



Original Research

Clinical Feasibility and Airway Deposition of Nebulized Voriconazole in Healthy Horses



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ARTICLE INFO

Article history:

Received 26 May 2020

Received in revised form

5 August 2020

Accepted 24 August 2020

Available online 3 September 2020

Keywords:

Equine

Voriconazole

Nebulization

Pneumomycosis

Fungal pneumonia

Fungal rhinitis

ABSTRACT

Voriconazole (VRC) is a potential treatment for pneumomycosis in horses. The objectives of this study were to determine if the delivery of Vfend using a Flexineb nebulizer produced clinically significant [VRC] in lower airways. The hypothesis was that [VRC] after delivery by nebulization would be greater in the pulmonary epithelial lining fluid than plasma. A secondary objective was to determine [VRC] in upper airways through the collection of nasopharyngeal wash (NPW) samples. Voriconazole solution [Vfend-6.25 mg/mL, 100 (n = 2), 200 (n = 3), 500 (n = 1) mg] was nebulized once in 6 healthy geldings. Clinical responses, duration of nebulization, and [VRC] at various time points (up to 8 hours) in plasma, bronchoalveolar lavage fluid (BALF) supernatant and cell pellet, and NPW samples were recorded. Voriconazole (Vfend-6.25 mg/mL, 200 mg) was nebulized in 5 additional, healthy geldings, and [VRC] was measured in NPW samples pre- and postnebulization at time points up to 8 hours. The antifungal activity of BALF and NPW samples was determined using agar disk diffusion. Concentrations of voriconazole were below detection in plasma, BALF supernatant, and cell pellets for all time points and doses except the BALF cell pellet (0.4 µg/g) immediately after nebulization of 500 mg. For 5 horses, administered 200 mg of Vfend, mean [VCR] in NPW at the end of nebulization and 1, 6, and 8 hours postnebulization were: 30.8 ± 29, 1.0 ± 0.84, 0.2 ± 0.19, and 0.34 ± 0.67 µg/mL, respectively. Only NPW samples obtained immediately postnebulization showed antifungal activity. A nebulized Vfend solution is not recommended for the treatment of pneumomycosis in horses.

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1. Introduction

Fungal pneumonia or pneumomycosis is an unusual, yet potentially life-threatening condition in horses. Fungal infections of the upper respiratory tract (URT) are more common than in the

lower respiratory tract (LRT) and may affect the nasal passages, paranasal sinuses, and guttural pouches [1]. These infections are acquired most frequently via inhalation of the causative organism; however, some cases are thought to arise by penetration of fungi through an inflamed gastrointestinal tract or open wound with subsequent hematogenous spread to the lungs [2]. In most cases, pneumomycosis develops secondary to the use of immunosuppressive drugs for the treatment of diseases such as inflammatory bowel disease and equine asthma [1,2].

In horses, one of the most common organisms isolated in fungal respiratory diseases is the species of the genus *Aspergillus*, which are ubiquitous in the environment [2]. Invasive pulmonary aspergillosis (IPA) is a fungal disease of the lungs associated with high mortality rates in immunosuppressed horses [3,4] and in people [5,6], despite treatment. Diagnosis and treatment of fungal

Animal Welfare/Ethical Statement: The study "Plasma, Pulmonary Epithelial Lining Fluid, and Nasopharyngeal Wash Concentrations of Voriconazole after Nebulization in Horses" was approved by the Institutional Animal Care and Use Committee (PRN 2018-3279).

Declarations of Interest: none.

Conflicts of interest statement: Not applicable.

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respiratory infections are challenging for equine veterinarians, and the prognosis for complete resolution of infection is often guarded [1]. Treatment requires long-term systemic administration of antifungal medication with potentially severe side effects, and the efficacy of treatment protocols for pneumomycosis in horses has not been thoroughly studied [3].

Voriconazole (VRC) is a broad-spectrum triazole antifungal agent used against a variety of clinically important yeasts and molds (*Aspergillus* spp., *Candida* spp.), and is considered the first-line treatment drug for IPA in people [7]. Voriconazole is usually well tolerated in humans, although there are dose and duration related side effects such as transient visual disturbance, urticaria, hepatotoxicity, and neuropsychiatric symptoms [8,9]. In horses, side effects associated with VRC administration have not been reported, but this may be due to a lack of information on long term (more than 14 days) systemic administration [10]. Rather than systemic administration, the most common use of VRC by equine practitioners in horses is the aqueous solution (Vfend) for topical or intralesional treatment of ocular infections.

