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Full Length Article

Managing Immunocompetence of Broiler Chicken through Vitamin E Supplementation at Low Ambient Temperature

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Abstract

This study evaluated the involvement of high dietary vitamin E (VE) supplementation in frosty environment on cell mediated and humoral immune response of broiler birds. The study was conducted on 200 Hubbard broilers, kept under cold stress (5±2°C) without or with VE supplementation (3 g/Kg feed) and sampling was carried out on 21st, 28th, 35th and 42nd days of age. Peritoneal macrophages were isolated and *in vitro* cytotoxic activity was assessed along with their nitric oxide (NO) production, blood heterophil to the lymphocyte ratio (H:L), mitogenic response to phytohemagglutinin-P (PHA-P), serum IgM and IgG titers and serum corticosterone concentration. The VE supplementation considerably ($P \leq 0.05$) enhanced macrophage engulfment percentage and nitric oxide production with the increase in age. The H:L and phytohemagglutinin-P provoked toe-web thickness was elevated ($P \leq 0.05$) in VE treated group as compared to control. A persistent boost ($P \leq 0.05$) in total antibody titer against SRBC along with IgM and IgG titers was observed in VE fed birds. Serum corticosterone was significantly lesser ($P \leq 0.05$) in VE supplemented birds; however, an abrupt decline in the temperature persuaded a rapid boost in corticosterone concentration. The study validates the utilization of improved VE level in feed to attain enhanced immuno-modulation throughout a cold stress. © 2013 Friends Science Publishers

Keywords: Poultry; Cold stress; Immunity; Vitamin E

Introduction

Stress is known as a state of vulnerable homeostasis and is aggravated by adverse environment or management system. Broiler production under cold temperature is a challenge to poultry farmers as in warm-blooded animals; cold stress (CS) is intended to modify the immune functioning. Broiler feed consumption increases in low temperature (May *et al.,* 1972) because thermoregulation requires an immense amount of energy in cold season, and is the reason behind suppressed immune responses in CS (Dietert and Golemboski, 1998). Macrophages play an important role in the immunity and are implicated in initiating the innate and specific immune response (Sandhu *et al.,* 2007a, b). They are tumoricidal along with the capability of engulfment and production of cytokines (Dietert and Golemboski, 1998; Sandhu *et al.,* 2012).

In chicken and other wild birds, the effect of CS on humoral and cellular immune response is not always the same. A chilled environment of 7°C augment antibody production, whereas decrease antibody responses to SRBC, as found by Regnier *et al*. (1980) but, not in the hens kept at 0°C (Hester *et al.*, 1996a). However, cell-mediated immunity is depressed in chicken attributed to cold exposure (Regnier and Kelley, 1981) without any effect of CS on humoral immunity in chicks (Hangalapura *et al.,* 2004). It is suspected that in stress higher production of serum corticosteroids causes decreased cell proliferation factor, or interleukin II (Siegel and Latimer, 1984). Vitamin E (VE), a lipid soluble substance, is one of the different substances of stress relievers. It is present in biological membranes and works as a chain-breaking antioxidant (Halliwell and Gutteridge, 1989) and protects the tissue from oxidative damage (Gallo-Torres, 1980; Ajakaiye *et al.,* 2011) owing to many types of stress factors.

There are different contradictory reports about CS related cell mediated and humoral immuno-modulation but less is known about the alleviation of this stress. Therefore, the rationale of the current study was to have a look at the immunological effects of cold environmental temperature and its therapy all the way through dietary VE/α-tocopherol supplementation.

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Materials and Methods

Poultry Husbandry and Experimental Design

For this study, 200 day-old Hubbard broilers were procured from an open market and kept at the Poultry Research Institute Rawalpindi, Pakistan. After brooding till 21st day of age, birds were kept at 5±2°C and allocated into two groups (a) Control (Group, I; N=100; without VE; Merck chemical Co, Darmstadt Germany, 64271) (b) With VE (Group, II; N=100). At 21st day of age, both groups were shifted to the climate chambers until the last experimental day and fed experimental feed. The birds were further subdivided into four replicates of 25 birds each according to their body weights. During this period, the birds were fed broiler finisher rations containing crude protein (CP-19) and water *ad libitum* with a 16 h lighting period per day. In this experiment, VE was used at 3 g/Kg feed, containing α-tocopherol acetate 50.0%, which is an excellent source of vitamin E owing to its stability (Dove and Ewan, 1991). On the 21st, 28th, 35th and 42nd days of age, we collected the blood of five randomly selected birds from each replicate, and abdominal cells were harvested after decapitation. All the experimental protocols were followed by the permission of the Institutional Animal Ethics Committee.

