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Evaluation of antioxidant status, oxidative stress and serum trace mineral levels associated with Babesia ovis parasitemia in sheep

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ABSTRACT

Ovine babesiosis is a fatal disease characterized by severe progressive hemolytic anemia. In order to clarify the causal mechanisms implicated in anemia, this study was aimed to assess the antioxidant status and erythrocyte oxidative stress in sheep suffering from ovine babesiosis. *Babesia* infection was confirmed both with Giemsa's staining blood smears and semi-nested PCR amplified region of 18S rRNA gene. Thirty-eight Iranian sheep, naturally infected with *Babesia* spp., were considered as the infected group and divided into four subgroups according to parasitemia rates (<1%, 1–2%, 2–3% and >3%), and the same number non-infected animals were selected as the control group. Blood samples were taken and hematological parameters, activities of antioxidant enzymes including erythrocyte glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), glucose-6-phosphate dehydrogenase (G6PD), total antioxidant capacity (TAC), median corpuscular fragility (MCF), and serum concentrations of some trace minerals (copper, iron, zinc, manganese, and selenium) were measured. In addition, as an index of lipid peroxidation, the level of malondialdehyde (MDA) was measured. The results revealed a significant decrease ($P < 0.01$) in RBC count, packed cell volume (PCV) and Hb concentration as well as the activities of erythrocyte GSH-Px, SOD, CAT, G6PD, TAC, MCF and serum concentrations of Cu, Zn, Mn and Se in the infected sheep. In contrast, significantly increased ($P < 0.01$) levels of MDA and erythrocyte osmotic fragility as well as serum concentration of iron were recorded in the infected animals. Overall, the observed remarkable decrease in the antioxidant enzyme activities, median corpuscular fragility and substantial elevated levels of lipid peroxidation associated with the notable increase in parasitemia indicate high exposure of RBCs to oxidative damage in *Babesia* infected sheep. These results indicate that the disturbed antioxidant defense mechanisms in ovine babesiosis can promote the development of anemia.

1. Introduction

Hemoparasites of the genus *Babesia* are protozoans which predominantly infect ruminants in tropical and subtropical regions (Hashemi-Fesharki, 1997) and impose

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heavy losses due to high rates of mortality and decreased productivity in affected animals. *Babesia ovis* is the most common species that causes sheep babesiosis in Iran (Rahbari et al., 2008).

According to previous studies, the invasion of piroplasms of *Babesia* species on erythrocytes could form a severe progressive anemia (Habibi et al., 2004; Sevinc et al., 2013). Although various mechanisms have been suggested for anemia in bovine babesiosis (Saleh, 2009), this process is not well-understood; however, as yet, little information has been rendered to indicate such mechanisms in ovine babesiosis. In addition to the involvement of mechanical damage in *Babesia argentina*-infected cattle (Callow and Pepper, 1974) or autoimmune phenomena in intravascular hemolysis in bovine babesiosis (Argon, 1976), some recent studies suggested that the oxidative damage of erythrocytes has a close relationship with anemia (Deger et al., 2009; Saleh, 2009; Esmaeilnejad et al., 2012). It was hypothesized that the alterations in the activity of antioxidant enzymes, particularly glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) (Chaudhuri et al., 2008; Esmaeilnejad et al., 2012), may play a role in the pathogenesis of anemia. On the other hand, some reports concluded that erythrocyte destruction resulting from oxidative injuries was related to lipid peroxidation of RBCs (Ambawat et al., 1999; Crnogaj et al., 2010). These processes might be a cause of the increase in morphological changes and osmotic fragility of RBCs (Crnogaj et al., 2010), making erythrocytes susceptible to phagocytosis.

Micronutrients, such as minerals, are essential components of the antioxidant defense against free radical-induced damage to tissues for the maintenance of health. Zinc, copper and selenium are utilized for synthesis of antioxidant enzymes (Evans and Halliwell, 2001). Due to lack of information on the status of alterations in these agents, and so the probable coordination to counteract the oxidative stress to erythrocytes in *B. ovis* infection, this study was conducted to measure the activities of the key antioxidant enzymes (GSH-Px, SOD, G6PD and catalase), total antioxidant capacity, the level of median corpuscular fragility and lipid peroxidation of erythrocytes, as well as serum trace mineral concentrations, to evaluate the probable interrelationship of these factors with anemia in different parasitemia rates during natural ovine babesiosis.

