

## EFFECT OF VITAMIN D ON CHRONIC BEHAVIORAL AND DENTAL TOXICITIES OF SODIUM FLUORIDE IN RATS

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**SUMMARY:** Adult female Wistar rats were treated daily for 60 days with sodium fluoride (500 ppm NaF = 226 ppm fluoride ion) in drinking water, alone or in combination with vitamin D (200 IU/kg by oral intubation). Throughout the period, food intake was measured daily. Body weight gain, exploratory motor activity (EMA) rota-rod motor coordination, dental structure, brain acetylcholinesterase (AChE) activity, and serum fluoride and serum calcium concentration were determined 24 hr after the last treatment. Serum fluoride concentration increased markedly in the NaF-treated animals and was accompanied by decreased food intake, reduced body weight gain, impairment of EMA and motor coordination, dental lesions, inhibition of brain AChE activity, and hypocalcemia. Administration of vitamin D along with NaF prevented hypocalcemia. However, the toxic action fluoride on motor coordination, brain AChE activity, and the teeth was not prevented in these animals, probably because vitamin D is not able to decrease the level of fluoride in the serum. Therefore, vitamin D has only limited value as a protective dietary factor against chronic toxic effects of fluoride.

**Keywords:** Dental lesions; Fluoride toxicity; Locomotor behavior; Rat toxicity; Serum calcium; Serum fluoride; Vitamin D.

### INTRODUCTION

Fluorides are naturally occurring contaminants in the environment.<sup>1</sup> Prolonged ingestion of drinking water containing 1–3 ppm of fluoride ion produces deleterious effects on skeletal, dental,<sup>1</sup> and soft tissues,<sup>2,3</sup> enzyme activities,<sup>4</sup> and locomotor behavior<sup>5</sup> in animals. Calcium supplementation acts to prevent toxic effects of fluoride in experimental animals.<sup>6</sup> Because vitamin D facilitates gastro-intestinal absorption of calcium,<sup>7</sup> it is likely to have a countering effect on the toxic effects of fluoride.

In the present study, food intake, body weight gain, exploratory motor activity (EMA), motor coordination, brain acetylcholinesterase (AChE) activity, and dental structure were investigated in animals treated with sodium fluoride (NaF) along with vitamin D daily for 60 days. Serum calcium and fluoride concentrations were also measured in these animals.

### MATERIALS AND METHODS

Colony-bred adult 4-5 month old female Wistar rats weighing 130–150 g were used. Since male rats were found in a previous study to be more susceptible than females to effects of chronic fluoride treatment,<sup>5</sup> only female rats were used in this work. Eight animals were chosen randomly for each

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test and control group. Except those used for recording food intake, the rats were caged in groups (4 per cage) and were maintained at room temperature (22–26°C) with a normal 12-hr light/dark cycle. The animals were fed a balanced commercially available pelleted rat chow (Gold Mohur, M/S Hindustan Lever Ltd., Mumbai, India). Sodium fluoride (LR, Qualigens Fine Chemicals, Mumbai, India) was administered *ad libitum* in the drinking (tap) water at a concentration of 500 ppm NaF (= 226 ppm of fluoride ion) *ad libitum*. *Guidelines for Breeding of and Experiments on Animals, 1998*, published by the ministry of Social Justice Empowerment, Government of India, were followed in this investigation.

Since NaF administered at a concentration of 226 ppm fluoride ion in the drinking water for 4 weeks produced growth retardation and skeletal fluorosis rats in a previous study,<sup>8</sup> this concentration was used in the present study designed for 60 days.

Another two groups of animals received vitamin D (cholecalciferol, Duphar Interferan Ltd., Mumbai, India), alone and in combination with NaF, for 60 days. The vitamin D (arachitol 300,000 IU per mL) was made into a fine emulsion with 1% gum acacia powder in distilled water. The emulsion was administered by oral intubation in a volume of 0.1 mL/100 g body weight at 200 IU/kg daily for 60 days. In a preliminary study in this laboratory, vitamin D at 100 IU/kg/day for 60 days did not change the toxic effects of fluoride. Hence, the higher dose was chosen for this study. Experiments were carried out 24 hr after the 60<sup>th</sup> day of treatment in test and control animals. Locomotor behavioral tests and sacrifice for biochemical determinations were conducted between 1100 and 1300 hr at the temperature of the housing.

