Black Tea Forestalls Sodium Fluoride-Induced Neurobehavioral Toxicity in Laboratory Rats

Mervat M. Kamel[1*], Heba S. El-lethey[1], Iman B. Shaheed[2], and Gehan M. Kamel[3]

[1]Department of Animal Hygiene and Management, [2]Department of Pathology, [3]Department of Pharmacology, Faculty of veterinary Medicine, Cairo University, Cairo, Egypt, mevy58@yahoo.com*

Abstract: The present study aimed to investigate the main effects as well as the interaction effect of supplemental Na-F and black tea on emotional reactivity and learning and memory capacities in rats using a variety of behavioural tasks. Eighty weanling 32-days old Wistar male rats randomly distributed into four groups of 20 animals each, were supplemented with Na-F at 100 ppm and 2% black tea in a factorial pattern to constitute 4 experimental treatments. Brain tissue specimens, representing all treatments, were taken for biochemical and histopathological investigations. In the open field test, Na-F-treated rats displayed higher levels of anxiety that were significantly reduced when black tea was concomitantly administered. Marked impairment in habituation was a significant remark for Na-F group. A superior learning and memory ability was recorded for black tea-supplemented rats during classic maze test, where black tea significantly recovered the intervention observed in Na-F-exposed rats. Moreover, black tea significantly enhanced spatial cognition learning ability and successfully alleviated Na-F-induced spatial memory impairment. Rats administered Na-F displayed distinct neurodegenerative changes of nerve cells especially in hippocampus, accompanied by inhibition of brain acetylcholinesterase (AChE) activity with increased oxidative stress. Administration of black tea along with Na-F was able to afford protection against these Na-F-induced alterations. Our findings suggest a profound ameliorative effect of black tea on Na-F-induced adverse alterations in the brain of rats as indicated by hindrance of learning and memory performance, and argue for concurrent administration of black tea to Na-F-exposed individuals in order to help alleviate fluoride intoxication.

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1. Introduction:

Toxic effects of excessive intake of fluoride are matters of serious international concern (Verma et al., 2006). Fluorides are naturally occurring contaminants in the environment and commonly involved in toothpastes, mouth rinses, processed beverages and food as well as public water in order to prevent dental caries (Buzalaf et al., 2004). In humans, exposure to elevated fluoride drinking water in endemic areas has been found to cause headache, followed by lethargy and insomnia with reduced Intelligence Quotient (IQ) of children and lowered levels of mental work capacity of adults (Lu et al., 2000, Xiang et al., 2003, Sharma et al., 2009).

Since fluoride was evidenced to cross blood brain barrier, a link between excessive exposure to fluoride and dysfunction of the central nervous system has established (Blaylock, 2004, Ge et al., 2005). High levels of fluoride in drinking water (3-11 ppm) are known to cause definite harm to the central nervous system as manifested in diminished mental acuity with alterations in learning and memory processes directly without first causing the physical deformities of skeletal fluorosis (Wu et al., 2006, Chioca et al., 2008, Zhang et al., 2008). Rats exposed to fluorosis showed neuroinflamation in the brain, including demylinization, a reduction in the number of Purkinje cells, thickening and disappearance of dentrites, swelling of mitochondria and dilation of endoplasmic reticulum in neurons (Guan et al., 1998). Moreover, disturbances in brain development in offspring rats with chronic fluorosis have been reported (Liu et al., 1989).

Prolonged exposure to sodium flouride has been reported to induce deleterious effects on soft tissues (Patel and Chinoy, 1997, Purohit et al., 1999) and changes in behaviour through locomotor activity impairment (Ekambaram and Paul, 2001, 2003).

The central nervous system is principally more susceptible to oxidative damage due to its high oxygen consumption along with high tissue concentrations of iron and comparatively low levels of some antioxidants system (DrÖge and Schipper, 2007; Korkmaz et al., 2007). It has been emphasized that higher levels of fluoride provoke oxidative stress in the brain through excessive production of reactive oxygen species (ROS) free radicals taxing the compensatory task of antioxidant system with increased levels of lipid peroxidation, accounting for neuronal dysfunction with cognitive decline (Guan et al., 1998, Chirumari and reddy, 2007). Moreover, fluoride intoxication has been shown to induce changes in central neurotransmission presenting inhibited acetylcholinesterase (AChE) activity (Gao

et al., 2009). Thus, prevention of fluoride-induced oxidative damage to the brain is likely to be beneficial for the maintenance of cognitive function.

There is a growing awareness of the role of certain nutritional components including flavonoid polyphenols, a group of dietary-derived phytochemicals, found in fruits, vegetables and beverages like tea in maintenance of health and prevention of chronic diseases (Youdim et al., 2002). Flavonoids have been reported to induce improvement in memory, learning and cognition (Spencer, 2009). Several studies suggested that flavonoids might improve cognitive function by protecting vulnerable neurons against injury induced by neurotoxins, suppressing neuroinflamation, and enhancing existing neuronal function or by stimulating neuronal regeneration (Mandel and Youdim, 2004, Spencer, 2008, Rendeiro et al., 2009). Most of these studies have been conducted in rodents as models for human in order to predict effects of flavonoids on human cognitive performance (Casadesus et al., 2004, Lee et al., 2005, Williams et al., 2008).

Although six types of teas are distinguished, the three main types are black tea (fully fermented), oolong tea (semi-fermented) and green tea (non-fermented). Black tea accounts for 80% of the world's total tea production (Krisnamoorthy, 1991). While black tea is the most common type of tea consumed in Egypt and widely consumed beverages, second to water, few studies have investigated the effects of black tea on cognitive performance.

The type of flavonoid polyphenols founds in different types of teas, and constitutes 93% of total tea phenolic compounds (Lakenbrink et al., 2000), depends on the degree of fermentation during manufacture. Black tea contains more complex flavonoids than green tea. Although catechin content of black tea is lower (3-10%), but it has a higher content of theaflavins and thearubigins as a result of catechins enzymatic polymerization under substantial oxidation during processing (Unno and Hoshino, 2007). In addition, tea leaf also contains theanine, caffeine and other chemical components (Xu and Chen, 2002).

Tea flavonoids are brain permeable (Nakagawa and Miyazawa, 1997) and have been reported to possess potent cognitive protective effect through antioxidative and radical scavenging properties that can help to ameliorate neurodegenerative disorders such as Alzheimers and Parkinson diseases (Weinreb et al., 2004, Mandel and Youdim, 2004, Sutherland and Rahman, 2006).

In a previous study, clear deleterious effects of Na-F on the brain of rats were revealed as indicated by impaired cognitive abilities (El-lethey et al., 2010). Therefore, the present study was undertaken to evaluate the possible ameliorative effect of black tea, an important source of dietary antioxidants, against Na-F-induced alterations in the brain of rats with negative manifestation on learning and memory performance. For further investigation for the mechanisms of Na-F-induced cognitive disorders, and the potential protective effect of black tea on brain oxidative stress, the later parameter was also quantified in the current study.

