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*Original article*

# Circulating immune complexes and markers of systemic inflammation in RAO-affected horses

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## Abstract

The aim of this study was to investigate the levels of circulating immune complexes (CICs) and concentration of haptoglobin, fibrinogen and C-reactive protein in the serum of horses with recurrent airway obstruction and healthy controls. The study was conducted on a group of 14 adult Polish Konik horses, kept in uniform environmental and living conditions. Horses were divided into two groups: 7 horses were not affected by any respiratory problem (control group) and 7 horses had a history of recurrent airway obstruction (RAO) (study group). A clinical and laboratory evaluation, endoscopic examination and bronchoalveolar lavage (BAL) were performed in all horses. Levels of circulating immune complexes were significantly ( $p=0.0057$ ) increased in heaves-affected horses compared to healthy controls (median [25<sup>th</sup> – 75<sup>th</sup> percentiles]) (3.96 [3.96 – 4.43] vs. 7.46 [5.13 – 11.9]). No significant difference was observed in the levels of the examined acute phase proteins between the groups. Moreover, all results were within the reference range established for horses. The results of this study indicate that heaves in horses is associated with the formation and high level of CICs. Haptoglobin, fibrinogen and C-reactive protein failed as markers of early stage systemic inflammation in the course of RAO.

**Key words:** heaves, horse, CICs, APPs, haptoglobin, fibrinogen, C-reactive protein

## Introduction

Circulating immune complexes (CICs) are the result of the host defense against endogenous or exogenous antigens. The elimination of a foreign antigen is carried out by the antibody, complement and phagocyte systems. When an immune complex is formed in the circulation, clearance is affected by cells

of the reticulo-endothelial system. This system normally provides the most efficient protection against disease, since animals and humans are continually faced with a variety of inhaled and ingested antigens (Navolotskaya 2014). In humans, CICs are detectable in a variety of systemic disorders such as autoimmune diseases, allergies and infectious diseases (Samuel et al. 1984, Pandey 2013, Wener 2014). Therefore, CICs

are acknowledged markers in the diagnosis of inflammatory diseases and provide useful aid in determining the immunopathology of the disease, help in determining the prognosis and are useful during the follow-up (Nowak-Łoś et al. 2013).

A number of environmental, immunologic, infectious and genetic factors play an important role in the pathogenesis of equine recurrent airway obstruction (RAO) (Léguillette 2003). However, the immunological basis of this disease is still poorly understood and requires further studies. The two hypersensitivity reactions – the immediate-type I and the immune complex formation type III – play a pivotal role in the development of airway inflammation (Leclere et al. 2011). Evidence suggests that an immune response contributes to the activation of inflammatory cells, with an increased expression of Th1 and Th2 cytokines, chemokines, adhesion molecules, receptors and a release of reactive oxygen species (ROS) (Leclere et al. 2011).

Although a lot of reports describe a wide range of immunological factors and biomarkers in equine respiratory diseases, circulating immune complexes have been poorly described in horses in the course of RAO. Moreover, the available literature on markers of systemic inflammation in horses with heaves is not well characterized and contains inconsistent results (Riihimäki et al. 2008, Leclere et al. 2011, Lavoie-Lamoureux et al. 2012). Apart from papers on the expression of different cytokines in the course of RAO in horses, only few papers contain results of levels of acute phase proteins (APPs), which are easy to determine *ex tempore*.

The present study hypothesized that alterations in the formation of CICs are present in horses with a clinical form of RAO. Therefore, the aim of this study was to characterize the levels of circulating immune complexes in horses affected with heaves. A simultaneous low-cost measurement of the concentration of selected APPs was carried out.

## Materials and Methods

### Animals

Seven Polish Konik horses (five geldings and two mares, 5-13 years old) that had no sign of airway or pulmonary diseases were used as control horses. Seven Polish Konik horses with RAO that had a history of chronic respiratory disease when fed hay and bedded on straw, were included in the study group (four geldings and three mares, 7-14 years old). All horses were placed in the groups based on their history and a thorough clinical examination, including

a blood gas analysis, hematology, screening biochemistry, endoscopy of the airways and the results of a bronchoalveolar lavage fluid (BALF) cytology. These procedures ruled out that horses suffered from RAO and had no other health problems. All horses did not receive any medical treatment during the 8 weeks preceding the study and during its course. RAO-affected horses were investigated during crisis. An acute crisis of heaves was induced by placing the horses in a poorly ventilated stable, bedding them on straw and feeding them hay with visible mold growth for 48 hours prior to examination. All experimental procedures were performed with the approval of the 2nd Local Ethics Committee on Animal Experimentation in Wrocław (resolution No. 1/2012).

