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Neurotoxicity of Sodium Fluoride in Rats

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MULLENIX, P. J., P. K. DENBESTEN, A. SCHUNIOR AND W. J. KERNAN. *Neurotoxicity of sodium fluoride in rats.* NEUROTOXICOL TERATOL **17**(2) 169–177, 1995.—Fluoride (F) is known to affect mineralizing tissues, but effects upon the developing brain have not been previously considered. This study in Sprague-Dawley rats compares behavior, body weight, plasma and brain F levels after sodium fluoride (NaF) exposures during late gestation, at weaning or in adults. For prenatal exposures, dams received injections (SC) of 0.13 mg/kg NaF or saline on gestational days 14–18 or 17–19. Weanlings received drinking water containing 0, 75, 100, or 125 ppm F for 6 or 20 weeks, and 3 month-old adults received water containing 100 ppm F for 6 weeks. Behavior was tested in a computer pattern recognition system that classified acts in a novel environment and quantified act initiations, total times and time structures. Fluoride exposures caused sex- and dose-specific behavioral deficits with a common pattern. Males were most sensitive to prenatal day 17–19 exposure, whereas females were more sensitive to weanling and adult exposures. After fluoride ingestion, the severity of the effect on behavior increased directly with plasma F levels and F concentrations in specific brain regions. Such association is important considering that plasma levels in this rat model (0.059 to 0.640 ppm F) are similar to those reported in humans exposed to high levels of fluoride.

Fluoride Neurotoxicity Central nervous system

DENTAL fluorosis has been on the rise since the 1950s, indicating that our total fluoride exposure is increasing (9). Fluoride, including sodium fluoride (NaF), has been added to public water supplies for over 40 years in the United States as a preventative measure against dental caries. Other sources of fluoride exposure include processed beverages, toothpastes, mouth rinses, dietary supplements, and food. Although dental fluorosis causes discoloration of teeth, it is not considered a public health concern because it does not hinder tooth function or oral health. In addition, no clear link has been established between fluoride and cancer risk, bone fractures, birth defects, or problems of the gastrointestinal, genito-urinary, or respiratory systems (1). Therefore, the impetus to limit total fluoride exposure in the United States is currently based on cosmetic concerns and a general desire not to expose the public to any more fluoride than the amount necessary to prevent dental caries.

One concern that has not been fully investigated is the link between fluoride and effects on the central nervous system (CNS). In vitro studies have shown that intracellular fluoride can alter the kinetic properties of calcium currents in hippocampal neurons (22). Fluoride is a normal component of cerebrospinal fluid (21), but it has not been found to accumulate there during endemic fluorosis or nervous system disease (21,41). Yet, there have been reports from Chinese investigators that high levels of fluoride in drinking water (i.e., 3–11 ppm) affect the nervous system directly without first causing physical deformations from skeletal fluorosis (13,20,40). One study of adult humans found attention affected by sublingual drops containing 100 ppm of sodium fluoride (39), an exposure level potentially relevant to humans because toothpastes contain 1000 to 1500 ppm fluoride (8,48) and mouthrinses contain 230–900 ppm fluoride (48).

Many years of ubiquitous fluoride exposure have not resulted in obvious CNS problems such as seizures, lethargy, salivation, tremors, paralysis, or sensory deficits. Still unexplored, however, is the possibility that fluoride exposure is linked with subtle brain dysfunction. The present study evaluates the neurotoxic potential of sodium fluoride in an animal model. It uses behavioral methodology that focuses on behavioral repertoire, responses to novelty and the temporal or sequential organization of spontaneous behavior, all important

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TABLE 1
EFFECTS OF PRENATAL FLUORIDE EXPOSURE IN 9-WEEK-OLD RATS

Gestational Age of F Exposure	Body Weight (g ± SD)		Plasma F (ppm ± SD)		Behavior (RS Statistic)
	Control	Exposed	Control	Exposed	
Females					
Days 14–18	228.6 ± 15.6	240.5 ± 15.6			0.037
	n = 18	n = 27			n = 18 pairs
Days 17–19	242.0 ± 15.0	244.0 ± 22.9	0.008 ± 0.002	0.008 ± 0.003	0.066
	n = 24	n = 24	n = 9	n = 10	n = 20 pairs
Males					
Days 14–18	347.4 ± 25.3	351.8 ± 38.7			0.082
	n = 20	n = 31			n = 20 pairs
Days 17–19	366.2 ± 37.9	371.0 ± 32.4	0.011 ± 0.003	0.008 ± 0.002	0.144*
	n = 24	n = 24	n = 10	n = 11	n = 20 pairs

*$p < 0.001$.

to the study of CNS function and cognitive processes (12, 32,35,46). Rats were exposed at various stages of development to determine critical periods of CNS susceptibility to fluoride. Also, effects on behavior were related to levels of fluoride found in plasma and in different regions of the brain.

METHOD

Animals

Five hundred and thirty-two pathogen-free Sprague–Dawley rats from the Charles River Laboratories (Kingston, RI) were evaluated in this study. All procedures were conducted under the auspices of Forsyth Dental Center's Animal Care and Use Committee. The animals were assigned randomly to either experimental or control groups and housed 2/cage/treatment and sex. Light cycles were maintained as 12L : 12D cycle (6:00 a.m. to 6:00 p.m.), and food and water were given ad lib except during the behavioral observation periods. Body weight was recorded once weekly and a t test was applied with a $p < 0.01$ required for significance. Further details of treatment protocols depended on the age at exposure.

Prenatal exposures. Twenty-nine timed pregnant dams were obtained on gestational day (GD) 8 (vaginal plug = day 1) and individually housed throughout gestation and lactation. At birth, litters were culled to 10 pups/dam with an equal number of males and females whenever possible. The diet of the dams consisted of Purina Rat Chow (Formulab), and the pups received Certified Purina Rat Chow (5002) after weaning on GD 21. Drinking water for both was deionized water throughout the study. On GDs 14–18 or 17–19, experimental dams (n = 7 and 9, respectively) received SC injections of 0.13 mg/kg sodium fluoride (in saline) two or three times daily (a total of 9 injections per group) at least 4 h apart. Control dams (13 total) received SC injection of an equal volume/body weight of saline on the same gestational days to match each experimental group. This route and concentration of fluoride exposure produces peak plasma fluoride levels of 0.15–0.20 ppm which return to control levels within 4 h in nongravid females (4). Beyond the prenatal period, these pups received no other experimental fluoride treatment.

