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Modulation of Fluoride Toxicity in Rats by Calcium Carbonate and by Withdrawal of Fluoride Exposure

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Abstract: In order to assess the effect of calcium on the toxic effects of fluoride, adult female Wistar rats were treated with sodium fluoride (NaF, 500 ppm in drinking water) alone or in combination with calcium carbonate ($CaCO_3$, 50 mg/kg by oral intubation) daily for 60 days. Food, water and fluoride intake were measured daily for 60 days. Body weight gain, exploratory motor activity, rota-rod motor coordination, dental structure, activities of acetylcholinesterase (AchE, brain and skeletal muscle) and Na^+ K^+ ATPase (erythrocyte membrane and skeletal muscle) and the concentrations of protein (serum and skeletal muscle), calcium (serum) and fluoride (serum) were determined in these animals 24 hr after the last treatment. The same parameters were tested in another group, 60 days after withdrawal of NaF exposure (500 ppm in drinking water daily for 60 days). NaF treatment decreased food and water intake, reduced body-weight gain and impaired exploratory motor activity and rota-rod performance. Dental lesions, inhibition of the activities of AchE and N^+ K^+ ATPase and a decrease in the concentration of protein, and serum calcium were also observed in these animals. These effects were accompanied by a marked elevation of fluoride concentration in the serum. $CaCO_3$ decreased the concentration of fluoride in the serum of NaF-treated animals. A decrease in serum fluoride concentration was found also after NaF withdrawal. A prevention of locomotor behavioural, biochemical and dental toxicities of fluoride was observed both in these groups. It is concluded that the dose of $CaCO_3$ used in the present study has a potential to prevent the toxicity of fluoride by maintaining serum fluoride at a less toxic level. Further, the toxic effects of fluoride are reversible if its exposure is withdrawn for 2 months.

Epidemiological studies have shown that prolonged ingestion of drinking water containing high levels of fluoride produces deleterious effects on skeletal (Gupta *et al.* 1993) dental (Hicks & Flaitz 2000) and soft tissues (Michael *et al.* 1996) in man. Locomotor (Spittle 1994) and biochemical (Trivedi *et al.* 1993) deficits have also been found in persons exposed chronically to fluoride. Experimental studies have demonstrated that chronic fluoride exposure is injurious to skeletal (Harrison *et al.* 1984; Choubisa 1999) dental (Boulton *et al.* 1995; Choubisa 1999) and soft tissues (Purohit *et al.* 1999). Locomotor (Paul *et al.* 1998) and biochemical (Chinoy *et al.* 1991) impairments have also been observed in animals receiving fluoride daily for several days.

The skeletal effects of fluoride was found to be reduced in rats when calcium was administered along with fluoride (Harrison *et al.* 1984). However, the protective effect of calcium on the toxicity of fluoride on locomotor behaviour, dental structure and biochemical parameters has not been investigated yet. Hence in the present study these parameters have been assessed in animals treated with sodium fluoride (NaF) along with calcium carbonate ($CaCO_3$) daily for 60 days. The present study has also been aimed at determining whether the changes produced by 60 day treatment of NaF on the above-mentioned parameters can be reversed upon withdrawal of exposure.

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Materials and Methods

Animals. Colony-bred 3–4 month old female Wistar rats weighing 130–150 g were used. Female rats were used in this study because in a previous study in this laboratory, mortality occurred in male and not in female animals during 60 day oral treatment with toxic doses of NaF (Paul *et al.* 1998). Eight animals were chosen randomly for each test and control group. Animals were caged singly, since food and water intake were recorded in both test and control groups. The animals were maintained at room temperature (22–26°) with normal 12 hr light/dark cycle and fed *ad libitum*, a balanced commercially available pelleted rat feed containing 1% of calcium (Gold mohur, M/s.Hindustan Lever Ltd., Mumbai, India) and NaF mixed tap water containing 40 ppm of calcium. The background concentration of fluoride in drinking water was 0.2 ppm. Guidelines defined by the Ministry of Social Justice Empowerment, Government of India (Singh 1998) was followed in this study.

