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Fluoride Toxicity and Status of Serum Thyroid Hormones, Brain Histopathology, and Learning Memory in Rats: A Multigenerational Assessment

Piler Mahaboob Basha · Puja Rai · Shabana Begum

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Abstract High-fluoride (100 and 200 ppm) water was administered to rats orally to study the fluoride-induced changes on the thyroid hormone status, the histopathology of discrete brain regions, the acetylcholine esterase activity, and the learning and memory abilities in multigeneration rats. Significant decrease in the serum-free thyroxine (FT4) and free triiodothyronine (FT3) levels and decrease in acetylcholine esterase activity in fluoride-treated group were observed. Presence of eosinophilic Purkinje cells, degenerating neurons, decreased granular cells, and vacuolations were noted in discrete brain regions of the fluoride-treated group. In the T-maze experiments, the fluoride-treated group showed poor acquisition and retention and higher latency when compared with the control. The alterations were more profound in the third generation when compared with the first- and second-generation fluoride-treated group. Changes in the thyroid hormone levels in the present study might have imbalanced the oxidant/antioxidant system, which further led to a reduction in learning memory ability. Hence, presence of generational or cumulative effects of fluoride on the development of the offspring when it is ingested continuously through multiple generations is evident from the present study.

Keywords Fluoride · Multigenerational effects

Fluorosis is a progressive degenerative disorder resulting from excess intake of fluoride either by natural (drinking water) or anthropogenic sources. Recent literature indicate that fluoride crosses the placental barrier and enter the fetus to cause toxic impact on the development and differentiation. Altered neuronal and cerebrovascular integrity [1], abnormal behavior patterns

P. M. Basha (✉) · P. Rai
Department of Zoology, Bangalore University, Bangalore 560 056, India
e-mail: pmbashabub@rediffmail.com

P. Rai
e-mail: pujarai04@gmail.com

S. Begum
Department of Zoology, Maharanis Science College for Women, Bangalore 560001, India
e-mail: pmbashabub@gmail.com

[2], metabolic lesions in the brain of fluoride-exposed animals have been reported [3–5]. Fluoride is known to interfere with thyroid gland function and cause degenerative changes in the CNS, impairment of brain function, and abnormal development in children [6, 7]. Thyroid hormone is an epigenic signal required to achieve the construction of a normal neuronal network during development [8]. Thyroid hormone deficiency, when established during the critical period of neural differentiation, produces permanent and severe alterations in the anatomy and function of the nervous system. This eventually results in anatomical alterations in the cerebellum, cerebrum, hippocampus, and other brain structures [9–11].

Accumulation of fluoride has been observed in the brain of experimental animals exposed to high doses of fluoride for a prolonged period [12]. The levels of mental work capacity of adults with chronic fluorosis and the intelligence quotient (IQ) of children born and raised in the areas with endemic fluorosis were found to be lower than normal [7, 13, 14]. These findings are also consistent with animal studies in rats [15, 16]. A number of histopathological changes, including demyelinization, a decrease in the number of Purkinje cells, thickening and disappearance of dendrites, swelling of mitochondria, and dilation of endoplasmic reticulum in neurons have been observed in the brains of experimental animals subjected to fluorosis [12]. Studies of Mullenix et al. [2] have suggested that the severity of the adverse effects of fluoride on the behavior of rats is also directly correlated with the concentrations of fluoride ion in the plasma and in specific regions of the brain. Acetylcholine esterase (AChE) activity in the brain is important for maintaining normal brain physiological function such as learning and memory ability, and it seems to be the most sensitive parameter for monitoring intoxication due to toxic compounds and drugs that affects mammals [17]. This study was done to determine any generational, possibly cumulative effects on the development of the offspring when fluoride was ingested continuously through multiple generations. The study of more than one generation allows detection not only of any effects on adult reproduction but also of effects on subsequent generations due to exposure in utero and early postnatally. The latest evaluation of sodium fluoride administration through multiple generations has been made previously in general aspects by Collins et al. [18, 19], in the myocardium, lung, kidney, and testis by Cicek et al. [20], Aydin et al. [21], Karaoz et al. [22], and Meral et al. [23] respectively, but brain tissue has not been studied specifically in multigeneration animals. Due to the lack of information on multigeneration studies concerning fluoride-induced changes on the thyroid hormone status, the histopathology of discrete brain regions, and the resultant alterations in acetylcholine esterase levels and the learning and memory abilities, the present study was undertaken.