Weanling exposures. At 19 days of age, male and female pups were shipped with dams having 10 pups/litter. When weaned on GD 21, the pups (n = 19–27/sex and treatment) were maintained thereafter on a low fluoride diet (< 10 ppm fluoride, Purina 5010, or Teklad L356, Madison, WI). When teeth were broken in either the control or experimental groups, this diet was given in powdered form for a few days until normal occlusion returned. Their drinking water contained either 0, 75, 100, 125, or 175 ppm fluoride (3.9, 5.3, 6.6, or 9.2 mM NaF in deionized water, respectively) for 6 or 20 weeks. The 175 ppm level, studied only in females, resulted in dehydration and the death of 10 of the exposed animals within 10 days. Therefore, the 175 ppm exposure was discontinued after 10 days, and the 11 survivors were given deionized water for the remainder of the study. Each fluoride treatment group had matching controls who received deionized water only. This range of fluoride exposures was selected because 100 ppm fluoride in drinking water produces dental fluorosis without other overt signs of toxicity in rats (11).

Adult exposures. Male and female rats (n = 21–24/sex/treatment) were obtained at 10 weeks of age and given 0 or 100 ppm sodium fluoride in deionized water for 5 to 6 weeks starting at 12 weeks of age. They were fed the same low fluoride diet as in the weanling exposure.

Plasma and Brain Fluoride

After the behavioral tests at the termination of each study, blood samples were collected by cardiac puncture under CO_2 anesthesia. When plasma fluoride determinations were needed at ages prior to termination of the study, blood samples were obtained from extra control and exposed animals not included in any behavioral study. All plasma fluoride concentrations were determined using an ion-specific electrode (Orion, Cambridge, MA), following the hexamethyldisiloxane diffusion method (HMDS) of Whitford and Reynolds (51). Brain fluoride concentrations also were determined for two treatment groups receiving weanling or adult exposures. After CO_2 euthanasia, these animals were decapitated and the brain removed, blotted, and chilled with further dissection performed on an ice-cooled glass plate. Seven regions of the rat brain were dissected:

1. Cerebellum,
2. Medulla oblongata,

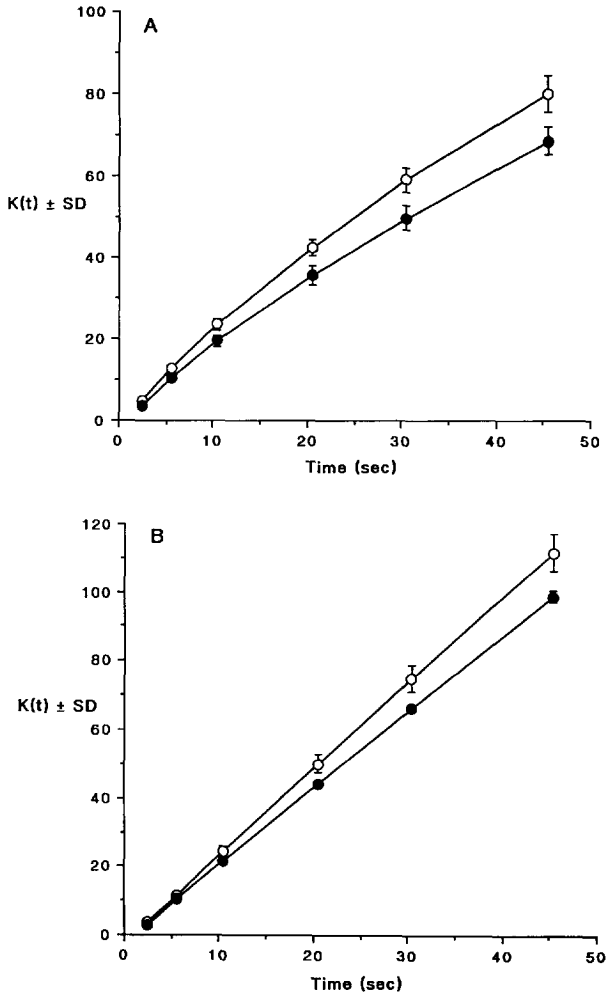

FIG. 1. Regardless of the gestational period of exposure or sex of the offspring, prenatal fluoride typically affected behavioral time structure but not behavioral initiations or total times. These example K functions illustrate the time-structure changes found in 9-week-old male rats significantly affected (RS = 0.144; $p < 0.001$) by fluoride exposure on GDs 17–19 (●) compared to respective controls (○). Significant dispersions (decreased K values) are shown for the behaviors groom/attention (A) and turn (B). Error bars indicate ± SD.

3. Hypothalamus,
4. Midbrain,
5. Striatum,
6. Hippocampus and
7. Cortex

The procedures described in detail by Glowinski and Iversen (17) were used. Each brain region was individually lyophilized and weighed, and the fluoride content was measured following diffusion of ionic fluoride (51).

Behavior

Methods to assess effects on behavior were the same as in prior studies of amphetamine induced hyperactivity and stereotypy (34), triethyltin induced hypoactivity (23), and cognitive deficits induced by CNS therapy (35,36). Except for

TABLE 2

EFFECTS ON BODY WEIGHT BY FLUORIDE EXPOSURE STARTED AT WEANING

F in Drinking Water	Exposure Duration	Age When Weighed	Controls g ± SD	Exposed g ± SD
Females				
75 ppm	6 weeks	9 weeks	248.1 ± 19.3 $n = 20$	257.0 ± 30.2 $n = 21$
100 ppm	6 weeks	9 weeks	247.0 ± 23.0 $n = 22$	242.0 ± 24.0 $n = 22$
125 ppm	6 weeks	9 weeks	237.7 ± 15.4 $n = 21$	208.3 ± 30.2† $n = 26$
	11 weeks	14 weeks	296.1 ± 25.8 $n = 21$	265.1 ± 26.2† $n = 27$
	16 weeks	19 weeks	329.0 ± 26.1 $n = 21$	300.4 ± 31.6* $n = 27$
	20 weeks	23 weeks	339.1 ± 30.1 $n = 21$	313.4 ± 33.4* $n = 26$
175 ppm	10 days	9 weeks	227.0 ± 20.8 $n = 19$	206.5 ± 11.5* $n = 11$
	10 days	18 weeks	352.9 ± 33.3 $n = 16$	331.4 ± 31.8 $n = 11$
Males				
75 ppm	6 weeks	9 weeks	381.1 ± 25.4 $n = 20$	380.9 ± 21.8 $n = 21$
125 ppm	6 weeks	9 weeks	380.1 ± 37.2 $n = 21$	330.1 ± 35.9‡ $n = 24$
	11 weeks	14 weeks	545.1 ± 56.1 $n = 18$	451.3 ± 30.1‡ $n = 23$
	16 weeks	19 weeks	656.6 ± 80.3 $n = 21$	553.6 ± 44.4‡ $n = 25$
	20 weeks	23 weeks	717.8 ± 90.8 $n = 21$	592.9 ± 66.3‡ $n = 25$

*$p < 0.01$, t-test; †$p < 0.001$, t-test; ‡$p < 0.0001$, t-test.

