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Correlations Among the Level of Homocysteine, Antioxidant Enzymes, Antioxidant Vitamins and Lipid Peroxidation of Erythrocytes in Malignant Ovine Theileriosis

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ABSTRACT

Malignant ovine theileriosis is a fatal disease of sheep caused by *Theileria hirci*. The present study was designed to estimate the levels of plasma Homocysteine (Hcy), antioxidant enzymes, antioxidant vitamins and lipid peroxidation of erythrocytes and also to evaluate their correlations in different parasitemia rates in naturally infected sheep. Fifty Iranian sheep, about 1-2 years old, naturally infected with *T. hirci* were selected and divided into 2 subgroups according to their parasitemia rates (<2%, 2-4%). Ten non-infected animals were also selected as controls. Blood samples were collected and Hcy, antioxidant enzymes, antioxidant vitamins and lipid peroxidation of erythrocytes were measured. Our results showed that the activities of erythrocytic antioxidant enzymes including SOD, GPX and catalase were substantially reduced in infected sheep. Furthermore, the animals with higher parasitemia rate showed remarkable declines in SOD, GPX and catalase activities. There was a significant rise in the level of MDA, particularly in the animals with the higher (2-4%) parasitemia rates. In addition, it was evident that coinciding with the elevation of the parasite-bearing erythrocytes in infected animals, the level of lipid peroxidation in RBC membrane was significantly increased. Although the concentration of vitamin C showed a significant decrease in infected sheep with <2% parasitemia rate, the concentrations of antioxidant vitamins (A and E) generally showed no remarkable alterations. In our study, a significant increase was evident in the level of homocysteine in sheep suffering from theileriosis. Our data also showed that the increase in of parasitemia was coupled with the elevation of homocysteine level ($r = 0.34$, $p<0.05$).

Key words: Malignant ovine theileriosis, homocysteine, antioxidant enzymes, antioxidant vitamins, malondialdehyde

INTRODUCTION

Theileria species are important tick-born protozoan parasites that infect wild and domestic animals. Malignant ovine theileriosis is a fatal disease of sheep that occurs due to highly pathogenic species of *Theileria* including *T. lestoquardi* (Morel and Uilenberg, 1981) and two described *Theileria* sp. in China (China 1 and China 2) (Schnittger *et al*., 2000). The disease has been reported from North Africa, Southern Europe, Asia minor and India (Salih *et al*., 2003) and has been considered as a fatal disease of sheep and goats in Iran from a long time ago, which imposes heavy losses due to mortality and decreased production in affected animals (Hooshmand-Rad, 1974).

Based on the studies on bovine theileriosis, it has been suggested that hemolytic anemia is a key feature of the disease (Aulakh *et al*., 1998; Omer *et al*., 2002), but there is a paucity of information on anemia and related mechanisms in ovine theileriosis. Also, despite previous studies on bovine tropical theileriosis pointing out an immune-mediated hemolysis indicated by the presence of a hemagglutinin in *T. annulata* infected calves (Hooshmand-Rad, 1976), some recent findings suggest that anemia could be a consequence of the oxidative damage of RBCs, as well as significant modulation of erythrocytic antioxidant enzymes activities (Shiono *et al*., 2003; Rezaei and Dalir-Naghadeh, 2006; Nazifi *et al*., 2008). On the other hand, other studies have suggested that erythrocyte destruction during oxidative stress was related to lipid peroxidation of the RBCs (Friedman, 1979; Grewal *et al*., 2005). This process might be the cause of morphological changes in erythrocyte surface (Saluja *et al*., 1999; Grewal *et al*., 2005), which increase erythrocyte susceptibility to phagocytosis.