Materials and Methods

Chemicals

Total ionic strength adjustment buffer and fluoride standards were procured from Thermo Orion while other chemicals (AR grade) were purchased from Merck Ltd.

Preparation of Fluoride Water

A stock of 1,000 ppm sodium fluoride solution was prepared by dissolving 2.21 g of sodium fluoride in 1 L of tap water. To prepare 100 and 200 ppm fluoride water, 100 and 200 mL of the stock solution was taken and made up to 1 L with tap water respectively.

Animals

The protocol of this study was approved by the Institutional Animal Ethics Committee, Bangalore University, Bangalore, India. Healthy adult female albino Wistar rats, weighing 170–200 g and male rats weighing 200–250 g were procured from Sri Raghavendra Enterprises, Bangalore, acclimated for a week, and maintained at room temperature of 25± 2°C with 12-h dark–light cycle. Animals were fed with standard rodent diet (Amruth feeds, India). There was no water and light restriction throughout the experiment.

Experimental Design

About 40 albino adult Wistar rats (F_0) were selected and maintained in cages. One male (weighing 200–250 g) and three females (weighing 170–200 g) were housed in a cage for a night and then the following day the vaginal plugs of each female were examined to confirm pregnancy. Only 24 of 30 females were identified as pregnant, and these 24 pregnant females were selected to obtain the first-generation rats (F_1). These 24 pregnant rats were then divided into three groups of eight rats each and treated with the following doses of fluoride: group I (control) was provided with tap water ad libitum (<1 ppm F), group II was given 100-ppm fluoride in drinking water, group III was given 200-ppm fluoride in drinking water during the gestation period.

The pups obtained were considered as F_1. The mothers of experimental group continued receiving fluoridated water throughout the lactation period, and thereafter the pups of experimental group had free access to fluoridated water. One-month-old pups from each group were used for assessing learning and memory by T-maze experiments, and thereafter were euthanized for estimating the serum thyroid hormone levels, acetylcholine esterase activity, fluoride levels, and histopathological studies in discrete brain regions viz., cerebral cortex, cerebellum, medulla oblongata, and hippocampus.

On attaining maturity, some of the first-generation rats were selected to continue the study on the basis of being healthy. They were then kept for breeding in a similar way as their parental generation (F_0). The pups obtained from the first generation were considered as the second generation (F_2). Some of the pups (F_2) which were 1-month old from each group were used for various studies as described above, the remaining pups were made to mature while continuing their respective dose of fluoride. On maturity, these F_2 were kept for breeding in a similar manner as described above. The pups obtained from this F_2 were considered as the third generation (F_3). One-month-old pups from each group were used for the various studies as described above.

Serum thyroid hormones (FT3 and FT4) were measured with the assistance of diagnostic laboratory, Thyrocare (Bombay, India) by using Bayer Centaur autoanalyser according to the method of Thomas [24]. Acetylcholine esterase activity was estimated spectrophotometrically by the method of Ellman et al. [25] and proteins by the method of Lowry et al. [26]. Fluoride in discrete brain regions was estimated by the modified method of Inkielewicz et al. [27] using ion meter (Thermo Orion 720A+ with fluoride ion-sensitive electrode 9609 BNWP). For the histopathology, discrete brain regions were fixed in Bouin's solution, embedded in paraffin, serially sectioned at 5 μm and stained with haematoxylin–eosin. Spatial learning and memory were tested in a T-maze according to the method of Bures et al. [28].

Statistical analysis for serum thyroid hormone levels, acetylcholine esterase activity, and fluoride levels were done by one-way analysis of variance with Duncan's multiple range test (DMRT) post hoc at $p<0.05$ level of significance by using SPSS software. The data of

mean number of alternations and the time taken to reach the goal area (latency) in rewarded alternation task were analyzed by two-factor ANOVA with repeated measures on one factor followed by Newman Keul's post hoc test. The data on sessions to reach the criterion (acquisition) and the number of errors committed in the retention test were subjected to one-way ANOVA followed by Duncan's post hoc test. The data are expressed as mean±SE and values of $p<0.05$ were considered statistically significant.

