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

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Purpose. To determine in vitro release profiles, transcorneal permeation, and ocular injection characteristics of a voriconazole-containing thermogel suitable for injection into the subconjunctival space (SCS).

Methods. In vitro release rate of voriconazole (0.3% and 1.5%) from poly (DL-lactide-co-glycolide-b-ethylene glycol-b-DL-lactide-co-glycolide) (PLGA-PEG-PLGA) thermogel was determined for 28 days. A Franz cell diffusion chamber was used to evaluate equine transcorneal and transscleral permeation of voriconazole (1.5% topical solution, 0.3% and 1.5% voriconazole-thermogel) for 24 hours. Antifungal activity of voriconazole released from the 1.5% voriconazole-thermogel was determined via the agar disk diffusion method. Ex vivo equine eyes were injected with liquid voriconazole-thermogel (4°C). Distension of the SCS was assessed ultrasonographically and macroscopically. SCS voriconazole-thermogel injections were performed in a horse 1 week and 2 hours before euthanasia and histopathologic analysis of ocular tissues performed.

Results. Voriconazole was released from the PLGA-PEG-PLGA thermogel for more than 21 days in all groups. Release followed first-order kinetics. Voriconazole diffused through the cornea and sclera in all groups. Permeation was greater through the sclerae than corneas. Voriconazole released from the 1.5% voriconazole-thermogel showed antifungal activity in vitro. Voriconazole-thermogel was easily able to be injected into the dorsal SCS where it formed a discrete gel deposit. Voriconazole-thermogel was easily injected in vivo and did not induce any adverse reactions.

Conclusions. Voriconazole-containing thermogels have potential application in treatment of keratomycosis. Further research is required to evaluate their performance in vivo.

Keywords: equine, voriconazole, subconjunctival, keratomycosis, thermogel, sustained-release, PLGA-PEG-PLGA

Fungal keratitis, or keratomycosis, is a vision-threatening disease well described in humans and animals worldwide.[1–3] In people, the reported incidence varies from 1.8% to 70.0% of microbial keratitis cases, and is strongly influenced by geographic and epidemiologic factors.[4–9] Keratomycosis occurrence is positively associated with warm, humid, subtropical to tropical climates, and is negatively associated with the degree of urbanization and gross domestic product of a region.[2,3] Keratomycosis is one of the most important causes of ocular morbidity and vision loss in developing nations, where it may account for more than half of corneal ulcers.[4–7] It has also recently been described as an emerging disease in more temperate climates and affluent countries, such as France and the United States, with case occurrence related to the presence of traditional risk factors (e.g., agricultural work and ocular trauma) and to the additional risk factor of soft contact lens wear.[3,10,11]

Keratomycosis is an emerging disease associated with severe morbidity 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.[12–15] Horses serve as the only naturally occurring, comparative animal model for human keratomycosis, and are thought to be particularly prone to fungal ocular infection due to their large globe size, unique orbital shape and resultant globe exposure, suspected tear film instability, and living environment.[16,17] Clinical manifestations of this disease in horses are very similar to those in people, and include corneal ulceration, superficial punctate keratitis, stromal abscessation, stromal plaque formation, and corneal perforation.[1,16,18,19] Fungal organisms and infiltrating immune cells are capable of initiating both vigorous inflammatory responses and protease overproduction in the cornea, which may lead to the development of acute complications, including rapid keratomalacia, unresolving ulceration, uncontrolled

anterior uveitis, endophthalmitis, and corneal perforation.[14,15,20] Long-term sequelae may include corneal scarring, cataract or synechiae formation, phthisis bulbi, and vision loss.[1,14]

Current treatment protocols for keratomycosis are expensive, prolonged, and labor-intensive. Voriconazole, a second-generation triazole antifungal drug, has recently become widely used for treatment of both human and equine keratomycosis due to its wide spectrum of antifungal activity, low minimum inhibitory concentrations (MICs) against common pathogens, and excellent corneal penetration.[13,21–23]

A number of barriers to ocular drug penetration, such as normal tear turnover, gravity-induced tear flow, blinking, induced lacrimation, and corneal and conjunctival epithelial tight junctions, limit the ability of topical medications to reach the anterior ocular segment.[24–28] Poor client/patient compliance can further reduce the volume and frequency of drug administration especially in horses due to their size, strong eyelid musculature, and lack of tolerance for repeated application of topical medications. 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 barriers.[27,29,30] 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 form a gel deposit upon reaching body temperature.[29,31] 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/patient compliance.[28,29,32–35]

The purpose of this study was to develop a thermogel suitable for injection into the subconjunctival space (SCS) in horses, as a natural animal model of human disease, that was capable of sustained-release and delivery of voriconazole to the cornea and anterior chamber.

