

Neurotoxicity of fluoride in ethanol fed rats: Role of oxidative stress, mitochondrial dysfunction and neurotransmitters

Shailender Singh Chauhan*, Sudarshan Ojha & Akhtar Mahmood
Department of Biochemistry, Panjab University, Chandigarh-160 014, India

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Prolonged exposure to fluoride or alcohol affects brain. However, the understanding about their interactions and neurotoxicity following co-exposures is still poor. The present study was designed to assess oxidative stress, mitochondrial dysfunctions, acetylcholinesterase (AChE) activity, neurotransmitter levels and morphological alterations in brain of fluoride or/and ethanol fed rats. Six and eighteen month old animals received sodium fluoride (NaF, 25 mg/kg) and 30% ethanol (EtOH, 1 mL/kg) individually and in combination for 90 days. Brain showed elevation in oxidative stress with age and NaF/EtOH treatment. There was increased lipid peroxidation; decreased glutathione, total and protein thiol content; along with declined activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase under these conditions. Mitochondrial functions were impaired significantly with age and NaF/EtOH treatment. The activities of NADH dehydrogenase, succinate dehydrogenase and cytochrome c oxidase along with mitochondrial respiration rate were decreased whereas the levels of nitric oxide and citrulline were increased in treated animals. Administration of NaF/EtOH showed altered neurotransmitter levels and increased AChE activity in brain. The levels of dopamine and 5-hydroxytryptamine were decreased while 5-hydroxyindoleacetic acid and homovanillic acid were increased significantly. Histological examination showed morphological alterations in treated animals compared to controls. Interestingly, the observed effects were more pronounced in rats co-exposed to NaF and EtOH. It is concluded that neurotoxic effects of fluoride are age dependent and further amplified by alcohol co-administration. These effects are mediated through elevated oxidative stress, mitochondrial dysfunctions and impaired neurotransmitter functions.

Keywords: Acetylcholinesterase (AChE) activity, Fluoride-alcohol interactions

Fluoride adversely affects brain and is reported to be a powerful neurotoxin^{1,40}. During long-term chronic exposures to fluoride, its entry into brain is no longer restricted by blood brain barrier, which adversely affect cellular architecture, metabolism, enzymes, the antioxidant status, neurotransmitters and overall tissue functions². Epidemiological studies have revealed that the intelligent quotient of children living in high fluoride areas is lower than the children living in low fluoride areas³. In addition, a decrease in learning ability and memory resulting in dysfunctions of central nervous system has been demonstrated in experimental animals⁴ and offspring⁵ exposed to high amount of fluoride. A common feature of many neurodegenerative diseases is increased generation of reactive oxygen species (ROS) and lipid peroxidation levels, which are suggested factors mediating

pathogenesis caused by fluoride⁶. The brain is especially sensitive to free radical induced oxidative damage due to the presence of high polyunsaturated fatty acids, relatively low antioxidant capacity, presence of redox metal ions and high oxygen utilization. The inferences drawn from previous literature indicate that animals treated with fluoride exhibit elevated lipid peroxidation levels and compromised antioxidant defense⁷. Fluoride can also exhibit its harmful effects on brain by inhibiting the release of melatonin from pineal gland. Melatonin, a hormone produced by the pineal gland, has been shown to have powerful neutralizing effects on free radicals and lipid peroxidation and to increase the levels of several antioxidants in brain.

Brain cells are mainly dependent on mitochondrial functions for meeting energy requirements. Mitochondrial defects resulting in decreased energy production leads to several deleterious consequences such as increased generation of superoxide and hydroxyl radicals, which are normally produced as by-products of oxidative metabolism⁸. The interaction

*Correspondence:

Phone: +1 3142656610 (Mob.)

E-mail: shailenderc@email.arizona.edu

Present add.: Department of Cellular and Molecular Medicine,
College of Medicine, University of Arizona, Tucson, USA

of superoxide radicals with nitric oxide (NO) produces peroxynitrite, which irreversibly inhibits mitochondrial respiration, reacts with proteins, lipids, carbohydrates and DNA, and causes DNA fragmentation and lipid oxidation. Fluoride is reported to induce NO generation and cause inhibition of cellular energy producing enzymes, including mitochondrial electron transport enzymes through peroxynitrite formation⁹. Thus, NO may be a critical component in mediating fluoride induced oxidative damage in brain.