2. Materials and methods

2.1. Animals and housing:

Since estrogen has been found to enhance memory in females, only male rats were employed in our study in order to eliminate estrogen related beneficial influence on memory (Wolf and Kirschbaum, 2002). Eighty weanling 32-days old Wistar male rats, approximately 45g weight were obtained from the Unit for Laboratory Animals at Faculty of Veterinary Medicine, Cairo University and used in our study. They were housed in standard polypropylene cages with stainless steel wire lids, bedded with wood shavings. Animals were maintained on a 12-h light/dark cycle at a room temperature of 20-22°c and 60% humidity with free access to feed (standard laboratory pellets) and water throughout the study. The procedures concerning animal care and experiment protocols were carried out in accordance with guidelines from Cairo University Policy on Animal Care and Use.

2.2. Experimental design:

All males were randomly distributed into four groups of 20 animals each, divided on 2 replicates and orally administered our treatments throughout the study till its completion at 106 days of age, in a 2 x 2 factorial design as follows:

Group (1) control (C), n=20: Weanling pups were administered plain water.

Group (2) Na-F group (F), n=20: Weanling pups were exposed to *ad libitum* supply of Na-F alone (Sigma Chemical Company) in drinking distilled water at 100 ppm on a mg/kg/day basis of 10.77 Na-F (Chioca et al., 2008).

Group (3) black tea group (T), n=20: Weanling pups were exposed to *ad libitum* supply of 2% black tea alone in drinking water (Trivedi et al., 2006). Twenty grams of black tea solids (Lipton Yellow label, Unilever Limited, India) and 1000 ml boiled drinking water were used to produce a 2% tea solution.

Group (4) ameliorated group (Na-F+T), n=20: Weanling pups were exposed to *ad libitum* supply of 100 ppm Na-F in combination with 2% black tea solution.

2.3. Behavioural assessment:

All behavioural testing were conducted by the same personnel throughout, started at 90 days and ended at 106 days of animals' age.

2.3.1. Open field test

The open field has been long established as an appropriate test for measuring situational anxiety in rodents (Millan, 2003). The process of habituation, a form of non-associative learning, was also measured in the open field test (Mello e Souza et al., 2000; Chioca et al., 2008). 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 treatment groups were tested at the same day in a random array. Rats were gently placed into a corner of the arena and allowed to confront this novel aversive situation (Belzung, 1999) for 3 minutes.

During the three minutes test duration, assessment of anxiety included measuring time spent freezing (immobility), exploratory behaviours in the form of ambulation (horizontal locomotion) and rearing (vertical activity) as well as non-exploratory measures comprising only vegetative behaviours in the form of number of faecal boluses (defecation) and number of times of urination (Kalueff et al., 2006). These parameters have all been labeled measures for anxiety (Ivinskis, 1970, Archer, 1973).

Ambulation (horizontal locomotion) is assessed in relation to lines drawn on the floor (the number of squares crossed). A crossed square was defined as the rat placing its two forepaws in the next square and moving forward (Chioca et al., 2008), whereas rearing 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 (Brown et al., 1999). Hand operated counters and stop watches were used to score the behaviour of animals.

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 between tests. 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.

2.3.2. Classic maze test

Associative learning was assessed using classic maze test. The base of the maze measured (100 x 100cm) 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 in a randomized order. 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 of the maze and numbers of entries of blind alleys (errors) were recorded according to Staddon (1983).

2.3.3. Spatial Y-maze memory

The test was conducted only once for all treatment groups randomly divided on two consecutive days, between 09:00 and 15:00 h. The Y-maze apparatus consisted of three arms (labeled A, B and C). Each arm was 40cm long, 30cm high, 15cm wide and positioned at an equal angle converged in an equilateral triangular central area with 15cm at its longest axis. The maze has no floor, but placed on a clean sheet of paper on a table-top, where the sheet has to be changed for each animal in order to prevent the use of odour cues in maze navigation. Each rat, was randomly placed at the end of one arm and allowed to move freely through the maze for eight minutes session. Entry was considered complete when the hind paws of the rat had completely entered the arm. The sequence of arm entries was recorded (i.e. ABCABABCACABACAB, etc.). A spontaneous alternation behaviour, a measure of spatial memory, was defined as consecutive entries into all three different arms without repetitions in overlapping triplet sets (Rasoolijazi et al., 2007). Percentage of alternation was calculated as the ratio of actual to all possible alternations (total number of arm entries minus two) multiplied by 100.

2.4. Biochemical and histopathological examination:

On completion of all behavioural assessments, five rats per treatment were sacrificed by cervical decapitation under ether anaesthesia for whole brain tissue extraction. Brain tissue specimens were dissected out carefully and cut into two sagital sections for execution of biochemical and histopathological examination.

2.4.1. Biochemical estimation

Brain tissue specimens were homogenized (10% w/v) in ice-cold 1.15% KCl-0.01M, sodium potassium phosphate buffer (pH 7.4) using a Teflon mechanical homogenizer. The homogenate was then centrifuged at 4000 rpm for 15 min at 4^{o}C and the supernatant was used for the studied enzymatic assay.

2.4.1.1. Determination of acetylcholinestrase (AChE) activity

AChE activity (µmoles/min/mg protein) was estimated in the brain by an improved Ellman's colrimetric method, employing acetylcholine iodide as a substrate for the reaction (Darreh-Shori et al., 2002).

2.4.1.2. Determination of super oxide dismutase (SOD) activity

SOD activity (µmoles/min/mg protein) was measured according to Giannopolitis and Ries (1977), by means of SOD assay kit (Cayman, MI, USA). The kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to produce 50% dismutation of superoxide radical. The SOD assay measures all three types of SOD; Cu/Zn, Mn, and Fe SOD. Enzyme activity was determined as the amount of the enzyme required to induce 50% inhibition of nitro-blue tetrazolium (NBT) reduction rate.

2.4.1.3. Determination of thiobarbituric acid reactive substance (TBARS) formation

The level of lipid peroxidation, in terms of TBARS formation (nmoles/min/mg protein) was determined (Esterbauer and Cheeseman, 1990). Tissue supernatant was mixed with 1ml of 20% trichloroacetic acid (TCA), 2ml of 0.67% thiobarbituric acid (TBA) and then heated at 100°C for 1h. After cooling, the precipitate was removed by centrifugation. The absorbance of the sample was measured at 535nm using a blank containing all the reagents except the sample. Since, 99% of TBARS was malondialdehyde (MDA), so TBARS concentrations of the samples were calculated using the extinction co-efficient of MDA, which is $1.56\times105\ M^{-1}cm^{-1.}$

2.4.1.4. Estimation of total protein

Total protein content (mg/100 mg fresh tissue weight) was identified in brain tissue homogenate as described by Lowry et al. (1951).

2.4.1.5. Estimation of lipids

Lipids were extracted from brain tissues using a mixture of (2:1 v/v) chloroform-methanol (Folch et al., 1957) and the contents were expressed as mg/100mg tissue.