Abdominal Exudate Cell Collection

The cell-mediated immunity after CS in broiler chicken was accessed by isolation of abdominal exudate cells (AEC). The AEC was collected in an aforementioned method (Sandhu *et al.,* 2012). Briefly, 3% (w/v) Sephadex© G-50 granules (Sigma Chemical Co., St. Louis, MO) were waterlogged in sterilized, deionized water overnight, re-suspended in 0.85% normal saline and injected intra-abdominal @ 1 mL/100g body weight. After 42 h of injection, the birds were sacrificed by decapitation, dipped in 70% alcohol; abdominal cavities were exposed and washed with 30 mL of chilled normal saline (0.85%) containing 0.5 U/mL of heparin. After placid massaging, approximately 20 mL of cell suspension was acquired into seliconized test tubes, left on ice for 10 min to settle down debris and non-engulfed sephadex granules. The cell suspension was centrifuged at 350xg for 10 min at 4°C. Recovered AEC pallet was washed twice with fresh MEM.

Macrophage Culture

The peritoneal macrophages from individual birds were counted in a hemocytometer and their livability was assessed by trypan blue dye. After mixing the macrophages from each replicate the average viability was 95% and their final concentration at $2.9x10^4$. A 20 µL of this concentration was poured on to round sterilized glass cover slips (18 mm) and given 30 min for macrophage attachment. These cover slips were given a washing with fresh MEM along with antibiotics (100 U/mL penicillin and 50 µg/mL streptomycin) supplemented with 5% heat inactivated fetal calf serum. The cover slips were placed in Petri dishes containing cell culture medium and incubated at 39.6°C in 5% humidified CO_2 incubator for one hour.

Preparation of Hyperimmune Serum

The hyper immune serum against SRBC was formed in layer birds. Briefly, 5% washed SRBC suspension in phosphate buffer saline (PBS) was assorted with an equivalent volume of Alsever's solution (Sigma Chemical Co., St. Louis, MO) and stored at 4°C. On day 1, the SRBC suspension (0.25 mL) was injected intravenously in the wing of five birds (each 3 months old). The amount (0.5 mL) was reiterated 7 days later, and the ultimate dose (0.5 mL) was injected after 15 days of the first inoculation. Seven days afterward blood was collected and serum was estranged aseptically (centrifuged at 400xg for 10 min) and reserved in barren polyethylene tubes at -20°C for further use in SRBC opsonization. Anti-SRBC antibodies were monitored by haemagglutination assay using 2.5% SRBCs, with the highest titer of 1:64 being obtained. For opsonization, one and the same parts of heat-inactivated antiserum (diluted to 1:128) was assorted with 2.5% SRBC and set aside for 2 h at 4°C. Following washing in PBS, the cells were adjusted to a 2.5% suspension in MEM. A 2.5% suspension of unopsonized SRBCs was separately made in MEM for cytotoxic bustle assessment of macrophages.

In vitro Phagocytic Activity

The macrophage suspension was subjected to evaluate their phagocytic activity as given by Sandhu *et al.* (2007b). Briefly, after 30 min of incubation in MEM (at 39°C with 5% CO_2) all the cover slips containing adherent macrophages were gently washed for removal of any non-adherent cells and were shifted to the fresh culture medium. The phagocytic capability of adherent macrophages was dogged by using 3% suspension of unopsonized and opsonized SRBC inoculation into the culture medium for the study of engulfment percentage and engulfment/cell competence of macrophages. After 1h, cover slips were removed from the culture medium, washed for removal of any free SRBC, fixed in methanol for 10 min, and stained with May-Grünwald-Giemsa stain, mounted with DPX on clean glass slides. A number of 200 adherent cells per cover slip were counted in randomly selected fields at 1000X. Macrophage engulfment percentage was calculated as under:

$$\text{Engulfing percentage} = \frac{\text{Number of macrophage engulfing}}{\text{Number of adherent macrophage}} \times 100$$

