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*Full Length Research Paper*

# Effect of dietary vitamin E on *Eimeria tenella*-induced oxidative stress in broiler chickens

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**An experiment was carried out to investigate the impact of high doses of dietary vitamin E on antioxidant status in broiler chickens (Ross 308) experimentally infected with *Eimeria tenella.* One day old chicks were assigned to five groups (25 each) and given basal diet (A and B) or basal diet supplemented with 100, 316 or 562 mg/kg of vitamin E (C to E), respectively. On the 21st day, all chicks except those in group A were inoculated with *E. tenella* and monitored for any change in blood vitamin E, malondialdehyde (MDA) and superoxide dismutase (SOD). Plasma vitamin E decreased by infection, but increased with dietary vitamin E ($p<0.05$). A significant rise of plasma and erythrocyte MDA was observed in infected birds ($p<0.05$), however, the chicks fed diet with 316 mg/kg added vitamin E had a lower MDA compared to infected controls ($p<0.001$). The erythrocyte SOD was not affected by infection ($p>0.05$), but it was significantly higher in group D than in groups B and E ($p<0.05$). In conclusion, addition of dietary vitamin E at 316 mg/kg can afford antioxidant protection to chickens infected with *E. tenella*, but at higher doses it may aggravate the unbalanced oxidant/antioxidant status.**

**Key words:** *Eimeria tenella*, oxidative stress, broiler chickens, vitamin E, malondialdehyde, superoxide dismutase.

## INTRODUCTION

Vitamin E (D-α-tocopherol; AT) is the most widely known natural antioxidant donating electrons to free radicals in biological membranes, thereby making them stable. This prevents free radicals from binding to fatty acids, inhibits oxidative reactions and therefore maintains cell membrane integrity (Leeson and Summers, 2001). AT is generally added as DL-α-tocopheryl acetate (VE-AC) to commercial poultry feeds at levels from 17 mg/kg (Erf et al., 1998) to 48 mg/kg (Allen and Fetterer, 2002). Some studies showed that high dietary supplements of AT can enhance humoral immune response of chickens to Newcastle disease (Franchini et al., 1986), infectious bursal disease (McIlroy et al., 1993), and *Escherichia coli* infection (Macklin et al., 2000).

Coccidiosis of chickens is caused by seven species of intracellular protozoan parasites of the genus *Eimeria*, responsible for major economic losses in poultry industry by increasing mortality and reducing growth rates (Guo et al., 2007). Free radical oxidative species are known to be generated during the host's cellular immune response to invasion by *Eimeria* sp. (Allen and Teasdale, 1994; Allen, 1997), which is important in defending against parasite infections. However, their high concentrations may be over the threshold of cell tolerance, causing tissue damage and cytotoxicity (Evans and Halliwell, 2001) and partly contributing to the pathology of infection. Since AT is a potent antioxidant, this experiment was performed on

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**Abbreviations : MDA,** Malondialdehyde; **SOD,** superoxide dismutase.

**Table 1.** Composition of base mash for experimental diet.

| Item | Broiler starter (1 to 20 day) | Broiler grower (21-29 day) |
|---|---|---|
| **Ingredients** | | |
| Corn | 58.80 | 61.20 |
| Soybean meal (44%) | 32.00 | 31.00 |
| Fish meal (60%) | 3.50 | 2.50 |
| Vegetable oil | 2.00 | 2.00 |
| Oyster shell (30% purity) | 1.80 | 1.50 |
| Di-calcium phosphate | 1.00 | 1.00 |
| Vitamins- minerals premix[1] | 0.50 | 0.50 |
| Salt | 0.19 | 0.15 |
| DL-methionine | 0.20 | 0.15 |
| L-lysine | 0.01 | -- |
| Total | 100.00 | 100.00 |
| **Calculated composition** | | |
| Metabolisable energy (kcal/kg) | 3,010 | 3,045 |
| Crude protein (%) | 21.4 | 20.4 |
| Fat (%) | 4.3 | 4.5 |
| Fibre (%) | 3.8 | 3.7 |
| Methionine (%) | 0.56 | 0.49 |
| Lysine (%) | 1.18 | 1.15 |
| Calcium (%) | 0.97 | 0.90 |
| Available P (%) | 0.47 | 0.45 |
| Sodium (mg/kg) | 1,550 | 1,400 |
| Chloride (mg/kg) | 1,850 | 1,600 |

