

Pulmonary epithelial lining fluid and plasma ascorbic acid concentrations in horses affected by recurrent airway obstruction

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Objective—To determine the pulmonary epithelial lining fluid (ELF) concentrations and degree of oxidation of ascorbic acid in horses affected by recurrent airway obstruction (RAO) in the presence and absence of neutrophilic airway inflammation.

Animals—6 RAO-affected horses and 8 healthy control horses.

Procedure—Nonenzymatic antioxidant concentrations were determined in RBC, plasma, and ELF samples of control horses and RAO-affected horses in the presence and absence of airway inflammation.

Results—ELF ascorbic acid concentration was decreased in RAO-affected horses with airway inflammation (median, 0.06 mmol/L; 25th and 75th percentiles, 0.0 and 0.4 mmol/L), compared with RAO-affected horses without airway inflammation (1.0 mmol/L; 0.7 and 1.5 mmol/L) and control horses (2.2 mmol/L; 1.4 and 2.2 mmol/L). Epithelial lining fluid ascorbic acid remained significantly lower in RAO-affected horses without airway inflammation than in control horses. Moreover, the ELF ascorbic acid redox ratio (ie, ratio of the concentrations of dehydroascorbate to total ascorbic acid) was higher in RAO-affected horses with airway inflammation (median, 0.85; 25th and 75th percentiles, 0.25 and 1.00), compared with RAO-affected horses without airway inflammation (0.04; 0.02 and 0.22). The number of neutrophils in bronchoalveolar lavage fluid was inversely related to the ELF ascorbic acid concentration ($r = -0.81$) and positively correlated with the ascorbic acid redox ratio ($r = 0.65$).

Conclusions and Clinical Relevance—Neutrophilic inflammation in horses affected by RAO is associated with a reduction in the ELF ascorbic acid pool. Nutritional supplementation with ascorbic acid derivatives in horses affected by RAO is an area for further investigation. (*Am J Vet Res* 2004;65:80–87)

Recurrent airway obstruction (RAO), formerly known as equine chronic obstructive pulmonary disease or heaves, is a respiratory condition in horses, with proposed similarities to human asthma.^{1,2} Recurrent airway obstruction is characterized by peri-

ods of acute neutrophilic airway inflammation and airway obstruction and periods of remission.

Oxidants produced by activated inflammatory cells are capable of damaging DNA, lipids, and protein and acting as inflammatory mediators. A complex system of enzymatic and nonenzymatic antioxidants exists to protect against oxidative injury. Recurrent airway obstruction-affected horses with airway inflammation have previously been shown to have evidence of pulmonary oxidative stress on the basis of the oxidation of glutathione in the pulmonary epithelial lining fluid (ELF).³ However, we have recently determined that ascorbic acid is present in the ELF of healthy horses at a concentration approximately 40 times greater than that of glutathione⁴ and 20 times the ELF ascorbic acid concentration of man.⁵ The high concentration of ELF ascorbic acid probably reflects the horse's ability to synthesize this antioxidant vitamin. Rats, which can also produce ascorbic acid endogenously, have a similar ELF ascorbic acid concentration to horses.⁶

Ascorbic acid has been identified as an important nonenzymatic antioxidant⁷ reacting with inhaled oxidants such as ozone⁸ and neutrophil-derived oxidants such as superoxide, hydrogen peroxide, and hypochlorous acid.⁹ Results of a number of studies^{10–12} have suggested that ascorbic acid may be beneficial in the treatment of asthma, airway obstruction, and airway hyper-reactivity. Ascorbic acid functions by donating electrons to reactive oxygen species and recycling the oxidized forms of other antioxidants such as α -tocopherol¹³ and glutathione,¹⁴ thereby restoring their antioxidant capability. Glutathione has also been implicated in the enzymatic¹⁵ and nonenzymatic¹⁶ reduction of the oxidized form of ascorbic acid (ie, dehydroascorbate [DHA]).

In mice, hyperoxia and infection with influenza A virus have been shown to result in the oxidation of ascorbic acid in bronchoalveolar lavage fluid (BALF)¹⁷ and lung tissue,¹⁸ respectively. As far as we are aware, however, there are no published reports of the concentration of DHA in ELF or plasma of human asthmatic patients or RAO-affected horses.

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The purpose of the study reported here was to examine the systemic and pulmonary concentrations of ascorbic acid and DHA in RAO-affected horses with and without airway inflammation and compare these findings with those of control horses maintained under similar management. We hypothesized that airway inflammation in RAO-affected horses would result in an increase in the oxidation of ascorbic acid in ELF, which would be reversed with the resolution of inflammation.