Materials and Methods

All protocols were approved by the Institutional Animal Care and Use Committee of Auburn University and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Thermogel Preparation

Two commercially available, thermosensitive PLGA-PEG-PLGA triblock copolymers (AK24 [molecular weight (Mw) ~1100–1000–1100, 3:1 lactide:glycolide] and AK19 [Mw ~1500–1500–1500, 1:1 lactide:glycolide]); Akina, Inc., West Lafayette, IN, USA) 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% wt/wt; Hospira, Inc., Lake Forest, IL, USA), vortexing for 1 minute every 8 hours for 48 hours and storage at 4°C (liquid form). The two thermogel solutions were combined in a 1:1 ratio to form a thermogel with an optimal gelation setpoint of 32–36°C[36] ("the thermogel"), concordant with that of the equine bulbar SCS (mean temperature ± SD: 34.5°C ± 0.6°C).[37] Test groups containing different concentrations of voriconazole were prepared as outlined below. In all cases voriconazole crystals (US Pharmacopeia, Rockville, MD, USA) alone or voriconazole crystals dissolved in ethanol (Sigma-Aldrich Corp., St. Louis, MO, USA) solution were added to the thermogel at 4°C and the resultant voriconazole-thermogel was vortexed for 1 minute every 8 hours for 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, USA) for ease of visualization.

In Vitro Release Rate of Voriconazole From the Thermogel

In vitro release rate determination was performed as described previously.[38,39] Six test groups were prepared, five consisting of 300-μL aliquots of the thermogel combined with voriconazole in the following concentrations: 0% (group 1; negative control group), 0.3% voriconazole (1 mg; group 2), 0.3% voriconazole (1 mg as a 10% solution in ethanol; group 3), 1.5% voriconazole (5 mg; group 4), and 1.5% voriconazole (5 mg as a 10% solution in ethanol; group 5), and one positive control group consisting of voriconazole crystals alone (5 mg; 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 gellation, 1 mL PBS (Lonza, Walkersville, MD, USA) was added to each vial. The PBS was collected from the vials and replaced with 1 mL 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 analysis of voriconazole concentration via reverse-phase HPLC. Observations of thermogel appearance were recorded daily. Each formulation was tested in triplicate.

In Vitro Corneal and Scleral Voriconazole Permeation

Five eyes were obtained from horses free from corneal and scleral disease that were euthanized at the J. T. Vaughan Large Animal Teaching Hospital (JTVLATH), Auburn University, AL, USA, 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, USA) and transported on ice to the laboratory within 20 minutes of enucleation, where two paracentral corneal and two dorsal scleral buttons were harvested from each eye via the use of a corneal trephine (Asico, LLC, Westmont, IL, USA) 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 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 (5 mL, pH 7.4), that was maintained at 34°C to mimic the equine SCS temperature[37] with a water circulation jacket (37°C) surrounding the lower part of the cell. Stirring in the receptor chamber was maintained by a magnetic bead. Three tests groups were determined on the basis of in vitro release study results and the donor compartment loaded with either 300 μL of a 1.5% voriconazole solution previously shown to penetrate equine corneas in vivo[13] (Vfend; Pfizer, New York, NY, USA; group 1), 300 μL 0.3% voriconazole-thermogel (group 2), or 300 μL 1.5% voriconazole-thermogel (group 3). PBS samples were taken from the receptor chamber and the receptor chamber was replenished with 1 mL fresh PBS at 0, 2, 4, 6, 8, 12, and 24 hours. Samples were stored at –80°C then analyzed for voriconazole content via reverse-phase HPLC. Each group was tested in triplicate.

