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# Effects of Vitamin E and C Supplementation on Performance and Immune Response of Broiler Chicks

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**Abstract:** In order to study the effect of different levels of dietary vitamin E (0, 50,75,IU $kg^{-1}$ diets)and L-Ascorbic Acid (0,500 and 1000ppm in drinking water) on performance, immune response and some blood parameters, 432 male commercial broilers were allocated to 9 treatments with 4 replications (12 broilers in each) for 42 days (starter, 0-21 day, grower, 2-42 day) in factorial arrangement (3×3) based on completely randomized design. Immunity was assessed as antibody production to Infectious Bronchitis Virus (IBV), Newcastle Disease Virus (NDV) and Sheep Red Blood Cell (SRBC). Chickens were inoculated (18day) intramuscular with 0.5 mL of 5% SRBC. The injection with SRBC was repeated 12 day later. Performance (0-21 day)was not influenced by Vitamin E (VE). Daily weight gain (22-42day) was improved in chicks fed diets with supplemented 75 IU$kg^{-1}$ VE. Lymphoid organ weights and antibody titer for primary and secondary responses to SRBC were increased by 50 IU $kg^{-1}$ VE. Humoral immunologic response showed that antibody titer to NDV and IBV were highest ($p<0.05$) in groups receiving 75 IU $kg^{-1}$ diet VE. Adding 1000 ppm Vitamin C (VC) in drinking water significantly increased ($p<0.05$) antibody responses to SRBC, daily weight gain (22-42day) and body weight gain in 42day. Secondary responses to SRBC, antibody titer to NDV and IBV were improved ($p<0.05$) by supplemented 500 ppm VC in drinking water. Overall, the results of this experiment showed that VE and VC supplemental, improved some of performance parameters and humoral immune response in broiler chicks, respectively. This data suggest that optimum growth and humoral immune response may be achieved at supplemental level of VE of 50 IU $kg^{-1}$ and VC at 500 ppm.

**Key words:** Vitamin E, vitamin C, immune response, performance, broiler chicks

## INTRODUCTION

Vitamin E (VE),a fat soluble vitamin of plant origin, is essential for integrity and optimal function of the reproductive, muscular, circulatory, nervous and immune response. VE is known for its role as an antioxidant, protecting unsaturated bonds of cellular membrane phospholipids against free radical attack (Tappel, 1972). VE also has been shown to be a requirement for normal development and function of the immune system. Growth and viability of chicks improve with vitamin E supplementation (Walter and Jensen, 1964; Serman *et al*., 1992). The immunomodulatory effects of VE have been demonstrated in humans and a variety of animal species and were most evident in very young, very old and immunocompromised individuals (Tengerdy *et al*., 1984; Meydani, 1995; Meydani *et al*., 1995).

In experimental models, many parameters of the immune system , including resistance to infection, specific antibody production, number of antibody producing cells and *in vitro* mitogenic responses of lymphocytes are altered by supplementing diets that are deficient or marginal in VE (Meydani and Blumberg, 1993). In Single Comb White Leghorn chickens, VE and Se deficiency significantly impaired bursal growth and reduced the number of lymphocytes in the bursa and the thymus gland (Marsh *et al*., 1986). Additionally, VE and Se deficiency impaired the proliferation response of T cells to mitogen stimulation and was shown to specifically affect the differentiation of T cells (Chang *et al*., 1994). Lastly, VE has been shown to enhance immunity to Escherichia coli infection (Tengerdy and Brown, 1977), coccidiosis (Colnago *et al*., 1984), infectious bursal disease (McIlroy *et al*., 1993) and Newcastle disease (Franchini *et al*., 1995) in chickens.

High intensity poultry production requires fast growing strains, usually at high stocking densities. With this type of husbandry, flocks are highly susceptible to infectious agents, either as a result of reduced immune potential (Lamont and Dietert, 1990). Stress increases

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ascorbic acid requirements, indicating that it should be provided to birds living in stressful conditions. Vitamin C (VC) has been demonstrated to improve immune responsiveness and increase disease resistance in chickens by optimizing the functions of the immune system (Pardue *et al*., 1985; Rund, 1989). Although poultry are renal synthesizers of ascorbic acid, synthesis is inadequate under stressful conditions such as high environmental temperature, high humidity, high egg and meat production rate and parasite infestation (McDowell, 1989). In the first line of defense against pathogens, phagocytosis by neutrophils involves increased consumption of both ascorbate and dehydroascorbate (Stankova *et al*., 1975; Rund, 1989). In addition, viral infections have been shown to cause depletion of leukocyte ascorbate, resulting in varying degrees of nonspecific immunosuppression (Thomas and Holt, 1978). Ascorbic acid can modulate the activity of B cells and addition of dietary ascorbate prior to immunization has been found to increase antibody production (McCorkle *et al*., 1980). Dietary supplementation with VC, therefore, may have beneficial effects on immuno responsiveness in chickens. Beneficial effects of ascorbic acid supplementation on live weight gain, feed intake and carcass characteristics were observed in stressed poultry (Pardue and Thaxton, 1986; Sahin and Kucuk, 2001).

The main objective of this study was to assess the effects of various levels of dietary VE supplementation and VC in drinking water on performance, humoral and cell mediate immune response. Humoral immunity was measured as invivo antibody production,cell mediated immunity was measured in vivo as Cotaneous Basophil Hypersensitivity (CBH) and *in vitro* as lymphocyte proliferation to Phytohemagglutinin A (PHA) and concanavalin A.

## MATERIALS AND METHODS

**Animals and diet:** Commercial broiler males (Ross 208) were obtained from a local hatchery. One-day-old chicks were weighed and randomly allotted to dietary and water treatments. The basal corn-soybean meal diet was prepared according to NRC (1994), except that VE was omitted from the vitamin premix. Thirty-six pens of chicks (12 chicks per pen) were assigned to each of nine dietary treatments consisting of standard commercial starter and grower diets supplemented. The experimental design was a 3×3 factorial arrangement of 3 levels of dietary VE (0, 50, 75 mg dl-a-tocopherol acetate $kg^{-1}$ of feed) and 3 levels of dietary VC ( 0, 500 and 1000ppm L-Ascorbic Acid in drinking water). The diet supplemented with VE at 50 and 75 (IU $kg^{-1}$) and VC at 500 and 1000 ppm was based on the findings of previous our research. Diets contained 11.5IU VE in starter and 12.2 IU VE in grower derived from natural source (corn, soybean and corn oil) and was considered to be marginal for meeting NRC (1994) VE requirements. Dietary VE concentrations were confirmed by HPLC analysis. VE nad VC was provided by Basf co LTD. Nutrient requirement was calculated by UFFDA softwore (Table 1).

