VETERINARSKI ARHIV 86 (3), 395-405, 2016

Evaluation of the blood oxidant-antioxidant interactions in pigeons naturally infected with *Haemoproteus columbae*

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RAZAVI, S. M., S. NAZIFI, M. AFSAR, Z. YAZDANPANAH, E. RAKHSHANDEHROO: Evaluation of the blood oxidant-antioxidant interactions in pigeons naturally infected with *Haemoproteus columbae*. Vet. arhiv 86, 395-405, 2016.

ABSTRACT

Haemoproteus columbae is a haemosporidian parasite that infects pigeons throughout the world. The present study was designed to elucidate more aspects of the antioxidant defense of the body against haemoparasite infections in pigeons. A total of 46 indigenous pigeons naturally infected with *H. columbae* were selected and subdivided into three subgroups based on their parasitemia rates (<1 %, 1-3 %, 3-5 %). 24 non-infected birds were also used as controls. Blood samples from both groups were taken, and haematological parameters were measured. Although our data demonstrated significant decreases in the red blood cell count (RBC), packed cell volume (PCV) and haemoglobin values of the infected animals ($P<0.05$), no remarkable changes were observed in the activities of antioxidant enzymes (including superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase), the level of malondiadehyde (MDA) as an index of lipid peroxidation, serum levels of lipids (cholesterol, triglyceride, HDL and LDL) and serum concentrations of antioxidant trace elements (copper, iron, zinc, manganese and selenium) and vitamins (A, E and C) in infected groups compared to controls. These findings show that anaemia may occur as a result of the infection with *H. columbae* in pigeons. In addition, the unchanged levels of different antioxidant agents, lipid peroxidation index (MDA) and the main lipid components of serum during the infection, may implicitly suggest that the parasite probably cannot induce significant effects on the antioxidant mechanisms protecting erythrocytes against oxidative agents. Also, oxidative shock may not play a significant role in the pathogenesis of the parasite in pigeons infected with *H. columbae*. Further studies to clarify the exact mechanism(s) of anaemia in this haemoparasitic infection need to be carried out.

Key words: *Haemoproteus columbae*, antioxidant enzymes, malondialdehyde trace elements, antioxidant vitamins

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ISSN 0372-5480
Printed in Croatia

Introduction

Haemoproteus columbae is a common and large avian haemosporidian parasite that infects pigeons widely in tropical and subtropical regions. Domestic pigeons (*Columba livia domestica*) and many species of wild pigeons are the natural hosts of this parasite (SOULSBY, 1982). The parasite, transmitted by a blood sucking hippoboscid fly (*Pseudolynchia canariensis*), invades red blood cells and develops crescent-shaped gamonts, which partially encircle the nucleus of the host cells and occupy them (ZAJAC and CONBOY, 2012).

Previous studies have suggested various explanations to describe the factors involved in the onset of anaemia, following the erythrocyte damage induced by infection with various haemoparasites. However, the exact mechanisms have long been a matter of debate. Recently, some researchers, who worked on piroplasms such as *Theileria* parasites, have claimed that the anaemia is probably a consequence of oxidative injuries to erythrocytes (SHIONO et al., 2003), and indicated significant reductions in the activity of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (SHIONO et al., 2003; NAZIFI et al., 2011; NAZIFI et al., 2013; RAZAVI et al., 2011; RAZAVI et al., 2012). Some investigations have also suggested that lipid peroxidation of the RBCs (GREWAL et al., 2005) and thus the morphological changes in the cell surface of erythrocytes (SALUJA et al., 1999; GREWAL et al., 2005) could cause an increase in the susceptibility of erythrocytes. Malondiadehyde (MDA), an end product of polyunsaturated fatty acid oxygenation, is a reliable and commonly used biomarker for assessing lipid peroxidation (MOORE and ROBERTS, 1998).

