

# Effects of Dietary Vitamin E on Immunological Stress of Layers and their offspring

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Abbreviated title: Vitamin E on Immunological Stress

## Summary

The applications of vitamin E (VE) in poultry production have been well documented. In the present study, two experiments were conducted to evaluate the effect of VE supplementation of a commercial layer diet on the laying performance and immunological stress responses of hens and their offspring. In Experiment 1, responses to increased dietary VE levels were evaluated on 180 White Leghorn layers between 20 and 35 week of age. There were 3 levels of VE in the diets (0, 40 and 100 IU/kg) and 5 replicates per treatment, each containing 12 hens. When the laying rate reached 70%, egg quality including shell thickness, shape index, yolk color and Haugh units were investigated. Results showed that the high level of VE supplementation (100IU/kg) had a beneficial effect on feed intake and feed efficiency of hens ( $P<0.05$ ), compared with the VE-deficient or low level group. In Experiment 2, 540 female progeny from the VE-treated hens in Experiment 1 were used. The experimental design consisted of 3 levels of VE supplementation (the same as their mothers')  $\times$  3 vaccinating routines, the first vaccination being administered on day 5, 8 or 11. All vaccines and the subsequent vaccinating intervals were identical. At the metaphase of the experiment, each bird was injected celiacly with *E. coli* lipopolysaccharide (LPS, 0.05 mg/bird). The results showed that antibody titers against Newcastle disease virus or avian influenza virus and the plasma concentration of IL-1 were increased by the high level of VE supplementation. There were significant effects of the day of initial immunization with IBD on the ND and AI antibody

titer, H/L ratio and plasma concentration of corticosterone and interleukin-1 before and after injecting LPS, suggesting the occurrence of immunological stress. There was also significant interaction between VE and vaccination routine on the immune functions of experimental birds. Considered together with the results of Experiment 1, vitamin E's biological function appeared to be dose-dependent, especially with regard to its positive effect on the immune responses of young chickens.

Keywords: Vitamin E, Laying Hens, Immunological Stress, initial immunization, LPS

## Introduction

During the growth of laying hens, they encounter various stressors, such as fright, toxic environmental gases, heat and cold stress, etc. Some stress conditions might be acute, but others might prevail throughout the growing and laying phase. In recent years, vaccines and vaccination have emerged as essential tools in disease control for poultry (Swayne and Kapczynski, 2008). There are 6-7 kinds of disease vaccines (Marek's Disease, Infection Bursal Disease, Newcastle Disease, Avian Influenza, Infectious Bronchitis, etc) and more than 12 vaccinations are given during 0 to 8 weeks post hatch. Although vaccinations can increase resistance to infection, prolonged external stimulation of the immune system evokes a stress-like response that may impair some aspects of the chicken's immune function (Immerseel *et al.*, 2006) and some kinds of vaccine, such as Infection Bursal Disease (IBD), induce moderate to severe bursal lesions and immunosuppression (Bublöt *et al.*, 2007). Immunization stress has become one of the main stressors during the production of chicken flock.

It is well documented that response to stress and immunocompetence of chickens are influenced by dietary vitamin E (Tengerdy and Nockels, 1973; Sell *et al.*, 1997; Erf *et al.*, 1998; Leshchinsky and Klasing, 2001; Heffels-Redmann *et al.*, 2001; Puthpong-siriporn, *et al.*, 2001). Stressors, such as heat stress and vaccination stress, could stimulate the release of corticosterone and catecholamines and initiate lipid peroxidation in cell membranes (Freeman and Crapo, 1982), including those of T and B lymphocytes. The antioxidative property of vitamin E is considered to have a role in the development of immune response and to protect cells, such as lymphocytes, macrophages, and plasma cells, to enhance the function and proliferation of these cells in chickens in face of oxidative damage (Franchini *et al.*, 1991; Meydani and Blumberg, 1993). Chickens, however, cannot synthesize vitamin E and requirements must be met from dietary sources (Chan and Decker, 1994). Haq *et al.* (1996) reported that chicks hatched from hens fed 0.03%  $\alpha$ -

tocopherol acetate had higher bursal and splenic lymphocyte proliferation when stimulated *in vitro* with tetrahydrofuran or concanavalin A (ConA) than did the control chicks hatched from hens without supplemental  $\alpha$ -tocopherol acetate. Panda *et al.* (2008) showed that during heat stress, Vitamin E increased the antibody response to Newcastle Disease vaccine while significantly enhancing laying rate and feed conversion rate.