In recent years, researchers in human medicine have focused attention on inhaled delivery of medications for the treatment of respiratory fungal infections, as administration via nebulization ensures local disposition of the drug in the affected areas, and reduces systemic dose requirements and the potential for systemic adverse effects [11]. Targeted drug delivery of Vfend demonstrated high tissue and plasma drug concentrations, as well as improved survival in a murine study of IPA [5,12], and detectable tissue and plasma drug concentrations in a human study [9]. Based on current literature, there are no studies in horses measuring the concentration of an antifungal drug delivered via nebulization. Recent developments in nebulizer technologies for use in horses have resulted in the development of mesh nebulizers, such as the Flexineb. This type of nebulizer has the advantage of being silent and battery-powered, making it easy to use and portable. The medication cup is comprised of a gold-plated metallic mesh containing very small holes that, when energized, produces high-frequency vibrations that generate a fine aerosol mist. The particle size distribution within the aerosol is largely determined by the size of the holes within the vibrating mesh [13]. This device is commonly used in equine practice and has successfully been used in several equine studies [14,15] to deliver medication into the lower airways.

The primary objective of this study was to determine the feasibility of delivery of a common, commercially available aqueous VRC solution (Vfend) to horses by nebulization using a common, commercially available equine nebulizer (Flexineb), and to determine concentrations of VRC in lower airway samples and plasma. The hypothesis was that VRC delivery by nebulization would be well-tolerated and would produce clinically significant concentrations (above the MIC for *Aspergillus* spp. 0.5 µg/mL) of VRC in the lower airways that were greater than plasma concentrations. In case of rejection of the hypothesis due to low VRC concentrations in the lower airways, a secondary objective was to measure VRC concentrations in the upper airway by collecting the nasopharyngeal wash fluid (NPW) samples.

2. Material and Methods

2.1. Horses

The study was approved by the Institutional Animal Care and Use Committee (PRN 2018-3279). University-owned, adult mixed-breed geldings were included in the study. Mean body weight was 552 kg (range: 461–639 kg). The mean age of the horses was 10 years (range: 6–12 years). The horses were determined to be healthy for inclusion in the study based on normal physical

examination (including re-breathing examination), complete blood count (CBC), serum biochemical analysis (including bile acids concentration), and bronchoalveolar lavage fluid (BALF) cytological examination. The data (BALF analysis) were used as baseline data for each individual and was obtained within 2 weeks prior to drug administration. Criteria considered for exclusion included abnormal findings in the physical examination, signs of inflammation or infection on the BALF analysis or bloodwork, or the receipt of any medication within 30 days of enrollment.

Horses were maintained on pasture and acclimated to an individual stall in the barn 24 hours prior to the study. Water and bermudagrass hay were available *ad libitum*. Physical examinations were performed twice daily throughout the study. Horses were returned to their regular pasture the day after the study was performed. Each horse stayed in a stall for a total of 3 days.

2.2. Voriconazole

2.2.1. Aerosol Particle Size

The particle size of the aerosol created by the equine nebulizer (Flexineb E2; JS Equine Division, Union City, TN) was measured by the manufacturer using a spray particle and spray droplet size analyzer (Spraytec; Malvern Panalytical Ltd, Enigma Business Park, Grovewood Road, Malvern, UK). Because there is usually ± 5% deviation from one measurement to another (in terms of % of particles), the analyzer takes measurements every 3 seconds and runs the test for a minute, thus getting 20 measurements per minute, and an average measurement is obtained at the end. For determining the flow rate obtained when nebulizing the Vfend solution, saline was used as a baseline for comparison, and two concentrations (10 mg/mL and 6.25 mg/mL) of Vfend solution were tested. Based on those results, the 6.25 mg/mL concentration was used for all nebulizations.

2.2.2. Preparation of VRC and Delivery by Nebulization

Voriconazole lyophilized sterile powder for injection 200 mg (Vfend; Pfizer Inc, New York, NY) was reconstituted using 32 mL of sterile water for injection to make a final concentration of 6.25 mg/mL [12]. Voriconazole was delivered using a portable mesh nebulizer (Flexineb E2; JS Equine Division, Union City). A standard (gray, Standard Flow Medication) cup and fast (green, Fast Flow Medication) cup (commercially available) designed to increase the speed of nebulization, were provided by the company for this study. The fast cup was designed to use with difficult to nebulize, high viscosity medications in order to maximize deposition, reduce condensation in the mask (decrease waste), and reduce the delivery time.

2.3. Samples

2.3.1. Plasma

Plasma samples for measurement of VRC and urea were collected through a 14-gauge, 2-inch intravenous (IV) jugular catheter (Surflo; Terumo Medical Corp, Somerset, NJ), which was placed aseptically in the horses on the day of drug administration. Blood samples were collected in sodium heparin tubes and kept on ice until centrifugation within 1 hour of collection. These samples were spun at 2,106 relative centrifugal force (rcf) for 10 minutes. The resultant plasma was stored in duplicate at –80°C until batch analysis.