### Collection and cytologic evaluation of BALF

Horses were held in stocks and sedated with 0.01 mg/kg of detomidine (Domosedan®, Orion Corporation, Finland) and butorphanol (Butomidor®, Richter Pharma AG, Austria). An endoscopy and BAL procedure was performed using methods previously described (Niedzwiedz et al. 2014). The amount of the recovered fluid was recorded and BALF for each individual horse was pooled in a sterile specimen cup, placed on ice and processed within 2h after collection. The samples were concentrated by cytopspin and stained with Wright's stain for the BALF cytology. Differential cell counts were obtained based on 400 leukocytes (Fernandez et al. 2013).

### Quantification of circulating immune complexes

The detection of circulating immune complexes was performed according to a modified method previously described by Haskova (1978). This generally consisted of adding 0.25 ml of the serum samples to 0.75 ml of 3.3% polyethylene glycol (PEG). The obtained mixture was then incubated at 4°C for 18 h, and centrifuged for 15 min at 15,000 min<sup>-1</sup> and 4°C. The supernatant was removed and the sediment was washed in 1 ml of 2.5% PEG. After centrifugation for 15 min at 15,000 min<sup>-1</sup> and 4°C, the supernatant was removed and the sediment containing the complexes was dissolved in 2.5 ml of 0.1% sodium hydroxide (NaOH). The absorbance was obtained using an ELx800UV Absorbance Microplate Reader (BioTek, Winooski, VT, USA), and the wavelength was set at 280nm. Results were expressed as mg/dl.

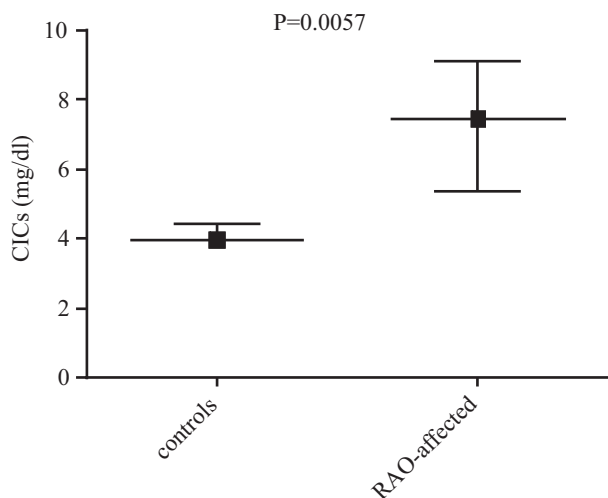


Fig. 1. Serum circulating immune complexes in RAO-affected horses and healthy controls. Central line represents median value and whiskers represent 25<sup>th</sup> and 75<sup>th</sup> percentiles.

### Quantification of acute phase proteins (APPs)

Plasma fibrinogen concentrations were determined using a chronometrical method (Coag Chrom 3003, Bio-Ksel, Gudziadz, PL). The C-reactive protein (CRP) concentrations were determined using a Konelab Prime 30 ISE automated analyzer (ThermoScientific, Waltham, MA, USA). The haptoglobin concentration was determined using an adapted guaiacol test according to Jones and Mould (1984).

### Statistical analysis

Data are presented as median and range. The differences between median values were estimated by use of a Mann-Whitney *U*-test. Correlations between APPs, CICs and selected parameters of lung function were assessed by the Spearman's rank test. All analyses were performed using STATISTICA v. 10.0 software (Stat Soft, Tulsa, USA). Figure 1 was created using Graphpad Prism V.5. (GraphPad Software, Inc., CA, USA). A *P* value <0.05 was considered as statistically significant.

## Results

### Results of clinical and BALF assessment

The results of the BALF analysis, clinical assessment and arterial blood gasometry are shown in Table 1. All heaves affected horses developed respiratory

signs typical of RAO after challenge with moldy hay and straw. As expected, control horses showed no or mild respiratory signs following stabling in a dusty environment, and, when present, nasal flaring, sporadic cough and abdominal lift were the most frequent abnormalities in this group. Only two individuals from this group had an increased amount of mucous, while the remaining horses had a very small amount of mucus or none at all.

Calculations using the Mann-Whitney *U*-test demonstrated a significant effect of a dusty environment on the percentage of BALF cell populations in heaves horses compared to control animals. The average BALF recovery in the control horses was 56.6% and 43.6% in the study group (*P*<0.005). The median percentage of neutrophils was significantly higher in the RAO-affected horses compared with controls (*P*<0.001), and the median percentage of macrophages was significantly higher in the control group compared with the study group (*P*<0.001). Also, the median percentage of lymphocytes in the control group was significantly higher compared with the study group (*P*<0.05).

### Circulating immune complexes

Circulating immune complexes were detected in all serum samples. Median concentrations were significantly higher in serum from heaves-affected horses compared with controls (*p*=0.0057) (Table 2). The median (25<sup>th</sup>-75<sup>th</sup> percentiles) serum concentration of circulating immune complexes in healthy animals versus RAO-affected horses was 3.96 (3.96-4.43) mg/dl and 7.46 (5.36-9.1) mg/dl respectively (Fig. 1).

