

## CHANGES IN FLUORIDE LEVELS IN THE LIVER, KIDNEY, AND BRAIN AND IN NEUROTRANSMITTERS OF MICE AFTER SUBACUTE ADMINISTRATION OF FLUORIDE

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**SUMMARY:** The effects of fluoride after subacute oral administration of NaF at levels of 0, 1, 5, 25, and 125 ppm F<sup>-</sup> were evaluated in adult male BALB/c mice. Fluoride levels in the murine liver, kidney, and cerebrum after one month were determined using a highly sensitive flow-injection apparatus with a fluoride ion selective electrode as the detector. To examine for neurotoxic effects, levels of regional brain neurotransmitters and their metabolites were determined by a high performance liquid chromatography procedure. Water intake increased among the groups administered 25 and 125 ppm F<sup>-</sup>, and the concentrations of fluoride in liver and kidney were significantly increased among the 125 ppm group compared to the control. On the other hand, the fluoride levels in the cerebrum were not significantly different among the groups. However, a significant difference was observed in homovanillic acid (HVA) in the hypothalamus. Significant differences in the levels of neurotransmitters and their metabolites were not observed in other brain regions.

Keywords: BALB/c mice; Brain fluoride; Kidney fluoride; Liver fluoride; Neurotransmitters; Subacute fluoride.

### INTRODUCTION

Extensive contamination of groundwater by fluoride has been reported in China<sup>1</sup> and India,<sup>2</sup> where endemic fluorosis continues to be prevalent. Recently, we have developed a highly sensitive method for determining fluoride in biological samples.<sup>3,4</sup> With this method, fluoride levels in internal organs of experimental animals can be accurately measured. In various studies, oral administration of chemicals to mice for one month has been used as a simple screening model of environmental exposure. For fluoride, subacute administration may also be adequate as a model for environmental exposure, and the determination of the resulting fluoride levels in internal organs afterwards is of interest and useful to evaluate the adequacy of the method as a model.

There are disagreements about the toxic effects of fluoride on internal organs. The kidney is known to be a target organ of fluoride among internal organs,<sup>5</sup> but the effects of fluoride on the liver and brain are not clear. Manocha et al.<sup>6</sup> administered fluoridated water to the squirrel monkeys for 18 months at the concentrations of 0, 1, and 5 ppm fluoride. Significant cytochemical changes were observed in the kidneys, especially of the monkeys on 5 ppm fluoride intake in

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their drinking water. For the liver, the activities of Krebs cycle enzymes were slightly enhanced in the groups administered fluoride. The nervous system appeared to be unaffected. On the other hand, Mullenix et al.<sup>7</sup> demonstrated that the exposure to fluoride via drinking water significantly altered the behavior of female rats compared to the controls. It is of interest, therefore, to know whether neurological effects can be induced in mice by oral exposure to fluoride. For such evaluation, adequate indexes are required, e.g., alterations in neurotransmitters (catecholamines, indoleamine) and their metabolites, which serve as indicators of toxic effects in the central nervous system.<sup>8-10</sup>

The purpose of this study was to determine the fluoride levels in organs (liver, kidney, and brain) of mice exposed to subacute levels of fluoride via drinking water for one month. The neurological effect of fluoride was also examined by determining neurotransmitter levels and their metabolites.

### MATERIALS AND METHODS

*Animals:* Adult male BALB/c mice (4-5 weeks of age) were obtained from Oriental Bioservice (Tokyo). The initial mean body weight of the mice was  $22.1 \pm 0.2$  g ( $\pm$ standard error). The mice were acclimated for one week in a housing facility and maintained on commercial rodent chow *ad libitum* at 21°C with a 12-hr light/dark cycle before treatment. The mice were randomly assigned to a treatment group (six per group) and housed in polycarbonate cages. The food and water consumption per group as well as the body weight of each mouse were monitored daily. The care and treatment of mice were in accordance with the guidelines established by Fukushima Medical University's Institutional Animal Care and were approved by the Use Committee.

