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
H2O2 hydrogen peroxide
HiCN hemoglobin cyanide
K2EDTA potassium versenate
MDA malondialdehyde
O2– superoxide radicals
OH− 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. 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-PGF₂α between the groups. Ascobic 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. Moreover, this study also demonstrated disturbances in GSSG and the glutathione redox ratio between study groups.

There are only a few studies on the activity of erythrocyte lysate glutathione peroxidase (GPx) in horses with RAO. 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. 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. 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 (TBARSSs), 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 Wroclaw (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. 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. Venous blood was additionally obtained to rule out evidence of pulmonary infection, based on blood count and acute phase protein concentration. 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 and 0.01 mg/kg of butorphanol. 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. 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 hours after collection. For cytologic analyses, the smear was prepared with 10 mL of sample aliquots, cytospun at 300 × g for 10 minutes using the cytopsin and stained with Wright’s stain. A 400-cell leukocyte differential count (×1000 magnification) was performed excluding epithelial cells. 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 (K₂EDTA) for a full blood analysis. The activity of SOD, CAT, GPx, and GR was 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. 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 mL of supernatant with 980 μL of 50 mM phosphate buffer (pH 7.5) containing 0.2% bovine albumin and 2 mM K₂EDTA. 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 at 530 nm. Hemoglobin concentrations were determined using Drabkin’s cyanmethemoglobin method to express the enzyme activities as units of activity per gram of hemoglobin.

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 cyanodervative. The absorbance of the cyanodervative 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 |

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. 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

Statistical Analysis

The most important product of lipid peroxidation reacting with thiobarbituric acid (TBA) is malondialdehyde (MDA). 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 GPs, SOD, CAT, and GR was expressed in U/g Hb.
There is clear evidence that oxidative stress is involved in the pathophysiology of airway inflammation.

### 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 (%)   | 2.0 (2 and 2)  | 6.0 (5 and 6)        |
| PaO2 (mmHg) (%)      | 96 (91 and 108)| 91 (85 and 107)      |
| PaCO2 (mmHg) (%)     | 45 (43 and 46)| 45.5 (44 and 50)     |
| BALF neutrophils (%) | 5.1 (4.1 and 5.3)| 59.8 (51.3 and 64.8)|
| BALF lymphocytes (%) | 41 (38.5 and 45.9)| 38.1 (34.8 and 41.1)|
| BALF macrophages (%) | 55.8 (49.8 and 59.1)| 32.8 (25.9 and 35.7)|
| BALF eosinophils (%) | 0.4 (0.2 and 0.5)| 0 (0 and 0)          |
| BALF mast cells (%)  | 0.1 (0 and 0.3)| 0 (0 and 0)          |

* * Differences statistically significant (P < .05)

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

| Antioxidant enzyme | Control Median 25th and 75th percentiles Range | RAO-affected Median 25th and 75th percentiles Range | Mann-Whitney U-test P-value |
|--------------------|-----------------------------------------------|----------------------------------------------------|----------------------------|
| SOD [U/g Hb]       | 489.1 470.3–677.3 328.2–943.1                  | 880.8 717.5–995.6 652.1–1191                         | .011                       |
| GPx [U/g Hb]       | 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 | MDAcellular [mol/g Hb] 40.18 39.74–44.14 37.77–47.66 | 41.28 38.86–45.68 38.86–49.2 | .89 |
|                    | MDAplasma [mol/mL] 4.8 4.5–6.6 3.9–6.1          | 5.9 4.9–6.7 4.8–7.2                                      | .06                        |

* * Differences statistically significant (P < .05).
in the course of RAO in horses. However, there is little information relating to antioxidants circulating in the blood of RAO-affected horses. 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. When activated, leukocytes release many mediators and proinflammatory cytokines that amplify the inflammation, such as reactive oxygen species (ROS), which include superoxide radicals (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH$^-$), and singlet oxygen (O$_2^*$). 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$^-$). Neutrophils present in the bronchial tree of horses with RAO can produce large amounts of superoxide radicals together with hydrogen peroxide. 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 thioarbitruric 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. In equine medicine, previous study performed on the plasma lipid hydroperoxides of RAO horses, revealed results similar to ours. Likewise, a slightly different study evaluating the effects of acute airway inflammation induced by ozone, showed no evidence of pulmonary lipid peroxidation. Increased formation of lipid peroxides has been observed in human asthma and chronic obstructive pulmonary disease (COPD). 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. 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. 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. 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. In both results, CAT activity was not significantly different in the 2 groups of horses.

