EXERCISE-INDUCED PUMONARY HEMORRHAGE: DETERMINATION OF MECHANISMS AND POTENTIAL TREATMENTS

by

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B.S., KANSAS STATE UNIVERSITY, 1993
M.S., KANSAS STATE UNIVERSITY, 1997
D.V.M., KANSAS STATE UNIVERSITY, 1998

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology
College of Veterinary Medicine

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Abstract

Exercise-induced pulmonary hemorrhage (EIPH) or epistaxis has been recognized in racehorses since the 16th century. Since this time, great strides have been made in terms of identifying the lungs as the source of the hemorrhage via the endoscope, utilization of bronchoalveolar lavage to quantify the hemorrhage, and the discovery of successful treatments such as furosemide and the nasal strip that ameliorate, but do not abolish EIPH. It has been determined that, in addition to extremely high pulmonary arterial pressures and the negative intrapleural pressures being the major physiologic forces causing pulmonary capillary stress failure, other factors have the potential for influencing the severity of EIPH including locomotory impact trauma, inflammatory airway disease (IAD), upper airway obstruction, coagulation anomalies, and high blood viscosity. It has been hypothesized that EIPH is detrimental to performance and this was recently confirmed by Hinchcliff *et al.* in 2004.

EIPH is a complex multi-factorial condition with much still unknown about the etiology, best method for diagnosis, and most effective form of treatment. Chapter one of this dissertation determined the effectiveness of a novel treatment, concentrated equine serum, in ameliorating EIPH via reduction of IAD. Chapter two refuted the hypothesis that herbal formulations commonly used in the field with anecdotal success would decrease EIPH by correcting coagulation deficits during exercise, as scientific efficacy was not evident, at least at the dose and duration used in our investigation. Chapter three addressed the dogma that EIPH only occurs during maximal intensity exercise, and in

demonstrating significant EIPH during sub-maximal exercise, emphasized the role that the airways play in contributing to the initiation and severity of EIPH. Chapter four examined the occurrence and severity of EIPH in the horse's canine counterpart, the racing Greyhound. The demonstrated presence of mild EIPH in the Greyhound, a physiologically similar yet different athlete in comparison to the horse sheds new light on the etiology of this condition in both species.

The results of these investigations have advanced the frontiers of our knowledge concerning EIPH. Specifically, they have generated novel information on the mechanistic bases of EIPH and have provided evidence supporting additional treatment options for reducing the severity of EIPH in horses.

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CHAPTER 1 - THE EFFECTIVENESS OF IMMUNOTHERAPY IN TREATING EXERCISE-INDUCED PULMONARY HEMORRHAGE

ABSTRACT

Inflammatory airway disease has been linked to exercise-induced pulmonary haemorrhage (EIPH), and consequently, we hypothesized that immunomodulation induced by concentrated equine serum (CES) treatment would reduce EIPH as determined by the number of red blood cells (RBCs) per mL of bronchoalveolar lavage (BAL) fluid. Separate groups of Thoroughbred horses were treated with either CES (n=6) or placebo (PL; 0.9% saline; n=4). All horses completed pre- and post-treatment (2 and 4 weeks after initiating treatment) maximal exercise tests on a 10% inclined treadmill (1 m/s/min increments to fatigue) over a 10 week period (2-3 weeks between tests), with BAL performed 30 minutes post-exercise in each case. Treatment ensued 10 days following the pre-treatment exercise test, with horses receiving a series of 5 CES or PL injections 24 hours apart (20 mL intratracheal and 10 mL intravenously), with subsequent weekly injections for 5 weeks thereafter. Treatment with neither CES nor PL altered any of the cardiorespiratory or metabolic variables measured during maximal exercise. No statistically significant changes were evident in EIPH or white blood cells in the PL group. However, after treatment with CES, both EIPH (pre-treatment: $61 \pm 24 \times 10^6$; 4-week post-treatment run: $29 \pm 11 \times 10^6$ RBCs/mL BAL fluid; $P < 0.05$) and white blood cells (WBCs; pre-treatment: $419 \pm 99 \times 10^3$; 4-week post-treatment run: $288 \pm 60 \times 10^3$ WBCs/mL BALF; $P < 0.05$) were reduced significantly by the 4 week post-treatment run. The 109 ± 75% (-26 to 466%) increase in the WBC/RBC ratio (WBCs per unit of haemorrhage; 4 of 6 horses) post-CES treatment may be a consequence of immune system stimulation. In conclusion, as EIPH was decreased significantly with the CES administration, therapeutic intervention involving the immune system may represent a viable approach to reducing the severity of EIPH.

Introduction

Exercise-induced pulmonary haemorrhage (EIPH) and inflammatory airway disease (IAD) constitute a significant proportion of the pulmonary disease encountered in racehorses, and this category of disorder is second only to musculoskeletal disease as a cause of poor performance and premature career termination[1-4]. It is important to note that epidemiologic[5] and post-mortem studies have identified an association between EIPH and IAD[6-8]. In addition, the development of performance decrements consequent to EIPH and associated IAD[9-14] often necessitate additional veterinary care, extended breaks from training, and/or permanent racetrack banishment in cases where horses continue to exhibit EIPH despite treatment[11,15]. Therefore, the pathophysiological and financial ramifications of recurrent EIPH mandate the development of a treatment that will significantly attenuate or eliminate this condition. The existing therapies available for EIPH (i.e. frusemide and the equine nasal strip) [16-20] are successful in reducing, but not completely abolishing EIPH, even when used concurrently. However, as EIPH has a complex and multifactorial etiology, it is not surprising that the current uni-directional approach to treatment (i.e. decreasing capillary transmural pressure) does not completely abolish EIPH. Hence, alternative strategies may provide additive or synergistic reductions in EIPH.

Preliminary data published by Ragland *et al.*[21] and Hamm *et al.*[22] suggests that the product being tested in the current study; concentrated equine serum (CES), and a similar product called caprine serum fraction (CSF), have the potential for reducing inflammation associated with lower airway disease (i.e. IAD and EIPH) via immunomodulation. In addition, empirical evidence gathered from field trials on the racetrack (Sera, Inc., Shawnee Mission Kansas, unpublished findings) also supports a role for CES in reducing IAD and EIPH. Furthermore, immunomodulation via intravenous immunoglobulin (using doses approximately two orders of magnitude higher than used in the current study) has been successfully used to treat asthma and systemic inflammatory conditions in man[23-27]. However, no studies have been conducted to specifically examine the effectiveness of CES as a treatment for EIPH in a scientifically controlled fashion.

The purpose of the present study was to determine whether CES could attenuate the extent of EIPH. We hypothesized that: 1) CES would reduce EIPH (as quantified by the number of red blood cells (RBCs)/mL bronchoalveolar lavage (BAL) fluid), 2) global pulmonary inflammation would be decreased (decreased total white blood cells (WBCs)/mL BAL fluid),

and 3) that RBC and WBC counts would not be altered after the horses were identically treated with saline as a placebo.

Materials and Methods

Animals

Ten Thoroughbred horses (4 to 12 years old; 470 to 620 kg) with a history of EIPH and vaccinated against Eastern and Western Equine Encephalomyelitis, tetanus, equine influenza, West Nile virus, Equine Herpes virus I, and rabies were used in these investigations. Six horses were included in the treatment group and a placebo (0.9% saline) was administered to 4 additional horses in an identical manner. The horses were trained on a high speed treadmill (SATO Inc., Uppsala, Sweden) 3 days/week, and had food, but not water withheld for at least 2 hours before experimentation. All procedures were approved by the Kansas State University Animal Care and Use Committee.

Animal Preparation

The horses were instrumented with two aseptically placed 7-F jugular introducer catheters and one 18-gauge 2" Abbocath (Abbott Laboratories, North Chicago, Illinois, USA) catheter placed in a previously elevated left carotid artery. The arterial catheter was utilized for the collection of arterial blood gas and plasma lactate samples. To monitor pulmonary arterial pressure (Ppa), a 7-F microtipped pressure transducer (Millar Instruments, Inc., 6001 Gulf Freeway, Houston, Texas, USA) was placed through one of the introducer catheters into the pulmonary artery, approximately 8 cm past the pulmonic valve. The location of the pressure transducer was verified by cardiac waveform evaluation using a computer based data acquisition system (DATAQ, Akron, Ohio, USA). The Millar pressure transducer was calibrated prior to and immediately following each experimental run (range 0-200 mmHg) with a Mercury manometer. No drift was detected in the transducer across any runs. A thermistor catheter (Columbus Instruments, Columbus, Ohio, USA) was advanced through the other introducer catheter into the pulmonary artery to measure pulmonary arterial temperature, allowing for temperature correction of blood gases and pH[28]. The thermistor was calibrated using a Physitemp thermocouple thermometer (Clifton, New Jersey, USA). A Fourier analysis of the pulmonary arterial pressure waveform was performed and the numerical value of the first peak

(~ 2 Hz) was multiplied by 60 cycles/second to obtain the respiratory rate[29] since this peak has been shown to correspond to the fundamental frequency for this variable. Heart rate was determined with a heart rate monitor (Polar®, Mill Valley, CA, USA).

Administration of CES or Placebo

The intravenous form of Seramune® Equine IgG (CES, Sera, Inc., Shawnee Mission, Kansas, USA) or placebo (PL; 0.9% saline) was used for the experiment. Concentrated equine serum (CES) is a biological aggregate composed of serum collected from multiple draft horse donors and contains high levels of immunoglobulins (IgG (8,000-11,000 mg/dL), IgA (700-2,500 mg/dL) and IgM (200-600 mg/dL)) and other serum proteins (i.e. iron binding proteins and complement). The dose used in this study was empirical and based on field studies completed at various racetracks across the country over a 5 year period where a reduction in EIPH was observed (Sera, Inc., Shawnee Mission, KS, unpublished observations). The PL was administered in a fashion identical to that employed for CES.

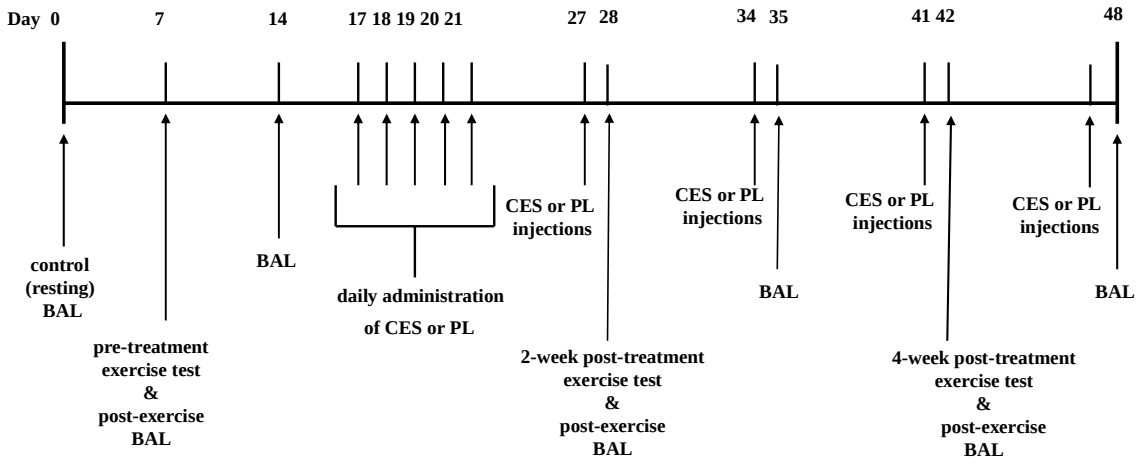
Before the intratracheal (IT) injection, the proximal one third of the trachea was clipped and surgically prepped, the tracheal rings were palpated, and an 18G 1.5 inch needle was inserted via sterile technique to approximately two thirds of its length in between 2 tracheal rings. The hub of the needle was firmly held in place to prevent exit from the trachea and 20 mL of CES or PL were administered following aspiration to insure proper location in the trachea. After administration, the horse's head was held up for approximately 10 minutes post-injection as to allow gravity dependent flow of CES or PL into the lungs. Ten mL of CES or PL were then administered slowly via a standard intravenous (IV) injection. The procedure was repeated every 24 hours for 5 days with weekly injections (both the IV and IT) given for 5 weeks thereafter . On the week of an exercise test, the CES or PL were administered 24 hours before exercise. No adverse effects were noted in any of the experimental animals.

Experimental Protocol

A timeline of the experimental protocol is provided in Figure 1. The horses were trained on a moderate-to-heavy intensity exercise regimen prior to and throughout the study ($\leq$ 10 m/s on flat; $\leq$ 7 m/s on inclined treadmill). On experimental days, subsequent to collection of resting cardiorespiratory measurements and blood samples, each horse performed a maximal exercise test. This test consisted of horses trotting for 800 m at 3 m/s (warm-up) followed by a 1

m/s/min incremental ramp, on a 10% incline, beginning at 4 m/s and continuing to the point of fatigue as judged by the inability of the horse to keep up with the treadmill despite humane encouragement. Horses were then allowed to trot for 800 m at 3 m/s (cool down). Pulmonary gas exchange was measured with a bias-flow system as described previously by Langsetmo *et al*.[30] and cardiorespiratory measurements (i.e. oxygen uptake (VO_2), carbon dioxide elimination (VCO_2), heart rate, and Ppa) were made continuously throughout the exercise test. Arterial blood samples were collected during the last 10 seconds at each speed, and time-to-fatigue was determined for each run. Approximately thirty minutes after the run BAL was performed to quantify EIPH[16, 18, 31].

Figure 1.1 Time line of experimental protocol.



BAL: bronchoalveolar lavage; CES: concentrated equine serum; PL: placebo (0.9% saline) Both CES and PL were administered at a dose of 20 mL intratracheally and 10 mL intravenously at each time point.

Bronchoalveolar Lavage

The horses were sedated with detomidine hydrochloride (Dormosedan®, Pfizer Animal Health, Exton, Pennsylvania, USA;10-20 µg/kg IV) and butorphanol tartrate (Torbugesic®, Fort Dodge Animal Health, Fort Dodge, Iowa, USA; 20-50 µg/kg IV) to facilitate BAL[18, 31]. A BAL

tube (3 m long, 10 mm in diameter; Bivona Medical Technology, Gary, Indiana, USA) was introduced into the right naris through the ventral meatus and into the lung until wedged in a subsegmental bronchus of the dorsal caudal portion of the lung[32]. The cuff was inflated to ensure lavage of the distal airway as well as maximize recovery of the lavage fluid. A total of 300 mL (in 50 mL aliquots) of 0.9% physiologic saline was infused. After a couple of breaths to allow mixing, the fluid was aspirated with gentle suction and placed on ice. The BAL fluid was centrifuged (Table Top Centrifuge, Beckman TJ-6, Beckman Instruments, Inc., Palo Alto, California, USA), the supernatant decanted, and the pellet resuspended in 0.9% saline[33]. The amount of saline used for resuspension ranged from 10-200 mL (depending on the severity of EIPH) and was chosen in order to avoid errors in cell counting due to widely differing RBC concentrations (i.e. haematocrit was standardized between runs and conditions). Red blood cells (RBCs) and total nucleated cells (TNCs) were counted using a haemocytometer (Fisher Scientific, 2000 Park Lane Drive, Pittsburgh, Pennsylvania, USA; Microscope, Nikon, Inc., Instrument Group, Garden City, New York, USA). Data are presented as RBCs and TNCs (WBCs) per milliliter of recovered BAL fluid minus tube dead space (17 mL). Differential WBC counts (Shandon Cytospin 3, Shandon, Inc., Pittsburgh, Pennsylvania, USA) were performed on the WBCs after staining with Hema 3 quick stain (Protocol® Fisher Scientific, Middletown, VA, USA) which stains cells similarly to the Wright-Giemsa stain.

Blood Analysis

Following anaerobic withdrawal, (into plastic, heparinized syringes) blood samples were placed immediately on ice. Within 1-2 hours of the experiment arterial blood gases, pH, and plasma lactate were analyzed (Blood Gas Analyzer - Nova Stat M, Nova Biomedical, Waltham, Massachusetts).

Statistical Methods

All data are presented as mean ± standard error (SE). Data was evaluated using one-way analysis of variance (ANOVA) with repeated measures. Where an *a priori* directional hypothesis was to be tested, a one-tailed test was utilized. The degree of relationship between certain pre-determined variables was determined via a Pearson Product-Moment correlation analysis. Significance for all variables was accepted at $p < 0.05$.

Results

No differences were noted for any variable between the pre-treatment and 2-week post-treatment run (11 days after initiation of CES treatment). Therefore, for clarity of presentation the BAL results focus on differences between the pre-and 4-week post-treatment means and between the CES and PL groups at these time points. However, for completeness, all BAL fluid data (RBC and WBC counts) are included in Tables 1 and 2.

Table 1.1 Pre- and post-saline treatment comparisons for cardiorespiratory and metabolic variables during maximal exercise and bronchoalveolar lavage variables post-exercise.

Variable	pre-PL treatment	2-week post-PL treatment	4-week post-PL treatment
VO_2max (L/min)	72.9 ± 2.0	75.4 ± 2.6	76.5 ± 2.3
VCO_2max (L/min)	82.8 ± 3.3	86.5 ± 1.3	85.9 ± 2.2
PaO_2 (mmHg)	63.4 ± 1.3	65.0 ± 3.0	60.9 ± 1.5
$PaCO_2$ (mmHg)	61.7 ± 3.9	62.0 ± 4.2	62.0 ± 2.6
HRmax (beats/min)	218 ± 2	219 ± 5	214 ± 4
RRmax (breaths/min)	115 ± 3	115 ± 2	115 ± 2
$[La^-]$ (mM)	21.9 ± 3.3	29.6 ± 1.7	26.5 ± 4.6
Ppa (mmHg)	87.3 ± 7.3	91.4 ± 3.1	89.2 ± 3.9
pH	7.22 ± .02	7.21 ± .02	7.18 ± .02
Hematocrit	59 ± 1	61 ± 1	61 ± 1
Time-to-Fatigue (s)	656 ± 21	673 ± 21	658 ± 20
Avg WBCs/mL BALF	301.00 ± 73.63 x 10^3	305.00 ± 37.81 x 10^3	219.97 ± 37.51 x 10^3
Avg RBCs/mL BALF	4.63 ± 2.29 x 10^6	9.08 ± .87 x 10^6	13.04 ± 3.74 x 10^6

Values are mean ± SE. Oxygen uptake at STPD: VO_2; carbon dioxide elimination at STPD: VCO_2; partial pressure of oxygen in the arterial blood: PaO_2; partial pressure of carbon dioxide in the arterial blood: $PaCO_2$; heart rate: HR; respiratory rate: RR; plasma lactate concentration: $[La^-]$; mean peak pulmonary arterial pressure: Ppa; average number of white blood cells per mL bronchoalveolar lavage fluid: Avg WBCs/mL BALF; average number of red blood cells per mL bronchoalveolar lavage fluid; Avg RBCs/mL BALF. No significant differences were found between the pre-treatment and either of the post-treatment runs for any of these variables.

