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Pulmonary Bioavailability of Ascorbic Acid in an Ascorbate-synthesising Species, the Horse

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Vitamin C (ascorbic acid) is a non-enzymatic antioxidant important in protecting the lung against oxidative damage and is decreased in lung lining fluid of horses with airway inflammation. To examine possible therapeutic regimens in a species with ascorbate-synthesising capacity, we studied the effects of oral supplementation of two forms of ascorbic acid, (each equivalent to 20 mg ascorbic acid per kg body weight) on the pulmonary and systemic antioxidant status of six healthy ponies in a 3×3 Latin square design. Two weeks supplementation with ascorbyl palmitate significantly increased mean plasma ascorbic acid concentrations compared to control (29 ± 5 and $18 \pm 7\,\mu$mol/l, respectively; $p < 0.05$). Calcium ascorbyl-2-monophosphate, a more stable form of ascorbic acid, also increased mean plasma ascorbic acid concentrations, but not significantly ($23 \pm 1\,\mu$mol/l; $p = 0.07$). The concentration of ascorbic acid in bronchoalveolar lavage fluid increased in five out of six ponies following supplementation with either ascorbyl palmitate or calcium ascorbyl-2-monophosphate compared with control (30 ± 10, 25 ± 4 and $18 \pm 8\,\mu$mol/l, respectively; $p < 0.01$). Neither supplement altered the concentration of glutathione, uric acid or α-tocopherol in plasma or bronchoalveolar lavage fluid. In conclusion, the concentration of lung lining fluid ascorbic acid is increased following ascorbic acid supplementation (20 mg/kg body weight) in an ascorbate-synthesising species.

Keywords: Plasma; Lung; Ascorbic acid; Antioxidants; Horse

INTRODUCTION

Lung lining fluid is exposed to reactive oxygen species (ROS) produced by activated inflammatory cells and from inhaled oxidants such as ozone and nitrogen dioxide. A number of non-enzymatic antioxidants, including ascorbic acid, glutathione and uric acid, are present in lung lining fluid and interact with ROS to prevent damage to the underlying lung tissue.[1,2] In human asthma, there is increased production of ROS from inflammatory cells[3] and a reduced concentration of ascorbic acid in the bronchoalveolar lavage fluid (BALF) compared to healthy control subjects.[4] Oxidation of antioxidants[5] and a reduction in the ascorbic acid concentration[6] in the lung lining fluid also occur in horses suffering from recurrent airway obstruction (RAO), a condition similar to human asthma.[7] Elevation of the antioxidant concentrations in the lung lining fluid by supplementation may therefore serve to reduce or prevent oxidative damage.

Snow and Frigg[8] have demonstrated that the increase in plasma ascorbic acid after oral supplementation with ascorbyl palmitate (AP; a lipid soluble derivative of ascorbic acid) was over 2-fold greater than after supplementing with L-ascorbic acid. Oral ascorbic acid supplementation has previously been shown to produce a significant increase in BALF ascorbic acid in guinea pigs[9] and to prevent airway hyper-responsiveness induced by nitrogen dioxide and decrease the degree of lipid peroxidation in healthy human subjects.[10,11] However, unlike man and guinea pigs, the horse has the ability to synthesise ascorbic acid, which may affect

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its response to ascorbic acid supplementation. In man, tissue ascorbic acid homeostasis is regulated by intestinal absorption and renal reabsorption. In contrast, in the horse, commercial diets commonly contain little or no ascorbic acid. Therefore, the aim of the present study was to evaluate the effects of oral supplementation with two forms of ascorbic acid on the pulmonary and systemic concentrations of ascorbic acid and other major non-enzymatic antioxidants in a species with the capacity to synthesise ascorbic acid.

MATERIALS AND METHOD

Animals

Six Welsh Mountain ponies [6 geldings; 3 years old; 229 ± 41 kg (mean ± SD)] free of respiratory disease as determined by resting clinical examination, tracheal wash cytology and bacteriology, routine haematology and serology were studied. All animals were regularly dewormed prior to the commencement of the study. The experimental protocol was approved by the Ethics Committee of the Animal Health Trust and conformed to the Home Office Animals (Scientific Procedures) Act 1986.

