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Evaluation of oxidative stress and antioxidant status, serum trace mineral levels and cholinesterases activity in cattle infected with *Anaplasma marginale*

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ABSTRACT

This study was undertaken to assess the influence of an *Anaplasma marginale* infection on oxidative stress and antioxidant status, trace elements and cholinesterase as markers of the inflammatory process and biomarkers of oxidative imbalance. An infected group comprised of 35 crossbred Holstein cattle, about 2–3 years old, naturally infected with *Anaplasma marginale*, were divided into 4 subgroups according to their parasitemia rates (< 1%, 1–10%, 10–20%, > 20%) and also 10 healthy cattle as control were selected. 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 corpuscularfragility (MCF) as well as acetylcholinesterase (AChE), and serum concentrations of antioxidant trace minerals (copper, iron, zinc, manganese, and selenium) and butyrylcholinesterase (BChE) were determined. In addition, as an index of lipid peroxidation, the level of malondialdehyde (MDA) was measured. The results revealed a significant decrease ($P < 0.05$) 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 AChE and serum concentrations of Cu, Zn, Mn, Se and BchE in the infected cattle. In contrast, significantly increased ($P < 0.05$) levels of MDA and erythrocyte osmotic fragility as well as serum concentration of iron were recorded in the infected animals. The significant decrease in antioxidant enzyme activities and substantial elevated levels of lipid peroxidation and erythrocyte osmotic fragility associated with the notable increase in parasitemia indicate increased exposure of RBCs to oxidative damage. Furthermore, decrease of cholinesterase in infection by *A. marginale* can and directly or indirectly lead to increase acetylcholine levels potent anti-inflammatory molecules, thereby inhibiting inflammation.

1. Introduction

Anaplasma marginale, the most widespread obligatory intracellular arthropod-borne pathogen of cattle worldwide is endemic regions of North, Central, and South America, as well as Africa, Asia, and Australia [41]. Ixodid ticks are the biological vector of *A. marginale*, while mechanical transmission can occur through fly bites and blood transfusion [40]. Fever, high levels of bacteremia ($\geq 10^7$ bacterial/ml of blood) and marked anemia are characteristics of bovine anaplasmosis [28,48].

Inadequate availability of antioxidant enzymes or uncontrolled production of free radicals in the body leads to the manifestation of oxidative stress [45]. Lipid peroxidation (LPO), a significant phenomenon in free radical induced tissue damage, has been implicated in many pathological conditions and in our study, higher levels of LPO and significant rise in osmotic fragility of RBCs occurred in infected cattle among different parasitemia rates. The rise in LPO of RBCs is an indicative of oxidative shock during parasitemia, whereby the production of reactive oxygen metabolites exceeds their removal by antioxidant mechanisms. Major targets of cellular damage induced by reactive oxygen species (ROS) are membrane lipids [36]. MDA is one of the final products of lipid peroxidation, due to the interaction between ROS and rich polyunsaturated fatty acids (PUFA) [26] at cell membrane level. The important indicator of oxidative damage of cell membrane, MDA is derived from LPO which interacts with cellular membrane

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elements, resulting in increased cellular permeability and enzyme activities. Higher MDA levels indicate inadequacy of antioxidant defense mechanisms to sufficiently neutralize oxidative stress [22]. Therefore, the damage to erythrocytes that stems from oxidative stress could relate to lipid peroxidation [20]. This process can also promote reduced membrane symmetry and increase membrane permeability that leads to elevated levels of osmotic fragility and causes morphological changes in the RBC cell surface [44] as a result. Consequently, these morphologically altered erythrocytes would be susceptible to erythrophagocytosis which is a phenomenon that occurs commonly in severe anemia [34]. Although some studies have recently revealed that erythrocytes oxidative damage may play a significant role in anemia caused by hemotropic protozoa such as *Theileria* spp., *Babesia* spp. and *Anaplasma ovis* in infected animals [18,27,34,38,39,42,43]; however, as yet, little information has been rendered to indicate such mechanisms in bovine anaplasmosis. This oxidative damage can affect the activity of antioxidant enzymes; especially glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT). Also, lipid peroxidation is reported to be related to oxidative destruction of erythrocytes and anemia, and may be the reason for the increase in morphological changes and osmotic fragility of RBCs that make them susceptible to phagocytosis by reticulo-endothelial cells [29].

Microminerals such as zinc, copper, and selenium as the essential components of the antioxidant defense system are utilized to synthesize antioxidant enzymes against free radical-induced damage to tissues [19]. Located in the cell cytosol [32], the copper-zinc SOD (Cu-Zn-SOD) catalyzes the conversion of $O2^-$ into H_2O_2; therefore contributing to the first line of antioxidant pathway. Therefore, the measurement of zinc and copper concentration in serum can demonstrate the status of mineral nutrients as well as representing their coordinated antioxidant role accompanied by antioxidant enzyme activities. Because of being a mineral with oxidative properties, assessment of serum iron level can also be beneficial in clarifying the oxidant/antioxidant status [9].

