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Effect of NaF on albino female mice with special reference to behavioral studies and ACh and AChE levels

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Abstract

The present study investigates the effect of the Sodium Fluoride (NaF) on Wistar Albino mice. The animals have been divided into three batches each containing five individuals among which 1st batch served as control while, remaining two batches were treated with 5 and 10ppm respectively. After completion of exposure, the animals were subjected to behavioural studies. Then the animals were sacrificed and the brain regions were used for the biochemical analysis. Significant behavioral changes were observed in the NaF treated animals when compared to the control animals. Among the two doses the animals exposed to high dose showed significant changes compared to low dose exposed animals. Among the biochemical parameters studied AChE activity was decreased in the brain region of the animals exposed to NaF compared to the control. Besides decrease in AChE activity, ACh content was found to be increased in the brain regions of the NaF exposed animals. Among the different brain regions studied Cerebral Cortex was found to be more vulnerable to the NaF toxicity. However 10pm NaF showed more inhibition both in terms of behavioral and biochemical alterations in the Mice compared to the 5ppm exposed animals.

Key-Words: Fluoride, AChE activity, Ach content, Behavioral alterations, Brain regions

Introduction

The toxic effects of Sodium fluoride (NaF) on mice are well documented[1]. The toxicity of Fluoride on female mice has been shown to depend not only on the concentration of fluoride but also on the pH, hardness and temperature of the water, and the presence of ion exchange minerals[2]. NaF toxicity on mice depends very much on increasing F concentration, exposure time, as well as water temperature and uptake of F directly via food[3,4]. NaF causes adverse biological effects such as changes in carbohydrate, lipid, and protein metabolism, reproduction, impairment, reduced embryonic and development of life stages, and alteration of size and growth[5] and is therefore a potent hazardous pollutant[6]. In the present study, an attempt has been made to study the chronic toxicity and behavioral responses induced by NaF in female mice. Indeed, levels of acetylcholinesterase (AChE)[7] and choline acetyltransferase (ChAT)[8], both presynaptic markers of cholinergic neurons, significantly decrease in the cortex and hippocampus of mice. Consistent with these observations, memory deficits are improved by stimulation of the cholinergic system[9,10] .

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Evidence that fluoride (F) crosses the blood brain barriers raises the possibility that F can affect the structure and functions of the central or peripheral nervous system. Significantly impaired learning and memory, shown in mice, reduced motor coordination and behavior symptoms like nervousness, depression, tingling sensations in fingers and toes, excessive thirst, and tendency to urinate frequently in human beings, after excess intake of fluorinated water suggest that not only the structure but functions of the central nervous system are also affected. The present investigation was under taken to study the effects of two different F concentrations in drinking water on cholinergic system and behavior studies.

Material and Methods

Procurement and maintenance of animals

Female adult Wistar mice weighing 35±5 grams were used as the experimental animals in the present investigation. The mice were purchased from the Indian Institute of Science (IISc), Bangalore, maintained in the animal house in polypropylene cages under laboratory conditions of 28±2^{0}C temperature with photoperiod of 12 hours light and 12 hours dark and 75% relative humidity. The mice were fed with standard pellet diet (Hindustan Lever Ltd., Mumbai) and water ad libitum. The mice were maintained

according to the ethical guidelines for animal protection and welfare bearing the CPCSEA.

The mice were randomly divided into 3 groups having 5 in each group and were treated as follows:

Group I: received saline (control).
Group II: 5ppm NaF.
Group III: 10 ppm NaF.

NaF was dissolved in distilled water and given to the animals for three months, through drinking water.

Isolation of tissues

After a stipulated duration of exposure, behavioral studies were performed for a period of three days and then the animals were sacrificed by cervical dislocation and different brain regions (cerebral cortex [CC], cerebellum [CB], pons medulla [PM] and hippocampus [HC]) were immediately isolated, frozen in liquid nitrogen and stored at -40^{0}C until analysis.

Behavioral studies

Open-Field Behavior

The open field test has been widely used to assess emotional reactivity/anxiety. It provides measures of locomotor activity. The horizontally directed activity (or locomotion) is measured by the number of line crossings, and vertically directed activity (or exploration) is measured by the frequency of rearings[11]. The open-field behaviour of one month age mice were assessed in a wooden box measuring 90 x 90 x 30 cm high. The floor of the arena is divided into 36 equal squares by black lines. Immediately after a mouse was placed in the centre of the open field, the movement of the animal was scored. The number of squares crossed with all paws (crossings), the standings on the hind legs (rearings), placing the nose against wall or floor (sniffing), wiping, licking, combing or scratching of any part of the body (grooming) are counted in all sessions. All the activities measured were combined together to assess the mean total behaviour in each session. Testing was carried out on three consecutive days in five minute sessions in control, NaF-exposed (both 5ppm and 10ppm NaF exposed) animals. The number of crossings indicates locomotor activity [12].