Results

Brain fluoride levels were directly related to the levels of fluoride exposure (Table 1). Rats treated with 200 ppm fluoride had more fluoride accumulation in the discrete brain regions than the 100 ppm fluoride-treated rats. Significant decrease in the levels of serum FT3 and FT4 was observed in the fluoride-treated rats when compared with the control. The decrease was maximum in the third-generation fluoride-treated rats followed by the second- and first-generation rats. In each generation, the extent of decrease was found to be more in 200 ppm than the 100 ppm fluoride group, but the difference was not statistically significant (Table 2).

Histological analysis of cerebral cortex, cerebellum and hippocampus of control and fluoride-treated rats are depicted in Figs. 1, 2, and 3. The histopathological changes in both doses of fluoride groups were more or less the same but differed only in their degree of severity. The cerebral cortex of control rats showed normal morphology with intact neuron and cytoplasm (Fig. 1). Fluoride treatment resulted in necrosis, which is depicted by hyperchromasia and disintegrated cytoplasm. Cytoplasm showed edema depicted by

Table 1 Multigenerational fluoride exposure and resultant fluoride retention in discrete brain regions

Fluoride (μg of F/g weight of the tissue)

		CC	CB	MO	HC
F_1	Control	0.672±0.005 a	0.318±0.005 a	0.358±0.008 a	0.258±0.006 a
	100 ppm F	2.28±0.018 b (+239.29)	1.83±0.049 b (+475.47)	2.12±0.062 b (+492.18)	0.993±0.028 b (+284.88)
	200 ppm F	3.43±0.084 e (+410.42)	2.32±0.062 d (+629.56)	3.09±0.049 d (+763.13)	1.93±0.043 e (+648.06)
F_2	Control	0.675±0.007 a	0.32±0.004 a	0.362±0.007 a	0.256±0.007 a
	100 ppm F	2.89±0.067 c (+328.15)	2.07±0.054 c (+546.88)	2.78±0.073 c (+667.96)	1.24±0.08 c (+384.38)
	200 ppm F	3.66±0.034 f (+442.22)	2.61±0.055 e (+715.63)	3.53±0.074 e (+875.14)	2.34±0.058 f (+814.06)
F_3	Control	0.673±0.005 a	0.319±0.003 a	0.359±0.007 a	0.26±0.006 a
	100 ppm F	3.11±0.031 d (+362.11)	2.51±0.035 e (+686.83)	3.03±0.031 d (+744.01)	1.65±0.059 d (+534.62)
	200 ppm F	3.92±0.071 g (+482.47)	2.87±0.058 f (+799.69)	3.84±0.037 f (+969.64)	2.73±0.03 g (+950.0)

Values are mean±SE of six observations, and values in parenthesis indicate percentage change. Mean values of different lower case letters (a–g) within each row are statistically significant ($P<0.05$) as determined by DMRT

(+) increase over control, F_1 first generation, F_2 second generation, F_3 third generation, *CC* cerebral cortex, *CB* cerebellum, *MO* medulla oblongata, *HC* hippocampus

Table 2 Multigenerational fluoride exposure and changes in serum FT3 and FT4 levels

		FT3 (ng/dL)	FT4 (pg/mL)
F_1	Control	3.04±0.052 d	1.89±0.066 d
	100 ppm F	2.54±0.048 b (−16.45)	1.86±0.053 d (−1.59)
	200 ppm F	2.83±0.055 c (−6.91)	1.69±0.08 c (−10.58)
F_2	Control	3.01±0.041d	1.9±0.062d
	100 ppm F	1.85±0.056 a (−38.54)	1.23±0.053 a, b (−35.26)
	200 ppm F	1.82±0.044 a (−39.54)	1.17±0.042 a, b (−38.42)
F_3	Control	3.0±0.058 d	1.88±0.056 d
	100 ppm F	1.85±0.054 a (−38.33)	1.33±0.058 b (−29.26)
	200 ppm F	1.73±0.056 a (−42.33)	1.09±0.032 a (−42.02)

Values are mean±SE of six observations, and values in parenthesis indicate percentage change. Mean values of different lowercase letters (a–d) within each row are statistically significant ($P<0.05$) as determined by DMRT

