Determination of the levels of serum oxidative indicator, cytokine and some biochemical parameters in horses naturally infected with *Theileria equi*

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Received date: 07.08.2019- Accepted date: 02.01.2020

Abstract: Equine theileriosis is considered as a serious problem because of harmful effects on the health and performance of equids. Despite the importance of this disease, there are few studies in related to clinical pathologic changes in equine theileriosis especially in horses. In this study, the oxidative stress biomarkers, cytokines, enzymes, lipid profile, electrolytes, minerals and some metabolites were evaluated in horses naturally infected with *Theileria equi* (n=22) and healthy (n=7). In infected horses, the serum concentrations of MDA, IFN-γ, total protein, globulin, bilirubin (total, direct, indirect), triglyceride, glucose, iron, chloride, sodium and copper with enzyme activities of ALP, AST and GGT were found significantly higher, however concentrations of GSH, albumin, total cholesterol, HDL, TIBC, calcium, phosphorus, magnesium, potassium, zinc and manganese with enzyme activity of CAT were found lower when compared to the healthy horses (P<0.05). The changes in TNF-α, creatinine, urea and LDL concentrations were not statistically significant (P>0.05).The results indicate that there are significant changes in the oxidative indicator, cytokine, and biochemical parameters of horses in *T. equi* infection and that these changes may be useful in the evaluation of the diagnosis, prognosis and treatment of theileriosis. In addition, comprehensive studies are needed to better understand the role of cytokines in the pathogenesis of theileriosis.

Keywords: Biochemical parameter, cytokine, oxidative stress, *Theileria equi.*

Theileria equi ile doğal enfekte atlarda serum oksidatif belirteç, sitokin ve bazı biyokimyasal parametre düzeylerinin belirlenmesi

Özet: Equine theileriosis equidelerin sağlığı ve performansı üzerindeki zararlı etkileri nedeniyle ciddi bir sorun olarak kabul edilir. Bu hastalığın önemine rağmen, atların equine theileriosisdeki klinik patolojik değişikliklerle ilgili az sayıda çalışma bulunmaktadır. Bu çalışmada, *T. equi* ile doğal enfekte olmuş (n = 22) ve sağlıklı atlarda (n =7) oksidatif stres biyobelirteçleri, sitokinler, enzimler, lipit profili, elektrolitler, mineraller ve bazı metabolitler değerlendirildi. Sağlıklı grup ile karşılaştırıldığında, enfekte atlarda istatistiki olarak anlamlı olacak şekilde, MDA, IFN-γ, total protein, globulin, bilirubin (total, direkt, indirekt), trigliserit, glukoz, demir, klor, sodyum ve bakır konsantrasyonları ile ALP, AST ve GGT enzim aktivitelerinin arttığı, bununla birlikte, GSH, albumin, total kolesterol, HDL, TDBK, kalsiyum, fosfor, magnezyum, potasyum, çinko ve mangan konsantrasyonları ile CAT enzim aktivitesinin azaldığı tespit edildi (P<0,05). TNF-α, kreatinin, üre ve LDL konsantrasyonlarındaki değişikliklerin istatistiki olarak anlamlı olmadığı belirlendi (P>0,05). Elde edilen sonuçlar, *T. equi* enfeksiyonunda atların oksidatif belirteç, sitokin ve biyokimyasal parametrelerinde önemli değişiklikler olduğunu ve bu değişikliklerin theileriosis'in tanı, prognoz ve tedavisinin değerlendirmesinde faydalı olabileceğini göstermektedir. Ayrıca theileriosisin patogenezinde sitokinlerin rolünün daha iyi anlaşılabilmesi için kapsamlı çalışmalara ihtiyaç vardır.

