Addis Ababa University

School of Graduate Studies
College of Health Sciences
School of Medicine
Department of Medical Anatomy

Project Paper on: Histological and Functional Effect of Fluoride on Cerebral Cortex of the Brain

A Project Paper Submitted to Addis Ababa University College of Health Sciences School of Medicine, Department of Anatomy in the Partial Fulfillment of the requirement for the Degree of Master of Science in Human Anatomy

By: Selemun Hagos

July, 2014

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Advisor: Dr. Girma Seyoum (PhD)

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DEDICATION

To late Dr. Emiru Seyoum (PhD, Associate professor of Insect Pathology) for his dedicated contribution as my advisor during my M.Sc. studies in Insect Science. His wonderful contribution and friendly approach is always in my mind.

Acknowledgement

First and foremost, all thanks are to almighty God who blessed my life with patience, strength and full health. He is my way, my life and my truth. For this I praise his name.

I wish to express my most sincere gratitude and appreciation to my advisor Dr. Girma Seyoum, for his consistent follow up, guidance and encouragement. My genuine appreciation also goes to my instructors Dr. Girmay Gebru, Dr. Mekbeb Afwerk and Dr. Amenu Tolera for their friendly cooperation and over all support throughout the academic year. Moreover, thanks are due to the Anatomy department staff members, for their sincere cooperation and moral supports.

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I am also forever grateful to my parents especially Crystal Clarke Barham (Mom) and her American families for their remarkable support and love. Thanks Mom for being a good mother. Finally, I am thankful for all those good people in my life, they have shown me exactly who I do want to be.

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List of Abbreviation

5-HIAA…….5-hydroxy-indoleacetic acid
AChE…….Acetyl-cholinesterase
ANOVA…….Analysis of variance
ATSDR… ……Agency for Toxic Substances and Disease Registry.
CA…….Cornu Ammonis
CAT……Catalase
cm…….....Centimeter
CNS……Central nervous system
DNA ……..Deoxyribonucleic acid
EMA……...Exploratory motor activities
g……....Gram
GPX……Glutathione peroxides
GST……Glutathione transferase
H&E……Haematoxylin and Eosin
h…….....Hour
HVA……..Homovanillic acid
IQ…….. Intelligence quotient
Kg…….Kilogram
L ……Liter
LPO……Lipid peroxidation
MDA……...Malondialdhyde
Mg ……..Milligram
NaF…….Sodium fluoriede
NE……..Norepinephrine
oc …….Degree Celsius
Ppm ……..Parts per million
RNA…….Ribonucleic acid
SOD…….Superoxide dismutase
US EPA….. ……....United States Environmental Protection Agency
WHO……..World Health Organization
XOD……...Xanthine oxidase

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Summary

Fluoride is omnipresent in our environment and has been added to drinking water supplies with a recommended dose. Drinks, tooth pastes, mouth rinses, dietary supplements and foods are also considered as sources of fluorides. This paper reviews the scientific literatures linking fluoride with its effect on histology and function of cerebral cortex of the brain. In this paper the role of fluoride in region specific and sub-cellular distribution of the brain with the relation of its neurotoxicity is highlighted. Studies were assessed by focusing on the dose of fluoride, duration of exposure, type of experimental animals used to measure the effect of fluoride. The literatures reviewed in the present paper used mice, rats and rat offspring as experimental animals. From the literatures reviewed in the present paper, fluoride showed to be neurotoxic chemical which affects the biochemical content of brain, cause weight loss, cause neurodegeneration, histological alternation in hippocampus and cerebral cortex of the brain, disrupting behavioral activities and cause reduction in cognitive and memory functions.

Keywords: Cerebral cortex, Histopathological effect, Weight loss, Biochemical change, Neurobehavioral toxicity and Fluoride

1. Introduction

1.1. Physical and chemical Properties of Fluorides

Fluorine is a naturally occurring, widely distributed element and a member of the halogen family which is most electronegative and one of the most reactive of all the elements (ATSDR, 2003 and Pratusha et al. (2011). Fluoride is a pale yellow-green, irritating gas with a sharp odor and chemically reactive (WHO, 2002). Fluorine is a common element that does not exist in the elemental state in nature because of its high reactivity. In combination it comprises for about 0.065% of the Earth's upper most crust and exists in the form of fluorides in a number of minerals, of which fluorspar, cryolite and fluorapatite are the most common (WHO, 2004 and Pratusha *et al.*, 2011). Fluorine gas reacts with most organic and inorganic substances; with metals, it forms fluorides and with water, it forms hydrofluoric acid (ATSDR, 2003 and Pratusha *et al.,* 2011).

Table 1: Some physical properties of fluoride compounds (WHO, 2002)

Appearance	Sodium fluoride is a fine dry powder or a dry crystalline material with no lumps
Molecular Formula	NaF
Molecular Formula	41.99
Ph at 20^{o}c	close to neutral (7.4)
Bulk density	1,000 – 1,400 kg/m3
Solubility in water	1.0 g/100 mL at 20°C or 4.3 g/100 mL at 25°C
Melting point	approx. 1818ºF (992ºC)
Flash point	saturated solution
Vapor Pressure	1971ºF (1070ºC)

1.2. Occurrence and Sources of Fluoride

Fluorine is the thirteenth most abundant element in the Earth's crust. It rarely occurs as the element but normally is found as the fluoride ion or as a variety of inorganic and organic fluorides. It occurs in varying concentrations in rocks, soil, water, air, plants and animals both naturally and as a consequence of human activity such as agricultural or industrial processes (WHO, 2002).

1.2.1. Water

The main source of fluoride for humans is the intake of groundwater contaminated by geological sources (maximum concentrations reaching 30–50mg/l). The level of fluoride contamination is dependent on the nature of the rocks and the occurrence of fluoride-bearing minerals in groundwater (Barbiera *et al*., 2010 and US EPA, 2010).

Fluoride in ground water is mainly derived from the weathering of rocks. In geothermal waters and volcanic zones, fluoride concentrations are related to the development of hyper alkaline volcanic rockes, the melts and volatile fractions of which accumulate large contents of fluoride. In sedimentary basins, the sources of fluoride are mainly fluorite (CaF_2), fluoroapetite ($Ca_5(PO4)_3F$), and marine clays on which fluoride may be adsorbed. Other sources of fluoride in ground water are anthropogenic inputs which increase fluoride concentrations in rain including chlorofluorocarbons (CFCs) and other industrial emissions (Pratusha *et al*., 2011).

1.2.2. Diet

Diet is the second largest source of fluoride exposure after drinking water for the population. The fluoride concentration of the food depends on soil where the food was grown, water and the use of fluoride containing fertilizers and pesticides. In addition, the fluoride level of water used to prepare or process food also contributes to the fluoride concentration of a particular food. Some food items such as tea and fish known to high in fluoride content and contribute to the elevated level of fluoride intake (ATSDR, 2003 and WHO, 2002). Generally, Fluoride intake from dietary sources is generally higher in areas with fluoridated water supplies or

where there are naturally occurring elevated concentrations of fluoride in drinking-water (WHO, 2002).

1.2.3. Soil and Air

Fluoride compounds in the air rank among the most air pollutants. They arise from the dusts of soils that contain fluoride, industrial gaseous effluents, the burning of coal and from volcanic gases and particulates (Pratusha *et al.,* 2011 and US EPA, 2010). The biggest natural source of hydrogen fluoride and other fluorides released to the air is volcanic eruptions (ATSDR, 2003). The airborne fluoride can indirectly contribute to human exposure as a result of secondary contamination of edible fruits and vegetables (US EPA, 2010).