Trace elements, such as zinc, copper and selenium, are utilized for synthesis of antioxidant enzymes (EVANS and HALLIWELL, 2001) and are assigned as essential components of the antioxidant defence of the body against free radical-induced damage. Also, previous studies proved that antioxidant vitamins, such as A, E, and C, could protect cells against free oxygen radicals in parasitic infections (MISHRA et al., 1994; DEDE et al., 2000). Accordingly, several studies have indicated that the antioxidant systems comprising such vitamins could have cellular protective action against oxidative stress during some parasitic invasions (DEDE et al., 2000). Despite a bulk of studies on antioxidant trace elements and vitamins, the role of non-enzymatic antioxidant defence has not been clearly investigated during the pathogenesis of blood parasites.

This study was designed to assess the pattern of antioxidant enzymes activities in erythrocytes, the level of lipid peroxidation in the RBC membrane, and the level of lipids and antioxidant trace elements and vitamins in the serum of pigeons (*Columba livia domestica*) naturally infected with *Haemoproteus columbae*, in order to obtain insight into the probable function of the antioxidant defence against haemoparasite infections in pigeons.

Materials and methods

Source of animals and samples. The study was performed in the southwest region of Iran (Fars province). Overall, seventy adult pigeons were obtained from different households and kept in a metal cage. The birds were fed on a commercial pigeon feed, consisting of mixed seeds, and water was provided *ad libitum*. Blood samples were taken by brachial vein puncture into EDTA containing tubes for evaluation of haematological parameters and erythrocyte oxidant-antioxidant measurements, and without anticoagulant to obtain serum.

Animal ethics. This experiment was conducted with the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran. Also, the recommendations of the European Council Directive (86/609/EC) of November 24, 1986 were followed, regarding the standards for the protection of animals used for experimental purposes.

Haematological procedures. Blood smears were prepared, air dried and fixed in absolute methanol for 5 minutes immediately after sample collection, and later stained with 10 % Giemsa stain for 45 min. The slides were then carefully examined microscopically (at a magnification of ×1000) to observe macro/micro gamonts of *Haemoproteus columbae*. Then the animals were divided into infected (n = 46) and controls (n = 24) according to the presence or absence of the gamonts of the parasite in their RBCs. In the infected group, the rate of parasitaemia (parasitized RBC rate) was also quantified. The erythrocytes were examined for each case on at least 15 microscopic fields and then the proportion of infected (parasite- bearing) erythrocytes to the total number of counted cells was calculated and expressed as the percentage of parasitaemia. Parasites were only counted in fields with a homogenous distribution of erythrocytes, which most frequently occurred in the tails of the smears. The infected pigeons were subdivided into three subgroups according to their parasitaemia rates (<1 %, 1-3 % and >3 %). Haematological parameters were also measured in all blood samples by standard routine procedures (JAIN, 1993).

Antioxidant enzymes activities. SOD activity was measured with a commercial kit (RANSOD kit, Randox Com, UK). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT), to form a red formazan dye. The enzyme activity was then determined by the degree of reaction inhibition, where one unit of SOD corresponded to 50 % inhibition of INT reduction under assay condition. GPX activity was measured by a commercial kit (RANSEL kit, Randox Com, UK) based on the method previously described (PAGLIA and VALENTINE, 1967). GPX catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidised glutathione is immediately converted to the reduced form, with the concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance

was measured at 340 nm. The values of both enzymes were expressed as units/gr of haemoglobin.

The activity of catalase was determined using the commercial catalase assay kit (Oxford Biomedical Research, Inc., USA) based on the colorimetric method, described by SLAUGHTER and O'BRIEN (2000) and the activities of the enzymes were expressed as U/g of haemoglobin. The analyses were accomplished using the standard auto-analyser with veterinary software (Cobas-Mira, ABX-Diagnostics, Japan).

Lipid peroxidation of RBCs. The lipid peroxidation level of the RBC membrane was evaluated by means of a modified HPLC method using UV-Visible spectrophotometry (Jasco, UV-975, Tokyo, Japan) based on Lykkesfeldt (LYKKESFELDT, 2001). The measurement was based on MDA reactions with thiobarbituric acid (TBA) to form a coloured MDA-TBA adduct, and the values were expressed as mmol/L of MDA.