Nitrite Determination

Nitrite in terms of NO was determined by colorimetric based assay as detailed by Green *et al.* (1982) and Sandhu

et al. (2012). In brief, 1ml of macrophage suspension was loomed discretely with 2.5% SRBC and *Escherichia coli* 0111:B4 lipopolysaccharide (LPS; Sigma Chemical Co., St. Louis, MO) independently in the polyethylene culture tubes. After 6 h interaction, 100 µL of culture supernatant from each tube was mixed with an equivalent quantity of Griess reagent [1% sulphanilamide (Sigma Chemical Co., St. Louis, MO) and 1% naphthylethylene-diamine dihydrochloride (Merck, Chemical Co., Darmstadt, Germany)] and incubated for 15 min at room temperature. The color change was indicative of nitrite presence and was quantified at A_{520} on an ELISA plate reader (BioTEK, ELX-800©). Nitrite concentration was dogged by sodium nitrite (10–100 mM) standard curve.

Heterophil to Lymphocyte Ratio (H:L)

At blood collection time, two thin blood smears were made from each bird for further differential leukocytic count (DLC). After fixing the slides in methanol the smears were stained with May-Grünwald-Giemsa stain. The H:L was ascertained by reckoning about hundred cells based on their morphology.

Immune Response against Phytohemaglutinin

In each group a number of five birds were injected with 100µg PHA-P (Himedia Laboratories, Mumbai, 86, India) in 100µl barren saline solution in the left foot pad. After 24 h of inoculation, the foot web thickness was deliberated by digital vernier caliper (Mitutoyo, Japan) ahead of and subsequent to injection. The pad bump was premeditated as the difference between the width of the foot pad prior to and following the injection.

Serum IgG and IgM Titer

The birds were injected intravenously with 1 mL of 0.25% suspension of washed SRBC in normal saline 14 days prior to blood collection. A booster amount of same dose was injected later than 7 days of prior shot. After 14 days passed to first inoculation, blood was harvested from wing vein of each bird; serum was gathered and stored at -4°C. The whole antibody titer was indomitable by haemagglutination test. The fractions of SRBC antibody (IgM and IgG) were dogged by mercaptoethanol sensitivity microtitration assay as described by Bartlett and Smith (2003). Briefly, 2-mercaptoethanol (2-ME) (Fluka Chemical Co., West St. Paul) was used to arrest the binding affinity of multivalent IgM antibody, while agglutination by IgG remained unaltered. A 25 µL of 0.15M 2-ME was additional to the foremost well of each row in regular antibody titers determination against SRBC. 2-ME was used to conclude the titer of IgG only and the titer of IgM was obtained by subtracting 2-ME resistant titers from total antibody titer against SRBC.

Serum Corticosterone Concentration

The serum corticosterone concentration (ng/mL) in VE supplemented and control broiler birds were measured through ELISA using a kit (DRG Diagnostics, Marburg, Germany, Ref. No. EIA-5186).

Statistical Analysis

The collected data from different categories was subjected to completely randomized design (SAS, 1995). If a difference was found between the means the data was subject to the Duncan Multiple Range Test (DMRT) to find out the significance of the data and the means in consideration (Duncan, 1995).

Results

The CS is unfavorable in broiler chicken concerning to production and respiratory infections. Enhanced meat production can be pulled off during better immune response and resistance from ubiquitous diseases.

***In vitro* Cell-mediated Immunity**

There was a significant boost ($P \leq 0.05$) in macrophage engulfment percentage of unopsonized SRBC throughout CS as compared to 21st days; however, supplementation of VE drastically enhanced the unopsonized SRBC engulfment percentage (Fig. 1A). Along with the increase in age (28th day to 42nd day) there was non-significant macrophage engulfment percentage. When the macrophages were co-incubated with opsonized SRBC, there was a significant increase ($P \leq 0.05$) in macrophage engulfment percentage at 42 days of age. While, VE supplementation significantly strengthened ($P \leq 0.05$) the opsonized SRBC engulfment proportion as compared to positive and negative control (Fig. 1B). Incessant VE dosage gradually increased the level of engulfment until 42nd day of age. The macrophage engulfment/cell hanged about non-significant ($P \geq 0.05$) until 42nd day of age; though, VE action significantly improved the engulfment amount at 42nd day as shown in Fig. 1C. There was a significant augment ($P \leq 0.05$) in macrophage nitric oxide fabrication after LPS co-treatment when we measure up to it with unopsonized SRBC co-incubation and without any type of treatment (alone macrophages). It is palpable from the results that NO production physiologically enhanced with the rise in age (Fig. 1D); nevertheless, LPS treatment increased the induced NO production significantly ($P \leq 0.05$) as compared to SRBC stimulated and without treated macrophages.