1.3. Route of Fluoride by Humans

1.3.1. Inhalation

Inhalation is one of the major routes of fluoride to humans. Industrial emissions such as from phosphate fertilizer plants, aluminum plants and coal-fired power plants are potential routes of fluoride exposure through inhalation (ATSDR, 2003). Mean plasma ionic fluoride concentrations among persons living in communities with non fluoridated drinking water supplies 0.3 mg/l. In optimally fluoridated communities is from 0.7 to 1.2 mg/l. In non industrial urban areas, the highest fluoride quantity available for inhalation is approximately 0,04mg/day but near fluoride emitting factories could increase to 4 mg/day (Pratusha *et al*., 2011).

1.3.2. Oral

Drinking water, diet and toothpaste are main route of fluoride intake (ATSDR, 2003 and US EPA, 2010). Fluoride toothpaste can provide another major source of intake particularly to children. Tooth pastes contain 1.0 to 1.5 mg fluoride per gm and based on estimates of an average ingestion of 0.5g toothpaste per use for 2-5 year old children could result in the intake of 0.5-0.75 mg fluoride per use. Fluoride containing mouth wash could contribute 0.2-0.4 mg fluoride per use. Fluoride tablets and topical gels represent additional sources of fluoride exposures (Pratusha *et al.,* 2011).

1.4. Major Uses

Fluoride are used in ware and cable insulations, pipe linings, as rocket propellants, rodenticides, refrigerants, aerosol propellants, polymers for plastics, in the separation of uranium isotopes, and in the aluminum, beryllium, antimony, superphosphate fertilizer(which contain an average of 3.8% fluorine), glass, electronic ceramics, brick industries and sodium fluoride are used in municipal water fluoridation schemes (Pratusha *et al.,* 2011).

1.5. Fate of Fluoride Absorbed by the Body

Fluoride is absorbed in to blood plasma, where it readily crosses the placenta and is absorbed by the fetus. It also crosses the blood brain barrier and accumulates in soft and hard tissues. Soluble fluoride compounds are rapidly and completely absorb to an extent of 90 to 95% across the gastrointestinal tract (Akinrinade, 2013a; Chauhun *et al.,* 2013; Pratusha *et al.,* 2011).

The large volume of extracellular body fluids dilutes the absorbed fluoride concentration; there by avoiding elevation in plasma, fluoride diffuses to the tissues throughout the body. Approximately 50% of the daily fluoride intake is deposited in calcified tissue which encompasses teeth and bone (Pratusha *et al*., 2011).

1.6. Effects of Fluoride on Humans Health

Fluoride is an essential trace element, plays an important role in preventing dental carries and reducing tooth decay (Brindha and Elango, 2011). However, higher exposure of fluoride causes different functional and histological health problems including: skeletal system, teeth, and brain (Basha *et al.,* 2014) and spinal cord and nerves (Reddy *et al.,* 2013). Long term exposure of fluoride can cause denser bones, joint pain, and a limited range of joint movement (Reddy, 2009 and ATSDR, 2003). In advanced stages of fluorosis, neurological manifestation such as paralysis of limbs, vertigo, spasticity in extremities, and impaired mental activity are observed in human beings. Additionally, Fluoride intake causes significant dose-dependent reduction in the content of acidic, basic, neutral, and total protein content in the cerebral hemisphere, cerebellum and medulla oblongata (Trivedi *et al*., 2007).

Studies have raised the possibility that prolonged exposure to fluoride in drinking water is capable of causing neurological impairments. Due to the possible chronic exposure to fluorides and their ability to readily crosses the blood-brain barrier , as result it induce altered neuronal and cerebrovascular integrity, abnormal behavioral patterns and metabolic brain lesions (Akinrinade, 2013a ; Chauhun *et al.*,2013; El-lethey *et al*., 2010).

Studies also show that fluoride exposure reveals great implications for Alzheimer's disease, Dementia and Attention deficit disorder. Moreover, fluoride exposure to schoolchildren causes decrease in intelligent quotient and an increase in the rates of mental retardation (Xiang *et al*., 2003; Xiang *et al*., 2011; Hamid *et al.,* 2013). Furthermore, chronic exposure to high concentrations fluoride has been reported to cross the placenta and induce disturbances in the development of human brain (Du *et al*., 2008 and Trabelsi *et al*., 2001).

2. Review and Analysis of Published Research Articles on the Histological and Functional Effects of Fluoride in the Cerebral Cortex of the Brain

In this project paper, several investigations conducted to study the histological and functional effect of fluoride on cerebral cortex of the brain are reviewed. Most of the studies reviewed here used mices and rats as animal model and the methods used were mostly similar. The published papers analyzed are presented as follows.

A study conducted by Nasir and Asad (2013) on the effect of fluoride on CA3 (Cornu Ammonis) region of the hippocampus of adult Albino rats, behavioral and histopatological alternations were reported. In this study, thirty apparently healthy adult male albino rats, weighing 200 – 250 g were used. Rats were randomized and assigned to two groups: control and treated groups, containing fifteen rats each.

The rats in the control group (Group I) were given food and water orally for seven weeks. The treated group (Group II) received 20 ppm sodium fluoride in water orally for 7 weeks. The experiment was conducted between 10-11 am to minimize diurnal variation/ circadian rhythm. After the last administration of fluoride to each in the treatment group, for duration of seven weeks, the rats were sacrificed following anesthesia by diethyl ether, and intra-cardiac perfusion was done with 10% formaldehyde. Brains were dissected out and hippocampus was identified.

Tissues were processed by different dilutions of alcohol, xylene, and paraffin embedding was done. Blocks were made and 5 micron thin sections were made of identical regions of different groups. Haematoxylin and Eosin (H&E) staining was used to demonstrate general cell structure and fluoride levels in the brain were determined with fluoride specific ionic electrode (Orion R 96-090). Neuronal density in the CA3 region of the hippocampus was compared in both groups using Motic 2.0 software (Nasir and Asad, 2013).

The result of Nasir and Asad (2013) showed that rats were sluggish and less reactive progressively with the administration of sodium fluoride as compared to control group. This reveals that the effect of sodium fluoride on motor activities. Furthermore, quantitative

estimate of neuronal density per unit area showed significant changes in NaF treated groups as compared to control group (Table - 2).

Table 2. Comparison of neuronal density in the CA3 region of animals in control and experimental groups (Nasir and Asad (2013).

Group	Control group(CG)	Experimental group(EG)
Neuronal Density	132.2 ± 4.1	84.2 ± 3.7

- Values are expressed as cells/mm2 ± SE.

Moreover, the result of Nasir and Asad (2013), demonstrated that rats' exposure to fluoride for duration of 7 weeks induce reduced neuronal density in CA3 region as shown in figure-1c.

