



Histamine bronchoprovocation does not affect bronchoalveolar lavage fluid cytology, gene expression and protein concentrations of IL-4, IL-8 and IFN- γ

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ABSTRACT

In diagnosing inflammatory airway disease (IAD) in performance horses, a histamine bronchoprovocation (HBP) test is often performed. In previously published studies, HBP is usually undertaken prior to cytological examination of the bronchoalveolar lavage (BAL) cells. The purpose of this study was to determine if HBP alters (1) the total nucleated cell numbers and distribution in BAL fluid (BALF) and (2) the mRNA and protein concentrations of selected cytokines in BAL cells and BALF, respectively. BALF was initially collected endoscopically from the right middle or diaphragmatic lung lobe in eight healthy young Standardbred horses. Five to six days later, HBP was performed by aerosolization of histamine (8 mg) over a 2 min period. BALF was again collected within 2–4 h of the HBP from the left middle or diaphragmatic lung lobe. In both samples, total and differential WBC counts were obtained. The gene expressions of interleukin-4 (IL-4), IL-8, interferon- γ (IFN- γ) and β -actin in BAL cells were measured using real-time RT-PCR. The cytokine protein concentrations were measured in the BALF using ELISA. HBP was not associated with either a change in the total BAL cell number or in the distribution of the BAL cells. BAL cell expression of IL-4, IL-8 and IFN- γ , detected in all samples with the exception of IL-4 in one horse (post-HBP), was not altered as a result of HBP. HBP was not associated with a significant change in IL-8 or IFN- γ concentrations in the BALF. IL-4 protein was undetectable in BALF either prior to or following HBP. We conclude that HBP can precede BALF collection performed within 2–4 h of the former without affecting selected parameters analysed in the BAL cells or BALF.

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1. Introduction

Inflammatory airway disease (IAD) is a pulmonary disorder of young to middle-aged athletic horses. Affected horses exhibit cough, a nasal discharge (at rest or post-

exercise), excessive tracheobronchial secretions and poor athletic performance (Hoffman et al., 1998; Hare and Viel, 1998; Mazan et al., 1999). Pulmonary alterations include the presence of (1) nonseptic inflammation detected by cytologic examination of bronchoalveolar lavage fluid (BALF) or (2) overt pulmonary dysfunction as evidenced by lower airway obstruction, airway hyper-responsiveness or impaired blood gas exchange at rest or during exercise (Couëtil et al., 2007). Cytological alterations in the BALF of horses affected with IAD are diverse and include increases in the total nucleated cell counts and increases in

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neutrophil, lymphocyte, mast cell or eosinophil percentages (Hare et al., 1994; Moore et al., 1995; Hare and Viel, 1998). Generally, in clinically healthy young athletic horses the distribution of nucleated cells in the BALF is 60% macrophages; 34% lymphocytes; <5% neutrophils; <2% mast cells or eosinophils (Hare et al., 1994). In diagnosing horses with IAD, a histamine bronchoprovocation (HBP) test, to detect airway hyper-reactivity, followed by a bronchoalveolar lavage, to obtain airway cells for cytological analysis, are typically performed. While this sampling order – HBP first followed by BALF – avoids the untoward effects that instillation of the lavage saline has on pulmonary function measurements (Leguillette and Lavoie, 2006), it is not known if the histamine aerosolization promotes inflammatory cell influx into the airways or alters BAL cell cytokine expression and/or release. Thus, the purpose of this study was to determine if HBP in healthy horses alters (1) the distribution of the nucleated cells in the BALF; (2) the gene expression of IL-4, IFN- γ , and IL-8 in the BAL cells or (3) the concentration of these 3 proteins in the BALF. Interleukin-4 and IFN- γ were examined because they represent the prototypical cytokines of allergic and cell-mediated responses, respectively (Mosmann and Sad, 1996). Interleukin-8 was examined because this neutrophil chemoattractant is up-regulated in equine pulmonary disorders (Franchini et al., 2000).

2. Materials and methods

Eight healthy Standardbred horses (mean \pm S.D.) 5 ± 0.6 years old consisting of five geldings and three mares were studied. Prior to the study, the horses had been on pasture for a minimum of 2 months and had been supplemented with hay and grain twice daily as needed. The horses were owned by the University of Guelph and the experimental protocol was approved by the Canadian Council on Animal Care and the IACUC at Cornell University. The base line BAL was performed in a stock at the research farm facility then 5 days later, the horses were transported to the Ontario Veterinary College and were housed for the day in an isolation stall unit. Following the BAL and histamine provocation test the horses returned to the research farm facility.

