THE IMMUNOTOXIC EFFECTS OF FLUORIDE ON MICE AFTER SUBACUTE ADMINISTRATION BY EVALUATING CYTOKINE mRNA EXPRESSION IN SPLENOCYTES

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SUMMARY: The fluoride ion (F), an environmental pollutant, affects the cytokine mRNA expression of macrophages *in vitro*, but it is not clear whether or not these effects occur *in vivo*. In this study, we examined the effects of F on cytokine mRNA expression in splenic macrophages of mice after subacute oral administration for 1 mo as well as determining serum F. BALB/c mice were administered F at 0, 1, 5, 25, and 125 ppm in their drinking water. At the end of the 1-mo administration, the serum F was determined. Each spleen was sampled, the lymphocytes were separated as non-adherent cells, and the macrophages were separated as adherent cells. The macrophages were activated by lipopolysaccharide and the lymphocytes were activated by phytohemagglutinin. After the activation and incubation, the RNA was extracted and cDNA was synthesized. We examined the mRNA expressions of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) for macrophages and those of interferon-γ (IFN-γ) and IL-2 for lymphocytes by RT-PCR (reverse transcription polymerase chain reaction). The mean intake of food or water per body weight was significantly lower in the 125-ppm group compared to the other groups. The relative weights of spleens in the 1- and 5-ppm groups were significantly lower than those in the control. The mRNA expression of TNFα in the macrophages was lower in the 125-ppm group. There were no differences among the groups for the mRNA expression of IL-1β in macrophages and those of IFN-γ and IL-2 in lymphocytes. Significant lower relative weights of the spleens were observed in a non-dose-dependent manner. Although there was no statistically significant difference in the expression of mRNA of TNFα in macrophages among the groups, the value in the 125-ppm group was lower. Although F may affect TNFα expression *in vivo*, the inability to demonstrate this in the present study may have been due to the F concentration in the blood not being sufficiently high compared with the value which affected mRNA expression in macrophages *in vitro*.

Keywords: Fluoride; Macrophages; Spleen; TNFα

INTRODUCTION

Endemic fluorosis, including skeletal fluorosis, occurs in many in India[1] and China,[2] and other areas in the world due to the presence in the drinking water or food of the fluoride ion (F). The formation of fluoroapatite is the main mechanism for the development of bone sclerosis with F.[3] While, as has been pointed out recently, cytokines are involved in bone metabolism, tumor necrosis factor-α

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(TNFα) is associated with bone resorption by the induction of differentiation to osteoclasts.[4] It is possible that bone metabolism is altered by cytokines.

The toxic effects of F on cytokine-producing cells such as macrophages have been demonstrated in several studies.[5,6] In the study by Hosokawa et al.,[6] the levels of relative mRNA expressions of interleukin-1β (IL-1β) and TNFα of the cells exposed to F at 1 mM and lipopolysaccharide were significantly higher than those of the control. The mRNA expression of TNFα exposed to 300 μM of F was rather lower than that of the control, and became significantly higher than the control with 1 mM of F.[6] It is of interest whether or not the gene expressions of IL-1β and TNFα in the macrophages from the mice exposed to F in their drinking water are altered as *in vitro*. In addition, F may be toxic for lymphocytes as it is for macrophages. The mRNA cytokine expression in lymphocytes may also be altered by F.

BALB/c mice have been used for screening the immune reaction to chemical compounds. In this study, the mRNA expression of cytokines in the splenic macrophages and lymphocytes from BALB/c mice exposed to F via drinking water for 1 mo were examined. In addition, for examining the systemic effects, the F concentration in plasma and the organ weights were determined for the BALB/c mice.

MATERIALS AND METHODS

In the present study, we used 4–5-week-old male BALB/c mice. The mean body weight on the first day of the experiment was 23.2±0.2 g (mean±standard error). Mice were housed in standard cages, on 12 hr light/dark cycle and air temperature was maintained at 22±2ºC.

The concentrations of NaF (MW=41.99, Nacalai Tesque, Kyoto, Japan) in the drinking water were 1, 5, 25, and 125 ppm. The number of mice for each group was six. The control group mice were given tap water (F<0.8 mg/L). The mice were administered F for 1 mo with free access to drinking water. CE-2 rodent chow (CLEA Japan, Tokyo, Japan) was fed *ad libitum*. The mice were euthanized by decapitation at the end of the observation period, and the blood and spleen of each mouse was sampled. The blood was centrifuged at 3000 rpm × 10 min and the F concentrations were determined by flow injection analysis with an F ion-selective electrode as a detector.[7] All the experiments were performed according to the guidelines for the care of laboratory animals of the Ethics Committee of Kitasato University of Medical Sciences.

