HIGH DIETARY FLUORINE ALTERATION OF SERUM CYTOKINE AND IMMUNOGLOBULIN IN BROILERS

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SUMMARY: As part of our study on the effects of high dietary fluorine (F) on organs and tissues of young chicken broilers, changes in the serum content of cytokines including interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and immunoglobulins (IgG, IgM, IgA) were investigated starting with one-day-old broilers fed diets containing 400, 800, and 1200 mg F/kg throughout an experimental period of 42 days. The results showed that the serum content of IL-2, IL-4, IL-6, TNF-α, IFN-γ, IgG, and IgM was lower (p<0.05 or p<0.01) in the three high F groups than in the control group. The serum IgA level was significantly increased (p<0.05 or p<0.01) in the high F groups II and III at 42 days of age and significantly decreased (p<0.01) in the same groups at 14 days or from 14 to 28 days of age. In conclusion, high dietary F in the range of 800–1200 mg/kg was found to reduce the aforementioned serum cytokines and immunoglobulins, which could ultimately adversely impact humoral and cellular immune functions in broilers.

Keywords: Chicken broilers; Cytokines; Enzyme-linked immunosorbent assay (ELISA); High dietary fluorine; Immunoglobulins.

INTRODUCTION

Fluorine (F) as the fluoride ion is well known to be toxic at high levels of intake.1 Various studies have shown that chronic ingestion of excess F damages teeth2,3 and bones4,5 and causes various undesirable changes and disorders in the functions of non-skeletal organs6-9 and body systems10-13 in humans as well as animals. Excess F also has adverse effects on the immune system.14-17

Among the many components of the animal immune system, cytokines play a key regulatory role.18 In our recent studies, we have found that dietary high F in the range of 800–1200 mg/kg reduces the percentages of T-lymphocyte subsets as well as the contents of interleukin-2 (IL-2),19 interleukin-4 (IL-4), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) in the cecal tonsil20 of broilers. The relationship between thymocyte population and serum IL-2 contents in broilers has also been observed in three high F groups and a control group.21 To date, numerous studies concerning the impact of F on the contents of cytokines have been conducted on other animals or cells,22-26 but reports remain scarce for broilers.

Because they provide key information on the humoral immune status, serum immunoglobulin levels are determined routinely in clinical practice.27 An early study demonstrated that NaF can inhibit antibody formation in rabbits.28 Other research showed that F can elicit an increase in antibody responses,29 and addition of NaF to the immunogen markedly improves immunological states in orally
immunized chickens. Recently, our newest study indicates dietary high F can reduce the contents of immunoglobulins in the intestinal mucosa of broilers. However, there do not appear to be any systematic reports on changes in the contents of serum immunoglobulins directly induced by dietary high F in broilers.

As a part of our studies on the effects of high dietary F on organs and tissues, the present research observed changes in the content of serum cytokines and immunoglobulins by enzyme-linked immunosorbent assay (ELISA) in the chicken broilers of those studies fed a high dietary F. The results are aimed at providing important information for the clinical pathological diagnosis and prevention of fluorosis in broilers.

MATERIALS AND METHODS

Broilers and diets: The same 280 one-day-old healthy avian broilers used in our recent studies were employed in this research. As indicated in those reports, the broilers were randomly divided into four groups of 70 broilers each and fed a corn–soybean diet as follows: a control group with 22.6 mg F/kg, and three high F groups I, II, and III with 400, 800, and 1200 mg F/kg diet, respectively. The broilers were housed in electrically heated cages and given ad libitum access to water and the above-mentioned diets for 42 days. All the dietary nutrition requirements were adequate according to the US National Research Council (NRC 1994). The Sichuan Agricultural University Animal Care and Use Committee approved the procedures and animal handling involved in these studies.

Serum samples: At 14, 28, and 42 days of age during the experiment, five broilers in each group were phlebotomized from the jugular vein to collect serum. Non-anticoagulative blood was clotted for 15 min and the serum was separated by centrifugation at 3000 rpm for 15 min to remove fibrinogens, white blood cells, and platelets. Sixty serum samples were collected and preserved at –20°C before assay.

