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Oxidant-Antioxidant Status in the Blood of Horses with Symptomatic Recurrent Airway Obstruction (RAO)

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Background: Systemic oxidative stress in horses with recurrent airway obstruction (RAO) is poorly characterized.

Objectives: The goal of this study was to investigate whether equine RAO is associated with systemic disturbances in the oxidant-antioxidant equilibrium.

Animals: Seven healthy horses and 7 horses with symptomatic RAO.

Methods: A prospective study. Healthy and RAO-affected horses were exposed to a 48-hour challenge with moldy hay and straw to induce clinical exacerbation of RAO. Venous blood was collected and the activities of the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) in equine erythrocyte lysates were measured. The concentration of thiobarbituric acid-reactive substances (TBARSs) was assessed both in erythrocyte lysates and in plasma.

Results: A significant increase in the activities of GPx and SOD was detected in RAO-affected horses compared with the control animals. There was no significant difference between groups in terms of the erythrocyte lysate activities of CAT, GR, or TBARs or the plasma concentration of TBARs.

Conclusion and Clinical Importance: Our results support the hypothesis that RAO in horses is associated with systemic oxidative stress. Future studies are needed to assess whether horses suffering from RAO can benefit from antioxidant supplementation.

Key words: Antioxidants; Horse; Oxidative stress; RAO.

Recurrent airway obstruction (RAO), formerly termed equine chronic obstructive pulmonary disease, is a naturally occurring asthma-like respiratory condition in horses. This disease is characterized by distal airway inflammation, reversible airway obstruction, and bronchial hyperresponsiveness.[1,2] The etiology of RAO is multifactorial, and a number of environmental, immunologic, and genetic agents play an important role in its pathogenesis, causing disturbances in the oxidant-antioxidant equilibrium.[3]

Oxidative stress has been demonstrated to occur in many human respiratory conditions, including chronic obstructive pulmonary disease (COPD) and asthma.[4,5] Reactive oxygen species derived from inflammatory cells (neutrophils, macrophages), large numbers of which migrate to the lungs, are crucial in the oxidant-antioxidant imbalance observed in the course of the above-mentioned diseases. Research on oxidative stress in horses with RAO has been conducted previously, although ambiguous results have been reported.[6–13] Moreover, most of the studies relate to the local effects of oxidative stress in the airways.[7–9] Only a few studies investigated systemic markers of oxidative stress in RAO-affected horses. Results from these studies are difficult to compare because of differences in methodologies used and type of biomarker investigated. One study showed no difference in plasma lipid hydroperoxides or in erythrocyte hemolysate reduced glutathione (GSH), oxidized glutathione (GSSG), or total glutathione (total GSH) between healthy horses and RAO horses in crisis or in remission.[10] Certain blood oxidant status variables in horses after exercise were examined, but when comparing baseline values before competition, this study showed significant increases in the concentration of GSH in both the RAO-affected horses in remission and in crisis compared to healthy

Abbreviations:

AA	ascorbic acid
BALF	bronchoalveolar lavage fluid
CAT	catalase
COPD	chronic obstructive pulmonary disease
GPx	glutathione peroxidase
GR	glutathione reductase
GRR	glutathione redox ratio
GSH	reduced glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
HiCN	hemoglobin cyanide
K2EDTA	potassium versenate
MDA	malondialdehyde
$O2^{\cdot}-$	superoxide radicals
$OH^{\cdot}$	hydroxyl radicals
RAO	recurrent airway obstruction
ROS	reactive oxygen species
SOD	superoxide dismutase
TBARSs	thiobarbituric acid-reactive substances
TBA	thiobarbituric acid
total GSH	total glutathione

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controls.[11] Moreover, the glutathione redox ratio (GRR) measured at rest before competition was significantly decreased in horses in crisis compared to healthy animals. However, no difference was observed in the concentration of uric acid, GSSG, or 8-iso-$PGF_{2\alpha}$ between the groups.[11]

Ascorbic acid (AA) has been identified as an important systemic and local nonenzymatic antioxidant in horses and showed decrease in the concentration of plasma AA and dehydroascorbate (DHA) was shown in horses with RAO.[6,7] Moreover, this study also demonstrated disturbances in GSSG and the glutathione redox ratio between study groups.[7]

