

# Serum lipid and protein oxidation and anti-oxidant status in horses naturally infected with *Theileria equi*

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## SUMMARY

Reactive oxygen and nitrogen species are important parts of cellular immune response involved in killing intracellular parasites. In order to determine if intracellular parasite infection may be associated with oxidative damage, circulating lipid peroxidation, protein oxidation and antioxidant status were investigated in horses naturally infected with *Theileria equi*. For that, the *T. equi* infection was confirmed in 9 horses using an indirect fluorescence antibody test and malondialdehyde (MDA), protein carbonyl (PCO), nitric oxide metabolites (NOx) and reduced glutathione (GSH) concentrations as well as the total antioxidant activity (AOA) were measured in sera from *T. equi* infected horses and from healthy controls (n = 9). Whereas GSH concentrations and serum AOA were significantly depressed in the infected horses compared to the controls, the serum MDA, PCO and NOx concentrations were markedly increased. These results show the occurrence of oxidative stress amplified by the antioxidant depletion probably due to overproduction of free radicals by activated inflammatory cells in horses naturally infected with *T. equi*.

**Keywords:** *Theileria equi*, horse, piroplasmosis, serum, lipid peroxidation, protein carbonyl, glutathione, total antioxidant activity, oxidative stress.

## RÉSUMÉ

**Oxydation des lipides et des protéines sériques, et statut sérique anti-oxydant chez des chevaux naturellement infestés par *Theileria equi***

Les radicaux oxygénés et azotés jouent un rôle important dans les réponses immunes cellulaires dirigées contre des parasites intracellulaires. Afin de déterminer si une infestation par un parasite intracellulaire peut être associée à l'apparition d'un stress oxydatif, les réactions d'oxydation des lipides et des protéines sériques ainsi que le statut antioxydant du sérum ont été explorés chez des chevaux naturellement infestés par *T. equi*. Pour ce faire, l'infestation par *T. equi* a été confirmée chez 9 chevaux par un test d'immunofluorescence indirecte et les concentrations sériques de MDA (malondialdéhyde), de PCO (protéines enrichies en groupements carbonyles), des métabolites du NO (NOx) et du glutathion réduit (GSH) ainsi que l'activité totale antioxydante (AOA) du sérum ont été mesurées chez les chevaux infestés par *T. equi* et chez 9 chevaux témoins. Alors que les concentrations de GSH et l'AOA ont été significativement diminuées chez les chevaux infestés par rapport aux témoins, les concentrations sériques de MDA, PCO et NOx ont été remarquablement augmentées. Ces résultats montrent l'existence d'un stress oxydatif amplifié par un déficit des systèmes antioxydants chez les chevaux naturellement infestés par *T. equi* probablement dû à une production exacerbée des radicaux libres par les cellules inflammatoires activées par la présence du parasite.

**Mots clés :** *Theileria equi*, cheval, piroplasmose, sérum, peroxydation lipidique, oxydation des protéines, glutathion, activité antioxydante totale, stress oxydatif.

## Introduction

Haemoparasites inflict losses to animals in term of morbidity and mortality due to their heavy incidence [12]. Equine piroplasmosis is one of the most important parasitic diseases of the equines and it causes great damage to animal health. Equine piroplasmosis is a tick-borne haemoprotozoan disease caused by *Theileria equi* (*T. equi*) and *Babesia caballi* (*B. caballi*) that affects horses worldwide. The disease caused by *T. equi* is more pathogenic and widespread in horses than this caused by *B. caballi*. Through infecting and destroying red blood cells, it can compromise tissue oxygenation, leading to loss of vitality and decrease in the performance of infected animals [5, 31, 38].

The possible role of the reactive oxygen and nitrogen species, (ROS and RNS) in the pathogenesis of parasitic infections has been commonly studied in recent years [1, 34]. Furthermore,

the microorganisms killed throughout cellular mechanisms have been the subject of intense research. Numerous studies demonstrated that a variety of inflammatory cells are activated to induce or activate various oxidant-generating enzymes to kill intra-cellular and extra-cellular parasites [16, 29].

The reactive species are produced primarily to attack invading microorganisms by reactions of nitration, oxidation and chlorination [36]. However, excess amounts of ROS and RNS can cause injury to host cells [21].

In this study, the role of ROS and RNS were discussed for generating macrophage efficiency as a first line of defence during phagocytosis in parasitic infection; therefore, total antioxidant activity (AOA), reduced glutathione (GSH), protein carbonyl (PCO), nitric oxide (NOx), and malondialdehyde (MDA) concentrations in plasma were compared between healthy and horses seropositive for *T. equi*.

## Material and Methods

### SUBJECTS AND PARASITOLOGICAL EXAMINATION

The present multi-centred, randomized and single blinded study was conducted in 18 racing, breeding and non-pedigree horses stemming from different parts of Turkey between 2008 and 2009. Blood samples were collected from jugular puncture into sterile microtubes without anticoagulant and after clotting for 12 hours at room temperature, they were centrifuged at 1500 g for 15 minutes at room temperature and sera were carefully harvested and stored at -20°C until assayed.