For the food intake study, the test and control animals were caged singly. A measured amount of feed was supplied every day and the leftover was measured 24 hr later. Thus, daily and total food intake for 60 days was measured. These animals were weighed on the day of starting treatment and then 24 hr after the last (60<sup>th</sup>) treatment. The percent body weight gain was determined.

EMA was measured using an activity monitoring cage. The capacitance sensors implanted in the floor of the cage were sensitive to the vibrations caused by the locomotor as well as scratching and grooming activities of the animals. Since exploratory locomotor activity of rats in a novel environment was being tested, no habituation time was allowed. The instrument was switched on, and one min later the animal was placed in the chamber to measure the activity over a period of 10 min.

A rota-rod apparatus<sup>9</sup> was used for testing motor coordination. The apparatus consisted of a horizontal rod with a roughened surface, 5 cm in diameter and 30 cm long with partitions for testing 3 animals at a time. The

rod rotated on its axis at 14 rpm. The rationale of this test was that the animal was forced to stand on the rotating rod and that the animals having defective motor function would drop off to a tray placed 20 cm below the rod. The test was carried out as described previously.<sup>5</sup> A test period of 90 s was allowed for each treated and test animal, and the endurance time was determined by measuring the time between placing the rat on the moving rod and the moment it fell off the rod. Ninety min with no standard error mean was allotted to animals that remained successfully for 90 s on the rotating rod.

The changes observed in the incisors were assessed using a 0–5 scoring method described previously.<sup>10</sup> Scoring was done as follows: 0 = normal shape of teeth and smooth, glossy orange-yellow colour of enamel; 1 = slight whitening of the enamel; 2 = faint horizontal banding of enamel chalky spots, slight erosion; 3 = chalky enamel, moderate erosion of tips, staining; 4 = pitting and chipped of edges, loss of enamel colour, heavy staining; 5 = cutting tips splayed and eroded to blunt stubby abnormal curvature.

AchE activity ( $\mu\text{moles}/\text{min}/\text{g}$ ) was measured in the brain by the method of Ellman *et al.*<sup>11</sup> Brain tissue was homogenized with 0.1 M phosphate buffer at pH 8.0. To 0.4 mL aliquot of the homogenate, 2.6 mL of phosphate buffer was added in a cuvette. Afterward, 0.1 mL of DTNB reagent was added to the reaction mixture. The absorbance was measured at 412 nm. Then, to the reaction mixture, 0.02 mL of acetylthiocholine iodide was added. Changes in the absorbance were recorded, and the change/min was calculated.

Serum fluoride (mg/L) was determined as described in the literature<sup>12</sup> using a fluoride ion specific electrode (Orion model 9409, Cambridge, MA, USA) and a Fisher “accumet” model 425 pH/mV digital meter (Fisher Scientific Co. Ltd., Don Mills, Canada). For the determination, 1.0 mL of serum was mixed and stirred with 10 mL of total ionic strength adjusting buffer in a small plastic beaker.

Serum calcium (mg/100 mL) was estimated by the method of Transeau and Freiere.<sup>13</sup> One mL of serum sample was fed into an inductively coupled plasma emission spectroscope (ARL, Model 2410). A standard wave length of 317.93 nm was used for calcium estimation.

Behavioral and biochemical data were analyzed by one way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Dental lesion scores were analyzed by the Mann-Whitney rank order test (U).

## RESULTS

*Effect of NaF:* Both food consumption and body weight gain decreased significantly in NaF-treated animals as compared to control animals (Figures 1A and B). Decreased EMA and shortening of rota-rod endurance time were also observed (Figures 1C and D), and significant decrease of AchE activity

occurred in the brains of NaF-treated animals (Figure 2A). Moderate to severe dental lesions (scale  $3.5 \pm 0.3$ ) were also observed in these animals (Figure 2B). In addition, NaF treatment produced a marked increase (790%) in the concentration of fluoride in the serum (Figure 2D), and the serum calcium concentration was 67% lower in these animals (Figure 2C).

