DECREASED PERCENTAGES OF CD4+CD25+ REGULATORY T CELLS AND Foxp3 EXPRESSION IN THE SPLEEN OF FEMALE MICE EXPOSED TO FLUORIDE

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SUMMARY: CD4+CD25+ regulatory T cells (CD4+CD25+ Tregs) play an important role in suppression of immune response in humans and animals. Moreover, the transcription factor foxhead box P3 (Foxp3) is considered to be the molecular marker of CD4+CD25+ Tregs. Therefore, in the present study, in order to evaluate the toxic effects of fluoride (F) on the immune-regulating function of CD4+CD25+ Tregs, the percentages of such cells and Foxp3 expression in the spleen were determined in female mice exposed to 50, 100, and 150 mg NaF/L in their drinking water for 90 days. Compared with the control, the percentage of CD4+CD25+ Tregs in CD4+ T cells was significantly reduced with highest exposure to F. The Foxp3 gene expression in the 50, 100, and 150 mg NaF/L groups decreased by 13.2%, 26.3%, and 26.9%, respectively, compared to the control. The Foxp3 protein expression in the 100, and 150 mg NaF/L groups was also significantly reduced. These findings indicate that F-induced reduction of CD4+CD25+ Tregs may be associated with the inhibition of Foxp3 expression.

Keywords: CD4+CD25+ Tregs; Female mice; Fluoride and immune response; Foxp3 expression; Immune suppression; Mouse spleen.

INTRODUCTION

There are numerous examples of fluoride (F) toxicity in humans1 and animals such as in mice,2 guinea pigs,3 and even donkeys4 that impairs many organs like the teeth,3,5 testis,2 lung,6 kidney,7 thymus gland,8 and spleen.9 Recently, an increasing number of studies have reported the adverse effect of F on various immunocytes.8-11 CD4+CD25+ Tregs, an important type of immunocyte accounting for 5~10% of CD4+ T cells in peripheral blood in mice and humans,12-16 is professionally considered to have unique immuno-modulatory/inhibitory effects on cells.17 In vivo experiments with BALB/c athymic nude (nu/nu) mice showed that removal of CD4+CD25+ Tregs caused a variety of autoimmune diseases that were restrained by resuming CD4+CD25+ Tregs.17 However, exactly how CD4+CD25+ Tregs works in vivo after exposure of the host to high F still remains unknown.

On the surface of CD4+CD25+ Tregs, there are many functional elements, one of which is Foxp3 (foxhead box p3), a molecular marker of such cells.14,16 Foxp3 has been shown to partly control the maturity of CD4+CD25+ Tregs16,18 and to be involved in the inhibitory function of CD4+CD25+ Tregs.15,16

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In the present study, female mice exposed to 0, 50, 100, and 150 mg NaF/L in their drinking water were used to measure Foxp3 gene and protein expression levels in their spleens to investigate the mechanism by which F affects the immune-regulating function of CD4+CD25+ Tregs in their peripheral blood.

**MATERIALS AND METHODS**

*Experimental animals:* For the experiments, the Chinese Academy of Medical Sciences of Beijing supplied 48 healthy 7-week-old female Kunming mice, and the Institutional Animal Care and Use Committee of China approved all protocols. The mice were randomly divided into the following four groups of 12 mice receiving ultrapure drinking water treated as follows: (1) Control group without NaF; (2) 50 mg NaF/L group; (3) 100 mg NaF/L group; (4) 150 mg NaF/L group.

*Conventional analysis:* The daily water intake was recorded. On the 90th day, the mice were anesthetized by injecting 20% urethane and sacrificed by breaking their necks. The spleens were collected, and spleen indexes (spleen weight/body weight, SW/BW) were calculated.

*Flow cytometry (FCM):* Blood was collected by enucleating the eyes and mixed with ethylenediamine tetraacetic acid (EDTA) to prevent coagulation. Afterward, 1 mL of the blood was diluted with an equal volume of Hanks solution. The diluted blood was then trickled slowly down the side of a centrifuge tube containing 3 mL of lymphocyte separation medium, and the mixture was centrifuged at 1000 rpm for 20 min. Mononuclear cells were sampled from the boundary between two liquid layers. Three mL of Hanks solution was then added to the mononuclear cell solution. After centrifugation at 1500 rpm for 5 min, the supernatants were discarded, and the mononuclear cells were collected. The cells were re-suspended in 0.5 mL RPMI-1640 medium, and the passaged cells at a density of 1.0×10^6/mL were washed in PBS and the supernatants discarded. Anti-mouse FITC-CD4/PE-CD25 antibody (Bioscience, San Diego, CA, USA) was added and incubated for 20 min at 25ºC. Next, 2 mL of the RBC lysate was stirred gently and then incubated for 10 min at 25ºC. Afterward, the lysate was washed in PBS, 300 µL of Binding Buffer was added, and the mixture was monitored by FCM in the dark at room temperature.