Total cholesterol was estimated by the method of Zlatkis et al. (1953), where 9.9ml of ferric chloride-acetic acid reagent was added to 0.1ml of lipid extract, allowed to stand for 15 min, centrifuged and the supernatant fluid was collected. 3ml of conc. H_2SO_4 were then added to 5ml of the collected supernatant fluid. The colour developed was red after 20 min at 560nm against a reagent blank and values were expressed as mg/100mg tissue.

Triglycerides were estimated by the method of Foster and Dunn (1973). An aliquot of lipid extract was evaporated till dryness in glass tubes, 0.1ml methanol was then added followed by 4ml isopropranol and 0.4g alumina and centrifuged at 3000rpm for 15 min. 2ml of the supernatant fluid was transferred to labeled tubes to which 0.6ml of a saponification reagent was added, and then placed in a water bath at 65°c for 15 min for saponificatioin. 0.5ml of acetyl acetone reagent was added, mixed and the tubes were kept in a water bath at 65°c for 1h. The contents were then cooled and the absorbance was red at 420nm. The triglyceride content was expressed as mg/100mg tissue.

Low-Density Lipoprotein-Cholesterol (LDL-c) was calculated using Friedewald formula (Friedewald et al., 1972), based on the assumption that LDL-c is present in a concentration equal to one-fifth of the triglycerides. Values are expressed as mg/100mg tissue.

2.4.2. Neurohistopathology

The dissected brain tissue specimens were fixed in 10% neutral buffer formalin, processed by paraffin embedding method, sectioned at 4-5µm and stained with Hematoxylin and Eosin stain (Bancroft et al., 1996). Stained sections were fixed on slides, and lesions were then confirmed by microscopic examination.

2.5. Statistical analysis

Data analysis for all variables were carried out by means of analyses of variance (ANOVA) to judge the effect of administration of Na-F, black tea to rats as well as session factor for behavioural tests using the general linear models procedure in SPSS® statistical software (SPSS, 2006). After confirmation of significant effects in the overall ANOVA, data for different groups were compared using post hoc Tukey HSD test. For all tests, the criterion for statistical significance was $p<0.05$. Results are reported as mean ± SEM.

3. Results

3.1. Open field test:

Anxiety state of the animals was assessed on first occurrence in the open field test as shown in Table 1. Administration of Na-F to rats produced an anxiogenic profile of behavioural changes as indicated by increased time spent freezing ($F_{(1, 36)} = 19.35$; $p = 0.00$), higher vertical activity (numbers of rearing; ($F_{(1, 36)} = 36.16$; $p = 0.00$) as well as elevated defecation scores ($F_{(1, 36)} = 21.92$; $p = 0.00$). Also,

rats administered black tea solution alone displayed higher levels of anxiety-related behaviours than others in the control group. However, the level of enhancement noted for anxiety measurements was significantly different in individuals of Na-F group compared to counterparts in T group. On the other hand, our treatments had no significant influence on horizontal activity (numbers of crossed squares) as well as urination scores. Furthermore, administration of black tea along with Na-F significantly reduced levels of Na-F-induced anxiety. Similar levels of anxiety were recorded for rats in the ameliorated group and those in T group.

A significant degree of formation of memory of habituation over three sessions in the open field test was noted regarding time spent freezing ($F_{(2, 117)} = 11.61$; $p = 0.00$), horizontal activities (numbers of squares, ($F_{(2, 117)} = 27.16$; $p = 0.00$), vertical activity (numbers of rearing, ($F_{(2, 117)} = 11.34$; $p = 0.00$) and defecation scores ($F_{(2, 135)} = 9.57$; $p = 0.00$) for all treatments but not for Na-F group. In the later group, an equivalent assessment were significantly noted in the first day of induction compared to second and third test sessions ($p = 0.91$ and 0.08), ($p = 0.78$ and 0.09), ($p = 0.66$ and 0.16), ($p = 0.62$ and 0.27) for all previous parameters, respectively.

3.2. Maze test:

The ability of learning and memory of animals over the five days of maze test was presented in Table 2. Administration of Na-F to rats significantly increased both latency to locate the feed at the end of the maze ($F_{(1, 180)} = 122.87$; $p = 0.00$) and frequencies to enter the blind alleys ($F_{(1, 180)} = 127.82$; $p = 0.00$) compared to control group. In contrast, learning and memory were superior in group of rats exposed to black tea solution compared to the control group, as shown by reduced latency to end the maze ($F_{(1, 180)} = 62.83$; $p = 0.00$) accompanied with less numbers of errors (numbers of entries for blind alleys, ($F_{(1, 180)} = 90.19$; $p = 0.00$). Moreover, Administration of black tea to Na-F-treated rats significantly improved the parameters studied to the level of control group.

On testing the retention of the task over five days of maze test, rats in all treatments required steadily less time to terminate the maze ($F_{(4, 180)} = 16.46$; $p = 0.00$) with reduced numbers of errors ($F_{(4, 180)} = 21.01$; $p = 0.00$), except for Na-F-treated rats. Concerning Na-F group, values for latency as well as numbers of errors weren't significantly different during acquisition on first day when compared to their retention values on the four consecutive days of testing ($p = 1.00$, 1.00, 1.00 and 0.97), ($p = 1.00$, 0.61, 0.33, and 0.33), respectively, indicating poorer memory retention in Na-F treated rats compared to all other treatments.

3.3. Spatial Y-maze memory:

Spatial Y-maze performance was illustrated in Table 3. Spatial memory on the Y-maze was seriously impaired in rats administered Na-F as indicated by reduced percentages of spontaneous alternation behaviour in comparison with rats in all other treatments ($F_{(1, 36)} = 49.31$; $p = 0.00$). On the other hand, rats supplemented with black tea solution significantly improved spatial cognition learning ability than controls ($F_{(1, 36)} = 23.08$; $p = 0.00$). Furthermore, administration of black tea significantly alleviated Na-F-induced spatial memory impairment, where rats in the ameliorated group exhibited as better performance in the Y-maze test as their counterparts in the control group.

3.4. Biochemical analysis:

3.4.1. Brain acetylcholinesterase (AChE) activity:

The activity of AChE in the brain of rats was demonstrated in Table 4. Na-F-treated rats have shown a significant reduction in AChE activity compared to their counterparts in the control group. Administration of black tea has no significant influence on AChE level in the fluoride group. No significant differences were detected between rats in tea group and those in the control one.

3.4.2. Oxidative stress parameters:

SOD activity, TBARs formation as well as total protein contents in the brain tissues of rats were presented in table 5. SOD activity was significantly lowered in Na-F-exposed rats compared to Na-F-free rats. Significant alleviation of this reduction was recorded when black tea was concomitantly administered. Black tea alone significantly enhanced SOD activity in rats compared to the control group.

The Level of TBARs formation was significantly higher in case of Na-F-treated rats. This elevation was significantly diminished by administration of black tea. Compared to control rats, treatment with black tea alone had no significant influence on brain tissue TBARs level .

Total protein content was significantly decreased in brain tissues of rats administered Na-F when compared to those in the control group. The later reduction was significantly improved when black tea was concomitantly administered. The observed increase in total protein contents in black tea-treated rats, compared to control one, was not statistically significant.