The ponies were stabled in pairs and bedded on shredded paper. Each stable had a floor area of 3.6 × 5.6 m^2 with a volume of 72 m^3. Water was provided *ad libitum*. The top stable door and rear window vents were permanently open and the stables had air vents in the apex of the roof. The ponies were turned out in grass paddocks with face muzzles to prevent ingestion of grass between 9 a.m. and 1 p.m. daily.

Experimental Design

The study conformed to a 3 × 3 Latin square design and the ponies were studied in their stable pairings. Following a two-week lead in period, each pony underwent tracheal wash and bronchoalveolar lavage (BAL) of the dorso-caudal region of the right lung. The six ponies were then divided into three groups of two and each group was initially assigned one of three treatments:

(1) Ascorbyl palmitate (AP; fed at 50 mg/day per kg body weight; 40% L-ascorbic acid).
(2) Calcium ascorbyl-2-monophosphate (CAP; fed at 57.2 mg/day per kg body weight; 35% L-ascorbic acid), a stabilised monophosphate ester of L-ascorbic acid.
(3) No treatment (control).

The two forms of ascorbic acid (supplied by Winergy®, Masterfoods, Waltham-on-the-Wolds, Melton Mowbray, Leics., UK) were supplemented at the equivalent dose of 20 mg L-ascorbic acid per day per kg body weight. The supplements were mixed with 210 g molassed soaked sugar beet and 20 g bran and fed at 8 a.m. The control ponies received the sugar beet and bran only, which contained no detectable ascorbic acid (limit of detection of 50 mg/kg dry mass). All groups were fed 210 g sugar beet and 20 g bran at 5 p.m. each day with no supplements added. After two weeks of supplementation the ponies underwent a two-week washout period. This protocol was repeated twice more so each pony received each treatment followed by a two-week washout period. During the lead in, supplementation and wash out periods the ponies were fed 210 g sugar beet, 20 g bran and 3 kg haylage (Marksway Horsehage, Marksway and Son, Paignton, Devon, UK) twice a day.

Two days prior to the end of each period (supplementation and washout periods), the ponies had a venous blood sample collected from the jugular vein and underwent BAL on the right dorso-caudal lung 4 h after being fed their respective treatments and haylage. On the final day of each supplementation period (after 13 days of treatment) a bioavailability test was performed. The ponies were given haylage at 6 a.m. and any haylage remaining after 1 h was removed. At 8 a.m., a 14G catheter was inserted into the right jugular vein. One hour after catheterisation, a blood sample was collected and placed in a tube containing EDTA for ascorbic acid analysis, following which, the ponies were given their respective supplements. A venous blood sample was collected *via* the intravenous catheter 1 h after supplementation and each subsequent hour for 8 h, after which the ponies received their evening feed.

Sample Collection

Tracheal wash and BAL were performed with a 12 mm × 1.8 m flexible fibre-optic endoscope (Pentax UK Ltd., Slough, Berkshire, UK) using 30 and 200 ml, respectively, of 0.9% saline at 37°C as described previously.[12,13]

Processing of Samples

Venous blood samples were placed on ice in separate tubes containing either lithium-heparin (15 ml) or EDTA (5 ml) and centrifuged at 400*g* for 10 min at 4°C within 5 min of collection. For haemolysate glutathione analysis, 0.5 ml red blood cells were added to 0.5 ml 0.9% NaCl containing 2 mM Na_2EDTA and stored at −196°C. For ascorbic acid analysis, 0.5 ml EDTA plasma was added to 0.5 ml of 10% metaphosphoric acid containing 1 mM Na_2EDTA to deproteinise the plasma. The sample was vortexed and snap frozen in liquid nitrogen and stored at −80°C. In

order to reduce the oxidised form of ascorbic acid (dehydroascorbate, DHA) to measure the total concentration of ascorbic acid, 0.3 ml EDTA plasma was added to 0.2 ml 10 mM dithiothreitol, vortexed and left to stand at room temperature for 10 min. Five hundred μl of 10% metaphosphoric acid/1 mM Na_2EDTA was added, the sample vortexed, snap frozen and stored at −80°C. Lithium heparin plasma aliquots (0.5 ml) were stored at −80°C for α-tocopherol, urea and uric acid analysis. BALF was deproteinised with methanol for total glutathione analysis and stored at −196°C. Aliquots of BALF were centrifuged at 800*g* at 4°C for 10 min and stored at −80°C for ascorbic acid, α-tocopherol and urea analysis, and at −196°C for uric acid.

