

CLINICAL AND BIOCHEMICAL STUDIES ON *THEILERIA ANNULATA* IN EGYPTIAN BUFFALOES (*BUBALUS BUBALIS*) WITH PARTICULAR EMPHASIS ON OXIDATIVE STRESS AND KETOSIS RELATIONSHIP

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ABSTRACT - This study was carried out on 68 naturally infected buffaloes with *Theileria annulata*, in addition to 25 parasite-free buffaloes, distributed in small herds at Dakahlia and Gharbya governorates, Egypt, to demonstrate the clinical picture associated with theileriosis, with particular emphasis on the oxidative stress and ketosis relationship. The clinical signs recorded in infected buffaloes were fever, enlargement of one or more lymph nodes, ocular discharge, corneal opacity, skin lesions, decreased milk yield, pale mucous membrane and anorexia. Blood and serum analysis revealed significant decrease in **red blood cells** (RBCS) and/or haemoglobin (HB) concentration in the infected animals, compared to the control ones. Moreover, a significant increase ($p \leq 0.05$) was found in the levels of Malondialdehyde (MDA), beta hydroxy butyric acid (BHBA) and non-esterified free fatty acid (NEFA) with a significant decrease ($p \leq 0.05$) in the levels of reduced glutathione (R.GSH), superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (TAC), nitric oxide (NO), glucose

and glucose-6-phosphate dehydrogenase (G6PD) in infected animals, compared to the control ones. It can be concluded that *Theileria annulata* played an imperative role in the occurrence of anaemia, oxidative stress and ketosis in Egyptian water buffaloes.

Key words: *Theileria*, oxidative stress, antioxidants, buffaloes, ketosis

Rezumat – Studii clinice și biochimice privind *Theileria Annulata* la bivoliul de apă egiptean (*Bubalus bubalis*), în relație cu stresul oxidativ și cetoza. Studiul a fost realizat pe 68 bivoli, infectați natural cu *Theileria annulata* și alți 25 bivoli neinfecțați, răspândiți în mici cirezi din provinciile Dakahlia și Gharbya, Egipt, pentru a prezenta imaginea clinică asociată teileriozei, punându-se accent pe influența stresului oxidativ și a cetozei. Semnele clinice observate la bivoli infectați au fost febra, mărirea unui sau mai multor limfonoduri, scurgeri oculare, opacitatea corneei, leziuni ale pielii, scăderea producției de lapte, paliditatea mucoaselor și

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anorexie. Din analiza sângelui și a serului s-a observat o scădere semnificativă a numărului de hematii și/sau hemoglobinei la animalele infectate, în comparație cu variantele martor. S-a mai remarcat și o creștere semnificativă ($p \leq 0.05$) a nivelului de malondialdehidă, beta-hidroxi butiric și de acizi grași liberi neesterificați, în același timp cu o scădere semnificativă ($p \leq 0.05$) a nivelului de glutatation redus, superoxid dismutază, catalază, capacitatea antioxidantă totală, oxid nitric, glucoză și glucoză-6-fosfat dehidrogenază la animalele infectate față de variantele martor. Concluzia la care s-a ajuns a fost că *Theileria annulata* joacă un rol important în apariția anemiei, a stresului oxidativ și cetozei la bivoul de apă egiptean.

Cuvinte cheie: *Theileria*, stres oxidativ, antioxidanți, bivou, cetoză

INTRODUCTION

Theileria annulata, a protozoan parasite of cattle and domestic buffaloes, is transmitted by ticks of the *Hyalomma* genus and causes a disease called Mediterranean or tropical theileriosis. It represents a major threat for Egyptian water buffaloes, causing significant economic losses as well as reduced production. There are some evidences that the oxidative stress and lipid peroxidation incorporate theileriosis in the pathogenesis of anaemia. Lipid peroxidation is a general mechanism, which, by free radicals, induces tissue damages and is involved under several pathological conditions (Halliwell and Gutteridge, 1999; Knight, 1995). Malondialdehyde (MDA), a product of polyunsaturated fatty acid oxygenation, is a reliable and commonly used biomarker for

assessing lipid peroxidation (Moore and Roberts, 1998). Recently, a growing interest was shown in the use of MDA as a marker of lipid peroxidation in various kinds of diseases (Sheu et al., 2003).