(−) sign indicate decrease over control, F_1 first generation, F_2 second generation, F_3 third generation, *CC* cerebral cortex, *CB* cerebellum, *MO* medulla oblongata, *HC* hippocampus

vacuoles and also eosinophilia (red neurons) visible at many instances. Fluoride-treated second and third-generation rats showed similar but more severe necrotic signs. Figure 2 shows normal morphology of the cerebellum of the control rats. Presence of intact granular layer, normal count and morphology of Purkinje cells were observed. Fluoride treatment resulted in a decrease in the number of granular cells, and Purkinje cells showed eosinophilic character (red neurons). There was a presence of gap/space between the Purkinje cells and the granular layer in the fluoride-treated rats, and this alteration was very prominent especially in third-generation rats treated with 200 ppm fluoride. Figure 3 shows normal morphology of the hippocampus of the control rats. Presence of degenerating neurons was observed in the fluoride-treated rats. The severity of the alterations was most pronounced in the third-generation fluoride-treated rats.

The acquisition and retention data subjected to two-way ANOVA with repeated measures revealed a significant difference between groups $F_{(6,\ 35)}=3.596$; $p<0.01$ and sessions $F_{(6,\ 210)}=39.254$; $p<0.001$. The latency data also revealed a significant difference between groups $F_{(6,\ 35)}=3.742$; $p<0.01$ and sessions $F_{(6,\ 210)}=20.831$; $p<0.001$. The third-generation fluoride-treated rats of both doses took more sessions to learn a task (acquisition), performed poorly, and took more time to reach the goal area (latency) when compared with the second- and first-generation rats. Memory in terms of the number of errors committed during a single session of 30 trials showed no significant difference between the three generation fluoride-treated rats (Fig. 4). Significant decrease in acetylcholine esterase activity was observed in fluoride-treated group when compared with the control. The decrease was most profound in third-generation fluoride-treated group when compared with first and second generation (Table 3).

Discussion

Extensive epidemiological and experimental studies have established that the biological responses of animals to fluoride are related to dosage and other factors that influence the