After 24 hours, each corneal button was rinsed thrice with PBS to remove any remaining voriconazole or thermogel, dried

and the "wet weight" (W_a) recorded. The corneal samples were then desiccated at 70°C for 8 hours and the "dry weight" (W_b) recorded. From these data, the percentage corneal hydration was calculated (% corneal hydration = $[1 - (W_a/W_b)] \times 100$), with a limit of 83% corneal hydration set for inclusion of results in the study.[40]

High-Performance Liquid Chromatography

PBS samples were analyzed by reverse-phase HPLC as previously described.[41] The HPLC system (Agilent 1200 series) consisted of pumps, an autosampler, UV/visible light absorption detector, column (4.6 mm · 15 cm, 5 μm; Thermo BetaBasic-18, Bellefonte, PA, USA), and computer interface. The flow rate of the mobile phase was 1.0 mL per minute and 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, showing retention times of 3.7 and 13.5 minutes, respectively. Calibration standards for voriconazole concentrations ranged from 0.02 to 10 μg/mL and were prepared in dissolution media and selected fluids. The lower limit of detection and quantification for voriconazole was 0.02 μg/mL and 0.05 μg/mL, respectively. Values of 1.96% and 5.01% were noted for intra- and interday variations, respectively.

Antifungal Activity of Voriconazole Following Release From the Thermogel

Antifungal activity of voriconazole released from group 4 thermogels was evaluated via the agar disk diffusion method, as previously described.[42–45] Briefly, *Aspergillus flavus* ATCC 204304 was grown on potato dextrose agar slants at 25°C with an aliquot subcultured onto Sabouraud dextrose agar (SDA) and passaged to ensure purity and viability. After 4 days of incubation, the slants were washed with sterile saline, Tween 20 (Sigma-Aldrich Corp.) was added to improve conidia dispersion and the suspension was adjusted to No. 1 MacFarland standard using sterile saline as the diluent. For the inoculum, the cell density of the suspension was adjusted until absorbance reading with a spectrophotometer (Genesys 10S UV-VIS spectrophotometer; Thermo Fisher Scientific, Waltham, MA, USA) was OD530 nm, which equates to 0.4×10^6 CFU/mL to 5×10^6 CFU/mL. Dilutions were then plated on SDA and incubated at 25°C for 48 hours to confirm density. The inoculum was then swabbed onto Mueller Hinton agar (MHA). Sterile paper disks were impregnated with 100 μL of the voriconazole-PBS solutions collected on hours 6 and 12 and days 1, 5, 7, 14, 21, and 27 of the drug release study and placed in triplicates on MHA plates, together with a voriconazole control disk (1 μg, BD Sensi-Disk; Becton Dickenson and Co., Franklin Lakes, NJ, USA). Plates were incubated at 35°C and examined at 24 and 48 hours, and the diameter of the zone of inhibition was measured for each disk.

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 JTVLATH for reasons not related to this study. Enucleation was performed immediately following euthanasia via a transpalpebral approach and the voriconazole-thermogel injected immediately following enucleation. The temperature in the ventral SCS was measured immediately before injection with a type T thermocouple (Cole-Palmer, Vernon Hills, IL, USA) to ensure normothermia was maintained. The thermogel (300 μL) 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. Ultrasound biomicroscopy, 50 MHz (UBM) (Aviso TM, 2016; Quantel Medical, Bozeman, MT, USA) was used 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 further described.