The Heterophil to the Lymphocyte (H:L) Ratio

When we put side by side H:L under CS, there was a significant elevation ($P \leq 0.05$) in VE supplemented groups.

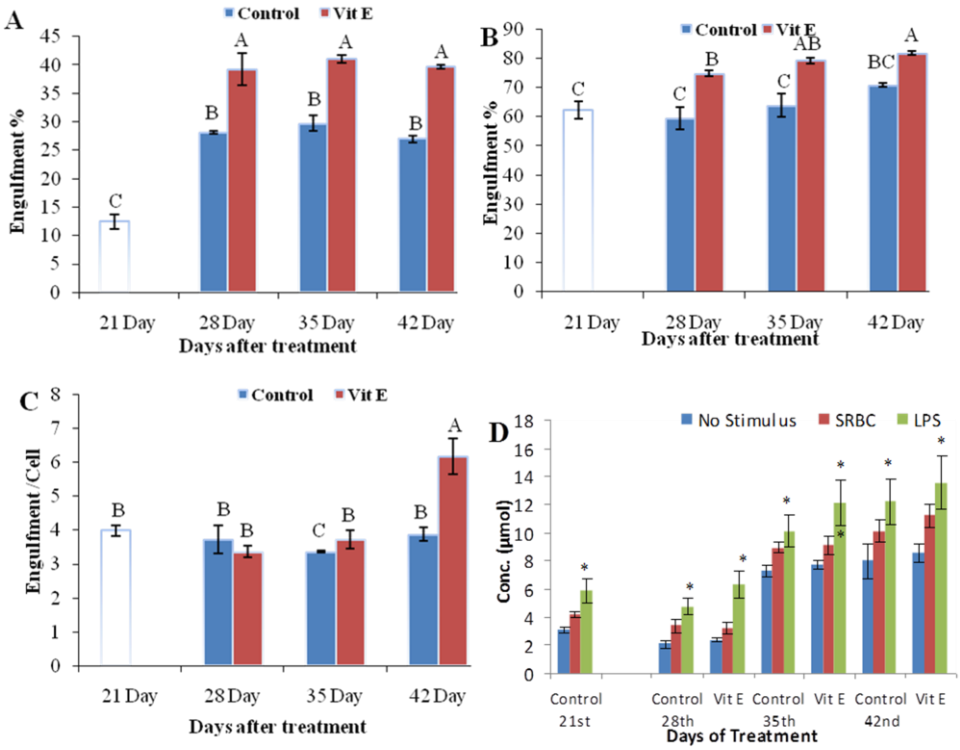

Fig. 1: After 21 days of brooding, birds were kept under cold environmental stress (CS) and were supplemented with vitamin E (VE). The part (A) shows the effect of CS and VE treatment on peritoneal macrophage SRBC (unopsonized) engulfment fraction at stipulated interval. In graph (B) peritoneal macrophage SRBC (opsonized) engulfment percentage throughout CS and VE treatment. In (C) effect of CS on peritoneal macrophage SRBC engulfment / cell after VE supplementation. The (D) shows the outcome of VE treatment in CS and peritoneal macrophage nitric oxide (µmol) creation subsequent to *in vitro* SRBC and LPS action at the fixed hiatus of treatment. A-C; similar alphabets do not differ significantly ($P \leq 0.05$). * indicates the significant difference ($P \leq 0.05$). 21st days indicates negative control, while control at 28th, 35th and 42nd day of age indicates positive control

However, a non-significant dissimilarity was observed between positive and negative control groups, while this decrease was non-significantly more with an increase in age (Fig. 2). It is noticeable the H:L remained enhanced in VE fed group in comparison to others.

Lymphoproliferative Response to PHA-P

Under CS condition, PHA injection induced a non-significant difference in toe web thickness at 21st and 28th days of age irrespective of VE treatment. Additionally, incessant CS statistically decreased the web thickness in positive control birds at 35th and 42nd day of age as compared to negative control and from 28th day or age. With VE treatment, toe web thickness was significantly elevated ($P \leq 0.05$) at 35th and 42nd day of age as compared to positive and negative control groups (Fig. 3).