[1]Vitamin and mineral premix provided per kilogram of diet are: vitamin A, 9,000 IU; vitamin $D_3$, 2,000 IU; vitamin E, 18 IU; vitamin $K_3$, 2 mg; thiamine, 1.8 mg; riboflavin, 6.6 mg; Ca pantothenate, 10 mg; niacin, 30 mg; pyridoxine, 3 mg; folic acid, 1.0 mg; vitamin $B_{12}$, 0.015 mg; biotin, 0.1 mg; choline chloride, 50 mg; Fe, 50 mg; Cu, 10 mg; Zn, 100 mg; Mn, 100 mg; I, 1.0 mg; Se, 0.2 mg.

the assumption that supplementing diet with high amounts of VE-AC might counteract the effectors of oxidative stress and improve the imbalanced oxidant/ antioxidant status induced by *E. tenella* infection in chickens. For this purpose, malondialdehyde (MDA), as a biomarker for radical-induced lipid peroxidation, and superoxide dismutase (SOD), an enzyme involved in endogenous antioxidant defence against free radicals were chosen to be assayed.

## MATERIALS AND METHODS

### Chickens, housing and diets

All chickens were Ross 308 of both sexes obtained at day 1 from a local hatchery and then reared in wire-floored cages with continuous lighting and in accordance with the principles presented in Guide for the Care and Use of Laboratory Animals (NRC, 2010). All chicks were free from coccidian infection, but vaccinated against Newcastle disease virus. Corn and soybean meal-based mash diet (Table 1) without any antibiotic additives, was formulated (NRC, 1994) to meet the nutrient requirements of broiler chicks and were provided *ad libitum* to all chicks with water.

### Parasites

The Houghton strain of *E. tenella* kindly provided by Dr. Damer Blake (Department of Pathology and Infectious Diseases, The Royal Veterinary College, University of London) was used in the current experiment. It was maintained by periodic passage through coccidia-free chickens, and the unsporulated oocysts isolated from cecum on the 7th day post-inoculation (pi) were purified and processed by standard method (Chapman and Shirley, 2003). Before challenge, the population of sporulated oocysts were determined by haemocytometer (Germany), and then adjusted to the desired infective dose in 1 ml of phosphate-buffered saline (PBS).

### Experimental protocol

The experimental chickens were randomly allocated to five groups of 25 chickens each. The chicks in groups A and B were fed basal diet and served as controls, but those in groups C to E received basal diet supplemented with 100, 316 or 562 mg/kg of VE-AC (Aras Bazar Pharmaceutical Co., Amol, Iran), respectively. To avoid accidental contamination, infected chicks were housed in separate but comparable conditions as uninfected chicks. At day 20, the excreta beneath all cages were examined for any unwanted coccidian infection. On the next day, all chicks except those in

**Table 2.** Effect of graded levels of dietary vitamin E on the blood levels[1] of vitamin E, malondialdehyde (MDA) and superoxide dismutase activity (SOD) in chickens infected with *Eimeria tenella*.

