A multiple-test study for the evaluation of the behavioural Alterations with Relation to Oxidative Stress Induced by Ingestion of Sodium Fluoride in Female Albino Rats

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Abstract: Fluoride is toxic to all living things.Living organisms are exposed to excess fluoride through their food, water, and air. Long-term exposure to excess fluorine can cause fluorosis in both animals and humans.Fluoride consumption has been associated to behavioural alterations in mice and rats, depending on the dosage, animal sex, and time of exposure. This property of fluoride shows high use in dental care. In the given study 30 female albino rats were divided into 5 groups, the control group received saline water and remaining groups received 5, 10, 15 and 20 mg NaF/kg bw for 45 days orally. Results showed that the decreased body weight,oxidative stress enzymes activities of Superoxide Dismutase and Catalase while increased Lipid Peroxidation as compared to control group.Open-field test and Morris water maze indexesof experimental animals also highly altered at 10, 15 and 20 mg NaF/kg bw while slightly in 5 mg NaF/kg bw.These findings show a link between oxidative stress enzymes with learning,memory and motor activity of animals.

Keywords: Albino rats (*Rattus norvegicus*), Behavioural activity, Organs weight, Oxidative stress enzymes, Sodium fluoride

I. Introduction:

Fluoride is a strongly electronegative anion, renowned for its ability to enhance the pace of hydroxyapatite production and remineralisation of carious lesions in teeth. Fluoride (F) is an important life element for human health. In small amounts, it is necessary for normal bone mineralization and the formation of dental enamel (Chouhan and Flora, 2010), but above the acceptable limit (WHO 1.5ppm), it becomes toxic in both animals and humans, causing dental and skeletal fluorosis (Hussain*et al.,*2002, 2004; Singh *et al.,*2007). F has a strong affinity for calcified tissues, so more than 90% of the total body burden of F is reserved in bones and teeth, with the remainder distributed in highly vascular soft tissues and blood (Fawell *et al.,* 2006).As a result, the individual with skeletal fluorosis experienced limb joint pain, tingling, numbness, and cramping, as well as back pain that was exacerbated by activity(Shashi *et al.,*2008).The most important sources of F intake are drinking water, foodstuffs, industrial dust, smoke, pesticides, and F-containing dental products. (Mittal and Flora,

(2006); Bouaziz *et al.,* 2007; Eraslan *et al.,* 2007; Kanbur *et al.,* 2009; Barbier *et al.,* 2010). The higher exposure of F increases the biochemical stress in the body by generating imbalance between reactive oxygen species (ROS) (Tribowo*et al.,* 2014). Elevated levels of such free radicals cause neuronal dysfunction (Spittle, 1994; Guan*et al.,* 1998; Yang *et al.,* 1998; Shao*et al.,*2000; Chirumari and Reddy, 2007)and abnormal behavioural patterns (Mullenix *et al.,*1995).Some data suggested that nervous system dysfunction associated with insomnia, lethargy and deterioration of learning, memory and locomotor activity (Spittle,1994; Gupta *et al.,*1993; Wu *et al.,* 2006; Sharma *et al.,* 2009). F can cross the blood-brain barrier, accumulate in the brain and damage the neural architecture (Mullenix, *et al.,* 1995; Vani and Reddy, 2000). Spontaneous activity, a motivated behaviour, is supposed to be the most common interpreter of CNS dysfunction (Mullenix and Kernan, 1989).

The current study aimed to investigate the effect of different sodium fluoride (NaF) concentrations on learning and memory tasks using the water maze test, open field method, Y-maze, and elevated plus maze to measure spontaneous activity, conditioned-response behaviour, to assess spatial memory and anxiolytic or anxiogenic effects in relation to oxidative stress in adult female albino rats.

