

## Malignant Ovine Theileriosis: Alterations in the Levels of Homocysteine, Thyroid Hormones and Serum Trace Elements

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### Abstract

**Introduction:** Malignant ovine theileriosis is a fatal disease of sheep caused by the pathogenic species of protozoans of the genus *Theileria*. This study was designed to estimate the levels of plasma homocysteine (Hcy), serum thyroid hormones, the serum trace elements and to evaluate their correlations in different parasitemia rates in naturally *Theileria* infected sheep.

**Materials and Methods:** 50 Iranian sheep, about 1-2 years old, naturally infected with *T. lestoquardi* were selected and divided into 2 subgroups according to parasitemia rates (<2%, 2-4%). 10 non-infected animals were also selected as controls. Blood samples were collected and Hcy, thyroid hormones and major trace elements were measured.

**Results:** Significant decrease in the values of red blood cell count (RBC), packed cell volume (PCV) and hemoglobin confirmed that anaemia had occurred in the infected sheep. Significant increase in Hcy and some trace elements (Mn, Fe and Zn), significant decrease in the values of thyroxine (T<sub>4</sub>) and free tri-iodothyronine (fT<sub>3</sub>) were observed.

**Conclusion:** Substantial elevations in plasma Hcy can potentially produce endothelial injuries and consequently help the formation of anaemia. On the other hand, significant decrease in T<sub>4</sub> and fT<sub>3</sub> and increase in some trace elements (Mn, Fe and Zn) besides the lack of any changes in the other related factors, indicate that the infection of sheep with lower than 4% parasitemia rates, can induce negative effects on the secretion and concentrations of thyroid hormones, but the infection could not cause reverse effects on important trace elements.

**Keywords:** Malignant ovine theileriosis; Homocystein; Thyroid hormones; Trace elements

### 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* [1] and the two newly described *Theileria* sp. in China (China 1 and China 2) [2]. The disease has been reported from North Africa, Southern Europe, Asia Minor and India [3], and has been considered in Iran since long time ago, as a fatal disease of sheep and goats that imposes heavy losses due to mortality and decreased production in affected animals [4].

Homocysteine (Hcy) is a highly reactive thiol-containing amino acid, produced by the intracellular demethylation of methionine. Endothelial cell injury in experimental animals [5] and also cardiovascular diseases in human [6], have been attributed to the alterations in the concentration of plasma Hcy. Increased Hcy could also exert pathological effects by promoting oxidative stress [7], which has been indicated as a mechanism involved in the formation of anaemia. Although, it is clear that the invasion of piroplasms of *Theileria* species on erythrocytes could induce anemia [8], the probable correlations of hyper and/or hypo-homocysteinemia with the occurred anaemia have not been clearly described.

Thyroid hormones have great impact on basic metabolic rate and are anabolic in physiological quantities. In theileriosis, schizont-infected cells may disseminate through the lymphoid tissues into thyroid glands and cause damage [9]. On the other hand, there are reports that changes in some of the micronutrients (selenium, copper, cobalt, zinc, manganese, and iodine) affect thyroid function [10-12]. In addition, recent studies evidenced that the decrease in the serum trace elements during ovine theileriosis, could interfere with the protective antioxidant mechanisms of RBCs [13], resulting in the anaemia.

This study was therefore designed, to estimate the levels of plasma Hcy, serum thyroid hormones and the levels of the serum trace elements, and also to evaluate their correlations in different parasitemia rates, in naturally *Theileria* infected sheep.

### Materials and Methods

#### Animals and samples

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

#### Hematological and parasitological measurements

Blood samples were collected from jugular vein into EDTA containing tubes for measuring hematological parameters and Hcy,

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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 ( $\times 1,000$ ), to observe intraerythrocytic forms of *T. lestoquardi*. Piroplasm parasitemia (parasited RBC rate) was also quantified by examination of at least  $1 \times 10^4$  RBC, at a magnification of  $\times 1000$  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. Hematological parameters were measured by routine standard procedures [8].

### Hcy and thyroid hormones

The blood samples were centrifuged at 1,200 g for 10 minutes 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). Triiodothyronine ( $T_3$ ), thyroxine ( $T_4$ ), free  $T_3$  ( $fT_3$ ) and free  $T_4$  ( $fT_4$ ) levels were measured in the sera specimens by radioimmunoassay kits  $T_3$  [ $^{125}I$ ],  $T_4$  [ $^{125}I$ ],  $fT_3$  [ $^{125}I$ ],  $fT_4$  [ $^{125}I$ ] (Izotop Co. Budapest, Hungary).

