INCREASED APOPTOTIC LYMPHOCYTE POPULATION IN THE SPLEEN OF YOUNG CHICKENS FED DIETS HIGH IN FLUORINE

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SUMMARY: Three hundred one-day-old avian broilers were divided into four equal groups of 75 that were fed for 42 days as follows: a control diet containing 23 mg F/kg and three high F diets containing 400, 800, and 1200 mg F/kg, respectively, for high F groups I, II, and III. Mitochondrial injury and increased numbers of apoptotic cells with condensed nuclei were observed in spleens of the high F groups II and III. As measured by flow cytometry, the percentage of apoptotic splenocytes was significantly increased (p<0.01) in the high F groups II and III when compared with that of control group. Immunohistochemistry tests showed increased frequencies of positive cells in Bax detection, and decreased Bcl-2 protein in the high F groups II and III. The results indicate that excess dietary F in the range of 800~1200 mg/kg caused histological lesions and cellular apoptosis of the spleen in chickens.

Keywords: Apoptosis of spleen; Broilers; Chickens; Flow cytometry; High dietary fluorine; Splenic lymphocytes.

INTRODUCTION

Fluoride (F) pollution is an international public health problem. Besides dental and skeletal fluorosis, the toxic effects of F include oxidative stress as shown by damage to liver, kidney, brain, and parathyroid glands in rats. In China, fluorosis in chicks leads to great economic losses due to the use of nondefluorinated calcium monohydrogen phosphate as a mineral supplement (average dietary F level reaches about 300 mg/kg and in some cases as high as 800 mg/kg). In their report on pathological changes in soft tissues caused by F and the mechanism of fluorosis in chicks, Liu et al. examined impact of diets containing 500, 1000, and 2000 mg F/kg. To our knowledge, however, there have been no systematic studies on the effect of excess F on the immune system in chickens. As part of a larger study, the present investigation examined the immunotoxicity of F in chickens by the methods of experimental pathology and flow cytometry (FCM), with emphasis on the impact of excess dietary F on cellular apoptosis of the spleen.

MATERIALS AND METHODS

Chickens and diets: Three hundred one-day-old healthy avian broilers were divided into four equal groups and fed on diets as follows: control group (23 mg F/kg) and high F group I (400 mg F/kg); high F group II (800 mg F/kg); and high F group III (1200 mg F/kg). The broilers were housed in electrically heated cages and were provided with drinking water (F ≤ 1 mg/L) as well as the aforementioned diets ad libitum for 42 days. Nutritional requirements were adequate according to the US National Research Council.
Pathological observation: Five birds in each group were humanely killed at 7, 14, 21, 28, 35, and 42 days of age. After gross examination, 5 µm-thick microtome sections of the spleens were fixed in 10% formalin solution and embedded in paraffin. Slides were stained with hematoxylin and eosin (HE) for examination under light with an Olympus microscope.

At 42 days of age, spleens of 4 chickens in each group were dissected and then prefixed with a mixed solution of 4% paraformaldehyde and 2.5% glutaraldehyde, followed by softening in 3% EDTA solution for 20 min. The tissue was then fixed in 1% osmium tetroxide, dehydrated in series with acetone, infiltrated in Epox 812 for 2 hr, and then embedded in epoxy resin. Ultrathin sections were cut with glass knives, stained with uranyl acetate and lead citrate and examined with a TEM H-600 electron microscope (Hitachi, Japan).

Annexin-V apoptosis detection: Five chickens in each group were humanely killed at 14, 28, and 42 days of the experiment, and spleens were immediately taken from each chicken and ground to form a cell suspension that was filtered through a 300-mesh nylon screen. The cells were washed twice with cold PBS (phosphate buffer solution, pH 7.2-7.4) and were then suspended in 1× binding buffer (Cat. No. 51-66121E) at a concentration of 1×10^6 cells/mL. One hundred-μL portions of the cell suspension were transferred 5-mL culture tubes, and 5 μL of Anexin V-FITC (Cat. No. 51-65874X) and 5 μL of PI (Cat. No. 51-66211E) were added. The mixture was gently vortexed and incubated for 15 min at 25ºC in the dark. Four hundred μL of 1× binding buffer was added to each tube, and analysis by flow cytometry (BD FACSCalibur) was conducted within 1 hr.

Bax/Bcl-2 Detection: Bax and Bcl-2 were detected by the immunohistochemical method (SABC) and stained with DAB as described by Wang et al. Anti-Bax and anti-Bcl-2 as well as DAB were purchased from Wuhan Boster Biological Technology Co., Ltd., China.

Statistical analysis: The significance of difference among four groups was analyzed by analysis of variance. The analysis was done with SPSS 12.0 for windows.

RESULTS

Clinical observation: The broilers in all the high F groups grew more slowly than in the control group. Broilers in the high F groups II and III exhibited decreased feed intake and slower movement, but there were no obvious clinical symptoms in the high F group I. Three chickens in group II and seven chickens in group III died during the 42-day experiment.

Pathological lesions: Macroscopically, the spleens were smaller in a dose- and time-dependent manner in the three high F groups than in the control group. There were no obvious histopathological lesions in high F group I during the experiment. At the same time, the number of lymphocytes was significantly decreased with the widened splenic sinuses in the high F groups II and III.
Ultrastructural changes: There were no obvious abnormal ultrastructural changes in the high F group I. Apoptotic lymphocytes were increased in the high F groups II and III compared with those of the control group (Figure 1). The apoptotic cells showed typical condensed nuclei with horseshoe-like or crescent shapes (Figures 2 and 3). The mitochondria of lymphocytes were enlarged and their cristae were broken or/and absent with vacuolar shapes in the high F groups II and III (Figure 4).

Figure 1. The splenocytes in the control group (bar=2.00µm).