Materials and Methods

Animals—The study was approved by the Ethics Committee of the Animal Health Trust and conformed to the United Kingdom Animal (Scientific Procedures) Act of 1986. Eight horses (5 geldings, 3 mares, 6 Thoroughbreds, and 2 Welsh ponies; age, 7 ± 3 years old [mean \pm SD]; weight, 425 ± 115 kg) served as control horses and were determined to be free from respiratory tract disease after 3 months at pasture on the basis of clinical examination findings, routine hematologic evaluation results, and findings on cytologic evaluation ($< 10\%$ neutrophils in BALF¹⁹) and bacterial culture of tracheal wash fluid and BALF. In addition, in response to environmental challenge, control horses did not develop increases in airway resistance.¹⁹

Six horses were also studied (5 geldings, 1 mare; 2 Thoroughbreds, 2 crossbred horses, and 2 ponies; age, 16 ± 5 years old; weight, 413 ± 83 kg) and determined to have RAO by demonstrating increases in airway resistance and $> 50\%$ neutrophils on differential cell counts of BALF after environmental challenge.¹⁹ Bronchoalveolar lavage was performed in each RAO-affected horse during naturally occurring airway inflammation and after 3 months at pasture when these horses had no substantial airway inflammation (defined as < 20 neutrophils/ μ L of BALF) or clinical signs of respiratory tract disease. Bronchoalveolar lavage in the right dorsocaudal lung lobe was performed by use of 200 mL of saline (0.9% NaCl) solution at 37°C , as described previously.⁴ All horses in the study were fed haylage.^a

Sample processing—Venous blood samples (20 mL), obtained by venipuncture immediately prior to sedation, were placed on ice in separate tubes containing either lithium-heparin (15 mL) or EDTA (5 mL) and centrifuged at $400 \times g$ for 10 minutes at 4°C within 5 minutes of collection. For hemolysate glutathione analysis, 0.5 mL of RBCs was added to 0.5 mL of saline solution containing 2mM Na₂EDTA and stored at -196°C . For ascorbic acid analysis, 0.5 mL of plasma in EDTA was added to 0.5 mL of 10% metaphosphoric acid containing 1mM Na₂EDTA to deproteinize the plasma. The sample was vortexed, snap-frozen in liquid nitrogen, and stored at -80°C . To reduce the oxidized form of ascorbic acid (ie, DHA) and measure the total concentration of ascorbic acid, 0.3 mL of plasma in EDTA was added to 200 μ L of 10mM dithiothreitol, vortexed, and left to stand at room temperature (approx 22°C) for 10 minutes. Next, 500 μ L of 10% metaphosphoric acid in 1mM Na₂EDTA was added, the sample vortexed, snap-frozen in liquid nitrogen, and stored at -80°C . Lithium heparin plasma aliquots (0.5 mL) were stored at -80°C for urea and uric acid analysis. Bronchoalveolar lavage fluid was deproteinized with methanol for total glutathione analysis and stored at -196°C . Aliquots of BALF were centrifuged at $800 \times g$ at 4°C for 10 minutes and stored at -80°C for ascorbic acid, α -tocopherol, and urea analysis and at -196°C for uric acid analysis. For malondialdehyde analysis, 1 mL of centrifuged BALF was added to the evaporated residue of 100 μ L of butylated hydroxy toluene (100 mg/mL in ethanol) and 100 μ L of desferal (100 mg/mL in water), vortexed, and stored at -80°C .

Sample analysis—Plasma and BALF ascorbic acid concentrations were analyzed by high-performance liquid chro-

matography (HPLC) with ultraviolet detection.²⁰ The ascorbic acid redox ratio was calculated by dividing the concentration of DHA by the total ascorbic acid concentration (sum of DHA and ascorbic acid). High-performance liquid chromatography with electrochemical detection was used to measure reduced glutathione (GSH) and oxidized glutathione (GSSG) concentrations in BALF and RBC haemolysates.²¹ Glutathione redox ratio was calculated by dividing GSSG by the total concentration of glutathione (sum of GSH and GSSG). The concentration of α -tocopherol in BALF was measured by use of HPLC according to the method described by Kelly et al.²² Plasma uric acid concentration and plasma and BALF urea concentrations were determined by use of commercial kits.^b Bronchoalveolar lavage fluid uric acid concentration was analyzed by use of HPLC with ultraviolet detection. Bronchoalveolar lavage fluid malondialdehyde concentration was determined by use of HPLC with fluorometric detection based on the method of Young and Trimble.²³ Bronchoalveolar lavage dilution factor was calculated by use of the urea method,²⁴ and the results were expressed per liter ELF.