Anahtar sözcükler: Biyokimyasal parametre, oksidatif stres, sitokin, *Theileria equi*

Introduction

Equine piroplasmosis is known as a protozoan disease which is caused by the *Babesia caballi* and *Theileria equi* (formerly *Babesia equi*) species and is transmitted by ticks. The disease is widespread all over the world, especially in tropical and subtropical regions and is considered as a major problem in transportation processes at national and international levels. *Theileria equi* infections are seen more common compared to *B. caballi* infections (29). The disease is peracute, acute and chronic and characterized by fever, anemia, icterus, and hepatosplenomegaly. Severe anemia and hemoglobinuria are commonly seen in *T. equi* infections (37). Oxidative stress is defined as the disruption of the balance existing between prooxidants and antioxidants in favor of prooxidants. If antioxidant systems are insufficient to withstand oxidative stress, oxidative damage occurs in cells, which in turn deteriorates the functions of the cells (28). The presence of oxidative stress is shown by measuring free radicals or oxidative biomarkers and antioxidants. Therefore, malondialdehyde (MDA) from oxidant biomarkers as well as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) from antioxidants are frequently used (14). In previous studies, it was indicated that equine piroplasmosis in horses was associated with antioxidant defense systems of cells such as oxidative stress and reactive oxygen/nitrogen types, lipid peroxidation and poor antioxidant state in pathogenesis (8, 14, 27, 30, 42).

Cytokines which are polypeptide produced and secreted by various cell types regulate immune and inflammatory events in host defense. These molecules have different functions. Interferon-gamma (IFN-γ) is synthesized from T lymphocytes with the stimulation of various factors such as viruses, other microorganisms which are intracellular parasites, bacterial endotoxins, and mitogens. It increases the ability of many cells to kill intracellular pathogens. Tumor necrosis factor-alpha (TNF-α) is synthesized by active macrophages and T lymphocytes. TNF-α production is increased by IFN-γ. Its most important effect is inflammatory immune response improvement and tumor necrosis (11). Although proinflammatory cytokines are important in host defense against blood parasites, their unstable and excessive production is harmful to the host. They cause shock, tissue damage, weight loss and lipid peroxidation (10, 15). TNF-α was determined to be the most important factor playing a role in pathogenesis of babesiosis (22, 44) and malaria (25).

Parasitic diseases make animals susceptible to trace element deficiencies. Trace elements function as cofactors or components of organic compounds in the activation of enzyme systems. Trace element metabolism must work properly for all living creatures to maintain a healthy life.

Deficiency or excess of some trace elements plays an important role in many diseases and carcinogenesis (1). The diagnosis and course of a disease and the response to treatment are monitored by the biochemical profile of the blood. Blood parameters such as glucose, urea, creatinine, cholesterol, triglyceride, albumin, globulin, total protein, total lipid, and bilirubin as well as electrolytes such as Ca, Na, K, P and enzymes such as AST, ALT, ALP, and CK are the most important biochemical parameters that determine the biochemical profile (23).

The aim of this study was to determine the changes in serum oxidative stress biomarkers, cytokines, enzymes, lipid profiles, electrolytes, minerals and some metabolites in horses infected with *T. equi*.

Material and Methods

Parasitological analysis: This study was conducted between June and August 2017 with 182 horses aged 1 year and over raised by the local people in Muş region. Additionally, it was conducted in compliance with the regulation issued by Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee (2019/06). Blood samples with EDTA taken from the jugular vein of the horses in vacuum gel biochemistry tubes in accordance with the technique were brought to the laboratory in the cold chain. Serum from biochemical tubes and plasma from blood samples with EDTA which were centrifuged at 3000 rpm for 15 minutes were obtained and stored at -80ºC for analysis. The obtained plasmas were screened with cELISA (*Babesia equi* Antibody Test Kit, cELISA, Vmrd, USA) test for *Theileria equi* antibodies. The c-ELISA test was performed according to the manufacturer's test procedure. *T. equi* was found to be positive in 12.1% (22/182) of the horses evaluated within the scope of the study (2).

