

EFFECT OF THE SEVERITY OF *THEILERIA ANNULATA*
INFECTION ON SOME HAEMATOLOGICAL PARAMETERS
AND ANTIOXIDANT ENZYMES IN NATURALLY
INFECTED CATTLE

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Summary

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This study was conducted to assess the effect of the severity of *Theileria annulata* infection on some haematological parameters and antioxidant enzymes in naturally infected cattle. The diseased group comprised 50 dairy Holstein cattle, 2–3 years old, naturally infected with *Theileria annulata* that was further divided into 4 subgroups according to parasitaemia rates (<1%, 1–3%, 3–5%, >5%). As a control group, 20 non-infected cattle were also investigated. Blood samples were obtained for measuring haematological parameters and activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX). There were significant differences in red blood cell counts (RBCs), packed cell volume (PCV), concentration of haemoglobin and methaemoglobin and activities of SOD and GPX between healthy cattle and those infected with *Theileria annulata* ($P < 0.05$). As parasitaemia increased in infected cattle, a significant decrease was observed in RBCs ($r = -0.934$, $P < 0.01$), PCV ($r = -0.955$, $P < 0.01$), haemoglobin concentration ($r = -0.950$, $P < 0.01$), and in SOD ($r = -0.962$, $P < 0.01$) and GPX ($r = -0.846$, $P < 0.01$) activities. In contrast, with increase in parasitaemia, a significant elevation in mean corpuscular volume (MCV) ($r = 0.466$, $P < 0.01$), mean corpuscular haemoglobin (MCH) ($r = 0.430$, $P < 0.01$) and concentration of methaemoglobin ($r = 0.944$, $P < 0.01$) was detected.

Key words: cattle, haematological parameters, oxidative stress, *Theileria annulata*, tropical theileriosis

INTRODUCTION

Tick-borne diseases are most prevalent, and of greatest impact in the tropical and subtropical regions (Bram, 1983; Minjauw & McLeod, 2003). Theileriosis is among the most important of these diseases. Tropical theileriosis is one of the commonest and economically important fatal diseases of cattle in Iran (Hashemi-Fesharki, 1988). It is a progressive lymphoproliferative disease of cattle caused

by the protozoan parasite *Theileria annulata* (Omer *et al.*, 2003a, b; Taylor *et al.*, 1992). The parasite is a serious constraint to cattle production in endemic areas causing lethal infections in exotic cattle and considerable mortality in indigenous and crossbred stock (Forsyth *et al.*, 1997). The disease is observed in south Europe, North Africa, middle and south Asia and the Middle East and threatens approxi-

mately 250 million cattle at all (Uilenberg, 1981; Young, 1981).

A significant feature of the disease is haemolytic anemia (Gill *et al.*, 1977; Aulakh *et al.*, 1998; Omer *et al.*, 2002) caused by an immune-mediated haemolysis which is indicated by the presence of a haemagglutinin (Hooshmand-Rad, 1976). Although various evidence have been presented to explain the mechanism of the anaemia, the exact underlying mechanism is currently unknown (Shiono *et al.*, 2004). It is suggested that the activity of antioxidant enzymes such as superoxide dismutase (SOD) is affected by the parasite and results in increased fragility of RBCs and thus, acceleration of erythrocyte clearance by phagocytic cells (Shiono *et al.*, 2003b). Shiono *et al.* (2003a) investigated the relationship between oxidative bursts of peripheral blood phagocytes (neutrophils and monocytes) and the oxidation of RBCs and the development of anaemia in cattle, experimentally infected with *Theileria sergenti*. Oxidating agents are responsible for the conversion of haemoglobin to methaemoglobin that results in decreased O₂ transmission. Yagi *et al.* (1989) studied the abnormality of osmotic fragility and morphological disorders of bovine erythrocytes infected with *Theileria sergenti*. Hiroki *et al.* (2001) reported that oxidative damage and loss of membrane symmetry in RBCs are related to the development of anaemia in *Theileria sergenti* infection. The oxidative damage to the RBCs might play an important role in the pathogenesis of anaemia in bovine theileriosis. Grewal *et al.* (2005) evaluated the status of erythrocyte lipid peroxidation, activities of some antioxidant enzymes and osmotic fragility of RBC in cattle naturally infected with *Theileria annulata*. Asri Rezaei & Dalir-Naghadeh (2006) reported that oxidative damage to

RBCs may contribute to the pathogenesis of anaemia in bovine tropical theileriosis. Nazifi *et al.* (2008) reported that with increasing of parasitaemia rate in cattle infected with *Theileria annulata*, an increase in erythrocyte osmotic fragility and a decrease in SOD activity occurred.

