**Research Note**

**Effect of Three Immunostimulants on Some of Indicators of Broilers’ Immune Response**

Seyed N. Khaleghi Miran¹, Mohammad A. Karimi Torshizi¹, Mohammad R. Bassami² and Hamid Jandaghi³

¹ Department of Poultry Science, Faculty of Agriculture, Tarbiat Modares University, Tehran, Islamic Republic of Iran
² Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Islamic Republic of Iran
³ Faculty of Veterinary Medicine, Comprehensive University of Applied and Practical Sciences, Khorasan Razavi, Mashhad, Islamic Republic of Iran

This study was conducted to determine the effect of *Echinacea purpurea*, levamisole and vitamin E on humoral and cell-mediated immunity of broilers. Total of 200 one-day-old male broiler chickens (Ross 308), were randomly distributed to experimental groups including control, vitamin E (150 mg/kg diet), 0.1% aqueous *Echinacea*, levamisole (15 mg/kg BW). Vaccination against Newcastle disease (ND) and avian influenza (AI; H9N2) were performed. The hemagglutination inhibition (HI) titers against both vaccines were determined on serum samples at days 21 and 42. Chicks were injected into breast muscle with sheep red blood cells (SRBC) at 14 and 35 days. Blood samples were drawn at 7 days after the first and the second injections. The antibody levels against SRBC were measured by hemagglutination test. The cell-mediated immunity was determined via phytohemagglutinin (PHA) and dinitrochlorobenzene (DNCB). Antibody titers against AI, ND, total anti-SRBC at 21 day and IgM at 21 and 42 days of age were not affected by the treatments (*P > 0.05*). Although cell-mediated immune responses by DNCB (31 day) and PHA (42 day), total anti-SRBC antibody, IgG and IgG/IgM ratio (42 day) (*P < 0.01*), IgG and IgG/IgM ratio (21 day) (*P < 0.05*), were affected by some of the used treatments. In conclusion, taking into account the public urge in withdrawal of chemicals, interest in nutraceuticals in animal production and observed results, use of *Echinacea* extract and vitamin E as immunomodulator feed adjuncts needs further study.

**Key words:** broiler, *Echinacea*, immune response, levamisole, vitamin E


**Introduction**

The use of immunomodulators in poultry production shows an increasing trend in the last few years. Since the present day poultry is subjected to a variety of stress associated immunosuppression, the use of immunomodulators would be one of the important practices in poultry industry. Numerous compounds such as vitamin E, levamisole and *Echinacea purpurea* are frequently used as immunomodulators. Levamisole originally is used as antihelminthic drug; however it is a promising agent for use in the immunotherapy of patients with deficient host defense mechanisms. Both in anergic patients and in experimental animals levamisole has been shown to stimulate cell mediated immunity probably through the enhanced maturation of cells (Amery, 1978; Soppi et al., 1979) or through the enhanced function of macrophages (Kelly, 1978). The results concerning the effect of levamisole on humoral immunity are controversial. In animal and human studies levamisole had generally little or no effect on existing serum immunoglobulin levels or on specific antibody production (Symoens and Rosenthal, 1977). In anergic patients, levamisole has been shown to increase the number of circulating B-cells and to enhance the antibody response to typhoid, but not to influenza vaccine or to diphtheria toxoid (Delespesse et al., 1977). Renoux et al. (1976) showed that the activation of murine T cells by levamisole was accompanied by a switch of anti-sheep erythrocyte antibodies from IgM to IgG. However, Lord and Stites (1977) demonstrated a failure of levamisole to increase delayed hypersensitivity or antibodies to SRBC in normal or T cell deprived mice. In the chicken, levamisole has been shown to increase the antibody response to Newcastle disease virus vaccine (Kulkarni et al., 1973). Vitamin E (VE) is the most widely known natural antioxidant, and alpha-tocopherol is the most active biological form of it for physiological functions, despite its limited...
efficiency as anti-oxidant. The delta and gamma forms are better anti-oxidants, but are less efficient to support animal growth and performance (Rutz and Lima, 1994). The National Research Council (NRC, 1994) identified the requirement for vitamin E as 10 IU/kg diet. Moderately higher levels of vitamin E (25–50 IU/kg) enhance antibody titres following a vaccination but still higher levels (> 150 IU/kg) are suppressive. According to Klasing (1998), in addition of being the first line of body defenses against the action of free radicals, protecting the host cells, VE modulates the immune response. It also decreases the synthesis of prostaglandins, leukotrienes, and cytokines, which regulate the inflammatory response, thereby reducing damage caused to the tissues by the inflammatory process.