*Real-time quantitative reverse transcriptase polymerase chain reaction (real-time QRT-PCR):* The spleens were crushed under liquid nitrogen, and total RNA was extracted as prescribed in the Trizol (Invitrogen, USA) manufacturer’s instructions for QRT-PCR. According to the alignments of the published mRNA sequences of β-actin gene and Foxp3 gene in mice from Genbank, two pairs of specific primers were used in this study designed by Primer 3 (Table 1.). The primers of Foxp3 gene are designed to amplify a 95-base pairs (bp) transcript. The endogenous housekeeping gene β-actin was used as control to normalize the quantity of Foxp3 transcripts with its primers designed to amplify an 83-bp transcript.
The expression level of Foxp3 gene was quantified by the method of Yan et al.\textsuperscript{19} QRT-PCR was conducted by using the Mx3000\textsuperscript{TM} QRT-PCR system (Stratagene, USA) and the Two-Step SYBR\textsuperscript{®} QRT-PCR kit (Takara, China). The QRT-PCR protocol included reverse transcription at 42°C for 5 min and an initial denaturation at 95°C for 10 sec. This was followed by 40 PCR cycles consisting of a denaturation step at 95°C for 5 sec, an annealing step at 62°C for 30 sec, and an extension step at 72°C for 10 sec. Finally, the melting curve analysis was performed at 95°C for 15 sec, at 60°C for 1 min, and at 95°C for 15 sec as in the protocol for the three reaction steps. The amplified products were analyzed by agarose gel electrophoresis.

\textit{Immunohistochemistry (IHC):} The protocol recommended for the Foxp3 immunohistochemistry kit was followed. Deparaffinized and rehydrated sections were selectively incubated separately using rabbit anti-Foxp3 polyclonal antibody (1:200, Bioss Co. LTD. Beijing, China) for 24 hr at 4°C and washed 3 times in PBS. Biotin-labeled anti-rabbit secondary antibody (Bioss Co. LTD. Beijing, China) was introduced, followed by incubation for 2 hr at 37°C. The specificity of the antibody was tested by omission of the primary antibody. After washing 3 times in PBS, strept avidin-biotin complex (SABC) was added followed by storage at 37°C for 20 min and washing 4 times by PBS for 5 min, after which the tissues were visualized with diaminobenzidine (DAB) and counterstained with hematoxylin. Finally, the sections were dehydrated in xylene and covered with a cover slide.

\textit{Statistical analysis:} Experimental data are expressed as mean values $\pm$ SD. The flow cytometer data were evaluated by T-test (Statistical Package for the Social Sciences, SPSS 11.5) to show a trend because there was no significant difference by using one-way ANOVA (analysis of variance). The other data, however, were analyzed by one-way ANOVA, and the significance between pairs was determined by Tukey’s Multiple Comparison Test. Levels with $p<0.05$ were considered statistically significant.

\textbf{RESULTS}

\textit{Water intake and SW/BW:} With increasing in F concentration, there was a decrease in water intake and the ratio of spleen weight to body weight (SW/BW).
However, compared with the control group, no statistically significant change was found (Table 2).

<table>
<thead>
<tr>
<th>NaF (mg/L)</th>
<th>Water intake (mL/day) (n=12)</th>
<th>Spleen index (g/kg) (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.86±1.04</td>
<td>3.34±0.32</td>
</tr>
<tr>
<td>50</td>
<td>5.43±0.99</td>
<td>3.13±0.31</td>
</tr>
<tr>
<td>100</td>
<td>5.28±1.01</td>
<td>3.01±1.04</td>
</tr>
<tr>
<td>150</td>
<td>5.19±0.92</td>
<td>2.91±0.92</td>
</tr>
</tbody>
</table>

*Table 2. Water intake and spleen index of mice in four groups (Values are mean±SD)*

*Flow cytometry (FCM):* The scatter diagrams of CD4⁺CD25⁺ Tregs are presented in Figure 1. As seen in Table 3, the percentages of CD4⁺CD25⁺ Tregs in the three F treatment groups decreased significantly in a dose-dependent manner.