Grewal et al. (2005) showed an increased interest in oxidative stress and lipid peroxidation in erythrocytes of cattle infected with *T. annulata*. They concluded that this might be the cause of increased erythrocyte fragility and membrane lysis. Recently, it was found that the levels of methemoglobin, used as an index of erythrocyte oxidation, have significantly increased at the onset of anaemia in the experimental *Theileria sergenti* infection (Shiono et al., 2003a) and an inverse relationship was noticed between the methemoglobin levels and PCV (Shiono et al., 2001). In addition, increased oxidation of proteins in the membrane of erythrocytes at the advanced stage of anaemia in *T. sergenti*-infected cattle has been reported (Yagi et al., 2002). Shiono et al. (2003b) indicated that the levels of antioxidants in RBC decreased during the progression of anaemia in the cattle infected with *T. sergenti*. They suggested that the oxidative damages of RBC had a close relationship with the onset of anaemia in bovine theileriosis. These results strongly support the hypothesis that the oxidative changes in erythrocytes are closely related to the pathogenesis of anaemia in theileriosis.

The diagnosis of *Theileria annulata* infection in buffaloes, based on clinical signs, is difficult, because of the wide variety of the disease

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clinical picture that may be mistaken with other diseases. Stained thin blood film and lymph node smears are accepted as a method of laboratory diagnosis in cattle and buffaloes (Ramazan and Ugur, 2006).

The prevalence of *Theileria annulata* infection in Egyptian buffaloes is high and a different clinical picture might be mistaken with other diseases. Therefore, this study aimed to throw the light on the clinical picture with special reference to oxidative stress and ketotic state of such infection.

MATERIALS AND METHODS

This study was carried out in Dakahlia and Gharbya governorates, Egypt, on 68 water buffaloes found in small groups and in contact with cattle. In addition, 25 parasite-free buffaloes, located in the same area and under the same level of nutrition and hygiene, were used as a control group. Infected buffaloes were selected according to the clinical examination and positive blood and/or lymph node smears.

Clinical examination was performed on all the animals. The signs of *Theileria annulata* infection were observed and recorded. Thin blood smears were prepared from the ear veins of all animals. Lymph node aspirates were collected from suspected cases suffering of enlarged superficial lymph nodes.

Sampling protocol. All the animals under study were subjected to ear vein puncture and lymph node aspiration. Blood samples were collected from all the infected buffaloes and the parasite-free control, through jugular vein puncture, in tubes contaminated with ethylenediamine-tetraacetic acid dipotassium salt (EDTA-K₂) for routine blood tests and in

heparinized glass-stoppered tubes for other analyses (Schalm et al., 1986).

Malondialdehyde (MDA) and Nitric Oxide (NO). MDA levels were estimated using commercially available test kits, according to the methods described by Satoh, (1978) & Okawa et al., (1979) and Montgomery & Dymock (1961), respectively. BHBA and NEFA, BHBA and NEFA were carried out using commercially available test kits, according to the methods described by Tietz (1999).

Superoxide dismutase (SOD). The activity of SOD was carried out using commercially available test kits, according to the method described by Nishikimi et al. (1972).

Reduced glutathione (R.GSH). The activity of R.GSH was carried out using commercially available test kits, according to the method described by Beutler et al., (1963).

Glucose-6-phosphate dehydrogenase. The activity of glucose-6-phosphate dehydrogenase was determined spectrophotometrically, according to the method described by Beutler et al. (1984).

Glucose levels were estimated using commercially available test kits, according to the method described by Young (2001).

Catalase (CAT). The activity of CAT was carried out using commercially available test kits, according to the method described by Aebi (1984).

Total antioxidant capacity (TAC). The activity of TAC was carried out using commercially available test kits, according to the method described by Koracevic et al. (2001).

Statistical analysis. The obtained data were analysed using the Student's t-test, according to the method described by Snedecor and Cochran (1989).

RESULTS AND DISCUSSION

The obtained data showed that the clinical signs of theileriosis in Egyptian water buffaloes were fever, superficial lymph node enlargement (Figure 3), lacrimation, respiratory manifestations, anorexia, skin lesion

(Figure 4), diarrhoea, corneal opacity (Figures 1 and 2), nasal discharge, pale mucous membrane and decreased milk production (Table 1). These clinical signs are in concern with those obtained by Al-Gaabary (1991) and Osman and AL-Gaabary (2007).