2.3.2. Bronchoalveolar Lavage

Horses were sedated with detomidine hydrochloride (Dormosedan; Zoetis, Parsippany, NJ; 0.01 mg/kg IV) and butorphanol tartrate (Torbugesic; Zoetis, Parsippany, NJ; 0.01 mg/kg IV) and a

bronchoalveolar lavage catheter (BAL 240: 10 mm OD, 2.5 mm ID, 240 cm; Mila International Inc, Florence, KY) was passed through the nasopharynx, lodged blindly in a bronchus, and the cuff was inflated. By using syringes, 60 mL of 0.5% lidocaine was instilled, followed by 250 mL of sterile 0.9% sodium chloride solution (saline). The fluid was retrieved immediately by syringe aspiration and placed on ice until processing within 1 hour of collection. The BALF was pooled in a sterile graduated cylinder, and the amount of fluid recovered was recorded. A portion of the BALF sample was placed into an EDTA tube for cytological examination by a board-certified clinical pathologist, and 6 mL of BALF was frozen at -80°C for batch evaluation of antifungal activity via the agar disk diffusion method [16]. The remainder of the BALF was centrifuged at 2,106 rcf for 10 minutes to separate the supernatant and cell pellet for [VRC] analysis. Once samples were obtained, the supernatant was frozen as triplicate at -80°C and the cell pellet was resuspended in 1 mL of phosphate-buffered saline (PBS) to be frozen as triplicate at -80°C . For BALF analysis, cytopspins were prepared, and 400 cells were counted. The percentages of inflammatory cells (macrophages, small lymphocytes, neutrophils, eosinophils, and mast cells) were recorded [17,18].

2.3.3. Nasopharyngeal Wash

The nasopharyngeal wash was performed using a 14-French X 16-inch red rubber catheter (Rusch; Teleflex, Morrisville, NC) and 60 mL of sterile 0.9% saline. The catheter was introduced through one of the nostrils to the nasopharynx, and 60 mL of sterile saline was infused. A sterile cup was used to collect the fluid flowing from the nostrils. This fluid was kept on ice until stored (within 1 hour of the collection) in triplicate at -80°C until batch analysis.

2.4. High-performance Liquid Chromatography (HPLC) Assay

Plasma, BALF supernatant and cell pellet, and NPW samples were analyzed by reverse HPLC as previously described [10]. Briefly, the HPLC system (Waters–Alliance HPLC; Waters Corporation, MA) consisted of a separation module (Waters e2695), photodiode array detector (Waters 2998), column (Thermo BetaBasic-18, 4.6 mm \times 15 cm, 5 μm ; Bellefonte, PA), silica guard column (4 mm \times 1.25 cm), and computer for data collection and analysis (Gateway 2000, P4D-66, Irvine, CA). The mobile phase consisted of 35% 0.1 M N, N', N'-tetramethylethylenediamine (Fisher Scientific, Inc, Waltham, MA) and methanol (Fisher Scientific, Inc), at a flow rate of 1.0 mL/min at room temperature. The injection volume was 100 μL . Voriconazole and ketoconazole (the internal standard), were detected at a wavelength of 254 nm, and the retention times were 3.7 and 13.5 minutes, respectively. Calibration standards with voriconazole concentrations in plasma, cell pellets and supernatant from BALF, and NPW samples ranging from 0.02 to 10 μg were prepared. Plasma, BALF, and NPW samples collected at baseline prior to the administration of voriconazole were used as the blank controls. A dilution correction using urea was applied to any BALF supernatant samples with detectable VRC concentrations in order to estimate pulmonary epithelial lining fluid (PELF) concentrations according to previously published methods [10]; this correction was not applied to NPW samples as there are no published assessments using this technique in that fluid in horses.

2.5. Extraction

To 500 μL of each sample, 15 μL of the internal standard solution was pipetted and vortexed for 10 minutes. Voriconazole and internal standard were extracted with 4 mL ethyl acetate, as previously described [10]. The dried residue was reconstituted with the mobile phase and transferred to an HPLC vial and inserted for

analysis. Recoveries from plasma and fluids were $>100 \pm 6\%$ for drug and internal standard. Calibration standards with voriconazole concentrations in plasma, cell pellets and supernatant from BALF, and NPW samples ranging from 0.02 to 100 $\mu\text{g}/\text{mL}$ (g) were prepared. Calibration curves of peak area ratios (voriconazole/internal standard) versus concentration appeared linear with linear regression analysis yielding R^2 values > 0.99 . The lower limit of detection for voriconazole was 0.02 $\mu\text{g}/\text{mL}$, and the lower limit of quantification was 0.05 $\mu\text{g}/\text{mL}$. Concentrations below 0.05 $\mu\text{g}/\text{mL}$ were reported as below detected. Based on results of at least 5 standard curves [0.05–100 $\mu\text{g}/\text{mL}$ (g)] and duplicates, the intraday and interday variations were 1.96% and 5.01%, respectively. There appeared no evidence of drug sample carryover in the HPLC methods based on the inclusion of blank samples.

