Evaluation of antioxidant status and oxidative stress in sheep experimentally infected with *Anaplasma ovis*

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Summary

Anaplasma ovis infections can cause severe anemia in the acute phase of the disease. In order to investigate the alterations of erythrocyte protective antioxidant mechanisms associated with anemia in sheep experimentally infected with *A. ovis*, 100 ml heparinized blood was collected from splenectomised sheep that showed 6% *A. ovis* parasitemia. Inoculums of 20 ml blood were administered intravenously to five male sheep without any blood parasite. Parasitological and haematological changes and the activities of erythrocyte superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were studied in experimentally infected animals on the 0-38 post infection days. Parasitemia increased significantly with the progress of infection and reached its maximum level on day 15 of the experiment. From this point to day 38, there was a gradual decline in parasitemia. A significant decrease in PCV, RBC and Hb concentration was evident coincidentally with peak parasitaemia in the infected sheep. On post infection day 15, the activities of all enzymes increased, the changes being significant for SOD activity. There was a significant positive correlation among parasitemia and the activities of erythrocyte SOD ($r = 0.644$, $P<0.0005$) and CAT ($r = 0.424$, $P<0.05$). Glutathione peroxidase activity declined significantly between post infection days 23-38. From the present study, it can be concluded that oxidative stress has an important role in anemia induced by anaplasmosis in sheep. It seems that SOD is a useful indicator of oxidative stress caused by *A. ovis* infection, due to its constant increasing means in the course of the disease.

Key words: *Anaplasma ovis*, Sheep, Oxidative stress, Anemia

Introduction

Ovine anaplasmosis is a tick-borne rickettsial disease widespread in tropical and subtropical areas (Torina *et al*., 2008; Jalali *et al*., 2013). It is usually a subclinical or mild condition; however, moderate to severe clinical disease is generally characterized by fever, variable degrees of anemia and icterus that may occasionally lead to death. Apart from the costs of additional veterinary care, this disease lessens the animal's body weight, causes abortions, and reduces milk production. Recovered animals become carriers (Stoltsz, 2004). Transmission is transplacental or by ticks, biting insects, contaminated instruments and inoculation of blood into susceptible animals (de Echaide *et al*., 1998). Splenectomy and intercurrent infections lower the resistance of the animals and render them more susceptible to the disease. A diagnosis of the individual animal is usually made on the basis of clinical signs, the presence of the organism in blood smears and haematological evidences of infection. Extravascular opsonization and phagocytosis of parasitised erythrocytes by reticuloendothelial cells leads to anemia. However, the severity of anemia may be a result of immune-mediated destruction of non parasitised as well as parasitised erythrocytes (Stoltsz, 2004). Erythrocytes are equipped with many defense systems representing their antioxidant capacity. This protective system includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione, glutathione-S-transferase, and glutathione reductase (Rauchova *et al*., 2005). Oxidative stress is generally described as an imbalance between oxidants and antioxidant levels. Some recent studies suggest that the oxidative damage of erythrocytes has a close relationship with anemia (Nazifi *et al*., 2008; De *et al*., 2012). Alteration of oxidative stress indices have been reported in hemoparasitic diseases such as theileriosis, and babesiosis (Nazifi *et al*., 2011; Esmaeilnejad *et al*., 2012).

Published reports describing erythrocytic oxidative damage and antioxidant defense in experimental ovine anaplasmosis are sparse to date. Thus, the present study was designed to determine the activities of erythrocyte GPx, SOD and CAT as hallmarks of oxidative damage to erythrocytes in sheep experimentally infected with *A. ovis*.

Materials and Methods

PCR analysis

DNA extraction was performed using a molecular biological system transfer kit (MBST, Iran), based on the manufacturer's instructions. A poly chain reaction (PCR) method was carried out to detect *Anaplasma* spp. (*A. ovis* and *A. marginale*) based on the major surface protein 4 gene (*msp4*) sequence, differentiated by PCR-RFLP using HpaII enzyme similar to Ahmadi-Hamedani *et al.* (2009).

Theileria and *Babesia* infections were ruled out by the PCR technique using specific primers for 18SrRNA gene (Shayan *et al.*, 2011).

Source of animals

An *Anaplasma ovis* infected sheep with no other blood parasites infection was diagnosed based on blood smear and PCR examination. This animal was selected as donor, on which splenectomy was performed to induce high levels of parasitemia.

Five local breed male sheep, about 5–6 months old, were selected from the Animal Research Institute, Faculty of Veterinary Medicine, Tehran University, Iran. All were tested negative for microscopic blood parasites infections, confirmed by the PCR method.

Selected animals were transferred to the Veterinary Research and Teaching Hospital, Faculty of Veterinary Medicine, Tehran University. All were treated against internal parasites, 14 days prior to the study.

Experimental infection

The donor was subjected to blood collection for inoculation three weeks after splenectomy while showing 6% *A. ovis* parasitemia. 100 ml blood was from donor, collected in a container with heparin anticoagulant, and an inoculum of 20 ml blood was administered intravenously to each test animal. This study was approved by the ethical committee of the Faculty of Veterinary Medicine, Tehran University.