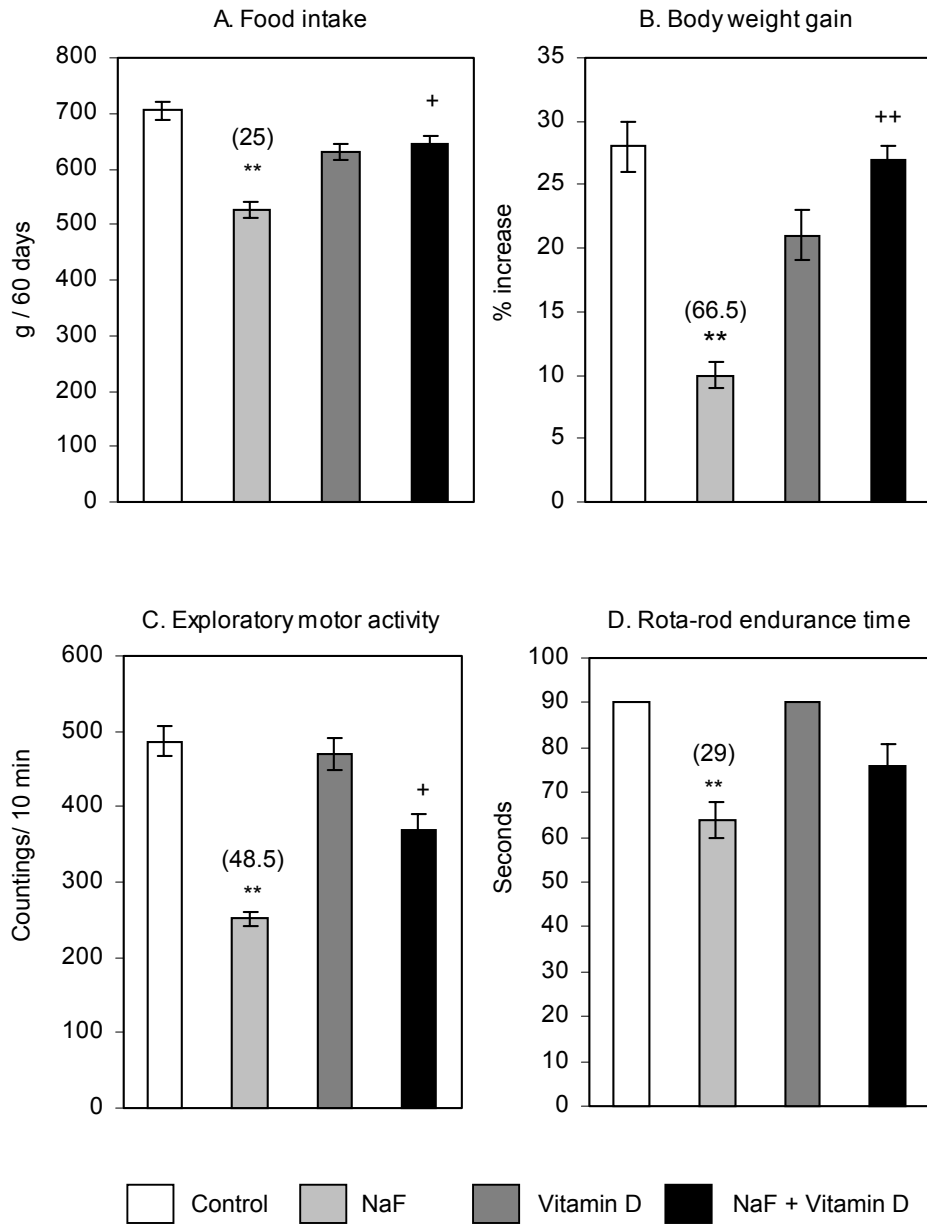
*Effect of vitamin D:* Administration of vitamin D alone did not produce significant changes in food intake (Figure 1A), body weight gain (Figure 1B), EMA (Figure 1C), and rota-rod endurance time (Figure 1D). AchE activity (Figure 2A) and dental structure (Figure 2B) were also not altered in these animals. Vitamin D alone increased the concentration of calcium in the serum (Figure 2C), but the effect was not statistically significant. Moreover, the concentration of serum fluoride was not altered significantly in these animals (Figure 2D).

*Effect of NaF + vitamin D:* Vitamin D prevented NaF from decreasing food intake body weight gain and EMA (Figures 1A, B, C). NaF-induced hypocalcemia was also prevented by vitamin D (Figure 2C). However, vitamin D failed to prevent the effects of NaF on motor coordination (Figure 1D), brain AchE activity (Figure 2A) and dental structure (Figure 2B). Serum fluoride concentration was only slightly decreased in these animals (Figure 2D).

