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Rapid, multiwell colorimetric assay for measuring neutrophil chemoattractant activity in bronchoalveolar lavage fluid of horses with recurrent airway obstruction

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Abstract. The criteria used to diagnose recurrent airway obstruction (RAO) in affected horses include demonstration of reversible lower airway obstruction and greater than 25% neutrophils in bronchoalveolar lavage fluid (BALF). Additional objective laboratory tests are needed to improve diagnostic accuracy and to monitor response to treatment. The goal of this study was to determine if neutrophil chemoattractant activity of BALF could be measured by using a previously described, rapid, multiwell colorimetric assay for chemotaxis. In this assay, neutrophils that have migrated through a membrane filter are collected into the bottom well of a disposable chemotaxis–cell migration chamber. The number of viable cells collected in the bottom well is quantified by measurement of the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide (MTT), which is reduced by dehydrogenase in mitochondria of live cells. The number of migrating cells corresponds to the amount of MTT reduced, which is measured with an enzyme-linked immunosorbent assay plate reader. Fourteen adult horses were enrolled in this study, 7 of which had owner histories consistent with RAO. Each horse was sedated, a bronchoalveolar lavage tube was passed, and saline was infused and immediately aspirated. An aliquot of BALF was used for differential cell count, and BALF supernatant was harvested to assess neutrophil chemoattractant activity. Normal control horses and RAO-affected horses were distinguished according to clinical signs and percent neutrophils in BALF. Neutrophil chemoattractant activity of BALF was significantly greater in RAO-affected horses ($P = 0.001$) compared with control horses. This assay may be useful in future studies for monitoring response to therapy in RAO-affected horses.

Key words: Chemotaxis; chronic bronchiolitis; equine; neutrophils.

Introduction

Recurrent airway obstruction (RAO), also known as *heaves* to horse owners and by the pathological-anatomical term *chronic bronchitis* or *bronchiolitis*, is the most common respiratory disease of adult horses.[7] It is characterized by reversible airway narrowing caused by bronchospasm,[22] which is mediated by muscarinic receptors and accompanied by airway hyperresponsiveness, neutrophilic inflammation in small airways, and hypersecretion of mucus, all leading to severe airway disease.[20,22–24] A diagnosis of RAO is usually made based on clinical signs, which include cough, respiratory distress (flared nostrils and forced expiratory effort), mucopurulent nasal discharge, abnormal lung sounds (harsh respiratory sounds, including wheezes and crackling heard especially on expiration), an enlarged lung percussion field, and a heave line. In clinically normal horses, small airway resistance contributes less than 20% of total airway resistance.[3,17,22] However, an extensive decrease in the caliber of small airways by mucus and smooth muscle contraction greatly increases resistance and, therefore, the work of breathing.

The etiology of RAO is likely multifactorial, though most researchers believe a hypersensitivity reaction to dusty or moldy hay, which is associated with indoor housing and poor ventilation, is a major cause.[20,22] Exposure of susceptible animals to hay and straw antigens can precipitate an inflammatory response, leading to bronchospasm and severe airway obstruction.[24] Researchers have shown that *in vitro* stimulation of equine bronchoalveolar macrophages results in the induction of expression of the chemokines interleukin (IL)-8 and macrophage inflammatory protein (MIP)-2, both of which are potent neutrophil chemoattractants.[11] Concentrations of IL-8 have also been shown to be increased in

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bronchoalveolar lavage fluid (BALF) of horses with RAO after exposure to hay.[12] Airway epithelial cells might also produce cytokines (e.g., IL-8) that result in neutrophil migration into airways and inflammation.[12] Equine neutrophils also have the ability to produce messenger RNA (mRNA) for IL-8 and MIP-2 and thus may significantly influence the evolution of the immune process through cytokine secretion.[15]

The purpose of the present study was to determine if neutrophil chemoattractant activity in BALF could be measured by using a rapid, multiwell colorimetric assay previously described.[27] The goal was to use this assay to determine if neutrophil chemoattractant activity was different in RAO-affected horses compared with healthy control horses.