There are only a few studies on the activity of erythrocyte lysate glutathione peroxidase (GPx) in horses with RAO.[12,13] An environmental challenge with dusty straw and hay induced a significant difference in the activity of cellular GPx between the control and RAO-affected horses, and the mean activity of GPx was higher at all sampling points in the RAO-affected horses.[12] A similar study assessed changes in the activity of intracellular antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase in RBCs of horses with symptomatic RAO in comparison to healthy horses.[13] Horses with RAO showed lower superoxide dismutase activity, whereas there was no difference in RBC glutathione peroxidase and catalase activity.

The literature contains limited data on the relationship between RAO and the activities of glutathione reductase (GR) and the so-called enzymatic triad (ie, superoxide dismutase [SOD], catalase [CAT], and glutathione peroxidase [GPx]) in equine erythrocytes. Therefore, the aim of this study was to compare the activity of the above-mentioned enzymes among horses with RAO in crisis and healthy controls. Moreover, the authors chose to analyze the effect of this disease on the concentration of thiobarbituric acid-reactive substances (TBARSs), given that these are some of the most frequently used indicators of lipid peroxidation.

Materials and Methods

This study was conducted with the approval of the 2nd Local Ethics Committee responsible for Animal Experimentation in Wrocaw (resolution No 1/2012).

Horses

Fourteen adult Polish Konik horses maintained in the same environmental and living conditions with regards forage, bedding, and housing were used in this study. Horses were assigned to groups based on their history. Seven horses were not affected by RAO (control horses) and another 7 horses had a history of RAO (RAO-affected horses). Horses were owned by the Polish Academy of Sciences Research Station for Ecological Agriculture and Preservation of Animal Breeding in Popielno. One horse was owned by a private breeder; however, conditions of maintenance were deemed comparable to those of the other animals and therefore exposure to dust and allergens was assumed to be similar. The control group consisted of 5 castrated males (geldings) and 2 mares (median ages: 8 years; range: 5–13) with no history of airway disease or evidence of lung pathology. The study group consisted of 4 geldings and 3 mares (median ages: 9 years; range: 7–14) with a history of RAO. Grouping was performed on the basis of medical history and the results of clinical examination and RAO scoring, conducted previously by the local veterinarian caring for the herd. The RAO group consisted of horses with histories of recurring signs of obstructive pulmonary disease that develop after exposure to moldy hay and straw, and which spontaneously resolve after removal of the animals from the adverse environmental conditions. The appropriate classification was furthermore confirmed by the authors using the methods described below. None of the horses received medication in the 2 months preceding the assessment.

Experimental Protocol

Before the study, all the horses were kept at pasture or in a stable with wood shavings and received good quality soaked hay for a minimum of 8 weeks to reduce exposure to unfavorable inhaled particles. An acute crisis of RAO was induced in selected animals by placing them in a poorly ventilated stable, bedding them on straw and feeding them hay with visible mold growth for 48 hours before the examination.[14] Disease exacerbation induced by the poor environmental conditions was confirmed by clinical examination, a modified clinical RAO score and an endoscopic examination as defined previously (see Appendix 1), together with cytology of the bronchoalveolar lavage fluid (BALF) and arterial blood gas analysis.[15] Venous blood was additionally obtained to rule out evidence of pulmonary infection, based on blood count and acute phase protein concentration.[16,17] A score of less than 10% of neutrophils in BALF was required for healthy horses, whereas >50% neutrophils on a differential cell count after environmental challenge was required to define RAO-affected horses in crisis.