Sample Analysis

Plasma and BAL ascorbic acid were analysed by HPLC with UV detection as described previously.[14] HPLC with electrochemical detection was used to measure GSH and GSSG in BAL and red blood cell haemolysates.[15] Plasma uric acid was determined using a commercial kit (Sigma kit 685-10). BAL uric acid was analysed by HPLC with UV detection. The concentration of α-tocopherol in plasma and BAL was measured by HPLC according to the method described by Kelly *et al.*[16] The chemicals were purchased from Sigma Chemical Co. (Dorset, UK) or Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK).

Tracheal wash 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 mucus in the trachea (0-none; 1-slight; 2-moderate; 3-severe), smear cell density (0-low; 1-medium; 2-medium-high; 3-high) and neutrophil proportion (0-none, few cells or low number and diffuse; 1-low number but aggregated; 2-moderate number; 3-neutrophil is predominant cell type). The scores for tracheal mucus were made at the time of endoscopy. Scores for cell density and neutrophil proportion were made by a cytologist blinded to animal and treatment. 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 were determined on cytospun preparations stained with haematoxylin and eosin.

Statistical Analysis

Tracheal wash scores are presented as median and range. All other data are presented as mean ± sd. ANOVA was used for crossover analysis of plasma and BALF concentrations with type 1 sums of squares to test for any carryover effect. If a significant effect of supplementation was indicated, further analysis was performed with a Student–Newman–Keuls test. Plasma bioavailability of ascorbic acid was analysed using a three-way ANOVA. Significant interactions between time and supplementation were investigated further by comparing the effect of supplementation at each time point separately using a two-way ANOVA and the Student–Newman–Keuls test. The plasma ascorbic acid concentration following feeding compared to prior to feeding was investigated using a two-way ANOVA followed by Dunnett's test, if significance was attained. P value of <0.05 was regarded as statistically significant.

RESULTS

All ponies ate all the meals and no adverse effects of supplementation were observed.

Tracheal Wash and Bronchoalveolar Lavage Cytology

Tracheal wash scores prior to supplementation for mucus (median 1, range 0–2), cell density (0, 0–2), neutrophil proportion (1, 0–3) and total inflammation score (2, 0–6) were not significantly different following supplementation with either form of ascorbic acid ($p > 0.05$). Neither form of ascorbic acid treatment affected the percentage of BAL macrophages (59 ± 18%), lymphocytes (37 ± 17%), neutrophils (2.4 ± 1.4%) or epithelial cells (5 ± 7%), or the total nucleated cell count (258 ± 88 cells/μl) or macrophage (127 ± 55/μl), lymphocyte (82 ± 65/μl), neutrophil (5.3 ± 4.5/μl) or epithelial cell counts (4 ± 7/μl) ($p > 0.05$). All tracheal wash and BAL samples were negative for bacterial culture. The tracheal wash and BAL cytology and bacteriology confirmed that the ponies had no significant airway inflammation throughout the period of the study.

Circulatory Antioxidant Status

Plasma ascorbic acid concentrations 4 h after feeding (immediately prior to the BAL) were increased significantly following supplementation with AP ($p < 0.05$) and showed a trend to increase with CAP ($p = 0.07$) compared to control (Fig. 1). Supplementation with AP produced a significantly greater plasma concentration of ascorbic acid at 4 h post feeding compared to with CAP ($p < 0.05$). There was a significant carryover effect on plasma ascorbic acid concentration between the different time points