TABLE 3

PLASMA FLUORIDE LEVELS AFTER FLUORIDE EXPOSURE STARTED AT WEANING

F in Drinking Water	Exposure Duration	Age When Measured	Controls ppm ± SD	Exposed ppm ± SD
Females				
75 ppm	6 weeks	9 weeks	0.009 ± 0.003 $n = 8$	0.066 ± 0.02† $n = 8$
100 ppm	6 weeks	9 weeks	0.007 ± 0.001 $n = 6$	0.150 ± 0.03‡ $n = 7$
125 ppm	6 weeks	9 weeks	0.006 ± 0.001 $n = 4$	0.107 ± 0.028‡ $n = 6$
	20 weeks	23 weeks	0.015 ± 0.006 $n = 8$	0.640 ± 0.308‡ $n = 8$
Males				
75 ppm	6 weeks	9 weeks	0.012 ± 0.002 $n = 10$	0.170 ± 0.097‡ $n = 10$
125 ppm	6 weeks	9 weeks	0.011 ± 0.011 $n = 4$	0.126 ± 0.031† $n = 7$
	20 weeks	23 weeks	0.013 ± 0.005 $n = 8$	0.408 ± 0.255* $n = 8$

*$p < 0.01$, t test; †$p < 0.001$, t test; ‡$p < 0.0001$, t test.

TABLE 4
EFFECTS ON BEHAVIOR BY FLUORIDE EXPOSURE STARTED AT WEANING

F in Drinking Water	Exposure Duration	Age When Measured	RS Statistic
Females			
75 ppm	6 weeks	9 weeks	0.052
			n = 20 pairs
100 ppm	6 weeks	9 weeks	0.359†
			n = 22 pairs
125 ppm	6 weeks	9 weeks	0.115*
			n = 20 pairs
	11 weeks	14 weeks	0.140†
			n = 20 pairs
	16 weeks	19 weeks	0.169†
			n = 20 pairs
Males			
75 ppm	6 weeks	9 weeks	0.024
			n = 20 pairs
125 ppm	6 weeks	9 weeks	0.086
			n = 20 pairs
	11 weeks	14 weeks	0.204†
			n = 20 pairs
	16 weeks	19 weeks	0.311†
			n = 20 pairs

*$p < 0.01$; †$p < 0.001$.

adult exposures, behavior was tested in animals at 9 weeks of age, and behavioral tests were repeated at 14 and 19 weeks when the period of exposure extended beyond 6 weeks. Behavioral tests were conducted in an isolated observation room between 0900 and 1300 h each day for consistent diurnal testing. Two video cameras taking 1 frame/s were used to monitor simultaneously the spontaneous behavior of 1 fluoride-treated rat and its matched control during a 15-min exploration of a novel environment. The novel environment consisted of a clear Plexiglas box, where the control and treated rats were separated by a clear partition with small holes that allowed them to see and smell each other during exploration. The video signals were transferred to a MICRO VAX I and a VAX 11/750 for pattern analysis and behavioral classification of the data. The behaviors identified by the computer consisted of five major body positions (stand, sit, rear, walk, and lying down) and eight modifiers (groom, head turn, look, smell, sniff, turn, wash face, and blank or no recognized activity). The system of cameras, computers, computer software, and novel environment has been described in detail (26).

Three measures of spontaneous behavior were taken: a calculation of behavioral initiations (BI), behavioral total time (BTT), and a measure of behavioral time structure (BTS) concerning the time distribution of the initiation of discrete acts and of sequences of joint acts. The BI, BTT, and BTS measures in this study were computed for 18–22 pairs (a pair consists of 1 fluoride-treated rat and 1 matched control) per treatment group.

Calculation of behavioral initiations (BI). The frames in which a specific behavior began were totaled for each act during the 15-min observation period for each rat. The mean number of initiations was determined for each control and experimental group of rats. A student's t test was applied and a $p < 0.05$ was required for statistical significance.

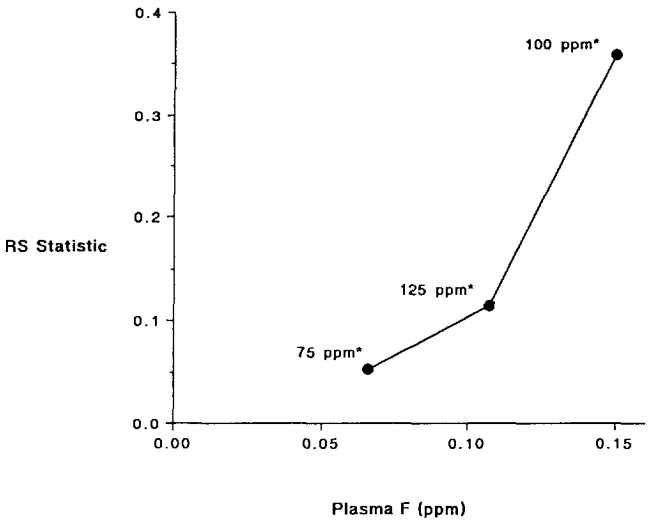

FIG. 2. Severity of behavioral disruption indicated by the RS statistic increased as plasma fluoride levels increased. Elevated plasma fluoride levels were induced by 6-week exposures to sodium fluoride in drinking water of female rats whose exposure began at 21 days of age. Concentrations of fluoride in drinking water associated with each plasma level are highlighted with asterisks.

Calculation of behavioral total time (BTT). The number of frames that a behavior continued, including the frame it was initiated, was totaled for the 15-min observation period. The mean total time for each act in control and experimental groups of rats was determined and statistical significance was evaluated using the student's t test, with a $p < 0.05$ required for a change to be considered significant.