II. Materials and Methods:

A. Experimental Animals and Treatment

Thirty female albino rats *Rattus norvegicus* was selected as animal model for the present work with an average body weight of 180-200gm obtained from Shree Animal Farms, Nimgao, District Bhandara, Maharashtra, India. Rats was kept in the animal house of Department of Zoology, RTM Nagpur University, Nagpur under strict care and hygiene having light: dark (12:12 hrs) cycle, fed with pellet and water twice daily to keep them normal and healthy condition. The experimental protocol was approved by Institutional Animals Ethical Committee (IAEC) and animal care was taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India (Registration No. 478/01/a CPCSEA). The rats were randomly divided into 5 groups with each group consists of 6 animals. The group I, served as a control and was provided with saline water while the remaining animals in groups II, III, IV and V were treated with doses of NaF (Sigma chemical company, USA) at 5, 10, 15, and 20 mg NaF/kg bw/day, respectively, orally via drinking water for 45 days. Oral administration was preferred in view of water being the main source of fluoride among the human population in endemic areas. During the experimental period rats weight were checked. Blood samples were collected on completion of experimental protocol by cardiac puncture to determine antioxidant enzymes in serum.

B. Antioxidant enzymes

1) Superoxide Dismutase (SOD)

Superoxide Dismutase activity was described by the method, involves generation of superoxide by pyrogallol autoxidation and the inhibition of superoxide dependent reduction of the tetrazolium dye

MTT [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide] to its formazan, measured at 570 nm (Madesh and Balasubramanian, 1998).

2) Catalase (CAT)

Catalase activity was measured by the decomposition of hydrogen peroxide was monitored by measuring the decrease in absorbance at 240 nm (Aebi, 1983).

3) Lipid Peroxidation (LPO)

Level of lipid peroxidation was estimated by measurement of thiobarbituric acid reactive substances (TBARS) using the method (Rehman, 1984). The pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde, a secondary product of lipid peroxidation was estimated at 535 nm.

C. Behaviour analysis

1) Open-field test (OFT)

The open-field test was used to measure the locomotor activity and habituation of rats (Colman, 2001). The rectangular arena (50×40×63 cm), made from wood (three walls and floor) and with a glass front panel (50×52 cm) were used. The floor was divided by lines into 20 small squares (10×10 cm). The rats were placed individually in the centre of the open-field and measures locomotion with index, rearing, grooming and squares crossing recorded for 2 min (Fig. 1).

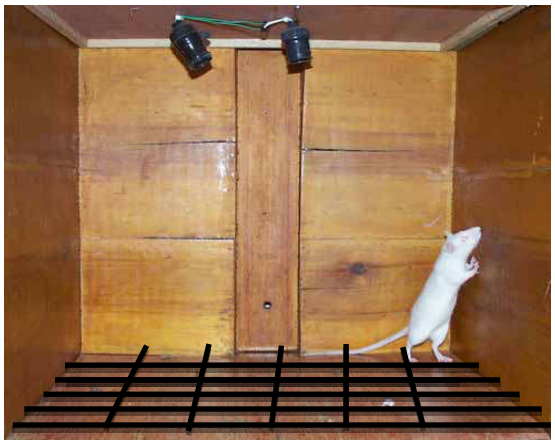


Fig. 1:Open field test arena for locomotion andanxiety

2) Morris water maze (MWM) test

Morris Water Maze (MWM) test was employed to assess learning and memory of rats (Morris, 1984). MWM performed in circular pool (150cm in diameter, 45cm in height, filled to a depth of 30 cm with water at 28±1^{0}C). The circular pool was divided into four equal quadrants with the help of two threads, fixed at right angle to each other and submerged platform (10cm) 1cm below surface of water inside the target quadrant. The position of platform was kept unaltered during the trial session. The water was made opaque with nontoxic white colour powder to hide the platform. Each animal

was subjected to four consecutive trials on each day with a gap of 5min. In test session the rat was gently placed in the pool between quadrants, facing the wall locate submerged platform. Allow it to swim for 120sec. if it failed to find the platform within 120sec, it was put gently onto the platform and allowed to remain there for 20sec. To locate the hidden platform in water maze was noted by an index, (1) escape latency time and (2) exploration in peripheral region (Fig. 2).

