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ORIGINAL ARTICLE

Effect of high dietary levels of α-tocopherol acetate on immune response of light and heavy weight male broiler breeders

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Abstract

The objective of this study was to investigate the effect of overdosing dietary α-tocopherol acetate (α-TOH) on immune response of heavy weight roosters. A total of 60 roosters (Ross 308) of light (LW, $n = 30$) and heavy weight (HW, $n = 30$) were kept in individual cages. Roosters were randomly allotted to ten treatment groups in a 2 × 5 factorial design. Each group received a basal diet supplemented with graded levels of α-TOH (0, 100, 200, 300, 400 mg/kg diet) for 10 weeks (from 25 to 35 week of age). Blood samples were collected at the fifth and tenth weeks of the experiment. Afterwards, humoral immune system function was evaluated by sheep red blood cell (SRBC) hemagglutination assay (HA) (at 35 weeks of age) and hemagglutination inhibition (HI) tests including antibody response to Newcastle disease viruses (NDV) and avian influenza viruses (AIV) (at 30 and 35 weeks of age). Cell-mediated immune response was evaluated by the cutaneous basophil hypersensitivity (CBH) test. A positive linear relationship was observed between incremental levels of dietary α-TOH and both humoral and cell-mediated immunity ($P \leq 0.05$), with the highest immune parameters recorded for dietary supplementation of 400 mg/kg α-TOH ($P \leq 0.05$). The results showed that CBH was significantly higher in LW roosters in comparison with HW roosters ($P \leq 0.05$). However, the interaction of BW and α-TOH doses did not have any significant effect on immune responses ($P \geq 0.05$). In conclusion, dietary supplementation of α-TOH at higher levels (four times more than the strain nutrients recommendation) had beneficial effects on the immune response of both HW and LW male broiler breeders.

Keywords Broiler breeder males · Immune response · α-tocopherol · SRBC · CBH · Avian influenza · Newcastle disease

Introduction

Vitamin E (VE) is a biological compound with multiple biological functions including antioxidant properties against free radicals, playing a role in normal development and function of immune system, regulation of heme biosynthesis, playing a specific role in intestinal amino acids transport and iron metabolism as well as steroidogenesis (Tappel 1962; Halliwell and Gutteridge 2015; Rengaraj and Hong 2015; Combs and McClung 2016). It has been demonstrated that oxidative stress is one of the major factors negatively affecting the performance of birds in the poultry industry (Xiao et al. 2011).

Undoubtedly, immune system function is the most important factor in the health situation of species, since many natural and synthetic products, as well as the different conditions, could interfere with its functions. As mentioned in early researches, VE is a natural compound which has immune modulatory effects in humans (Rizvi et al. 2014; Ghanem et al. 2017), ewes (Anugu et al. 2013) and birds (Sakamoto et al. 2006; Liu et al. 2014). Also, it has been reported that VE could enhance both cells mediated and humoral immune ability of birds followed by the increase of their immunity against several diseases including *Escherichia coli* infection, coccidiosis, infectious bursal disease, and Newcastle disease (Erf et al. 1998; Pekmezci 2011; Zhao et al. 2011; Liu et al. 2014).

Presence of lipids in the poultry's diet makes it susceptible to oxidation; therefore, the addition of an extra level of antioxidants may be needed (Tavárez et al. 2011). Despite the fact

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that NRC (1994) has recommended 10 IU/kg vitamin E in the diet as nutritional requirements of broilers, Pompeu et al. (2018) stated that broilers VE requirements have not been updated since 1994; whereas, the nutritional requirements have been changed due to the drastic genetic selection and increased metabolism in response of the high producing ability and reduced immune-competence in broiler flocks (Singh et al. 2006). It seems, therefore, that the producers need to optimize the immune responses of broiler flocks by nutritional manipulation. In this regard, Kuttappan et al. (2012) demonstrated that the determination of poultry's exact vitamin E requirements is very difficult due to the interactions between different factors that may alter the requirements of the broiler chickens (Kuttappan et al. 2012).