Blood samples were collected from the jugular vein into EDTA-containing tubes from each test animal prior to inoculation, as control (day 0), and weekly thereafter (on days 8, 15, 23, 30 and 38 post inoculation) for haematological and parasitological analysis. Additionally, heparinized blood samples were obtained at the same days, for estimations of antioxidant enzyme activities. All the animals were treated against anaplasma infections at the end of the study.

Parasitological and haematological assessment

Blood smears were prepared and fixed with methanol for 5 min, stained with 5% Giemsa solution for 30 min and then examined for the presence of *Anaplasma* inclusion bodies under oil immersion lens (×100).

Parasitemia ratio was assessed by counting the number of infected red blood cells after examining at least 200 microscopic fields. The number of infected cells was then expressed as a percentage (Heidarpour

Bami *et al.*, 2010).

Haematological parameters including, red blood cell count (RBC), packed cell volume (PCV) and hemoglobin concentration (Hb), were estimated as described by Meyer and Harvey (2004).

Antioxidant enzymes activities

Glutathione peroxidase activity was evaluated by Paglia and Valentine's method (1967), using (RANSEL Kit, Randox, UK). Glutathione peroxidase catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured.

Superoxide dismutase activity was measured by a iodophenyl nitrophenol phenyltetrazolium chloride modified method (RANSOD Kit, Randox, UK). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl-tetrazolium chloride (INT) to form a red formazan dye. SOD activity was then measured by the degree of inhibition of this reaction. One SOD unit was considered as that which caused a 50% inhibition of the reduction rate of INT under the assay condition.

Catalase activity was measured in hemolysate according to Aebi (1984). Decomposition of H_2O_2 was followed directly by monitoring the decrease of absorbance at 240 nm. Enzyme activity was calculated as catalytic content of a sample and expressed as k/g Hb.

Statistical analysis

All values were expressed as means ± standard error of means (SEM). Analysis of variance (ANOVA) and Tukey tests were used to evaluate statistical differences between groups and $P<0.05$ was considered as statistically significant. The relationship between antioxidant enzymes and severity of parasitemia were assessed by computing Pearson's correlation coefficient.

Results

The values of parasitological examinations and haematological parameters during the days of the experiment are presented in Table 1.

Microscopic examination of blood smears obtained from test animals revealed that the percentage of *Anaplasma* inclusion bodies increased significantly with the progress of infection and reached a peak of 1.38 ± 0.31% on day 15 of the experiment. From this point to day 38, there was a gradual decline in the percentage of parasitemia to a minimum of 0.18 ± 0.03%.

The data showed that the values of PCV, RBC and Hb decreased soon after the appearance of parasitaemia, reaching their lowest levels coincidentally with the peak of parasitaemia (day 15). After that, there was a slight rise in these parameters until the last day of experiment.

Table 1: Mean values ± SEM for haematological indices and anaplasma % in sheep infected with *A. ovis*

Days post infection	0	8	15	23	30	38
Anaplasma (%)	0.00 ± 0.00	0.50 ± 0.08	$1.38 \pm 0.31^*$	$0.73 \pm 0.08^*$	0.38 ± 0.09	0.18 ± 0.03
Hb (g/L)	110.7 ± 7.9	87.7 ± 5.1	$58.30 \pm 9.6^*$	$65.9 \pm 2.8^*$	$75.4 \pm 2.8^*$	$80.6 \pm 2.9^*$
PCV (L/L)	0.32 ± 0.07	0.27 ± 0.07	$0.18 \pm 0.30^*$	$0.20 \pm 0.07^*$	$0.23 \pm 0.10^*$	$0.26 \pm 0.06^*$
RBC ($\times10^{12}$/L)	11.00 ± 0.71	8.48 ± 0.45	$5.26 \pm 0.31^*$	$5.34 \pm 1.05^*$	$7.06 \pm 0.31^*$	$8.12 \pm 0.36^*$

* Each column denotes significant differences relative to the first day (P<0.05)

Table 2: Mean values ± SEM for catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities in sheep infected with *A. ovis*

Days post infection	0	8	15	23	30	38
CAT (k/g Hb)	0.34 ± 0.06	0.22 ± 0.02	0.54 ± 0.10	0.53 ± 0.08	0.42 ± 0.03	0.29 ± 0.05
GPX (IU/mg Hb)	176.14 ± 6.42	176.38 ± 13.24	187.57 ± 22.57	$77.87 \pm 12.42^*$	$47.22 \pm 5.87^*$	$66.78 \pm 8.81^*$
SOD (IU/mg Hb)	892.01 ± 52.89	1311.82 ± 74.07	$2235.76 \pm 338.37^*$	$1747.23 \pm 44.32^*$	1554.12 ± 90.97	1472.09 ± 54.88

* Each column denotes significant differences relative to the first day (P<0.05)

The statistics of the CAT, SOD and GPx activities are presented in Table 2.