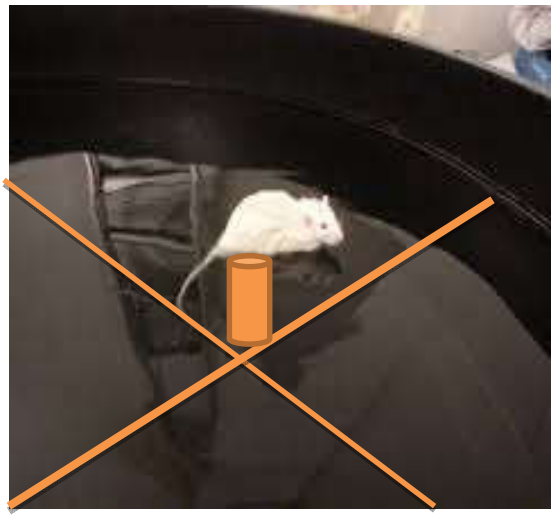


Fig. 2: Morris water maze test for memory

3) Y-maze test

Short-term memory was assessed by spontaneous alternation behavior in the Y-maze task. The Y-shaped maze is typically used as an enclosed maze in which testing occurs with three identical white, opaque wooden arms at a 120° angle from each other, 41cm long and 15cm high and 5cm wide to accommodate mice, rats, and small primates (M-1). Each rat was placed in one of the arm compartments and was allowed to move freely until its tail completely enters another arm. The sequence of arm entries was manually recorded. For each animal the Y-maze testing was carried out for 5 minutes. The purpose of the Y-Maze test was to assess spatial memory and learning in animals, in a control vs. disease model/intervention group, by observing their ability to remember which arm they have previously entered. This test can provide information regarding hippocampal-dependent learning, specifically spatial memory (Fig. 3).

The percentage alternation was calculated by following formula

{(actual alternations /maximum alternations) x 100}.

Fig. 3: Y- Maze test for memory or spontaneous alternation behaviour

4) Elevated plus maze (EPM)

Anxiolytic or anxiogenic effects of vehicle were evaluated using the elevated plus maze. Elevated maze with four arms (two open and two enclosed) were arranged to form plus shape and was described by Handley and Mithani, (1984). This assay essentially determines a preference between a comparatively safe and comfortable environment (closed arms) and a risky environment (elevated open spaces). This was often discussed in terms of avoidance or fear, but this was not strictly accurate. This was technically a preference test; one portion of the arm was avoided only in comparison to the other portion. Rat was more “anxious”, the less likely they were to explore an uncomfortable, risky, or threatening environment. The assessment of anxiety behaviour of rodents assessed by the ratio of time spend on the open arms to the time spend on the closed arms (Fig. 4).

Fig. 4: Elevated plus maze test for anxiety

Each NaF treatment group had matching controls who received defluoridated water only. This range of NaF exposures was selected because this ranged showed toxicity in reproductive organs, uterus (Thakare and Dhurvey, 2014), thyroid (Patil and Dhurvey, 2015)and estrous cycle and reproductive hormones (Dhurvey and Thakare, 2016) of rats.

D. Statistical analysis

The data were statistically analyzed and expressed as mean ± SEM. Statistical analysis of the variance between control and experimental values was done using Student's 't' test with the help of graph pad calculator (GraphPad, 2000).