An increase in poultry's body weight is positively correlated with increased body metabolism, which in turn, increases the nutrient requirements. It has been reported that several factors including strain, age, dietary levels, and also body weight, have some obvious effects on poultry's immune system (Leshchinsky and Klasing 2001; van der Most et al. 2011; Ao et al. 2012). Although it is suggested that the amount of vitamin E in the diet be adequate for poultry needs (Pompeu et al. 2018), the requirements during poultry body weight increase have not yet been studied. However, it is reported that VE deficiencies in the diet of overweight broilers make them susceptible to a variety of diseases (van der Most et al. 2011). Thus, this information will be very useful to optimize the inclusion levels of VE in broilers diets, because of their importance in the poultry industry.

Genetic selection for growth parameters in meat-type chickens gives rise to a parent stock (broiler breeders) that tends to lack the ability to self-regulate feed intake. As a result, their high body mass is associated with excessive fat deposition and reproductive disorders. Zaghari et al. (2013) reported that feeding vitamin E four times above that recommended by the strain management guide decreased fractional yolk weight and blood triacylglycerol concentration and changed the metabolic direction of cholesterol in heavy broiler breeder hens.

With regard to the potential benefits of determining the exact dietary amount of VE on the health condition of heavy broiler breeder males, the immune modulatory effects of VE on broiler males also need to be further investigated. In this scenario, the main objective of this study was to assess the effects of α-tocopherol acetate as a vitamer of VE on cell-mediated immune response of heavy weight broiler breeder males.

Materials and methods

Chemicals and reagents

Chemicals were purchased from Sigma-Aldrich Co. (St. Louis, Mo, USA) unless otherwise indicated.

Ethical approval

This study was conducted after approval by the care and welfare committee of department of our institute.

Animals and experimental design

Sixty male broiler breeders (Ross 308) were engaged in a 2 × 5 factorial experiment from 20 to 35 weeks of age. Factors included two categories of body weight (Light weight (LW) and heavy weight (HW)) and five levels of α-tocopherol acetate (0, 100, 200, 300, and 400 mg/kg) which were added to the basal diet. The basal diet lacked any vitamin E supplementation; however, it met all other nutrient requirements recommended by the strain management guide of 2011 (Table 1). Ten treatments were replicated six times in the individual wire cages (40 × 40 × 50 cm). The mean initial body weights of LW and HW groups were 2672 and 4038 g, respectively, and the mean final body weights were 2878 and 4516 g, respectively. The HW group on average received 20% more daily feed than

Table 1 Ingredients and nutrients composition of experimental basal diet

Ingredients	g/100 g
Corn grain	67.31
Soybean meal (44%)	6.20
Wheat bran	23.28
Dicalcium phosphate	1.36
Limestone	0.81
Sodium chloride	0.32
Sodium bicarbonate	0.10
Vitamin and mineral premix	0.5
D-L- methionine (99%)	0.11
L-Lysine HCL (99%)	0.01
Calculated nutrient composition (%)	
AME_n (kcal/kg)	2750
Crude protein	11.5
Crude fat	3.65
Crude fiber	4.292
Ca	0.7
Available phosphorus	0.35
Na	0.18
Lysine	0.45
Methionine	0.31
Methionine + cysteine	0.33
Threonine	0.48

*Vitamins premix supply below amounts per kg feed; A 11,000 IU; K3 5 mg; B12 0.03 mg; D 3500 IU; riboflavin 7.5 mg; niacin 55 mg; pantothenic acid 15 mg; pyridoxine 4 mg; biotin 0.25 mg and folic acid 2 mg and minerals; Fe 50 mg; Mn 120 mg; Zn 110 mg; I 2 mg and Se 0.3 mg (without α-tocopherol acetate)

the LW group from the beginning until the end of the experiment. The HW roosters were 5% heavier, and the LW counterparts were 6% lighter than the body weight recommended by the Ross 308 management guide (4300 g at week 35). Average body weight and daily feed allocation of LW and HW groups are shown in Table 2. A 5-week depletion period was considered (from weeks 20 to 25) before roosters received the experimental diets. A uniform and controlled environment (21–23 °C and a 14:30 L–9:30 D photoperiod schedule) was provided throughout the experimental period.