Paired samples were collected 3 days apart from 12 horses (6 controls and 6 RAO-affected horses). Measurement precision for paired samples was as follows: for plasma samples, ascorbic acid concentration of 1.4 μ mol/L, DHA concentration of 0.4 μ mol/L, total ascorbic acid concentration of 1.9 μ mol/L, and an ascorbic acid redox ratio of 0.04; for RBC samples, GSH concentration of 40 μ mol/L, GSSG concentration of 4 μ mol/L, total GSH concentration of 44 μ mol/L, and a glutathione redox ratio of < 0.01 ; and for ELF samples, ascorbic acid concentration of 0.20 mmol/L, DHA concentration of 0.08 mmol/L, total ascorbic acid concentration of 0.20 mmol/L, GSH concentration of 28 μ mol/L, GSSG concentration of 2.4 μ mol/L, total GSH concentration of 29 μ mol/L, an ascorbic acid redox ratio of 0.09, and a glutathione redox ratio of 0.03. Bronchoalveolar lavage fluid differential cell counts on 200 cells were determined manually on cytospin preparations stained with H&E. Bronchoalveolar lavage fluid nucleated cell counts were determined manually on a hemocytometer. Measurement precision for paired BALF samples were as follows: total nucleated cell count of 34 cells/ μ L, neutrophil count of 7 cells/ μ L or 4%, macrophage count of 20 cells/ μ L or 8%, and lymphocyte count of 20 cells/ μ L or 9%.

Statistical analysis—Data are presented as median and 25th and 75th percentiles. The Kruskal-Wallis test was used to compare the data of the control group with that of RAO-affected horses with and without airway inflammation. If significance was attained, further analyses were performed with the Mann-Whitney test. Comparison between RAO-affected horses with and without airway inflammation was investigated by use of the Wilcoxon signed rank test. Relationships between pulmonary and systemic antioxidants and findings on BALF cytologic evaluation were investigated by use of the Spearman's Rank correlation in control horses and RAO-affected horses with airway inflammation, but not RAO-affected horses without airway inflammation to remove the effect of repeated measures. Values of $P < 0.05$ were considered significant. Analysis was performed by use of a software program.^c

Results

Airway inflammation—In BALF, neutrophil percentage (Table 1) and count (Fig 1) were significantly higher in RAO-affected horses with airway inflammation, compared with RAO-affected horses without airway inflammation and control horses. Macrophage percentage and count were significantly lower in BALF of RAO-affected horses with and without airway

Table 1—Median and 25th and 75th percentile values from cytologic evaluation of bronchoalveolar lavage fluid in 8 healthy horses (control horses) and 6 recurrent airway obstruction (RAO)-affected horses with and without airway inflammation

BALF variables	Control horses		RAO-affected horses with inflammation		RAO-affected horses without inflammation	
	Median	Percentiles*	Median	Percentiles*	Median	Percentiles*
Total NC (cells/ μ L)	250 ^a	223 and 285	160 ^{ab}	105 and 200	110 ^b	80 and 125
Neutrophils (cells/ μ L)	8 ^a	5 and 12	29 ^b	23 and 33	3 ^a	1 and 4
Neutrophil (%)	4 ^a	2 and 4	16 ^b	12 and 21	3 ^a	1 and 4
Macrophages (cells/ μ L)	140 ^a	131 and 179	29 ^b	26 and 74	38 ^b	26 and 38
Macrophage (%)	60 ^a	56 and 62	25 ^b	17 and 40	35 ^b	31 and 37
Lymphocytes (cells/ μ L)	90 ^a	72 and 100	69 ^a	56 and 118	59 ^a	51 and 78
Lymphocyte (%)	33 ^a	30 and 36	48 ^{ab}	36 and 57	56 ^b	54 and 62

*25th and 75th percentiles.
^{a,b}Median values with different superscript letters indicate significant ($P < 0.05$) differences in BALF variables among groups.
 NC = Nucleated cells.

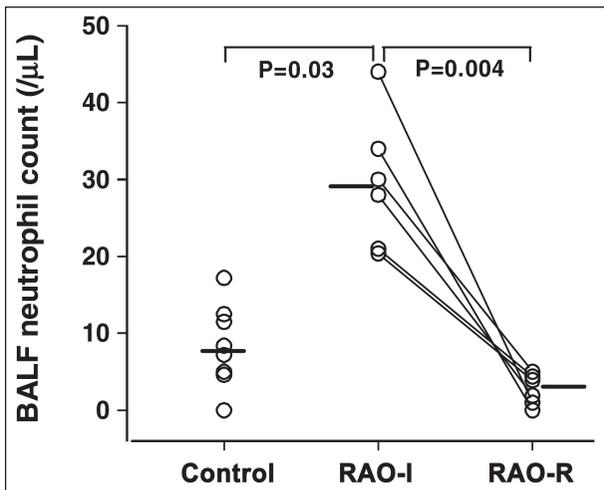


Figure 1—Individual neutrophil counts in bronchoalveolar lavage fluid (BALF) samples from clinically healthy horses ($n = 8$; control) and horses affected by recurrent airway obstruction (RAO; 6) with marked airway inflammation (RAO - I) and without airway inflammation (RAO - R; < 20 neutrophils/ μ L). Solid horizontal bars indicate the median for each group.

inflammation, compared with BALF of control horses. No growth of potentially pathogenic bacteria in excess of 10^3 colony-forming units/mL was detected in any BALF samples.