Endoscopic Examination and Bronchoalveolar Lavage Fluid Collection and Cytology

Endoscopy of the airways and bronchoalveolar lavage was performed after sedation with 0.01 mg/kg of detomidine[a] and 0.01 mg/kg of butorphanol.[b] The nares of each horse were cleaned using a chlorhexidine sponge. A 1.8-m long endoscope was passed through the nasal passage into the trachea. Changes in the airways were graded by 2 clinicians using a modified RAO staging scale.[15] The endoscope was passed further using visual guidance, until it wedged in the peripheral, right dorsal bronchial tree. Bronchoalveolar lavage was performed by instilling 250 mL of sterile saline (0.9% NaCl) at body temperature through the endoscope working channel into a bronchus using successive 60-mL boluses, and reaspirating BALF through gentle suction using a 60-mL syringe until no further fluid was obtained. The amount of recovered fluid was recorded and BALF for each individual horse was pooled in a sterile specimen cup, placed on ice and processed within 2 hour after collection. For cytologic analyses, the smear was prepared with 10 mL of sample aliquots, cytospinned at 300 × *g* for 10 minute using the cytospin[c] and stained with Wright's stain. A 400-cell leukocyte differential count (×1000 magnification) was performed excluding epithelial cells.[18] Cell counts were always performed by the same diagnostician, who was unaware of the clinical status of the horse or the group to which it belonged.

Measurement of Antioxidative Enzymes and Malondialdehyde

Venous blood was collected from the external jugular vein using a disposable syringe and needle, and transferred into tubes

containing potassium versenate (K_2EDTA) for a full blood analysis. The activity of SOD, CAT, GPx, and GR were determined in the erythrocyte lysates. The levels of TBARS were determined in both erythrocytes and plasma.

After centrifugation at 3,000 g at 4°C, plasma was removed and a quantity of 1 mL of packed red blood cells (RBC) was suspended in 1 mL of a 1% solution of a double density peroxide-free Triton X-100 detergent.[d] After being homogenized and incubated at 25°C for 10 minutes, the hemolysate was centrifuged at 13,000 g for 10 minutes. The supernatant was prepared for measurement of SOD, CAT, GPx, and GR activities by diluting 10 μL of supernatant with 980 μL of 50 mM phosphate buffer (pH 7.5) containing 0.2% bovine albumin and 2 mM K_2EDTA. Blood cell hemolysates were diluted with PBS containing 0.1% bovine albumin to determine the enzyme activity. The measurement of the enzyme activity and TBARS level was carried out according to the assay kit instructions and employing the kinetic spectrophotometric method. The absorbance was read with an ELx800UV Absorbance Microplate Reader,[e] and the wavelength was always adjusted according to assay kit instruction. All assays were run in duplicate according to the manufacturer's instructions, and the mean of the duplicate values was used. The agreement between duplicate measures was good, and only 4 of 98 test pairs (including hemoglobin) were discordant, and this was because of technical reasons. The detailed method description and reagent kits for each parameter are summarized in Table 1.

The hemoglobin concentration of the hemolysates was determined using Drabkin's cyanmethemoglobin method to express the enzyme activities as units of activity per gram of hemoglobin.[19] Drabkin's solution, consisting of potassium ferricyanide, potassium cyanide, and sodium bicarbonate, was prepared, and a quantity of 20 μL of blood was added to 5 mL of the solution. Readings were obtained by spectrophotometry at a wavelength of 530 nm. Hemoglobin concentrations were calculated from a hemoglobin curve prepared using hemoglobin cyanide (HiCN) standard[f] calibrated to international standards.

The most important product of lipid peroxidation reacting with thiobarbituric acid (TBA) is malondialdehyde (MDA).[20] Therefore, the levels of TBARS were expressed as nmol MDA/mL in plasma and as nmol MDA/g Hb in erythrocyte lysates. The activity of GPx, SOD, CAT, and GR was expressed in U/g Hb.

Statistical Analysis

Data are presented as median, 25th and 75th percentiles. The nonparametric Mann-Whitney *U*-test was performed to evaluate the results. Dependencies between the analyzed parameters were assessed using correlation matrices. A statistical hypothesis of the significance of the correlation coefficients (*r*) was tested. All analyses were performed using STATISTICA v. 10.0 software.[g] $P < .05$ was considered statistically significant.

Results

Clinical Signs and BALF Cytology

The detailed anamnesis, together with the results of the clinical, hematologic, and biochemical examinations as well as the basic acute phase protein content, did not

Table 1. Methods used to measure the enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), thiobarbituric acid-reactive substances (TBARSs), and hemoglobin (Hb).