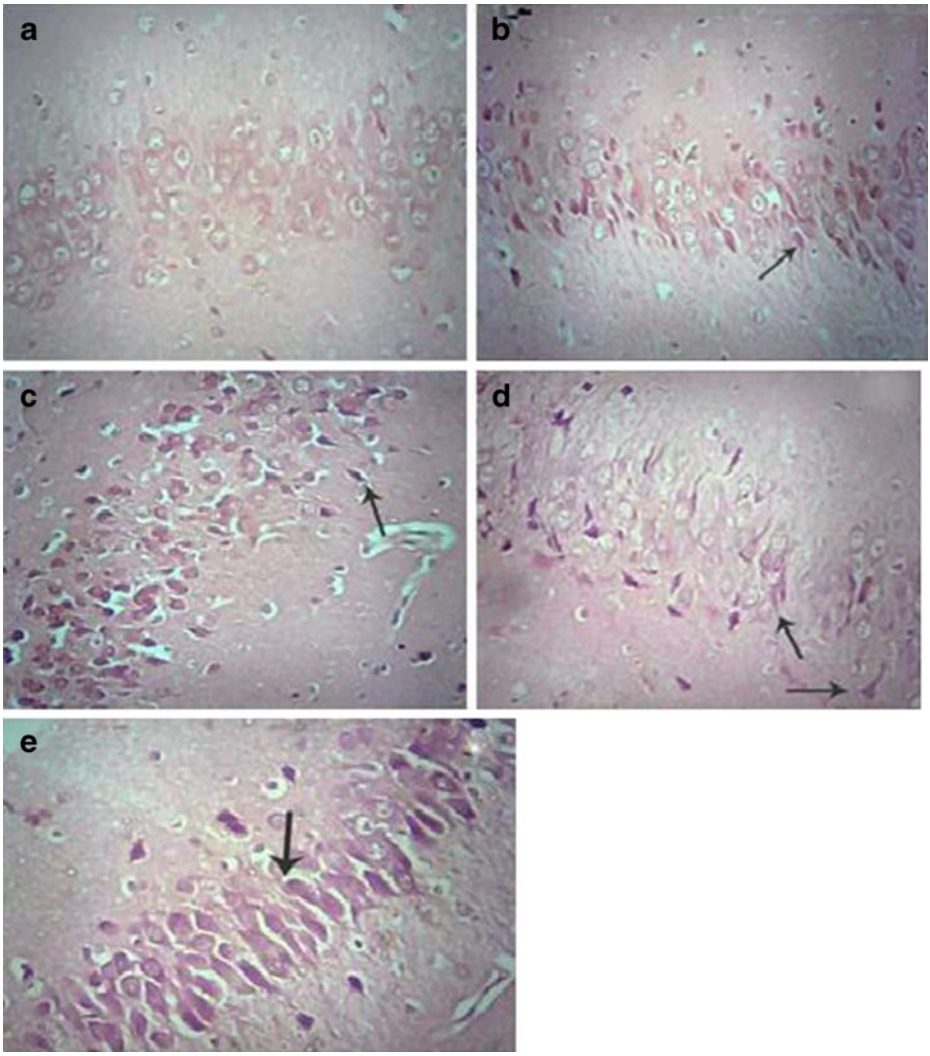

Fig. 1 Histological changes in cerebral cortex: **a** control rats, **b** F2 100 ppm F, **c** F2 200 ppm F, **d** F3 100 ppm F, and **e** F3 200 ppm F. Note the presence of degenerating neurons (*arrows*) in the fluoride-treated group. (H & E, ×40). (*F2* second generation, *F3* third generation)

animal's physiological and anatomical responses [29]. In the present study, a significant decrease in thyroid hormones (FT3 and FT4) was observed in fluoride-treated rats which are in agreement with the results of Zhan et al. [30] who reported decreased level of serum FT3 and FT4 in young pigs fed with 100, 250, and 400 mg fluoride/Kg diet. Trabelsi et al. [31] also reported a significant decrease in the plasma free T4 level in 14-day-old mice whose mothers had been treated with 0.5 g NaF/L in drinking water. Wu et al. [32] have suggested that the decreased levels of thyroid hormones might be due to the inhibition of the absorption of iodine through fluoride interaction, insufficient synthesis, and secretion of thyroglobulin and oxidized iodides from the thyroid gland owing to follicle injury after excessive intake of fluoride. Additionally, the iodothyronine deiodinases, D1,