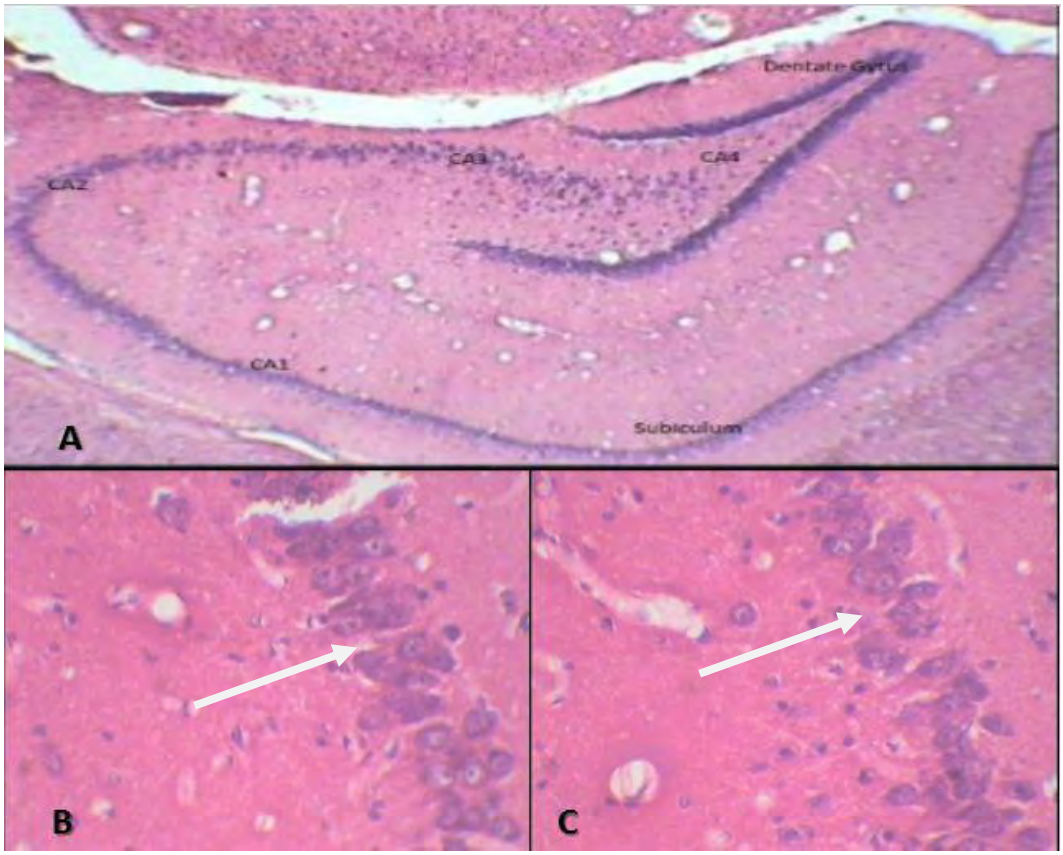

Figure 1. Morphological changes of Hippocampus (Nasir & Asad, 2013).

- A= Hippocampus
- B= CA3 region of control group (arrow head showing neuronal density)
- C= CA3 region of experimental group (arrow head showing reduced neuronal density)

A study conducted by Chauhan *et al*. (2013) investigated the toxic effect of fluoride on lipid peroxidation system on rat brain. In this study 32 female Sprague Dawley rats (6 month old) weighing 190-200g were used. The animals were housed in propylene cages and maintained at 22±3 ^{o}c, on a 12:12 h light dark cycles and a minimum 40% RH. Standard pellet diet and water were given *ad libitum*. The animals were acclimatized to the laboratory conditions for 1 week before initiating the experiments. For the experimental protocol, 32 rats were distributed in to 4 groups of 8 animals each. The animals in the control group were given a normal diet. Treatment group animals received intragastric treatment of NaF (25mg/kg) for 3 and 6 weeks. The body weight, food and water intakes were recorded during the experimental period. For overnight fasted animals from each group were sacrificed by deception under light ether anesthesia after 3 or 6 weeks of treatment periods. Brain was quickly removed and washed in ice cold 0.9% NaCl solution, dried and weighed. A section of brain was fixed in buffered 10 % formaldehyde for histological examination. After fixation in 10 % formaldehyde, tissues were embedded in paraffin; solid sections were cut at 5µm thickness and stained in H&E. The analyses were done by SPSS 14.0.Values were considered statistically significant at $P<0.05$.

The results of this study are presented in figure 2 and 3. The result of the study showed that rats treated with 25mg NaF for 6 weeks were similar in body weight with the control group (Figure 2). The decline in body weight gain was noticed from the onset of 3rd week of treatments and continued till remaining treatment period. After six weeks, gain in body weight was 29% in control group and 26% in sodium fluoride treated group.

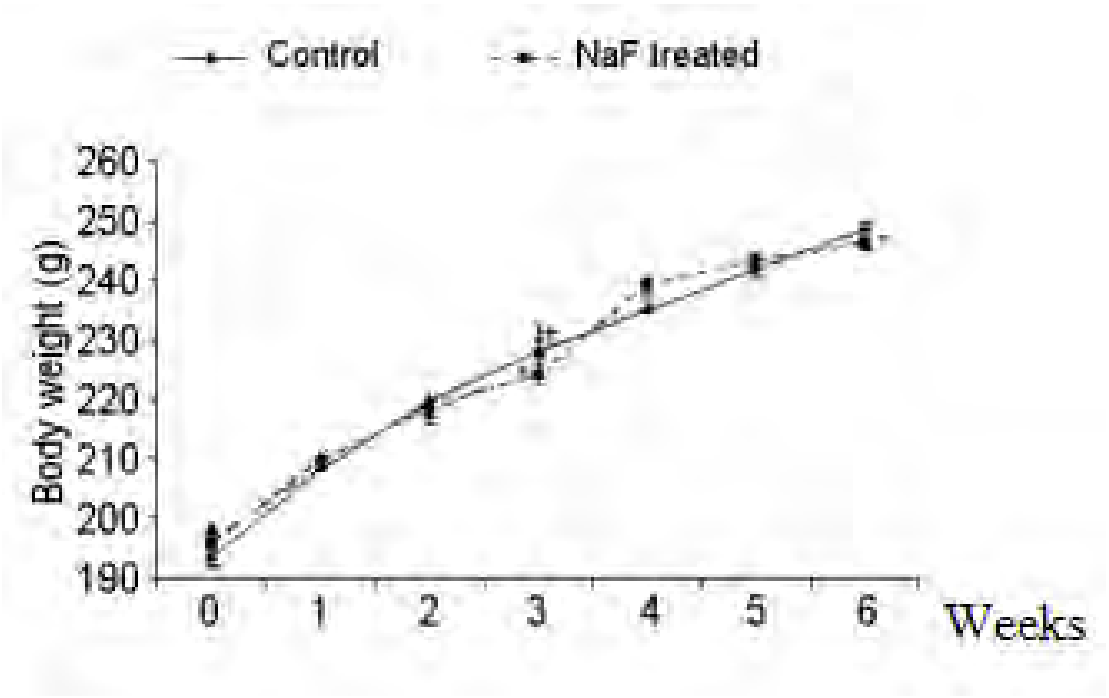

Figure 2. Effect of sodium fluoride (NaF) administration on body weight (Chauhan *et al.*, 2013).

The cortical regions of brain tissue also showed morphological alterations in NaF treated rats when compared to control rats (figure 3). Exposure to NaF showed excessive lymphocytes and mild spongiogenesis after three weeks (figure 3b) and edema along with spongiosis after six weeks (figure 3d) in cortical regions of the brain.