Enzyme	Method	Reagents	Quality control	Between-day imprecision CV	Reference
CAT	Measurement of absorbance decrease, measured at a wavelength of 240 nm, as hydrogen peroxide is decomposed by CAT	Cayman Chemical Item No. 707002	Cayman Chemical (Catalase Control – Item No. 707013)	9.8%	Johansson et al[34]
GPx	Reduction of oxidized glutathione catalyzed by reduced glutathione and connected with a NADP formation and decrease in absorbance at 340 nm	Cayman Chemical Item No. 703102	Cayman Chemical (Glutathione Peroxidase Control - Item No. 703114)	7.2%	Paglia and Valentine[35]
SOD	Utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine	Cayman Chemical Company Item No. 706002	Cayman Chemical (SOD Standard - Item No. 706005)	3.7%	Misra and Fridovich[36]
GR	NADP formation during reduction of oxidized glutathione	Cayman Chemical Item Number 703202	Cayman Chemical (GR Control - Item No. 703214)	9.3%	Carlberg and Mannervik[37]
TBARS	Creation of a colored complex between lipid peroxidation products and thiobarbituric acid (TBA) at the temperature of 100˚C and in an acidic environment	Cayman Chemical Item Number 10009055	Cayman Chemical (TBA Malondialdehyde Standard - Item No. 10009202)	5.9%	Buege and Aust[38]
Hemoglobin	Total hemoglobin at alkaline pH is rapidly converted to the cyanoderivative. The absorbance of the cyanoderivative is determined at 530 nm	Sigma-Aldrich (Product Code D 5941)	HiCN standard calibrated to international standards, StanBio Laboratory, (cat. nr 0330-302)	5,5%	van Lerberghe et al[19]

Table 2. Results of clinical scores, blood gas analyses, and BAL fluid cytology in healthy horses and RAO-affected horses. Values are expressed as median and 25th and 75th percentiles.

	Healthy(n = 7)	RAO-affected (n = 7)
Clinical score[a]	2.0 (2 and 2)	6.0 (5 and 6)
PaO2 (mmHg)[a]	96 (91 and 108)	91 (85 and 107)
PaCO2 (mmHg)	45 (43 and 46)	45.5 (44 and 50)
BALF neutrophils (%)[a]	5.1 (4.1 and 5.3)	59.8 (51.3 and 64.8)
BALF lymphocytes (%)[a]	41 (38.5 and 45.9)	38.1 (34.8 and 41.1)
BALF macrophages (%)[a]	55.8 (49.8 and 59.1)	32.8 (25.9 and 35.7)
BALF eosinophils (%)[a]	0.4 (0.2 and 0.5)	0 (0 and 0)
BALF mast cells (%)	0.1 (0 and 0.3)	0 (0 and 0)

[a]Differences statistically significant ($P < .05$)

indicate any systemic disorder in either group of horses (data not shown). No significant correlation was detected between the BALF cytology results and the RAO score ($r = 0.12$) in the control horses. All control horses had a clinical score below 1 according to the cough score, nostril flare, and abdominal lift. The only abnormalities observed in these horses were a sporadic cough and slightly flared nostrils. There was a significant difference ($P < .05$) between all variables in the control and study group (Table 2). The RAO-affected horses had a median clinical score of approximately 6. There was a positive linear correlation ($r = 0.67$) between the neutrophil percentage and the RAO score results.

The percentage recovery of the 250 mL of physiologic saline infused into the bronchi of each horse was determined. The average recovery in the healthy control horses was 56.6%, whereas it was 43.6% in the RAO-affected horses. The difference in the percentage recovery between the 2 groups was statistically significant ($P < .001$). The median (25th and 75th percentiles) differential BALF counts in healthy horses were 5.1% (4.1 and 5.3%) neutrophils, 41% (38.5 and 45.9%) lymphocytes, and 55.8% (49.8 and 59.1%) macrophages, and in RAO horses were 59.8% (51.3 and 64.8%) neutrophils, 38.1% (34.8 and 41.1%) lymphocytes, and 32.8% (25.9 and 35.7%) macrophages. As expected, the median percentage of neutrophils was significantly higher in all RAO-affected horses compared with controls ($P < .001$). The median percentage of macrophages and lymphocytes was significantly higher in the healthy horses compared to the study group: ($P < .001$) and ($P < .05$), respectively (Table 2).