The activities of all enzymes increased on post infection day 15, with the progress of infection along with the peak of parasitemia; these changes were significant for SOD activity. There were rises of 2.51 and 1.96 times in SOD activity on post infection days 15 and 23, respectively, as compared to prior inoculation on day 0 (as control). Compared to the first day, a continuous increase was observed in SOD activity until the last day of the experiment, while CAT activity increased up to day 30 and then returned approximately to its normal activity on day 38 post infection.

Glutathione peroxidase activity declined significantly between days 23-38 post infection, to such a degree that on days 23 and 30 its activity reduced to approximately a half and a fourth of its initial value, respectively.

Parasitemia percentage was found to be positively correlated with erythrocyte SOD (r = 0.644, P<0.0005) and CAT (r = 0.424, P<0.05) activities. Furthermore, an insignificant positive correlation was observed between the GPx activity and parasitema percentage.

Discussion

Erythrocytes are permanently in contact with potentially harmful levels of oxygen, but their metabolic activity is qualified for reversing this injury under normal conditions. However, oxidative stress can occur as a consequence of an increased reactive oxygen species (ROS) generation and/or an antioxidant system depression. Erythrocytes are supplied by numerous defense systems representing their antioxidant capacity (Rauchova *et al.*, 2005). This protective system includes SOD, CAT, GPx, reduced glutathione, glutathione-S-transferase, and glutathione reductase. The major reactive oxygen species (ROS) produced in aerobic organisms is the superoxide radical which is a highly reactive cytotoxic agent. Superoxide dismutase is a primary line of defense agents against oxygen-derived free radicals, which catalyses the dismutation of superoxide radical to hydrogen peroxide. Catalase and GPx share in H_2O_2 detoxification and convert it to molecular oxygen and water. Glutathione peroxidase can reduce lipid peroxidase and other organic hydro-peroxides that are highly cytotoxic products (Sindhu *et al.*, 2004).

In this experiment, on post infection day 15, coincidentally with the parasitemia peak and the lowest levels of PCV, RBC and Hb, the activity of all enzymes increased and these changes were significant for SOD. Djordjevic *et al.* (2010) reported that in the combined (acute and chronic) stress, SOD and CAT activities increased significantly but GPx remained unchanged. Increased SOD activity in both acute and chronic phases of the disease may be a result of its important enzymatic defense function against superoxide radical generation in infected erythrocytes. Catalase and GPx reacted with red cell hydrogen peroxide which accumulated after SOD activity. The affinity of GPx for H_2O_2 was stronger than that of CAT, which make it more efficient at low levels of H_2O_2 (Kalpakcioglu and Senel, 2008). Nagababu *et al.* (2003) delineated that GPx was responsible for the initial phase of the reaction against hydrogen peroxide. As mentioned previously, GPx activity increased in the early stage of infection, which might be a consequence of its preliminary role in removing hydrogen peroxide from infected erythrocytes. In our experiment, GPx activity dropped significantly between 23-38 days post infection, which may be a result of GSH depletion in this phase. In normal conditions, CAT was of no great importance in most cell types, but in the presence of oxidative stress, it was the most adaptive antioxidant enzyme in animals. Mueller *et al.* (1997) demonstrated that CAT was the predominant H_2O_2-removing enzyme in human erythrocytes and that H_2O_2 decomposition depended linearly on its concentration. Glutathione peroxidase activity declined with the progress of infection, therefore the activity of CAT increased to compensate for this deficiency and protect erythrocytes from dangerous accumulations of H_2O_2 generated by increased SOD. Extracts of erythrocytes infected with *A. marginale* were found to contain more CAT activity than normal erythrocytic preparations. The increase in CAT activity appeared concurrently with increases in the number of erythrocytes containing *Anaplasma* bodies (Wallace and Dimopoullos, 1965). Asri-Rezaei and Dalir-Naghadeh (2006) reported that the activity of erythrocyte CAT significantly increased in naturally infected cattle with *Theileria annulata*. In many studies, the levels of these

enzymes have shown varying concentration levels and reports from various laboratories have been controversial (Sindhu *et al*., 2004). While investigators such as Nazifi *et al*. (2011), De *et al*. (2012) and Esmaeilnejad *et al*. (2012) reported lower levels of these enzymes in naturally haemoparasited diseases, Grewal *et al*. (2005) found higher GPx and unchanged SOD and CAT levels. On the other hand, More *et al*. (1989) demonstrated that SOD and GPx activities increased in the patent period relative to the pre patent stage of bovine anaplasmosis, but this change was not significant for GPx activity.

In brief, the results of experimental anaplasmosis in sheep demonstrated that the peak of parasitemia and the lowest levels of RBC indices on day 15 post infection coincided with highest levels of SOD and CAT enzymes activities, when they increased 2.51 and 1.59 times more than their prior inoculation values, respectively. It can be concluded that oxidative stress has an important role in anemia induced by anaplasmosis in sheep. SOD, among all assessed enzymes, seems to be a useful indicator of oxidative stress caused by *A. ovis* infection, due to its constant increase in the course of the disease.

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