2. Materials and methods

2.1. Source of animals and samples

The study was done in the North-West of Iran (East-Azerbaijan province), where babesiosis due to *B. ovis* is prevalent during the summer season (June–September 2011). An infected group composed of 38 Iranian sheep naturally infected with *Babesia* spp. was selected and divided into four subgroups according to parasitemia rates (<1%, 1–2%, 2–3% and >3%). Similar number ($n = 38$) non-infected sheep selected from the same farms served as control group. The selection of animals was implemented according to a criterion based on a thorough clinical examination performed on all animals. As such, those sheep with acute or subacute clinical signs of *Babesia* infections including; a history of tick infestation, anorexia, prolonged listlessness, increased rectal temperature, dyspnea, tachycardia, pale mucous membrane, stages of icterus were selected. Further analysis was carried out to confirm the infection through Giemsa staining of the collected blood samples as well as semi-nested PCR amplified region of 18S rRNA gene. Moreover, the presence of anemia was confirmed through measurement of different hematological parameters, i.e., RBC count, the values of packed cell volume (PCV), and hemoglobin concentration. However, other potential causes of anemia were investigated by the evaluation of hematological measurements and molecular, biochemical and microbiological tests. On the other side, those clinically healthy animals without parasitemia (according to the blood smears) with normal hematological profile were assigned as controls.

2.2. Sampling and parasitological examination

From the infected and control sheep, blood samples were taken from the jugular vein into vacutainers (Kendall Company, Covidien, USA) containing EDTA-K_2 as anticoagulant for determination of hematological parameters and without EDTA-K_2 for isolation of serum samples and further biochemical analysis.

Thin blood smears were prepared of the ear vein from both the healthy and infected sheep and then stained with Giemsa for microscopic observation of *B. ovis* in erythrocytes. Parasitemia was assessed by counting the number of infected red blood cells through examination of 20 microscopic fields (approximately 15×10^3 cells). The number infected was then expressed as a percentage (Sevinc et al., 2013). The smear was recorded as negative if no parasites were observed in 100 oil-immersion fields. Apart from morphological characteristics of piroplasms differentiation between *B. ovis*, *Babesia motasi*, *Babesia crassa* and *Theileria lestoquardi* was done by using semi-nested PCR assay.

2.3. PCR reaction

In order to detect the presence of *B. ovis* in blood of the infected animals and to rule out the presence of *Babesia* spp. infection in the healthy animals semi-nested PCR was performed as described previously by Esmaeilnejad et al. (2012). Then, differential detection of *B. ovis* from *T. lestoquardi* was performed according to the procedure described by Shayan and Rahbari (2005).

2.4. Hematological examination

RBC count, Hb concentration and packed cell volume (PCV) were determined by automated hematology analyzer (Autolyser AL 820, Switzerland) (Schalm et al., 1986). For determination of MDA and antioxidant enzymes, blood samples were centrifuged at $700 \times g$ for 15 min; plasma separated and packed cells washed three times with normal saline solution. The washed erythrocytes were then hemolysed with nine volumes of ice-cold distilled water to prepare 10% RBC hemolysate.

2.5. Osmotic fragility test (OFT)

Osmotic fragility test (OFT) was determined by the method of Chanarin (1989). Briefly, washed erythrocytes incubated with saline buffer in a series of hypotonicity ranging in concentration from 1.0 g/L (0.1%) to 9.0 g/L (0.9%) sodium chloride. The percentage of hemolysis at each concentration of NaCl was calculated and a graph of hemolysis percent against concentration of NaCl was plotted. The results were expressed as the concentration of NaCl causing 50% haemolysis, i.e. the median corpuscular fragility (MCF).