Antibody Response against SRBC

There was an abrupt decrease ($P \leq 0.05$) in total Ab, IgG and IgM titer production against SRBC at 28th day of age in both VE supplemented and positive control groups as compared to negative control (Fig. 4A, B. C and D). However, the fall out was less in dietary VE given group and remained significantly high ($P \leq 0.05$) at 28th, 35th and 42nd day of age in contrast to the positive control. The domino effect of serum IgG titer uncovered a hasty reduction after the 21st day of age at the initiation of CS. The prototype of IgG antibody titer remained similar at all experimental days viz. 21st, 28th, 35th and 42nd days of age in equally positive and VE enhancement groups. It is remarkable that the maximum prevailing antibody's post seven days of SRBC injection were of IgM type.

Serum Corticosterone Concentration

As it is obvious from the Fig. 5 that CS persuaded an important raise ($P \leq 0.05$) in serum corticosterone concentration in the positive control group all the way through the trial, but there was an abrupt lift up with the onset of CS. The supplementation of VE illustrated a

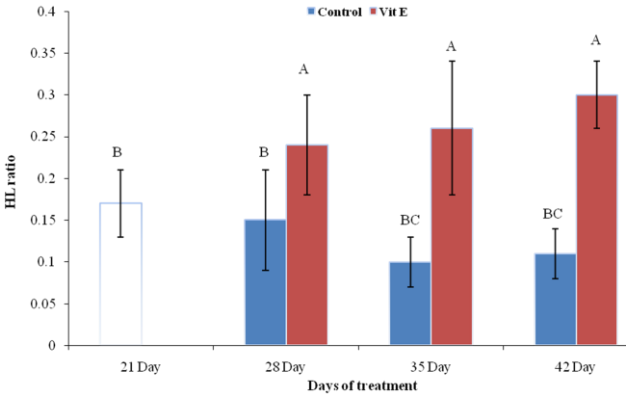

Fig. 2: The result of cold stress and vitamin E supplementation on blood heterophil to the lymphocyte ratio (H:L). A-C; similar alphabets do not differ significantly (P≤0.05). 21st days indicates negative control, while control at 28th, 35th and 42nd day of age indicates positive control

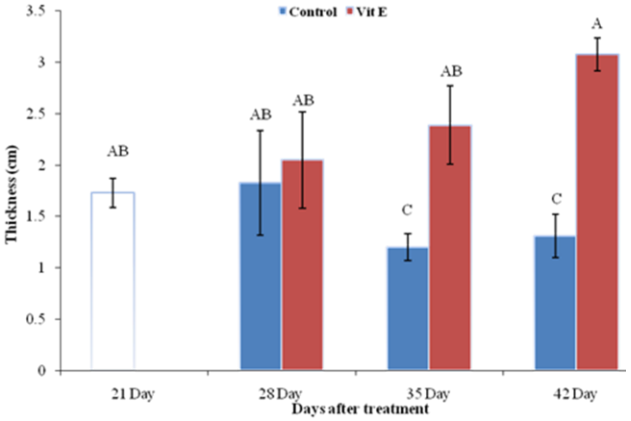

Fig. 3: The end results of vitamin E supplementation in cold stress on tow web thickness following PHA inoculation. A-C; similar alphabets do not differ significantly (P≤0.05). 21st days indicates negative control, while control at 28th, 35th and 42nd day of age indicates positive control

significantly lower (P≤0.05) serum corticosterone concentration that was non-significant with a negative control group. The lowest serum corticosterone was pragmatic at 42nd day of age. An important feature was that, there is a step-wise decrease in serum corticosterone concentration with the increase in age.

Discussion

In the environment, every individual has to tolerate different stresses and one of them is CS. However, these stress conditions have their own response regarding physical or physiological disturbances and go side by side to each other.