There is little information about oxidative damage to RBCs and resulting anaemia in theileriosis. The autoimmune mechanism has a considerable role in extravascular haemolysis in theileriosis. However, it is probable that other factors such as oxidating agents play role in anemia. This study was conducted to assess the effect of the severity of *Theileria annulata* infection on some haematological parameters and antioxidant enzymes in naturally infected cattle.

MATERIALS AND METHODS

Animals

This study was carried out in the southwest region of Iran (Fars province) where theileriosis due to *Theileria annulata* is prevalent. The diseased group comprised 50 dairy Holstein cattle, 2–3 years old, naturally infected with *Theileria annulata*, divided into 4 subgroups with different parasitemia rates (<1%, 1–3%, 3–5%, >5%). As a control group, 20 non-infected cattle were also sampled.

Theileria annulata identification and parasitaemia rates

The animals had not been treated for disease prior to sampling and were screened for other potential causes of anaemia by determination of haematological parameters, epidemiological ground, clinical signs and biochemical and microbiological tests and were sampled once only in the course of the disease. Blood samples

were taken from the jugular vein into vacutainers containing EDTA for measuring haematological parameters and with heparin for measurement of methaemoglobin and activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX). Thin blood smears were prepared, fixed with absolute methanol (5 min), stained with 10% Giemsa solution (30 min) and examined under oil immersion ($\times 1000$) to observe intraerythrocytic forms of *Theileria annulata*. Identity of *T. annulata* was determined on morphological, clinical and epidemiological grounds. After examining more than 50 microscopic fields of blood films at a magnification of $\times 1000$, the parasitaemia rate was quantified and expressed as percentage of infected erythrocytes (Shiono *et al.*, 2003a).

Blood parameters

Haematological parameters: red blood cell counts (RBCs), packed cell volume (PCV), haemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were measured by routine procedures (Jain, 1986). SOD activity was measured with a commercial kit (RANSOD kit, Randox Com, UK). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity was then determined by the degree of inhibition of this reaction. One unit of SOD corresponded to 50% inhibition of INT reduction under the conditions of the assay. GPX was measured by means of a commercial kit (RANSEL kit, Randox Com, UK) based on the method of Paglia & Valentine (1967). GPX catalyses the

oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidised glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The decrease in absorbance was measured at 340 nm. Measurement of methaemoglobin was carried out by the method of Makerem (Chanarin, 1989).

Statistical analysis

Student's t-test was used for comparison between 2 groups (control and diseased cattle). Analysis of variance (ANOVA) and Tukey tests were used for comparisons carried out in subgroups with different parasitaemia rates and Pearson's correlation coefficients were calculated to determine relationships among parameters at different parasitaemia rates. All values were expressed as mean and standard error of mean (SEM), and $P < 0.05$ was considered as statistically significant.

RESULTS

The values of haematological parameters and methaemoglobin concentrations in healthy cattle and cattle infected with *Theileria annulata* at different parasitaemia rate are presented in Table 1. The activities of GPX and SOD are illustrated in Fig. 1 and 2, respectively. There were significant differences in RBCs, PCV, concentration of haemoglobin and methaemoglobin and activities of SOD and GPX between healthy cattle and those infected with *Theileria annulata* with different parasitaemia rate ($P < 0.05$). As parasitaemia increased in infected cattle, a significant decrease was observed in RBCs ($r = -0.934$, $P < 0.01$), PCV ($r = -0.955$, $P < 0.01$), haemoglobin concentration ($r = -0.950$, $P < 0.01$) and activities of SOD

Table 1. Mean \pm SEM of red blood cell counts (RBC), packed cell volume (PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and methemoglobin (MetHb) concentration in uninfected cattle and cattle infected with *Theileria annulata* with different parasitemia rates

	Parasi- taemia, %	MetHb, %	RBC, 10 ¹² /L	PCV, L/L	Hb, g/L	MCV, fl	MCH, pg	MCHC, g/L
Cont- rol	0 (n=20)	0.82 ± 0.11 a	6.64 ± 0.23 a	0.29 ± 0.01 a	99.10 ± 5.40 a	43.84 ± 0.97 a	14.83 ± 0.31 a	338.5 ± 2.0 a
	<1 (n=18)	2.42 ± 0.09 b	5.84 ± 0.11 b	0.25 ± 0.00 b	86.10 ± 0.90 b	43.37 ± 0.46 a	14.76 ± 0.14 a	340.5 ± 0.8 a
Diseased	1-3 (n=14)	3.39 ± 0.10 c	5.16 ± 0.13 c	0.22 ± 0.00 c	75.60 ± 0.90 c	42.82 ± 0.75 a	14.71 ± 0.24 a	343.7 ± 0.9 ab
	3-5 (n=10)	4.26 ± 0.06 d	3.95 ± 0.07 d	0.19 ± 0.00 c	66.20 ± 0.80 d	48.81 ± 0.56 b	16.74 ± 0.22 b	342.9 ± 1.4 a
	>5 (n=8)	5.02 ± 0.07 e	2.52 ± 0.09 e	0.11 ± 0.00 d	40.30 ± 0.40 e	45.81 ± 1.17 ab	16.11 ± 0.51 b	351.4 ± 5.1 b

