topical dexamethasone hydrogel for

treatment of uveitis and a ganciclovir hydrogel for intravitreal injection for management of cytomegalovirus retinitis.<sup>51, 52</sup> These polymeric “smart” materials 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, including gene delivery and tissue engineering.<sup>53</sup> Thermosensitive biodegradable hydrogels (‘thermogels’), such as poly (DL-lactide-co-glycolide-b-ethylene glycol-b-DL-lactide-co-glycolide) (PLGA-PEG-PLGA) are one such group of polymers. They are administered as a liquid but convert to form a gel deposit upon reaching body temperature.<sup>50, 54</sup> 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.<sup>50</sup> PLGA-PEG-PLGA thermogels consist of hydrophobic poly(lactic-co-glycolic acid) (PLGA) segments, which link together to form micelles at low temperatures, and hydrophilic poly(ethylene glycol) (PEG) segments which provide bridges between micelles. At low temperatures the hydrogen bonding between hydrophilic PEG segments of the copolymers and water molecules dominates, and therefore the copolymer takes on a liquid form.<sup>55</sup> As the temperature rises, the hydrophobic interactions among PLGA segments predominate and the transition to a gel occurs.<sup>55</sup> PLGA-PEG-PLGA copolymers can entrap both hydrophilic and hydrophobic compounds, with the hydrophobicity/hydrophilicity of the polymer able to be controlled by altering the ratio of the molecular weight of PLGA to that of PEG and the ratio of lactic acid to glycolic acid of PLGA.<sup>55, 56</sup> Release of hydrophobic (lipophilic) drugs is dependent on degradation of the hydrogel matrix, and is therefore a sustained process.<sup>55</sup> Degradation kinetics and gel modulus can also be altered



by altering the ratio of the molecular weight of PLGA to that of PEG and the ratio of lactic acid to glycolic acid of PLGA in the copolymer.<sup>56</sup>

## **Section 9: Justification of the Study**

### *Subsection 9a: Choice of Drug, Polymer and Route of Administration*

Voriconazole was selected as the antifungal agent in this project as a result of its unique characteristics in the treatment of keratomycosis.<sup>21, 30</sup> Voriconazole has been shown to adequately penetrate the cornea following topical administration in horses, as previously discussed<sup>4, 31</sup> It is a drug with a broad spectrum of antifungal activity, and in a recent study of *Aspergillus* sp. and *Fusarium* sp. 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.<sup>30</sup> These *in vitro* findings are supported in the literature, with voriconazole now recommended as a first line agent for treatment of keratomycosis.<sup>25, 27, 30, 31</sup> Subconjunctival administration of voriconazole has not been extensively evaluated in horses, however, 2 case reports have described subconjunctival and intrastromal administration of voriconazole which, in combination with topical antifungal therapy, resulted in resolution of keratomycosis in three horses without adverse effects.<sup>25, 27</sup> The safety of voriconazole has been demonstrated in equine, leporine and human eyes and in human cultured corneal endothelial cells.<sup>4, 21, 57, 58</sup> Voriconazole 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  $\leq 1\%$ , the concentration typically used in equine practice.<sup>4, 6, 21, 58</sup> Mild transitory blepharospasm and epiphora with no residual adverse

effects have, however been described in equine eyes at concentrations of 3%.<sup>4</sup> Systemic side effects of ocular administration of voriconazole are unlikely.<sup>4, 21</sup> A peak plasma concentration of 0.03µg/ml was achieved following topical ocular application of 1-3% voriconazole in horses, which is significantly lower than that achieved following administration of the recommended oral dose (1.47 µg/ml).<sup>4, 59</sup>

PLGA-PEG-PLGA is a biocompatible, biodegradable, thermogel that has been investigated as a drug delivery vehicle for human chemotherapeutic and ocular medications. It was selected for use in the proposed project due to its demonstrated safety in other species, ease of administration and its previously demonstrated sustained-release characteristics when combined with other small molecular weight, lipophilic drugs.<sup>51, 52, 60</sup> Following administration *in vivo*, the final degradation products of PEG-PLGA-PEG copolymers are lactic acid, glycolic acid, and PEG.<sup>56</sup> All of these substances are approved as safe materials by the United States Food and Drug Administration.<sup>37</sup> Previous ocular studies using PLGA-PEG-PLGA hydrogels have demonstrated successful sustained-release of selected medications and safety of PLGA-PEG-PLGA when used in the eye.<sup>35, 51, 54</sup> Histopathology of rabbit corneas following application of pure PLGA-PEG-PLGA as a corneal bandage revealed healthy epithelial cells and keratocytes and an absence of invading inflammatory cells.<sup>54</sup> Intravitreal administration of PLGA-PEG-PLGA containing ganciclovir was not associated with retinal toxicity in rabbits.<sup>51</sup> Subconjunctival injection of a protein loaded PLGA-PEG-PLGA hydrogel in rats resulted in delivery of the protein to the sclera, choroid and retina over 14 days without evidence of inflammation, infection or irritation in treated eyes.<sup>35</sup> Additional toxicity studies of PLGA-PEG-PLGA, when combined with

paclitaxel (a chemotherapeutic agent), revealed no toxicity following intramedullary spinal cord injection and intracranial injection in rats or following intralesional administration in human patients with esophageal cancer.<sup>60-62</sup>

The subconjunctival route of drug administration has many advantages over topical therapy and more invasive intraocular injection techniques. Topical and subconjunctival routes are often combined in treatment of human keratomycosis and occasionally in cases of equine keratomycosis, and this combination therapy is associated with more favorable outcomes than topical therapy alone in people, and anecdotally, in horses.<sup>6, 22, 25</sup> Subconjunctival drug administration provides higher and more prolonged drug concentrations in the anterior segment of the eye than topical therapy, with therapeutic concentrations achieved for up to 12 hours post-injection. This property is of particular benefit in cases of keratomycosis, where fungal organisms reside within the corneal stroma and anterior chamber. Several mechanisms have been proposed to explain the movement of drugs from the subconjunctival site of injection to the anterior segment of the eye. Transconjunctival permeation followed by corneal absorption, systemic absorption followed by return via the vascular bed, reflux out of the injection site, and, finally direct penetration of the globe of the eye all contribute to aqueous humor drug levels following subconjunctival injection.<sup>32, 63</sup> Bulbar subconjunctival injections are well tolerated by horses and can be performed by veterinarians in the field, thus making the proposed product available to a wider horse population than alternative techniques requiring specialty training and associated higher costs.<sup>25, 27</sup>

*Subsection 9b: Impact on Equine Health, the Alabama Economy and the Welfare of Alabama's Citizens*

A sustained-release voriconazole thermogel for subconjunctival injection would display many advantages over current topical therapy options and have great potential to improve comfort and ultimate outcome for horses with keratomycosis. It would provide a treatment which would be available to all horses, regardless of budget, as it could be administered in the field. It would negate the need for frequent topical application of voriconazole, thus minimizing stress in patients and improving client compliance by decreasing the volume, frequency and cost of medication required. The proposed voriconazole thermogel thus has the potential to positively impact global equine health.

The Alabama horse industry is a significant contributor to Alabama's economy with an estimated \$2.3 billion of the Gross State Product derived from this industry in 2004.<sup>64</sup> Horses contribute to Alabama's economy through direct horse expenses, healthcare, maintenance costs, equipment, competition, tourism and employment.<sup>64, 65</sup> Alabama's estimated horse population grew from 83,710 in 1989 to 186,871 in 2006 and the state has several factors which encourage continued growth.<sup>64</sup> These include climactic factors (mild climate, long growing seasons, good rainfall), low land costs and taxes, an abundance of established breeding and training facilities, and readily available, high-quality health support.<sup>65</sup> Horse ownership plays a role in the lives of many Alabama citizens, with 1 in 20 households in Alabama reporting a connection to horse ownership in 2005, as opposed to 1 in 63 households nationwide.<sup>64</sup> The health benefits of pet ownership

are widely recognized and Alabama's horses provide an important source of recreation for horse owners and riders, as well as improving the state's landscape for all of its citizens.<sup>64</sup>

Equine keratomycosis occurs most frequently in tropical and subtropical climates and this climactic effect is reflected in the cases seen at our institution.<sup>2</sup> Keratomycosis accounted for 40% of AULATH keratitis cases in 2012-13; an incidence considerably higher than the 25% reported nationally.<sup>5</sup> Scaring, blindness or globe loss resulting from keratomycosis can decrease the economic value of horses used for performance by preventing them from performing their intended use, decreasing their success in appearance-driven disciplines and diminishing their resale value. Vision loss can also negatively impact the horse-owner relationship as it may change horses' temperaments and owners' comfort around their horse, and in rare cases, may cause horses to become a danger to handlers or themselves. 90% of Alabama's horses are estimated to be of low economic value, therefore expensive veterinary conditions are likely to go untreated or generate economic hardship in this State.<sup>64</sup> The project outlined below therefore has great potential to positively impact the health of Alabama's horse population, and thus indirectly its economy and the welfare of its citizens, by providing a cost effective means of treating equine keratomycosis with improved success in terms of maintenance of vision in a population of horses where keratomycosis is prevalent. Shorter treatment duration, increased success and decreased costs in cases of equine keratomycosis would positively impact Alabama's horses, horse-owners and veterinarians.

## **Chapter 2 - Preliminary Research**

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.

### **Section 1: Equine Bulbar Subconjunctival Space Temperature Determination**

#### *Purpose*

Thermogels injected into the subconjunctival space (SCS) show promise as a sustained delivery system targeting the cornea and anterior chamber in horses. The aim of this experiment was to describe the bulbar SCS temperature in normal horse eyes in order to identify the optimal gelation temperature set point of thermogels intended for bulbar SCS injection in horses.

#### *Materials and Methods*

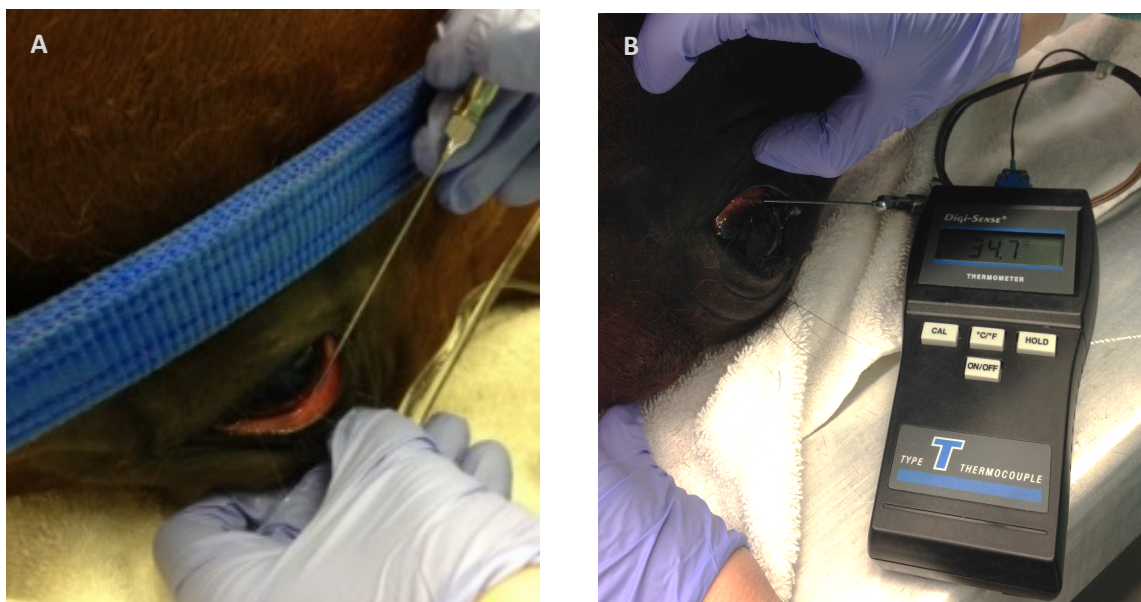
Ten adult horses participating in student surgical laboratories at the AULATH from April 2014 to December 2014 were enrolled in this study. Horses were pre-medicated with xylazine (Anased®, Akom, Inc, Decatur, IL) then general anesthesia was induced with ketamine (Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA) and maintained via inhalation of isoflurane (Fluriso®, Vet One, Boise, ID). Temperature measurements were collected within 10 minutes of anesthetic induction. Dorsal and ventral SCS temperatures were measured in the left and right eye using a Type T

thermocouple (Cole-Palmer, Vernon Hills, IL) (Figure 1), and rectal temperatures were measured using an electronic rectal thermometer (Provet, Sydney, NSW). Microsoft Excel 2013 (Microsoft, Redmond, WA) was used for statistical analysis of results. Temperatures in the left and right SCS, the dorsal and ventral SCS and rectal and mean SCS temperatures were compared using paired t-tests. Results were reported as range and mean  $\pm$  standard deviation and significance was set at a value of  $P < 0.05$ .

### *Results*

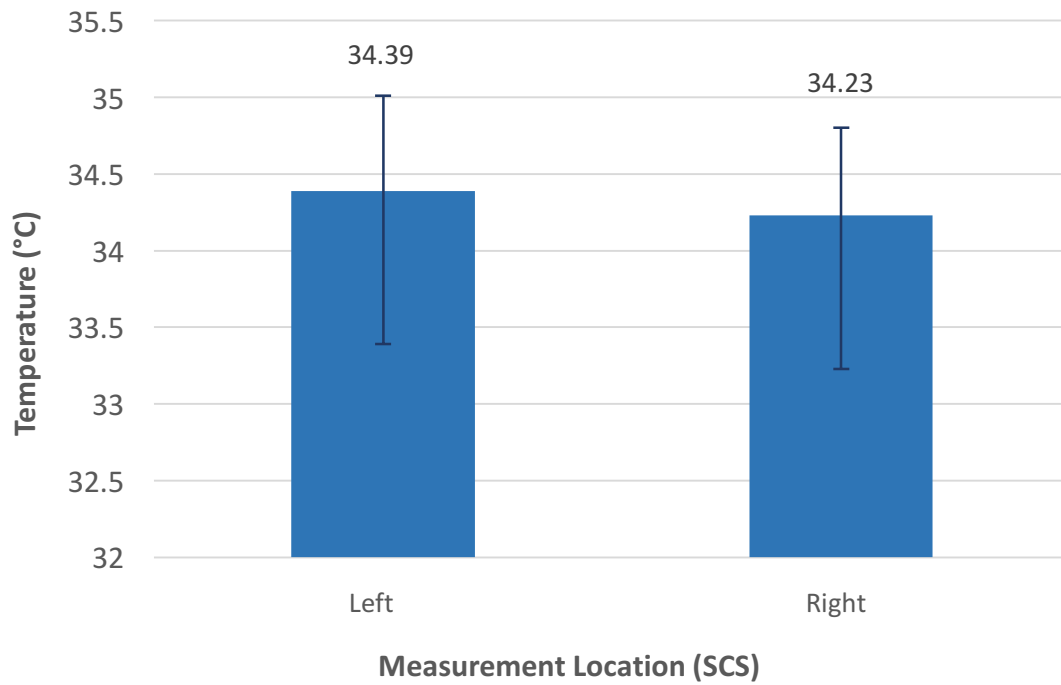
Horses were of mixed breed (4 Thoroughbreds, 5 Quarter Horses, 1 Trakehner) and gender (3 mares, 6 geldings, 1 stallion). The average age of the horses enrolled in the study was 14.4 years (range: 3-25 years), and their average weight was 466.2 kg (range: 385-444 kg).