Determination of serum cytokine and immunoglobulin levels: Before the assay, the serum samples were thawed, the contents of serum cytokines and immunoglobulins were determined by enzyme-linked immunosorbent assay (ELISA) as described by Gaça et al. ELISA Kits (R&D Systems, USA) specially designed for use in chicks were IL-2 (I039-09; LOT: 201211), IL-4 (I042-09; LOT: 201211), IL-6 (I046-09; LOT: 201211), TNF-α (T041-09; LOT: 201211), IFN-γ (I015-09; LOT: 201211), IgA (I018-09; LOT: 201211), IgM (IO33-09; LOT: 201211), and IgG (I032-09; LOT: 201211). The final cytokine and immunoglobulin contents were assayed by standard curves and expressed as picogram per milliliter (pg/mL) or microgram per milliliter (µg/mL).

Statistical analysis: Significance of difference among four groups was analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc test. Numerical results are given as means ± standard deviation (±SD), and a p value of less than 0.05 (p<0.05) was considered to indicate
statistical significance. The analysis was done using SPSS Statistics 17.0 for Windows.

RESULTS

Changes in the serum IL-2 content: The serum IL-2 content was significantly lower (p<0.05 or p<0.01) in the high F groups I, II, and III than in the control group from 14 to 42 days of age (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>14 days</th>
<th>28 days</th>
<th>42 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>229.32±2.64</td>
<td>244.15±5.85</td>
<td>289.22±6.60</td>
</tr>
<tr>
<td>High F Group I</td>
<td>219.74±3.10</td>
<td>193.78±4.47*</td>
<td>202.56±5.27*</td>
</tr>
<tr>
<td>High F Group II</td>
<td>165.53±8.22*</td>
<td>101.78±6.27*</td>
<td>91.93±2.32*</td>
</tr>
<tr>
<td>High F Group III</td>
<td>161.18±7.24*</td>
<td>93.57±3.66*</td>
<td>87.95±4.54*</td>
</tr>
</tbody>
</table>

Compared with the control *p<0.01, †p<0.05.

Changes in the serum IL-4 content: The serum IL-4 content was significantly decreased (p<0.01) in the high F groups II and III from 14 to 42 days of age and was significantly lower (p<0.05 or p<0.01) in the high F group I at 14 and 42 days of age in comparison with the control group (Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>14 days</th>
<th>28 days</th>
<th>42 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>268.92±6.41</td>
<td>286.64±6.49</td>
<td>298.51±7.75</td>
</tr>
<tr>
<td>High F Group I</td>
<td>260.91±3.40†</td>
<td>275.71±8.79</td>
<td>270.68±9.97*</td>
</tr>
<tr>
<td>High F Group II</td>
<td>218.81±3.97*</td>
<td>236.79±6.19*</td>
<td>190.15±9.09*</td>
</tr>
<tr>
<td>High F Group III</td>
<td>142.63±3.42*</td>
<td>164.51±7.13*</td>
<td>169.59±7.48*</td>
</tr>
</tbody>
</table>

Compared with the control *p<0.01, †p<0.05.

Changes in the serum IL-6 content: The serum IL-6 content was markedly reduced (p<0.01) in the high F groups II and III from 14 to 42 days age compared with the control group (Table 3).

<table>
<thead>
<tr>
<th>Group</th>
<th>14 days</th>
<th>28 days</th>
<th>42 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>105.37±1.01</td>
<td>93.76±1.23</td>
<td>71.64±1.68</td>
</tr>
<tr>
<td>High F Group I</td>
<td>104.55±0.79</td>
<td>91.96±0.68</td>
<td>69.10±1.23†</td>
</tr>
<tr>
<td>High F Group II</td>
<td>101.72±1.22*</td>
<td>86.41±3.46*</td>
<td>60.23±1.02*</td>
</tr>
<tr>
<td>High F Group III</td>
<td>92.88±1.21*</td>
<td>77.27±2.15*</td>
<td>54.58±1.99*</td>
</tr>
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</table>

Compared with the control *p<0.01, †p<0.05.

Changes in the serum TNF-α content: The serum TNF-α content decreased (p<0.05 or p<0.01) in the high F groups I, II, and III from 14 to 42 days of age compared with the control group (Table 4).
Changes in the serum IFN-γ content: The serum IFN-γ content was significantly lower (p<0.01) in the high F groups II and III than in the control group from 14 to 42 days of age. Moreover, the serum IFN-γ content was lower (p<0.01) in the high F group I at 42 days of age (Table 5).

Changes in the serum IgA content: The serum IgA content was significantly lower (p<0.01) in the high F group II at 14 days of age, in the high F group III at 14 to 28 days of age than those of the control group. However, the serum IgA contents were significantly increased (p<0.05 or p<0.01) at 42 days of age in the high F groups II and III (Table 6).

