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Dietary Vitamin E Influences the Levels of Nitric Oxide and Cytokines in Broiler Chickens

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ABSTRACT : The study investigated the effects of dietary Vitamin E (VE) on nitric oxide (NO) metabolism, immune function and analyzed the correlation between NO free radical and cytokines (IL-2 and IL-6) in broilers. One hundred and fifty 2-week-old broilers were randomly divided into three groups. Control group and lower VE (VE^-) group were provided with a basic diet supplemented with 12.55 mg/kg VE and 2.55 mg/kg VE for 30 days, respectively. Higher VE (VE^--VE^+) group was supplemented with 2.55 mg/kg VE in the first 15 days and then 32.55 mg/kg VE in the next 15 days. Five broilers in each group were then sacrificed on the 5th, 10th, 15th, 20th, 25th and 30th days, respectively, and the content of NO free radical, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA) and cytokines, IL-2 and IL-6, were measured. The results showed that lower VE could decrease growth performance of broilers while higher VE could increase growth performance and eliminate differences resulted from feeding lower VE dietary in early stages ($p<0.05$). Compared with the control group, lower VE could increase significantly NO and MDA concentration, and increase IL-2 concentration in serum ($p<0.05$). Higher VE could significantly increase activities of SOD and glutathione GSH-Px ($p<0.05$). IL-2 is positively correlated with NO in heart ($p<0.05$) and IL-6 is negatively correlated with NO in liver ($p<0.05$) and heart ($p<0.01$). These results indicate that dietary VE could regulate antioxidant capacity and NO metabolism of broilers and higher VE-supplemented diet could directly decrease production of IL-2. (**Key Words :** Vitamin E, Nitric Oxide, Free radical, Cytokines, Broiler)

INTRODUCTION

Nitric oxide (NO) is one of the highly reactive biological nitrogen free radicals (Fang and Li, 1989). Since discovered as a gaseous messenger, it has been a focus of frontline research on free radicals in addition to reactive oxygen species (Koshland, 1992). The pleiotropic functions of NO depend on its source, concentration, localization and body oxidative status.

Vitamin E (VE) is an indispensable cellular antioxidant (Liu and Qu, 2001; Brigelius-Flohé et al., 2002). It can effectively block free radical chain reactions and consequently impede unsaturated fatty acid oxidation and lipid peroxidation (Roy and McCord, 1982), and therefore plays a role in the repair of membranes damaged by free radicals and in free radical metabolism (Zhang et al., 1998). It has been reported that tissue NO concentration is negatively related to dietary VE content, and higher dietary VE is related to lower NO concentration (Xu et al., 2007). VE also participates in the regulation of immune functions of tissues that secrete cytokines when activated by pathogens.

Cytokines are signaling proteins and peptides including interleukin (IL), interferon, tumor necrosis factor, erythropoietin and macrophage colony-stimulating factor. They are critical to the immune responses, cell differentiations, hematopoietic regulations, tumor immunities and other physiological and pathological processes (Stasi et al., 1995).

In the present study, we investigated the effects of dietary VE on free radical NO concentration, antioxidant indices, cytokine contents, and elucidated the correlations among VE, NO and immune functions in broiler chickens.

MATERIALS AND METHODS

Animals and experiment design

150 two-week old broilers were randomly divided into

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three groups. Each group had 5 replicates of 10 broilers with 5-males and 5-females. The experiment was performed in two 15-day stages. A corn-soybean meal basic diet was formulated according to the broilers requirement of NRC (1994) and the dietary compositions are listed in Table 1. The amount of VE in the basic diet was 7.45 mg/kg. The control group was provided with the basic diet supplemented with 12.55 mg/kg VE for 30 days. The lower VE (VE^-) group was provided with the basic diet supplemented with 2.55 mg/kg VE for 30 days. The VE^--VE^+ group was fed with the basic diet supplemented with 2.55 mg/kg VE in the first 15 days and then 32.55 mg/kg VE in the next 15 days. The supplemented VE was Vitamin E500 (BASF Vitamins Co., Ltd.) in the form of VE acetate powder containing 50% of VE.