2.6. Antifungal Activity of VRC from BALF and NPW

Antifungal activity of VRC within the BALF and NPW samples was evaluated via the agar disk diffusion method, as described previously [19]. 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 four 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 McFarland 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) was OD530 nm, which equated 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 swabbed onto Mueller Hinton agar (MHA). Sterile paper discs were impregnated with 100 μL of the unseparated BALF and NPW samples and were placed in triplicate on MHA plates with a voriconazole control disk (1 μg , BD Sensi-Disk; Becton Dickinson and Co, Franklin Lakes, NJ). Plates were incubated at 35°C and examined at 24 and 48 hours. The diameter of the zone of inhibition around each disc was measured in millimeters. A diameter equal to or greater than 17 mm was considered the epidemiologic cutoff value for the detection of molds that were susceptible [16,20].

2.7. Study Protocol

2.7.1. Objective 1: Clinical Feasibility of VRC Nebulization and VRC Concentrations in BALF and Plasma

Clinical feasibility was assessed in 6 horses by examining the dose of VRC required to attain detectable concentrations in BALF and PELF, duration of nebulization, tolerance of nebulization, and cytological abnormalities in BALF after nebulization. The duration of nebulization and the residual fluid volume accumulated in the reservoir at the end of nebulization were recorded for all horses. A fast flow cup was used for delivery of VRC in all horses, except the first horse for whom the standard flow cup was used. Information regarding VRC dose, administration, and sample collection time points for each horse is summarized in Table 1. Two hundred milligram of VRC (32 mL at 6.25 mg/mL) was delivered by nebulization to 3 horses (horse 1, 5, 6). This dose was selected based on studies performed in humans using a 40 mg dose [9,12]. Additional VRC doses evaluated for plasma and BALF cell pellet and supernatant VRC concentrations included 500 mg (85 mL at 6.25 mg/mL, horse 2) and 100 mg (16 mL at 6.25 mg/mL, horse 3 and 4). All horses were monitored for coughing, head tossing or shaking, and pawing during nebulization.

Table 1
Voriconazole (VRC) concentrations in BALF and plasma and cytological analysis before and after nebulization in each horse tested.

VRC Dose (mg)	Horse ID	Duration of Neb (min)	Time-Point	Plasma [VRC] (µg/mL)	BALF [VRC]		Anti-Fungal Activity in BALF	NP in BALF (%)	Lymph in BALF (%)	MP in BALF (%)
					Cell Pellet (µg/g)	SNT (µg/mL)				
200 (STD CUP)	1	90	SCRN	—	BD	BD	—	1	32	68
			0	BD	—	—	—	—	—	—
			EON	BD	—	—	—	—	—	—
			1 hr PN	BD	BD	BD	—	0	45	55
			4 hr PN	BD	—	—	—	—	—	—
500	2	115	8 hr PN	BD	BD	BD	—	—	—	—
			SCRN	—	BD	BD	—	6	60	34
			0	BD	—	—	—	—	—	—
			EON	BD	0.40	BD	Neg	3	62	34
			2 hr PN	BD	—	—	—	—	—	—
100	3	30	6 hr PN	BD	BD	BD	—	—	—	—
			SCRN	—	BD	BD	—	3	44	53
			0	BD	—	—	—	—	—	—
			EON	BD	BD	BD	Neg	2	61	37
			1 hr PN	BD	—	—	—	—	—	—
100	4	18	6 hr PN	BD	—	—	—	—	—	—
			SCRN	—	BD	BD	—	9	60	31
			0	BD	—	—	—	—	—	—
			EON	BD	BD	BD	Neg	6	66	26
			1 hr PN	BD	—	—	—	—	—	—
200	5	60	6 hr PN	BD	—	—	—	—	—	—
			SCRN	—	BD	BD	—	^a	^a	^a
			0	BD	—	—	—	—	—	—
			EON	BD	BD	BD	Neg	8	62	30
			1 hr PN	BD	—	—	—	—	—	—
200	6	30	6 hr PN	BD	—	—	—	—	—	—
			SCRN	—	BD	BD	—	10	40	50
			0	BD	—	—	—	—	—	—
			EON	BD	BD	BD	Neg	5	41	54
			1 hr PN	BD	—	—	—	—	—	—
			6 hr PN	BD	—	—	—	—	—	

Abbreviations: BALF, bronchoalveolar lavage fluid; BD, below detection; EON, end of nebulization; ID, identifier; Lymph, lymphocyte; MP, macrophage; Neg, negative; neb, nebulization; NP, neutrophil; PN, postnebulization; SNT, supernatant.