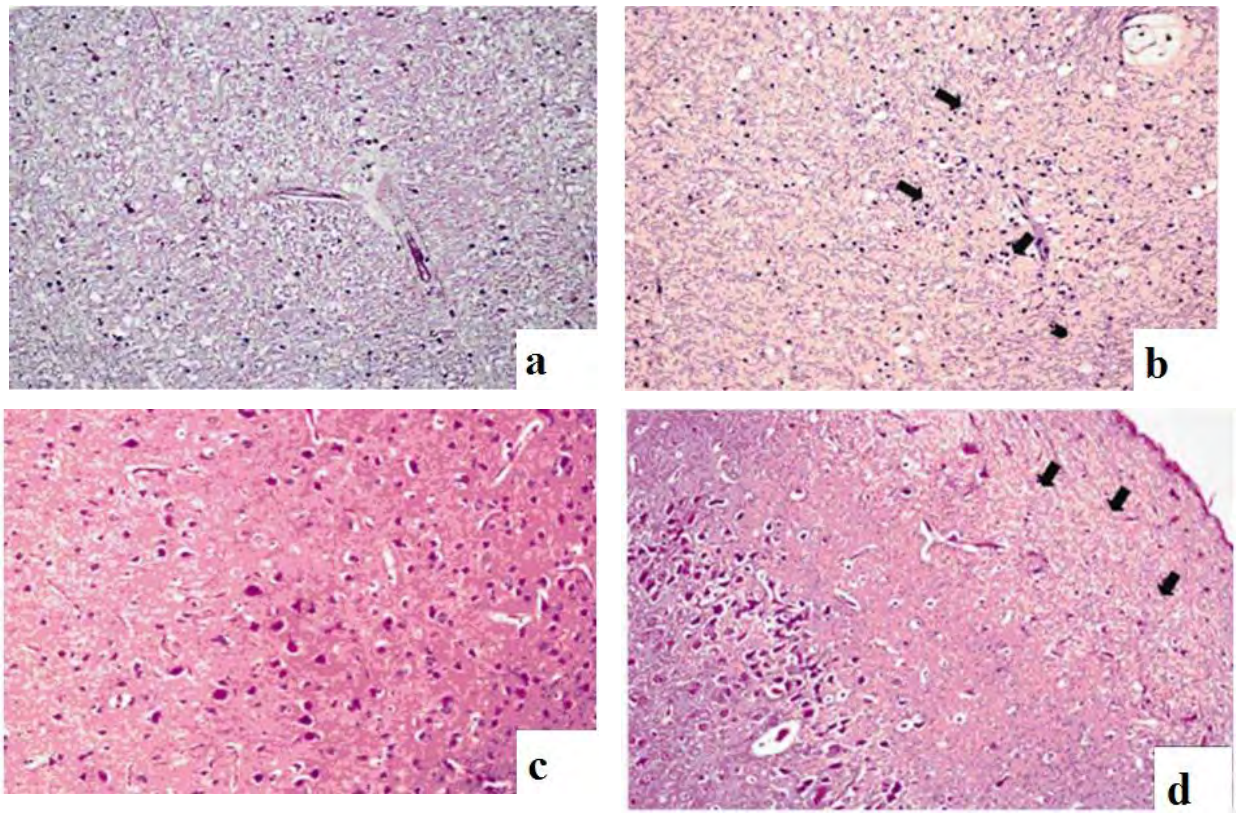

Figure 3. Morphology changes of cortical regions brain rat (Chauhan *et al*., 2013).

- a and c = Control group
- b = NaF treated group (3 weeks) arrowheads showing excessive lymphocytes and mild spongiogenesis
- d = NaF treated group (6 weeks) arrowheads showing edema along with spongiosis

A study carried out by Hamid *et al*. (2012) investigated the histopathological effects of different concentrations of fluoride on rat cerebrum. The study was conducted on 60 albino rats with 120-140 grams body weight. Rats were randomly divided into four groups of 15 animals each that were given different dose of fluoride as shown in table 3.

Table 3. Dividing experimental animals (albino rats) into four groups & the dosage of drug to be administered correspondingly. Hamid *et al.*, (2012)

Group	A	B	C	D
No. of Animals	15	15	15	15
Dosage	10ppm	100ppm	500ppm	Plain water (control)

Fluorinated water was prepared by dissolving sodium fluoride in tap water. Both the control and experimental groups of animals were kept in identical standard laboratory conditions and fed on standard laboratory diet which comprised of grams, vegetables and flour. The total duration of fluoride administration was 90 days. Then animals were studied for gross changes after 30, 60 and 90 days of fluoride administration. For body weight and gross observation 5 animals were weighed and examined every 30 days from each groups. Furthermore, after end of treatment of different doses of fluoride, the rats were anaesthetized using chloroform and scarified. Sections of the cerebrum were prepared from the control and fluoride treated rats.

The result of Hamid *et al*. (2012) revealed that, significant weight loss in treated animals as compared to control group. This change in weight in the animals appeared to be directly proportional to the strength and dose of fluoride in their drinking water and the time period for which fluorinated water was given. The results of this study revealed that animals of the groups B and C that receiving higher concentration of fluoride were most affected as depicted by weight loss (Figure 3) and looked lethargic, particularly after 60 and 90 days of fluoride administration.

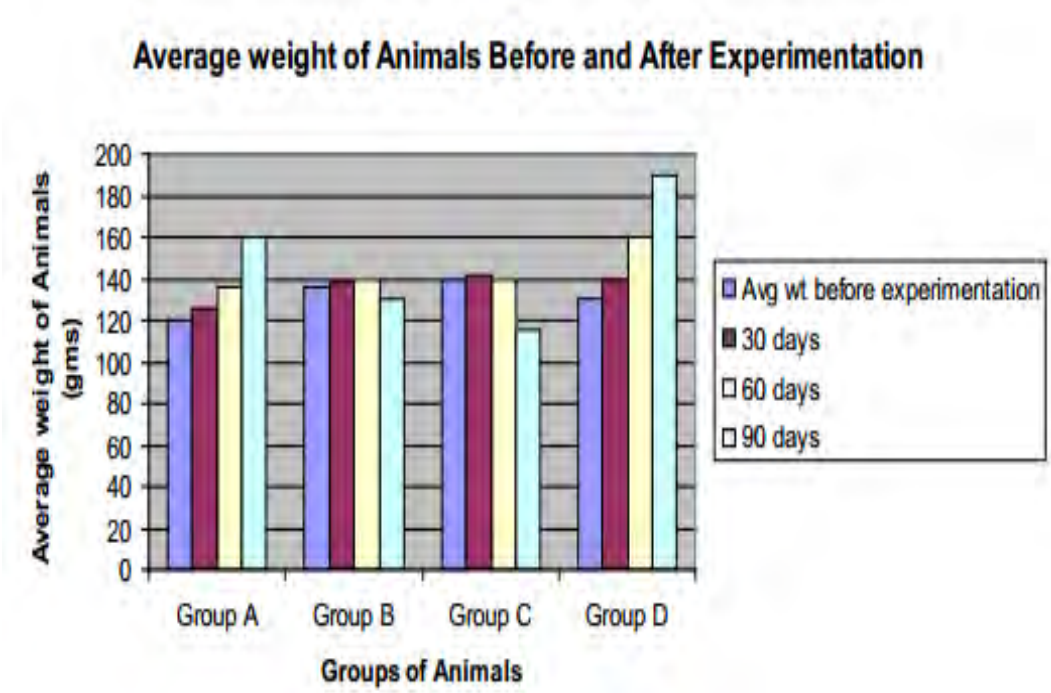

Figure 4. Average weight of animale before and after experimentation (Hamid *et al.*, 2012)

In experimental animals with low concentration of fluoride (10 ppm), the histological architecture of the cerebrum showed no change. However, with increasing concentrations of fluorides (100 ppm and 500 ppm) and increase in duration of exposure (60 and 90 days), there were marked histological changes in cerebrum such as neuronal swelling, signs of chromatolysis, vacoulation (Figure 5A), pyknotic changes (Figure 5B), decrease in neuronal density (Figure 5C) and gliosis (Figure 5D) (Hamid *et al.*, 2012).