2.6. Biochemical analysis

Lipid peroxidation in the RBC hemolysate was determined as thiobarbituric acid reactive substance (TBARS) according to Placer et al. (1966). The method is dependent on forming a color complex between the products of lipid peroxidation and thiobarbituric acid (TBA). Briefly, 0.2 ml of RBC hemolysate was added to 1.3 ml of 0.2 M Tris–KCl buffer (pH 7.4) and incubated at 37 °C for 30 min after which 1.5 ml of TBA was added. The mixture was immediately heated (60 min at 95 °C) and cooled with running water. Then, 3 ml of pyridine/n-butanol (3:1, v/v) and 1 ml of 1 N NaOH was added and mixed by shaking. The absorbance was read at 532 nm versus distilled water as a blank. In this assay, 1,1,3,3-tetramethoxypropane was used as a standard. Lipid peroxidation in the RBC hemolysate was expressed as MDA nanomoles per grams of hemoglobin (nmol/g Hb).

The activity of catalase was measured by colorimetric method, described by Slaughter and O'Brien (2000), which involves two steps. Since the rate of dismutation of hydrogen peroxide to water and oxygen is proportional to the concentration of catalase, samples were first incubated with a known amount of hydrogen peroxide (2.950 ml). The remaining hydrogen peroxide, following a fixed incubation period (22–25 °C), was then determined by the oxidative coupling reaction of 4-aminophenazone (4-aminoantipyrene, AAP) and 3,5-dicholo-2-hydroxybenzenesulfonic acid (DHBS) in the presence of H_2O_2 and catalyzed by horseradish peroxide. The resulting quinoeimine dye was measured at 520 nm (Catalase Assay Kit, Oxford Biochemical Research, Inc., USA). Activities of the enzymes were expressed as Katal/gHb.

The activity of GSH-Px, in the RBC hemolysate was measured using the method of Beutler et al. (1963); this method is based on the development of a stable yellow color when 2-nitrobenzoic acid is added to sulfhydryl compounds. The amount of reduced product, thionitrobenzene, was measured by commercially available kits (Ransel Kit, Randox Laboratories Ltd. G.B.) at 412 nm. Activity of the enzyme was expressed as IU/mg Hb.

To measure the activity of SOD, superoxide radicals generated by the xanthine oxidase reaction convert 1-(4-iodophenyl)-3-(-4-nitrophenol)-5-phenyltertrazolium chloride quantitatively to a formazan dye (Ransel Kit, Randox Laboratories Ltd. G.B.). Conversion of superoxide radicals to hydrogen peroxide by superoxide dismutase inhibits dye formation and serves as a measure of superoxide dismutase activity. Activities of the enzymes were expressed as IU/mg Hb.

The activity of glucose-6-phosphate dehydrogenase was determined according to reaction described by Beutler (1984). In this reaction, glucose-6-P is oxidized to gluconate-6-P and $NADP^+$ is reduced to $NADPH + H^+$. The NADPH production in this reaction was determined by spectrophotometrically at 340 nm at 37 °C and was expressed as international units per gram of hemoglobin (IU/g Hb). Hemoglobin determination was carried out by cyanomethemoglobin method (Chanarin, 1989).

The activity of total antioxidant capacity (TAC) was determined in the RBC hemolysate according to the methods described by Koracevic et al. (2001). In this reaction, $ABTS^{\bullet+}$ (2,2′-Azino-di-[3-ethylbenzthiazoline sulphonate]) radicals are produced by incubation of ABTS® with a peroxidase (metmyoglobin) and H_2O_2. The resulting stable blue-green color was measured at 600 nm (Ransel Kit, Randox Laboratories Ltd. G.B.). Activity of the enzyme was expressed as mmol/l.

2.7. Measurement of serum trace mineral levels

For determination of the serum trace mineral levels, serum samples were digested by a mixture of perchloric and nitric acid (in a ratio of 3:7, respectively). Manganese, copper, iron, selenium and zinc were measured using an atomic absorption spectrophotometer (Shimadzu Asc-6100, Japan). Argon was used as the purging gas, and the background absorption was automatically corrected by the Zeeman effect. Standard solution (1000 μg/ml) of zinc, copper, cobalt, manganese, and selenium were used in measurements (Bouman et al., 1986; Nazifi et al., 2011). The values of serum trace mineral levels were expressed in μmol/L of serum.

2.8. Statistical analysis

The packaged SPSS program for windows Version 17 (SPSS, Chicago, IL, USA) was used for statistical analysis. Data were expressed as standard error of mean (means ± SEM). Differences between groups were determined by one way analysis of variance (ANOVA) followed by pair-wise comparisons using the Duncan test. Pearson's correlation (r) and linear regression analysis (R^2) were performed on the paired data obtained by individual infected cases. All values were expressed as mean and standard error of mean (SEM), and $P < 0.01$was considered as statistically significant.