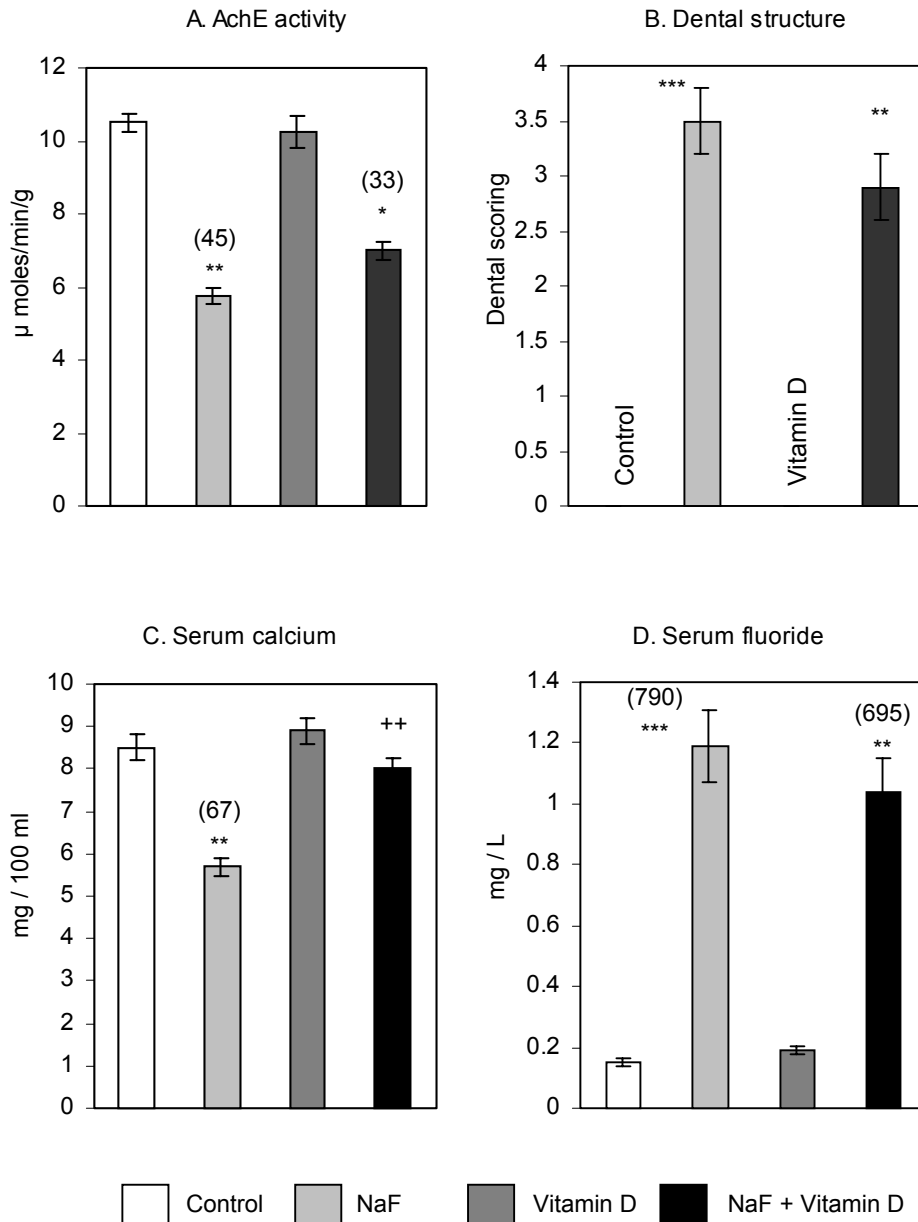
#### DISCUSSION

In the present study, in agreement with previous work,<sup>14,15</sup> serum fluoride concentration was increased substantially after oral administration of NaF, thereby suggesting a steady rate of absorption of fluoride from the gastrointestinal tract following NaF intake from the drinking water. A decreased urinary excretion of fluoride resulting from fluoride-induced impairment of renal function may also contribute to an elevation of fluoride concentration in the serum.<sup>16</sup>