Cholinesterase, as low-grade inflammatory markers can be used to detect cell lesions [25]. The cholinergic system is a major modulatory pathway in the central nervous system (CNS), and composes an essential part of several vital functions, such as regulation of cerebral blood flow [33]. Acetylcholine (ACh), the main component of the cholinergic system is mainly hydrolyzed by cholinesterase (AChE). AChE is a widely distributed enzyme in CNS, but can also be found in erythrocytes, platelets and lymphocytes [13]. Since AChE activity represents functional properties, similar to that of synaptic cleft, it is considered a good marker of CNS peripheral changes in blood. Additionally, butyrylcholinesterase (BChE) may also hydrolise ACh, particularly when AChE is inhibited. The consequent decreases in the levels of ACh is the result of increase in the activity of AChE and BChE, and reducing its anti-inflammatory effects which leads to the activities of AChE and BChE being considered as intrinsic regulators of inflammation [3,14].

There is paucity of information regarding oxidant/antioxidant interactions and alterations of essential micronutrients of antioxidant defense as well as involvement of cholinesterase's inflammatory process in bovine anaplasmosis, this study was, therefore, conducted to clarify the cholinesterases inflammatory process associated with oxidative stress by measuring the activities of the key antioxidant enzymes (GSH-Px, SOD, G6PD and catalase), total antioxidant capacity, the level of median corpuscular fragility, lipid peroxidation of erythrocytes and concentrations of some antioxidant trace elements (Zn, Se and Cu) as well as cholinesterases activity in different parasitemia levels in cattle naturally infected by *A. marginale*. In addition, the probable correlation of these factors with degree of parasitemia has been evaluated. The present study was also discussed the contributory role of oxidative damage and inflammatory process (i.e., Cholinesterases) as serious consequences of the pathogenic action of *A. marginale*.

2. Materials and methods

2.1. Animals and samples

This study was done in the five different rural areas of Iran (Ahvaz, Hamedam, Ilam Kermanshah and Urmia), where bovine tick-borne diseases are prevalent during the seasons (early May - late September 2015, 2016). A group of thirty-five 2–3 years-old crossbred cattle (Holstein Friesian × Saraby breeds) naturally infected with *Anaplasma* spp. was divided into four subgroups according to parasitemia rates (< 1%, 1–10%, 10–20% and > 20%). Here, it should be noted that despite the fact that the species of the parasites was not definitively identified, it seems that the infective agent was likely *Anaplasma* spp. based on the morphological structure on the blood smears, particularly the predominant existence of the inclusion bodies are located peripherally or marginally inside the RBCs [50]. The control group (n = 10) was selected from the same farms and matched the treated ones in age. The infected cattle showed signs of the disease upon clinical examination which included dullness, low feed intake, rise in rectal temperature (39.5–41 °C), dyspnea, tachycardia and stages of icterus and also evidences of existing stages of anemia (determined through measurement of different hematological parameters, i.e., RBC count, the values of packed cell volume (PCV), and hemoglobin (Hb) concentration) without hemoglobinuria. However; apart from precise evaluation of blood smears, a multiplex PCR was employed to detect of the presence of *A. marginale* in blood of the infected animals and rule out of other potential causes of anemia (like some parasitic infections such as theileriosis and babesiosis) as described previously by Ref. [7]. Also, routine microbiological assessments such as direct microscopical blood examination with differential staining (Gram's staining method), conventional pure culturing (streaked onto blood agar medium, Merck, Germany) and routine biochemical tests and acid-fast staining methods were done as well. No treatment was administered on the infected animals prior to sampling and the sampling was only carried out once in the course of their disease. On the other hand, clinically healthy animals without parasitemia (according to the blood smears) with normal hematological profile and negative in molecular assessment were considered as controls.

2.2. Sampling and parasitological examination

To determine the hematological parameters, blood samples were drawn from the jugular vein of infected and control group into vacutainers (Kendall Company, Covidien, USA) that contained EDTA-K_2 as anticoagulant. Isolation of serum samples and further biochemical analysis was carried out without EDTA-K_2. Thin blood smears of both groups were prepared from the ear vein and stained with Giemsa for microscopic observation of *A. marginale* inside erythrocytes. The number of infected red blood cells was counted at a magnification of × 1000 for each case through examination of at least 1×10^4 RBCs to assess parasitemia. The result was expressed as a percentage [15]. Observing no parasites in 100 oil-immersion fields in the blood smear was recorded as negative.

2.3. Routine hematological examination

Hematological analysis was performed within 5–6 h of blood collection on EDTA. Hematological parameters like hemoglobin (Hb) concentration, erythrocyte count, and packed cell volume (PCV) were determined by automated hematology analyzer (Autolyser, Al 820, Swiss) [46]. Blood samples were centrifuged at 700 × g for 15 min, plasma separated and packed cells washed three times with normal saline to determine MDA and antioxidant enzymes. Next, the washed erythrocytes were hemolysed using nine volumes of ice-cold distilled water to prepare 10% RBC hemolysate.