In the present study, the effects of different levels of Vitamin E added to diets of laying hens and their offspring chickens, were investigated using different initial times when an intermediate IBD vaccination was given and then after stressing by injecting lipopolysaccharide (LPS). This study will aid in better understanding mechanisms of immunological stress and in providing the theoretical basis for production practice.

## **Materials and methods**

### **Experimental animals and treatments**

#### ***Exp.1***

Healthy Leghorn hens (180) with similar bodyweights from the same hatch and fed the same, were assigned randomly at 19 wk to 3 treatment groups with 5 replicates, each containing 12 hens.

From 20 to 35 wks, the hens were fed a basal diet (Table 1), supplemented with 0, 40 or 100 IU/kg of vitamin E (VE).

The birds had been vaccinated with inactivated oil-emulsified vaccines against avian influenza (AI), Newcastle disease (ND) and Infection Bursal Disease (IBD) at the same day in the 22<sup>th</sup> week. Serum was obtained at 35 wk to determine titers against AI and ND. During this treatment interval, egg weight, egg output, and feed intake were recorded. When laying rate exceeded 70%, eggs from one day were collected and egg quality, including albumen height, egg shell strength, longitudinal diameter, horizontal diameter, yolk color, etc. were determined.

The hens were artificially inseminated at 35 wk and eggs were grouped within each dietary treatment, incubated and hatched for Experiment 2.

## **Exp. 2**

Hatchlings (540 total), within each of the same 3 VE treatments, were assigned to 3 immunization treatments (3 x 3 arrangement) with 5 replicates, each of 12 chickens.

The 3 immunization stress treatments differed only on the day of the first vaccination (day 5, 8 or 11 as Treatments A, B and C respectively). In the case of Treatment A, vaccination against IBD was on day 5, then ND and IB on day 8, and AI and ND on day 12. The vaccines and subsequent intervals between them (3 and 4 days) were identical for Treatments B and C. Each chicken was injected with 0.05 mg of LPS into the abdominal cavity 36 d later (i.e. at 41, 44, or 47 d) and blood samples were taken 12 h before injecting LPS, then 12, 24, 36, 48, and 72h after. Plasma and serum were prepared from 4 randomly selected birds in each replicate. Feed consumption was recorded daily by replicates.

## **Analytical methods**

**AI and ND titer:** Antibody titers for AI and ND were measured on sera collected 12h before injecting LPS, according to the prescripts of State Standard Diagnostic Techniques for Highly Pathogenic Avian Influenza of the People's Republic of China (GB/T18936-2003) and State Standard Technical Code of Quarantine for Newcastle Disease of the People's Republic of China (GB/T16550-1996). Blood coagulation and hemagglutination inhibition tests were also performed.

**H/L cell ratio:** Blood samples were taken from the wing vein 12h before and 72h after injecting LPS. Blood smears were stained by the Wright-Giemsa method and evaluated according to the leukocyte classification method of Alfred, *et al.*, (1961), with counting and calculating according to Campo and Davila (2002).

**Corticosterone and interleukin-1:** For each group, plasma samples taken 12h before and 12, 24, 36, 48 h after injecting LPS were used to measure the contents of corticosterone and interleukin-1 by ELISA methods.

## **Data processing**

Experiment data were analysed using the GLM (general liner model) of SAS (Version8e, SAS Institute, 1998) and are expressed as means  $\pm$  standard errors ( $\bar{X} \pm SE$ );  $P < 0.05$  was used as the threshold for significance.

## **Results**

### **Effects of vitamin E on laying performance, egg quality and antibody titers of laying hens**

Table 2 shows the results of dietary vitamin E supplementation on hen performance in Exp 1. Feed intake and ratio of feed intake to egg yield in the laying hens were significantly affected by dietary Vitamin E level. With the increase of VE from 40 to 100 IU/kg, feed intake and ratio of feed intake to egg yield were reduced significantly ( $P<0.05$ ). The Vitamin E level had no significant effects on laying rate, or mean egg weight in the experimental period, nor any significant effects ( $P>0.05$ ) on eggshell strength, yolk color, Haugh unit and egg shape index.