3. Results

Precise investigations on blood smears of all infected animals with different parasitemia rates (0.01–3%) revealed a wide range of abnormal erythrocytes including reticulocytosis, anisocytosis, and basophilic stippling cells (Table 1).

All of the infected animals were identified as positive samples by PCR using *B. ovis*-specific primers. In contrast, no PCR products were seen with *B. motasi*, *B. crassa* and *T. lestoquardi* DNAs.

Table 1
RBCs, hemoglobin (Hb) concentration and packed cell volume (PCV) in uninfected sheep and those infected with *B. ovis* with different parasitemia rates.

	Parasitemia (%)	RBC ($\times 10^{12}$/L)[1]	PCV (L/L)[2]	Hb (g/L)[3]
Control	0 ($n=38$)	8.64 ± 0.035^{a}	0.31 ± 0.0017^{a}	101.1 ± 0.184^{a}
Infected	<1 ($n=11$)	7.01 ± 0.019^{b}	$0.26 \pm 0.0.002^{b}$	97.1 ± 0.206^{b}
	1–2 ($n=9$)	$5.14 \pm 0.0.086^{c}$	$0.22 \pm 0.0.0018^{c}$	91.7 ± 0.168^{c}
	2–3 ($n=10$)	3.50 ± 0.027^{d}	0.17 ± 0.0019^{d}	82.3 ± 0.161^{d}
	>3 ($n=8$)	2.01 ± 0.043^{e}	0.11 ± 0.0073^{e}	70.6 ± 0.247^{e}

Different superscript in each column denote significant differences ($P<0.01$).
[1] Reference value: 9–15 $\times 10^{12}$/L.
[2] Reference value: 0.27–0.45 L/L.
[3] Reference value: 90–150 g/L (Blood, 1997).

The mean values of the hematological parameters decreased significantly ($P<0.01$) in the infected sheep compared to the control group. According to evaluated hematological parameters, our data showed remarkable declines in RBCs, Hb concentration, and PCV in the infected sheep rather than in the controls ($P<0.01$), which strictly confirmed anemia in the infected group. In addition, parasitemia rate was negatively correlated ($r=-0.98$) with the value of PCV (Table 3).

MDA and MCF levels in both control and affected sheep are presented in Table 2. The level of MDA showed a significant elevation in the infected group. In addition, with an increase in parasitemia rates, the level of MDA production was significantly increased ($r=0.99$, $P<0.01$). Furthermore, corpuscular MDA concentration was negatively correlated with PCV ($r=-0.98$) (Table 3). Thus, taking into account that the determination of MDA allows the detection of the degree of lipid peroxidation and the level of free oxygen radicals indirectly (Esterbauer, 1996; Yagi, 1998; Owen, 1996), this study showed that erythrocytic lipid peroxidation in affected sheep increased significantly ($P<0.01$). In a similar way, median corpuscular fragility of RBCs was significantly higher in infected sheep compared to those of the control group ($P<0.01$). The measured MCF showed a significant positive correlation with parasitemia rate ($r=0.89$) and a negative correlation with activities of SOD ($r=-0.89$), GSH-Px ($r=-0.87$), catalase ($r=-0.88$), G6PD ($r=-0.87$) and TAC ($r=-0.87$) (Table 3).

According to our data, the activities of antioxidant enzymes including SOD, GSH-Px, G6PD and catalase as well as levels of TAC in infected sheep were substantially reduced compared to those of healthy ones ($P<0.01$) (Table 2). As in animals with higher parasitemia rates, a significant decrease was observed in the activities of SOD ($r=-0.89$, $P<0.01$), GSH-Px ($r=-0.88$, $P<0.01$), catalase ($r=-0.78$, $P<0.01$), G6PD ($r=-0.98$, $P<0.01$) and TAC ($r=-0.78$, $P<0.01$) (Table 3).

Accordingly, the Table 3 shows the strong negative correlations of antioxidant enzyme activities (SOD, GSH-Px, catalase, G6PD and TAC) with different parasitemia levels. By contrast, the significant rise in the lipid peroxidation index (MDA) in higher parasitemias as well as the strong positive correlation of the level of erythrocytic osmotic fragility with the percentage of parasitemia in infected sheep was clearly evident.