Changes in the serum IgM content: The serum IgM content showed a significant decrease (p<0.01) in high F groups II and III from 14 to 42 days of age in comparison with those of the control group (Table 7).
Changes in the serum IgG content: The serum IgG content dramatically reduced (p<0.01) in the high F group II at 14 and 42 days of age and in the high F group III from 14 to 42 days of age compared with those of the control group (Table 8).

Table 7. Changes in the serum IgM content in broilers (Values are means±SD, n=5)

<table>
<thead>
<tr>
<th>Group</th>
<th>14 days</th>
<th>28 days</th>
<th>42 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>8.86±0.27</td>
<td>10.19±0.87</td>
<td>9.98±0.85</td>
</tr>
<tr>
<td>High F Group I</td>
<td>8.46±0.41</td>
<td>9.85±0.54</td>
<td>9.70±0.72</td>
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<tr>
<td>High F Group II</td>
<td>6.65±0.83*</td>
<td>8.35±0.62*</td>
<td>4.23±0.55*</td>
</tr>
<tr>
<td>High F Group III</td>
<td>6.45±0.98*</td>
<td>7.49±0.45*</td>
<td>3.93±0.42*</td>
</tr>
</tbody>
</table>

Compared with the control *p<0.01.

Table 8. Changes in the serum IgG content in broilers (Values are means±SD, n=5)

<table>
<thead>
<tr>
<th>Group</th>
<th>14 days</th>
<th>28 days</th>
<th>42 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>41.76±0.12</td>
<td>33.22±1.47</td>
<td>23.84±0.73</td>
</tr>
<tr>
<td>High F Group I</td>
<td>41.08±0.20</td>
<td>31.34±1.78</td>
<td>22.85±0.54</td>
</tr>
<tr>
<td>High F Group II</td>
<td>33.15±0.49*</td>
<td>34.62±0.90</td>
<td>18.34±1.06*</td>
</tr>
<tr>
<td>High F Group III</td>
<td>24.75±1.14*</td>
<td>25.66±0.86*</td>
<td>12.57±1.06*</td>
</tr>
</tbody>
</table>

Compared with the control *p<0.01.

DISCUSSION

It is fairly clear that the innate and adaptive immune systems cooperate to provide optimal host defense, and a defect in either system can have significant consequences. The state of the immune system is evaluated by the number of T or B cells, the quantitative or qualitative measure of the cytokine and antibody levels, or cellular function such as phagocytic activity.

Effects of high dietary F on serum cytokines in broilers: The production of cytokines is largely dependent on the state of differentiation of the T cells. Two different types of differentiated T cells can be characterized according to the pattern of cytokine production of T cells. IL-2, TNF-α and IFN-γ, which play an important role in cell-mediated immune response, are produced by T helper 1 (Th1) cells. On the other hand, IL-4, and IL-6 belong to T helper 2 (Th2) cells and enhance humoral immunity. Standard laboratory methods for assessing T-lymphocyte function in vitro include assessment of cytokine secretion after T-cell activation, which specializing in evaluating immune function.

IL-2 is widely considered to be a key cytokine in T-cell-dependent immune responses. It promotes the generation, proliferation, and differentiation of T cells and the production of antibodies by B cells. IL-4, which was known as B lymphocyte growth factor-1 (BCGF-1), can promote mast cell, B and T lymphocyte proliferation, differentiation and the process of forming sIgA. IL-6,
produced by antigen-presenting cells and non-hematopoietic cells in response to external stimuli, was originally identified as a B cell differentiation factor and was believed to play an important role in antibody production \textit{in vivo}.\textsuperscript{45} However, it is now recognized as a typical multifunctional cytokine involved in the regulation of the immune response, hematopoiesis, and inflammation.\textsuperscript{46} Our recent studies have shown F decreases the contents of IL-4 and IL-6 in the cecal tonsil\textsuperscript{20} and intestinal mucosa\textsuperscript{34} of broilers, which are consistent with Li’s report on contents of serum IL-6 in dairy goat.\textsuperscript{23} These results demonstrate that F can not only inhibit the secretion of cytokines and the differentiation of B and T cells, but it also has adverse effects on antibody-mediated immunity response as in broilers.