Vitamin E supplementation had no significant effects ( $P>0.05$ ) on antibody titers against AI or ND, but titers of the 40 and 100 IU/kg groups were higher than those of the control group (data not shown).

### **Effects of vitamin E on performance and immune function in young chickens**

#### ***Effects on food intake***

There were no significant effects of Vitamin E level or different days of initial immunization on feed intake of chickens ( $P<0.05$ ), though daily feed intake was reduced as dietary VE increased (see Table 3).

#### ***Effects on antibody titers and H/L ratio***

Vitamin E supplementation and different day of initial immunization did have significant effects on antibody titers against AI and ND of the chickens (see Table 3). With increases in the amount of Vitamin E, titers against AI and ND both increased ( $P<0.01$ ). Titters against AI were significantly higher in chickens first immunized against IBD on day 5 compared to those immunized on days 8 or 11 but the reverse was true for titers against ND. A significant interaction existed between the Vitamin E level and the day of initial immunization.

While there were no significant effects of either Vitamin E supplementation or the day of initial immunization stress on the control H/L value (before injecting LPS), there were highly significant effects on the H/L response to challenging with LPS (Table 3). After the LPS stress, the H/L value of the 0 IU/kg VE chickens was significantly higher than those fed 40 IU/kg and 100 IU/kg VE. The response in chickens first immunized on day 8 was significantly higher than those first immunized on day 5 or day 11; the latter not being different from each other. The LPS stress test showed that Vitamin E at 40 IU/kg, or first vaccination on days 5 or 11 could significantly reduce the H/L value.

### ***Effects on plasma concentration of interleukin-1***

As shown in Table 4, there was no significant effect ( $P>0.05$ ) of VE supplementation on plasma IL-1 concentrations at 5 time points before and after injecting LPS. In contrast, IL-1 levels at each time point after injecting LPS were the highest ( $P<0.05$ ) in chickens that were first immunized with IBD on day 11. Concentrations of IL-1 rapidly increased at 12h after injecting LPS then declined and remained relatively stable between 24 and 48h.

### ***Effects on plasma concentrations of corticosterone***

There were significant effects ( $P<0.01$ ) of Vitamin E supplementation on the concentrations of corticosterone in plasma at 12 h after the LPS stress was administered with levels in the VE-deficient chickens being significantly higher than those in birds provided with 40 or 100 IU/kg VE. There were significant effects of the day of initial immunization with IBD on the plasma concentration of corticosterone at 12, 24, 48 h after injecting LPS ( $P<0.01$ ); chickens first immunized on day 11 had significantly higher levels of corticosterone, 24 and 48 h after LPS, than did those vaccinated on days 5 or 8. The pattern of the corticosterone responses was similar to that described for IL-1.

## **Discussion**

### **Effects of Vitamin E on performance and egg quality**

Many studies have reported effects of Vitamin E on production performance during different phases. Siegel *et al.* (2006) discovered that 300 IU/kg of Vitamin E could increase bodyweight at 28 d but show little effect on feed conversion at 28 and 42 d. Puthongsiriporn *et al.* (2001) found that 25, 45 and 60 IU/kg VE supplementation did not affect feed consumption of laying of hens before and under heat stress. In the present study, Vitamin E

supplementation from 20 to 35 wks significantly affected feed intake and the ratio of feed intake to egg yield but there was no significant effect on feed intake during the brood time (0-7 weeks age).

Whitehead *et al.* (1998) reported that dietary vitamin E at 250 mg/kg provided for optimum egg production compared to 10 mg/kg fed to control hens by vitamin E promoting the release of vitellogenin from liver by protecting cell membranes of hepatocytes from oxidative damage. Puthongsiriporn *et al.*, (2001) found that egg yolk was significantly increased when hens were fed 45 and 65 IU/kg compared with the control vitamin E level (25 IU/kg). Haugh units were higher for hens fed 65 IU of vitamin E/kg compared to 25 and 45 IU/kg. Inconsistent with these findings, there were no significant effects of Vitamin E on egg quality characteristics in the present experiment.