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. 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. 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. 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. 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$_2$O$_2$ 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. Lower GPx activity in human COPD and asthmatic patients as well as in laboratory animals has been widely described previously. 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-xB and Nrf2 that are responsible for triggering various genes, including mitochondrial GSH-GPx.

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. 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. 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. 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. 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 a mechanism causing increased susceptibility to airway infections in susceptible horses. 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

1. Domosedan, Orion Corporation, Finland
2. Butomidor, Richter Pharma AG, Austria
3. Beckman Coulter Allegra x-22, Beckman Coulter, Inc, CA
4. Sigma, St. Louis, MO
5. BioTek, Winoski, VT
6. StanBio Laboratory, Boerne, TX
7. StatSoft, Tulsa, OK

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Conflict of Interest Declaration: Authors disclose no conflict of interest.

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

References

1. Léguillette R. Recurrent airway obstruction – heaves. Vet Clin North Am Equine Pract 2003;19:63–86.
2. Davis E, Rush BR. Equine recurrent airway obstruction: Pathogenesis, diagnosis, and patient management. Vet Clin North Am Equine Pract 2002;18:453–467.
3. Williamson KK, Davis MS. Evidence-based respiratory medicine in horses. Vet Clin North Am Equine Pract 2007;23:215–227.
4. Zuo L, Otenbaker NP, Rose BA, Salisbury KS. Molecular mechanisms of reactive oxygen species-related pulmonary inflammation and asthma. Mol Immunol 2013;56:57–63.
5. Kirkham PA, Barnes PJ. Oxidative stress in COPD. Chest 2013;144:266–73.
6. Deaton CM. The role of oxidative stress in an equine model of human asthma. Redox Rep 2006;11:46–52.
7. Deaton CM, Marlin DJ, Smith NC, et al. Breath condensate hydrogen peroxide correlate with both airway cytology and epithelial lining fluid ascorbic acid concentration in the horse. Free Radic Res 2004;38:201–208.
8. Deaton CM, Marlin DJ, Deaton L, et al. Comparison of the antioxidative status in tracheal and bronchoalveolar epithelial lining fluids in recurrent airway obstruction. Equine Vet J 2006;38:417–422.
9. Kirschvink N, Marlin D, Delvaux F, et al. Collection of exhaled breath condensate and analysis of hydrogen peroxide as a potential marker of lower airway inflammation in cats. Vet J 2005;169:385–396.
10. Art T, Kischvink N, Smith N, Lekeux P. Indices of oxidative stress in blood and pulmonary epithelium lining fluid in horses suffering from recurrent airway obstruction. Equine Vet J 1999;31:397–401.
11. Kirschvink N, Smith N, Fievez L, et al. Effect of chronic airway inflammation and exercise on pulmonary and systemic antioxidant status of healthy and heaves-affected horses. Equine Vet J 2002;34:563–571.
12. Kramaric P, Pavlica Z, Koklic T, et al. Membrane switch hypothesis. 2. Domain structure of phagocytes in horses with recurrent airway obstruction. J Chem Inf Model 2005;45:1708–1715.
13. Tan RH, Thatcher CD, Buechner-Maxwell V, et al. Measurement of ascorbic acid concentration and glutathione peroxidase activity in biological samples collected from horses with recurrent airway obstruction. Am J Vet Res 2010;71:1500–1507.

14. Moran G, Buechner-Maxwell VA, Folch H, et al. Increased apoptosis of CD4 and CD8 T lymphocytes in the airways of horses with recurrent airway obstruction. Vet Res Commun 2011;35:447–456.

15. Tilley P, Sales Luis JP, Branco Ferreira M. Correlation and discriminant analysis between clinical, endoscopic, thoracic X-ray and bronchoalveolar lavage fluid cytology scores, for staging horses with recurrent airway obstruction (RAO). Res Vet Sci 2012;93:1006–1014.

16. Cywinska A, Gorecka R, Szarska E, et al. Serum amyloid A level as a potential indicator of the status of endurance horses. Equine Vet J Suppl 2010;42:23–27.

17. Stopyra A, Sobiech P, Waclawksa-Matyjasik A. Acid-base indicators in the venous and arterial blood of horses affected by recurrent airway obstruction (RAO). Pol J Vet Sci 2012;15:463–467.