Echinacea purpurea is a well known medical herb. It is widely used around the world to treat common cold and other infectious disorders with the claim to have immunity after DNCB treatment was measured using a digital caliper (Mitutoyo, Japan). Treatment by DNCB solution (mg/m²) was applied on the right side of the two birds per pen. Similar position on the left side of the bird treated by the solvent alone (acetone: olive oil, 4:1 v/v) to correct the solvent effect. The second treatment by DNCB solution (1 mg/ml) was applied on the 42 day. The skin swelling was calculated as the difference between the thickness of the skin before and after DNCB treatment was measured using a digital caliper (Mitutoyo, Japan).

Mitogen Injection
Response induced in vivo by mitogen was evaluated by injection of phytohemagglutinin (PHA) into the toe webs between the second and third digits. At 6 weeks of age, two chicks from each pen (the same birds challenged previously with DNCB) were intradermally injected in the toe web of the left foot with 0.1 ml phytohemagglutinin M-form (100 μg dissolved in 100 μl of sterile phosphate-buffered saline (PBS), Gibco, USA). Sterile PBS (0.1 ml) was injected to the right toe web as a control. At 24 h post PHA injection, the toe web swelling was calculated as the difference between the thickness of the toe web before and after injection was measured by a digital caliper (Mitutoyo, Japan).

Humoral Immune Response
Vaccination against Newcastle disease (ND) and Avian influenza H9N2 were performed on day 7 using eye drop (Live B1 strain, Vetrina, Croatia) and subcutaneous injection (Newcastle and Avian influenza killed vaccines, Pasouk, Iran) to all chicks. As a booster, the second live Newcastle disease vaccine (La Sota strain) was performed via drinking water at day 23. The immune response was assessed by hemagglutination inhibition (HI) test. The HI titers against both vaccines were determined on serum samples of the same birds at days 21 and 42.

The sheep red blood cells (SRBC) were collected and washed 3 times in PBS. The packed cells were brought to a 5% v/v solution in sterile PBS. Chicks were injected into breast muscle with SRBC (5% suspension in PBS, 0.1 ml/chick) followed by a booster injection at 14, 35 days respectively. Blood samples were drawn at 7 days after the first and second injections of the same birds previously used for NDV and AI antibody titer determination. Plasma was stored at −20°C until tested. The antibody levels against SRBC were measured by hemagglutination test. Plasma was heat inactivated at 56°C for 30 min and then analyzed for total, mercaptoethanol-sensitive (Presumably IgM) and mercaptoethanol-resistant (MER-
IgG) anti-SRBC antibodies as previously described (Qureshi and Havenstein, 1994). Briefly, 25μl of plasma was added to an equal amount of PBS in the first column of a 96 wells U-shaped bottom microplate and serial dilution was then made and 25μl of 1% SRBC suspension was added to each well. Total antibody titers were then read after 120 min of incubation at 37°C. The well immediately preceding a well with a distinct SRBC button was considered as the endpoint titer for agglutination. For MER-IgG response, 25μl of 0.02 M mercaptoethanol in PBS was used instead of PBS alone, followed by the previous mentioned procedure. The difference between the total and IgG response was considered to be equal to the IgM antibody level.

**Statistical Analysis**

The data were analyzed by GLM procedure for completely randomized experimental design with 4 treatments and 5 replicates with using the SAS software (SAS Institute, 1990), and the means were compared by LSD test ($P < 0.05$).

**Results**

There was not significant difference ($P > 0.05$) between experimental groups in antibody production against AI and ND vaccines (Table 1). The highest concentration was related to Echinacea (21 day) and levamisole (42 day) for ND. The levamisole, numerically produced the highest antibody titers for AI (21, 42 days). There was significant difference for total anti-SRBC antibody (on 42 day), IgG and IgG/IgM ratio at 21 and 42 day of age ($P < 0.05, P < 0.01$ respectively) (Table 2). All three dietary treatments increased the toe web response to PHA in comparison to control. The highest increase in skin thickness was observed in levamisole group ($P < 0.01$). There was a significant lower increase in skin thickness of Echinacea drank birds compared to the rest of the experimental groups for DNCB at 31 day ($P < 0.01$), while DNCB at 42 day was not affected by the treatments ($P > 0.05$) (Table 3).