Chemicals and treatment. A dose of NaF (500 ppm daily in drinking water) that produced growth retardation in a previous study (Harrison *et al.* 1984) was chosen for the present study. Four groups of animals received NaF (LR, Qualigens Fine Chemicals, Mumbai, India; 500 ppm with drinking water) or $CaCO_3$ (GR Sarabhai M Chemicals, India; 50 mg/kg), both together or tap water daily for 60 days. $CaCO_3$ was chosen because of its ready absorption from the gastrointestinal tract in comparison to other salts of calcium (Gilman *et al.* 1992). Harrison *et al.* (1984) who assessed the effect of calcium on skeletal fluorosis, fed the rats a diet containing 2% calcium. A smaller dose was chosen for the present study because rats were fed a balanced diet containing 1% calcium. A fine suspen-

sion of $CaCO_3$ was prepared with 1% gum acacia powder (an inert substance used to make suspensions of water insoluble compounds) in distilled water, so as to administer by oral intubation in a volume of 0.1 ml/100 g body weight. Animals received a dose of $CaCO_3$ daily in the morning 10.00–10.30 a.m. Animals receiving tap water for 60 days served as untreated control. Food and water intake were measured in all test and control animals daily for 60 days. The tests described below were carried out 24 hr after the last treatment. The locomotor behavioural tests and sacrifice for biochemical determination were done between 10.00–12.00 a.m. at room temperature.

In another study, animals received 500 ppm of NaF with drinking water daily for 60 days, and the next 60 days NaF was withdrawn from the drinking water. Food and water intake were measured daily during these (withdrawal) 60 days. The tests described below were carried out 24 hr after the 60th day of withdrawal of NaF treatment. The respective control animals received balanced diet and tap water for 120 days and tests were carried out 24 hr later as in the withdrawal group.

Food, water and fluoride intake and body weight gain. A measured amount of feed and water were supplied and the left-over was weighed 24 hr later. Thus, daily and total food and water intake for 60 days were recorded in NaF, $CaCO_3$ and $NaF+CaCO_3$ treated and in NaF withdrawn animals as well as in controls. Since intake of water was measured daily, it was convenient to calculate the amount of fluoride consumed by test and control animals from drinking water, during the 60 days treatment.

The animals were weighed on the day of starting treatment and then 24 hr after the last (60th) treatment. Body-weight was determined on the 1st and 24 hr after the last (60th) day of withdrawal. Percent body weight gain was calculated.

Exploratory motor activity was measured using an activity monitoring cage (Paul *et al.* 1998). 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 animal. Since, exploratory locomotor activity of rats in a novel environment was tested, no habituation time was allowed. The instrument was switched on and one min later the animal was placed in the chamber and the activity was measured for a duration of 10 min.

Motor coordination was tested using a rota-rod apparatus designed by Dunham & Miya (1957). The apparatus consisted of a horizontal rod (5 cm diameter, 30 cm long with partitions for testing 3 animals at a time) with a roughened surface. The rod moved on its axis at 14 rpm. The rationale of this test was that the animal was forced to stand on the moving rod and that animals having defective motor functions would drop off from the moving rod to a tray placed 20 cm below the rod. The test was carried out as described previously (Paul *et al.* 1998). Prior to the treatment, each animal was given a one min. exposure to the moving rod to get accustomed. Animals that stood successfully were chosen for the study. The rota-rod test was conducted 24 hr after the last (60th day) treatment. A test period of 90 sec. was allowed to each animal and the endurance time was determined by measuring the time between the placing of the rat on the moving rod and the moment the animal fell down. Separate groups were used for exploratory motor activity and rota-rod motor coordination studies.

