Effect of Clenbuterol on Tracheal Mucociliary Transport in Horses Undergoing Simulated Long-Distance Transportation


Background: Pneumonia is observed in horses after long-distance transportation in association with confinement of head position leading to reduction in tracheal mucociliary clearance rate (TMCR).

Hypothesis/Objectives: Clenbuterol, a beta-2 agonist shown to increase TMCR in the horse, will ameliorate the effects of a fixed elevated head position on large airway contamination and inflammation in a model of long-distance transportation model.

Animals: Six adult horses.

Methods: A cross-over designed prospective study. Horses were maintained with a fixed elevated head position for 48 hours to simulate long-distance transport, and treated with clenbuterol (0.8 µg/kg PO q12h) or a placebo starting 12 hours before simulated transportation. TMCR was measured using a charcoal clearance technique. Data were collected at baseline and 48 hours, and included TMCR, tracheal wash cytology and quantitative culture, rectal temperature, CBC, fibrinogen, and serum TNFα, IL-10, and IL-2 levels. There was an 18–21 day washout between study arms, and data were analyzed using regression analysis and Wilcoxon rank-sum tests.

Results: Tracheal mucociliary clearance rate was significantly decreased after transportation in both treatment (P = .002) and placebo (P = .03) groups. There was a significant effect of treatment on TMCR, with the treatment group showing half the reduction in TMCR compared with the placebo group (P = .002). Other significant differences between before- and after-transportation samples occurred for serum fibrinogen, peripheral eosinophil count, quantitative culture, tracheal bacteria, and degenerate neutrophils, though no treatment effect was found.

Conclusions and Clinical Importance: Treatment with clenbuterol modestly attenuates the deleterious effects of this long-distance transportation model on tracheal mucociliary clearance.

Key words: Beta-2 agonist; Pneumonia; Transport; Transportation fever.

Pneumonia is a frequently life-threatening complication of long-distance transportation in the horse, where case fatality is as high as 55.8% and fewer than 26% of surviving horses return to racing. Long-distance transport of horses commonly involves the horses traveling with their head in a fixed upward position in a poorly ventilated environment that results in airway inflammation and often subsequent bacterial pneumonia. Even without transportation, confining horses with elevated head position leads to an increased number of bacteria and inflammatory secretions in tracheal fluid. Clenbuterol, a beta-2 agonist, increases the mucociliary clearance in a variety of species including the horse and could potentially aid in the clearance of particulate matter and infectious organisms from the airway of the transported horse.

The effect of the beta-2 agonist, clenbuterol, on the mucociliary clearance rates of horses during long-distance transportation, either real or simulated remains unclear. We hypothesized that the positive effect of clenbuterol on TMCR will negate the deleterious effects of the fixed elevated head position on the contamination of the proximal airway with organic and microbial debris.

Materials and Methods

Six adult horses from the university research herd were used for this randomized, placebo-controlled, blinded, cross-over design clinical trial. Horses were Thoroughbreds between the age of 7 and 14 years and weighed 450–500 kg. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. The long-distance transportation model used (horses remaining cross-tied for 48 hours) has been previously evaluated and validated in several studies, and has been shown to induce physiologic and clinico-pathologic changes similar to horses undergoing long-distance transport.

Each of the 6 horses was brought in from pasture at least 1 day before start of the trial and acclimated to the stall and cross-ties, and randomized using paper lottery to a control (n = 3) or experimental (n = 3) group. On the morning of day 1 (time = 0), horses remained cross-tied in their stalls so that their muzzles could drop no lower than the point of the elbow. They were allowed free-choice

Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ETA</td>
<td>endoscopically acquired tracheal aspirate</td>
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<td>TMCR</td>
<td>tracheal mucociliary clearance rate</td>
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Submitted May 9, 2012; Revised May 8, 2013; Accepted July 17, 2013.

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10.1111/jvim.12166
hay from a net, and were offered water every 6 hours. The horses were supervised directly throughout the study period, and rectal temperatures were obtained every 6 hours. The stalls were bedded with straw, and the barn doors remained open during the study. After 48 hours, horses were restested as described above. The ETA was obtained approximately 1–2 hours after the last administration of clenbuterol or placebo, and the TCMR obtained exactly 1 hour after the ETA in each horse; horses remained cross-tied until the TMC was completed. After an 18–21 day washout period, the study was repeated in a cross-over fashion. All investigators were blinded to group allocation until conclusion of the study.