Short- and Mid-Term Toxicity Pilot Study in a Single Live Horse

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

The horse was sedated with 5 mg detomidine hydrochloride (Dormosedan; Zoetis, Inc., Florham Park, NJ, USA) and 5 mg butorphanol tartrate (Torbugesic; Zoetis, Inc.) IV, and local ocular anesthesia was supplied through administration of perineural 2% lidocaine hydrochloride (Hospira, Inc., Lake Forest, IL, USA) around the auriculopalpebral and frontal nerves and topical 0.5% proparacaine hydrochloride (Akorn, Inc.). The eye was prepped with sterile diluted 5% betadine, irrigated with sterile eyewash (Purdue Products, L.P., Stamford, CT, USA) and 300 μL 1.5% voriconazole-thermogel was injected into the right dorsal SCS through a 30-gauge needle. Flunixin meglumine (1.1 mg/kg IV; Banamine: Intervet International, Intervent, Inc., Madison, NJ, USA) was administered immediately post injection and topical ocular neomycin-polymixin-bacitracin ointment (Vetropolycin; Dechra Veterinary Products, Overland Park, KS, USA) was applied to the treated eye every 6 hours for 48 hours following injection. A complete ophthalmic examination, including Schirmer tear test (Schering-Plough, Charlotte, NC, USA), biomicroscopy examination (SL-14; Kowa, Tokyo, Japan), tonometry (Tonovet, iCare, Vantaa, Finland), fluorescein test (Akorn, Inc.), and funduscopy, was performed 1 day before and 7 days after the SCS injection. After pupillary dilation with tropicamide 1% (Akorn, Inc.), the ocular fundus was photographed (Optibrand Clearview; Optibrand Ltd., Fort Collins CO, USA). 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.[46] 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.[46] Seven days postinjection the horse was anesthetized with xylazine (1.1 mg/kg IV; Anased; Akorn, Inc.), ketamine (2.2 mg/kg IV; Ketaset; Fort Dodge Animal Health, Fort Dodge, IA, USA), and inhaled isoflurane (Fluriso; Vet One, Boise, ID, USA), then euthanized for reasons not related to this project with pentobarbital sodium and phenytoin sodium (0.2 mL/kg; Beuthanasia-D; Intervet, Inc., Madison, NJ, USA). Following anesthetic induction (2 hours before euthanasia) 300 μL of a 1.5% voriconazole-thermogel was injected into the left dorsal SCS. Following euthanasia, both of the horse's eyes were enucleated and submitted for histopathologic examination.

Data Analysis

Descriptive statistics (mean, median, range) were calculated for measurements of thermogel deposit dimensions obtained following ex vivo SCS injection and percentage corneal

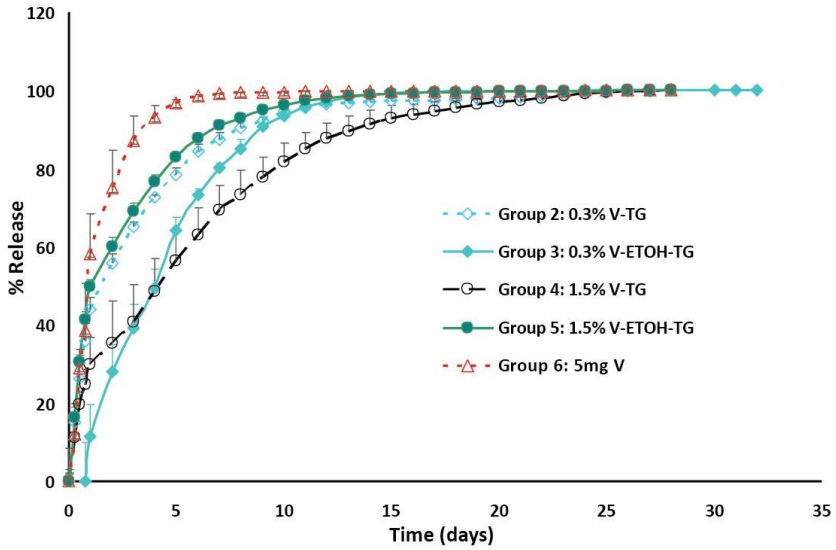

FIGURE 1. Percent cumulative voriconazole release from the thermogels. Voriconazole was sustainedly released from the thermogels throughout the study period. Addition of ethanol inconsistently altered voriconazole release. TG, thermogel; V, voriconazole; ETOH, ethanol.

hydration following in vitro permeation studies (Microsoft Excel 2013; Microsoft, Redmond, WA, USA). All data were expressed as mean ± SD. 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 coordinates. 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 data was presented as flux or permeation rate (μg/cm^2/h). Data were analyzed by 1-way ANOVA followed by Dunnett's test to determine the level of significance between various groups. The mean differences were considered significant at $P < 0.05$. Data were analyzed using a commercial statistical analysis program (GraphPad Prism software version 5; GraphPad, La Jolla, CA, USA). For antifungal activity assessment, the diameters of the growth inhibition zones around each disk were measured in millimeters. A zone diameter equal to or greater than 17 mm is the epidemiologic cutoff value for detection of moulds with reduced susceptibility.[45]

RESULTS

Thermogel Preparation

Voriconazole appeared to combine with the thermogel in groups 2, 3, and 5 to form an aqueous solution. Voriconazole crystals appeared to disperse within the thermogel forming a suspension in group 4. The thermogel retained its thermosensitive properties following combination with voriconazole alone, voriconazole in ethanol solution, and methylene blue.

