Biochemical studies on crossbreed cattle infected with Theileria in New Valley;

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Abstract

Bovine tropical 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 Theileriosis in cattle. 39 crossbred cattle, about 1-3 years old, naturally infected with T. annulata were selected and divided into 3 subgroups according to parasitemia rates (<2%, 2-4%, 4-8%). 10 non-infected crossbred cattle were also selected as controls (clinical and laboratory healthy). Blood samples were collected and Hcy, thyroid hormones and major trace elements were measured.Significant decrease in the values of red blood cell count (RBC), packed cell volume (PCV) and hemoglobin confirmed that anemia had occurred in the infected cattle. Significant increase in Hcy, significant decrease in some trace elements (selenium and Zn), significant decrease in the values of thyroxine (T4) and (T4) but no remarkable changes in (FT3) and (FT4) were observed. Substantial elevations in plasma Hcy can potentially produce endothelial injuries and consequently help the formation of anemia. On the other hand, significant decrease in T4 and T3 and in some trace elements (selenium and Zn) besides the lack of any changes in the other related factors, indicate that the infection of cattle with different degree of parasitemia rates, can induce negative effects on the secretion and concentrations of thyroid hormones, some trace element but the infection could cause positive effects on important homocysteine.

Keyword ; *Crossbred cattle, Natural theileriosis, some blood parameters, New-Valley Governorate.*

Introduction;

Bovine Tropical theileriosis (BTT) is a tick-borne hem protozoan disease caused by *Theileria annulata which* is transmitted by tick of Hyalomma species. *Theileria annulata* is one of the most devastating blood parasite affecting cattle and causing serious economic losses through mortality and loss of productivity [1].Also causing devastating impact on small farmers which represent the majority of livestock owners in endemic areas and the methods currently used to protect against tropical theileriosis are expensive and all have serious limitations in efficacy and sustainability [2].

Theileria parasites infected the cattle during infected tick with sporozoitic form feeding on host body, which instantly enters in leukocytes of mononuclear lineage, where they got mature into macroschizont form which develop further into microschizonts and ultimately into merozoites, which are liberated from the mononuclear cells and invade erythrocytes and

develop into piroplasms, the prevalence, morbidity and mortality of theileriosis are considerably high [3].

The main symptoms shown by affected cattle are high fever and long lasting anemia due to intra-erythrocyte formation of piroplasms where the parasites become infected for tick [4, 5]. General debility, weight loss, anorexia, high body temperature, petechial hemorrhages on conjunctiva mucosa, enlarged lymph nodes, anemia and cough are the predominant clinical symptoms ,also the disease is lymph proliferative in its early phases resulting enlargement of lymph nodes and later on causing lymph destructive phase which is associated with pronounced leucopenia [6].

In theileriosis, schizont-infected cells disseminate through the lymphoid tissues into pituitary and thyroid glands and cause injury [7]. Moreover, the thyroid metabolism may deteriorate as a result of the decrease in oxygen transport due to anemia where these conditions affect the concentration of thyroid hormones [8].
Trace element (micronutrient) in biology, any chemical element required by living organisms in minute amounts (that is less than 0.1 percent by volume [1,000 parts per million]), usually as part of a vital enzyme (a cell-produced catalytic protein) ,where some changes in trace element following theileriosis [9,10]. Moreover [11, 12], they said that the changes in some microelements (selenium, copper, cobalt, zinc and manganese) affected thyroid function.

Thyroid hormones have great impact on basic metabolic rate and are anabolic in physiological quantities, working in conjunction with growth hormone and insulin where protein synthesis is stimulated and nitrogen excretion is reduced, in this way growth and metabolism and finally their production may be affected [13, 14], among domestic animals, thyroid function and its diseases are well known in companion animals but less so in livestock [15]. The importance of thyroid function and its diseases has also become progressively more important as the production of livestock has increased [13].
In theileriosis, schizont-infected cells disseminate through the lymphoid tissues into pituitary and thyroid glands and cause injury [7]. Following damage to these organs, their functions may alter and their secretions reduce. It has been reported that following experimental infection with *T. annulata*, thyroid hormones decreased significantly by day 20 [16].