Blood was obtained on all horses before more invasive sampling, and was submitted for a complete blood count and serum fibrinogen analysis by the hospital clinical laboratory. Serum was frozen at −80°C for subsequent cytokine analysis. The ETA was obtained using a 1-m endoscope sterilized in a glutaraldehyde disinfectant according to the manufacturer’s instructions and the exterior and biopsy channel were thoroughly rinsed with sterile saline. Each horse was restrained chemically with xylazine (200 mg IV) administered approximately 5 minutes before the procedure. The endoscope was passed into the trachea to a distance of 80 cm from the external naris, and a triple-guarded catheter advanced through the endoscope instrument portal for an additional 5 cm. Sterile saline (10 mL) was injected through the catheter, and then aspirated into a sterile syringe. The sample was divided into 2 aliquots, which were immediately submitted for cytologic analysis and semiquantitative culture.

One hour after the ETA, the TMC was measured. The horses were resedated with xylazine (150 mg IV), and the endoscope was again passed into the trachea to a distance of exactly 80 cm from the caudal aspect of the alar fold of the naris as measured by the external markings of the endoscope. A length of polyethylene tubing preloaded with a small volume of powdered charcoal suspension was advanced through the endoscope portal, and a small drop of charcoal suspension deposited on the ventrum of the trachea, as described by Sweeney et al. A stopwatch was then activated, and after 10 minutes the endoscope was reintroduced into the trachea, and distance of travel of the leading edge of the charcoal determined using the endoscope markings. The TMC was thus calculated by dividing the distance of travel by exact time of transit from the stopwatch.

Semiquantitative cultures of the ETA samples were performed using standard techniques. Briefly, a 0.01 mL calibrated loop was placed into the specimen, and streaked on an agar plate in a grid pattern. After 24 hours of incubation, the colonies were counted, and the number multiplied by 100 to obtain the number of colonies per milliliter.

All ETA samples were prepared as either sediment or direct preparations depending on a subjective assessment of cellularity, with four slides made per case. Twenty-two samples were sediment preparations and 1 sample was made using direct preparation. For each aspirate, 3 slides were stained with Wright–Giemsa and 1 slide was stained with Prussian blue. All slides were evaluated by a single clinical pathologist (KJ).

Wright–Giemsa stained slides were scanned at 4x and 10x and a subjective quantification (0–3+) of all of the following were made: overall cellularity; respiratory epithelial cells; goblet cells; inflammatory cells; squamous epithelial cells; mucus; presence of any foreign material; and presence of bacteria. The quantification of overall cellularity was subjective and was performed by scanning all 3 of the Modified Wright–Giemsa slides at 4x and 10x and making an overall judgment of nucleated cellularity with 0 representing no nucleated cells, 1+ representing less than 50% of nucleated cells, 2+ representing 50% of the nucleated cells as degenerate, and 3+ representing greater than 50% of the neutrophils as degenerate. The field with the most foreign material, which typically corresponded to the most mucin laden area, was chosen on at least 2 slides per aspirate. At 50x the number of pieces of plant material, pollen, Alternaria spp., yeast, and saprophytic fungal hyphae were averaged over 10 fields, and reported as a whole number. Bacteria when present were subjectively quantified and their location (eg, adherent to squamous epithelial cells, extracellular or intracellular) and morphology (eg, cocci, bacilli, pairs, chains) were noted. Lastly, the Prussian blue–stained slide was evaluated for the presence of hemosiderin and a subjective quantification (0–3+) was made based on the number of hemosiderin-laden macrophages.

The concentrations of equine TNF-α, IL-10, and IL-2 were measured in serum samples by sandwich ELISA using manufacturer’s instructions. Briefly, capture antibody was coated onto 96-well ELISA plate overnight at room temperature followed by intensive wash to remove unbound protein, 50–100 μL serum or ETA and 7 points of standard proteins were applied to the plate, the plate was incubated 2 hours at room temperature to ensure efficient binding of antigens to the coating antibody, followed by intensive wash to remove unbound proteins. A biotinylated detection antibody was applied to bind the antigens in the plate and followed by 2-hour incubation and intensive wash to remove unbound antibody, and finally the cytokines were measured by applying streptavidin-conjugated horseradish peroxidase and detection substrate reagents (H2O2 and tetramethylbenzidine) in the microplate reader (BioRad 3550 Microplate Reader). The concentrations were automatically calculated based on a four-parameter logistic (4-PL) of the standard curve by the commercial software. The coefficient ($R^2$) was 0.998 for TNF-alpha and 1.000 for the other 2 cytokines.

