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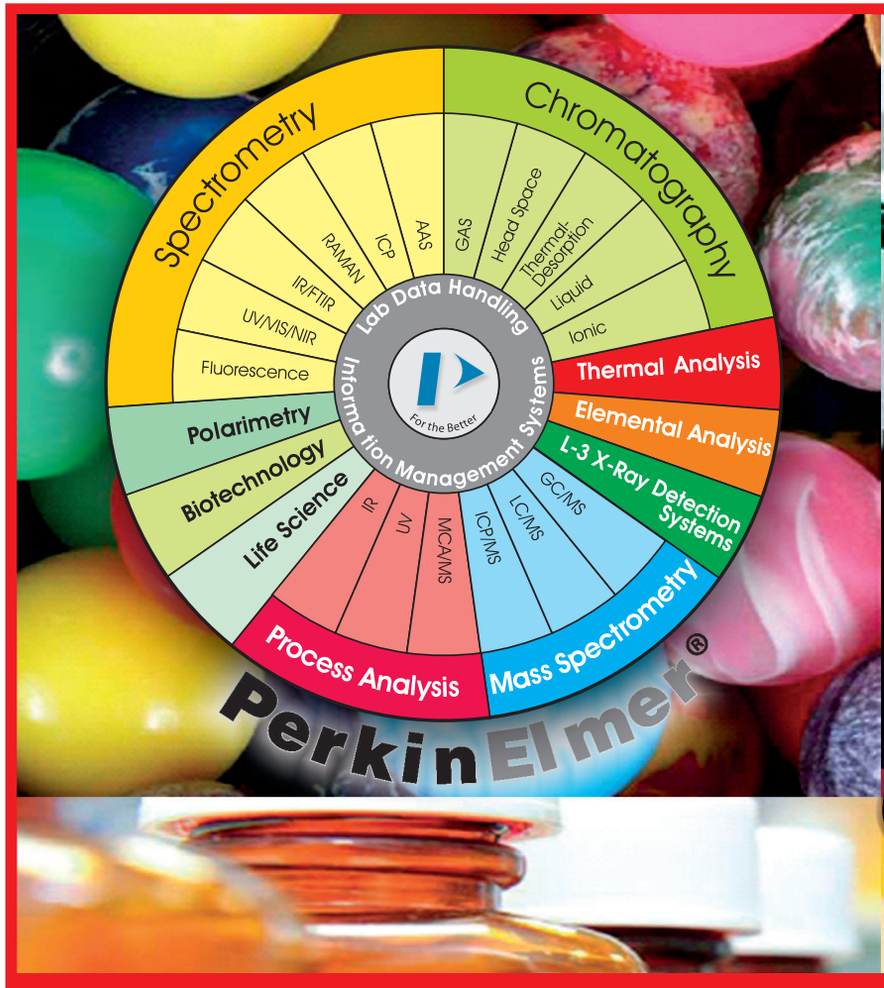