Dental lesion. The changes observed in the incisors were assessed using a 0–5 scoring method as described by Boulton *et al.* (1995). Scoring was done as follows: normal shape of teeth and smooth, glossy orange-yellow colour of enamel – 0; slight whitening of the enamel – 1; faint horizontal banding of enamel; chalky spots, slight erosion – 2; chalky enamel, moderate erosion of tips, staining – 3; pitting and chipped off edges, loss of enamel colour and heavy staining – 4; cutting tips splayed and eroded to blunt stubby abnormal curvature – 5.

Sample collection and biochemical study. Blood (from neck wound) and tissue samples (brain, gastrocnemeus muscle) were collected soon after decapitation. Blood was collected in centrifuge tubes and left undisturbed in a slanting position. After clotting, the sample was centrifuged at 3000 rpm for 10 min. and the serum was collected and processed immediately for biochemical determinations.

Isolation of erythrocyte and skeletal muscle membranes for Na^+ K^+ ATPase activity. The erythrocyte membrane was isolated as described by Quist (1980). Blood, collected with EDTA as an anticoagulant, was used. Plasma was separated by centrifugation at $1000\times g$ for 20 min. The packed cells, remaining after the removal of plasma, were washed with isotonic saline to remove the buffy coat. Four ml packed cells were then washed three times with isotonic Tris-HCl buffer (0.31 M, pH 7.4). Haemolysis was performed by pipetting out the washed red blood cell suspension into polypropylene centrifuge tubes which contained hypotonic buffer (Tris-HCl buffer 0.015 M, pH 7.2). Erythrocyte ghosts were sedimented in a high speed refrigerated centrifuge at $35{,}000\times g$ for 40 min. The supernatant was decanted and the pellet of the erythrocyte membrane was resuspended in 10 ml Tris-HCl buffer (0.31 M, pH 7.4). Aliquots of this reconstituted membrane preparation was taken for the estimation of Na^+ K^+ ATPase. Skeletal muscle tissue was taken in ice-cold 0.1 M Tris-HCl buffer of pH 7.4 (100 mg in 1 ml) homogenized and the plasma membrane bound activity of Na^+ K^+ ATPase was estimated by the method of Bonting (1970). A standard method was used for the determination of protein concentrations in serum and skeletal muscle (Lowry *et al.* 1951).

Acetylcholinesterase activity was measured in brain and skeletal muscle using the method of Ellman *et al.* (1961). The tissues were homogenised with 0.1 M phosphate buffer at pH 8.0. Tissue homogenate of 0.4 ml, 2.6 ml of phosphate buffer, and 0.1 ml or dithio bisnitro benzoic acid was added to the reaction mixture. The absorbance was measured at 412 nm. Then to the reaction mixture

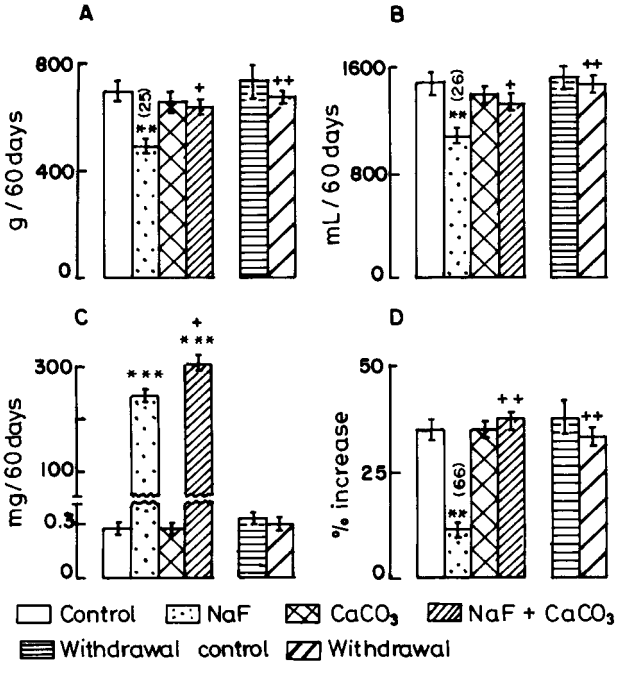

Fig. 1. Food intake (A), water intake (B), fluoride intake (C) and body weight gain (B) in test and control animals. Each bar represents mean±S.E.M. of 8 animals. Percent change from control value in parenthesis. $^{**}P<0.01$, $^{***}P<0.001$, compared to control. $+P<0.05$, $++P<0.01$ compared to NaF-treated group (one way ANOVA followed by Tukey's multiple comparison test).