Indirect fluorescence antibody test (IFAT, Fuller Laboratories, Fullerton CA, USA), often used to diagnose equine piroplasmiasis [24, 38], was performed according to the manufacturer's instructions. All serum samples were negative against *B. caballi* but 9 sera were positive against *T. equi* and constituted the infected group whereas the other 9 sera were negative against *T. equi* and constituted the control group.

### BIOCHEMICAL ANALYSES

The malondialdehyde (MDA), reduced glutathione (GSH), protein carbonyl (PCO), nitric oxide (NOx) concentrations and total antioxidant activity (AOA) were determined for all samples.

The MDA concentration, an index of lipid peroxidation, was measured by the double heating method of DRAPER and HADLEY [9], which is based on spectrophotometric measurement of the purple colour generated by the reaction of thiobarbituric acid (TBA) with MDA. Protein carbonyl concentrations were measured using the method of LEVIN *et al.* [25]. The carbonyl content was calculated based on the molar extinction coefficient of 2,4-dinitrophenylhydrazine (DNPH) ( $\epsilon$ : 2.29104 cm<sup>-1</sup> M<sup>-1</sup>) and expressed as  $\mu\text{mol/g}$  protein. The GSH concentration was measured using the method described by TIETZE [42]. Nitric oxide decomposes rapidly in aerated solutions to form stable nitrite/nitrate products (NOx). Nitrite/nitrate concentration was measured by a modified method of Griess assay described by MIRANDA *et al.* [27]. The principle of this assay is the reduction of nitrate by vanadium combined with detection by the Griess reaction. Nitrite/nitrate concentration was calculated using a NaNO<sub>2</sub> standard curve and expressed in  $\mu\text{mol/L}$ . The total AOA was determined using the method described by KORACEVIC *et al.* [23]. The assay measures the capacity of the serum to inhibit the production of TBA-reactive substances (TBARS) from sodium benzoate under

the influence of the oxygen-free radicals derived from the Fenton's reaction. The reaction was measured spectrophotometrically at 532 nm. Antioxidants from the added sample suppressed the production of TBARS, and the inhibition of colour development was defined as AOA.

### STATISTICAL ANALYSIS

All data were separately presented as mean  $\pm$  standard error (SE) for the infected and control groups. The comparisons of parameters were performed with Student's t-test. Data were analyzed using the SPSS for Windows computing program (Version 10.0) and  $P < 0.05$  were considered statistically significant.

## Results

Biochemical parameters measured in infected and control horses were summarized in Table I. It was observed significant increases in circulating oxidant (MDA, NOx and PCO) concentrations in *T. equi* infected horses ( $P < 0.05$ ); in seropositive animals, the mean MDA, NOx and PCO concentrations were increased by 18.93%, 90.50% and 48.95%, respectively. By contrast, the serum GSH concentrations and the total antioxidant activity were significantly lower in infected animals than in controls ( $P < 0.05$ ), the variation rates being -67.01% and -25.57%, respectively.

## Discussion

Equine piroplasmiasis is microscopically diagnosed by evidencing the parasites in stained blood smears. However, serological testing is actually recommended for detecting carrier animals [24, 38].

Infection and inflammation activate a variety of inflammatory cells [21] that play important roles in the host defence [8]. These cells are capable of generating large amounts of highly toxic molecules such as ROS (including superoxide anion, hydrogen peroxide and hydroxyl radicals) and RNS (including nitric oxide) [21]. In addition, ROS and RNS are capable of degrading numerous biomolecules including DNA, carbohydrates, lipids and proteins [2, 17, 41]. ROS-induced oxidation of polyunsaturated fatty acids in biological systems results in the formation of lipid peroxidation products. One of the most frequently used ROS biomarkers which provide

Biochemical parameter	Control group	<i>T. equi</i> infected group	P value
MDA ( $\mu\text{mol/L}$ )	1.32 $\pm$ 0.06	1.57 $\pm$ 0.05 (+18.93%)	< 0.05
PCO ( $\mu\text{mol/g}$ )	3.58 $\pm$ 1.07	6.82 $\pm$ 0.50 (+90.50%)	< 0.05
NOx ( $\mu\text{mol/L}$ )	4.29 $\pm$ 0.32	6.39 $\pm$ 0.54 (+48.95%)	< 0.05
GSH ( $\mu\text{mol/L}$ )	12.52 $\pm$ 2.09	4.13 $\pm$ 0.45 (-67.01%)	< 0.05
AOA (mmol/L)	3.95 $\pm$ 0.17	2.94 $\pm$ 0.34 (-25.57%)	< 0.05

MDA: malondialdehyde; PCO: Protein carbonyl; NOx: nitrite/nitrate products; GSH: reduced glutathione; AOA: total antioxidant activity.