3.4.3. Lipid profile:

Total cholesterol (TC), triglycerides (TG) and low density lipoproteins cholesterol (LDL-c) were significantly increased when Na-F was administered to rats (Table 6). Administration of black tea significantly lightened this noted increase in Na-F-exposed rats. Rats administered black tea alone have revealed a significant decrease in lipid profile parameters when compared to their counterparts in the control group.

3.5. Histopathological examination:

No histopathological changes could be detected in the brain of rats in both of the control and tea-treated groups.

Examination of fluoride-treated rats revealed severe pathological alterations as evidenced by congestion of the meningeal, cerebral and cerebellum blood capillaries, in addition to congestion of choroid plexus in the ventricle (Fig. 1). Large hemorrhagic areas were also detected in the cerebral cortex, cerebellum white matter as well as in the ventricles around choroid plexuses (Fig. 2). Neurodegenerative changes were noticed in nerve cells especially at hippocampus and large nerve cells of cerebral cortex, as represented by the accumulation of neurofilaments in the cytoplasm of nerve cells and axons (Fig. 3).

In addition, nerve cells of cerebral cortex revealed sever edema, central chermatolysis, atrophy, necrosis and neuronophagia (Fig. 4), whereas the pyramidal cells of Ammon's horn of hippocampus showed atrophy and necrosis (Fig. 5). Either focal or diffused Gliosis were noticed in the cerebral cortex with severe demylination of the nerve fibers in the neuropil shown in the cerebrum, and accompanied with axonal swelling (Fig. 6). Glial fibers was detected under the ependymal cells lined the ventricles. The cerebellum showed necrosis of Purkinje cells and edema with necrosis in the granular cell layer.

Concomitant administration of tea to fluoride-intoxicated rats resulted in mild to moderate pathological changes compared to their counterparts exposed to fluoride alone. Small hemorrhagic areas were noticed in the ameliorated group, especially in the ventricle around the choroid plexuses (Fig. 7). Only edema was detected in the large nerve cells of the cerebral cortex (Fig. 8). Few numbers of large pyramidal cells of hippocampus appeared slightly atrophied (Fig. 9). Focal glial cells and fibers were detected in few numbers of the ameliorated examined cases (Fig. 10).

Table 1. Effect of Na-F and its amelioration by black tea on anxiety measurements on first occurrence in open field test in rats.

	Experimental Groups			
	(C) Group	**(Na-F) Group**	**(T) Group**	**(Na-F+T) Group**
Freezing time(s)	**2.00±0.37[a]**	**7.30±3.82[b]**	**4.50±3.09[c]**	**4.70±3.09[c]**
Horizontal activity	**50.3±3.93[a]**	**55.8±1.26[a]**	**46.9±4.44[a]**	**51.8±3.14[a]**
Vertical activity	**6.80±7.36[a]**	**15.80±3.82[b]**	**10.20±3.09[c]**	**11.60±3.09[c]**
Defecation scores	**0.50±7.36[a]**	**5.40±3.82[b]**	**2.70±3.09[c]**	**3.00±3.09[c]**
Urination scores	**0.2±0.13[a]**	**0.6±0.22[a]**	**0.4±0.16[a]**	**0.5±0.22[a]**

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F.
(T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 10 animals per treatment.

Table 2. Effect of Na-F and its amelioration by black tea on measurements of maze test over the course of five days in rats.

	Experimental Groups			
	(C) Group	**(Na-F) Group**	**(T) Group**	**(Na-F+T) Group**
Latency (s)	**87.48±21.25[a]**	**215.38±7.91[b]**	**49.1±18.79[c]**	**115.1±24.05[a]**
No. of entries of blind alleys	**2.32±0.56[a]**	**6.38±0.44[b]**	**1.32±0.52[c]**	**2.76±0.74[a]**

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F.
(T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 50 animals per treatment.

Table 3. Effect of Na-F and its amelioration by black tea on spatial Y-maze memory in rats.

	Experimental Groups			
	(C) Group	**(Na-F) Group**	**(T) Group**	**(Na-F+T) Group**
Spontaneous alternation behaviour (%)	**71.08±4.07[a]**	**23.10±9.22[b]**	**95.00±4.48[c]**	**57.59±5.13[a]**

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F.
(T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 10 animals per treatment.

Table 4. Effect of Na-F and its amelioration by black tea on the level of AChE activity in the brain of rats.

	Experimental Groups			
	(C) Group	**(Na-F) Group**	**(T) Group**	**(Na-F+T) Group**
AchE (µmoles/min/mg protein)	**0.18±0.01[a]**	**0.10±0.01[b]**	**0.18±0.01[a]**	**0.11±0.01[b]**

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F. (T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 5 animals per treatment.

Table 5. Effect of Na-F and its amelioration by black tea on various parameters of oxidative stress in the brain of rats.

	Experimental Groups			
	(C) Group	**(Na-F) Group**	**(T) Group**	**(Na-F+T) Group**
SOD (µmoles/min/mg protein)	**0.07±0.01[a]**	**0.02±0.00[b]**	**0.09±0.01[c]**	**0.04±0.01[d]**
TBARs (nmoles/min/mg protein)	**0.10±0.01[a]**	**0.18±0.01[b]**	**0.10±0.01[a]**	**0.15±0.01[c]**
Total protein (mg%)	**71.20±6.31[a]**	**43.15±4.68[b]**	**73.28±7.00[a]**	**56.28±4.27[c]**

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F.
(T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 5 animals per treatment.

Table 6. Effect of Na-F and its amelioration by black tea on lipid profile in the brain of rats.

	Experimental Groups			
	(C) Group	(Na-F) Group	(T) Group	(Na-F+T) Group
TC (mg/100mg tissue)	1038.59±59.25[a]	1582.47±70.14[b]	823.18±55.47[c]	1283.55±67.22[d]
TG (mg/100mg tissue)	293.18±13.94[a]	474.37±15.22[b]	242.08±12.83[c]	315.92±15.47[a]
LDL-c (mg/100mg tissue)	58.64 ±2.31[a]	74.88±3.64[b]	48.42±2.56[c]	63.18±2.32[a]

(C) Group: Animals received plain water without any treatment and served as a control.
(Na-F) Group: Animals received 100 ppm Na-F.
(T) Group: Animals received 1% black tea solution alone.
(Na-F+T) Group: Animals received 100 ppm Na-F + 1% black tea solution.
[a-c]Values within row with unlike superscripts differ significantly **($p<0.05$)**, according to ANOVA.
Values represent mean±SEM of 5 animals per treatment.

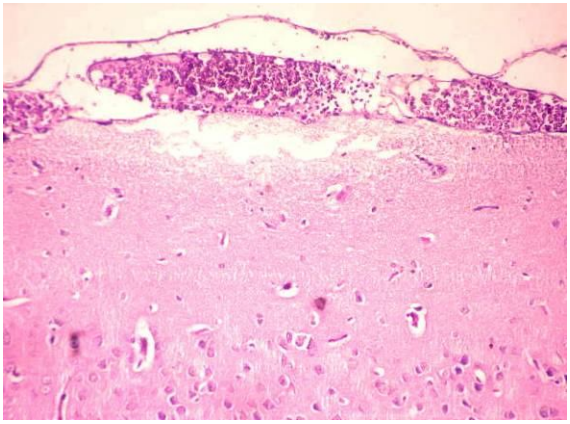

Figure 1: Brain of fluoride-treated rats showing congestion of meningeal blood vessels. H&E X 200.