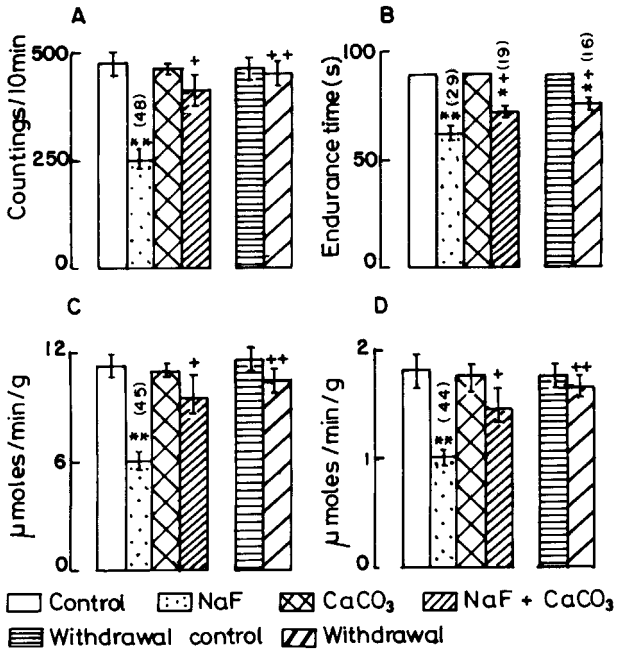

Fig. 2. Exploratory moter activity (A), rota-rod endurance time (B), AchE activity in brain (C) and skeletal muscle (D) of test and control animals. Each bar represents mean±S.E.M. of 8 animals. All untreated control animals stood on the moving rod till the end of allotted 90 sec. Therefore, these groups did not show S.E.M. Percent change from control value in parenthesis. *P<0.05, **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).

0.02 ml of acetylthiocholine iodide was added. Changes in absorbance were recorded and the change in the absorbance per min. was calculated.

Serum fluoride was determined by the method of Hall *et al.* (1972) using 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). One ml of serum was mixed with 10 ml of total ionic strength adjusting buffer (concentrated acetic acid of 57.5 ml, 58 g sodium chloride, 4 g of cyclohexane dinitrilo tetra acetic acid, and 37.5 g of sodium hydroxide in distilled water with a total volume of one liter, pH 5.25) in a small plastic beaker. The solution was mixed thoroughly by using a magnetic stirrer. Then the electrode was immersed in the sample solution. The millivolt reading was recorded.

Serum calcium was estimated by the method of Trandeau & Freiere (1967). One ml of serum sample was fed into inductively coupled plasma emission spectroscope (ARL, Model 2410). A standard wave length of 317.933 was used for calcium estimation.

Statistics. The behavioural and biochemical data were analyzed using the one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Non-parametric Mann-Whitney rank order (U) test was used to analyse dental lesions scoring data.

Results

Food (25%; P<0.01) and water (26%; P<0.01) consumption were decreased significantly in NaF-treated animals as compared to control (fig. 1A, B). Fluoride intake was increased substantially in these animals (P<0.001) compared to control (fig. 1C). A marked reduction in body weight gain (66%; P<0.01) was observed in these animals (fig. 1D).