The alteration of serum trace mineral levels in the control sheep and those infected with *B. ovis* are presented in Table 4. Serum concentrations of copper, zinc, manganese, and selenium showed a marked decrease in infected sheep ($P<0.01$). In contrast, serum concentration of iron was significantly higher in infected sheep compared to those of the control group ($P<0.01$). In order to show relevant significant correlation, the level of some determinative antioxidant trace elements in relation to the increase in the level of parasitemia in sheep suffering from babesiosis are depicted in Fig. 1. As Fig. 1 shows, parasitemia rate was negatively correlated with serum level of copper, zinc, manganese, and selenium, whereas it was positively correlated with serum concentration of iron.

4. Discussion

The comparison between control and *B. ovis*-infected sheep showed that the number of RBCs, PCV, and hemoglobin content in ovine babesiosis were significantly reduced ($P<0.01$) confirming anemia in the infected animals. Most obviously, the severity of the anemia increased in animals with higher parasitemia rates. Although similar findings in the cases of ovine babesiosis corroborate our data (Esmaeilnejad et al., 2012; Sevinc et al., 2013), the underlying mechanisms of such a progressive anemia regarding alterations of cooperative antioxidant trace elements have not been described. However, low levels of RBCs, PCV, and hemoglobin concentration in ovine babesiosis due to *B. ovis* have been attributed to erythrophagocytosis by activated macrophage (Shoda et al., 2000; Saleh, 2009).

In this study, higher levels of lipid peroxidation (LPO) as well as a significant rise in osmotic fragility of RBCs occurred in infected animals and similarly among different parasitemia rates. The rise in LPO of RBCs pointed to an oxidative shock during parasitemia, whereby the reactive metabolites of oxygen exceed their removal by antioxidant mechanisms. Membrane lipids are major targets for cellular damage induced by reactive oxygen species (ROS) (Esterbauer, 1996; Owen, 1996). Interaction of ROS with rich polyunsaturated fatty acids (Halliwall and Chirico, 1993) at the cell membrane level results in the formation of several lipid peroxidation products like MDA. Higher MDA levels of RBCs indicate that antioxidant defense mechanisms were not sufficient to neutralize oxidative stress (Grewal et al., 2005). Therefore, erythrocytic damage resulting from oxidative stress could relate to lipid peroxidation (Friedman, 1979). Consequently, this process