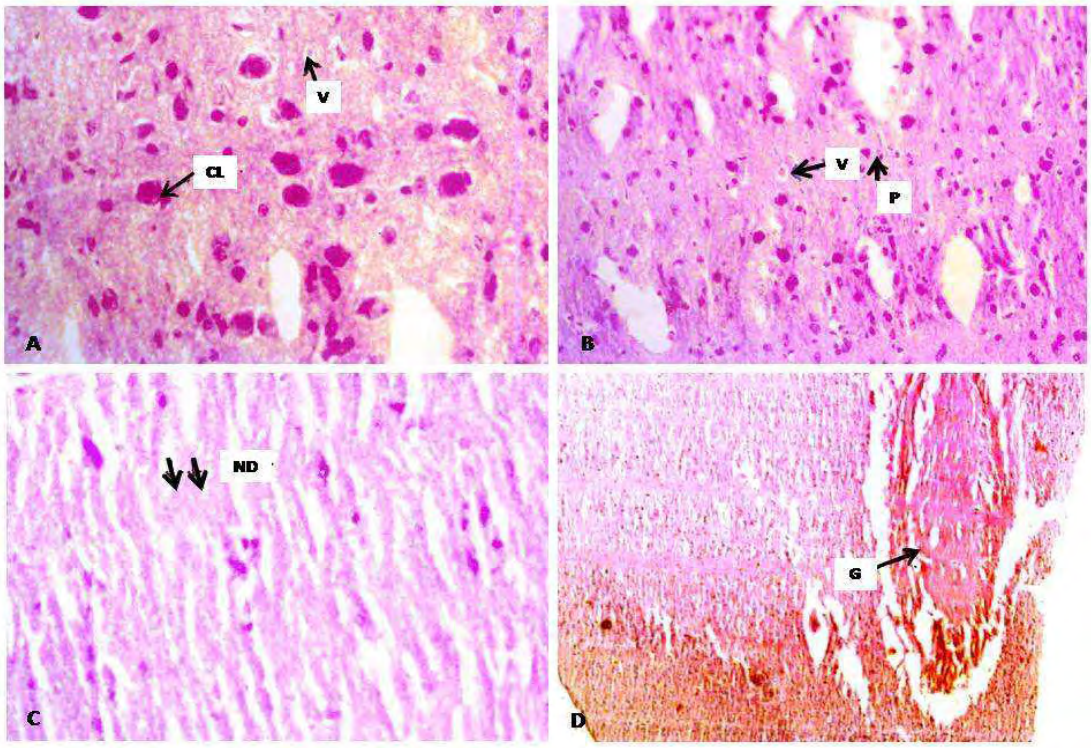

Figure 5. Histological changes in cerebrum (Hamid *et al*., 2012).

- A= Arrow heads showing neuronal swelling, signs of Chromatolysis, Vacoulation
- B= Arrow heads showing Pyknotic change
- C= Arrow heads showing decrease in Neuronal Density
- D= Arrow heads showing Gliosis

Another study conducted by Shah and Chinoy (2004) also demonstrated the adverse effects of fluoride on the cerebral hemisphere of mice. In this study ten adult male mice (Mus musculus) of Swiss strain were used. The animals were divided into two groups (control and treated).

Treated groups received 5 mg/kg body weight NaF for 30 days. After cessation of treatment the animals were sacrificed by cervical dislocation. The cerebral hemisphere of all control and treated animals was dissected out carefully, blotted free of blood. The harvested brain parts were then utilized to study histopathologial alternation as well as biochemical analysis. The histology of cerebral hemisphere of control and all treated groups of animals was studied by using the standard haematoxylin-eosin (HE) method. The stained slides were used for histocytometric analyses using an ocular (scaled) eyepiece and a micrometer scale.

In addition, standard techniques were used to determine biochemical parameters such as acetyl-cholinesterase (AChE) and nucleic acids in the cerebral hemisphere of control and treated mice. The statistical analysis for each biochemical parameter were assayed and the data were statistically analyzed by Student's t test and ANOVA.

The result of the experiment revealed that, the histology of cerebral hemisphere of control mice showed well developed neurons (Figures 6). However, the brain of 30-day NaF-treated mice revealed vacuolization and pyknosis of nuclei (Figure 7).

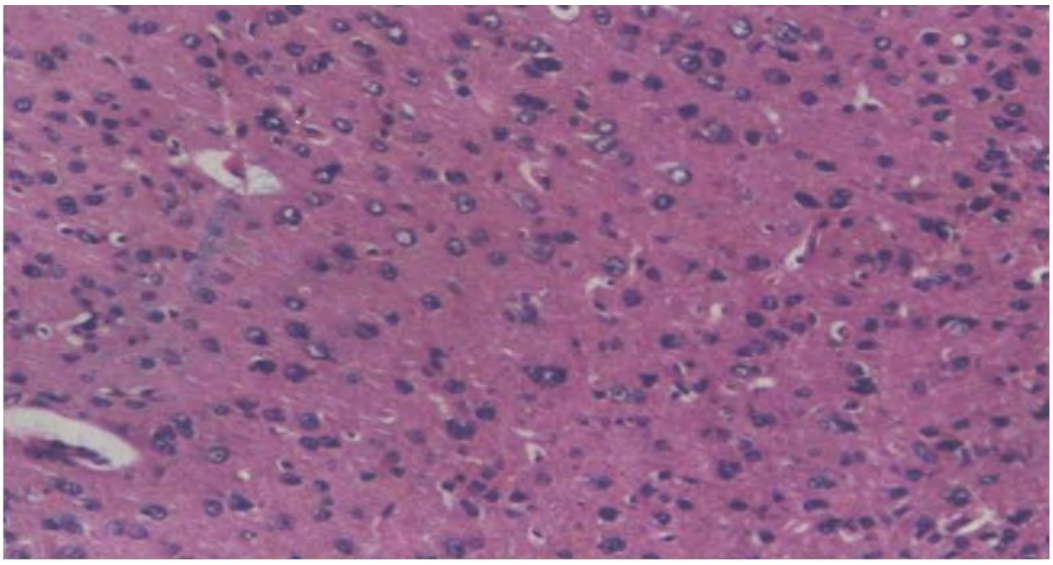

Figure 6.Transverse section of cerebral hemisphere of control mice (Shah and Chinoy, 2004).

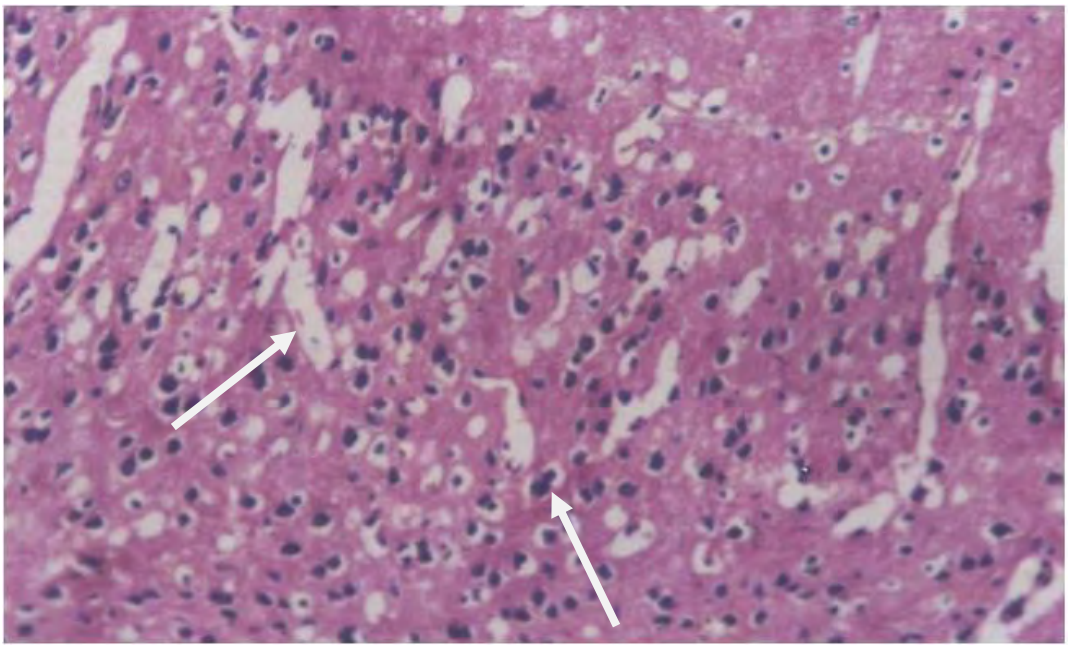

Figure 7.Transverse section of cerebral hemisphere of NaF treated mice (Shah and Chinoy, 2004)

- Arrow heads showing vacuolization and pyknosis

Furthermore, the activity of acetylcholinesterase, DNA and RNA levels in the cerebral hemisphere decreased significantly (P<0.001) after 30 days of NaF treatment in the treated groups as compared to control groups (table- 4).