III. Results

A. Body weight measurement

NaF treatment group decreased body weight as compared to the control groups, the body weight of rats was significantly reduced at the end of protocol as the dose increased. We found that higher dose treatment the decreased body weight at 10 ($p \leq 0.05$), 15 ($p \leq 0.001$) and 20 ($p \leq 0.0001$) mg NaF/kg bw/day groups. There was slight change in body weight at the 5 mg NaF/kg bw/day group (Table I).Altered antioxidant enzymes in serum

The activity of SOD, Cat and LPO in the serum of NaF treated rats were depicted in Table II. The results showed that, decreased activities of SOD and Cat at 10 ($P \leq 0.05$), 15 ($P \leq 0.05$) and 20 ($P \leq 0.001$) mg NaF/kg bw/day groups while increased activity of LPO at 10 ($P \leq 0.05$), 15 ($P \leq 0.05$) and 20 ($P \leq 0.001$) mg NaF/kg bw/day groups as compared to control groups. In low dose group (5 mg NaF) showed slight changed in above parameter.

Table I: Effect of NaF on body weight of rats for 45 days duration

Values are mean ± SEM, n=6 in each group, P≤0.05*, P≤0.001**, P≤0.0001***

Groups	**Initial body weight (gm)**	**Final body weight (gm)**
Control	222.83 ±6.76	218.89 ± 1.18
5mg/kg bw	216 ± 2.51	208 ± 1.23
10mg/kg bw	220.12 ± 1.73	195.67 ± 1.36*
15mg/kg bw	209.23 ± 2.66	180 ± 1.54**
20mg/kg bw	215 ± 2.4	175 ± 2.65***

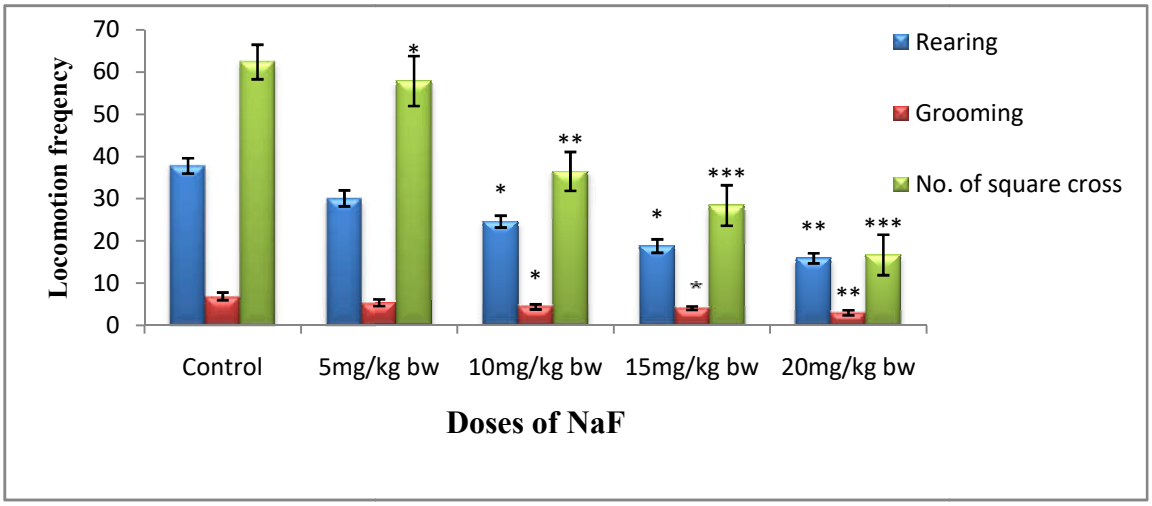
Table II: Effects of NaF on antioxidant enzymes of rats for 45 days duration

Values are mean±SEM, n=6 in each group, P≤0.05*, P≤0.001**

Groups	SOD (U/mg protein)	Cat (U/mg protein)	LPO (nanomoles /mg protein)
Control	462.5 ± 14.16	82.91 ± 1.316	1.96 ±3.2
5mg/kgbw	396.32 ± 13.17	80.84 ± 1.174	2.01± 4.1
10mg/kgbw	295.63 ± 14.22*	76.66 ± 1.124*	2.85 ± 4.7*
15mg/kgbw	261.17 ± 14.32**	74.76 ± 2.285*	3.35± 3.9*
20mg/kgbw	212.55 ± 12.84**	66.96 ± 2.34**	5.42 ± 3.6**