Circulatory antioxidants—Horses affected by RAO had significantly lower plasma concentrations of total ascorbic acid (sum of ascorbic acid and DHA) in both the presence and absence of airway inflammation, compared with control horses (Fig 2). Concentrations of plasma DHA were not significantly different among the groups. The plasma ascorbic acid redox ratio was significantly higher in RAO-affected horses without airway inflammation, compared with control horses. The plasma ascorbic acid redox ratio was higher in RAO-affected horses with airway inflammation, compared with control horses, but this difference was not significant ($P = 0.07$). Concentrations of total GSH in RBC hemolysate were not significantly different among the groups; however, the concentration of GSSG and the glutathione redox ratio were significantly higher in RAO-affected horses with airway inflammation, com-

pared with RAO-affected horses without airway inflammation and control horses (Fig 3). No significant differences were found in the concentration of plasma uric acid between RAO-affected horses with (median, 8.9 μ mol/L; 25th and 75th percentile, 7.2 and 13.1 μ mol/L) or without (9.2 μ mol/L; 9.2 and 10.0 μ mol/L) airway inflammation, and neither group had plasma uric acid concentrations that differed significantly from control horses (10.5 μ mol/L; 9.5 and 11.8 μ mol/L).

Pulmonary antioxidants—Epithelial lining fluid ascorbic acid concentration was significantly lower in RAO-affected horses with airway inflammation, compared with RAO-affected horses without airway inflammation and control horses. Recurrent airway obstruction-affected horses with airway inflammation also had a significantly higher ascorbic acid redox ratio in ELF, compared with RAO-affected horses without airway inflammation ($P = 0.02$) and control horses ($P = 0.07$). In the absence of inflammation, the concentration of ELF ascorbic acid remained significantly ($P = 0.03$) lower in RAO-affected horses, compared with control horses, but no significant difference was found in the ascorbic acid redox ratio between the 2 groups (Fig 4). The median concentration of total GSH was significantly increased in the ELF of RAO-affected horses without airway inflammation, compared with control horses (Fig 5). Recurrent airway obstruction-affected horses without airway inflammation had a significantly higher ELF GSH concentration, compared with RAO-affected horses without airway inflammation and control horses. The concentration of ELF GSSG was significantly higher in RAO-affected horses with airway inflammation, compared with control horses. The glutathione redox ratio was greatly increased in 4 of 6 RAO-affected horses with airway inflammation, compared with RAO-affected horses with airway inflammation and control horses. No significant differences were found in the concentration of ELF uric acid between RAO-affected horses with (median, 2.4 μ mol/L; 25th and 75th percentiles, 1.2 and 2.6 μ mol/L) or without (2.4 μ mol/L; 2.0 and 2.8 μ mol/L) airway inflammation or control horses (2.6 μ mol/L; 2.1 and 3.1 μ mol/L). In BALF, α -tocopherol was not detected in samples from any horse (limit of detection, 0.01 μ mol/L). Recurrent airway

obstruction-affected horses both with (median, 2.4 $\mu\text{mol/L}$; 25th and 75th percentiles, 2.3 and 3.0 $\mu\text{mol/L}$) and without (3.7 $\mu\text{mol/L}$; 3.2 and 3.8 $\mu\text{mol/L}$) airway

inflammation had significantly higher concentrations of malondialdehyde in ELF, compared with control horses (1.0 $\mu\text{mol/L}$; 0.9 and 1.7 $\mu\text{mol/L}$).