**Discussion**

**Cell-mediated Immunity**

It has been reported that levamisole enhances macrophage and T-lymphocyte function and reduces suppressor T-cell function (Hersey and Werkmeister, 1981). Because antibody formation to most infectious agents is T-lymphocyte dependent, the augmentation of the helper functions of these cells could enhance antibody production (Babiuk and Misra, 1981). In the present study levamisole enhanced cellular immune responses in chickens. It has been shown that levamisole increase the lymphocyte responses to PHA mitogen, and the protein synthesis of T-lymphocytes suggesting selective activation of T cells (Whitcomp, 1977; Soppi et al., 1978). Our in vivo observations confirm and extend these previous findings (Lichtenfeld et al., 1976). The present findings suggested that levamisole may affect different populations of lymphocytes because PHA and Con A have been shown to stimulate at least partially different lymphoid cell populations (Jacobsson and Blomgren, 1974). The long-lasting stimulatory effect suggests that levamisole may affect the ratio of lymphocyte populations in the blood. The direct mobilization of large amounts of lymphocytes from thymus or bursa seems improbable, since levamisole had no effect on the weights of these organs. On the other hand, levamisole is proposed to affect the migration of lymphocytes (Amery, 1978). Alkamids in Echinacea are discussed by Woelkart and Bauer (2007) to be considered as a class of cannabinomimetics as possible mode of action

### Table 1. Comparison between antibody titrations against ND and H9N2 vaccine viruses by HI method on 21 and 42 days age of broilers (N=10)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Newcastle (log2 HI)</th>
<th>Avian influenza (log2 HI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21 day</td>
<td>42 day</td>
</tr>
<tr>
<td>Control</td>
<td>3.00</td>
<td>4.60</td>
</tr>
<tr>
<td>Echinacea</td>
<td>3.60</td>
<td>5.00</td>
</tr>
<tr>
<td>Levamisole</td>
<td>3.20</td>
<td>5.20</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>3.00</td>
<td>5.00</td>
</tr>
<tr>
<td>SEM</td>
<td>0.15</td>
<td>0.18</td>
</tr>
<tr>
<td>P-value</td>
<td>0.46</td>
<td>0.73</td>
</tr>
</tbody>
</table>

### Table 2. Total anti-SRBC antibody, IgG, IgM titers (log2 HA) and IgG/IgM ratio by HA method on 21 and 42 days age of broilers (N=10)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Total anti-SRBC</th>
<th>IgG</th>
<th>IgM</th>
<th>IgG/IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21 day</td>
<td>42 day</td>
<td>21 day</td>
<td>42 day</td>
</tr>
<tr>
<td>Control</td>
<td>3.92</td>
<td>4.34</td>
<td>2.65</td>
<td>3.25</td>
</tr>
<tr>
<td>Echinacea</td>
<td>4.40</td>
<td>4.50</td>
<td>2.20</td>
<td>3.20</td>
</tr>
<tr>
<td>Levamisole</td>
<td>3.80</td>
<td>5.76</td>
<td>2.82</td>
<td>4.76</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>3.00</td>
<td>4.48</td>
<td>1.80</td>
<td>3.52</td>
</tr>
<tr>
<td>SEM</td>
<td>0.153</td>
<td>0.157</td>
<td>0.130</td>
<td>0.162</td>
</tr>
<tr>
<td>P-value</td>
<td>0.1089</td>
<td>0.0002</td>
<td>0.0112</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

*bc* Values with the different superscripts are statistically different.
Table 3. **Effect of experimental groups on relative lymphoid organ weights and cell-mediated immunity by response of skin to DNCB and toe web swelling by PHA (N=10)**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Increase in skin thickness (%) to PHA and DNCB</th>
<th>Relative lymphoid organ weights (g/100g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA - DNCB (31 day)</td>
<td>DNCB (42 day)</td>
</tr>
<tr>
<td>Control</td>
<td>0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.95&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Echinacea</td>
<td>0.57&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Levamisole</td>
<td>0.69&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.86&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM</td>
<td>0.017</td>
<td>0.053</td>
</tr>
<tr>
<td>P-value</td>
<td>0.0001</td>
<td>0.010</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup>Values with the different superscripts are statistically different.

