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Breath Condensate Hydrogen Peroxide Correlates with Both Airway Cytology and Epithelial Lining Fluid Ascorbic Acid Concentration in the Horse

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The relationship between hydrogen peroxide (H_2O_2) concentration in expired breath condensate (EBC) and cytology of the respiratory tract obtained from tracheal wash (TW) or bronchoalveolar lavage (BAL), and epithelial lining fluid (ELF) antioxidant status is unknown. To examine this we analysed the concentration of H_2O_2 in breath condensate from healthy horses and horses affected by recurrent airway obstruction (RAO), a condition considered to be an animal model of human asthma. The degree of airway inflammation was determined by assessing TW inflammation as mucus, cell density and neutrophil scores, and by BAL cytology. ELF antioxidant status was determined by measurement of ascorbic acid, dehydroascorbate, reduced and oxidised glutathione, uric acid and α-tocopherol concentrations. RAO-affected horses with marked airway inflammation had significantly higher concentrations of breath condensate H_2O_2 than control horses and RAO-affected horses in the absence of inflammation ($2.0 \pm 0.5\,\mu mol/l$, $0.4 \pm 0.2\,\mu mol/l$ and $0.9 \pm 0.2\,\mu mol/l$ H_2O_2, respectively; $p < 0.0001$). The concentration of breath condensate H_2O_2 was related inversely to the concentration of ascorbic acid in ELF ($r = -0.80$; $p < 0.0001$) and correlated positively with TW inflammation score ($r = 0.76$, $p < 0.0001$) and BAL neutrophil count ($r = 0.80$, $p < 0.0001$). We conclude that the concentration of H_2O_2 in breath condensate influences the ELF ascorbic acid concentration and provides a non-invasive diagnostic indicator of the severity of neutrophilic airway inflammation.

Keywords: Airway inflammation; Horse; Lung; Epithelial lining fluid

INTRODUCTION

Activated inflammatory cells such as neutrophils, macrophages and eosinophils, produce hydrogen peroxide (H_2O_2) through a respiratory burst.[1] H_2O_2 and other reactive oxygen species (ROS) protect the airways from invading micro-organisms. However, inflammation of the airways is also a common finding in a range of respiratory diseases in the absence of airway infection. Under normal circumstances the lung is protected from the damaging effects of H_2O_2 by an array of antioxidants present in pulmonary epithelial lining fluid (ELF). The major non-enzymatic antioxidants in human and equine ELF are ascorbic acid, glutathione and uric acid.[2,3] Patients with mild asthma have reduced concentrations of ascorbic acid in their airways compared to healthy controls.[4]

Elevated concentrations of H_2O_2 in expired breath condensate (EBC) have been detected in human subjects suffering from a variety of inflammatory lung disorders including adult respiratory distress syndrome (ARDS),[5] asthma,[6] bronchiectasis,[7] COPD[8,9] and unstable cystic fibrosis.[10] Long-term antioxidant supplementation with *N*-acetylcysteine decreased the H_2O_2 concentration in EBC of COPD subjects,[11] however, as far as we are aware there is

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no published evidence of a direct relationship between pulmonary antioxidant status and EBC H_2O_2 concentration.

Asthmatics using anti-inflammatory medication have lower concentrations of expired H_2O_2, suggesting that the concentration of H_2O_2 in EBC is related to the severity of airway inflammation.[12] A relationship between increased breath condensate H_2O_2 concentration and numbers of eosinophils and neutrophils in induced sputum has been demonstrated.[13,14] However, induced sputum contains cells and materials from both the trachea and lower airways and therefore, the contribution of these respiratory regions to EBC H_2O_2 is unclear. As far as we are aware, no published study has investigated the correlation between the number of neutrophils derived by bronchoalveolar lavage (BAL) and the concentration of H_2O_2 in EBC.

Recurrent airway obstruction (RAO; previously referred to as equine COPD or "heaves") is a respiratory condition in the horse, which has been used as an animal model of asthma.[15] RAO is characterised by periods of acute airway obstruction and inflammation, and periods of remission.[16] Periods of marked airway inflammation have been demonstrated most commonly by an increase in the number of neutrophils and less commonly eosinophils, in tracheal wash (TW) and bronchoalveolar lavage (BAL) fluid, compared to clinically healthy horses and RAO-affected horses in clinical remission.[17] In the current study we therefore used TW and BAL samples to obtain a more accurate identification of the region of lung involved in H_2O_2 production.