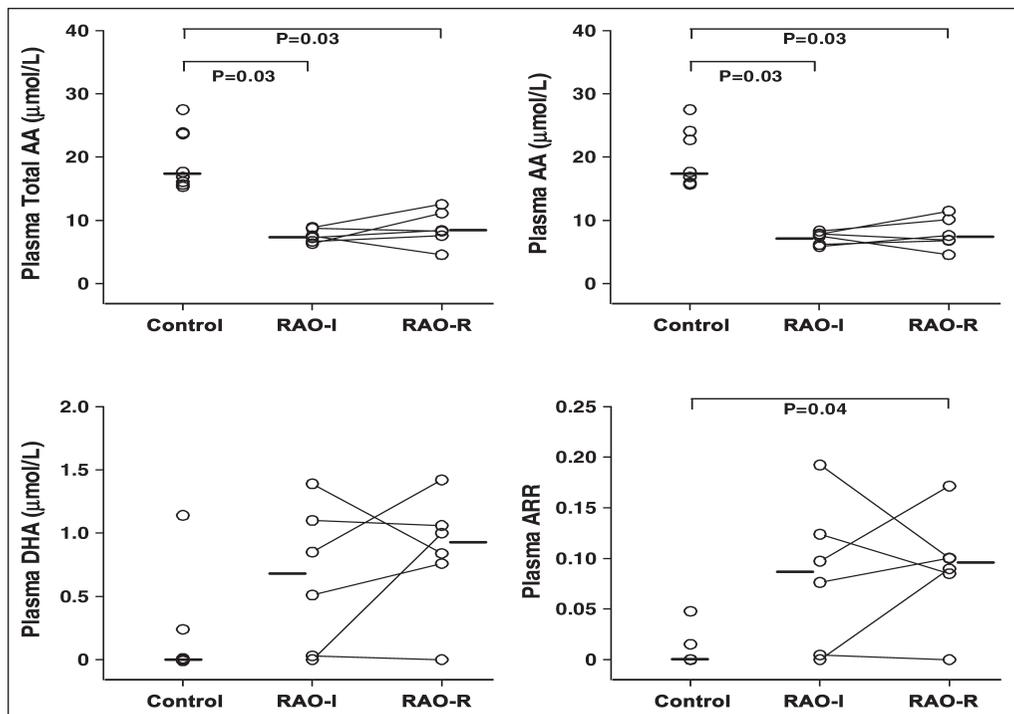


Figure 2—Individual concentrations of total and reduced ascorbic acid (AA) and dehydroascorbate (DHA) and the ascorbic acid redox ratio (ARR) in plasma samples of horses. See Figure 1 for remainder of key.

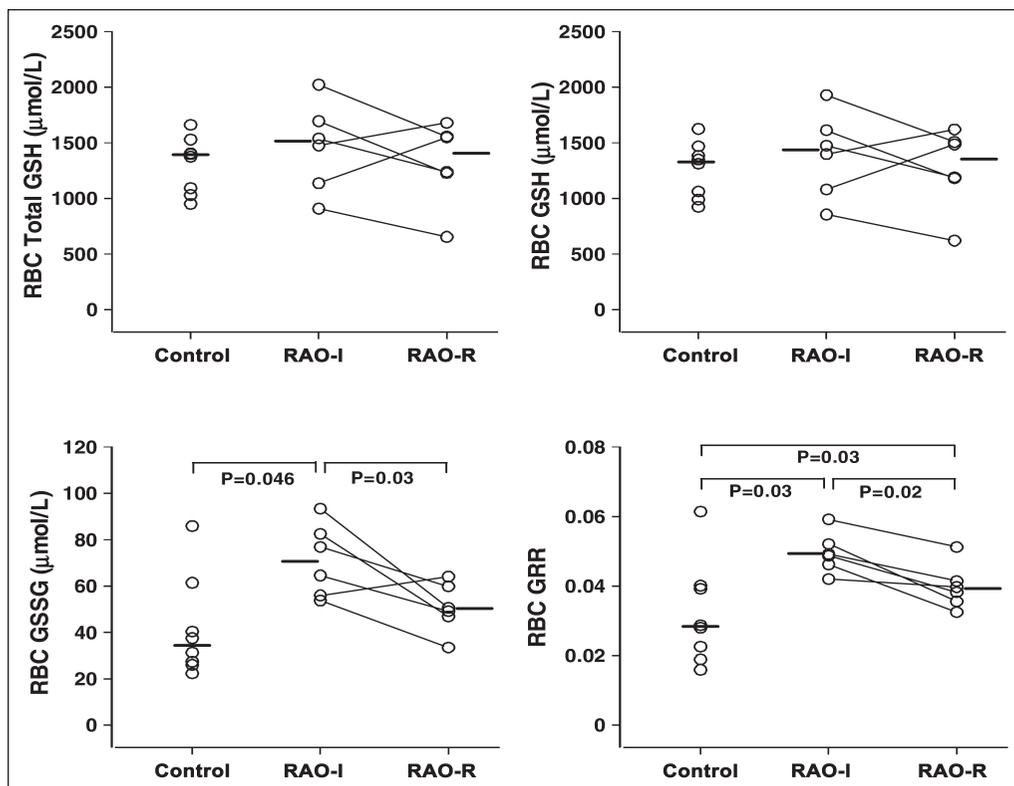


Figure 3—Individual concentrations of total glutathione (total GSH), reduced glutathione (GSH), and oxidized glutathione (GSSG) and the glutathione redox ratio in RBC hemolysate samples of horses ELF= Epithelial lining fluid. See Figure 1 for remainder of key.