Materials and methods

Animals. Seven client-owned horses previously diagnosed with RAO were entered into this study (mean age 17 yr, range 12–21 yr; 5 Quarter horses, 1 Appaloosa, and 1 Mustang). Horses were referred by local veterinarians. None of these horses had been treated with corticosteroid therapy, though 1 horse had received bronchodilator[a] therapy 3 days before examination. Seven clinically normal adult horses were selected from the Oregon State University College of Veterinary Medicine teaching herd to be used as healthy controls (mean age 11 yr, range 4–17 yr; 3 Thoroughbreds, 2 Quarter horses, 1 Holsteiner, and 1 Appaloosa). A thorough physical examination was performed on all horses with recording of body temperature; heart rate; respiratory rate; lung sounds; expiratory effort; and presence of coughing, nostril flair, nasal discharge, abdominal movement, and a heave line. A clinical scoring system previously described elsewhere[25,26] was used to assign a numerical score for nasal flaring and abdominal movement on a scale of 1–4. For nasal flaring, the scores were 1 = no flaring; 2 = slight, occasional flaring of nostrils; 3 = moderate nostril flaring; and 4 = severe, continuous flaring during each respiration. For the abdominal component of breathing, the scores were 1 = no abdominal component to breathing; 2 = slight abdominal movement; 3 = moderate abdominal movement; and 4 = severe, marked abdominal movement. The results of the 2 scores were summed to provide the total clinical score, so a score of 2 = no signs, 3 or 4 = mild signs, 5 or 6 = moderate signs, and 7 or 8 = severe signs. The experimental protocol was reviewed and approved by the Oregon State University Animal Care and Use Committee according to principles outlined by the National Institutes of Health.[19]

Bronchoalveolar lavage. Bronchoalveolar lavage fluid was collected to measure neutrophil chemoattractant activity. Horses were sedated with 5 mg of detomidine[b] given intravenously, and a bronchoalveolar lavage tube[c] was introduced through a nostril and into the nasopharynx and trachea and was then advanced until it wedged in a bronchus, where it was sealed by inflating a balloon cuff. At the same time the tube was advanced, 10 ml of 1% lidocaine[d] was administered through the tube to anesthetize the bronchial surface to prevent coughing. The bronchus was infused 3 times with physiological saline (100-ml aliquots), and fluid was manually aspirated immediately after each infusion. An aliquot of BALF was submitted for cytological examination for a cell differential count (cytospin), which was performed by a certified medical technologist by way of microscopic examination of 100 cells previously stained with Wright-Giemsa stain. The remaining BALF was kept on ice until centrifuged for 20 min at 4°C and 400 × *g*. Aliquots of supernatant were frozen at −70°C for later analysis.

Neutrophil chemoattractant activity. Equine neutrophils, collected from a healthy horse that was not included in this study, were purified from whole blood with heparin added as an anticoagulant, using a histopaque 1119 density gradient centrifugation protocol as previously described.[14] More than 93% of total cells isolated were neutrophils, and cell viability was greater than 90% based on trypan blue exclusion. These neutrophils were resuspended in Earle's minimum essential medium (EMEM; with 25 μm N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer without L-glutamine and phenol red) with 5% heat-inactivated fetal calf serum (FCS).

Approximately 300 μl of each BALF supernatant were placed into lower compartment wells of a 96-well ChemoTx Disposable Chemotaxis System[e] (5-μm pore size; 6.0-mm-diameter wells). After the microplate was loaded with BALF, a framed polycarbonate membrane filter was positioned over the plate. Next, 70 μl of the neutrophil cell suspension (2.5 × 10^5 cells) was pipetted over each well. A lid was placed over the filter of the 96-well plate apparatus, and the plate was incubated at 37°C in 5% CO_2 for 1 hr. After incubation, and with the filter still in place, cells were gently wiped off the top of the filter with cotton Q-tips. The plate and attached filter were held at a 45° angle while medium was carefully flushed over the surface of the filter to remove nonmigrated cells from the top surface without disturbing cells that had migrated through the filter. Next, 40 μl of 2 mM EDTA in phosphate-buffered saline (PBS) was pipetted onto the filter over each well, removing cells that had not traveled into or through the membrane filter, and the plate was incubated at 4°C for 30 min. After incubation, excess fluid and leftover cells were removed with cotton Q-tips. The plate was centrifuged at 500 × *g* at 18–21°C for 10 min to pellet cells that had migrated into or through the filter into the lower compartment wells. The filter was removed, and 230 μl of supernatant were discarded and replaced with 120 μl fresh medium (EMEM with 5% FCS) plus 10 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide (MTT).[f] The plate was incubated at 37°C in 5% CO_2 for 4 hr. All the fluid from the lower wells was carefully removed, leaving behind insoluble purple crystals. Next, 100 μl of room-temperature acid-isopropanol in 2 mM hydrochloric acid were added to the wells, and the purple crystals were resuspended by repeated pipetting. The plate was read immediately with an ELISA plate reader at a wavelength of 540 nm.