Exploratory Behavior

Exploratory behavior was evaluated in the hole board. The apparatus was an open-field arena with four equally spaced holes (3 cm in diameter) in the floor. Each mice was placed individually in the centre of the arena for 5 min, during which we recorded head-dip count and head-dipping duration, in seconds. A head dip was scored if both eyes disappeared into the hole. Head-dipping duration data are expressed as total duration during the 5-min session. The results for head dip are expressed as number of counts, and for head-dipping duration in seconds.

Total Locomotor Activity

Total locomotor activity of the mice was studied with Opto-Varimex mini (Columbus Instruments, USA). The data was taken as total duration 30minutes to each animal during the 5 minutes session interval.

Biochemical Analysis

Isolation of synaptosomal fraction

Synaptosomal fractions from brain homogenates were separated using ficoll-sucrose gradients[13]. The tissues were weighed and homogenized in 10 ml of ice-cold homogenizing buffer and the volume was brought up to 25 ml with homogenizing buffer. The homogenates were centrifuged at 750g for 10 minutes. The pellets were discarded and the supernatants were centrifuged at 17,000g for 20 minutes. The pellets were suspended in 10ml of 0.32M sucrose and were layered on a two step discontinuous Ficoll-sucrose gradient consisting 13% and 7.5% Ficoll and centrifuged at 65,000g for 45 minutes. A milky layer was formed at the interface of 13% and 7.5% Ficoll. The pellet consisting of mitochondrial fraction formed at the bottom of the centrifuge tube was taken and suspended in homogenizing buffer. The milky layer fraction was diluted with 9 volumes of 0.32M sucrose and centrifuged again at 17,000g for 30 minutes. The supernatant was discarded and the pellet (synaptosomal fraction) was suspended in 0.32M sucrose.

Estimation of Acetylcholine (ACh)

The acetylcholine content was estimated by the method of Metcalf [14] as given by Augustinson[15]. The synaptosomal fractions of cerebral cortex, hippocampus, cerebellum and medulla oblongata were placed in boiling water for 5 minutes to terminate the AChE activity and also to release the bound ACh. To the synaptosomal fractions 1 ml of alkaline hydroxylamine hydrochloride followed by 1 ml of 50% hydrochloric acid were added. The contents were mixed thoroughly and centrifuged. To the supernatant 0.5 ml 0.37M ferric chloride solution was added and the brown colour developed was read at 540nm against a reagent blank in a spectrophotometer

Estimation of Acetyl cholinesterase Activity (AChE)

AChE specific activity was determined following the method of Ellman *et al.* [16]. The reaction mixture contained 3.0ml of phosphate buffer (pH 8.0), 20 µl of 0.075M acetylthiocholine iodide (substrate) and 100µl of 0.01M DTNB (5, 5-Dithiobis-2-Nitrobenzoic acid). The reaction was initiated with the addition of 100µl of synaptosomal fraction. The contents were incubated for 30 min at room temperature and the color absorbance was measured at 412 nm in spectrophotometer (Hitachi, Model U-2000). The enzyme activity was

expressed as µ moles of ACh hydrolyzed/mg protein/hr.

Statistical analysis

All observations were carried out with six separate replicates from each group. The mean, standard error (SE) and Analysis of Variance (ANOVA) were done using SPSS statistical software (11.5 ver.) for different behavioral parameters. Difference between control and experimental were considered as significant at $P<0.05$.

Results and Discussion

The behavioral changes were monitored and were given in Table 1.

Records of the behavioural responses including Crossings, Rearings, Sniffings, Groomings and Locomotor Activity.

During the three months study period, the NaF exposed mice (10ppm and 5ppm) were more active and restless compared to the control group. With regard to Crossings, Rearings, Sniffings, Groomings of open field behavior in the NaF exposed group have shown significant decrease. These observations are well supported by earlier findings from other toxicity studies[17].The variation in toxicity of a toxic substance to attributable to specific characteristics of mice such as variations in size and weight, sex, and maturity, species biological behaviour, and physico-chemical conditions of ambient water. It is clear that NaF can exert adverse effects on female mice.

The underlying mechanisms of NaF and cognition defects are still elusive, but several possible pathways have been described. Possible mechanisms of action for NaF based on the proposed underlying signaling pathways[18]. Both cAMP and cGMP have been strongly implicated in hippocampal LTP[19]. A very recent study suggested another pathway through which cGMP can influence cognitive processes. The hippocampal NO/cGMP pathway directly stimulates the postsynaptic cAMP/PKA/CREB pathway[20]. However, the present study involves prefrontal cortex-dependent behavior, and it remains to be proven that the above mentioned hippocampal of synaptical strengthening are also applicable in the prefrontal cortex. The effects of NaF on prefrontal cortex performance were explained[21].