Table 1.2 Pre- and post-CES treatment comparisons for cardiorespiratory and metabolic variables during maximal exercise and bronchoalveolar lavage variables post-exercise.

Variable	pre-CES treatment	2-week post-CES treatment	4-week post-CES treatment
VO_2max (L/min)	73.7 ± 1.9	67.7 ± 5.6	71.9 ± 3.1
VCO_2max (L/min)	81.0 ± 2.4	76.5 ± 6.1	81.0 ± 3.7
PaO_2 (mmHg)	64.9 ± 2.7	64.1 ± 2.0	62.4 ± 1.7
$PaCO_2$ (mmHg)	64.2 ± 4.1	63.8 ± 3.7	67.5 ± 4.0
HRmax (beats/min)	219 ± 4	224 ± 8	216 ± 4
RRmax (breaths/min)	121 ± 2	120 ± 2	121 ± 2
[La^-] (mM)	25.3 ± 3.2	28.8 ± 3.2	32.6 ± 1.7
Ppa (mmHg)	96.1 ± 6.2	95.7 ± 6.9	97.0 ± 4.6
pH	7.18 ± .04	7.21 ± .03	7.19 ± .04
Hematocrit	63 ± 1	63 ± 1	63 ± 1
Time-to-Fatigue (s)	711 ± 14	678 ± 9	681 ± 13
Avg WBCs/mL BALF	412.00 ± 1.02 x 10^3	359.67 ± .95 x 10^3	287.67 ± .61 x 10^3
Avg RBCs/mL BALF	61.08 ± 24.66 x 10^6	144.00 ± 103.37 x 10^6	29.05 ± 11.58 x 10^6

Values are mean ± SE. Concentrated equine serum: CES; oxygen uptake at STPD: VO_2; carbon dioxide elimination at STPD: VCO_2; partial pressure of oxygen in the arterial blood: PaO_2; partial pressure of carbon dioxide in the arterial blood: $PaCO_2$; heart rate: HR; respiratory rate: RR; plasma lactate concentration: [La^-]; mean peak pulmonary arterial pressure: Ppa; average number of white blood cells per mL bronchoalveolar lavage fluid: Avg WBCs/mL BALF; average number of red blood cells per mL bronchoalveolar lavage fluid; Avg RBCs/mL BALF. *Mean is significantly different from pre-CES group.

Indices of Maximal Effort

Tables 1.1 and 1.2 show the average data for cardiorespiratory and metabolic variables measured during maximal exercise for both the PL and CES treatment experiments, respectively. No significant differences were noted for any of these variables between pre- and either of the post-treatment exercise runs.

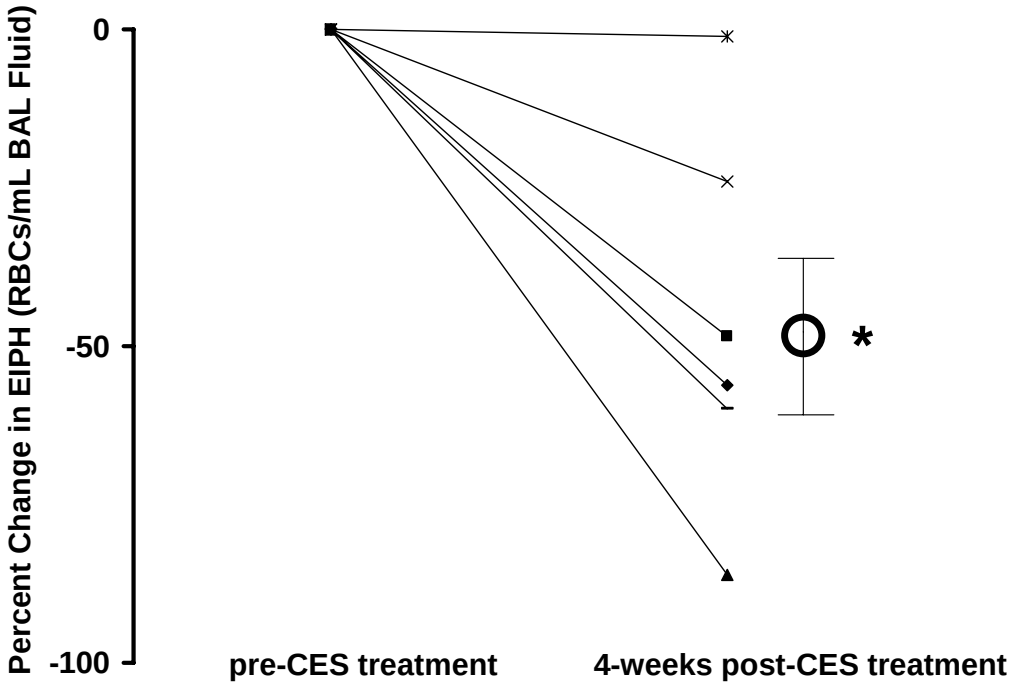
BAL Fluid Evaluation Pre- and Post-Treatment

The percentage of BAL fluid recovered for the pre-treatment as well as the 2 and 4-week post-treatment runs was (55.6± 5.5, 50.9 ± 5.7, and 69.7 ± 7.2%; $p > 0.05$) for the PL group, and (62.1 ± 3.8, 55.2 ± 7.0, and 56.1 ± 5.8%; $p > 0.05$) for the CES treated horses, respectively. The number of RBCs/mL of BAL fluid decreased 46 ± 12% ($p < 0.05$) between the pre-treatment and the 4-week post-CES treatment run (pre-treatment run: 61 ± 25 x 10^6; 4-week post-treatment run: 29 ± 12 x 10^6 RBCs/mL, $p < 0.05$), whereas the number of RBCs/mL BAL fluid did not change over the same time period in the PL group (pre-treatment run: 5 ± 1 x 10^6; 4-week post-treatment run: 13 ± 4 x 10^6 RBCs/mL BALF, $p > 0.05$). In fact, the percent change in RBCs/mL BAL fluid tended to increase from control levels, (albeit not significant statistically due to high variability; $p=0.14$) by the 4-week post-treatment run in horses treated with saline (PL) whilst

this variable evidenced a significant decrease (46 ± 12%) in horses treated with CES (Figure 1.2).

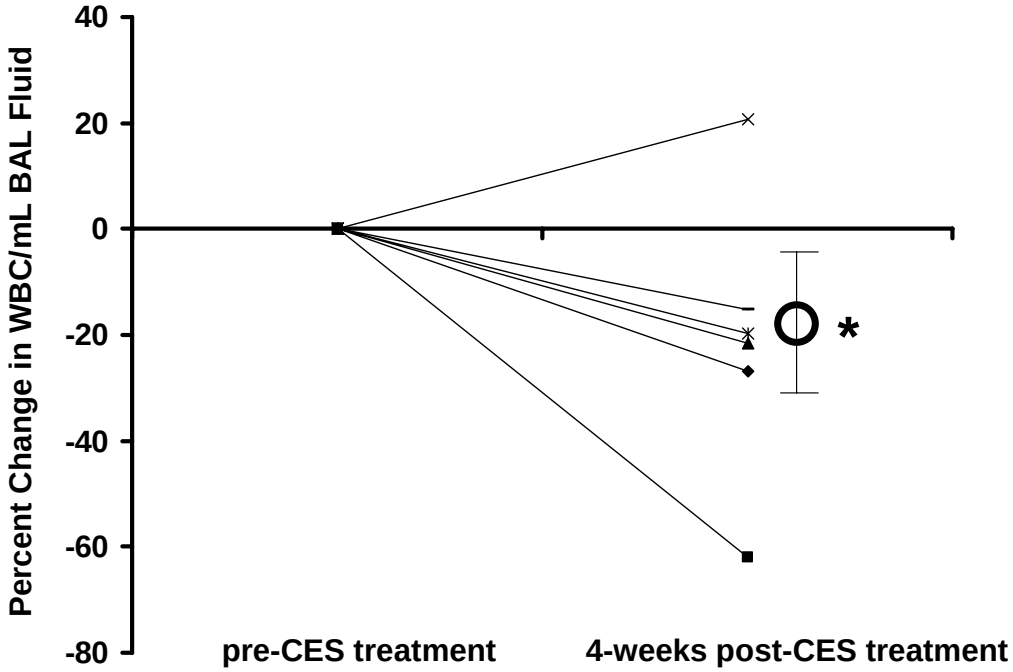
Overall, WBCs declined 21 ± 11% (pre-treatment run: 412 ± 102 x 10^3; 4-week post-treatment run: 288 ± 61 x 10^3 WBCs/mL BALF; $p < 0.05$) by the 4-week post-treatment run in the CES treated horses (Figure 1.3). The WBC counts from the PL group were not significantly different between pre- and either of the post-treatment runs (pre-treatment run: 301 ± 74 x 10^3; 4-week post-treatment run: 220 ± 38 x 10^3 WBCs/mL BAL fluid, $p > 0.05$). In addition, no significant differences were detected in the differential counts (Figure 1.4) for either pre- or post-run lavages of the PL or CES treated horses. The baseline and one week post-run lavages served the purpose of illustrating that RBC and WBC counts had returned to baseline by 1 week post-maximal exercise, eliminating any question of residually elevated RBC levels that may confound the bleeding in subsequent runs for horses given either PL or CES. No significant differences from baseline were detected in the total WBC count (data not shown), RBC count (data not shown), or WBC differential counts (Figure 1.4) for any of the non-exercising (1 week post-run) lavage periods.

Figure 1.2 Relative changes in exercise-induced pulmonary hemorrhage (EIPH; RBC counts) at 4 weeks following initiation of concentrated equine serum (CES; n=6) treatment.



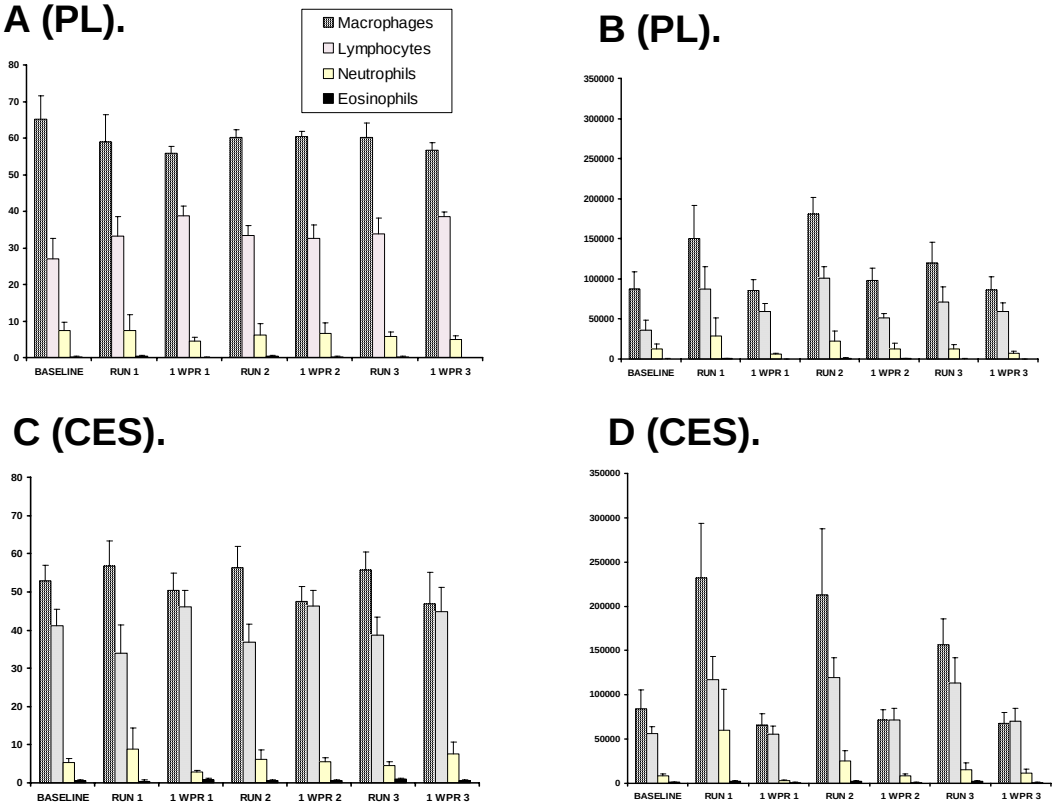
Individual horse data are presented as a percent decrease in EIPH following maximal exercise after 4 weeks of treatment with CES, where pre-CES treatment levels are set to 0. Individual horses are represented by different symbols and the mean (± SE) percent decrease in EIPH of all horses is displayed as a large open circle. *Mean is significantly different from pre-treatment run, $p < 0.05$.

Figure 1.3 Relative change in inflammation (WBC counts) at 4 weeks following initiation of concentrated equine serum (CES; n=6) treatment.



Relative change in Inflammation (WBC counts) at 4 weeks following initiation of concentrated equine serum (CES; n=6) treatment. Individual horse data are presented as a percent decrease in inflammation following maximal exercise after 4 weeks of treatment with CES, where pre-CES treatment levels are set to 0. Individual horses are represented by different symbols and the mean (± SE) percent decrease in inflammation of all horses is displayed as a large open circle. *Mean is significantly different from pre-treatment run, $p < 0.05$.Level.

Figure 1.4 Differential white blood cell (WBC) counts for placebo and concentrated equine serum (CES) treated horses.



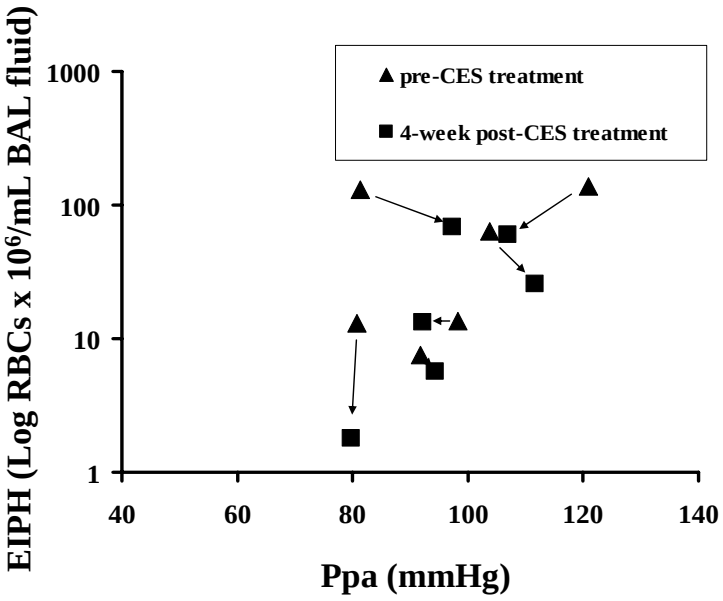
Panels A and B represent the percentage and number/mL of each cell type evaluated in the WBC differentials (300 cell count) of the placebo (PL; saline) treated horses (n=4), respectively. Panels C and D represent the percentage and number/mL of each cell type evaluated in the WBC differentials (300 cell count) of the Concentrated Equine Serum (CES) treated horses (n=6), respectively. The x-axes represents the different time periods evaluated during the study, including resting levels (baseline), 3 exercise periods (runs 1, 2, 3), and one-week post-run periods (1WPR 1, 2, 3). No differences were observed in the differential WBC counts for any of the time periods under either PL or CES

Discussion

The novel finding of the current study is that immunotherapy with CES significantly reduced EIPH and associated inflammation following maximal exercise in Thoroughbred horses. Specifically, by the end of the 4 week treatment period, CES resulted in a 46% reduction in the immediate post-exercise BAL RBC count, while concurrently demonstrating a 21% decrease in the WBC count. In comparison, no significant changes in EIPH or WBC counts were observed in PL horses over the same time period. The CES-induced mitigation of EIPH in the absence of pulmonary arterial and presumably capillary transmural pressure reduction[16-18, 29], indicates that EIPH severity can be modulated independently of changes in vascular pressures (Figure 1.5). It

is worth noting that the differences observed in this study may not have been detectable without the use of BAL, since rigorous quantification of haemorrhage is not possible with endoscopy. The technique of BAL has proven to be a reproducible and sensitive method for evaluating treatment effectiveness and degree of EIPH. For example, under controlled laboratory conditions (in which horses ran identical protocols with no treatment), we have demonstrated that the recovery of BAL fluid and the number of RBCs per milliliter of lavage fluid is highly reproducible between runs (i.e. coefficient of variation = ~ 5%)[18]. The finding that CES can cause a reduction in EIPH and inflammation suggests that immunomodulation may provide an effective therapeutic tool for reducing EIPH.

Figure 1.5 Exercise-induced pulmonary hemorrhage (EIPH) versus mean peak pulmonary artery pressure (Ppa).



Diamonds represent the Ppa and associated EIPH of horses during the pre-concentrated equine serum (CES) treatment run and the squares represent the Ppa and associated EIPH of the horses during the 4 week post-CES treatment run, with the arrows connecting the 2 respective values for each individual horse. The log scale is used simply as an expedient to better visualize the data. The coefficient of determination for the relationship between the severity of haemorrhage (logRBCs/mL BALF) versus Ppa (linear scale; $r^2 = .14$, $P > 0.05$) indicates that associated decreases in EIPH and pulmonary arterial pressure were not observed as a result of CES treatment (i.e. some horses exhibited decreased logRBCs/mL BALF at the same or higher Ppa), and therefore, some variable other than Ppa is accounting for the severity of haemorrhage observed in these horses.

CES Versus Traditional Prophylactic Therapies for EIPH

Currently, frusemide and the equine nasal strip are the only scientifically established treatments for EIPH[16-20]. When administered 4 hours prior to maximal exercise (at a dose of 1 mg/kg), frusemide has been shown repeatedly to be effective in reducing pulmonary haemorrhage. This occurs via a significant reduction in the pulmonary vascular pressures during near maximal and maximal exercise[17-19], thus implicating pulmonary arterial pressure as a principal causative mechanism for EIPH[34, 35]. On the other hand, the equine nasal strip ameliorates EIPH[16-20] by maintaining nasal patency, and thereby reducing airway resistance[36] and presumably capillary transmural pressure. The only contradictory evidence suggesting that either of these treatments (i.e. frusemide and the nasal strip) do not invoke a significant reduction in EIPH is that of Goetz et al.[37] and Birks et al.[38]. However, no rigorous attempt was made to quantify EIPH in either of these studies (i.e. endoscopy was performed). In contrast to mitigating EIPH via a direct reduction in capillary transmural pressure (as is the case with frusemide and the equine nasal strip), the results of the current study suggest that CES may act indirectly to reduce the deleterious effects of blood in the airways by an (as yet) undetermined time-dependent mechanism. This was substantiated in the current study by the fact that EIPH was significantly reduced after treatment with CES in the face of unaltered pulmonary arterial pressures and oxygen uptake measurements (Table 1.2). One major difference between treatments which acutely impact transmural pressures (i.e. equine nasal strip (instantly) and frusemide (hours)) and CES (4 weeks) is the time necessary to observe the reduction in EIPH.