2.4. Osmotic fragility test (OFT)

[10] method was used to perform osmotic fragility test (OFT). Saline buffer in ranging hypotonicity concentrations from 1.0 g/L (0.1%) to 9.0 g/L (0.9%) sodium chloride was used to incubate the washed erythrocytes briefly. Hemolysis percentage of each NaCl concentration was calculated. A graph was plotted using hemolysis percentage against concentration of NaCl. The median corpuscular fragility (MCF) (the concentration of NaCl causing 50% haemolysis) was used to express the results.

2.5. Oxidant/antioxidant assessment

As a thiobarbituric acid reactive substance (TBARS), lipid peroxidation was determined in the RBC hemolysate according to [37]; which depends on forming a color complex that results from the reaction 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), incubated at 37 °C for 30 min, then 1.5 ml of TBA was added to the mixture which was immediately heated (60 min at 95 °C) and cooled after using running water. After, 3 ml of pyridine/n-butanol (3:1, v/v) plus 1 ml of 1 N NaOH was added to the mix and shaken. The absorbance was read at 532 nm versus distilled water was used as a blank.1,1,3,3-tetramethoxypropane was used as a standard. Lipid peroxidation of RBC hemolysate was expressed as MDA nanomoles per grams of hemoglobin (nmol/g Hb).

Colorimetric method, described by Ref. [49] was used to measure catalase activity and involves two steps. Due to the rate of dismutation of hydrogen peroxide to water and oxygen being proportional to the catalase concentration, samples were incubated with 2.950 ml of hydrogen peroxide. The remaining amount of hydrogen peroxide was determined by the oxidative coupling reaction of 4-aminophenazone (4-aminoantipyrene, AAP) and 3,5-dicholo-2-hydroxy-benzenesulfonic acid (DHBS) in the presence of H2O2and using horseradish peroxide as the catalyst in a fixed incubation period (22–25 °C). The resulting quinoeimine dye was measured at 520 nm (Catalase Assay Kit, Oxford Biochemical Research, Inc., USA) and enzymes activities were expressed as Katal/gHb.

[5] method was used to measure the activity of GSH-Px in the RBC hemolysate; a method based on the development of a stable yellow color that appears when 2-nitrobenzoic acid is added to sulfhydryl compounds. Thionitrobenzene amount, a reduced product, was measured by commercially available kits (Ransel Kit, RandoxLaboratories Ltd. G.B.) at 412 nm. Enzyme activity was expressed as IU/mg Hb.

In order to measure the SOD activity, superoxide radicals generated by the xanthine oxidase reaction convert 1-(4-iodophenyl)-3-(-4-nitrophenol)-5-phenyltertrazoliumchloride quantitatively to a form azan dye (Ransel Kit, Ran-dox Laboratories Ltd. G.B.). Superoxide dismutase converts superoxide radicals to hydrogen peroxide and inhibits dye formation; serving as a measure of superoxide superoxidedismutase activity. Enzyme activities were expressed as IU/mg Hb.

Glucose-6-phosphate dehydrogenase (G6PD) activity was determined based on the reaction described by Ref. [6] in which glucose-6-P is oxidized to gluconate-6-P and NADP + is reduced to NADPH + H^+. Spectrophotometry at 340 nm and 37 °C was used to determine NADPH production and it was expressed as international units per gram of hemoglobin (IU/g Hb). Hemoglobin determination was performed by cyanomethemoglobin method [10].

Total antioxidant capacity (TAC) of RBC hemolysate was assesed according to the [30] method. In this method, incubation of ABTS® with a peroxidase (metmyoglobin) and H_2O_2 produces $ABTS^{®*+}$ (2,2_-Azino-di-[3-ethylbenzthiazolinesulphonate]) radicals and results in a stable blue-green color that can be measured at 600 nm (Ransel Kit, Randox Laboratories Ltd. G.B.). Activity of the enzyme was expressed as mmol/l.

2.6. Measurement of levels of serum trace minerals

To determine the serum trace mineral levels, perchloric and nitric acid mixture (in a ratio of 3:7, respectively) was used to digest serum samples. Using an atomic absorption spectrophotometer (Shimadzu Asc-6100, Japan), manganese, copper, iron, selenium and zinc were measured. The purging gas used was argon; and the background absorption was automatically corrected by the Zeeman effect. The standard solution consisted of (1000 g/ml) of zinc, copper, manganese, and selenium [35]. The values were expressed in mol/L of serum.