Lipid Peroxidation Biomarkers and Antioxidant Enzyme Activity

No significant differences were observed either in the cell lysates or in the plasma ($P = .89$ and .06, respectively) between the healthy and RAO-affected horses. TBARS concentration in RBCs was almost 10 times higher than in plasma in both groups (Table 3).

The difference between the activity of glutathione peroxidase and superoxide dismutase in healthy and RAO-affected horses was significant (Table 3). The median (25th and 75th percentiles) activity of GPx was 59.48 U/g (53.33 and 67.05 U/g) in the healthy controls versus 72.74 U/g (69.32 and 86.27 U/g) in RAO horses ($P = .029$). The activity of SOD was 489.1 U/g (470.3 and 717.5) in the control group and 880.9 U/g (452.1 and 995.6 U/g) in the study group ($P = .011$).

There were no significant differences between the activity of catalase and glutathione reductase in the RAO-affected horses compared to the control animals ($P = .159$ and .44, respectively) (Table 3).

There was no significant correlation between the different variables of lipid peroxidation biomarkers and antioxidant enzyme activity either in the RAO-affected or healthy horses.

Discussion

There is clear evidence that oxidative stress is involved in the pathophysiology of airway inflammation

Table 3. Activities of enzymatic antioxidants and lipid peroxidation products in 7 control and 7 RAO-affected horses.

	Control			RAO-affected			Mann-Whitney *U*-test
	Median	25th and 75th percentiles	Range	Median	25th and 75th percentiles	Range	*P*-value
Antioxidant enzymes							
SOD [U/g Hb][a]	489.1	470.3–677.3	328.2–943.1	880.8	717.5–995.6	652.1–1191	.011
GPx [U/g Hb][a]	59.48	53.33–67.05	43.72–76.5	72.74	69.32–86.27	64.62–91.43	.029
CAT [U/g Hb]	157.6	151.2–205.5	142.3–293	205.09	176.1–250.3	158.4–284.9	.159
GR [U/g Hb]	34.89	26.02–39.38	21.51–40.1	29.72	27.96–35.92	25.77–37.53	.44
Lipid peroxidation products							
$MDA_{cellular}$ [nmol/g Hb]	40.18	39.74–44.14	37.77–47.66	41.28	38.86–45.68	38.86–49.2	.89
MDA_{plasma} [nmol/mL]	4.8	4–5.6	3.9–6.1	5.9	4.9–6.7	4.8–7.2	.06

Data are expressed as median, percentiles, and range.
[a]Differences statistically significant ($P < .05$).

in the course of RAO in horses.[6] However, there is little information relating to antioxidants circulating in the blood of RAO-affected horses.[10–13] The aim of this study was to investigate whether oxidative stress, measured as a difference in the activity of SOD, CAT, GPx, GR, and the concentration of lipid peroxidation products, is associated in RAO horses in crisis. Moreover, to date, no study has been published concerning the analysis of the antioxidant enzymatic triad in RAO-affected horses living in uniform environmental conditions with regard to forage, beddings, and housing.

In the present study, we demonstrated significant differences in the activities of glutathione peroxidase and superoxide dismutase in RAO-affected horses in crisis compared to healthy controls. The levels of catalase, glutathione reductase, and the concentration of erythrocyte and plasma TBARS were not statistically significant.

Exacerbation of RAO is manifested by airway inflammation, which involves the influx of circulating white blood cells (predominantly nondegenerate neutrophils) into the bronchial lumen.[1] When activated, leukocytes release many mediators and proinflammatory cytokines that amplify the inflammation, such as reactive oxygen species (ROS), which include superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), and singlet oxygen ($^1O_2$).[21] Acute airway inflammation can be caused by a release of hydrogen peroxide in the presence of iron salts, either alone or as a newly generated hydroxyl radical ($OH^{\cdot}$). Neutrophils present in the bronchial tree of horses with RAO can produce large amounts of superoxide radicals together with hydrogen peroxide.[7] Moreover, because of the extreme reactivity of the mentioned compounds, they can cause damage to membrane lipids, lipid components of the bronchial lining fluid, and lipid-containing mediators. Following oxidant injury in the airways, they may also lead to the peroxidation of these structures and the formation of malondialdehyde (MDA) and thiobarbituric acid-reactive substances (TBARs).