*Figure 1. Effects of F on CD4⁺CD25⁺ Tregs in peripheral blood of female mice. Adult female mice exposed to 0 (A), 50 (B), 100 (C), and 150 mg NaF/L (D) for 90 days. After the treatment, scatter diagrams of CD4⁺CD25⁺ Tregs were obtained by FCM.*
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Quantification of Foxp3 gene expression: The relative expression level of Foxp3 mRNA in the spleen of the mice is shown in Figure 2. Compared with the control group, the expression level of Foxp3 gene in the 50 mg NaF/L group did not decrease significantly. However, the expression levels of Foxp3 gene in the 100, 150 mg NaF/L groups were significantly decreased (p<0.05).

![Figure 2](image)

**Figure 2.** Effects of F on Foxp3 mRNA expression in spleen of adult female mice exposed to 0, 50, 100, and 150 mg NaF/L for 90 days. After the treatment, the expression levels of Foxp3 gene were analyzed by real time QRT-PCR, calculated on the basis on the threshold cycle (Ct) for each well using the provided software and normalized to β-actin for SYBE Green assays as endogenous controls. *p<0.05.

Foxp3 protein expression: As seen in Figure 3, Foxp3 protein was predominantly localized in the cytoplasm in spleen. The Foxp3 protein expression level in the spleen is demonstrated in Table 4. Compared with the control group, the Foxp3 protein expression levels in the 50, 100, and 150 mg

<table>
<thead>
<tr>
<th>NaF (mg/L)</th>
<th>Percentage (%) (n=12)</th>
<th>Relative to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.13±1.38</td>
<td>1</td>
</tr>
<tr>
<td>50</td>
<td>6.98±1.52*</td>
<td>0.765</td>
</tr>
<tr>
<td>100</td>
<td>6.90±1.92*</td>
<td>0.756</td>
</tr>
<tr>
<td>150</td>
<td>3.82±1.01*</td>
<td>0.418</td>
</tr>
</tbody>
</table>

*Compared with the control, *p<0.05
NaF/L groups were significantly different \((p<0.01)\). Moreover, in the 100 mg NaF/L group, they also differed significantly from the 50 mg NaF/L group \((p<0.01)\). They were also significantly different \((p<0.05)\) between the 100 and 150 mg NaF/L group.

**DISCUSSION**

In our previous study, serious toxic disturbances in specific immune function organs of rabbits occurred from excessive F intake.\(^8\) Recently, Liu et al. reported that F adversely affected the proliferation and development of T cells in chicken broilers.\(^10\) Based on these earlier investigations, this study, for the first time, focused on whether the alteration of \(\text{CD}^4\text{CD}25^+ \text{T}_{\text{regs}}\), one of the most important regulatory T cells,\(^10-12\) occurs through alterations in Foxp3 expression in the spleen of mice treated with high F. Results indicate a negative dose-dependent effect of F on the percentages of \(\text{CD}^4\text{CD}25^+ \text{T}_{\text{regs}}\), and gene and protein levels of Foxp3, suggesting that F may threaten the immunoregulatory function.

Since immune organ indexes can reflect the state of immune function, the degree of spleen damage reflected by the ratio of spleen weight to body weight (SW/BW) was chosen here for study. Results showed that SW/BW decreased with increased concentration exposure to F compared with the control group. Moreover, F
exposure also puts a curb on the growth of the mice. Thus the reduced SW/BW ratio suggests that the immune lymphocyte function might be adversely impacted.

CD4+CD25+ Tregs, representing 5–10% of the total CD4+ T cells in the peripheral circulation, plays an important role in controlling immune response. Butler et al. showed that excessive F caused changes in T cell subsets and CD4+/CD8+ ratios, thus disrupting cell immunity. In the present study, the percentages of CD4+CD25+ Tregs in CD4+ T cells decreased in a dose-dependent manner after exposure of the mice to 50, 100, and 150 mg NaF/L in their drinking water. These changes in CD4+CD25+ Tregs may be due to the inhibition of T cell proliferation induced by F as suggested in the report by Butler et al.

Foxp3 is known to be specifically expressed in CD4+CD25+Tregs of peripheral blood and is therefore viewed as the molecular marker of such cells. Excess expression of Foxp3 gene may lead to the increased production of regulatory T cells and can convert CD4+CD25–Tregs into CD4+CD25+Tregs to achieve immunomodulatory effects. After knockout of the Foxp3 gene, CD4+CD25+ Tregs are lacking and can cause autoimmune lymphoproliferative diseases. In the current study, the expressions of Foxp3 mRNA and protein were significantly decreased in the F treatment groups, thereby suggesting that the quantity and function of CD4+CD25+ Tregs are suppressed in female mice administrated increasing doses of F.

ACKNOWLEDGEMENTS

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