to modulate immune functions. This is in agreement with Cundell et al. (2003) who found an increase of lymphocytes after one week in rats fed with dried *Echinacea* preparations. Probably the ethanolic extract used in our study has a higher concentration of alkamids and different ratio of components causing this effect.

The studied VE level presented similar behavior as to cell immune response cutaneous basophilic hypersensitivity (CBH). Corrier and DeLoach (1990) evaluated the effect of different PHA-P doses on cutaneous hypersensitivity of broilers in the starter period (up to 14 days of age), and observed the presence of T-helper cells already in 3 day old birds which skin thickness was first observed 6 hours post-inoculation reached its maximum level after 12 hours, and decreased 24 hours post-inoculation. Conversely Leshchinsky and Klasing (2001) did not find any effect of VE supplementation on CBH of broilers.

**Humoral Immune Response**

The effect of treatments on antibody titers against SRBC was more eminent compared to ND and/or AI. Although the exact reason of observed results is unclear, it seems that the short term life of broiler chicks may encourage the interfering influences of ill-uniform presence of maternal antibodies, along with another possible causes like as uncontrolled natural exposure to field viruses, uneven administration of vaccine or its inactivation.

It has been generally agreed that levamisole has no effect on the humoral immune response (Symoens and Rosenthal, 1977), although some challenging reports have been presented (Lods et al., 1975). The lack of uniform results is apparently due to the differences in the doses and administration schedules of levamisole (Neveu, 1978). One reason for the contradictory results may also be the differences in the methods used for the estimation of the humoral immune response. In this study levamisole increased both the total anti-SRBC and IgG antibody responses at 42 day. However, based on the present data no conclusion can be drawn about the possible effect of levamisole on the switch from IgM to IgG (Renoux et al., 1976). Regarding antibody titers effect of the *Echinacea* supplementation was not evident. Zhai et al. (2007) also found an increased antibody response in mice administered with alcoholic extracts of *Echinacea*. So far, there are no studies available on the possible mode of action of activating the specific immune systems by plant components. Likely presence of pathogen associated molecular patterns in plant components, which are recognized by toll-like receptors and other pattern recognition receptors, can contribute to affect the specific immune system. The potential of *Echinacea* to stimulate adaptive immune functions can be helpful in practical animal husbandry to enhance the effects of vaccinations. Alkamids in *Echinacea* preparations seemed to have additional mechanisms such as anti-inflammatory and anti-viral effects by reducing NO, TNF-α, various interleukins, and other parameters (Woelkart and Bauer, 2007), however the level of alkamids has not determined in used extract.

Gore and Qureshi (1997) injected VE in Dl-α-tocopherol vitamer in turkey embryos at 18 days of incubation, and observed that birds receiving 10IU VE/kg presented higher anti-SRBC titers as compared with control (4.2 vs 3.0). This suggests that VE may have an immunomodulator effect, increasing the resistance to diseases. The results of the present experiment were similar to those observed by Boa-Ampsonem et al. (2000) who did not find significant effect of vitamin E (levels of 10 and 300 IU/kg) on average anti-SRBC titers. On the other hand, Leshchinsky and Klasing (2001) found an increase in the antibody titers of broilers supplemented with 50IU VE/kg and concluded that moderate VE levels (25 to 50IU/kg) promoted better immunomodulation than high VE levels (100 to 200 IU/kg) which correspond to the VE levels needed for the inhibition of lipid peroxidation, and for the protection of liver mitochondria against oxidative stress.

In conclusion, all immunomodulators used in present study have shown to some degree enhancement of antibody production against viral vaccines and cell mediated immunity response to PHA. Finally taking into account the public urge in withdrawn of chemicals, interest in nutraceuticals in animal production and observed results,
use of *Echinacea* extract and vitamin E needs further studies before commercially application as immunomodulator feed adjuncts.

**References**


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