Broiler management

Broilers were kept in two-tier cages with proper ventilation, put in 23 h/1 h day/night cycle with light intensity of 5 to 10 Lx, and given *ad libitum* access to feed and water. On the 14th and 28th day of the experiment, broiler's bodyweights and food consumptions were recorded to calculate the average daily gain, daily feed intake and feed-weight ratio.

Sample collection and preparation

Broilers from one replicate in each group were taken out every 5 days and weighed. The blood was collected from jugular vein and serum was separated and frozen at -75°C for measurement. After blood sampling, the broilers were sacrificed and livers, hearts, thymuses, spleens and bursals were collected, washed with cold 0.9% NaCl and weighed immediately. About 1 gram of liver and heart from each group was immediately homogenized with a 0.9% NaCl solution and centrifuged at 3,000 rpm for 10 minutes at 4°C, the supernatant was collected and used to detect proteins, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and alondialdehyde (MDA). The remaining liver and other tissues were stored at -20°C for further testing.

Table 1. Composition and ingredients of the experimental basis diet

Item	Amount
Ingredients (%)	
Corn	63.30
Soybean meal	28.80
Fish meal	2.00
$CaHPO_4$	1.40
Limestone	0.90
Soybean	2.30
NaCl	0.30
Premix[1]	1.00
Composition	
ME (Kcal/kg)	3,205
Crude protein (%)	19.78
Calcium (%)	0.92
Available phosphorus (%)	0.36
Methionine (%)	0.49
Lysine (%)	1.12

[1] Premix per kg contains followings: 9 mg Cu, 44 mg Zn, 50 mg Fe, 66 mg Mn, 0.4 mg I, 0.20 mg Se, 7,000 IU VA, 875 IU VD_3, 3 1 mg VK, 2 mg VB_l, 4.5 mg VB_2, 2.5 mg VB_6, 0.6 mg VB_{12}, 50 mg nicotinic acid, 12 mg D-calcium pantothenate, 0.15 mg biotin, and 0.8 mg folic acid.

Detection of NO

NO produced in the liver and heart was detected using electron spin resonance (ESR) spectroscopy according to the method of Guo et al. (2002) with modification. In detail, 0.5 g fresh heart or liver from broilers sacrificed at day 15 and 30 were homogenized with 0.9 ml 0.1 mol/L spin trapping reagent N-tert-butyl-α-phenylnitrone (Aldrich Chemical Company, USA) in a glass tube for 2 min. The homogenates were immediately (within 15 s) transferred into an electron paramagnetic tube and snap frozen in liquid nitrogen. The ESR spectra were obtained within 1.5 hours using a Bruker-ER200D-SRC ESR instrument (Bruker Corporation, Germany) under the following settings: microwave frequency of 9.74 GHz, microwave power of 20 mW, modulation frequency of 100 KHz, modulation amplitude of 5Gs, center field strength of 3,470Gs, scan field width of 100Gs and magnification fold of 4×10^5. ESR spectra were recorded at 297 K (24°C). The free electron values g were calculated. In general, g value reflects the internal characteristics of molecules in the magnetic field and is widely used as an important parameter to determine the molecular structure (Han and Wang, 2002). G value between 2.0050-2.0073 corresponds to free radical NO (Fang and Zheng, 2002). In ESR, the free radical concentration is proportional to the peak height and the square of the peak width (Rossi, 1986). Therefore, when the peak width is consistent, the relative concentration of free radical can be expressed as the peak height. To avoid the interferences from other peaks, the height of the second peak was used as a quantitative indicator of NO content.

Measurements of IL-2, IL-6

Serum IL-2 and IL-6 concentration were measured by radioimmunoassay kit (Beijing Beimian Dongya Biotech Institute) according to the protocols provided by the manufacturer.