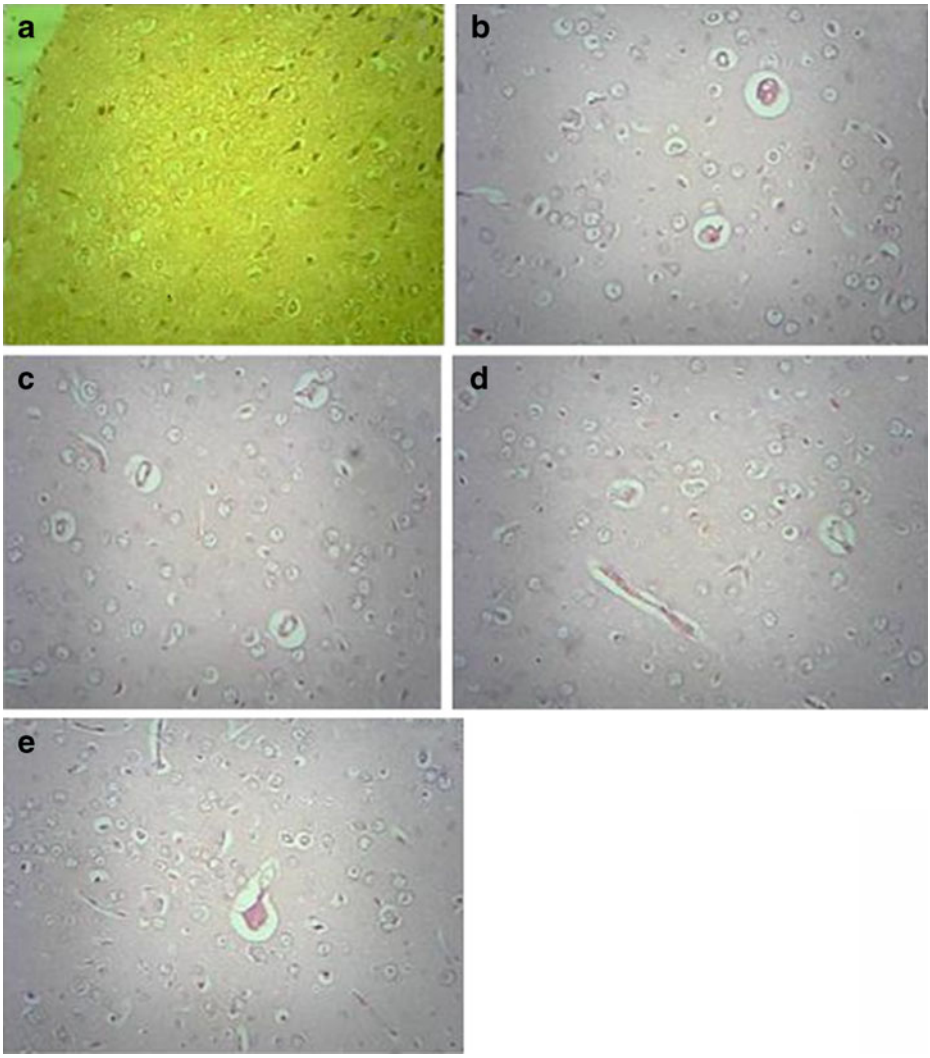

Fig. 2 Histological changes in cerebellum: **a** control rats, **b** F2 100 ppm F, **c** F2 200 ppm F, **d** F3 100 ppm F, and **e** F3 200 ppm F. Note the presence of eosinophilic Purkinje cells (*eP*) and the presence of gap between the Purkinje cells (*P*) and granular layer (*GL*) in the fluoride-treated group. (H & E. ×40). (*F2* second generation, *F3* third generation)

D2, and D3, as well as some glutathione peroxidases do contain selenium in the form of selenocysteine at their active sites; they play crucial role in determining the circulating and intracellular levels of the active thyroid hormone, T3. Thyroid hormones control the body's entire oxidant/antioxidant system [33] and fluoride may manipulate deiodinases directly as a thyroid stimulating hormone (TSH) analogue since TSH levels directly correlate with malondialdehyde and superoxide dismutase activity [31]. Perhaps, changes in the thyroid hormone levels in the present study might have imbalanced the oxidant/antioxidant system [34], which further led to a reduction in learning memory ability.