The levels of TBARs observed in the erythrocytes and blood plasma in both groups of horses were not statistically different. The 10-fold higher TBARs concentration in RBCs versus plasma can indicate a physiologically increased amount of lipid peroxidation reactions in red blood cells, as the same ratio was found between both groups. In human medicine, lipid peroxidation and oxidation of proteins of the erythrocytes and platelets has been reported in patients with COPD and with asthma.[22,23] In equine medicine, previous study performed on the plasma lipid hydroperoxides of RAO horses, revealed results similar to ours.[10] Likewise, a slightly different study evaluating the effects of acute airway inflammation induced by ozone, showed no evidence of pulmonary lipid peroxidation.[24] Increased formation of lipid peroxides has been observed in human asthma and chronic obstructive pulmonary disease (COPD).[25] However, this study, together with the other studies, confirms that RAO in horses does not affect the systemic and local formation of lipid peroxides either in RBCs or in plasma.[10,24] This phenomenon could be explained by the high level of efficiency of the chemical scavenging of lipid peroxides using the organism's antioxidative defenses. Further studies should be performed to better elucidate this mechanism.

In an aerobic organism, the most important first-line antioxidant defense mechanism is the enzymatic triad, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). These 3 enzymes mainly control the biological effect of reactive oxygen species; therefore, the key objective was to assess their activity in symptomatic RAO horses. In the present study, RAO-affected horses had a significantly increased RBC SOD and GPx activity. This was not the case for CAT and GR, which did not differ in the symptomatic RAO horses compared with the controls. In terms of GPx, our findings are in agreement with the other study, which also showed a higher activity of this enzyme in RBC of RAO-affected horses before, during, and after environmental challenge.[13] Nevertheless, these results are in contrast to those of a previous study, which failed to reveal differences in the activity of the erythrocyte hemolysate GPx.[12] Moreover, the authors demonstrated differences in the antioxidant balance in RAO-affected horses in terms of a lowered SOD activity. Although the present experiment did not allow us to confirm all the previous results, we did achieve comparable results with respect to catalase.[12] In both results, CAT activity was not significantly different in the 2 groups of horses.[12]

Prolonged exposure of susceptible horses to dust and noxious gases results in an infiltration of neutrophils into the bronchial lumen and thereby leads to a greater oxidative load.[6] When hydrogen peroxide levels rise as a consequence of the neutrophil respiratory burst, homeostasis requires increased production of antioxidant enzymes, especially the enzymatic triad, to maintain the equilibrium between oxidant production and the antioxidant system.[26] The SOD and GPx results of our study demonstrate different enzymatic activity in RAO-affected horses compared to healthy animals. Superoxide dismutase is an important factor in the metabolism of superoxide radicals that results in the formation of hydrogen peroxide.[27] Thus, while SOD is an important factor in the defense against oxidative stress, it also accelerates the formation of hydrogen peroxide, which also occurs during RAO exacerbation.[6] Therefore, it has been suggested that higher SOD activity in horses with RAO can to a large extent be explained by the stimulating effect of increased H_2O_2 production.

GPx, which catalyzes the reduction in hydrogen peroxide and organic peroxides, together with reduced GSH, constitutes one of the most important antioxidant defenses in living organisms.[28] Lower GPx activity in human COPD and asthmatic patients as well as in laboratory animals has been widely described previously.[23,29] The results of these studies confirmed the presence of disturbances in the functioning of the antioxidant enzyme barrier. Our results and the results of

other studies conducted in horses reveal higher GPx activity in RBCs lysates. The variance of results obtained in horses and in other species, in the course of similar diseases, may be explained by equine-specific adaptation factors. Prolonged inflammation, which occurs in RAO, can activate transcription factors such as NF-κB and Nrf2 that are responsible for triggering various genes, including mitochondrial GSH-GPx.[30]

Despite the above findings, it still remains unclear whether oxidative stress in RAO is simply a consequence of chronic airway inflammation or whether it is one of the contributors to the development of allergy and bronchial hypersensitivity. In human asthma, oxidative stress in the airway precedes the development of allergic inflammation, airway hyperresponsiveness, and other important features of asthma, such as increased mucus secretion.[29] Therefore, there is a strong hypothesis that an increased level of ROS plays a pivotal role as a critical contributor to the induction of allergic airway inflammation. Because of some similarities between RAO and human asthma, it is conceivable that local disturbances in oxidant-antioxidant balance may contribute to RAO development.