The mRNA was extracted from the splenic macrophages and lymphocytes according the method described by Kido et al.[8] Briefly, to prepare the splenic cell suspension, the spleen from each mouse was homogenized with RPMI 1640 medium containing 100 U of penicillin and 100 μg of streptomycin (Nikken, Kyoto, Japan) with 5% heat inactivated fetal bovine serum (Equitec Bio, Kerrville, TX) using a stomacher (BD Falcon, Tokyo, Japan). The red blood cells were removed by adding 5 mL of red blood cells lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA [ethylenediaminetetraacetic acid]). The splenic cells

were incubated at 37ºC, 5% CO_2 for 1 hr in a 24-well culture plate, and were divided into non-adherent cells (macrophages) and adherent cells (lymphocytes). The lymphocytes were transferred to another a 24-well plate. The cell number was adjusted to 1×10^6 in each well.

The macrophages were activated by lipopolysaccharide at 100 ng/mL and cultured for 6 hr, after which, the RNA of the macrophages was extracted using TRIzol (Life Technologies, Grand Island, N.Y.). The lymphocytes were activated with PHA (phytohemagglutinin) at the concentration of 0.5 μg/mL and cultured for 12 hr. The mRNA of the lymphocytes was extracted by TRIzol after cultivation. The cDNA was synthesized from extracted RNA by using a First-Strand cDNA synthesis Kit (Amersham Pharmacia Biotech, Picataway, NJ). The relative mRNA expression of TNFα and IL-1β in the splenic macrophages and of interferon-γ (IFN-γ) and interleukin-2 (IL-2) in the splenic lymphocytes were examined by RT-PCR. β-actin was used for the internal standard. The primers used were:[9]

(i) for TNFα:

5'GTTCTATGGCCCAGACCCTCACA-3' and 5'-TCCCAGGTATATGGGTTTCATACC-3'

(ii) for IL-1β:

5'-GCAACTGTTCCTGAACTCA-3' and 5'-CTCGGAGCCT-GTAGTGCAG-3'

(iii) for IFN-γ:

5'-AACGCTACACACTGCATCT-3' and d5'-AGCTCATTGAATGCTTGG-3'

(iv) for IL-2:

5'-CTCGCATCCTGTGTCACATT-3' and 5'-ATCCTGGGGAGTTTCAGGTT-3'

(v) for β-actin:

5'-ATGGATGACGATATCGCT-3' and 5'-ATGAGGTAGTCTGTCAGGT-3'

The thermal cycles consisted of denaturation at 94ºC for 30 sec, annealing at 54ºC for TNFα, 56ºC for IL-1β, 72ºC for β-actin, 48ºC for IFN-γ and IL-2 for 30 sec, and extension at 72ºC for 1 min, at 5 min. The number of cycles was optimized for 35 followed by final extension at 72ºC. The amplified products were fractionated on 2% agarose gel containing 0.476 μM ethidium bromide. The bands were quantified using the Print Graph Image Saver and Lane & Spot Analyzer Ver.5.0 (ATTO, Tokyo). The quantified value for each cytokine, was adjusted with the respective β-actin band.

The mean water intake of each mouse and the mean daily water intake per body weight for the groups were calculated. The mean F intake was calculated based on the water intake. The mean serum F body weight and the relative spleen weight were also calculated for the groups. The mean value of relative mRNA expression of each cytokine was compared among the groups. These mean values were compared by one-way ANOVA (analysis of variance) using Statview 5.02 v (SAS, Cary, CA), followed by the Fisher PLSD test (significance level, $p < 0.05$).

RESULTS

No mice died during the observation period. Based on the water intake, the mean value of F intake per body weight for the 4-week period was calculated for the groups as follows: 0.17 mg/kg in the 1-ppm group, 0.83 mg/kg in the 5-ppm group, 4.52 mg/kg in the 25-ppm group, and 22.91 mg/kg in the 125-ppm group. Figure 1 shows the serum F concentration in the mice at the end of the treatment period. The mean serum F concentration in the 125-ppm group was significantly higher than that in the control, 1-, and 5-ppm groups. The serum F level of the 125-ppm group was 29.3 μg/L. The serum F level in the 25-ppm group was also was significantly higher than that in the control, 1-, and 5-ppm groups.