Fluoride toxicity is markedly influenced by the co-presence of any other xenobiotics. Although, fluoride and ethanol are two well documented neurotoxicants co-exposing alcoholic population residing in high fluoride endemic areas, little is known about their combined effects¹⁰⁻¹³. Previously, we reported for the first time that female rats co-exposed to fluoride and ethanol exhibit compromised antioxidant defense in brain⁷. To better understand fluoride-alcohol interactions, we designed the present investigation to assess oxidative stress, mitochondrial dysfunctions, AChE activity, neurotransmitter functions and morphology in rat brain co-exposed to fluoride and ethanol.

Materials and Methods

Chemicals

Sodium fluoride (NaF) and ethanol (EtOH) were procured from Sisco Research Laboratories (SRL) Pvt. Ltd. Mumbai, India and Changshu Yangyuan Chemicals, China, respectively. Dopamine (DA), 5-hydroxytryptamine (5-HT), 5-hydroxyindole acetic acid (5-HIAA) and homovanillic acid (HVA) standards were procured from Sigma Chemicals, USA. All other analytical grade chemicals and reagents were purchased from Merck (Germany), Sigma or SRL Chemicals (India). Ultrapure water prepared by lab-PURE-Series Analytica 6 & Ultraplusuf was used throughout the experimental period.

Animals and Treatment

Six and 18 month old female Sprague Dawley rats weighing 200-220 g and 280-300 g respectively were procured from the Central Animal House of Panjab University, Chandigarh, India. They were housed in propylene cages and maintained at 22±3°C, on a 12:12 h light/dark cycle and a minimum 40% RH. Standard pellet diet and water were given *ad libitum*. After one week of acclimatization, age matched animals were separately subjected to random group division: Control (untreated); NaF treated (25 mg/kg);

30% EtOH treated (1 mL/kg); and NaF+EtOH co-treated (a combination of NaF and EtOH as mentioned above). There were four animals in each group and all the treatments were given orally using Ryle's tube daily at 9 am for 90 days. Body weight along with food and water intake was recorded. Overnight fasted rats were euthanized under light ether anaesthesia. Brain was removed, rinsed with ice-cold isotonic saline (0.9% w/v NaCl) and processed as required. The experimental protocol was approved by the Institute's Ethical Committee, in accordance with the guidelines issued for the use of laboratory animals.

Oxidative stress analysis

The standard spectrometry protocols were followed to quantify parameters relating to oxidative stress including lipid peroxidation (LPO); reduced glutathione (GSH); total (T-SH) and protein thiols (Pt-SH); superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GPx); glutathione reductase (GR) and glutathione-S-transferase (GST) as described previously⁶.

Mitochondrial dysfunction analysis

Brain mitochondria were isolated¹². Briefly, a 10% (w/v) tissue homogenate was prepared in buffer A (0.44 M sucrose, 10 mM Tris, 10 mM ethylenediaminetetraacetic acid, 0.1% bovine serum albumin, pH 7.4) and centrifuged at 600 ×g for 15 min. The supernatant was further spun at 14,000 ×g for 15 min. The pellet was separated and washed with buffer A followed by centrifugation at 7,000 ×g for 15 min. Finally, mitochondrial pellet was suspended in 2 mL of buffer B (0.44 M Sucrose, 10 mM Tris-HCl, pH 7.4) and purity was checked by measuring succinate dehydrogenase activity¹⁴. The parameters depicting mitochondrial functions including NADH dehydrogenase¹⁵, succinate dehydrogenase, cytochrome c oxidase¹⁶, NO¹⁷, citrulline¹⁸, and respiration rate¹⁹ were quantified in brain mitochondria.

AChE assay

AChE activity was determined in post mitochondrial supernatant (PMS)²⁰. The assay mixture consisted of 2.8 mL of phosphate buffer (0.1 M, pH 8.0), 0.1 mL of Ellman's reagent (10 mM, 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) and requisite amount of PMS. The reaction was initiated by the addition of 0.1 mL of acetylthiocholine iodide (14.9 mM) and change in absorbance was followed at 412 nm for 2 min. The activity was calculated using

molar extinction coefficient of 5-thio-2-nitrobenzoic acid ($13.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