Table 1: Composition of the basal corn-soybean diets (starter: 1-21 day, grower: 21-42day)

| Ingredient (%) | Starter 0-21day | Grower 21-42day |
|---|---|---|
| Corn, yellow | 64.32 | 72.2 |
| Soybean meal | 27.56 | 19.55 |
| fish meal | 5 | 5 |
| Dicalcium phosphate | 1.09 | 1.09 |
| Limestone,ground | 1.1 | 1.11 |
| Lysine | 0.1 | 0.06 |
| Salt | 0.45 | 0.45 |
| Vitamin and mineral premix[1] | | |
| Nutrient analysis, calculated | | |
| ME, kcal.kg | 2900 | 2900 |
| Protein | 20.85 | 18.12 |
| Lysine, % | 1 | 0.9 |
| Metionine% | 0.45 | 0.35 |
| Ca, % | 0.906 | 0.815 |
| Available P, % | 0.41 | 0.35 |

[1]Vitamin mineral premix provided (per kilogram of diet) = vitamin A, 7,500 IU; cholecalciferol, 1,500 IU;; vitamin B2, 5.28 mg; pantothenic acid, 8 mg; vitamin B6, 1.84 mg; folic acid, 0.5 mg; vitamin B12, 0.0125 mg; choline, 350 mg; Se, 0.15 mg; I, 1.9 mg; Co, 0.2 mg; Cu, 6 mg; Fe, 30.8 mg; Zn, 50 mg; Mn 80 mg; S, 232 mg

Unconsumed feed from each cage was weighed back a week to monitor average feed consumption. Chicks were weighed at week's intervals to calculate average chick weight by cage.

**Humoral immune response:** Chicks were vaccinated against, respectively Infectious Bronchitis Virus (IBV) and Newcastle Disease Virus (NDV) by intraocular on day 6 (attenuated live virus) and i.m inoculation on 18 day of age. Blood was collected from the wing vein at 6 and 12 day after vaccination. Serum was isolated and stored at -20C until analyzed. Pooled samples representative of the pen, were obtained by mixing equal volumes of the serum from vaccinated birds from the same pens (n = 3). Serum antibody titers were determined by ELISA antibody test kit according to the manufacturer's directions.

Antibodies specific for NDV were detected in the serum of chicks by means of a Haemagglutination Inhibition (HI) test (Allan *et al*., 1978). In brief, the HI test was conducted by 2-fold serial dilutions of serum made in Normal Saline Solution (NSS) in microtitre plates. Four HA units of NDV in equal volume (25 μL) were added to each serum dilution. Then 1% chicken Red Blood Cells (RBCs) in 25 μL volume was added to each well and incubated at

37°C for 45 min. The HI titre was expressed as the log2 reciprocal of the highest serum dilution producing 100% inhibition of HI activity.

SRBC were washed three times in PBS and diluted in PBS to 5% (vol/vol). Three chicks per dietary replicate were injected (18day) with either 0.5 mL of 5% SRBC intramuscularly into each breast muscle. The injection with SRBC was repeated 12 day later. Heparinized blood was collected by venipuncture 6 and 12 day after immunization with SRBC. Plasma was stored at -20°C until further analysis. The hemagglutination assays were performed as described by Munns and Lamont (1991) for SRBC. Before analysis, complement was heat inactivated (56°C, 30 min). Each well of a 96-well plate received 0.05 mL of diluent buffer containing PBS. The initial well received 0.05 mL of plasma, which was serially doubly diluted by transferring 0.05 mL to the next well. Then, 0.05 mL of 2% SRBC in PBS was added to each well. The plates were shaken for 1 min, incubated for 1 h, shaken again for 1 min, incubated for 24 h at room temperature and then scored.

The agglutination titer was expressed as the log2 of the highest titer with 50% agglutination. Antibody titers were also determined after the treatment of samples with 0.2 *M* 2-mercaptoethanol for 30 min at 37°C, to inactivate IgM.

***In vitro* proliferation of isolated blood lymphocytes in response to Phytohemagglutinin A (PHA) and Concanavalin A (Con A):** At 26 days of age, 3 birds per pens were randomly assigned for lymphoblastogenesis assay *in vitro*. Blood samples (3mL chick$^{-1}$) were withdrawn with a EDTA syringe and diluted with equal volume of PBS. Blood from all birds in each pens was pooled and The Peripheral Blood Lymphocytes (PBLs) were separated from the whole blood by Ficoll (Atlanta Biologicals, Norcross, GA) density gradient by centrifuging at 2000 rpm for 30 min. The cells were washed twice and cell count was adjusted to $2\times10^5$ mL$^{-1}$ in RPMI-1640 growth medium (Fisher, Norcross, GA) supplemented with 5% heat inactivated fetal bovine serum and 1% antibiotic. One hundred μL PBLs, containing $2\times10^5$ cells from each sample were added to each of the 4 wells of a 96-well plate. In a preliminary experiment, the dose response of the mitogen-induced lymphocyte proliferation was determined by incubating cells with different concentration of ConA or pHA. The PHA and ConA concentrations that gave maximal proliferation responses were choosen for testing experimental samples. All experimental samples were incubated in triplicate in 96-well flat-bottom plates with either PHA-P (25 μgmL$^{-1}$), Con A (15 μg mL$^{-1}$), or cell culture media (nonstimulated). The plates were incubated at 37°C in a humidified incubator in the presence of 5% CO. After 72 h of incubation, At the end of the incubation period, 20 μL of MTT was added to each well. The plate was incubated again for 4 h at 37°C with 5% CO2, after which 150 μL of supernatant was pipetted from each well.