| Parameter | Sample[2] | Group[3] | | | | |
|---|---|---|---|---|---|---|
| | | A | B | C | D | E |
| Plasma vitamin E (mg/dl) | 1 | 0.29 ± 0.06$^{b}$ | 0.17 ± 0.03$^{c}$* | 0.21 ± 0.04$^{c}$* | 0.29 ± 0.04$^{ab}$* | 0.34 ± 0.03$^{a}$* |
| | 2 | 0.35 ± 0.03$^{c}$ | 0.25 ± 0.02$^{d}$ | 0.36 ± 0.03$^{c}$ | 0.42 ± 0.03$^{b}$ | 0.47 ± 0.04$^{a}$ |
| Plasma MDA (nmol/ml) | 1 | 1.12 ± 0.07$^{c}$ | 1.98 ± 0.14$^{a}$ | 1.88 ± 0.07$^{a}$ | 1.41 ± 0.09$^{b}$ | 2.00 ± 0.03$^{a}$ |
| | 2 | 1.13 ± 0.07$^{d}$ | 1.87 ± 0.06$^{ab}$ | 1.78 ± 0.10$^{b}$ | 1.33 ± 0.08$^{c}$ | 1.93 ± 0.10$^{a}$ |
| Erythrocyte MDA (nmol/g-Hb) | 1 | 64.22 ± 4.91$^{d}$ | 90.82 ± 6.25$^{b}$ | 85.37 ± 7.39$^{b}$ | 75.98 ± 3.54$^{c}$ | 105.25 ± 5.72$^{a}$ |
| | 2 | 65.59 ± 4.86$^{d}$ | 87.44 ± 5.37$^{b}$ | 84.43 ± 5.30$^{b}$ | 74.29 ± 4.48$^{c}$ | 103.63 ± 4.11$^{a}$ |
| Erythrocyte SOD (Unit/mg-Hb) | 1 | 2.79 ± 0.10$^{ab}$ | 2.70 ± 0.02$^{b}$ | 2.72 ± 0.11$^{b}$ | 2.93 ± 0.12$^{a}$ | 2.68 ± 0.08$^{b}$ |
| | 2 | 2.80 ± 0.12$^{ab}$ | 2.64 ± 0.10$^{b}$ | 2.70 ± 0.10$^{ab}$ | 2.92 ± 0.12$^{a}$ | 2.62 ± 0.11$^{b}$ |

[1]Values are means ± SEM from 8 chicks per group at each sample. [2]First and second samples were taken on days 4 and 8 after infection, respectively. [3]Group A: non-infected and fed basal diet; group B: infected and fed basal diet; groups C to E: infected and fed 100, 316 or 562 mg/kg vitamin E, respectively. [a-d]Means in the same row with no common superscript are significantly different ($P$<0.05).
*Indicates a significant difference between the first and the second sample in each group.

group A were challenged via crop intubation with $4 \times 10^4$ sporulated oocysts of *E. tenella* per bird, whereas the chicks in group A were sham-inoculated with PBS. All birds were starved overnight for 8 h before inoculation. All groups were bled for any change in plasma vitamin E, plasma and erythrocyte MDA, and erythrocyte SOD activity.

### Samples

On days 4 and 8 pi, blood samples were taken via the wing vein from 8 birds in each group. After bleeding, the chicks were colour-marked so that they were not reused for blood collection. Each sample was transferred into 5-ml sterile EDTA-K3 containing tubes and immediately centrifuged at 2,500 rpm for 10 min at 4°C. Then, the obtained plasma was aliquoted into microtubes and stored at -80°C until tested. Next, the erythrocyte pellet was processed for preparation of hemolysate (Ivanov, 1999). It was washed three times with PBS, and then 0.5 ml of the cell suspension was diluted with 2 ml of cold water to lyse the erythrocytes. To 0.2 ml lysate, 1.8 ml water and ethanol/chloroform (3:5/ v: v) were added to precipitate haemoglobin. The tubes were shaken vigorously for 5 min and centrifuged at 2,500 rpm for 20 min. The supernatants were used for the assessment of MDA and SOD.