Fig. 1 - Corneal opacity



Fig. 2 - Corneal opacity 2



Fig. 3 - Enlarged lymph node



Fig. 4 - Skin lesion

Table 1 - Percentage of different clinical signs in *Theileria annulata* infected buffaloes

Clinical signs	Number of affected animals/total sick ones	%
Fever	68/68	100%
Enlarged lymph node	68/68	100%
Lacrimation	18/68	26.47%
Respiratory manifestation	18/68	26.47%
Anorexia	68/68	100%
Skin lesion	2/68	0.3%
Diarrhoea	5/68	0.7%
Corneal opacity	25/68	36.7%
Nasal discharge	6/68	0.8%
Pale mucous membrane	68/68	100%
Decreased milk	18/68	26.47%

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The haematological examination (Table 2) revealed significant decreases ($p \leq 0.05$) in the Hb content, PCV%, RBCs and WBCs counts in sick buffaloes, compared to the control ones. Neutropenia, eosinopenia, lymphopenia, mono-

cytopenia with a significant increase ($p \leq 0.05$) in the numbers of thrompocytes were recorded. These results were in agreement with those obtained by Osman and AL-Gaabary (2007).

Table 2 - Blood picture in *Theileria annulata* free and infected buffaloes

Variable	Parasite-free buffaloes (No = 25)	Infected buffaloes (No = 68)
Hb gm/dl	12.36 $\pm$ 0.29	5.2 $\pm$ 0.18*
RBCs ($10^6/\mu\text{l}$)	9.48 $\pm$ 0.13	5.82 $\pm$ 0.21*
PCV%	38.49 $\pm$ 0.042	27.11 $\pm$ 0.169*
TLC($10^3/\mu\text{l}$ -1)	8.65 $\pm$ 0.34	5.46 $\pm$ 0.33*
Neutrophils ($10^3/\mu\text{l}$ -1)	2.8 $\pm$ 0.018	2.2 $\pm$ 0.047*
Basophiles ($10^3/\mu\text{l}^{-1}$)	0.0128 $\pm$ 0.0001	0.0128 $\pm$ 0.0001
Eosinophils ($10^3/\mu\text{l}^{-1}$)	0.98 $\pm$ 0.10	0.145 $\pm$ 0.0003*
Lymphocytes ($10^3/\mu\text{l}^{-1}$)	3.78 $\pm$ 0.025	2.72 $\pm$ 0.028*
Monocytes ($10^3/\mu\text{l}^{-1}$)	0.39 $\pm$ 0.001	0.35 $\pm$ 0.002*
Thrombocytes ($10^3/\mu\text{l}^{-1}$)	277.25 $\pm$ 2.4	177.8 $\pm$ 3.2*

*Means are significantly different at the level ($p \leq 0.05$)

According to Stockham et al. (2001) and Omer et al. (2002), the decrease in RBC could be caused by increased levels of activated complement products. Additionally, since the oxidised erythrocytes may be destroyed easily by erythrophagocytosis, oxygen radicals may also be involved in the pathogenesis of the resultant anaemia (Clark et al., 1986; Mbassa et al., 1994; Yagi et al., 2002).

Moreover, erythrophagocytosis, caused by an immune-mediated mechanism, might be responsible for the erythrocyte destruction (Uilenberg 1981). The removal of piroplasm-infected erythrocytes by macrophages in the organs of the reticulo-

endothelial system has been suggested as a cause of anaemia (Campbell and Spooner, 1999; Singh et al., 2001). In addition, pro-inflammatory cytokines, particularly TNF-a, have been implicated in mediating anaemia, associated with tropical theileriosis (Forsyth et al., 1999; Graham et al., 2001).

Some researchers have demonstrated that the leucocyte number in cattle increased immediately after theileria infection and then, significantly decreased within several days (Can et al., 1980; Mehta et al., 1988; Sandhu et al., 1998). *T. annulata* induced leucopenia in cattle, which was mainly mediated by TNF-a (Forsyth

et al., 1999). This decrease was related to the destruction of lymphocytes in lymphoid organs and infiltration of these cells into various organs (Sandhu et al., 1998; Omer et al., 2002).