Forty SCS temperature and 10 rectal temperature measurements were recorded. SCS temperatures ranged from 33.3°C-35.3°C ( $34.5^{\circ}\text{C} \pm 0.6^{\circ}\text{C}$ ) in the dorsal bulbar SCS and 33.3°C-35.3°C ( $34.5^{\circ}\text{C} \pm 0.6^{\circ}\text{C}$ ) in the ventral bulbar SCS. Rectal temperatures ranged from 36.1-38.5°C ( $37.3^{\circ}\text{C} \pm 0.7^{\circ}\text{C}$ ). There was no significant difference between SCS temperatures in the left and right eye ( $P=0.276$ ) (Figure 2) or between temperatures in the dorsal bulbar SCS and the ventral bulbar SCS ( $P=0.739$ ) (Figure 3). There was a significant difference between the temperatures measured in the rectum and the bulbar SCS ( $P=0.0000001$ ) (Figure 4). The temperature difference between rectal and SCS temperatures ranged from 2.0°C-4.1°C ( $2.8^{\circ}\text{C}; \pm 0.6^{\circ}\text{C}$ ).

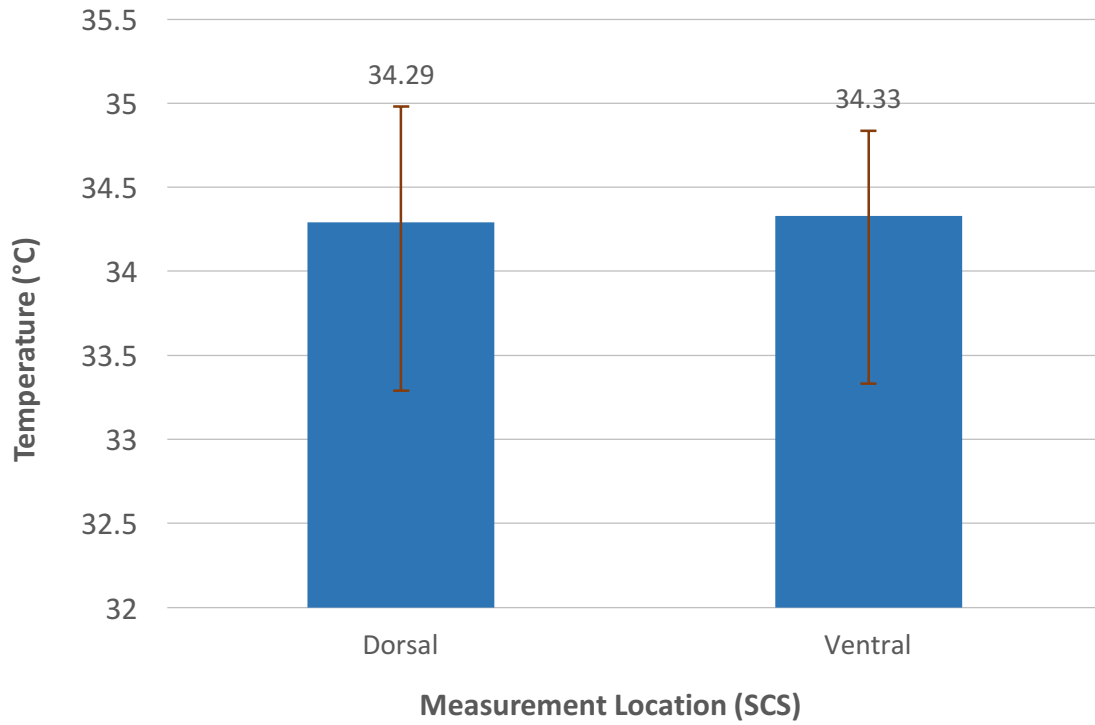


**Figure 1.** Measurement of the temperature in the **A.** dorsal and **B.** ventral bulbar subconjunctival space of a horse under general anesthesia using a Type T thermocouple.

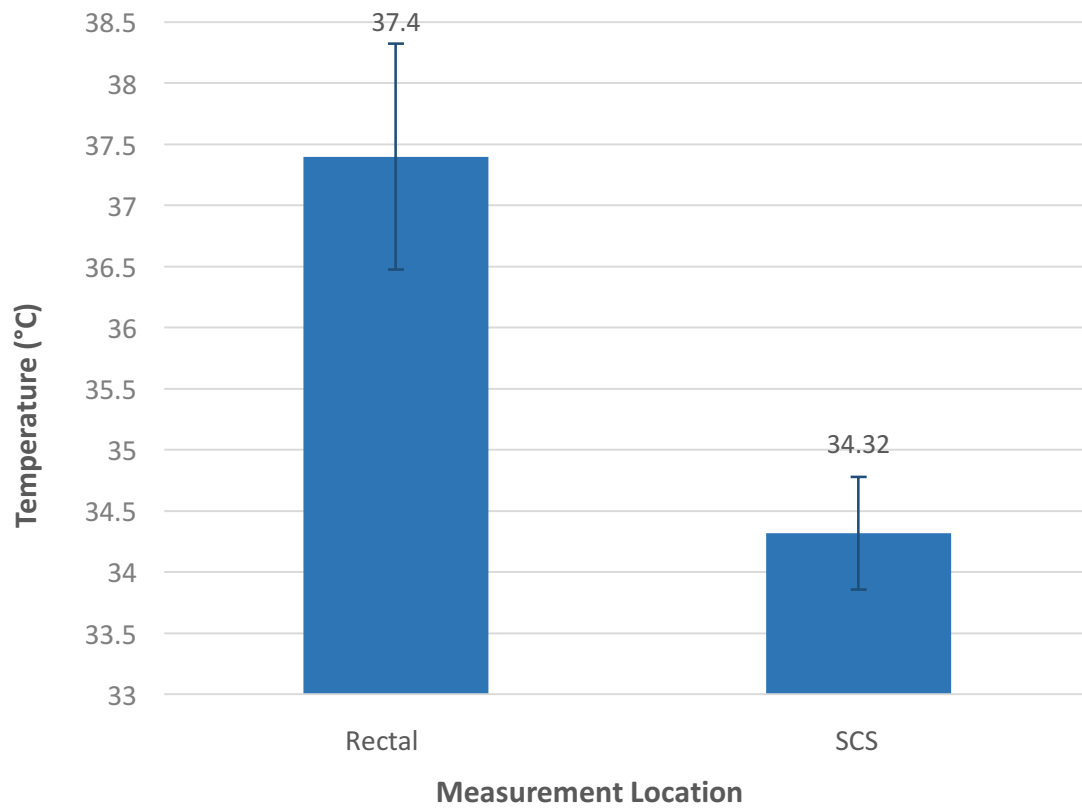




**Figure 2.** Mean temperature  $\pm$  SD ( $^{\circ}$ C) in the equine bulbar subconjunctival space by location. There was no significant difference between mean temperatures in the SCS of the left and right eyes.



**Figure 3.** Mean temperature  $\pm$  SD ( $^{\circ}$ C) in the equine bulbar subconjunctival space by location. There was no significant difference between mean temperatures in the dorsal and ventral SCS.



**Figure 4.** Mean rectal and subconjunctival space temperatures  $\pm$  SD ( $^{\circ}$ C) in anesthetised adult horses. There was a significant difference between rectal and SCS temperatures.

## *Conclusions*

The mean and range of temperatures in the bulbar SCS of adult horses are 34.5°C and 33.3°C-35.3°C respectively. There is no significant difference between the temperature in the dorsal and ventral bulbar SCS of individual eyes or between the bulbar SCS of contralateral eyes in horses. The mean difference between the rectal and SCS temperature in horses is 2.8°C, however the SCS temperature can range from 2.0°C-4.1°C lower than the rectal temperature in horses. The above findings are consistent with previously reported bulbar SCS temperatures in humans and rabbits, when adjusted for species-specific core temperature ranges.<sup>66-68</sup> They indicate that the ideal thermogel for injection in the SCS of horses would have a gelation set point of 33°C-36°C.

## **Section 2: Thermogel Selection**

### *Purpose*

Different thermogels have unique rheological properties.<sup>69</sup> This experiment aimed to identify the best PLGA-PEG-PLGA thermogel for injection into the SCS in horses and to determine whether or not the gel retained its thermosensitive properties following combination with voriconazole and methylene blue.

### *Materials and Methods*

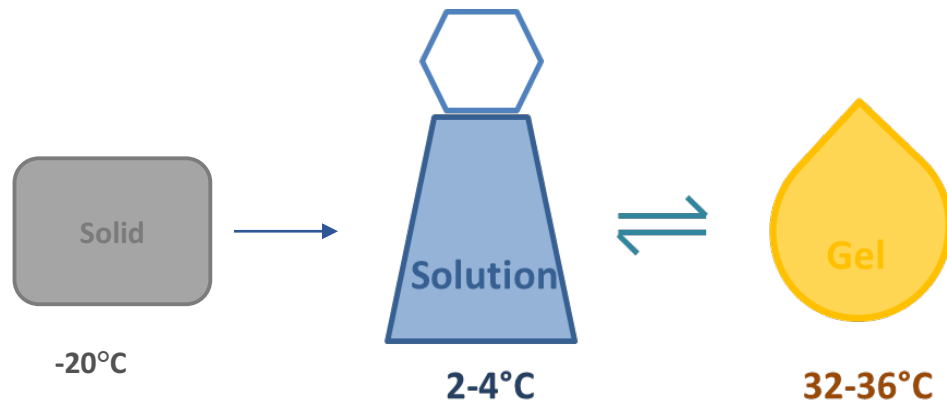
Two commercially available PLGA-PEG-PLGA triblock copolymers (AK24/'thermogel A' (MW 1100-1000-1100, 3:1 lactide:glycolide) and AK19/'thermogel B' (MW 1500-1500-1500, 1:1 lactide:glycolide); Akina Inc, West Lafayette, IN) were converted from a solid state (-20°C) to a liquid state through addition of 0.9% sodium chloride (Hospira Inc., Lake Forest, IL), vortexing

every 8 hours for 48 hours and maintenance at 4°C. Five thermogels were tested: thermogel A, thermogel B and thermogels combined in A:B ratios of 1:1, 2:1 and 3:1. Each thermogel tested was divided into 4 x 300µL aliquots (Groups 1,2 3 and 4). Group 1 aliquots were placed in glass vials which were placed in a water bath at 21°C for 30 minutes, stored overnight at 4°C, placed in a water bath at 35°C for 30 minutes, then again stored at 4°C, and conversions from liquid to solid state reported. Group 2 aliquots were placed in glass vials to which 1mg voriconazole (U. S. Pharmacopeia, Rockville, MD) was added, and group 3 aliquots were placed into glass vials to which 10µL of methylene blue (Akorn Inc., Lake Forest, IL) was added; the vials were maintained at 4°C, vortexed every 8 hours for 48 hours and then subjected to the temperature changes described above. Group 4 aliquots were injected through a 30 gauge needle into the bulbar subconjunctival space of a horse under general anesthesia that was enrolled in a terminal student surgical laboratory at the AULATH.

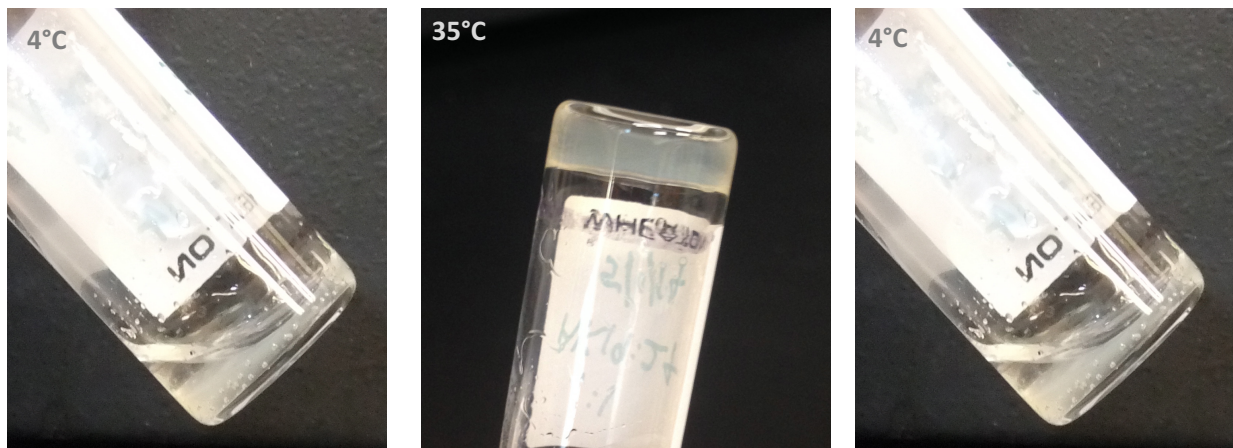
### *Results*

Thermogel A formed a firm gel within 20 seconds at 21°C but did not gel at 35°C. Thermogel B did not gel at 21°C but underwent partial gelation at 35°C. Thermogels in A:B ratios of 1:1, 2:1 and 3:1 did not gel at 21°C but formed firm gels within 30, 45 and 60 seconds respectively at 35°C (Figure 5). All thermogels returned to liquid state when vials were returned to a 4°C environment. Voriconazole was able to be combined with all five thermogels, and all thermogels retained their thermosensitive characteristics following combination with voriconazole and methylene blue. All thermogels were able to be injected through a 30 gauge needle into the bulbar SCS of a horse under general anesthesia. Thermogel A did not firm a discrete deposit in the SCS.