Lipid components analysis. The blood samples were centrifuged at 750 *g* for 15 min, and the sera separated and kept at -20 °C until analysis. The samples with haemolysis were discarded. The analysis of the sera for total cholesterol was done using a commercial kit (Ziest Chem Diagnostics, Tehran, Iran) by a modified Abell-Kendall/Levey-Brodie (A-K) method (BURTIS and ASHWOOD, 1994), and the measurement of serum triglyceride was accomplished on the basis of the enzymatic procedure (McGOWAN et al., 1983) using a commercial kit (Ziest Chem Diagnostics, Tehran, Iran).

Lipoproteins, including HDL-cholesterol (mmol/L) and LDL-cholesterol (mmol/L), were analysed by the quantitative enzymatic colorimetric method, using test kits supplied by STANBIO Laboratories, Boerne, TX, USA. All reactions were measured using a Digital VIS/Ultraviolet Spectrophotometer (CE 292, series 2, Cecil instruments, Cambridge England) (FRIEDEWALD et al., 1972).

Trace element measurement. To evaluate the serum concentration of trace elements, digestion of serum was performed by a mixture of perchloric and nitric acid (3:7 ratios respectively). Manganese, copper, iron, selenium and zinc were then measured using an atomic absorption spectrophotometer (Shimadzo AA-670, Kyoto, Japan).

Antioxidant vitamins. The concentrations of vitamins A, E and C were evaluated using the HPLC method with Ultraviolet detection. Vitamins A and E were measured on the basis of the protocol described by JOHNSON-DAVIES et al. (2002), and vitamin C using a commercial kit (ALPCO Diagnostics, USA). The HPLC system used consisted of a solvent delivery pump (JASCO 980-PU, Tokyo, Japan), a reversed-phase column (Luna C18, 250 mm × 4.6 mm, Phenomenex, CA, USA), and a UV-Vis detector (Jasco, UV-975, Tokyo, Japan).

Statistical analysis. Analysis of variance (ANOVA) and Tukey tests were applied to find statistical differences between control values and the infected subgroups, and

Pearson's correlation coefficients to determine relationships among parameters at different parasitaemia rates. All values were expressed as mean and standard error of mean (SEM), and $P<0.05$ was considered as statistically significant.

Results

The infection was confirmed by the presence of *Haemoproteus columbae* gametocytes in RBCs (Fig. 1). The blood smears prepared from all infected animals with different parasitaemia rates revealed a range of abnormal erythrocytes, including reticulocytosis and macrocytosis. The values of haematological parameters, antioxidant enzymes activities and Malondialdehyde (MDA) in non-infected birds and those naturally infected with *H. columbae* with different parasitaemia rates, are depicted in Table 1. The haematological values revealed the occurrence of anaemia in the infected pigeons at all levels of parasitaemia. This phenomenon was evidenced by significant decreases in erythrocyte numerations, haemoglobin and the haematocrit (PCV) values, compared to non-infected controls ($P<0.05$). However, correlation analyses showed that there was no remarkable relationship between the severity of anaemia and the degree of parasitaemia.

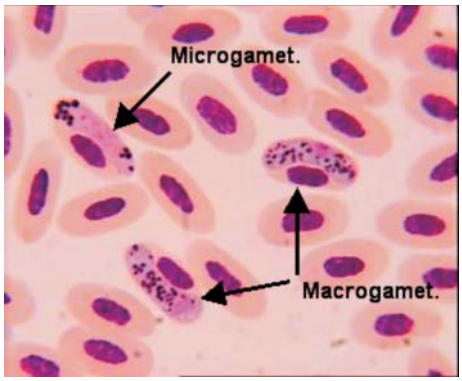

Fig. 1. Blood smear from an infected bird showing micro- and macrogametocytes of *Haemoproteus columbae* (×1000).

According to our data (Table 1), the activities of antioxidant enzymes, including SOD, GPX and catalase and also MDA concentration, did not show any substantial changes in infected pigeons compared to healthy ones ($P<0.05$).

Table 1. Haematological parameters, antioxidant enzymes activities and the level of MDA in non-infected pigeons and those infected with *H. columbae* in different parasitaemia rates (values are presented as mean ± SEM).