In this study, significant decrease ($p \leq 0.05$) was recorded in neutrophil, eosinophil and lymphocyte number in *T. annulata* infected buffaloes, compared to those from the control ones. Similar findings were reported by Omer et al. (2002) in cattle and Osman and AL-Gaabary (2007) in buffaloes. However, an insignificant difference in absolute basophile and monocyte number between healthy and infected cattle was recorded by Omer et al. (2002), significant decreases in monocyte number was

recorded in our study. This variation could be attributed to the differences in the stage and severity of the disease.

Significant increases ($p \leq 0.05$) were found in the levels of MDA in *Theileria annulata* infected water buffaloes, compared with healthy buffaloes. In contrast, there was a significant reduction ($p \leq 0.05$) in the levels of NO, R.GSH, SOD, CAT, and TAC in *Theileria annulata* infected buffaloes, compared with healthy buffaloes (Table 3). According to authors, there are no available data concerning these levels in Egyptian water buffaloes. However, some of these levels were estimated in cattle by Asri-Rezaei and Dalir-Naghadeh (2006).

Table 3 - Levels of oxidants and antioxidants in *Theileria annulata*-free and infected buffaloes

Variable	Parasite-free buffaloes (No = 25)	Infected buffaloes (No = 68)
MDA (nmol/g Hb)	24.68 $\pm$ 0.19	104.45 $\pm$ 2.16*
NO (mmol/ml)	25.8 $\pm$ 0.24	18.78 $\pm$ 0.21*
R.GSH (mmol/L)	7.23 $\pm$ 0.21	2.85 $\pm$ 0.23*
SOD (u/g Hb)	9.24 $\pm$ 0.1	6.37 $\pm$ 0.07*
CAT (u/ml)	2.69 $\pm$ 0.02	0.96 $\pm$ 0.03*
TAC (mmol/L)	1.46 $\pm$ 0.011	0.62 $\pm$ 0.03*

*Means are significantly different at the level $p \leq 0.05$

Increased MDA concentration with decreased levels of NO, R.GSH, SOD, CAT and TAC in affected buffaloes may be an indicator of high oxidative stress in theileriosis. The oxidative stress appears when the production of free radicals and reactive metabolites of oxygen exceeds their safe disposal by

antioxidant mechanisms. Free oxygen radicals cause lipid peroxidation and gave MDA as the finished product. The determination of MDA is the mirror for the degree of lipid peroxidation and the level of free oxygen radicals, indirectly (Esterbauer, 1996; Yagi, 1998; Owen, 1996). The erythrocyte membrane is

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rich in polyunsaturated fatty acids, a primary target for reactions involving free radicals and is very susceptible to lipid peroxidation (May et al., 1998; Devasena et al., 2001).

Similar findings have been reported in theileriosis, caused by *T. sergenti*. Shiono et al. (2003a) showed that the levels of MDA began to increase remarkably in proportion to the decrease of packed cell volume and the increase of parasitemia in *T. sergenti* infected cattle during the onset of anaemia. During the serious stage of anaemia, this oxidative index reached its maximum value. They concluded that the oxidative damage to the RBCs might play an important role in the pathogenesis of anaemia in bovine theileriosis. Moreover, similar picture was previously reported in cattle by Asri-Rezaei and Dalir-Naghadeh (2006). Based on our results, the same pathogenic mechanism may also be involved in case of tropical theileriosis in Egyptian buffaloes.

The significant decrease in the activity of G6PD in affected buffaloes suffering of severe anaemia is an indicator of a metabolic disturbance of erythrocytes. This enzyme has a key role in the pentose phosphate pathway, which has critical significance in the survival of erythrocytes (Beutler, 1984). G6PD enzyme is the principal source of NADPH, which helps in maintaining glutathione at reduced state, thus protecting erythrocytes from oxidative stress. G6PD serves as an antioxidant enzyme and the low activity of G6PD

has been associated with increased haemolysis in cattle affected by theileriosis (Singari et al., 1991) and increased oxidative stress in endothelial cells (Leopold et al., 2003).