Homocysteine (Hcy) is a highly reactive thiol-containing amino acid, produced by the intracellular demethylation of methionine. Endothelial cell injury in experimental animals (Harker *et al*., 1983) and also cardiovascular diseases in human (Arnesen *et al*., 1995) have been attributed to the alterations in plasma Hcy. Increased Hcy could also exert pathological effects by promoting oxidative stress (Perna *et al*., 2003), which has been indicated as a mechanism involved in anemia. Although it is clear that the invasion of piroplasms of *Theileria* species on erythrocytes could induce anemia (Jain, 1993), the probable correlations of hyper and/or hypo-homocysteinemia with the occurred anemia have not been clearly described.

Antioxidant vitamins such as A, E and C could protect the cells from damage against free oxygen radicals. The antioxidant systems comprising such vitamins could have a cellular protective action against the oxidative stress during some parasitic infections (Dede *et al*., 2000), but it has not been clearly investigated whether or not these vitamins have a role in preventing oxidative damage to the erythrocytes during malignant ovine theileriosis.

This study was therefore, designed to estimate the levels of plasma Hcy, the activities of the key antioxidant enzymes (SOD, GPX and catalase), antioxidant vitamins (A, E and C) and lipid peroxidation of erythrocytes (MDA) and also to evaluate their correlations in different parasitemia rates in malignant ovine theileriosis.

MATERIALS AND METHODS

Animals and samples: Fifty Iranian fat-tailed sheep suffering from theileriosis due to *T. hirci* were selected in the southwest region of Iran (Fars province) and divided into 2 subgroups according to different parasitemia rates (<2%, 2-4%). Ten non-infected sheep were also selected from the same herds and used as controls. The animals had not been treated for disease prior to sampling and were screened for the other potential causes of anemia by determination of hematological parameters, clinical signs and routine microbiological tests.

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

Hematological and parasitological measurements: Blood samples were collected from jugular vein into EDTA containing tubes for measuring hematological parameters and Hcy and into plain tubes without anticoagulant for conducting serum assays. Thin blood smears were prepared, fixed

with absolute methanol (5 min), stained with 10% Giemsa solution (30 min) and examined under oil immersion (×1,000) to observe intraerythrocytic forms of *T. hirci*. The parasitemia rate was quantified by examination of at least 1×10^4 RBC, at a magnification of ×1000 for each case and expressed as the percentage of parasitemia. Identity of the parasite was determined on the basis of morphological, clinical and previous epidemiological studies. The hematological parameters were measured by routine standard procedures (Jain, 1993).

Hcy measurement: The blood samples were centrifuged at 1,200 g for 10 min at 37°C and the plasma was obtained. The Enzyme Immunoassay (EIA) for the measurement of plasma total homocysteine was performed, using the AXIS Homocysteine EIA Kit (Axis-Shield Diagnostic Ltd. Dundee, UK).

Antioxidant enzymes activities: The activity of SOD was measured with a commercial kit (RANSOD kit, Randox Com, UK). In this method, xanthine and xanthine oxidase were employed to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophe-nol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity in hemolysate was then determined by the degree of inhibition of this reaction, as one unit of SOD corresponded to 50% inhibition of INT reduction under assay condition. Finally, the enzyme activity was expressed as U g^{-1} of hemoglobin. The activity of GPX was measured by a commercial kit (RANSEL kit, Randox Com, UK) based on the method of Paglia and Valentine (1967). Briefly, the GPx in hemolysate catalyzes the oxidation of Glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The absorbance was measured at 340 nm and the enzyme activity was expressed as U g^{-1} of hemoglobin. The activity of catalase was determined with a commercial catalase assay kit (Oxford Biomedical Research, Inc., USA), based on the colorimetric method described by Slaughter and O'Brien (2000). Since the rate of hydrogen peroxide dismutation is proportional to the catalase concentration, the samples were first incubated with a known amount of hydrogen peroxide. The remaining hydrogen peroxide, following a fixed incubation period, was then determined by the oxidative coupling reaction of 4-aminophenazone (4-aminoantipyrene, AAP) and 3, 5-dichloro-2-hydroxy-benzenesulfonic acid in the presence of H_2O_2 and catalyzed by horseradish peroxidase. The absorbance of the resulting quinoneimine dye was measured at 520 nm and the enzyme activity was expressed as U g^{-1} Hb.