One hundred fifty microliters of 10% saponin in PBS was added into each well to lyse the cells. The plate was shaken by a plate shaker for 10 min and centrifuged at 1.500 rpm for 10 min. One hundred seventy-five microliters of supernatant was pipetted from each well,175 μL of acid isopropanol solution was added and the plate was shaken again for 10 min. The solutions in each well were then mixed by a multichannel pipette until sedimentation disappeared and the plate was then centrifuged at 1,500 rpm for 10 min. One hundred microliters of supernatant from each well was transferred to a new 96-well flat-bottomed plate. The Optical Density (OD) was read on a plate reader at 540 nm wavelength. Proliferative responses of lymphocytes were expressed as absorbance as described by Maslak and Reynolds (1995).

***In vitro* PhytohemagglutininA (PHA) and Concanavalin Exposure in whole blood:** At 26 days of age, 3 birds per pens were randomly assigned for lymphoblastogenesis assay *in vitro*. Blood samples (3mL/chick) were withdrawn with a EDTA syringe and diluted blood from all birds in each pens was pooled. Pooled whole blood (4μL) and PHA (25 μg mL$^{-1}$) or Con A(10 μg mL$^{-1}$) were added to microtiter plates containing a 200 μL of RPMI 1640 and antibiotic. The cell were plated, incubated, harvested and analyzed as described for isolated lymphocytes.

**CBH response:** The CBH response elicited in chickens by an intradermal injection of PHA-P was used to assess the *in vivo* cell-mediated immune response (Goto *et al*., 1978; Corrier and DeLoach, 1990; Kean and Lamont, 1994). Briefly 100 μg PHA-P in 0.1 mL saline (0.85%) was injected intradermally in the interdigital skin between the second and third digits of the right foot. The left foot received 0.1 mL saline and served as the control. Thickness was measured at 24 h later using a constant tension micrometer with its spring removed in order to obtain measurements without compressing the swollen tissues. The swelling response was determined by subtracting the preinjection thickness from the postinjection thickness. Data is reported as difference between skin thickness of the PHA and saline injected sites.

**Lymphoid organ weights:** Three Birds in pens were slaughtered on day 42 and thymus, spleen, liver and bursa

of Fabricius were collected and weighed. Individual lymphoid organ weights ratio was calculated by dividing lymphoid organ weights by body weight and multiplying by 1,000.

**Statistical analysis:** Data were analyzed using the SAS software computer package (SAS, 1989). A complete random design was used. Arcsine transformation of all percentage and ratio data before ANOVA analysis was done in order to normalize data. Untransformed means of percentage and ratio data are presented in the tables. Comparisons among the means were made using a multiple range test.

## RESULTS AND DISCUSSION

**Performance:** The results of the present study identified no significant differences in body weight gain in chicks fed supplemental VE in 42day (Table 2). Addition of VE had no effect on the cumulative food consumptions of chicks. Chicks given supplementary vitamin E (50 and 75 IU $kg^{-1}$) consumed significantly higher dairy weight gain (21-42day). There was no difference in food consumption between the chicks provided with 50 and 75 IU additional vitamin E per kg diet. The increase in dairy weight gain(21-42day) attributable to vitamin E supplementation in the present study confirms previous reports (Walter and Jensen, 1964; Serman *et al.*, 1992). However, previous reports found numerical increases in body weight (Serman *et al.*, 1992) when vitamin E was included at 60 and 90 mg $kg^{-1}$ to a basal diet containing 30 and 25 mg $kg^{-1}$ vitamin E, respectively. In agree to our observations Serman *et al.* (1992) found that vitamin E addition in the diet of chicks did not influence food efficiency of broilers at the different stage. Raza *et al.* (1997) who found that supplementary vitamin E at 300 IU $kg^{-1}$ had beneficial effects on weight gain, food intake and food conversion efficiency.

VC addition did not influence food consumption (0-21day). Chicks fed additional VC (1000ppm) exhibited a significant beneficial effect for body weight gain (42day), daily weight gain and feed consumption (21-42day). VC is involved in growth by promoting collagen synthesis, calcium and vitamin D3 metabolism, carnitine synthesis for oxidation of fatty acids, oxidation of amino acids, electron transport in the cells and scavenging of free radicals (Combs, 1992). The present study indicates that chickens fed a diet supplemented with VC (1000 ppm) had higher body weight gains as compared to those fed a diet not supplemented with VC. This finding is compatible with a previous report that chickens benefited from dietary supplementation of VC and gained weight faster than controls (Schildknecht *et al.*, 1986). However, no significant differences in body weight gains were observed between chickens drank VC-supplemented and those drank not supplemented with VC (21 day old). The reason for this finding is unclear. However, kidneys, which are the principal organs for chickens to synthesize AA, cannot synthesize adequate amounts of AA until after 15dayof age (Puls, 1994). Therefore, kidneys of chickens at 21 day of age are functionally and morphologically competent to synthesize sufficient amounts of VC to supply the tissues.

The combination of 50 IU vitamin E/kg and 1,000 ppm vitamin C supplementation induced the highest body weight gain in 42 day ($p<0.0002$), indicating an additive effect of vitamins E and C. Hoehler and Marquardt (1996) showed that VC enhance the biological efficacy of VE, thus the interaction between VC and VE affected performance in broiler chicks.

**Humoral immune response:** Antibody titers to live IBV were greater on day 12 compared with day 6. The level of dietary VE also affected titers. On day 6 postvaccination antibody titers increased when VE was supplemented (75 IU $kg^{-1}$). Twelve days postvaccination, titers did increase significantly ($p< 0.05$) as levels increased from 0-50 and 75 IU $kg^{-1}$ added VE (Table 3). In general, NDV antibody titers were higher at 6 day postvaccination than at 12 day. Titers generally increased as VE level increased from 0-50 and 75 IU $kg^{-1}$ of added vitamin E, peaking at 75 IU $kg^{-1}$ and decreasing afterward. In this experiment, antibody titers to SRBC were significantly lower on day 12 compared with day 6. The level of dietary VE also affected titers. On day 6 post injection antibody titers increased when VE was supplemented, with 50 and 75 IU of added VE/kg being significantly higher than 0 IU $kg^{-1}$ ($p< 0.05$). Twelve days post injection, titers did not difference significantly ($p< 0.05$) as levels increased from 0-50 IU $kg^{-1}$ added VE (Table 3). Previous studies using dietary VE supplementation have also shown similar increases in anti-SRBC antibody levels. Because VE deficiency is shown to depress bursal, thymic and spleen growth and reduce circulating lymphocytes in chicks, VE availability would seem to have a beneficial effect on the ontogeny of immune response in terms of humoral immunity (Tengerdy *et al.*,1984; Marsh *et al.*, 1986).