Homocysteine is an intermediary amino acid which does not exist in proteins and formed by methionine metabolism [17, 18, 19], homocysteine is a sulfhydryl which contains amino acid produced by methionine demethylation [20,21], where methionine is an essential amino acid produced by breakdown of endogenous proteins and additionally by breakdown of diet proteins [18]. Homocysteine leads to pathological disorders due to lowering of nitric oxide, increasing thrombocyte and collagen amount for vascular endothelium and give rise to excessive production of homocysteine reactive molecules (Homocysteine -tiolahton) [22]. Homocysteine has been accepted as an independent risk factor for premature cardiovascular disease and is valuable for the diagnosis and follow up of cobalamin or folate deficiencies.

The present study was carried out to evaluate the relationship between homocysteine, thyroid hormones and some serum trace element in crossbred cattle suffering from theileriosis.

Materials and Methods';

Study area;

New Valley Governorate (Muḥāfẓet El Wādī El Ǧedīd) is one of the governorates of Egypt. It is located in the southwestern part of the country, in Egypt's Western Desert, part of the Sahara Desert – between the Nile, northern Sudan, and southeastern Libya. Consisting of roughly a third of Egypt's area, the New Valley Governorate is the country's largest governorate and one of the biggest on the African continent. The capital is at the Kharga Oasis.

Animals;

Forty nine crossbreed cattle of both sex, aged from 1-3 year,39 crossbreed cattle infected with theileria species and classified into three group according to the degree parasitemia % (+ < 2% parasitemia(n=13), ++ 2-4% parasitemia(n=13) and +++ 4-8% parasitemia(n=13) ,the rest number, Ten crossbred cattle were clinically and laboratory healthy and consider as a control group.

Samples;

1-Drop of blood from visible ear marginal vein to make three blood smear (thin and thick blood smear) from each animals, where the animal consider negative with three blood smear are negative.

2- Lymph sample from each animal to detect schizont stage in each animal.

3- 5 ml blood from jugular vein from each animal in vacutainer tube with EDTA as anticoagulant to carry of polymerase chain reaction (PCR) and hematological analysis.

4- 5 ml blood from jugular vein from each animal in vacutainer tube without, EDTA to carry of biochemical analysis.

Parasitological analysis;

1-**Direct smear**;

Thin and thick blood smears and lymph smear were prepared from each animal, left in air to dry and fixed in absolute methyl alcohol for 1 – 2 min and staining with freshly filtered and diluted 10% Giemsa stain for 30 – 45 minutes then washed with distal water to remove excess of stain, the Slides were left to dry, then put one drop of cider oil on the slide and examined under oil immersion lens at 1000× Magnification for the presence of Theileria piroplasms in blood smears and schizont stage in lymph smear[23,24].Animal can be considered negative if the three slides were negative.

2-**Quantitative evaluation of parasitemia;**

Percentage of infected RBCs was assessed by counting of the number of parasite erythrocytes present per 1000 cells at a magnification of×1000 then divided by ten and expressed as parasitemia percentage. Similarly, the deformed RBC were counted, and expressed as echinocytosis percentage [25].

3-**DNA Extraction from Blood;**

The DNA was extracted from each sample by chloroform/isoamyl extraction method (All buffers used according to [26]. Blood samples typically were obtained as 1 ml of whole blood stored in EDTA vacutainer tubes. To each 1 ml sample, add 0.8 ml 1X SSC (saline-sodium citrate) buffer, mix and centrifuge for 1 minute at 12,000 rpm in a micro-centrifuge tube. Remove 1 ml of the supernatant and discard into disinfectant. Add 1 ml of 1X SSC buffer, vortex and centrifuge as above for 1 minute, and remove all of the supernatant. Add 375 µl of 0.2M NaOAc to each pellet, vortex briefly and add 25 µl of 10% SDS and 5 µl of proteinase K (20 mg/ml H2O) (Sigma P-0390), vortex briefly and incubate for 1 hour at 55°C. Add 120 µl phenol/chloroform/ isoamyl alcohol and vortex for 30 seconds. Centrifuge the sample for 2 minutes at 12,000 rpm in a micro-centrifuge tube, carefully remove the aqueous layer to a new 1.5 ml micro-centrifuge tube, add 1 ml of cold 100% ethanol, mix, and incubate for 15 minutes at -20°C. Centrifuge for 2 minutes at 12,000 rpm in a micro-centrifuge. Decant the supernatant and add 180 µl 10:1 TE buffer, vortex, and incubate at 55°C for 10 minutes. Add 20 µl 2 M sodium acetate and mix. Add 500 µl of cold 100% ethanol, mix, and centrifuge for 1 minute at 12,000 rpm in a micro-centrifuge. Decant the supernatant and rinse the pellet with 1 ml of 80% ethanol. Centrifuge for 1 minute at 12,000 rpm in a micro-centrifuge. Decant the supernatant, and dry the pellet in a Speedy-vortex for 10 minutes (or until dry). Re-suspend the pellet by adding 200 µl of 10:1 TE buffer. Incubate overnight at 37C, vortex periodically to dissolve the genomic DNA. Store the samples at -20°C