The horses were sedated with 15 mg of intravenous romifidine (Boehringer Ingelheim, St Joseph, MO). A 180 cm long, 13 mm diameter fiberoptic endoscope (Olympus, Tokyo, Japan) was passed nasally into the trachea. At the level of the carina, 60 mL of 0.2% lidocaine solution was sprayed through the biopsy channel to desensitize the airways. The endoscope was then passed into the right mainstem bronchus and wedged in a subsegmental bronchus of the right middle or diaphragmatic lung lobe. The lavage was performed by infusing and then immediately aspirating two 250 mL aliquots of warmed (37 °C) sterile physiological saline. Aspiration was achieved with a medical suction pump (Power Air Division, Sheboygan, WI) calibrated to deliver a constant vacuum of 10 cm H₂O. The volume of the BALF was recorded, divided into three aliquots and placed on ice. A 50 mL aliquot was frozen at –80 °F for subsequent cytokine protein determination. A 5 mL aliquot was used to determine (1) the total nucleated cell count, performed

by an automated cell counter (Beckman Coulter Inc., Fullerton, CA) and (2) the differential cell count. For the latter, the sample was first cytocentrifuged and automatically stained with a Wright-Giemsa before a differential count was performed on 500 cells. Based upon the total nucleated cell count, the volume of fluid necessary to obtain a total of 10^7 cells (the third aliquot) was calculated. This third sample was then centrifuged at $2400 \times g$ for 10 min and the supernatant was removed. The cell pellet was resuspended and frozen at –80 °F for subsequent gene expression studies.

Five to six days after the initial BAL was performed, the same horses were re-tested. Pulmonary function tests were first obtained on the standing, unsedated horse. Esophageal pressures were measured as previously described using a balloon catheter (110 cm long with 3 mL of air injected, attached to a 2 m polyethylene catheter, 2.8 mm inner diameter, 3.5 mm outer diameter), inserted to mid-thoracic esophagus. Horses wore an airtight plastic face mask to which was attached a Fleisch #4 pneumotachograph (Gould Electronics, Bitholve, The Netherlands) for air flow measurement. Volume was derived by the electronic integration of the flow signal (Buxco Electronics Inc., Sharon, CT). Transpulmonary pressure was calculated as the difference between the airway opening pressure (measured in the face mask) and the esophageal pressure detected by the latex balloon catheter. The flow-volume and pressure-volume loops of 5–10 breaths were analysed using a commercial software program (Buxco Electronics Inc., Sharon, CT) which calculated the maximum change in pleural pressure ($\Delta P_{PI \text{ max}}$), the pulmonary resistance (R_L) and the lung compliance (C_{dyn}). Baseline pulmonary function parameters were recorded prior to aerosolization of a 0.9% saline solution through a nebulizer (Wilder Medical, Kitchener, Ontario, Canada) using a gas flow of 4.5 L/min generated by a compressor (AFP Medical, Rugby, United Kingdom). At this flow rate, the nebulizer output was 0.13 ± 0.05 mL/min. Each horse was then aerosolized with 8 mg of histamine diphosphate solution (Sigma-Aldrich Co., St Louis, MO) and function tests were again recorded (Klein and Deegen, 1986). A second BAL of the left middle or diaphragmatic lung lobe was performed within 2–4 h of completion of the HBP. A heparinized venous blood sample was also obtained at this time for serum urea determinations.