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 for all voriconazole-thermogel groups (groups 2–5; Figs. 1, 2). 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). The concentrations released exceeded the target MIC (0.5 μg/mL) for 28 days in groups 2 and 4 and 16 days in groups 3 and 5 (Fig. 2). 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 (Fig. 1).

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

In Vitro Corneal and Scleral Voriconazole Permeation

Permeation of voriconazole through the equine cornea and sclera was observed for all formulations (Fig. 4). The sclera was 2-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. The permeation rate through the sclera or cornea for 1.5% voriconazole-thermogel was at least 3-fold higher than that of 0.3% voriconazole-thermogel ($P < 0.001$); 1.5% voriconazole-thermogel showed significantly lower permeation as compared with the 1.5% voriconazole solution through both the sclera and cornea, suggesting the sustained release character of the gel formulation. Corneal hydration was less than 83% in all cases (81.5% ± 1.9%, 78.3%–82.9%).

Antifungal Activity of Voriconazole Following Release From the Thermogel

The results of the antifungal activity testing of voriconazole released from 1.5% voriconazole-thermogel (group 4) in comparison with those of the reference compound, voriconazole (1 μg), are given in the Table. Voriconazole released from 1.5% voriconazole-thermogel showed moderate to good inhibitory activity against *Aspergillus flavus* ATCC 204304

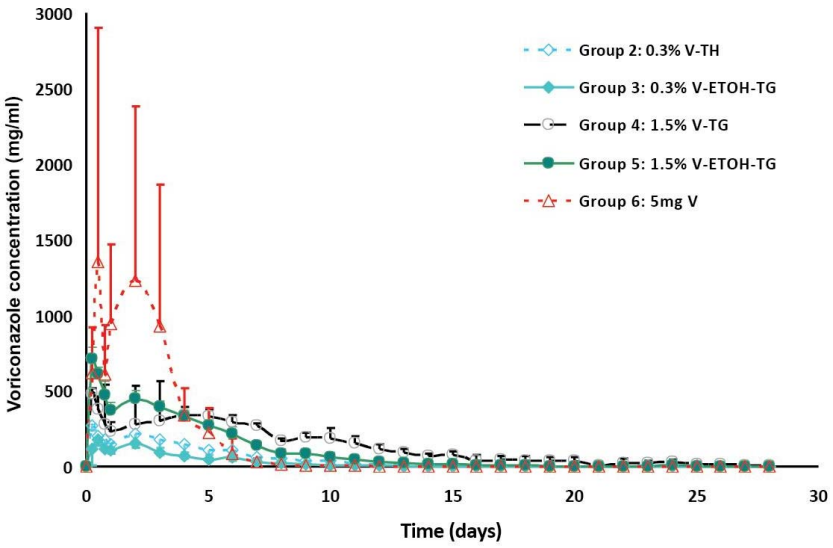

FIGURE 2. Voriconazole concentration in PBS over time. Voriconazole release exceeded the target MIC (0.5 μg/mL) for 28 days in groups 2 and 4 and 16 days in groups 3 and 5.

(24–50 mm inhibition zone diameters) at every studied time point.

Characterization of Subconjunctival Injection of the Thermogel in Ex Vivo Equine Eyes

The thermogel was able to be easily injected into the dorsal SCS through a 30-gauge needle. 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 (Fig. 5). The deposits formed were small and ovoid with length 15.1 ± 1.37 mm, width 10.1 ± 1.65 mm, and maximum depth 1.3 ± 0.25 mm (0.83–1.72 mm). Gelation occurred rapidly, with a mean time from initiation of the injection to gelation of 16.4 ± 1.65 seconds.

Short- and Mid-Term Toxicity Pilot Study in a Single Live Horse

Dorsal SCS injection of the voriconazole-thermogel was easily performed and well tolerated in vivo following administration of IV sedation and local anesthesia (Fig. 6). The thermogel remained in liquid form when stored in a syringe in a cooler containing ice 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 7 days following injection, with the exception of self-limiting chemosis and conjunctival hyperemia on days 2 and 3 postinjection. No significant difference was found from baseline in the cumulative daily inflammatory scores 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 after thermogel injection (Fig. 7).