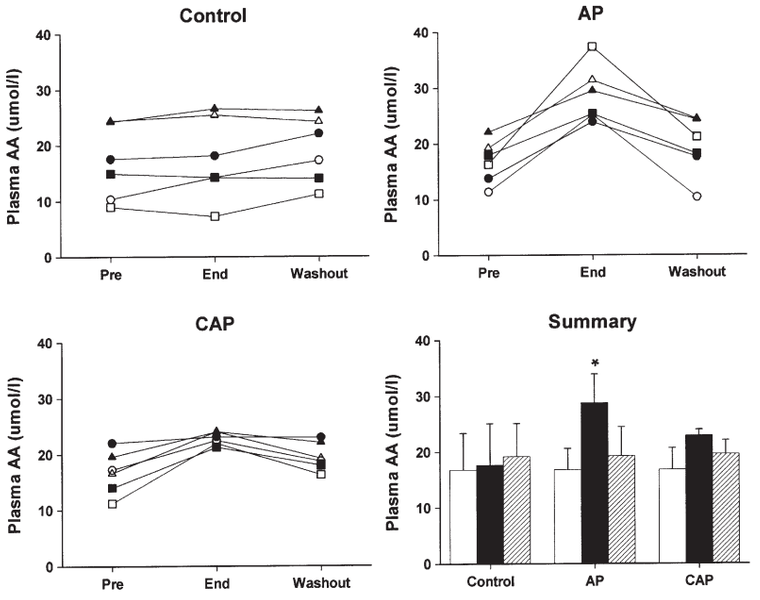

FIGURE 1 Individual plasma concentrations of ascorbic acid (AA) prior to treatment (Pre), after 2 weeks supplementation (End) with control, ascorbyl palmitate (AP) or calcium ascorbyl-2-monophosphate (CAP) and after 2 weeks washout (Washout) in blood samples collected 4 h post feeding. On the summary graph (mean ± SD, $n = 6$) open bar: Pre; solid bar: End; hatched bar: Washout. * Denotes significantly different to control and CAP ($p < 0.05$).

($p = 0.03$). The coefficient of variation (CV) for plasma ascorbic acid was 1.9 μmol/l during the control treatment. Compared to animals on the control diet, plasma concentrations of dehydroascorbate (DHA; Table I), uric acid (8.4 ± 1.4 μmol/l) and α-tocopherol (7.5 ± 2.9 μmol/l) and the percentage of total ascorbic acid (AA + DHA) present as DHA (4.9 ± 7.3%) were not affected by supplementation with either AP or CAP. Similarly, supplementation did not alter the red blood cell haemolysate concentrations of GSH or GSSG (Table I) or the percentage of total glutathione present as GSSG (4.5 ± 1.4%).

Plasma Ascorbic Acid Bioavailability

Plasma ascorbic acid bioavailability was determined after 13 days of supplementation. Following feeding of the supplements or control, the plasma ascorbic acid concentrations were significantly increased in all three groups (Fig. 2). The concentration of plasma ascorbic acid was significantly greater ($p < 0.05$) in

TABLE I Pulmonary and systemic concentrations of antioxidants before (Pre) and after two weeks treatment (End) with ascorbic acid supplements* and after two weeks washout

Antioxidants†		Supplement‡	Pre	End	Washout
GSH (μmol/l)	RBC	Control	1163 ± 282	1144 ± 238	1086 ± 224
		AP	1342 ± 374	1180 ± 246	1165 ± 277
		CAP	1121 ± 217	1210 ± 200	1324 ± 389
	BALF	Control	1.0 ± 0.6	0.9 ± 0.4	0.9 ± 0.5
		AP	1.3 ± 0.8	1.0 ± 0.6	1.0 ± 0.5
		CAP	0.5 ± 0.5	1.5 ± 0.5	1.7 ± 0.6
GSSG (μmol/l)	RBC	Control	52 ± 12	47 ± 11	49 ± 19
		AP	65 ± 37	52 ± 21	52 ± 12
		CAP	52 ± 15	53 ± 8	66 ± 36
	BALF	Control	0.19 ± 0.13	0.25 ± 0.27	0.24 ± 0.16
		AP	0.13 ± 0.14	0.09 ± 0.07	0.15 ± 0.13
		CAP	0.31 ± 0.12	0.13 ± 0.12	0.07 ± 0.02
DHA (μmol/l)	Plasma	Control	0.9 ± 1.4	0.7 ± 0.9	1.0 ± 0.8
		AP	0.1 ± 0.3	2.2 ± 1.7	1.0 ± 1.3
		CAP	1.0 ± 0.8	0.9 ± 1.2	0.3 ± 0.5
	BALF	Control	1.9 ± 3.6	0.8 ± 1.1	4.3 ± 3.9
		AP	1.0 ± 1.0	1.6 ± 2.6	2.2 ± 3.4
		CAP	7.5 ± 6.2	1.3 ± 2.3	1.7 ± 1.1

*Blood samples (red blood cell haemolysate, RBC, and plasma) and bronchoalveolar lavage fluid (BALF) were collected 4 h post feeding. †GSH (reduced glutathione), GSSG (oxidised glutathione) and DHA (dehydroascorbate) presented as mean ± SD. ‡AP (ascorbyl palmitate) and CAP (calcium ascorbyl-2-monophosphate) supplemented at the equivalent of 20 mg ascorbic acid per day per kg body weight.