For the gene expression studies on the BAL cells, total RNA was extracted (Qiagen Inc., Valencia, CA), genomic DNA was destroyed (Invitrogen Inc., Grand Island, NY), cDNA was synthesized (Invitrogen Inc., Grand Island, NY) and the gene expressions of IL-4, IL-8, IFN- γ and β -actin were measured using real-time RT-PCR (Applied Biosystems, Foster City, CA) as previously described (Ainsworth et al., 2003). Reaction mixtures had a final volume of 27.5 μ L consisting of 2.5 μ L of cDNA and 25 μ L of master mix. Amplification conditions were identical for all samples: 2 min at 50 °C, 10 at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. For each cDNA sample, duplicate reactions were performed on each plate for detection of target genes. Also included on each plate were a positive (cDNA from PWM-stimulated lymphocytes or LPS-stimulated macrophages) and a negative control

(water). Gene expression was normalized to the house-keeping gene, β -actin. The endpoint used in the real-time RT-PCR quantification, C_T , is the cycle number corresponding to the detection of product formation; C_T ranged from 0 to 40. Gene expression was reported as the normalized cycle threshold ($\Delta C_T = C_T$ target gene – C_T housekeeping gene). In general, the smaller the value for ΔC_T the more cDNA (i.e. mRNA) contained in a sample.

After concentrating the BALF 10-fold, IL-4, IL-8 and IFN- γ protein concentrations were measured using an ELISA (Wagner et al., 2003). Briefly, equine IL-8 was detected using rat anti-equine IL-8 antibodies which were produced in rats immunized with recombinant equine IL-8 (Courtesy of Dr. Judith Ball, Texas A&M University, College Station, TX) ELISA plates were coated with rat anti-equine IL-8 antibody and incubated overnight at 4 °C. After washing the plate, the BALF was added to each well and plates were incubated for 15 h at 4 °C. A 1:2000 dilution of biotinylated rabbit α -equine IL-8 (Courtesy of Dr. Marco Franchini, University of Zurich, Zurich, Switzerland) was added and plates were incubated for 45 min. Plates were again washed and streptavidin–peroxidase applied at a 1:50,000 dilution. The plates were washed and filled with substrate buffer, containing tetramethylbenzidine and hydrogen peroxide and incubated for 20 min in the dark until the reaction was stopped by adding one volume of 0.5 M H_2SO_4 . Plates were evaluated in an automatic microplate reader (Biotek Instruments, Winooski, VT) at 450 and 630 nm absorbance. The IL-4 cytokine ELISA was performed as previously described (Wagner et al., 2005). For the IFN- γ ELISA, a mouse anti-bovine IFN- γ (Serotec, Raleigh, NC) antibody coated the ELISA plate, a biotinylated goat anti-murine IFN- γ (R&D Systems, Minneapolis, MN) was used as the secondary antibody followed by the addition of streptavidin–peroxidase and substrate for detection. Positive controls for IFN- γ and IL-4 consisted of recombinant equine IFN- γ and recombinant equine IL-4 (Wagner et al., 2005). Samples were run in duplicate and the average OD of the two time samples at each time point was used for analysis. The BALF dilution factor was calculated by measuring the urea nitrogen concentration (Stanbio Laboratory, Boerne, TX) in the pre- and post-HBP samples and comparing them to the serum urea concentration taken at the post-HBP sampling time point (Kirschvink et al., 2001).

The pre- and post-HBP pulmonary function tests, BAL total and differential cell counts, the mRNA and protein concentrations of IL-4, IL-8 and IFN- γ were compared using the Wilcoxon signed-rank test with a Bonferroni adjustment for multiple comparisons. Statistical significance was considered at $P \leq 0.017$. All computations were performed by a statistical software program (Statistix 8.0, 2003 Analytical Software, Tallahassee, FL) and a graphics program was used to generate Bland–Altman graphs in the BALF protein analyses (MedCalc 9.3.2.0, Mariakerke, Belgium).

3. Results

Shown in Table 1 are the results of the pulmonary function tests obtained prior to and following saline and

Table 1

Median (25th, 75th quartile) pulmonary function variables for the horses measured at baseline, following saline and following histamine aerosolization

	Baseline (n = 8)	Saline (n = 8)	Histamine (n = 7)
$\Delta P_{PI \max}$ (cm H_2O)	8.0 (7.3, 8.6)	8.2 (7.3, 8.9)	7.8 (6.7, 10.7)
C_{dyn} (L/cm H_2O)	1.5 (1.3, 2.1)	1.4 (1.3, 2.0)	1.5 (1.3, 1.6)
R_L (cm $H_2O/L/s$)	0.7 (0.4, 1.0)	0.5 (0.4, 0.8)	0.4 (0.4, 0.9)

Note: During the HBP, the esophageal balloon broke in one horse preventing data to be obtained. No significant differences were found.

histamine aerosolization. No significant differences between the three time points were found for the $\Delta P_{PI \max}$, in R_L or in C_{dyn} .