Impaired OFT behaviour

OFT measured with index like rearing, grooming and square crossing by experimental animals with different doses of NaF treatment for 45 days. Rearing (standing on rear limb) of rat are used to detecting behavioural changes, related vigilance and reflex. The treatment showed decrease in rearing activity, animals completed the task sluggishly and didn't follow the time required to it at 10 (P≤0.05), 15 (P≤0.05) and 20 (P≤0.001) mg NaF/kg bw/day groups significantly (24.6 ± 1.4, 20.8 ± 1.6, 15.9 ± 1.2). Grooming is inborn frequently performed behaviour activities in rodents that evaluates complex sequential pattern of action. The treatment showed decrease in grooming activity significantly at 10 (P≤0.05), 15 (P≤0.05) and 20 (P≤0.001) mg NaF/kg bw/day groups (4.4 ± 0.6, 4.1 ± 0.4, 3.0 ± 0.6) respectively while at 5 mg NaF/kg bw/day groups showed non-significant changes in both rearing and grooming. Square crossing measurement also key factor for anxiety and locomotion, showed unable to cross the squares significantly at 10 (P≤0.001), 15 (P≤0.0001) and 20 (P≤0.0001) mg NaF/kg bw/day groups (36.5 ± 10.6, 28.4 ± 11.8, 16.7 ± 9.8) respectively as compared to control group (Fig. 3).



B. Altered MWM test

The MWM test is spatial learning task measured with index like escape latency time and exploration in peripheral region. In MWM test indexing significantly increased escape latency time (89.4 ± 11.2sec, 98.9 ± 13.2, 105.8 ± 12.9sec) days and exploration in peripheral region (74.4 ± 11.6sec, 78.9 ± 13.2sec and 91.12± 14.8sec) at 10 (P≤0.05), 15 (P≤0.05) and 20 (P≤0.001) mg NaF/kg bw/day groups treatment for 45 days respectively as compared to control group while there were slight changes in 5 mg NaF (Fig. 4).

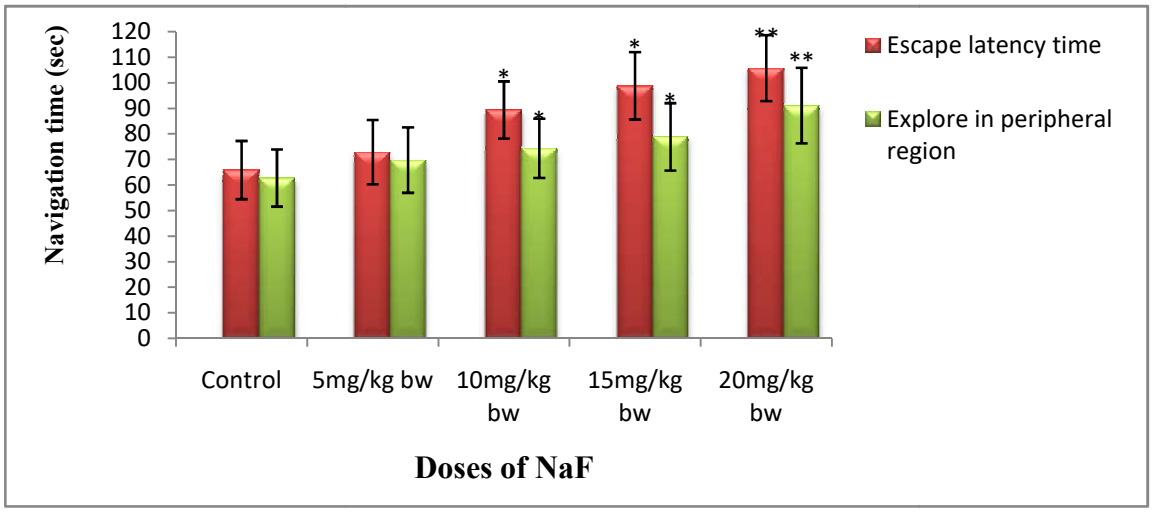


Fig. 4: Effect of NaF on MWM activity of rats for 45 days duration (mean±SEM), P≤0.05*, P≤0.001**

