TOXIC EFFECTS OF FLUORIDE ON PRIMARY LYMPHOID ORGANS AND WHITE BLOOD CELLS IN FEMALE MICE

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SUMMARY: Our previous studies showed that excessive fluoride (F) induced thymus apoptosis in female rats. Here, we investigated the toxic effects of F on lymphocytes and the complete blood count in female mice. The results showed that excessive F reduced weight gain and induced ultrastructural changes and DNA damage in the immunocytes. Compared to the control group, in the F group, in the thymus, bone marrow, and blood lymphocytes, typical comet configurations were present and the ratio of tailing and the tail length of the immunocytes were significantly increased. Decreases occurred in the blood in the total white blood cell (WBC) count (p<0.01), lymphocytes (p<0.05), middle cells (p<0.05), granulocytes (p<0.05) and the thrombocytocrit (p<0.05). In conclusion, in female mice, excessive F reduces weight gain, seriously damages the DNA and ultrastructure of immunocytes, and reduces blood total WBC count, lymphocytes, middle cells, granulocytes, and the thrombocytocrit.

Keywords: Blood lymphocytes; Bone marrow; DNA damage; Fluoride; Mice; Thymus

INTRODUCTION

Immunocytes, a class of cells involved in the immune response, include lymphocytes (T lymphocytes, B lymphocytes, K cells, and NK cells), accessory cells (mononuclear phagocytes and dendritic cells), and other immune cells like granulocytes. With the exception of the T lymphocytes, whose stem cells mature in the thymus, the other immunocytes originate and mature in the bone marrow. As an irreplaceable primary lymphoid organ, bone marrow replenishes the immune system using a pool of hematopoietic stem cells, and it is a critical site for the sustained production of common lymphoid progenitor cells.1 It is well known that bone marrow serves as the primary site for development of B cells, NK cells, monocytes, and granulocytes, and that the thymus is the site that provides the main venue for the development and education of T cell progenitors. However, accumulated evidence also suggests that the bone marrow can also function as a secondary lymphoid organ for CD4 and CD8 cells, as well as a preferential homing site for memory T cells.2 Damage to the two primary lymphoid organs can reduce the production of immunocytes and induce immune dysfunction.1,3-7

After differentiation in the bone marrow or thymus, immunocytes migrate to the blood and perform their functions. Lymphocytes are responsible for the specific immune responses to infection, while the other immunocytes are considered to be non-specific. A complete blood count (routine or full blood screen) can indicate the immune status of the body. Some studies have shown that a higher total white blood cell (WBC) count is an independent predictor of all-cause mortality in older adults, and that the monocyte subtype provides even greater predictive ability.8

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Disorders of the WBC subpopulations can also be indicative of some diseases. Moreover, some of the factors which are able to cause immune suppression can also decrease the WBC count.

Fluoride (F) is not an essential trace element in the body and, when present in a high concentration, it can be harmful to teeth, bones, heart, liver, kidneys, gastrointestinal tract, lungs, brain, blood, hormones, the immune system, and various biochemical parameters in both experimental animals and humans. Our previous studies showed that excessive F ingestion can seriously damage immune function and induce thymus apoptosis. However, the full effects of F-induced damage to the bone marrow and peripheral blood remained unclear. The aim of this study was to further investigate, in female mice, the effects of F-induced immune system impairment on thymus ultrastructure, hematology, and the immunocytes.

**MATERIALS AND METHODS**

*Experimental animals:* A total of 24 healthy female Kunming mice (female: male = 3:1) were obtained from the Experimental Animal Center of Zhengzhou University, and were kept in a standard animal house at 22–25°C with ventilation and hygienic conditions.

*Experimental design:* The mice were randomly divided into two groups, of nine female and three male mice. The mice in the control group were reared on a standard diet and given *ad libitum* access to water. The mice in the F group were given a standard diet and drinking water containing 100 mg F/L. After three months of establishing the animal model, the female experimental animals were allowed to become pregnant by natural mating. During and after nursing, the offspring were reared in the same conditions as their parents. On the 21st day, the female mice were separated. On the 70th day, the separated female offspring mice were randomly selected for further study. The experimental design was approved by the Institutional Animal Care and Use Committee of China.

*Transmission electron microscopic observation of thymus cells:* To study the effect of F on thymus ultrastructure, the fresh thymus in mice was removed and fixed in 0.1% sodium arsenate dimethyl (pH 7.4) for observation with a transmission electron microscope (TEM). The sections were treated with 1% osmic acid and embedded in Araldite resin. About 50 nm thick sections were processed, stained with uranyl acetate and lead citrate, and examined with an H-7650 TEM.

*Analysis of DNA damage in bone marrow cells, thymus cells, and blood lymphocytes:* To analysis the effect of F on DNA damage, the fresh thymus in mice was removed and treated as reported in our earlier study. Using single cell gel electrophoresis (SCGE), the ratio of tailing was calculated by counting the percentage of cells showing tailing. The DNA damage was assessed from the length of DNA migration derived by subtracting the diameter of the nucleus from the total length of the image.