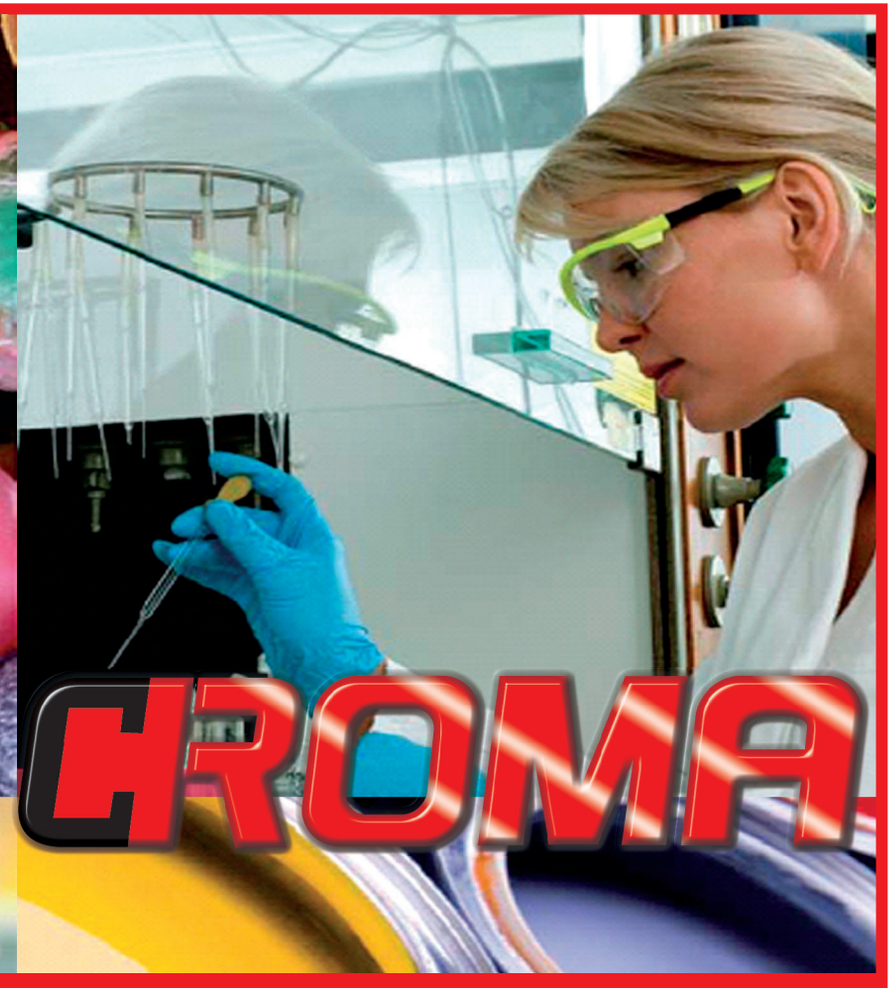

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Combined effect of Cygro® and vitamin E on oxidative stress status of broiler chickens infected with *Eimeria tenella*

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Abstract. *We measured plasma concentrations of malondialdehyde (MDA) as indices of the levels of lipid peroxidation products. In addition, we studied the activities of the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), in broiler chickens infected with Eimeria tenella and treated with a combination of Cygro® (1% maduramycin ammonium) and vitamin E. Before treatment with this drug combination there were significantly higher levels of MDA, decreased SOD and increased CAT erythrocyte activities in the infected chickens compared to the healthy controls (P<0.001). Treatment with Cygro® alone showed some beneficial effects on lipid peroxidation in broiler chickens infected with Eimeria tenella. However, a combination of Cygro® (5 ppm) and vitamin E, at a dose 50 mg per chicken for 11 days, did not attenuate oxidative stress (increased plasma MDA) and did not normalize the ecological oxidative balance (EOB) in the chickens (decreased SOD and increased CAT). The observed changes in the economical parameters (weight gain and FCR) were indicative for a severe infection, in which the oxidative stress probably was also involved during pathogenesis.*

Keywords: *Eimeria tenella*, maduramycin, vitamin E, lipid peroxidation, antioxidant enzymes

Abbreviations: CAT– catalase, EOB – ecological oxidative balance, FCR – feed conversion rasios, MDA – malondialdehyde, ROS – reactive oxygene species, SOD – superoxide dismutase

Introduction

Free radicals, including reactive oxygen species (ROS), such as $O_2^{\cdot-}$, H_2O_2, $^{\cdot}OH$, are products of activated macrophages and other phagocytic leukocytes and are known to be toxic to bacteria and some parasites (Rosen et al., 1995). They perform a significant role in resistance and immunity to infectious diseases (MacMicking et al., 1997; Tappel and Tappel, 2004). In order to avoid the damages caused by the ROS, multiple defence systems, collectively called antioxidants, are present in serum as well as in erythrocytes (Patterson and Leake, 1998). Erythrocytes are excellently equipped to handle intracellular oxidative stress through the combined activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione (GSH) (Stern, 1986). In a number of studies it has been demonstrated that concentrations of ROS are increased in parasitoses (Allen, 1997; Lillehoj and Li, 2004). One of the most frequently encountered parasite species in chickens is *Eimeria tenella* (Lawn and Rese, 1982) whose sporozoites are transported to the crypt epithelium, where they undergo development. Several polyether ionophores, such as monensin, lasalocid, salinomycin, narasin and maduramicin, are thought to minimise the possible negative effects of infection, the latter acting through general mechanisms of ion transport alteration and disruption of osmotic balance (Folz et al., 1988). These ionophores are important in coccidiosis control (Yadav and Gupta, 2001). ROS are produced also from exogenous sources including drugs (Sas, 1993; Hirose et al., 1997; Alistar and Boxall, 2004). According to an earlier proposition (Georgieva, 2005) the state of equilibrium between ROS generation and the detoxifying capacity of the endogenous antioxidant defence system could lead to a state of ecological oxidative balance (EOB) in the biological system. In a state of EOB, biological systems are maximally protected against oxidative damages of ROS (Georgieva and Gadjeva, 2005).