### Acute phase proteins

Serum concentrations of all the measured acute phase proteins were detectable in all serum samples, both in the control and study group (Table 2). There was no significant difference between groups with regard to the median serum fibrinogen, haptoglobin and CRP concentrations. Moreover, results of all measured acute phase proteins remained within reference values established for horses (Pollock et al. 2005, Cywińska et al. 2012).

### Correlation analysis

The measured lung function parameters such as the % BALF neutrophils, % of BALF recovery,

Table 1. Results of BAL fluid cytology, clinical assessment and blood gas analysis in healthy horses and RAO-affected horses. Values are expressed as median and 25<sup>th</sup> and 75<sup>th</sup> percentiles.

	Healthy n=7	RAO-affected n=7
Clinical score <sup>a</sup>	2.0 (2 and 2)	6 (5 and 6)
PaO <sub>2</sub> (mmHg) <sup>a</sup>	91 (85 and 107)	96 (91 and 108)
PaCO <sub>2</sub> (mmHg) <sup>b</sup>	45 (43 and 46)	45.5 (44 and 50)
BALF Neutrophils (%) <sup>a</sup>	5.1 (4.1 and 5.3)	59.8 (51.3 and 64.8)
BALF Lymphocytes (%) <sup>a</sup>	41 (38.5 and 45.9)	38.1 (34.8 and 41.1)
BALF Macrophages (%) <sup>a</sup>	55.8 (49.8 and 59.1)	32.8 (25.9 and 35.7)
BALF Eosinophils (%) <sup>a</sup>	0.4 (0.2 and 0.5)	0 (0 and 0)
BALF Mast cells (%) <sup>b</sup>	0.1 (0 and 0.3)	0 (0 and 0)

<sup>a</sup> Differences statistically significant (P<0.05)

<sup>b</sup> NS

Table 2. Serum CICs and APPs concentrations in RAO-affected horses and healthy controls. Values are expressed as median, 25<sup>th</sup> – 75<sup>th</sup> percentiles and range.

	Control			RAO-affected			p-value
	median	25 <sup>th</sup> – 75 <sup>th</sup> percentiles	range	median	25 <sup>th</sup> – 75 <sup>th</sup> percentiles	range	
CICs (mg/dl)	3.96	3.96-4.43	3.5-5.83	7.46	5.36-9.1	5.13-11.9	0.0057
CRP (mg/ml)	8.9	5.9-9.9	5.1-11.3	12.2	9-12.9	7.9-16.9	NS
Fibrinogen (g/l)	3.84	3.78-5.49	3.13-5.77	3.85	3.06-5	3.55-4.66	NS
Haptoglobin (g/l)	0.92	0.82-0.93	0.35-1.04	0.93	0.91-1.1	0.24-1.33	NS

Table 3. Spearman correlations and P values between lung functions parameters and CICs and APPs in RAO-affected horses. The Spearman correlation  $r_s$  and corresponding (P values) are indicated.

	%BALF Neutrophils	%BALF recovery	PaCO <sub>2</sub>	PaO <sub>2</sub>
CICs	-0.035 (0.93)	-0.071 (0.87)	-0.36 (0.42)	-0.50 (0.24)
Haptoglobin	-0.28 (0.53)	0.14 (0.75)	-0.59 (0.15)	0.32 (0.47)
Fibrinogen	-0.25 (0.58)	0.57 (0.18)	0.59 (0.15)	-0.07 (0.87)
CRP	0.66 (0.10)	-0.34 (0.45)	0.63 (0.12)	-0.21 (0.63)

PaCO<sub>2</sub> and PaO<sub>2</sub> were not significantly correlated with serum APPs or CICs (Table 3).

## Discussion

The objectives of this study were to quantify circulating immune complexes (CICs) and acute inflammatory phase proteins (APPs) in heaves-affected horses and compare the results with those of healthy individuals. The APPs chosen in this study were fibrinogen, haptoglobin and C – reactive protein (CRP), since they are commonly used in equine practice and their evaluation is relatively inexpensive (Cywińska et al. 2012). CICs were selected since it is necessary to establish additional marker of inflammation that would

be useful in early stage of diseases exacerbation. Moreover, to the authors' knowledge, the concentration of CICs in heaves horses has not been previously described in literature.