### **The effects of dietary Vitamin E level on immune function**

Vitamin E is a lipid soluble antioxidant, and its deficiency results in increased free radical-induced membrane damage to red blood cells (Basu, 1996). Supplementation with vitamin E of birds enhanced mitogen-induced lymphocyte proliferation and IL-2 production, and improved the antibody responses to vaccines, while decreasing the synthesis of the immunosuppressive eicosanoid PGE2 (Meydani *et al.*, 1990;1997). Bassiouni, *et al.* (1990) gave hens high doses of Vitamin E and significantly increased coagulation-inhibiting antibody titer against Newcastle disease. Groe, *et al.* (1997) injected 10 IU of Vitamin E into 18-d chick embryos, and increased immunoglobulin IgG concentrations and macrophage phagocytosis of injected sheep red blood cells (SRBC). When SRBC were injected again, the immunoglobulin IgG and overall phagocytic activity of macrophages were higher than those of the control group. In the present experiment, Vitamin E had no significant effect on laying hens during the laying period, but did have effects on their offspring as assessed during the 7th week of age; antibody titers against AI and ND were significantly increased with increased dietary VE. As the immune system is still developing in the young growing chickens, its sensitivity to VE might well differ from that of adult birds.

### **The effects of dietary Vitamin E level on immunity stress**

Infectious bursal disease is an acute and highly contagious disease in chickens 3 weeks of age and older ( Berg *et al.*, 2000). A strategy for control of this disease is effective vaccination against IBD, including vaccination of chickens with inactivated oil-emulsified vaccines or with live attenuated vaccines (Tayade *et al.*, 2006). Some chickens immunized

with live attenuated IBD vaccines, especially the "intermediate" and "hot" vaccines, showed some degree of bursal atrophy and immunosuppression that interfered with the effectiveness of other vaccinations (Muller *et al.* 2003; Tsukamoto *et al.*, 1995). Lipopolysaccharides are cell wall components of Gram-negative bacteria that cause inflammation and sickness (Verdrengh and Tarkowski, 1997). In the present study, different initial vaccination times of "intermediate" IBD and LPS injection were used as stress model. The results showed that there were significant effects of the day of initial immunization with IBD on the ND and AI antibody titer, H/L ratio and plasma concentration of corticosterone and interleukin-1 after injecting LPS.

The multiple mechanisms by which VE affects lymphocytes might explain the complex relationship between dietary VE level and indices of immunocompetence. Leshchinsky and Klasing (2001) demonstrated that the level of dietary VE influenced lectin-induced proliferation of peripheral blood lymphocytes and the influx of heterophils into the blood during inflammation. In other study of Leshchinsky and Klasing (2003) showed that dietary VE decreased mRNA expression of the proinflammatory cytokine MGF after LPS administration, which might explain the anti-inflammatory effect of VE. In the present study, 40 and 100 IU/kg of Vitamin E significantly reduced the H/L value, concentrations of corticosterone and increased the IL -1 level in plasma at 12 h after the LPS stress. The results of these experiments support supplementation of vitamin E in young chickens to reduce the negative effects of immunization stress.

## **Conclusion**

Vitamin E supplementation of chickens had significant effects on production performance, antibody titers and was able to partly offset the extent of the stress response provoked by injection of LPS. Different days of initial immunization with IBD had significant effects on the ND and AI antibody titer, H/L ratio and plasma concentration of corticosterone and interleukin-1 before and after injecting LPS. Considered together with the results of Experiment 1 and 2, vitamin E's biological function appeared to be dose-dependent, especially with regard to its positive effect on the immune responses of young chickens.

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Table1 Composition of the basal diet<sup>1</sup>

Ingredients	Experiment 1	Experiment 2
Corn, yellow	66.35	63.28
soybean meal	22.58	28.94
Fish meal	-	3.00
Limestone meal	8.07	1.78
Salt	0.30	0.30
3% premix	3.00 <sup>2</sup>	3.00 <sup>3</sup>
Nutrient levels		
ME(MJ/kg)	11.90	11.70
Protein, %	17.00	19.00
Ca, %	3.06	0.90
Available P, %	0.31	0.31
Lys, %	0.90	1.13
Met, %	0.28	0.44
Met+Cys, %	0.57	0.74

<sup>1</sup>The basal diet was fed during the entire experiment.