DISCUSSION

This series of studies 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 in vivo animal model. We demonstrated that voriconazole when directly combined with a PLGA-PEG-PLGA thermogel, is released in a sustained manner from the thermogel in concentrations exceeding the MIC of common ocular fungal pathogens for 28 days in vitro and is capable of permeating the isolated equine cornea and sclera and effecting sustained antifungal activity, following release. Furthermore, we demonstrated that subconjunctival injection of the thermogel is feasible in ex vivo and in vivo equine eyes under sedation and local anesthesia and in a single horse was not associated with adverse systemic, or local tissue, reaction.

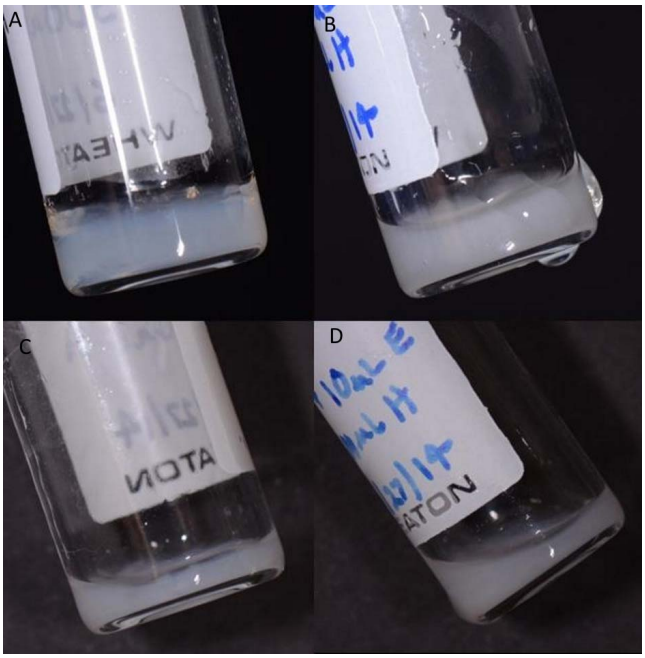

FIGURE 3. Thermogel degradation over time. Addition of ethanol slowed the degradation of the thermogel, with more thermogel remaining in groups 3 and 5 than in groups 1, 2, and 4 at study completion. (**A**) Group 1, day 1. (**B**) Group 3, day 1. (**C**) Group 1, day 28. (**D**) Group 3, day 28.

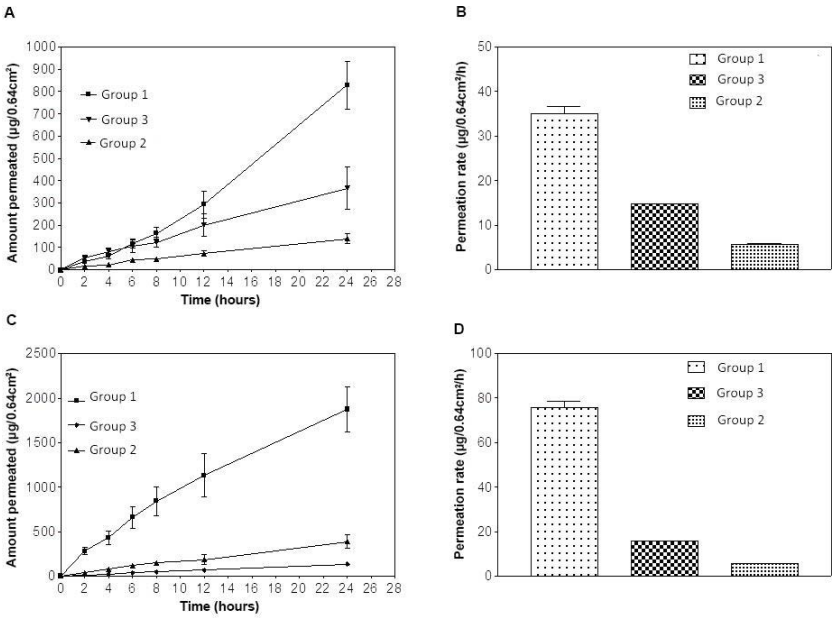

Figure 4. Permeation of voriconazole across the (**A**) and (**B**) cornea and (**C**) and (**D**) sclera over time. A concentration-dependent increase in the permeation of voriconazole from the thermogel formulations was noted, with the permeation rate through the sclera or cornea for group 3 at least 3-fold higher than that of group 2 ($P < 0.001$). Group 3 showed significantly lower permeation as compared with the 1.5% voriconazole solution, suggesting the sustained release character of the thermogel.