Chicks drank 500 ppm of added VC had significantly ($p<0.05$) higher IBV and NDV antibody titers than chicks drank normal water or 1000 ppm added VC. This finding was compatible with those of previous reports that chickens supplemented with AA at 500 ppm had

Table 2: Effect of vitamin E and C supplementation on performance in broiler chicks

| Treatment | | weight gain (g/chicks/day) | | Feed consumption (g/chicks/day) | | Feed conversion ratio | | Chick body weight(g) | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0-21 | 21-42 | 0-21 | 21-42 | 0-21 | 21-42 | 21 d | 42d |
| Vitamin E (I.U) | | Vitamin C (ppm) | | | | | | | |
| 0 | 0 | 27 | 70 | 44 | 142.9 | 1.64 | 2.04 | 608 | 2078.2 |
| 0 | 500 | 26.5 | 68.9 | 45.2 | 139 | 1.71 | 2.02 | 598 | 2045.5 |
| 0 | 1000 | 27.6 | 73.5 | 44.6 | 146.8 | 1.61 | 1.99 | 620 | 2165.5 |
| 50 | 0 | 27.3 | 70 | 42.3 | 142.3 | 1.55 | 2.03 | 614 | 2086.5 |
| 50 | 500 | 25.8 | 70 | 43.5 | 140.7 | 1.69 | 2.01 | 583 | 2056 |
| 50 | 1000 | 26.8 | 74.95 | 42.8 | 150 | 1.59 | 2 | 603 | 2177.7 |
| 75 | 0 | 25.7 | 71.9 | 43.4 | 142.3 | 1.69 | 1.97 | 581 | 2093.2 |
| 75 | 500 | 26.1 | 73.9 | 43.2 | 146.7 | 1.66 | 1.99 | 589 | 2140.2 |
| 75 | 1000 | 27 | 71.6 | 44.2 | 144.7 | 1.64 | 2.02 | 608 | 2112.7 |
| Main effect | | | | | | | | | |
| Vitamin E | | | | | | | | | |
| 0 | | 27.05 | 70.8b | 44.61 | 142.9 | 1.65 | 2.01 | 609.03 | 2096.4 |
| 50 | | 26.65 | 71.7ab | 42.86 | 144.3 | 1.61 | 2.01 | 600.7 | 2106.7 |
| 75 | | 26.29 | 72.48a | 43.58 | 144.6 | 1.66 | 1.99 | 593.1 | 2115.4 |
| Vitamin C | | | | | | | | | |
| 0 | | 26.69 | 70.7[b] | 43.25 | 142.5[b] | 1.62 | 2.01 | 601.53 | 2086[a] |
| 500 | | 26.16 | 71[b] | 43.96 | 142.2[b] | 1.68 | 2 | 590.36 | 2080.6[b] |
| 1000 | | 27.14 | 73.4[a] | 43.84 | 147.11[a] | 1.61 | 2 | 611.08 | 2152[a] |
| SEM | | 0.481 | 0.453 | 0.705 | 1.287 | 0.034 | 0.019 | 10.11 | 8.668 |
| | d.f | p Value | | | | | | | |
| Vitamin E | 2 | 0.22 | 0.04 | 0.54 | 0.62 | 0.54 | 0.68 | 0.54 | 0.31 |
| Vitamin C | 2 | 0.74 | 0.0004 | 0.36 | 0.01 | 0.34. | 0.87 | 0.36 | 0.0001 |
| Vitamin E* C | 4 | 0.94 | 0.0005 | 0.8 | 0.11 | 0.68 | 0.73 | 0.8 | 0.0002 |

[a-b]Means in a column with no common superscript differ significantly ($p < 0.05$)

Table 3: Effect of vitamin E and C supplementation on Anti-NDV and IBV 6 and 12daypost vaccination and primary and secondary antibody response of SRBC

| Treatment | | Anti-IBV antibody ($Log_{10}$) | | Anti- NDV antibody ($Log_2$) | | Primary anti-SRBC antibody | | Secondary anti-SRBC antibody | |
|---|---|---|---|---|---|---|---|---|---|
| | | 6 d | 12 d | 6 d | 12 d | 6d | 12d | 6d | 12d |
| Vitamin E (I.U) | Vitamin C (ppm) | | | | | | | | |
| 0 | 0 | 3.49 | 3.67 | 3.85 | 3.6 | 1.87 | 0.9 | 3.9 | 2.95 |
| 0 | 500 | 3.48 | 3.7 | 4.5 | 3.97 | 1.95 | 0.85 | 4.37 | 2.95 |
| 0 | 1000 | 3.48 | 3.72 | 4.52 | 4.02 | 2.7 | 1.17 | 4.12 | 2.82 |
| 50 | 0 | 3.48 | 3.72 | 4.67 | 3.62 | 2.95 | 0.9 | 4.97 | 3.47 |
| 50 | 500 | 3.46 | 3.76 | 5.37 | 4.92 | 4.02 | 1.22 | 7 | 4.2 |
| 50 | 1000 | 3.47 | 3.72 | 5.57 | 5.15 | 3.97 | 1.1 | 5.87 | 4 |
| 75 | 0 | 3.51 | 3.74 | 5.1 | 4.4 | 2.92 | 1.15 | 5.07 | 3.47 |
| 75 | 500 | 3.54 | 3.74 | 5.75 | 5.42 | 4.07 | 1.4 | 5.02 | 3.1 |
| 75 | 1000 | 3.54 | 3.72 | 5.02 | 4.25 | 3.85 | 1.3 | 4.95 | 3.27 |
| Main effect | | | | | | | | | |
| Vitamin E | | | | | | | | | |
| 0 | | 3.5 | 3.69[b] | 4.29[b] | 3.86[c] | 2.17[b] | 0.97[b] | 4.13[c] | 2.9[c] |
| 50 | | 3.5 | 3.73[a] | 5.2[a] | 4.56[b] | 3.65[a] | 1.07[b] | 5.95[a] | 3.89[a] |
| 75 | | 3.49 | 3.73[a] | 5.29[a] | 4.69[a] | 3.61[b] | 1.29[a] | 5.01[b] | 3.28[b] |
| Vitamin C | | | | | | | | | |
| 0 | | 3.5 | 3.7[c] | 4.54[c] | 3.87[c] | 2.58[c] | 0.98[b] | 4.65[c] | 3.3 |
| 500 | | 3.5 | 3.73[a] | 5.2[a] | 4.77[a] | 3.35[b] | 1.15[a] | 5.46[a] | 3.41 |
| 1000 | | 3.49 | 3.72[b] | 5.04[b] | 4.47[b] | 3.5[a] | 1.2[a] | 4.98[b] | |
| SEM | | 0.009 | 0.002 | 0.035 | 0.031 | 0.047 | 0.036 | 0.054 | 0.041 |
| | d.f | P Value | | | | | | | |
| Vitamin E | 2 | 0.0002 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |
| Vitamin C | 2 | 0.93 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0005 | 0.0001 | 0.162 |
| Vitamin E* C | 4 | 0.67 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0073 | 0.0001 | 0.0001 |