NaF-treatment inhibited (48%) exploratory motor activity (P<0.01; fig. 2A). NaF-treated animals dropped from the rod after a median of 62 sec. (P<0.01) as compared to 90 sec. fixed control value (fig. 2B). A decreased activity of AchE was found in brain (45%; P<0.01) and skeletal muscle (44%; P<0.01) of these animals (fig. 2C, D). NaF treatment decreased the concentration of protein in the serum (41%, P<0.01) and skeletal muscle (30%; P<0.01) (fig. 3A, B). An inhibition of Na^+ K^+ ATPase was found in erythrocyte (56%; P<0.01) and skeletal muscle (50%; P<0.01) membranes of these animals (fig. 3C, D). A moderate to severe degree of dental lesions were observed in NaF-treated animals (fig. 4A). Serum fluoride concentration was increased (790%) markedly (P<0.001) in these animals (fig. 4B). The concentration of calcium was decreased in the serum (65%; P<0.01) of NaF-treated animals (fig. 4C).

$CaCO_3$ treatment alone produced no significant changes in any of the parameters tested here. However when it was administered along with NaF, water intake was increased as compared to NaF-treated group (P<0.05; fig. 1B). As a result, consumption of fluoride by these animals increased significantly (P<0.05) as compared to NaF-treated animals (fig. 1C). However, the concentration of fluoride was decreased markedly (P<0.01) in the serum of these animals in comparison to that measured in NaF-treated animals. But

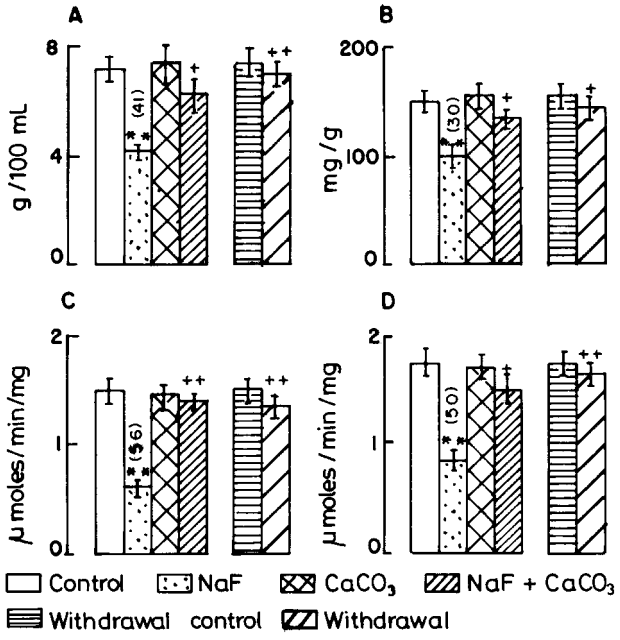

Fig. 3. Protein concentrations in serum (A), skeletal muscle (B), Na^+ K^+ ATPase activity in erythrocyte (C) and skeletal muscle (D) membranes of test and control animals. Each bar represents mean±S.E.M. of 8 animals. Percent change from control value in parenthesis. **P<0.01 compared to control. +P<0.05, ++P<0.01 compared to NaF-treated group (one way ANOVA followed by Tukey's multiple comparison test).