Neurotransmitter analysis

Biogenic amines including DA, 5-HT, 5-HIAA and HVA were estimated by High Performance Liquid Chromatography (HPLC) with electrochemical detector as described by Church²¹. Briefly, brain slices were homogenized (5%, w/v) in perchloric acid (0.1 M) and were centrifuged at $12000 \times g$ for 5 min. The supernatant was filtered through $0.25 \mu\text{m}$ nylon filters, before injecting in the HPLC injection pump. Data was recorded and analysed with the help of Empower software. Waters standard system consisting of a high pressure isocratic pump, a $20 \mu\text{L}$ sample injector valve, C18 reverse phase column (type: Waters symmetry C18 ($5 \mu\text{m}$); length and diameter: $4.6 \times 250 \text{ mm}$) and electrochemical detector were used. Mobile phase consisted of citric acid (2%), potassium hydrogen phosphate (2%), ethylenediaminetetraacetic acid (1 mM), methanol (1.2%) and sodium octyl sulphate (70 mg/mL). The pH of the mobile phase was adjusted to 3 with hydrochloric acid (6 N). Electrochemical conditions for the experiments were + 0.800V, sensitivity ranges from 5-50 nA. Separations was carried out at a flow rate of 1.0 mL/min. Sample ($20 \mu\text{L}$) were injected manually. The level of neurotransmitters was determined using appropriate standards and results were expressed as nanogram/mg tissue.

Tissue morphology

After fixation in 10% formaldehyde, tissues were embedded in paraffin, solid sections were cut at $5 \mu\text{m}$ and stained with haematoxylin and eosin. The sections were examined under light microscope and photomicrographs were taken.

Statistical analysis

Data were analysed using SPSS/14.0 software package program for windows. Hypothesis testing methods included one way analysis of variance followed by least significant difference (LSD) and post-hoc Dunnett test. $P < 0.05$ were considered to indicate statistical significance. All the results were expressed as mean ($n=4$) \pm S.D.

Results

Administration of NaF or/and EtOH significantly reduced the gain in body weight along with food and water intake, when compared to age matched controls. However, the effect was more pronounced in co-treated animals (Fig. 1).

Effect on oxidative stress

Oxidative stress in brain was increased significantly with age and with treatment to NaF or/and EtOH (Fig. 2 A-C). The LPO levels were high while thiol (GSH, T-SH and Pt-SH) content and enzyme (SOD, CAT, GPx, GR and GST) activities were low in 18 month old animals, compared to 6 month old rats. These changes were further elevated by NaF or/and EtOH administration. Irrespective of the age, the effect on brain oxidative stress was more pronounced in animals receiving NaF together with EtOH. After 90 days, LPO levels in co-treated animals were significantly increased by 141% in 6 and 162% in 18 month old rats. Brain GSH, T-SH and Pt-SH content showed 59, 65 and 66% reduction, respectively in 6 month old and 66, 72 and 72% decline, respectively in 18 month old animals, when compared to age matched controls. Enzyme (SOD, CAT, GPx, GR and GST) activities were significantly decreased in 6 month (85, 69, 58, 73 and 63%) and 18 month (90, 75, 69, 74 and 73%) month old co-treated animals under similar experimental conditions.

Effect on mitochondrial dysfunction

Brain mitochondrial dysfunction showed a significant increase with age which were further aggravated by NaF or/and EtOH administration (Fig. 2 D and E). However, the observed effect was maximum in rats receiving NaF together with EtOH in both age groups. Mitochondrial NADH dehydrogenase, succinate dehydrogenase and cytochrome c oxidase activities were decreased by 29, 46 and 30%, respectively in 6 month old and by 56, 55 and 31%, respectively in 18 month old animals co-exposed to NaF and EtOH, when compared to age matched controls (Fig. 2D).

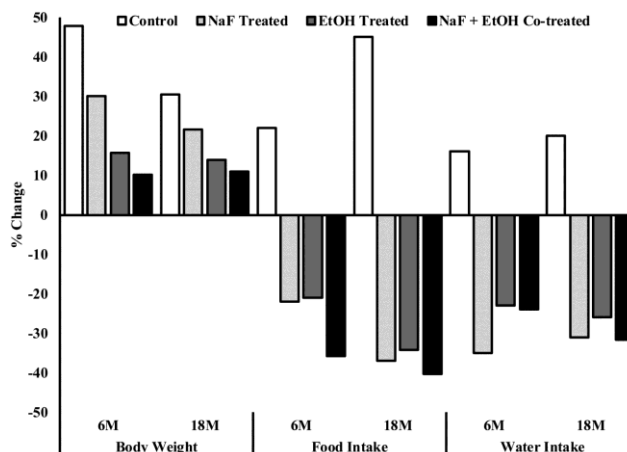


Fig. 1—Effect of NaF or/and EtOH administration for 90 days on body weight, food and water intake in rats.