### Trace elements measurement

Blood samples were collected into vacutainers and serum was separated by centrifugation at 750 g for 15 min and stored at -20°C, until use. The samples with haemolysis were discarded. Digestion of serum was performed by a mixture of perchloric and nitric acid (3:7 ratios respectively). Manganese, copper, iron, selenium and zinc were measured, using an atomic absorption spectrophotometer (Shimadzu AA-670, Kyoto, 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 between 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

### Hematological parameters

The values of hematological parameters in non-infected sheep and those naturally infected with *T. lestoquardi* 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 anaemia in infected group. In addition, with the increase in the level of parasitemia, marked decrease was observed

	Parasitemia, %	RBC $\times 10^{12}/L$	PCV L/L	Hb g/dL	WBC $\times 10^9/L$	Neutrophil $\times 10^9/L$	Lymphocyte $\times 10^9/L$
Control	0 (n=10)	6.03 <sup>a</sup> $\pm 1.26$	0.348 <sup>a</sup> $\pm 0.008$	12.20 <sup>a</sup> $\pm 0.25$	10.45 <sup>a</sup> $\pm 1.03$	8.36 <sup>a</sup> $\pm 2.61$	2.04 <sup>a</sup> $\pm 0.51$
Diseased	<2 (n=23)	3.79 <sup>b</sup> $\pm 1.18$	0.215 <sup>b</sup> $\pm 0.007$	7.78 <sup>b</sup> $\pm 0.23$	9.42 <sup>a</sup> $\pm 0.90$	6.64 <sup>b</sup> $\pm 1.55$	2.57 <sup>ab</sup> $\pm 0.15$
	2-4 (n=16)	2.21 <sup>c</sup> $\pm 0.8$	0.12 <sup>c</sup> $\pm 0.005$	4.60 <sup>c</sup> $\pm 0.16$	9.59 <sup>a</sup> $\pm 1.07$	5.75 <sup>b</sup> $\pm 2.15$	3.67 <sup>c</sup> $\pm 0.99$

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

**Table 1:** Mean  $\pm$  SEM of hematological parameters in non-infected sheep and those infected with *Theileria lestoquardi* with different parasitemia rates.

	Parasitemia, %	Homocyst. $\mu\text{mol/L}$	$T_3$ nmol/L	$T_4$ $\mu\text{g/dL}$	$fT_3$ pg/mL	$fT_4$ ng/dL
Control	0 (n=10)	7.29 <sup>a</sup> $\pm 0.54$	0.73 <sup>a</sup> $\pm 0.12$	4.32 <sup>a</sup> $\pm 0.30$	4.76 <sup>a</sup> $\pm 0.31$	1.29 <sup>a</sup> $\pm 0.05$
Diseased	<2 (n=36)	12.11 <sup>b</sup> $\pm 0.50$	0.71 <sup>a</sup> $\pm 0.07$	1.78 <sup>b</sup> $\pm 0.20$	2.42 <sup>b</sup> $\pm 0.22$	1.18 <sup>a</sup> $\pm 0.17$
	2-4 (n=14)	11.10 <sup>b</sup> $\pm 0.57$	0.58 <sup>a</sup> $\pm 0.9$	1.18 <sup>b</sup> $\pm 0.22$	2.43 <sup>b</sup> $\pm 0.20$	0.94 <sup>a</sup> $\pm 0.10$

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

**Table 2:** Mean  $\pm$  SEM of the concentrations of homocysteine and thyroid hormones in non- infected and infected sheep.

	Parasitemia, %	Homocyst. $\mu\text{mol/L}$	$T_3$ nmol/L	$T_4$ $\mu\text{g/dL}$	$fT_3$ pg/mL	$fT_4$ ng/dL
Control	0 (n=10)	7.29 <sup>a</sup> $\pm 0.54$	0.73 <sup>a</sup> $\pm 0.12$	4.32 <sup>a</sup> $\pm 0.30$	4.76 <sup>a</sup> $\pm 0.31$	1.29 <sup>a</sup> $\pm 0.05$
Diseased	<2 (n=36)	12.11 <sup>b</sup> $\pm 0.50$	0.71 <sup>a</sup> $\pm 0.07$	1.78 <sup>b</sup> $\pm 0.20$	2.42 <sup>b</sup> $\pm 0.22$	1.18 <sup>a</sup> $\pm 0.17$
	2-4 (n=14)	11.10 <sup>b</sup> $\pm 0.57$	0.58 <sup>a</sup> $\pm 0.9$	1.18 <sup>b</sup> $\pm 0.22$	2.43 <sup>b</sup> $\pm 0.20$	0.94 <sup>a</sup> $\pm 0.10$

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

**Table 3:** Mean  $\pm$  SEM of the serum trace elements in non-infected and infected sheep.

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. On the contrary, no substantial changes were found in white blood cell (WBC) count between the control and the infected groups. In addition, differential analysis of WBCs in non-infected and *Theileria* infected sheep, proved substantial decrease in the rate of neutrophils in the diseased groups relative to the controls; however, lymphocytes evidenced significant elevations in the diseased animals.