Horses with heaves exhibit oxidative stress in their systemic circulation, particularly during exacerbations of the disease. In asthmatic patients, systemic oxidative stress is explained as a result of increased superoxide generation by peripheral neutrophils.[4] In RAO in horses, neutrophils circulating in the blood have also been shown to produce increased amounts of superoxide anions during exposure to organic dust.[31,32] Thus, it is possible that a systemic imbalance in oxidant-antioxidant status in RAO-affected horses may be a consequence of the disease and activation of peripheral neutrophils.

Disturbances of the oxidative-antioxidative balance have been described in horses in the course of many conditions, without any specificity to particular respiratory diseases.[26] Therefore, the usefulness of these tests both in disease monitoring or detection of disease is negligible.

In conclusion, our results show that RAO crisis is associated with systematic oxidative stress, and differences in the systemic oxidant-antioxidant balance seem to be a consequence of the disease. The effect of an augmentation of GPx and SOD activities on the health and welfare of affected horses is currently unknown. The altered activity of these enzymes may be 1 mechanism causing increased susceptibility to airway infections in susceptible horses.[33] Anti-inflammatory treatment using corticosteroids and appropriate environmental changes remains the mainstay of RAO treatment. The effect of current RAO treatment in preventing oxidative stress is not yet clear and needs broader study. However, our studies prepare the way for such research, which in the future may provide a therapeutic effect. To our knowledge, this study is the first to examine the antioxidative enzymatic triad together with lipid peroxidation products in RAO horses.

Footnotes

[a] Domosedan, Orion Corporation, Finland
[b] Butomidor, Richter Pharma AG, Austria
[c] Beckman Coulter Allegra x-22, Beckman Coulter, Inc, CA
[d] Sigma, St. Louis, MO
[e] BioTek, Winooski, VT
[f] StanBio Laboratory, Boerne, TX
[g] StatSoft, Tulsa, OK

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Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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Appendix 1 Modified clinical staging of RAO in horses according to Tilley et al.[15]

Parameter	0	1	2	3	4	5
Clinical assessment[a]						
Cough score	None	Coughs at specific times of day (feeding/exercising/ making beds)	Frequent cough with periods of no coughing	Very frequent cough		
Nostril flare	None	Flares during inspiration (returns to normal at end inspiration)	Flares on inspiration and exhalation (slight movement can still be seen)	Flares on inspiration and expiration (no movement can be seen)		
Abdominal lift	None	Slight flattening of ventral flank	Obvious abdominal flattening and "heave line" extending no more than half way between cubital joint and *tuber coxae*	Obvious abdominal lift and "heave line" extending beyond halfway between cubital joint and *tuber coxae*		
Airway endoscopy[b]						
Mucus accumulation	None, clean	Little, multiple small blobs	Moderate, larger blobs	Marked, confluent or stream-forming	Large, pool-forming	Extreme, profuse amounts
Mucus color	None, clean	Colorless	White	Thick White	Yellow	Thick yellow
Mucus localization and stickiness	None, clean	1/2 Ventral	2/3 Lateral	3/4 Dorsal	Threading	Threading
Mucus apparent viscosity	None, clean	Very fluid	Fluid	Intermediate	Viscous	Very viscous

[a]Final Clinical Score (CS): 0 (CS final score <2), 1 (2 ≤ CS final score ≤4), 2 (5 ≤ CS final score ≤6), 3 (7 ≤ CS final score ≤9)
[b]Final airway Endoscopy Score: 0 (ES final score <8.5), 1 (8.5 ≤ ES final score ≤12), 2 (12 < ES final score ≤16) 3 (ES final score >16)
Final RAO Stage:
Stage 0 – No RAO (Total Score = 0);
Stage 1 – Mild RAO (1 ≤ Total Score <2);
Stage 2 – Moderate RAO (3 ≤ Total Score ≤4);
Stage 3 – Severe RAO (5 ≤ Total Score = 6)