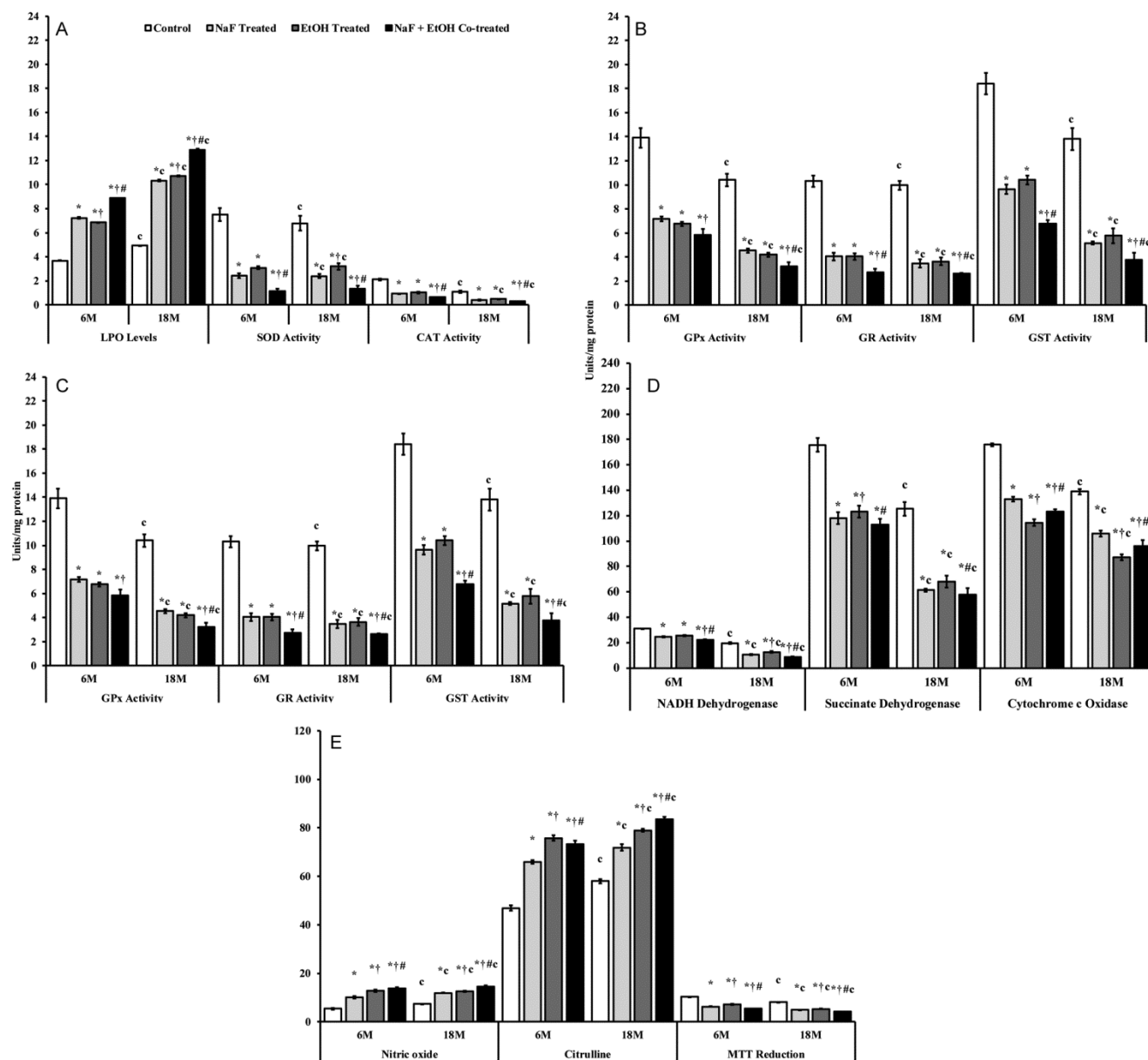


Fig. 2—Effect of NaF or/and EtOH administration for 90 days on parameters relating to oxidative stress (A-C); and mitochondrial function (D & E). [Values are mean \pm S.D.; (n = 4); * $P < 0.05$ vs. control group, † $P < 0.05$ vs. sodium fluoride (NaF) treated group, # $P < 0.05$ vs. 30% ethanol (EtOH) treated group, ^c $P < 0.05$ vs. 6 month (M). Units: Lipid peroxidation (LPO), nmol malondialdehyde; Superoxide dismutase (SOD), one unit is defined as the amount of enzyme inhibiting the rate of reaction by 50%; Catalase (CAT), mmol H₂O₂ decomposed/min; Glutathione peroxidase (GPx), n moles NADPH oxidized/min; Glutathione reductase (GR), n moles NADPH oxidized/min; Glutathione-S-transferase (GST), n moles GSH-CDNB conjugate formed/min; Total thiols (T-SH), Reduced glutathione (GSH), Protein thiols (Pt-SH), μ moles; NADH dehydrogenase, n moles NADH oxidized/min; Succinate dehydrogenase, n moles succinate oxidized/min; Cytochrome c oxidase, n moles n moles cytochrome c oxidized/min; Nitric oxide, n moles of NO₂ accumulated in sample/well; Citrulline, μ moles citrulline; MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5 Diphenyltetrazolium Bromide) reduction, μ g formazan formed/min]

The levels of NO and citrulline in brain mitochondria were increased significantly in treated animals, compared to age matched controls. At the end of experimental period, NO and citrulline levels were increased by 158 and 56%, respectively in

6 month and by 97 and 44%, respectively in 18 month old animals co-exposed to NaF and EtOH (Fig. 2E). Brain mitochondrial respiration rate in 18 month old animals was lower ($P < 0.05$) than 6 month old animals. Treatment to NaF or/and EtOH showed a

significant decline in mitochondrial respiration compared to age matched controls. NaF exposure resulted in 39-40% decrease in respiration rate in animals. EtOH administration showed decreased respiration rate in 6 month (30%) and 18 month old (35%) animals. However, co-treated animals showed 48% reduction in brain mitochondrial respiration rate both age groups (Fig. 2E).

Effect on AChE