TNF-$\alpha$, a multi-functional cytokine secreted mainly by macrophages/monocytes and T lymphocytes, has a variety of functions in the regulation of inflammation and cellular immune responses.\textsuperscript{47,48} IFN-$\gamma$ is a strong activator of macrophages and plays a major role in adaptive cell-mediated immune responses produced by Th1 lymphocytes and cytotoxic T lymphocytes.\textsuperscript{49}

In the present study, the serum content of IL-2, IFN-$\gamma$, and TNF-$\alpha$ decreased (p<0.01) in the high F groups II and III, thereby indicating that F impaired cell-mediated immunity response in the broilers. These results are similar to those in Zhou’s research in rabbits,\textsuperscript{22} Hosokawa’s study in the J774.1 murine macrophage cell line,\textsuperscript{24} Chen’s study on serum IL-2 contents in chickens,\textsuperscript{21} and Luo’s\textsuperscript{34} results on intestinal mucosa of broilers.

\textbf{Effects of high dietary F on serum immunoglobulins in broilers: In vitro studies of B-cell immune function are used primarily in research settings to evaluate immunoglobulin secretion.}\textsuperscript{50} IgY is the major antibody produced by chickens\textsuperscript{51} and is the functional equivalent of IgG in birds, reptiles, and amphibians.\textsuperscript{52} IgG antibodies mediate a wide range of functions including transplacental passage and opsonization of antigens through binding of antigen/antibody complexes to specialized Fc receptors on macrophages and other cell types.\textsuperscript{53} IgM provides the initial response to foreign antigens and plays a regulatory role in subsequent immune response development, accelerating the production of high-affinity IgG.\textsuperscript{54} IgA, as the principal antibody class in the secretions, named sIgA that bathe these mucosal surfaces, acts as an important first line of defense and also as an important serum immunoglobulin, mediating a variety of protective functions through interaction with specific receptors and immune mediators.\textsuperscript{55}

As seen in the present study, the contents of serum IgG, IgM decreased (p<0.05 or p<0.01) in the high F groups II and III, thereby demonstrating that excessive dietary F intake inhibited the secretion of IgG, IgM. It should also be noted that the serum IgA contents were significantly enhanced (p<0.05 or p<0.01) in the high F groups II and III at 42 days of age but were significantly decreased (p<0.01) in the same groups at 14 days or from 14 to 28 days of age. Low Ig levels define some humoral immunodeficiencies.\textsuperscript{56} In contrast, high Ig levels are observed in liver diseases, chronic inflammatory diseases, haematological disorders, infections, and malignancies.\textsuperscript{57} In rats, antigens penetrating the mucosal surfaces have been found
to stimulate the humoral immune system and induce a serum IgA response.\textsuperscript{58} However, since these findings are limited by the research methods, research samples, and research models, the elevated content of serum IgA in the present research should be given greater in-depth study.

It is also well known that the thymus is where T-cells proliferate and mature.\textsuperscript{14} In poultry, the bursa of Fabricius is the primary lymphoid organ responsible for the establishment and maintenance of the B lymphocyte compartment.\textsuperscript{59} The spleen also has a close relationship with B and T lymphocytes.\textsuperscript{60} Studies have reported that excess dietary F can not only impair the development of the spleen,\textsuperscript{16} the bursa of Fabricius\textsuperscript{61} and the thymus\textsuperscript{14} of broilers, but it can also induce differentiation and proliferation impairment of lymphocytes in the spleen,\textsuperscript{21} the bursa of Fabricius\textsuperscript{61} and the thymus\textsuperscript{14} in broilers.

Based on the results in the present study and the foregoing discussion, the declining contents of serum IL-2, IL-4, IL-6, IFN-\(\gamma\), TNF-\(\alpha\), IgM, and IgG are closely related to the reduction of T- and B-lymphocytes population and/or activation in the immune organs. At the same time, the content of serum IgA in chickens is less than 4 percent of total immunoglobulins.\textsuperscript{62} Consequently, its rise incurred a comparatively small influence on the total contents of immunoglobulins. The total contents of serum immunoglobulins in high F groups II and III were still lower than those in the control group. We conclude that high dietary F in the range of 800–1200 mg/kg can significantly impair the humoral and cellular immune functions in broilers. Thus the present results are helpful to increase our understanding of the effects of F on the immune system in broilers.

**ACKNOWLEDGEMENTS**

This research was supported by the program for Changjiang Scholars and the University Innovative Research Team (IRT 0848) of the Sichuan Agricultural University and also the Education Department of Sichuan Province (09ZZ017).

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