### Statistical Analysis

Data were analyzed using clustered regression clustered on horse, admitting precondition (before versus after intervention) and treatment (placebo versus clenbuterol) and the interaction of these factors. In addition, differences between before and after samples by treatment were evaluated using the Wilcoxon signed-rank test. All data were analyzed using commercial software (BioRad Microplate Manager Software 6.0) with significance set at $P \leq .05$.

### Results

Horses tolerated the simulated transportation scenario well and all sample collections were successful. Tracheal mucociliary clearance rate was ascertained in each attempt without complication and there was adequate yield on tracheal aspirates to perform all testing.
No adverse reactions were seen with clenbuterol or placebo administration.

Mean after-transportation TMCR values were 1.55 ± 0.82 cm/min and 1.71 ± 0.64 cm/min for placebo and clenbuterol groups, respectively (Table 1). In both the treated and untreated groups, the TMCR was lower after transportation than the baseline (before) measurement. The group receiving clenbuterol had 50% less of a reduction in TMCR (mean = 0.14 cm/min) than the placebo group (mean = −0.28 cm/min), and a significant interaction of treatment group and condition was identified (P = .002), indicating that treatment with clenbuterol increased the difference between before- and after-treatment TMCR. There was no significant effect detected for treatments (P = 0), but there were differences before and after head elevation for plasma fibrinogen concentration (P = .027), bacterial counts from the ETA semiquantitative culture (P = .045), and number of degenerate neutrophils (P = .05) and nondegenerate neutrophils (P = .046) on the ETA. The overall cellularity of the tracheal cytology showed a significant interaction of treatment group and condition (P = .019), with horses receiving placebo treatment having more cellular ETA samples than those receiving clenbuterol. When hematology (Table 1) and serum inflammatory markers (Table 2) were analyzed, there was a significant difference seen only between before- and after-transportation measurements of eosinophils in the placebo group (P = .03) and the clenbuterol group (P = .046). There was no effect of treatment on this parameter.

Two horses did display clinical signs associated with inflammation and respiratory disease. One horse in both legs of the study became dull, febrile (maximum temperature −39.3°C), and developed a cough and purulent nasal discharge. This horse was broken from the study (2nd set of data collected earlier than the planned 48-hour mark) 4 and 3 hours early in each leg. These data were still included in the analysis. The 2nd horse became dull and febrile (maximum temperature −39.3°C) in the placebo leg of the study, but was neither tested early nor removed from the study. Neither of these cases had obviously different values for any of the parameters, including inflammatory cytokines, serum fibrinogen, or peripheral white blood cell count, when compared with the group.

Discussion

Treatment with oral clenbuterol improved tracheal mucociliary clearance in the face of simulated long-distance transportation. We hypothesized that treatment with clenbuterol would not only increase mucociliary clearance but that this increased movement of debris cranially up the trachea would lead to reduced contamination of the airway as evidenced by lower quantitative cultures of bacteria and lower concentrations of inflammatory cytokines. Both placebo and treatment groups showed a decrease in the mean TMCR after simulated transportation and clenbuterol attenuated this reduction in transport time compared with placebo. Treatment only modestly decreased the overall cellularity of the airway contamination. Bacterial and fungal counts were increased after transportation in both groups and treatment did not attenuate these changes. The increase in concentration of fibrinogen and eosinophils between before- and after-transportation samples were the only significant differences seen in the blood analyses performed. These changes were seen in both the treatment and placebo group, but with no effect of treatment on the parameters, and these changes are not considered clinically significant. The increased peripheral eosinophil count may reflect an increase in allergic inflammation because of excessive debris in the airways after transportation that could not be counteracted by the effects of clenbuterol. In addition, it could represent mucosal inflammation translating into peripheral eosinophilia. Humans with mucosal airway inflammation have been shown to have increased serum levels of eosinophilic markers, although interleukin-4 and interleukin-5 were not increased peripherally with horses with eosinophilic bronchial alveolar lavage samples.

There are several limitations of this study. The timing of the after-transportation samples may have not been ideal to reflect certain acute inflammatory changes. Serum fibrinogen concentrations did increase significantly between before- and after-transportation samples in both groups. Although all values measured were within the reference range and not clinically significant, it does represent an increase in acute phase proteins stimulated by our transportation simulation and had samples been taken further out after transportation, a clinically significant difference may have been detected. Leukopenia seen with acute toxic events may have been missed in the initial 24–48 hour period and whereas fibrinogen is an acute phase protein, this index may have peaked after the last sampling time. Sample
size may have adversely affected the statistic significance of the results such as the overall cellularity, which showed a significant difference that was overshadowed by wide standard deviations; alternatively, the trends and minor associations that were seen, but not found to be statistically significant, may have been statistically significant if there were greater numbers in each group. Although xylazine decreases TMCR, this sedative was used in all horses at all TMCR sampling times, and the effects would not have altered our comparison of before- and after-transportation parameters.