In our previous studies we have shown changes in blood levels of malondialdehyde (MDA) - a marker of radical-induced damage, as well as altered erythrocyte activities of the antioxidant enzymes SOD and CAT in broiler chickens, infected with *Eimeria acervulina* (Gabrashanska et al., 2005; Koinarski et al., 2005) and *Eimeria tenella* (Georgieva et al., 2006). Thus the changes in the antioxidant status of *Eimeria tenella* - infected chickens compared to healthy controls (Georgieva et al., 2006) have formed the basis for further studies on decreasing oxidative stress in broiler chickens infected with *Eimeria tenella*. This is with a view to fashioning out a possible future treatment regime using a combination of a coccidiostatic agent and an antioxidant agent. As of now, the use of a combination therapy, involving Cygro® (1% maduramycin ammonium) and vitamin E, in this direction is unknown.

Accordingly, the aim of the present study was to determine the effect of a combined application of the ionophore antibiotic Cygro® and vitamin E on the oxidative stress status of broiler chickens infected with *Eimeria tenella.*

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Materials and methods

Compounds tested

Cygro® was obtained from Alpharma - Animal Health Divisoin (USA). Vitamin E - DL-α-tocopherol acetate was obtained from Merck KGaA (Darmstadt, Germany), hydrosoluble 1mL = 500 mg DL-α-tocopherol acetate.

Experimental animals

The experiment was approved by the Committee on Animal Experimentation at the Trakia University, Stara Zagora, Bulgaria and was performed according to the recommendation of Directive 86/609/EC from November 24, 1986.

The study was performed on 80 clinically healthy broiler chickens, Cobb 500 hybrids, aged 20 days, weighing 335-370 g. Up to 11 days, the birds were housed in cages on slat floors under conditions excluding further *Eimeria* infection. The chickens received a standard diet without antibiotics or coccidiostats. At 11 days of age, four experimental groups of 20 birds each were established. The first experimental group comprised healthy, untreated and non-infected (negative control) birds. The birds from the groups II, III and IV were infected once with $8x10^4$ sporulated *Eimeria tenella* oocysts on day 13. The fodder of chickens from the groups III and IV was supplemented with Cygro® (5 ppm) beginning from two days prior to the infection (day 11) to the end of the period of the study (post infection day 7). The birds from the group IV received also vitamin E at a dose of 50 mg per chicken x day^{-1} with fodder, beginning two days prior to the infection (day 11) to the end of the period of the study (post infection day 7).

Infectious material

Eimeria tenella oocysts were obtained from naturally infected chickens, passed through 2-weeks-old broiler chickens and stored in 2.5% potassium bichromate solution in a refrigerator (4 °C). The sporulated oocysts were administered via an ingluvial tube.

Biochemical investigations.

Blood for biochemical analyses (5 mL) for MDA, SOD and CAT assays was sampled from the *cutaneous ulnar or brachial vein* (of every chicken from the experimental groups) on post infection day 7. Ethylenediaminetetraacetic acid (EDTA) was used as anticoagulant.

Peripheral blood processing. Collected blood was centrifuged at 2000 - 3000 *g* for 15 min and plasma was separated. Then, the plasma was deproteinized with 25% trichloroacetic acid by continuous mixing for 5 min and centrifugation at 2000 *g* for 15 min.