C. Impaired Y- Maze Test

The natural behaviour of animals consists of their choice of goal arm alternation and counting of arm entries is an important indicator of animal locomotor activity. Y- Maze test measured with index like spontaneous alternation and arm entries. On 45 days treatment showed decrease of alternation percentage activity at 15 mg/kg bw and 20 mg/kg bw (27.95 ± 0.66, 25.28 ± 1.27), while decreased activity of total arm entries at 10 mg/kg bw, 15 mg/kg bw and 20 mg/kg bw (12.33 ± 0.81, 11 ± 1.26, 9.83 ± 0.75) respectively (Fig. 5).

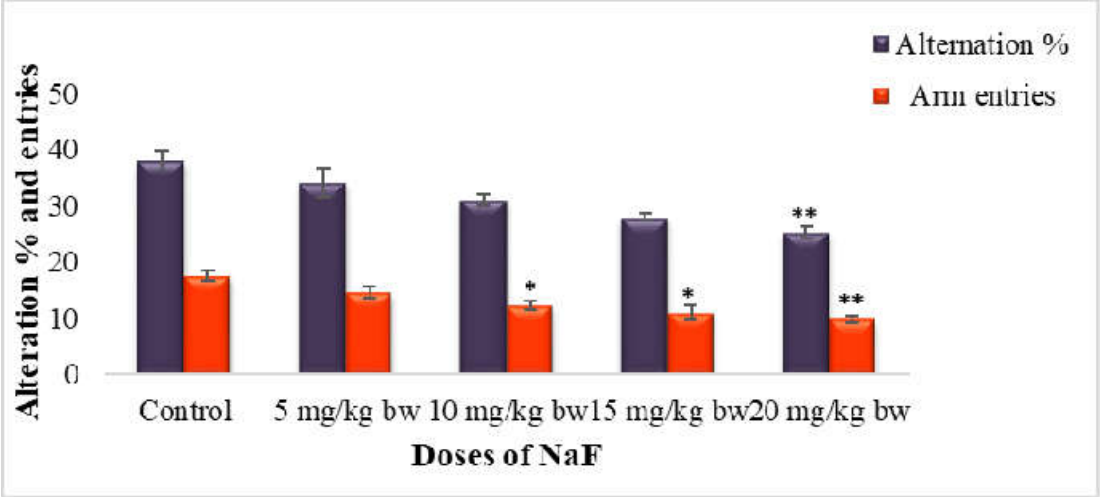


Fig. 5: Effect of NaF on Y- maze activity of rats for 45 days duration (mean±SEM), P≤0.05*, P≤0.001**

D. Altered EPM test

Animals underwent the EPM test to measure their anxiety like levels. The EPM test indexing decreased with increased in duration for 45 days of NaF treatment in open arm entries (34.83 ± 0.75, 31.83 ± 1.47, 29.83 ± 0.78) and time spent in open arm (40.33 ± 1.5, 31.83 ± 2.13, 28.33± 1.86) at 10 mg/kg bw, 15 mg/kg bw and 20 mg/kg bw respectively as contrast increased closed arm entry at 10 mg/kg bw, 15 mg/kg bw and 20 mg/kg bw (13.5 ± 1.04, 17.33 ± 0.81,19.17 ± 1.16) respectively when comparing to control group (Fig. 6).