Two months of oral administration with NaF decreased AchE activity in the brain. As in a previous study,<sup>5</sup> this decrease was accompanied by an inhibition of motor activity. A modulation of the central cholinergic mechanism probably accounts for decreased EMA in NaF-treated animals. Since a defect in motivated locomotor behavior may lead to suppression of eating, this behavioral impairment may in part account for a decreased food intake in the present and previous<sup>5</sup> studies on animals treated daily with NaF for several days. Atrophic gastritis produced by chronic oral treatment of NaF<sup>15</sup> may also contribute to decreased food intake in these animals. As expected, dental lesions were observed in the present study in NaF-treated animals. The incisors became white and chalk-like with broken tips. This effect, as proposed earlier,<sup>17</sup> may impair the ability of animals to masticate food prior to swallowing and therefore contribute to a decreased food intake with a decrease in body weight gain.



**Figure 1.** Effects of NaF (226 ppm F in drinking water for 60 days) on food intake (A), body weight gain (B), exploratory motor activity counts (C), and rota-rod endurance time (D) in adult female Wistar rats. Each bar represents mean  $\pm$  SEM of 8 animals. Percent change from control value in parenthesis. \*\*  $P < 0.01$  compared to control. +  $P < 0.05$ , ++  $P < 0.01$  compared to NaF-treated group (one way ANOVA followed by Tukey's multiple comparison test).



**Figure 2.** Effects of NaF (226 ppm F in drinking water for 60 days) on brain AChE activity (A), dental structure (B), serum calcium (C), and serum fluoride levels (D) in adult female Wistar rats. Each bar represents mean  $\pm$  SEM of 8 animals. Percent change from control value in parenthesis. \*P<0.05, \*\*P<0.01 compared to control. ++ P<0.01 compared to NaF-treated group. (one way ANOVA followed by Tukey's multiple comparison test).

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A significant hypocalcemia was also observed in NaF-treated animals. Poor gastrointestinal absorption of calcium resulting from formation of slightly soluble calcium fluoride (CaF<sub>2</sub>) or fluorapatite<sup>18</sup> and promotion by fluoride of uptake of calcium by bone<sup>18</sup> may account for hypocalcemia in these animals. Inadequate food intake can also be a contributing factor for hypocalcemia.

Hypocalcemia was largely prevented in the present and in a previous study<sup>8</sup> when animals received vitamin D along with NaF. We attribute this effect to the fact that vitamin D facilitates absorption of calcium from the gastrointestinal tract.<sup>7</sup> However, vitamin D failed to prevent the deleterious effects of fluoride on motor coordination, the teeth, and AchE activity, thus indicating that vitamin D-induced facilitation of calcium absorption affords very little protection against these forms of fluoride toxicity.

Calcium supplementation has been found to be protective against the toxic effects of fluoride in rats.<sup>6,19</sup> In these experiments the serum fluoride concentration decreased considerably.<sup>6,19</sup> Orally administered calcium was suggested to form insoluble CaF<sub>2</sub> with fluoride available in the gastrointestinal tract. Thus, absorption of fluoride seems to be prevented in these animals and may account for the protective effect of calcium on the toxic effects of fluoride.<sup>6,19</sup> Since vitamin D is known to facilitate absorption of calcium,<sup>7</sup> supplementation of this vitamin is likely to decrease availability of calcium in the gastrointestinal tract and that absorption of fluoride cannot be prevented in the gastrointestinal tract. This effect may account for the serum fluoride concentration being nearly as high as in animals treated with NaF alone. Thus, the deleterious effects of fluoride on skeletal muscle, dental structure, and AchE activity were not prevented in animals treated with vitamin D and NaF together.

In the present study, vitamin D did not independently increase food intake, body weight gain, or EMA. However, the deleterious effects of NaF on these parameters were significantly prevented when animals received vitamin D along with NaF, thus indicating that vitamin D can inhibit the toxic effects of NaF on food intake, body weight gain, and EMA. In previous studies, vitamin D ameliorated the toxic effects of NaF on reproductive function in male mice<sup>20</sup> and on fetal growth in pregnant rats.<sup>21</sup>

In conclusion, vitamin D was unable to prevent fluoride-induced toxicities on motor co-ordination, dental structure, and AchE activity in rats, although it did prevent hypocalcemia. This failure may be attributed to the inability of vitamin D to decrease serum fluoride concentration through of its enhancement of calcium absorption from the gastrointestinal tract. Thus vitamin D affords only limited protection against the chronic toxic effects of fluoride.

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