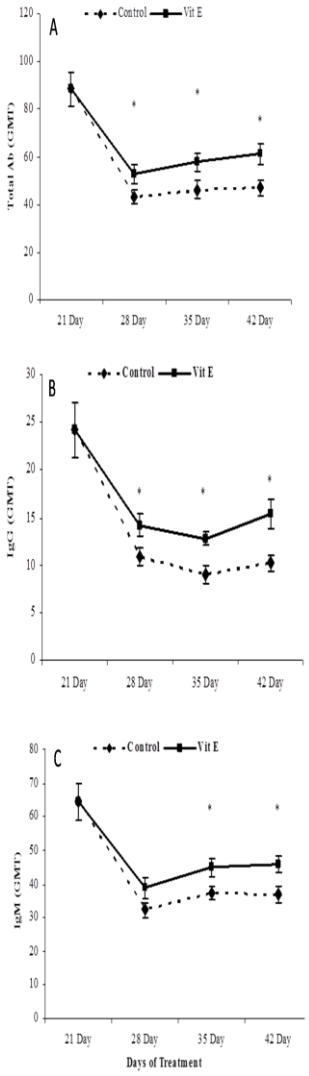

Fig. 4: The sway of dietary vitamin E on cold stress against SRBC and the type of antibody titers. Data are mean ± SD of total antibody titers to SRBC (A), the subtype IgM (B), and IgG antibody titers to SRBC (C). The effect of vitamin E was significant for both isotypes of total Ab. *indicates the significant difference (P≤0.05)

In Pakistan, we have diverse environmental conditions, very hot and very cold conditions. Poultry farming at high altitudes always suffer from very stressful cold environment in winter season. This cold stress has a negative impact on the immunity. One of the foremost antigen presenting cells in the body is macrophages and formulate first line of defense. Chicken do not encompass abdominal macrophages, however; the injection of Sephadex G-50 can elicit the macrophage population (Qureshi *et al.*, 1986).

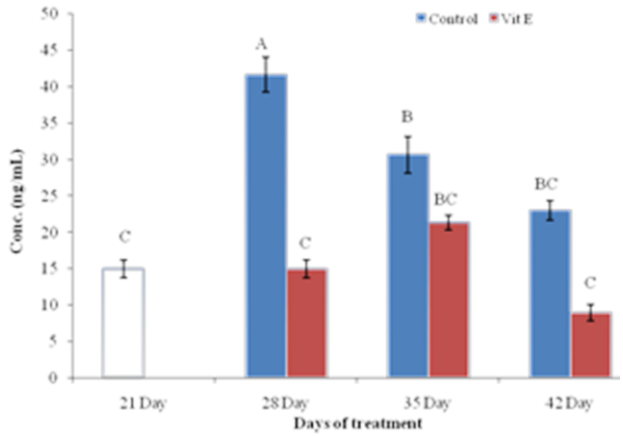

Fig. 5: Serum corticosterone concentration (ng/mL) in cold stressed and vitamin E supplemented broilers. 21st days indicates negative control, while control at 28th, 35th and 42nd day of age indicates positive control. A-C; similar alphabets do not differ significantly (P≤0.05)

These macrophages execute their utility by binding, internalizing and degrading unfamiliar elements (Qureshi and Dietert, 1995). The study indicates that macrophage engulfment reduced in low temperature stress, while there was no effect of acute CS. This may be due to decrease in receptors actively involved in phagocytosis. There are some contradictory reports that activated macrophage are more phagocytic in CS by activation of Fcγ receptor, mannose and β-Glucagon receptors in mice (Baccan *et al.*, 2010). Fc-receptor presence amplifies the phagocytic action along with carnage method of opsonized antigens by macrophages (Yamamoto and Johnston, 1984). Supplementation of VE induced increase in macrophage engulfment percentage and this possibly owing to up-regulation of receptors or caused by less adrenal glucocorticoid's. The engulfment percentage was significant with opsonized SRBC and VE treatment; this might be due to strengthening of FC receptors. These receptors are present on the surface of macrophages and are responsible to engulf the antigen bound with their specific antibodies (Qureshi, 2003) and due to these FC receptors, SRBC form rosette around macrophages. In cattle, blood macrophages function, interleukin-I production and expression of major histocompatibility complex type II expressions are potentiated by VE supplementation (Stabel *et al.*, 1992; Politis *et al.*, 1995). Until 21st days of age, meat type chickens are fully immunocompromised as they fall back on maternal antibodies and innate immunity for resistance alongside the environmental pathogens (Konjufca *et al.*, 2004). An additional role of Fc receptors is to develop the phagocytic and lysosomal hydrolysis mechanisms through reactive oxygen intermediates (ROI) production against engulfed antigens (Yamamoto and Johnston, 1984). Macrophages have the capability to fabricate free radicals for destroying the engulfed material by ROI. It is important that the oxidative pathway is important to format ROI, and NO creations are particularly dissimilar (Nathan and Gabay, 1992); these are associated to antimicrobial actions (Ribeiro-Dias *et al.*, 1999; Nascimento *et al.*, 2002). After SRBC stimulus, the production of macrophage NO was lower than LPS and this may be correlated to less engulfment or FC-receptor non-stimulation. The LPS, an outer membrane component of gram-negative bacteria initiates cellular inflammatory responses (Elder *et al.*, 2000) and is most persuasive activators of NO production (Sandhu *et al.*, 2012). This shows an undeviating association between macrophage engulfment and NO production. There is advanced macrophage NO production following one-hour incubation with LPS rather than lacking stimulation or SRBC stimulation. This may be as a result of enhanced responsiveness of VE; similar results have been narrated by Gore and Qureshi (1997) after *in-ovo* VE injection. It is noteworthy that Type-I INF cannot provoke NO synthesis, nevertheless, chicken INF-δ persuade its synthesis (Digby and Lowenthal, 1995). In live body, micro-organism resistance or vulnerability induces the diversity in macrophage ROI production (Tasat *et al.*, 2003). Our laboratory has previously reported similar results and correlation of LPS with NO production (Sandhu *et al.*, 2007b; Sandhu *et al.*, 2012).