Blood sampling and sera preparation

Blood samples (60 samples) were collected from the roosters at 30 and 35 weeks of age in non-heparinized tubes. The samples were centrifuged at 1500×*g* for 15 min at 25 °C to obtaining the sera. Then the sera were stored at − 20 °C until evaluation.

Measurement of serum α-TOH concentration

Due to the vulnerability of α-TOH to light, all serum samples were stored in the covered tubes. The empty space of the tube was filled with nitrogen gas, and samples were kept at − 20 °C until the time of analyses. After thawing the samples, 200 μl of serum placed into a 2-ml micro tubes then 50 μl of internal standard (vitamin E), 200 μl of ethanol, and 200 μl of methanol were added to the serum. Sample vertexed for 10 s, then 500 μl hexane was added and again vertexed for 60 s. The mixture was then centrifuged at 1500 g for 5 min. In the next step, the supernatant was removed, and 500 μl hexane was added to the mixture and vortexed for 60 s, and again was supernatant removed. Both removed supernatant phases were evaporated at 45 to 50 °C under nitrogen gas to completely loss the hexane. Then 200 μl of methanol was added to the dried portion and dissolved. Finally 50 μl of solution was injected into the HPLC apparatus (Lkb Pharmacia, USA), and the output was used to analyze the data (Cuesta and Castro 1986).

Table 2 Average body weight and daily feed allocation of LW and HW groups

Age (week)	Body weight (g)		Daily feed allocation (g/bird)	
	LW	HW	LW	HW
21	2672 ± 47.8	2878 ± 37.4	105	137
22	2784 ± 40.9	3111 ± 41.8	108	140
23	2912 ± 38.7	3194 ± 35.8	110	120
24	2992 ± 32.1	3369 ± 30.8	110	130
25	3109 ± 30.4	3510 ± 30.0	115	135
26	3217 ± 29.4	3636 ± 30.0	115	135
27	3322 ± 27.2	3782 ± 32.5	118	140
28	3437 ± 29.02	3910 ± 36.5	119	141
29	3535 ± 30.7	3999 ± 42.7	121	143
30	3632 ± 31.7	4120 ± 45.4	122	144
31	3750 ± 34.01	4230 ± 49.3	124	148
32	3837 ± 37.1	4294 ± 55.2	126	150
33	3941 ± 51.4	4353 ± 59.7	128	150
34	3976 ± 44.1	4401 ± 73.9	128	150
35	4038 ± 46.3	4516 ± 66.4	128	150
Average	3410	3820	118	141

LW light weight, *HW* heavy weight

Newcastle disease virus and avian influenza virus

All roosters were intramuscularly vaccinated against Newcastle disease virus (NDV) and avian influenza virus (AIV) at 22 weeks of age. To evaluate the antibody response to NDV and AIV, the blood samples were collected from 60 roosters at 30 and 35 weeks of age, respectively. Afterwards, their sera were subjected to hemagglutination inhibition (HI) tests to determine the antibody titers of NDV and AIV (Wallerström et al. 2014; Park et al. 2016).

Anti SRBC antibody response

A suspension of SRBC (5%) and phosphate-buffer saline (PBS; pH = 7.5) was intramuscularly injected (0.1 ml/kg BW) to major pectoral muscle of all roosters at 31 weeks of age. Roosters received a booster injection at 32 weeks of age. Seven days after each injection, the roosters were bled from the brachial vein and the samples were subjected to sera preparation. Total anti-SRBC antibody level was evaluated through using the method described by Van Der Zijpp and Leenstra (1980) with a minor modification (Sharideh and Zaghari, 2017). Briefly, serum was inactivated in a 56 °C water bath for 30 min, and then 50 μL of PBS and 50 μL of serum were placed in the first row of wells in a 96-well V-bottom micro titration plate (Corning Glass, Corning, NY, USA), and the solution was incubated for 30 min at 37 °C. Afterwards, 50 μL of PBS was added to the remaining wells to make a twofold serial dilution for each sample on consecutive rows. Finally, 50 μL of 5% SRBC suspension was added to each well and incubated for 30 min at 37 °C. The titers were expressed as log2 of the reciprocal of the highest dilution giving visible agglutination (Van der Zijpp and Leenstra, 1980).