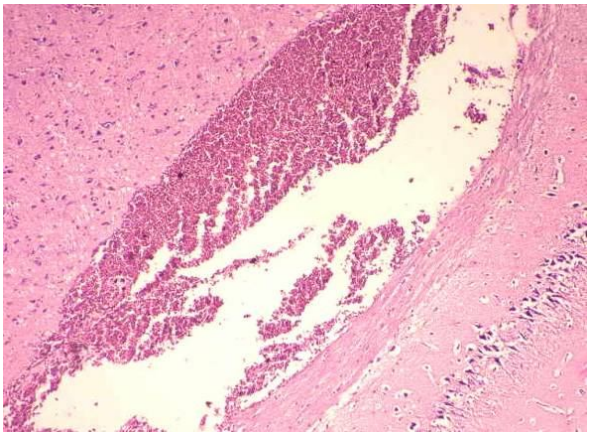

Figure 2: Brain of fluoride-treated rats showing large area of hemorrhage. H&E X 200.

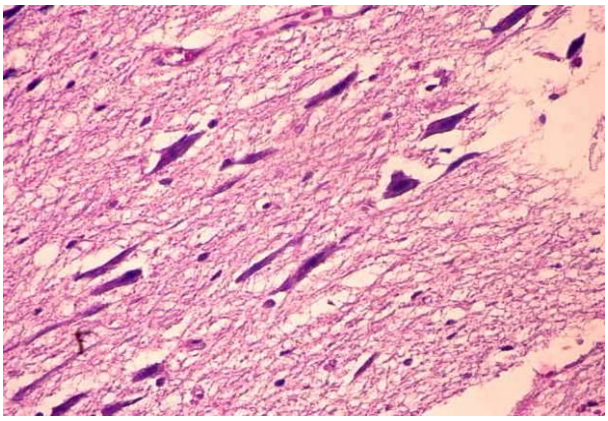

Figure 3: Brain of fluoride-treated rats showing neurofilaments accumulation in the cytoplasm of nerve cells and axons. H&E X 200

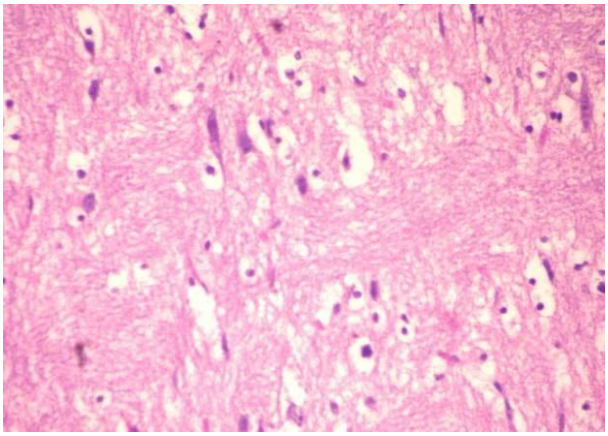

Figure 4: Brain of fluoride-treated rats showing cellular edema, atrophy and necrosis. H&E X 400.

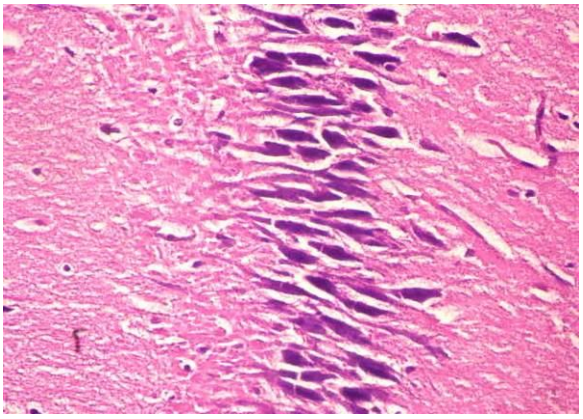

Figure 5: Brain of fluoride-treated rats showing atrophy and necrosis of pyramidal cells of Ammon's horn of hippocampus. H&E X 200.

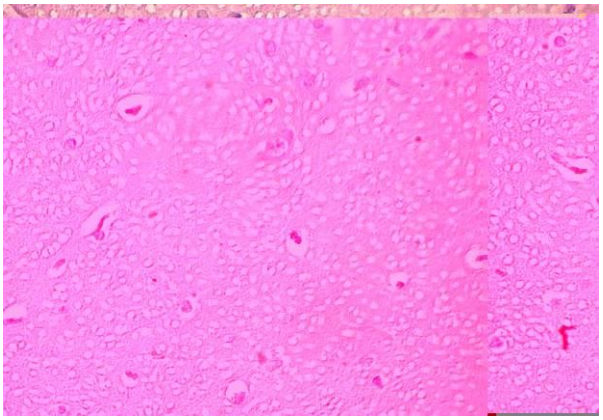

Figure 6: Brain of fluoride-treated rats showing demyelination of nerve fibers. H&E X 100.

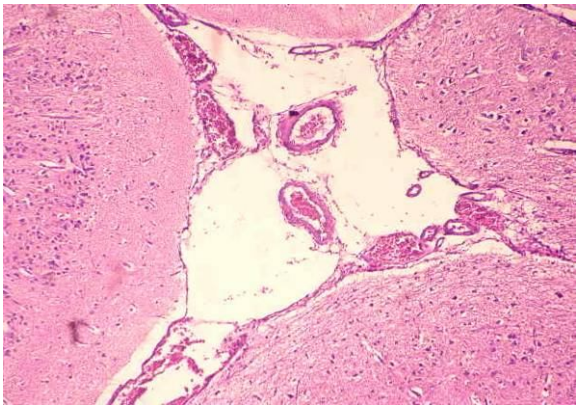

Figure 7: Brain of fluoride-treated rats concomitantly administered black tea showing few numbers of free RBC's around chroid plexuses. H&E X 200.

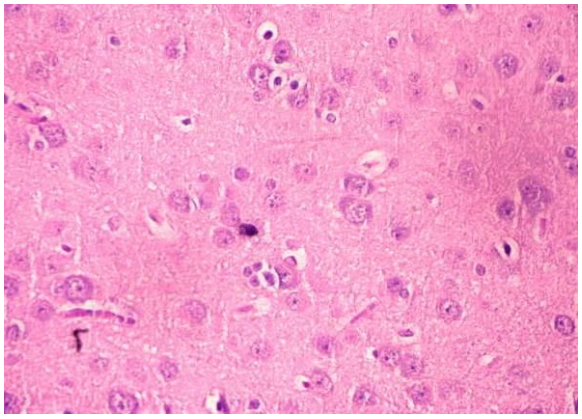

Figure 8: Brain of fluoride-treated rats concomitantly administered black tea showing edema in few numbers of large nerve cells of cerebrum. H&E X 400.