In avian species, immunosuppression is associated with involution of immune organs that roots poorer immunity with amplified morbidity (Lin *et al.*, 2006; Virden *et al.*, 2007; Shini *et al.*, 2008). The H:L changes were not obvious in birds under CS conditions. These results are in disagreement with (Campo *et al.*, 2008) that heterophil number increased with CS. The difference in results may be due to temperature variation and species of birds used. Another imperative justification is the stress-related glucocorticoids' production. High circulating glucocorticoids hold back antibody production, T-cell proliferation and phagocytosis (Engler and Stefanski, 2003). Similar results of increased plasma corticosterone levels under CS were reported by Hester *et al.* (1996a). Nevertheless, Hangalapura *et al.* (2004) stated an oppressive effect of CS (10°C for 7 d) on corticosterone levels. In our results, we have distinguished that this change in H:L is due to a decreased number of lymphocytes (not given). We hypothesize that this decline in the lymphocyte number is due to the higher levels of circulating corticosteroids and nuclear receptors of steroids may have hampered the promoter regions of numerous genes (Almawi and Melemedjian, 2002). This perhaps is attributable to suppression of transcription gene expression and decreased lymphocyte number (Campo *et al.*, 2008). Our hypothesis supported Shini *et al.* (2010) that by exogenous injection of corticosterones drops-off peripheral lymphocyte number and thus total leukocytic count. The decrease in the lymphocyte number can also be correlated to VE insufficiency (Dietert *et al.*, 1983). Treatment with VE induced a significant increase in H:L and can be correlated

with the deposition of vitamin E in the white marrow of birds due to its lipid soluble nature thus increasing the number of lymphocytes.

The response of PHA-P is a wonderful measure of T-cell operation (Qureshi *et al.,* 1997). The significant decrease in toe web thickness with sustained CS indicates clear suppression of immunity. Our study indicates a stress-related decrease in tow web swelling and the swelling gradually augmented with the chronic CS and VE supplementation. The older T-cells have greater lympho-proliferative function, possibly as VE has a positive function on cell-mediated immunity (Bayyari *et al.,* 1997). Our results are in line with (Regnier and Kelley, 1981) that chronic CS impairs the functional expression of PHA *in vivo*.

In humoral immunity of avian species, the key component is the bursa of Fabricius and VE deficiency is involved in spleen, bursal and thymic growth (Marsh *et al.,* 1986). There is a permanent decline in total Ab production against SRBC. Our results show a noteworthy effect of CS on total antibody and its isotypes IgG and IgM production. This may be due to adverse effects of CS on broiler lymphoid organs and corticosterone over production. The suppressed humoral immunity is due to CS rather than genetic character (Hester *et al.,* 1996b). We also observed an increase in antibody production after VE fortified diet provision. Our results are further strengthened by Gore and Qureshi, (1997) that VE mediated antibody response is more pronounced in IgM production than IgG. The change in antibody response may be due to type of antigen. However, our results do not show any significant change in type of antibody production and with increased IgM and IgG titers.

In conclusion, we can state that the use of VE is beneficial during low environmental temperature stress. The highest doses of VE as compared to National Research council (NRC) recommendations are beneficial to boost the immune system in domestic poultry during harsh climate to sustain a better production.

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