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

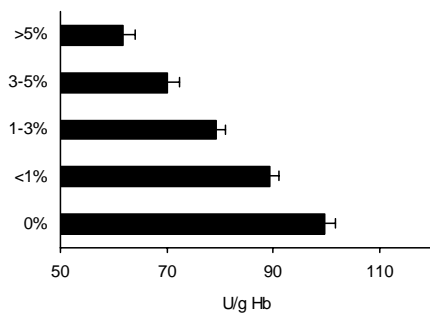


Fig. 1. Activity of glutathione peroxidase (U/g Hb) in different parasitaemia rates (%) in *T. annulata*-infected cattle.

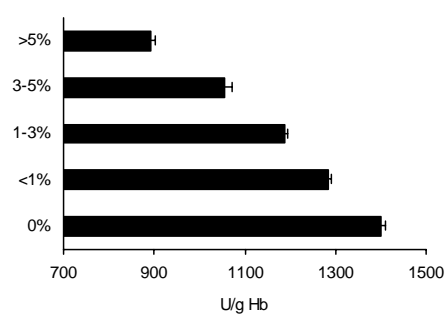


Fig. 2. Activity of superoxide dismutase (U/g Hb) in different parasitaemia rates (%) in *T. annulata*-infected cattle.

($r = -0.962$, $P < 0.01$) and GPX ($r = -0.846$, $P < 0.01$). In contrast, with increase in parasitaemia, a significant increase in MCV ($r = 0.466$, $P < 0.01$), MCHC ($r = 0.430$, $P < 0.01$) and methaemoglobin ($r = 0.944$, $P < 0.01$) was detected.

DISCUSSION

In the present study, the number of RBCs, PCV and haemoglobin level in cattle with

theileriosis were significantly lower in comparison with healthy ones ($P < 0.05$). Also, with progression of the severity of parasitaemia, a significant decrease in RBCs, PCV and haemoglobin was observed. Most obviously, the severity of the anaemia increased as parasitaemia progressed. The decline in RBCs, PCV and haemoglobin may be attributed to destruction of erythrocytes by macrophages in the lymph nodes, spleen and

other organs of the monocyte-macrophage system (Singh *et al.*, 2001). Sandhu *et al.* (1998) evaluated haematological and biochemical factors in experimental *Theileria annulata* infection in crossbred calves. They reported a significant progressive decrease in haemoglobin concentration, PCV and RBCs, whereas the total leukocyte count showed an initial non-significant leukocytosis followed by a significant leukopaenia. Dhar & Gautam (1979), Sharma (1979), Rayula & Hafeez (1995) and Mehta *et al.* (1988) reported a progressive decrease in haemoglobin, PCV and RBCs in acute *Theileria annulata* infection. A progressive decrease in the haemoglobin and PCV, along with a marked reticulocytosis was also reported by Singh *et al.* (2001).

As parasitaemia increased, the observed elevation in MCV and MCHC indicated a macrocytic hyperchromic anaemia. Macrocytosis, polychromasia and basophilic stippling in blood smears indicated a regenerative anaemia in infected animals. Regenerative anaemia is accompanied by the presence of reticulocytes which explains the elevation of MCV, and increase in MCHC may be due to extravascular haemolysis of infected erythrocytes. Stockham *et al.* (2000) reported a macrocytic normochromic anaemia in cattle infected with *Theileria buffeli*. Yagi *et al.* (1989) reported that by increasing parasitaemia in calves infected with *Theileria sergenti*, the osmotic fragility of the erythrocytes and haemolysis increased significantly. Abnormality of osmotic fragility and morphological disorders of erythrocytes occurred not only in parasitized erythrocytes but also in intact ones in *Theileria sergenti* infection. Mbassa *et al.* (1994) reported that haematopoietic precursor cells in bone marrow were attacked by *Theileria parva* merozoites resulting in erythroid hypopla-

sia and severe anaemia. In another study, Yagi *et al.* (1991) stated a significant decrease in lifespan of parasitized and non-parasitized erythrocytes in cattle, infected by *Theileria sergenti*. Hooshmand-Rad (1976) reported an immune-mediated haemolysis and a kind of haemagglutinin in calves, infected with *Theileria annulata*. Shizonts induced an indirect effect on RBCs resulting in production of haemagglutinin antibody. The RBCs exposed to antibodies are changed to spherocytes whose presence indicated marked immune-mediated hemolysis (Stockham & Scott, 2002; Latimer *et al.*, 2003).