For positive controls for chemotaxis, 10-fold serial dilutions of IL-8[g] reconstituted in PBS with 0.1% bovine serum albumin (BSA) were placed into a series of lower

compartment wells. Wells with PBS and 0.1% BSA without IL-8 served as negative controls for chemotaxis. In addition, wells containing PBS with 0.1% BSA but without IL-8 and without cell suspension pipetted on the filter sites above these wells were used as negative controls for MTT. Additionally, a serial dilution of neutrophil cell suspension was pipetted into lower compartment wells for use as positive controls for MTT. Earles minimum essential medium with 5% FCS was also placed into a series of lower wells. One such set had cells placed on the filter above and served as media positive controls for chemotaxis. Another set of wells had no cells placed on the filter above and served as media negative controls for MTT.

Statistical analysis. Data are reported as mean ± SEM. Data were found to be normally distributed by the Kolmogorov-Smirnov test and were analyzed by the unpaired *t*-test. Significance was set at $P < 0.05$. Statistical analyses were performed by Number Cruncher Statistical System software.[h]

Results

Clinical signs and BALF cytologies. Signalment, stabling conditions, time elapsed since last treatment, clinical scores for nasal flaring and abdominal movement according to physical examination findings, and percent neutrophils in BALF are summarized in Table 1. On the basis of these findings, normal horses were considered as having histories free from respiratory disease, total clinical scores of 2, and less than 10% neutrophils on BALF cytology. The only abnormal clinical sign noted in the healthy control horses was increased respiratory rate (>20 breaths/minute) in 4 of 7 horses. Horses affected by RAO had previous diagnoses of RAO and total clinical scores greater than 5, except for the horse that had received bronchodilator therapy 3 days before examination. This horse had a total clinical score of 2. Six of 7 RAO-affected horses had clinical signs of coughing, increased respiratory rate (>20 breaths/minute), increased respiratory effort, increased adventitious respiratory sounds, and nostril flare; 4 of 7 had nasal discharge; and 3 of 7 had a heave line. The horse receiving bronchodilator therapy 3 days before examination did not have an increased respiratory rate (>20 breaths/minute), increased respiratory effort, nostril flare, nasal discharge, or a heave line. Horses affected by RAO also had greater than 25% neutrophils on BALF cytology, except for the previously treated horse, which had 20% neutrophils on BALF cytology.

Percent recovery of the 300 ml of physiological saline infused into bronchi of each horse was determined. For the healthy control horses, 220 ± 7 ml (mean ± SEM) or 73% of infused BALF was obtained from each horse. For RAO-affected horses, 146 ± 7 ml (mean ± SEM) or 49% of infused BALF was obtained from each horse. The difference in percent recovery between the 2 groups was statistically significant ($P < 0.001$).

For normal horses, the differential cell counts on BALF, reported as mean (range), were 4% (1–7%) neutrophils, 88% (81–92%) macrophages, and 6% (2–15%) lymphocytes. Differential cell counts for RAO-affected horses were 52% (20–83%) neutrophils, 40% (17–71%) macrophages, and 7% (0–14%) lymphocytes. Mean percent neutrophils were significantly higher in RAO-affected horses compared with normal horses ($P < 0.001$), and mean percent macrophages were significantly higher in normal horses compared with RAO-affected horses ($P < 0.001$). There were no significant differences in percent lymphocytes between the 2 groups of horses.

Neutrophil chemoattractant activity. The IL-8 standard curve shown in Fig. 1 demonstrates a typical bell-shaped curve for chemokine activation, similar to the standard curve obtained from the classical multiwell chamber chemotactic assay.[4] The IL-8 diluent alone (PBS with 0.1% BSA) did not cause migration of cells. The relationship between MTT reduction and number of cells is shown in Fig. 2. This standard curve was prepared from a serial dilution of the neutrophil cell suspension and showed a linear relationship between absorbance and cell numbers, from 3,900 to 250,000 cells ($R^2 = 0.920$). Intra-assay coefficient of variation for the neutrophil serial dilution standard curve ranged from 1 to 15%. Neutrophil migration was higher when using plates that had membrane filters with 5-µm pore size compared with membrane filters with 2- or 3-µm pore size (data not shown). When plates that had membrane filters of 8-µm pore size were used, migration was greater than 60% in all wells, and there was no difference in neutrophil chemoattractant activity between the 2 groups of horses (data not shown). Neither the IL-8 diluent (PBS with 0.1% BSA) nor the medium (EMEM with 5% FCS) reacted with MTT.