Groups	RBC $\times 10^{12}$/L	PCV L/L	Hb g/dL	SOD U/gr Hb	GPX U/gr Hb	Catal U/gr Hb	MDA nmol/g Hb	Parasitaemia, %
Control	4.87[a] ± 0.22	0.46[a] ± 0.007	16.23[a] ± 0.36	87.1[a] ± 3.80	19.46[a] ± 2.64	182.23[a] ± 1.38	3.73[a] ± 0.12	0 (n = 24)
Infected	3.13[b] ± 0.21	0.38[b] ± 0.006	13.94[b] ± 0.3	90.49[a] ± 3.72	18.07[a] ± 4.37	182.84[a] ± 1.32	4.05[a] ± 0.16	<1 (n = 20)
	3.76[b] ± 0.22	0.40[b] ± 0.009	14.22[b] ± 0.32	80.84[a] ± 3.25	20.9[a] ± 4.28	180.95[a] ± 1.73	3.83[a] ± 0.17	1-3 (n = 16)
	3.07[b] ± 0.32	0.39[b] ± 0.011	13.94[b] ± 0.2	83.134[a] ± 5.74	20.24[a] ± 4.08	184.87[a] ± 2.43	3.97[a] ± 0.20	3-5 (n = 10)

MDA: malondialdehyde; Catal: catalase; GPX: glutathione peroxidase; SOD: superoxide dismutase; Hb: haemoglobin; PCV: packed cell volume; RBC: red blood cells count. *Different letters in each column indicate statistical significance ($P<0.05$).

Interestingly, similar to the pattern of antioxidant enzymes, no significant alterations occurred at the serum level of lipid contents (cholesterol, triglyceride, HDL and LDL) (Table 2), antioxidant trace elements (copper, iron, zinc, manganese and selenium) and antioxidant vitamins (A, E and C) (Table 3) in the infected animals. Moreover, our data did not reveal any significant correlations between the rate of parasitaemia and the concentrations of different lipid compartments, antioxidant trace elements and vitamins. Similarly, no remarkable correlations were seen between the haematological parameters (RBC count, PCV and haemoglobin) and the antioxidant agents measured in the present study.

Table 2. The values of the main lipid components of serum in non-infected pigeons and those infected with *H. columbae* in different parasitaemia rates (values are presented as mean ± SEM).

Groups	Cholesterol nmol/L	Triglyceride nmol/L	HDL nmol/L	LDL nmol/L	Parasitaemia, %
Control	384.67[a] ± 8.11	217.5[a] ± 20.87	189[a] ± 24.58	205.33[a] ± 8.47	0 (n = 24)
Infected	373.9[a] ± 15.62	219.1[a] ± 21.82	132.85[a] ± 25.66	225[a] ± 9.4	<1 (n = 20)
	383.69[a] ± 12.34	210.63[a] ± 21.69	139.8[a] ± 23.46	222.94[a] ± 14.06	1-3 (n = 16)
	373.7[a] ± 23.17	190.8[a] ± 22.84	155.9[a] ± 38.09	225.3[a] ± 17.9	3-5 (n = 10)

LDL: low density lipoprotein; HDL: high density lipoprotein. *Different letters in each column indicate statistical significance ($P<0.05$).

Table 3. The concentrations of the antioxidant serum trace elements and vitamins in non-infected and diseased pigeons in different parasitaemia rates (values are presented as mean ± SEM).

Groups	Cu µmol/L	Fe µmol/L	Zn µmol/L	Mn µmol/L	Se µmol/L	Vit A µmol/L	Vit E µmol/L	Vit C µmol/L	Parasitaemia %
Control	0.73[a] ± 0.03	167.13[a] ± 12.84	2.97[a] ± 0.12	0.01[a] ± 0.001	0.03[a] ± 0.001	27.64[a] ± 3.2	0.74[a] ± 0.042	0.41[a] ± 0.03	0 (n = 24)
Infected	0.43[a] ± 0.01	172.25[a] ± 23.76	2.4[a] ± 0.02	0.01[a] ± 0.001	0.03[a] ± 0.002	35.39[a] ± 3.74	0.83[a] ± 0.052	0.41[a] ± 0.03	<1 (n = 20)
	0.46[a] ± 0.01	166.06[a] ± 18.63	2.81[a] ± 0.12	0.01[a] ± 0.001	0.03[a] ± 0.0001	28.62[a] ± 3.95	0.78[a] ± 0.044	0.43[a] ± 0.05	1-3 (n = 16)
	0.46[a] ± 0.01	139.1[a] ± 19.19	2.9[a] ± 0.36	0.01[a] ± 0.002	0.03[a] ± 0.0001	28.42[a] ± 4.83	0.97[a] ± 0.068	0.31[a] ± 0.04	3-5 (n = 10)

*Different letters in each column indicate statistical significance (P<0.05).