*Complete blood count examination (routine or full blood screen):* On the 70th day of the treatment, 6 female mice were selected randomly from each group and
were deprived of food for 12 hr. Blood was collected from the peri-orbital sinus and the complete blood counts performed with a blood cell analyzer.

**Statistical analysis:** Data are expressed as mean±SD. Statistical analyses were performed by Student’s t test. Values of p< 0.05 were considered significant.

**RESULTS**

**Body weight of mice:** Compared with the control group, the growth of the mice was significantly inhibited by the excessive F ingestion in the F group after 42 days of treatment (Figure 1).

![Figure 1. Effect of fluoride on body weight in mice. Compared to the control group: *p<0.05, †p<0.01.](image)

**TEM observation of thymus cell:** In the control group, the thymus cells showed normal ultrastructural features under TEM while, in the F-exposed groups, significantly more apoptotic thymus cells were present (Figure 2).

![Figure 2. Effect of fluoride on ultrastructural changes in thymus of mice. a: Normal thymus cells of mice in the control group, b: Damaged thymus cells of mice in the fluoride group.](image)

The apoptotic cells had morphological changes with the nuclear chromatins lessened, condensed, marginalized against the nuclear envelope, and aggregated
into large dark, compact masses. The cytoplasm was concentrated and associated with multiple cytoplasm vacuolization. The bilayer structure of the nuclear membrane of the apoptotic thymus cells was less distinct and interrupted in places. The mitochondria showed irregularities and swelling with dissolution and disappearance of the cristae (Figure 2b).

**Analysis of DNA damage:** In comparison with the control group, F-induced DNA damage occurred in the blood lymphocytes and the thymus and bone marrow immunocytes with the F-group cells showing typical comet configurations and significant increases in both the ratio of tailing and the tail length. These results indicate that F induced DNA damage in the mice immunocytes. (Tables 1 and 2).

**Table 1.** Ratio of tailing (% of cells showing tailing) and tail length in mice cells induced by fluoride (Values are mean±SD; n = 4)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group</th>
<th>Ratio of tailing (%)</th>
<th>Tail length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Marrow</td>
<td>Control group</td>
<td>13.21±0.75</td>
<td>3.24±0.85</td>
</tr>
<tr>
<td></td>
<td>Fluoride group</td>
<td>27.37±1.38†</td>
<td>16.26±2.93†</td>
</tr>
<tr>
<td>Blood lymphocytes</td>
<td>Control group</td>
<td>18.22±1.31</td>
<td>3.48±0.74</td>
</tr>
<tr>
<td></td>
<td>Fluoride group</td>
<td>38.67±3.07†</td>
<td>18.26±3.49†</td>
</tr>
<tr>
<td>Thymus</td>
<td>Control group</td>
<td>15.63±1.26</td>
<td>3.39±0.73</td>
</tr>
<tr>
<td></td>
<td>Fluoride group</td>
<td>32.71±2.46†</td>
<td>18.97±2.93†</td>
</tr>
</tbody>
</table>

Compared to the control group: *p<0.05, †p<0.01

**Table 2.** Undamaged and migrated cells according to severity by grade (%)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group</th>
<th>Normal</th>
<th>Grade I</th>
<th>Grade II</th>
<th>Grade III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Marrow</td>
<td>Control group</td>
<td>86.79</td>
<td>11.78</td>
<td>1.19</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Fluoride group</td>
<td>72.63</td>
<td>9.02</td>
<td>10.42</td>
<td>7.92</td>
</tr>
<tr>
<td>Blood Lymphocytes</td>
<td>Control group</td>
<td>81.78</td>
<td>15.28</td>
<td>2.31</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>Fluoride group</td>
<td>61.33</td>
<td>11.71</td>
<td>12.42</td>
<td>14.54</td>
</tr>
<tr>
<td>Thymus</td>
<td>Control group</td>
<td>84.37</td>
<td>13.39</td>
<td>1.83</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>Fluoride group</td>
<td>67.29</td>
<td>8.97</td>
<td>10.98</td>
<td>12.76</td>
</tr>
</tbody>
</table>

**Complete blood count examination:** Compared with the control group, in the F group the blood counts were reduced for the total WBCs, lymphocytes, middle cells, and granulocytes (Figure 3). The lymphocyte proportion increased from 46% to 51% while that for granulocytes decreased from 39% to 34% (Figure 4). In comparison with the control group, in the F group MCHC, MCV, MCH, RDW HCT, MPV, and HGB did not change significantly while the PCT decreased (p<0.05). The results indicate that F induced WBC changes in the blood of mice (Tables 3 and 4).
Toxic effects of fluoride on primary lymphoid organs and white blood cells in female mice

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Figure 3. Effect of fluoride on white blood cell count in mice. Compared to the control group: *p<0.05, †p<0.01.