TABLE I: Serum oxidant and antioxidant status in horses infected with *T. equi* (n = 9) and in healthy horses (n = 9). Results are expressed as mean  $\pm$  standard error (SE) and the rate of variation for each parameter compared to the control values are indicated into parenthesis.

an indication for the overall lipid peroxidation intensity is MDA, which is a by-product of lipid peroxidation [30]. Many studies demonstrated that the amount of reactive oxygen radicals which cause lipid peroxidation is increased in the cells of hosts infected with different species of parasites (distomatosis, leishmaniasis and malaria), resulting in cell and tissue damage [7, 21, 37]. SALEH [34] reported that erythrocyte lipid peroxidation increased in cattle naturally infected with *B. bigemina*. Moreover, around the time of peak parasitaemia, it was observed that peripheral bovine monocytes and neutrophils were involved in the oxidative burst and production of oxidative radicals [3]. SHIONO *et al.* [40] and REZAEI and DALIR-NAGHADEH [33] detected a noteworthy increase in MDA concentrations in parallel with the decrease in haematocrit and the increase in parasitaemia in bovine theileriosis. In this study, increased serum MDA concentrations in horses naturally infected with *T. equi* were consistent with the findings of other studies and might also support the use of serum MDA as a marker of oxidative stress in horses.

The oxidative inactivation of enzymes and the oxidative modification of proteins cause the formation of protein carbonyl derivatives [25]. Alteration of the protein structures and functions induced by free radicals may contribute to carcinogenesis. Free radicals react with proteins and modify amino acid residues by oxidation, nitrosation, and carbonylation [20]. KOCYIGIT *et al.* [21] demonstrated that serum protein carbonyl (PCO) amounts were higher in patients with cutaneous leishmaniasis compared to the healthy group. However, there is no report on protein oxidation in horse naturally infected with *T. equi*. The results obtained in the present study also showed that the PCO concentrations were significantly higher in horses naturally infected with *T. equi* compared to the controls.

The free radical nitric oxide (NO) is an important mediator of both physiological and pathophysiological processes [28]. Macrophages, neutrophils and mast cells are all indicated as major producers of this molecule. NO produced by iNOS (inducible NO synthase) has an antimicrobial activity and may be involved in killing tumour cells. In this regard, it is a part of the non specific host defence system [11, 26]. DEDE *et al.* [6] demonstrated that the concentration of nitrate significantly increased in goats infected with parasites (*Trichostrongylidae sp.*, *Protostrongylidae sp.*, *Eimeria sp.*, *Babesia sp.*). HANAFUSA *et al.* [18] reported that the production of NO in horses infected with *B. caballi* was significantly increased before death although the parasitaemia level remained very low. In horses naturally infected with *T. equi*, NOx concentrations were found higher than in healthy subjects.

The total AOA of body fluids suggests a simultaneous interaction between various antioxidants, and it is crucial for the maximum suppression of free radical production in extracellular compartments [14]. Whereas the activity or the concentration of a given enzymatic or non enzymatic antioxidant indicates its specific implication (induction of depletion) in the oxidative burst, the total antioxidant activity represents the aggregate antioxidant characteristics of all antioxidants in the serum or plasma. In addition to AOA, reduced GSH and its metabolizing enzymes constitute the major defence against ROS-induced cellular damage [15]. GSH acts as a reducing agent in oxidation reactions resulting in the formation of GSSG. GSH can protect

cells against the damage from ROS and free radicals that arise under oxidative stress conditions [13]. Therefore, reduced GSH amounts may reflect the depletion of the antioxidant reserve. As a consequence of GSH deficiency, a number of related functions may be impaired by a decrease in reducing capacity, protein biosynthesis, immune function, accumulations of lipid peroxidation products and detoxification capacity [19, 39]. DAS *et al.* [4] proposed that since the parasites cause damage to the cells which synthesize anti-oxidative agents, a decrease in the numbers of such cells is natural. In the present study, the serum AOA in horses naturally infected with *T. equi* were found significantly lower than in the controls. Therefore, *T. equi* infection was observed to cause a significant reduction in AOA. On the other hand, serum GSH concentrations were also significantly lowered in the infected group compared to the control group. In agreement, GSH concentrations were also reported to be decreased in hosts infected with parasite species (*Schistosoma mansoni*, *Fasciola hepatica* and *Dicrocoelia*) compared to the healthy control animals [10, 22, 35]. In this context, the observed insufficiency in antioxidant activity could be caused by antioxidant defence mechanisms directly modified by the *T. equi* infection.

As a conclusion, according to the findings of the present study, serum concentrations of MDA, PCO and NOx were higher in horses naturally infected with *T. equi* compared to the healthy controls, indicating the occurrence of oxidative damage due to the *T. equi* infection. Furthermore, the serum GSH concentration and the total antioxidant activity were significantly depressed in the infected group. The overproduction of reactive oxygen and nitrogen species by activated neutrophils and macrophages in horses naturally infected with *T. equi* may result in oxidative stress amplified by the consecutive antioxidant depletion, leading to intense lipid peroxidation and protein oxidation.

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