All were nebulized with the fast cup except for horse 1, where indicated. The “—” indicates that samples were not collected or analyzed at that time point. Screening (SCRN) indicates initial BALF collected within 2 wk of giving drugs by nebulization. Time 0 is immediately before nebulization.

^a Low cellularity sample due to processing issue; cell population was normal but was not quantified.

Whole blood was collected for VRC plasma concentration analysis immediately prior to nebulization (time 0) in all horses, immediately following nebulization (n = 6), and at 1 hour (n = 5), 2 hours (n = 1), 4 hours (n = 1), 6 hours (n = 5) and 8 hours (n = 1) postnebulization. Samples of BALF were obtained immediately following nebulization (n = 5), and at 1 hour (n = 1), 6 hours (n = 1) and 8 hours (n = 1) post nebulization. All BALF samples were processed for the evaluation of supernatant and cell pellet VRC concentrations and were also analyzed for antifungal activity using the agar disk diffusion method. For each horse, a cytological examination was performed on the first BALF sample collected after nebulization and compared to samples collected within 2 weeks prior to drug delivery at the time of screening.

2.7.2. Objective 2: Concentrations of VRC in NPW Samples

Information regarding VRC dose and sample collection time points is summarized in Table 2. From the horses used in objective 1, an NPW sample was collected at the end of nebulization from horses 2–6, and at 1 and 6 hours post nebulization in horses 3–6. NPW samples were analyzed for VRC concentrations by HPLC.

An additional 5 horses were administered 200 mg (32 mL at 6.25 mg/mL of VRC solution using the fast cup. Plasma and NPW samples were obtained immediately prior to nebulization, at the end of nebulization, and at 1, 6, and 8 (NPW only) hours after nebulization. Plasma and NPW samples were analyzed for VRC concentrations by HPLC and NPW samples for antifungal activity using the agar disk diffusion method.

2.8. Data Analysis

Descriptive statistics were calculated where appropriate for measurements of VRC concentration in plasma, BALF cell pellet, BALF supernatant, and NPW. Continuous data were expressed as mean ± SD. For antifungal activity assessment, a positive inhibition was determined if the diameter of the zone of inhibition around each disk was equal to or greater than 17 mm. Data were evaluated for normality using the Shapiro–Wilk test. The percentages of neutrophils, lymphocytes, and macrophages in BALF samples were compared between samples collected before and after nebulization of VRC using a Wilcoxon matched-pairs signed-rank test. Concentrations of VRC in NPW samples from 5 horses after nebulization of 200 mg of VRC were compared across time points using a Friedman’s test with Dunn’s multiple comparison test. P < .05 was considered significant. Data were analyzed with a commercial software program (GraphPad Prism v. 6, La Jolla, CA).

3. Results

3.1. Particle Size

The particle size for voriconazole using the standard cup was not determined. The authors contacted the manufacturer after horse 1 was nebulized, and due to the difficulties nebulizing the medication with the standard cup, it was determined that the fast cup would be more appropriate to use with this medication. The

Table 2
Voriconazole (VRC) concentrations in the nasopharyngeal wash (NPW) and plasma after nebulization.

VRC Dose (mg)	Horse ID	Number of Horses (n)	Time Point	Plasma [VRC] ($\mu\text{g/mL}$)	NPW [VRC] ($\mu\text{g/mL}$)	Anti-Fungal Activity in NPW
500	2	1	0	BD	BD	—
			EON	BD	47.4	—
			2 hr PN	BD	—	—
			6 hr PN	BD	—	—
100	3	1	0	BD	BD	—
			EON	BD	19.9	—
			1 hr PN	BD	1.31	—
			6 hr PN	BD	0.66	—
100	4	1	0	BD	BD	—
			EON	BD	17.9	—
			1 hr PN	BD	0.51	—
			6 hr PN	BD	0.39	—
200	5	1	0	BD	BD	—
			EON	BD	5.77	—
			1 hr PN	BD	1.15	—
			6 hr PN	BD	BD	—
200	6	1	0	BD	BD	—
			EON	BD	16.5	—
			1 hr PN	BD	2.81	—
			6 hr PN	BD	2.23	—
200	a	5	0	^b BD	BD	Neg
			EON	^b BD	30.8 \pm 29	Pos
			1 hr PN	^b BD	1.02 \pm 0.84	—
			6 hr PN	^b BD	0.2 \pm 0.19	Neg
			8 hr PN	—	0.34 \pm 0.67	—

Abbreviations: BD, below detection; EON, end of nebulization; ID, identifier; Neg, negative; PN, postnebulization; Pos, positive.

The “—” indicates that samples were not collected or analyzed at that time point.