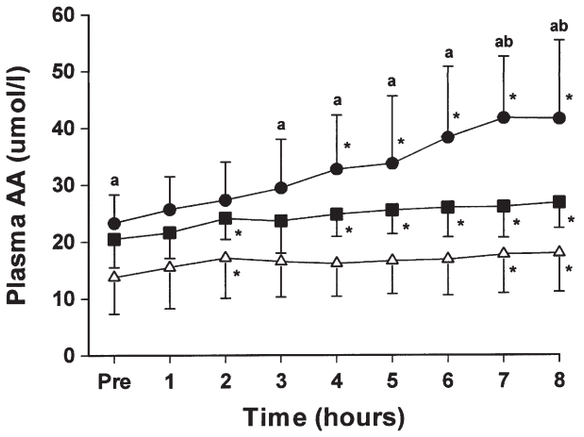

FIGURE 2 Plasma concentrations of ascorbic acid (AA) after 13 days of supplementation prior to (pre) and 1, 2, 3, 4, 5, 6, 7 and 8 h after supplementation with control (△), ascorbyl palmitate (● AP) or calcium ascorbyl-2-monophosphate (■; CAP) (mean ± SD, $n = 6$). *: significantly different from pre concentration ($p < 0.01$); a: significantly different from control ($p < 0.05$); b: significantly different from CAP ($p < 0.05$).

the AP group prior to supplementation and between 3 and 8 h post supplementation compared to the control, and at 7 and 8 h post supplementation compared to following CAP supplementation (Fig. 2).

Pulmonary Antioxidant Status

The concentrations of BALF ascorbic acid increased in five out of six ponies following either supplement (Fig. 3). One pony did not respond to either of the treatments and was considered to be an outlier. When the data were re-analysed with this pony excluded, the concentration of BALF ascorbic acid was increased significantly following supplementation with AP or CAP compared with the control (30 ± 10, 25 ± 4 and 18 ± 8 μmol/l, respectively; $p < 0.01$). Compared to the control, BALF concentrations of DHA, GSH, GSSG (Table I) and uric acid (0.027 ± 0.016 μmol/l) and the percentage of total ascorbic acid present as DHA ($5.1 \pm 7.0\%$) and the percentage of total GSH present as GSSG ($24 \pm 27\%$) were not changed by either supplement. α-tocopherol was not detected in BALF (limit of detection of 0.01 μmol/l) before or following supplementation.

DISCUSSION

Ascorbic acid is quantitatively the most important non-enzymatic antioxidant in equine lung lining fluid.[13] In the present study, two forms of ascorbic acid, ascorbyl palmitate and calcium ascorbyl-2-monophosphate provided at a dose of 20 mg ascorbic acid per kg body weight, increased the plasma concentration of ascorbic acid in healthy ponies. Interestingly despite the inherent capacity for ascorbic acid production, the BALF concentration of ascorbic acid was also increased in five out of six ponies following supplementation with either form of ascorbic acid. In one pony, supplementation with