*Treatment and sampling:* The mice were given sodium fluoride dissolved in distilled water at the concentrations of 0, 1, 5, 25, 125 ppm fluoride ion in their drinking water *ad libitum* for one month. Following the treatment period, the mice were euthanized, and the liver and kidney were removed and weighed. Brain samples were dissected into six regions (cerebrum, cerebellum, medulla oblongata, midbrain, corpus striatum and hypothalamus) according to the method of Glowinski and Iversen.<sup>11</sup> Each cerebrum sample was further divided into two portions for the analyses of fluoride and neurotransmitters. Tissue sampling was conducted at midmorning to avoid possible diurnal alterations in neurotransmitter levels.<sup>12</sup> Immediately after dissection, brain samples were soaked in ice-cold 0.05 M perchloric acid (Wako, Osaka) with 0.1% cysteine (Nacalai Tesque, Kyoto) in tared vials. The ratio of brain tissue to extraction solvent was approximately 1:4 (tissue weight/volume). After weighing, each brain sample was homogenized for the extraction of the neurotransmitters and their metabolites, and centrifuged using a 0.2  $\mu$ m pore-size filter (Millipore, Bedford, MA). The filtrate was stored at  $-80^\circ\text{C}$  until analysis.

*Determination of fluoride levels in cerebrum, liver, and kidney:* Fluoride in each organ was isolated by use of a pyrohydrolysis apparatus (Daiwa Denshi, Kyoto) and recovered into water.<sup>3</sup> The levels of isolated fluoride in the water were determined by a flow-injection apparatus with a fluoride ion selective electrode as the detector (Daiwa Denshi) by the method previously described.<sup>3,4</sup>

*Determination of neurotransmitters and their metabolites:* The levels of the catecholamines norepinephrine (NE) and dopamine (DA), DA metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), indoleamine serotonin (5-hydroxytryptamine, 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were simultaneously determined in each sample by high performance liquid chromatography (HPLC) with an electrochemical detector by a modification of the manufacturer's protocol (GL Science, Tokyo). The analysis system consisted of a GL Science ED623 electrochemical detector (GL Science), an Hitachi L-6250 pump (Hitachi, Tokyo), a GL Science DG660 degasser, and a Sugai U620V #50 column heater with a temperature controller (Sugai Chemie, Wakayama). A reversed phase column, Inertsil ODS-3, 4.6×150 mm, particle size 5 µm (GL Science) was employed for chromatography. The mobile phase was composed of 9.6 g/L citric acid, 100 mg/L sodium octane sulfate, 40 mg/L EDTA, and 15% methanol. Samples were eluted at 35°C for 40 min at a flow rate of 0.75 mL/min. A calibration standard (100 ng/mL) containing NE bitartrate, DA hydrochloric acid, DOPAC, HVA creatinine sulfate, and 5-HIAA dicyclohexylammonium salt (Sigma) in 0.05 M perchloric acid with 0.1% cysteine was employed.

*Statistical analyses:* The mean values of fluoride, neurotransmitters, and their metabolites of the groups were compared by one-way ANOVA followed by Fisher's PLSD test as a *post hoc* test.

## RESULTS

Table 1 shows the mean daily water and food consumption by the mice over the one-month exposure period. The group administered 25 ppm fluoride in their drinking water had a significantly higher mean value of water consumption compared to those in the control, the 1 ppm, and the 5 ppm groups. The group administered 125 ppm fluoride also had significantly higher mean values of water consumption compared to those in the control and 1 ppm group. There was no significant difference in food consumption among the groups.

**Table 1.** Water and food consumption by mice administered fluoride in drinking water (mean ± standard error; n = 6)

Group	Water consumption (mL/mouse/day)	Food consumption (g/mouse/day)
Control	4.58±0.13	3.444±0.054
1 ppm F	4.61±0.11	3.528±0.040
5 ppm F	4.71±0.07	3.638±0.038
25 ppm F	5.10±0.12***†††‡	3.697±0.106
125 ppm F	4.93±0.12*†	3.733±0.123

\*p<0.05, \*\*\*p<0.001 compared to the control.

†p<0.05, †††p<0.001 compared to the 1 ppm group.

‡p<0.05 compared to the 5 ppm group.