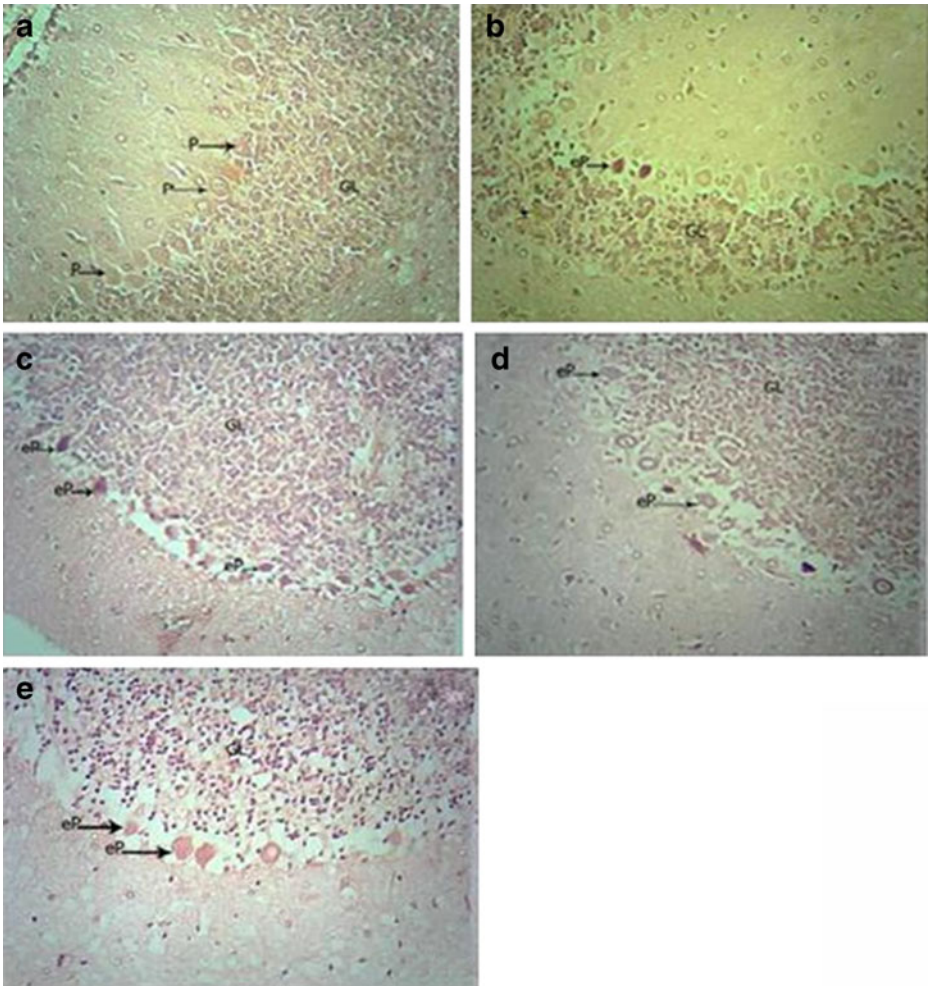

Fig. 3 Histological changes in hippocampus: **a** control rats, **b** F2 100 ppm F, **c** F2 200 ppm F, **d** F3 100 ppm F, and **e** F3 200 ppm F. Note the presence of degenerating neurons (*arrows*) in the fluoride-treated group. (H & E, ×40). (*F2* second generation, *F3* third generation)

In the T-maze experiments, the third-generation fluoride-treated rats performed poorly and took much longer time to reach the goal area when compared with the second- and first-generation fluoride-treated rats. Both the doses of fluoride exposure significantly impaired the acquisition and retention in the present study. Decreased learning and memory abilities induced by fluoride have been reported by various authors [16, 35–37]. Since fluoride is known to be neurotoxic, greater accumulation of fluoride in the discrete brain regions in the third-generation rats as found in the present study may have made the subsequent generations more vulnerable to the toxic effects of fluoride. Learning and memory has been associated with hippocampal activity and cholinergic neurotransmission [38, 39]. Thiel et al. [40] related the increased release of acetylcholine in the hippocampus to sodium fluoride intoxication in rats, where fluoride may get through the blood brain barrier and accumulate in rat hippocampus resulting in inhibition of cholinesterase activity. Nicotinic acetylcholine receptors (nAChRs)

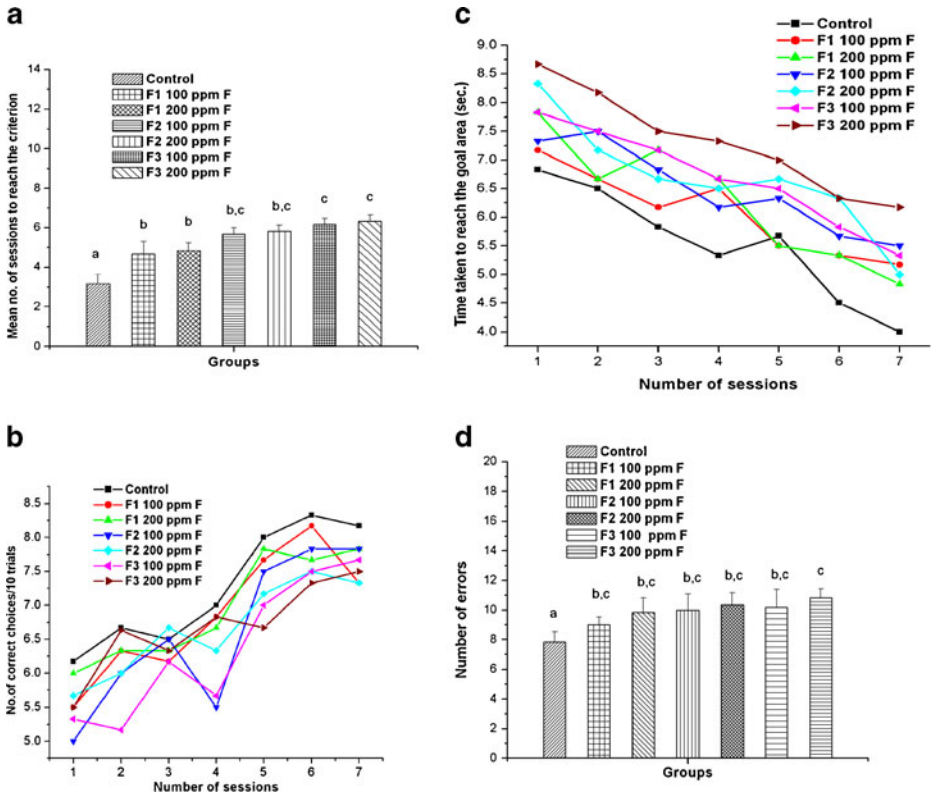

Fig. 4 Multigenerational fluoride exposure induced changes in learning and memory indices in the rewarded alternation task conducted in rats. **a** mean number of sessions to reach the criterion **b** number of correct choices per session **c** time taken to reach the goal area **d** number of errors committed in the retention test (*F1*, *F2*, *F3* represent first, second and third generation respectively)