Lipid peroxidation of RBCs: Malondialdehyde (MDA), an end product of polyunsaturated fatty acid oxygenation, as a reliable and commonly used biomarker for assessing lipid peroxidation (Moore and Roberts, 1998) was evaluated by a modified HPLC method with UV-visible spectrophotometry based on Lykkesfeldt (2001). The method was based on MDA reactions with Thiobarbituric Acid (TBA) to form a colored MDA-TBA adduct. The HPLC system consisted of a solvent delivery pump (JASCO 980-PU, Tokyo, Japan), a reversed-phase column (Luna C18, 250×4.6 mm, Phenomenex, CA, USA) and a UV-Vis detector (Jasco, UV-975, Tokyo, Japan) operated at 532 nm.

Antioxidant vitamins: The concentrations of vitamins A, E and C were determined by HPLC method. Vitamins A and E were measured based on the protocol described by Johnson-Davis *et al*. (2009) and vitamin C by a commercial kit (ALPCO Diagnostics, USA). The

HPLC system consisted of a solvent delivery pump (JASCO 980-PU, Tokyo, Japan), a reversed-phase column (Luna C18, 250×4.6 mm, Phenomenex, CA, USA) and a UV-visible detector (Jasco, UV-975, Tokyo, Japan).

Statistical analysis: Student's t-test was used for comparison of measured parameters between control and diseased group. Analysis of Variance (ANOVA) and Tukey tests were used for statistical differences among subgroups and Pearson's correlation coefficients to determine relationships among parameters at different parasitemia rates. All values were expressed as mean and Standard Error of Mean (SEM) and $p<0.05$ was considered as statistically significant.

RESULTS

In the present study, a wide range of abnormal RBCs including reticulocytosis, macrocytosis, basophilic stippling and anisocytosis, except for spherocytosis, were evident in the infected sheep. This phenomenon corroborates the occurrence of the regenerative or compensatory responses of bone marrow to anemia in infected animals. The values of hematological parameters in non-infected sheep and those naturally infected with *T. hirci* with different parasitemia rates are presented in Table 1. Significant declines in Red Blood Cells (RBCs), Hemoglobin (Hb) concentration and Packed Cell Volume (PCV) were clearly seen in the infected sheep ($p<0.01$). These data confirm the occurrence of anemia in infected group. In addition, with the increase in the level of parasitemia, marked decrease was observed in RBC count ($r = -0.97$, $p<0.01$), Hb concentration ($r = -0.94$, $p<0.01$) and PCV values ($r = -0.94$, $p<0.01$), which means higher parasitemia levels coincided with the higher degrees of anemia.

Our results showed that the activities of erythrocytic antioxidant enzymes including SOD, GPX and catalase were substantially reduced in infected sheep. Also, animals with the higher parasitemia rate showed remarkable declines in SOD, GPX and catalase activities (Table 1). The concentration of antioxidant vitamins (A, E and C) in the control and the diseased sheep are presented in Table 2. As shown in Table 1, there was a significant rise in the concentration of MDA, particularly in the animals with the higher (2-4%) parasitemia rates. In addition, it was evident that coinciding with the elevation of the parasite-bearing erythrocytes in the infected animals, the occurrence of lipid peroxidation in the RBC membrane was significantly increased (Table 1). In contrast, although the concentration of vitamin C showed a significant decrease in the infected sheep with <2% parasitemia rate, the concentrations of the other antioxidant vitamins generally showed no remarkable alterations (Table 2).