Measurements of antioxidant capacity

The activities of SOD and GSH-Px and the content of MDA and proteins in liver and heart were assayed using colorimetric methods with a spectrophotometer (Thermo Company, British). The assays were conducted using the

Table 2. The effects of dietary VE on growth performance in broilers when the diet changed at day 15

Item	15th day			30th day		
	Control	VE^-	VE^--VE^+	Control	VE^-	VE^--VE^+
Average daily gain (g)	50.79±0.91[a]	39.78±0.12[b]	39.78±0.12[b]	49.00±0.94[a]	35.21±0.62[b]	46.83±0.15[a]
Average feed intake (g/d)	83.65±0.60[a]	70.22±0.36[b]	70.22±0.36[b]	116.04±1.23[a]	98.79±1.06[b]	113.56±0.64[a]
Feed-weight ratio	1.65±0.02[b]	1.77±0.03[a]	1.77±0.03[a]	2.37±0.04[b]	2.80±0.05[a]	2.42±0.04[b]

[a,b] Means in the same row with different superscripts differ ($p<0.05$).

assay kits purchased from Nanjing Jiancheng Insititute of Bioengineering (Nanjing, Jiangsu, China) according to the instructions of the manufacturer. The activity of SOD was measured by the xanthine oxidase method, which monitored the inhibition of reduction of nitro blue tetrazolium by the sample (Winterbourn et al., 1975). The activity of GSH-Px was detected with 5,5'-dithiobisp-nitrobenzoic acid, and the change of absorbance at 412nm was monitored using a spectrophotometer (Hafeman et al., 1974). The MDA content was analyzed with 2-thiobarbituric acid, monitoring the change of absorbance at 532 nm with the spectrophotometer (Placer et al., 1996). The protein concentration was determined by the biuret method. Enzyme activity and MDA content was expressed as units per milligram of protein for liver and heart tissue.

Statistical analyzes

Results were expressed as mean and standard deviation (SD). Statistical analysis was carried out using the SPSS program (version 12.0 software, SPSS Inc. Chicago, Illinois, USA). For the comparison of groups, variance analysis (oneway ANOVA) and the Pearson correlation test were used. p Values of less than 0.05 were regarded as significant.

RESULTS

Effects of dietary VE on growth performance in broilers

Dietary VE has significant effect on broiler's growth performance. As shown in Table 2, on the 15th day, average daily feed intake, daily gain and feed conversion efficiency in VE^- group and VE^--VE^+ group were significantly lower than those in control group ($p<0.05$). On the second stage, growth performance of broilers in VE^- group were the lowest among the groups ($p<0.05$). Compared with VE^- group, daily feed intake, daily gain and feed conversion efficiency in VE^--VE^+ group significantly increased ($p<0.05$). No significant difference was observed between control group and VE^--VE^+ group ($p<0.05$).

Effects of dietary VE on free radical NO concentration in liver and heart

The ESR spectra of groups in hearts and livers on the 15th and 30th day, respectively, were obtained and showed in Figure 1. The Spectra showed typical patterns of triple peak of ESR signals. The g value of the second peak, the major peak, was 2.0061, indicating that the captured free radical was NO. As shown in Table 3, on the 15th day, compared with control group, low VE significantly increased NO concentration in both the liver ($p<0.05$) and the heart ($p>0.05$). On the 30th day, NO concentration was highest in VE^- group and lowest in VE^--VE^+ group both in the liver and the heart ($p<0.05$).

Effects of dietary VE on antioxidation ability of broilers

We studied the effects of dietary VE on SOD and GSH-Px activities and MDA contents. As shown in Table 4, SOD activities in the liver and heart from the control group were higher than those from the VE^- and VE^--VE^+ groups on the 10th and 15th day, respectively ($p<0.05$). When VE concentration changed from low VE to high VE in the diet of the VE-VE^+ group, the SOD activities in the liver and heart gradually increased and reached the same concentration of those in the control group on the 30th day ($p>0.05$), while SOD activities in the VE^- group were lowest among groups ($p<0.05$).

