EFFECT OF DIETARY ARGININE AND VITAMIN E ON HUMORAL AND CELL MEDIATED IMMUNE RESPONSE IN EXPERIMENTAL T-2 MYCOTOXICOSIS IN BROILER CHICKEN

RAMESH, B. K., NARAYANASWAMY, H. D., SATYANARAYANA, M. L. AND RAO, S.

Department of Veterinary Pathology, Veterinary College, Bengaluru 560 024, India.
E. mail: samuditha2014@gmail.com, Cell: 094801 26363

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Abstract: Day old 144 broiler chicks were randomly divided into 6 groups. Groups I, II, III and IV received 0.0, 0.25, 0.50 and 1ppm of dietary T-2 toxin respectively. Arginine (22 g/kg) and vitamin E (80 mg/kg) were supplemented to Groups V and VI which respectively had 0.0 and 1 ppm of dietary T-2 toxin. The test diets were fed for 0-28 days. On day 28, humoral immunity was measured as an antibody response to B1 strain of Newcastle disease virus (NDV). CMI response was assessed by measuring percent CD4+ and CD8+ cells in peripheral blood and by measuring delayed foot web reaction (DFWR) to kill S. aureus antigen in sensitised birds at different post challenge intervals. A significant (P ≤ 0.05) decrease both in HI titres to NDV and percent CD4+, CD8+ cells was noticed in Group IV whereas the decrease was seen only for HI titres in Group III. A significantly (P ≤ 0.05) high HI titres and percent CD4+ cells was observed in Group V whereas HI titres, percent CD4+, CD8+ cells were similar to control in Group VI birds. The foot web thickness peaked at 24h post challenge. A reduction in DFWR was significant (P ≤ 0.05) in Group IV but was similar in Group VI when compared to control birds. The study revealed that dietary inclusion of T-2 toxin for 0-28 days was immunotoxic to broiler chickens at levels equal and higher than 0.5ppm. Dietary ARG and VE supplementation above the recommended NRC levels was found to be immunostimulatory.

Key words: T-2 toxin, Arginine, Vitamin E, Immune response, Broiler chicken

INTRODUCTION

The commercial broiler chickens depend on compounded feed. The ingredients, chiefly the grains, used in feed formulation come from various sources. The contamination of feed with mycotoxins becomes imminent when practical hurdles faced by farmers during agricultural practices get coupled with favourable climatic conditions for fungal growth. Hence, mycotoxin contamination is still posing a threat to the poultry industry despite the awareness created among poultry farmers on the quality of feed.

T-2 toxin, a trichothecene mycotoxin is produced by several species of genus Fusarium. Adverse effect of T-2 toxin on production performance of poultry has been well established. Further, it is a potent immunotoxicant affecting both humoral and cell mediated arms of immune system and its immunosuppressive effects are the result of direct or indirect inhibition of protein synthesis [1]. The knowledge gained by these findings and the rapid strides made in T-2 toxin detection systems have addressed the issue to such an extent that incidences of overt T-2 toxicosis are waning. However, the prime concern in the present scenario is the harm
T-2 toxin may cause on immune system if it is present in levels which otherwise could cause no overt toxicity symptoms in poultry birds [2].

On the other hand, efforts have been made to combat the problem with the use of certain critical nutrients which would boost the immune status of the birds and this has been referred to as 'Nutritional Immunomodulation' [3]. Arginine (ARG) and vitamin E (VE) are two such nutrients which have been evaluated for their role in improving immune response in birds. ARG is an essential amino acid in chickens, as they do not possess a complete urea cycle and VE is a potent antioxidant compound in biological systems. Their supplementation either individually or in combination has been observed to improve immune function [4-6]. The major mechanisms of nutrient immunomodulation for ARG and VE have been identified [7]. ARG, as a substrate for immune system, regulates T cell development and function, B cell maturation and helps to generate nitric oxide as an effector molecule in activated tissue macrophages. Vitamin E as an antioxidant, protects cells against reactive oxygen species. However, the immunomodulatory properties of these nutrients are achieved when their levels in the diet are included above their requirement for the growth [8].