2.7. Assessment of cholinesterase activity

In order to measure AChE activity, whole blood was collected in tubes containing EDTA and centrifuged for 10 min at 1000 g. The plasma was discarded. The sediment of erythrocytes was washed three times with tenfold isotonic NaCl solution and after each procedure; the mixture was centrifuged at 1000 g for 10 min. In the end, the erythrocytes obtained were used to assess AChE activity by the method of [17]. Prior to the addition of substrate, the mixture was incubated for 10 min to achieve temperature equilibration and complete reaction of sample matrix sulfhydryl groups with 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). Enzyme activity was corrected for spontaneous hydrolysis of the substrate and DTNB degradation. Ethopropazine inhibited the activity of butyryl cholinesterase (BChE; EC 3.1.1.8). The AChE activity was measured at 436 nm from the quotient between the AChE activity and the hemoglobin content (Hb). Zijlstra-modified solution was used to calculate Hb value. Results were then expressed as mU/umolHb. [17]; spectrophotometric method was used to perform the BChE enzymatic assay in serum.100 mM potassium phosphate buffer, pH 7.5, and 1.0 mM DTNB made up the reaction mixture (2 ml final volume) and formation of the yellow anion, DTNB is the basis of this method is based which is measured by absorbance at 412 nm during 2 min of incubation at 25 °C. Enzyme activity was expressed in mol BuSCh/h/mg of protein.

2.8. Statistical analysis

The packaged SPSS program for windows (Version 22. 2013, 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 $P < 0.05$ was considered as statistically significant.

3. Results

The level of parasitemia ranged from less than 1% to more than 24.8%. Of the 35 infected animals, 8 (22.85%), 11 (31.42%), 10 (28.57%) and 6 (17.14%) cattle had < 1%, 1–10%, 10–20% and > 20 of parasitemia, respectively. All of the infected animals were identified positively infected animals by using *A. marginale*-specific primers (an expected 265 bp fragment), meanwhile, no PCR products were observed to have *Theileria* or *Babesia* DNAs. As well, no sample showed microscopic evidence of pseudo-morulae of *A. phagocytophilum* in granulocytes or of inclusion bodies of *A. centrale* in erythrocytes. Also, no growth was seen on blood agar medium after culture of samples.

Table 1 represents the mean values of hematological parameters in control and infected animals with different parasitemia rates. According to evaluated hematological parameters, the data showed remarkable decline in RBC, Hb concentration and PCV in the infected cattle compared to controls ($P < 0.05$); strictly confirming anemia in the infected group. Also, parasitemia rate was negatively correlated ($r = -0.60$)

Table 1
Mean ± SEM of hematological parameters in control cattle and those infected with *A. marginale*.

	Parasitemia (%)	RBC ($\times 10^{12}$/L)[a]	PCV (L/L)[b]	Hb (g/L)[c]
Control	0 (n = 10)	7.18 ± 0.24[a]	0.318 ± 0.01[a]	102.6 ± 0.06[a]
Infected	< 1 (n = 8)	5.76 ± 0.13[b]	0.269 ± 0.04[b]	90.40 ± 0.04[b]
	1-10 (n = 11)	4.98 ± 0.14[c]	0.238 ± 0.02[c]	81.00 ± 0.03[c]
	10-20 (n = 10)	3.96 ± 0.24[d]	0.201 ± 0.01[d]	67.00 ± 0.01[d]
	> 20 (n = 6)	2.83 ± 0.20[e]	0.173 ± 0.01[e]	57.70 ± 0.04[e]

Different superscript in each column denotes significant differences ($P < 0.05$).
[a] Reference value: 5–10 × 1012/L.
[b] Reference value: 0.24–0.46 L/L.
[c] Reference value: 80–150 g/L [8].

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 cattle and those infected with *A. marginale* with different parasitemia rates.

	Parasitemia (%)	MDA (nmol/gHb)	GSH- Px (IU/mgHb)	SOD (IU/mgHb)	Catalase (Katal/gHb)	TAC (mmol/L)	MCF (g/dL)	G6PD (IU/mgH)
Control	0 (n = 10)	4.16 ± 0.2[e]	211.14 ± 1.2[a]	916.64 ± 0.4[a]	154.19 ± 0.56[a]	1163.3 ± 2.1[a]	0.47 ± 0.001[e]	23.16 ± 0.4[a]
Infected	< 1 (n = 8)	7.54 ± 0.4[d]	201.17 ± 1.6[b]	911.13 ± 0.1[b]	148.15 ± 0.14[b]	1014.1 ± 1.6[b]	0.51 ± 0.001[d]	20.65 ± 0.1[b]
	1-10 (n = 11)	11.00 ± 0.16[c]	191.19 ± 1.3[c]	901.13 ± 0.1[c]	131.21 ± 0.01[c]	916.14 ± 1.2[c]	0.55 ± 0.002[c]	16.89 ± 0.3[c]
	10-20 (n = 10)	15.25 ± 0.3[b]	178.14 ± 1.1[d]	890.12 ± 0.3[d]	111.17 ± 0.21[d]	773.19 ± 1.1[d]	0.61 ± 0.003[b]	13.14 ± 0.2[d]
	> 20 (n = 6)	22.58 ± 0.3[a]	156.12 ± 1.4[e]	874.22 ± 0.2[e]	91.19 ± 0.13[e]	423.17 ± 1.3[e]	0.66 ± 0.001[a]	9.16 ± 0.1[e]

Different superscript in each column denotes significant differences ($P < 0.05$).

with the value of PCV (Table 3).