[a-c]Means in a column with no common superscript differ significantly ($p<0.05$)

Table 4: Effect of vitamin E and C supplementation on the proliferation lymphocyte in purified lymphocyte and whole blood (OD in 540 nm)

| | | Purified lymphocyte | | | Whole blood | | |
|---|---|---|---|---|---|---|---|
| | | Without mitogen | Mitogen | | Without mitogen | Mitogen | |
| Treatment | | (Control) | PHA | Con.A | (Control) | PHA | Con.A |
| Vitamin E (I.U) | Vitamin C (ppm) | | | | | | |
| 0 | 0 | 0.26 | 0.34 | 0.29 | 0.25 | 0.41 | 0.35 |
| 0 | 500 | 0.43 | 0.56 | 0.51 | 0.23 | 0.38 | 0.33 |
| 0 | 1000 | 0.29 | 0.55 | 0.53 | 0.25 | 0.49 | 0.43 |
| 50 | 0 | 0.46 | 0.59 | 0.55 | 0.24 | 0.44 | 0.34 |
| 50 | 500 | 0.52 | 0.79 | 0.7 | 0.22 | 0.43 | 0.37 |
| 50 | 1000 | 0.41 | 0.65 | 0.51 | 0.26 | 0.52 | 0.5 |
| 75 | 0 | 0.27 | 0.47 | 0.4 | 0.25 | 0.57 | 0.53 |
| 75 | 500 | 0.18 | 0.33 | 0.25 | 0.27 | 0.59 | 0.61 |
| 75 | 1000 | 0.25 | 0.29 | 0.32 | 0.24 | 0.36 | 0.35 |
| Main effect | | | | | | | |
| Vitamin E | | 0.33$^{b}$ | 0.48$^{b}$ | 0.44$^{b}$ | 0.24 | 0.42$^{c}$ | 0.37$^{b}$ |
| 0 | | 0.46$^{a}$ | 0.67$^{a}$ | 0.58$^{a}$ | 0.24 | 0.46$^{b}$ | 0.4$^{b}$ |
| 50 | | 0.23$^{c}$ | 0.36$^{c}$ | 0.32$^{b}$ | 0.25 | 0.5$^{a}$ | 0.5$^{a}$ |
| 75 | | | | | | | |
| Vitamin C | | | | | | | |
| 0 | | 0.33$^{b}$ | 0.47$^{c}$ | 0.42$^{c}$ | 0.25 | 0.47 | 0.41 |
| 500 | | 0.38$^{a}$ | 0.56$^{a}$ | 0.48$^{a}$ | 0.24 | 0.47 | 0.44 |
| 1000 | | 0.32$^{b}$ | 0.49$^{b}$ | 0.45$^{b}$ | 0.25 | 0.45 | 0.43 |
| SEM | | 0.007 | 0.008 | 0.006 | 0.012 | 0.008 | 0.008 |
| | d.f | P Value | | | | | |
| Vitamin E | 2 | 0.0001 | 0.0001 | 0.0001 | 0.67 | 0.0001 | 0.0001 |
| Vitamin C | 2 | 0.0001 | 0.0001 | 0.0001 | 0.93 | 0.19 | 0.34 |
| Vitamin E* C | 4 | 0.0001 | 0.0001 | 0.0001 | 0.72 | 0.0001 | 0.0001 |

$^{a-b}$Means in a column with no common superscript differ significantly ($p<0.05$)

increased antibody titers to infectious bronchitis virus (Tuekam *et al*., 1994). The increase of antibody titer VC-supplemented chickens may have been due to the antioxidant property of VC in that VC was able to protect immature lymphocytes from damage by free radicals due to oxidation, thus enhancing the immune response.

Table 3 shows Anti-SRBC antibody production. Titer antibody was increased significantly ($p< 0.05$) by levels of VC in 12daypost injection. At both 6 and 12 day after the second inoculations were improved SRBC antibody titers in groups received 500 ppm VC. Although chickens can synthesize VC, certain conditions may cause stress that depletes the VC levels in the body. These include exposure to hot or cold temperatures, starvation and infectious diseases. Vaccination is also a stressor that can interfere with adequate biosynthesis of VC in chickens (Gross, 1988; Satterlee *et al*., 1989). Thus, supplementing the diet with VC becomes beneficial (McDowell, 1989; Kutlu and Forbes, 1993). Table 3 shows effects of vitamins E and C supplementation and interaction on IBV, NDV and SRBC antibody titers. Supplementation of 50 IU of vitamin E kg$^{-1}$ and 500 ppm vitamin C both significantly enhanced SRBC antibody titers ($p<0.0001$).

In the case of the humoral immune response, the effect of added VE depended on the nature of the antigen. We observed an increase in the antibody response to SRBC (6 day) when VE (50IU kg$^{-1}$) was added to the diet and there was a non significant effect for a similar response for IBV. Previously, Leshchinski and Klassing (2001) demonstrated that VE enhanced the response to killed IBV but impaired the response to live IBV vaccine.