Circulating immune complexes are protein structures that are formed as a consequence of an immune response of the organism to the antigens of various origin (Navolotskaya 2014). Results of our study indicate the presence of significantly elevated levels of circulating immune complexes in the serum of horses with RAO exacerbation compared with healthy controls. There is no quantitative data available in literature concerning CICs in heaves-affected horses. Lack of the above mentioned data creates difficulties in assessing and comparing the results with those of other authors. In humans, many insights have been made

into the pathogenesis of diseases such as rheumatoid arthritis and glomerulonephritis, using the analysis of the levels of circulating immune complexes levels (Nowak-Łoś et al. 2013, Navolotskaya 2014, Wener 2014). The incidence of circulating immune complexes has been ascertained for many disease conditions (Haymen 1990, Pandey 2013, Maheswari et al. 2014) and the level of circulating immune complexes has been correlated with the activity of certain diseases. Moreover, the assays have also been used to evaluate the effect of pharmacological therapy on the levels of circulating immune complexes (Murunikkara and Rasool 2014). CICs were the only biomarkers significantly associated with a heaves exacerbation, while the evaluated APPs failed to show any abnormality. Therefore, it seems reasonable to look deeper into the association between CICs and RAO in horses. The obtained results should be considered as an introduction into further studies using CICs involving a larger group of horses. Currently, sensitive and specific tests useful in routine laboratory diagnostics are being sought in horses with recurrent airway obstruction. Finding a new test would be particularly important in horses with dyspnea in the acute stage of the disease, where bronchoalveolar lavage is contraindicated. A simple biomarker would help in distinguishing between animals with lower airway infections and those with allergic problems.

Fibrinogen, haptoglobin and the C – reactive protein are acute phase proteins of moderate importance in horses (Cywińska et al. 2012). During the acute inflammation phase their concentrations increase 5-10x and are maintained at such a level up to a few weeks after the remission of the disease. Moreover, maximum concentrations of fibrinogen and haptoglobin are achieved after 72 h, and even 120 h as regards CRP, after tissue damage (Cray 2012). The results of our study did not show any disturbances in the concentration of acute phase proteins. The C – reactive protein was the sole acute phase proteins, whose concentration increased. However, the results remained within the reference range established for horses, and were not significantly different when compared to healthy animals. The obtained results indicate that systemic inflammation response in the first 72 hours after exposure to an allergen is poor in horses. In addition, these results confirm that strict environmental dust control leads to complete remission of inflammation. Our study supports the report of Lavoie-Lamoureux et al., regards only CRP (Lavoie-Lamoureux et al. 2012). In their study, the C-reactive protein remained undetected in serum samples from 1 control and 1 RAO-affected horse and there was no significant difference in the mean serum concentration of CRP both before and after

RAO exacerbation. However, the authors did find statistically significant differences in the haptoglobin concentration between RAO and control horses. The measurement of the concentration of haptoglobin before and after an allergic challenge showed a significant increase in the heaves-affected horses compared with control animals (Lavoie-Lamoureux et al. 2012). A lack of increase of haptoglobin concentrations in our study may be explained by too short a time from the exacerbation of RAO to carrying out the measurements. However, RAO-affected horses in the study of Lavoie-Lamoureux et al. had elevated haptoglobin concentrations even before the allergic challenge, which may suggest that the animals were not in complete clinical remission, despite a normal percentage of BALF neutrophils. Alternatively, the normal results in our study may be explained by a complete remission of RAO in the examined animals or an adaptation of these animals to a higher hemoglobin concentration as a consequence of chronic inflammation and erythrocyte damage. In spite of this, haptoglobin failed as a marker of early stage RAO exacerbation in our study. We can agree with other authors that monitoring of this APP during long-term treatment can help in selecting a therapy and monitoring recovery. However, future studies are needed to clearly assess a relationship between the concentrations of haptoglobin in various stages of recurrent airway diseases.

In our study, neither CICs, haptoglobin, fibrinogen nor C-reactive protein were correlated with the results of pulmonary function, such as BALF percentage neutrophils, BALF recovery, PaCO<sub>2</sub> or PaO<sub>2</sub>. This lack of correlation suggests that the studied biomarkers are not directly involved in the neutrophil influx to the bronchial lumen, bronchoconstriction and the deterioration of pulmonary gas exchange.

The limitations of this study include a relatively small study population of horses and a lack of continuous measurement of CICs and APPs concentrations over a longer period of time. Also, due to financial constraints, we chose to study only the basic acute phase proteins. Studying a broader spectrum of these biomarkers may help to better characterize this disease and choose a parameter that best correlates with a RAO exacerbation.

Recently, due to a growing prevalence of allergic diseases in horses throughout the world, a lot of effort has been put into understanding their pathophysiology. The results of our study lead to the following conclusion: (1) the determination of circulating immune complexes in equine serum is technically possible and generally not expensive; (2) elevated circulating immune complexes are associated with RAO exacerbation in horses; (3) further studies are needed to establish a marker of systemic inflammation in the

course of RAO, since ambiguous results are obtained in different studies.

### Competing interests

The authors declare that they have no competing interests.

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