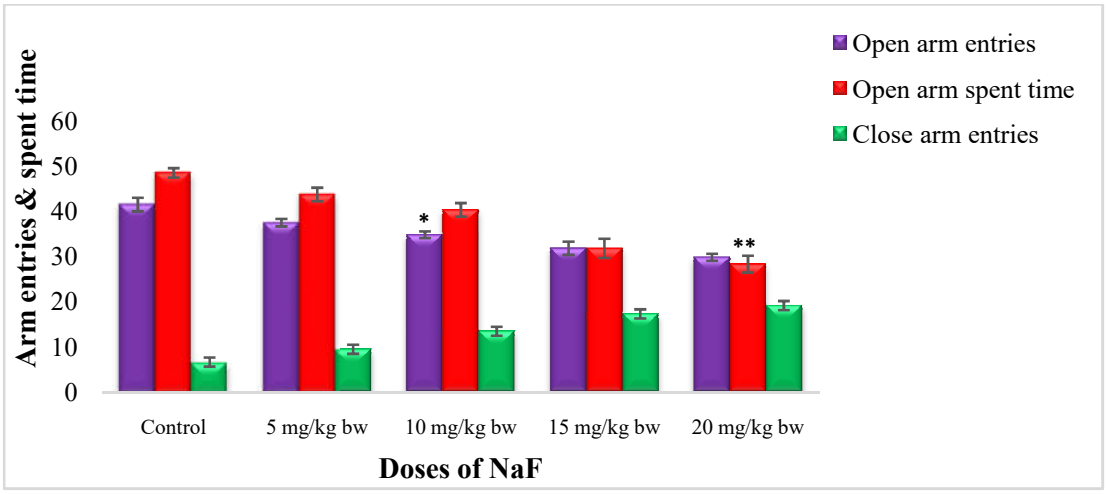


Fig. 6: Effect of NaF on EPM activity of rats for 45 days duration (mean±SEM), P≤0.05*, P≤0.001**

IV. Discussion

This study investigated a link between the different concentrations of NaF exposures and behavioural disruption in the female rats. In the present study, a reduced body weight at low dose, whereas more significant decreased at high dose of NaF treatment group. In various study, NaF treatment brought about a reduction in body weight of rats (Agrawal and Sharma, 2008; Agrawal *et al.,* 2004) and in mice (Vani and Reddy, 2000). The lower body weight which could be attributed to very low food consumption, altered protein and energy metabolism (Chinoy, 1991; Trivedi *et al.,* 2012), electrolyte imbalance (Das and Susheela, 1991) may impaired the behaviour Ross and Daston, 1995) in fluorotic animals.

It is well known that reactive oxygen species denatured total proteins, lipid peroxidation and altered the permeability of cell membranes (Polasa, 1992; Devasagayam and Kamat, 1999). Free radical production and elimination are controlled by antioxidant defence enzymes mechanism (marks *et al.,* 1996). In the present study, fluoride caused a decrease in the activities of SOD and Cat levels, but increased lipid peroxidation in the serum of female rats the results are in agreement with ovary of mice (Chinoy and Patel, 1998) in liver, kidney and testis (Sun *et al.,* 1994; Sharma and Chinoy, 1998). SOD activity also decreased in the erythrocytes of children with endemic skeletal fluorosis (Shivarajashankara*et al.,* 2001)and inthe brain and gastrocnemius muscle of mice(Vani and Reddy, 2000).

The key findings of the present study showed impairment in the OFT and MWM test with higher dose of NaF ingestion by female rats. The results revealed that high NaF intake induced a decreased capacity in learning and memory of rats. The results are agreement with (Wu *et al.,* 2006) showed that reduced learning and memory capacity through sub-chronic and chronic and impaired step-down inhibitory avoidance in animals. The MWM test is best for studying the spatial learning and memory ability of animals (Gozal*et al.,* 2001). The present findings also indicate that the latency was significantly prolonged in MWM test at higher dose treatment as compared to the control group and it did not shorten till the end of protocol this suggesting that the spatial learning ability of these experimental animals was impaired in which a possible mechanism might be connected to the high level of oxidative stress resulting from fluorosis. Some studies reported reduced learning capacity and memory ability with increased oxidative stress (Lu *et al.,* 2000; Xiang *et al.,* 2003; Trivedi *et al.,* 2007; Gao *et al.,* 2009), damage cellular membrane structure by lipid peroxidation and alteration in the membrane lipid composition (Guan*et al.,* 1998) might influence the insertion and turnover of neuronal nicotinic acetylcholine receptors and weakened cholinergic nervous system responses.