Discussion

The findings of this study clearly show that infection with *Haemoproteus columbae* in pigeons may promote the development of anaemia. However, our observations could indicate the compensatory response of bone marrow to emerging and progressive anaemia in infected pigeons. In addition, our conclusions ruled out the occurrence of oxidative stress during infection with *H. columbae*.

Although the process of anaemia has been investigated to some extent in a few haemoparasitic diseases, the exact mechanisms have long been a matter of debate. Some previous studies indicated that the rupture or phagocytosis, the removal of uninfected red cells due to antibody sensitization or other physicochemical membrane changes, could be the potential causes of RBC destruction and haemolysis in infection with *Plasmodium falciparum* (PHILLIPS and PASVOL, 1992). More recently, some studies have focused on anaemia in ruminants infected with different species of another haemoparasite, *Theileria,* and suggest that this feature is probably a consequence of oxidative damage to erythrocytes (SHIONO et al., 2003; NAZIFI et al., 2011; NAZIFI et al., 2013; RAZAVI et al., 2011). Parasite-induced modulations of the activity of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase, could result in the acceleration of the erythrocyte clearance by phagocytic cells (SHIONO et al., 2003; GREWAL et al., 2005; NAZIFI et al., 2011; RAZAVI et al., 2011).

Despite some investigations on theileriosis, revealing significant reductions in antioxidant enzyme activity in the RBCs of infected animals (ASRI REZAEI and DALIR-NAGHADEH, 2006; NAZIFI et al., 2011; RAZAVI et al., 2011), GREWAL et al. (2005) reported that GPX activity exhibited a significant rise in cattle naturally infected with *T. annulata*, whereas SOD and catalase showed no substantial changes. They concluded that GPX has

a pivotal activity for neutralizing the lipid peroxides, rather than SOD or catalase activity. Considering the data obtained here, it may be argued that although infection with *H. columbae* in pigeons may promote the production and activation of antioxidant enzymes to protect erythrocytes against oxidative agents, it seems that a balance may be developed between the production and consumption of these enzymes, which allows them to remain unchanged during parasite invasion of host erythrocytes.

In the present work, the lack of any significant changes in the levels of lipid peroxidation (LPO) and major lipid components of the serum of the infected animals, along with the lack of any remarkable correlation with the parasitaemia rate showed that the parasites could not induce significant alterations in erythrocytic membrane peroxidation or in lipid metabolism. These results again confirm that *H. columbae* probably could not induce oxidative shock during parasitaemia. Membrane lipids are major targets for cellular damage induced by reactive oxygen species (ROS) (DAVIES and GOLDBERG, 1987). Interaction of ROS with rich polyunsaturated fatty acids (HALLIWELL and CHIRICO, 1993) at the cell membrane level, results in the formation of several lipid peroxidation products such as MDA. The unchanged MDA levels of RBCs indicate that antioxidant defence mechanisms were sufficient to neutralize oxidative stress, a process which consequently may promote reduced membrane symmetry and increase membrane permeability, causing morphological changes in the RBC cell surface (SALUJA et al., 1999) and eventually anaemia.