Erythrocyte Processing. The erythrocyte pellet was washed three times with saline and lysed. In brief: after the erythrocyte pellet was washed three times with saline, and 0.5 mL of the cell suspension was diluted with 2 mL cold water to lyse the erythrocytes. To 0.2 mL lysate, 1.8 mL water and ethanol/chloroform (3:5/v:v) were then added to precipitate hemoglobin. The tubes were shaken vigorously for 5 min and centrifuged at 2500 *g* for 20 min. The supernatants were used for determine of enzyme activity.

Determination of lipid peroxidation products. The deproteinized plasma was used for lipid peroxidation products determination. The total amount of lipid peroxidation products in plasma was assayed using the thiobarbituric acid reactive substance (TBARS) method, measuring spectrophotometrically malondialdehyde at 532 nm (Placer et al., 1966).

Determination of superoxide dismutase activity. Erythrocyte lysates were assayed for CuZn-SOD activity by method of Sun et al., (1988) with minor modifications. Briefly, the xanthine/xanthine oxidase system was used to generate the superoxide anion (O2.–)x. This anion reduces nitroblue tetrazolium (NBT) to formazan, which is monitored at 560 nm. SOD in the sample removes the O2.– and inhibits the reduction. The level of this reduction is used as a measure of SOD activity. One unit of enzymatic activity is defined as the amount of enzyme causing 50% inhibition of the reduction of NBT to formazan observed. Results were expressed as units per g hemoglobin (U/gHb).

Determination of catalase activity. CAT activity was assessed in the erythrocyte lysats by the method described by Beers and Sizer (1952) with minor modifications. Briefly, hydrogen peroxide (30Mm) was used as a substrate and the decrease in H_2O_2 concentration at 22°C in phosphate buffer (50 Mm, pH 7.0) was followed spectroscopically at 240 nm. One unit of CAT activity is defined as the amount of enzyme that degrades 1M H_2O_2 per min. Results are presented as units per g hemoglobin (U/gHb).

Hemoglobin concentrations. Hemoglobin concentrations of lysates were analysed by cyanmethaemoglobin method (Mahoney et al., 1993).

Statistical analysis

The results are reported as mean SD. Statistical analysis was

Table 1. Plasma MDA concentrations, erythrocyte SOD and CAT activities in broiler chickens

Groups	Group I	Group II	Group III	Group IV
Parameters	Non-infected, untreated	Infected, untreated	Infected + Cygro®	Infected + Cygro® + vit. E
MDA (μmol/L)	2.56 ± 0.05	3.00 ± 0.09^{1c}	2.59 ± 0.07^{2b}	3.21 ± 0.07^{1c}
SOD (U/g Hb)	3046.4±147.9	2434.0 ± 91.4^{1b}	1978.7 ± 129.3^{1c}	$2461.1 \pm 141.5^{1b,3b}$
CAT (U/g Hb)	1367.1±68.9	2307.4 ± 80.6^{1c}	2432.1 ± 100.7^{1c}	$7264.0 \pm 253.4^{1c,2c,3c}$

Values are presented as mean ± SD ; n = 20 chickens in each group; $^aP<0.05$; $^bP<0.01$; $^cP<0.001$; 1 – *vs*. non-infected, untreated (negative controls) ; 2 – *vs*. infected, untreated (positive controls) ; 3– *vs*. infected and treated with Cygro® (Maduramicin, Alpharma-Animal Health Division)

performed using the Mann-Whitney *U*-test. *P*-values of < 0.05 were considered statistically significant.

Results

Six days after the *Eimeria tenella* inoculation, five birds from positive controls (group II) died. Blood MDA concentrations and the activities of antioxidant enzymes SOD and CAT in the birds are presented in Table 1. A significant increase in average MDA concentrations was observed in those infected with *Eimeria tenella* (positive controls), compared to the healthy ones-negative controls ($P<0.001$). Average MDA levels were found to be slightly higher (not significant) in chickens infected with *E. tenella* and treated with the coccidiostatic Cygro®, than in negative controls ($P>0.05$) and were significantly reduced, compared to the positive controls ($P<0.01$). Plasma MDA concentrations remained increased in infected birds, than in healthy birds, after combined application of Cygro® and vitamin E ($P<0.001$).