As shown in Table 4, on the 15th day, GSH-Px activities in both liver and heart from the VE^- and VE^--VE^+ groups were significantly lower than those from the control group ($p<0.05$). As the dietary VE content increased in the VE^--VE^+ group, GSH-Px activities in the liver and heart significantly increased. On the 30th day, GSH-Px activities in the liver and heart in the VE^--VE^+ group were higher than those in the VE^- group and control group ($p<0.05$). GSH-Px activities in the liver and heart from VE^- group were lowest among groups ($p<0.05$).

On the 15th day, the MDA contents in the liver of the VE^- and VE^--VE^+ groups were significantly higher than those of the control group ($p<0.05$), and there was no significant difference of MDA content in the heart among three groups ($p>0.05$). On the 20th day, when the diet changed from low VE to high VE, MDA content in the liver and heart of the VE^--VE^+ group decreased by 52.05% and 28%, respectively and was significantly lower than that in the VE^- group ($p<0.05$). On the 30th day, MDA content in both the liver and heart from the VE^--VE^+ group was lowest and VE^- was highest among groups ($p<0.05$).

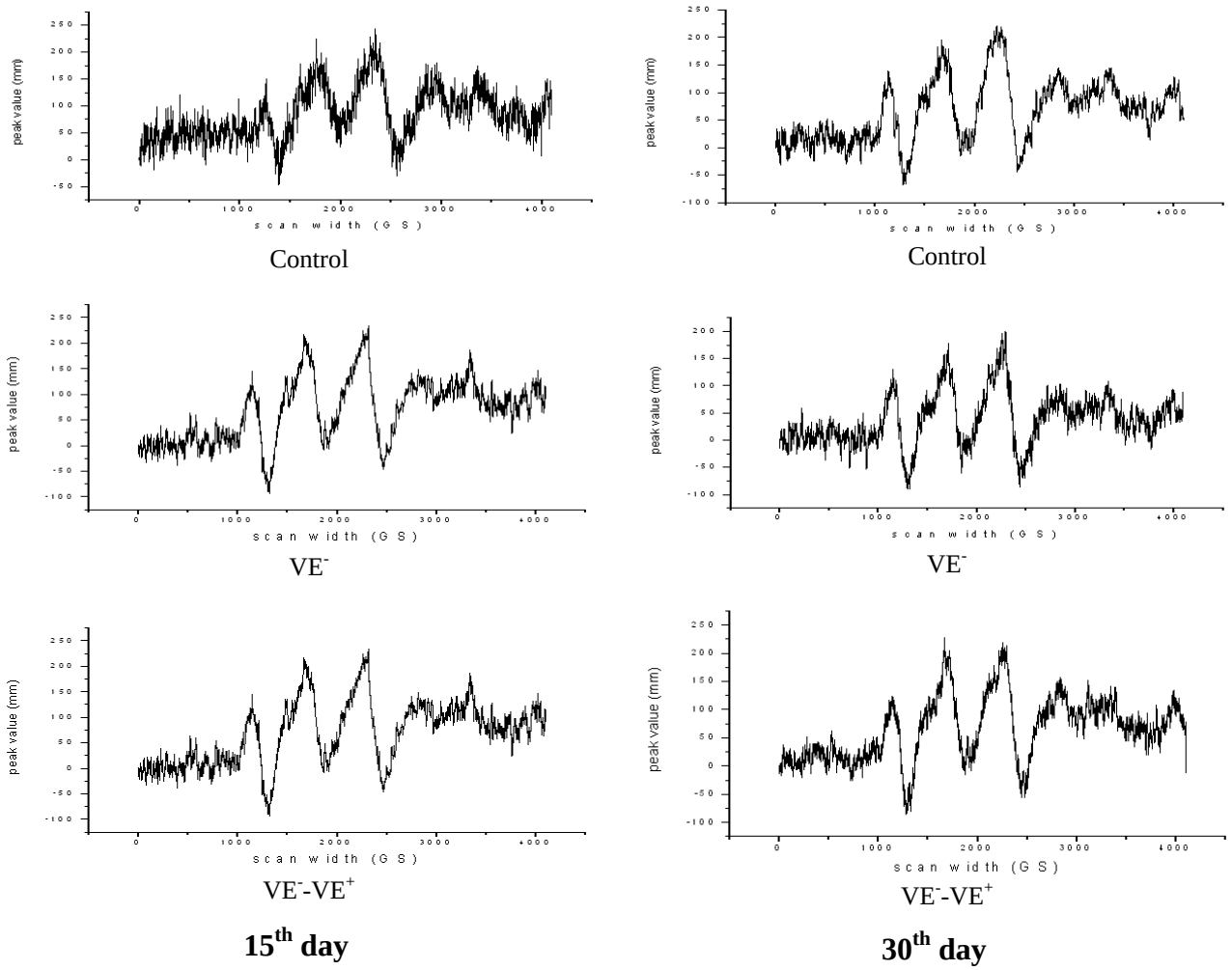

Figure 1. The ESR spectra of the hearts and livers in control group, VE^- group and VE^--VE^+ group on 15th day and 30th day.

Table 3. Effects of dietary vitamin E on the free radical NO concentration (mM) in the liver and heart of broilers when the diet changed at day 15

Treatment	Control	VE^-	VE^--VE^+
Liver			
15th day	162.33±10.64[b]	180.81±8.84[a]	180.81±8.84[a]
30th day	137.48±11.25[b]	157.37±10.35[a]	106.35±11.25[c]
Heart			
15th day	168.04±7.08	172.33±9.20	172.33±9.20
30th day	137.77±8.47[b]	151.42±11.26[a]	98.45±1.98[c]

[a, b, c] Means in the same row with different superscripts differ ($p<0.05$).