Targeting IAD with CES

The idea that EIPH and IAD were interrelated was first published by O'Callaghan *et al.*[6, 7] and later by McKane and Slocombe[8, 34, 39], who provided indirect evidence that the extravasation of blood into the airways may be at least partially responsible for the IAD observed on post-mortem examination in EIPH affected areas. These findings are in agreement with the recent epidemiologic studies of Newton and Wood[5], which identified a link between IAD and EIPH. The horses in the present investigation all exhibited evidence of IAD: specifically, higher than normal TNCs (> 200 cells/μL), mild neutrophilic inflammation (5-20% neutrophils), and lymphocytosis in BAL fluid[40] which may in turn exacerbate future EIPH episodes. Objective measurements demonstrated reduced inflammation following CES treatment as evidenced by

decreased WBC counts (21%) in BAL fluid by 4 weeks post-treatment in the current study. Furthermore, our findings substantiate the subjective reductions in both inflammation (decreased airway mucus) and EIPH (endoscopic grading) observed following field use of CES in 187 horses at three racetracks (Fonner Park, NE; Prescott, AZ; and Marshall, VA) during the years 1995-99 (Sera, Inc., Shawnee Mission Kansas, unpublished observations). Moreover, unpublished data from our laboratory (n=4) suggests at least an equivalent reduction in EIPH with CES (68%) versus frusemide (58%), the nasal strip (42%), or the concurrent use of the nasal strip and frusemide (46%) in the same maximally exercising horses. These data strongly suggest the possibility that mitigation of IAD may address an important component of EIPH that is at least equivalent to the reduction of intravascular (frusemide) [18, 19] and extravascular (equine nasal strip) [16-20] pressures. In light of the present findings, it is possible that CES might benefit the long term pulmonary health of racehorses since the consequences of a prolonged inflammatory reaction to the blood in the alveoli and the subsequent fibrosis could decrease pulmonary compliance and increase shear forces within the alveoli of the lungs[9, 10, 12, 39]. These mechanical alterations are likely to progressively weaken the blood-gas barrier, and predispose a larger population of the capillaries to stress failure with each succeeding EIPH episode,[9, 10] an effect that may be magnified by insufficient healing time between insults[39].

Potential Mechanistic Bases for the Reduction of EIPH with CES

The exact mechanism through which CES therapy reduces EIPH remains speculative, however, the present results are consistent with a time-dependent phenomenon related to the reduction of inflammation through immunomodulation. The delayed nature of this response was evident when the pre- and post-treatment runs were considered. Specifically, EIPH increased from pre-treatment to the 2-week post-treatment run, before significantly decreasing by the 4-week post-treatment run in CES-treated horses. In the PL group of horses, a much different pattern was observed, whereby, a trend for increasing EIPH was noted by the 4-week post-treatment run. Though not statistically significant ($p=0.14$), the pattern of bleeding observed with the PL group is in agreement with the current belief that sequentially repeated maximal exercise bouts will result in increased severity of hemorrhage[38, 41]. This intuitively makes sense, especially if the factors (i.e. capillary transmural pressures, IAD) involved in initiating and perpetuating EIPH are not addressed by some form of intervention (i.e. frusemide, equine nasal

strip, or CES), regardless of whether the effects of treatment are immediate or require time to become evident.

The immunomodulation hypothesis is based on research by Ragland *et al.* (CES administration)[21] and Hamm *et al.* (caprine serum fraction administration)[22] demonstrating that a reduction in pulmonary inflammation and a more rapid recovery of horses from suppurative lower airway disease occurred as a result of "non-specific" stimulation of the immune system. In agreement with these two studies, the current study demonstrated a reduction in WBCs and a concurrent and marked increase (109 ± 75%) in the WBC/RBC ratio as a result of CES treatment. In contrast, horses treated with PL demonstrated a decreased WBC/RBC ratio (66 ± 9%). In addition, the correlation between the WBC/RBC ratio and the level of haemorrhage found pre-treatment ($r^2 = 0.71$, $P<0.05$) disappeared post-treatment ($r^2 = 0.34$, $P>0.05$) in the horses treated with CES. These findings suggest that the additional recruitment of WBCs into the lung post-CES treatment may expedite clearance of blood from the lungs. This further supports the premise that immune modulation in response to CES treatment may be responsible for the observed reduction in EIPH, in a fashion independent of changes in vascular transmural pressures.

It has been shown that alveolar macrophage function (phagocytosis and oxidative burst) becomes suppressed for 2-3 days when challenged by alveolar haemorrhage[8, 39, 42-48] as a consequence of the limited ability of alveolar macrophages to effectively metabolize/detoxify the iron in RBCs[49, 50]. Concentrated equine serum contains high levels of immunoglobulins (Sera, Inc., Shawnee Mission, Kansas) and complement[51, 52] compared with normal horse serum (IgG (8,000-11,000 vs. 1000-1500 mg/dL), IgA (700-2,500 vs. 60-350 mg/dL) and IgM (200-600 vs. 100-200 mg/dL)[51, 52]. Increased levels of these components and other mediators in the CES may act directly (result of intratracheal administration) or indirectly (intravenous administration) to enhance WBC recruitment to the lung and expedite the clearance of RBCs in the interim between exercise bouts. This may occur through enhancement of phagocytic ability (complement and immunoglobulin mediated opsonization)[51-54] and oxidative burst activity (metabolize/detoxify the iron overload more efficiently)[45, 49, 50, 55] of alveolar macrophages. The combination of a more rapid and enhanced functional response of the phagocytes towards clearing the haemorrhage would markedly reduce the vicious and self-perpetuating inflammatory cycle incited by the prolonged presence of blood in the lung[8]. If this is indeed true, the degree of

subsequent tissue injury and associated fibrosis, as well as prolonged inflammation may in turn be reduced as a result of CES treatment. Even though immunomodulation takes time to become manifest, our results together with those discussed above, suggest that CES is a powerful tool that can be used to reduce the severity of EIPH through immune system stimulation.

Future Studies with CES

Future investigations are needed to elucidate the mechanisms of action of CES, to optimize the route of administration and dose, and to determine whether additive or synergistic benefits may be gained by the concurrent use of CES with frusemide and/or the nasal strip. The mechanism of action is still unclear and may in fact be dose dependent[23-27]. I*n vitro* CES treatment of alveolar macrophages obtained from BAL samples of resting and exercising horses suggests that CES may enhance phagocytosis and oxidative burst function (unpublished data; Epp, Wilkerson, Myers, Erickson). However, *in vivo* studies are needed to confirm that these effects are observed in horses treated systemically with CES.

Conclusions

After 4 weeks of treatment, CES reduced the EIPH response to maximal exercise by ~ 50%. Quantitatively, this reduction is equivalent to that demonstrated by frusemide and the nasal strip after the same exercise challenge. As changes in pulmonary arterial pressure and pulmonary gas exchange were not detected after treatment, CES presumably operates via a mechanism that is not dependent upon a reduction in either intra- or extravascular pulmonary pressures. Consequently, we speculate that CES reduces EIPH through an immune-mediated mechanism and that this may improve the lung tissue healing, maintain lung function, and increase athletic career longevity.

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CHAPTER 2 - THE EFFECT OF HERBAL SUPPLEMENTATION ON THE SEVERITY OF EXERCISE-INDUCED PULMONARY HEMORRHAGE

ABSTRACT

Exercise-induced pulmonary haemorrhage (EIPH) is a serious condition that affects the health and possibly the performance of all racehorses. However, only two treatments, frusemide and the Flair™ equine nasal strip, both of which reduce capillary transmural pressure, have been successful in reducing EIPH. Alternatively, transient impairment of platelet function and coagulation during exercise have been considered as additional contributors to EIPH. Consequently, herbal formulations designed to enhance platelet function, and hence coagulation, are hypothesized to reduce EIPH. To investigate the validity of this hypothesis, five Thoroughbred horses completed 3 maximal incremental exercise tests on a 10% inclined treadmill in a randomized crossover design experiment. Treatments included twice daily oral administration (for 3 days) of a placebo (PL; cornstarch) and 2 herbal formulas, *Yunnan Paiyao* (YP) or *Single Immortal* (SI). Blood samples for coagulation profiles, complete blood counts, and chemistry profiles were collected before each exercise test. During each test, pulmonary arterial pressure, oxygen uptake, arterial blood gases, plasma lactate, and time-to-fatigue were measured. Severity of EIPH was quantified via bronchoalveolar lavage (BAL) 30-60 minutes post-exercise. These herbal formulations were not effective in decreasing EIPH (PL, 27.1 $\pm$ 11.6 x 10^6; YP, 33.2 $\pm$ 23.4 x 10^6; SI, 35.3 ± 15.4 x 10^6 RBCs/mL BAL fluid, $P > 0.05$) or in changing any of the other measured variables with the exception of time-to-fatigue, which was slightly but significantly prolonged by *Single Immortal* when compared to the placebo and *Yunnan Paiyao* (PL, 670 ± 9.6; YP, 665 ± 5.5; SI, 685 ± 7.9 sec, $P < 0.05$). Thus, these results do not support the use of these herbal formulations in the prevention of EIPH.

Introduction

Exercise-induced pulmonary haemorrhage (EIPH) is a ubiquitous phenomenon in equine athletes exercising at or near maximal effort[1, 2]. Since the condition was first documented in 16th century racehorses[3], research efforts have focused on mitigation of the haemorrhage. The development of effective treatments that will prevent and/or diminish the severity of EIPH has become paramount in importance due to the potentially serious consequences of recurrent pulmonary haemorrhage on the health and welfare of performance horses. These negative consequences and cumulative effects of EIPH include inflammation, scarring[4-7], and (possibly) decreased performance[8-10]. However, only 2 treatments to date have demonstrated scientific efficacy in the amelioration of EIPH. Frusemide, which significantly lowers pulmonary arterial pressure[11], a primary factor in the initiation of EIPH[12], reduces EIPH by 50-90% in strenuously exercising horses[13-15]. Unfortunately, the use of frusemide is problematic, in part, because its effects are variable, but also due to it's ability to enhance performance[16, 17]. Furosemide has also been considered to mask the detection of illegal drugs[16, 17], however, given the state-of-the-art detection methods available this is probably not a valid concern today. The Flair™ equine nasal strip decreases EIPH by 30-50%[13-15, 18, 19] as a result of maintaining nasal patency and reducing upper airway resistance during inspiration[20], thereby reducing the work of breathing, and ultimately, lowering the capillary transmural pressure. Unfortunately, neither of these treatments alone or in combination has been able to completely eliminate EIPH[15], and both are banned from use in many racing jurisdictions.

Early studies[21-26] investigating a proposed relationship between a coagulation anomaly in exercising horses and the potentiation of EIPH suggested that horses may have decreased clotting ability during exercise, thus increasing the amount of haemorrhage observed consequent to an impaired ability to rapidly plug the breaks that occur in the blood gas barrier during strenuous exercise. In light of this data, these herbal formulations have been anecdotally reported to be helpful in addressing this problem and are in widespread use on the race track. However, controversy arises since the only proof of benefit to this point has been clinical impressions of efficacy, and no scientific evidence exists to support the use of herbal formulations for the prevention or reduction of EIPH. In fact, the most current literature refutes the validity of inferences made from the earlier studies (suggesting that platelet function defects may be present in exercising horses) as a result of the discovery that the anticoagulant used may

in and of itself inhibited platelet function[27, 28]. Therefore, it seems unlikely that the severity of EIPH would be reduced by shortened coagulation and enhanced platelet function.

The purpose of this investigation was to determine the effectiveness of two herbal formulations (i.e. *Yunnan Paiyao* and *Single Immortal*) on coagulation and EIPH in the horse. The individual ingredients in these formulations have been shown to enhance coagulation by decreasing the bleeding time in other species (i.e. rats and rabbits)[37-39], and *Single Immortal* has anecdotally been reported to be effective in reducing EIPH in horses[29]. We hypothesized that EIPH would not be reduced following maximal exercise when evaluated by bronchoalveolar lavage (BAL), whether or not coagulation variables or bleeding times were shortened as a result of treatment with the herbal formulations.

Materials and Methods

Animals

Five Thoroughbred horses, aged 5-14 years and weighing 470-600 kg with a documented history of EIPH, were used in this study. The animals were housed on a dry lot with loafing sheds and free access to water and salt. They were fed alfalfa and free-choice grass hay, as well as concentrate (Strategy, Purina Mills Inc., St. Louis, MO, USA) twice daily. They were dewormed at three month intervals, rotating ivermectin with oxibendazole, and vaccinated against Eastern and Western Encephalomyelitis, tetanus, equine influenza, West Nile virus, rabies, and Equine Herpes virus I. The horses were trained on a high-speed treadmill (SATO Inc., Uppsala, Sweden) three days/week and had food, but not water withdrawn for at least two hours before experimentation. All procedures were approved by the Kansas State University Animal Care and Use Committee.

Treatments

Treatment order was randomized in a crossover design. The investigators were blinded to which treatment the experimental subjects had received. Cornstarch (5 tablespoons) was administered as the placebo by mouth twice daily for three days prior to the exercise test, as well as the morning of the maximal exercise test. A patented Chinese herbal formula called *Yunnan Paiyao* (Mayway Corporation, Oakland, CA, USA) and another herbal formulation called *Single Immortal* (Jing-Tang Herbal Company, Reddick, FLA, USA) were tested. The doses utilized

were those either recommended by the manufacturer (*Single Immortal)* or considered effective from widespread administration on the racetrack (*Yunnan Paiyao*). Two full weeks were allowed for washout time between treatments. *Yunnan Paiyao* was administered to the horses at a dose of 4 g of powder by mouth twice daily for three days before the trial, and on the morning of the maximal exercise test. *Single Immortal* was administered for 3 days before exercise testing at a dose of 50 g of powder by mouth twice daily, including the morning of the maximal exercise test[29]. Blood samples were obtained by jugular venipuncture for complete blood counts (CBC), chemistry profiles (CP) coagulation assays (prothrombin time and partial thromboplastin time) and platelet counts immediately before all maximal exercise tests. Control blood samples (from normal, healthy horses not on the experiment) were also submitted with the experimental coagulation assay samples.

Animal Preparation

Prior to the exercise test, each horse had two 7-F introducer catheters placed in the right jugular vein and one 18-Guage, 2" catheter (Safelet, NIPRO Medical Corporation, Miami, FL, USA) placed in either a previously elevated left carotid artery or the transverse facial artery (1 horse). These procedures were performed under local anesthesia (2% lidocaine) using aseptic techniques. A carotid arterial cannula (polyethylene; 1.6 mm inner diameter and 3.2 mm outer diameter) was connected to the arterial catheter to facilitate withdrawal of arterial blood. A 7-F microtipped Millar (Millar Instruments, Inc., Houston, TX, USA) pressure transducer was placed into the pulmonary artery through one of the 7-F introducer catheters, approximately 8 cm past the pulmonic valve to monitor pulmonary arterial pressure. A Fourier analysis of the pulmonary arterial pressure waveform was performed and the numerical value of the first peak (~ 2 Hz) was multiplied by 60 cycles/second to obtain the respiratory rate[30] since this peak has been shown to correspond to the fundamental frequency for this variable. The location of the pressure transducer and the thermistor was verified by cardiac wave form evaluation via a data analysis system (DATAQ, Akron, OH, USA) and viewed on a monitor. The Millar pressure transducer was calibrated prior to and immediately following each experimental run in 50 mmHg increments (range 0-200 mmHg) with a Mercury manometer. A thermistor (Columbus Instruments, Columbus, OH, USA) was advanced through the other 7-F introducer catheter into the right pulmonary artery to measure pulmonary arterial temperature, allowing for temperature

correction of blood gases and pH[31]. The thermistor was calibrated using a Physiotemp (BAT-10, Physitemp, Clifton, NJ, USA) thermocouple thermometer. Oxygen consumption was measured with an bias flow system as previously described by Kindig *et al.* [32]. Heart rate was determined with a heart rate (Polar®, Mill Valley, CA, USA) monitor.

Maximal Exercise Test

Each horse completed one maximal exercise test on the inclined treadmill (10% incline) after each of the following conditions: placebo, *Yunnan Paiyao*, and *Single Immortal*. After resting measurements were made with the horses standing quietly on the treadmill, each horse was warmed up at 3 m/s for two minutes. The horses then performed an incremental exercise test (speed increasing by 1 m/s per minute) beginning at 4 m/s to volitional fatigue (maximal oxygen uptake; VO_{2max}), then recovered at a trot (3 m/s for four minutes). Cardiorespiratory measurements [heart rate, pulmonary arterial pressure, oxygen uptake (VO_2), and carbon dioxide production (VCO_2)] were collected continuously throughout exercise and cool-down, and arterial blood samples were collected during the last 10 seconds at each speed, as well as during recovery at two and four minutes post-maximal exercise. Thirty to 60 minutes after the exercise test, BAL was performed to quantitate EIPH as described below[2, 15].

Blood Analysis

Following anaerobic withdrawal, (into plastic, heparinized syringes) blood samples were placed immediately on ice. Within one-two hours of the experiment, arterial blood gases were quantified by means of blood gas analysis (Nova Stat M, Nova Biomedical, Waltham, MA, USA) and corrected to the horse's pulmonary arterial blood temperature[31]. The blood gas analyzer was calibrated before running the samples according to manufacturer's standards.

Bronchoalveolar Lavage (BAL)

The horses were sedated using detomidine hydrochloride (Dormosedan®, Pfizer Animal Health, Exton, PA, USA; 5-10 µg/kg IV) and butorphanol tartrate (Torbugesic®, Fort Dodge Animal Health, Fort Dodge, IA, USA; 5-10 µg/kg IV) to facilitate BAL and to quantify the severity of EIPH 30-60 minutes post-exercise[2, 15]. A Bivona tube (Bivona Medical Technology, Gary IN, USA; 3 m long, 10 mm in diameter) with an inflatable cuff was introduced into the right naris through the ventral meatus, and into the lung until wedged in a subsegmental

bronchus of the dorsal caudal portion of the lung[33]. The Bivona tube with a cuff created a seal within the airway, which ensured lavage of the distal airway and maximized recovery of lavage fluid. A total of 300 mL (in 50 mL aliquots) of 0.9% physiologic saline was infused. After approximately two breaths, the fluid (a percentage of the entire 300 mL) was aspirated with gentle suction. Fluid recovery averaged approximately 60% of instilled volume; no significant differences in recovery existed between trials. The BAL fluid was centrifuged (Beckman TJ-6, Beckman Instruments, Inc., Palo Alto, CA, USA), the supernatant decanted, and the pellet was resuspended in 0.9% saline[34]. Centrifugation, washing, and resuspending BAL fluid prior to cell counts results in no significant difference in total cell counts[34] and was utilized for the following reasons: 1) to reduce mucus and debris that interfere with counting, and 2) to achieve a similar final RBC concentration across runs and horses to avoid errors in counting due to widely differing RBC concentrations. The amount of saline used for resuspension ranged from 10-200 mL depending on the severity of EIPH to maintain a relatively similar RBC-to-saline solution ratio (i.e. lavage fluid haematocrit). Red blood cells (RBCs) and total nucleated cells (TNCs) were counted using a haemocytometer (Fisher Scientific No. 02-671-5, Pittsburg, PA, USA) and a microscope (Nikon Instrument Group, Inc., Garden City, NY, USA). Data are presented as RBCs and TNCs per milliliter of recovered BAL fluid minus tube dead space (17 mL). We consider this technique to be valid and reliable for evaluating treatment effectiveness since our laboratory has demonstrated that under controlled laboratory conditions in which horses run identical protocols, the recovery of BAL fluid and the number of RBCs per milliliter of lavage fluid is highly reproducible between runs (i.e. coefficient of variation = ~ 5%)[13]. Resting/control BAL samples were taken from the horses under light-moderate training with no strenuous high intensity exercise performed within 10 days, and were evaluated for the RBCs and TNCs at the initiation of the study.