Neutrophil chemoattractant activity of BALF was significantly higher in the RAO-affected horses compared with the normal horses (Table 1; $P < 0.001$). Of the 2.5×10^5 cells pipetted onto the membrane filter over each BALF supernatant, there was 0% migration for normal horses and 9.1% migration for RAO-affected horses. The horse in the RAO-affected group that had received bronchodilator therapy 3 days before examination had a similar value for neutrophil chemoattractant activity of BALF compared to the other horses in this group.

Discussion

By using the MTT assay, neutrophil chemoattractant activity of BALF was found to be significantly

Table 1. Signalment, history, clinical scores, and percent neutrophils in BALF of RAO-affected horses and healthy control horses.*

Group	Breed	Age (yr)	Sex	History (time elapsed since last treatment)	Stabling conditions; feed and pasture	Clinical scores: Nasal	Clinical scores: Abdominal	Total clinical score	BALF recovered (ml)	BALF neutrophils (%)	Chemotaxis result (OD)
RAO											
R1	QH	15	F	>1 yr	Hay in winter; outside	3	3	6	146	58	0.41
R2	QH	20	G	3 mo	Grass hay; outside	3	4	7	170	44	0.33
R3	App	15	G	1.5 wk	Alfalfa, cob; stall	3	2	5	130	83	0.42
R4	QH	19	G	Several years	Alfalfa, cob; outside–day, inside–night	4	3	7	144	50	0.48
R5	Mustang	12	M	1 yr	Grass hay, Allegra Sr; inside-outside	2	3	5	155	28	0.38
R6	QH	15	G	>1 yr	Hay in winter; outside	2	3	5	124	81	0.45
R7	QH/App	21	G	3 days	Stall	1	1	2	155	20	0.40
Mean ±SEM		16.7 ± 1.2							146 ± 7	52 ± 9	0.41 ± 0.02
Control											
C1	QH	14	G	None	Outside	1	1	2	220	3	0.30
C2	QH	4	G	None	Outside	1	1	2	187	7	0.29
C3	App	17	F	None	Hay; inside–night	1	1	2	239	5	0.28
C4	TB	12	F	None	Hay; inside–night	1	1	2	220	7	0.33
C5	TB	10	G	None	Hay, cob and beet pulp; stall, paddock and pasture	1	1	2	223	5	0.33
C6	Holsteiner	9	G	None	Hay; inside–night	1	1	2	228	1	0.21
C7	TB	12	G	None	Hay, cob and beet pulp; stall, paddock and pasture	1	1	2	222	3	0.23
Mean ±SEM		11.1 ± 1.5							220 ± 7	4 ± 1	0.28 ± 0.02

* BALF = bronchoalveolar lavage fluid; RAO = recurrent airway obstruction; OD = optical density; QH= Quarter horse; F= female; G = gelding; App = Appaloosa; M = male; TB = Thoroughbred.

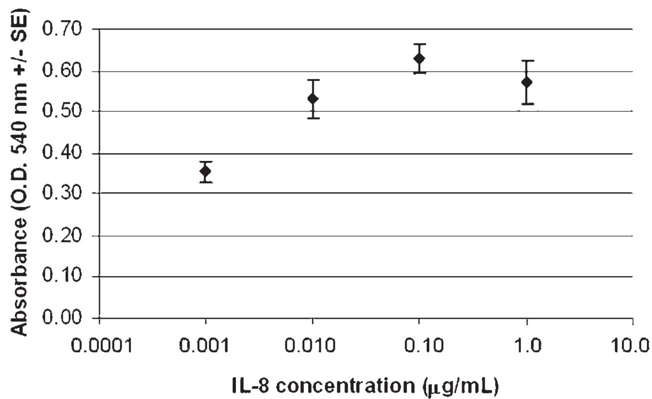

Figure 1. Dose-response curve for human IL-8 with a chemotaxis chamber and MTT reduction. Serial dilutions of IL-8 were placed in lower compartment wells of a 96-well ChemoTx Disposable Chemotaxis System[e] (5-μm pore size; 6.0-mm diameter wells), and neutrophil migration was assessed by MTT reduction. Each point represents the mean of 3 replicates.

greater in RAO-affected horses compared with normal horses. Even the horse that had received bronchodilator therapy, but not corticosteroid therapy, 3 days before examination had increased neutrophil chemoattractant activity in BALF similar to the other RAO-affected horses. Because the pharmacological mechanism of action of albuterol is predominantly that of a ß2-agonist, such bronchodilator therapy probably did not alter production of neutrophil chemokines and thus did not alter neutrophil chemoattractant activity of BALF as assessed by the MTT assay.