**A**

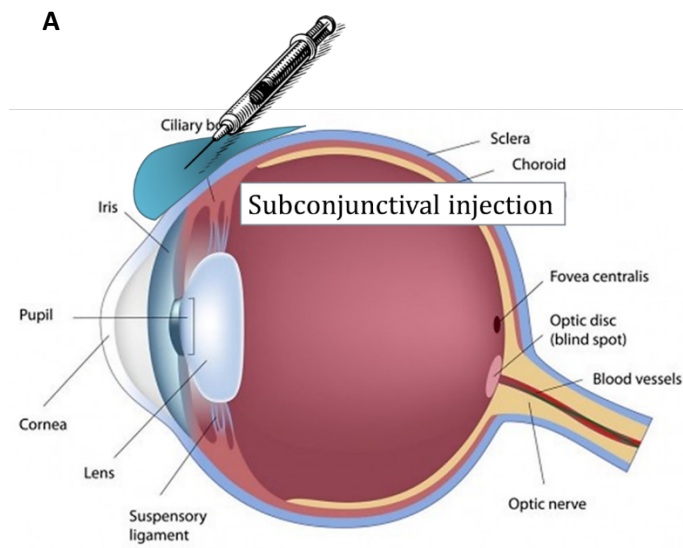


**B**

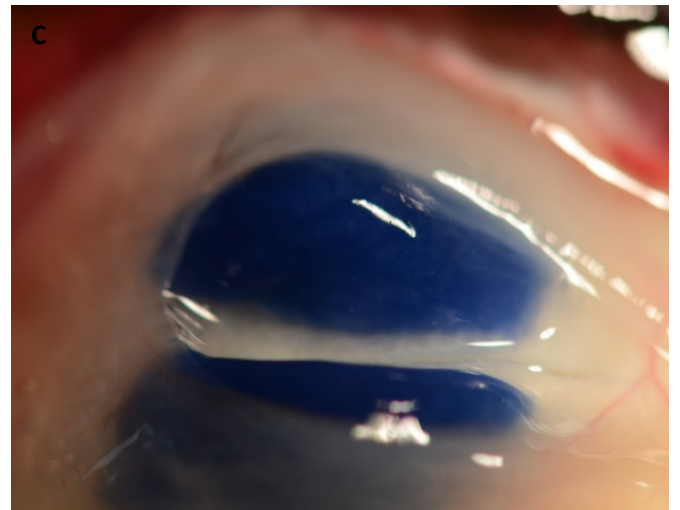


**Figure 5. A.** Schematic depiction of conversion of thermogel with a gelation set point of  $32-36^{\circ}\text{C}$  from its solid to liquid state to gel state. **B.** Reversible liquid to gel transition of a thermogel consisting of a 1:1 mixture of PLGA-PEG-PLGA (Mw 1100:1000:1100) and PLGA-PEG-PLGA (Mw 1500:1500:1500).

Thermogel B and thermogels with A:B ratio of 2:1 and 3:1 formed soft, poorly-defined deposits in the bulbar SCS. The thermogel with 1:1 A:B ratio formed a firm, discrete gel deposit in the equine bulbar SCS (Figure 6).



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**Figure 6. A.** Schematic depiction of conversion of thermogel with a gelation set point of 32-36°C from its liquid state to its solid state following SCS injection. **B.** Photograph of injection of a 1:1 mixture of PLGA-PEG-PLGA (Mw 1100:1000:1100) and PLGA-PEG-PLGA (Mw 1500:1500:1500) into the dorsal bulbar SCS of a horse under general anesthesia. **C.** Dissection of the bulbar conjunctiva to show the thermogel deposit within the dorsal bulbar SCS.



### *Conclusions*

The optimal gelation set points of the thermogels tested were consistent with published rheological data.<sup>69</sup> The thermosensitive characteristics of the tested thermogels were not changed by addition of voriconazole or methylene blue. A thermogel with 1:1 ratio of PLAG-PEG-PLGA (1100:1000:1100):PLGA-PEG-PLGA (1500:1500:1500) is most suitable for injection in the equine bulbar SCS.

### **Section 3: Preliminary Voriconazole Release Data**

#### *Purpose*

A voriconazole-containing thermogel for subconjunctival injection could allow for sustained drug release into target tissues. This experiment aimed to determine the solubility of voriconazole in crystalline form and in ethanol solution in a volume of thermogel suitable for SCS injection (300  $\mu$ l) and to determine if different thermogels containing 1mg voriconazole would release the drug in concentrations exceeding the MIC of common equine fungal pathogens (0.5  $\mu$ g/ml) for at least 7 days *in vitro*.

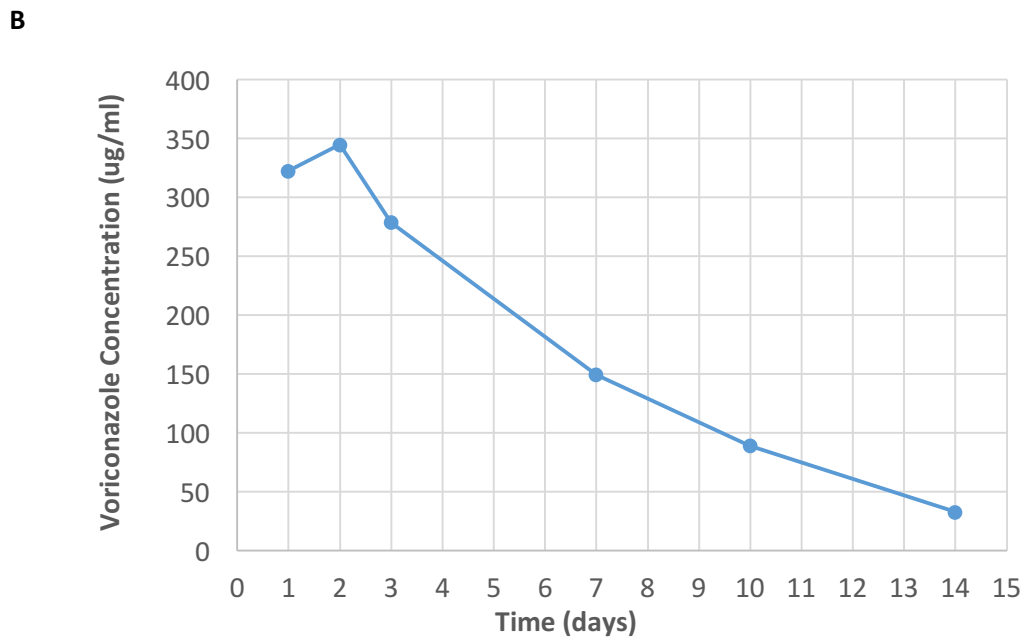
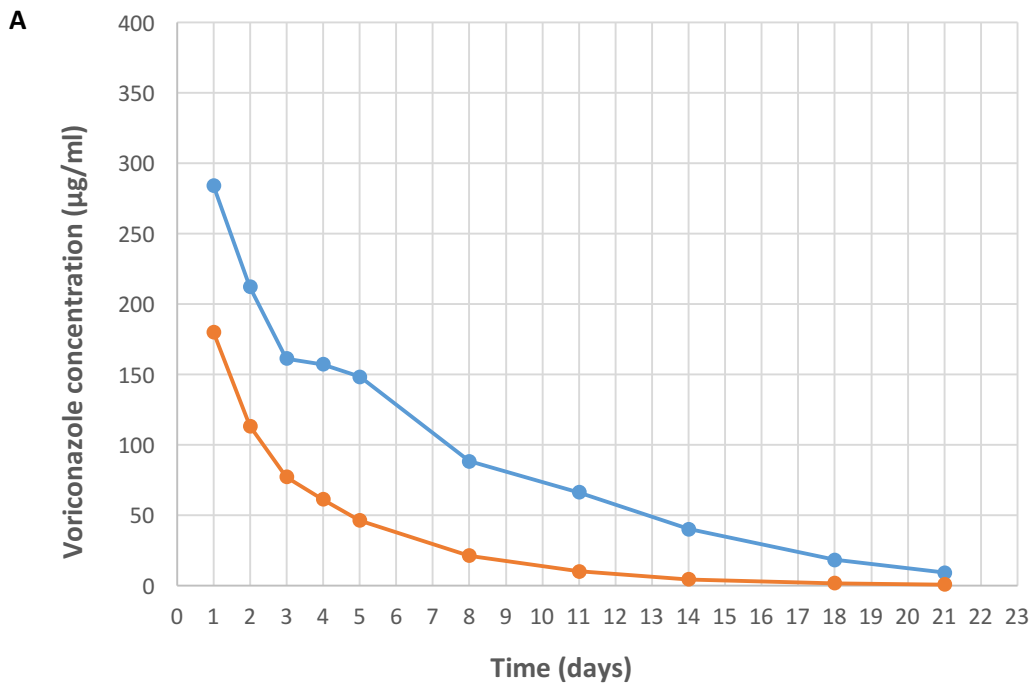
#### *Materials and Methods*

Two PLGA-PEG-PLGA hydrogels were converted from a solid state (-20°C) to a liquid state through addition of 0.9% sodium chloride, vortexing every 8 hours for 48 hours and maintenance at 4°C. Three thermogels were then prepared: Thermogel 1 - 1mg voriconazole + 300 $\mu$ l PLGA-PEG-PLGA (MW 1100:1000:1100), Thermogel 2 - 1mg voriconazole in 10 $\mu$ l ethanol (Sigma-Aldrich, St Louis, MO) + 300 $\mu$ l PLGA-PEG-PLGA (MW 1100:1000:1100), Thermogel 3 - 1mg voriconazole + 300 $\mu$ l

1:1 PLGA-PEG-PLGA (MW 1100:1000:1100):PLGA-PEG-PLGA (MW 1500:1500:1500). Each thermogel sample was stored in a glass vial and was maintained at its optimal gelation temperature (23°C for Thermogel 1&2; 35°C for Thermogel 3) together with 1ml phosphate buffered saline (PBS) in a water bath for 21 days. Twice daily visual inspection of samples was performed for the 21 days. All PBS was collected daily and replaced with fresh PBS for 14 days and voriconazole content determined via liquid chromatography-tandem mass spectrometry.

### *Results*

Voriconazole was successfully combined with two PLGA-PEG-PLGA hydrogels in liquid form. Both hydrogels retained their thermosensitive properties when combined with voriconazole in crystalline form and in ethanol solution. Phase separation of the thermogels occurred after 12 hours, with less than a quarter of the thermogel aliquot entering an aqueous phase and the balance remaining a discrete gel. Voriconazole was released from the thermogels over 14 days with concentrations achieved in excess of the target MIC at all time points (Figure 7). Maximum drug release occurred on days 1 and 2. The concentrations achieved *in vitro* likely significantly underestimate the concentrations which would be achieved following *in vivo* administration, therefore 1mg voriconazole is the minimum volume which should be added to 300 µl thermogel for injection. All 3 thermogels underwent a degree of breakdown over the 21 days, but there was thermogel remaining in all test vials at the conclusion of the experiment.



**Figure 7. A.** Voriconazole release from PLGA-PEG-PLGA (Mw 1100:1000:1100). Release rate was greater for voriconazole in crystal form (blue line; Thermogel 1) than in ethanol solution (orange line; Thermogel 2). **B.** Voriconazole release from a 1:1 mixture of PLGA-PEG-PLGA (Mw 1100:1000:1100) and PLGA-PEG-PLGA (Mw 1500:1500:1500).

### *Conclusions*

Voriconazole is able to be combined with, and is released in a sustained manner from, thermosensitive PLGA-PEG-PLGA thermogels *in vitro* for at least 14 days. Investigation into solubility of voriconazole at higher concentrations and subsequent *in vitro* drug release is indicated.

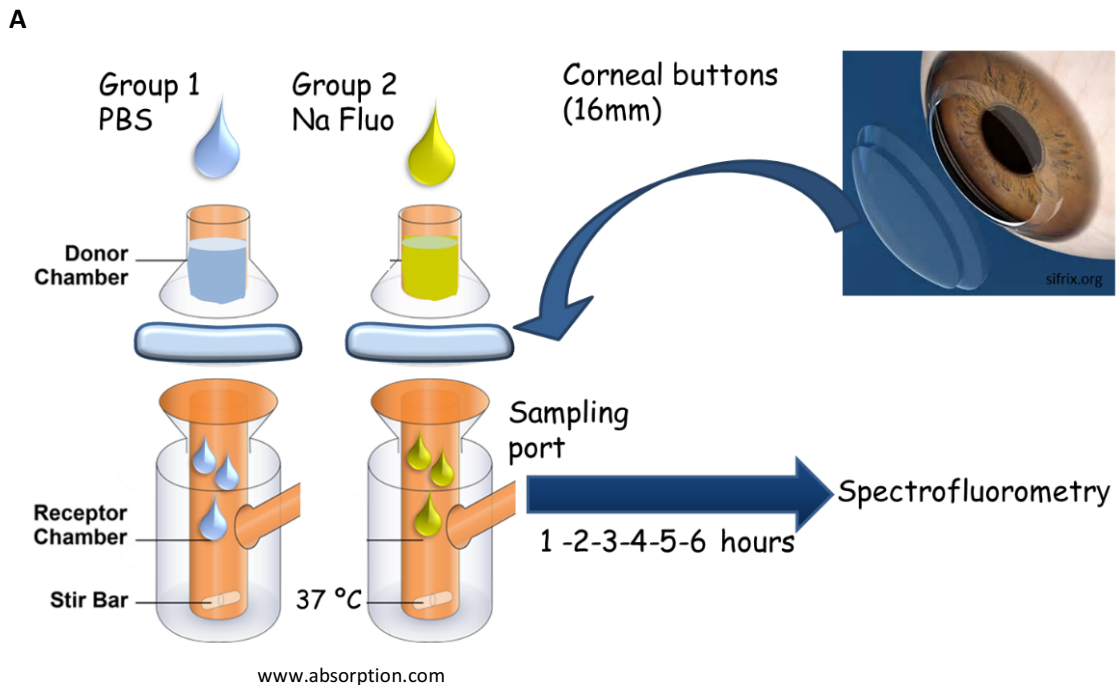
## **Section 4: Development of an ex-vivo Trans-Corneal Permeation Model in Horses**

### *Purpose*

Franz cell diffusion studies have been previously used in evaluation of permeation of topical ocular medications through human, goat and sheep corneas. Use of the Franz cell method with equine corneas has not been previously described. The aim of this experiment was firstly to describe an ex-vivo trans-corneal drug permeation model for use in equine corneas, and secondly to present evidence on the integrity of the equine epithelial barrier function for 6 hours.

### *Materials and Methods*

Fresh equine corneas used in the experiment were obtained from horses enrolled in terminal laboratories performed at the AULATH. Corneoscleral buttons (16 mm diameter) were dissected using standard eye bank technique within 2 hours of enucleation. Fluorescent permeation experiments using a Franz diffusion cell method were performed to examine the integrity of the epithelial barrier function during the times studied (2 and 6 hours). Corneas were mounted horizontally between the donor and the receiving compartments of the diffusion cells (exposure window  $0.64\text{cm}^2$ ), which were maintained at  $37^\circ\text{C}$  (Figure 8).