Calculation of behavioral time structure (BTS). The time

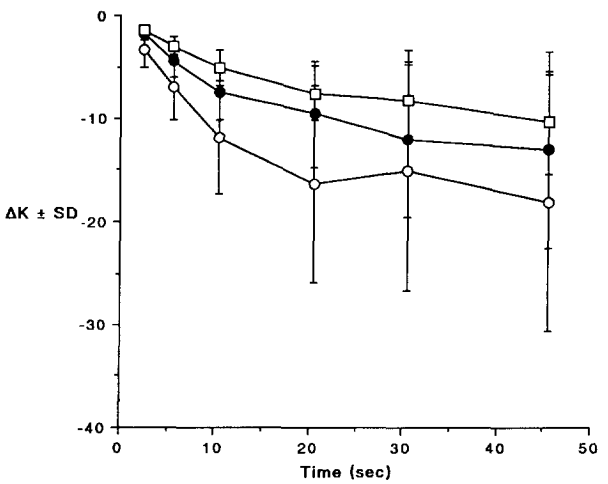

FIG. 3. The ΔK's [the difference between K(t) for a control and an exposed group at the first six time points evaluated] are shown for 100 ppm fluoride for 6 weeks starting at 3 months of age in females (●) and 125 ppm fluoride for 20 weeks starting at 21 days of age in females (○) and males (□). Regardless of the different doses, exposure durations, and ages when exposure began, the fluoride in drinking water caused the time structure of the behavior sit to cluster (negative ΔK values) by 5 months of age. Error bars indicate ± SD.

TABLE 5

CONSISTENT BEHAVIORAL EFFECTS OF FLUORIDE EXPOSURE STARTED AT WEANING

Behavior	Females		Females		Males	
	Control	100 ppm F for 6 Weeks	Control	125 ppm F for 16 Weeks	Control	125 ppm F for 16 Weeks
Sit						
BI (±SE)	20.7 ± 3.0	16.0 ± 1.8	22.4 ± 2.2	15.2 ± 1.8*	57.7 ± 3.3	42.8 ± 3.5†
BTT (±SE)	76.6 ± 19.3	37.9 ± 5.1	66.8 ± 10.9	37.7 ± 5.5*	245.6 ± 21.8	174.4 ± 23.6*
Groom						
BI	13.8 ± 2.6	6.5 ± 1.1*	8.1 ± 2.0	5.2 ± 1.2	30.0 ± 5.5	14.3 ± 2.2*
BTT	29.8 ± 6.3	9.4 ± 1.8†	20.1 ± 6.2	11.4 ± 3.4	70.3 ± 16.1	35.8 ± 8.9
Turn						
BI	123.5 ± 5.5	123.1 ± 5.1	110.7 ± 3.8	105.9 ± 5.5	97.2 ± 5.0	81.7 ± 5.4*
Head turn						
BI	61.9 ± 3.5	57.4 ± 2.9	67.3 ± 2.2	58.6 ± 1.5†	68.1 ± 4.0	58.5 ± 5.0
BTT	75.8 ± 5.3	66.8 ± 3.7	78.8 ± 2.9	69.4 ± 1.9†	84.9 ± 5.5	72.7 ± 7.0
Groom/explore (cluster)						
BI	12.6 ± 3.1	8.5 ± 0.9	15.4 ± 1.9	8.6 ± 1.0†	37.5 ± 3.0	23.7 ± 2.9†
BTT	17.3 ± 4.8	10.7 ± 1.2	19.5 ± 2.4	11.5 ± 1.4†	49.5 ± 4.3	32.5 ± 4.6†
Groom/attention (cluster)						
BI	26.5 ± 4.4	16.1 ± 2.4*	20.4 ± 2.9	12.6 ± 1.8*	72.1 ± 5.2	46.1 ± 4.1‡
BTT	60.7 ± 11.8	43.2 ± 9.0	40.3 ± 8.6	21.5 ± 3.6*	184.9 ± 19.8	131.4 ± 18.7
Groom (cluster)						
BI	10.4 ± 2.5	4.6 ± 0.9*	6.9 ± 1.6	4.3 ± 1.1	22.6 ± 3.8	11.7 ± 2.0*
BTT	22.7 ± 6.2	7.0 ± 1.6*	13.9 ± 3.7	8.2 ± 2.4	42.4 ± 7.8	23.6 ± 4.5*
Stand						
BTT	576.0 ± 22.1	607.9 ± 12.0	608.1 ± 14.9	629.2 ± 17.7	532.5 ± 20.2	599.0 ± 22.0*
Attention (cluster)						
BTT	494.9 ± 19.9	529.5 ± 13.4	505.3 ± 14.8	528.7 ± 20.0	418.4 ± 21.4	499.3 ± 20.9*

*$p < 0.05$, t test; †$p < 0.01$, t test; ‡$p < 0.001$, t test.

distribution and time sequence of behavioral acts were calculated using equations for $K(t)$ as previously reported (27,28,33). The K function was calculated for specific behavioral acts (e.g., sit, rear) or sequences of specific behavioral acts (e.g., sit . . . rear) (33) and for combined acts (e.g., attention or attention/groom) or sequences of combined acts (e.g., attention . . . explore or attention/explore . . . groom/attention) (28). For each of these, a $\Delta K(t)$ [the difference between $K(t)$ for the fluoride animals and matched controls] was calculated for eight time points (2,5,10,20,30,45,100, and 200 s). At any one time point, when K values increase (compared to controls) for a behavior, it means that that particular behavior (or sequence) is "clustering" in time (as seen in hypoactivity), while a decrease means it is "dispersing" in time (it had increased regularity of timing between initiations as seen in hyperactivity). Whenever a behavioral act was initiated less than 10 times on average per animal, control or experimental, $K(t)$ values were not determined for that behavior and related sequences. The bootstrap technique was used for estimating SD at each time point of the K-function for a behavior, and the ad hoc criteria for significance of a difference between control and exposed groups have been described (23,25,27,28,33,34).

An RS statistic was determined for each fluoride treatment. The ad hoc RS statistic distinguishes low level behavioral ef-

TABLE 6

EFFECTS OF 100 ppm FLUORIDE FOR 6 WEEKS STARTING IN 3 MONTH-OLD-RATS

	Body Weight (g ± SD)		Plasma F (ppm ± SD)		Behavior (RS Statistic)
	Control	Exposed	Control	Exposed	
Females	331.8 ± 41.6	319.8 ± 36.1	0.010 ± 0.002	0.077 ± 0.040*	0.200‡
	$n = 21$	$n = 22$	$n = 5$	$n = 5$	$n = 20$ pairs
Males	620.3 ± 45.3	609.0 ± 72.1	0.012 ± 0.005	0.059 ± 0.027†	0.053
	$n = 24$	$n = 22$	$n = 6$	$n = 5$	$n = 18$ pairs

*$p < 0.05$, t test; †$p < 0.01$, t test; ‡$p < 0.001$.

fects from noise (24). This statistic encompasses all data produced in an experiment into one simple statistic. This is an advantage considering that the computer system generates over 100 behavioral measures of three distinctly different types (initiations, total times, and time structures) per experiment. The RS statistic indicates whether behavior is changed overall and the confidence level associated with that change. Statistical significance was set at the $p < 0.01$ level.