have been also established to play a major role in cognitive processes such as learning and memory. In a fluoride toxicity study, Long et al. [41] observed a decreased number of nAChRs resulting in brain dysfunction. In the present study, acetylcholine esterase activity was inhibited in fluoride-treated rats, which is in agreement with previous reports [16, 42, 43]. Significant decline in brain regional AChE activity during learning process was observed in animals exposed to both doses of fluoride. The inhibition was found to be at a maximum in the third-generation fluoride-treated rats. Earlier studies showed decreased AChE activity in brain tissues of rats treated with fluoride as well as in the blood of the patients with fluorosis and also in the gastrocnemius muscle and liver of fluorosed mice [43–46]. Fluoride is also known to have adverse effects on cholinesterase activity involved in the hydrolysis of esters of choline [5]. This toxic effect may lead to altered utilization of acetylcholine, thus affecting the transmission of nerve impulses in brain tissue [47, 48].

Purkinje cells of cerebellar cortex are vital for the expression of behavior and other motor functions [49]; they undergo necrosis or apoptosis followed by oxidative stress [50]. In the present study, there was a decrease in the number of granular cells in fluoride-treated rats which is in agreement with the studies of Trabelsi et al. [31]. They postulated that the destruction of granular layer could be a consequence of fluoride interfering in the mitosis of these cells, and the loss of granular cells in these animals might have resulted due to lack of

Table 3 Multigenerational fluoride exposure and changes in the AChE activity in discrete brain regions

AChE (Units/mg protein)					
		CC	CB	MO	HC
F_1	Control	77.18±3.2 d	83.25±2.27 e	81.69±2.01 d	96.26±2.13 d
	100 ppm F	69.23±2.35 d (−10.3)	73.31±1.4 d (−11.94)	72.64±1.53 c (−11.08)	85.19±1.52 c (−11.5)
	200 ppm F	61.35±2.62 c (−20.51)	70.67±2.43 d (−15.11)	68.73±1.93 c (−15.87)	81.13±1.79 c (−15.72)
F_2	Control	76.3±3.34 d	82.5±1.63 e	80.21±1.28 d	95.3±0.94 d
	100 ppm F	53.32±1.76 b (−30.12)	64.36±1.63 c (−21.99)	60.77±2.08 b (−24.24)	73.33±1.3 b (−23.05)
	200 ppm F	50.63±1.39 b (−33.64)	61.82±1.68 b, c (−25.07)	54.22±2.55 a (−32.4)	70.26±2.25 b (−26.28)
F_3	Control	76.5±3.66 d	84.61±2.97 e	82.73±2.6 d	93.5±2.09 d
	100 ppm F	47.4±2.25 a, b (−38.04)	57.37±1.89 a, b (−32.2)	51.5±1.6 a (−37.75)	60.26±1.71 a (−35.55)
	200 ppm F	42.05±1.62 a (−45.03)	54.07±1.34 a (−36.1)	49.41±2.01 a (−40.28)	58.28±2.69 a (−37.67)

Values are mean±SE of six observations, and values in parenthesis indicate percentage change. Mean values of different lower case letters (a–e) within each row are statistically significant ($P<0.05$) as determined by DMRT

(−) sign indicate decrease over control, F_1 first generation, F_2 second generation, F_3 third generation, *CC* cerebral cortex, *CB* cerebellum, *MO* medulla oblongata, *HC* hippocampus

proper contact between granular cells and Purkinje cells. Since the cerebral cortex and the hippocampus are the key tissues related to learning and memory, it is highly plausible that there is a direct relationship between IQ and histopathological changes of brain. These changes may form the neural basis for impaired learning and memory, abnormal behavior patterns, and disturbed overall body physiology [51].

The main conclusion of the present study is that fluoride exposure causes cumulative effects not only in parental generation but also in subsequent generations, resulting in decreased thyroid hormone associated learning and memory impairments in addition to variation in neuronal cytoarchitecture.

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