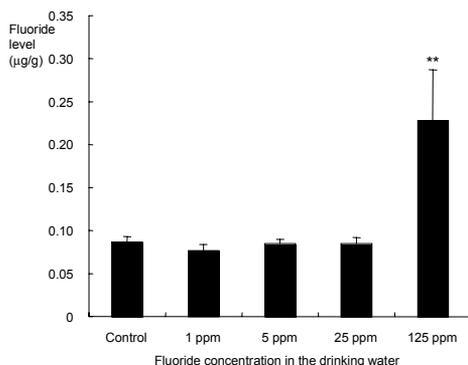
Table 2 shows the mean body and relative organ weight of the mice of each group of mice. There were no differences in mean body weight and relative organ

weights of the kidney and liver among the groups. The mean intake of fluoride of each group, based on the mean body weight on each day and daily water consumption of each group, was 0.17 mg/g body weight for the 1 ppm group, 0.83 mg/g body weight for the 5 ppm group, 4.52 mg/g body weight for the 25 ppm group, and 22.91 mg/g body weight for the 125 ppm group.

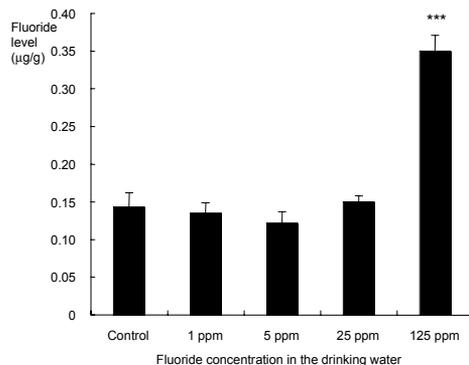
**Table 2.** Body weight (g) and relative organ weights (mg/g b.w.) of mice treated with fluoride (mean ± standard error; n = 6)

Group	Final body weight (g)	Liver (mg/g b.w.)	Kidney (mg/g b.w.)
Control	27.60±1.17	55.55±1.62	15.40±0.30
1 ppm F	26.93±0.35	55.10±1.17	15.29±0.28
5 ppm F	28.15±0.48	55.18±0.97	15.16±0.14
25 ppm F	28.20±0.46	55.74±1.53	14.91±0.31
125 ppm F	26.90±0.56	56.28±0.74	15.28±0.32

Figure 1 illustrates the mean values of fluoride accumulation in the liver, showing a slight, nonsignificant downward trend to 1 ppm, followed by slight increases at 5 and 25 ppm, and then a significantly higher mean value at 125 ppm compared to the other groups. A similar pattern was observed in the kidney (Figure 2) with a nonsignificantly different lowest value at 5 ppm and a significantly higher value at 125 ppm compared to the other groups.

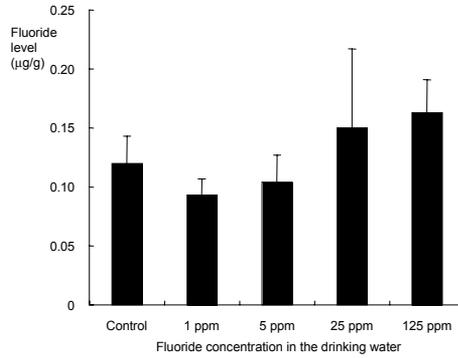


**Figure 1.** Fluoride levels in the liver from mice administered fluoride through drinking water. Each bar represents mean value. Error bars represent standard error.  $p < 0.01$  by ANOVA.  $**p < 0.01$  compared to the other groups by Fisher's PLSD test.