In light of the above considerations, the present study was taken up to assess the effect of dietary T-2 toxin on immune system at levels which are commonly encountered in the field conditions and that cause no overt toxicity symptoms in broiler birds. The possible immunomodulatory effect, the ARG and VE supplementation could have on T-2 toxin fed birds was also studied.

**MATERIALS AND METHODS**

One hundred and forty four unsexed day old commercial broiler chicks (Cobb) were procured from a reputed hatchery. The chicks were wing banded, weighed and housed in battery brooder with *ad libitum* supply of feed and water. They were randomly divided into six groups of 24 birds each. Groups I, II, III and IV received 0.0, 0.25, 0.50 and 1ppm of dietary T-2 toxin respectively. Arginine (22 g/kg) and vitamin E (80 mg/kg) were supplemented to Groups V and VI which respectively had 0.0 and 1 ppm of dietary T-2 toxin. The test diets were fed for 28 days from the day of hatch. All the chicks were vaccinated against Newcastle disease (ND) using Lentogenic B1 strain (Ventri Biologicals, Bangalore, India) by intraocular route on the seventh day.

**T-2 toxin production:** The T-2 toxin was produced on whole wheat using *Fusarium sporotrichoides* MTCC 1894 [9] and quantified by thin layer chromatography at Animal Feed Analytical and Quality Assurance Laboratory (AFAQAL), Veterinary College and Research Institute, Namakkal, Tamilnadu.

The experimental trials were approved by the Institutional Animal Ethics Committee, India and were conducted under its guidelines. The broiler mash containing no toxin binders and free from mycotoxins was used in the experimental study. Weighed amounts of powdered wheat culture material containing known amounts of T-2 toxin was incorporated in the feed to yield three dietary levels of 0.25 ppm, 0.5 ppm and 1 ppm. L-Arginine (Sigma Aldrich) and VE (Tocopheryl acetate adsorbed on precipitated silicon dioxide from Mercks Pvt. Ltd., Goa) were mixed in the intended feed to have final supplementation rate of 22 g/kg (2.2%) and 80 mg/kg respectively. The test diets were fed for 28 days from the day of hatch. All the chicks were vaccinated against Newcastle disease (ND) using Lentogenic B1 strain (Ventri Biologicals, Bangalore, India) by intraocular route on the seventh day.

**EVALUATION OF IMMUNE SYSTEM**

**Humoral immune response:** Blood samples were collected in non-heparinized tubes from six birds of each group at the end of 28 day experimental trial. Humoral immunity was measured as an antibody response to Newcastle disease virus (NDV). Haemagglutination inhibition (HI) test was performed for determining HI titres to NDV in both the control and treated sera [10].

**CELL-MEDIATED IMMUNE RESPONSE**

**Flow cytometric (FACS) analyses of CD4+ and CD8+ cells in peripheral blood:** For enumeration of CD4+ and CD8+ T-cells, flow cytometric analysis of peripheral lymphocytes was performed on FACS Calibur® instrument from BD Bioscience. The monoclonal antibodies (mice anti-chicken CD4+ and CD8+ conjugated with FITC and RPE, respectively) used in the study were procured from AbD Serotech, USA.

The peripheral blood mononuclear cells (PBMC) were separated by Ficoll density centrifugation...
Equal volume (2.5 ml) of blood samples from each of six chickens of all the groups were collected aseptically from jugular vein into EDTA vacuccatiners at the end of 28 day toxicity trial. Each 2.5 ml of blood sample was diluted with equal volume of PBS-azide-BSA and layered carefully over 1.5 ml of histopaque in screw capped 15 ml plastic centrifuge tubes. The tubes were centrifuged at 200 x g for 30 min at room temperature. The interface layer ring rich in mononuclear cells was collected carefully and washed twice in cold PBS by centrifugation at 4°C for 5 min at 100 x g each time. Washed cells were re-suspended in 2 ml of RPMI-1640 growth media. The cells were counted based on dye exclusion method using trypan blue (0.4% in PBS) as vital stain. Number of lymphocyte cells (1x10^6 cells/ml) was adjusted.