Table 4.Acetylcholinesterase (AChE activity/mg protein), DNA and RNA levels (g/100mg fresh tissue weight) in cerebral hemisphere of mice Bhatnagar *et al.* (2006).

Group	Treatment	AChE	DNA	RNA
I	Control + distilled water	10.44 ± 0.09	575.65 ± 1.7	220.77 ± 1.05
II	NaF	7.50 ± 0.06§	375.34± 1.3§	120.14 1.80§

- Results are expressed as mean ± SD
- § Values were significant at **P<0.001**

Another study conducted by El-lethey *et al*. (2010) investigated the neurobehavioral toxicity of fluoride on rats. The researcher was used forty five mature Wistar female rats weighing about 200-220g. Animals had free access to feed and water. They were housed at a room temperature of 20-22°c and 60% humidity on a 12h light: dark cycle. All females were mated with males of the same strain.

For the administration of NaF, pregnant females were divided at random into three groups of 15 animals each. Group I (control) received non fluoridated water and treated groups (Group II and Group III) were received 50 and 100 ppm of NaF respectively. The NaF was incorporated in drinking distilled water and administered to pregnant rats for a 44 days period (from day 8 of gestation till termination of lactation and weaning of pups at 30 days of age).

After weaning, 200 pups were then collected and distributed into five groups of 20 animals each, divided on 2 replicates, as following: Group 1(control), weaning pups were derived from control dams receiving no NaF. These pups served as a control group, where NaF-free water was administered throughout the study till completing all assessments of learning and memory behaviours at 105 days of age. Groups 2 (low-discontinued), weaning pups were derived from dams receiving low dose of NaF. Pups were then exposed to low dose of NaF in drinking water, only till weaning at 30 days of age. Groups 3 (low-continued), weaning pups were derived from dams receiving low dose of NaF. Pups were then continually exposed to supply of low dose of NaF in drinking water till completing all assessments of learning and memory behaviors at 105 days of age. Groups 4 (high-discontinued), weaning pups were derived from dams receiving high dose of NaF. Pups were then exposed to supply of high dose of NaF in drinking water, only till weaning at 30 days of age. Groups 5 (high-continued), weaning pups were derived from mothers receiving high dose of NaF. Pups were then continually exposed to supply of high dose of NaF in drinking water till completing all assessments of learning and memory behaviors at 105 days of age.

After end of the treatment, data for all variables were subjected to ANOVA to assess the effect of different doses of Na-F and duration of administration on rats. Session factor for behavioral tests was also assessed using the general linear models procedure in statistical software (SPSS, 2006). A probability of $p<0.05$ was considered significant for all evaluations.

All data are expressed as mean ± SEM. Therefore, the behavioral assessments were involved as follows:

I. Open Field Habituation Test

The locomotor activity and habituation, a form of non-associative learning, were measured in the open field test. The open field used was a square wooden arena measured 90 x 90 x 25cm. The wood of the apparatus is covered with a plastic laminate (formica), which prevents absorption of fluids (urine of rats). The floor was divided by black lines into 36 small squares (15 x 15cm). All testing was conducted between 09:00 and 15:00 h. All treatments groups were tested at the same day in a random order. Rats were gently placed into a corner of the arena and allowed to explore the apparatus for 3 minutes. During the three minutes of exploration, the time spent freezing (no movement) was quantified.

Exploratory measures as well as non-exploratory behaviors were recorded by the observer. Exploratory motor activity (EMA) measures included horizontal locomotion (the number of squares crossed) as well as vertical activity (rearing). A crossed square was defined as the rat placing its two forepaws in the next square and moving forward. The vertical activity was defined as the number of times an animal stood erect on its hind legs with its fore legs in the air or leaning against the wall of the open field. Non-exploratory measurements comprised only the vegetative behaviours (numbers of urination episodes and defecation). After the 3 minutes test session, the rat was returned to its home cage and the open field was cleaned using 70% ethyl alcohol (to avoid odour cues) and permitted to dry. To assess the process of habituation to the novelty of arena, rats were exposed to the apparatus for a 3 minutes test session, on three consecutive days.

Therefore, the result of open field habituation test revealed a significant influence of dose of Na-fluoride on exploratory motor activities (EMA) and emotionality with marked impairment in habituation in rats exposed to high Na-F (El-lethey *et al*., 2010).

II. Classic Maze Test

Associative learning was also assessed using classic maze test. The base of the maze measured (100 x100cm) with walls height of 25cm. The entire maze was made of plywood

with a glass cover in order to prevent escape of animals and allow observation. Testing was carried out between 09:00 and 15:00 h, where all groups were randomly allowed for testing at the same day. Rats were deprived from feed for a 23 hours period before start of testing. Rats were given their daily feed amount as a reward at the end of the maze. Animals were given one trial per day for five consecutive days. Time elapsed to locate the feed at the end and numbers of entries of blind alleys were recorded. Therefore, result of classic maze test demonstrated that learning and memory assessed during maze test showed reduced memory retention in rats exposed to high fluoride for long periods (El-lethey *et al*., 2010).

III. Novelty Acquisition for Exploration Test

El-lethey *et al*. (2010) also designed to investigate the exploratory activity, where a mini-holeboard consisted of a dark platform (40 x 40cm) containing a hole (5.5cm diameter x 5cm depth) in each quadrant was used. This mini-holeboard was inserted into the base of the recording chamber (40 x 40 x 40cm). A small object, which differed in scent and texture, was placed in each hole (stimulus rich). Exploratory behaviour of rats including numbers of rears as well as head dips (to examine the interior of, or the objects within the four holeboard holes) were counted during a 15-min exposure period of the rats to the holeboard. Numbers of both rearing and head dipping were significantly lower during re-exposure. To assess whether learning has occurred in rats, reexposure of rats to high Na-F concentrations for long period was conducted. Thus, in novelty acquisition test, a reduced degree of habituation was noticeably shown in rats with long time exposure to high doses of Na-F compared to other treatments.

A study conducted by Chirumari and Reddy (2007) also demonstrated the dose-dependent effects of fluoride on neurochemical milieu in the hippocampus and neocortex of rat brain. Adult female Wistar rats about 6 to 7 weeks old, weighing 100–120 g, were used for this study. The rats were housed in polypropylene cages at 25±2ºC on a 12 hr light/dark cycle. They were also maintained on a standard rat pellet diet with water supplied *ad libitum*.

Rats were divided into 5 groups of 6 each. Each rat was administered intraperitoneally 1 mL of solution containing required quantities of NaF/100 g body weight each day for 14 days. Group I served as controls that were treated with 1 mL of mammalian physiological saline, group II received 1 mg NaF/kg bw, group III 5 mg NaF/kg bw, group IV 10 mg NaF/kg bw, and group V 20 mg NaF/kg bw. After 14 days the rats were sacrificed by decapitation. The brains were rapidly removed and dissected out, blotted free of blood. The hippocampus and neocortex were separated. Free radical enzymes such as Xanthine oxidase (XOD), Catalase (CAT), Superoxide dismutase (SOD), Glutathione transferase (GST), lipid peroxidation (LPO) level as malondialdehyde (MDA) and Glutathione peroxidase (GPX) were assayed. In addition, the levels of catecholamines, serotonin, dopamine, Epinephrine, 5-Hydroxyindoleacetic acid (5-HIA) and homovanillic acid (HVA) were determined. Statistical analysis was carried out using one way ANOVA followed by Tukey's multiple comparison test and significance set at $p<0.05$.