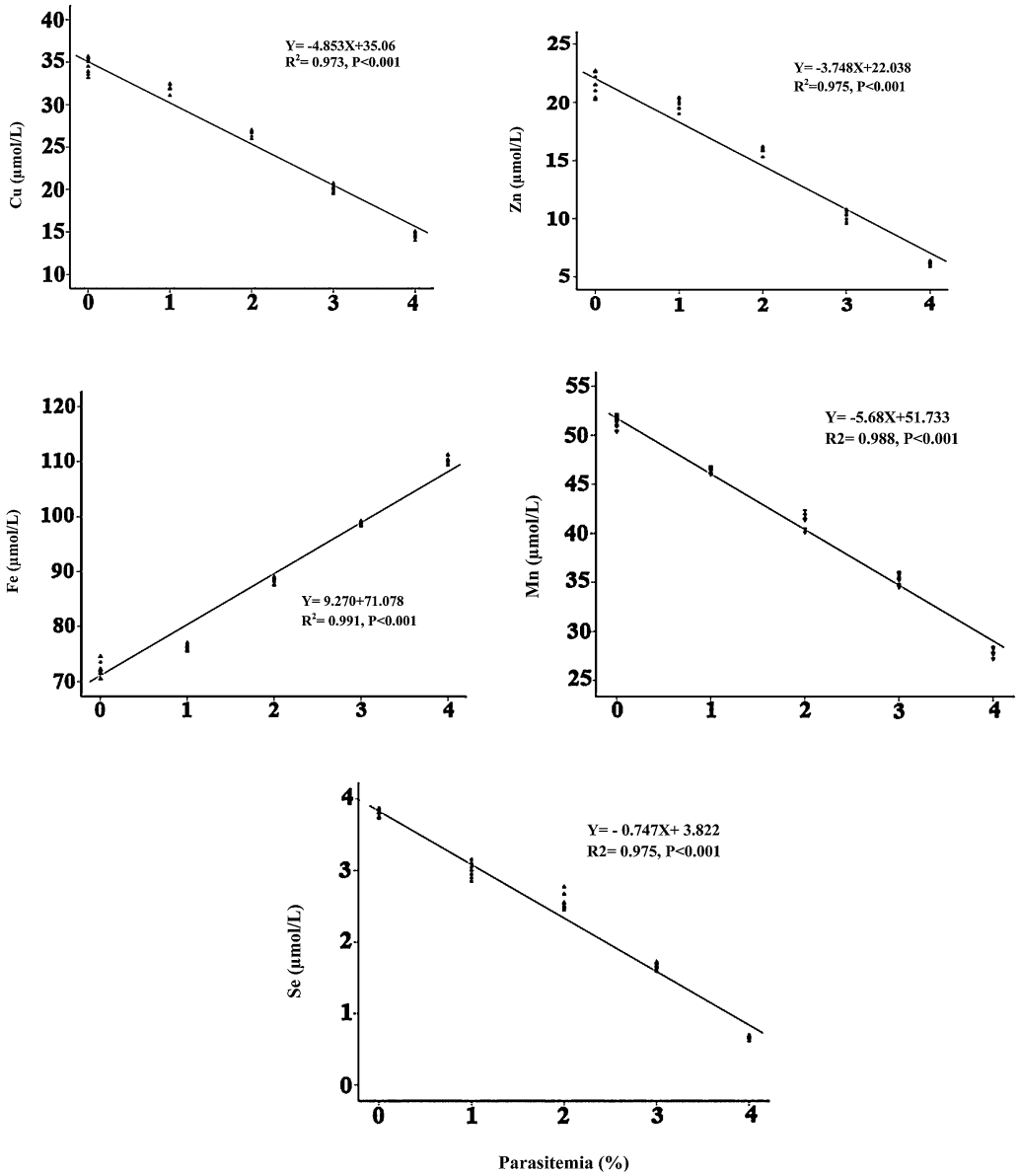

Fig. 1. Linear regression (R^2) analysis of different levels of parasitemia in *B. ovis*-infected sheep with serum trace minerals copper (Cu), zinc (Zn), iron (Fe), manganese (Mn) and selenium (Se).

can promote reduced membrane symmetry and increased membrane permeability (leading to elevated levels of osmotic fragility), causing morphological changes in the RBC cell surface (Saluja et al., 1999). Hence, morphologically altered erythrocytes would be susceptible to removed from the body through erythrophagocytosis, which commonly occurs in severe anemia (Winterbourn, 1990). Moreover, it can be speculated that significant negative correlations between the osmotic fragility of RBCs with the activity of SOD, GPX, and catalase, as well as the significant positive correlation of fragility with lipid peroxidation of RBCs, can prove that membrane lipid peroxidation of RBCs plays a considerable role in the loss of membrane stability, permeability and structural integrity, which indicates disturbed antioxidant activities during parasitemia.

In the present study, concurrent with increasing the severity of parasitemia, a significant decline was observed in the level of the activity of erythrocytic antioxidant enzymes including SOD, GSH-Px and catalase. In other words, the negative significant correlation between parasitemia rate and the activities of GSH-Px, SOD and catalase indicates increase exposure of RBCs to oxidative stress products at high parasitemias. GSH-Px activity is a major mechanism for intracellular decomposition of lipid peroxidase (Flohe, 1971). Hafeman and colleagues (1974) also proposed that GSH-Px plays a crucial role in preventing

Table 2
Concentration of malondialdehyde (MDA), activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase, total antioxidant capacity (TAC), glucose-6-phosphate dehydrogenase (G6PD) and median corpuscular fragility (MCF) in uninfected sheep and those infected with *B. ovis* with different parasitemia rates.