The median (25th, 75th quartiles) total nucleated cell count and percentages of each cell type in the BALF obtained prior to and following HBP are shown in Table 2. The distribution of the nucleated cell types prior to HBP was within normal limits, demonstrating that the horses were free of IAD. No significant changes in the cell number or in the differential counts occurred following HBP (all P values >0.22). Note that of the 500 mL of saline instilled during each BAL procedure, a median (25th, 75th quartiles) volume of 413 (321, 492) mL was recovered prior to HBP and was not significantly different from the median volume of 404 mL (314, 485) recovered post-HBP. The BALF dilution factor did not differ between the two collection times ($P = 0.1$).

Shown in Fig. 1 are the box and whisker plots of the normalized IL-4, IL-8, and IFN- γ expression in the BAL cells prior to (white boxes) and following (hatched boxes) HBP. Transcripts of IL-4, IFN- γ and IL-8 were detected in all samples with the exception of one sample in which IL-4 expression was not detected in the BAL cells post-HBP. There were no significant changes in the BAL cell expression of IL-4, IFN- γ and IL-8 as a result of HBP.

Protein concentrations of IL-4, IL-8 and IFN- γ were measured in the BALF fluid prior to and following HBP. Interleukin-4 protein was not detected in any of the samples of the BALF at any time point. There were no significant differences in the median BALF protein concentrations of IL-8 or of IFN- γ as a result of HBP ($P = 0.55$, $P = 0.03$, respectively). Shown in Figs. 2 and 3 are Bland–Altman plots for IL-8 and IFN- γ , respectively. The Bland–Altman plot is a graphical way to assess the amount of change in the BALF protein concentrations after histamine

Table 2

Median (25th, 75th percentiles) BALF volumes and nucleated cell distributions prior to and following histamine bronchoprovocation test

	Pre-HBP BAL	Post-HBP BAL
Total WBC (cells/ μ L)	770 (525, 954)	688 (516, 789)
Macrophages (%)	58 (51, 63)	54 (43, 60)
Lymphocytes (%)	41 (36, 45)	44 (38, 51)
Neutrophils (%)	1 (0, 1)	2 (1, 4)
Mast cells (%)	2 (1, 2)	2 (1, 2)
Eosinophils (%)	0 (0, 1)	0 (0, 0)

No significant differences were found.

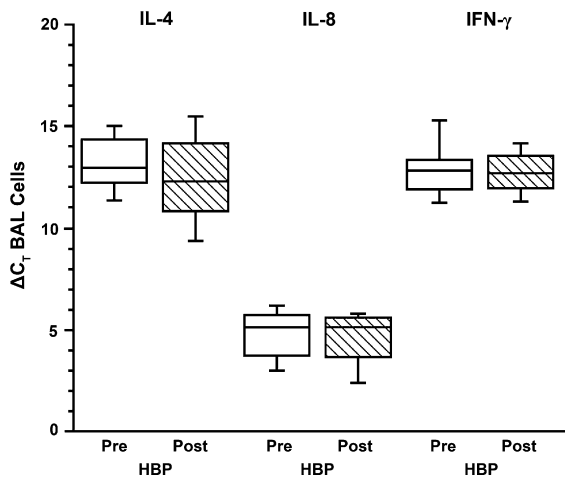


Fig. 1. Boxed plots depicting the normalized gene expression of IL-4, IL-8 and IFN γ in the BAL cells prior to (empty box) and following (hatched box) HBP in eight healthy Standardbred horses. In general, the smaller the ΔC_T , the greater the gene expression. No significant differences in the gene expression were found between pre- and post-HBP.

treatment as a function of the cytokine concentration in the individual horse. The change in protein following HBP is plotted (y-axis) against the average concentration of the protein obtained at the two sampling times (pre + post-HBP/2), shown on the x-axis (Bland and Altman, 1986; Schoonjans et al., 1995). If there is no difference following the treatment, the plot shows a random scatter of points as noted in Figs. 2 and 3. Also, note that most points are centered along the 0.0 of the y-axis which implies that there was little difference between the pre- and post-protein measurements, especially at lower mean values of IL-8. However, the mean bias for both IL-8 and IFN- γ was less than 0.0—implying that the pre-measurement typically was greater than the post-measurement. The graphs also suggest that there was greater discrepancy between the pre- and post-measurement (of both proteins) when the mean values were larger.