As shown in table 5 and 6, the result of Chirumari and Reddy (2007) revealed that the activities of superoxide dismutase (SOD), glutathione S-transferase (GST) and catalase (CAT) were decreased significantly as compared to the control ($p<0.05$). The level of lipid peroxidation (LPO) and the activities of glutathione peroxidase (GPX) and xanthine oxidase (XOD) in the NaF treated groups were increased significantly in a dosage dependent manner in comparison with control groups. In addition, Dopamine, serotonin, 5-hydroxyindoleacetic acid and homovanillic acid levels were increased, whereas norepinephrine and epinephrine levels decreased significantly ($P<0.05$)in the hippocampus and neocortex of the NaF treated rats compared with the controls.

Table 5. Free radical enzymes in hippocampus and neocortex of brain in control and NaF treated rats. Chirumari and Reddy (2007)

Neocortex					
Free radical enzymes Mean±SD	Group I (Control)	Group II	Group III	Group IV	Group V
Catalase	5.22±0.10	4.73±0.08	4.12±0.10	3.42±0.08	2.88±0.10
GPX	4.06±0.13	4.92±0.10	5.29±0.10	5.72±0.10	6.11±0.13
GST	8.33±0.09	7.69±0.11	7.17±0.10	6.83±0.09	6.10±0.12
LPO(MDA)	4.16±0.11	4.79±0.11	5.18±0.12	5.73±0.09	6.03±0.15
SOD	5.79±0.11	5.18±0.11	4.73±0.10	3.76±0.13	3.30±0.12
XOD	1.43±0.09	2.04±0.14	2.31±0.08	2.82±0.11	3.13±0.11
Hippocampus					
Free radical enzymes Mean±SD	Group I (Control)	Group II	Group III	Group IV	Group V
Catalase	3.73±0.09	3.12±0.12	2.74±0.11	2.17±0.10	1.73±0.12
GPX	2.47±0.42	2.81±0.09	3.23±0.10	3.85±0.12	4.29±0.10
GST	6.73±0.10	5.80±0.10	5.22±0.10	4.83±0.09	4.33±0.09
LPO(MDA)	2.26±0.11	2.85±0.11	3.10±0.12	3.32±0.09	3.69±0.10
SOD	3.37±0.09	2.81±0.09	2.25±0.11	1.80±0.12	0.91±0.10
XOD	0.21±0.08	0.78±0.11	1.05±0.13	1.33±0.09	1.73±0.09

- Values are expressed as Mean±SD of six animals per group.
- Values were significant at $p<0.05$
- The values of multiple comparison test were significant ($p<0.05$) among group I, II, III, IV and V.

Table 6. Neurotransmitter levels in hippocampus and neocortex of brain in control and NaF treated rats with variable doses of NaF. Chirumari and Reddy (2007)

Neocortex					
Neurotransmitters Mean±SD	Group I (Control)	Group II	Group III	Group IV	Group V
Dopamine	0.52±0.08	1.10±0.10	1.72±0.10	2.17±0.09	2.90±0.11
NE	0.94±0.08	0.68±0.08	0.52±0.09	0.38±0.09	0.11±0.07
Epinephrine	2.59±0.12	2.23±0.08	1.93±0.08	1.53±0.09	0.82±0.09
5-HIAA	2.32±0.11	2.82±0.10	3.22±0.08	3.69±0.11	4.05±0.12
HVA	0.31±0.10	1.31±0.09	1.74±0.11	2.18±0.11	2.84±0.13
Serotonin	1.20±0.12	1.82±0.09	2.26±0.08	2.78±0.11	3.31±0.08
Hippocampus					
Neurotransmitters Mean±SD	Group I (Control)	Group II	Group III	Group IV	Group V
Dopamine	0.12±0.09	0.78±0.10	1.11±0.09	1.53±0.08	1.89±0.12
NE	1.91±0.10	1.57±0.07	1.07±0.09	0.62±0.09	0.25±0.07
Epinephrine	1.92±0.10	1.62±0.08	1.35±0.10	0.68±0.38	0.49±0.10
5-HIAA	1.48±0.08	1.91±0.09	2.24±0.11	2.74±0.10	2.97±0.10
HVA	0.16±0.10	0.42±0.08	0.92±0.08	1.11±0.10	1.29±0.10
Serotonin	0.33±0.08	0.92±0.09	1.25±0.11	1.81±0.11	2.06±0.12

- Values are expressed as Mean±SD of six animals per group.
- The values were significant at $p<0.05$
- The values of multiple comparison test were significant ($p<0.05$) among group I, II, III, IV and V.

Another research conducted by Bhatnagar *et al*. (2006) investigated the toxic effects of fluoride on biochemical changes on brain mice from fluoride in their drinking water. In this study 40 adult (one-month old) female Swiss albino mice with weighing 25±5 gm were used.

The mice were divided into three groups: control (n=10), experimental group E1 (n=15), and experimental group E2 (n=15). Each mouse was caged separately. Control group mice were given deionized, defluoridated water. Mice in the E1 group were given 60 ppm F (from 132.6 mg NaF/L) in their drinking water while those in the E2 group were given 120 ppm F (from 265.2 mg NaF/L) in their drinking water.

After 30 days of treatment all the mice were weighed and sacrificed by cervical decapitation. The brain was dissected out and cut in to two sagittal pieces. Then the hippocampus was dissected out under a stereomicroscope. Tissues of at least three animals were pooled to prepare enough samples for biochemical estimation. Samples were then diluted tenfold and the homogenate was spun at 10,000 rpm for 15 min and the supernatant was used for enzymatic assay.

Biochemical estimation by standard methods was conducted for AchE, BchE, SOD, CAT, MDA, ascorbic acid, and total protein in brain (hippocampus) of control and Fluoride treated animals. Finally, the data were statistically analyzed by Student's t test.

The results of the Bhatnagar *et al*. (2006) showed that dose-dependent decrease on the activities of AchE and BchE and total protein (Table - 7).

Table 7.Effects of fluoride in drinking water on various parameters in the hippocampus of mice Bhatnagar *et al*. (2006)

Group	**Control**	**60 ppm (E1)**	**120 ppm (E2)**
AchE	0.11± 0.01	0.062±0.01*	0.022±0.004§
BchE	0.09± 0.009	0.027±0.011‡	0.017±0.023
SOD	56.55± 1.30	49.99±0.91§	38.10±0.862§
CAT	53.57± 6.26	27.38±4.06§	10.71±2.27§
MDA	1.85± 0.32	1.21±0.28	4.67±0.68‡
Ascorbic acid	0.87± 0.20	1.83±0.17	2.24±0.22‡
Total protein (mg/mL)	0.10± 0.00	0.09±0.00	0.032±0.005§

- Results are expressed as **Mean±SD**
- ***** Values were significant at **P<0.05**
- ‡ Values were significant at **P<0.01**
- § Values were significant at **P<0.001**

3. Discussion

Scientific literatures reviewed in the present paper showed that exposure to fluoride produces histopathological effect, neurotoxicity, biochemical change, impairment of motor activities and impairment in habituation on cerebral cortex.

The study conducted by Hamid et al. (2012) on the histopathological effects of fluoride on cerebrum showed physical weakness as compared to the control group in albino rats. This was evidenced by a definite loss of body weight in animals of the experimental group as compared to the control group. The weight loss in the animals appeared to be directly proportional to the strength of fluoride in their drinking water and the time period for which fluorinated water was given. The rats treated with high dose of luoride for long period of time were most affected as depicted by weight loss and looked lethargic. This finding is comparable with that of El-lethey *et al*. (2010), Hamid *et al*. (2012) and Reddy *et al*. (2011) who reported similar toxic effects of fluoride in rats. In addition, the study conducted by Trabelsi *et al*. (2001) on mice reported a 35% decrease in body weight. The weight loss might be due to inhibition of protein synthesis and breakdown of protein by fluorides (Trivedi *et al*., 2012). It could also be attributed to the toxic effect of fluorides on the tissues and the metabolism of the body (Hamid *et al*., 2012).