Statistical Analysis

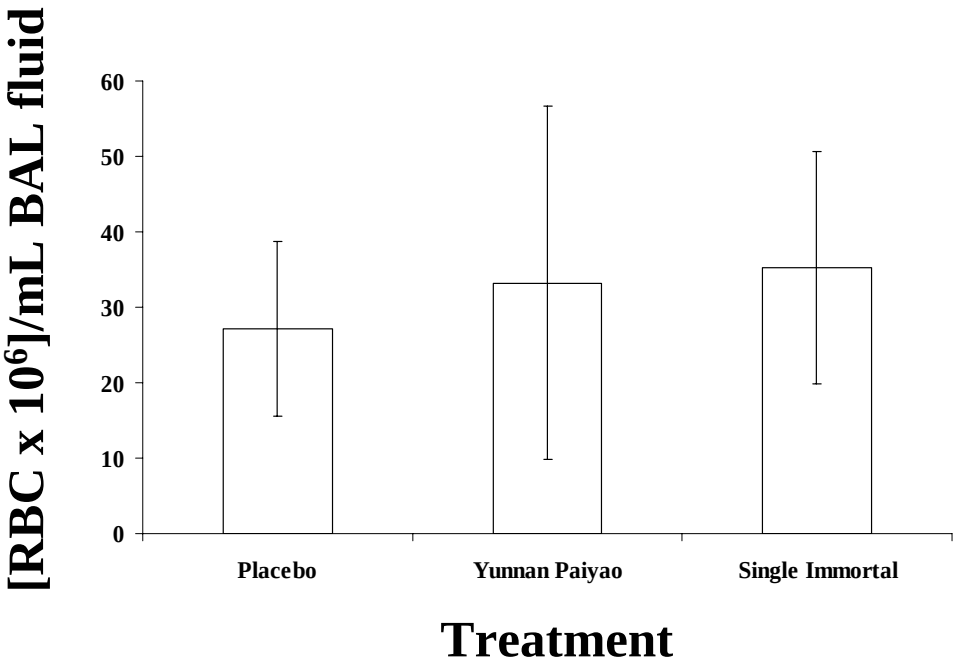
A one-way ANOVA for repeated measures was utilized to determine if differences existed between treatment variables measured across experimental conditions, with the exception of platelet numbers and VO_{2max}. These latter variables were normally distributed (Kolmogovov Smirov Test for normality), but did not have equal variances, and were therefore analyzed with one-way repeated measures ANOVA on ranks. If significance was revealed with ANOVA, a

Student-Newman Keuls post-hoc test was used to determine where the differences were located. Pre and post-test variables (i.e. cutaneous bleeding times) were examined using a paired t-test. Statistical significance was accepted at the $P<0.05$ level.

Results

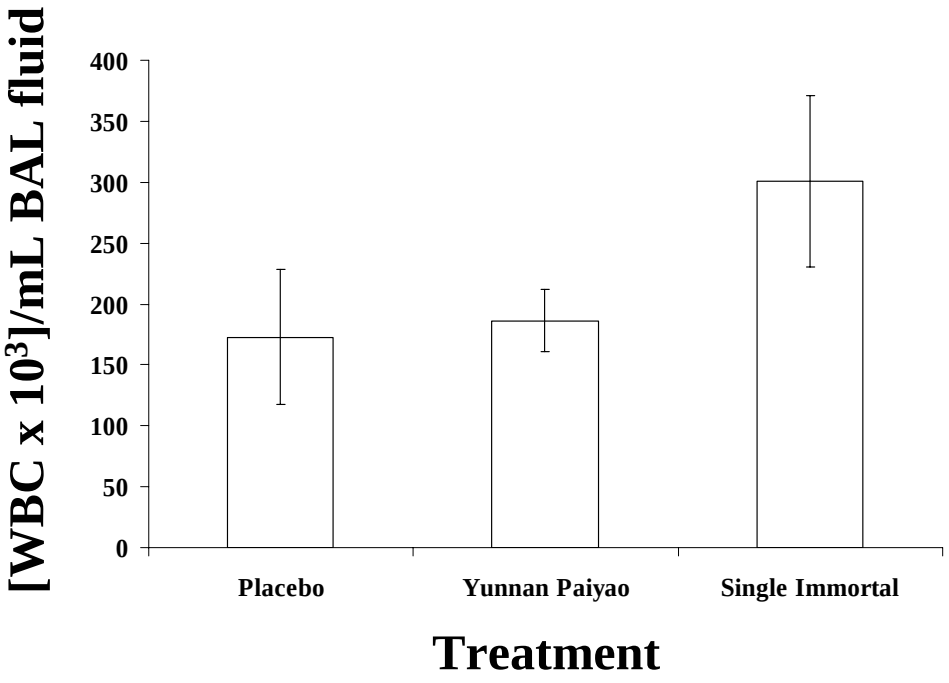
Neither RBCs/ml BAL fluid (Figure 2.1) nor WBCs/ml BAL fluid (Figure 2.2) were altered after treatment with *Yunnan Paiyao* or *Single Immortal*. However, treatment with *Single Immortal* increased the time-to-fatigue by a small but significant amount over both Yunnan Paiyao and placebo exercise tests (PL, 670 ± 10 s; YP, 665 ± 6 s; SI, 685 ± 8 s, $P <0.05$; Table 1). None of the other variables measured at maximal exercise (Table 2.1), including cardiorespiratory variables (heart rate, pulmonary arterial pressure, VO_2, and VCO_2, arterial blood gases, plasma lactate, acid-base variables, or hematological variables) were altered with treatment. Of the original five horses, one was dropped from the VO_2 and VCO_2 analysis because of technical difficulties during data collection. Coagulation variables including platelet numbers, fibrinogen, partial thromboplastin time and prothrombin time were not altered as a result of treatment with the herbal formulations (Table 2.2).

Figure 2.1 Exercise-induced pulmonary haemorrhage (EIPH) following maximal exercise and after treatment with either placebo or herbal formulations.



Severity of exercise-induced pulmonary haemorrhage following maximal exercise (n=5) as determined from the concentration of red blood cells (RBCs) in lung lavage fluid after treatment with placebo, *Yunnan Paiyao*, and *Single Immortal*. There were no significant differences between conditions (P>0.05).

Figure 2.2 Inflammatory response in bronchoalveolar lavage (BAL) fluid following maximal exercise and treatment with either placebo or herbal formulations.



White blood cells (WBCs) in lung lavage fluid following maximal exercise (n=5) after treatment with placebo, *Yunnan Paiyao* and *Single Immortal*. There were no significant differences between conditions (P>0.05).

Table 2.1 Time-to-fatigue, cardiorespiratory variables, arterial blood gas, and acid-base data at maximal exercise.

Variable	Control	Yunnan Paiyao	Single Immortal
Total Time-to-Fatigue (seconds)	670 ± 10	665 ± 6	685 ± 8*
Hematocrit (%)	63 ± 1	64 ± 1	63 ± 1
PaO_2 (mmHg)	61.8 ± 1.6	63.2 ± 1.5	64.4 ± 3.1
$PaCO_2$ (mmHg)	61.2 ± 2.8	62.0 ± 2.8	64.3 ± 2.3
pH	7.2 ± 030	7.18 ± 030	7.2 ± 020
Mean Peak Ppa (mmHg)	93.2 ± 5.9	94.0 ± 5.9	92.3 ± 5.2
VO_2max (L/min)	75.8 ± 1.5	76.2 ± 3.5	74.15 ± 2.0
VCO_2max (L/min)	80.7 ± 4.6	85.3 ± 3.4	77.9 ± 5.2
Heart Rate (beats/min)	212 ± 4	215 ± 5	213 ± 4
Respiratory Rate (breaths/min)	117 ± 4	117 ± 3	117 ± 3
Plasma Lactate peak;mmol/L)	26.6 ± 2.4	23.3 ± 2	24.3 ± 2.4

Values are means ± SE; * Significantly different from control and *Yunnan Paiyao* ($P < 0.05$). Partial pressure of oxygen (PaO_2); partial pressure of carbon dioxide ($PaCO_2$); pulmonary arterial pressure (Ppa); maximal oxygen uptake at STPD (VO_2max); maximal carbon dioxide production at STPD (VCO_2max).

Table 2.2 Coagulation Variables

Variable	Control	Yunnan Paiyao	Single Immortal	Statistical Significance
Platelets (x $10^3/\mu l$)	146,400 ± 11,587	146,750 ± 9035	136,600 ± 9415	NSD
Fibrinogen (mg/dl)	220 ± 20	180 ± 20	240 ± 40	NSD
Partial Thromboplastin Time (sec)	39.08 ± 1.99	37.84 ± 2.17	37.62 ± 2.12	NSD
Partial Thromboplastin Time Control (sec)	36.06 ± 0.99	36.56 ± 1.23	35.28 ± 1.10	NSD
Prothrombin Time (sec)	9.26 ± 0.21	9.16 ± 0.16	9.40 ± 0.26	NSD
Prothrombin Time Control (sec)	9.18 ± 0.16	9.22 ± 0.25	9.28 ± 0.21	NSD

Values are means ± SE; No significant differences (NSD) found in any variable except a possible trend for shortened bleeding times.

Discussion

This is the first study to investigate the impact of herbal formulations upon the severity of EIPH. These compounds (i.e. *Yunnan Paiyao* and *Single Immortal*) did not reduce the severity of EIPH in maximally exercising horses. Specifically, the RBC counts in the bronchoalveolar lavage fluid of horses treated with the herbal formulations did not differ significantly from that of horses treated with a placebo. There was, however, a small but statistically significant increase in the time-to-fatigue after herbal treatment with *Single Immortal* that may suggest the presence of some performance enhancing properties that are unrelated to the severity of EIPH per se.

Rationale for using herbal formulations to treat EIPH

Prolonged blood coagulation during exercise has been cited as a possible contributing factor to EIPH[21-24, 26]. Thus, increased clotting times following exercise-induced injury to the blood-gas barrier could theoretically exacerbate the severity of EIPH as a consequence of delayed sealing of damaged microvessels, thereby allowing an increased volume of blood to be extravasated. Indeed, exercise has been shown to diminish the ability of equine platelets to respond to platelet aggregating factors (i.e. adenosine diphosphate, collagen, and platelet activating factor) in both "EIPH-positive" and "EIPH-negative" horses[21-24]. Moreover, Bayly *et al.* [23] have shown that furosemide reduces the exercise-induced inhibition in platelet-induced aggregation to adenosine diphosphate, which was hypothesized to contribute to frusemide's ability to decrease EIPH.

Based on the theory that a haemostatic defect would exacerbate EIPH, herbal formulations that are purported to enhance coagulation have been considered as potential treatments for EIPH. The main herbal ingredient of *Yunnan Paiyao* is *Notoginseng*, which reduces bleeding time[37-39], thrombin time[39], clotting time[37, 38], and coagulation times[40], as well as initiating platelet release[35, 36] and decreasing fibrinogenemia[41]. The main herbal ingredients of *Single Immortal* are *Notoginseng* and *Bletillae*. *Bletillae* is effective as a vascular embolizing agent[42] (promotion of thrombin formation) as well as in decreasing bleeding time and thrombin time[39]. The rationale from a conventional "Western" medicine perspective that herbal formulations may be effective for treating EIPH is built on the premise that platelet function may be enhanced, since ingredients in these formulations have been shown to shorten bleeding time[37-39].

Possible explanations for the ineffectiveness of herbal formulations

The inability of herbal formulations to reduce EIPH in the present study may indicate that either impaired haemostasis is not a primary factor in the etiology of EIPH or that these formulations were not effective in addressing the specific coagulation problem. It should also be realized that diagnoses made by Traditional Chinese Medicine differ from "Western" medicine. Traditional Chinese Medicine depends on a holistic system of relationships between externally observed symptoms and internal organs to ultimately determine a pattern of illness versus addressing individual symptoms and diseases separately (i.e. EIPH). Therefore, a veterinarian utilizing the Traditional Chinese methods may assign different diagnoses to each individual horse in this population, and choose slightly different formulations to suit the individual evaluation of each animal. However, veterinarians trained in conventional "Western" medicine would diagnose all horses as having EIPH and treat them the same (as was done in the current study) with one of the herbal formulations empirically indicated for the reduction of pulmonary haemorrhage without taking into consideration the individual patterns of illness expressed by each horse[43].

Again, the contention that coagulation is compromised in horses with EIPH should be considered cautiously since horses in the coagulation studies[21-25] were designated as "bleeders" based upon a history of epistaxis with or without endoscopic evaluation. It has been shown by McKane *et al.* [1] and Meyer *et al.* [2] using BAL, that all strenuously exercised horses bleed to some

degree. In accordance with this, the statistical analysis of most data in the current literature has shown no difference in coagulation profiles between "bleeders" and "non-bleeders." With regard to demonstrating conclusively that impaired coagulability exists in exercising horses, major confounding variables include the timing of blood sample collection[44] (before, during, or after exercise, since alterations in coagulation are only evident transiently during exercise), the alteration of coagulation parameters as a result of increased fitness and exercise intensity (which tends to augment fibrinolysis[44] and decrease platelet function[23]), and the fact that *in vitro* processing of samples can artificially alter measurements of coagulation variables themselves[45] (i.e. partial thromboplastin time, prothrombin time, and platelet function).

Furthermore, Kociba *et al.*[46], in direct contrast to previous studies (that demonstrated a more pronounced decrease in the response of equine platelets from horses during exercise and those known to be "bleeders"[21, 24] to aggregating agents such as adenosine diphosphate[22-24, 26], collagen[26], and platelet activating factor[26]), found no association between exercise status and decreased platelet aggregation by adenosine diphosphate, or any alteration in coagulation variables including prothrombin time, partial thromboplastin time, and bleeding time. Instead, increases in platelet and fibrinogen concentrations and increased platelet retention with maximal exercise were observed in all horses. Moreover, there was no difference between frusemide and placebo with respect to augmenting any of the haemostatic values (prothrombin time, partial thromboplastin time, bleeding time, platelet and fibrinogen concentration, and platelet retention). In addition, recent data published by Kingston *et al.*[27, 45] demonstrated enhanced platelet aggregation in response to supramaximal exercise. Discrepancies between the results of the coagulation studies in exercising horses may be partially explained by the noteworthy finding in the Kingston *et al.*[27, 28] studies, that sodium citrate is not the anticoagulant of choice for evaluating the effects of exercise on equine platelet function since it clearly inhibits platelet aggregation. Therefore, it would be reasonable to consider the possibility that studies using sodium citrate as the anticoagulant may have falsely implicated diminished platelet function in exercising horses in the etiology of EIPH. This may also explain why herbal formulations designed to address this proposed coagulation problem failed to mitigate the haemorrhage.

Although no alterations have been demonstrated in prothrombin and partial thromboplastin times following exercise with either "bleeders" or "non-bleeders"[22-24, 26, 44], shortened whole blood clotting times and a trend for shortened prothrombin time and thrombin

time[23, 44] have been shown. This is also in agreement with the data of Kingston *et al.* [27, 45] which suggests that horse blood becomes transiently hypercoagulable during exercise. However, an increased fibrinolysis that is transient with exercise[44] has been observed and may attenuate or counterbalance the effects of this hypercoagulability.

The data collected from resting horses in our study demonstrated that coagulability variables including, platelet number, prothrombin time, and partial thromboplastin time were not altered with the herbal formulations, so they would not be expected to be further altered with exercise. Inferences concerning the effects of herbal treatments on cutaneous template bleeding times (which is dependent on the number and functional ability of circulating platelets responding to vascular injury)[47] would be speculative, since only a limited number of horses were evaluated using the standardized technique of Kopp *et al.*[48] (TS Epp, P McDonough, DJ Padilla, JH Cox, HH Erickson, and DC Poole unpublished data) 3 times before treatment (n=6; bleeding time 483 ± 31 seconds) and after administration of *Single Immortal* (n=2; bleeding time 413.75 ± 21.75 seconds; 2 observations), *Yunnan Paiyao* (n=1; bleeding time 585.0 ± 0.0 seconds; 1 observation), or cornstarch (n=3; bleeding time 615.0 ± 74.41 seconds; 3 observations). Bleeding times were only performed on a few horses since the primary objective of this study was to determine the efficacy of specific herbal formulations in reducing EIPH as evidenced by RBC counts in BAL fluid. In addition, our data along with that from several studies using this technique have reported high variability and large standard errors between and within animals[48] that make treatment effects if they exist, hard to detect[47]. However, from a retrospective point of view, conclusive evidence for shortened bleeding times is of little importance mechanistically since the herbal formulations did not diminish the severity of EIPH.

The overwhelming evidence from the current scientific literature suggests that a primary haemostatic deficiency is not present in the exercising horse[27, 28, 45]. This is also supported by the data from the current study, especially since herbal formulations designed to shorten coagulation did not reduce EIPH. Rather, the potential exists for an exercise-induced hypercoagulability with the formation of platelet-neutrophil aggregates[45, 49, 50] (evaluated by spontaneous echocardiographic contrast)[51] that may act to increase pulmonary arterial pressure by lodging in the microvasculature and consequently increasing EIPH. In fact, the tendency for EIPH to be increased with herbal treatments in the current study (Figure 1) suggests that the blood-gas barrier ruptures are not sealed more effectively with the current treatment.

Further evidence to suggest that a haemostatic deficiency does not exist in exercising horses comes from the work of Elliot *et al.* [52], in which rapid reversibility of capillary breaks (within three minutes of decreasing capillary pressure, which is less than the clotting time for horses) was observed and inferences made from the morphologic data suggested this to be the result of mechanical phenomenon (release of tension) rather than biological repair by cells (i.e. platelets). Subsequent data from West *et al.* [12] supported this work, since breaks in the blood-gas barrier of post-maximally exercised horses were difficult to find and were frequently associated with platelets and leukocytes plugging the breaks (implying no problem with coagulation). It has been shown that once the threshold pressure required to cause stress failure of the capillaries[53, 54] is reached, the incidence and severity of capillary breaks does not change as a function of time, suggesting that the degree of haemorrhage is determined simply by the amount of time the "critical pressure" is exceeded[52, 15] and not a delay in platelet plug formation.

