Effects of ascorbic acid on cell mediated, humoral immune response and pathophysiology of white blood cell in broilers under heat stress

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Abstract
Aengwanich,W., Sridama, P., Phasuk, Y., Vongpralab, T., Pakdee, P., Katawatin, S. and Simaraks, S.
Effects of ascorbic acid on cell mediated, humoral immune response and pathophysiology of white blood cell in broilers under heat stress

The purpose of this study was to conduct an experiment related to the effects of chronic heat stress on total white blood cell changes, pathophysiology of leukocyte and effects of ascorbic acid on lymphocytes, lympholytic cells and humoral immunity of New-castle disease of broilers under chronic heat stress. Randomized complete block was the design. One hundred-forty-four chickens were maintained at 33±1 °C environmental temperature and on four levels of added ascorbic acid i.e. 0 (control group), 200, 400 and 800 mg/kg in diets for 21 days. On days 1, 3, 7, 14 and 21 of the experimental period, total white blood cells count, lympholytic
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Cell and HI titer for Newcastle disease were determined. On day 21, histopathology of lung, liver, kidney, heart and bursa of fabricius of randomly selected broilers (n=36; 3 birds per experimental unit) were studied. Total white blood cells (TWBC) of the birds were significantly increased on day 3 (P<0.05) and were highest on days 7 and 14 then significantly decreased on days 21 (P<0.05). Monocytes were significantly increased on day 3 (P<0.05). Lymphocytes were significantly increased on day 7 and were highest on day 14 (P<0.05). On day 21, the value of lymphocyte was significantly lower than on days 7 and 14 (P<0.05), respectively. Lympholytic cells were significantly increased on day 3 (P<0.05). Heterophils were significantly increased on day 3 (P<0.05). Tissue injury and hemorrhage in broilers under chronic heat stress caused leukocytosis, heterophilia, lympholysis and monocytosis. The size of lobules within the bursa of fabricius in broilers receiving ascorbic acid at 800 mg/kg in the diet were larger than in birds that received added ascorbic acid at 400, 200 and 0 mg/kg in their diets, respectively. Lymphocytes and lympholytic cells were not significantly different among the ascorbic acid treatment groups. Besides, HI titers of Newcastle disease at 800 mg/kg in the diet were significantly higher than the others (P<0.05). Apparently, adding ascorbic acid at 800 mg/kg in the diet could improve humoral immunity in broilers under heat stress.

Key words: ascorbic acid, immune response, white blood cell, broilers, heat stress
When broilers were exposed to acute heat stress, the percentage of monocyte and lymphocyte decreased while the percentage of the heterophil increased (Altan et al., 2000). Moreover, Borges et al. (1999) reported that heat stress increased the percentage of heterophils and decreased the percentage of lymphocytes. Total white blood cell of broilers under stress increased (Puvadolpirod and Thaxton, 2000). Corticosteroid administration results in a lymphopenia and an increase in circulating heterophils in chicken. Therefore, it appears that chickens have a “stress leukogram” similar to that of mammals (Harmon, 1998). Jain (1993) reported that corticosteroid caused lympholysis in blood and lymphoid tissue, increased shift of lymphocytes from blood to other body compartments, or both in mammals. Moreover, T cell in blood and tissues are most sensitive to the lympholytic effect. After broilers were exposed to high ambient temperature, their body temperature increased more than the normal body temperature (Reddy, 2000), corticosterone stored in adrenal cortex was released into the blood circulation to help broilers increase metabolism (Richard, 1998). This hormone might cause cell mediated and humoral immunity failure because changes in the plasma concentrations of corticosteroides and ACTH affected the lymphoid tissues; for example, a diminution in the mass of the spleen, thymus and bursa of fabricius (Daghir, 1995).

Ascorbic acid has been widely used to reduce the stress in chickens, because this vitamin could decrease corticosterone level in the blood circulation (Nockel et al., 1973; Sheila and Cheryl, 1978). However, information concerning the effects of ascorbic acid on cell mediated, humoral immune response and pathophysiology of white blood cell in broilers under heat stress has not been reported. Therefore, the purpose of this study was to conduct an experiment related to the effects of ascorbic acid on lymphocyte, lympholytic cell, humoral immunity, white blood cell changes and pathophysiology of leukocyte in broilers under heat stress.