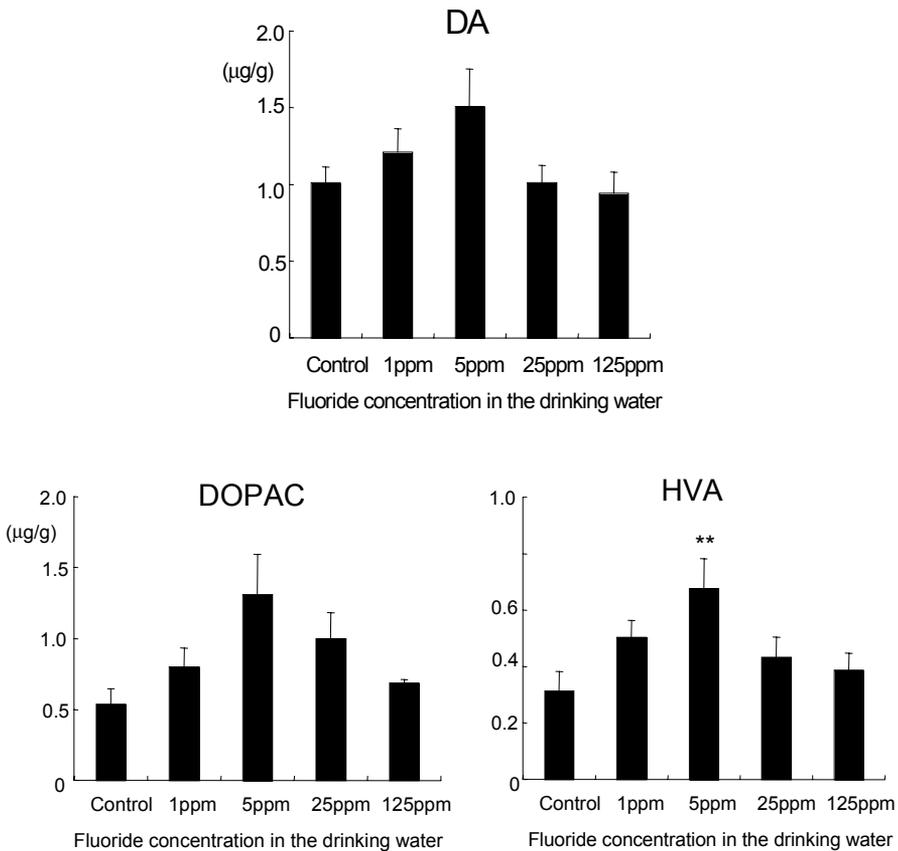
**Figure 2.** Fluoride levels in the kidney from mice administered fluoride through drinking water. Each bar represents mean value. Error bars represent standard error.  $p < 0.0001$  by ANOVA.  $***p < 0.0001$  compared to the other groups by Fisher's PLSD test.

Figure 3 illustrates the fluoride levels in the cerebrum among groups showing nonsignificantly lowest and highest values at 1 ppm and 25 ppm, respectively. There were, however, no significant differences for fluoride concentration in the cerebrum among the groups.



**Figure 3.** Fluoride levels in the cerebrum from mice administered fluoride through drinking water. Each bar represents mean value. Error bars represent standard error.

For the results of the neurotransmitter analyses, Figure 4 illustrates the concentrations of dopamine (DA) and its metabolites in the hypothalamus among the different groups. In each assay, a peak level occurred at 5 ppm, but it was significant only for HVA (homovanillic acid) compared to the control.



**Figure 4.** The levels of dopamine (DA) and its metabolites in the hypothalamus from the mouse treated by fluoride through drinking water. Mean values  $\pm$  S.E. are indicated.  $p < 0.05$  by ANOVA. \*\* $p < 0.01$  compared to the control, 25 ppm and 125 ppm by Fisher's PLSD test.

Table 3 reports the concentrations of DA and DA metabolites in the other five brain regions. There were no significant differences for them among the different exposure groups. There were also no significant differences for NE, 5-HT, and 5-HIAA levels in any brain regions (data not shown).

**Table 3.** Concentrations of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in various brain regions of mice treated with sodium fluoride