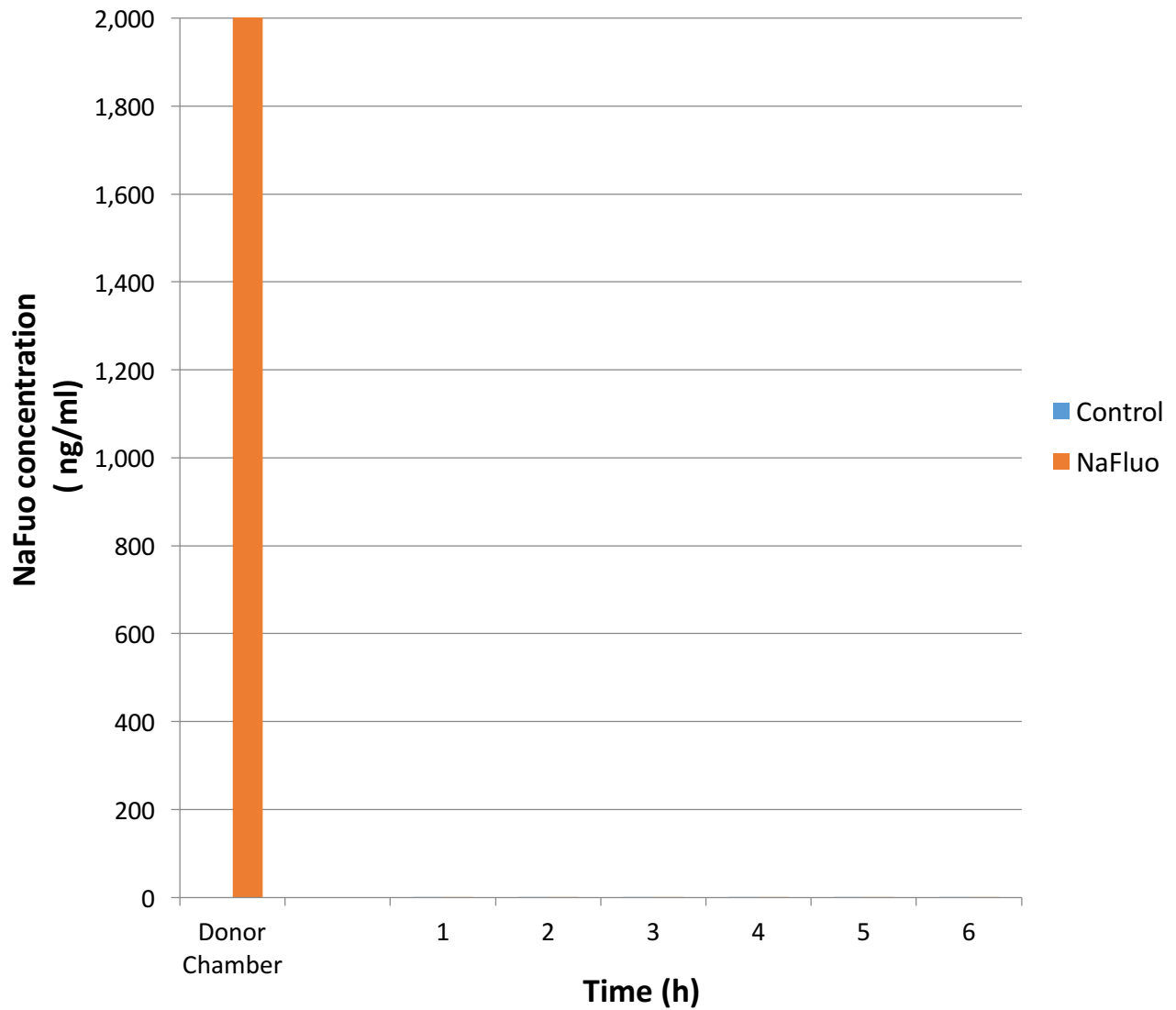


**Figure 8. A.** Schematic and **B.** photographic depiction of the Franz cell diffusion apparatus used to develop a permeation model for equine corneas.

Two test groups were defined: a control group in which the donor compartment was filled with 1ml PBS pH 7.4 (Group 1) and the fluorescein group in which the donor compartment was filled with 1ml of a solution containing PBS pH 7.4 and 10 $\mu$ M sodium fluorescein (NaF) (Group 2). Samples (1ml PBS pH 7.4) were removed from the receiving compartment hourly and replaced with fresh receiving fluid. The fluorescent intensities of the receiving solution samples were analyzed using a microplate spectrofluorometer (Fluostar, BMG Labtech, Ortenberg, Germany) and a 96 well plate (Costar, Corning, NY). A standard solution of NaF was prepared in PBS with a concentration range of 30  $\mu$ g/ml to 0.12  $\mu$ g/ml. Fluorescence was determined in a spectrofluorometer at excitation and emission wavelengths of 490 nm and 512 nm, respectively. All groups were tested in triplicate. The results were expressed as mean value  $\pm$  standard error of the mean (SEM). The differences between values for Group 1 and Group 2 were assessed using a one-way analysis of variance (ANOVA) ( $p < 0.05$ ).

### *Results*

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 experiment at any time point, showing that the epithelial barrier function was maintained (Figure 9).



**Figure 9.** Concentration of fluorescein (NaFluo) measured in the donor chamber and receiving compartment over 6h of the *in vitro* permeation study; mean  $\pm$  SD, n=3. Fluorescent concentrations detected in the receiving compartment were not significantly different than those obtained from the negative control experiment at any time point.

### *Conclusions*

This work presented a simple Franz diffusion cell-type modification for use in ex-vivo ocular drug delivery investigations in the equine eye. The results showed that the diffusion cell was able to maintain the integrity of equine epithelial corneal barrier function throughout the 6 hours of the permeation experiment.



## **Chapter 3 - Development of a Voriconazole-containing PLGA-PEG-PLGA**

### **Thermogel for Subconjunctival Administration**

#### **Section 1: Statement of Hypotheses and Objectives**

*Specific Aim 1: In vitro release of voriconazole from a PLGA-PEG-PLGA thermogel*

- A. Hypothesis Aim 1: Voriconazole when combined with a PLGA-PEG-PLGA thermogel will be sustainably released from the thermogel over a period of at least 2 weeks in an *in vitro* model in concentrations exceeding the MIC of common fungal organisms.
- B. Objectives Aim 1:
  - a. Describe the release of voriconazole from the thermogel over a 4 week period.
  - b. Determine concentrations of voriconazole released from the thermogel and compare to the MIC for common fungal organisms implicated in equine keratomycosis i.e. *Fusarium*, *Aspergillus* and *Candida* spp.

*Specific Aim 2: Corneal and scleral penetration of voriconazole released from a PLGA-PEG-PLGA thermogel in vitro*

- A. Hypothesis Aim 2: Voriconazole released from a PLGA-PEG-PLGA thermogel will penetrate the intact isolated equine cornea and sclera in an *in vitro* model.
- B. Objectives Aim 2:
  - a. Demonstrate diffusion of voriconazole out of the PLGA-PEG-PLGA thermogel and

across isolated equine corneas and sclerae using an *in vitro* Franz Cell diffusion model.

- b. Compare the rate of diffusion of voriconazole (topical 1.5% vs thermogel) across isolated equine corneas and sclerae in an *in vitro* Franz Cell diffusion model.

*Specific Aim 3: Characteristics of voriconazole-PLGA-PEG-PLGA thermogel injected subconjunctivally in ex vivo equine eyes*

- A. Hypothesis Aim 3: Subconjunctival injection of the thermogel will result in a discrete, well defined gel deposit in the bulbar subconjunctival space of equine eyes.
- B. Objectives Aim 3:
  - a. Describe the technique for injecting voriconazole-PLGA-PEG-PLGA thermogel subconjunctivally in the horse in an *ex vivo* and *in vivo* model.
  - b. Demonstrate formation of a discrete gel deposit in the SCS following injection of liquid (4°C) voriconazole-PLGA-PEG-PLGA thermogel into equine eyes via ultrasound biomicroscopy (UBM) examination and tissue dissection.

*Specific Aim 4: Short- and mid-term ocular toxicity pilot study in a live horse*

- A. Hypothesis Aim 4: Subconjunctival injection of the thermogel in the dorsal bulbar subconjunctival space of equine eyes will be well tolerated and will not be associated with ocular toxicity.
- B. Objectives Aim 4:
  - a. Describe the technique for injecting voriconazole-PLGA-PEG-PLGA thermogel subconjunctivally in the horse in an *in vivo* model.

- b. Demonstrate safety of the injection through clinical and histopathologic examination of treated eyes 2 hours and 7 days post injection of the thermogel.

## **Section 2: Materials and Methods**

### *Subsection 2a: Thermogel preparation*

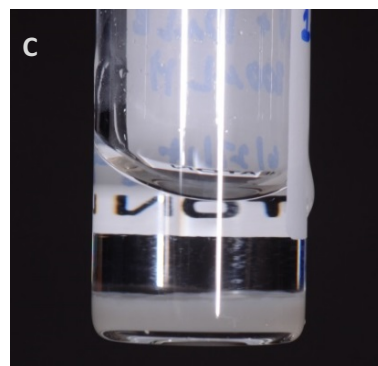
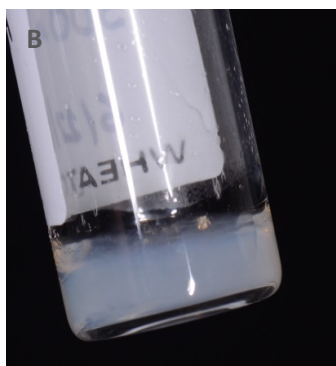
Two commercially available, thermosensitive PLGA-PEG-PLGA triblock copolymers (AK24 (MW 1100-1000-1100, 3:1 lactide:glycolide) and AK19 (MW 1500-1500-1500, 1:1 lactide:glycolide); Akina Inc, West Lafayette, IN) were purchased in solid form (-20°C). Both PLGA-PEG-PLGA thermogels were converted to an aqueous solution via addition of 0.9% sodium chloride (80% w/w; Hospira Inc., Lake Forest, IL), vortexing for 1 minute every 8 hours for a period of 48 hours and storage at 4°C (liquid form). The two thermogel solutions were then combined in a 1:1 ratio to form a thermogel with an optimal gelation setpoint of 32-36°C (hereafter referred to as ‘the thermogel’), concordant with that of the equine bulbar SCS (average temperature 34.5°C; preliminary research, page 19).<sup>69</sup> Test groups containing different concentrations of voriconazole were prepared as outlined below. In all cases voriconazole crystals (U. S. Pharmacopeia, Rockville, MD) or voriconazole in ethanol (Sigma-Aldrich, St Louis, MO) solution were added to the thermogel at 4°C and the resultant voriconazole-containing thermogel was vortexed for 1 minute every 8 hours for a period of 48 hours to ensure even distribution of the drug within the thermogel. The thermogel used for *ex vivo* injection was stained with methylene blue (Akorn Inc., Lake Forest, IL) for ease of visualization.

*Subsection 2b: In vitro release of voriconazole from the thermogel*

*In vitro* release rate determination was performed as described previously.<sup>70, 71</sup> Six test groups were prepared, five consisting of 300µl aliquots of the thermogel combined with voriconazole in the following concentrations: 0% (Group 1; negative control group), 0.3% voriconazole (1mg; Group 2), 0.3% voriconazole (1mg in ethanol solution; Group 3), 1.5% voriconazole (5mg; Group 4) and 1.5% voriconazole (5mg in ethanol solution; Group 5), and one positive control group consisting of voriconazole crystals alone (5mg; Group 6). The thermogels were prepared in glass vials at 4°C (liquid state). Conversion to a gel state was facilitated by suspension of the glass vials in a water bath set at a constant temperature of 34.5°C, where they were maintained for 28 days. Immediately following conversion of the thermogel to a gel state, 1ml 0.067M PBS (Lonza, Walkersville, MD) was added to each vial (Figure 10). The PBS was collected from the vials and replaced with 1 ml of fresh PBS at each sampling time point. Samples were collected at 6, 12, 18 and 24 hours on day 1, then every 24 hours for a further 27 days. Samples were stored at -80°C until the end of the testing interval and then analyzed for voriconazole concentration via reverse phase high-performance liquid chromatography (HPLC). Observations of thermogel appearance were recorded daily. Each formulation was tested in triplicate.

*Subsection 2c: In vitro corneal and scleral voriconazole permeation*

Five eyes were obtained from horses free from corneal and scleral disease that were euthanized at the AULATH for reasons not related to this study. Enucleation was performed immediately following euthanasia. Enucleated eyes were placed in 0.9% saline (Baxter Healthcare Corporation, Deerfield, IL) and transported on ice to the laboratory within 20 minutes of



**Figure 10.** Voriconazole release study. Six test groups were tested in triplicate. **A.** Glass vials containing test samples and PBS were maintained at 34.5C in a waterbath. **B.** Samples were allowed to gel, then **C.** 1ml PBS was added to each glass vial. PBS was removed for HPLC analysis and fresh PBS replaced daily.

enucleation, where 2 paracentral corneal and 2 dorsal scleral buttons were harvested from each eye via the use of a 16 mm corneal trephine (Asico, LLC, Westmont, IL) and Castroviejo corneal and Westcott tenotomy scissors.

*In vitro* permeation studies were performed on vertical static Franz diffusion cells (PermeGear, Hellertown, PA, USA). The harvested corneal and scleral buttons were rinsed free of proteinaceous material with 0.9% saline and placed horizontally between the donor and receptor halves of individual cells (diffusion area  $0.64\text{cm}^2$ ), with the epithelial surface of the corneal buttons and the external surface of the scleral buttons facing the donor compartment. The receiver chamber contained PBS (5ml, pH 7.4), that was maintained at  $34^\circ\text{C}$  with a water circulation jacket surrounding the lower part of the cell. Stirring in the receptor was maintained with the help of a magnetic bead. Three tests groups were determined and the donor compartment loaded with either  $300\mu\text{l}$  of a 1.5% voriconazole solution previously shown to penetrate equine corneas *in vivo* (Vfend, Pfizer, New York, NY; control group),  $300\mu\text{l}$  thermogel combined with 1mg voriconazole (Gel A; 0.3% voriconazole-thermogel) or  $300\mu\text{l}$  thermogel combined with 5mg voriconazole (Gel B; 1.5% voriconazole-thermogel).<sup>4</sup> PBS samples were taken from the receptor chamber and the receptor chamber was replenished with a fresh 1ml PBS at 0, 2, 4, 6, 8, 12, 24 hours. Samples were stored at  $-80^\circ\text{C}$  until the completion of sampling and then analyzed for voriconazole content via reverse phase HPLC. Each group was tested in triplicate.