Evaluation of the cell-mediated cutaneous basophil hypersensitivity (CBH) responses

CBH response was evaluated by intra dermal injection of phytohemagglutinin-P (PHA-P, Sigma Aldrich, USA) (at 33 weeks of age). In this regard, the CBH response to PHA-

P was assessed by determining the thickness of the interdigital skin before and at 24 h after injection with a digital caliper. The CBH response was calculated according to the method described below (Da Silva et al. 2011):

(a) Response = post-PHA-P injection thickness of the right foot − pre-PHA-P injection thickness of the right foot (mm)
(b) PBS control response = post-PBS injection thickness of the right foot − pre-PBS injection thickness of the left foot (mm)
(c) Therefore, cell reaction at each evaluation time was calculated as

CBH = (a) − (b).

Statistical analysis

All data were analyzed by GLM procedure in a (2 × 5) factorial arrangement of SAS 9.1 (SAS Institute 2002) and followed by Duncan's multiple comparison tests at 5% probability level for mean comparison. α-TOH requirement was determined with NLIN procedure of SAS with linear and nonlinear regression of performance criteria on dietary vitamin E content.

Results

The effects of overdosing dietary inclusion of α-TOH on its serum concentration are presented in Fig. 1. Results showed that the increased level of dietary α-TOH significantly elevated the concentration of α-tocopherol in the serum, with the highest amount recorded for the groups received the diet containing 400 mg/kg α-TOH (Fig. 1).

Results presented in Fig. 2 showed that cell-mediated immunity response (CBH) of LW roosters was significantly stronger than that of HW roosters ($P < 0.05$). However, the humoral immunity (SRBC) response of LW and HW roosters showed no significant difference.

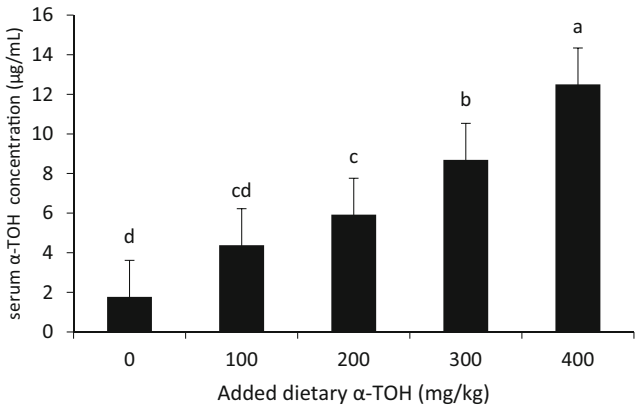

Fig. 1 Influence of dietary α-TOH on serum concentration of α-tocopherol acetate

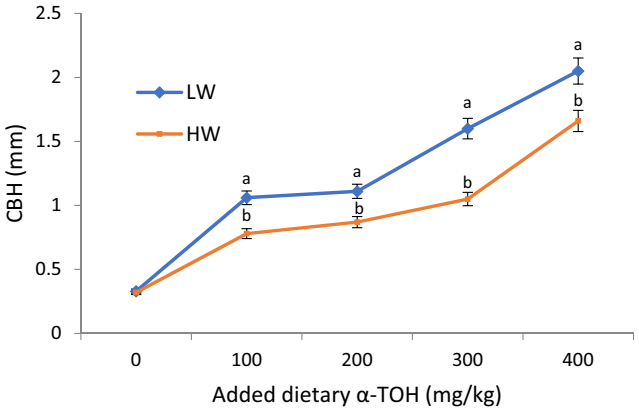

Fig. 2 Effect of dietary graded levels of α-TOH and body weight of roosters on cell-mediated immunity (CBH)

Table 3 represents the data concerning the effects of dietary inclusion of α-TOH on immune performance of heavy and light weight male broiler breeders. There were no significant differences in antibody titers against AIVw5, AIVw10, NDVw5, and NDVw10 between LW and HW roosters ($P > 0.05$). In contrast to the negative effect of excess body weight on cell mediated immunity response, α-TOH had positive effects on CBH, SRBC, and antibody production against AIV, NDV 5 weeks after the onset of consumption of the diet fortified with vitamin E ($P < 0.05$).