MDA and MCF levels in both control and affected cattle are presented in Table 2. The MDA level showed significant enhancement in the infected group. Moreover, the levels of MDA showed elevation with increasing parasitemia rates. In addition, corpuscular MDA concentration was positively correlated with parasitemia (r = 0.96, $P < 0.05$) and negatively correlated with PCV (r = −0.64, $P < 0.05$) (Table 3). Therefore, taking into account that MDA determination allows for the detection of lipid peroxidation value and the level of free oxygen radicals indirectly [54], the research indicates that lipid peroxidation in erythrocytes of affected cattle increased significantly ($P < 0.05$). Similarly, MCF of RBCs of infected cattle was significantly higher compared to that of the control group ($P < 0.05$) and showed a significant positive correlation with parasitemia rate (r = 0.96).

Based on our findings, antioxidant enzymes activities including SOD, GSH-Px, G6PD and catalase as well as levels of TAC in infected cattle reduced significantly compared to those control ($P < 0.05$) (Table 2). In animals with higher parasitemia rates, significant decrease in the activities of SOD (r = −0.97, $P < 0.05$), GSH-Px (r = −0.95, $P < 0.05$), catalase (r = −0.98, $P < 0.05$), G6PD (r = −0.95, $P < 0.05$) and TAC (r = −0.95, $P < 0.05$) was observed (Table 3). Accordingly, Table 3 shows the strong negative correlations of antioxidant enzyme activities (SOD, GSH-Px, catalase, G6PD and TAC) to different parasitemia levels. The significant rise in the lipid peroxidation index (MDA) was clearly evident in higher parasitemias; the strong positive correlation of the level of erythrocytic osmotic fragility with the percentage of parasitemia in infected cattle was observed as well.

The change in serum levels of antioxidant trace elements of the control and infected cattle with *A. marginale* are presented in Table 4. Concentrations of copper, zinc, manganese and selenium in serum

Table 3
Correlation between oxidative stress markers, activities of antioxidant enzymes and parasitemia in cattle infected with *A. marginale*.

	PCV	parasitemia	MDA	GSH-Px	Catalase	SOD	G6PD	MCF	TAC
PCV									
PC[b]	–	- 0.609[a]	−0.648[a]	0.574[a]	0.631[a]	0.638[a]	0.615[a]	−0.641[a]	0.617[a]
Parasitemia									
PC		–	0.962[a]	−0.950[a]	−0.981[a]	−0.977[a]	−0.953[a]	0.963[a]	−0.955[a]
MDA									
PC			–	−0.970[a]	−0.981[a]	−0.990[a]	−0.964[a]	0.977[a]	−0.988[a]
GSH-Px									
PC				–	0.968[a]	0.978[a]	0.959[a]	−0.956[a]	0.971[a]
Catalase									
PC					–	0.995[a]	0.983[a]	−0.988[a]	0.972[a]
SOD									
PC						–	0.980[a]	−0.985[a]	0.985[a]
G6PD									
PC							–	−0.976[a]	0.959[a]
MCF									
PC								–	−0.963[a]
TAC									
PC									–

[a] Correlation is significant at the 0.05 level (2-tailed).
[b] Pearson correlation.

Table 4
Concentration of serum trace elements in uninfected cattle and those infected with *A. marginale* with different parasitemia rates.

	Parasitemia (%)	Cu (μmol/L)	Zn (μmol/L)	Fe (μmol/L)	Mn (μmol/L)	Se (μmol/L)
Control	0 (n = 10)	21.19 ± 1.13^{a}	19.22 ± 0.01^{a}	42.17 ± 0.05^{e}	0.68 ± 0.05^{a}	2.92 ± 0.02^{a}
Infected	< 1 (n = 8)	18.17 ± 1.08^{b}	16.96 ± 0.03^{b}	44.16 ± 0.02^{d}	0.62 ± 0.02^{b}	2.68 ± 0.01^{b}
	1-10 (n = 11)	14.13 ± 0.62^{c}	12.14 ± 0.01^{c}	50.01 ± 0.01^{c}	0.56 ± 0.01^{c}	2.10 ± 0.06^{c}
	10-20 (n = 10)	9.00 ± 0.37^{d}	8.01 ± 0.05^{d}	58.15 ± 0.02^{b}	0.49 ± 0.02^{d}	1.71 ± 0.02^{d}
	> 20 (n = 6)	5.15 ± 0.24^{e}	2.14 ± 0.1^{e}	66.10 ± 0.03^{a}	0.40 ± 0.03^{e}	1.00 ± 0.03^{e}

Different superscript in each column denotes significant differences ($P < 0.05$).