Erythrocyte SOD activity was decreased in the *Eimeria tenella*-infected but non-drug treated chickens, compared to the negative controls (Table 1). On the other hand, erythrocyte SOD activity in the chickens infected with *Eimeria tenella* and treated with Cygro® was decreased, compared to the negative ($P<0.001$) and to the positive ($P<0.001$) controls. Ultimately, the erythrocyte SOD activity in the group of chickens treated with a combination of Cygro® + vitamin E was decreased in comparison with the negative control group ($P < 0.001$) and was increased in the group treated with only Cygro® ($P<0.01$). The SOD activity was found to be significantly lower in all groups of chickens (groups II, III and IV) infected with *Eimeria tenella,* compared to the negative controls (group II *vs.* group I, $P<0.01$; group III *vs.* group I, $P<0.001$ and group IV *vs.* group I, $P<0.01$). In contrast, significant increase in the erythrocyte CAT activity was evidenced in all *Eimeria tenella* infected birds, compared to healthy ones ($P<0.001$). CAT activity in the infected and Cygro®-treated chickens did not show any significant change compared to the positive controls ($P>0.05$). However, the level of CAT in infected chickens treated with a combination of Cygro® + vitamin E was markedly increased, compared to the healthy ($P < 0.001$), compared to the infected but non-drug treated ($P<0.001$), and compared to the infected and Cygro®-treated ($P<0.01$) groups.

Table 2 presents the lesion scores and the oocyst index. Our results of lesion scores (2.8±0.7) showed that 7 days after the challenge, *Eimeria tenella* produced substantial injuries in the caeca manifested by visible erosions of the mucosa, extremely thinned mucosa and blood on the intestinal lumen as well as enlargement in the size of the intestine. In contrast, lesion scores in the chickens infected with *Eimeria tenella* and treated with only Cygro® (group III) and Cygro® + vitamin E (group IV) were decreased, compared to the positive controls ($P<0.05$). The oocyst index (31.0±6.3) in infected but non-treated birds (group II), as expected, indicated that oocysts had been released in significant amounts by chickens

Table 2. Changes in lesion scores and in oocyst index in broiler-chickens

Groups	Lesion scores	Oocyst index
I. Healthy (-) controls	–	–
II. *E.tenella* (+) controls	2.8 ± 0.7	31.0 ± 6.3
III. *E.tenella* + Cygro®	1.1 ± 0.3[a]	5.2 ± 1.1[a]
IV. *E.tenella* + Cygro® + vit. E	1.0 ± 0.2[a]	4.8 ± 1.5[a]

Values are presented as mean ± SD ; n = 10 chickens in each group; aP<0.05 vs. infected untreated (positive controls).

infected with *Eimeria tenella*, demonstrating the development of infection. It was find that the oocyst index in infected and treated 20-day-old chickens (group III and IV) significant decreased, compared to the positive controls ($P<0.05$).

Table 3 presents the live body weights, the weight gains and the feed conversion ratios (FCR) in chickens, infected with *Eimeria tenella* and healthy chickens. The results evidenced that at the beginning of the experiment, the body weight of all groups was equal but at the end (7 days after infection), it was significantly lower in infected birds (group II) compared to the healthy chickens ($P<0.001$). This decrease was obviously due to the relatively low

Table 3. Changes in the live weight, the weight gain and FCR in healthy and *Eimeria tenella* infected broiler-chickens

Groups	Initial live weight, g	Final body weight, g	Weight gain, g/chicken	Weight gain, %	FCR
I. Healthy (-) controls	170.0±2.1	362.0±5.8[2]	192.0±11.8[1]	100	0.98
II. *E.tenella* (+) controls	173.0±3.1	233.0±4.6[b]	60.0±3.8[a]	31.2	3.10
III. *E.tenella* + Cygro®	170.0±1.8	310.0±3.8[a]	140.0±10.5[a]	72.9	1.10
IV. *E.tenella* + Cygro® + vit. E	175.0±1.3	340.0±10.8	140.0±11.5	72.9	1.20

Results are presented as mean ± SD (20 chickens in group). ; [a]$P<0.05$; [b]$P<0.001$ vs. negative controls [1]$P<0.05$; [2]$P<0.001$ vs. positive controls

weight gain (31.2 – 72.9, %) in all infected chickens compared to healthy controls. After treated with maduramycin (group III) weight gain was found to be significantly higher, compared to infected non-treated birds (P<0.001). Similar results were found to the chickens treated with the combination of Cygro® + vitamin E (group IV). At the same time, FCR in infected birds was higher, compared to healthy controls (3.1 vs. 0.98). After treated with only maduramycin, FCR was found to be lower, compared to infected non-treated birds (1.1 vs. 3.1). FCR in infected birds and combined treated (group IV) was remaining lower to FCR in positive control group (group II) (1.20 vs. 3.1).