In the present study, as the parasitaemia increased, a progression in the severity of anaemia was found out as well as in haemolysis, which resulted from increased RBC fragility and decreased SOD and GPX activities. As parasitaemia progressed, an increase in morphological alterations of RBCs and abnormal RBCs forms such as spherocytes with more fragile membrane in peripheral blood did occur. Spherocytes are phagocytosed too rapidly by the spleen to be detectable in blood smears of infected cattle (Stockham *et al.*, 2002). The presence of other abnormalities in erythrocytes' shape such as basophilic stippling, Howell Jolly body and macrocytes, indicate the increase of haematopoiesis and bone marrow reaction to anaemia (Stockham *et al.*, 2002; Latimer *et al.*, 2003).

In the present study, as parasitaemia progressed, the activities of SOD and GPX were significantly decreased that indicated increased haemolysis and increased exposure of RBCs to oxidative stress products, with consequent loss of the symmetrical structure of lipids in cell membrane, decrease in flexibility, disturbance in water and ion exchange, swallowing and destruction of RBC (Glass & Gershon, 1984; Edwards & Fuller, 1996).

The fragility of RBCs exposed to oxidants increases and results in acceleration of RBC clearance by phagocytosis. Also, decreased SOD and GPX induce changes in haemoglobin structure that leads to decrease in O₂ transmission (Vacha, 1983). There is a positive correlation between oxidative stress and RBC life cycle. The more oxidative stress affects the RBCs, the less GPX and SOD could play their antioxidant role (Saik *et al.*, 1982).

SOD and GPX are responsible for RBC protection whereby free O₂ radicals are converted to hydrogen peroxide by SOD and hydrogen peroxide gets catabolized by GPX (Edwards & Fuller, 1996). Grewal *et al.* (2005) evaluated the status of RBC lipid peroxidation, activities of some antioxidant enzymes and osmotic fragility of RBC in cattle naturally infected with *Theileria annulata*. Diseased cattle exhibited a higher erythrocytic lipid peroxidation and osmotic fragility. Activities of antioxidant enzymes, viz. glucose 6-phosphate dehydrogenase (G6PD) and GPX were also significantly increased in clinical cases. However, SOD and catalase did not show significant changes. Infection with *Theileria* led to increased oxidative stress in animals, and even a significant rise in the activities of antioxidant enzymes. G6PD and GPX could not lower this oxidative stress. However, the increase in the activities of antioxidant enzymes pointed towards the body's defense mechanism against lipid peroxidation during oxidative stress in theileriosis. Shiono *et al.* (2003) found that oxidative damage and asymmetry in RBC membrane lipid had a significant correlation with progression of anaemia in infection with *Theileria sergenti*. Asri Rezaei & Dalir-Naghadeh (2006) reported that with increase in parasitaemia and severity of anaemia, lipid peroxidation in RBC membrane of infected cattle increased signifi-

cantly. However, the activities of antioxidant enzymes, SOD and GPX, showed a significant decrease. It was suggested that oxidative damage to RBCs may contribute to the pathogenesis of anaemia in bovine tropical theileriosis. Nazifi *et al.* (2008) reported that by increasing parasitaemia rate, in cattle infected with *Theileria annulata*, an increase in severity of anaemia, erythrocyte osmotic fragility and lactate dehydrogenase (LDH) activity and a decrease in SOD activity occurred. In this study as parasitaemia progressed, a significant increase in methaemoglobin was observed. Hiroki *et al.* (2001) reported a significant negative correlation between methaemoglobin and PCV in theileriosis. Haemoglobin oxidation can be considered as a factor that increases the severity of anaemia in *Theileria sergenti* infection. Shiono *et al.* (2003) found that a positive correlation between H₂O₂ production of phagocytes and each of the oxidative indices of methaemoglobin and malondialdehyde (MDA) during the onset of anaemia. The level of antioxidants, namely reduced glutathione and G6PD in RBC also decreased with the progression of anaemia. It was suggested that oxidative damage of RBC was closely related to the onset of anaemia in bovine theileriosis, and that the oxidative burst of phagocytes may play an important role in the pathogenesis of anaemia in tropical theileriosis.

In conclusion, as parasitaemia progressed in theileriosis, an increased severity of anaemia and haemolysis were established. Also, with increase in parasitaemia, a significant decrease in activities of the antioxidant enzymes SOD and GPX and significant increase in concentration of methaemoglobin were observed. These results reveal that antioxidant mechanisms that protect RBC against oxidative damage can be dis-

turbed by infection with *Theileria annulata*, that induces lipid peroxidation in RBC membrane and anaemia in infected cattle.

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