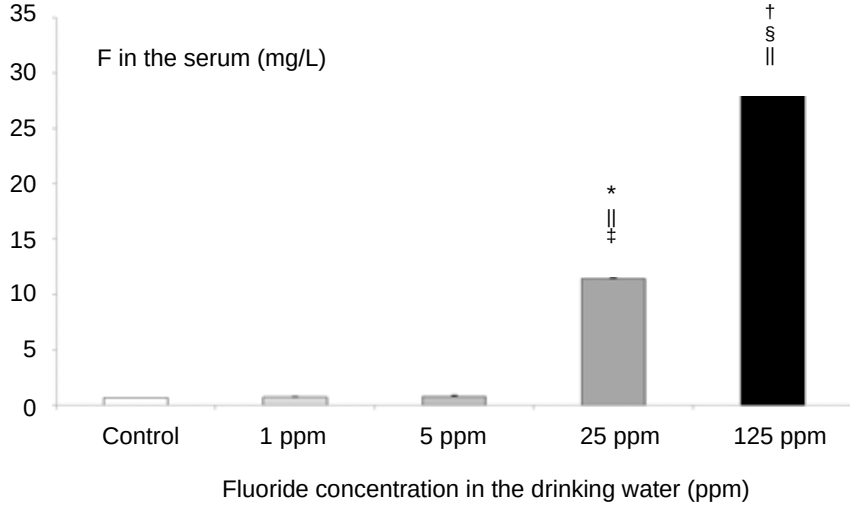

Figure 1. The serum F concentration in the mice at the end of the treatment period. Each bar represents the mean value, and error bars represent standard errors. p=0.0001 by ANOVA for BALB/c mice. *p<0.01 compared to the control, †p<0.001 compared to the control, ‡p<0.01 compared to the 1 ppm, §p<0.001 compared to the 1 ppm, ‖p<0.001 compared to the 5 ppm by the Fisher's PLSD test.

The mean values of body weight and the relative spleen weight for mice at the end of the observation period are shown in Table 1.There was no significant difference in body weight among the groups. The mean relative spleen weight in the 1-ppm group was significantly lower compared with that in the control. The mean relative spleen weight in the 1-ppm group was also significantly lower compared with those in the 25- and 125-ppm groups. Table 2 shows the relative mRNA expressions of cytokines in splenic lymphocytes. There were no significant differences among the groups for either IL-2 or IFN-γ.

Table 1. Body weight and relative organ spleen weight (mg/g bw) of mice treated with fluoride (F) via their drinking water

Group	Final body weight (g) (mean±SE)	Spleen (mg/g bw) (mean±SE)
Control	27.60±1.17	4.20±0.15
1 ppm	26.93±0.35	3.71±0.06*
5 ppm	28.15±0.48	3.88±0.11
25 ppm	28.20±0.46	$4.52\pm0.08^{\ddagger,\S}$
125 ppm	26.90±0.56	$4.14\pm0.14^{\dagger,\|\|}$

Mean values of the body and relative organ weights of BALB/c mice exposed to F at the end of the observation period. The final body weights and spleen weights are given as the mean value and standard errors. p=0.0001 by ANOVA for spleen, *p<0.01 compared to the control; †p<0.05 compared to the 1-ppm group; ‡p<0.001 compared to the 1-ppm group; §p<0.01 compared to the 5-ppm group; $^{\|\|}$p<0.05 compared to the 25-ppm group by Fisher's PLSD test.

Table 2. The relative mRNA expression for IL-2 and IFN-γ in splenic lymphocytes for BALB/c mice exposed to F for 1 month

Group	IL-2/β-actin	IFN-γ/β-actin
Control	0.66±0.23	0.13±0.03
1 ppm	1.05±0.49	0.24±0.17
5 ppm	1.07±0.33	0.27±0.08
25 ppm	1.38±0.52	0.14±0.08
125 ppm	0.58±0.31	0.44±0.10

Mean±SE are indicated (n=6). p=0.6228 by ANOVA for IL-2, p=0.1847 by ANOVA for IFN-γ.