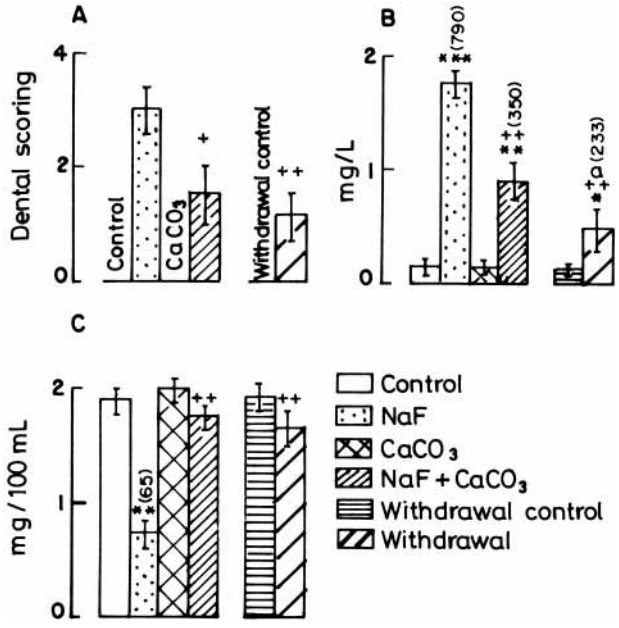

Fig. 4. Dental (structure (A), serum concentrations of fluoride (B) and calcium (C) in test and control animals. Each bar represents mean±S.E.M. of 8 animals. Percent change from control value in parenthesis. *P<0.05, **P<0.01, ***P<0.001 compared to control. +P<0.05, ++P<0.01 compared to NaF-treated group. a P<0.05 compared to NaF+$CaCO_3$ treated group (one way ANOVA followed by Tukey's multiple comparison test).

the concentration was still 350% (P<0.01) higher than in the controls (fig. 4B). However, the toxic effects of fluoride were prevented significantly in these animals.

The effects of NaF on rota-rod performance (P<0.05; fig. 2B) and dental structure (P<0.05; fig. 4A) were less pronounced in animals which also recieved $CaCO_3$ along with NaF. However, these parameters were still significantly different from control animals indicating that the effects of fluoride on motor coordination and dental structure were not completely prevented by $CaCO_3$. But all other effects of NaF were completely prevented when animals received $CaCO_3$ along with it (fig. 1A, B, D; fig. 2A, C, D; fig. 3A, B, C, D; fig. 4C).

Sixty days after withdrawal of NaF treatment, the concentration of fluoride was decreased markedly (P<0.01) in the serum. The fluoride ion concentration was still greater than the control value (233%; P<0.05), but below that measured in animals treated with NaF+$CaCO_3$ (P<0.05) (fig. 4B).

In the NaF-withdrawn group the rota-rod endurance time was prolonged as compared to that observed in the NaF-treated animals (P<0.05; fig. 2B). Dental scorings were decreased in these animals in comparison to NaF-treated animals (P<0.01; fig. 4A). However, these effects of NaF were not reverted completely in these animals. All the other effects of NaF were reverted completely 60 days after withdrawal of NaF (fig. lA, B, C, D; fig. 2A, C, D; fig. 3A, B, C, D; fig. 4C).

Discussion

In the present study, consistent with a previous report (Susheela & Bhatnagar 1993), the concentration of fluoride was increased substantially in the serum after oral administration of NaF daily for several days. This result suggests that there is a steady-rate of absorption of fluoride in the gastrointestinal tract. A decreased urinary excretion of fluoride resulting from fluoride-induced impairment of renal function (Schiffl & Binswanger 1980) may also contribute to an elevation of its concentration in the serum.

Since, fluoride penetrates into the brain (Mullenix *et al.* 1995), NaF can produce neurotoxic actions, if it is administered daily for several days. In the present study and the previous (Paul *et al.* 1998) study, NaF inhibited exploratory motor activity and the effect was accompanied by a decreased activity of AchE in the brain. Therefore, a derangement of central cholinergic activity may account for a deficit in motivated locomotor behaviour in NaF-treated animals. A suppression of eating and drinking processes caused by a defect in motivated locomotor behaviour may account for a decreased food and water intake. Atrophic gastritis produced by chronic oral treatment of NaF (Das *et al.* 1994), may also contribute to decreased food and water intake in these animals.

NaF-treatment produced marked dental lesions in the present study. Since fluoride is known to interact with calcium in the saliva to form insoluble calcium fluoride (Ekstrand & Oliveby 1999), fluoride-induced calcium deficiency in the saliva may account for dental lesions observed in NaF-treated animals. Dental lesions may inevitably impair the ability of animals to masticate food prior to swallowing, as proposed earlier (Shupe *et al.* 1984). This may also contribute to a decreased food intake in NaF-treated animals. Thus poor food and water consumption may be a contributing factor for the failure of NaF-treated animals to gain body weight. However, in the present study NaF-treatment decreased body weight gain more markedly than food and water intake. Therefore, biochemical impairment produced by fluoride may also account for the failure of these animals to gain body weight. Protein synthesis could have been impaired by fluoride because in the present study an inhibition of Na^+ K^+ activated ATPase which is essential for the uptake of amino acids by tissues (Opit *et al.* 1966), was observed in the red blood cells and skeletal muscle of NaF-treated animals.