Each PBMC sample was stained with mice anti-chicken CD4\(^+\) and CD8\(^+\) monoclonal antibodies. The cells (1x10^6) were re-suspended in 0.3 ml of PBS in 1.5 ml microcentrifuge tube and 7.5 µl of Mab were added to each tube. After proper mixing (vortexing), the cells were incubated for 1 hour at 37°C. The cells were washed with PBS at 1174 x g for 5 min and re-suspended in 200 µl of PBS for acquisition in 5ml round bottom FACS tubes. All the above mentioned steps involving Mab were carried out in dark. Sample acquisition after the instrument setting was decided by using unstained and stained cells and 5,000 events were analysed for positive staining with FITC and PE. Sample acquisition was carried out at Centre for Cellular and Molecular Platforms (C-CAMP), National Centre for Biological Sciences (NCBS), Bengaluru.

**Delayed type hypersensitivity (DTH) response:** The method described previously [12] in eliciting DTH reaction in broiler chicken using killed *Staphylococcus aureus* antigens was followed in the present study for eliciting delayed foot web reaction (DFWR). Six identified birds in all the groups were sensitized on days 14 and 21 of toxicity trial. For each sensitizing dose, chicks were injected subcutaneously in the neck region with 0.2 ml of killed *S. aureus* (3 x 10^6 organisms per bird) diluted 1:1 with polyethylene glycol. On day 28, each chick was challenged intradermally in the toe web between the 3rd and 4th digits of the right foot with 0.1 ml of 1.5 X 10^8 of *S. aureus* diluted 1:1 with sterile PBS. Corresponding toe web of the left foot was injected with 100 µl of sterile PBS alone.

The thickness of toe web was measured at 0, 6, 24, and 48 h post challenge. Delayed foot web reaction was recorded as the difference between toe web thickness of the *S. aureus* and the PBS injected sites.

**Statistical analysis:** The data generated for different parameters were systematically classified and subjected to one way analysis of variance [13] using SPSS 17 statistical package.

### RESULTS

**Humoral immunity:** Mean (± SE) HI titres (log 2) to NDV in broiler chickens of different treatment groups at the end of 28 days toxicity trial are presented in the Table 1. A significant (P ≤ 0.05) decrease in HI titres was noticed in birds fed with 0.5 ppm and 1 ppm of T-2 toxin when compared to HI titres of control birds. However, HI titres in birds fed with 0.25ppm of T-2 toxin were statistically comparable to control birds.

Supplementation of ARG and VE to the diet containing 1 ppm of T-2 toxin (Group VI) resulted in HI titres which were similar to titres in control birds. However, HI titres in birds fed with 0.5 ppm and 1 ppm of T-2 toxin when compared to HI titres of control birds. However, HI titres in birds fed with 0.25ppm of T-2 toxin were statistically comparable to control birds.

A significantly (P ≤ 0.05) high HI titre to NDV was

### Table 1: Mean (±SE) HI titres to NDV and peripheral blood CD4\(^+\) and CD8\(^+\) cells in broiler chicken in different treatment groups at the end 28 days sub acute trial. Note: Means bearing different superscripts within a column differ significantly (P < 0.05).

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<table>
<thead>
<tr>
<th>Groups</th>
<th>T-2 toxin (µg/g)</th>
<th>ARG (22g/kg)</th>
<th>VE (80 mg/kg)</th>
<th>HI titres (log2)</th>
<th>CD4(^+)</th>
<th>CD8(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>II</td>
<td>0.25</td>
<td></td>
<td></td>
<td>6.83±0.17</td>
<td>11.34±0.34</td>
<td>5.18±0.20</td>
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<tr>
<td>III</td>
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<td></td>
<td></td>
<td>6.30±0.21</td>
<td>10.44±0.54</td>
<td>4.16±0.26</td>
</tr>
<tr>
<td>IV</td>
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<td></td>
<td></td>
<td>5.83±0.31</td>
<td>10.07±0.86</td>
<td>3.78±0.65</td>
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<tr>
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<td>+</td>
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<td>4.0±0.25</td>
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</tr>
<tr>
<td>VI</td>
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<td>+</td>
<td>+</td>
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<td>14.14±1.42</td>
<td>6.10±0.60</td>
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Fig. 7: Mean (±SE) foot web thickness (mm) at 24h post challenge. Means bearing different superscripts differ significantly (P < 0.05)
seen in ARG and VE supplemented group (V) when compared to control birds.