Results of correlation analyses performed on data from control horses and RAO-affected horses with airway inflammation indicated that the ascorbic acid redox

ratio and glutathione redox ratio in ELF were significantly correlated ($r = 0.95$; $P < 0.001$) and the number of neutrophils in BALF was inversely related to the ELF

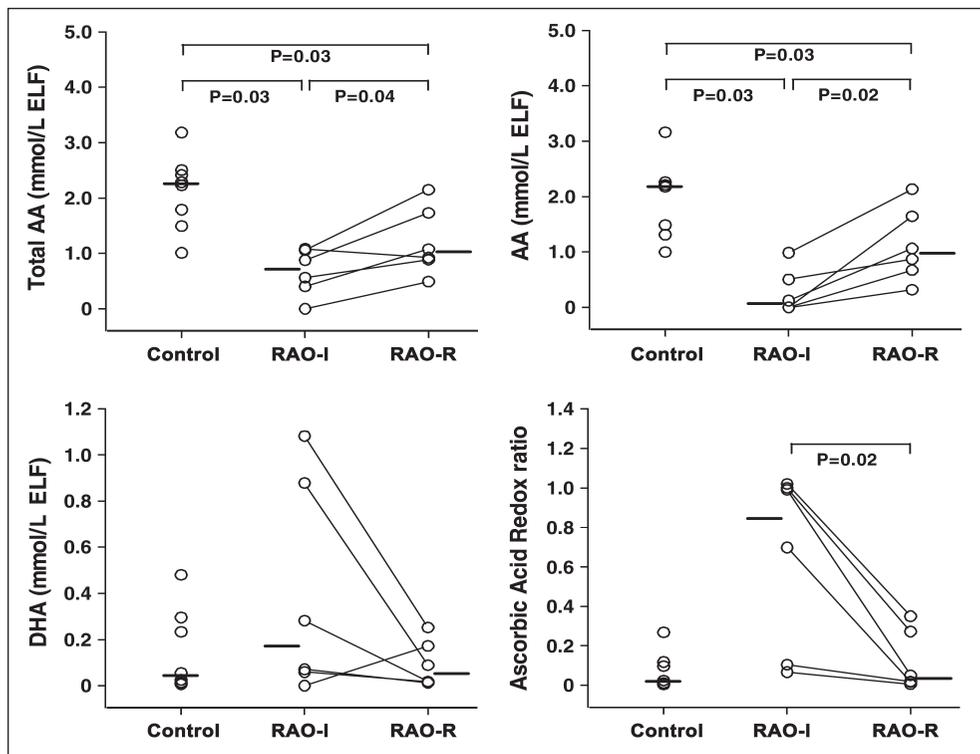


Figure 4—Individual concentrations of total and reduced AA and DHA and the ascorbic acid redox ratio in pulmonary ELF samples from horses. See Figure 1 for remainder of key.