Brain AChE activity in 18 month old animals was lower ($P < 0.05$) than 6 month old animals. Treatment to NaF or/and EtOH showed a significant increase in enzyme activity compared to age matched controls. NaF exposure resulted in 65 and 86% increase in AChE activity in 6 and 18 month old animals, respectively. EtOH administration showed increased AChE activity in 6 month (65%) and 18 month old (86%) animals. However, co-treated groups showed 70 and 90% increase in brain AChE activity in 6 and 18 month old animals respectively (Table 1).

Effect on neurotransmitters

The alterations in brain neurotransmitter levels are shown in Fig. 3. There was a significant decline in the neurotransmitter levels with age and different treatments except 5-HIAA which were increased following NaF or/and EtOH treatment. The levels of

dopamine, 5-HT and HVA were decreased by 58, 51 and 35%, respectively in 6 month old and by 52, 43 and 40%, respectively in 18 month old animals with NaF treatment. Animals receiving EtOH showed decreased neurotransmitter (dopamine, 5-HT and HVA) levels in 6 month old (50, 36 and 29%) and 18 month old (46, 43 and 30%) under these conditions. However, co-exposure to NaF and EtOH decreased dopamine, 5-HT and HVA levels by 42, 41 and 49%, respectively in 6 month old and by 59, 61 and 59%, respectively in 18 month old animals. NaF exposure resulted in 61 and 75% increase in 5-HIAA levels in 6 and 18 month old animals, respectively. EtOH administration showed increased 5-HIAA levels in 6 month (44%) and 18 month old (55%) animals. However, co-treated groups showed 48 and 92% increase in brain 5-HIAA levels in 6 and 18 month old animals, respectively.

Table 1— The effect of NaF or/and EtOH administration for 90 days on acetyl cholinesterase activity in rat brain

	Control	NaF Treated	EtOH Treated	NaF+EtOH Co-treated
6M	11.10±0.06	18.28±0.24*	18.08±0.28*	18.89±0.67*#
18M	7.92±0.10 ^c	14.77±0.23* ^c	13.50±0.39* ^{†c}	15.08±0.51* ^{#c}

[Values are mean ± SD from 4 observations. Units:- nmoles/min/mg protein. * $P < 0.05$ vs. control group; [†] $P < 0.05$ vs. sodium fluoride (NaF) treated group; # $P < 0.05$ vs. 30% ethanol (EtOH) treated group; and ^c $P < 0.05$ vs. 6 month (M)]