Despite the ubiquity of transport-associated pneumonia in the equine performance equine population, relatively little is known about the pathogenesis of this disease, nor, more importantly, how to prevent it. It has been shown that a fixed elevated head position leads to increased contamination in the "tracheal toilet", and that this deleterious effect is not attenuated by prophylactic antimicrobials. One explanation for accumulation of debris and bacteria in the trachea is decreased mucociliary function. Again, Raidal et al proved that restricting a horse's head position, mimicking that of transportation, led to a significant decrease in mucociliary transport rate. Decreased mucociliary clearance has been shown to result in pneumonia in humans and dogs with both primary and acquired ciliary dyskinesia. In both the dog

### Table 1. Mean ± SD hematology results for before- and after-transportation simulation under the effects of clenbuterol or a placebo.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo Before</th>
<th>Placebo After</th>
<th>Treatment Before</th>
<th>Treatment After</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Blood Cells (×103/μL)</td>
<td>6.84 (±1.24)</td>
<td>8.41 (±1.35)</td>
<td>6.22 (±0.15)</td>
<td>7.64 (±2.00)</td>
</tr>
<tr>
<td>Neutrophils (×103/μL)</td>
<td>4.45 (±1.21)</td>
<td>6.36 (±1.61)</td>
<td>3.91 (±0.68)</td>
<td>5.47 (±2.05)</td>
</tr>
<tr>
<td>Lymphocytes (×103/μL)</td>
<td>1.83 (±0.48)</td>
<td>1.69 (±0.49)</td>
<td>1.76 (±0.49)</td>
<td>1.66 (±0.52)</td>
</tr>
<tr>
<td>Eosinophils (×103/μL)</td>
<td>0.27 (±0.19)</td>
<td>0.07 (±0.11)</td>
<td>0.17 (±0.12)</td>
<td>0.33 (±0.34)</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
<td>290 (±28.35)</td>
<td>377 (±96.20)</td>
<td>316 (±65.60)</td>
<td>329 (±42.04)</td>
</tr>
</tbody>
</table>

*Within a row, values differ significantly (P < .05).

### Table 2. Mean ± SD serum inflammatory marker concentrations for before- and after-transportation simulation under the effects of clenbuterol or a placebo.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo Before</th>
<th>Placebo After</th>
<th>Treatment Before</th>
<th>Treatment After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Protein (mg/mL)</td>
<td>34.15 (±22.47)</td>
<td>28.71 (±11.68)</td>
<td>25.17 (±18.51)</td>
<td>43.53 (±19.04)</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>2,931.11 (±3,191.40)</td>
<td>2,727.47 (±3,196.74)</td>
<td>2,796.13 (±3,246.69)</td>
<td>3,001.45 (±3,289.87)</td>
</tr>
<tr>
<td>IL-2 (pg/mL)</td>
<td>195.00 (±426.04)</td>
<td>110.00 (±224.05)</td>
<td>35.03 (±48.74)</td>
<td>122.33 (±264.17)</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>1,340.33 (±1,920.30)</td>
<td>1,425.33 (±2,085.13)</td>
<td>1,944.33 (±2,282.61)</td>
<td>1,947.67 (±2,903.87)</td>
</tr>
</tbody>
</table>

*Within a row, values differ significantly (P < .05).