An increase in EBC H_2O_2 is indicative of increased ROS production and or impaired mechanisms of removal. This study examined the hypothesis that the concentration of H_2O_2 in exhaled breath condensate would be related to both the degree of airway neutrophilic inflammation (as assessed by cytology) and to the concentration of antioxidants in the ELF.

MATERIALS AND METHODS

Animals

Eight horses (7 geldings, 1 mare); 6 Thoroughbreds (TB), 2 Welsh mountain ponies; 429 ± 125 kg (mean ± s.d.); age 9 ± 4 years old), free of respiratory disease as determined by resting clinical examination, TW (cytology and bacteriology), routine haematology and serology were studied (Control). The horses were kept at pasture 24 h a day. After a period of at least two months at pasture, EBC was collected, followed by TW and BAL. In addition, ten horses were studied, which were diagnosed as suffering from RAO by demonstrating marked increases in airway resistance and more than 50% neutrophils in BAL fluid after environmental challenge.[18] Two groups of horses affected by RAO were studied. The first group had significant airway inflammation as determined by TW and BAL (RAO-I; $n = 8$; 5 geldings, 3 mares; 2 TB, 3 mixed breed, 3 ponies; weight 410 ± 105 kg; 13 ± 5 years). They were sampled on arrival at our clinic. The second group (RAO-R; $n = 8$; 7 geldings, 1 mare; 2 TB, 3 mixed bred, 3 ponies; weight 429 ± 117 kg; age 15 ± 5 years) was studied in the absence of airway inflammation (defined as BAL neutrophil count less than 20 cells/μl or BAL neutrophil percentage less than 10%) and after a period of at least two months at pasture.

Collection of Expired Breath Condensate

EBC was collected indoors between 8 and 11 am from conscious unsedated horses and always prior to TW and BAL. A collection method for horses was developed using a 5-l "U"-shaped stainless steel condensing tube surrounded by ice and water (volume of ice water 85 l; temperature 0°C). For collection, the horse was fitted with a mask (modified large Equine AeroMask™, Trudell Medical International, Veterinary division, London, Ontario, Canada) with a tight fitting rubber shroud and a non-rebreathing valve (Model 7200, Hans Rudolph, Kansas City, Missouri, USA). A flexible pipe (Superflex PU-R medium weight Polyurethane ducting, RS Components Ltd., England; volume 5 l, 50 mm diameter, 2.5 m long) connected the expiratory port of the valve to the condensing tube. The flexible pipe was placed in the centre of a larger pipe (10 cm diameter, 2.4 m in length, volume 18.8 l) and heated to 30°C by the use of a regulated heating device ("Comfi-Grip" hair dryer, Morphy Richards, Swinton, England) controlled by a 230 V 5 amp fan speed controller (ZIEHL-ebm, RS Components Ltd., England). The warm air was blown down the exterior pipe and around the connecting pipe from the end attached to the stainless steel condensing tube. This method maintained a temperature of 30°C within the connecting pipe as determined by direct measurements of temperature inside the exterior and interior pipes in pilot studies. All horses were familiarised with the mask before the collections commenced. The horse breathed through the circuit for 10 min and 2–4 ml of breath condensate was recovered from the condensing tube.

TW and BAL

Prior to endoscopy, the horses were sedated with romifidine intravenously (40–50 μg/kg; Sedivet, Boehringer Ingelheim, Bracknell, Berkshire, England). TW and BAL in the right dorso-caudal

lung were performed using 30 and 200 ml, respectively of 0.9% saline at 37°C as described previously.[19]