In this work, our data clearly show that the parasites cannot cause any marked effect on the status of antioxidant mineral nutrients and vitamins. Micronutrients, such as zinc, copper and selenium, are essential components of the body's antioxidant defence, that play an important role in the prevention of free-radical induced damage (EVANS and HALLIWELL, 2001), for instance, copper-zinc SOD (Cu, Zn-SOD) and manganese SOD (Mn-SOD), which are located in the cell cytosol (McCORD and FRIDOVICH, 1969) and the mitochondria (WEISIGER and FRIDOVICH, 1973). On the other hand, a number of previous studies which studied haemoparasitic diseases (such as the genus *Babesia*) demonstrated the decreased blood levels of such vitamins in the serum of the affected animals, and suggest that this phenomenon was a consequence of elevation of the level of oxidative damage (DEGER et al., 2009). Corroborating our data, NAZIFI et al. (2013) concluded that there were no statistical differences in the levels of antioxidant vitamins in sheep infected with *Theileria lestoquardi*. This may be attributed to the occurrence of equilibrium between the vitamin supplies (via their dietary uptake, and indirectly from tissue stores, particularly in the liver) and their consumption in damaged invaded cells.

In conclusion, anaemia was a critical aspect of the infection of pigeons with *Haemoproteus columbae*. Also, the unchanged levels of antioxidant enzyme activities, the lipid peroxidation index (MDA), the major lipid content of the serum, and antioxidant

trace elements and vitamins in the infected birds, may reveal that the parasite most likely cannot induce remarkable changes to the antioxidant mechanisms which protect RBCs against oxidative agents. Also, the oxidative shock to RBCs may not play a significant role in the pathogenesis of the infection with *H. columbae* in pigeons. Further studies to elucidate the exact mechanism(s) of anaemia in this haemoparasitic infection are necessary.

Acknowledgements

The authors would like to thank the Research Council of Shiraz University and School of Veterinary Medicine, Shiraz University for financial and technical support of this study (Grant No.71-GR-VT-5).

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Received: 25 February 2015
Accepted: 10 December 2015

RAZAVI, S. M., S. NAZIFI, M. AFSAR, Z. YAZDANPANAH, E. RAKHSHANDEHROO: Vrednovanje međusobnog djelovanja oksidant-antioksidant u krvi golubova prirodno zaraženih vrstom *Haemoproteus columbae*. Vet. arhiv 86, 395-405, 2016.

SAŽETAK

Haemoproteus columbae jest hemosporidij koji napada golubove diljem svijeta. Ovo istraživanje poduzeto je u namjeri da se s više gledišta rasvijetli antioksidacijska obrana organizma od hemoparazitskih infekcija u golubova. Ukupno je bilo odabrano 46 autohtonih golubova prirodno invadiranih vrstom *H. columbae* te podijeljeno u tri podskupine na osnovi stupnja njihove parazitemije (<1%, 1-3%, 3-5%). Kao kontrola poslužile su 24 neinficirane ptice. Uzeti su uzorci krvi od obiju skupina te su određeni hematološki pokazatelji. Iako su rezultati pokazali značajno smanjenje broja crvenih krvnih stanica, vrijednosti hematokrita i hemoglobina u invadiranih životinja ($P<0,05$), nisu ipak zapažene značajne promjene u aktivnostima antioksidacijskih enzima (uključujući superperoksidnu dismutazu, glutationsku peroksidazu i katalazu), razine malondialdehida (MDA) kao pokazatelja lipidne peroksidacije, razine lipida u serumu (kolesterola, triglicerida, HDL i LDL) i serumskih koncentracija antioksidacijskih elemenata u tragovima (bakra, željeza, cinka, mangana i selena) te vitamina (A, E i C) u uzorcima seruma invadiranih skupina u odnosu na kontrolnu skupinu. Ti nalazi pokazuju da se anemija može javiti kao rezultat invazije vrstom *H. columbae* u golubova. Povrh toga, nepromijenjene razine različitih antioksidacijskih tvari, indeks lipidne peroksidacije i sadržaj glavnih lipida u serumu tijekom invazije može značiti da parazit vjerojatno ne može potaknuti značajne učinke na antioksidacijske mehanizme koji bi zaštitili eritrocite od oksidacijskih tvari. Također, oksidacijski šok nema značajnu ulogu u patogenezi invazije vrstom *H. columbae* u golubova. Potrebna su daljnja istraživanja za točno rasvjetljavanje mehanizama nastanka anemije kod hemoparazitskih invazija.

Ključne riječi: *Haemoproteus columbae*, antioksidacijski enzimi, malondialdehid, elementi u tragovima, antioksidacijski vitamini