Possible explanation for increased time-to-fatigue by Single Immortal

Time to fatigue was significantly increased when *Single Immortal* was administered to the horses versus *Yunnan Paiyao* or the placebo. It therefore appears that *Single Immortal* may have some ergogenic properties that are unrelated to the severity of EIPH. In a recent study, the treatment of horses with *Echinacea*[55] was found to have potential ergogenic effects as a haematinic agent, due to the increased size and concentration of RBCs in the peripheral circulation and the associated increased concentration of haemoglobin that may enhance oxygen transport and thus performance. However, similar effects were not observed in this study. Huang[56] reported that endurance was prolonged in animals treated with *Ginseng* root, and it may be that *Single Immortal* contains a larger proportion of the plant (whole herb versus the active ingredient affecting hemostasis) than *Yunnan Paiyao*. It is also possible that an ingredient in *Single Immortal* may have effects that are currently undetermined.

Conclusions

In conclusion, the herbal formulations *Yunnan Paiyao* and *Single Immortal* when used at the dosage and for the duration evaluated herein, were not effective in reducing EIPH severity in maximally exercising horses. The results of our experiment can be interpreted in at least two different ways. Specifically: 1) Haemostatic impairment (i.e. platelet function defect) does not contribute significantly to EIPH severity, or alternatively 2) The herbal treatment did not

successfully address the coagulability anomaly that is actually present. The potential performance enhancing capability of *Single Immortal* is worthy of further study in order to determine 1) if a longer time-to-fatigue may have offset any EIPH benefit derived from herbal treatment, and 2) the mechanism for this phenomenon. The findings of the present investigation should not be interpreted beyond the immediate context of the results obtained after treatment with Yunnan Paiyao and Single Immortal at this particular dose and duration. There also remains the possibility that other herbal formulations may be successful in reducing EIPH.

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CHAPTER 3 - EXERCISE-INDUCED PULMONARY HEMORRHAGE DURING SUB-MAXIMAL EXERCISE

ABSTRACT

Reasons for performing the study: Maximally exercising horses achieve mean pulmonary artery pressures (Ppa_{mean}) that exceed the minimum threshold (75 mmHg) estimated for pulmonary capillary rupture and exercise-induced pulmonary hemorrhage (EIPH). EIPH is not expected to occur during moderate sub-maximal exercise (i.e. 40-60% VO_2max) since Ppa_{mean} remains well below this threshold. **Hypothesis:** We tested the hypothesis that prolonged sub-maximal exercise (trotting) would precipitate locomotory respiratory uncoupling and cause EIPH. We expected EIPH to be present as a result of the most negative intrapleural pressures (as estimated by the minimum esophageal pressure (Pes_{min})) occurring simultaneously with the most positive Ppa (Ppa_{peak}) to produce estimated maximal pulmonary artery transmural pressures ($PATMP_{max}$) that surpass the EIPH threshold. **Methods:** Five Thoroughbred horses trotted to fatigue (~25 min) at 5 m/s on a 10% incline. Ventilation (V_E), Pes, and Ppa were measured at 5 minute intervals, and bronchoalveolar lavage (BAL) red blood cells (RBCs) were quantified 45 minutes post-exercise. **Results:** BAL revealed an increased number of RBCs/mL BALF (EIPH; rest: $2.0 \pm 1 \times 10^5$, exercise: $17 \pm 10 \times 10^5$ RBCs/mL BALF; $p < 0.05$). This occurred despite the highest Ppa_{mean} reaching only 55 ± 3 mmHg, whilst V_E (1197.5 ± 77.0 L/min), tidal volume (12.7 ± 0.5 L), and Pes_{min} (-31 ± 6 cmH_2O) approached 70-80% of the values achieved at maximal running speeds (10% incline: 12-13 m/s) by these same horses. The resulting $PATMP_{max}$ reached 110 ± 11 mmHg, well above the level considered causative of EIPH. **Conclusions:** The finding of significant EIPH during sub-maximal exercise broadens the spectrum of performance horses susceptible to EIPH and supports studies which suggest that extravascular factors are of primary importance in the etiology of EIPH. **Potential Relevance:** Consideration of strategies such as the equine nasal strip for reducing negative extravascular pressures is warranted even for exercise at moderate intensities.

Introduction

Exercise-induced pulmonary hemorrhage (EIPH) consequent to pulmonary capillary stress failure (West *et al.* 1993) occurs in almost all horses during short bouts of maximal intensity exercise (e.g. horse races; McKane *et al.* 1993, Meyer *et al.* 1998, Langsetmo *et al.* 2000). The major factor implicated in EIPH has been the prodigiously high pulmonary artery pressures (Ppa's), especially since diuretic treatment (i.e. furosemide) lowers both mean Ppa (Ppa_{mean}) and EIPH (Kindig *et al.* 2001a; Geor *et al.* 2001; McDonough *et al.* 2004). However, pulmonary artery transmural pressures (PATMP's; i.e. intravascular – extravascular forces) may be of greater importance than Ppa_{mean} alone since extremely negative alveolar and intrapleural (i.e. extravascular) pressures summate with the highly positive intravascular pressures to provide large distending forces across the delicate blood-gas barrier. In fact, there is strong evidence that PATMP's greater than 75-100 mmHg precipitate pulmonary capillary stress failure and EIPH (Birks *et al.* 1997; Langsetmo *et al.* 2000). Therefore, conditions such as laryngeal hemiplegia that obstruct the airway and increase the negativity of extravascular pressures (Jackson *et al*. 1997; Ducharme *et al.* 1999) exacerbate EIPH (Cook *et al.* 1988) whereas reducing nasal passage collapse and therefore upper airway resistance by means of the nasal strip (Holcombe *et al.* 2002) reduces EIPH (Poole *et al.* 2000; Geor *et al.* 2001; Kindig *et al.* 2001a; McDonough *et al.* 2004, Valdez *et al.* 2004).

Given that horses performing sub-maximal (moderate) exercise bouts maintain Ppa_{mean} (< 60 mmHg; Hopkins *et al.* 1998) well below the threshold mentioned above, the tacit assumption has been that such exercise bouts do not cause horses to suffer from EIPH. However, this assumption only takes into consideration the Ppa_{mean} and ignores the effects of the extravascular pressures on the PATMP. Consequently, almost all EIPH investigations in equids have focused upon maximal or near-maximal exercise. The one exception to this notion is the post-mortem study of Oikawa (1999) which suggested that EIPH may occur in horses exercised at sub-maximal speeds.

The current investigation utilized a sub-maximal exercise intensity protocol (brisk trot at 5 m/s on inclined treadmill until gait and head carriage were indicative of fatigue) known to generate high levels of ventilation (Bayly *et al.* 1995; Hopkins *et al.* 1998) and potentially high PATMP's whilst eliminating suprathreshold Ppa_{mean} (by keeping $Ppa_{mean} < 60$ mmHg) to test the hypothesis that such exercise could induce EIPH (as assessed by number of red blood cells

(RBCs) in the bronchoalveolar lavage (BAL) fluid). The presence of EIPH following moderate exercise as demonstrated herein may influence the design of training regimes, the frequency with which horses compete, and prophylactic measures taken when exercising horses.

Methods

Animals

Five fit Thoroughbred geldings with a history of EIPH, but otherwise certified as healthy by the attending veterinarian were utilized for this study; they ranged in age from 4-10 years and weighed 450-600 kg. The horses had been acquired from the racetrack 2-7 years prior to the study and were confirmed bleeders of varying degrees. The horses were housed in dry lots with shelters, fed concentrate (Strategy)[1] in addition to brome and alfalfa hay twice daily, and had free access to water and salt blocks. All horses were rotationally dewormed and vaccinated against rabies, Eastern/Western Encephalomyelitis, tetanus, Rhinopneumonitis, equine influenza, and West Nile virus. Each horse was conditioned to run on a high speed treadmill (Sato)[2] 3 times a week. Workouts were endurance and interval based protocols beginning several months prior to the experiment and involved extended periods of trotting and slow cantering on both the flat and inclined treadmill. All procedures used in this investigation were approved by the Kansas State University Institutional Animal Care and Use Committee.

Animal preparation, measurements, and calculations

Two 7-F introducer catheters were aseptically inserted into the right jugular vein in order to utilize a microtipped pressure transducer (Model SPC-471A)[3] and a thermistor catheter (Model 08407 Thermal Dilution Catheter)[4] to monitor Ppa and core body temperature, respectively. Pulmonary artery mean pressure (Ppa_{mean}) as well as the highest Ppa (Ppa_{peak}) were analyzed. Arterial blood samples were obtained from an aseptically placed 18 G catheter into either a previously elevated carotid artery or the transverse facial artery. Placement and calibration methods have been described previously (Meyer *et al.* 1998; Kindig *et al.* 2001a, b, 2003; McDonough *et al.* 2004). An air-filled esophageal balloon catheter connected to a differential pressure transducer (Model MC1-3-871)[5] was placed at the mid-thoracic level at the base of the heart to obtain an indirect estimate of intrapleural pressure (esophageal pressure (Pes); Art *et al.* 1988) in the same horizontal plane as the dorsal portion of the lung (Langsetmo

et al. 2000; Kindig *et al.* 2003), which is the predominant site of EIPH. The Pes waveforms were subsequently analyzed to resolve the highest positive Pes (Pes_{peak}), the most negative Pes (Pes_{min}), and the magnitude of positive to negative change in Pes ($Pes_{peak} - Pes_{min}$; Pes_{swing}) during breathing. The maximal estimated value for pulmonary artery transmural pressure ($PATMP_{max}$) was calculated by subtracting the Pes_{min} from the Ppa_{peak} after converting Pes_{min} from cmH_2O to mmHg. Horses were also instrumented with a heart rate monitor (Polar®)[5] for determination of heart rate (HR) during exercise.

Ventilation, pulmonary gas exchange, and blood gases

Ventilation and inspiratory airflow were measured on a breath-by-breath basis using ultrasonic phase-shift flowmeters (Model FR-41eq)[7] and expired O_2 and CO_2 concentrations for gas exchange were determined using a mass spectrometer (Perkin-Elmer Medical Gas Analyzer Model 1100)[8], as described previously (McDonough *et al.* 2002a, b). All systems were interfaced with a computer-based data acquisition system (Po-Ne-Mah Data Acquisition System)[9] and standard equations were used for calculation of minute ventilation (V_E) and gas exchange variables including oxygen uptake (VO_2), carbon dioxide production (VCO_2), and ventilatory equivalent for CO_2 (V_E/VCO_2), (Kindig *et al.* 2001a; McDonough *et al.* 2002a, b; Padilla *et al.* 2004, McDonough *et al.* 2004). Anaerobically collected arterial blood samples were analyzed (Nova Stat Profile)[10] for blood gases including partial pressure of arterial oxygen (P_aO_2) and carbon dioxide (P_aCO_2), pH (corrected to core body temperature; Fedde 1991), plasma lactate [La-], and hematocrit (Hct).

Experimental protocol

Maximal HR (HR_{max}; highest HR achieved during exercise bout) and maximal VO_2 (VO_{2max}; highest VO_2 achieved during a maximal incremental exercise bout) was available for all horses from prior maximal exercise test runs. Each of the five horses completed a sub-maximal exercise test on a 10% incline consisting of a 4 minute warm-up at 3 m/s followed by an increase in speed to 5 m/s (~60% of VO_{2max} and ~ 75% of HR_{max}; unpublished data McDonough and Epp) until the horses reached 42˚C core body temperature or fatigued (24.7 ± 1.6 minutes). Since horses performing this type of exercise do not tend to drop to the back of the treadmill when fatigued, the point of fatigue was judged as when the horses changed to a shuffling gait, eyes became dull, and/or ears began to droop. To offset potential dehydration, 5 L of lactated Ringers

solution was administered intravenously during recovery, while the horses were cooling down at a trot on the treadmill. Ventilatory (inspiratory flow, V_E, V_T (tidal volume), and b_f (breathing frequency)), cardiorespiratory (VO_2, VCO_2, and HR), core body temperature, and pressures (Ppa and Pes) variables were collected continuously throughout the test and averaged every five minutes, while blood samples (blood gases, lactate) were collected every 5 minutes during the exercise bout.

Brochoalveolar lavage

The technique of BAL was chosen over endoscopy to evaluate the quantity of EIPH due to the increased sensitivity of the technique in detecting significant, but modest amounts of hemorrhage. We have also demonstrated that the soft and flexible lavage tube does not damage the airway epithelium and cause bleeding (Meyer et al. 1998). The pre-exercise baseline BAL samples were taken from the horses rested for at least 10 days before the initiation of the study. The presence of RBCs in the BAL as a result of exercise is not detectable after 7-10 days post-exercise (Meyer *et al.* 1998). A minimum of 7 days was allowed to elapse from the baseline BAL to the BAL obtained 45 minutes after the exercise test since this has been shown by (Clark *et al.* 1995) to allow baseline cellular populations to re-establish between lavages. For all lavages, the horses were sedated using detomidine hydrochloride (Dormosedan®; 5-10 µg/kg IV)[11] and butorphanol tartrate (Torbugesic®; 5-10 µg/kg IV)[12] to facilitate BAL (Meyer *et al.* 1998; Kindig *et al.* 2001a). A BAL tube (Bivona VBAL30)[13] with an inflatable cuff was introduced into the right naris through the ventral meatus, and into the lung until wedged in a sub-segmental bronchus of the dorsal caudal portion of the lung (McKane and Rose 1993). The Bivona tube with a cuff created a seal within the airway, which ensured lavage of the distal airway and maximized recovery of lavage fluid. A total of 300 mL (in 50 mL aliquots) of 0.9% physiologic saline was infused. After approximately two breaths, the fluid (a percentage of the entire 300 mL) was aspirated with gentle suction. The BAL fluid was centrifuged (Beckman TJ-6)[14], the supernatant decanted, and the pellet was resuspended in 0.9% saline (Lapointe *et al.* 1994). The amount of saline used for resuspension ranged from 10-25 mL depending on the severity of EIPH. The goal was to maintain a relatively similar RBC-to-saline solution ratio (i.e. lavage fluid hematocrit) to decrease any variability inherent in the counting procedure. Red blood cells (RBCs) were counted (Meyer *et al.* 1998; Kindig *et al.* 2001a, McDonough *et al.*

2004) using a hemocytometer (Fisher No. 02-671-5)[15] and a microscope (Nikon)[16]. EIPH data are presented as RBCs per milliliter of recovered BAL fluid minus tube dead space (17 mL).

Statistical Analysis

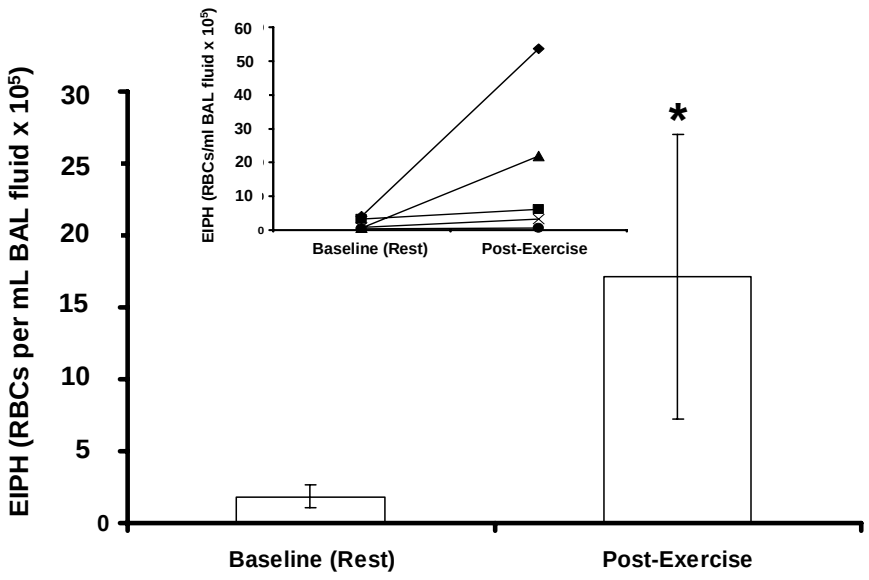
A one-way analysis of variance with repeated measures was used to determine whether differences existed between the different time periods of the sub-maximal exercise bout (i.e. 5, 10, 15, 20, and 25 minutes or end-exercise) for cardiorespiratory and metabolic variables. When significance was revealed, the point of significance was identified using a Student-Newman-Kuels or Least Squares post-hoc test. EIPH, represented by RBCs/mL BAL fluid was analyzed using a student's paired t-test. Pearson Product Moment Correlations were used to determine relationships between variables. Statistical significance was accepted at $p \leq 0.05$ level. The Sigma Stat 3.0[17] statistical package was used to analyze the data.

Results

Exercise-induced pulmonary hemorrhage

The exercise challenge resulted in an increased EIPH ($17 \pm 10 \times 10^5$ RBCs per mL BAL fluid) over baseline ($2 \pm 1 \times 10^5$ RBCs per mL BAL fluid) values (Fig 3.1). The BAL recovery volume averaged 55.5 ± 3.1 and $53.5 \pm 7.2\%$ for baseline and exercise testing, respectively. A non-significant association was observed between $PATMP_{max}$ versus EIPH ($r = 0.84$; $p = 0.07$).

Figure 3.1 Exercise-induced pulmonary hemorrhage (EIPH) following sub-maximal exercise bout.

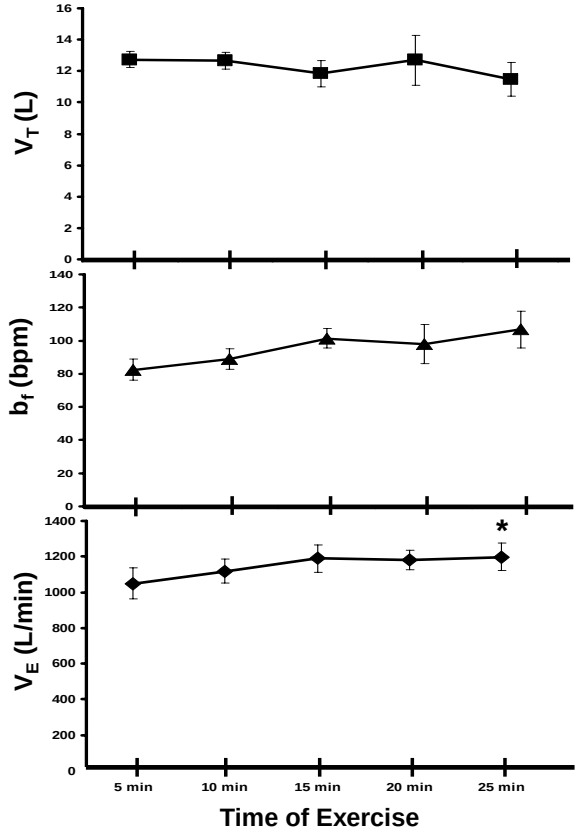


This bar chart demonstrates the increase (number of horses = 5) in exercise-induced pulmonary hemorrhage (EIPH) following sub-maximal exercise as determined from average number of red blood cells (RBCs) in lavage fluid. Data are presented as mean ± SE. *Post-Exercise RBCs/mL > Baseline (Rest) RBCs/mL; ($p < 0.05$). Inset illustrates individual horse results.