After 24 hours, each corneal button was rinsed 3 times with PBS to remove any remaining voriconazole or thermogel, blotted dry and the 'wet weight' ( $W_a$ ) recorded. The corneal samples were then desiccated at 70°C in an incubator for 8 hours and the 'dry weight' ( $W_b$ ) recorded. From this data the percentage of corneal hydration was calculated ( $\% \text{ corneal hydration} = [1 - (W_a/W_b)] \times 100$ ), with a limit of 83% corneal hydration for individual corneal buttons set for inclusion of results in the study.<sup>72</sup>

#### *Subsection 2d: High-performance liquid chromatography*

The drug release and permeation test PBS samples were analyzed by reverse phase HPLC as previously described.<sup>73</sup> Briefly, the HPLC system (Agilent 1200 series) consisted of pumps, an autosampler, UV/visible light absorption detector, column (Thermo BetaBasic-18, 4.6 mm · 15 cm, 5 $\mu$ ; Bellefonte, PA, USA), and computer interface. The mobile phase consisted of 35% 0.1 M N, N, N $\zeta$ , N $\zeta$ -tetramethylenediamine (Fisher Scientific, Inc., Waltham, MA, USA) and methanol (Fisher Scientific, Inc.), at a flow rate of 1.0 ml/min at room temperature. The injection volume was 100  $\mu$ l. Voriconazole and ketoconazole, the internal standards, were detected at a wavelength of 254 nm, and the retention times were 3.7 and 13.5 min respectively. Calibration standards with voriconazole concentrations in dissolution and selected fluids ranging from 1.0 to 100  $\mu$ g/ml were prepared. The lower limit of detection for voriconazole was 1  $\mu$ g/ml, and the lower limit of quantification was 0.5  $\mu$ g/mL. Intra- and inter-day variations were 4.0 % and 5.1% respectively.

*Subsection 2e: Characterization of subconjunctival injection of the thermogel in ex vivo equine eyes*

Ten eyes were obtained from horses free from corneal and scleral disease that were euthanized at the AULATH, for reasons not related to this study. Enucleation was performed immediately following euthanasia via a transpalpebral approach, and the thermogel was injected immediately following enucleation. The temperature in the ventral bulbar SCS was measured immediately prior to injection to ensure normothermia was maintained following enucleation. The thermogel (300 $\mu$ l) in its liquid state (4°C) was injected under the dorsal bulbar conjunctiva through a 30-gauge needle and the ease of injection and time taken to gel formation recorded. High-frequency (50MHz) UBM (Aviso TM, 2016 Quantel Medical, Bozeman, US) was performed in order to visualize the thermogel deposit, describe its location and shape, and measure its dimensions. The eyes were then frozen with liquid nitrogen, sectioned using a microtome blade and the gross appearance of the gel deposit was further described.

*Subsection 2f: Short- and mid-term toxicity pilot study in a single live horse*

Following validation of procedures *ex vivo*, the purpose of this portion was to evaluate short-term (2 hours) and mid-term (7 days) clinical tolerance of, and histopathologic changes following, SCS injection of voriconazole-containing thermogel in a pilot study in a healthy adult horse.

The horse was sedated with 5mg detomidine hydrochloride (Dormosedan; Zoetis Inc., Florham Park, NJ) and 5mg butorphanol tartrate (Torbugesic; Zoetis Inc., Florham Park, NJ) intravenously and local ocular anesthesia was supplied through administration of perineural 2% lidocaine



hydrochloride (Hospira Inc., Lake Forest, IL) around the auriculopalpebral and frontal nerves and topical 0.5% proparacaine hydrochloride (Akorn Inc., Lake Forest, IL). The eye was prepped with sterile diluted 5% betadine solution, irrigated with sterile eyewash (Purdue Products L.P., Stamford, CT) and 300µl 1.5% voriconazole thermogel was injected into the right dorsal bulbar SCS through a 30-gauge needle. Flunixin meglumine (1.1mg/kg IV; Banamine®, Intervet International, Germany) was administered immediately post injection and topical ocular neomycin-polymixin-bacitracin ointment (Vetropolycin®, Dechra Veterinary Products, Overland Park, KS) was applied to the treated eye every 6 hours for the 48 hours following injection. A complete ophthalmic examination including 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) and funduscopy was performed 1 day before and 7 days after the SCS injection. After pupillary dilation with tropicamide 1% (Akorn Inc., Buffalo Grove, IL), the ocular fundus was photographed (Optibrand Clearview, Optibrand Ltd., Fort Collins CO). The horse was examined twice daily and monitored closely for signs of inflammation, reaction or pain in the treated eye. A modified Hackett-McDonald microscopic ocular inflammatory scoring system was used to evaluate the ocular anterior segment and anterior vitreous.<sup>74</sup> Scores of the conjunctiva (congestion; swelling; discharge; 0-4); aqueous flare (0-3); pupillary light reflex (0-2); iris involvement (0-4); cornea (involvement and area; 0-4); pannus (vascularization; 0-2); and anterior vitreal cellular infiltrate (0-4) were summed to provide a single inflammatory score for each examination.<sup>74</sup> Seven days post-injection the horse was placed under general anesthesia then euthanized for reasons not related to this project. Following anesthetic induction (2 hours prior to euthanasia) 300µl of a 1.5% voriconazole thermogel was

injected into the left dorsal bulbar SCS. Following euthanasia, both of the horse's eyes were enucleated and submitted for histopathologic examination.

#### *Subsection 2g: Data Analysis*

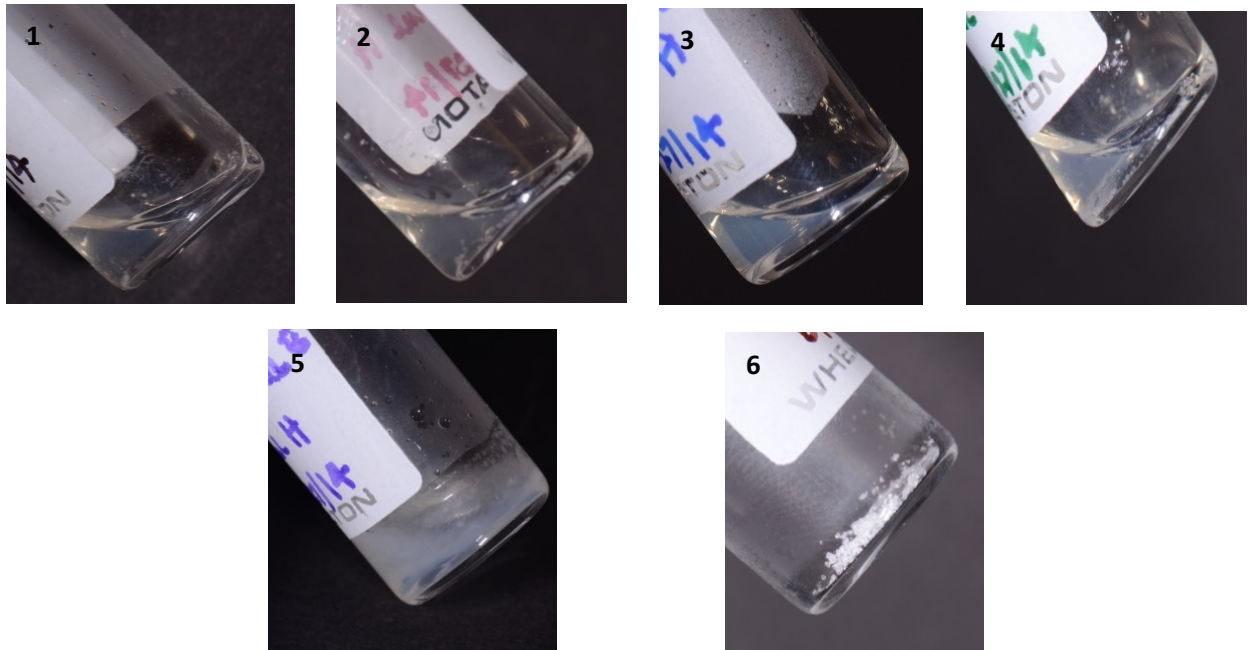
Descriptive statistics (mean, median, range) were calculated for measurements of thermogel deposit dimensions obtained following *ex vivo* SCS injection and percentage corneal hydration following *in vitro* permeation studies (Microsoft Excel 2013, Microsoft, Richmond, WA). All data were expressed as mean  $\pm$  standard deviation. For the release study, rates of release and transfer were obtained from slopes of cumulative amounts released, unreleased, and transferred based on linear and log co-ordinates. For first-order release, half-life ( $t_{1/2}$ ) was estimated as 0.693 divided by the first-order rate constant. For the permeation study, the cumulative amount of voriconazole released from the thermogel as well the amount permeated across cornea and sclera were plotted as a function of time. The slope of the linear portion of the permeation plot was presented as permeation rate ( $\mu\text{g}/\text{cm}^2/\text{h}$ ). Data were analyzed by one-way ANOVA followed by Dunnett's test to determine the level of significance between various groups. The data were considered significant at  $P < 0.05$ . Data were analyzed using a commercial statistical analysis program (GraphPad Prism software version 5, La Jolla, CA).

### **Section 3: Results**

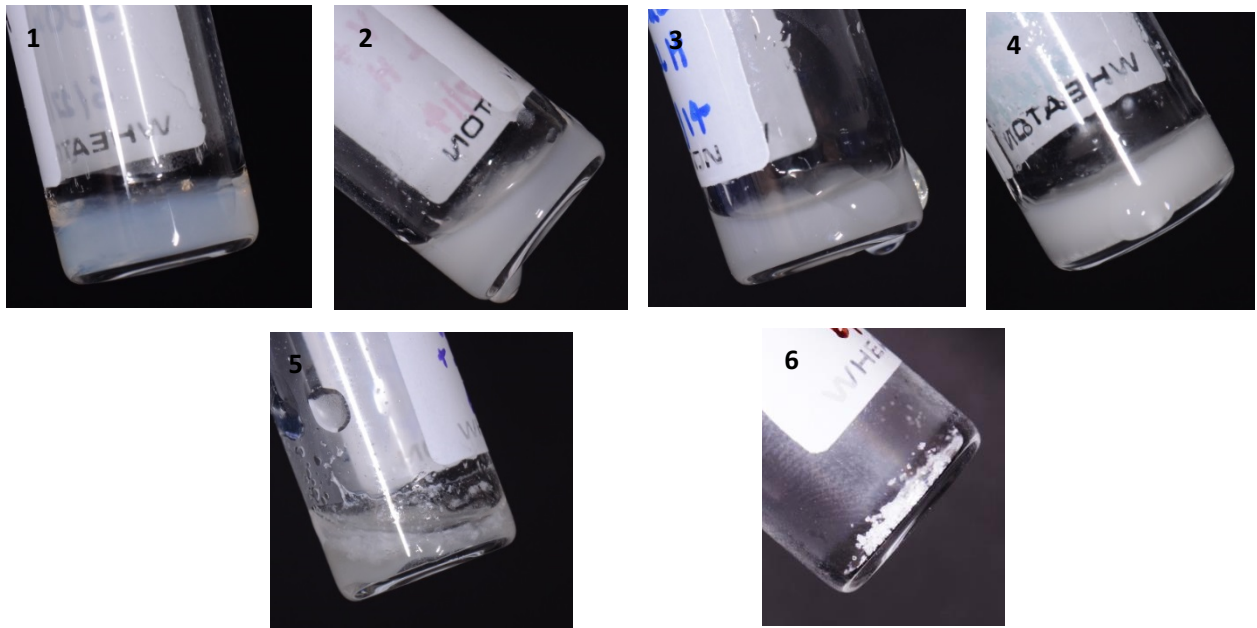
#### *Subsection 3a: Thermogel preparation (Figure 11)*

Voriconazole completely dissolved within the thermogel in Groups 2, 3, and 5 to form an aqueous solution. Voriconazole dispersed well within the thermogel, forming a suspension, in Group 4.

A



B



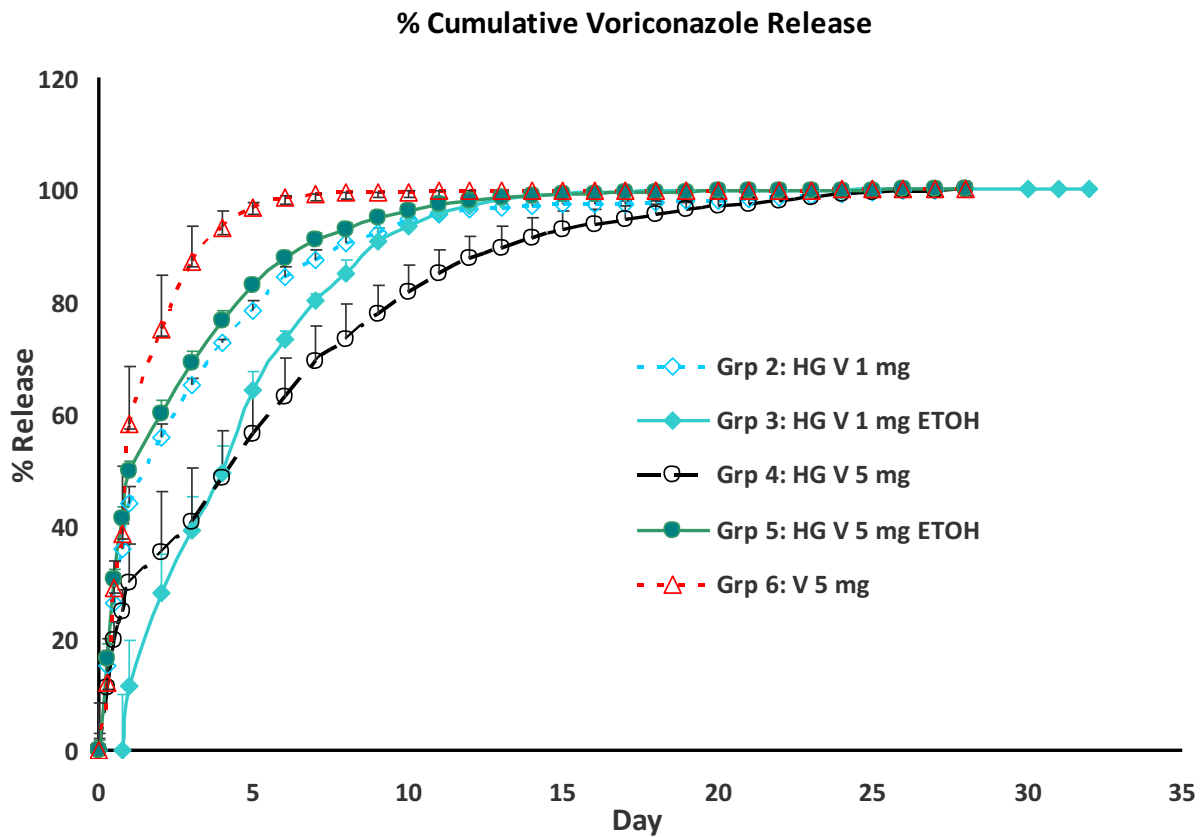
**Figure 11.** Tests groups 1-6 at **A.** 4°C and **B.** 34.5°C. All thermogel samples retained their thermosensitive properties after addition of voriconazole (Groups 2-5).