**Cell mediated immunity - CD4+ Cells:** Results obtained from flow cytometric cell segregation showed that percent CD4+ cells (Table 1 and Figures 1-6) were significantly (P ≤ 0.05) lowered in birds fed with 1 ppm of T-2 toxin (Group IV) when compared to control birds (Group I). However, 0.25 and 0.5 ppm of T-2 toxin in the diet did not significantly alter CD4+ cells when compared to control, although a numerical decrease was noticed at both these levels.

The birds of Group V (ARG+VE) recorded significantly (P ≤ 0.05) higher percent of CD4+ cells than the values observed in control birds. Birds in Group VI (T2+ARG+VE) showed significantly (P ≤ 0.05) higher CD4+ cells compared to birds fed 1 ppm of T-2 toxin (Group IV) but the values were comparable to the values in control group.

**CD8+ cells:** The percent CD8+ cells (Table 1 and Figures 1-6) observed in Group I (control) and Group V (ARG+VE) birds were statistically comparable but were significantly (P ≤ 0.05) higher compared to percent CD8+ cells in birds that consumed 1 ppm of T-2 toxin (Group IV). Numerically lower CD8+ values observed in birds fed with 0.25 and 0.5 ppm of T-2 toxin did not differ significantly (P > 0.05) with the values of control birds.

However, CD8+ cells in Group VI (T2+ARG+VE) were statistically lower (P ≤ 0.05) compared to the values in Group V (ARG+VE) but were similar (P > 0.05) to that of Group I (Control).

**Delayed foot web reaction (DFWR):** An increase in mean (± SE) foot web thickness was noticed in birds of all the groups at 6 h post challenge and reached peak by 24 h. At 24 h post challenge (Fig. 7), Group IV birds fed with 1 ppm of dietary T-2 toxin recorded a significantly (P ≤ 0.05) lower foot web thickness compared to the thickness in birds of Groups I and V. The trend remained same even at 48 h post challenge. However, the Group VI birds (T2+ARG+VE) showed foot web thickness which did not differ significantly (P > 0.05) with the values recorded in control birds at all the post challenge intervals.

HI titres to NDV were significantly (P = 0.05) higher in ARG and VE supplemented Group V compared to control (Group I). This enhanced humoral response due to ARG and VE supplementation either individually or in combination was evaluated earlier by several researchers. VE supplementation at levels higher than NRC recommendation was reported earlier to have enhanced humoral immune response in chicken to non-proliferating antigens namely killed IBV and SRBC [8] and to NDV [18]. The immunomodulatory VE levels corresponded to the levels necessary for the inhibition of lipid peroxidation against oxidative stress [19]. This effect of VE on fatty acid stability was in itself immune regulatory as this maintained proper membrane fluidity and cellular communication [20].

The effect of dietary supplement of ARG (2 to 2.5 times the NRC recommendation) on protective humoral immune responses against IBDV and HPSV in broiler chicken were respectively evaluated [21,22] and these levels of ARG supplementation were found safe and free from any side effects. Dietary ARG supplementation at 2.2% was found to increase the proportion of B-cells in peripheral blood 9 days after vaccination with IBDV when compared to B-cell proportion at 1.2% of ARG supplementation [23]. ARG role in immunomodulation was evaluated considering its essentiality as a substrate for immune system [24]. The functional aspects of T-cells, B-cells and monocytes are reliant on ARG utilization. B-cells are released from bursa in growing chicks [25] and ARG deficiency arrests B-cells at the pro to pre-B cell transition [26]. Thus, ARG plays a key role in regulating the percentage of B cells in peripheral
blood by affecting B-cell maturation.

As the effects of ARG and VE have been individually evaluated for their role in improving humoral response, there are few studies to assess their role on immune status of birds when supplemented together in different combination. A significantly higher antibody titre to sheep red blood cells (SRBC) was recorded when a high level of ARG (1.5%) was supplemented with VE at 80 IU/kg of feed [27]. A combined effect of dietary ARG at 22 g/kg and VE at 80 mg/kg was found to improve the humoral immune response of broilers to IBDV and SRBC [28].