The assay described in this study has been shown to be more sensitive than other techniques, both in terms of response to low titers of chemoattractant and of the numbers of cells required for detection.[27] The MTT assay is sensitive in that as few as 500 cells can be measured, and the absorbance is highly correlated

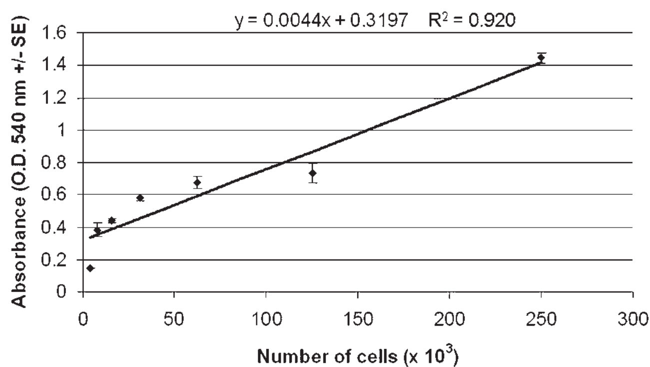

Figure 2. The relationship between MTT reduction and cell number. After incubation at 37°C for 4 hr, the medium was decanted and the reduced MTT crystals were dissolved and quantitated with an ELISA reader. Each point represents the mean of 3 replicates.

(r = 0.98) to the actual count of cells in the lower compartment wells.[27] In the study reported here, the relationship between absorbance and cell number was linear down to as few as 3,900 cells (r = 0.96). The advantage of using MTT is that it provides a simple, sensitive, reproducible, and rapid procedure that utilizes standard laboratory equipment. It is especially useful for large numbers of samples.[27]

The MTT assay serves as an estimate of the number of mitochondria and, hence, the number of living cells in the sample.[8] Because the MTT process also depends on enzymatic cellular activities, which can be upregulated in stimulated cells, differences in MTT assay results in some studies may reflect differences in metabolism of activated cells. For example, inducible nitric oxide synthase has been shown to reduce MTT in the presence of nicotinamide adenosine dinucleotide phosphate (NADPH).[21] This would suggest that cell activation may cause interference in this assay, leading to overestimation of cell numbers. However, in this study, the neutrophils used in all assays were isolated from a healthy horse that was not included in either group of horses reported here. Thus, differences in the MTT assay results reflect differences in neutrophil chemoattractant activity of the BALF between the healthy control and the RAO-affected horses.

Before the MTT assay, RAO-affected and healthy control horses were distinguished according to clinical scores and percent neutrophils in BALF. The RAO-affected horses met the "heaves" phenotype criteria for such animals as defined by the International Workshop on Equine Chronic Airway Disease.[22] To document pulmonary neutrophilia, BALF was collected, processed, and analyzed according to these guidelines. Six of 7 horses met the guideline of greater than 25% neutrophils in BALF during airway obstruction. The seventh horse had 20% neutrophils in BALF but was coughing and had abnormal lung sounds in addition to a history of reversible lower airway obstruction. This horse had been given bronchodilator therapy 3 days before presentation. At the time of examination, none of the horses were provocatively challenged to accentuate RAO. Normal horses met the guidelines in that they did not have a history of RAO and had less than 10% neutrophils in BALF.[22] The ages of these RAO-affected horses were consistent with the epidemiological pattern of RAO, whereby affected horses are usually older than 7 years. The volume of lavage fluid retrieved usually ranges from 40 to 60%, but this may decrease with RAO.[22] The volume retrieved was 73% in the control horses and 49% in the RAO-affected horses. Thus, these results are consistent with what has been previously reported.