Minute ventilation, tidal volume, and breathing frequency

The ventilatory variables including V_E, V_T, and b_f are illustrated in Figure 3.2. From 5 minutes to end-exercise at 5 m/s there was an increase in V_E from 1048.9 ± 88.1 L/min to a final value of 1197.5 ± 77.0 L/min. No differences were noted for in b_f or V_T, (Fig 2) whilst V_E/VCO_2 increased (Table 3.1) from 5 minutes to end-exercise. Inspiratory flow increased over the last 15 minutes of the exercise bout (Table 3.1).

Figure 3.2 Ventilatory variables during sub-maximal exercise bout.



Minute ventilation (V_E; solid diamonds), tidal volume (V_T; solid squares) and breathing frequency (b_f; solid triangles) plotted as a function of exercise time in minutes. Sub-maximal exercise was performed at 5 m/s. Number of horses = 5. *Mean V_E at 25 minutes is greater than that at 5 minutes, $p < 0.05$.

Table 3.1 Cardiorespiratory and metabolic variables during sub-maximal exercise.

Variable	5 minutes	10 minutes	15 minutes	20 minutes	25 minutes
VO_2 (L/min)	49.6 ± 2.9	51.2 ± 3.5	51.1 ± 4.9	48.5 ± 4.6	45.4 ± 2.2
VCO_2 (L/min)	47.2 ± 3.2	50.8 ± 2.4	44.7 ± 3.6	40.8 ± 4.0b	39.9 ± .3b
V_E/VCO_2	22.2 ± 1.3	22.0 ± .5	26.9 ± 1.4	29.8 ± 2.7a,b	30.0 ± 1.7a,b
Inspiratory Flow (L/s)	34.0 ± 2.6	35.8 ± 1.6	39.8 ± 2.4a,b	40.2 ± 1.0a,b,c	41.2 ± 1.6a,b,c
PaO_2 (mmHg)	80 ± 5	86 ± 2a	89 ± 2a,b	92 ± 3a,b,c	97 ± 2a,b,c,d
$PaCO_2$ (mmHg)	45.4 ± 1.3	47.6 ± 1.8	46.9 ± 2.4	46.6 ± 2.5	45.6 ± 2.9
Ppa_{peak} (mmHg)	82 ± 6	85 ± 6	85 ± 6	87 ± 7	85 ± 2
Pes_{swing} (cmH_2O)	56.2 ± 4.3	58.7 ± 3.2	60.2 ± 5.7	64.5 ± 11.9	60.6 ± 12.3
Pes_{min} (cmH_2O)	-23.7 ± 2.7	-28.1 ± 1.9	-29.1 ± 3.0	-31.3 ± 6.4	-29.3 ± 5.0
HR (bpm)	161 ± 3	161 ± 3	161 ± 4	166 ± 5	167 ± 7
Plasma [La^-] (mmol/L)	2.2 ± .4	1.6 ± .3	2.0 ± .5	3.3 ± 1.1	5.4 ± 1.8a,b,c
Hct (%)	55.8 ± 1.4	54.2 ± 1.7	53.4 ± 1.7	53.8 ± 1.3	55.6 ± 2.2
CBT (˚C)	38.2 ± .4	39.3 ± .4a	39.9 ± .4a,b	40.8 ±.4a,b,c	41.4 ± .5a,b,c,d
pH	7.47 ± .01	7.45 ± .01	7.46 ± .01	7.46 ± .02	7.46 ± .02

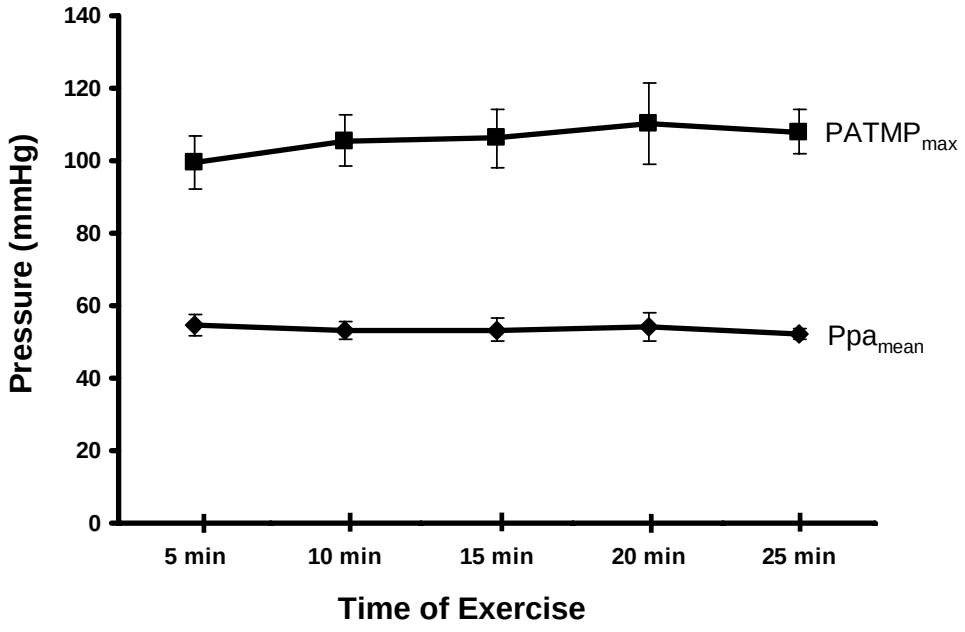
The data for 5 horses are included in the table. Values are 5 minutes averages over the 25 minutes exercise period at 5 m/s and are presented as mean ± SE. VO_2: oxygen uptake, STPD; VCO_2: carbon

dioxide elimination, STPD; V_E/VCO2: ventilatory equivalent for CO_2; PaO_2: temperature corrected partial pressure of oxygen in the arterial blood; $PaCO_2$: temperature corrected partial pressure of carbon dioxide in the arterial blood; Ppa_{peak} highest pulmonary arterial pressure; Pes: esophageal pressure; Pes_{min}: most negative Pes; Pes_{swing} (maximum change in esophageal pressure) = Pes_{peak} (most positive Pes) – Pes_{min}); [La^-]: plasma lactate concentration; HR: heart rate; Hct: hematocrit; and CBT: core body temperature. Significant differences are indicated by superscripts: a=different from 5 minutes, b=different from 10 minutes, c=different from 15 minutes, and d=different from 20 minutes. Values with the same superscript were not different from each other.

Pulmonary artery and maximum pulmonary artery transmural pressure

The average Ppa_{mean} for the 25 minute duration at 5 m/s was 54 ± 1 mmHg (Fig 3.3), and both Ppa_{mean} and Ppa_{peak} were stable throughout exercise. The average $PATMP_{max}$ (Ppa_{peak} – Pes_{min}) was 106 ± 8 mmHg for the same time period (Fig 3.3).

Figure 3.3 Pulmonary artery (Ppa) and pulmonary artery transmural ($PATMP_{max}$) pressures during sub-maximal exercise.



The maximum pulmonary artery transmural pressure ($PATMP_{max}$ = Ppa_{peak} (highest Ppa) – Pes_{min} (lowest esophageal pressure (Pes)); solid squares) and mean pulmonary artery pressure (Ppa_{mean}; solid diamonds) at each time point during sub-maximal exercise. Number of horses = 5. Exercise was performed at 5 m/s.

Cardiorespiratory, metabolic, and hematological variables

VO_2, $PaCO_2$, Pes_{min}, Pes_{swing}, pH, HR, and Hct, remained relatively constant throughout the 25 minute duration of the 5 m/s exercise test (Table 3.1). PaO_2 and core body temperature progressively increased throughout the exercise bout. The peak VCO_2 at 10 minutes (5 m/s; 50.8 L/min) was greater than that measured at end-exercise (39.9 L/min; Table 3.1). Plasma lactate significantly increased from 5 minutes to end-exercise (Table 3.1).

Discussion

Contrary to the widely held belief that EIPH can only be induced by maximal or near-maximal exercise, this investigation demonstrates that a significant level of EIPH occurs during sub-maximal exercise in known bleeders. This finding supports Oikawa's (1999) post-mortem observations that a group of 1-2 year old Thoroughbred horses exercised at speeds not exceeding 8.5 m/s, displayed EIPH lesions in the dorsocaudal lung region.

Contribution of pulmonary artery pressure to EIPH

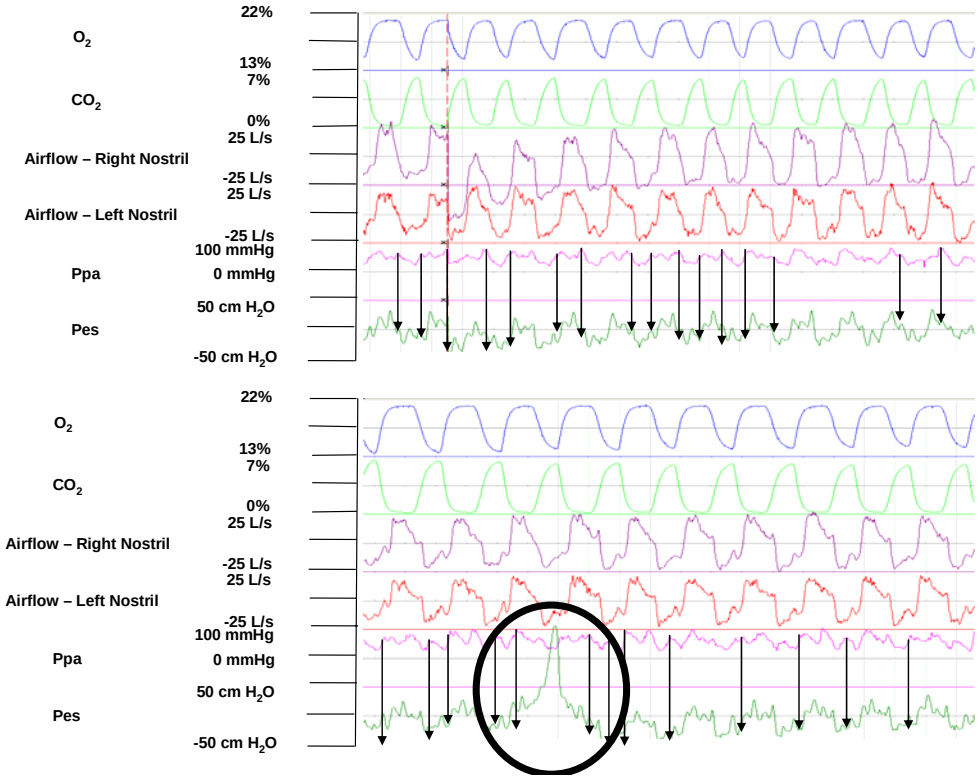
The most widely accepted mechanism for acute mechanical failure of the blood-gas barrier is the achievement of extraordinarily high Ppa_{mean} in maximally exercising horses (Erickson *et al.* 1990, 1992; West *et al.* 1993). However, when considering the contribution of the Ppa by itself in the sub-maximally exercised horses used in the current investigation, Ppa_{mean} only reached 55 mmHg, which is far below the Ppa_{mean} commonly measured at maximal exercise (90 - 120 mmHg; Erickson *et al.* 1990; Kindig *et al.* 2001a, b). Pressures above 90 mmHg are high enough without taking the extravascular forces into account to cause rupture of the pulmonary capillaries and thus EIPH according to the hypothesized PATMP threshold (Birks *et al.* 1997; 75-100 mmHg; Langsetmo *et al.* 2000; ~ 90 mmHg). It is pertinent, however, that the significant EIPH evidenced herein was well below that typically observed in these same horses at maximal exercise (EIPH = 4-64 x 10^6 RBCs/ml BAL fluid; Kindig *et al.* 2001a, b, 2003; Hildreth *et al.* 2003; McDonough *et al.* 2004; Epp *et al.* 2005). One potential reason for this lower EIPH follows from the Elliot *et al.* (1992) study which suggested that once the critical pressure has been reached (presumably either by elevation of intravascular or by a combination of intravascular and extravascular pressures), the severity of the hemorrhage will be dependant upon the amount of time the pressures are sustained, since breaches seal rapidly once pressures are lowered. In other words, EIPH of mild-to-moderate severity may be observed during

moderate intensity sub-maximal exercise because tears in the blood-gas barrier that occur as a result of isolated instances of high PATMP are rapidly sealed as the mean intravascular pressure is far below that necessary to cause continued extravasation. In fact, EIPH may occur along a continuum as exercise intensity increases, as the Ppa_{mean} will be elevated above the critical pressure for progressively longer periods of time as treadmill speed is increased (i.e. Poole *et al.* 2000 [12 m/s; 18 x 10^6 RBCs/mL BAL fluid], Kindig *et al.* 2001a [14 m /s; 55 x 10^6 RBCs/mL BAL fluid], McDonough *et al.* 2004 [15-16 m/s; 64 x 10^6 RBCs/mL BAL fluid]).

Contribution of the airways to EIPH

Trotting (sub-maximal exercise), as opposed to galloping (maximal exercise), does not evoke 1:1 locomotory-respiratory coupling (Attenburrow and Goss 1982; Hornicke *et al.* 1983; Bramble and Carrier 1983; Bayly *et al.* 1995; Hopkins *et al.* 1998) and allows for variable breathing strategies, primarily associated with changes in the ratio of inspiratory to expiratory time (Bayly *et al.* 1995; Hopkins *et al.* 1998). This alteration in inspiratory timing allows for horses to take larger breaths which in turn causes the Pes_{min} to become even more negative and this behavior may be repeated over a longer time period during prolonged exercise at sub-maximal intensities. These larger and longer breaths ultimately result in an increased incidence of pressure waveform (i.e. Ppa_{peak} and inspiratory Pes_{min}) superimposition or summation (Fig 3.4). This occurrence is in contrast to maximal exercise when uncoupling rarely (if ever) occurs (Erickson *et al.* 1990; Langsetmo *et al.* 2000), and may be critical in achieving the high $PATMP_{max}$ found herein. Moreover, the V_E (Fig 3.2) required for sub-maximal exercise is increased out of proportion to the metabolic rate (increased V_E/VCO_2), especially at end-exercise (Table 3.1). The resulting elevation of V_E in these moderately-exercised horses was such that the inspiratory effort (Hopkins *et al.* 1998) and associated Pes_{min} (Table 3.1) were sufficient when combined with the intravascular pressures (especially when in-phase with Ppa_{peak}; $PATMP_{max}$ versus EIPH, r=.84 (p=0.07); Fig 3.3) to exceed the PATMP threshold, initiating rupture of the pulmonary capillaries. In other words, sub-maximal exercise levels increased the opportunity for extravascular (i.e. V_T, V_E, and Pes_{min}) rather than intravascular (Ppa) pressures to cause EIPH.

Figure 3.4 Cardiorespiratory data tracings from a representative horse during sub-maximal exercise bout.



Tracings from representative horse including inspired/expired O_2%, inspired/expired CO_2%, inspiratory flow (right and left nostrils; L/min), pulmonary arterial pressure (Ppa; mmHg), and esophageal pressure (Pes; cmH_2O). Arrows represent points on tracing where peak or near peak Ppa's (Ppa_{peak}) occur concurrently with highly negative inspiratory Pes (minimum Pes; Pes_{min}) when horses are not coupled in a 1:1 locomotory respiratory coupling and taking occasional larger breaths. Note: Absolute superimposition of peak Ppa and end inspiratory Pes leads to maximum pulmonary arterial transmural pressures ($PATMP_{max}$), as $PATMP_{max} = Ppa_{peak} – Pes_{min}$. Circle helps visualize maximization of PATMP's on either side of an outstandingly large breath.

Further support for an extravascular mediator of EIPH in the present investigation relates to the relatively large V_T values achieved during sub-maximal exercise (i.e. greater than would be observed at the same speed during an incremental exercise test; McDonough *et al.* 2002b). Fu *et al.* (1992) demonstrated a greater incidence of capillary rupture at higher versus lower lung volumes in rabbit lungs. That publication considered that the greater alveolar and intrapleural pressures associated with large lung volumes caused increased stretching and therefore fragility of the capillaries which ultimately resulted in increased capillary rupture. Furthermore, Kindig *et al.* (2003) showed that during maximal exercise on an inclined treadmill the increased V_T was associated with greater EIPH, even in the presence of slightly lower Ppa_{mean}. The horses in the

current study evidenced V_T's similar to those measured at maximal exercise by Kindig *et al.* (2003) whilst demonstrating much lower Ppa_{mean}, and attenuated EIPH. Therefore, the present novel finding that sub-maximal exercise induces EIPH, combined with the nasal strip and upper airway obstruction studies, supports the hypothesis that extravascular factors are important in initiating and modulating EIPH.

Diagnosis of EIPH with bronchoalveolar lavage (BAL)

Sweeney and Soma (1983) as well as Hopkins *et al.* (1998) found no evidence that bleeding occurs in competitive endurance horses or horses exercised at sub-maximal levels and this may be due to less sensitive diagnostic methods being employed: epistaxis and endoscopy versus bronchoalveolar lavage. The small but significant level of bleeding observed in the current study did not cause epistaxis and would most likely have been undetectable by bronchoscopy alone (Meyer *et al.* 1998; Langsetmo *et al.* 2000), thus requiring the sensitivity of the BAL technique for diagnosis (Kindig *et al.* 2001a). Notwithstanding the inability to assess global lung blood volumes, BAL allows reproducible sampling (Kindig *et al.* 2001a) from the dorsocaudal lung region (McKane *et al.* 1993) which is the predominant location of EIPH.

Future studies

The knowledge that bleeding occurs during sub-maximal exercise is important and relevant to equine health because even small amounts of blood may, over time, result in a chronic inflammatory response, consequent scarring (McKane and Slocombe 2002; Robinson and Derksen 1980), and associated sequelae (bronchiolitis and fibrosis; O'Callaghan *et al.* 1987a, b). Therefore, future studies investigating non-pharmacological alternatives such as the nasal strip to mitigate EIPH during sub-maximal training may prove to be beneficial to the overall pulmonary health of the equine athlete.

In summary, significant levels of EIPH occurred during prolonged sub-maximal exercise in a population of horses with a history of EIPH, despite only modest increases in pulmonary intravascular pressures. This knowledge identifies EIPH as a potential problem in horses performing at lower intensities (i.e. endurance horses) and may alter perceptions regarding the need for prophylactic measures and alterations in training regimes in these horses.

Manufacturers' addresses

[1] Purina Mills Incorporated, St.Louis, Missouri, USA.

[2] Sato Incorporated, Uppsala, Sweden.