Analysis

A spectrophotometric assay based on the oxidation of tetramethylbenzidine (TMB) by hydrogen peroxide and catalysed by horseradish peroxidase adapted from Gallati and Pracht,[20] was used to assay for H_2O_2 concentration. Two hundred and fifty microlitres of 840 μmol/L TMB in 0.42 M citrate buffer pH 3.8 and 50 μl of 52.5 U/ml horseradish peroxidase were added to 500 μl breath condensate and the peak absorbance read at 370 nm after approximately 10 s. The limit of detection was 0.1 μmol/l with an intra-assay coefficient of variation $< 4\%$. All samples were assayed in duplicate. Snap freezing and thawing the samples caused a reduction in H_2O_2 concentration of $57 \pm 16\%$ compared to analysis within 5 min of collection (0.81 ± 0.35 μmol/l and 1.63 ± 0.98 μmol/l, respectively). Therefore all samples were analysed immediately following collection. The combined coefficient of variation for three consecutive breath condensate collections performed in each of the three horses was $6.6 \pm 6.1\%$, which is similar to that reported in humans ($9 \pm 3\%$;[21]). Recovery of H_2O_2 in condensate samples ($n = 3$) spiked with 2.5 μmol/l H_2O_2 was $99 \pm 3\%$.

TW samples were centrifuged at 1000 rpm for 10 min. A smear was made of the sediment on a poly-L-lysine coated slide and stained with haematoxylin and eosin. The degree of tracheal inflammation was assessed on the basis of an inflammation score out of nine, consisting of the sum of scores for visible mucus in the trachea (0 – none; 1 – slight; 2 – moderate; 3 – severe; assessed immediately prior to the TW), smear cell density (0 – low; 1 – medium; 2 – medium–high; 3 – high) and neutrophil proportion (0 – none, few cells or small number and diffuse; 1 – small number but aggregated; 2 – moderate number; 3 – neutrophil is predominant cell type). The visual scores for tracheal mucus were made at the time of endoscopy (i.e. were not made blind) and by the same investigator (C.M.D.). Scores for cell density and neutrophil proportion were made by a cytologist blinded as to the status of the horses sampled. Scoring of neutrophil number and cell density were made as opposed to direct counts due to the frequent occurrence of clumping in the presence of increased mucus. Inflammation scores of 2 or below (out of nine) are considered to be within normal or acceptable limits for our laboratory. Scores of 3 or above are considered to indicate increasing severity of disease. BAL differential and nucleated cell counts on at least 200 cells were determined on cytospun preparations stained with haematoxylin and eosin.

Aliquots of BAL fluid were centrifuged at 800*g* at 4°C for 10 min, within 10 min of collection, and stored at −80°C for ascorbic acid, uric acid and urea analysis. BAL fluid was deproteinised with methanol and stored at −196°C for glutathione analysis. BAL ascorbic acid and dehydroascorbic acid were analysed by HPLC with UV detection as described previously.[3,22] HPLC with electrochemical detection was used to measure reduced (GSH) and oxidised (GSSG) glutathione in BAL.[23] BAL uric acid, and plasma and BAL urea were determined using commercial kits (Sigma kits 685-10 and 66-20, respectively). The concentration of alpha-tocopherol in BAL was measured by HPLC according to the method described by Kelly *et al.*[24] BAL dilution factor was calculated using the urea method[25] and the results were expressed per litre of ELF. All samples were analysed within 4 weeks of collection. Concentrations of ascorbic acid and dehydroascorbate in ELF stored at −80°C were stable for at least 2 months (1.5 ± 0.4 mmol/l versus 1.5 ± 0.3 mmol/l for ascorbic acid and 0.19 ± 0.11 mmol/l versus 0.16 ± 0.11 mmol/l for dehydroascorbate).

Substances with a molecular weight of less than or equal to 62 were measured in EBC (Hydrogen ions, H_2O_2, nitrite and nitrate). Molecules with higher molecular weights were not detected in EBC (uric acid, ascorbic acid, glutathione and protein). When 40 μmol/l ascorbic acid was added to EBC samples, no H_2O_2 could be detected. However, addition of 4 μmol/l ascorbic acid did not alter the concentration of H_2O_2 in EBC, which is above the limit of detection for ascorbic acid (0.06 μmol/l).

Statistical Analysis

TW scores are presented as median and range. All other data are presented as mean ± SD, unless stated otherwise. One-way analysis of variance (ANOVA) with the Newman–Keuls test for multiple comparisons was used to compare the EBC H_2O_2 concentration and the BAL data for the different groups of horses, and the H_2O_2 concentration as a function of TW scores. Four RAO-affected horses were sampled in both the presence (RAO-I group) and absence (RAO-R group) of inflammation. However, the remaining RAO-affected horses were not sampled in both states. The Newman–Keuls test was used to compare the groups. The differences in TW scores (a category variable) between the groups of horses were analysed using the Mann–Whitney test. Spearman's Rank correlation was used to evaluate the relationship between TW inflammation score and H_2O_2 concentration. The relationship between BAL cell counts, and ELF antioxidant status with H_2O_2

concentration in EBC was investigated using Pearson's correlation.