Time 0 is immediately before nebulization.

^a Additional group of 5 horses that only had NPW and plasma samples collected and analyzed.

^b Plasma values were BD in all 5 horses.

flow rate of the fast cup with saline was 2.5 mL/minute, and the volume median diameter 50 (VMD 50) was 10 microns, and thus, 50% of the particles measured below 10 microns and 50% were greater. When the same cup was used with 10 mg/kg Vfend, the flow rate was reduced to 0.4 mL/minute. In comparison, when used with 6.25 mg/mL of Vfend, the flow rate was 1.05 mL/min, and the VMD 50 was 6–7 microns, and thus, 50% of the particles were less than 6–7 microns, and 50% were > 7 microns. If the medication was nebulized too fast and at a high concentration, the mesh cup created large size particles. Thus, 6.25 mg/mL was considered to be the best concentration to use.

3.2. Clinical Feasibility of Vfend Nebulization and VRC Concentrations in BALF and Plasma

The results are summarized in Table 1. VRC concentrations were undetectable in all plasma and BALF supernatant samples for all doses in all horses; further details about the delivery and clinical feasibility are expanded below. Since VRC concentrations were below detection in BALF supernatant, urea dilution to calculate PELF concentrations was not performed. All horses tolerated the nebulization of VRC well (no coughing, snorting, head tossing, or flehmen responses), but became restless with long-duration nebulization of some doses.

In horse 1, the duration of nebulization was 90 minutes, and voriconazole concentrations in plasma, BALF supernatant, and cell pellet were below detection at all time points (Table 1). Based on the duration of nebulization, the makers of the Flexineb provided fast cups to improve the speed of nebulization, and the other doses of Vfend were tested using this type of cup.

Due to the lack of detectable concentrations of VRC in BALF after 200 mg, the dose was increased to 500 mg, but the duration of nebulization was again very long at 115 minutes. VRC

concentrations in plasma were below the level of detection in all time points. Voriconazole concentrations obtained from the BALF cell pellet were detectable (0.4 $\mu\text{g/g}$) only at the end of nebulization (Table 1), but below the MIC for *Aspergillus* spp., and concentrations in the BALF supernatant at all time points were below detection (Table 1). There was no anti-fungal activity in the unseparated BALF fluid sample. After delivery of 500 mg, there was 20 mL of waste liquid in the aerosol chamber with a VRC concentration of 712.74 $\mu\text{g/mL}$, showing that 485 mg of the dose was delivered with 14.25 mg left in the chamber. For all other doses and horses, the fluid left in the chamber was very low (<500 μL), so VRC was not quantified in the residual.

For horses 3–6, the duration of nebulization was faster, but again, concentrations of VRC in plasma, BALF cell pellet, and supernatant were below detection (Table 1). There was no anti-fungal activity in unseparated BALF samples.

Bronchoalveolar lavage fluid samples collected after nebulization of all VRC dosages showed no cytological changes consistent with inflammation, and there were no significant differences in the percentage of neutrophils, lymphocytes, and macrophages between samples collected at screening sampling (within 2 weeks of nebulization) and samples collected after nebulization (Table 1). Eosinophils and mast cells were considered very rare in all samples, so they were not quantified.

3.3. Voriconazole Concentrations After Nebulization in NPW

VRC concentrations were detectable in NPW samples at multiple time points and with multiple doses but were below 0.5 $\mu\text{g/mL}$ by 6 hours in most horses. Horse 2 (nebulized with 500 mg) had VRC concentrations in the NPW at the end of the nebulization of 47.4 $\mu\text{g/mL}$. Due to those very high concentrations and the long duration of nebulization (115 minutes), a lower dose of 100 mg was tested in

horses 3 and 4, and the VRC concentrations in NPW samples were far above the MIC for *Aspergillus* spp. immediately postnebulization, but concentrations dropped considerably by 1 and 6 hours postnebulization (Table 2). In horses 5 and 6 (nebulized with 200 mg), the VRC concentrations in NPW samples were above the MIC for *Aspergillus* spp. immediately and at 1 hour postnebulization. At 6 hours, concentrations were below detection in horse 5, but right at 0.5 µg/mL in horse 6 (Table 2), so an additional 5 horses were nebulized with 200 mg VRC to evaluate NPW concentrations up to 8 hours (Table 2, Fig. 1). In these 5 horses, the duration of nebulization was 38 ± 8 minutes. Plasma voriconazole concentrations were below detection at all time points. Mean VRC concentrations obtained from NPW samples immediately postnebulization were significantly greater ($P = .0074$) than at 8 hours, but other time points were not different (Fig. 1). Concentrations were below 0.5 µg/mL in most horses after 6 hours (Fig. 1) and did not show antifungal activity.