Neurochemical	NaF (ppm)	Concentrations ( $\mu\text{g/g} \pm$ standard error; $n = 6$ ) in				
		cerebrum	cerebellum	medulla	midbrain	striatum
DA	0	1.621 $\pm$ 0.147	0.027 $\pm$ 0.004	0.052 $\pm$ 0.004	0.238 $\pm$ 0.019	3.761 $\pm$ 0.386
	1	1.845 $\pm$ 0.146	0.031 $\pm$ 0.006	0.062 $\pm$ 0.006	0.238 $\pm$ 0.015	3.671 $\pm$ 0.554
	5	1.748 $\pm$ 0.105	0.042 $\pm$ 0.010	0.053 $\pm$ 0.003	0.229 $\pm$ 0.008	4.586 $\pm$ 0.408
	25	1.560 $\pm$ 0.107	0.026 $\pm$ 0.006	0.061 $\pm$ 0.006	0.259 $\pm$ 0.036	4.504 $\pm$ 0.921
	125	1.418 $\pm$ 0.162	0.036 $\pm$ 0.009	0.044 $\pm$ 0.004	0.346 $\pm$ 0.080	4.257 $\pm$ 0.739
DOPAC	0	0.212 $\pm$ 0.034	0.092 $\pm$ 0.009	0.024 $\pm$ 0.003	0.087 $\pm$ 0.005	0.507 $\pm$ 0.055
	1	0.201 $\pm$ 0.015	0.110 $\pm$ 0.030	0.032 $\pm$ 0.007	0.091 $\pm$ 0.011	0.672 $\pm$ 0.195
	5	0.186 $\pm$ 0.003	0.106 $\pm$ 0.023	0.019 $\pm$ 0.003	0.092 $\pm$ 0.006	0.883 $\pm$ 0.157
	25	0.172 $\pm$ 0.011	0.095 $\pm$ 0.024	0.024 $\pm$ 0.005	0.102 $\pm$ 0.016	0.653 $\pm$ 0.131
	125	0.237 $\pm$ 0.082	0.085 $\pm$ 0.018	0.021 $\pm$ 0.003	0.103 $\pm$ 0.016	0.951 $\pm$ 0.269
HVA	0	0.284 $\pm$ 0.021	0.029 $\pm$ 0.002	0.047 $\pm$ 0.006	0.167 $\pm$ 0.011	0.814 $\pm$ 0.102
	1	0.335 $\pm$ 0.028	0.077 $\pm$ 0.045	0.060 $\pm$ 0.009	0.185 $\pm$ 0.018	0.855 $\pm$ 0.099
	5	0.307 $\pm$ 0.025	0.038 $\pm$ 0.009	0.041 $\pm$ 0.004	0.178 $\pm$ 0.006	1.060 $\pm$ 0.111
	25	0.271 $\pm$ 0.025	0.030 $\pm$ 0.002	0.046 $\pm$ 0.009	0.188 $\pm$ 0.020	0.900 $\pm$ 0.163
	125	0.339 $\pm$ 0.054	0.032 $\pm$ 0.003	0.043 $\pm$ 0.005	0.216 $\pm$ 0.032	1.100 $\pm$ 0.145

## DISCUSSION

The fluoride levels used in this study ranged from the 1-ppm concentration recommended for the prevention of dental caries to that known to induce kidney damage after several months in experimental animals.<sup>5</sup> Since people in China and India suffer from endemic fluorosis mainly when exposed to fluoride in their drinking water,<sup>1,2</sup> we conducted our study by exposing the mice to fluoride in their drinking water.

Among the mice treated with fluoride at 25 and 125 ppm in the water, their water intake was increased compared to the controls. In the study by Manocha et al.,<sup>6</sup> water consumption was significantly higher in squirrel monkeys exposed to fluoride in their drinking water. Juncos and Donadio<sup>13</sup> reported that two patients with renal insufficiency and systemic fluorosis imbibed large amounts of water

since early childhood or infancy. Although Collins et al.<sup>14</sup> found no significant differences in the water intake of rats exposed to fluoride in drinking water at 0, 25, and 100 ppm during a 10-week growth period, rats exposed to fluoride at 175 and 250 ppm showed a significant decrease in water intake. From our results, it appears that fluoride stimulates water intake in mice at the levels in the range of 25 to 125 ppm.

Water intake is important since humans ingest fluoride mainly through drinking water. In tropical areas like India, high temperatures stimulate water intake, and there are reports that mottled teeth are observed even with relatively low fluoride water concentrations.<sup>15,16</sup> Yamada et al.<sup>17</sup> reported that comparatively low fluoride concentrations in drinking water ( $0.85 \pm 0.84$  ppm) caused mottled teeth in China, in which the average temperature is 5°C lower than in hotter countries such as in India. Although endemic fluorosis in China is caused in part by high fluoride briquette-type coal,<sup>17</sup> fluoride in drinking water may stimulate water intake even at relatively lower levels.