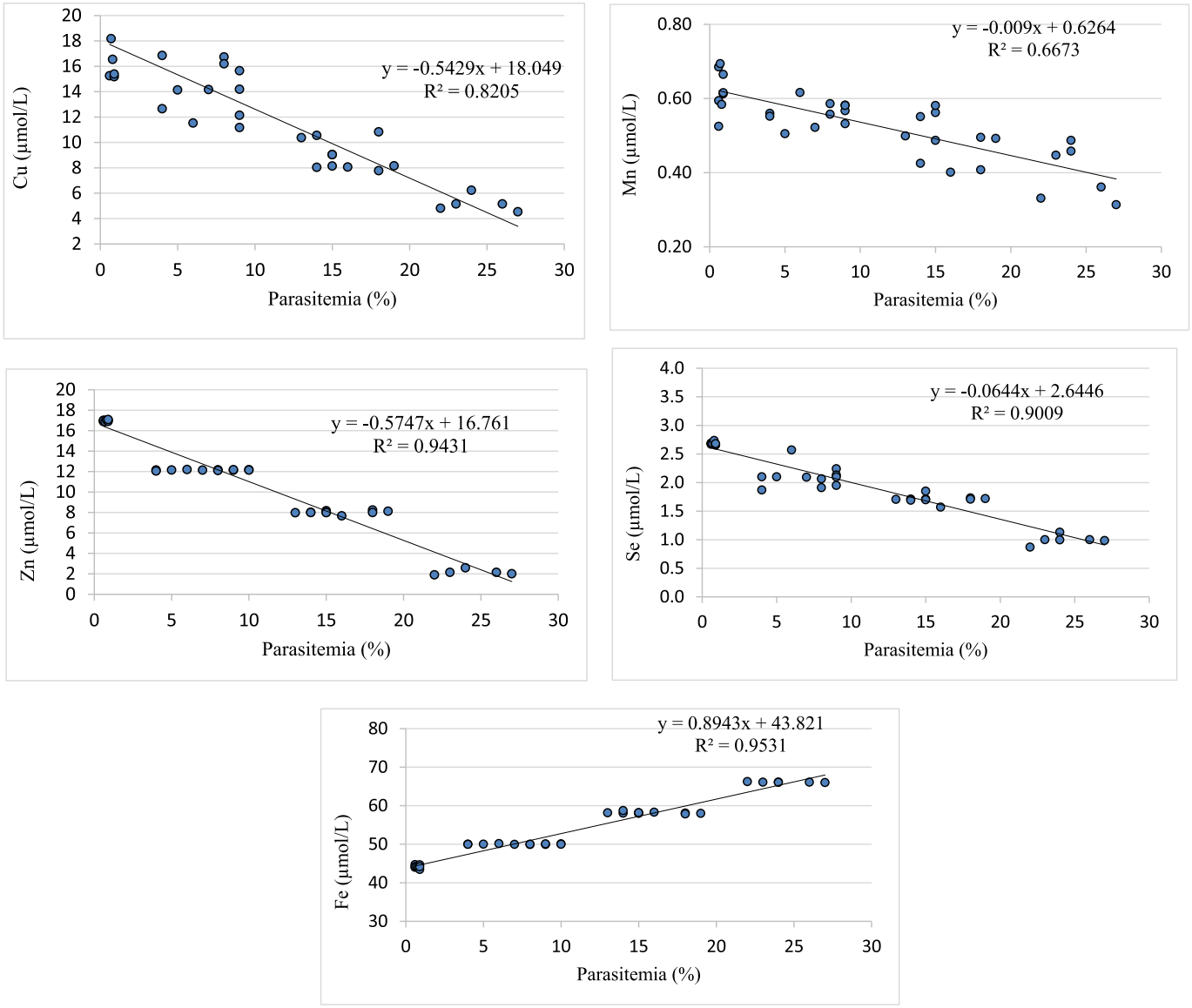

Fig. 1. Linear regression (R2) analysis of different levels of parasitemia in *A. marginale*-infected cattle with serum trace minerals copper (Cu), zinc (Zn), iron (Fe), manganese (Mn) and selenium (Se).

showed a remarkable decrease in infected cattle ($P < 0.05$); adversely, serum concentration of iron was significantly higher in infected cattle compared to that of control group ($P < 0.05$). The level of antioxidant trace elements in relation to the increase in the parasitemia level in cattle suffering from anaplasmosis are depicted in Fig. 1 to show relevant significant correlation. As can be seen in Fig. 1, parasitemia rate was negatively correlated with the serum level of copper, zinc, manganese, and selenium, meanwhile being positively correlated with serum concentration of iron in contrast.