### Table 3. Tracheal aspirate mean ± SD quantitative cytologic parameters for before- and after-transportation simulation under the effects of clenbuterol or a placebo.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo Before</th>
<th>Placebo After</th>
<th>Treatment Before</th>
<th>Treatment After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitative culture (cfu)</td>
<td>20,000 (±16,958)</td>
<td>98,000 (±41,856)</td>
<td>20,166 (±30,622)</td>
<td>105,000 (±42,680)</td>
</tr>
<tr>
<td>Overall cellularity</td>
<td>2.83 (±0.75)</td>
<td>3.83 (±0.41)</td>
<td>3.17 (±0.75)</td>
<td>3.5 (±0.55)</td>
</tr>
<tr>
<td>Inflammatory cell score</td>
<td>2.67 (±0.82)</td>
<td>3.83 (±0.41)</td>
<td>2.67 (±0.52)</td>
<td>3.33 (±0.52)</td>
</tr>
<tr>
<td>Goblet cells (/PHF)</td>
<td>0.50 (±0.55)</td>
<td>0.33 (±0.52)</td>
<td>1.00 (±0.63)</td>
<td>0.33 (±0.52)</td>
</tr>
<tr>
<td>Neutrophil degeneration score</td>
<td>0.00 (±0.00)</td>
<td>2.33 (±1.37)</td>
<td>1.50 (±1.22)</td>
<td>2.50 (±1.52)</td>
</tr>
<tr>
<td>Fungi (/HPF)</td>
<td>0.50 (±0.55)</td>
<td>0.50 (±0.55)</td>
<td>0.33 (±0.52)</td>
<td>1.00 (±0.63)</td>
</tr>
<tr>
<td>Bacteria (/HPF)</td>
<td>0.5 (±0.84)</td>
<td>3.17 (±0.98)</td>
<td>1.33 (±0.82)</td>
<td>3.33 (±1.63)</td>
</tr>
<tr>
<td>Nondegenerate neutrophils (%)</td>
<td>50.17 (±31.44)</td>
<td>27.5 (±43.33)</td>
<td>58.67 (±24.50)</td>
<td>20.33 (±38.14)</td>
</tr>
<tr>
<td>Degenerate neutrophils (%)</td>
<td>0.00 (±0.00)</td>
<td>63.00 (±48.13)</td>
<td>0.00 (±0.00)</td>
<td>57.16 (±45.67)</td>
</tr>
</tbody>
</table>

*Within a row, values differ significantly (P < .05).
and the unrestrained horse, administration of \( \beta_2 \)
adrenergic drugs, such as clenbuterol, leads to increased mucociliary clearance.\(^5,6\)

The cytokines TNF\(\alpha\), IL-2 and IL-10 were chosen as the inflammatory and anti-inflammatory markers investigated in this study. TNF\(\alpha\) stimulates acute phase reactions in systemic inflammation, and is an endogenous pyrogen that induces the production of the inflammatory cytokine IL-1. Changes in TNF\(\alpha\) should reflect the initiation of inflammation in the system. IL-2 is a signaling cytokine that functions to attract lymphocytes to sites of microbial infection and was expected to be present in tracheal secretions when bacterial contamination and high concentrations of inflammatory cells are found. IL-10 is an anti-inflammatory cytokine produced by monocytes and lymphocytes. It counters excessive responses of the immune system by down-regulating the expression of Th1 cytokines, MHC class II antigens, and macrophage-stimulating hormones. Despite clinical evidence of a systemic inflammatory response (fever, nasal discharge, and cough) in some cases, there were no consistent changes seen with any of these cytokines in the systemic circulation.

In conclusion, treatment with the oral \( \beta \)-adrenergic agonist clenbuterol prior to simulated long-distance transportation does improve the mucociliary transport rate of the equine trachea, but does not completely ameliorate the negative effects seen with transportation. Additional investigation should be performed on the effect of clenbuterol on the TMCR in horses with known respiratory compromise, such as recurrent airway obstruction and recent viral respiratory tract infection, as well as the combination of management changes and pharmacologic interventions in the prevention of transport-associated pleuropneumonia.

### Footnotes

\(^a\) Aeropulmin Syrup; Phoenix Pharmaceuticals, St Joseph, MO  
\(^b\) Cidex Plus; Advanced Sterilization Products, Irvine, CA  
\(^c\) 0.9% preservative-free saline; Hospira Inc, Lake Forent, IL  
\(^d\) AnaSed; Akorn Inc, Decatur, IL  
\(^e\) Triple Stage Catheter 190 cm (EMAC800); Mila International Inc, Ehlanger, KY  
\(^f\) Toxiban Activated Charcoal Suspension; Lloyd Inc, Shenandoah, IA  
\(^g\) DuoSet ELISA; Research & Diagnostics Systems Inc, Minneap-
ol, MN  
\(^h\) GSI Equine ELISA; Genorise Scientific, Paoli, PA  
\(^1\) BioRad, Hercules, CA  
\(^1\) Stata 11.0; StataCorp, College Station, TX  

### Acknowledgments

The authors thank Dr Lawrence Soma for his contributions in the design and funding of this study, and Sue Lindborg, Marcy Ippolito, and Ann Hess for all their excellent technical assistance in performing.

**Conflict of interest:** Authors disclose no conflict of interest.

**Support:** Supported by the McCabe Fund (2008) and the Equine Research Endowment Grant, University of Pennsylvania (2009).

### References