	Parasitemia (%)	MDA (nmol/g Hb)	GSH-Px (IU/mg Hb)	SOD (IU/MG Hb)	Catalase (Katal/g Hb)	TAC (mmol/L plasma)	MCF (g/dL)	G6PD (IU/g Hb)
Control	0 ($n=38$)	28.47 ± 0.322^{e}	64.13 ± 0.10^{a}	14.43 ± 0.059^{a}	98.26 ± 0.175^{a}	0.91 ± 0.002^{a}	0.41 ± 0.0010^{e}	25.19 ± 0.101^{a}
Infected	<1 ($n=11$)	41.43 ± 0.46^{d}	60.33 ± 0.21^{b}	11.17 ± 0.093^{b}	93.26 ± 0.176^{b}	0.85 ± 0.001^{b}	0.44 ± 0.0017^{d}	23.01 ± 0.053^{b}
	1–2 ($n=9$)	55.31 ± 0.13^{c}	55.63 ± 0.22^{d}	8.15 ± 0.125^{d}	86.34 ± 0.267^{d}	0.78 ± 0.002^{d}	0.49 ± 0.0019^{c}	21.15 ± 0.074^{d}
	2–3 ($n=10$)	68.42 ± 0.21^{b}	44.17 ± 0.08^{e}	5.16 ± 0.070^{e}	79.86 ± 0.163^{c}	0.65 ± 0.002^{c}	0.53 ± 0.0014^{b}	16.14 ± 0.151^{c}
	>3 ($n=8$)	83.3 ± 0.334^{a}	39.19 ± 0.17^{e}	2.43 ± 0.138^{e}	60.65 ± 0.235^{e}	0.51 ± 0.006^{e}	0.56 ± 0.0011^{a}	13.28 ± 0.124^{e}

Different superscript in each column denotes significant differences ($P<0.01$).

membranes from peroxide damage induced by lipid peroxides. In addition, it appears that SOD plays a crucial role to protect erythrocytes against oxidative damage (Chaudhuri et al., 2008). The results indicate that enhanced oxidation of the erythrocytes could significantly increase the generation of free radicals as well as exacerbate by the inefficient antioxidant capacity, a condition in which RBCs encounter the invasion of oxidant agents, which results in RBC injury and, virtually, hemolysis and occurrence of anemia.

Despite several studies in agreement with our results of a negative correlation of antioxidant enzyme activities of RBCs with the level of parasitemia and also a significantly higher activity of those enzymes in uninfected animals (Bicek et al., 2005; Deger et al., 2009), Chaudhuri et al. (2008) reported that SOD and catalase activities exhibit a significant rise in dogs naturally infected with *Babesia gibsoni*. They concluded that the increased level of SOD during parasitemia could be due to the high percentage of reticulocytes in the infected dogs, since the activity of enzyme is higher in reticulocytes than in mature erythrocytes (Yamasaki et al., 2000). In contrast, in this study, the significantly decreased level of catalase activity in the infected group compared to controls, its substantial positive correlation with the activity of enzymes GSH-Px and SOD, and also, the decrease in the activity of these enzymes with an increase in the level of parasitemia indicated that all of these antioxidant agents could act as determinative factors; however, the cited enzymes are consumed to scavenge H_2O_2 to protect erythrocytes against oxidative injuries and thus declined in the infected animals. Moreover, these results were not in agreement with the findings of Chaudhuri et al. (2008), as they obtained no significant difference in catalase activity in both healthy and *B. gibsoni*-infected dogs and believed that this enzyme might have a role in concert with SOD activity to scavenge peroxides.

There appears to be no report on the alteration of G6PD activity in *B. ovis* infection to compare with present study. However, low activity of G6PD has been reported in *Theileria annulata*-infected cattle (Asri-Rezaei and Dalir-Naghadeh, 2006). Our results suggest that, G6PD serves as an antioxidant enzyme and decreased activity of G6PD has been associated with increased hemolysis. However, in contrast with our results, Grewal et al. (2005) reported a significant rise in the activity of G6PD in infected cattle. The different results of two studies may be related to that G6PD similar to GSH-Px and SOD is the major mechanism for intracellular decomposition of lipid peroxides (Agar and Board, 1983). Reduction in the level of TAC in the infected sheep may probably be ascribed to the consumption of antioxidant enzymes as free radical scavengers during the oxidative process in natural *B. ovis* infection in sheep.