Discussion

Based on the previous and present studies we could show the role of SOD, CAT and MDA as markers of oxidative stress and EOB in protozoal infections. The results shown in this paper indicated that the infection of chickens with *Eimeria tenella* was accompanied by oxidative stress and disrupted EOB in the host. The significant changes in plasma concentrations of MDA and erythrocyte SOD and CAT activities (Table 1) in chickens infected with *Eimeria tenella,* compared to healthy controls, were indicative of a severe infection. This was supportive of our proposition to the effect that oxidative stress with the attendant disruption of the EOB could be pathognomonic of *eimeriosis*. The oxidative stress and injuries are considered to be a component of virtually every disease process (Tappel and Tappel, 2004). Biochemical evidence of oxidative injury can be shown through the detection of lipid peroxidation product, such as MDA (de Zwart et al., 1999). The present results showed that *Eimeria tenella* infection leads to increased levels of lipid peroxidation products measured by the formation of MDA (P<0.001). Lipid peroxidation is an autocatalytic mechanism leading to oxidative destruction of cellular membranes (Cheeseman, 1993; Kühn and Borchert, 2002; Popova and Popov, 2002). Probably the increased levels of MDA in blood of infected non-treated chickens showed a structural modification of complex lipid protein assemblies, such as biomembranes and lipoproteins, which usually associated with cellular malfunction and formation of free radicals. The results showed decreased MDA concentration in the infected and Cygro®-treated chickens compared to the infected but non-drug treated ones (P<0.01), and no significant change, compared to the healthy controls (P>0.05). Oral Cygro® treatment produced a decrease in MDA level and an increased imbalance of antioxidant enzyme system. The impaired enzyme antioxidant system may favour accumulation of ROS, which probably induced *Eimeria tenella* infection. Free radicals including ROS are known to be toxic to some parasites (Rosen et al., 1995). Probably ROS may also be useful in fight against other skin infections and maduramycin could reduce the negative effects of *Eimeria tenella.* This agrees with our observation of decreased level of MDA – marker of oxidative stress, in plasma of chickens treated with Cygro®. After combined application of Cygro® and vitamin E, MDA in plasma of chickens infected with *Eimeria tenella* was found to remain higher. We concluded that addition of vitamin E at dose 50 mg per chicken every day in the diet did not protect against *Eimeria tenella*. Probably, its presence did not affect the early events in parasite development. Similar results of Vitamin E supplement in chickens infected with *Eimeria maxima have been* reported by Allen and Fetterer (2001, 2002). Vitamin E may cause beneficial or harmful effects on health depending on various modifying factors (Hemilä et al., 2006).

According to the results, increased MDA concentration was accompanied by an imbalance of the activity of the antioxidant enzymes SOD and CAT during the infection. Erythrocytes are equipped with a highly effective antioxidant defence system (Frei, et al., 1988). Superoxide dismutase is believed to play a major role in the first line of antioxidant defence (Fridovich, 1975). In the present study the activity of SOD was found to be significantly lower in all groups infected with *Eimerea tenella* compared to the healthy controls. Based on the decreased SOD activity, Isler et al., (2002) proposed that ROS, especially hydrogen peroxide, inhibited SOD activity. Probably *Eimeria tenella* infection produced ROS (including $O_2^{\cdot-}$) in Cygro®-treated, group treated with Cygro® + vitamin E and the non-treated group, thus leading to inhibition of SOD activity. Furthermore, decreased SOD activity may contribute to the free radical production. Therefore, we can speculate that $O_2^{\cdot-}$ may be converted rapidly to hydrogen peroxide by SOD. Hydrogen peroxide is not especially toxic to cells, but it can cross cellular membranes and, in the presence of trace amounts of transition metal ions, can interact with $O_2^{\cdot-}$ to produce hydroxyl radicals (˙OH)x. The hydroxyl radicals will react with whatever biological molecule in its vicinity. It will damage proteins, cause DNA strand breakage, and initiate lipid peroxidation. The ultimate damaging species should be considered whenever superoxide is formed. It would agree with our observation about the higher MDA concentration.