RESULTS

Airway Inflammation

BAL total and differential cell counts and TW cytology for the control and RAO horses are presented in Table I and are similar to those previously reported.[26–29] RAO-affected horses with inflammation (RAO-I) had higher TW inflammation scores and BAL neutrophil counts compared to control horses and RAO-affected horses without inflammation (RAO-R) (Table I; $p \leq 0.01$). TW inflammation scores and BAL neutrophil counts were not different between RAO-affected horses without inflammation and control horses.

Effect of Disease Status

The H_2O_2 concentration in EBC (Fig. 1) was significantly greater in RAO-affected horses with inflammation (RAO-I; 2.0 ± 0.5 μmol/l) and without inflammation (RAO-R; 0.9 ± 0.2 μmol/l), compared to healthy control horses (Control; 0.4 ± 0.2 μmol/l; $p < 0.0001$). In the absence of inflammation, RAO horses (RAO-R) had a significantly lower concentration of H_2O_2 than those with inflammation (RAO-I; $p < 0.0001$). The H_2O_2 concentrations measured in EBC of healthy control horses (Control) were similar to those reported previously in healthy human subjects.[5,13,30,31]

Hydrogen Peroxide and Airway Inflammation

The H_2O_2 concentration in EBC as a function of TW mucus, cell density and neutrophil scores is shown in Fig. 2. There were no significant differences in H_2O_2 concentration between the mucus score groups ($p = 0.07$). Neutrophil scores and cell density scores of two or above were associated with an elevation in H_2O_2 concentration compared to scores of less than two and less than one, respectively ($p = 0.04$). In addition, a cell density score of 3 was associated with significantly higher H_2O_2 concentrations than a score of 1 ($p = 0.007$). EBC H_2O_2 concentration was significantly correlated with TW inflammation score ($r = 0.76$, $p < 0.0001$; Fig. 3).

H_2O_2 concentration did not correlate with BAL total nucleated cell, lymphocyte or macrophage counts. However, there were significant correlations between H_2O_2 concentration and BAL neutrophil percentage ($r = 0.71$, $p < 0.0001$) and the number of BAL neutrophils ($r = 0.80$, $p < 0.0001$; Fig. 3).

Hydrogen Peroxide and ELF Antioxidant Composition

The concentrations of antioxidants in ELF are presented in Table II. The concentration of H_2O_2 in EBC was inversely correlated with the ELF concentration of ascorbic acid ($r = -0.80$, $p < 0.0001$; Fig. 4), but was not related to the concentration of dehydroascorbate, reduced or oxidised glutathione, or uric acid ($p > 0.05$). Alpha-tocopherol was not detected in BAL fluid from any horse (limit of detection of 0.01 μmol/l).

DISCUSSION

The findings of the present study demonstrate that the concentration of H_2O_2 in EBC correlates positively with BAL neutrophil number and TW inflammation score, but negatively with the concentration of ascorbic acid in the pulmonary ELF. These new data provides both practical help, as the concentration of EBC H_2O_2 can differentiate RAO horses in the presence and absence of airway inflammation, as well as providing new mechanistic information regarding ELF antioxidant status and airway H_2O_2 concentration.

TABLE I Tracheal wash (TW; median and range) and bronchoalveolar lavage (BAL; mean ± SD) cytology in clinically healthy horses (Control; $n = 8$) and horses affected by recurrent airway obstruction (RAO) with airway inflammation (RAO-I; $n = 8$) and without airway inflammation (RAO-R; $n = 8$)