RESULTS

Prenatal Exposures

No maternal or offspring toxicity was indicated by reduced body weight in dams during treatment or in their pups soon after birth. Yet, prenatal exposure to sodium fluoride altered behavioral outcome in male offspring when exposure occurred on GDs 17–19 (Table 1). This effect consisted entirely of time structure changes in 11 behaviors and behavioral sequences, 10 of which were significantly dispersed compared to matching controls as illustrated in Fig. 1. These behavioral effects did not coincide with reduced body weight nor elevated plasma fluoride levels at 9 weeks of age (Table 1). At 3 weeks of age, plasma fluoride levels also were not elevated despite prenatal exposure on GD 17–19; plasma fluoride levels were no different in prenatal fluoride females (0.007 ppm ± 0.003 SD; $n = 7$) compared to matched controls (0.006 ppm ± 0.002 SD; $n = 7$) or in prenatal fluoride males (0.004 ppm ± 0.002 SD; $n = 8$) compared to controls (0.004 ppm ± 0.003 SD; $n = 8$).

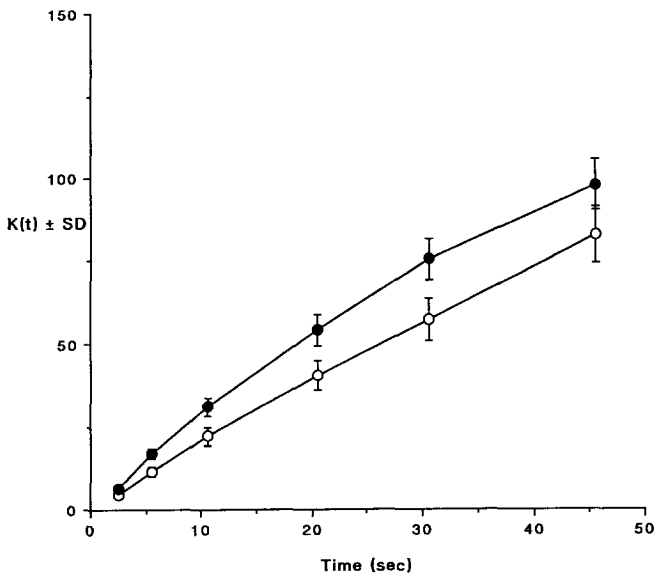

FIG. 4. Exposure to fluoride at the adult stage significantly altered (RS = 0.200; $p < 0.001$) behavior of female rats (●) compared to respective controls (○). This example K function illustrates time-structure changes typical of the adult F effect. Significant clustering (increased K values) is shown for the behavior groom/attention, which prenatal F, in contrast, significantly dispersed (Fig. 1A). Error bars indicate ± SD.

Weanling Exposures

When fluoride exposures began at 21 days of age, effects on body weight depended on the fluoride concentration in the drinking water (Table 2). Concentrations below 125 ppm did not affect body weight gain at any time during a 5- to 6-week exposure. In contrast, at 125 ppm body weight was reduced throughout 20 weeks of exposure in both sexes. The 11 survivors of a 10-day exposure to 175 ppm F also had stunted growth compared to matched controls at 9 weeks of age. However, by 18 weeks of age, stunting among the 175 ppm female survivors was ameliorated (Table 2).

Plasma fluoride levels were significantly increased in all exposed animals, but again the increase depended upon the fluoride concentration given in the drinking water (Table 3). At 75 and 100 ppm fluoride in drinking water of females for 6 weeks, plasma fluoride levels increased respective of dose. When concentration in the drinking water was 125 ppm for 6 weeks, plasma fluoride levels increased compared to controls but not to levels expected considering results observed at lower drinking water concentrations (Table 3).

Fluoride in drinking water of weanlings altered behavior in both sexes (Table 4). The duration and concentration of exposure determined whether significant effects occurred. In females, a 6-week exposure to 100 or 125 ppm was sufficient to alter behavior, whereas in males an 11-week exposure to 125 ppm in drinking water significantly affected behavior. Too few 175 ppm fluoride females (11 in total) survived after a 10-day exposure to determine an RS statistic for that group. A relationship between behavioral effects and plasma fluoride levels was observed in females exposed for 6 weeks to 75, 100, or 125 ppm fluoride. Figure 2 illustrates that as plasma fluoride levels increased, the RS statistic increased, with significant behavioral impact estimated to occur at a plasma fluoride level of approximately 0.107 ppm. Significant behavioral impact in males, however, did not occur until plasma levels exceeded 0.126 and 0.170 ppm (Table 3 and Table 4).

Regardless of sex, duration of exposure, or the fluoride concentration in the drinking water of weanlings, a common pattern among behavioral disturbances developed. Table 5 includes all behaviors that were significantly affected in BI and/or BTT by at least one fluoride exposure. Age and sex influenced the BI and BTT of these behaviors in controls, but still a general effect of fluoride emerged. Whether exposure lasted 6 or 16 weeks, at the 100 or 125 ppm level, in males or females, the same direction of change with respect to controls occurred for a certain array of behaviors and related behavioral clusters. Whereas the act of standing and the related attention cluster tended to increase in total time, the other acts consistently decreased in initiations and total times.

Adult Exposures

Male and female adult rats given 100 ppm fluoride for 6 weeks had significantly increased plasma fluoride levels with no effect on body weight, whereas behavior was affected only in females (Table 6). Compared to females exposed at weaning, females exposed as adults had a lower plasma fluoride level (0.077 ppm) associated with significant behavioral impact. However, the same pattern of BI and BTT changes seen with weanling exposures (Table 5) also developed in females exposed as adults. For example, initiations of sitting, the groom/attention cluster, and the groom/explore cluster in adult female controls (42.9 ± 3.0; 50.4 ± 5.4; 23.9 ± 2.4 SE, respectively) were more frequent than in adult exposed females (30.2 ± 3.0; 34.7 ± 3.7; 15.3 ± 1.8 SE, respectively; $p < 0.01$). Another similarity appeared among BTS effects when adult and weanling exposed rats approached 5 months of age (Fig. 3). Other BTS effects appeared to differ