Figure 2. The apoptotic lymphocytes in the high F group II (bar=1.33 µm).

Figure 3. Typical condensed nucleus with horseshoe-like or crescent shapes of apoptotic lymphocytes in the high F group III (bar=1.07µm).

Figure 4. The mitochondria of lymphocytes are enlarged and their cristae are broken and absent in the high F group III (bar=1.6 µm).
**Annexin-V-FITC staining assay by flow cytometry:** As seen in the Table and in Figure 5, the percentage of apoptotic cells in the spleen of the high F group I was not appreciably different from that in the control group, but it was significantly greater in the high F groups II and III, except at 28 days for group II. In the Table, the apoptotic rates of splenocytes are the sum total of early and end-stage apoptosis.

<table>
<thead>
<tr>
<th>Group</th>
<th>14 days (%) (n=5)</th>
<th>28 days (%) (n=5)</th>
<th>42 days (%) (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>5.24±0.913</td>
<td>7.26±1.083</td>
<td>7.25±0.366</td>
</tr>
<tr>
<td>High F group I</td>
<td>6.14±1.941</td>
<td>7.06±0.642</td>
<td>5.94±0.840</td>
</tr>
<tr>
<td>High F group II</td>
<td>9.89±1.384*</td>
<td>8.19±1.675</td>
<td>13.91±1.437*</td>
</tr>
<tr>
<td>High F group III</td>
<td>16.50±1.577*</td>
<td>18.95±1.484*</td>
<td>20.32±2.062*</td>
</tr>
</tbody>
</table>

*Compared with the control group, p<0.01.

**Figure 5.** Scattergram of apoptotic splenocytes. A, B, C, and D represent the control group and the high F groups I, II, III, respectively. Cells that stain positive for Annexin V-FITC and negative for PI are undergoing apoptosis. Cells that stain positive for both Annexin V-FITC and PI are in the end-stage of apoptosis.
**Bax detection:** No obvious changes were observed in the high F group I. The number of positive cells containing Bax protein (brown-stain), but its frequencies of occurrence were increased in the high F groups II and III compared with those of control the group (Figures 6 and 7).

![Figure 6. Spleen in control group stained for Bax protein (brown-stain). (bar=16.00 µm).](image1)

![Figure 7. Spleen in high F group III stained for Bax protein (brown-stain). (bar=16.00 µm).](image2)

**Bcl-2 detection:** No obvious changes were observed in the high F group I, but the number of positive cells containing Bcl-2 protein (brown-stain) and its frequencies of occurrence were significantly lower in the high F groups II and III than in the control group (Figures 8 and 9).

![Figure 8. Spleen in control group stained for Bcl-2 protein (brown-stain). (bar=16.00 µm).](image3)

![Figure 9. Spleen in high F group III stained for Bcl-2 protein (brown-stain). (bar=16.00 µm).](image4)

**DISCUSSION**

According to data summarized by Weinstein and Davison, the dietary tolerance level of F for chickens is 400-mg F/kg dry weight with greater toxicity in younger avians. In the present 42-day study, however, no obvious clinical symptoms were observed in the high F 400-mg F/kg diet group I, in agreement with similar findings by Liu et al. In the Liu’s report, one-day-old chickens fed a 500-mg F/kg diet did not show any obvious clinical symptom before 50 days of age. Moreover, in the serum of chickens fed the 500-mg F/kg diet, there were no significant changes before 120 days of age in antioxidative activity compared to the control group.
As is well known the spleen is the principal peripheral immune organ and plays an important role in protective immune reactions. In the present study, excessive dietary F intake led to decreased food intake and lower body weight gain in a dose-dependent manner, similar to what Paul et al. found with rats. Histopathologically, we observed widened splenic sinuses and loosely packed lymphocytes in the two higher F 800 and 1200 mg F/kg diet groups II and III. These changes suggest DNA damage occurred leading to inhibition of cell proliferation. Studies conducted by Zhang et al. on young pigs showed that high concentrations of NaF in the diet inhibited the synthesis of DNA and RNA in the pancreas. Other studies confirm that F can cause DNA damage.

The main object of the present study was to determine if excess dietary F increased the percentage of splenocytes in growing broilers. AnnexinV-FITC staining assay showed that the percentage of apoptotic cells in the spleen was not obviously changed in the high F 400-mg F/kg diet group I, but it was significantly increased (p<0.01) in the two higher F 800 and 1200 mg F/kg diet groups II and III compared with that of control group (Figure 5). Ultrastructurally, the population of apoptotic splenocytes was higher in the high F groups II and III than in the control group. At the same time, enlarged mitochondria were observed in the high F groups II and III, which is believed to be a compensatory process due to ATP deficiency that finally leads to apoptosis.

With the demonstration of deleterious effects of F on anti-oxidative systems as in the rat kidney and in peripheral blood erythrocytes of oxen, it is reasonable to propose that oxidative damage could occur in mitochondria and lead to apoptosis through a mitochondria-mediated pathway. Members of the Bcl-2 family of proteins are known to affect mitochondrial function and to regulate the release of apoptosis activating factors. The ratio of Bcl-2 to Bax determines survival or death of the cell. In the present study, Bax proteins were overexpressed while Bcl-2 proteins were depressed in the high F groups II and III when compared with the control group, similar to the findings of Xu et al. on rat renal tubular cells, thus resulting in an increase of apoptotic lymphocytes in these two groups. These findings are similar to those on DNA damage and apoptosis of ox peripheral blood lymphocytes induced by fluoride.

From the results of this study, together with above discussion, we conclude that a 400-mg F/kg diet intake is relatively safe for the spleen of young chickens. However, a higher intake of 800 and 1200 mg F/kg diet inhibited the development of the spleen by inducing lymphocyte apoptosis.

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