Biochemical analysis: In the serum of horses diagnosed with theileriosis, MDA level was determined according to Placer et al.,'s (26) method. For this purpose, the concentration of MDA reacted with thiobarbituric acid was measured as 532 nm in spectrophotometer. 1.1.3.3-tetramethoxypropane was used as standard in MDA measurement. GSH level was determined according to the method reported by Sedlak and Lindsay (32). For this purpose, the color change occurred by using 5.5'dithio-bis-2-nitrobenzoic acid was measured as 412 nm in spectrophotometer (32). Catalase activity was measured according to the method of Goth (19). For this purpose, the absorbance of the colored complex formed by ammonium molybdate and hydrogen peroxide was measured as 405 nm. Tumor necrosis factor-alpha (Equine, ELISA Kit, catalogue no: 201-03-0015, USA) and IFN-γ (Equine, ELISA Kit, catalogue no: 201-03-0117, USA) analyses were conducted on the ELISA device (Avarenes, Stat Fax 2100, USA) by using a

commercial kit. Analysis of biochemical parameters (total protein, albumin, globulin, bilirubin (total, direct, indirect), triglyceride, cholesterol, TIBC, iron, ALP, AST and GGT) were performed at an autoanalyzer (Abbott Architect® i6000 and c8000, USA) by using the commercial kit and macro (Ca, K, P, Cl, Na, Mg) and micro mineral (Cu, Zn, Mn) analysis was carried out by using an atomic absorption spectrophotometer (Unicam 929, UK).

Statistical analysis: The statistical calculations of the data were made by using SPSS 22 program. Statistical differences between groups were evaluated using Mann-Whitney "U" test. Spearman's rank correlation was used to test relation between IFN- γ, TNF-α, GSH, MDA and CAT. The obtained results were given as Mean±SEM. The value of P<0.05 was accepted as statistically significant.

Results

It was determined that MDA, IFN-γ, total protein, globulin, bilirubin (total, direct, indirect), triglyceride, glucose, iron, chlorine, sodium and copper concentrations and ALP, AST and GGT enzyme activities statistically significantly increased; however, GSH, albumin, cholesterol, HDL, TIBC, calcium, phosphorus, magnesium, potassium, zinc and manganese concentrations and CAT enzyme activity statistically significantly decreased in horses naturally infected with *T. equi* compared to the healthy group (P<0.05). It was determined that changes in TNF-α, creatine, urea and LDL concentrations in horses naturally infected with *T. equi* were not statistically significant compared to the healthy group (P>0.05) (Table 1).

Table 1. Serum oxidative indicator, cytokines and some biochemical parameter levels of healthy horses and horses naturally infected with *T. equi*