Figures 3 and 4 demonstrated the linear relationship between graded levels of α-TOH and cell mediated immunity (CBH) and humoral immunity (SRBC) ($P < 0.05$). The quadratic and exponential relationships between immune responses, as dependent variables and independent variable (dietary α-TOH), were not significant ($P > 0.05$). Also, the interactions of treatment factors on measured traits were not statistically significant.

Discussion

Generally, environmental stresses such as temperature instability, nutritional deficiency, and extreme farm conditions may weaken the defense system of birds and consequently make the birds susceptible to infectious diseases. Vitamin E is a biological antioxidant with the ability to suppress free radicals and therefore, it can limit lipoperoxidation (Urano and Matsuo 1976). Besides, studies have demonstrated that increased vitamin E levels in the diet (above NRC recommendation) could better the performance of broilers raised under subclinical infectious conditions (McIlroy et al. 1993). Also, it has been reported that dietary inclusion of vitamin E can improve immune system capability in different species (Heinzerling et al. 1974; Marsh et al. 1981; Corwin et al. 1981; Reddy et al.

Table 3 Analysis of the relationship between dependent (CBH, AIV, NDV, and SRBC) and independent variable α-TOH. The main effect of two BW type (LW and HW) and five different levels of α-TOH (0, 100, 200, 300, and 400 mg/kg diet) on rooster's immune parameters (Means ± SE)

Factors		Parameters					
		CBH (mm)	AIV^{2}w5	AIV^{2}w10	NDV^2 w5	NDV^{2}w10	SRBC^2
BW	SW	$1.23^{a} \pm 0.15$	4.46 ± 0.16	5.93 ± 0.20	4.67 ± 0.12	6.40 ± 0.21	4.10 ± 0.20
	HW	$0.93^{b} \pm 0.10$	4.57 ± 0.15	6.13 ± 0.29	4.58 ± 0.15	6.46 ± 0.32	4.00 ± 0.20
α-TOH	0	$0.33^{d} \pm 0.05$	$4.91^{d} \pm 0.23$	$5.00^{c} \pm 0.32$	$4.08^{b} \pm 0.08$	$5.16^{c} \pm 0.30$	$2.75^{e} \pm 0.17$
	100	$0.92^{c} \pm 0.15$	$5.33^{c} \pm 0.22$	$5.50^{bc} \pm 0.34$	$4.53^{ab} \pm 0.20$	$5.83^{c} \pm 0.30$	$3.75^{d} \pm 0.16$
	200	$0.99^{c} \pm 0.11$	$5.33^{c} \pm 0.14$	$6.33^{ab} \pm 0.21$	$4.66^{ab} \pm 0.18$	$6.50^{bc} \pm 0.22$	$4.12^{cd} \pm 0.22$
	300	$1.33^{b} \pm 0.10$	$5.85^{ab} \pm 0.29$	$6.50^{a} \pm 0.22$	$4.75^{a} \pm 0.14$	$7.16^{ab} \pm 0.16$	$4.62^{bc} \pm 0.18$
	400	$1.85^{a} \pm 0.21$	$6.16^{a} \pm 0.16$	$6.83^{a} \pm 0.47$	$5.08^{a} \pm 0.25$	$7.50^{a} \pm 0.22$	$5.00^{ab} \pm 0.17$
P value	BW	0.0175	0.5804	0.4992	0.6418	0.7661	0.5319
	α-TOH	0.0001	0.0013	0.0039	0.0222	0.0001	0.0001
	BW × α-T	0.6907	0.2593	0.9897	0.8532	0.2532	0.9615
	Linear BW	0.0175	NS	NS	NS	NS	NS
	Linear α-TOH	0.0001	0.0001	0.0002	0.0023	0.0001	0.0001
	R-square	0.6618	0.3504	0.5302	0.2231	0.7662	0.7648
	Quadratic α-TOH	NS	NS	NS	NS	NS	NS
	Exponential α-TOH	NS	NS	NS	NS	NS	NS