	Control	RAO-I	RAO-R
TW mucus score (out of 3)	1.0 (0.0–2.0)	2.0* (1.0–3.0)	1.0 (0.0–2.5)
TW cell density score (out of 3)	0.0 (0.0–1.0)	2.0* (0.0–3.0)	0.0† (0.0–1.0)
TW neutrophil score (out of 3)	0.0 (0.0–1.0)	3.0* (2.0–3.0)	2.0† (0.0–3.0)
TW inflammation score (out of 9)	1.0 (0.0–3.0)	6.0* (3.0–9.0)	2.0† (0.0–6.5)
Total nucleated cell count (/μl)	248 ± 93	226 ± 67	128 ± 38*,†
BAL % macrophages	56 ± 6	47 ± 10*	43 ± 12*
BAL % lymphocytes	35 ± 6	36 ± 9	47 ± 11*,†
BAL % neutrophils	5 ± 3	16 ± 6*	6 ± 3†
BAL macrophage count (/μl)	141 ± 65	109 ± 49	56 ± 26*,†
BAL lymphocyte count (/μl)	84 ± 26	84 ± 34	59 ± 19
BAL neutrophil count (/μl)	11 ± 8	34 ± 11*	8 ± 6†

*$p \leq 0.02$ from control. †$p \leq 0.02$ from RAO-I.

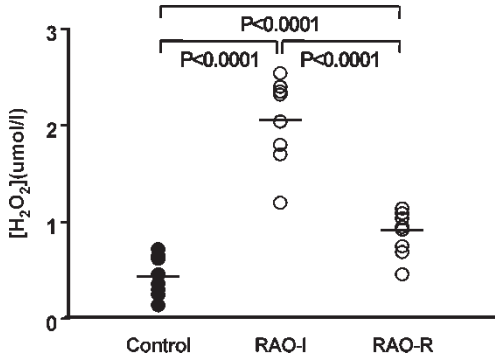

FIGURE 1 Hydrogen peroxide concentrations ([H_2O_2]) in breath condensate of clinically healthy horses (Control) and horses affected by recurrent airway obstruction (RAO) with airway inflammation (RAO-I) and RAO horses without inflammation (RAO-R). The mean for each group is indicated by the horizontal bar.

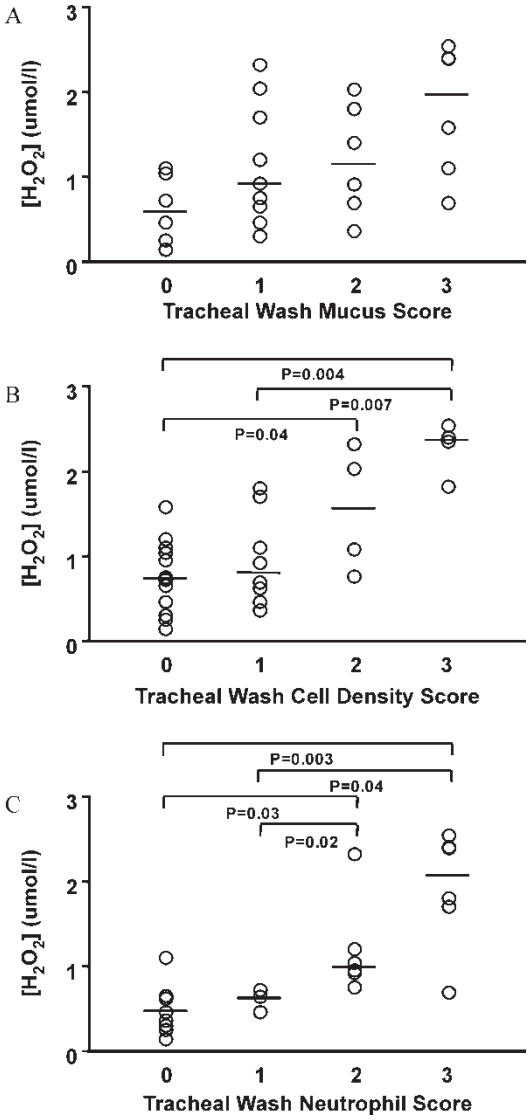

FIGURE 2 Hydrogen peroxide concentration ([H_2O_2]) in expired breath condensate as a function of TW mucus (A), cell density (B) and neutrophil proportion (C) scores in healthy control horses and in horses with recurrent airway obstruction in the presence and absence of inflammation. For each group, the median is indicated by the horizontal bar.