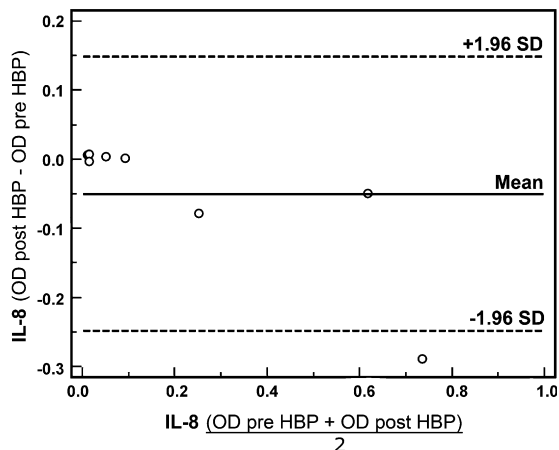


Fig. 2. Bland–Altman graph depicting the difference between IL-8 protein concentration in the BALF before and after HBP in eight healthy Standardbred horses.

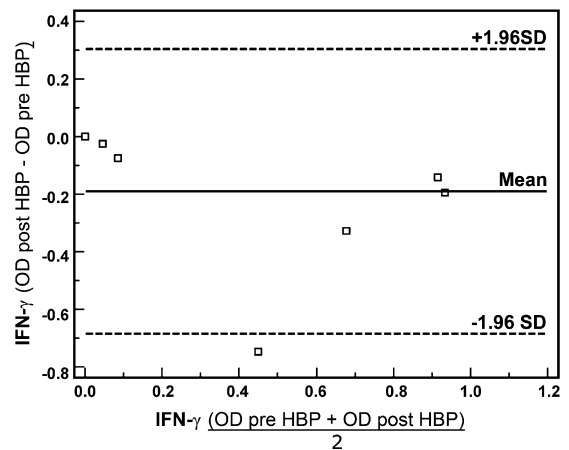


Fig. 3. Bland–Altman graph depicting the difference between IFN- γ protein concentration in the BALF before and after HBP in eight healthy Standardbred horses. Note that data from two horses were located at 0, 0.

4. Discussion

Our results demonstrate that within 2–4 h of performing a histamine bronchoprovocation test, significant changes in the cytological composition of the BALF; in the BAL cell mRNA expression of IL-4, IL-8 or IFN- γ or in the BALF protein concentrations of IL-4, IL-8 or IFN- γ does not occur in healthy horses.

4.1. The effect of HBP on pulmonary function tests

The histamine bronchoprovocation test is typically performed in human and in veterinary patients in order to detect nonspecific airway hyper-reactivity (Klein and Deegen, 1986; Hare and Viel, 1998; Cockcroft, 2003). The bronchoprovocation test is just one of the methods that have been recently recommended by veterinary pulmonologists for use in identifying horses with IAD (Couëttil et al., 2007). When histamine is inhaled, it interacts with histamine receptors (H-1) located on cells within the respiratory tract to cause sensorineural stimulation (naso-nasal reflexes in upper respiratory tract; vago-vagal reflexes in lower respiratory tract), vascular dilation, increased vascular permeability and airway smooth muscle contraction (Prendiville et al., 1987; Togias, 2003). In the upper respiratory tract, the clinical manifestations of histamine challenge include the development of rhinorrhea, nasal congestion and pruritis. In the lower respiratory tract, bronchoconstriction ensues and may reflect the direct actions histamine on airway smooth muscle (constriction) as well as the indirect effects of histamine that cause vagally induced smooth muscle contraction. In healthy horses, the inhalation of 8 mg of histamine fails to induce significant alterations in pulmonary function tests. However, in horses with pulmonary inflammation and airway hyper-reactivity, 8 mg of histamine is sufficient to cause significant increases in R_L and decreases C_{dyn} (Klein and Deegen, 1986). The lack of physiological or cytological changes in the pulmonary function tests following HBP confirmed that the horses were healthy and free of airway disease.