Statistically similar HI titres to NDV observed in Group VI (T2+ARG+VE) birds compared to Group I (control) could be due to protection extended by VE against T-2 toxin induced lipid peroxidation coupled with abundance of ARG which was available for proper B cell maturation in bursa.

The flow cytometric fluorescent activated cell sorting revealed significantly lowered per cent of CD4+ and CD8+ cells in peripheral blood of birds fed with 1ppm of T-2 toxin when compared to control. A significant reduction in per cent thymic and splenic CD4+ and CD8+ lymphocytes was earlier reported in broiler chicken fed 1 ppm of T-2 toxin for 0-28 days [15]. Similarly, a significant reduction in peripheral blood CD4+ and CD8+ ratio in broiler chicken fed T-2 toxin (0.5 and 1ppm) in combination with ochratoxin (0.25 and 0.5 ppm) [29] and lowering of peripheral blood CD4+ and CD8+ cells in broiler chicken fed 2 ppm of T-2 toxin for 28 days [17] were reported earlier. The result of the present study also showed lower CD4+ and CD8+ cells in peripheral blood and adequately supported these earlier findings. The immunosuppressive potential of T-2 toxin due to its cytotoxic effect on lymphoid cells might be responsible for decreased lymphocyte population. A pronounced lymphoid necrosis and depletion observed microscopically in thymus and spleen in the present study substantiated these findings.

A significant increase in per cent CD4+ cells and a non-significant increase in CD8+ cells were observed in ARG and VE supplemented Group V. Similar observations of increased T-lymphocyte population have earlier been reported when ARG and VE supplementation was made to broiler birds either individually or in combination [4,6].

Furthermore, higher relative thymic weights recorded in the present study with distinctly larger thymic lobes and uniform sheet of thickly populated lymphoid cells microscopically substantiated the increase in values of CD4+ and CD8+ cells in peripheral blood of ARG and VE supplemented birds.

A non-significant increase in CD4+ and CD8+ values were observed in Group VI (T2+ARG+VE) in comparison to control birds. The antioxidant property of VE against free radical damage might have helped in maintaining cellular integrity, which is key to receive and respond to the messages needed to co-ordinate the immune response [20]. High levels of VE, 10 times greater than the required level have been found to be immunostimulatory [30]. Thus, 80 mg/kg of VE used in the present study in combination with 2.2% ARG which helps in T-cell development and function could have protected the birds against immunotoxic effect of T-2 toxin.

Birds fed with 1 ppm of T-2 toxin (Group IV) recorded a significantly (P ≤ 0.05) low mean foot web thickness compared to control birds at 24h and 48h post challenge. This could be attributed to cytotoxic and immunosuppressive effect of T-2 toxin. A similar reduction in DTH response was recorded previously [16] in T-2 toxin treated birds.

The present study indicated enhanced DTH response in Group V. Group VI (T2+ARG+VE) birds recorded foot web thickness which was comparable to thickness recorded in control. A beneficial effect of ARG and VE combination against the immunosuppressive effect of T-2 toxin was thus observed. ARG (2%) supplementation enhancing cutaneous basophil hypersensitivity (CBH) response, a CMI response to phytohemagglutinin A (PHA) response, a CMRI response to phytohemagglutinin A (PHA) in birds was previously [33] observed. However, earlier results with respect to effect of VE on CBH response to PHA were not consistent. A study [8] with different dietary supplements of VE (0, 10, 17.5, 25, 37.5, 50, 100 and 200 IU/kg) indicated that levels of VE did not influence CBH response. Whereas another [31] observed a reduction in CBH response at higher dietary VE level (300 mg/kg) compared to NRC recommended VE level of 10 mg/
kg. While, a considerable protection against in vitro T-2 toxin inhibition of lymphocyte proliferation in response to mitogens was shown by water soluble form of VE [32].

Thus, the study revealed immunotoxic nature of T-2 toxin in broiler chickens that received dietary levels of 0.5 ppm and above for 0-28 days and affecting both the humoral and cell mediated immune functions. The supplementation of ARG and VE to birds at levels higher than NRC recommendation was immunostimulatory and protected birds against T-2 toxin damage.

REFERENCES