Our results showing no significant differences in body weight and relative organ weights among the groups are in agreement with those of previous studies. In the study of Collins et al.,<sup>14</sup> male and female rats exposed to fluoride for 10 weeks at levels up to 175 ppm in drinking water did not show any significant differences in body weight gain among groups. Only males exposed to 250 ppm fluoride in drinking water showed a significant decrease in body weight gain compared to the controls. Relative organ weights were also not affected in that study. The administration of 100 ppm of fluoride for 6 weeks starting in 3-month-old rats also did not cause significant changes in body weight compared to controls.<sup>7</sup>

The higher concentration of fluoride in the kidney among the 125 ppm group in our study may be related to renal damage reported previously. Fluoride at levels of 100 ppm and over in drinking water induced renal damage.<sup>5</sup> For longer periods of administration, 5 ppm fluoride in drinking water induced renal damage.<sup>6</sup> In endemic fluorosis areas, people may be exposed to fluoride at a level comparable to more than 25 ppm, considering intake from water and food. Many hot springs in China contain more than 10 ppm fluoride, and the ground water there is contaminated with fluoride.<sup>18</sup> The renal function of people living in endemic fluorosis areas needs to be monitored.

A significant increase in the fluoride concentration in the liver was also observed in the 125 ppm group in our study. In a previous study on goats exposed for over three years to airborne fluoride, the mean fluoride level in the kidney was approximately three times higher than in the control, whereas the mean fluoride level in the liver was similar to the control.<sup>19</sup> The higher level of exposure to fluoride in the present study may affect metabolism and increase the fluoride level in the liver. Further studies are required to examine whether the liver was damaged after subacute administration of fluoride at 125 ppm in drinking water.

In contrast to the kidney and liver, the fluoride levels in the cerebrum were not significantly different among the groups, although there was a small but nonsignificant lower level in the cerebrum among the 1 and 5 ppm groups than in the control group (Figure 3). Mullenix et al.<sup>7</sup> found that fluoride levels increased in the medulla oblongata in rats of both sexes and in the hippocampus of females

exposed to 100 ppm fluoride in drinking water for 6 weeks compared to the controls. In addition, the fluoride levels were higher in the cerebellum, basal ganglia, midbrain, and hippocampus of male rats and in the cortex of female rats. The fluoride levels in brain regions may vary among species or sexes.

A significant difference in the 5 ppm exposure group was observed for HVA in the hypothalamus, and the pattern was similar for DA and DOPAC in a non-dose-dependent manner. The maximum levels of DA and the DA metabolites DOPAC and HVA occurred at the 5 ppm exposure level. Thus fluoride may have “paradoxical” concentration or dose-response effects on the metabolism of DA in agreement with results of Isaacson and co-workers<sup>20,21</sup> on anomalous central nervous system concentration effects of fluoride as suggested by Burgstahler.<sup>22</sup> In addition, alteration in neurotransmitters induced by toxic substances is not always dose-dependent. The effects of aluminum<sup>10</sup> and benzene<sup>23</sup> on neurotransmitters and their metabolites were only observed in the low-dose group. Since regions of the brain devoid of a blood-brain barrier include the median eminence of hypothalamus,<sup>24</sup> it may be easier for fluoride to reach the hypothalamus.

For our experimental conditions, exposure for a month may not be enough to evaluate the neurotoxicity of fluoride. Longer exposure may result in different effects on neurotransmitters by fluoride, since fluoride first accumulates in hard tissue such as the skeleton and teeth.<sup>5,17</sup>

For the evaluation of neurotoxicity of fluoride, other parameters may be more useful. The administration of fluoride resulted in a significant decrease in acetylcholine esterase in the brains of rats<sup>25</sup> and mice.<sup>26</sup> The usefulness of the determination of acetylcholine esterase as an index of neurotoxicity induced by fluoride should be examined further.

In conclusion, fluoride was found to increase water intake of mice at levels of 25 and 125 ppm in their drinking water. At 125 ppm it significantly increased the concentrations of fluoride in the liver and kidney but not in the brain. A significant increase in regional brain neurochemicals was observed only for HVA in the hypothalamus of the mice administered 5 ppm of fluoride.

#### ACKNOWLEDGEMENTS

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