### Biochemical analysis

The plasma vitamin E was measured according to Martinek (1964) in an oxidimetric colour reaction in which ferrous ion, produced through the reduction of ferric ion by vitamin E (tocopherols), forms a ferrous-TPTZ (2,4,6-tripyridyl-1,3,5-triazine) colour complex and this is used as an index for the determination of vitamin E concentration. Briefly, each plasma sample (1 ml) was saponified in the presence of 1 ml absolute ethanol and 1 ml xylene and then centrifuged at 1,500 × g for 5 min. The supernatant (500 µL) was removed and mixed with 500 µL of TPTZ (Sigma) and 100 µL of 0.12% ferric chloride. Then, absorbance was rapidly measured spectrophotometrically (Shimadzu, UV-120-12; Kyoto, Japan) at 600 nm, and the concentration was expressed as mg/dl.

The total amount of lipid peroxidation in plasma and erythrocyte lysate was estimated using thiobarbituric acid (TBA) method, by measuring malondialdehyde reactive products (Placer et al., 1966). In this method, 1 ml of TBA-TCA solution was added to 0.5 ml of each specimen in test tube and then it was placed in a boiling water bath for 15 min. After cooling in tap water, the tube was centrifuged at 1,000 × g for 10 min, and the absorbance of the supernatant was measured spectrophotometrically at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the MDA–TBA complex (absorbance coefficient $\varepsilon = 1.56 \times 105\ cm^{-1}M^{-1}$) and was expressed as nmol/ml. For erythrocyte lysate, the total haemoglobin (Hb) of each sample was determined using commercial kit (ZiestChem Diagnostic, Iran), and the MDA was expressed as nmol/g-Hb.

The SOD activity in erythrocyte lysate was assayed by commercial assay kit (Randox, England), which was based on the inhibition of the reduction of idonitrotetrazolium chloride by SOD in a xanthine/xanthine oxidase system. All measurements were done in duplicate.

### Statistical analysis

Differences among groups were tested by one-way ANOVA (Systat Software Inc., Point Richmond, Version 2.03, CA, USA). Values were compared using Tukey's post hoc test when they passed the normality test and Dun's post hoc test in case of failure to pass normality. Differences were considered significant at $p$ <0.05.

## RESULTS

The alterations of biochemical parameters are presented in Table 2. As demonstrated, infection with *E. tenella* resulted in a remarkable reduction of plasma vitamin E in chickens of group B ($p$<0.05). However, its plasma concentration increased when diet was supplemented with

VE-AC. Furthermore, infected birds had a significantly higher level of plasma and erythrocyte MDA in relation to healthy chickens ($p$<0.05). Moreover, the chicks fed diet with 316 mg/kg added VE-AC had a noticeably lower concentration of MDA compared to infected controls ($p$<0.05). Regarding SOD activity, challenge with *E. tenella* had no significant effect ($p$>0.05), but it was significantly higher in group D than in groups B and E ($p$<0.05).

## DISCUSSION

As shown in Table 2, plasma vitamin E in group B was significantly depressed by infection ($p$<0.05), but increased with dietary VE-AC in other infected groups. Allen and Fetterer (2002) also found that in *E. maxima*-infected chickens, supplementing diet with high amounts of VE-AC (13 to 200 mg/kg) increased plasma D-α-tocopherol (AT); however, regardless of its dietary level, infected chicks had lower plasma AT compared with the diet-matched uninfected chickens. This reduction was postulated by the authors to be due to AT malabsorption in mid-intestine, which is parasitized by *E. maxima*. *E. tenella* infects the cecal mucosa and is not a major effector of lipid malabsorption, but it was associated with an increase of nitric oxide species (Wang et al., 2008) and a decrease of antioxidant enzyme SOD (Georgieva et al., 2006), suggesting that depressed appetite and the bleeding observed in *E. tenella* infection as well as increased tissue demand for vitamin E to neutralize free radicals may account for the decreased plasma vitamin E in the current experiment. A significant drop in plasma level of vitamin E was observed in heat-stressed laying hens (Yardibi and Turkay, 2008) and was attributed to the decrease of feed intake and the increased demand for antioxidants.