Different superscripts (a and b) for BW, (a–e) for VE levels within the same line differ significantly

*AIVw*5 avian influenza virus week 5, *AIVw*10 Avian influenza virus week 10, *CBH* cutaneous basophil hypersensitivity, *NDVw*5 avian Newcastle virus week 5, *NDVw*10 avian Newcastle virus week 10, *SRBC* sheep red blood cell

1986). Accordingly, it is suggested that inclusion of vitamin E in the birds' diet may also have immunoregulatory effect.

In the present study, the findings showed that light weight roosters had significantly higher CBH response in comparison with the heavy weight roosters. In a study on turkey, (Li et al. 2001) reported that increased body weight resulted in decreased immune system activity such as phagocytic activity. They claimed that susceptibility to some diseases in selected heavy lines might be related to reduced phagocytic activity. Furthermore, it has been indicated that in humans, obesity affects the immune system negatively (Painter et al. 2015). It is well established that adipose tissue is an endocrine organ (Trayhurn and Wood 2004), and in obese men, inflammatory cytokines and hormones can cause suppressed antibody responses to vaccines (Bouwman et al. 2009). Therefore, in the present study, higher amounts of adipocyte tissues in heavy weight roosters presumably would have caused a negative effect on CBH response compared to the standard weight roosters. In the case of dietary supplementation of vitamin E, the results of the present study showed that increased dietary levels of vitamin E significantly elevated antibody response to CBH. This finding was similar to the results reported by (Singh et al. 2006). They revealed that supplementation of chick's diet with 200 and 0.2 mg/kg diet VE and selenium, respectively, resulted in significantly higher CBH titers. Also, they stated that this increase was associated with an increase in serum concentration of total immunoglobulins and circulatory immune complexes. Accordingly, it has been suggested that

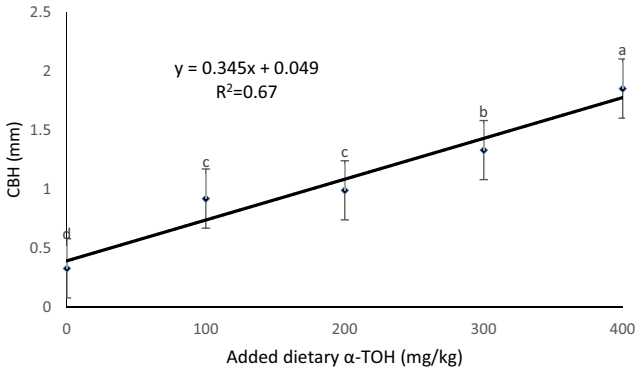

Fig. 3 The relationship between graded levels of α-TOH and cell-mediated immunity (CBH)

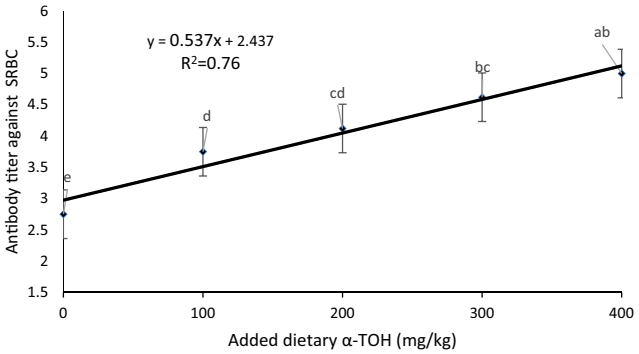

Fig. 4 The relationship between graded levels of α-TOH and humoral immunity (SRBC)

VE immune functions in broiler chicks are associated with membrane fluidity of lymphoid cells as well as an increase in bursal B cells due to the decreased oxidative stress, which in turn, lead to an improvement in antibody responses (Duthie et al. 1996; Singh et al. 2006; Niu et al. 2009).