When compared with controls, animals with NaF-treated had significantly elevated levels of ACh in all brain regions, with the highest increase noted in Hippocampus (Table 2). In animals treated with 5ppm and 10ppm, levels of AChE were decreased in different brain regions compared with levels in control animals (Table 2). The effect of 10ppm was greater than the effects of 5ppm (Table 2). Acetyl cholinesterase activity decreased significantly in all brain regions in NaF-induced mice, with the highest decrease noted in the 10ppm concentation at Hippocampal region (Table 2). When neuronal damage is taken into consideration as reported earlier, it is clear that the ACh accumulates as a result of the inhibition of AChE and subsequently causes specific neurobehavioral symptoms. It has been reported that several cholinergically mediated behavior would be disrupted if AChE activity in brain fall below 40% of the normal level[22]. The alterations in ACh content and AChE activity observed in NaF-induced mice, however, would tend to indicate that a direct relationship exists between activities of the cholinergic pathway[23].

It has been suggested that changes in the expression of key cholinergic proteins and the associated cholinergic dysfunction are key factors in the basic mechanisms underlying central nervous system-related NaF toxicity[24]. Although a direct role of cholinergic pathways induced NaF has not been demonstrated, these results indicate that there exists a mechanism involving summation of excitatory neurotransmission in the central nervous system during NaF toxicity. In the present study, the changes in ACh content and AChE activity were maximal with 10ppm NaF induced. Hence, it may be concluded that these two concentrations 5ppm and 10ppm of NaF have nerotoxic activity and cause perceptible changes in the cholinergic system.

Conclusion

The present study suggests that chronic F intoxication markedly affects cholinergic system, which may result in dysfunctions of neurotransmission in brain, and the F induced neurotoxicity is highly region specific, finally leading to the perturbations in the behavior responses.

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Table 1: Behavioral changes in Control and NaF Exposed Female Albino Mice

S. No	Parameters	Control	NaF (5ppm)	NaF (10ppm)
1	Open Field Behavior			
	a. Crossings	127.61±22.67	94.55*±13.95 (-25.9)	59.22*±23.86 (-53.59)
	b. Rearings	25.33±7.39	18.77*±3.00 (-25.89)	10.0*±3.97 (-60.52)
	c. Sniffings	22.55±6.372	18.0*±4.801 (-20.17)	8.00*±4.029 (-64.52)
	d. Groomings	24.772±7.152	20.555*±3.958 (-17.02)	9.5*±4.743 (-61.65)
2	ExploratoryBehavior (Number of Dippings)	10.222±1.895	6.277*±1.406 (-38.59)	2.72*±2.108 (-73.39)
3	Exploratory Behavior (No of Dipping Duration)	23.22±6.08	13.444*±4.368 (-42.1)	5.166*±3.853 (-77.75)
4	Total Locomotor Activity	660.77±92.27	529.33*±72.31 (-19.89)	261.83*±89.92 (-60.37)

All the mean values Significant at P < 0.05 compared with saline control in Bartlett's test.
± Standard deviation on values. (SD); Values in parentheses are percentages of change over control

Table 2: Changes in acetylcholine (ACh) content (µmole of acetylcholine/g wet wt of tissue) and acetylcholinesterase (AChE) activity (µmole of acetylthiocholine iodide hydrolyzed/mg protein/h) of different brain regions of control and NaF treated albino female mice

Brain region/ Experimental	Cerebral cortex		Cerebellum		Hippocampus		Medulla oblangata	
	ACh	***AChE***	***ACh***	***AChE***	***ACh***	***AChE***	***ACh***	***AChE***
Control	3.053	4.903	2.989	4.314	3.094	4.945	2.972	4.167
	±0.148	±0.214	±0.104	±0.289	±0.086	±0.156	±0.262	±0 .210
5ppm	3.29	3.875*	3.215	3.692*	3.306	3.948*	3.113	3.483*
	±0.051	±0.079	±0.135	±0.097	±0.053	±0.246	±0.124	±0 .140
	(7.76)	(-20.97)	(7.56)	(-14.42)	(6.85)	(-20.16)	(4.74)	(-16.41)
10ppm	3.5	3.043*	3.331	2.997*	3.751*	3.125*	3.31	2.974*
	±0.118	±0.597	±0.071	±0.362	±0.209	±0.569	±0.081	±0 .223
	(6.38)	(-21.47)	(3.61)	(-18.82)	(13.46)	(-20.85)	(6.33)	(-14.61)

Note: All values are means ± SD of six individual observations; Values in parentheses are percent change over control; *Significant at P < 0.05 compared with saline control in Bartlett's test.

Abbreviations

NaF : Sodium Fluoride
F : Fluoride
ACh : Acetyl Choline
AChE : Acetyl Choline esterase
CC : Cerebral Cortex
CB : Cerebellum
PM : Pons Medulla
HC : Hippo campus