<sup>2</sup>Provided per kilogram of diet :

Fe, 60mg ; Se 0.15mg ; I, 0.35mg ; Zn, 35mg ; Mn, 30mg ; Cu, 5mg ;  
 VA, 4000IU ; VD<sub>3</sub>, 300IU ; VK<sub>3</sub>, 0.5mg ; VB<sub>1</sub>, 0.80mg ; VB<sub>2</sub>, 2.5mg ; VB<sub>6</sub>, 3.0mg ; VB<sub>12</sub> 0.004mg ; Nicotinic acid, 11  
 mg ; Pantothenate, 10.0mg ; Folic acid, 0.3mg ; Biotin 0.10mg, Choline chloride, 800mg.

<sup>3</sup> Provided per kilogram of diet :

Fe, 80mg ; Se 0.20mg ; I, 0.35mg ; Zn, 40mg ; Mn, 60mg ; Cu, 9mg ;  
 VA, 8000IU ; VD<sub>3</sub>, 200IU ; VK<sub>3</sub>, 0.5mg ; VB<sub>1</sub>, 1.0mg ; VB<sub>2</sub>, 3.0mg ; VB<sub>6</sub>, 3.0mg ; VB<sub>12</sub> 0.003mg ; Nicotinic acid,  
 11mg ; Pantothenate, 10.0mg ; Folic acid, 0.4mg ; Biotin 0.15mg. Choline chloride, 1000mg.

Table 2 Effects of dietary vitamin E supplementation on performance and egg quality of hens<sup>1</sup>

Vitamin E (IU/kg)	Feed consumption (g/hen/day)	kg Feed /kg Eggs (20-35wks)	Shell strength	Egg colour	Haugh unit	Egg shape index
0	97.56 <sup>a</sup>	2.29 <sup>a</sup>	3.28	8.77±0.35	96.33±0.94	1.33±0.02
40	97.18 <sup>a</sup>	2.21 <sup>a</sup>	3.45	8.55±0.57	97.45±1.95	1.36±0.02
100	94.58 <sup>b</sup>	2.13 <sup>b</sup>	3.29	8.75±0.45	92.46±1.43	1.33±0.02
SEM	1.05	0.06	0.19	0.46	1.44	0.02

<sup>1</sup>Means within a parameter with different superscripts differ significantly (P<0.05).

Table 3 The effects of supplemental vitamin E levels on feed in take, antibody titers of NDV, AIV and heterophil to lymphocyte ratio of young chickens

Vitamin E (IU/kg)	Treat- ment	Feed in take	ND	AI	H/L ration	
			Pre-stress	Pre-stress	Pre-stress	Post-stress
0	A	21.77	4.80	4.80	0.08	0.13
0	B	22.40	10.00	4.20	0.13	0.74
0	C	21.78	9.80	3.60	0.11	0.24
40	A	21.99	9.20	4.60	0.14	0.15
40	B	22.48	10.00	5.20	0.09	0.20
40	C	21.16	9.40	3.80	0.15	0.18
100	A	21.37	9.00	7.00	0.16	0.17
100	B	21.77	10.00	5.00	0.13	0.29
100	C	21.44	9.80	5.00	0.16	0.21
SEM		0.60	1.31	1.07	0.03	0.08
0 IU/kg		21.98	8.20 <sup>b</sup>	4.20 <sup>b</sup>	0.11	0.37 <sup>a</sup>
40 IU/kg		21.87	9.53 <sup>a</sup>	4.53 <sup>b</sup>	0.13	0.18 <sup>b</sup>
100 IU/kg		21.53	9.60 <sup>a</sup>	5.67 <sup>a</sup>	0.15	0.22 <sup>b</sup>
SEM		0.58	1.93	1.89	0.05	0.09
A		21.71	7.67 <sup>b</sup>	5.47 <sup>a</sup>	0.13	0.15 <sup>b</sup>
B		22.22	10.00 <sup>a</sup>	4.80 <sup>ab</sup>	0.12	0.41 <sup>a</sup>
C		21.46	9.67 <sup>a</sup>	4.13 <sup>b</sup>	0.14	0.21 <sup>b</sup>
SEM		0.95	1.76	1.63	0.02	0.10
	d.f.			(P)		
Vitamin E	2	NS	<0.0001	0.01	NS	<0.0001
Treatment	2	NS	<0.0001	0.0325	NS	<0.0001
Vitamin E × Treatment	8	NS	<0.0001	0.3245	NS	<0.0001

<sup>a,b</sup>Means within a parameter with different superscripts differ significantly (P<0.05).