4- **PCR amplification;**

Theileria annulata piroplasm DNA was purified from bovine blood with approximately 25 % parasitemia. Genomic DNA extracted with a Genomic DNA extraction kit (Accu Prep, BIONEER). Aliquots of extracted DNA were kept at 20 °C.PCR was performed using one set of primers (Table 1) (N516GTAACCTTTAAAAACGT 234–250, *T. annulata* specific and N517 GTTACGAACATGGGTTT 954–938, *T. annulata* specific) in a final reaction volume of 100 µl containing 50 m M KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 200 µM deoxynucleoside triphosphate, 2.5 U of *Taq* polymerase (Biozyme, England), 20 pmol of primers and 5 µl of template DNA. The reactions were performed in an automatic DNA thermal cycler (Biorad, USA) for 35 cycles. Each cycle consisted of a denaturing step of 1 min at 94 °C, an annealing step of 1 min at 55 °C or 1 min at and an extension step of 1 min at 72 °C.

5-**Hematological analysis;**

2.5 mL Blood mixed with EDTA of calved aged 1-10 days(groups a and b) used to determine erythrocyte count (ER), hemoglobin (HB), packed cell volume (PCV), Mean corpuscular volume (MCV),Mean corpuscular hemoglobin concentration (MCHC), total leukocyte counts (TLC) on an automatic full digital cell counter (Beckman, USA) . Giemsa-stained blood smears were used for differential leukocyte counts and detection of periplasm of parasites [27].

6- **Biochemical analysis;**

The blood samples were centrifuged at 1200 g for 10 minute at 37°C and the plasma 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 (T3), thyroxine (T4), free T3 (fT3) and free T4 (fT4) levels were measured in the sera specimens by radioimmunoassay kits T3 [125I], T4 [125I], fT3 [125I], fT4 [125I] (Izotop Co. Budapest, Hungary).

7-**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. Analyses were performed using SPSS software (SPSS Inc., Chicago, USA) version 11.5. All values in the tables were expressed as mean and Standard Error of Mean (SEM) and $p<0.05$ was considered as statistically significant.

RESULTS;

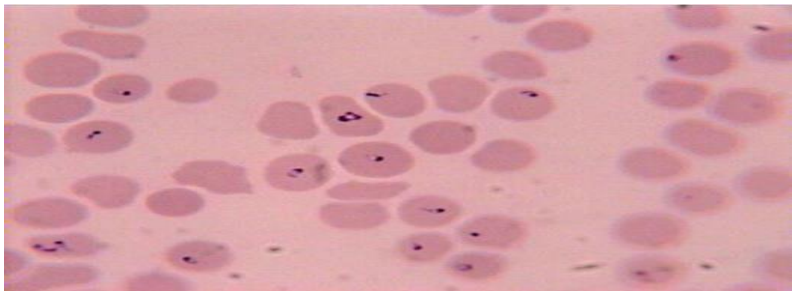

Figure 1.Bovine blood smear stained by giemsa stain showing erythrocytes periplasm.

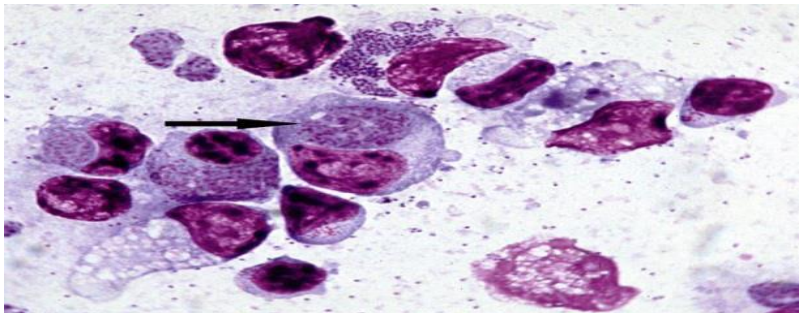

Figure 2.Lymph smear stained with Giemsa, showing theileria shizoint.