4.2. HBP and cytokine expression

In addition to its classical effects on pulmonary function, histamine, by virtue of its interaction with histamine receptors on lymphocytes, monocytes, macrophages and dendritic cells, histamine has the potential to influence the nature of the adaptive and innate immune responses (Cameron et al., 1986; Mazzoni et al., 2001, 2006; Packard and Khan, 2003; Kitawaki et al., 2006). For example, histamine pre-treatment of human or murine T cell cultures inhibits mitogen-induced proliferation of T helper 1 (Th-1) cells (which are involved in cell-mediated responses) and inhibits increases in the Th-1 associated cytokines, IFN- γ and IL-2 (Dohlsten et al., 1986; Beer and Rocklin, 1987; Packard and Khan, 2003). In contrast, histamine pre-treatment augments mitogen-induced proliferation of Th-2 cells (which are involved in allergic responses) and increases production and release of the Th-2 associated cytokines, IL-4, IL-5, IL-10 and IL-13 (Packard and Khan, 2003). These cytokine alterations are mediated through activation of histamine type-1 receptors which secondarily increase intracellular cAMP (Beer and Rocklin, 1987). These observations have led some to conclude that histamine may bias the immune system towards a Th-2 response. However, data from other investigations suggest that this paradigm may be overly simplistic. Krouwels et al. (1998) studied the responses of T cell clones derived from “activated” airway T cells (obtained by means of bronchoalveolar lavage) and “non-activated” T cells (obtained from the peripheral blood) of asthmatic and healthy, non-asthmatic humans. Stimulation with physiological concentrations of histamine (10^{-4} M) inhibited IFN- γ production in only 40% of the T cell clones, had no effect on 44% of the clones and augmented IFN- γ in the remaining 16% of the clones. More impressive was the observation that histamine failed to affect IL-4 production in 87% of the T cell clones (Krouwels et al., 1998). Furthermore, the investigators were unable to discern any significant differences between the cytokine responses when the observations were partitioned according to the anatomical source of the T cells (“activated” versus “non-activated” T cells) or disease status of the individual (“healthy non-asthmatic” versus “asthmatic”). In the present study, we were also unable to detect significant changes in either the BAL cell expression or in the BALF concentrations of either IL-4 or IFN- γ following histamine. Future studies will need to be conducted to determine if BAL cells or BALF obtained from horses with IAD demonstrate changes in these (or other cytokines) following HBP.

4.3. HBP and BALF cytology

Although histamine bronchoprovocation test has been performed as a research tool for several years in horses, we are unaware of any previous studies that have examined the effects of this test on total or nucleated cell percentages recovered in the bronchoalveolar lavage fluid. In primary cell cultures established from human bronchial epithelial airway cells (BEAS), histamine treatment significantly up-regulates proinflammatory cytokine production (Matsubara et al., 2005; Holden et al., 2007).

Histamine binding of the H1 receptor on epithelial cells activates phospholipase C which initiates an intracellular signaling cascade that eventually results in the nuclear translocation of NF- κ B and secondary inflammatory gene transcription. Within 1 h of histamine challenge, there is a detectable up-regulation of granulocyte-macrophage colony stimulating factor (GM-CSF), IL-6 and IL-8 in the BEAS cultures. This stimulatory effect on proinflammatory gene transcription continues for at least 35 h but can be suppressed by the prior addition of a histamine antagonist (Matsubara et al., 2005). As equine epithelial cells, examined ex vivo (biopsies) or in vitro (primary cell cultures) up-regulate IL-8 mRNA and protein concentrations in response to various inhaled stimuli (Franchini et al., 2000; Ainsworth et al., 2006; DeLuca et al., 2008), we predicted that HBP would be associated with the development of airway neutrophilia. The surprising lack of effect of histamine on IL-8 mRNA or protein concentrations and thus on BAL cytologies may simply reflect the time points examined in the present study. Perhaps had samples been obtained 24–36 h after HBP, an increase in either neutrophil chemokines or in the neutrophil percentages in the BALF might have developed. Nevertheless, the lack of effect on airway cytology (or chemokines expression) 2–4 h following HBP validates and supports the existing chronological order of these two techniques when evaluating the pulmonary health in horses.

5. Conclusions

As clinical and research investigations of IAD become more prevalent, investigators can be assured that HBP will not alter BALF cytological analyses or the expression of selected cytokines at the mRNA or protein level within 4 h of performing the HBP.

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