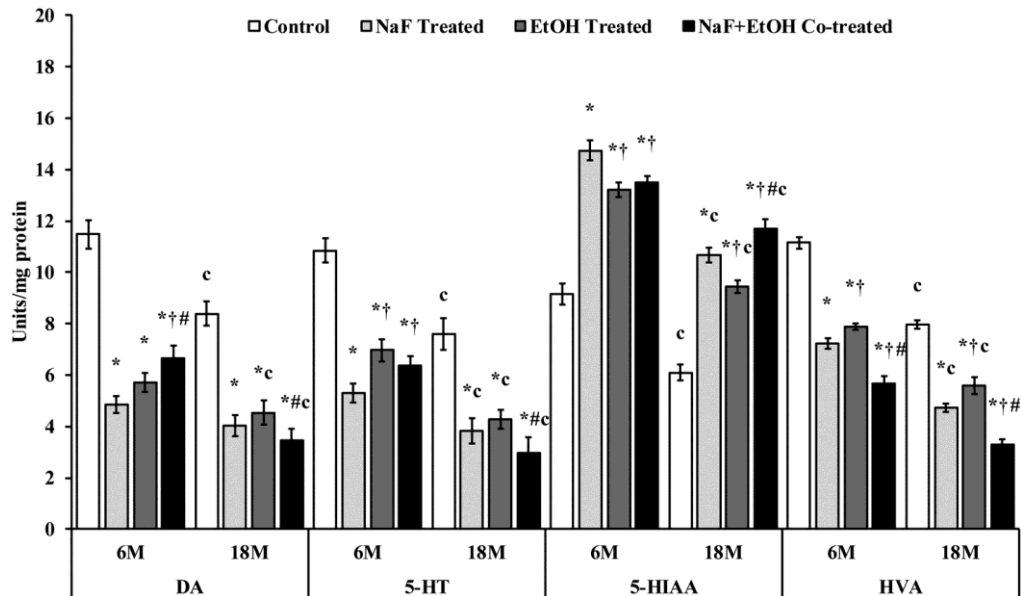


Fig. 3—Effect of NaF or/and EtOH administration for 90 days on neurotransmitters in rat brain. [Values are mean ± S.D.; (n = 4); * $P < 0.05$ vs. control group, [†] $P < 0.05$ vs. sodium fluoride (NaF) treated group, # $P < 0.05$ vs. 30% ethanol (EtOH) treated group, ^c $P < 0.05$ vs. 6 month (M); dopamine (DA), units: nano gram; 5-hydroxytryptamine (5-HT), units:- n gram; 5-hydroxyindoleacetic acid (5-HIAA); units: n gram; homovanillic acid (HVA), units:- n gram]