Table 1: Mean±SEM of hematological parameters, antioxidant enzymes activities and the level of malondialdehyde in non-infected sheep and those infected with *Theileria lestoquardi* with different parasitemia rates

Groups	Parasitemia (%)	RBC×10^{12} (L^{-1})	PCV ($L\ L^{-1}$)	Hb ($g\ L^{-1}$)	SOD ($U\ g^{-1}$ Hb)	GPX ($U\ g^{-1}$ Hb)	Catal ($U\ g^{-1}$ Hb)	MDA (nmol g^{-1} Hb)
Control	0 (n = 10)	6.04±0.12^{a}	0.35±0.00^{a}	122.0±2.5^{a}	1572.4±36.6^{a}	393.6±12.7^{a}	1873.4±61.4^{a}	2.00±0.07^{a}
Non-infected	<2 (n = 36)	4.69±0.11^{b}	0.19±0.00^{b}	95.8±2.2^{b}	1298.1±25.9^{b}	306.3±7.9^{b}	1359.1±13.9^{b}	3.35±0.09^{b}
Infected	2-4 (n = 14)	2.45±0.98^{c}	0.14±0.00^{c}	50.8±1.9^{c}	753.1±15.8^{c}	167.6±4.8^{c}	1197.9±21.2^{c}	3.69±0.09^{c}

Different letters in each column indicate statistical significance ($p<0.05$)

Table 2: Mean±SEM of homocysteine and antioxidant vitamins in non-infected sheep and those infected with *Theileria lestoquardi* with different parasitemia rates

Groups	Parasitemia (%)	Homocysteine	Vit. A	Vit. E	Vit. C
		---------- ($\mu mol\ L^{-1}$) ----------			
Control	0 (n = 10)	7.29±0.54^{a}	21.41±0.35^{a}	0.23±0.00^{a}	16.05±0.36^{a}
Non-infected	<2 (n = 36)	12.18±0.50^{b}	20.18±0.85^{a}	0.19±0.01^{ab}	14.08±0.69^{ab}
Infected	2-4 (n = 14)	11.16±0.57^{b}	20.86±1.86^{a}	0.20±0.01^{a}	15.41±0.44^{ab}

In each column, different letters indicate statistical difference and the same letters show no statistical difference

The alterations of homocysteine in the infected and non-infected groups are presented in Table 2. In our study, a significant increase was evident in the level of homocysteine in infected sheep. Our data showed that the increase in parasitemia rate was coupled with increasing the homocysteine level in the infected animals ($r = 0.34$, $p<0.05$).

DISCUSSION

Significant decrease in hematological parameters including RBC count, PCV and hemoglobin were confirmed in the infected sheep. This finding is in line with the cases discussed in bovine (Shiono *et al*., 2003; Razavi *et al*., 2011) and ovine theileriosis (Nazifi *et al*., 2011), however, the underlying mechanisms of anemia are still a matter of debate. Low levels of RBCs, PCV and hemoglobin concentration in bovine theileriosis due to *T. annulata* have been attributed to erythrocytes destruction by macrophages in the lymph nodes, spleen and other organs of the monocyte-macrophage system (Singh *et al*., 2001). One recent hypothesis indicates that disturbed antioxidant defense mechanisms can promote the development of anemia in ovine theileriosis (Nazifi *et al*., 2011; Nazifi *et al*., 2013). Our study was designed to obtain more detailed information on the status of erythrocytic antioxidant mechanisms in malignant ovine theileriosis to find out the probable interrelationships among the mechanisms involved in erythrocytic damage during parasitemia. The remarkable reductions in the activity of erythrocytic antioxidant enzymes including SOD, GPX and catalase and also the strong negative correlations between the parasitemia rate and the activities of such enzymes indicate an increased exposure of RBCs to oxidative stress products. Thus, it can be suggested that the invasion of RBCs by the parasites could markedly affect key antioxidant defense barriers, resulting in significant RBC damage and finally leading to extravascular hemolysis. Our data corroborate the previous studies on bovine tropical theileriosis (Rezaei and Dalir-Naghadeh, 2006; Nazifi *et al*., 2009), but Grewal *et al*. (2005) reported that the GPX activity was significantly elevated whereas the enzymes SOD and catalase showed no remarkable changes in cattle infected with *T. annulata*. They concluded that the increased level of GPX during the infection could be due to the fact that this enzyme activity is the major mechanism for the intracellular destruction of lipid peroxides rather than SOD or catalase activity, while our findings showed that, parallel to the role of GPX, the activities of SOD and catalase could also be important factors for RBCs to scavenge the oxidant agents during the parasitemia.