[3] Millar Instruments, Incorporated, Houston, Texas, USA.

[4] Columbus Instruments, Columbus, Ohio, USA.

[5] Validyne, Northridge, California, USA

[6] Polar Horse Heart Monitors, Mill Valley, California, USA.

[7] Flowmetrics-BRDL, Birmingham, UK

[8] Perkin-Elmer, Pomona, California, USA.

[9] Gould Instrument Systems, Valley View, Ohio, USA.

[10] Nova Biomedical, Waltham, Massachusetts, USA.

[11] Pfizer Animal Health, Exton, Pennsylvania, USA.

[12] Fort Dodge Animal Health, Fort Dodge, Iowa, USA.

[13] Smiths Medical, Philadelphia, Pennsylvania, USA.

[14] Beckman Instruments, Incorporated, Palo Alto, California, USA.

[15] Fisher Scientific, Pittsburgh, Pennsylvania, USA.

[16] Nikon, Incorporated Instrument Group, Garden City, New York, USA.

[17] SPSS, Incorporated, Chicago, IL, USA.

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CHAPTER 4 - THE OCCURRENCE AND SEVERITY OF EXERCISE-INDUCED PULMONARY HEMORRHAGE IN RACING GREYHOUNDS

Abstract

Exercise-induced pulmonary hemorrhage (EIPH) has been recognized as a major health concern in performance horses, but the incidence and severity of this condition in racing Greyhounds has received little attention. Equids and Greyhounds share many physiological characteristics related to their prodigious athletic ability. However, there are structural and functional differences that may protect the racing Greyhound from the extreme EIPH experienced by horses. Thus, we hypothesized that EIPH would be present, but the severity reduced in racing dogs as compared to horses. To test this hypothesis, we had Greyhound dogs (n=6) run a simulated 5/16 mile race on two occasions. Bronchoalveolar lavage (BAL) was performed at weekly intervals throughout the investigation to examine the number of red blood cells (RBCs), white blood cells (WBCs)/differentials, and hemosiderophages in the lungs of Greyhounds before, immediately after, and for 4 weeks following the 5/16 mile race. Maximal heart rate for the Greyhounds during the race was 230 ± 1 bpm and 10 minute post-exercise venous lactate was 18.6 ± 0.4 mmol/L. No epistaxis or pink froth was observed at the nose or mouth of any of the dogs. The RBCs in the lavage fluid demonstrated a significant increase immediately post-exercise (Baseline = 109.6 ± 11.7 x 10^3 RBCs/mL BAL fluid; Run = 292.3 ± 69.9 x 10^3 RBCs/mL BAL fluid), returning to baseline by 1 week post-exercise (149.2 ± 46.2 x 10^3 RBCs/mL BAL fluid). Percent hemosiderophages were not different for any of the measurement periods. The number of WBCs/mL BAL fluid decreased from baseline and run values at 2, 3, and 4 weeks post-exercise. Along with the decreased WBC counts during the weeks post-exercise, alveolar neutrophil numbers were decreased from baseline and run values for 4 weeks post-exercise. Despite the reduced number of WBCs, macrophage percentages were increased from baseline and run 1 by one week post-exercise and remained elevated for 2 weeks before returning to baseline. These results (i.e. increased RBC numbers and non-elevated WBC counts) demonstrate that these Greyhounds did not experience EIPH nor inflammatory airway

disease to the same degree described in horses, and therefore this condition may not be as detrimental to racing canines as to horses.

Introduction

Exercise-induced pulmonary hemorrhage (EIPH) has been reported to occur in nearly all horses[1-4], as well as camels[5], humans[6, 7], and dogs[8, 9] to some degree following strenuous exercise. Although this condition has been well documented in the Thoroughbred horse since the 16th century[10] and researched extensively, only two preliminary reports provide evidence that Greyhounds experience EIPH[8, 9].

In horses it has been determined that the etiology of EIPH is multifactorial, with high pulmonary artery pressures[11, 12], large airway pressure swings[3, 13-22], inflammatory airway disease[1, 23-29], and ground impact forces[30-32] thought to be the major contributing factors. The exercise response of Greyhounds is physiologically similar to that of the horse in many ways. The Thoroughbred horse and racing Greyhound are both exceptional sprinters[33-35] (~ 18 m/s) with massive cardiac outputs[33-36] (0.6 L/kg/min, 1.0 L/kg/min), and exceptional elevations in packed cell volume (PCV; 70, 66 %)[34, 35, 37, 38] during maximal exercise, respectively. The pulmonary capillary transmural pressure required for rupture in the canine pulmonary capillary (66-70 mmHg)[39, 40] is similar to the mean pulmonary artery pressure (Ppa) reported for the Greyhound during sub-maximal exercise at 11m/s (approximately 55 mmHg)[41]. Thus, it is likely that the Greyhound approaches or exceeds this "threshold for capillary rupture" during maximal exertion. One important difference between canine and equine athletes is that horses are obligate nasal breathers during exercise. This, in combination with a very long trachea, causes the development of exceptionally negative extravascular pressures, which summate with the intravascular pressures to create very high transmural pressures in the horse. As the Greyhound is not an obligate nasal breather (as is the horse) during exercise and has a much shorter trachea, these factors may act to limit the fall in alveolar pressure[42-44] experienced during maximal effort ventilation and reduce the potential for severe EIPH in this species.

While pulmonary capillary transmural pressures may be lower than that in the horse, the strength of the pulmonary capillary alveolar interface is lower in the dog compared with that in the horse[40]. Specifically, subtracting esophageal pressure (Pes) from mean Ppa to estimate pulmonary artery transmural pressure, a value of greater than 65 mmHg (close to canine stress

failure threshold) is estimated during sub-maximal exercise. Thus, it should not be surprising that preliminary data suggests that Greyhounds do experience EIPH during maximal sprint exercise[8, 9].

This investigation was designed to determine whether Greyhounds run over the standard 5/16 mile course demonstrate significant EIPH, and if present, outline the timeline for resolution. Specifically, we tested the hypothesis that Greyhounds would exhibit significant EIPH and that the time course of recovery (i.e. disappearance of red blood cells (RBCs), elevation of WBCs, and hemosiderophage emergence, peak, and decline) would follow a similar pattern to that documented for the horse.

Methods

Animals

Six healthy Greyhound dogs that had been raced on the track were acquired for the study. The group consisted of 4 intact females and 2 intact males ranging in age from 2-4 years and weighing 29.0 ± 1.2 kg. They were housed in a temperature controlled building (70-74˚ F with 30-70% humidity). They had 3 square foot inside their pen with free access to a 792 square foot sand run. Indoor conditions were on a 12:12 hour light:dark cycle. The dogs were fed Iams (Iams Mini Chunks®, Iams Company, Dayton, OH, USA) adult dog food (minimum of 26.0% crude protein, minimum of 15.0% crude fat, maximum of 5.0% crude fiber, and maximum of 10.0% moisture) once daily in the morning and had free access to water. The Greyhounds were current on vaccinations including Distemper Virus, Adenovirus, Parainfluenza Virus, Parvovirus, and Bordatella Bronchiseptica as well as being on monthly Heartguard Plus. All procedures used were approved by the Kansas State University Animal Care and Use Committee.

Animal Preparation

The Greyhounds were fed 3 hours before estimated run time. The dogs were transported in a climate-controlled dog trailer approximately 50 miles to a local training track. The Greyhounds were outfitted with heart rate monitors (Polar®; Polar Horse Heart Monitors, Mill Valley, CA, USA) attached by a harness made of elastic and secured with racing slinkys in order to determine maximal heart rate during sprinting. The heart rate monitors have data storage capacity and this data was downloaded to a personal computer for post-run analysis.

Exercise Protocol

Greyhounds were divided into 2 heats with 3 dogs each heat and run on the track in a race-simulated timed run (5/16 mile distance) around a track, chasing a lure. Within 10 minutes of completing the run a venous blood sample was obtained from the jugular vein with a 22 gauge one inch needle attached to a 3 cc heparinized syringe with a stopcock for post-exercise plasma lactate analysis. Greyhounds were not formally exercised post-run, but did have access to their runs. A second identical run (run 2) was performed two to three weeks after RBC and WBC counts had stabilized near baseline levels (~7 weeks post-run 1).

Bronchoalveolar Lavage

The initial baseline BAL was performed and cultures obtained after the dogs had not been formally exercised or raced for 1-2 weeks. The initial post-run lavage was performed approximately 2 hours post-exercise under general anesthesia in order to quantify the number of RBCs in the lung, and therefore, the severity of EIPH. Briefly, after intramuscular pre-medication with atropine (0.025 mg/kg), morphine (0.25 mg/kg), and valium (0.2 mg/kg) had taken effect, the dogs were induced with intravenous propofol (6 mg/kg; PropoFlo™, Abbott Laboratories, North Chicago, IL, USA), intubated and placed on supplemental oxygen. Dogs remained in sternal recumbency for the procedure. They were maintained with an intravenous infusion of propofol (0.4 mg/kg/min) to facilitate BAL. Heart rate, systemic arterial pressure, oxygen saturation, respiratory rate/character, mucus membrane color, and depth of anesthesia were monitored by a technician. After the animals were stabilized under anesthesia, a flexible modified foal stomach tube[45] (Argyle stomach tube, Sherwood Medical, Company, St. Louis, MO, USA) was wedged in a sub-segmental bronchus of the caudal dorsal lung lobe after insertion through the endotracheal tube. Before insertion, the distal end was transected with a sterile blade above the most proximal fenestration (approximately 6 cm from the distal end). A sterile, hand-held metal pencil sharpener was used to round the distal end of the tube and create a slight taper to enhance formation of a tight seal when the tube was wedged in a bronchus of the caudal lung lobe. During insertion once resistance was felt, an attempt was made to gently withdraw and reposition the catheter, ensuring placement in the most distal airway possible. A Christmas tree adapter was placed on the proximal end of the BAL catheter. Following placement of the catheter, 50 mL aliquots of sterile, isotonic saline (0.9%), warmed to body

temperature, were slowly infused. After a short delay (approximately 2 breaths), the fluid was aspirated with gentle suction while chest copage was performed bilaterally. This procedure was repeated 3 times, equivalent to a dose of 5 mL/kg of instilled fluid/dog (approximately 125-150 mL total per dog). Supplemental oxygen was administered after the procedure until extubation and provided by mask thereafter until the dog recovered. The dogs were monitored every 4-6 hours after the procedure for signs of respiratory distress, pale mucus membranes, or prolonged capillary refill time, and twice daily for the next 48 hours for signs of respiratory distress, cough, lethargy, or loss of appetite. The BAL samples were placed on ice immediately after collection and remained chilled throughout analysis. The BAL fluid was centrifuged (10 minutes at 600 x g; TJ-6 Table Top Centrifuge, Beckman Instruments, Incorporated, Palo Alta, CA, USA), after which the supernatant was removed via gentle suction and the pellet resuspended in sterile 0.9% saline. RBCs and WBCs were then quantified manually with standard hemocytometer counts (Fisher Scientific, Pittsburgh, PA, USA; Nikon, Incorporated Instrument Group, Garden City, NY, USA), expressed as cells/mL BAL fluid, and differential slides were made (Cytospin 2, Shandon, Pittsburg, PA, USA) and evaluated. Differential slides were stained with a dip quick stain (Hema-Stain, Fisher Scientific, Pittsburgh, PA, USA) that stains cells similar to the Wright-Giemsa stain. Cytospin slides were also stained with Perl's Prussian Blue Stain as previously described by Meyer et al.[2] and the percentage of hemosiderophages were determined from the entire slide which had a uniform concentration of 1×10^6 cells/slide.

This lavage procedure was then performed at one week intervals following the race to determine the time course of recovery from EIPH. There was a two week interval between the 4^{th} week post-run lavage and baseline 2. The post-run 2 lavage was done one week after the second baseline in a similar manner to run 1.

Blood Analysis

Following venous blood withdrawal (jugular vein ~10 minutes post-race) into plastic, heparinized syringes, blood samples were placed immediately on ice. Within two hours of the experiment, plasma lactate was quantified using a Nova Stat M blood-gas analyzer (Nova Biomedical, Waltham, MA, USA). This machine was calibrated according to the manufacturer's standards immediately prior to running the samples.

Statistics

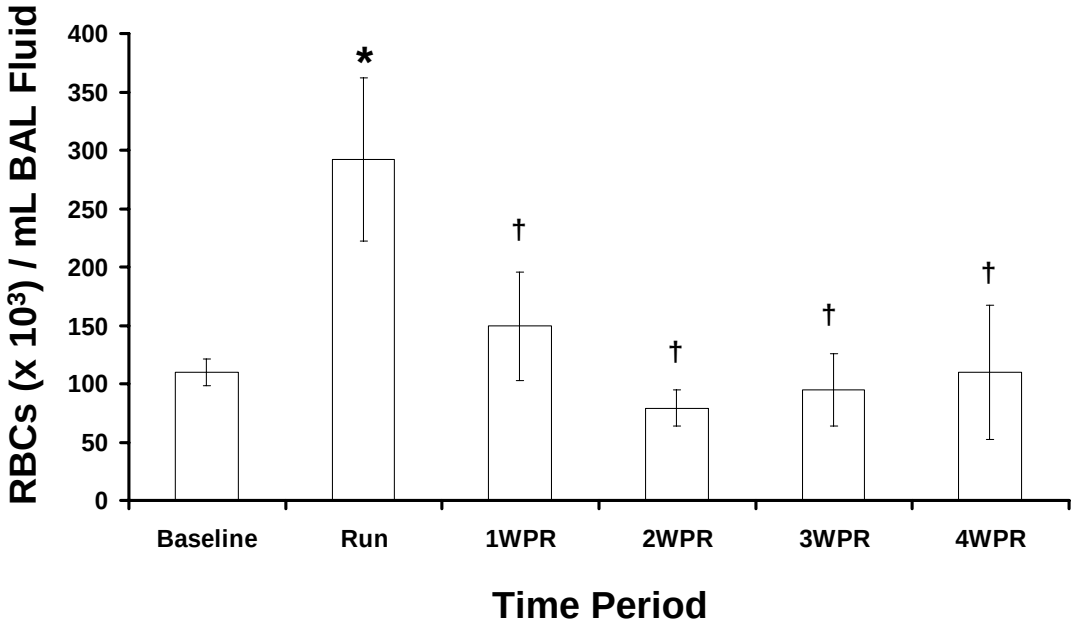
Differences in measured variables were analyzed using a mixed effects model where time is a fixed effect and dog and dog*time are random effects. The response equals overall effect + dog effect + time effect + dog*time interaction + error, where dog and dog* time are random effects. When significant differences were found, a Least-Square Means post-hoc test was used to determine where differences existed. The Statistical Analysis System Program 9.1.2 statistical package (SAS Institute, Incorporated, Cary, NC, USA) was used to analyze the data. Significance was accepted at the $p \leq 0.05$ level.

Results

Exercise-induced pulmonary hemorrhage

The Greyhounds exhibited a significant increase in EIPH ($p = 0.025$; as defined by the number of RBCs in the BAL fluid) when determined after both run 1 ($p = 0.036$) and run 2 ($p = 0.016$). Baseline and post-run 1 values (mean ± SE) were 144.5 ± 21.9 x 10^3 RBCs/mL BAL fluid and 313.8 ± 70.5 x 10^3 RBCs/mL BAL fluid, respectively. Baseline and post-run 2 values (mean ± SE) were 74.7 ± 13.1 x 10^3 RBCs/mL BAL fluid and 270.7 ± 120.1 x 10^3 RBCs/mL BAL fluid, respectively. The mean levels of RBCs for the baselines and runs were consistent (no significant differences existed between baseline 1 and baseline 2 or run 1 and run 2), so these 2 data points were averaged (Baseline = 109.6 ± 11.7 x 10^3 RBCs/mL BAL fluid; Run = 292.2 ± 69.9 x 10^3 RBCs/mL BAL fluid) for ease of interpretation (Fig 4.1). The number of RBCs/mL BAL fluid had returned to baseline by one week post-exercise (149,233 ± 46,239 RBCs/mL BAL fluid; Fig 4.1).

Figure 4.1 Exercise-induced pulmonary hemorrhage (EIPH) in racing Greyhounds as assessed by bronchoalveolar lavage (BAL).



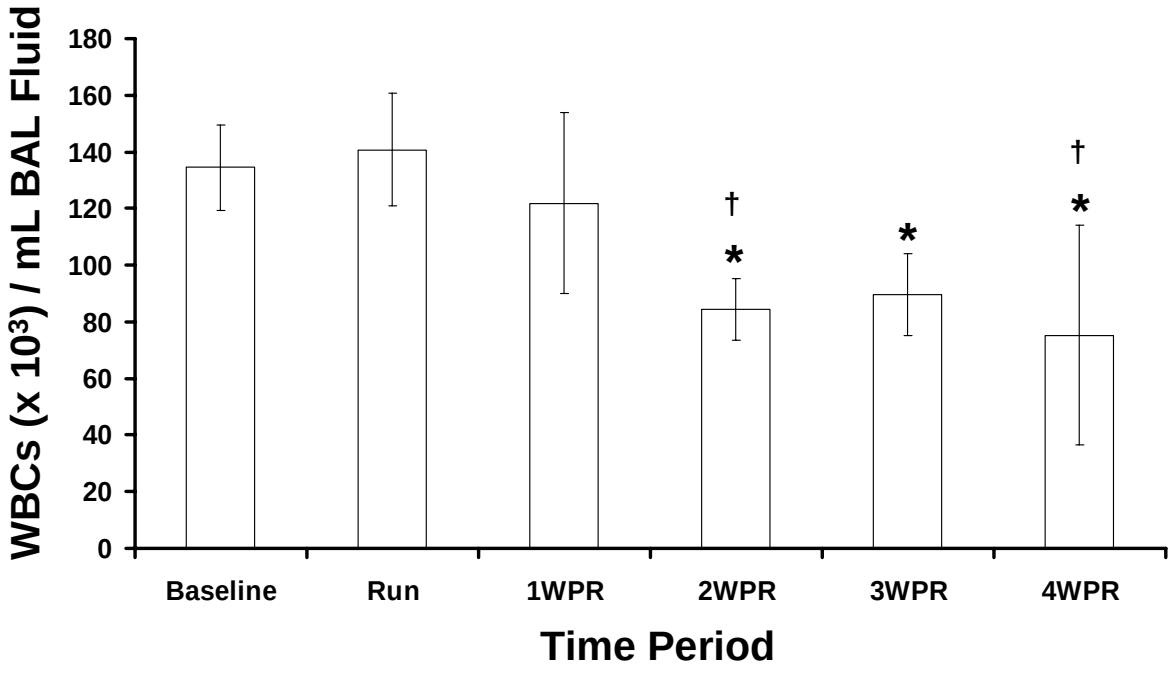
Exercise-induced pulmonary hemorrhage (EIPH) in Greyhounds as assessed by bronchoalveolar lavage (BAL; n = 6) after a 5/16 mile race. EIPH is expressed as the number of red blood cells (RBCs) per mL of BAL fluid. Data are presented as mean ± SE of the average of the baselines (1 and 2) and runs (1 and 2). Time periods on the x axis are abbreviated as follows: Baseline = resting BAL; Run = 2 hour post-run BAL; 1WPR = 1 week post-run BAL (resting); 2WPR = 2 weeks post-run BAL (resting); 3WPR = 3 weeks post-run BAL (resting); 4WPR = 4 weeks post-run BAL (resting). Lavages were performed 1 week apart. * Indicates significant increase in EIPH (increased number of RBCs) from baseline and † indicates significant decrease in the number of RBCs/mL BAL fluid post-run.