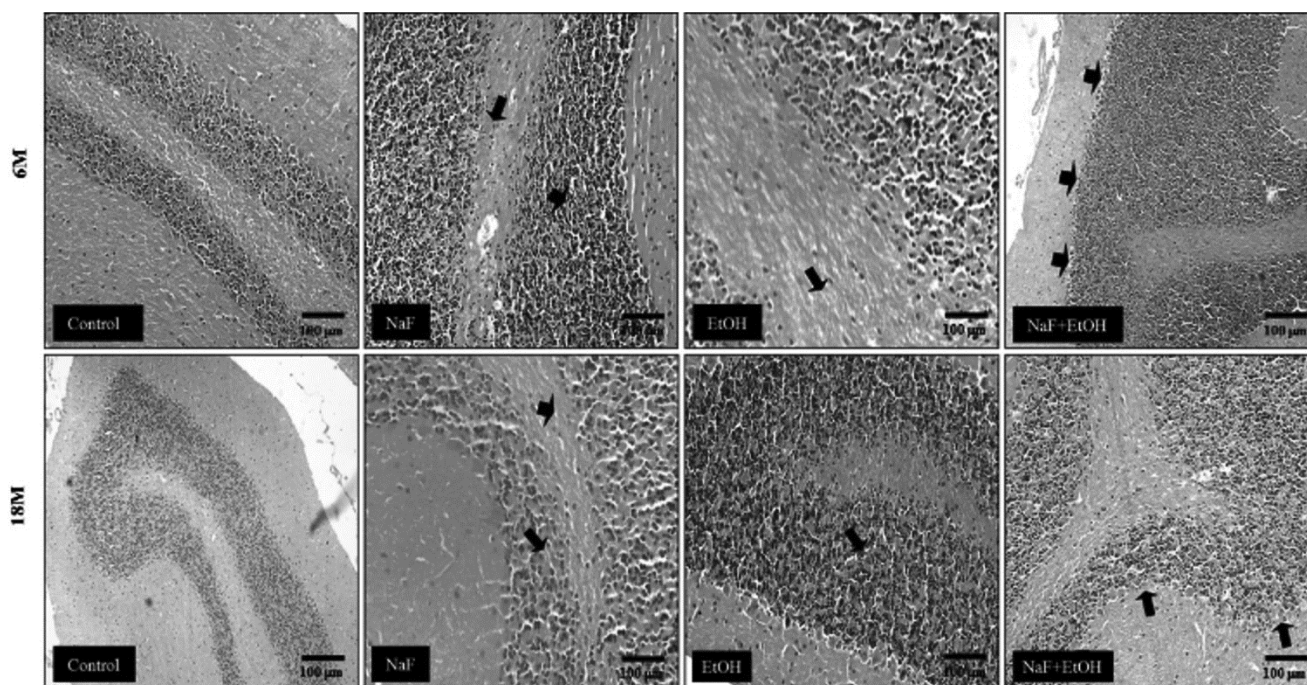


Fig. 4—Effect of NaF or/and EtOH administration for 90 days on histology of rat brain; Month (M); Magnification X40; arrows indicate changes in the morphology.

Effect on tissue morphology

Morphological changes in brain following NaF or/and EtOH treatment for 90 days are shown in Fig. 4. In 6 month old animals, there was cellular infiltration of granular layer and white matter with NaF exposure. Administration of EtOH resulted in demyelination of white matter. Animals co-exposed to NaF and EtOH revealed loss of Purkinje cells. In 18 month old animals, NaF treatment resulted in thinning of granular layer along with demyelination of white matter. EtOH administration revealed infiltration in the granular layer. Co-exposure to NaF and EtOH showed necrosis along with the loss of Purkinje cells in the cerebellum region of brain.

Discussion

This is the first report on neurotoxicity of fluoride in ethanol fed rats. Our data demonstrated that the neurotoxic effects of fluoride are age-related and amplified by ethanol co-administration. These effects are mediated through elevated oxidative stress, mitochondrial dysfunctions and impaired neurotransmitter functions. Fluoride also affected body weight gain, food and water intake as previously reported²².

Fluoride can stimulate lipid peroxidation in membranous structures. However, the mechanism of fluoride-induced lipid peroxidation is not fully

understood. The current data suggest that the mechanism may involve a decline in $-SH$ groups and antioxidant activities along with impaired mitochondrial functions resulting in increased NO production. This can induce a peroxidative state in biological systems and, in turn, lead to peroxidation of polyunsaturated fatty acids. Aldehyde oxidase can also act on ethanol metabolites i.e. acetaldehyde and NADH to form superoxide anion radical ($O_2^{\cdot-}$) and may contribute to elevated lipid peroxidation in co-treated animals under these conditions. Fluoride binds to the active site of copper on SOD and causes its inhibition. A decreased activity of SOD and CAT in treated rats indicates inefficient scavenging of ROS which might be implicated to oxidative inactivation of enzyme. Unlike CAT, GPx activity depends on the balance between the levels of GSH and GSSG. Melatonin is a free radical scavenger and an antioxidant which stimulates the activity of GPx in brain. Fluoride accumulation in pineal gland impairs the secretion of melatonin which might be responsible for the fluoride related decrease in brain GPx activity²³. Following ethanol administration, GPx provides defense against oxidative damage to various tissues. Ethanol associated decline in GPx activity could be due to increased peroxidative stress and might reflect the decline in the production and availability of GSH to overcome the increased

production of H_2O_2 ²⁴. Glucose-6-phosphate dehydrogenase is one of the key enzymes of pentose phosphate pathway responsible for maintaining cellular NADPH levels. The activity of this enzyme is impaired directly by fluoride ingestion and indirectly by niacin deficiency caused during ethanol consumption. This results in diminished levels of NADPH which is required by GR to convert GSSG into GSH. The results obtained from the earlier studies suggest that depletion of GSH leads to a decrease in GR activity²⁵. Reduction in GST activity in treated animals may be attributed to decrease in GSH levels, because GSH is required as a substrate for GST activity. In this context, a decrease in the GST activity in fluoride²⁶ or ethanol²⁷ exposed animals has been reported. The depletion of GSH, GPx and GST promotes generation of ROS and oxidative stress with cascade of effects thereby affecting functional as well as structural integrity of cell or organelle membranes.