The results of the present study showed that plasma and erythrocyte concentrations of MDA were significantly higher in infected chicks than in the controls and the differences were significant either 4 or 8 days after infection ($p$<0.05). MDA is used as a biomarker for radical-induced damage of biological membranes (Day, 1996), increasing in numerous physiological (Yardibi and Turkay, 2008) and pathological states. Therefore, the higher concentration of MDA in infected chicks could be attributed to lipid peroxidation resulting from increased reactive-oxygen species (ROS). In previous studies, increased plasma levels of MDA were observed in chickens infected with *E. acervulina* (Koinarski et al., 2005) and *E. tenella* (Georgieva et al., 2006; Wang et al., 2008). An interesting finding in this experiment was that MDA level in plasma and erythrocyte was significantly lower in group D than in infected control ($p$<0.001), while the chicks given 562 mg/kg dietary VE-AC had higher erythrocyte MDA ($p$<0.05). This indicates that dietary VE-AC at 316 mg /kg had the best antioxidant effect; while at higher level, it aggravated the oxidative stress induced by infection. In a similar study, Wang et al. (2008) added grape seed proanthocyanidin extract (GSPE) as an antioxidant to the diet of *E. tenella*-infected chickens at 5 to 80 mg/kg and found that its lower concentration (10-20 mg/kg) was able to restore the balance of oxidant/antioxidant system that was exerted by the oxidative stress after the parasite infection. Fridman et al. (1998) also reported that incorporation of vitamin E at 150 mg/kg to the broiler's diet was detrimental to antibody production and so it was a prooxidant at this level. The prooxidative effect of high doses of vitamin E on human lipoprotein was also documented by Bowry and Stocker (1993). According to Leshchinsky and Klashing (2001), deficiency or excess of vitamin E decreases the activity of glutathione peroxidase, unbalancing the antioxidant action in the cells and enabling the increase in the formation of free radicals in the cytosol, thus damaging the immunomodulatory system of the birds.

In the current study, erythrocyte SOD was not affected by infection ($p$>0.05); however, it was significantly higher ($p$<0.05) in group D (2.92 Unit/mg-Hb) than in group B (2.64 Unit/mg-Hb) or E (2.62 Unit/mg-Hb). The lack of significant difference in SOD activity between infected and non-infected chicks is likely due to mild infection. A significant decrease of blood SOD activity probably due to enhanced ROS production has been previously reported in chickens infected with high doses of *E. tenella* (Georgieva et al., 2006) or *E. acervulina* (Koinarski et al., 2005). In accordance with our findings, Wang et al. (2008) did not observed a significant decrease in SOD activity when broiler chicks were infected with the greatest dose of $1 \times 10^5$ oocysts of *E. tenella*; however in their study, infected chicks fed on diet supplemented with a natural antioxidant (GSPE) had a significantly higher plasma SOD activity when compared with infected controls.

In view of the obtained results, it may be concluded that infection with *E. tenella* enhances lipid peroxidation in broiler chickens and that dietary VE-AC at a rate of 316 mg/kg can improve the oxidant/antioxidant system in challenged chicks, but at high doses the situation can be reversed. It is known that inflammatory cytokines elaborated by immune system during an infection can stimulate a number of cell types to synthesize large quantities of nitric oxide (Liew and Cox, 1991). Nitric oxide, a free radical oxidative species, promotes vasodilation and hemorrhage in coccidian infections, and could be toxic to coccidia as well as host cells harboring the parasite (Ovington and Smith, 1992). Because AT functions to maintain the integrity of biological membranes by antioxidant properties, we assume that further studies to determine the oocyst shedding, severity of pathological lesions and the degree of colonization of *E. tenella* in cecal tissue could be useful to explain the interaction between the vitamin and the infection by the parasite.

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