**Materials and Methods**

One hundred and forty four, symptomatically disease - free day - old broiler chicks were obtained from a commercial hatchery. They were incubated for 21 days before being placed in layer cages. Live Newcastle disease virus (Lasota strain) was administrated by ocularnasal instillation of 0.1 ml. on day 14 of age. Experiments began after 7 days adaptation period in the cages at 26-28 °C environmental temperatures. The chicks were fed on standard broiler starter (commercial feed) with continuous light and water supply.

The experiment was designed as a Randomized complete block design (RCBD) with four treatments i.e. supplementation of diets with ascorbic acid at 0, 200, 400 and 800 mg/kg. On day 1 of the experimental period (28 days of age), broilers were transferred into an environmentally controlled housing and kept in wire - floored layer
cages. All broilers were subjected to 5-hour episode of heat stress at 33±1 °C each day. Relative humidity was 60-70%. The total mixed diet (Table 1) with the four levels of ascorbic acid was fed ad libitum. On day 1, 3, 7, 14 and 21 of the experimental period, blood samples (via wing vein: 3.75 ml.) from randomly selected five birds per the experimental unit were collected and transferred to vial tubes containing EDTA as anticoagulant and without anticoagulant for white blood cell parameters and NDV-HI titers determination, respectively. Air-dried blood films were stained with Giemsa-Wright’s stain. Differential WBC counts and lympholytic cell were performed by using standard avian guidelines introduced by Ritchie, et al. (1994). Total white blood cells were determined by the Unopett method (Campbell, 1995). NDV-HI titers were investigated using the method described by Wongwatcharadumrong (1990).

All data were analysed by using repeated measurement of the ANOVA procedure of Statistical Analysis System (SAS, 1990). Influence of time on parameter changes when broilers were maintained under prolonged heat stress and treatments were considered. Means were separated by Duncan’s multiple range tests (Duncan, 1955). The level of significance was determined at P < 0.05.

On day 21 of the experimental period (49 days of age), three randomly selected broilers per experimental unit were killed by cervical dislocation. Lung, liver, kidney, heart and bursa of fabricius of each bird were collected. These organs were fixed in 10% buffered formalin, then sectioned, and stained with Hematoxylin and Eosin (H&E) for microscopic examination (Luna, 1968).

### Results

**Effects on white blood cell and differential counts**

When the broilers were subjected to 33±1 °C temperature episode during 1 - 21 days of experimental period, total white blood cells (TWBC) of the birds were significantly increased on day 3 (P<0.05) and were highest on days 7 and 14, then significantly decreased on day 21 (P<0.05). Monocytes were significantly increased on day 7 (P<0.05). Lymphocytes were significantly increased on day 7, and were highest on day 14 (P<0.05). On day 21, the value of lymphocyte was significantly lower than on days 7 and 14 (P<0.05), respectively. Lympholytic cells were significantly increased on day 3 and 7 and then decreased on day 14 (P<0.05). Heterophils were significantly increased on days 3 and 7 and then decreased on day 14 (P<0.05) (Table2). The patterns of white blood cell parameters are presented in Figure 1. Level of added ascorbic acid produced no effect on white blood cells and differential counts.

### Table 1. Total mixed feed ration for growing broilers.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Percentage of mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn#2</td>
<td>62.60</td>
</tr>
<tr>
<td>Fish meal</td>
<td>10.00</td>
</tr>
<tr>
<td>Soy bean meal china</td>
<td>23.00</td>
</tr>
<tr>
<td>Rice bran oil</td>
<td>2.96</td>
</tr>
<tr>
<td>Premix*</td>
<td>0.5</td>
</tr>
<tr>
<td>Alimethionine</td>
<td>0.25</td>
</tr>
<tr>
<td>L - Lysine</td>
<td>0.14</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.50</td>
</tr>
<tr>
<td>Salt</td>
<td>0.20</td>
</tr>
<tr>
<td>D.C.P. (Rock 16%)</td>
<td>0.30</td>
</tr>
<tr>
<td>D.C.P. (Rock 18%)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Each kg contains 50 Vitamin AD3E 500/1000 (400mg); Vitamin E (2,000 mg); Vitamin B (180 mg); VitaminB; (100 mg); VitaminB; (310 mg); VitaminB; 1% (120 mg); Vitamin K; 51% (100 mg); Niacin, B (2, 700 mg); D - calcuim pentothinate (1,000 mg); Folic acid (50 mg); Biotin, 2% (750 mg); Chlorine chloride 50% (20,000 mg); Magnesium sulphate (19,600 mg); Potassium iodide, KI (44 mg); Cobalt chloride (35 mg); Zinc Oxide, ZnO (1,980 mg); Copper sulphate, Cu 2+ (5 H2O (210 mg); ferrous sulphate, Fe 7H O (40,600 mg); Selenium (150 mg); Dicalcium phosphate (406.37g).
Figure 1. White blood cell parameters pattern of broiler response to heat stress for 21 days
(A: total white blood cell; B: lymphocyte; C: heterophil; D: lympholytic cell; E: monocyte)