Our results showed that CAT activity in the infected birds (group II) was higher compared to the healthy control group (P<0.001). The activity of CAT was found to be higher in all groups of treated, infected chickens compared to the negative control group (P<0.001). The concomitant increase of CAT activity would be a compensatory mechanism in the infected birds. This finding was compromised by reduction of the SOD activity in chickens of all infected groups (treated and untreated). Even though CAT is not essential for some cell types under normal conditions it, however, plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells (Turrens et al., 1984). This suggests the pivotal role of CAT for cell adaptation to oxidative stress. Such a role of CAT has been previously reported (Mates et al., 1999). Thus the antioxidant status of chickens may play an important role in their response to the Cygro® and vitamin E application.

Our data with regard to the lesion scores and the oocyst index values (Table 2) showed that by day 7 post infection, *Eimeria tenella* provoked significant injuries to the intestinal mucosa. Alterations in the caecum and oocyst production were indicative of a severe infection involving pathogenic oxidative stress and impaired EOB.

The established changes in caeca and the oocyst production in Cygro®-treated group of chickens (Group III) were indicative of beneficial effect of maduramycin on *eimeriosis* infection. The combined effect of Cygro® + vitamin E on the lesion scores and the oocyst index did not significant difference than the effect of only Cygro® (group IV vs. group III, P>0.05). Probably complex interaction among maduramycin, *Eimeria tenella* and vitamin E at a dose 50 mg per chickens did not attenuate oxidative injury resulting from infection of chickens with *Eimeria tenella.*

The World Association for the Advancement of Veterinary Parasitology (WAAVP) requires the determination of the parameters live body weight, weight gain and FCR for evaluation of the severity of infection (Holdsworth et al., 2004). In compliance with those necessities we observed a stunted growth in chickens infected with *Eimeria tenella* and the increased FCR (Table 3) indicated a severe infection. The lower live weight in infected birds was due on one part

to the worsened FCR and probably to impaired endogenous enzymatic systemic defense, on the other. The statistically significant changes in blood SOD, CAT and MDA levels (Table 1) in chickens, infected with *Eimeria tenella* compared to healthy controls, also supported our hypothesis.

The results of the live body weights, the weight gains and the FCR in chickens, infected with *Eimeria tenella* and healthy chickens evidenced that at the beginning of the experiment, the body weight of all groups was equal but at the end, it was significantly lower in all infected birds (Table 3). This decrease was obviously due to the relatively low weight gain in these chickens compared to healthy controls. At the same time, FCR in infected birds was higher than in negative control group. The observed changes in the economical parameters (weight gain and FCR) were indicative for a severe infection, in which the oxidative stress probably was also involved during pathogenesis.

Conclusion

Present results suggest that *Eimeria tenella* infection provoked the lipid peroxidation and imbalance in the enzyme antioxidant defence system in the infected chickens. It is suggested that *Eimeria tenella* would be reflected as disruption of the EOB in infected broiler chickens. Our results are suggestive of the beneficial effects of Cygro® on lipid peroxidation. However, this did not restore the EOB characteristic of the antioxidant defence system in chickens infected with *Eimeria tenella*. Cygro® after combined application with vitamin E did not prevent or lessen the oxidative stress caused by *Eimeria tenella* infection in chickens.

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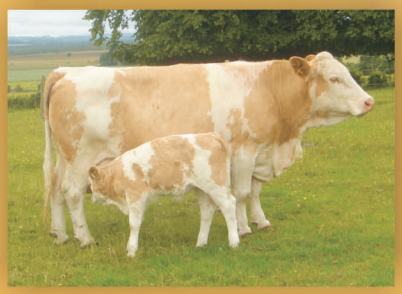

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