Parameters	Healthy group (n=7) (Mean ± SEM)	Infected group (n=22) (Mean ± SEM)	P
MDA (nmol/mL)	4.14±0.16	7.95±0.22	0.001
IFN- γ (pg/mL)	4.06±0.72	15.30± 0.47	0.001
TNF-α (pg/mL)	16.75±2.03	18.06±2.67	0.960
GSH (nmol/mL)	6.47±0.70	5.16±0.25	0.001
CAT (ku/L)	59.32±3.29	55.81±2.11	0.008
Creatinine (mg/dL)	084±0.04	0.83±0.03	0.791
Urea (mg/dl)	41.33±0.47	41.00±1.00	0.419
Total protein(g/L)	67.83±0.37	72.33±2.21	0.001
Albumin (g/L)	38.00±0.01	37.50±0.50	0.010
Globulin (g/L)	30.00±0.58	33.00±0.00	0.001
AST(U/L)	229.50±2.60	263.00±1.63	0.001
ALP (U/L)	140.17±0.37	187.83±1.11	0.001
GGT(U/L)	14.00±0.01	14.83±0.90	0.010
Total bilirubin (mg/dL)	0.26±0.01	0.74±0.02	0.001
Direct bilirubin (mg/dL)	0.13±0.01	0.37±0.01	0.001
Indirect bilirubin(mg/dL)	0.13±0.01	0.38±0.02	0.001
Triglyceride (mg/dL)	23.64±0.24	25.74±0.35	0.001
Cholesterol (mg/dL)	75.07±0.32	72.43±0.30	0.001
LDL (mg/dL)	25.57±0.97	24.11±0.99	0.220
HDL (mg/dL)	44.77±0.81	43.17±0.85	0.047
Glucose (mg/dL)	53.33±0.47	54.50±0.50	0.001
TIBC (µg/dL)	388.00±2.00	302.50±9.01	0.001
Iron (µg/dL)	113.83±0.37	118.50±0.50	0.001
Calcium (mmol/L)	1.58±0.11	1.24±0.5	0.001
Phosphate (mg/dL)	3.02±0.13	2.55±0.05	0.001
Magnesium (mg/dl)	2.34±0.03	2.18±0.01	0.001
Potassium (mmol/L)	5.03±0.13	4.85±0.05	0.003
Chloride (mmol/L)	97.40±0.49	98.50±0.50	0.001
Sodium (mmol/L)	130.71±1.25	132.50±0.50	0.001
Copper (µmol/L)	7.6±1.2	15.5±3.6	0.001
Zinc (µmol/L)	8.8±0.3	8.2±0.7	0.016
Manganese (µmol/L)	0.7±0.00	0.5±0.00	0.001

*P<0.05 shows the significance between the parameters on the same row. MDA: Malondialdehyde, GSH: Glutathione, CAT: Catalase, IFN-γ: *Interferon-gamma,* TNF-α: *Tumor necrosis factor-alpha*, ALP: Alkaline phosphatase, AST: Aspartate aminotransferase, GGT: Gamma glutamyl transferase, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, TIBC: Total iron binding capacity, T: Total, D: Direct, I: Indirect

Table 2. Correlation analysis of serum MDA, IFN-γ, TNF-α, GSH, and CAT values in the horses naturally infected with *T. equi* (n = 22)

	MDA	IFN-γ	TNF-α	GSH	CAT
MDA	1	r=0.240 P=0.605	r=0.149 P=0.750	r=-0.158 P=0.736	r=-0.419 P=0.349
IFN-γ		1	r=0.111 P=0.813	r=-0,052 P=0.913	r=-0.094 P=0.842
TNF-α			1	r=-0.224 P=0.629	r=-0.476 P=0.280
GSH				1	r=-0.667 P=0.102
CAT					1

In the infected group, positive correlations with MDA concentrations, IFN-γ and TNF-α (r=0.240, P=0.605 and r=0.149, P=0.750, respectively) as well as negative correlations with GSH and CAT (r=-0.158, P=0.736 and r=-0.419, P=0.349, respectively) were determined to be insignificant. In the infected group, positive correlations with IFN-γ concentrations and TNF-α (r=0.111, P=0.813) as well as negative correlations with GSH and CAT (r=-0.052, P=0.913 and r=-0.094, P=0.842, re pectively) were determined to be insignificant. In the infected group, negative correlations between TNF-α concentrations, GSH and CAT (r=-0.224 r=-0.052, P=0.629 and r=-0.667 P=0.102, respectively) were determined to be insignificant (Table 2).

Discussion and Conclusion

Parasitic agents cause significant changes in the biochemical profile of the host on which they live. *Theileria* spp. which are an intraerythrocytic parasite have detrimental effects on hemoglobin and erythrocytes (13, 36). Reactive oxygen types lead to oxidation in polyunsaturated fatty acid in biological membranes and initiate lipid peroxidation. Measurement of MDA which is formed as a result of lipid peroxidation in body fluids and tissues is used as an indicator of oxidative damage. High fever, inflammation, oxidative stress and cellular damage seen in most inflammatory diseases, such as blood parasite result in the formation of compounds with aldehyde structure such as MDA (14). It was determined in the studies that MDA level increased in donkeys and horses infected with *T. equi* (27, 42), in donkeys infected with *B. equi* (3), and in horses infected with *T. equi* and *B. caballi* (14). In this study, MDA level was found to be higher in horses infected with *T. equi* than health horses. This increase may be due to the lipid peroxidation and oxidative damage in erythrocytes caused by the interaction of polyunsaturated fatty acids in the structure of erythrocyte membrane with ROS.