Additional Bronchoalveolar Lavage Fluid Analysis

The percent BAL fluid recovery was consistent for all lavages (run and resting lavages) and ranged from 59-68% with an average of 63 ± 1%. Cultures obtained from the first lavage were negative for any pathogens. As there was no significant difference between the mean levels for the remaining lavage variables, the baseline and run lavages were averaged as for the RBC data. An inflammatory response also was not evident (other than an occasional Curshman's spiral) in the Greyhounds, as there was no change in the WBC numbers by one week post-exercise followed by a decrease in the counts from 2-4 weeks post-run (Fig 4.2; Table 4.1). There was also no difference among hemosiderophage counts for any of these periods (Table

4.1). However, despite the absence of an inflammatory response, there were significant differences in the differential counts. Specifically, the number of neutrophils in the BAL fluid was decreased during the entire 4 weeks post-exercise (Table 4.1). Conversely, the alveolar macrophage percentages (not numbers) increased one week post-run and remained significantly elevated for 2 weeks (occasionally containing vacuolation), returning to baseline levels at 4 weeks post-run (Table 4.1).

Figure 4.2 Pulmonary inflammatory response (WBC counts/mL BAL fluid) of Greyhounds measured 2 hours post-run and at weekly intervals thereafter.



White blood cells (WBCs) measured in the bronchoalveolar lavage (BAL) fluid 2 hours after Greyhounds ran a 5/16 mile race and at weekly intervals thereafter. Inflammatory response is presented as the number of WBCs per mL of BAL fluid. Data are presented as mean ± SE of the average of the baselines (1 and 2) and the runs (1 and 2). Time periods on the x axis are abbreviated as stipulated in the legend for Fig 1. Lavages were performed 1 week apart. No significant differences occurred from baseline to the run. * Indicates significant decrease in WBCs/mL BAL fluid post-run and † indicates significant decreases in the number of WBCs/mL BAL fluid from baseline.

Table 4.1 Bronchoalveolar Lavage (BAL) Data

Variable	Baseline	Run	1WPR	2WPR	3WPR	4WPR
Total WBCs/mL BALF	134,350 ± 14,947	140,767 ± 19,878	121,900 ± 32,068	84,433 ± 10,909*†	89,733 ± 14,507*†	75,183 ± 3,882*†
% Return BALF	66.3 ± 3.5	60.7 ± 3.1	63.6 ± 4.4	59.5 ± 3.4	60.3 ± 6.0	66.7 ± 4.3
Macrophages	93,129 ± 9,329	95,642 ± 14,181	95,484 ± 4,961	64,591 ± 2,085	66,851 ± 3,096	4 9,997 ± 3,962
(cells/mL BALF)	(70.7 ± 3.3)	(68.6 ± 2.9)	(78.3 ± 4.1)*†	(76.5 ± 2.5)†	(74.5 ± 3.5)	(66.5 ± 5.3)
Neutrophils	16,964 ± 3,253	16,360 ± 4,020	7,314 ± 2,670*†	7,743 ± 1,469*†	7,923 ± 1,256*†	9,774 ± 2,992*†
(cells/mL BALF)	(12.6 ± 2.5)	(11.3 ± 2.1)	(6.0 ± 2.2)	(9.2 ± 1.7)	(8.8 ± 1.4)	(13.0 ± 4.0)
Lymphocytes	19,850 ± 3,838	26,628 ± 5,076	18,687 ± 3,596	11,821 ± 2,043	14,806 ± 2,243	13,781 ± 2,218
(cells/mL BALF)	(14.3 ± 1.7)	(18.7 ± 2.4)	(15.3 ± 3.0)	(14.0 ± 2.4)	(16.5 ± 2.5)	(18.3 ± 3.0)
Eosinophils	4,406 ± 2,917	2,251 ± 1,657	610 ± 610	279 ± 279	153 ± 153	1,631 ± 1,631
(cells/mL BALF)	(2.4 ± 1.7)	(1.6 ± 1.0)	(.5 ±. 5)	(.3 ± .3)	(.2± .2)	(2.2 ± 2.2)
Mast Cells	214 ± 163	0 ± 0	0 ± 0	144 ± 144	0 ± 0	0 ± 0
(cells/mL BALF)	(0.2 ± 0.1)	(0.0 ± 0.0)	(0 ± 0)	(. 2 ± .2)	(0 ± 0)	(0 ± 0)
Hemosiderophages	41 ± 10.2	64.2 ± 38.1	108.0 ± 40.1	80.3 ± 35.9	21.2 ± 4.1	76.5 ± 37.7

The data in the table represents bronchoalveolar lavage (BAL) variables for 6 Greyhounds that were lavaged 2 hours after a 5/16 mile race. The differential counts are presented with the actual number of white blood cells (WBCs) by type (i.e. macrophage, neutrophils, lymphocytes, eosinophils, and mast cells) on the top and the differential percentage directly below it. Hemosiderophage numbers represent the number on a slide with 1 x 10^6 WBCs per slide. Time periods across the top of the table are abbreviated as follows: Baseline = resting BAL; Run = 2 hour Post-Run BAL; 1WPR = 1 week post-run BAL (resting); 2WPR = 2 weeks post-run BAL (resting); 3WPR = 3 weeks post-run BAL (resting); 4WPR = 4 weeks post-run BAL (resting). Lavages were performed 1 week apart from Baseline - 4WPR. *Indicates significant difference ($p < 0.05$) from Baseline; †Indicates significant difference ($p < 0.05$) from Run.

Cardiac and Metabolic Variables

Maximal heart rates were 231 ± 2 bpm for run 1 and 229 ± 2 bpm for run 2 ($p > 0.05$) with the average being 230 ± 1 bpm. Venous blood lactates were 19.2 ± 0.4 and 18.0 ± 0.5 mmol/L ($p > 0.05$) for 10 minute post-exercise draws for run 1 and run 2, respectively, with the average being 18.6 ± 0.4 mmol/L. Ten minute post-exercise rectal temperatures were 106.0 ± 0.3˚ for run 1 and 104.5 ± 0.4˚ for run 2 ($p > 0.05$), with the average being 105.2 ± 0.3˚. Run times for the 5/16 mile race were 32.1 ± 0.3 seconds and 32.6 ± 0.4 seconds for runs 1 and 2 ($p > 0.05$), respectively, with the average time being 32.2 ± 0.1 seconds.

Discussion

The principal original findings of this investigation are that: 1) Greyhound dogs run under simulated race conditions at 5/16 mile experience EIPH that appears to be of a reduced magnitude compared with that described in horses. 2) Recovery from an EIPH episode in Greyhounds follows a similar pattern to horses when looking at RBCs, but does not result in prolonged elevation of hemosiderophages or an extended inflammatory response in the lung.

Comparison with the Current Literature

A very limited amount of information exists concerning EIPH in racing Greyhounds (see preliminary reports)[8, 9]. King[8, 9] reported significant EIPH in Greyhounds racing 5/16 mile, ranging from 1-71million RBCs/mL BALF with an occasional dog displaying endoscopic evidence of EIPH. The data from the current study are in agreement with the studies by King et al.[8, 9] suggesting that Greyhounds do demonstrate pulmonary hemorrhage during exercise. However, the severity of bleeding detected in the current study was much lower than that found by King[8, 9]. Between-study differences in the severity of bleeding may be due to methodological factors including time from run to lavage (20 minutes in King et al.[8, 9] studies versus 2 hours in present study), induction of iatrogenic hemorrhage, lung differences (right versus left lung predominance in Greyhounds), and pre-selection of dogs for the study (certain populations of animals may be more prone to severe EIPH).

The Greyhounds in the current study appeared to follow a similar time course for recovery from a bleeding episode as the horses displayed in the Meyer et al.[2] study in that the RBCs/ml BALF detected 2 hours post-exercise returned to baseline by one week post-exercise. However the amount of hemorrhage detected is widely different with respect to values typically measured in the horse (EIPH = 4-64 x 10^6 RBCs/mL BAL fluid) [19, 22, 23, 45-48]. Hemosiderophages were also examined in that study[2] as an indicator of past pulmonary hemorrhage. In horses, hemosiderophages are commonly around 7% at baseline and respond by significant increases (10-20%) one week post-exercise, remaining elevated for 3-4 weeks post-exercise. Hemosiderophages have been considered a good indicator of past pulmonary hemorrhage[1, 2, 49, 50]. This equine profile is in contrast to extremely low, almost undetectable levels of hemosiderophages that we found in Greyhound dogs. Due to extremely sparse numbers of hemosiderin-laden macrophages, it was impossible to obtain a percentage hemosiderophage count for the dog slides. Therefore, the total number of hemosiderophages per million cells (entire slide) were counted to increase accuracy of comparison within dogs and among BAL periods. The small amount of blood detected in our study did not induce an increase in hemosiderophages 1-3 weeks post-exercise as observed in the horse[2]. The increases and decreases in the hemosiderophage counts for the Greyhounds were erratic with respect to pre- and post-run evaluations, and this profile may potentially be explained by mild iatrogenic bleeding initiated during the lavage procedure. This data is in agreement with King et al.'s[9]

observation that 7/10 dogs showed evidence of hemosiderin deposits within alveolar macrophages indicating past hemorrhage.

Inflammatory airway disease (IAD) in the horse is thought to contribute to the initiation and severity of EIPH as well as being a response to the condition[1, 24-28]. Long standing inflammatory reaction to blood within the alveoli results in a self-perpetuating phenomenon of gradually worsening hemorrhage as horses continue to train and race[24, 51]. However, in the racing Greyhound a much smaller amount of blood was detected in the lung after a race which may not be sufficient to overwhelm pulmonary defenses. Moreover, these RBCs may be cleared rapidly enough to avoid any prolonged inflammatory response in the Greyhounds. It is also possible that IAD itself may not induce the long-term pulmonary damage that exacerbates EIPH in the horse[27, 29]. The present investigation did not find abnormally elevated WBC counts in the lavage fluid at baseline (in comparison to the literature)[53-62] nor in response to exercise or the blood in the lungs at 2 hours post-exercise. However, an unexpected decrease occurred in the WBC counts at weeks 2, 3, and 4 after exercise. A decrease in the number of neutrophils was detected for four weeks post-exercise which follows the WBC counts and corroborates the absence of an inflammatory response to the mild hemorrhage. It is possible that weekly use of propofol (an anesthetic drug with anti-inflammatory properties) could aid in masking a mild inflammatory response[63, 64]. On the other hand, a significant rise in the percentage of macrophages (not actual numbers) from one to three weeks post-exercise occurred before returning to baseline by four weeks post-exercise. One explanation for this occurrence may be that this response was adequate for clearing the small amount of blood present without requiring an increased number of macrophages or WBCs in the BAL fluid. These results are also in agreement with EIPH studies of King et al.[8, 9]. However, this is different from the horse in that WBC counts typically elevate after an episode of EIPH and either remain elevated or decrease slowly, depending on the independent contribution from inflammatory airway disease. Again, the Greyhound dogs in the present investigation did not appear to have true inflammatory airway disease at any time (i.e. elevation in WBC counts with concurrent increase in macrophages, neutrophils, and/or lymphocytes). Other conditions cause inflammation in the lower airways of dogs, but are totally unrelated to the current study and include "ski-asthma" in Alaskan sled dogs, allergic bronchitis, and eosinophilic airway disease[52]. Much variation exists in the canine literature when looking at WBC counts and differentials from BAL fluid. However, when

examining publications containing information on BAL's done in healthy dogs, the WBC counts and differentials that we obtained from the dogs in the current study fall well within the published ranges[53-62].

Mechanisms of Exercise-induced Pulmonary Hemorrhage

Exercise-induced pulmonary hemorrhage has been studied most extensively in the Thoroughbred horse. Several mechanisms have been proposed to contribute to the pathophysiology of EIPH. In order of potential importance as considered in the literature these include elevated pulmonary arterial pressures[11, 12], airway contributions[3, 13-22], inflammatory airway disease[1, 23-29], blood viscosity[65], and locomotory impact forces[30-32].

Greyhounds may be expected to demonstrate EIPH as they have a number of physiologic attributes in common with the horse. Both species are exceptional sprinters[33, 34], have large heart to body weight ratios[66-69], large contractile spleens (high PCV)[34, 37, 38, 65], high pulmonary vascular pressures[12, 41] during exercise, and very high maximal oxygen consumptions[33, 35, 36, 70, 71] (R Pieschl and MR Fedde unpublished data). In addition Greyhounds have a thinner blood-gas barrier than the horse that requires a smaller transmural pressure gradient to cause capillary rupture (Birks et al. 1997, dog 0.795 ± 0.084 μm; 66 mmHg; horse 0.930 ± 0.044μm; 70-100 mmHg)[3, 39, 40, 72]. Greyhounds have high cardiac outputs[33, 34, 36] that play a large role (in combination with their highly elevated PCV) in generating both exceptional levels of oxygen uptake[33, 36] and pulmonary arterial pressures during maximal exercise. Based on Ppa pressures of Greyhounds measured during sub-maximal exercise (55 mmHg at 11m/s), peak exercising pressures at speeds approaching 20 m/s are expected to be much higher.

Notwithstanding the above, these are features specific to the Greyhound that may protect this animal from the severe EIPH observed in the horse. Specifically, the dog is not an obligate nasal breather and has a much shorter trachea than the horse (airway resistance is proportional to the length of the airway and inversely proportional to the radius to the 4^{th} power), so the work of breathing and pleural pressure swings generated[42] may not be as high as those seen in the horse. Saibene et al.[43] found that dogs during exercise experienced a pronounced dilation of the respiratory tract which reduces airflow resistance. The horse also has a much stiffer chest wall that limits the thoracic contribution to ventilation during exercise[73]. By forcing a proportionally greater diaphragmatic contribution to ventilation, horses may be predisposed to more negative

intrapulmonary pressures and also shear forces that damage the dorsocaudal lung lobes. The more compliant chest wall of the dog may well limit these effects if it permits lateral chest wall expansion during exercise. Impact forces are thought to be a significant cause of EIPH in horses[30-32]. However, these forces will be reduced in the dog in proportion to their body frames and weights, along with the consideration that they are not carrying a rider and saddle. Lastly, the severity of EIPH may depend on the duration over which the threshold capillary transmural pressure is exceeded[74]. Thus, the shorter distance (5/16 mile versus 1-1.5 miles) and reduced time (25-35 seconds for Greyhounds versus 1-2 minutes for horses) at which maximal effort is sustained by the Greyhound versus the horse may help constrain the magnitude of EIPH. However, racing Quarter Horses can demonstrate extreme EIPH in races of similar distance to Greyhounds.[75]

Methodological Considerations

Bronchoalveolar lavage has been implemented for a little over a decade in horses to evaluate the presence and degree of EIPH. Despite inherent limitations, it has been considered to be the most accurate[2, 49] and sensitive technique available to detect and quantify pulmonary hemorrhage. The EIPH observed in the current study would not likely have been detectable or tractable over time with endoscopy[1, 2, 49, 76]. However, King et al.[8, 9] did visualize small amounts of bleeding in 2 dogs with endoscopy. It has been repeatedly shown that a good correlation exists between BAL cytology and histopathology in horses with EIPH[49, 76] . It also has been shown in the horse that blind placement of the BAL tube typically results in wedging it in the dorsocaudal region[77] of the lung (side predominance of tube undetermined) where the majority of EIPH occurs[25, 26]. Unpublished radiographic data (TS Epp, AM Buchannan, L Gates, DC Poole, and HH Erickson) from our laboratory suggests a right caudal lung lobe predominance when a BAL tube is placed blindly (5 right versus 2 left). This is in agreement with Hawkins et al.[45], who radiographically demonstrated right caudal lung lobe predominance when a BAL tube was blindly placed (7 dogs right versus 2 dogs left). Therefore, it may be of importance that King et al.[8, 9] showed that the left lung may hemorrhage to a greater degree than the right which is in contrast to what has been observed in the horse[2, 78]. It is also important to keep in mind that the Greyhounds utilized in this study were not selected based upon a prior history of severe EIPH, as is often the case in horse studies[3, 19, 22, 23, 46-48].

Bronchoalveolar lavage (BAL) is becoming more popular as a diagnostic tool for pulmonary disorders in dogs. However, BAL results can be difficult to interpret. Disadvantages or problems potentially encountered in dogs (but not in horses) include the possibility that alveoli are non-uniform and intermittent collapse occurs, not allowing lavage fluid into the distal airways as well as an increased rate of mucociliary clearance in the dog versus the horse (3.3 cm/min. versus 2 cm/min.)[80-82]. In addition, there may be mild trauma to the airways obtained while trying to ensure wedging of the tube[45, 57, 58] and there is the potential for airway collapse[58] distal to the catheter if too much suction is inadvertently applied, resulting in possible trauma and a smaller recovery volume. Considerable variation and lack of standardization exists in the volume infused, technique employed (i.e. number of aliquots, interval between instillation and aspiration, lobes lavaged)[61, 79], and the processing of fluid (i.e. centrifugation, washing, counting)[53, 55, 57, 61]. The techniques utilized in this study were chosen after careful perusal of the available literature as well as some trial and error, and were found to combine the most consistent results with the least amount of iatrogenic trauma possible. Finally, depending specifically on volume and number of aliquots, normal numbers and differential percentages of WBCs in the lavage fluid are less defined than those available for equids[83]. Variations in the WBC counts as well as the differential cell counts[55, 61] may possibly be due to lavaging of different levels (i.e. bronchial versus alveolar)[62], different concentrations obtained[45, 53], and the lung lavaged (i.e. left or right)[8].

Implications of EIPH in Greyhounds

Data collected from Greyhounds in the current investigation suggest that these athletes display significant EIPH following maximal running, albeit at very low levels compared with equids. If these Greyhounds are typical of the entire racing population, it does not appear that the consequences of that bleeding are as pernicious and clinically worrisome as is the case for the horse. This conclusion is based on the fact that the levels of WBCs, hemosiderophages, and differential counts did not indicate a prolonged response to the blood (i.e. large inflammatory response and elevations in hemosiderophages over time) as is typically seen with the horse. Therefore, these dogs may not suffer the long term lung damage and performance deficits created by inflammation and fibrosis. However, validation of this hypothesis will necessitate post-mortem pulmonary anatomical studies similar to those done in horses. Such studies could

confirm or refute the notion that EIPH is not of major clinical significance in the Greyhound as it is in the horse.

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