Our results revealed a significant decrease ($p \leq 0.05$) in G6PD activities. This result differs from that reported by Grewal et al. (2005), who showed a significant increase in the activity of this enzyme in cattle naturally infected with *T. annulata*. They considered that this increase was a safeguard mechanism for protecting the erythrocytes from oxidative stress, in response to increased lipid peroxidation in erythrocytes. The variation of the G6PD activities might be related to the severity of the anaemia. Low G6PD activity can be followed by reduced activities of SOD and GSH, because of the dependence of the activity of these enzymes on NADPH + H levels in the cell.

In concurrence with Agar and Board (1983), we have also found a direct relationship between the erythrocyte G6PD activity and the activity of R.GSH and SOD in infected buffaloes. We considered as very significant the decrease in the levels of R.GSH in affected buffaloes, which is in agreement with the findings of Ozan et al. (1999) in cattle naturally infected with *T. annulata*. On the other hand, Grewal et al. (2005) reported a significant increase in the activity of GSH-Px in infected cattle. The activity of GSH-Px is a major mechanism for the intracellular decomposition of lipid peroxides.

Christophersen, 1966; Flohe, 1971; Hafeman et al., 1974 also proposed that GSH-Px played a crucial role in preventing membranes from peroxide damage induced by lipid peroxides. Reduced glutathione is required for the disposal of H_2O_2 from erythrocytes by a reaction catalyzed by GSH-Px. This reaction is important because the accumulation of H_2O_2 might decrease the lifespan of erythrocytes by increasing the rate of oxidation of haemoglobin to methemoglobin (Winterbourn, 1985).

According to the results of this study, the catalase levels were significantly decreased ($p \leq 0.05$) in affected buffaloes. It has been reported that catalase is of equal importance to GSH-Px in the defence of human erythrocytes against H_2O_2 generating reactions (Harvey, 1989).

The evaluation of SOD levels in affected buffaloes showed a significant ($p \leq 0.05$) reduction, compared to the control ones. The reduced SOD levels were accompanied by decreased G6PD activity in infected erythrocytes. It appears that, during theileriosis, SOD (similarly to GSH) plays an important role in the protection of erythrocytes against oxidative stress. Similar findings were reported in other parasitic infections. It has been reported that *Plasmodium* infected erythrocytes showed a decreased capacity of their antioxidant enzymes, including superoxide dismutase (Friedman, 1979; Wozencraft, 1986;

Erel et al., 1997), catalase, glutathione peroxidase (Greve et al., 1999), G6PD (Roth et al., 1988), methemoglobin reductase (Stocker et al., 1985) and antioxidant substances, such as Vitamin E (Griffiths et al., 2001). Moreover, there was a significant reduction in the levels of TAC in infected buffaloes. This reduction may be attributed to the reduction in antioxidant enzymes as they are consumed by excessive free radicals in the infected animals.

The results revealed a significant decrease ($p \leq 0.05$) in the levels of glucose in *Theileria annulata* infected water buffaloes, compared with clinically healthy buffaloes. Moreover, there was a significant increase ($p \leq 0.05$) in the levels of NEFA and BHBA in *Theileria annulata* infected buffaloes, in comparison with healthy buffaloes, indicating the ketotic state of these cases. According to our knowledge, there are no available data concerning the correlation between *Theileria annulata* and ketosis (Table 4). These results may be attributed to the abnormalities in metabolism and anorexic state of affected buffaloes or may be due to the abnormalities in liver functions.

It could be concluded that *Theileria annulata* had an imperative task as an anemic, oxidative and ketotic stressor on the Egyptian Buffaloes.

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Table 4 - Levels of glucose, BHBA, NEFA and G6PD in *Theileria annulata* free and infected buffaloes

Variable	Control (No = 25)	Infected buffaloes (No = 68)
Glucose (mg/dl)	63.0 ± 0.6	37.2 ± 2.1*
BHBA (mmol L ⁻¹)	1.08 ± 0.03	1.9 ± 0.01*
NEFA (mmol L ⁻¹)	363.6 ± 5.5	536 ± 10.8*
G6PD (u/g Hb)	22.45 ± 0.15	17.28 ± 0.29*

*Means are significantly different at the level ($p \leq 0.05$)

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