PLGA-PEG-PLGA is a biocompatible, thermoactive polymer. It was selected due to its proven safety, ease of injectable administration through a minimally traumatic 30-gauge needle, and sustained-release characteristics when combined with small molecular weight, lipophilic drugs.[30,47,48] As a biodegradable substance, PLGA-PEG-PLGA demonstrates advantages over current alternate sustained-release devices available for treatment of keratopathies, such as cyclosporine-containing silicone-based matrix implants,[38,49–51] as it is broken down by endogenous enzymes and therefore does not require surgical removal when it ceases releasing the loaded medication. The mode of breakdown of bioactive polymers varies greatly; for example, a PLGA and poly(d,l-lactide-co-caprolactone) microfilm surgically implanted in the SCS of rabbits was found to degrade via a combination of surface erosion and mass degradation.[52] The PLGA-PEG-PLGA thermogels used in this study were noted to undergo phase separation within the first 24 hours. This physical characteristic may explain the high voriconazole concentrations measured on days 1 to 2 of the release study, with a large amount of voriconazole being released from the hydrophilic thermogel fraction on day 1 and the remainder undergoing very slow release together with physical degradation of the hydrophobic thermogel matrix.[33] This property may connote a clinical advantage, with high concentrations of voriconazole achieved in the target tissues during the first 48 hours following injection, at the time of first exposure of the fungal pathogen to this medication.

Voriconazole is a triazole antifungal agent that is recommended as a first-line agent for treatment of keratomycosis.[21,23,53,54] Despite effective corneal penetration, its topical effect is short lived, with studies in rabbits demonstrating the need for administration of voriconazole every 30 minutes to

Table. Antifungal Activity

Time	Inhibition Zone Diameter, mm	
	Control: Voriconazole	Voriconazole-1.5% Thermogel
6 h	30	47.3 ± 2.3
12 h	28	43 ± 3.5
1 d	30	40 ± 0.0
5 d	34	50 ± 0.0
7 d	36	50 ± 0.0
14 d	32	38 ± 2
21 d	36	30 ± 4.6
27 d	35	24 ± 5.2

Antifungal activity of voriconazole in PBS released at several time points over a 28-day period from 1.5% thermogel (group 4) against *Aspergillus flavus* ATCC 204304. The value obtained for each time point represents the mean of three independent measurements ± SD.

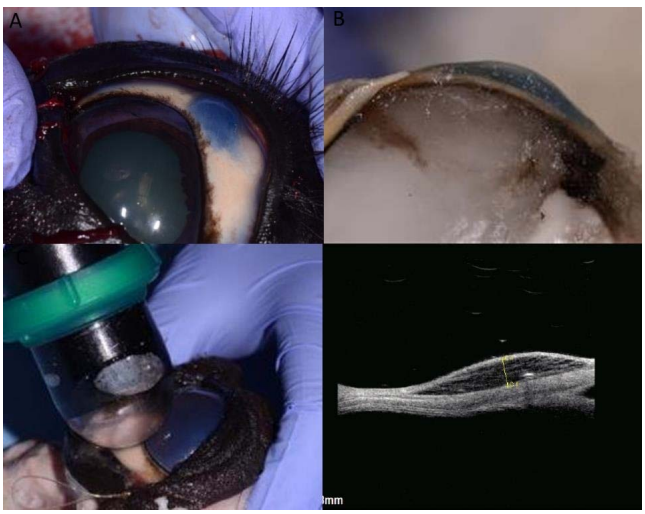

Figure 5. A 300-µL PLGA-PEG-PLGA thermogel deposit in the dorsal SCS of an ex vivo equine eye as identified by (**A**) visual inspection, (**B**) visual inspection following freezing with liquid nitrogen and sectioning, (**C**) performing UBM after thermogel injection, and (**D**) sonographic image obtained (50 MHz).