Figure 2 illustrates relative mRNA expression of IL-1β in splenic macrophages

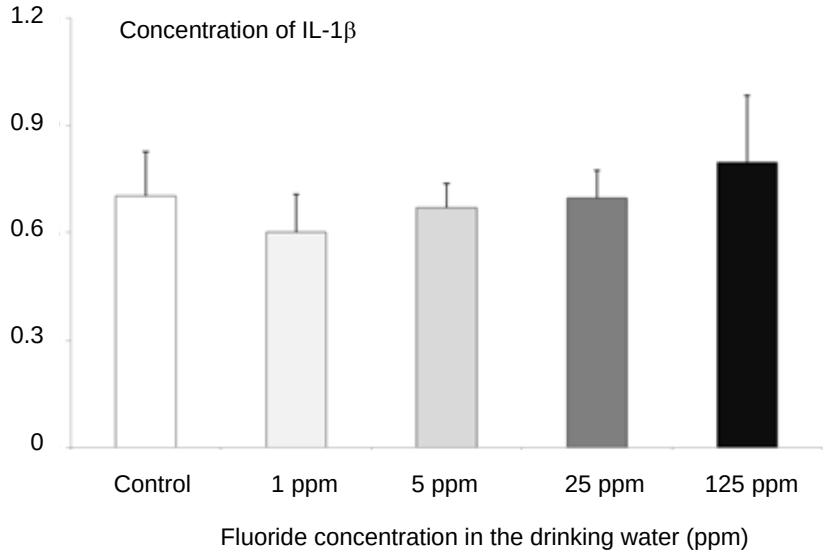

Figure 2. The mRNA expression for IL-1β in the splenic lymphocytes from the mice exposed to F in drinking water for 1 month. p=0.8276 by ANOVA. Each bar represents the mean value, and error bars represent standard errors (n=6).

Figure 3 illustrates the mRNA expression of TNFα. There were no significant differences among the groups for either IL-1β (p=0.8276) or TNFα (p=0.192). However, the relative expression of mRNA of TNFα for the 125-ppm group was lower than that in the control.

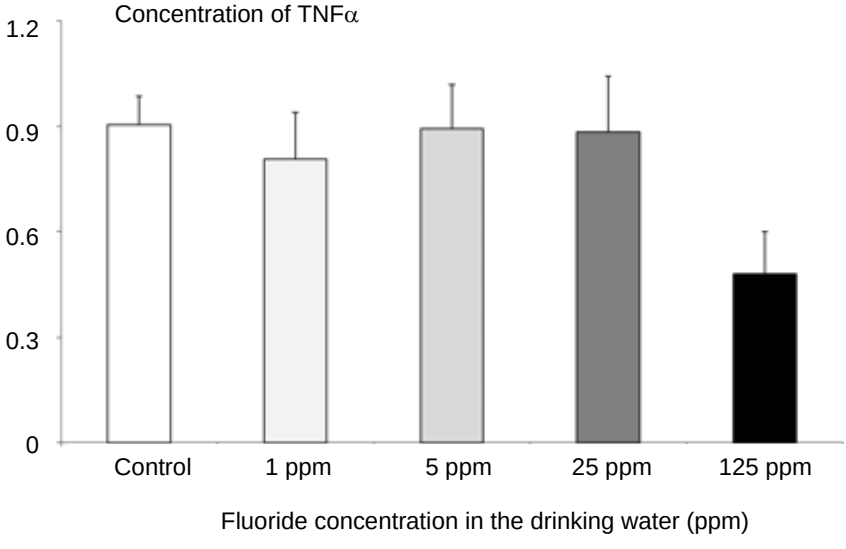

Figure 3. The mRNA expression for TNFα in the splenic lymphocytes. p=0.1923 by ANOVA. Each bar represents the mean value, and error bars represent standard errors (n=6).

DISCUSSION

In an aqueous solution, fluorine exists as an ion, F. The detrimental health effects of F have been reported extensively in China, India, and in other countries. In the Rajasthan region of India where endemic fluorosis has been reported,[10] the F concentrations in the drinking water were 1.5 mg/L or more in 13 regions and 3.0 mg/L or more in 5 regions. Fluorosis and bone sclerosis were observed among the people in the Sirsi village of India, where the F concentration in the drinking water was 5.91 mg/L. In the municipality of São João do Rio do Peixe, located in the tropical semiarid lands of Brazil, in a total of 111 groundwater samples the F concentration varied from 0.11 to 9.33 mg/L with 30% percent of the samples showing values above 1.5 mg/L.[11] Mapping of the fluoride distribution indicated that approximately 2,465 people in this region could be affected by dental fluorosis and 1,057 people might be affected by skeletal fluorosis.

The mechanism of the development of bone fluorosis has been explained by the formation of fluoroapatite. However, in recent years, the role of the immune system for bone metabolism has attracted attention. In a previous study,[5] F affected the macrophages and their production of TNFα which is related to bone metabolism. The mean value of cell viability of J774.1, a macrophage-lineage cell, in 2 mM (380 μg/L) of F was significantly lower than that in the control. The levels of mRNA expressions of IL-1β and TNFα of the cells exposed to F at 1 mM (190 μg/L) were significantly higher than those in the control.[6] The production of these cytokines were significantly lower in these macrophages at 1 mM of F. Hosokawa et al.,[6] found that at exposure to 300 μM of F, the mRNA expression was lower than that in the control. If these alterations in the mRNA expression or production of these cytokines was observed *in vivo*, they could alter bone metabolism. In addition, for the evaluation of general condition and specific effects on the spleen, it is necessary to measure both body weight and spleen weight.