Inhibition by fluoride of the activity of AchE in the skeletal muscle is likely to impair cholinergic transmission at neuromuscular junction. This and muscle weakness caused by decreased protein synthesis in the muscle accounted for a shortening of rota-rod endurance time in NaF-treated animals.

In the present study a significant hypocalcaemia was observed in NaF-treated animals. A poor gastrointestinal absorption of calcium that resulted from formation of a poorly soluble calcium fluoride or fluorapatite (Boink *et al.* 1994) and a promotion by fluoride of uptake of calcium by

bone (Farley *et al.* 1983), accounted for hypocalcaemia in these animals. Inadequate food intake can also be a contributing factor for hypocalcaemia.

Fluoride consumption was promoted when animals received $CaCO_3$ along with NaF, as a result of an increased water intake. However, the toxic effects of fluoride were prevented in these animals suggesting that calcium has a potential to manage effectively the body burden of fluoride. In support of this suggestion in the present study $CaCO_3$ prevented signifcantly the NaF-induced increase in fluoride concentration in the serum. This result indicates that calcium is able of preventing absorption of fluoride from the gastrointestinal tract. In support of this suggestion, calcium-supplemented diet has been found to decrease absorption of fluoride (Harrison *et al.* 1984; Whitford 1994). Formation of insoluble calcium fluoride accounted by these investigators for a poor gastrointestinal absorption of fluoride.

$CaCO_3$ supplementation completely prevented NaF from producing hypocalcaemia. An increased absorption of calcium as a result of the presence of surplus amounts in the gastrointestinal tract and a promotion of food (contains 1% of calcium) and water (contains 40 ppm of calcium) intake may account for this result.

Withdrawal of NaF for 2 months can decrease availability of fluoride in the gastrointestinal tract. As a result, absorption of calcium may not have been disturbed in these animals. This and adequate intake of food and water accounted for a reversal of serum calcium concentration in these animals. Serum fluoride concentration was not reverted to control level in these animals. A release of fluoride that was stored in the bone during 2 month NaF exposure can account for this result. In support of this suggestion, fluoride deposited in the bone has been found to be released slowly into the blood after withdrawal of exposure (Spencer *et al.* 1980). However, the behavioural, dental and biochemical impairments caused by fluoride were reverted significantly 2 months after withdrawal of its exposure. A significant reversal of endemic fluorosis has been reported upon changing the source of drinking water (Trivedi *et al.* 1993; Chen *et al.* 1993). Further, NaF-induced depletion of total protein, accumulation of glycogen and histological changes were found to be restored in reproductive organ of male mouse after withdrawal of its exposure (Chinoy & Sequeira 1989). Taken together, these results indicate that the changes produced by fluoride on locomotor behaviour, dental and soft tissues and biochemical parameters are transient and that the effects can be reverted if fluoride exposure is decreased to an acceptable level.

A much greater reduction in serum fluoride concentration was found in the NaF-withdrawn group in comparison to that measured in NaF+$CaCO_3$-treated animals. However, in both groups, except for neuromuscular and dental effects, all other impairments were completely inhibited. This result indicates that the rat can tolerate a 233 to 350% increase in the concentration of fluoride in the serum and that fluoride at this level is moderately injurious to neuromuscular system and dental tissue.

In conclusion, the dose of $CaCO_3$ used in the present study has a potential to maintain serum fluoride concentration at a less toxic level when it was administered along with NaF. In rats which were exposed to NaF for 2 months, withdrawal of NaF exposure resulted 2 months later in a reduction in serum fluoride to a lesser toxic concentration. As a result, animals in both the groups were protected significantly from the locomotor behavioural, biochemical and dental toxicities of fluoride.

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