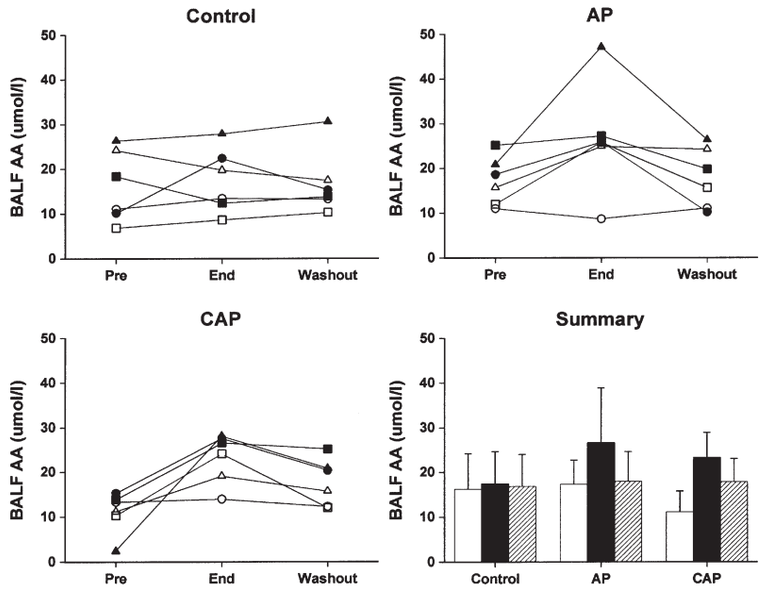

FIGURE 3 Individual concentrations of ascorbic acid (AA) in bronchoalveolar lavage fluid (BALF) prior to treatment (Pre), after 2 weeks supplementation (End) with control, ascorbyl palmitate (AP) or calcium ascorbyl-2-monophosphate (CAP) and after 2 weeks washout (Washout) in bronchoalveolar lavage samples collected 4 h post feeding. The symbols correspond to the same animals as in Fig. 1. On the summary graph, open bar: Pre; solid bar: End; hatched bar: Washout. (mean ± SD, $n = 6$).

either supplement had no effect on BALF ascorbic acid concentration.

Plasma ascorbic acid concentrations were similar to those reported previously in the horse,[8] and are similar to, although 10–20 μmol/l lower than concentrations measured in dogs,[17] calves[18] and camels,[19] all of which are lower than in the rat.[20] A small but significant increase in plasma ascorbic acid concentration was observed 2 h following supplementation with the control diet, which contains no ascorbic acid. This increase may reflect diurnal variation in ascorbic acid synthesis, fluid redistribution and haemoconcentration following feeding[21] or ascorbic acid synthesis in response to feeding.

BAL was performed 4 h post supplementation and therefore plasma ascorbic acid concentrations at 4 h were also presented to allow a direct comparison. Peak plasma ascorbic acid concentrations following both AP and CAP occurred after more than 4 h. If there was an immediate redistribution of ascorbic acid between plasma and lung lining fluid, the BALF ascorbic acid concentrations presented will clearly be an underestimate. Oral ascorbic acid supplementation has previously been shown to produce an approximately 4-fold increase in BALF ascorbic acid in guinea pigs, which are unable to synthesise ascorbic acid.[9] The greater increase following supplementation compared to the present study may be a reflection of the higher dose used (approximately 10-fold higher than in the present study).

When expressing the BALF ascorbic acid concentrations in terms of lung epithelial lining fluid (ELF), using the urea dilution method,[22] the concentrations in the present study are similar to those that we have reported previously in adult horses (~1500 μmol/l).[13] This concentration of ascorbic acid in equine ELF is approximately 100 times greater than the plasma ascorbic acid concentration, suggesting that passive diffusion alone into the lung lining fluid is unlikely. In one pony, plasma ascorbic acid concentrations increased by over 220 and 130% following supplementation with AP and CAP, respectively. However, the concentration of ascorbic acid in the BALF of this pony did not change following treatment with either supplement, unlike the other five ponies. This led us to regard this pony as an outlier. The absence of an increase in BALF ascorbic acid in this pony could be interpreted as being due to a defect in the active uptake mechanism from the plasma into the lung.

Separate transport mechanisms exist for DHA and ascorbic acid.[23] DHA uptake is via facilitated-diffusion glucose transporters, however, high plasma glucose concentrations compared to DHA (5 mmol/l versus 1 μmol/l)[24]) will effectively block these transporters.[25] In addition, the majority of ascorbic acid is in the reduced form (approximately 95% in the present study) limiting the effectiveness of these transporters under physiological conditions. Ascorbic acid in contrast is transported *via* a sodium dependent transport mechanism, which has been identified in rat alveolar macrophages and type II cells.[23,25,26] As no evidence of an increased requirement for ascorbic acid was found, for example underlying inflammation, we postulate that the absence of any increase in BALF ascorbic acid following supplementation in one pony may possibly have been due to a defect in this sodium dependent ascorbic acid transport mechanism.

Ascorbic acid supplementation had no effect on the other major non-enzymatic antioxidants in plasma, blood or BALF. Interactions between ascorbic acid, α-tocopherol and glutathione have been previously demonstrated to enable the recycling of the oxidised forms of the different antioxidants.[27,28] However, in the present study the concentrations of oxidised glutathione (GSSG) and ascorbic acid (DHA) were low. An effect of ascorbic acid supplementation on the concentrations of the other antioxidants may therefore only become apparent during periods of oxidative challenge when there are increased concentrations of antioxidants in their oxidised forms.

In conclusion, supplementation of ascorbic acid in the form of AP or CAP increased the concentration of BALF ascorbic acid, but did not effect either the systemic or pulmonary concentrations of the other major non-enzymatic antioxidants.

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