### Homocysteine and thyroid hormones

The alterations in the concentrations of homocysteine and thyroid hormones, 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 sheep suffering from theileriosis. Also, our data demonstrated the increasing severity of parasitemia coupled with the elevation in the level of homocysteine, in the diseased sheep ( $r = 0.34, P < 0.05$ ). In addition, correlation analysis represented remarkable negative correlations between the values of homocysteine and serum  $T_3$  ( $r = -0.28, P < 0.05$ ) and  $T_4$  ( $r = -0.36, P < 0.05$ ).

Among serum thyroid hormones,  $T_4$  and  $fT_3$  evidenced remarkable decreases in the infected sheep; however, no apparent alterations were seen in  $T_3$  and  $fT_4$  during different levels of parasitemia in infected sheep, compared to the controls.

### Trace elements

The concentrations of the serum trace elements related to the infected and non-infected sheep are compared in Table 3. Accordingly, serum levels of manganese, iron and zinc increased significantly in the infected sheep ( $P < 0.05$ ), however, the serum concentration of copper and selenium showed no significant changes. In addition, trace elements showed no significant difference with the progression of parasitemia in the diseased groups. Moreover, despite the significant relationship between the concentrations of zinc and  $fT_3$  ( $r = -0.28, P < 0.05$ ), no

substantial correlations were seen among trace elements, homocysteine and thyroid hormones.

## Discussion

This study represents the impact of natural theileriosis on some blood parameters in sheep. It can be stated that *Theileria lestoquardi* can trigger mechanisms to enhance the level of homocysteine, and some trace elements (manganese, iron and zinc) and alleviate thyroid hormones ( $T_4$  and  $fT_3$ ). Increase in the production of homocysteine (hyperhomocysteinemia), has been proven to be involved in cardiovascular diseases [6] or endothelial cell damage [5].

Significant decreases in hematological parameters including RBC count, PCV and hemoglobin (anaemia) were confirmed in the infected sheep. This finding is in line with the cases discussed in bovine [14,15] and ovine theileriosis [13], however, 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 [16]. One recent hypothesis indicates that disturbed antioxidant defense mechanisms, can promote the development of anemia in ovine theileriosis [13,17].

Marked enhances were seen in the concentration of plasma homocysteine (hyperhomocysteinemia) 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 [5] and cell culture [18]. Elevated total plasma homocysteine (tHcy) has been stated as an independent risk factor for peripheral vascular, cerebrovascular and coronary artery disease [19,20]. Although, there have been no documented reports on homocysteine changes in blood parasites of animals, our results suggest both hyperhomocysteinemia and anaemia are positively associated with the *Theileria* infection.

In the present study, decrease in the level of  $T_4$  and  $fT_3$  along with lack of any considerable changes in concentration of  $T_3$  and  $fT_4$ , does fit with preceding investigations. Some reports stated that schizont-infected cells may disseminate through the lymphoid tissues into pituitary and thyroid glands, causing injury and reducing their secretions [9]. Also, it has been reported that following experimental [21,22] and natural [23] infections with *T. annulata* in cattle, thyroid hormones decreased significantly. It has been postulated that the lower level of  $T_3$  and  $T_4$ , could partly be due to the anorexia condition, prevailing in the disease. In addition to the occurrence of the anaemia in the diseased groups in our study, it can be concluded that the thyroid gland activity cannot compensate the probable transient decrease in its secretions in low parasitemia rates (lower than 4%). In other words, parasitemias lower than 4 percent, are likely capable of producing significant reverse effects on the thyroid activities.

Our data proved substantial rises in serum levels of manganese, iron and zinc in the *Theileria* infected sheep. Micronutrients such as zinc, manganese, copper and selenium are essential components of the body's antioxidant defense [24]. The copper-zinc SOD (Cu,Zn-SOD) and the manganese SOD (Mn-SOD), which are located in the cell cytosol [25] and the mitochondria [26], respectively, contribute to the first line of the antioxidant pathway. Thus, it can be postulated that *T. lestoquardi* in lower than four percent parasitemia rates, could not impose a negative effect on the status of mineral nutrients. Also, increasing the levels of manganese, iron and zinc in our study, represents their coordinated

antioxidant role accompanied by antioxidant enzymes activities, to prevent parasite-induced damages. In contrast with our results, preceding works reported deficiencies in trace element in naturally occurring bovine [15,27] and ovine [13] theileriosis. However, it seems that this discrepancy may be a consequence of excessive consumption of the important trace elements, to help the emerged antioxidant activity in the animals invaded with high (higher than 4%) parasitemia rates.

Our study inferred that the anaemia is a main characterization of ovine theileriosis. Marked increase in the plasma homocysteine (hyperhomocysteinemia) during parasitemia in infected animals could be assigned as a risk factor, for probable endothelial injuries and help to form the anaemia. In addition, in sheep with malignant theileriosis, significant elevations in some trace elements (manganese, iron and zinc) were coupled with the decrease in some thyroid hormones ( $T_4$  and  $fT_3$ ). These data imply that despite the infections with lower than four percent parasitemia rates in sheep could not make reverse effects on critical trace elements, but may have negative influences on the activity and concentrations of thyroid hormones.

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