Microscopic changes
In all birds on day 21 of the experimental period, generalized edema and hemorrhage were observed in the kidney especially in renal papillae and renal tubulae. In addition, leukocytes accumulated in many inflammatory areas (Figure 2A). Fatty degeneration was mostly found in renal tubular epithelial cells. Most liver cells accumu-
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Effects of ascorbic acid in broiler chickens under chronic heat stress.

Table 2. White blood cell parameters changes in broiler chickens under chronic heat stress.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Days after heat exposure</th>
<th>SEM (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day1</td>
<td>Day3</td>
</tr>
<tr>
<td>Total WBC (10^4 cell/µl)</td>
<td>1.68</td>
<td>2.79</td>
</tr>
<tr>
<td>Monocyte (10^3 cell/µl)</td>
<td>10.05</td>
<td>21.00</td>
</tr>
<tr>
<td>Lymphocyte (10^3 cell/µl)</td>
<td>8.50</td>
<td>10.05</td>
</tr>
<tr>
<td>Lympholytic cell (10^3 cell/µl)</td>
<td>3.16</td>
<td>6.67</td>
</tr>
<tr>
<td>Heterophil (10^3 cell/µl)</td>
<td>6.13</td>
<td>13.69</td>
</tr>
</tbody>
</table>

\(^1\) SEM = Standard error of the mean

\(^{a,b,c,d}\) within row, mean with no common superscript differ significantly (P<0.05).

Figure 2. Microscopic examination of kidney, liver, lung and cardiac muscle in broilers under heat stress. (A= kidney, B= liver, C= lung and D= cardiac muscle)

Lated fat by vacuolation of fat with dilation of sinusoid. Necrosis with leukocyte granulation tissue was seen in some parts of the liver (Figure 2B). Lung tissue with massive congestion and hemorrhage was largely observed in alveolar duct and alveolar sac (Figure 2C). Massive myofibrillar degeneration with hemorrhage, vacuolation of myofibers and diffuse myocarditis containing white blood cells was found in some areas (Figure 2D). The lobules within the bursa of fabricius of broilers were atrophied when exposed to heat stress (Figure 3A and 3B). Whereas, only the size of lobules in broilers receiving ascorbic acid at 800 mg/kg in the diet were larger and active than this birds that received ascorbic acid at 400, 200 and 0 mg/kg in their diets, respectively (Figure 3A, 3B, 3C and 3D).
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Figure 3. Microscopic examination of lobules within bursa of fabricius in broilers with added
ascorbic acid in their diets after exposed to heat stress for 21 days (A = 0 mg/kg;
B=200 mg/kg; C= 400 mg/kg; D=800 mg/kg).

Table 3. Effects of ascorbic acid on lymphocyte, lympholytic cell and NDV-HI titers in
broilers chickens under chronic heat stress.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Levels of ascorbic acid</th>
<th>SEM(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0mg/kg</td>
<td>200mg/kg</td>
</tr>
<tr>
<td>Lymphocyte (x10^3 cell/µl)</td>
<td>17.10</td>
<td>18.60</td>
</tr>
<tr>
<td>Lympholytic cell (x10^2 cell/µl)</td>
<td>10.38</td>
<td>10.62</td>
</tr>
<tr>
<td>NDV - HI titer</td>
<td>1.30(^a)</td>
<td>1.52(^a)</td>
</tr>
</tbody>
</table>

\(^a\) and \(^b\) within row, mean with no common superscript differ significantly (P<0.05).