According to the literature, MDA is a reliable biomarker for assessing lipid peroxidation that takes place in the cell membrane (Moore and Roberts, 1998). Our data confirmed a significantly increased lipid peroxidation in the RBC membrane in the infected sheep, particularly in the highly infected animals. In the present study, higher levels of lipid peroxidation in the infected animals confirmed that erythrocytes encounter an oxidative shock during parasitemia. This situation may imply that the parasites can disturb RBC antioxidant mechanisms during infection and so erythrocytic damage could relate to the lipid peroxidation (Friedman, 1979). In addition, MDA, a highly reactive bifunctional molecule, has been shown to be able to cross-link RBC phospholipids and proteins, affecting membrane fluidity and impairing various membrane functions, which causes a decrease in RBC survival (Sugihara *et al*., 1991). Consequently, this process can reduced membrane symmetry and increased membrane permeability; leading to morphological changes in the RBC cell surface (Saluja *et al*., 1999). Thus, it can be argued that the significant negative correlation between the activity of SOD, GPX and catalase and the lipid peroxidation of RBCs in the infected animals showed that the membrane lipid peroxidation of RBCs can play a considerable role in the loss of membrane fluidity and permeability due to disrupted antioxidant activities

during parasite invasion of erythrocytes. The significant correlations between hematological parameters (RBC count, PCV and the hemoglobin concentration) and the activity of antioxidant enzymes support the hypothesis that the oxidative damage of erythrocyte could play an important role in the development of anemia in malignant ovine theileriosis. In the present study, no statistical difference was evident in the concentrations of the antioxidant vitamins (A, E and C) between the healthy and diseased sheep. Although there are no documented reports on the status of antioxidant vitamins in malignant ovine theileriosis, some studies on other hemoparasitic diseases (such as babesiosis) showed decreased serum levels of such vitamins in infected animals and this phenomenon may be a consequence of oxidative damage (Deger *et al*., 2009; Bicek *et al*., 2005; Dede *et al*., 2002). It can be proposed that despite being evident that antioxidant systems comprised of vitamins have a cellular protective action against oxidative stress (Dede *et al*., 2000), the unchanged level of antioxidant vitamins in our work may be attributed to the occurrence of an equilibrium between the level of vitamin uptake in the diet and the level in the serum and body tissues, particularly the liver storage. On the other hand, it can be postulated that such vitamins unlikely to elicit effective responses to the oxidative damage during ovine malignant theileriosis.

This study represents the impact of natural theileriosis on some blood parameters in sheep. It can be stated that *Theileria hirci* can trigger mechanisms to enhance the level of homocysteine. Increased production of homocysteine (hyperhomocysteinemia) has been proven to be involved in cardiovascular diseases (Arnesen *et al*., 1995) or endothelial cell damage (Harker *et al*., 1983). Marked rises were seen in the concentration of plasma homocysteine during parasitemia in *Theileria* infected sheep. Homocysteine is a highly reactive amino acid derived from methionine metabolism and is known to produce endothelial cell injury in experimental animals (Harker *et al*., 1983) and cell culture (Wall *et al*., 1980). Elevated total plasma homocysteine (tHcy) has been stated as an independent risk factor for peripheral vascular, cerebrovascular and coronary artery disease (Boushey *et al*., 1995; Nygard *et al*., 1997). Although, there have been no documented reports on homocysteine changes in blood parasites of animals, our results suggest both hyperhomocysteinemia and anemia are positively associated with the *Theileria* infection. Our study inferred that the anemia is a main manifestation of ovine theileriosis. Significant increase in the plasma homocysteine during parasitemia could be assigned as a risk factor for probable endothelial injuries and thus help to develop the anemia.

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