Lower AChE and BChE activity was observed in infected animals in comparison to uninfected group ($P < 0.05$) (Table 5). A significant decrease was observed in AChE and BChE activities in cattle with higher parasitemia levels. MDA level indicated significant negative correlation with enzyme activities of AChE and BChE (r = 0.92, 0.85, respectively) (Fig. 2).

Table 5
Mean ± SEM of blood acetilcholinesterase (AChE) activity and serum butyrylcholinesterase (BChE) activity in control cattle and those infected with *A. marginale*.

	Parasitemia (%)	AchE (mU/μmol)	BchE (μmolBuSch/mg protein)
Control	0 (n = 10)	122.7 ± 2.89[a]	0.1262 ± 0.0053[a]
Infected	< 1 (n = 8)	119.8 ± 1.37[a]	0.1381 ± 0.0017[b]
	1-10 (n = 11)	95.73 ± 3.22[b]	0.1149 ± 0.0032[c]
	10-20 (n = 10)	59.00 ± 3.30[c]	0.0698 ± 0.0033[d]
	> 20 (n = 6)	23.51 ± 3.62[d]	0.0228 ± 0.0038[e]

Different superscript in each column denotes significant differences ($P < 0.05$).

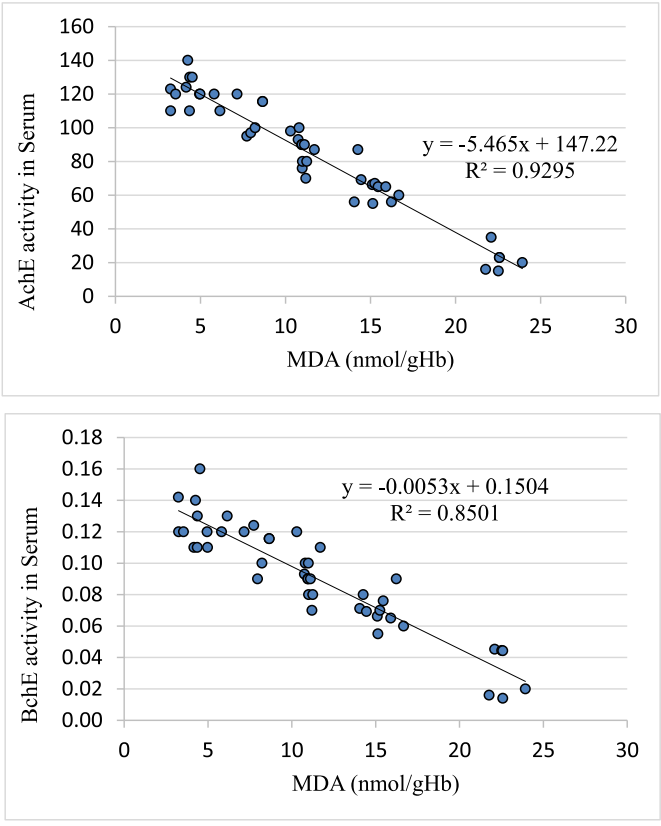

Fig. 2. The correlations among cholinesterases activity (AChE and BChE) with the level of lipid peroxidation index (MDA) in cattle infected with *A. marginale*.

4. Discussion

The significant feature of anaplasmosis is extra vascular hemolytic anemia [31]. The comparison between control and *A. marginale*-infected cattle showed a significant reduction ($P < 0.05$) in the number of RBCs, PCV, and hemoglobin content in bovine anaplasmosis that confirmed anemia in the infected animals. The severity of the anemia increased in animals that showed higher parasitemia rates. Similar findings on bovine anaplasmosis are consistent with our data [15], but the exact underlying mechanisms of such a progressive anemia in regards to the change in cooperative antioxidant trace elements have not been described. However, destruction of non-parasitized erythrocytes besides parasitized erythrocytes due to autoimmune phenomena has been considered as the cause of hemolytic anemia in bovine anaplasmosis due to *A. marginale* [34,47].

Considering that the cholinesterase's act regulating the inflammatory immune response, which composes a fundamental defense barrier against hemoparasites. Since blood cholinesterases activity indicates the integrity and functional state of erythrocytic membrane and serves as markers of systemic low-grade inflammation, little is known about a lot of studies have investigated it [14]. Our data represent the first study where the cholinesterase activities were assessed in bovine anaplasmosis. According to literature, low AChE activity has been reported in cattle experimentally infected with *Babesia bigemina* [16] as well as in *Trypanosoma evansi*-infected erythrocytes [53]. Consistently, our results suggest that reduction in cholinesterase activity could occur as a compensatory mechanism during hemoparasitic diseases and lead to inhibition of systemic inflammation (act by inhibiting the release of IL-1 and TNF-α) mediated by an increase in serum ACh concentration [14,16]. Present study showed an inverse relationship between cholinesterase activity and MDA levels, concurrent with increasing the severity of parasitemia. Similar finding have been reported that corroborate our data in the case of bovine theileriosis [16]. It seems like lipid peroxidation can easily lead to cell damage and therefore, can interfere with the activity of enzymes anchored to the cell membrane [25].