**Cell-mediated immunity**

**Lymphocyte proliferation:** Proliferation of purified lymphocytes in mitogen-free cell culture medium (control) was affected by the level of dietary VE fed to chicks (Table 4). Mitogenic responses to PHA and Con A were significantly altered by VE. The effect of VE on the proliferative responses to Con A were not similar to those with PHA. Mitogenic responses to Con A (15 μg mL$^{-1}$) in pure lymphocyte were not affected at 75IU kg$^{-1}$ added VE and proliferation at 50 IU of added VE kg$^{-1}$ was higher than at 0 and 75 IU kg$^{-1}$. proliferation of whole blood lymphocytes in the absence of mitogens was not affected with 50 and 75 IU kg$^{-1}$ of added VE compared to the control.

Chicks drank 500 and 1000 ppm of VC had significantly greater ($p<0.05$) *in vitro* lymphocyte proliferative responses to ConA and PHA than those of chicks drank normal water. Proliferation with mitogen and

control (without mitogen) of whole blood lymphocytes in the mitogens PHA and Con A was not affected with VC (Table 4).

Lymphocyte proliferation in response to mitogens is correlated with the ability of the host to mount a cellular immune response. It has been suggested that differential reactivity to mitogens reflects either maturational or functional differences in the responsive lymphocytes. These results support previous research reporting vitamin E enhancement of mitogenic responses of lymphocytes (Meydani and Blumberg, 1993; Haq *et al*., 1996).

We observed a large increase in proliferation of lymphocytes in response to PHA when VE supplementation increased 50 IU $kg^{-1}$ in purified lymphocytes. It was shown that many factors, including cell preparation, cell culture medium and duration of the assay, might influence the effects of dietary factors on *in vitro* responses. Specifically, several studies demonstrated that VE becomes quickly depleted in cell cultures and the addition of serum supplements to the media could introduce different levels of VE in *in vitro* experiments (Kelley *et al*., 1995; Leist *et al*., 1996). The measurement of lymphocyte proliferation in whole blood is considered by many researchers to be a more valid measure of T-cell function than the assays with purified or isolated lymphocytes, as it preserves the lymphocyte microenvironment (Talebi *et al*., 1995). Nevertheless, When lymphocytes are purified from whole blood they are separated from hormones and from VE-containing lipoproteins, which might modulate in vivo responses.

VC enhances lymphocyte proliferation by improving the responsiveness of T lymphocytes to mitogens (Johnston and Huang, 1991) and its antioxidant activity (Jacob, 1995; Retsky and Frei, 1995). Bendich *et al*. (1984) reported that the responses of T and B lymphocytes of guinea pigs were enhanced by diets containing higher-than-standard levels of vitamin C and E supplementation.

Table 4 shows that supplementation of 50 IU of vitamin E $kg^{-1}$ and 500 ppm vitamin C both significantly enhanced *in vitro* lymphocyte proliferative responses to Con A ($p<0.0001$) and PHA ($p<0.0001$) in pure lymphocyte. The combination of 75 IU vitamin E $kg^{-1}$ and 500 ppm vitamin C supplementation induced the highest *in vitro* lymphocyte proliferative responses to Con A and PHA in whole blood.

**Cutaneous basophil hypersensitivity:** The chicks fed the 50 and 75 I.U $kg^{-1}$ VE in diet had significantly ($p<0.05$) higher CBH response than the control. This observation implies that VE supplementation may increase T cell-mediated immunocompetence in chicks. Drinking of high dietary VC resulted in a improve in CBH responses

Table 5: Effect of vitamin E and C supplementation on CBH (Cutaneous Basophil Hypersensitivity) in 24 h post Phytohemagglutinin (PHA) injection

| Treatment | | |
|---|---|---|
| Vitamin E (I.U) | Vitamin C (ppm) | CBH (mm) |
| 0 | 0 | 0.41 |
| 0 | 500 | 0.41 |
| 0 | 1000 | 0.45 |
| 50 | 0 | 0.4 |
| 50 | 500 | 0.45 |
| 50 | 1000 | 0.51 |
| 75 | 0 | 0.56 |
| 75 | 500 | 0.61 |
| 75 | 1000 | 0.59 |
| Main effect | | |
| Vitamin E | | |
| 0 | $0.42^{c}$ | |
| 50 | $0.46^{b}$ | |
| 75 | $0.59^{a}$ | |
| Vitamin C | | |
| 0 | $0.46^{c}$ | |
| 500 | $0.49^{b}$ | |
| 1000 | $0.52^{a}$ | |
| SEM | 0.007 | |
| | d.f | P value |
| Vitamin E | 2 | 0.0001 |
| Vitamin C | 2 | 0.0001 |
| Vitamin E* C | 4 | 0.01 |

$^{a-c}$Means in a column with no common superscript differ significantly ($p<0.05$)

(Table 5). Chickens drank supplemented with VC (500 and 1000 ppm) had CBH responses level significantly ($p<0.05$) higher than that of chickens drank the diet without supplementation of VC. Supplemental of VC increased CBH response, although the exact mechanism is unclear. Pardue (1983) has theorized that VC either limits adrenocortical hormone release or through its antioxidant or other properties protects immunobiological tissue from the destroy.

The inflammatory response is considered to be the cornerstone of innate immunity with heterophils and macrophages as the first lines of defense during inflammation (Corrier and Deloach, 1990; Klasing, 1991). The main mediators of inflammation are pro-inflammatory cytokines interleukin-1, interleukin-6, myelomonocytic growth factor and tumor necrosis factor-$\alpha$ released by activated macrophages; these cytokines mediate changes in glucocorticoids, acute-phase proteins and recruitment of monocytes and heterophils from the bone marrow (Klasing, 1991; Siatskas and Boyd, 2000).

**Lymphoid organ weights:** Chickens fed the diet supplemented with 50 IU $kg^{-1}$ VE had significantly ($p< 0.05$) higher spleen and thymus ratios than those of chickens fed the diet without supplemented with VE.