Horses affected by RAO have been shown to have a similar percentage of neutrophils in BALF compared with control horses during clinical remission in a nonchallenge environment (pasture).[22] One study[12] reported that the percentages of neutrophils in BALF of RAO-affected horses when asymptomatic were 14.0 ± 2.7% but increased to 41.7 ± 9.5% when horses were exposed to hay dust for 2 weeks, during which time clinical signs of RAO developed. Another study[16] reported that the percentages of neutrophils in BALF of RAO-affected horses pre- and poststabling were 15.0 ±10.0% and >60%, respectively. In a third study,[1] when RAO-affected horses were housed in a natural challenge environment (stabled, fed dusty hay), the percentages of neutrophils in BALF were increased 4- to 6-fold (52 ± 8%) compared with healthy horses (8 ± 1%). The horses in this study were housed in a healthy environment; however, according to their BALF cytology results, neutrophilic inflammation of small airways was present, indicating that they were not in clinical remission. The clinical scores in the RAO-affected horses were judged to be of moderate severity in 4 horses and severe in 2 horses. All control horses were in the "no signs" category.

Chemotaxis is defined as directed cell locomotion toward a soluble extracellular gradient. Chemotaxis to a specific site is often associated with wound healing and immunologically mediated processes.[10] Chemokines are cytokines with chemotactic activity. There are 2 distinct neutrophil-specific chemokines: IL-8 and MIP-2. In a previous study, it was shown that supernatants of equine respiratory secretions (tracheal mucus) enhanced migration of equine neutrophils into the lower compartments of Boyden chambers, yet none of the 9 cell-free supernatants of undiluted BALF tested in that study induced significant neutrophil migration.[13] Researchers in another study failed to show a significant increase in chemotactic activity in BALF from RAO-affected horses compared with healthy horses, and the chemotactic activity did not correlate with the percentage of neutrophils in BALF.[11] However, in that study, RAO-affected horses exposed to dust had significantly higher chemotactic activity than did RAO-affected horses with low dust exposure. *In vitro* stimulation of BALF cells obtained from an RAO-affected horse in clinical remission showed an increase in mRNA specific for IL-8 and MIP-2, which was associated with an increase in chemotactic activity in the supernatant. Thus, a chemokine-specific mRNA increase was followed by an increase in chemotactic activity in the supernatant of stimulated cultures.[11] In a subsequent study, these same researchers showed that IL-8 concentration and chemotactic activity were greater in RAO-affected horses compared with healthy horses but only when hay-fed horses from each group were compared, and that IL-8 concentration and chemotactic activity were greater in RAO-affected horses exposed to hay dust compared with nonexposed RAO-affected horses.[12] In that study, a modified Boyden chemotaxis chamber was used to determine chemotactic activity, which was expressed as neutrophils per magnification field. The latter was determined by counting the number of neutrophils attached to the lower side of the 5-μm pore membrane separating the upper and lower compartments.

In addition to IL-8 and MIP-2, leukotriene (LT) B_4 has also been shown to be a neutrophil chemoattractant. One study showed that inhaled LTB_4 in horses causes neutrophil recruitment.[18] Authors of another study concluded that increased concentrations of LTB_4 in airways of RAO-affected horses contributes to neutrophil infiltration and the ongoing inflammation associated with RAO.[16] In humans, histamine stimulation of bronchial epithelial cells involves binding at H1 receptors, production of LTB_4, activation of the transcription factor NF-κB, and increased expression of IL-8.[2] Activity of NF-κB is increased in bronchial cells of RAO-affected horses and is highly correlated to the percentage of neutrophils present in the bronchi.[5,6] Selective blockage of NF-κB activity results in marked attenuation of allergic lung inflammation in antigen-sensitized mice.[9] On the basis of the results of the present study, whereby neutrophil chemoattractant activity in BALF was assessed with the MTT assay and found to be higher in RAO-affected horses compared with normal horses, this assay may be useful for monitoring the response to therapy of RAO-affected horses in future studies, regardless of the underlying chemoattractant.

Sources and manufacturers

a. Albuterol (Torpex in a metered dose inhaler, 360 μg twice per day), Boehringer Ingelheim, Vetmedica Inc., St. Joseph, MO.
b. Dormosedan, SmithKline Beecham Animal Health, Exton, PA.
c. Bivona Medical Technologies Inc., Gary, IN.
d. Lidocaine injection 2%, VEDCO, St. Joseph, MO.
e. Neuro Probe Inc., Gaithersburg, MD.
f. MTT, Molecular Probes, Eugene, OR.
g. IL-8, human recombinant, Sigma, St. Louis, MO.
h. Version 2004 (www.ncss.com), Kaysville, UT.

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