Acute phase response, which is a nonspecific defense reaction, is stimulated as a result of tissue damage caused by parasites or other infectious agents. The acute phase response is stimulated by proinflammatory cytokines such as IL-1, IL-6, and TNF-α released from leukocytes activated at the area of tissue damage. Macrophages are activated by TNF-α (18, 34) and IFN-γ (41) and NO eliminates the parasites by producing toxic mediators such as peroxynitrite and superoxide. In the previous studies conducted with horses, TNF-α, IFN-γ, IL-1, IL-4, and IL-6 in infections with neurospora species (10), IFN-γ, TNF-α, IL-1 and IL-6 in infections with *Trypanasoma vivax* (11), IFN-γ, TNF-α and IL-2 in infections with *B. caballi* (20) and TNF-α, IFN-γ, IL-1, IL-4, and IL-6 cytokines in the infections with *T. gondii* (16) were determined to increase. It was determined in this study that IFN-γ level from proinflammatory cytokines was significantly higher in horses infected with *T. equi* compared to the healthy group but the increase in TNF-α level was not statistically significant. This increase in IFN-γ and TNF-α levels may have occurred to control the disease and can be considered as an indicator of the development of immunity against the disease. The presence of important intracellular enzymatic antioxidants such as SOD, CAT, GSH R and GSH Px as well as endogenous non-enzymatic antioxidants such as GSH, ascorbic acid and alpha tocopherol in erythrocytes is important in ensuring and maintaining oxidative balance. In parasitic diseases, continuous and increased free radical production decreases antioxidant defense systems (4, 5). It was found in the studies that CAT enzyme activity in *T. equi* infection (27) and GSH concentration in *T. equi* and *B. caballi* infection (14) were decreased. Similar to the result of the mentioned studies, it was also determined in this study that GSH concentration and CAT enzyme activity decreased significantly in infected horses. This decrease in antioxidants may be caused by their usage in eliminating oxidants. The increase of oxidant parameters and the

decrease of antioxidant parameters due to *T. equi* infection indicate that oxidative stress occurs in erythrocytes of horses.

In the present study, a positive and/or negative correlation was determined between serum MDA, IFN-γ, TNF-α, GSH and CAT levels although it was not statistically significant. This result showed that lipid peroxidation, inflammation and antioxidant system changes in erythrocytes were simultaneously reflected in peripheral blood.

Compared to healthy animals, significant increases were determined in serum AST, ALP and GGT activities, which are specific to liver, in horses infected with *T. equi* (7, 31, 35, 39, 42). This increase in enzyme activity may be due to damage in hepatocytes caused by hypoxia associated with anemia. Besides, it was determined in some studies that there was no change in liver enzyme levels in theileriosis infections in horses *(36, 43)*. It was determined in the present study that total protein and globulin levels significantly increased but albumin level decreased in the infected group compared to healthy group. The increase in globulin fraction in response to antigenic stimulation and chronic inflammatory disorder in liver may be responsible for the increased protein level. It is thought that the decrease in albumin level is due to acute phase response and/or liver dysfunction (6). There are also studies reporting that total protein level increased (36, 39, 42) or no significant change occurred *(*17, 43) *in the horses infected with Theileria equi.*

In the present study, a significant increase in serum bilirubin (total, direct, indirect) concentrations was found in infected horses. Hemolysis and hepatic dysfunction of parasitic erythrocytes may be responsible for this significant increase in bilirubin levels (31). The studies found different results stating that total bilirubin levels increased (31, 42, 43) or did not change (17, 39). In addition, Takeet et al., (36) reported a decrease in direct bilirubin level; whereas, Salib et al., (31) reported an increase in indirect bilirubin level.