The findings of the current study showed that vitamin E treatments had significant positive effects on AIV and NDV at all levels, in a dose-dependent manner. Similarly, in a meta-analysis study about the VE functions in broilers immune system, Pompeu et al. (2018) suggested that dietary supplementation of VE improved broilers immune responses by reducing the free radical-induced pathology during both normal metabolic states and inflammation, modulating the expression of genes, and by affecting free radical-mediated signal transduction. Also, they concluded that the heterophil to lymphocyte (H/L) ratio, an indicator of stress in birds, was kept balanced after dietary supplementation of VE. It seems that stressful conditions could decrease the number of circulating lymphocytes due to their membrane oxidation, leading to an increase in the H/L ratio (Liu et al. 2014). Therefore, based on the results of the current study, it has been concluded that vitamin E supplementation may improve immune response because of its antioxidative properties; and whenever the vitamin E supplementation is increased to three or four times higher than that recommended by ROSS 308 broiler breeder nutrition specifications, it leads to better results. Although our results are in agreement with previous studies which have reported that vitamin E supplementation (200 mg/kg or 25 IU/kg) is advantageous for immune system and disease resistance in birds (Niu et al. 2009; Leshchinsky and Klasing 2001), there exist some other studies which have demonstrated the neutralized effect of vitamin E treatments on immune responses of poultry. Marsh et al. (1981) and Qureshi and Taylor (1993) showed that dietary inclusion of vitamin E (100 or 250 IU/kg) had no effect on antibody production in broilers. This contradiction in the results might be explained by differences in age, dietary levels, environmental conditions, and strain of the birds used in different studies.

Macrophages, as a first line of immunological defense, arise in the bone marrow; and after they enter the blood circulation, they play a key role in antigen processing (Qureshi et al. 2000). It has been reported that inclusion of 200 mg/kg vitamin E to the diet of broilers exposed to heat stress significantly increased the percentage of macrophages (Niu et al. 2009). Also, Konjufca et al. (2004) have reported that dietary supplementation of vitamin E (110 and 220 mg/kg diet) has resulted in more SRBC phagocytized in comparison with the control group. Similarly, the results of the current study showed that dietary supplementation of vitamin E at all levels had resulted in higher antibody response to SRBC compared to those with no vitamin E supplementation. Phagocytosis of antigens is the function of macrophages and has been proposed to be a membrane-mediated phenomenon (Qureshi et al. 2000). Because vitamin E has antioxidant properties, it can protect the cellular membrane phospholipids against free radical-induced damages (Tappel 1972). It is supposed that inclusion of vitamin E to the diet of cockerels has protected the membrane of macrophages from lipoperoxidation. Therefore, it has resulted in higher amounts of intact macrophages, and, consequently, higher antibody response to SRBC. Another possible reason for higher antibody response in vitamin E supplemented groups may be the role of vitamin E in downregulating of prostaglandin production. It has been suggested that high levels of prostaglandins may be immunosuppressive (Sheffy and Schultz 1979). Also, it has been reported that macrophages are responsible for prostaglandin production (Aderem et al. 1985). Release of arachidonic acid from the plasma membrane of macrophages can form the prostaglandins (Ricciotti and FitzGerald 2011). In a study, it was documented that vitamin E can inhibit 5-lipoxygenase, an enzyme by which arachidonic acid turns into some biologically active compounds such as prostaglandins (Reddanna et al. 1985). Thus, vitamin E, through inhibition of arachidonic acid cascade, may limit the formation of prostaglandins, and consequently limit the immunosuppressive effect of prostaglandins. In a like manner, Likoff et al. (1981) have shown that chickens fed a vitamin E supplemented diet (300 mg/kg) had a greater phagocytic function compared to those fed a diet without vitamin E supplementation, and they claimed that the increase in phagocytic function was derived from a decrease in prostaglandin levels.

In conclusion, α-TOH dietary supplementation of 300 or 400 mg/kg diet, 3–4 times more than the required amount of broiler breeder males, showed beneficial effects on immune responses parameters. However, the interaction effects of body weight and α-TOH α-T levels did not have any significant effect on immune responses of broiler breeder males, and body weight had only affected CBH.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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