In the present study, we examined whether the effects of F observed *in vitro* on mRNA expression and cytokine production also occurred *in vivo.* In addition, we looked for effects of F on body and spleen weight. The BALB/c mice were exposed to up to 125 ppm F in their drinking water for 1 mo, and no mice died. This result was in accordance with the results of the ICR mice, which were exposed to 150 ppm in their drinking water in our previous study.[12] Although there were no significant differences in the body weights among the groups, the mean relative spleen weight in the 1-ppm group was significantly lower compared with that in the control, 25-, and 125-ppm groups. The mean relative spleen weight of 25-ppm group was significantly higher than those in the 1- and 125-ppm groups. The effect of F on the relative spleen weight was not dose dependent. In the previous studies of Hosokawa et al.,[12,13] there was no significant difference in the relative spleen weight between the ICR mice exposed to 0 and 150 ppm F. However, the mean relative spleen weight of the ICR-derived glomerulonephritis (ICGN) mice (the renal insufficiency model) exposed to 150 ppm F was significantly lower compared to those in the 0-, 25-, and 50-ppm groups. Because

of the renal insufficiency, the F levels in the serum of the ICGN mice were comparatively high, and the spleen weight may have decreased because of the high level of F. In the present study, the decrease in relative spleen weight was only observed in the groups exposed to lower doses. The reason for this was not clear. The serum F level started to elevate in the group exposed to 25 ppm F. However, the level of F in the 125-ppm group did not reach the level to kill splenocytes. The serum F in the 125-ppm group was 29.3 μg/L, which was low compared with 1 mM F, equal to 190 μg/L.

For the mRNA expression of cytokines in the splenic lymphocytes, there was no significant difference for the expressions of IL-2 or IFNγ in splenic lymphocytes between the groups. F may not affect lymphocytes seriously.

For the relative mRNA expressions of cytokines in the splenic macrophages, although there was no significant difference among the groups for either TNFα or IL-1β, the mean value of the mRNA expression of TNFα was low. In previous *in vitro* studies, the mRNA expression of TNFα in the J774.1 macrophages linage cells exposed to 1 mM were analyzed by RT-PCR and real time PCR.[5,6] In a study in 2009,[5] the mRNA expression of TNFα was low in the J774.1 cell in which the viability was low, but the difference did not reach a significant level. In another study in 2011,[6] the mRNA expression of TNFα was significantly higher in the macrophages exposed to 1 mM than that in the control. The viability of the macrophages exposed to 1 mM was significantly lower than the control. Although there was inconsistency between the studies, the increase in mRNA of TNFα expression *in vitro* may be reaction to the death of splenocytes and a decrease in the production in TNFα.[6] In this *in vivo* study, a low mRNA expression of TNFα in the 125 ppm group was observed. In Hosokawa et al.,[6] although it was not significantly different, the mRNA expression of TNFα was lower in the cells exposed to 300 μM (29.3 mg/L) F. F may decrease mRNA expression of TNFα in the macrophages at the level in body induced by drinking water with 150 ppm F. In the present study, the F concentration in the body might not be high enough for the increase in the mRNA expression of TNFα. It is possible that F accumulates in bone, where the immune system is involved in metabolism, and the accumulated F alters the immune system in the bone. In the present study, the observation period was a relatively short-term of 1 mo. In the future, longer observations will be necessary to examine whether or not cytokine expression in bone might be decreased by F.

In addition to a relatively shorter period of observation, there were other limitations to this study. We only determined the mRNA expressions by semi-quantitative RT-PCR and we did not determine the protein levels of cytokines in the supernatant of the cultured splenocytes. For mRNA analyses, a quantitative real time PCR is better to determine the expression. Also, the determination of the protein levels of cytokines produced by splenocytes from mice after the administration of F would substantially strengthen the results of this study. In future studies, these limitations should be reduced by using real time PCR for mRNA analyses and the determination of protein level by ELISA (enzyme-linked

immunosorbent assay). In addition, although we determined the spleen weights of mice as a screening measure, we did not examine the pathological changes in the spleen. In further studies, the pathological examination of the spleen of mice exposed to F would also strengthen the data.

CONCLUSION

In conclusion, the administration to rats of F for 1 mo at 125 ppm in drinking water induced an elevation in the serum F but the increased level was not sufficient to induce significant alterations in mRNA expression of cytokines in splenocytes. However, a lower mRNA expression of TNFα was observed suggesting that the effects of F on macrophages are different at different doses.

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