The source of the exhaled H_2O_2 appears to be the neutrophil, based on the strong correlation between BAL neutrophil counts and the H_2O_2 concentration in EBC, and the significant increase in TW neutrophil score with the H_2O_2 concentration. The lack of correlation between exhaled H_2O_2 and macrophage and lymphocyte counts supports this conclusion. Increased exhaled H_2O_2 indicates pulmonary oxidative stress and if neutrophils are the main source of this H_2O_2 then the oxidative stress results from the lung inflammation.

EBC H_2O_2 concentration will depend, in part, on neutrophil activity, which cannot be determined from cell counts alone. Therefore, determining EBC H_2O_2 concentration may be a more accurate measure of the inflammatory status of the respiratory tract than BAL cell counts and TW inflammation scores. Exhaled H_2O_2 has previously been correlated with the early steps of neutrophil activation in asthmatic subjects.[32] Neutrophils from RAO horses are known to be hyperstimulated[33] and similarly, neutrophils from human asthmatics release greater amounts of H_2O_2 than cells from healthy subjects.[34] Therefore the increased concentration of H_2O_2 in EBC may be due to increased neutrophil activation as well as increased number of neutrophils.

In the present study, H_2O_2 in EBC was inversely related to the ascorbic acid concentration, the major non-enzymatic antioxidant in equine ELF. This relationship may reflect a direct interaction between neutrophil generated H_2O_2 and ascorbic acid in ELF. Alternatively, superoxide anions, the main ROS generated by activated neutrophils, readily oxidised extracellular ascorbic acid forming H_2O_2 and dehydroascorbate.[35] Furthermore at sites of inflammation, hydrogen peroxide may be produced following the oxidation of ascorbic acid by transition metal ions.[36] The concentration of ELF dehydroascorbate did not however correlate with the concentration of H_2O_2 in EBC. This is most likely due to the rapid metabolism of dehydroascorbate to diketo-L-gulonic acid[37] or its rapid uptake by cells.[38,39]

The measurement of exhaled H_2O_2 may be useful in providing an indicator of airway inflammation in association with the standard techniques of TW and BAL, which have a number of associated problems. First, BAL induces a localised pulmonary neutrophil influx that persists for at least 48 h[40] and the technique requires sedation, limiting its repeated use over a short time period. The same applies to the technique of sputum induction.[41] Second, both TW and BAL involve washing the surface of the lung, which induces dilution problems. Third, tracheal samples are not necessarily representative of the bronchoalveolar region, therefore the use of both procedures is advantageous.[27] In contrast,

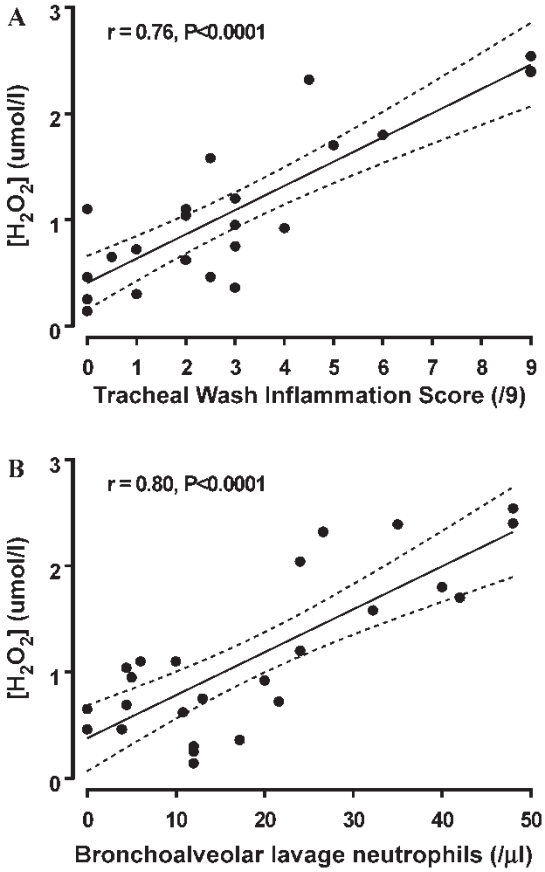

FIGURE 3 Correlation between TW inflammation score (Spearman, A) or BAL neutrophil number (Pearson, B) and expired breath condensate hydrogen peroxide concentration ($[H_2O_2]$). The regression line (solid line) and 95% confidence intervals (dotted line) are shown in each graph.

collection of EBC allows a global evaluation of the inflammatory status of the lung.