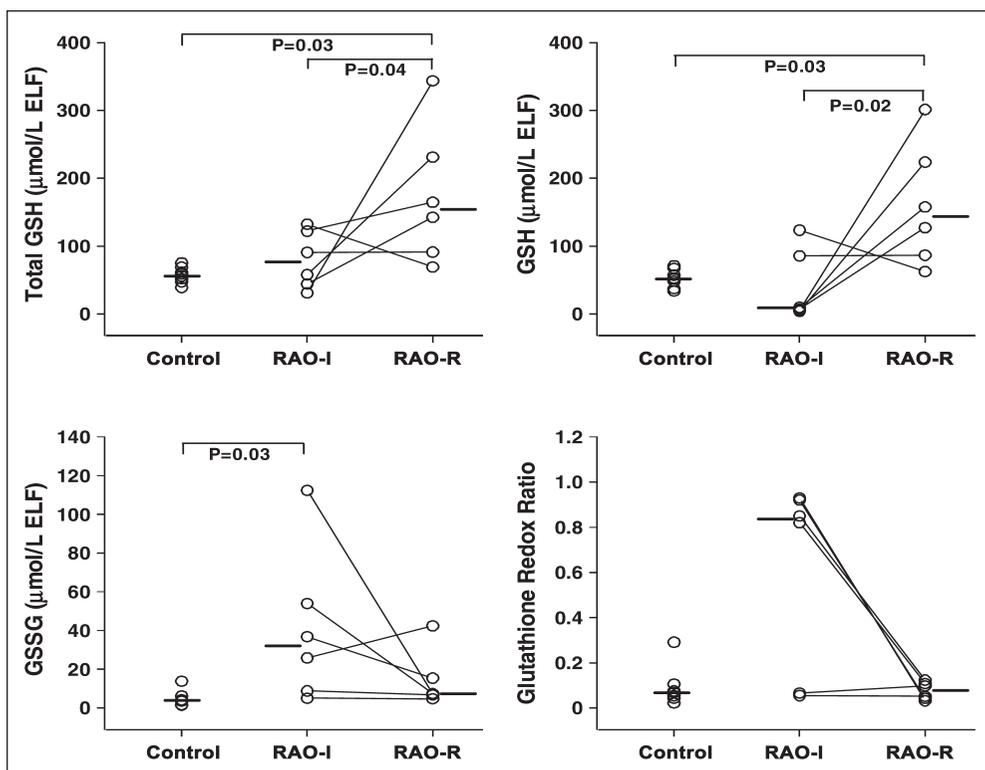


Figure 5—Individual concentrations of total GSH, GSH, and GSSG and the glutathione redox ratio in pulmonary ELF samples from horses. See Figure 1 for remainder of key.

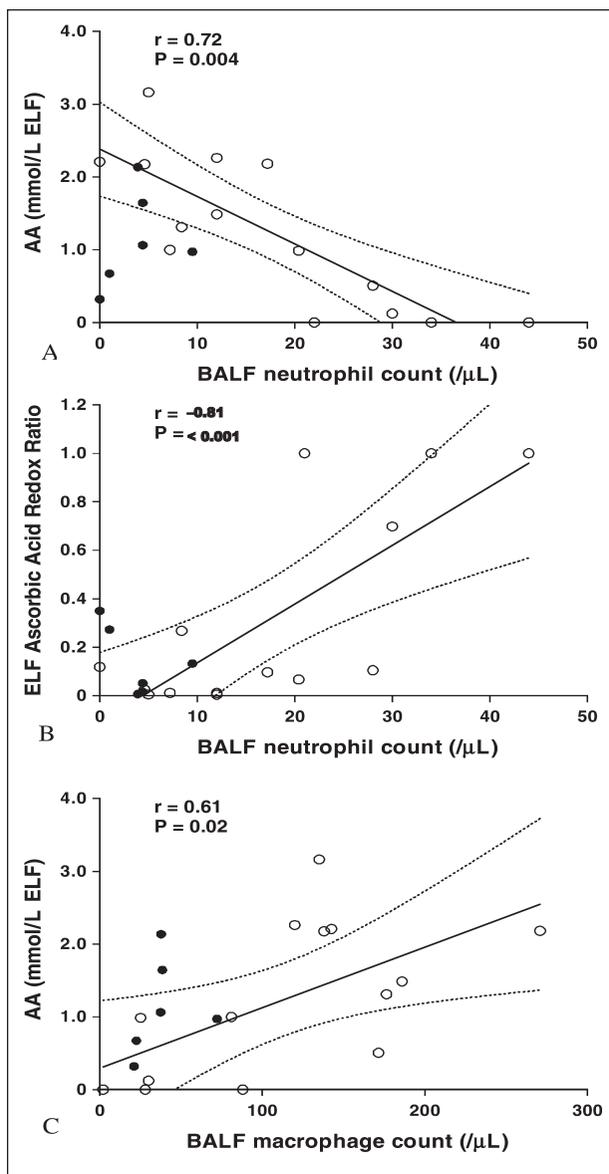


Figure 6—Correlations between BALF neutrophil count and pulmonary ELF ascorbic acid concentration (panel A), between BALF neutrophil count and the ascorbic acid redox ratio (panel B), and between BALF macrophage count and pulmonary ELF ascorbic acid concentrations (panel C) in control horses ($n = 8$) and RAO-affected horses with airway inflammation (6; open circles). Linear regression line (solid line) and 95% confidence limits are indicated (dashed lines). Data for RAO-affected horses without airway inflammation is also presented (closed circles); however, because of the effect of repeated measures, these data were not included in the correlation analyses.

concentration of ascorbic acid ($r = -0.81$; $P < 0.001$; Fig 6) and positively correlated with the ELF ascorbic acid redox ratio ($r = 0.65$; $P = 0.01$) and glutathione redox ratio ($r = 0.58$; $P = 0.03$). In addition, the BALF macrophage count was correlated with the ELF ascorbic acid concentration ($r = 0.61$; $P = 0.02$). Although simple linear regression was applied, visual observation of data indicated that in some instances, the relationships might have been nonlinear and better described by other regression functions. However, these were not applied because of the low number of horses in the study.

Discussion

Results of our study indicate that RAO-affected horses have a significantly lower concentration of reduced ascorbic acid in both ELF and plasma when afflicted with neutrophilic airway inflammation, compared with healthy unaffected horses, and this is not fully reversed with the cytologic resolution of airway inflammation. Overall greater variability was found in the concentrations of DHA, with considerable overlap among groups. This may reflect the labile nature of oxidized antioxidants.