The thermogel retained its thermosensitive properties following combination with voriconazole alone, voriconazole in ethanol solution and methylene blue.

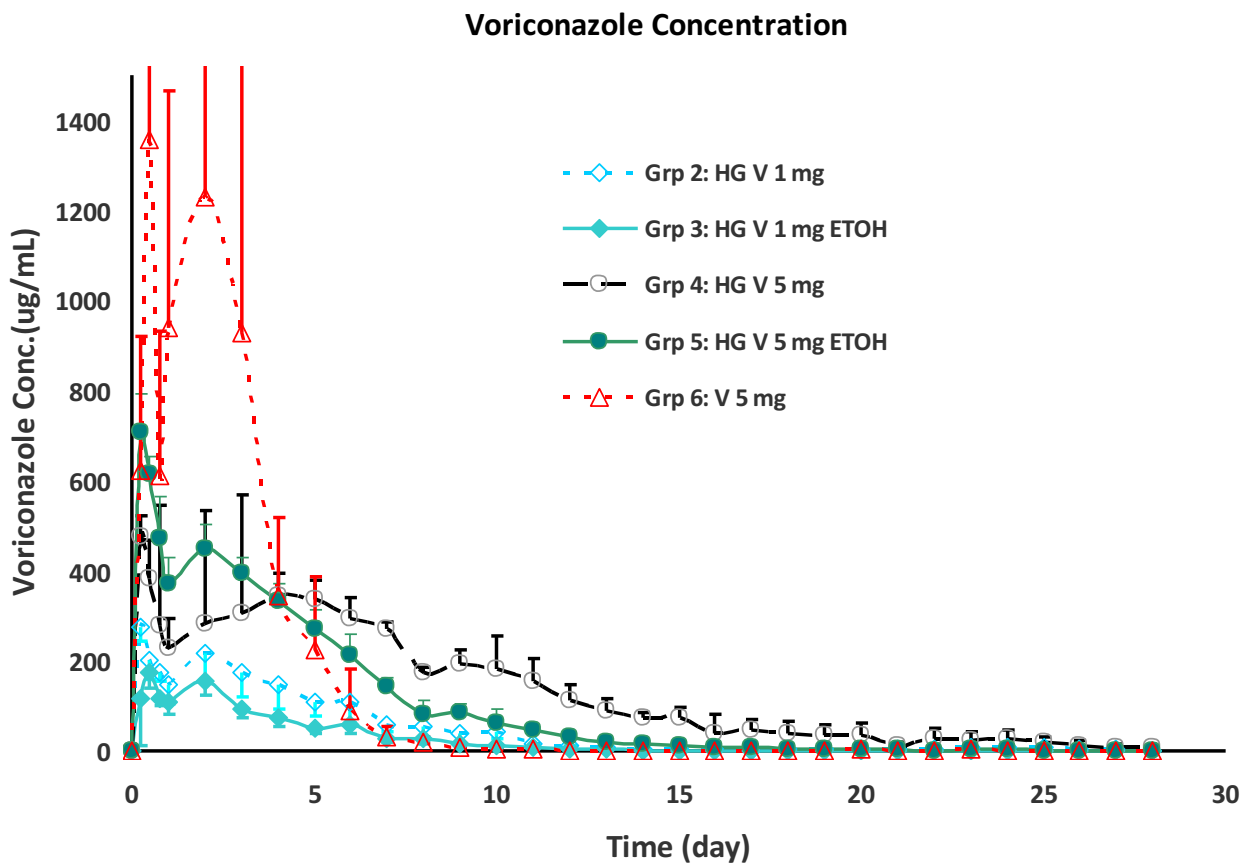
*Subsection 3b: In vitro release of voriconazole from the thermogel*

Voriconazole displayed sustained release from the thermogel throughout the study period for all formulations, with peak release occurring on day 1 (Figures 12 and 13). Voriconazole release from the thermogel followed first-order kinetics with release  $t_{1/2}$  ranging from 1.82 (Group 3) to 4.22 days (Group 2) (Table 1; Figure 14). The concentrations released exceeded the target MIC (0.5 $\mu$ g/ml) for 28 days in Groups 2 and 4 and 16 days in Groups 3 and 5 (Figure 12). There was no voriconazole detected at any time in samples from the negative control group (Group 1), and voriconazole was undetectable by day 6 in the positive control group (Group 6). The addition of ethanol inconsistently altered the nature of voriconazole release from the thermogel.

Phase separation of the thermogel into liquid and gel components occurred within the first 24 hours in all groups. Visual degradation of the thermogel occurred in all test groups from day 9 of the study. Greater thermogel breakdown occurred in the groups without ethanol (groups 1, 2 and 4), in which approximately 30% of the original volume remained at day 28 compared to those with ethanol (groups 3 and 5), in which approximately 50% remained at day 28 (Figure 15).



**Figure 12.** % cumulative voriconazole release from the thermogels. Voriconazole was sustainedly released from the thermogels throughout the study period. Addition of ethanol inconsistently altered voriconazole release.

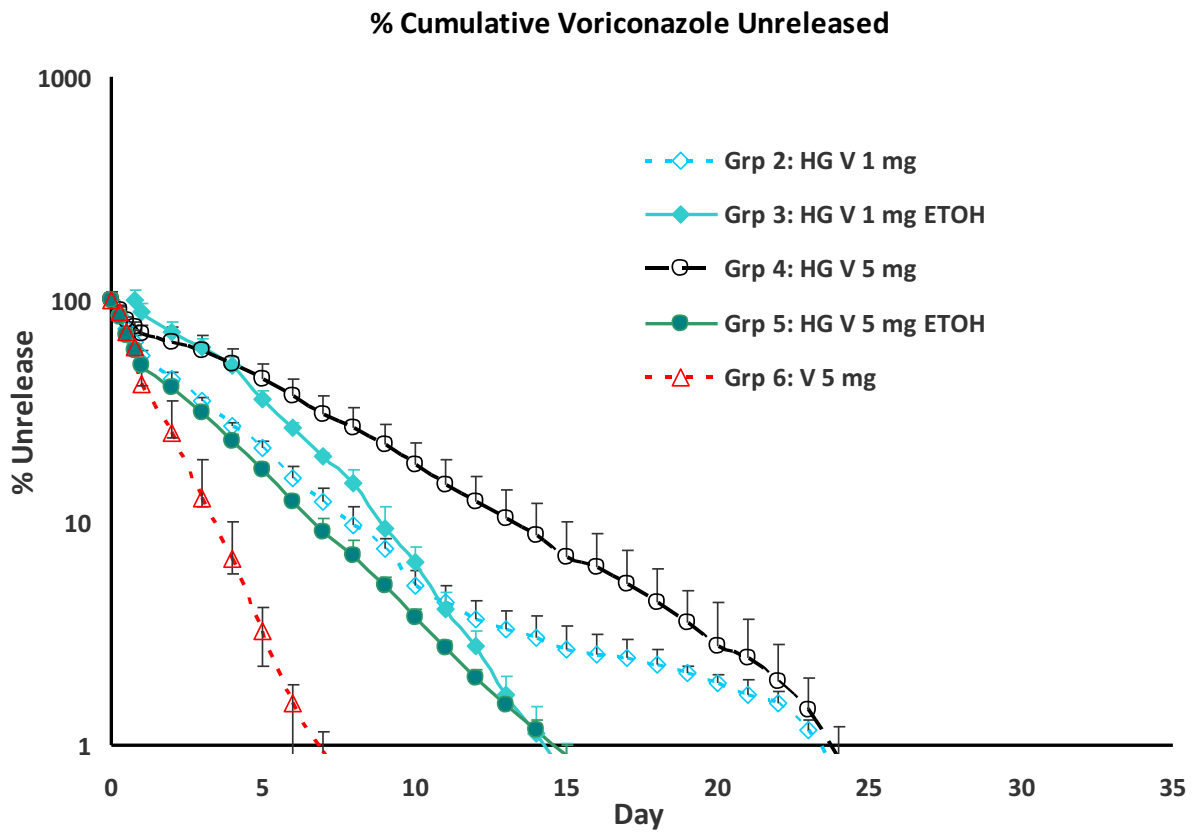


**Figure 13.** Voriconazole concentration in PBS over time. Voriconazole release exceeded the target MIC ( $0.5\mu\text{g/ml}$ ) for 28 days in Groups 2 and 4 and 16 days in Groups 3 and 5.

		Group 2	Group 3	Group 4	Group 5	Group 6
		Mean	Mean	Mean	Mean	Mean
		SD	SD	SD	SD	SD
<b>AUC24</b>	(ug*day/mL)	1276	696	3583	2871	4133
		309	88	1066	258	2498
<b>AUC28</b>	(ug*day/mL)	1293	696	3637	2877	4135
		312	88	1098	257	2498
<b>Krelease</b>	(1/day)	0.1642	0.3968	0.1915	0.2613	0.7558
		0.0021	0.0931	*	0.0101	*
<b>r</b>		0.9646	0.0029	0.9949	0.9939	0.9847
		0.0207	0.0029	0.0030	0.0041	0.0032
<b>t1/2release</b>	(day)	4.220	1.823	3.678	2.654	1.050
		0.056	0.487	*	0.100	*
						0.506

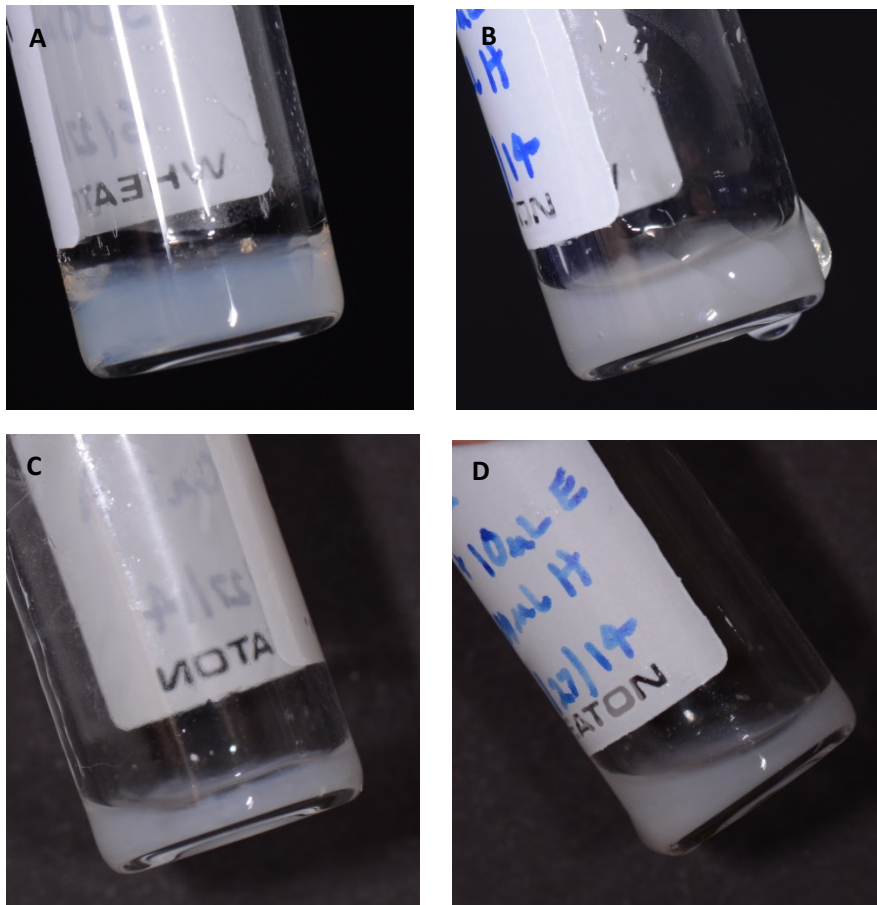
\* - p<0.05 on comparing with and without etoh

**Table 1.** Release kinetics of voriconazole from the thermogel *in vitro*.



**Figure 14.** % Cumulative voriconazole unreleased from the thermogels over time. Addition of ethanol hastened drug release.