Chickens drank the water supplemented with 500 ppm VC had the highest spleen, thymus and bourse ratio (Table 6). Although this study indicates a marked effect

Table 6: Effect of vitamin E and C supplementation on lymphoid organ weight (lymphoid organ weight*1000/body weight gain 42 d)

| Main effect | Organs | | |
|---|---|---|---|
| Vitamin E | Thymus | Spleen | Bursa |
| 0 | 3.15[b] | 1.25[b] | 3.31[a] |
| 50 | 3.5[a] | 1.65[a] | 3.02[ab] |
| 75 | 2.92[b] | 1.34[b] | 2.96[b] |
| Vitamin C | | | |
| 0 | 3.52[a] | 1.37 | 2.7[b] |
| 500 | 3.14a[b] | 1.52 | 3.87[a] |
| 1000 | 2.92[b] | 1.34 | 2.72[b] |
| SEM | 0.316 | 0.071 | 0.549 |

[a-b]Means in a column with no common superscript differ significantly ($p<0.05$)

of VE or VC on lymphoid organ development as well as body growth in chickens, it remains unclear whether the beneficial effects of this vitamin intake on the immune organs of chickens occur through a direct vitamin-lymphoid organ interaction or through indirect effects caused by changes in neuroendocrine status. The protective effects of VE and VC on Lymphoid Organ Weights may partially be a result of reducing circulating levels of glucocorticoids (McDowell, 1989).

In summary, our study demonstrated that dietary VE enhances the antibody response to some antigens, improve the proliferative response of lymphocytes to polyclonal mitogens. VC supplementation (500 and 1000 ppm) may improve body weight gain and antibody response to antigens, respectively. Interaction between VC and VE in immune response and performance was clearly. Moderate additions of combination VE (50 IU $kg^{-1}$) and VC affected indices of performance and immune response more than higher levels of supplementation. We hypothesize that moderate and high dietary levels of VE and VC may have different effects on the cellular free radical antioxidant balance, which results in different signal transduction events and activation states of the immune cells.

## REFERENCES


Allan, W.H., J.E. Lancaster and B. Toth, 1978. Newcastle disease vaccines, their production and use. FAO Anim. Prod. Health Series, pp: 10.

Bendich, A., P.D. Apolito, E. Gabriel and L.J. Machlin, 1984. Interaction of dietary vitamin C and vitamin E on guinea pig immune responses to mitogens. J. Nutr., 114: 1588-1593.

Chang, W.P., J.S.H. Hom, R.R. Dietert, G.F. Combs and J.A. Marsh, 1994. Effect of dietary vitamin E and selenium deficiency on chicken splenocyte proliferation and cell surface marker expression. Immunopharm. Immunotoxicol., 16: 203-223.

Colnago, G.L., L.S. Jensen and P.L. Long, 1984. Effect of selenium and vitamin E on the development of immunity to coccidiosis in chickens. Poul. Sci., 63: 1136-143.

Combs, G.F., 1992. Vitamin C. *in* Vitamins. Academic Press, Inc., New York, pp: 223-249.

Corrier, D.E. and J.R. DeLoach, 1990. Evaluation of cellmediated, cutaneous basophil hypersensitivity in young chickens by an interdigital skin test. Poul. Sci., 69: 403-408.

Franchini, A., S. Bertuzzi, C. Tosarelli and G. Manfreda, 1995. Vitamin E in viral inactivated vaccines. Poul. Sci., 74: 666-671.

Goto, N., H. Kodama, K. Okada and Y. Fujimoto, 1978. Suppression of phytohemagglutinin skin response in thymectomized chickens. Poul. Sci., 57: 246-250.

Gross, W.B., 1988. Effect of environmental stress on the responses of ascorbic-acid-treated chickens to Escherichia coli challenge infection. Avian Dis., 32: 432-436.

Haq, A., C.A. Bailey and A. Chinnah, 1996. Effect of betacarotene, canthaxanthin, lutein and vitamin E on neonatal immunity of chicks when supplemented in the broiler breeder diets. Poul. Sci., 75: 1092-1097.

Hoehler, D. and R.R. Marquardt, 1996. Influence of vitamin E and C on the toxic effects of ochratoxin A and T-2 toxin in chicks. Poul. Sci., 75: 1508-1515.

Jacob, R.A., 1995. The integrated antioxidant system. Nutr. Res., 15: 755-766.

Johnston, C.S. and S.N. Huang. 1991. Effect of ascorbic acid nutriture on blood histamine and neutrophil chemotaxis in guinea pig. J. Nutr., 121: 126-130.

Kean, R.P. and S.J. Lamont, 1994. Effect of injection site on cutaneous basophil hypersensitivity response to phytohemagglutinin. Poul. Sci., 73: 1763-1765.

Kelley, E. E., G.R. Buettner and C.P. Burns, 1995. Relative α-tocopherol deficiency in cultured cells: Free radical-mediated lipid peroxidation, lipid oxidizability and cellular polyunsaturated fatty acid content. Arch. Biochem. Biophys., 319: 102-109.

Klasing, K.C., 1991. Avian inflammatory response: Mediationby macrophages. Poul. Sci., 70: 1176- 1186.

Kutlu, R. and J.M. Forbes, 1993. Changes in growth and blood parameters in heat-stressed broiler chicks in response to dietary ascorbic acid. Livest. Prod. Sci., 36: 335-350.

Lamont, S.J. and R.R. Dietert, 1990. New Directions in Poultry Genetics. In: Poultry Breeding and Genetics. R.D. Crawford, (Ed.) Elsevier, Amsterdam, The Netherlands, pp: 497-541.

Leist, M., B. Raab, S. Maurer, U. Rosick and R. Brigelius-Flohe, 1996. Conventional cell culture media do not adequately supply cells with antioxidants and thus facilitate peroxideinduced genotoxicity. In: Free Radical Biology and Medicine, Elsevier Science Inc., New York, 21: 297-306.

Leshchinsky, T.V and K.C. Klasing, 2001. Relationship between the level of dietary vitamin E and immune response of broiler chickens. Poul. Sci., 80: 1590-1599.

Marsh, J.A., G.F. Combs, M.E. Whitacre and R.R. Dietert, 1986. Effect of lenium and vitamin E dietarydeficiencies on chick lymphoid organ development. Proc. Soc. Exp. Biol. Med., 182: 425-436.

Maslak, D.M. and D.L. Reynolds, 1995. Mitogenic responses of the head-associated lymphoid tissues of the chicken. Avian Dis., 39: 1-8.