Some of the variation in the ELF-reduced ascorbic acid pool was related to variation in the number of neutrophils in this compartment. Activated neutrophils may influence the ELF ascorbic acid pool by inducing the oxidation of ELF ascorbic acid via the production of reactive oxygen species and accumulating ascorbic acid via the uptake and subsequent reduction of DHA.^{25,26} Most RAO-affected horses with airway inflammation had increases in plasma and ELF ascorbic acid redox ratios, indicating the oxidation of ascorbic acid. Dehydroascorbate is much less stable than ascorbic acid and is rapidly and irreversibly hydrolyzed to 2,3-diketogulonic acid at physiologic pH and temperature [leading to a decrease in the ascorbic acid pool] because 2,3-diketogulonic acid has no antiscorbutic effect.²⁷ The greater variation in DHA concentration may be attributable to the smaller and more labile nature of the DHA pool. The considerable variation in the extent of oxidation of both ascorbic acid and glutathione in RAO-affected horses with airway inflammation also suggests that the number of neutrophils in BALF may not be the sole determinant of the degree of oxidative stress. The neutrophil count gives no indication of the activation state of the cells, which will obviously affect the rate of production and release of reactive oxygen species.

In contrast to neutrophils, the number of macrophages in BALF was positively related to the ELF ascorbic acid concentration, a finding that has previously been demonstrated in human subjects following ozone exposure.⁸ Macrophages display increased adherence to alveolar structures²⁸ and increased ascorbic acid accumulation upon activation.²⁹ Therefore, the positive correlation may simply be a result of a decrease in the number of macrophages recovered in BALF in the presence of inflammation and a simultaneous decrease in ELF ascorbic acid as a result of increased utilization of ascorbic acid or accumulation by macrophages or neutrophils. Increased intracellular concentrations of ascorbic acid could be considered beneficial to an active phagocytic cell, protecting it from its own respiratory burst.

The lower ELF and plasma ascorbic acid concentrations of RAO-affected horses in the absence of inflammation, compared with control horses, may have been the result of decreased production or increased systemic or pulmonary utilization, sequestration, or metabolism of ascorbic acid. A subsequent oxidative challenge might reasonably be expected to result in earlier or greater oxidative damage in an RAO-affected horse, compared with a healthy horse, as the antioxidant capacity is reduced. The RAO-affected horses were weight-matched

but considerably older than the control horses, and the concentration of plasma ascorbic acid has previously been shown to be lower in older horses.³⁰ However, in our study, no relationship was found between age and plasma or ELF ascorbic acid concentration within each group, suggesting that the reduction in plasma and ELF ascorbic acid concentration in RAO-affected horses, compared with the control horses, was not predominately an age effect. The increase in ELF GSH concentration in RAO-affected horses without airway inflammation, compared with control horses, may reflect a compensatory mechanism to counteract the reduced concentration of ascorbic acid. Supplementation of GSH in ascorbate-deficient guinea pigs significantly delayed the appearance of the signs of scurvy and spared tissue ascorbic acid, supporting an overlap in the function of these 2 antioxidants.³¹ We have previously demonstrated that dietary supplementation with ascorbic acid derivatives increased both the pulmonary and systemic concentrations of ascorbic acid in control horses, even though horses can synthesize this vitamin.³² Therefore, supplementation with ascorbic acid derivatives warrants investigation in RAO-affected horses without airway inflammation to determine whether the ELF ascorbic acid concentration can be increased to the concentrations in control horses and, if so, whether this prevents or reduces the neutrophilic inflammatory response and bronchoconstriction following exposure to hay and straw.

Recurrent airway obstruction-affected horses without airway inflammation continued to have a decreased concentration of plasma reduced ascorbic acid and an increase in ascorbic acid and glutathione redox ratios, indicative of continuing systemic oxidative stress, despite the resolution of airway neutrophilic inflammation and oxidative stress. This suggests that the lower plasma ascorbic acid concentration and increased systemic ascorbic acid and glutathione redox ratios are not solely the result of airway neutrophilic events, but may be related to decreased production or recycling or increased metabolism of tissue, airway cell, or extrapulmonary ascorbic acid. In humans, smoking, chronic obstructive pulmonary disease, and asthma have all been demonstrated to induce systemic oxidative stress.³³ In asthmatics, this may be the result of increased superoxide generation by circulating neutrophils.³⁴

Ascorbic acid and DHA have been reported as unstable during sample preparation, limiting their usefulness as markers of oxidative stress.³⁵ However, in our study, bronchoalveolar lavage was performed by use of saline solution (pH, 5.5), reducing the likelihood of ascorbate oxidation and DHA hydrolysis.³⁵ In addition, we observed a close correlation between the ascorbic acid redox ratio and glutathione redox ratio in ELF.

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^cStatMost for Windows, DataMost Corp, Salt Lake City, Utah.

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