18. Fernandez NJ, Hecker KG, Gilroy CV, et al. Reliability of 400-cell and 5-field leukocyte differential counts for equine bronchoalveolar lavage fluid. Vet Clin Pathol 2013;42:92–98.

19. Van Lerbergh W, Keegels G, Cornelis G, et al. Haemoglobin measurement: The reliability of some simple techniques for use in a primary health care setting. Bull World Health Organ 1983;61:957.

20. Lykkesfeldt J. Malondialdehyde as biomarker of oxidative damage to lipids caused by smoking. Clin Chim Acta 2007;380:50–58.

21. Varani J, Ward PA. Mechanisms of neutrophil-dependent and neutrophil-independent endothelial cell injury. Biolog Signals 1994;3:1–14.

22. De Castro J, Hernández-Hernández A, Rodríguez MC, et al. Comparison of changes in erythrocyte and platelet phospholipid and fatty acid composition and protein oxidation in chronic obstructive pulmonary disease and asthma. Platelets 2007;18:43–51.

23. Woźniak A, Górecki D, Szpinda M, et al. Oxidant-antioxidant balance in the blood of patients with chronic obstructive pulmonary disease after smoking cessation. Oxid Med Cell Longev 2013;2013:897075.

24. Deaton CM, Marlin DJ, Smith NC, et al. Antioxidant and inflammatory responses of healthy horses and horses affected by recurrent airway obstruction to inhaled ozone. Equine Vet J 2005;37:243–249.

25. Jacob KD, Noren Hooten N, Trzeciak AR, Evans MK. Markers of oxidant stress that are clinically relevant in aging and age-related disease. Mech Ageing Dev 2013;134:139–157.

26. Soffler C. Oxidative stress. Vet Clin North Am Equine Pract 2007;23:135–157.

27. Skółmowska M, Kmiec M. Antioxidant enzymosomes – properties and application. Postepy Hig Med Dosw (Online) 2011;67:640–646.

28. Steinbrenner H, Sies H. Protection against reactive oxygen species by selenoproteins. Biochim Biophys Acta 2009;1790:1478–1485.

29. Ahmad A, Shameem M, Husain Q. Relation of oxidant-antioxidant imbalance with disease progression in patients with asthma. Ann Thorac Med 2012;7:226–232.

30. Brigelius-Flohe R, Flohe L. Basic principles and emerging concepts in the redox control of transcription factors. Antioxid Redox Signal 2011;15:2335–2381.

31. Marr KA, Foster AP, Lees P, et al. Effect of antigen challenge on the activation of peripheral blood neutrophils from horses with chronic obstructive pulmonary disease. Res Vet Sci 1997;62:253–260.

32. Marr KA, Lees P, Cunningham FM. Antigen challenge increases adherence of circulating neutrophils in horses with chronic obstructive pulmonary disease. Equine Vet J 2002;34:65–70.

33. Albini S, Abril C, Franchini M, et al. Stenotrophomonas maltophilia isolated from the airways of animals with chronic respiratory disease. Schweiz Arch Tierheilkd 2009;151:322–328.

34. Johansson LH, Borg LA. A spectrophotometric method for determination of catalase activity in small tissue samples. Anal Biochem 1988;174:331–336.

35. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967;70:158–169.

36. Misra PH, Fridovich I. Superoxide dismutase: A biochemical augmentation assay. Arch Biochem Biophys 1971;156:271–280.

37. Carlberg I, Mannervik B. Purification and characterization of glutathione peroxidase from calf liver. An improved procedure for affinity chromatography on 2′, 5′-ADP-Sepharose 4B. Anal Biochem 1981;116:531–536.

38. Svingen BA, Buege JA, O’neal FO, Aust SD. The mechanism of NADPH-dependent lipid peroxidation. The propagation of lipid peroxidation. J Biol Chem 1979;254:5892–5899.
Appendix 1 Modified clinical staging of RAO in horses according to Tilley et al.\textsuperscript{15}

| Parameter                           | 0               | 1            | 2               | 3               | 4               | 5               |
|-------------------------------------|-----------------|-------------|-----------------|-----------------|-----------------|-----------------|
| **Clinical assessment\textsuperscript{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 \textit{tuber coxae} | Obvious abdominal lift and “heave line” extending beyond halfway between cubital joint and \textit{tuber coxae} |                 |                 |
| **Airway endoscopy\textsuperscript{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    |

\textsuperscript{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)

\textsuperscript{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)