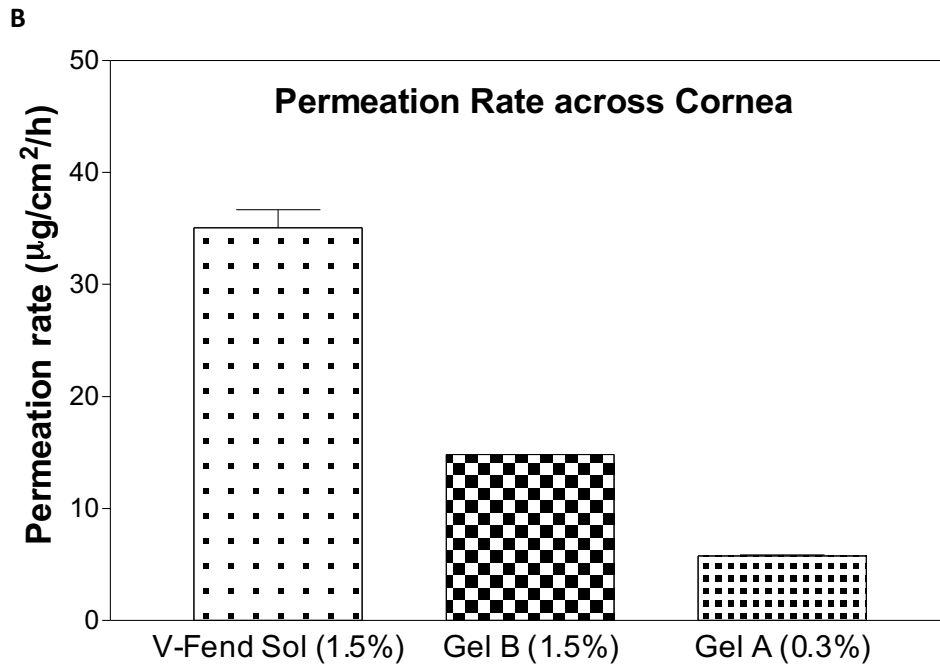
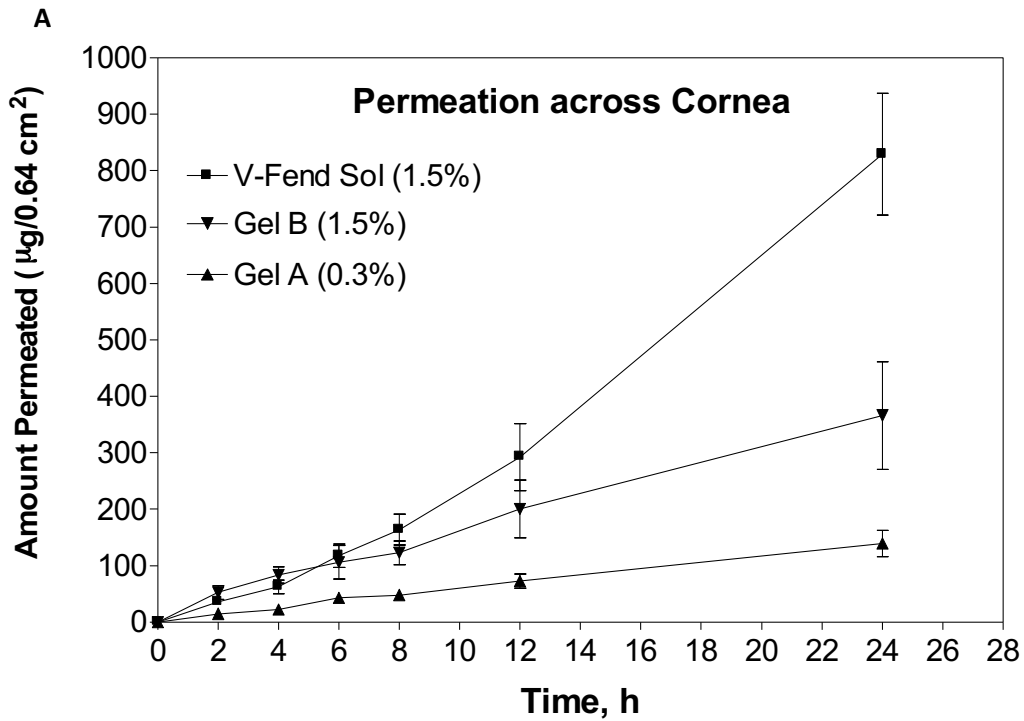
**Figure 15.** Thermogel degradation over time. Addition of ethanol slowed the degradation of the thermogel, which more thermogel remaining in Groups 3 and 5 than Groups 1, 2 and 4 at study completion. **A.** Group 1 day 1 **B.** Group 3 day 1 **C.** Group 1 day 28 **D.** Group 3 day 28.

### *Subsection 3c: In vitro corneal and scleral voriconazole permeation*

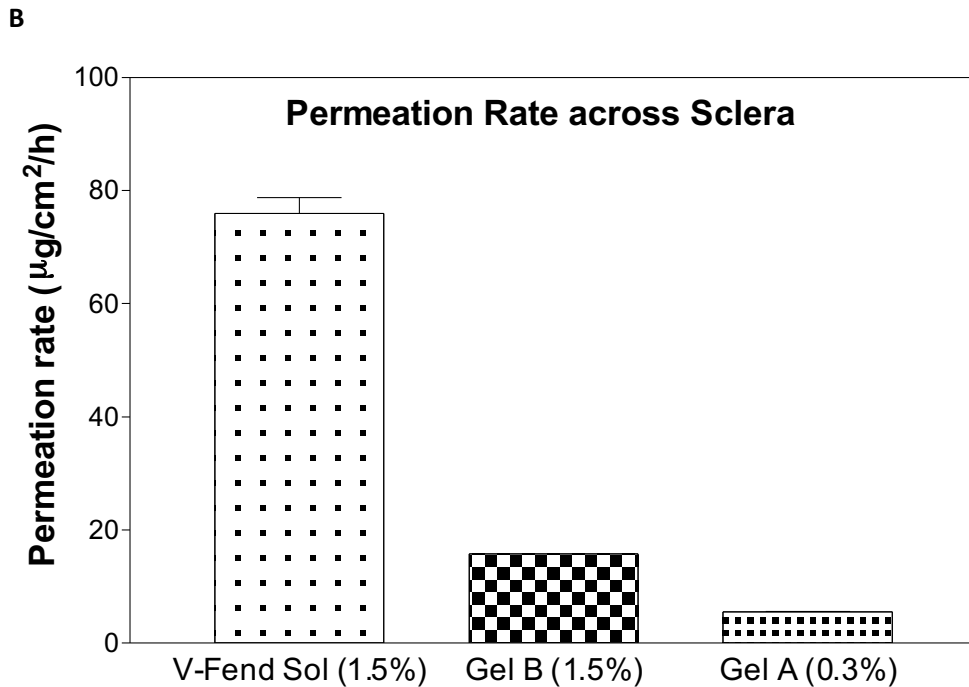
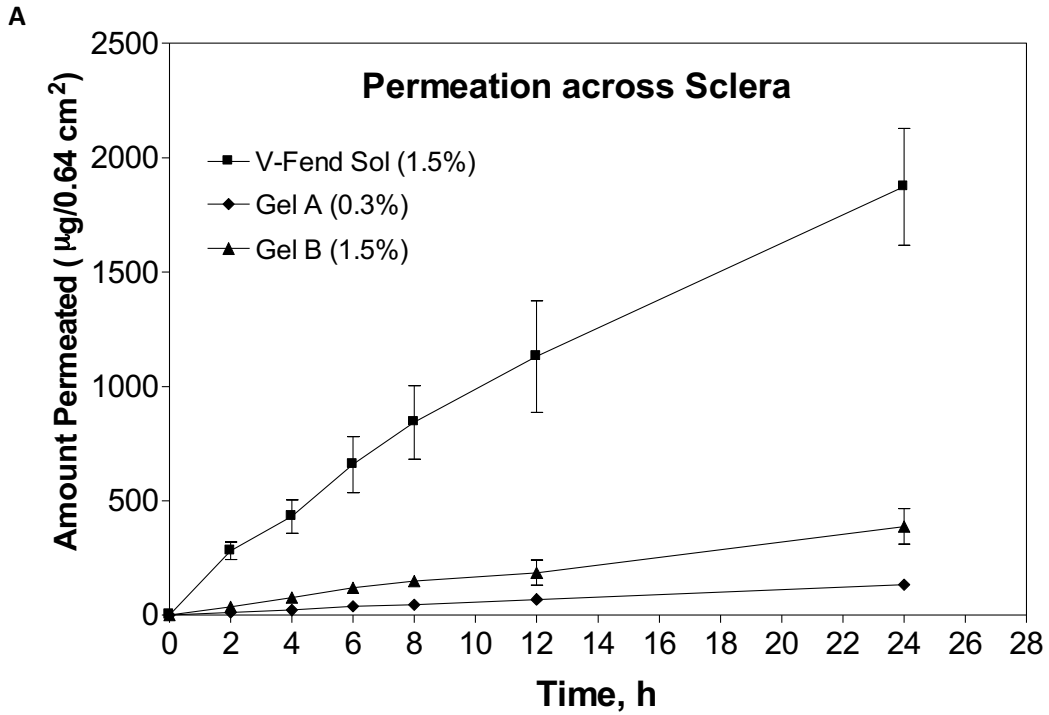
Permeation of voriconazole through the cornea and sclera was observed for all formulations (Figures 16 and 17). The sclera was two-fold more permeable than the cornea to the 1.5% voriconazole solution ( $P < 0.001$ ). Both corneal- and scleral- permeation data demonstrated a concentration dependent increase in the permeation of voriconazole from the thermogel formulations. The permeation rate through the sclera or cornea for Gel B (1.5% voriconazole-thermogel) was at least 3-fold higher than that of Gel A (0.3% voriconazole-thermogel) ( $P < 0.001$ ). Gel B (1.5% voriconazole-thermogel) showed significantly lower permeation as compared to the 1.5% voriconazole solution through both the sclera and cornea. Corneal hydration was less than 83% in all cases (range: 78.3-82.9%; mean  $\pm$  standard deviation:  $81.5\% \pm 1.9\%$ ) (Table 2).<sup>72</sup>

### *Subsection 3d: Characterization of subconjunctival injection of the thermogel in ex vivo equine eyes*

The thermogel was able to be easily injected into the dorsal bulbar SCS through a 30-gauge needle in all cases. A discrete gel deposit was formed in the SCS which was able to be observed both grossly and as a well-demarcated hypoechoic structure via UBM (Figure 18). The deposits formed were small and ovoid with length  $15.1 \pm 1.37$ mm, width  $10.1 \pm 1.65$ mm and maximum depth  $1.3 \pm 0.25$ mm (0.83-1.72mm). Gelation occurred rapidly, with a mean time from initiation of the injection to gelation of  $16.4 \pm 1.65$  seconds (14-19 seconds) (Table 3).



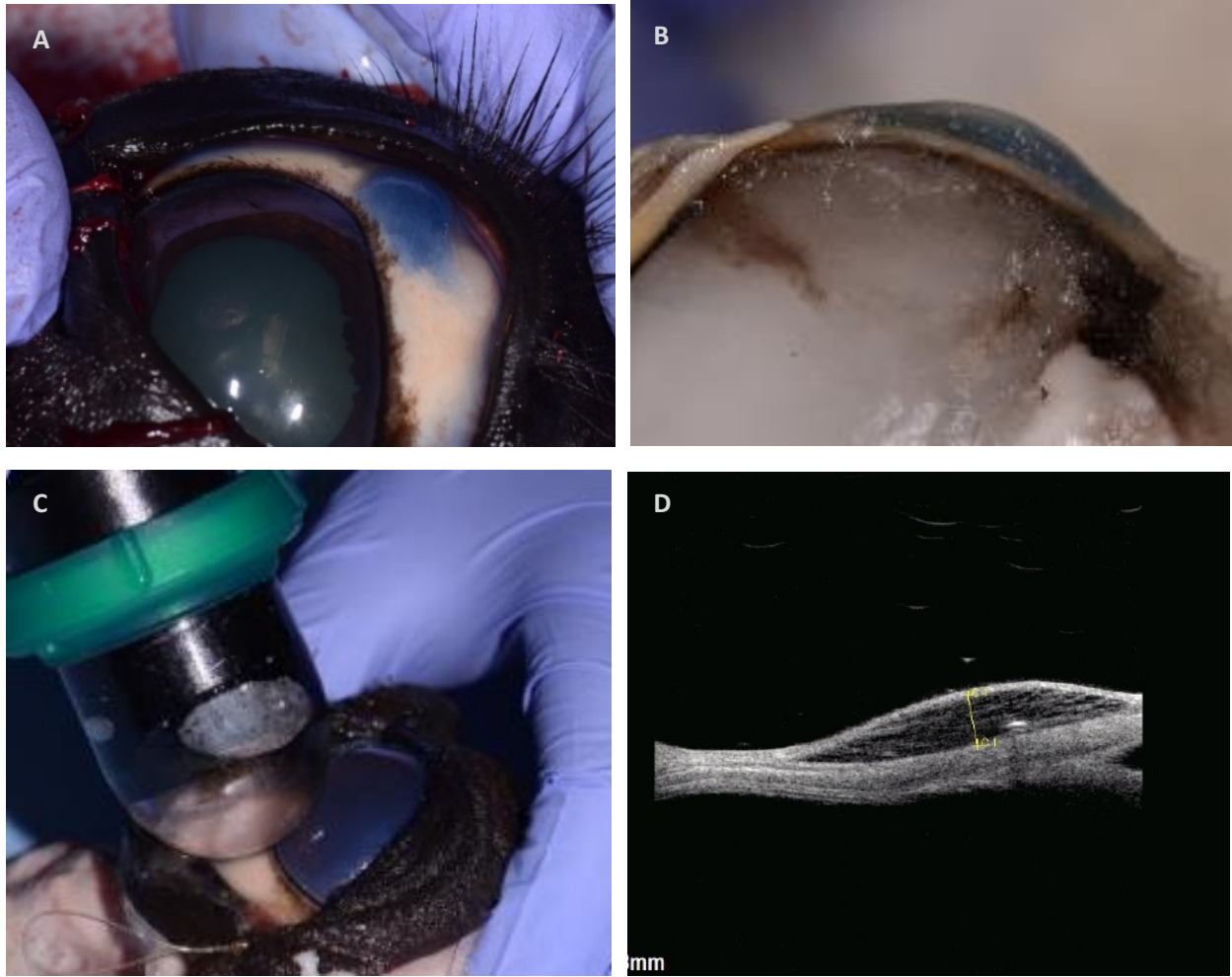
**Figure 16.** Permeation of voriconazole across the cornea over time. A concentration dependent increase in the permeation of voriconazole from the thermogel formulations was noted. Gel B (1.5% voriconazole-thermogel) showed significantly lower permeation as compared to the 1.5% voriconazole solution.



**Figure 17.** Permeation of voriconazole across the sclera over time. A concentration dependent increase in the permeation of voriconazole from the thermogel formulations was noted. Gel B (1.5% voriconazole-thermogel) showed significantly lower permeation as compared to the 1.5% voriconazole solution.

Sample number	Wet weight	Dry weight	% reduction
C1	95.4	20.7	78.3
C2	82.5	17.8	78.4
C3	114	19.5	82.9
C4	113.2	21.4	81
C5	142.3	24.5	82.7
C6	101.7	18.1	82.2
C7	115.8	20.2	82.6
C8	100.5	17.2	82.9
C9	106.4	18.3	82.8

**Table 2.** Corneal hydration values following the Franz cell diffusion study. C1-3: 1.5% voriconazole solution, C4-6: Gel A (0.3% voriconazole thermogel), C7-9: Gel B (1.5% voriconazole thermogel).



**Figure 18.** 300µl PLGA-PEG-PLGA thermogel deposit in the dorsal bulbar SCS of an *ex vivo* equine eye as identified by **A**. Visual inspection **B**. Visual inspection following freezing with liquid nitrogen and sectioning **C & D**. 50MHz ultrasound examination.

Eye number	Caliper measurements (mm)		U/S measurement (mm)	Bulbar SCS Temperature	Time to gel (secs)
	Length	Width	Max depth		
1	15	9	1.48	31	15
2	14	10	1.44	33	18
3	13	8	1.72	31.6	19
4	16	10	1.13	33	16
5	16	14	0.83	31.4	14
6	17	11	1.45	35.2	15
7	14	10	1.46	34.5	17
8	17	11	1.16	33.4	18
9	14	10	1.33	33.2	17
10	15	12	1.47	33.2	15

**Table 3.** Measurements of dorsal bulbar SCS thermogel deposits obtained after injecting ten *ex vivo* equine eyes with 300 $\mu$ l PLGA-PEG-PLGA thermogel.