TABLE 7

BRAIN REGION FLUORIDE LEVELS (ppm ± SE) IN 5- TO 6-MONTH-OLD RATS

	Hypothalamus	Cerebellum	Medulla Oblongata	Basal Ganglia	Mid-Brain	Cortex	Hippocampus
Females							
Control[+]	0.396 ± 0.073	0.358 ± 0.055	0.609 ± 0.107	0.406 ± 0.103	0.634 ± 0.213	0.479 ± 0.107	0.258 ± 0.043
	n = 12	n = 13	n = 14	n = 14	n = 14	n = 14	n = 12
125 ppm F for 20 wks in weanlings	1.685 ± 0.565†	3.120 ± 0.968†	3.281 ± 1.054†	1.281 ± 0.229†	1.091 ± 0.194	1.830 ± 0.383†	0.993 ± 0.168†
	n = 8	n = 8	n = 7	n = 8	n = 8	n = 8	n = 8
100 ppm F for 6 wks in adults	0.308 ± 0.043	0.325 ± 0.052	1.280 ± 0.445	0.252 ± 0.043	0.306 ± 0.087	0.602 ± 0.195	0.790 ± 0.328*
	n = 5	n = 6	n = 6	n = 6	n = 5	n = 6	n = 4
Males							
Control	0.364 ± 0.052	0.292 ± 0.055	0.281 ± 0.044	0.273 ± 0.036	0.246 ± 0.035	0.372 ± 0.069	0.287 ± 0.048
	n = 14	n = 13	n = 14	n = 14	n = 14	n = 14	n = 14
125 ppm F for 20 wks in weanlings	0.839 ± 0.130†	2.133 ± 0.573†	1.875 ± 0.334	0.697 ± 0.101†	0.770 ± 0.145†	1.727 ± 0.435†	0.834 ± 0.104†
	n = 7	n = 8	n = 8	n = 8	n = 8	n = 8	n = 8
100 ppm F for 6 wks in adults	0.340 ± 0.049	0.412 ± 0.095	3.922 ± 2.379†	0.422 ± 0.146	0.378 ± 0.106	0.350 ± 0.057	0.411 ± 0.088
	n = 5	n = 6	n = 5	n = 5	n = 6	n = 6	n = 6

[+]Controls pooled from 100 and 125 ppm fluoride exposures; *$p \leq 0.05$; one-factor ANOVA followed by Fisher's procedure for multiple comparisons; †$p \leq 0.01$; one-factor ANOVA followed by Fisher's procedure for multiple comparisons.

depending on the age when exposure occurred; the significant dispersing of groom/attention after prenatal fluoride (Fig. 1) is in contrast with the significant clustering of the same behavior by fluoride exposures started in adults (Figure 4). Future studies will have to determine if this variation is a function of the age at exposure or the age when tested.

Fluoride Levels in Brain

Fluoride exposure via drinking water elevated the fluoride levels in various brain regions (Table 7). In male and female rats exposed to 125 ppm fluoride for 20 weeks starting at weaning, brain fluoride levels increased in all seven brain regions examined. In rats exposed to 100 ppm fluoride for 6 weeks starting at 3 months of age, fluoride levels increased in the medulla oblongata in both sexes and in the hippocampus of females, the sex with significant behavioral disturbances.

DISCUSSION

This study demonstrates a link between certain fluoride exposures and behavioral disruption in the rat. The effect on behavior varied with the timing of exposure during CNS development. Behavioral changes common to weanling and adult exposures were different from those after prenatal exposures. Prenatal exposure on GDs 17-19 dispersed many behaviors as seen in drug-induced hyperactivity (34), while weanling and adult exposures led to behavior-specific changes more related to cognitive deficits (35,36). Prenatally induced behavioral effects were unaccompanied by changes in body weight or elevated plasma fluoride levels. Rather, the most obvious hypothesis is that the effects relied on transient peaks in maternal plasma fluoride levels, fluoride passing the placenta, and fluoride penetrating the blood-brain barrier of the fetus. Fluoride has been reported to pass the placenta in rats (45), and on GD 17-19 the blood-brain barrier is immature and readily penetrable (52). In contrast, the behavioral effects induced by weanling and adult exposures were accompanied often by weight reduction and always by elevated plasma fluoride levels. In fact, effects on behavior related directly to plasma fluoride levels and the fluoride accumulation in the brain. This contradicts findings from short-term fluoride kinetic studies, which found that the adult blood-brain barrier was relatively impermeable to fluoride when whole brain fluoride levels were measured within 1 h following IV injection (49,50). Considering the brain fluoride accumulations found in this study, such impermeability does not apply to chronic exposure situations.

Hyperactivity and cognitive deficits are generally linked with hippocampal damage (3), and in fact, the hippocampus is considered to be the central processor which integrates inputs from the environment, memory, and motivational stimuli to produce behavioral decisions and modify memory (12). GDs 17-19 in the rat is a period when pyramidal cells of the hippocampus are forming (6), and granule cells of the dentate gyrus of the hippocampus form at the ages when weanling and adult exposures were administered (7). Involvement of different cell types would explain variation in behavioral outcomes between prenatal, weanling, and adult exposures. The hypothalamus and the hippocampus in normal female rat brains have the lowest concentrations of fluorine, the element which was found to be the most regionally distributed by instrumental neutron activation analysis (10). The method used for ionic fluoride analysis in the present study also revealed that the brain region containing the lowest fluoride concentrations was the hippocampus of controls but only in females. This hippocampal selectivity was disrupted when adult females were exposed for 6 weeks to 100 ppm fluoride; hippocampal fluoride levels increased and behavior was affected. Adult males receiving the same fluoride exposure did not have significantly elevated fluoride levels in the hippocampus, nor did they have significant behavioral disturbances. Sex differences in hippocampal function have been described recently in other studies (2,47). Overall, the behavioral changes from fluoride exposure are consistent with interrupted hippocampal

development. Whether the hippocamus is indeed the brain region most susceptible to fluoride is a possibility deserving consideration in future studies.

Interruption of normal brain development often results in responses that are sex-dependent. The brain responds differently to drugs depending on which hormones are present at the time and whether the brain is male or female (30). In male primates the orbital cortex matures earlier than in females, and such developmental differences are thought responsible for the consequences of perinatal injuries appearing more frequently in males (18). This type of developmental difference might explain why transient peaks of fluoride on prenatal days 17–19 affected males and not females. The effects of chronic fluoride exposures at weanling and adult stages may have involved still other sexual dimorphisms. There are developmentally regulated sexual dimorphisms in hypothalamic somatostatin and growth-hormone-releasing factor signaling, growth hormone secretion and even hepatic metabolism (5,29,38). The sexually dimorphic control of growth would be especially important to fluoride distribution. The rate of fluoride uptake by bone depends on age or the stage of skeletal development; fluoride is deposited in mineralizing new bone more readily than in existing bone (49). As males experience greater and more prolonged growth spurts than females, their plasma fluoride might be directed more to bone than to brain, perhaps explaining why longer exposures and higher plasma fluoride levels were needed in males to affect behavior. Fluoride's tendency to seek developing bone may also explain why adult female rats had behavioral effects at a lower plasma fluoride concentration than did weanling female rats. Levels of fluoride in plasma and bone must be correlated with those in specific brain regions of both sexes to fully understand behavioral consequences.