4. Discussion

Nebulization of sterile VRC solution (Vfend) using a common, commercial nebulizer (Flexineb) did not achieve adequate concentrations of VRC in the lower airways at the doses tested (100–500 mg of VRC), so delivery of Vfend by this method is unlikely to be clinically useful for the treatment of pneumomycosis. This is important information for equine practitioners that may encounter fungal pneumonia cases and have access to a Flexineb nebulizer in their practice. Detectable concentrations of VRC were found in the upper airway using this delivery mechanism, and further study is needed to determine the dosage interval for clinical applicability in upper respiratory fungal infections.

The initial dose tested of 200 mg was based on human [9] and murine [12] studies, but did not result in detectable BALF drug concentrations using this nebulizer. Increasing the dose to 500 mg and using the fastest delivery cup available for this nebulizer still did not achieve adequate concentrations in BALF, and the duration of nebulization (almost 2 hours) was not clinically practical. The clinical significance of very low concentrations was reinforced in this study by the lack of antifungal activity from BALF samples evaluated via the agar disk diffusion method.

The low BALF concentrations could have been impacted by multiple factors. Vfend solution for injection was not easy to nebulize and required 18–115 minutes (depending on the amount

given and cup used) for delivery. The difficulty in nebulizing was likely due to the physical characteristics of the medication, such as low water solubility and high viscosity. Vfend contains sulfobutyl ether-β-cyclodextrin, an excipient that should improve the water solubility of the medication, but that did not improve the ability to nebulize this preparation. High viscosity drugs, or those with large particle size, may not nebulize well as they may clog the small holes of the membrane within the medication cup. For this reason, after the first horse was nebulized with a standard cup and analysis was performed by the nebulizer manufacturer, the fast cup was used with the rest of the horses. The clogging also explains why the high dose of 500 mg had so much residual volume left in the cup and explains some of the variability in the duration of nebulization despite the same volume of drug administration. Strict cleaning of the cup according to manufacturer's recommendations or replacement of the cups frequently would be required for clinical use of these cups with Vfend since more difficult to nebulize medications can decrease the lifespan of the cups.

In addition, the VMD 50 for this nebulizer and using the fast cup showed a particle size that may have been too large for a therapeutic amount of drug to reach the alveoli and be subsequently absorbed. A previous report in horses [21] determined that the particle size needed to reach the alveoli for absorption should measure between 1–5 µm. The particle size obtained in this study had a VMD 50 of 6–7 µm, meaning that 50% of the particles were that size (6–7 µm) or greater, and 50% of the particles were that size or smaller; thus, the amount of medication that reached the alveoli was likely lower than ideal.

Further, as the dose elected was determined based on similar murine [12] and human [9] studies using nebulized Vfend solution and previous equine studies that used orally administered VRC [10,22], it is possible that the dose was lower than the dose needed to detect VRC in the BALF. The pharmacokinetics of the drug could have also contributed to the low VRC concentrations in plasma and BALF samples. Based on a human study [23], this drug shows rapid absorption and nonlinear pharmacokinetics due to its capacity-limited elimination, and thus, it is considered a dose-dependent medication. The volume of distribution is high (2–4.6 L/kg), thus indicating an extensive distribution into extracellular and intracellular compartments. Lung biopsies with measurement of drug in the tissues would be required to investigate this mechanism but do not seem warranted based on not even finding detectable drug immediately after nebulization in the BALF supernatant or cell pellet of most doses.

When VRC is administered orally in people, a loading dose is recommended to reach plasma concentrations close to a steady-state within the first 24 hours of dosing. Without the loading dose, the accumulation occurs after multiple-dosing with steady-state plasma VRC concentrations being achieved by day 6 [8]. In a murine study [12,24], a high concentration of VRC was achieved following multiple inhaled doses of VRC solution. In the present study, the horses were nebulized only once, and the concentrations of VRC were undetectable in the BALF supernatant and plasma, which may indicate that multiple doses per day may be necessary to obtain greater VRC concentrations in the BALF and plasma. Costs of drugs change frequently and should not preclude investigation, but at current rates, 200 mg of Vfend is \$60–\$80 USD, so greater and more frequent doses may not be cost-effective for many clinical cases in some parts of the world for the duration of treatment that is typically needed for pneumomycosis.