It was determined in the present study that there was no significant change in serum urea (42) and creatine (36) levels in infected horses. However, Vidhyalakshmi et al., (39), found an increase in creatine level. Like the results of the study (39), we found change in serum glucose levels in infected horses compared to the control group in the present study. The increase in glucose level in infected horses may be due to stress or increased cortisol level or due to increased glucose mobilization. However, Zaeemi et al., (42), found no significant change in glucose level.

Different data were obtained about lipid profile in theileria infection in horses. Zaeemi et al., (42) found that triglyceride, cholesterol, LDL and VLDL levels increased and there was no significant change in HDL level and Takeet et al. (36) reported that the change in triglyceride level was not significant. In this study, it was found that triglyceride levels significantly increased, cholesterol, HDL and LDL levels decreased and the changes in only HDL was significant in infected animals. High triglyceride level in the infected group may indicate a problem in the increased production or removal of triglycerides in liver due to fatty tissue lipolysis. Low levels of total cholesterol may be due to the inability of providing normal synthesis of cholesterol by hepatocytes due to liver damage caused by the parasite. Thus, lipoproteins such as HDL and LDL that contain cholesterol in its structure are not synthesized sufficiently (33).

Trace element deficiencies seen in parasitic and infectious diseases are generally the result of a complex mechanism. Copper, zinc, magnesium and manganese are essential trace elements for antioxidant systems that resist damage caused by free radicals. The reduction of these elements in infected animals can provide conditions for the formation of oxidative stress. The effect of these elements on host immune function depends on the severity, duration and seriousness of parasitic infection. In this study, it was determined that manganese level was low and copper level was high in infected horses significantly (13). It was also determined that there was an significant decrease in zinc level. Decreased zinc levels may have occurred as a result of hormonal changes (13) as well as indirect effect of host-parasite relationship or increased zinc demand of the parasite itself (20). During inflammatory processes, ceruloplasmin levels increase which leads to elevated copper levels. Increased serum copper levels may be a result of the acute phase response of parasitic infection. Zaeemi et al., (42) determined in their study that both zinc and copper levels decreased.

It was determined in the present study that serum calcium, phosphorus, magnesium and potassium concentrations decreased (12, 21, 36, 42, 43), sodium and chlorine concentrations increased (36, 42) significantly in infected horses compared to the control group. The decrease in the serum levels of minerals may be due to malnutrition, decreased dietary absorption, and bowel and kidney disorders (9). In contrast to results of the present study, it was determined that calcium and phosphorus levels did not change in infection with theileriosis (17, 39).

TIBC is a negative acute phase protein and a measure of serum transferrin concentration. TIBC falls below normal physiological limits in inflammatory diseases (24). When the serum iron and TIBC levels were examined in infected horses, it was determined that the iron level increased and TIBC level decreased significantly in this study. The increase in iron level may be due to intravascular hemolysis of RBCs depending on infection (38) and the decrease in TIBC level may be due to the inflammation related to disease (40). In contrast to the

result of the present study, it was determined in the studies that iron levels did not change Zaeemi et al. (42) or decreased (35, 36, 43) in theileriosis.

In conclusion, the current data demonstrate that there were significant changes in oxidative indicator, cytokines and biochemical parameters of the horses in *T. equi* infection and these changes may be useful in the evaluation of diagnosis, prognosis and treatment of theileriosis. In addition, more extensive studies are needed to better understand the role of cytokines in the pathogenesis of theileriosis.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study was approved by the Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee (2019/06).

Conflict of Interest

The authors declared that there is no conflict of interest.

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