Rats ingested 75–125 ppm fluoride for weeks to attain plasma fluoride levels of 0.059–0.640 ppm. Six weeks of consuming 75 and 100 ppm fluoride produced higher plasma fluoride levels than did 125 ppm. Perhaps a taste aversion limited water consumption at the 125 ppm level, prolonging the period needed to attain plasma levels that were achieved in 6 weeks by the two lower exposure levels. Regardless, it was fluoride levels in plasma, not fluoride levels of exposure, which best predicted effects on behavior. Similar plasma fluoride levels of 0.076–0.25 ppm have been found in humans ingesting 5–10 ppm fluoride in drinking water (19,37,42), and plasma levels as high as 0.28 to 0.43 ppm have been measured in children drinking water containing 16 ppm fluoride (44). This plasma fluoride range also occurs in certain therapies. Fasting serum fluoride levels of 0.2 to 0.3 ppm are used in the treatment of osteoporosis (31), and plasma fluoride levels as high as 1.44 ppm are found in children 1 h after receiving topical applications of an acidulated phosphate fluoride (1.23%) gel (14,15).

Because humans occasionally are exposed to high amounts of fluoride and plasma levels as high as those found in this rat study, neurotoxic risks deserve further evaluation. This is the first laboratory study to demonstrate that CNS functional output is vulnerable to fluoride, that the effects on behavior depend on the age at exposure and that fluoride accumulates in brain tissues. Experience with other developmental neurotoxicants prompts expectations that changes in behavioral function will be comparable across species, especially humans and rats (16,43). Of course behaviors per se do not extrapolate, but a generic behavioral pattern disruption as found in this rat study can be indicative of a potential for motor dysfunction, IQ deficits and/or learning disabilities in humans. Substances that accumulate in brain tissue potentiate concerns about neurotoxic risks, but the conditions leading to fluoride deposits in any species are still not clear such that quantitative extrapolations are not possible at this time. Thus, conclusions concerning the neurotoxic potential of fluoride require further rat and human studies, both focused on the relationship of plasma fluoride levels with the brain, behavior, and skeletal growth.

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REFERENCES

1. Ad hoc Subcommittee on Fluoride of the Committee to Coordinate Environmental Health and Related Programs. Review of fluoride: Benefits and risks. Washington, DC: Public Health Service; 1991.
2. Ahima, R. S.; Lawson, A. N. L.; Osei, S. Y. S.; Harlan, R. E. Sexual dimorphism in regulation of type II corticosteroid receptor immunoreactivity in the rat hippocampus. Endocrinology 131: 1409–1416; 1992.
3. Altman, J. Morphological and behavioral markers of environmentally induced retardation of brain development. Environ. Health Perspect. 74:153–168; 1987.
4. Angmar-Månsson, B.; Whitford, G. M. Plasma fluoride levels and enamel fluorosis in the rat. Caries Res. 16:334–339; 1982.
5. Argente, J.; Chowen, J. A.; Zeitler, P.; Clifton, D. K.; Steiner, R. A. Sexual dimorphism of growth hormone-releasing hormone and somatostatin gene expression in the hypothalamus of the rat during development. Endocrinology 128:2369–2375; 1991.
6. Bayer, S. A. Development of the hippocampal region in the rat. I. Neurogenesis examined with ^{3}H-thymidine autoradiography. J. Comp. Neurol. 190:87–114; 1980.
7. Bayer, S. A.; Yackel, J. W.; Puri, P. S. Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life. Science 216:890–892; 1982.
8. Beltran, E. D.; Szpunar, S. M. Fluoride in toothpastes for children. Suggestion for change. Pediatr. Dent. 10:185–188; 1988.
9. Centers for Disease Control. Public Health Service Report on fluoride benefits and risks. MMWR 40 (No. RR-7):1–8; 1991.
10. Chan, A. W. K.; Minski, M. J.; Lai, J. C. K. An application of neutron activation analysis to small biological samples: Simultaneous determination of thirty elements in rat brain regions. J. Neurosci. Methods 7:317–328; 1983.
11. DenBesten, P. K.; Crenshaw, M. A. The effects of chronic high fluoride levels on forming enamel in the rat. Archs. Oral Biol. 29: 675–679; 1984.
12. DeLong, G. R. Autism, amnesia, hippocampus, and learning. Neurosci. Biobehav. Rev. 16:63–70; 1992.
13. Ding, L. I. The nervous systemic complications of chronic fluorosis. Chinese J. Endemiology 2:97–98; 1983.
14. Ekstrand, J. Pharmacokinetic aspects of topical fluorides. J. Dent. Res. 66:1061–1065; 1987.
15. Ekstrand, J.; Koch, G.; Lindgren, L. E.; Peterson, L. G. Pharmacokinetics of fluoride gels in children and adults. Caries Res. 15:213–220; 1981.
16. Francis, E. Z.; Kimmel, C. A.; Rees, D. C. Workshop on the qualitative and quantitative comparability of human and animal developmental neurotoxicity: Summary and Implications. Neurotoxicol. Teratol. 12:285–292; 1990.
17. Glowinski, J.; Iversen, L. L. Regional studies of catecholamines in the rat brain. I. The disposition of [^{3}H] norepinephrine, [^{3}H]