Figure 4. Effect of fluoride on the differential white blood cell count in mice. a: control group, b: fluoride group.

Table 3. Effect of fluoride on mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and red cell distribution width (RDW) in mice (Values are mean±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>MCHC (g/L)</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>RDW (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>340.00±7.04</td>
<td>51.05±1.26</td>
<td>17.32±0.45</td>
<td>15.08±1.07</td>
</tr>
<tr>
<td>Fluoride group</td>
<td>338.00±6.84</td>
<td>51.88±0.48</td>
<td>17.48±0.48</td>
<td>14.43±0.59</td>
</tr>
</tbody>
</table>

Table 4. Effect of fluoride on hematocrit (HCT), mean platelet volume (MPV), hemoglobin (HGB), and thrombocytecrit (PCT) in mice. (Values are mean±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>HCT (%)</th>
<th>MPV (fL)</th>
<th>HGB (g/L)</th>
<th>PCT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>40.20±3.99</td>
<td>9.62±0.43</td>
<td>136.83±13.59</td>
<td>0.24±0.04</td>
</tr>
<tr>
<td>Fluoride group</td>
<td>39.10±1.61</td>
<td>9.62±0.74</td>
<td>132±33±3.78</td>
<td>0.17±0.05*</td>
</tr>
</tbody>
</table>

Compared to the control group: *p<0.05
DISCUSSION

The development, differentiation, and metabolism of almost every tissue in the body of mammals requires not only nutrients but also a variety of hormones. In this study, growth in mice was significantly inhibited by 100 mg/L F in drinking water, which is consistent with the results of our previous studies in rabbits. The F-induced decrease in body weight gain may be due to a reduction in food intake together with a depletion of soft tissue protein. Protein and calcium supplementation can alleviate these F-induced body weight changes. Thyroid hormone plays an important role in regulating body growth, and excessive F can induce thyroid dysfunction with decreased serum thyroid hormone levels. Thus F-induced inhibition of weight gain may be due to a decreased food intake, a depletion of protein in soft tissues and thyroid dysfunction.

Caspases are a class of apoptosis-related proteins, which can be divided into apoptotic initiators, including caspase-9, and apoptotic effectors, such as caspase-3. In the present study, the F-treated mice had ultrastructural changes in the thymus with a significant increase in apoptotic thymus cells. This may due to a F-induced up-regulation in the thymus in the relative expression levels of caspase-3 and caspase-9. Numerous studies have shown that F can induce apoptosis in different tissues. Matsui et al. demonstrated that NaF may induce apoptosis-like necrosis in rat thymocytes with increases in the population of shrunken cells and cells positive to annexin V, both of which are associated with an early stage of apoptosis. Miao et al. and Cheng et al. found that F-induced apoptosis was correlated with increased levels of Fas/FasL and the high expression of Bcl-2 and Bax. These mechanisms may account for the occurrence of F-induced apoptosis in thymocytes.

As is well known, DNA is the main genetic material of life, and DNA damage may alter cell functions, disrupt protein generation, and lead to carcinogenesis and mutagenesis. Our study showed that excessive F can lead to DNA damage in immune cells. The comet assay images showed that the DNA damage in bone marrow cells, blood lymphocytes, and thymocytes in F-treated mice was more serious than in the control group. F-induced apoptosis is apparently associated with the DNA damage. It is also well documented that oxidative stress is one of accepted mechanisms of F-induced toxicity. Moreover, a large number of studies have shown that DNA damage and oxidative stress are inextricably linked. Thus we can safely conclude that F-induced DNA damage in bone marrow cells, blood lymphocytes, and thymocytes is closely related to oxidative stress.

The total and differential WBC count is an important auxiliary diagnostic test as changes occur with systemic inflammation and other diseases. An increased granulocyte count has been associated with an increased risk of heart failure in apparently healthy men, while a decreased risk has been associated with an increased monocyte count. Since the mature immunocytes migrate from lymphoid organs to the peripheral blood and then exert their effects, a complete blood count is necessary to assess the immune status of the body. Huyan et al. showed that the immunosuppressive effect of cyclophosphamide can decrease the...
counts of WBC and lymphocyte in Balb/c mice. Moreover, systemic lupus erythematosus patients have decreased peripheral WBC numbers with low lymphocytes, and there is evidence that these reductions are caused by autoantibody-related apoptosis. In the present study, the total blood WBC count, together with the subpopulations of lymphocytes, middle cells, and granulocytes, were significantly decreased in the F group which is consistent with a F-induced apoptosis of immune cells.

In conclusion, excessive F can inhibit the growth and general health in mice and impair their immune functions by destroying the ultrastructure of the thymus, damaging the DNA in the thymus, bone marrow, and blood lymphocytes, and by decreasing the total and differential counts of WBC in female mice.

ACKNOWLEDGEMENT

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