The EBC collection system was designed so that the volume of the stainless steel condensing tube and the connecting pipe approximated the resting tidal volume of the horse (approximately 5 l). With this approach, approximately one breath is held in the heated connecting pipe before being moved into the condensing tube by the following breath. This design was used to try and ensure that one part of the breath

TABLE II Concentrations of ascorbic acid, dehydroascorbate, reduced (GSH) and oxidised (GSSG) glutathione and uric acid in ELF obtained by bronchoalveolar lavage (mean ± SD) in clinically healthy horses (Control; $n = 8$) and horses affected by recurrent airway obstruction (RAO) with airway inflammation (RAO-I; $n = 8$) and without airway inflammation (RAO-R; $n = 8$)

	Control	RAO-I	RAO-R
Ascorbic acid (mmol/l)	2.1 ± 0.6	0.6 ± 0.4*	1.7 ± 0.6†
Dehydroascorbate (mmol/l)	0.2 ± 0.2	0.3 ± 0.3	0.2 ± 0.3
GSH (μmol/l)	71 ± 36	90 ± 58	124 ± 60
GSSG (μmol/l)	6 ± 6	11 ± 8	10 ± 9
Uric acid (μmol/l)	3.1 ± 1.7	1.9 ± 0.5	2.3 ± 1.7

*$p < 0.0001$ from control. †$p < 0.0001$ from RAO-I.

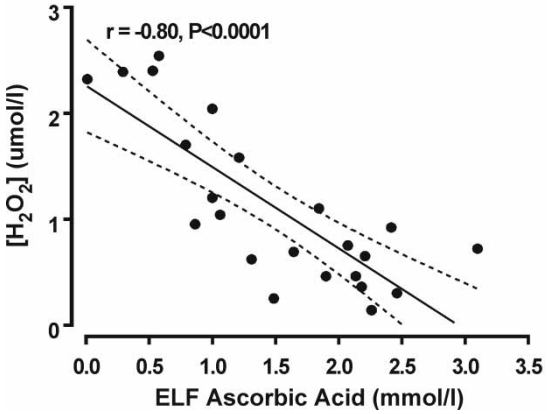

FIGURE 4 Correlation between ELF ascorbic acid (AA) concentration (Pearson) and expired breath condensate hydrogen peroxide concentration ($[H_2O_2]$). The regression line (solid line) and 95% confidence intervals (dotted line) are shown in each graph.

is not preferentially condensed but prevents anatomical identification of the site of H_2O_2 production. However, the correlations between EBC H_2O_2 and indicators of TW and BAL inflammation suggest that both the trachea and the bronchoalveolar regions of the respiratory tract are sites of H_2O_2 production. Schleiss and colleagues[42] suggested that H_2O_2 originated from the airways rather than the alveoli. The potential contribution of H_2O_2 produced from the nasal passages was not investigated in the present study. In human studies, nasal contamination is avoided by subjects wearing a nose clip and breathing through the mouth. Nowak and colleagues[21] reported a 20% increase in expired H_2O_2 in two human subjects when wearing a nose clip compared to without wearing it. However in another study, the same group reported no effect of wearing the nose clip.[31] The horse is an obligate nasal breather, therefore the contribution of H_2O_2 produced in the nasal passages in the condensate cannot be excluded. However, the strong correlations between EBC H_2O_2 concentrations and TW and BAL cytology and ELF ascorbic acid concentration, suggests that the contribution of nasal H_2O_2 production in this study was minimal.

Although minute ventilation was not recorded in the present study, there were no obvious changes in breathing pattern or frequency in any horse during EBC collection. Moreover, it is unlikely that any change in minute ventilation would influence the concentration of expired H_2O_2, as a rise would increase the volume of condensate produced as well as the amount of expired H_2O_2.[43]

In summary, the concentration of H_2O_2 in EBC differentiated RAO-affected horses in the presence of airway inflammation from healthy horses and RAO-affected horses without inflammation. The concentration of EBC H_2O_2 was strongly related to neutrophilic lower airway inflammation

on the basis of TW and BAL cytology and was inversely correlated with the ELF concentration of ascorbic acid.

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