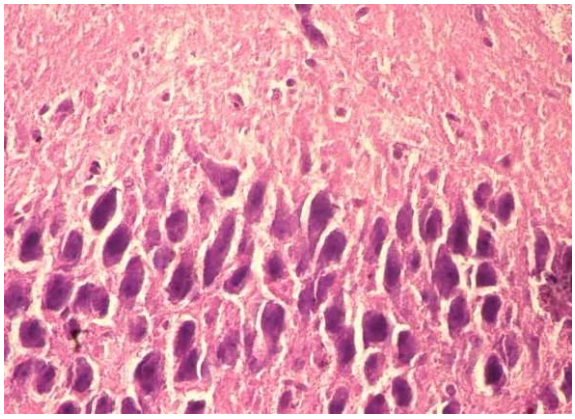

Figure 9: Brain of fluoride-treated rats concomitantly administered black tea showing atrophy of few numbers of pyramidal cells of hippocampus. H&E X 200.

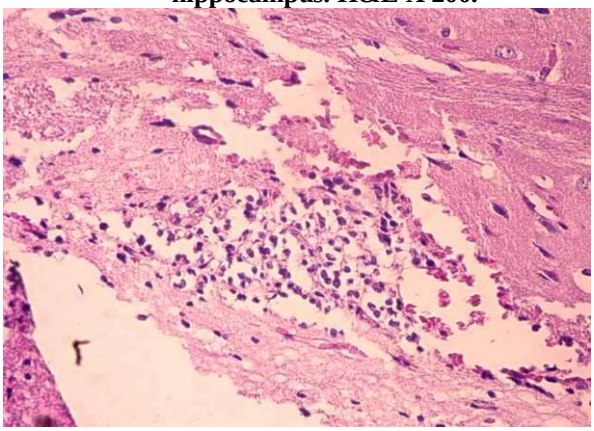

Figure 10: Brain of fluoride-treated rats concomitantly administered black tea showing glial cells and glial fibers in the cerebral cortex. H&E X 200.

4. Discussion:

For assessment of emotionality, the open field is one of the simplest and most popular tests currently in use (Weiss and Greenberg, 1996; Brain & Marrow; 1999; Crawley, 1999). The test is employed to assess anxiety-related conflict arising between the drive to explore by venturing into the center of the arena and safety by remaining in a corner or along a wall (Weisstaub et al., 2006).

To the best of our knowledge, few researches have implemented open field test to investigate Na-F influence on anxiety levels in rats. In compliance with our earlier study, rats exposed to Na-F spent more time freezing on the first exposure to open field test (El-lethey et al., 2010). Increased immobility in the open field is characteristic for increased levels of anxiety (Homberg et al., 2002; Fromm et al., 2004; Kalueff & Tuohimaa, 2004). Unlike horizontal locomotion, higher levels of vertical activity were detected in Na-F-treated rats. This finding supports the fact that vertical exploration is more sensitive to anxiety than horizontal locomtion (Lapin et al., 1995). Although the locality of the activities were not recorded here, the enhancement in vertical activity at first exposure might be on account of novelty-provoked more fear-related behaviour in rats such as activity in the corners and walls of the open field (Choleris et al., 2001). Moreover, rearing at the perimeter of the enclosure has been evidenced to indicate anxiety and an animal's attempts to escape; whereas rearing in the inner arena may be more indicative for curiosity, namely exploration of the upper parts of the apparatus (Anderson & Hughes, 2008). Although urination scores were not influenced by Na-F, our results revealed a profound increase in faecal boluses deposited in the field. These data were in agreement with Ivinskis (1968) who suggested that defecation is a more reliable measure than urination to assess emotionality in the open field. Also, Brain & Marrow (1999) have confirmed defection to be a satisfactory measure for anxiety, with increased defecation indicating higher anxiety. Taken together, these reports and the results presented here suggest a Na-F-induced increase of anxiety-like behaviour.

Where the decrement in exploratory response to successive exposure to a novel environment is taken as an index for memory of habituation, a non-associative learning task (Bolivar et al. 2000**;** Izquierdo et al., 2001; Winogard & Viola, 2004), an impairment in habituation was shown in Na-F-treated rats. Consistent with our finding, a decrement in habituation signifying learning and memory impairment was previously shown in rats exposed to 100 ppm Na-F, the identical concentration employed in the current study (Chioca et al., 2008; Pereira et al., 2009; El-lethey et al., 2010).

Tea consumption in many cases is the main source of caffeine (Haider et al., 1998). Caffeine content in black tea is greater than that in a green one, 3.86% versus 2.04%, respectively (Komes et al., 2009). The anxiogenic effect of caffeine has been documented in a substantial literature (Brice & Smith, 2002; Botella & Parra, 2003). Caffeine has been reported to elicit a dose-dependent, subjective feeling of anxiety, even at low doses (Kaplan et al., 1997). The stimulation of anxiety in response to caffeine may be as a result of increased levels of lactate in the brain (Tancer et al., 1991, 1994). Here, the enhancing effect of tea on rearing activity might be explainable in terms of caffeine-induced anxiety in a novel environment. In agreement with our findings, increased activity of animals was experienced after administration of low or moderate doses of caffeine (Buckholtz and Middaugh, 1987). Moreover, Haleem et al. (1994) have observed an increase in both home cage and open field activity in rats following low doses of caffeine. Enhancement of activity was not entirely due to caffeine per se, where presence of theophylline, another alkaloid present in tea, might be also attributable for promoting locomotor activity (Haider et al., 1998). In agreement with our data, no change on numbers of squares crossed in the open field was reported after four weeks of tea administration in rats (Haider et al., 1998). The stress effect of novelty on exposure to open field could suppress locomotor enhancing effects of stimulants present in tea, resulting in lack of effect on horizontal locomotion in our study. In addition, partial tolerance to the locomotor-enhancing effects of caffeine has been evidenced to develop following administration of high doses of caffeine for about a week resulting in lack of effect on locomotion (Haleem et al. (1994). Administration of tea to Na-F-treated rats brought about recovery in anxiety parameters almost to tea administration level alone, suggesting the potential ameliorative effect of tea on Na-F-induced symptoms of anxiety.

Although associative processes have been evidenced to play a real role in spatial learning, almost no studies have employed classic maze to address spatial memory (Leising, 2009). The animal must build a cohesive spatial representation of the maze to end with the food. On repeating the maze experiment several times, the changes in latency, and errors made to reach the food are indicators for learning and memory abilities of the rat. In the present study, the defective ability of Na-F-treated rats to navigate the maze for food reinforcement with higher errors on successive days of testing signifying inferior memory retention compared with other

treatments. Consistent with these findings, impairment in the learning capacity during classic maze was formerly experienced in Na-F-exposed rats (El-lethey et al., 2010). Bhatnagar et al. (2002) and Gao et al. (2009) have also reported poor performance of fluoride-intoxicated animals during maze test with increased inability to perform well with higher fluoride concentration in drinking water.