McCorkle, F.M. and B. Glick, 1980. The effect of aging on immune competence in the chicken: Antibody mediated immunity. Poul. Sci., 59: 669-672.

Mcdowell, L.R., 1989. Vitamins in Animal Nutrition, In: Mcdowell, L.R. (Ed.), Comparative Aspects to Human Nutrition. Vitamin C, Folacin, (London, Academic Press), pp: 365-387, 298-322.

McIlroy, S.G., E.A. Goodall, D.A. Rice, M.S. McNulty and D.G. Kennedy, 1993. Improved performance in commercial broiler flocks with subclinical infectious bursal disease when fed diets containing increased concentrations of vitamin E. Avian Pathol., 22: 81-94.

Meydani, S.N. and J.B. Blumberg, 1993. Vitamin E and the Immune Response. In: Nutrient Modulation of the Immune Response. S. Unningham-Rundles, (Ed.), Marcel Dekker, New York, pp: 223-238.

Meydani, S.N., 1995. Vitamin E enhancement of T cellmediated function in healthy elderly: Mechanisms of action. Nutr. Rev., 53: 52-58.

Meydani, S.N., D. Wu, M.S. Santos and M.G. Hayek, 1995. Antioxidants and immune response in aged persons: Overview of present evidence. Am. J. Clin. Nutr., 62: 1462-1476.

Munns, P.L. and S.J. Lamont, 1991. Effects of age and immunization interval on the anamnestic response to T-cell-dependent and T-cell-independent antigens in chickens. Poul. Sci., 70: 2371-2374.

National Research Council, 1994. Nutrient Requirements of Poultry. (9th Edn.), National Academy Press, Washington, DC.

Pardue, S.L., 1983. Relationship of Ascorbic Acid to physiological stress in domestic fowl .Ph.d. Diss. North Carolina state University, Raleigh, N.C.

Pardue, S.L., J.P. Thaxton and J. Brake, 1985. Role of ascorbic acid in chickens exposed to high environmental temperature. J. Applied Physiol., 58: 1511-1516.

Pardue, S.L. and J.P. Thaxton, 1986. Ascorbic acid in poultry. A Rev. World's Poul. Sci., 42: 107-112.

Puls, R., 1994. Vitamin levels in animal health. In: Diagonistic Data and Bibliographies. Sherpa International, Clearbrook, BC, Canada, pp: 78.

Raza, F.K., S.A. Khan, A. Raza, M.A. Saeed and M.N. Bashir, 1997. Effect of vitamin E deficiency and excess on immune system of b roilers. Int. J. Anim. Sci., 12: 39-41.

Retsky, K.L. and B. Frei, 1995. Vitamin C prevents metal iondependent initiation and propagation of lipid peroxidation in human low-density lipoprotein. Biochem. Biophys. Acta., 1257: 279-287.

Rund, B., 1989. Vitamin C plays a role in immunity. Poul. Dig., 48: 44-55.

Sahin, K. and O. Kucuk, 2001. Effects of vitamin C and vitamin E on performance, digestion of nutrients and carcass characteristics of Japanese quails reared under chronic heat stress (34_C). J. Anim. Physiol. Anim. Nutr., 85: 335-342.

SAS Institute, Inc, 1989. SAS/STAT User's Guide. Version 6, Vol. 1. SAS Institute Inc., Cary, NC.

Satterlee, D.G., I. Aguilera-Quintana, B.J. Munn and B.A. Krautmann, 1989. Vitamin C amelioration of the adrenal stress response in broilers being prepared for slaughter. Comp. Biochem. Physiol., 94: 469-574.

Schildknecht, E., T. Curtis, G.G. Untawale, A. Bendich and C. Gerenze, 1986. Effects of High Levels of Ascorbic Acid in Broiler Chickens Infected with Coccidiosis During Stress. In: Research in Avian Coccidiosis. Proceedings of the Georgia Coccidiosis Conference. L. R. McDougald, L. P. Joyner and P. L. Long, (Eds.), University of Georgia, Athens, GA., pp: 203.

Schrama, 1996. Divergent antibody responses to vaccines and divergent body weights of chicken lines selected for high and low humoral responsiveness to sheep red blood cells. Avian Dis., 40: 634-644.

Serman, V., N. Mas, H. Mazila and Z. Mikulec, 1992. 1. Immune response as a marker of needs for vitamin E in chickens. 2. The influence of Vitamin E on fattening chicks productivity. Krimva, 34: 65-69.

Siatskas, C. and R. Boyd, 2000. Regulation of chicken haemopoiesis by cytokines. Dev. Comp. Immunol., 24: 37-59.

Stankova, L., N.B. Gerherdt, L. Nagel and R.H. Bigley, 1975. Ascorbate and phagocyte function. Infect. Immun., 12: 252-255.

Talebi, A., P. Torgerson and G. Mulcahy, 1995. Optimal conditions for measurement of blastogenic responses of chickens to concanavalin A in whole blood assay. Vet. Immunol. Immunopathol., 46: 293-301.

Tappel, A.L., 1972. Vitamin E and free radical peroxidation of lipids. Ann. N.Y. Acad. Sci., 203: 12-28.

Tengerdy, R.P. and J.C. Brown, 1977. Effect of vitamin E and A on humoral immunity and phagocytosis in E. coli infected chickens. Poul. Sci., 56: 957-963.

Tengerdy, R.P., M.M. Mathias and C.F. Nockels, 1984. Effect of vitamin E on immunity and disease resistance. In: Vitamins, Nutrition and Cancer. K. N. Prasad, Ed. Karger, Basel, Switzerland, pp: 118-122.

Thomas, W.R. and P.G. Holt, 1978. Vitamin C and immunity: An assessment of the evidence. Clin. Exp. Immunol., 32: 370-379.

Tuekam, T.D., R.D.Miles and G.D. Butler, 1994. Performance and humoral immune response in heat-stressed broilers fed an ascorbic acid supplementation. J. Applied Anim. Res., 6: 121-130.

Walter, E.D. and L.S. Jensen, 1964. Serum glutamic oxaloacetictransaminase levels, muscular dystrophy and certain haematologicalmeasurements in chicks and poults as influenced by Vitamin E, selenium and methionine. Poul. Sci., 43: 919-926.292.