Table 4. Effects of dietary vitamin E on the activities of super oxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) content in the liver and heart of broilers when the diet changed at day 15

Item	Time	Liver			Heart		
		Control	VE^-	VE^--VE^+	Control	VE^-	VE^--VE^+
SOD (U/mg of protein)	5th day	32.49±4.53	32.30±5.57	32.30±5.57	180.41±5.1	182.63±5.81	182.63±5.81
	10th day	33.66±0.65^a	28.85±4.97^b	28.85±4.97^b	182.72±6.08^a	173.97±8.14^b	173.97±8.14^b
	15th day	36.41±0.57^a	28.84±5.01^b	28.84±5.01^b	202.81±6.96^a	158.63±9.77^b	158.63±9.77^b
	20th day	44.04±0.65^a	29.46±4.71^b	41.76±0.83^a	204.38±8.35^a	162.38±8.26^b	205.79±14.95^a
	25th day	52.10±0.83^a	31.12±4.84^c	45.73±0.80^b	204.71±8.42^a	165.67±8.34^b	212.99±23.52^a
	30th day	41.10±0.67^a	29.85±5.29^b	40.56±0.78^a	192.62±8.15^a	158.46±7.94^b	202.24±8.06^a
GSH-Px (U/mg of protein)	5th day	8.59±0.96	8.34±0.90	8.34±0.90	149.89±9.60	170.22±17.95	170.22±17.95
	10th day	8.33±0.29	8.63±0.31	8.63±0.31	193.65±4.89^a	170.33±14.32^b	170.33±14.32^b
	15th day	11.17±0.44^a	7.87±0.69^b	7.87±0.69^b	269.73±4.53^a	241.06±22.47^b	241.06±22.47^b
	20th day	12.65±0.24^a	8.24±0.36^b	12.37±0.20^a	263.15±8.78^a	218.35±19.48^c	300.32±7.59^b
	25th day	10.43±0.23^a	7.93±0.71^b	11.05±0.34^a	256.04±9.17^a	209.58±21.06^b	300.05±6.35^b
	30th day	11.59±0.22^b	7.23±0.68^c	13.44±1.00^a	328.28±6.44^a	217.57±23.17^c	341.44±11.3^b
MDA (nmol/mg of protein)	5th day	1.16±0.065	1.01±0.066	1.01±0.066	3.73±0.28	3.59±0.48	3.59±0.48
	10th day	1.18±0.063	1.17±0.066	1.17±0.066	3.75±0.48	4.06±0.50	4.06±0.50
	15th day	1.16±0.053^b	1.46±0.062^a	1.46±0.062^a	3.53±0.49	4.00±0.56	4.00±0.56
	20th day	0.69±0.091^b	1.66±0.071^a	0.70±0.083^b	3.78±0.36^b	4.67±0.67^a	2.88±0.40^c
	25th day	1.00±0.055^b	1.71±0.076^a	0.86±0.080^c	5.11±0.51^b	6.34±0.72^a	4.04±0.40^c
	30th day	1.09±0.034^b	1.85±0.082^a	0.80±0.079^c	4.10±0.21^b	6.63±0.65^a	3.62±0.33^c