Normally, electron transport chain (ETC) generates free radicals in small fraction which are efficiently handled by cellular defense system to regulate different signalling pathways. However, with age and exposure to certain toxic xenobiotics this free radical production is increased beyond the capacity of cellular defense and cause damage to cell organelles. Also, ETC itself is vulnerable to damage by free radicals which indicates that the oxidative stress observed in the current study may be either cause or the consequence of the mitochondrial dysfunction. Fluoride can pass through inner mitochondrial membrane and cause inhibition of succinate dehydrogenase and cytochrome oxidase. Stachowska *et al.*²⁸ reported that succinate dehydrogenase activity is inhibited by both competitive and non-competitive binding of fluoride to the active site. Impaired mitochondrial functions due to ethanol induced reduction in the activities of NADH dehydrogenase, succinate dehydrogenase and cytochrome c oxidase have been reported previously²⁹. NO is potentially a physiological regulator of mitochondrial respiration. At nanomolar levels it competes with oxygen and cause reversible inhibition of cytochrome oxidase. Previous studies have shown increased NO production in rats with age²⁹ and fluoride or/and ethanol treatment^{31,32}. In the presence of mitochondrial respiration inhibitors, superoxide production is increased which can cause irreversible inhibition of NADH dehydrogenase, succinate dehydrogenase and cytochrome c reductase through

formation of peroxyxynitrite. Under these conditions of sustained mitochondrial inhibition, peroxyxynitrite levels would increase continuously, causing depletion of antioxidants (especially GSH) and initiation of lipid peroxidation. Moreover, membrane-bound localization of ETC enzymes enhances their sensitivity to the lipid microenvironment and could be responsible for the impaired functions upon oxidative damage to inner mitochondrial membrane. Thus, depletion of brain GSH levels during fluoride or/and ethanol exposure may be critical for observed decrease in ETC enzyme complexes and overall mitochondrial dysfunction.

The current results agree with the findings of Jha and Rizvi³³ who reported an age-related decline in the enzyme activity and other investigations which indicated increased brain AChE activity in high fluoride³⁴ or ethanol³⁵ exposed animals leading to learning and memory impairment. Increased activity of AChE in fluoride treated animals may be due to interaction of fluoride with two or more hydrogen donors required for enzyme catalysis, whereas ethanol related elevation in the enzyme activity may be attributed to modifications in the protein glycosylation process thereby affecting distribution of soluble and membrane-bound AChE in the tissues. It has been reported that aging is associated with a decrease in the concentration of various neurotransmitters in the brain³⁶. Fluoride can cross the blood brain barrier and alter levels of biogenic amines¹. The decrease in the levels of DA and its metabolite HVA in fluoride treated animals may be due to the decreased activity of enzymes involved in their synthesis like tyrosine hydroxylase, DOPA decarboxylase and dopamine β -hydroxylase. Yuan *et al.*³⁷ have shown that the content of 5-HT in the brain is decreased during chronic fluorosis. The decline in 5-HT levels in fluoride treated animals in this study may be attributed to enhanced monoamine oxidase activity resulting in neurotransmitter degradation. This is further corroborated by elevated 5-HIAA (metabolic product of 5-HT) levels in the brain of rats exposed to fluoride³⁸. Alterations in brain neurotransmitter levels in ethanol treated animals observed in present study agree with the previous literature reporting decreased levels of dopamine³⁹ and 5-HT⁴⁰. Chronic ethanol administration increases the activity of monoamine oxidase, which could be responsible for the degradation of 5-HT and thereby resulting in elevated 5-HIAA levels. These results suggest that the accumulation of fluoride and ethanol in the brain

tissue can disrupt the synthesis and release of certain neurotransmitters and receptors in nerve cells, leading to neural damage. Recent studies conducted in different settings have also described neurotoxic effects of high fluoride exposures⁴¹⁻⁴³. The effects of fluoride or/and ethanol were also evident on brain morphology which may be associated with increased oxidative stress and mitochondrial dysfunction under these conditions. This would lead to enhanced formation of ROS, NO, and ONOO⁻ in brain and induce oxidative damage.

Conclusion

The present study demonstrated that the neurotoxic effects of fluoride are age-related and further amplified by ethanol co-administration. These effects are mediated through elevated oxidative stress, mitochondrial dysfunction and impaired neurotransmitter functions. Experimental observations suggest increased susceptibility of alcoholics residing in endemic fluoride areas toward age-related neurological disorders.

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Conflict of interest

The authors declare no conflict of interest.

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