However, the finding of Hamid *et al*. (2012) is inconsistent with (Chauhan *et al*., 2013) who showed that rats treated with fluoride revealed body weight pattern similar to the controls. This might be due to difference in experimental animal species and duration of the exposure. Chauhan *et al*. (2013) used Sprague Dawley rats whereas Hamid *et al*. (2012) used albino rats. The duration of time used by Chauhan *et al*. (2013) was 6 weeks that was less than the duration of time used by Hamid *et al*. (2012) which was 30- 90 days.

The study conducted by Hamid *et al*. (2012) on Albino rats treated with high dose of fluoride induced significant neurodegenerative changes in the motor cortex. In this study, decrease in size and number of neuron, signs of chromatolysis and gliosis were noticed as major neurodegenerative changes.

Similar study carried out by Chauhan *et al*. (2013) on rats treated with NaF also showed morphological alterations such as excessive lymphocytes and mild spongiogenesis. Comparable study was also conducted by Nasir and Asad (2013) and Reddy *et al*. **(**2013**)** on rats which revealed that poisoning of rats to fluoride induce reduced neuronal density in hippocampus. The results of these studies are supported by Shivarajashank *et al.,* 2002; Bhatnagar *et al*., 2002; Shashi (2003) and El-lethey *et al*. (2010) who reported significant neurodegenerative effects of fluorides in the hippocampus, amygdala, motor cortex, and cerebellum. Moreover, other similar findings are reported by Ge *et al*. 2005; Reddy *et al*., 2011; Trivedi *et al.* (2012) and Akinrinade *et al*. (2013b) who reported neurodegenerative and neurotoxic changes vacuolated in hippocampus and degenerated cell bodies, shrunken neurons, pyknotic, and decrease the overall number of neurons.

The consistencies of the results of the above investigations could be because the animal model and methods used were similar. The neurodegenerative and neurotoxic effects of fluoride might be due to the toxic effect of high-fluoride intake during the early developing stages that affects growth, differentiation, and subcellular organization of brain cells. Likewise, fluoride can pass through the blood-brain barrier. The fluoride accumulated in brain tissue might interfere with the metabolism of brain phospholipids, which is related with the degeneration of neurons (Du *et al*., 2008). The most probable mechanism for the neurodegenerative effects of fluoride are likely related to excitotoxicity by free radicals and lipid peroxidation. This impairs the glutamate removal and by activating microglia which contain abundant stores of glutamate (Chirumari and Reddy, 2007; Akinrinade *et al*., 2013b and Basha *et al*., 2014). Lipid peroxidation and apoptosis may co-exist at the beginning when human tissues are exposed to excessive fluoride. Lipid peroxidation generates a lot of free radicals that may be sufficient to cause apoptosis (Wang *et al*., 2004). It was also thought that NaF increases nitric oxide synthesis activity. This nitric oxide which plays a major role in all neurodegenerative diseases, primarily by damaging mitochondrial energy production, inhibiting glutamate reuptake and stimulating lipid (Chauhan *et al.,* 2013).

In another study conducted by Chirumari and Reddy (2007) on rats revealed that, high fluoride intake decreases the levels of antioxidant enzymes in the hippocampus and neocortex. The decrease in antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione transferase (GST), accompanied with an increase in the pro-oxidative markers lipid peroxidation (LPO) and xanthine oxidase (XOD) in the NaF treated Wistar rats is suggestive of oxyradical release. This study was in agreement with study of (Vani and Reddy, 2000; Wang *et al*., 2004 and Chauha *et al*., 2007) who demonstrated that fluoride exposure induces impaired function of the SOD, GST, and catalase enzymes in mice.

Chirumari and Reddy (2007) also reported exposure of rats to high level of fluorided induced significant increase in the levels of dopamine, serotonin, 5-hydroxyindoleacetic acid and homovanillic acid in the hippocampus and neocortex of the NaF treated rats. On the other hand, norepinephrine and epinephrine levels were decreased significantly in the hippocampus and neocortex of the NaF treated rats as compared to the controls.

Comparable findings were also reported by Bhatnagar *et al*., (2006) and Shah and Chinoy (2004) on the cerebral cortex of mice treated with fluoride. The findings of these studies revealed significant decrease in the activity of acetylcholinesterase in brain and the DNA and RNA levels in the cerebral cortex. Similarly Vani and Reddy (2000) and Sandeep *et al*., (2013) reported that AChE activity was significantly decreased in brain of fluoride treated mice. This might be due to fluoride intoxication markedly affects cholinergic system, which may result in dysfunctions of neurotransmission in brain, and the fluoride induced neurotoxicity.

In another study conducted by El-lethey *et al*. (2010) on open field test revealed a significant influence of exploratory motor activities (EMA) and emotionality with marked impairment in habituation in rats exposed to high dose of NaF. Besides, learning and memory assessed during maze test showed reduced memory retention in rats exposed to high Na-F for long periods. In novelty acquisition test, a noticeable reduced degree was demonstrated in rats continued to administer high Na-F for long duration. This is in line with investigation of

Chioca *et al*. (2007) who suggests that 50ppm and 100ppm NaF intake impaired habituation of the rats in the open- field box and exhibited a decrease in avoidance frequency.
In the study carried out by El-lethey *et al*. (2010) learning and memory assessed over five days of maze test showed that groups of animals exposed to high concentrations of Na-F for long period took longer time to locate feed and showed poor memory retention. Likewise, Y-maze test on Wistar albino rats treated with 150 mg NaF/l showed significantly increased number of error during the days of training and number of days in meeting the learning standard of the Y-maze test (Dui *et al*., 2008). In addition, Bera *et al*. (2007) reported that novel object exploration test for 40 days exhibited lower levels of exploratory activity. Furthermore, the active avoidance task revealed a significant impairment in 60 day old male rats at the dose of NaF 5.0 mg.

Similarly, the studies of Xiang *et al*., (2003) and Xiang *et al*., (2011) also reported that as the fluoride level in drinking water increased the IQ of school children fell and mental retardation increased. Another comparable finding conducted by Shashi (2003) also reported that, exposure to 20 mg of NaF causes hemiplegia and the gait was unsteady and voluntary movements of the animals were misdirected and jerky. Moreover, in animals administered 50 mg of NaF, spastic paraplegia, quadriplegia, tremors, and seizures were recorded. Furthermore, Fluoride intake at 50 or 100ppm, may lead to the impairment of cholinergic neurotransmission, thus causing memory impairment (Long *et al*., 2002). Thus, rats exposed to high concentrations of NaF in drinking water demonstrated impairment in habituation and active avoidance, which indicates potential memory impairment, might be due to fluoride intoxication.

4. Conclusion

Most of the research articles reviewed in the present paper indicated that, excessive consumption of fluoride affects the functions and histology of cerebral cortex. The effect of fluoride is assessed in different species of animal model (mice, rats and neonatal rats) for different doses and exposure of time. Studies of toxicity of fluoride in the animal models used provided evidence of damage to the brain histology and function. Results of various studies reviewed in the present paper also reported that fluoride causes extensive damage to the brain by accelerating oxidative damage to biomolecules like lipid, protein and nucleic acids, accumulation of fluoride levels in the brain as well as cognitive deficits and other changes, including decreased maze-learning ability, altered general motor activity, loss of body weight and histopathological alternation in the brain. All the investigations reviewed revealed similar toxic effects of fluoride even when the doses used and duration of the experiment in some cases different. However, results regulating body weight of the experimental animals were not consistently similar. These differences could probably be the result of variation in methodology, for example in the dosage, duration of exposure and animal models used were not the same for all the studies.

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