\(^1\) Standard error of the mean

Effects of ascorbic acid on lymphocytes, lympholytic cells and HI titer of ND

Birds were fed with four levels of added ascorbic acid i.e. 0, 200, 400 and 800 mg/kg diets
and subjected to 33±1 °C temperature episode to determine lymphocytes, lympholytic cells and
HI titer of Newcastle disease on day 1, 7, 14 and 21 of experimental period. Lymphocytes and
lympholytic cells showed no significant difference among the treatments. However, HI titer of
Newcastle disease at 800 mg/kg diets were significantly higher than others. Apparently, adding
ascorbic acid at 800 mg/kg in the diet could induce humoral immunity in broilers under heat stress
(Table 3).
Discussion

Generally, normal total leukocyte counts in chickens (Gallus gallus domesticus) were 1.2-3.0 × 10⁴ cell/µl (average 1.2) (Jain, 1993). A count that is greater than normal range is considered suggestive leukocytosis. General causes of leukocytosis include infection, trauma, toxicities, hemorrhage into a body cavity, rapidly growing neoplasm and leukemias. The leukocyte count aids in the assessment of the leukocytosis, because a heterophilia is usually present in leukocytosis caused by inflammation, (Ritchie et al., 1994). Typically, heterophil infiltration predominates in the first 6 to 12 hrs of the inflammatory response, but macrophages, lymphocytes, and event giant cells are present at 48 h and there are numerous giant cells at 72 h (Harmon, 1998).

In this study, heterophils started increasing on day 3 and returned to the normal level on day 14 of the experimental period, while total lymphocytes increased on day 7 and day 14, but subsequently decreased. The magnitude of the heterophilia usually indicates severity of the initial inflammatory process. Ritchie et al. (1994) reported that birds that normally had high numbers of circulating lymphocytes and may develop leukopenia and lymphopenia in the initial stress response, but macrophages, lymphocytes, and event giant cells are present at 48 h and there are numerous giant cells at 72 h (Harmon, 1998).

After the broilers were exposed to high ambient temperature, there was an increased release of corticosterone, stored in the adrenal cortex, into the blood circulation, (Richard, 1998). Furthermore, Jain (1993) reported that corticosteroid induced lymphopenia attributed to lympholysis in blood and lymphoid tissue, increased shift of lymphocytes from blood to other body compartments, or both. T-cells in blood and tissues are most sensitive to the lympholytic effect. Lymphocytes have high affinity receptors for corticosteroids in their cytoplasm. After ligand receptor interaction in the cytoplasm, the ligand receptor complexes bind to specific DNA sequences and induce the synthesis of mRNA, which in turn triggers the synthesis of protein that inhibits intracellular glucose transport and lipid synthesis. In addition, an endonuclease may become activated, causing DNA fragmentation. Glucocorticoids also markedly inhibit the synthesis of IL-1 by macrophages and IL-2 by activated T cell, thereby thwarting an immune response (an immunosuppressive effect), while ascorbic acid could decrease corticosterone level in the circulation (Nockel et al., 1973; Sheila and Cheryl, 1978). However, in this study, lympholytic cells started increasing on day 3, then significantly increased on days 7, 14 and 21 of the experimental period. Four levels of ascorbic acid could not reduce lympholytic cells, but could improve HI titer of Newcastle disease. Similarly Gross (1992) reported that ascorbic acid could improve immune response in birds under stress and disease condition. But the pathogenesis of heat stress in broilers in this study caused different physiological changes from stress under infection. Besides, the NDV-HI titer of broilers that received ascorbic acid at 800 mg/kg in the diet was related with the largest size of the lobules within their bursa of fabricius. This illustrates that tissue injury and hemorrhage in broilers under chronic heat stress
cause leukocytosis, heterophilia, lymphocytosis and monocytosis. Lympholytic cells were also increased. Adding ascorbic acid at 0, 200, 400 and 800 mg/kg in diets had no effect on lympholytic cells and lymphocyte numbers. However, adding ascorbic acid at 800 mg/kg in the diet could induce the highest humoral immunity in broilers under chronic heat stress because ascorbic acid at this level could protect the bursa of fabricius from the effect of glucocorticoid that were released during broilers under heat stress.

References
Wongwatcharadumrong, R. 1990. Laboratory for Veterinary Virology. Department of Pathology, Faculty of Veterinary Medicine, Chulalongkorn University. (in Thai)