$^{a, b, c}$ Means in the same row with different superscripts differ ($p<0.05$).

Effects of dietary vitamin E on IL-2 and IL-6 concentrations in serum

Table 5 shows IL-2 and IL-6 contents in the serum. On the 5th day, IL-2 concentration of the VE^- and VE^--VE^+ groups was significantly lower than that of the control group. Five days after changed to a high VE diet, the content of IL-2 in the VE^--VE^+ group decreased to 0.56±0.19 ng/ml, was lower than that in the control group and VE^- group ($p<0.05$) and kept in low concentration during the whole experiment. By contrast, dietary VE had no significant effect on the content of IL-6 ($p>0.05$). Pearson analysis indicates that NO concentration both in liver ($p<0.05$) and heart ($p<0.01$) is negatively correlated with IL-6 concentration in serum, and NO in the heart is positively correlated with IL-2 concentration ($p<0.05$), as shown in Table 6.

DISCUSSION

The current studies reveal that the content of dietary VE has a dramatic impact on the concentration of free radical NO in broilers. Following the increased dietary VE level, the NO concentration significantly decreased, and vice versa, indicating that dietary VE level is negatively correlated with the free radical NO concentration, which is regulated by dietary VE in broilers.

Free radicals are the source of redox reactions. Under normal physiological conditions, their generation and

Table 5. Effect of dietary vitamin E on the serum IL-2 and IL-6 concentrations in broilers when the diet changed at day 15

Item	IL-2 (ng/ml)			IL-6 (pg/ml)		
	Control	VE^-	VE^--VE^+	Control	VE^-	VE^--VE^+
5th day	0.80±0.18^a	0.64±0.09^b	0.64±0.09^b	333.89±89.45	354.74±76.73	354.74±76.73
10th day	0.72±0.15	0.73±0.09	0.73±0.09	328.76±38.79	363.20±42.17	363.20±42.17
15th day	0.84±0.36	0.87±0.42	0.87±0.42	317.94±67.30	328.45±49.60	328.45±49.60
20th day	1.00±0.20^a	0.84±0.36^a	0.56±0.19^b	357.84±44.09	333.73±45.62	381.86±20.79
25th day	0.49±0.23^b	0.76±0.25^a	0.44±0.07^b	337.83±48.29	340.63±48.39	370.31±76.15
30th day	0.59±0.09^b	0.75±0.31^a	0.49±0.27^b	353.81±46.27	357±53.47	386.91±56.22

$^{a, b}$ Means in the same row with different superscripts differ ($p<0.05$).

Table 6. Effects of dietary vitamin E on the correlation between NO level in liver or heart and cytokines

	NO in the liver	NO in the heart	IL-2	IL-6
NO in the liver	1			
NO in the heart	0.918(**)	1		
IL-2	0.347	0.411(*)	1	
IL-6	-0.398(*)	-0.524(**)	-0.297	1

*, ** Indicates that the correlation is significant at the 0.05 and 0.01 level (two-tailed), respectively.