Table 4 The effects of supplemental vitamin E levels on plasma interleukin-1 (IL-1) concentration of young chickens (pg/mL)

Vitamin E	Treat	Prestress12h	Post-stress 12h	Post-stress	Post-stress	Post-stress
0	A	59.8	171.2	56.3	58.3	45.0
0	B	48.1	125.9	41.7	40.0	54.8
0	C	37.5	972.9	64.7	57.8	53.1
40	A	47.4	187.3	60.4	53.6	41.5
40	B	60.6	129.7	46.6	56.5	52.7
40	C	49.5	1022.7	59.7	62.6	64.9
100	A	57.5	145.6	53.4	51.0	36.9
100	B	44.8	127.5	56.1	57.4	62.4
100	C	50.0	1117.1	80.5	67.3	71.3
SEM		3.94	27.33	4.55	4.11	4.23
0 IU/kg		47.43	423.39	54.30	52.08	51.00
40 IU/kg		52.25	446.62	55.73	57.61	53.07
100 IU/kg		50.72	463.45	63.36	58.62	56.89
SEM		2.35	7.01	3.70	2.23	1.87
A		54.93 <sup>a</sup>	168.08 <sup>b</sup>	56.88 <sup>b</sup>	54.30	41.16 <sup>b</sup>
B		51.21 <sup>ab</sup>	127.75 <sup>c</sup>	48.17 <sup>c</sup>	51.37	56.65 <sup>a</sup>
C		45.73 <sup>b</sup>	1037.64 <sup>a</sup>	68.33 <sup>a</sup>	62.63	63.14 <sup>a</sup>
SEM		3.26	21.22	5.44	3.21	3.60
	d.f.	----- (P) -----				
Vitamin E	2	NS	NS	NS	NS	NS
Treatment	2	NS	<0.0001	0.0014	NS	<0.0001
Vitamin E ×	8	0.03	0.01	NS	NS	0.05

<sup>a,b</sup>Means within a parameter with different superscripts differ significantly (P<0.05).

Table5 The effects of supplemental vitamin E levels on plasma corticosterone concentrations of young chickens (ng/mL)

Vitamin E	Treat	Pre-	Post-stress	Post-stress	Post-stress	Post-stress
0	A	3.90	6.53	3.85	4.48	4.58
0	B	3.63	3.98	3.63	5.03	4.88
0	C	4.28	5.85	5.58	5.93	8.50
40	A	3.93	5.33	4.03	4.60	4.68
40	B	4.53	3.60	4.38 <sup>c</sup>	4.35	4.90
40	C	4.33	5.18	5.75	5.10	8.10
100	A	4.43	5.45	4.35	4.35	3.83
100	B	4.10	4.95	4.38	4.35	4.08
100	C	3.80	5.40	5.38	4.80	8.18
SEM		0.66	1.49	0.31	0.40	1.79
0 IU/kg		3.97	5.45 <sup>a</sup>	4.35	5.14	5.98
40 IU/kg		4.27	4.70 <sup>b</sup>	4.72	4.68	5.89
100 IU/kg		4.08	5.27 <sup>a</sup>	4.70	4.50	5.36
SEM		0.55	1.03	0.23	0.41	0.45
A		4.09	5.77 <sup>a</sup>	4.08 <sup>b</sup>	4.48	4.36
B		4.09	4.18 <sup>b</sup>	4.13 <sup>b</sup>	4.58	4.62
C		4.13	5.48 <sup>a</sup>	5.57 <sup>a</sup>	5.28	8.26
SEM		0.31	1.28	0.26	0.38	1.54
	d.f.	----- (P) -----				
Vitamin E	2	NS	0.0048	NS	NS	NS
Treatment	2	NS	<0.0001	<0.0001	NS	<0.0001
Vitamin E ×	8	NS	0.0096	NS	NS	NS

<sup>a,b</sup>Means within a parameter with different superscripts differ significantly (P<0.05).