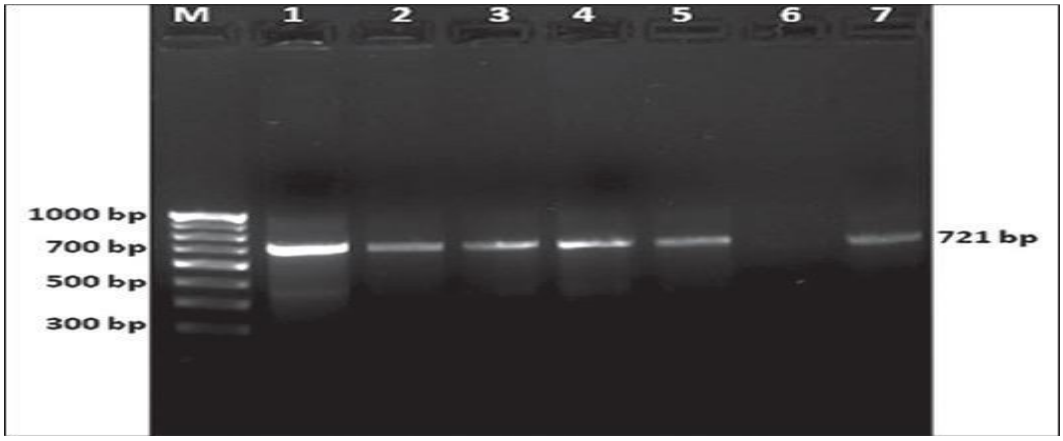

Figure 3; Detection of 721bp amplified DNA from T.annulata infected blood samples using direct blood polymeras chain reaction method and resolved in 1.5% agarose gel.M;100bp DNA ladder; Lane 1;positive control (where >80% RBCs were infected.

Primer	Sequence	Position	Amplified DNA fragment (bp)
N516	GTAACCTTTAAAAACGT	234–250	721
N517	GTTACGAACATGGGTTT	954–938	

Table 1.Oligonucleotide primers used in PCR.

Animals / Parameters	Control cattle	Infected cattle	
		Parsitemia (%)	Parameters
RBCs((×1012/ L)	6.14±0.23	+	5.21±0.11*↓
		+ +	4.15±0.18**↓
		+ + +	2.43±0.21***↓
PCV (%)	0.293±0.016	+	0.242±0.022*↓
		+ +	0.200±0.023**↓
		+ + +	0.140±0.021***↓
HB(g/l)	95.1±2.4	+	80.1±1.1*↓
		+ +	67.4±1.3**↓
		+ + +	44.1±1.7***↓
WBCs(×109 LG1)	5.49±0.38	+	4.84±0.62
		+ +	4.07±0.18
		+ + +	4.16±0.52
Neutrophil (%)	2.14±0.18	+	1.93±0.21*↓
		+ +	1.79±0.15*↓
		+ + +	1.68±0.18*↓
Lymphocytes (%)	4.02±0.21	+	4.15±0.2*↑
		+ +	4.28±0.13*↑
		+ + +	4.65±0.19*↑

Table 2 ; Mean and SEM of haematological parameters in control and infected cattle with Theileria annulata with defferent parasitemia rat (+=<2%,++=2-4%,+++=4-8% parasitemia ,n= number of animals).

+ < 2% parasitemia(n=13), ++ 2-4% parasitemia(n=13) and +++ 4-8% parasitemia(n=13)

Animal Parameters.	Control group	Infected group	
		Parasitemia %	Parameters
T3 (nmol/l)	1.11±0.07	+	0.84±0.05*↓
		+ +	0.76±0.09**↓
		+ + +	0.57±0.11**↓
T4 (nmol/l)	4.43±0.34	+	3.24±0.33*↓
		+ +	3.13±0.52**↓
		+ + +	3.03±0.45**↓
FT3 (ng/l)	1.28±0.18	+	1.18±0.21
		+ +	1.02±0.07
		+ + +	1.03±0.33
FT4 (ng/l)	3.4±1.14	+	3.22±0.59
		+ +	2.99±0.52
		+ + +	2.87±0.45
Homocysteine (µmol/l)	7.34±0.18	+	11.79±0.18*↑
		+ +	12.43±0.17**↑
		+ ++	12.51±0.21**↑

Table 3; Mean and standard error of the homocystein concentration and thyroid hormones in control and theileria infected cattle. *p<0.05; within each row, in each group, values differ significantly.