elimination are in dynamical equilibrium. Excessive free radicals cause oxidative damage to the body. VE is an antioxidant reacting with free radicals, disrupting the extension of the lipid peroxidative chain and consequently inhibiting or slowing down the formation of free radicals. SOD and GSH-PX are the two main antioxidant enzymes. SOD plays a vital role in the balance between oxidation and antioxidation. It dismutates superoxide anions to produce hydrogen peroxide and eliminates the cytotoxic effects of the superoxide anion (Tian et al., 2005). GSH-Px catalyzes H_2O_2 into H_2O and oxygen and prevents toxic OH generation. It has been proposed that the physiological function of GSH-Px is to reduce the toxic byproducts of the synthetic pathway by catalyzing the perioxidative intermediates to the intermediates used in metabolism (Reiss and Gershon, 1976). Lipid peroxidations amplify the effects of reactive oxygen species through a chain reaction and produces harmful chemicals such as MDA, the end products of lipid peroxidation. The content of MDA indirectly reflects the amount of free radicals produced and the degree of lipid peroxidation and is an important indicator of free radical induced damage. VE is one of the essential micronutrients that share a common biological antioxidant function to balance the internal reactive oxygen species (ROS) environment, scavenge exorbitant ROS, and maintain redox balance, and immunoenhancing functions (Zhu et al., 2010; Leshchinsky and Klasing, 2001). In this experiment, 15 days after switching from low diet to high VE diet, the activity of SOD and GSH-Px increased to a level significantly higher than in the control groups and decreased MDA in liver and heart indicating that diet VE can increase the total antioxidant ability to protect the body from damage by free radicals through eliminating ROS and inhibiting the lipid peroxidation chain reaction. Our results are consistent with a previous report showing that an increase of dietary VE enhances antioxidant capacity *in vivo* and in the carcass (Morrissey et al., 1997).

Many studies have shown the influence of VE on immune functions, but the mechanisms are not yet fully understood. It is known that a variety of external antigens induce NO production upon the activations of macrophages and polymorphonuclear leukocytes and the secretions of cytokines by lymphocytes. NO generated by macrophages can transmit cell signaling to lymphocytes and regulate the secretion of cytokines by nitrosylation. As an immune effector, NO participates, mediates and regulates various immune responses and connects many components in the body's immune system. Under resting conditions, lymphocytes can continuously produce a small amount of NO; and under the active status they produce a large amount of NO, which in turn can further induce T cells to secrete more cytokines, therefore sustaining the homeostasis.

IL-6 is involved in T-cell activation and represents an essential competence factor that synergizes with IL-1 to control the initial steps of T-cell activation, including induction of IL-2 and enhancement of responsiveness to IL-2 (Van Snick, 1990). In this study, IL-2 concentration decreased when lower the VE diet transformed to a higher VE diet at day 15. The immunostimulatory effects of VE are mediated, in part, by reduced prostaglandin E2 (PGE2) synthesis. PGE2 was shown to have a direct inhibitory effect on an early stage of T-cell activation, resulting in deeased IL-2 production, decreased IL-2 receptor expression, decreased responsiveness to exogenous IL-2 and decreased proliferation (Vercammen, and Ceuppens, 1987).

Our results indicate that decreased NO content is correlated with increased IL-2 and decreased IL-6 (Table 6), indicating that NO has pleiotropic effects in immunoregulation. It is possible that NO regulates IL-2 through NF-κB pathway. NF-κB has been reported to regulate the expression of IL-2 and other genes directly or coordinately with other transcription factors (Huang and Wang, 2000; Jin and Liu, 2000). NF-κB acts as a trans-element to regulate IL-6 functions by activation, translation and induction. Peng et al. (1995) have reported that NO inhibits TNF-α-induced NF-κB binding to DNA in human endothelial cells. Immunoprecipitation also shows that NO increases IκBα mRNA level and inhibits IκBα protein degradation (Lander et al., 1995). It has been reported that NO can affect the process of thymocyte apoptosis through p53 and induce apoptosis of macrophages, pancreatic β-cells, tumor cells, and cartilage cells, etc. (Peng et al., 1995; Geng et al., 1996).

The present study demonstrated that supplementing VE in diet can increase the antioxidant abilities and immune

function, which was manifested as increased SOD and GSH-Px activities, decreased MDA content, and eliminated NO free radicals which have a pleiotropic effects in immunoregulation. These results indicate that supplementing VE protects chickens from damage by preoxidation.

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