In the present study rearing and grooming task were decreased in OFT, the results are similar with Ekambaram and Paul, 2001, 2003; Niu *et al.,* 2008; Pereira and Dombrowski, 2011) showed that rats administered with high doses of NaF showed deterioration of exploratory motor activities and emotionality in habituation during the OFT and reduced memory retention during the maze test (El-

letheyet al., 2010). The decreased in grooming activity with increased anxiety, the results are in concerned with (Mullenix *et al.,* 1995) observed diminished locomotor activity on high F intake was proportional to increased F concentrations in the brain. Oxidative stress can have altered neurotransmitter, neuronal function and overall brain activity may cause anxiety (Bouayed*et al.,* 2009; Bouayed, 2010).

In the present study during all treatment periods, the NaF exposed rats were less active compared to control group. With regard to spontaneous alternation behaviour and number of arm entries of Y-Maze test in the NaF exposed group have shown significant decrease. These observations are well supported by Niu et al., (2008) found significant decreased in learning ability of animals in the HiF + HiPb group. Olakunle et al., (2012) noticed that in Y-maze spatial memory task at 1.0 and 1.5 mg/kg dose of monosodium glutamate causes a reduction in spatial learning and memory. Niu et al., (2008) observed activity chamber and a Y-maze test to measure the spontaneous activity and conditioned response behaviour in rats, showed that F significantly increased the error number of rats during the days of training. In rat hippocampus, part of brain is involved in learning and memory (Riedel et al., 1999; Vianna et al., 2000; Pittenger et al., 2002) but its most regions affected by F intoxication because F is classified as neurotoxic substance. F may interfere with oxygen metabolism and generate oxygen free radicals, which are responsible for diminished learning and memory (Chirumari and Reddy, 2007) and also alters cognitive responses and behaviour (Wu et al., 2006).

In the present data NaF treated groups showed significant anxiety like behaviour, and the percentage of time spent in open arm as well as open arm entries significantly decreases, while close arm entries increases after intoxication of different doses of NaF when compared to the control group. This result consistent with Liu et al., (2013) reported that high F group was significantly lower the percentage of time spent in open arms but in contrast to close arm entry and open arm entry no significant difference was observed. El-lethey et al., (2010) observed F intoxication causes anxiety like behaviour. Arsenic toxicity at 10 mg/L also show the increased anxiety like behaviour in the elevated plus maze and this behaviour were enhanced after 4 and 8 weeks of exposure (Chang et al., 2015). Pereira et al., (2011) found that NaF induced memory impairment was associated with NA and 5 HT increases in discrete rat brain regions and also affect the activity of some enzymes in the brain. In female wistar rats, Paul et al., (1998) showed F exposure inhibited spontaneous motor activity, due to alternations in the function of neurotransmitters.

The present study revealed that NaF exerts its toxic effects on memory and learning ability and causes cognitive dysfunctions may be due to oxidative stress.

V. Conclusion

On the basis of above findings, it may conclude that F is a potent health hazard. Prolonged treatment with NaF altered antioxidant level affectingbehavioural pattern, memory ability, learning

capacity and locomotion responses of rats may be due to neuronal dysfunction induced by oxidative stress.

Compliance with ethical standards

This study was performed following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, Registration no. (478/01/a/CPCSEA).

Acknowledgement

VI. References

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