After determining that Vfend delivery by this method would not be feasible for the treatment of fungal pneumonia, we investigated VRC deposition in the upper airways. VRC concentrations in NPW samples immediately after nebulization of 100 mg, 200 mg, and 500 mg of VRC were well over the MIC for *Aspergillus* spp. (≥ 0.5 µg/

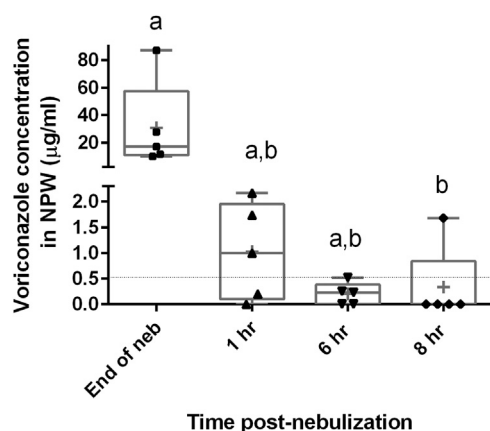


Fig. 1. Voriconazole concentrations in nasopharyngeal wash samples (NPW) after nebulization of 200 mg VRC in 5 horses. Boxes represent the interquartile range, the whiskers are the minimum and maximum, the mean is a + sign, and the symbols are individual values. The dashed line represents the MIC for *Aspergillus* spp. at 0.5 µg/mL. Different letters are significantly different at $P < .05$.

mL), but generally decreased to below the MIC by 6 hours. Fungal infections of the URT, such as fungal rhinitis and guttural pouch mycosis (which is commonly caused by *Aspergillus* spp.) are uncommon but occur more frequently than pneumomycosis, and treatment remains challenging [25,26]. Nebulized VRC may introduce a new treatment option for fungal diseases affecting the URT that could have the benefits of reaching a greater concentration at the site of infection and decreasing the incidence of side effects associated with systemic therapy [3,27]. Results from the current study could provide a framework to further investigate the appropriate dose and frequency of VRC nebulization for the treatment of URT fungal infections. More research is needed to make clinical dosing recommendations, such as measuring the VRC concentrations reached in the paranasal sinuses and guttural pouches and determination of an appropriate dose and dose interval since samples were not collected between 1- and 6 hours postnebulization. It may be that a lower dose can be given more frequently. Also, as mentioned previously, it is possible that multiple daily dosing via nebulization may yield greater concentrations in the upper airways. The same cost limitations, as described earlier, could apply in some cases.

A central component of the primary objective of this study was to determine whether concentrations of VRC in PELF achieved MIC levels for *Aspergillus* sp. The PELF is a thin layer of fluid that covers the epithelium of the small airways and alveoli, and drug concentrations within the PELF are thought to best predict antimicrobial activity against extracellular pathogens associated with pneumonia [28,29]. When a BAL is performed, the PELF becomes diluted within the BALF, and thus, a dilution correction must be performed to calculate the actual PELF drug concentration. Urea is most often used as an endogenous marker for this dilution correction, where it is assumed that the urea concentration within the PELF approximates urea concentration within the plasma [28,30,31]. It is this relationship that allows for the calculation of the volume of PELF within the BALF volume. Once the PELF volume is determined, the drug concentration within the PELF can be derived from the measured drug concentration within the BALF. Thus the determination of PELF drug concentration relies on achieving detectable drug concentrations within BALF. This method has been described in several species for a variety of antimicrobials, and although not without limitations [32,33], it is considered the method of choice for determining PELF drug concentration. In this study, because the concentrations of VRC were below the limit of detection within the BALF for all horses, urea dilution was not performed. Similar dilution methods have not been validated or described for fluids obtained from upper airway washes in horses, and thus, although drug concentrations were detected within the NPW samples, we did not perform urea dilution. For the BAL procedures and for the NPW, a similar volume of fluid was obtained from each horse after the wash, which should reduce the variability in the lining fluid dilution between samples.

This study has limitations that need consideration. The results obtained in this study can only be applied to the Flexineb, and other nebulizers such as those used in human studies (e.g., jet nebulizers) could be more effective at delivering this particular medication; however, this would require a further study that was beyond the scope of this project. The small group of horses included in the study and the lack of correction for dilution of the NPW could have affected the variable drug concentrations obtained in the NPW fluid.

5. Conclusions

The concentrations of VRC after nebulization of Vfend solution with a Flexineb nebulizer in plasma and BALF samples were

clinically insignificant at all time points, so delivery of Vfend by this method is not recommended for treatment of fungal pneumonia in horses. The concentrations and antifungal activity in NPW samples suggest that nebulization of Vfend solution may be more beneficial when treating upper respiratory tract fungal infections; however, further investigation into dose and dosing frequency is needed before this can be recommended.

Acknowledgments

The authors would like to thank Jessica Brown and Qiao Zhong (from AU), as well as Matt Walsh, Gavan O'Sullivan, and Clint Joiner (from Nortev Ltd/Flexineb) for technical assistance.

Financial Disclosure

This study was performed with the financial support of the Veterinary Comparative Respiratory Society (VCRS) and the Auburn University Department of Clinical Sciences. Material support (nebulizer unit) and particle size analysis was provided by Nortev Ltd/Flexineb.

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