Animals Parameters.	Control group	Infected group	
		Parasitemia %	Parameters
Selenium(nmol/l)	349±17.4	+	278±19.4*↓
		++	258±17.4**↓
		+++	238±15.4**↓
Zinc (µmol/l)	15.1±3.1	+	10.6±2.9*↓
		++	10.38±38**↓
		+++	9.9±3.1**↓
Copper (µmol/l)	14.3±3.4	+	13.86±2.14
		++	13.91±2.16
		+++	13.52±2.1
Cobalt (µmol/l)	0.30±0.06	+	0.24±0.06
		++	0.23±0.015
		+++	0.22±0.11
Manganese(µmol/l)	3.5±0.12	+	3.4±0.13
		++	3.3±0.17
		+++	3.1±0.15

Table 4; Mean and standard errors of serum tracelement in control and infected cattle with theierleria.

*p<0.05; within each row, in each group, values differ significantly.

1-Parasitological analysis;

A-**Direct smear;**

- Direct blood and lymph smear carried on crossbred cattle in the study, revealed *Theileria* priplasme stage in erythrocytes(fig.1) and theileria shizont stage in lymph smear (fig,2) . Additionally thick and thin blood smear indicated different degree of parasitemia (+ < 2% parasitemia(n=13), ++ 2-4% parasitemia(n=13) and +++ 4-8% parasitemia(n=13).

B- **PCR amplification;**

PCR amplification test carried on crossbred cattle in the study revealed all selected animals were positive to *Theileria parasites* (fig.3).

C- **Parasitemia and erythrocyte morphology;**

Parasitemia detected in infected cattle ranged from < 2% parasitemia(n=13), ++ 2-4% parasitemia(n=13) and +++ 4-8% parasitemia(n=13), by direct thick and thin blood smear.

2- Hematological analysis;

The mean values of hematological parameters in control healthy cattle and those naturally infected with *T. annulata* with different parasitemia rates are shown in Table., 2.According to the presented values, our data depict remarkable declines in Red Blood Cells (RBCs),hemoglobin concentration (HB) and Packed Cell Volume (PCV) in infected cattle rather than controls ($p<0.05$). This result confirms the occurrence of anemia in infected group. In addition correlation analysis revealed that with the increase in the level of parasitemia, marked decreases were observed in RBC count, HB concentration and a PCV value, which means higher parasitemia levels, coincided with the higher degrees of anemia. On the other hand, no substantial change was found in WBC count between the control and infected groups but with differential leukocytes revealed significant decrease in neutrophils and significance increase in lymphocytes.

3-Biochemical analysis;

a- Homocysteine and thyroid hormones:

The variations occurred in the concentrations of homocysteine and thyroid hormones in the infected and healthy cattle are presented in Table., 3, where our study revealed a significant increase was evidenced in the level of homocysteine in all infected group compared to control group. Also, with an increase in the rate of parasitemia coupled with increase in the level of homocysteine in affected cattle. According to presented data serum thyroid hormones (T3, T4) had significant decrease alterations during different levels of parasitemia, no apparent alteration were seen in fT3 and fT4 during the different level of parasitemia in infected cattle compared to the control. Additionally, correlation analysis revealed negative correlation between homocysteine and T3 and T4.

b- Trace element levels;

Concentrations of the serum trace elements related to the infected and non-infected sheep are compared in Table, 4. Accordingly, serum levels of selenium and zinc decreased significantly in the infected cattle ($P<0.05$), however, the serum concentration of copper, Cobalt and Manganese 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, selenium and T3 and T4 ($P<0.05$), no substantial correlations were seen among trace elements, homocysteine.

Discussion;

Bovine tropical theileriosis is a serious hemo-protozoan disease of cattle in tropical and sub-tropical countries. The parasite acts as a serious constraint to cattle production in endemic areas, causing lethal infections in exotic cattle and considerable mortality in indigenous and crossbred stock [28].

Hematological analysis revealed significant decreases in hematological parameters including RBC count, PCV and hemoglobin (HB) in the infected cattle. 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, HB concentration and PCV values, which mean 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. Additionally, differential analysis of WBCs in non-infected and infected group, 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. The finding coincided with the previous studies on bovine theileriosis [29, 30, and 31], the mechanisms of such a progressive anemia are still not clearly understood. However, 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 [32], while one recent hypothesis indicates the interference of the parasite with protective antioxidant mechanisms of RBCs against oxidative damages [29, 30].