The alternation behavior observed during spatial Y-maze memory testing has been motivated by the detection of novelty, where animals are attracted to a stimulus that is novel relative to previous stimuli experienced, being a sign for the degree of learning ability in rats (Gaffan and Davies, 1982). Confirming the results of classic maze, more evidences for impaired cognitive capacity were derived from the data for Y-maze test, where Na-F provoked a remarkable hindrance in spatial memory of rats as indicated by a notable decrease in spontaneous alteration behaviour. Our findings are in accordance with previous reports from Niu et al. (2008) that have demonstrated a spatial memory deficit in rats during Y-maze after Na-F exposure.

Consumption of flavonoid-rich foods, such as tea that makes a significant contribution to dietary intake of flavonoids by 82% (Hertog et al., 1997), throughout life holds a potential to limit the neurodegeneration associated with a variety of neurological disorders and to prevent or reverse normal or abnormal deteriorations in cognitive performance. Interestingly, there is evidence to suggest that tea polyphenols can localize within the brain following dietary consumption and thus be available to promote actions resulting in cognitive improvements (Matsuoka et al., 1995; Suganuma et al., 1998). Our results are in line with these earlier reports, where administration of black tea has greatly improved cognitive performance during the two tasks applied here for evaluating spatial memory. Also, the observed attenuation in cognitive capacity in Na-F-exposed rats has been blunted to normal level when black tea was concomitantly administered. Accordingly, the potential cognitive protective effect of tea has been reported in various experimental animal models (Kim et al., 2004; Unno et al., 2004). Long-term administration of green tea catechins has been reported to improve the performance in radial maze tasks (Haque et al., 2006). Likewise, the effectiveness of tea extract in enhancing learning and memory, and hence, reversing age-related deficits in aged rats has been proved, as signified in performance in elevated maze and passive avoidance tests (Kaur et al., 2008).

The neuroprotective effect of dietary flavonoids involves a number of actions within the brain, including a potential to protect neurons against neurotoxins-induced injury, an ability to suppress neuroinflamation, and to promote memory, learning and cognitive function (Spencer, 2009). This multiplicity of effects appears to be attained by two processes. Firstly, flavonoids interact with important neuronal signaling cascades leading to an inhibition of neurotoxins-triggered apoptosis, as well as a promotion of neuronal survival and differentiation. Secondly, they induce peripheral and cerebral vascular blood flow in a manner leading to induction of angiogenesis, and new nerve cell growth in the hippocampus.

Complex mechanisms are underlying the neuroprotective effect of tea.

As regards tea catechins, possible mechanisms might involve their antioxidant and iron-chelating properties, as well as modulation of cell-signaling and cell survival pathways (Mandel and Youdim, 2004; Weinreb et al., 2004). As for black tea, since catechins undergo enzymatic transformation during fermentation, their amount is generally lower than in green tea (Luczaj and Skrzydlewska, 2005; Unno and Hoshino, 2007). Nonetheless, the conversion of catechins to theaflavins and thearubigins during fermentation does not significantly alter their free radical-scavenging activity, and both black tea and its components possess strong antioxidative properties (Leung et al., 2001; Henning et al., 2004)**.** In addition to tea polyphenols, theanine, an amino acid uniquely found in tea leaf, might also possess neuroprotective effect and enhance the positive cognitive effects (Nathan et al., 2006; Haskell et al., 2008)**.** Because tea leaf contains various other phytochemicals, including vitamin C, it is likely that the cognitive protective effect of tea is not due to a single compound but rather to the synergistic effect of many of its chemical components (Tze-Pin et al., 2008).

Acetylcholine is an important neurotransmitter present at cholinergic nerve terminal and plays a key role in cholinergic neurotransmission (Garcia-Ayllon et al., 2006). A tight correlation has been established between acetylcholinestrase (AChE) activity and cognition. Estimation of AChE, a substrate specific enzyme degrading neurotransmitter acetylcholine in nerve synapses, provides an easy and valuable assessment of cholinergic function. 90% of AChE in the brain is membrane bound and only 10% is in a soluble form (Atack et al., 1986; Mortensen et al., 1998). Since, estimation of the soluble form of AChE is a relatively simple and sufficiently reliable indicator of relative changes of AChE activity in the brain (Muller et al., 1985; Zakut et al., 1985), quantifying the soluble form of this enzyme was only considered in our study.

Here, the marked reduction in AChE activity experienced in brain tissue of Na-F-treated rats is in

agreement with previous findings in rats (Wang et al., 2004; Wu et al., 2006). Further studies have reported a significant decline in brain AChE activity after excessive intake of Na-F in mice (Vani and Reddy, 2000; Bhatnagar et al., 2006).

The observed inhibition of AChE might be attributable to the loss of neuron cell bodies in the hippocampus as well as loss of synaptic structures (Bhatnagar et al., 2003; Ge et al., 2005). Anion hydrolysis products of methyl phosphoric difluoride were also implicated to cause an inhibition of AChE in both rats and guinea pigs (Dahl et al., 1987). Toxic inhibition of the AChE will cause high concentration of acetylcholine to accumulate in the body, whereas excessive induction of the acetylcholine will lead to down-regulatory effects through more hydrolysis of the acetylcholine into acetate and choline in order to reduce the concentration of acetylcholine in the body and counteract excess acetylcholinergic activity (Teh et al., 2010). Therefore, the diminution in AChE level may lead to altered utilization of acetylcholine, thus interfering with the synaptic transmission in brain, being accountable for cognitive dysfunctions observed in our study (Wang et al., 2004).

Contrary to our findings, an elevated level of AChE activity was detected with severe brain tissue damage accompanied by high fluoride intake in rats and mice (Chen and Bai 1995; Sun et al., 2000). This contradiction in Na-F effect on AChE activity might be due to the exposed level of Na-F, where the inhibited activity of AChE activity is mostly induced with moderate fluorosis, whereas severe fluorosis may exert a stimulatory effect on the activity of AChE (Gao et al., 2009).

There are numerous reports about inhibitory effect of antioxidants, namely polyphenols, on AChE acivity. Kim et al. (2004) showed that tea polyphenols exhibit a dramatic inhibitory effect on AChE activity and might be useful in the treatment of Alzheimer's disease (AD). Additionally, Kulisic-Bilusic et al. (2008) reported about high inhibitory activity of aqueous tea infusions on AChE activity. In contrast to these earlier findings, there was a lack of influence of black tea on brain tissue AChE activity in our study. This discrepancy might be on account of different polyphenols production as a result of different degrees of fermentation during tea manufacturing as well as variation in place of origin.