Increased severity of parasitemia coupled with a significant decline in the activities of erythrocytic antioxidant enzymes including SOD and GSH-Px was observed in present study. In other words, the negative significant correlation between parasitemia rate and GSH-Px and SOD activities is an indicative of increased exposure of RBCs to oxidative stress products at high parasitemias. GSH-Px activity is a major mechanism in intracellularly decomposing lipid peroxidase [21] [24]. also proposed a crucial role for GSH-Px in preventing peroxide damage to membranes induced by lipid peroxides. Moreover, it appears that SOD has a crucial role in protecting erythrocytes against oxidative damage [12], indicating that enhanced oxidation of the erythrocytes could increase the generation of free radicals significantly as well as strengthen with inefficient antioxidant capacity. This is a condition in which oxidant agents invade RBCs which results in damages to RBC and hemolysis and occurrence of anemia.

Despite several studies that are in agreement with our results of a negative correlation of antioxidant enzyme activities of RBCs and the level of parasitemia, also activity of those enzymes in uninfected animals being significantly higher [12,15,27,34,38] reported a significant rise in SOD and catalase activities of dogs naturally infected with *Babesia gibsoni*. They reached the conclusion that increased level of SOD could be the result of high reticulocyte percentage in the infected dogs, because compared to mature erythrocytes; the activity of enzyme is higher in reticulocytes [55]. In contrast, the significant decrease in catalase activity in infected group compared to controls and its substantial positive correlation with enzyme activities of GSH-Px and SOD, also, the decrease in the activity of these enzymes along with the increase in the parasitemia level in this study, indicated that all of these antioxidant agents could act as determinative factors; however, to scavenge H_2O_2 and protect erythrocytes against oxidative injuries, the cited enzymes are consumed, hence, declined in the infected animals. Moreover, these results were not consistent with reports of [12] findings, since they observed no significant difference in catalase activity in both healthy and *B. gibsoni*-infected dogs and believed that this enzyme works with SOD to scavenge peroxides.

To our knowledge, there are no published reports on the alteration of G6PD activity in *A. marginale* infection to compare with present study. However, there are reports on low activity of G6PD in bovine and ovine piroplasmosis [4,18]. Our results suggest that G6PD acts as an antioxidant enzyme and decreased activity of G6PD has been associated with increased hemolysis. However, in opposition to results [22], reporteda 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 [2]. Reduction in the TAC level of infected cattle can be attributed to the consumption of antioxidant enzymes that serve as free radical scavengers during the oxidative process in natural *A. marginale* infection of cattle [1,23].

According to the our findings, low serum levels of antioxidant minerals levels of copper, zinc, manganese and selenium accompanied by the raised levels of iron in the infected cattle convenes another alternative aspect of oxidative shock. Being essential components of the body's antioxidant defense, micronutrients, such as Cu, Zn and Se play an important role in prevention of free radical-induced damage [19]. The Cu–Zn-SOD and Mn-SOD, respectively located in the cell cytosol [32] and mitochondria [52] form the first line of antioxidant pathway by catalyzing the conversion of O_2- into H_2O_2. The conversion of H_2O_2 to H_2O is catalyzed by Se-containing GPX through the oxidation of R-GSH [11]. It is speculated that the decrease in the level of serum trace minerals of infected cattle proves that species of pathogenic *Anaplasma* impose a marked effect on the status of mineral nutrients; representing their coordinated antioxidant role along with antioxidant enzyme activities during the infection with these parasitesin cattle. In the present study elevated serum iron in infected cattle is most likely due to hemolytic anemia. Abnormal RBCs are specifically detected in extravascular hemolysis, by macrophages in the marrow, spleen, and/or liver, and are phagocytosed after. Macrophages degrade hemoglobin to globin, heme, and iron; therefore increasing serum iron level [51].

In conclusion, hemolytic anemia served as the major clinical aspect of the infection with the pathogenic *A. marginale* in cattle. Significant decrease in antioxidant enzyme activities and some associated antioxidant trace elements and considering the significant rise in lipid peroxidation of erythrocytes shows that these parasites disturb antioxidant mechanisms that protect RBCs against oxidative stress. This interference might be the cause of the increased erythrocyte fragility due to damage caused to membrane by oxidative stress. In addition, our results indicate that *A. marginale* infection in cattle can directly or indirectly change the activities of AChE and BChE, therefore, it is possible to conclude that cattle infected by *A. marginale* underwent in the oxidative stress process, concomitant with the reduction of cholinesterase'ıs activity. These factors had influenced the pathogenesis of this infection. Nevertheless, future molecular and biochemical studies are required to reach a better understanding about the pathogenesis of anaplasmosis, also investigate possible markers that can act as a tool to improve the early diagnosis of this parasitic disease.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.micpath.2018.07.039.

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