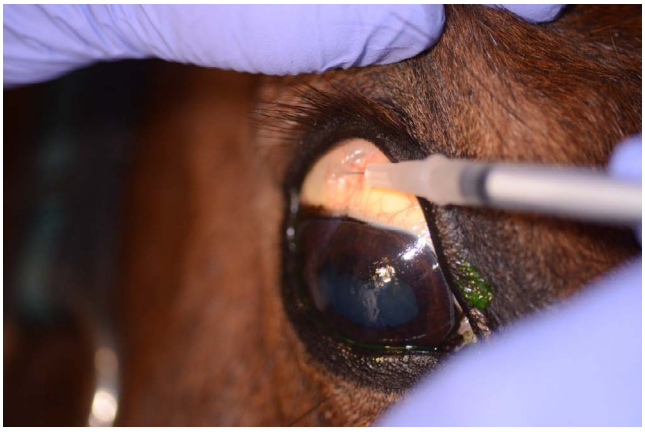

FIGURE 6. In vivo injection of 300 μL thermogel in the right eye of a horse following administration of sedation and local anesthetic agents.

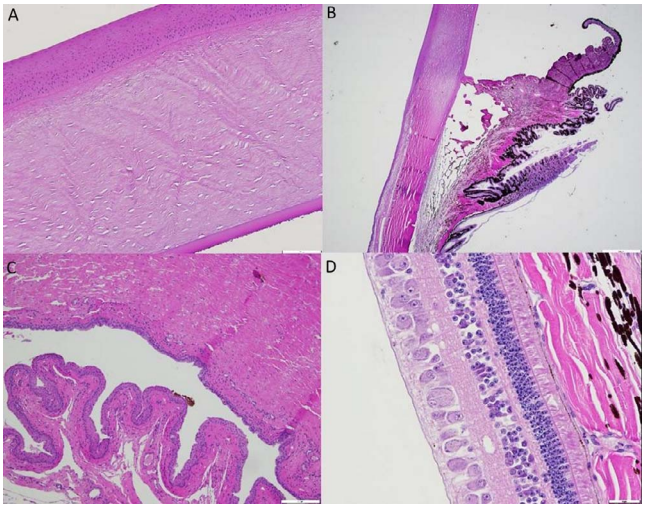

FIGURE 7. Histopathologic sections (hematoxylin and eosin) from the right eye of a horse injected with 300 μL thermogel in the right dorsal SCS 7 days before enucleation: (**A**) cornea (×10); (**B**) ciliary body, iris, limbus (×2); (**C**) conjunctiva (×10); (**D**) retina (×40). No evidence of inflammation or tissue damaged was observed on histopathologic examination of either eye following injection of the thermogel.

achieve a sustained high concentration of voriconazole in the anterior chamber.[55] Previous research has been conducted into lisosomal, nanoparticle-encapsulated, and microemulsion formulations of voriconazole, and contact lenses impregnated with alternative antifungal agents, to try to increase the contact time with the cornea, and therefore provide more sustained therapeutic drug levels following administration, whereas this study aimed to develop a depot thermogel formulation to prolong drug delivery to the anterior segment following injection.[56–59] In this study, voriconazole was shown to permeate isolated 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 with 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 that may have improved the permeation of the voriconazole in solution was the presence of cyclodextrins, an excipient used to increase solubility of voriconazole, in the commercial voriconazole solution.[60] Cyclodextrins have been shown to increase the permeability of lipophilic drugs through the cornea in other in vitro Franz diffusion cell studies.[60]

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.[61,62] Scleral permeation was 2-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.[63] 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.[64] 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. These thermogels show significant potential for use in the treatment of fungal ocular infections as the sustained achievement of therapeutic concentrations of 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.

A number of limitations existed in this study. First, drug release was measured only in vitro. Although the results were promising, marked discrepancies in drug release kinetics and longevity 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.[65] Second, two samples were taken from each equine cornea to create the corneal buttons used in the permeation study. Corneal thickness varies by region in the equine cornea; therefore, taking a single, central sample from each cornea harvested would have increased uniformity of tissues tested and reduced potential variability induced by disparities in tissue thickness in the results.[66] In this study, two samples were taken from each cornea in an effort to reduce mortality and each test group used 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.[67–69] 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 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 corneas in in vitro permeability studies.[40] 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.

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Conclusions

To the authors' knowledge, this study constitutes the first reported application of thermosensitive hydrogel technology to develop an injectable agent for treatment of keratomycosis. Results from this study are promising; however, further research is required, and is currently under way, to assess the safety, longevity, and performance of these thermogels 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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