dopamine and [^{3}H]DOPA in various regions of the brain. J. Neurochem. 13:655-669; 1966.
18. Goldman, P. S.; Crawford, H. T.; Stokes, L. P.; Galkin, T. W.; Rosvold, H. E. Sex-dependent behavioral effects of cerebral cortical lesions in the developing rhesus monkey. Science 186: 540-542;1974.
19. Guy, W. S.; Taves, D. R. Relation between F in drinking water and human plasma. J. Dent. Res. 52:238; 1973.
20. Hu, Y. H. Direct damage on nervous system by fluorosis. The Compilation of the First Conference on Neuropsychiatric Diseases in Xinjian. 1982:86-88.
21. Hu, Y. H.; Wu, S. S. Fluoride in cerebrospinal fluid of patients with fluorosis. J. Neurol. Neurosurg. Psychiatry 51:1591-1593; 1988.
22. Kay, A. R.; Miles, R.; Wong, R. K. S. Intracellular fluoride alters the kinetic properties of calcium currents facilitating the investigation of synaptic events in hippocampal neurons. J. Neurosci. 6:2915-2920; 1986.
23. Kernan, W. J.; Hopper, D. L.; Bowes, M. P. Computer pattern recognition: Spontaneous motor activity studies of rats following acute exposure to triethyltin. J. Am. Coll. Toxicol. 10:705-718; 1992.
24. Kernan, W. J.; Meeker, W. Q. A statistical test to assess changes in spontaneous behavior of rats observed with a computer pattern recognition system. J. Biopharm. Stat. 2:115-135; 1992.
25. Kernan, W. J.; Mullenix, P. J. Stability and reproducibility of time structure in spontaneous behavior in male rats. Pharmacol. Biochem. Behav. 39:747-754; 1991.
26. Kernan, W. J.; Mullenix, P. J.; Hopper, D. L. Pattern recognition of rat behavior. Pharmacol. Biochem. Behav. 27:559-564; 1987.
27. Kernan, W. J.; Mullenix, P. J.; Hopper, D. L. Time structure analysis of behavioral acts using a computer pattern recognition system. Pharmacol. Biochem. Behav. 34:863-869; 1989.
28. Kernan, W. J.; Mullenix, P. J.; Kent, R.; Hopper, D. L.; Cressie, N. A. Analysis of the time distribution and time sequence of behavioral acts. Intern. J. Neuroscience 43:35-51; 1988.
29. Legraverend, C.; Mode, A.; Wells, T.; Robinson, I.; Gustafsson, J.-A. Hepatic steroid hydroxylating enzymes are controlled by the sexually dimorphic pattern of growth hormone secretion in normal and dwarf rats. FASEB J. 6:711-718; 1992.
30. McEwen, B. S. Actions of sex hormones on the brain: "Organization" and "activation" in relation to functional teratology. Prog. Brain Res. 73:121-134; 1988.
31. Mohan, S.; Stauffer, M.; Baylink, D. J. Clinical use of fluoride in osteoporosis. In: Kanis, J. A., ed. Calcium metabolism. Basel: Karger; 1990:137-164.
32. Mullenix, P. J. Evolution of motor activity tests into a screening reality. Toxicol. Indust. Health 5: 203-219; 1989.
33. Mullenix, P. J.; Kernan, W. J. Extension of the analysis of the time structure of behavioral acts. Intern. J. Neuroscience 44:251-262; 1989.
34. Mullenix, P. J.; Kernan, W. J.; Tassinari, M. S.; Schunior, A. Generation of dose-response data using activity measures. J. Am. Coll. Toxicol. 8:185-197; 1989.
35. Mullenix, P. J.; Kernan, W. J.; Tassinari, M. S.; Schunior, A.; Waber, D. P.; Howes, A.; Tarbell, N. J. An animal model to study toxicity of central nervous system therapy for childhood acute lymphoblastic leukemia: Effects on behavior. Cancer Res. 50:6461-6465; 1990.
36. Mullenix, P. J.; Kernan, W. J.; Schunior, A.; Howes, A.; Waber, D. P.; Sallan, S. E.; Tarbell, N. J. Interactions of steroid, methotrexate, and radiation determine neurotoxicity in an animal model to study therapy for childhood leukemia. Pediatr. Res. 35:171-178; 1994.
37. Paez, D.; Dapas, O. Biochemistry of fluorosis X: Comparative study of fluoride levels in biological fluids. Fluoride 15:87-96; 1982.
38. Painson, J.-C.; Tannenbaum, G. S. Sexual dimorphism of somatostatin and growth hormone-releasing factor signaling in the control of pulsatile growth hormone secretion in the rat. Endocrinology 128:2858-2866; 1991.
39. Rotton, J.; Tikofsky, R. S.; Feldman, H. T. Behavioral effects of chemicals in drinking water. J. Appl. Psychol. 67:230-238; 1982.
40. Shung-Guan, C. M.; Luo, J. Y.; Wang, W. G.; Wang, K. M.; Ge, Z. H.; Sun, G. Y. The nonskeletal lesions of endemic fluorosis. Chinese J. Int. Med. 21:217-219; 1982.
41. Singer, L.; Armstrong, W. D.; Lavender, D. R. Fluoride levels of plasma and cerebrospinal fluid. J. Dent. Res. 46:455; 1967.
42. Singer, L.; Ophaug, R. Ionic and nonionic fluoride in plasma (or serum). CRC Crit. Rev. Clin. Lab. Sci. 18:111-140; 1982.
43. Stanton, M. E.; Spear, L. P. Workshop on the qualitative and quantitative comparability of human and animal developmental neurotoxicity, work group I report: Comparability of measures of developmental neurotoxicity in humans and laboratory animals. Neurotoxicol. Teratol. 12: 261-267; 1990.
44. Strivastava, R. N.; Gill, D. S.; Moudgil, A.; Menon, R. K.; Thomas, M.; Dandona, P. Normal ionized calcium, parathyroid hypersecretion, and elevated osteocalcin in a family with fluorosis. Metabolism 2:120-124; 1986.
45. Theuer, R. C.; Mahoney, A. W.; Sarett, H. P. Placental transfer of fluoride and tin in rats given various fluoride and tin salts. J. Nutr. 101:525-532; 1971.
46. Tucker, D. M.; Williamson, P. A. Asymmetric neural control systems in human self-regulation. Psychol. Rev. 91:185-215; 1984.
47. Turner, B. B. Sex differences in the binding of type I and type II corticosteroid receptors in rat hippocampus. Brain Res. 581:229-236; 1992.
48. Whitford, G. M. The metabolism and toxicity of fluoride. Monogr. Oral Sci. 13:1-160; 1989.
49. Whitford, G. M. The physiological and toxicological characteristics of fluoride. J. Dent. Res. 69:539-549; 1990.
50. Whitford, G. M.; Pashley, D. H.; Reynolds, K. E. Fluoride tissue distribution: Short-term kinetics. Am. J. Physiol. 236:F141-F148; 1979.
51. Whitford, G. M.; Reynolds, K. E. Plasma and developing enamel fluoride concentrations during chronic acid-base disturbance. J. Dent. Res. 58:2058-2065; 1979.
52. Woodbury, D. M. Maturation of the blood-brain and blood-CSF barriers. In: Vernadakis, A.; Weiner, N., eds. Drugs and the developing brain. New York: Plenum Press; 1974:259-280.