Biochemical analysis revealed significant elevations in the concentration of plasma homocysteine (hyper-homocysteine) in *T. annulata* infected cattle.The finding harmonic with the previous studies by [31, 33]. Although there have been no documented or incisive investigations on homocysteine changes in blood parasites of animals, several publications on human cardiovascular diseases (CAD) correlate hyper-homocysteine with coronary, cerebral and peripheral artery disease, as well as venous thrombosis [34]. In addition, the pathogenesis of the vascular injury caused by an increase of homocysteine (Hcy), includes damage to the endothelial cell, increased oxidation of LDL-cholesterol with deposits in the vessel wall and direct activation of the coagulation cascade [35].

Hyper-homocysteine has been demonstrated to increase oxidative stress through autoxidation of homocysteine, yielding hydrogen peroxide. Thus, the elevation of homocysteine in parasitized cattle in our study coincided with the results of previous studies that attributed anemia due to the role of oxidative stress on damaging erythrocytes, as well as

emphasize the probable formation of endothelial injuries and coagulation disorders (like disseminated intravascular coagulation) due to occurred hyper-homocysteine, which, in turn, could help the appearance of anemia [36, 30].

The present study showed that the cattle suffering from theileriosis had significantly lower concentrations of T3 and T4 in their sera. Reduced T3 and T4 levels in cattle in this study are also in corroborated with those reported by others [37, 16 and 31] in calves and disagreements with [7,33], they revealed did not considerable changes in theileriosis cattle occur. In addition to that reduced thyroid secretion rate during feed deprivation has also been reported in farm animals and it has been postulated that the lower level of T3 and T4 could partly be due to the anorexia condition prevailing in the disease [37]. In spite of these facts, other factors interfering with the function of the thyroid should not be overlooked. In addition, several trace elements are needed for the normal function, synthesis and metabolism of thyroid hormones. In particular, it has been indicated that deficiencies in the levels of selenium [38] and zinc [39] have a suppressing effect on thyroid hormones. Thus, it seems that having a good nutrition could be a reason for unchanged levels of thyroid hormones in the infected animals. Our study reported that the cattle suffering from theileriosis had significantly lower concentrations of zinc and selenium coupled with degree of parasitemia in their sera as compared to healthy controls. The finding agreements with the previous studies revealed that trace element deficiencies occur in cattle suffering from theileriosis [9, 40 and 31]. Incompatible to that several trace elements are needed for the normal function, synthesis and metabolism of thyroid hormones where the thyroid gland contains more selenium per gram of tissue than any other organ, Selenium acts as an antioxidant and is essential for normal thyroid function and thyroid hormone homeostasis [38]. Selenium is an essential component of ID-I (Type I iodothyronine deiodinase).The type I iodothyronine deiodinase, a thiol-requiring propyl-thiouracil-sensitive oxidoreductase, is found mainly in liver and kidney and is the enzyme which converts T4 to T3 [41], since a consequence of selenium deficiency is that conversion of T4 to T3 by hepatic and renal ID-I decreased by over 90% [42], Therefore the above mechanism for selenium may account partly for the lowered T4 and T3 in our study on theileriosis affected cattle. On the other hand zinc deficiency has a suppressing effect on thyroid hormones, whereas zinc supplementation has an opposite effect [43, 39]. But [12], concluded that copper and zinc alterations, induced by D-penicillamine administration, can significantly affect pituitary sex hormones, thyroid stimulating hormone and hypothalamic – pituitary– thyroid axis in rats, therefore zinc plays a role in the thyroid function. It is very important in thyrotropin-releasing hormone synthesis [44]. Zinc is essential for thyroxine (T4)-to-T3 conversion [45] and is required for the biological functioning of the thyroid hormone and related receptors [46]. Therefore it seems that significantly lower zinc concentration in our theileriosis affected cattle could affect T4 and T3 concentrations which might be due to multiple functions of zinc on thyroid gland.

Conclusion;

The results demonstrates that the infection of cattle with *Theileria annulata* is mainly characterized by the anemia. Also, evidenced elevation in the level of homocysteine (hyper-homocysteine) in parasitized cattle can result in oxidative stress on erythrocytes and the probable endothelial injuries.

The parasite cannot implement significant influences on the thyroid hormones in the affected cattle it seems likely that the hypothyroid state in *T. Annulata* infected cattle may be multifactorial and includes anorexia, thyroid gland injury due to parasite itself, trace element deficiencies (selenium and zinc) or hepatic involvement.

Our study inferred that the anemia is a main characterization of theileriosis. Marked increase in the plasma homocysteine (hyper-homocysteine) during parasitemia in infected animals could be assigned as a risk factor, for probable endothelial injuries and help to form the anemia.

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