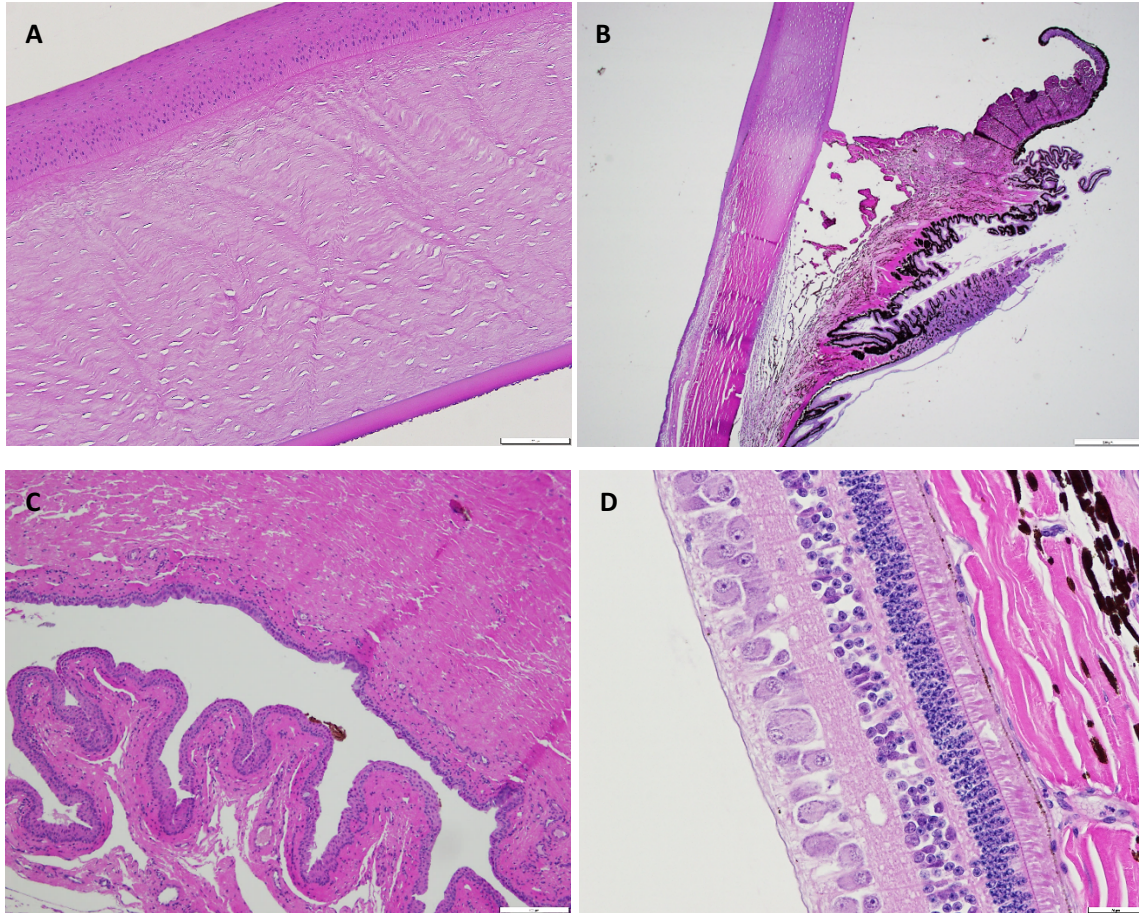
*Subsection 3e: Short- and mid-term toxicity pilot study in a single live horse*

Dorsal bulbar SCS injection of the voriconazole-containing thermogel was easily performed and well tolerated *in vivo* following administration of intravenous sedation and local anesthesia (Figure 19). The thermogel remained in liquid form when stored in syringes in a cooler during the time it took to administer local anesthesia and perform a sterile preparation of the eye, and converted to a gel state within 10 seconds of completing the injection. No local or systemic adverse reactions were noted in the treated eye in the 7 days following injection, with the exception of self-limiting chemosis and conjunctival hyperemia on days 2 and 3 post-injection. Twice daily ocular examination from a distance, physical examination and complete ophthalmic examination 7 days post-injection, all returned normal findings, and the treated horse exhibited normal attitude, feed and water intake and fecal and urine output throughout the entire study period. The conjunctiva, sclera, cornea, lens, retina and optic nerve of both eyes were normal on histologic examination, with no evidence of inflammation or tissue damage observed either 7 days or 2 hours post-thermogel injection (Figure 20).





**Figure 19.** In vivo injection of 300 $\mu$ l thermogel in the right eye of a horse following administration of sedation and local anesthetic agents.



**Figure 19.** Histopathologic sections (hematoxylin and eosin) from the right eye of a horse injected with 300 $\mu$ l thermogel in the right dorsal bulbar SCS seven days prior to enucleation: **A.** Cornea (10x), **B.** Ciliary body, iris, limbus (2x), **C.** Conjunctiva (10x), **D.** Retina (40x); no evidence of inflammation or tissue damaged was observed on histopathologic examination of either eye following injection of the thermogel.

## Chapter 4 – Discussion and Conclusions

Keratomycosis is a severe disease in which diagnosis can be challenging, response to medical therapy slow and outcome poor, and can therefore result in severe ocular morbidity.<sup>21, 22</sup> The incidence, clinical manifestations and treatment of equine keratomycosis are remarkably similar to those seen in human keratomycosis, and it is hoped that investigation into new, affordable treatment modalities in horses may influence development of parallel human therapies, therefore benefiting both horses and people with this devastating disease.<sup>3, 30</sup>

Topical and trans-scleral drug delivery are the least invasive and most commonly employed methods of treating anterior eye diseases.<sup>75</sup> Achievement of sustained delivery of lipophilic drugs to the anterior segment of the eye in therapeutic concentrations poses a challenge, however, due to the poor aqueous solubility of these drugs, and thus decreased ability to enter cells of the cornea and rapid clearance by the conjunctival and choroidal circulatory systems.<sup>76</sup> In addition for drugs with low solubility, such as voriconazole, a decrease in trans-scleral transport has been observed with increasing drug lipophilicity. This decreased transport is a result of drug binding in the melanin-rich choroid-retinal pigment epithelium layer or sclera.<sup>76</sup> Therefore, for any drug intended for trans-scleral delivery following subconjunctival injection, a depot form is preferred.<sup>76</sup>

This body of work describes the development of a novel, biodegradable, sustained-release ocular drug delivery system for treatment of keratomycosis and its evaluation *in vitro*, *ex vivo* and in a pilot animal model. We demonstrated that voriconazole is released in a sustained manner from a PLGA-PEG-PLGA thermogel in concentrations exceeding the MIC of common ocular fungal pathogens for over 21 days *in vitro* and is capable of permeating the equine cornea and sclera following release. Furthermore, we demonstrated that bulbar subconjunctival injection of a voriconazole-containing PLGA-PEG-PLGA thermogel is feasible in equine eyes and is not associated with adverse systemic or local tissue reaction.

PLGA-PEG-PLGA is a biocompatible, biodegradable, thermoactive polymer. It was selected for use in this study due to its proven safety in other species, ease of injectable administration and previously demonstrated sustained-release characteristics when combined with other small molecular weight, lipophilic drugs.<sup>51, 52, 60</sup> As a biodegradable substance PLGA-PEG-PLGA demonstrates advantages over current sustained-release devices available for treatment of equine ocular disease, such as cyclosporine-containing silicone-based matrix implants, as it is broken down by endogenous enzymes and therefore does not require surgical removal when it ceases releasing the loaded medication.

An additional advantage of PLGA-PEG-PLGA thermogels is their ability to increase the solubility of poorly soluble drugs.<sup>77</sup> This study demonstrated the advantages of this property, with a sufficient volume of voriconazole to exceed the target MIC for common organisms implicated in

keratomycosis following release able to be combined with very small volumes of thermogel. Voriconazole was found to be released continuously for 28 days from a volume of PLGA-PEG-PLGA thermogel suitable for injection in the bulbar SCS, with peak release occurring on day 1 and concentrations in PBS remaining above the target MIC of 0.5µg/ml for up to 21 days, consistent with previous findings testing alternate lipophilic drugs in PLGA-PEG-PLGA thermogels.<sup>52, 60, 78</sup> The PLGA-PEG-PLGA thermogels used in this study were noted to undergo phase separation within the first 24 hours into liquid and gel components. This physical characteristic may explain the high voriconazole concentrations measured on days 1-2 of the *in vitro* release study, with a large amount of voriconazole being released with the hydrophilic thermogel fraction on day 1 and the remainder undergoing very slow release together with physical degradation of the hydrophobic thermogel matrix.

Voriconazole is a triazole antifungal agent that is recommended as a first line agent for treatment of keratomycosis.<sup>25, 27, 30, 31</sup> In this study, voriconazole was shown to permeate excised equine corneas and sclerae in aqueous solution, and following release from the thermogel, *in vitro*. The 1.5% voriconazole-thermogel showed significantly lower permeation as compared to the 1.5% voriconazole solution, consistent with exposure of the tissues to a lower concentration of voriconazole in the thermogel groups. This finding was expected as the voriconazole in solution was immediately available to the tissues for permeation, however the voriconazole in the thermogel was sequestered from the tissues until it underwent release from the thermogel matrix. An additional factor which may have improved the permeation of the voriconazole in solution through the cornea *in vitro* was the presence of cyclodextrins in the commercial

voriconazole solution. Cyclodextrins have been shown to increase the permeability of lipophilic drugs through the cornea in other *in vitro* Franz diffusion cell studies.<sup>79</sup> Additionally, while the PLGA-PEG-PLGA used in preparation on the thermogels in this study increased the permeability of voriconazole sufficiently to enable preparation of a 1.5% voriconazole-thermogel, once the voriconazole was released following breakdown of the hydrogel matrix this influence on solubility, and by extrapolation, permeability into cells, would have been removed.

Transconjunctival permeation followed by corneal absorption, reflux out of the injection site, and direct penetration through the sclera are all proposed mechanisms of transport of medication to the anterior chamber following subconjunctival injection.<sup>32, 63</sup> Scleral permeation was two-fold higher than corneal permeation for the 1.5% voriconazole solution in this study, indicating that successful delivery of voriconazole through the sclera following subconjunctival injection is possible. This discrepancy is likely the result of structural and physiological differences between the tissues tested. The sclera has 10 times fewer glycosaminoglycans than the cornea, and the scleral stroma has a greater degree of fibrillar interweave than the corneal stroma.<sup>80</sup> Diffusion across the sclera as well as lateral diffusion within the sclera has been studied extensively as a function of molecular weight and other parameters, and greater permeation of drugs through the sclera than the cornea is well reported.<sup>81</sup> Similar permeation rates were identified across both tissues in the 1.5% and 0.3% voriconazole-thermogel formulations, further supporting the validity of these thermogels as vehicles for sustained ophthalmic drug delivery, as permeation is concentration dependent and this finding likely reflects lower exposure of the sclera to voriconazole in the thermogel groups as it was slowly being released from the thermogels than

in the solution group. Despite the lower permeation of the voriconazole through tissues following release from the thermogel than when applied as a solution alone, the thermogels still show significant potential for use in the treatment of fungal ocular infections as the slow and sustained achievement of therapeutic concentrations of the medication in the cornea and anterior chamber is the goal of therapy and this can be achieved in this case by a single injection of thermogel as opposed to multiple topical applications of voriconazole solution.

Subconjunctival injection of antifungal medications has been shown to improve treatment success in both equine and human patients with keratomycosis.<sup>22, 25, 27, 82</sup> In this study the thermogel proved easy to inject into the dorsal bulbar SCS of normothermic *ex vivo* equine eyes through a 30 gauge needle, in a manner mimicking current practices of SCS injections in the clinical setting, and formed a discrete gel deposit within 20 seconds of injection. This deposit was identifiable both grossly and via UBM. This method was easily repeatable in a single live pilot horse under standing sedation and local anesthesia and was able to be performed by an equine veterinarian without ophthalmology specialty training, following demonstration of the technique by a veterinary ophthalmologist. Furthermore, the presence of the small gel deposit in the dorsal bulbar SCS was well tolerated by the horse, and no evidence of toxicity was identified on clinical or histopathologic ocular examination either 2 hours or 7 days post-thermogel injection. These findings suggest that SCS injection of the voriconazole-containing hydrogel developed in this study could be performed practically and safely by veterinarians in equine keratomycosis patients.

A number of limitations existed in this study. Firstly, drug release was only measured *in vitro*. While the results were very promising, marked discrepancies in drug release kinetics and longevity from hydrogels are often noted between *in vitro* experiments and *in vivo* studies, when the hydrogel deposit is subjected to biologic influences, such as constant blood flow and enzyme degradation.<sup>56</sup> Further research is required to assess *in vivo* release of voriconazole from the thermogels and longevity of therapeutic voriconazole concentrations in the cornea and anterior chamber following injection. Secondly, two samples were taken from each equine cornea to create the corneal buttons used in the permeation study in this experiment due to the limited numbers of horses from which eyes were able to be harvested. Corneal thickness varies by region in the equine cornea, therefore taking a single, central sample from each cornea harvested would have increased the uniformity of tissue tested in the corneal permeation model and reduced potential variability induced by variation in tissues thickness in the results.<sup>83</sup> In this case 2 samples were taken from each cornea in an effort to reduce mortality. Each test group used 3 corneal buttons from the one horse in an effort to reduce the impact of additional individual factors, such as breed and age, on corneal thickness. Dissection of the corneas may have also impacted the results as opposed to placing an entire cornea with an intact scleral ring surrounding it into the Franz Cell chamber as occurs in human and small ruminant corneal drug permeation studies. Corneal hydration was calculated to evaluate the damage to corneal tissue and to determine the validity of samples for inclusion in the study. Corneal hydration was less than 83% in all cases, the percentage correlated with damage to the endothelium and or/epithelium of mammalian cornea in *in vitro* corneal permeability studies.<sup>72</sup> Finally, the safety assessment was a pilot study



in one horse, therefore further assessment of potential toxicity of the voriconazole thermogel in a larger number of horses is essential before being used in a clinical setting.

To the author's knowledge this study constitutes the first reported application of thermosensitive hydrogel technology to treatment of an equine ophthalmic disease. Results from this study are promising for future application of this thermogel in treatment of both equine and human keratomycosis, however further research is required to determine whether or not the voriconazole-containing thermogel will retain its sustained-release properties *in vivo*. Research is currently underway to assess the safety, longevity and performance of these thermogels in horses *in vivo*. If results of the *in vivo* experiments are positive, this thermogel could reduce cost of treatment, improve compliance and increase treatment success rates for keratomycosis patients.

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