The brain and nervous system are prone to oxidative stress, and also inadequately equipped with an antioxidant defense system to prevent ongoing oxidative damage (Halliwell, 2006). In addition, the brain consumes large quantities of oxygen that contributes to the formation of reactive oxygen species (ROS). In the current study, rats exposed to Na-F showed a significant reduction in the activity of antioxidant enzyme (SOD) and a concomitant enhancement in lipid peroxidation (TBARS), in accordance with earlier reports in brain tissues of fluoridated rats and mice (Mullenix et al., 1995; Vani and Reddy, 2000; Shivarajashankara et al., 2001; Bhatnagar et al., 2006; Chirumari and Reddy, 2007; Bharti and Srivastava, 2009). These detected alterations suggest Na-F-induced-oxidative stress in the brain thereby disturbing the antioxidant defense, in compliance with earlier findings (Chlubek, 2003; Inkielewicz and Krechniak, 2004). Increased oxidative stress could therefore be one of the mediating factors in the pathogenesis of Na-F-induced neurotoxicity experienced in the present work.

Tea polyphenols can pass through the brain-blood barrier to exert antioxidant activity and potent neuroprotective effects (Nie et al., 2002). One of the possible mechanisms may be their directly scavenging ROS produced either outside or inside the cell or both. Earlier data have shown that tea polyphenols can scavenge different kinds of ROS and organic free radicals, for example, superoxide anion, hydroxyl radical, singlet oxygen, and lipid free radicals (Zhao et al., 1989; Guo et al., 1996; Guo et al., 1999). Here, the elevated level of free radical enzyme (SOD) with lowered level of lipid peroxidation when black tea was administered to Na-F treated rats suggests the role of black tea in amelioration of Na-F-induced oxidative stress. This ameliorative effect of black tea may be due to the presence of monomeric catechins that affect plasma antioxidant biomarkers and energy metabolism (Williamson and Manach, 2005; Trivedi et al., 2006; Verma et al., 2006). Moreover, it has been reported that quercetin, a unique flavanol present in black tea extract, can reduce free radicals (Pietri et al., 1997). Polyphenols are also well known for their ability to reduce membrane lipid peroxidation that can prevent Na-F-induced-oxidative damage. Further support derived from former studies, where oral administration of a flavonoid-rich tea extract prevented iron-salt-induced lipid peroxide accumulation and suppressed age-related accumulation of neurotoxic lipid peroxides in rat brain (Inanami et al., 1995; Yoneda et al., 1995).

Brain protein is important to maintain normal brain physiological function and learning-memory ability (Liu et al., 1999). In the present study, protein contents have been shown to be reduced in the brains of rats subsequent to fluoride exposure. Decreased protein contents have been formerly reported in the brain of rats treated with Na-F (Wang et al., 2004; Wu et al., 2006). The Na-F-induced reduction in brain protein contents might be as a result of either increased proteolysis or decreased

rate of cellular protein synthesis through impairment of peptide chain initiation (Trivedi et al., 2007). Moreover, fluoride has been evidenced to inhibit oxidative decarboxylation of branched chain amino acids and simultaneously promote protein breakdown. Reduced activities of glutamine synthetase that catalyzing certain stages of amino acid biosynthesis as well as methionine activating enzymes of the liver have been also implicated in fluorosis-induced disturbance in protein synthesizing system (Zahvaronkov and Strochkova, 1981).

Brain triglycerides levels as well as cholesterol content were highly elevated in rats exposed to Na-F in the present study. In a study on rabbits, fluoride induced alterations in brain lipid metabolism similar to lipidosis, a disorder of lipid metabolism leading to abnormal fat accumulation in the body tissues, particularly in the liver and brain (Shashi, 1992). Fluoride intoxication-induced hyperlipidemia might occur due to enzymatic defect and inability of brain to degrade the lipid in the body. Furthermore, as lipids are transported in association with a carrier protein, a defect in lipoprotein metabolism as evidenced by reduced LDL-c in the current study might be on account for hyperlipidemia. Deficiency of a lipotropic agent has been evidenced to cause triglycerides to accumulate, leading to a decrease in free fatty acids synthesis during fluoride intoxication (Shashi, 1988; Shashi et al., 1989). Further evidence derived from a study for Trivedi et al. (2009) where accumulation of cholesterol and total lipids was reported in Na-F administered mice. Here, the observed hypercholesterolemia may be due to Na-F-induced deficiency of liposomal lipase which hydrolyzes cholesterol esters taken up by the cell, reducing the release of free fatty acids and glycerol as well as enhancing lipogenesis. (Shashi, 1992). The hypercholesterolemia and hypertriglyceridemia have been reported in previous studies to indicate excessive fat immobilization (Vatassery et al., 1980; Shashi et al., 1989). In our study, the alteration in lipid metabolism might also be due to increase in lipid peroxidation activity (Shivarajashankara et al., 2001).

In line with findings of Trivedi et al. (2009), amelioration of fluoride-induced rise in brain triglycerides and cholesterol contents by administration of black tea was recorded in our study. Black tea has been evidenced to inhibit the synthesis of cholesterol and decrease its concentration in the brain (Maron et al., 2003). Since the antilipogenic effect of black tea might be due to its radical scavenging activity, the alleviation of Na-F-induced alteration in brain by black tea might be due to its potent antioxidative properties (Katiyar and Mukhtar, 1997; Du Toit et al., 2001).

Given the crucial involvement of hippocampal activity in many aspects of learning and memory (Strack et al., 2000; Leussis & Bolivar, 2006), and since fluoride is known to accumulate in various parts of rat brain, especially in the hippocampus (Burgstahler and Colquhoun, 1996), detection of neurotoxic alterations in the hippocampus was monitored in the present study. Here, the demonstrated histopathological changes triggered by fluoride intoxication in the brain, especially hippocampus and cerebrum regions, are attributed to neurotoxic effect of fluoride. Previous studies have evidenced that accumulation of fluoride in the hippocampus generates neuronal dysfunction by mechanisms involving decreased aerobic metabolism with elevated levels of free radicals (Spittle, 1998; Chirumari and Reddy, 2007). Fluoride binds to antioxidants in the body such as N-acetyl cysteine and glutathione and other free-radical destroying enzymes, thereby disturbing the antioxidant defense, triggering oxidative stress that initiates nerve cell damage especially cell membrane and even cell apoptosis (Anuradha et al., 2001; Gao et al., 2009).

In the current study, presence of neurofilament in the nerve cells might be attributable to the interference of fluoride with various steps of protein synthesis inside nerve cells (Miu et al., 2003). Fluoride may also accumulate in both neurons and astrocytes, resulting in strong morphological changes such as clustering, degeneration and finally death (Mullenix et al., 1995).

In our study, the ameliorative effect of black tea on Na-F-induced histopathological alterations in the brain of rats could be explained on the basis of flavanoids-exhibited antioxidant activity via radical scavenging capacity (Trivedi et al., 2007).

In conclusion, our study demonstrated a potential effect of black tea drinking in protection against Na-F intoxication-induced cognitive decline in rats. Since, black tea is cheap, non toxic, and widely consumed, thus its regular consumption is greatly recommended in order to promote cognitive health and lessen the risk of cognitive impairment elicited by neurotoxins, particularly fluorides.

Corresponding author
Mervat M. Kamel
Department of Animal Hygiene and Management, Faculty of veterinary Medicine, Cairo University, Cairo, Egypt
mevy58@yahoo.com

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