According to the results of this study, low levels of serum trace minerals including copper, zinc, manganese and selenium along with the raised levels of iron in the infected sheep implicate another alternative aspect of oxidative shock. Micronutrients, such as Cu, Zn and Se are essential components of the body's antioxidant defense that play an important role in prevention of free radical-induced damage (Evans and Halliwell, 2001). The Cu–Zn-SOD and Mn-SOD, which are located in the cell cytosol (McCord and Fridovich, 1969) and the mitochondria (Weisiger and

Table 3
Correlation between oxidative stress markers, activities of antioxidant enzymes and parasitemia in sheep infected with *B. ovis*.

Parameter	PCV	Parasitemia	MDA	GSH-Px	Catalase	SOD	G6PD	MCF	TAC
PCV									
PC[a]	–	−0.987**	−0.986**	0.774**	0.779**	0.880**	0.773**	−0.875**	0.776**
Parasitemia									
PC		–	0.997**	−0.883**	−0.787**	−0.897**	−0.982**	0.892**	−0.783**
MDA									
PC			–	−0.980**	−0.887**	−0.893**	−0.780**	0.786**	−0.883**
GSH-Px									
PC				–	0.878**	0.775**	0.990**	−0.877**	0.787**
Catalase									
PC					–	0.982**	0.878**	−0.880**	0.792**
SOD									
PC						–	0.977**	−0.891**	0.876**
G6PD									
PC							–	−0.878**	0.888**
MCF									
PC								–	−0.876**
TAC									
PC									–

** Correlation is significant at the 0.01 level (2-tailed).
[a] Pearson correlation.

Table 4
Concentration of serum trace elements in uninfected sheep and those infected with B. ovis with different parasitemia rates.

	Parasitemia (%)	Cu (μmol/L)	Zn (μmol/L)	Fe (μmol/L)	Mn (μmol/L)	Se (μmol/L)
Control	0 ($n=38$)	34.54 ± 0.138^{a}	21.54 ± 0.169^{a}	72.13 ± 0.197^{e}	51.49 ± 0.082^{a}	3.81 ± 0.005^{a}
Infected	<1 ($n=11$)	31.98 ± 0.113^{b}	19.98 ± 0.142^{b}	76.23 ± 0.148^{d}	46.44 ± 0.074^{b}	3.00 ± 0.026^{b}
	1–2 ($n=9$)	26.68 ± 0.110^{c}	15.92 ± 0.091^{d}	88.31 ± 0.199^{c}	41.36 ± 0.232^{d}	2.55 ± 0.034^{d}
	2–3 ($n=10$)	20.13 ± 0.127^{d}	10.29 ± 0.128^{c}	98.81 ± 0.104^{b}	35.31 ± 0.169^{c}	1.67 ± 0.013^{c}
	>3 ($n=8$)	14.63 ± 0.128^{e}	6.16 ± 0.059^{e}	110.37 ± 0.227^{a}	27.74 ± 0.149^{e}	0.66 ± 0.0106^{e}

Different superscript in each column denotes significant differences ($P<0.01$).

Fridovich, 1973), respectively, contribute to the first line of antioxidant pathway by catalyzing the conversion of O_2^- into H_2O_2. Se-containing GPX catalyzes the conversion of H_2O_2 to H_2O through the oxidation of R-GSH (Chance et al., 1979). Thus, it is speculated that the decreased level of trace minerals in the serum of infected sheep proved that pathogenic *Babesia* species can impose a marked effect on the status of mineral nutrients and also represents their coordinated antioxidant role accompanied by antioxidant enzyme activities during the infection with these parasites in sheep.

In the present study, elevated serum iron in the infected sheep can probably be ascribed to hemolytic anemia. In intravascular hemolysis, parasitized-RBCs are directly ruptured inside vasculature and so hemoglobin is degraded to globin, heme and iron; therefore, serum iron is increased (Harvey, 1989; Stockham and Scott, 2002).

In conclusion, hemolytic anemia was the main feature of the infection with the pathogenic *B. ovis* in sheep.

The substantially decreased levels of antioxidant enzyme activities and some cooperative antioxidant trace elements, accompanied by a significant rise in lipid peroxidation of erythrocytes indicate that the antioxidant mechanisms which protect RBCs against oxidative damages can be disturbed by infection with these parasites, whereby this interfering might be the cause of the increased erythrocyte fragility due to oxidative membrane damage.

Therefore, it can be concluded that oxidative shocks to RBCs may play an imperative role in the pathogenesis of anemia in ovine babesiosis caused by *B. ovis*.

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