Neuroprotective effects of *Abelmoschus moschatus* seed extract on fluoride-induced myelin degeneration in developing brain of rats

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**Abstract**

**Aim:** Fluoride at higher concentrations affects various soft tissues, including brain, heart, kidney, and other tissues in addition to dental and skeletal systems. Particularly, in brain it induces various complications such as oxidative stress, alters in the levels of neurotransmitters, and histological changes. The aim of the present study is to report the protective effects of *Abelmoschus moschatus* seed extract against sodium fluoride-induced neurohistological changes with particular emphasis on myelin degeneration, cell shape, size, and Gamma-aminobutyric acid (GABA) as well as aspartate alterations. In addition, antioxidants (glutathione peroxidase [GSH-Px] and superoxide dismutase [SOD]), spatial navigation, and learning ability were observed.

**Materials and Methods:** The pregnancy confirmed Wistar rats were segregated into six groups, five subjects for each and doses started from 1st day of pregnancy. Control group received normal tap water, fluoride group fed on 20 ppm fluoridated water, 3rd group treated with NaF (20 ppm) + *A. moschatus* aqueous extract (AMAE) (300 mg/kg b. wt.), 4th group received NaF (20 ppm) + *A. moschatus* ethanolic extract (AMEE) (300 mg/kg b. wt.), and 5th and 6th groups treated with AMAE and AMEE alone. Treatment continued for 51 days (21 gestational and 30 postnatal days [PND]). On PND 1, 7, 14, 21, and 30 rat pups were sacrificed, dissected out the brain and used to assess antioxidants, GABA, aspartate and also used for histological studies. Days 21 and 30, rats were used to behavioral studies before they sacrificed.

**Results and Discussion:** The decreased learning ability is observed in NaF exposed rats compared to control and protective groups of rats. GSH-Px activity is increased and SOD activity is decreased in fluoride received rats. Moreover, GABA and aspartate levels are increased (*P* < 0.001). The GABA, aspartate, and myelin have a crucial role in the maturation of brain. Decreased neural connections, networks, dendritic branches, and degenerating myelin sheath are observed in NaF intoxicated rats through H and E stain and luxol fast blue stain. These all are reverted on the administration of AMAE and AMEE toward NaF toxicity. AMEE showed good results over AMAE.

**Conclusion:** It is concluded that the seed extract of *A. moschatus* possesses neuroprotective effects against fluoride toxicity.

**Key words:** *Abelmoschus*, aspartate, fluoride, Gamma-aminobutyric acid, histology, myelin

**INTRODUCTION**

Fluoride is widely distributed in nature, readily available in various sources and enters into the body through drinking water, food, toothpaste, mouth rinses, and other dental products such as drugs. In addition, fluoride dust and fumes from industries using fluoride containing salt and hydrofluoric acid also are sources for fluoride. Fluorosis is caused in human beings predominantly through fluoride in drinking water and also burning coal, drinking tea, and supplementing food with additives such as calcium monohydrogen phosphate containing high levels of fluoride contribute to fluorosis.[1] High levels of fluoride consumption are known to cause structural changes, altered activities of enzymes, and metabolic lesions in the brain and influence the metabolism of lipids.[2]

The fluoride-induced alterations in the central nervous system (CNS) include morphological and functional

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High levels of fluoride exposure during gestational periods have an adverse effect on the levels of fetal neurotransmitters. GABA is principal excitatory neurotransmitter in developing brain (in contrast to the mature brain in which it is inhibitory NT). Influences the processes of cell proliferation, migration, and differentiation, regulate the development of excitatory synapses at early stages of cortical circuit formation. Altered NTs levels and their abnormal functioning on NaF administration is resulted in decreased learning and memory ability. Increased free radicals from fluoride initiate oxidative stress pathway which leads to damage in the neuronal cell membranes, destruct them and in turn leads to the release and subsequent extracellular accumulation of glutamate, contributing to excitotoxicity and further promoting reactive oxygen species (ROS) generation. In our earlier report, increased glutamate levels were observed in NaF treated rats and thus, the altered levels of GABA, glutamate, and aspartate lead to the excitotoxic mechanism and alter the brain maturation process.

Myelin sheath is a plasma membrane of neural cells enriched with phospholipids. It is formed by oligodendrocytes in CNS and by Schwann cells in PNS and acts as an insulator and increase the speed of propagation in conducting action potentials. Myelin sheath, in addition to rapid signal conduction, is important for axon maintenance and function. Myelin degeneration is associated with many CNS pathological conditions such as congenital, autoimmune, and metabolic disorders. Structural perturbations also result in axonal degeneration and it occurs due to disruption in axo-oligodendrocytic signaling. Reddy et al. reported that the destruction of the myelin sheath in fluoride (20 ppm) exposed rats.

Most recent researchers are now looking at natural antioxidants as persuasive therapeutic agents against several neurological disorders, as they have the proficiency to combat by neutralizing free radicals. The primary source of antioxidants is our diet. However, the medicinal herbs are catching attention to be a commercial source of antioxidants at present. In earlier studies, researchers attempted to treat a variety of diseases with antioxidants, which acts through scavenging free radicals. In previous reports, for example, Ginkgo biloba extract, quercetin, resveratrol, Vitamin E, Vitamin C, curcumin, tamarind fruit pulp, and silymarin were used to scavenge free radicals. All these observations suggesting that the involvement of ROS in the pathogenesis of several diseases including neurodegenerative disorders and a possibility of the therapeutic use of free radical scavengers and antioxidants in the prevention of free radical-mediated neurological disorders. Many research studies evidenced that free radical scavenging substances inhibit the toxic effect of β-amyloid or hydrogen peroxide on cell cultures and organotypic hippocampal cultures.

Three free radical scavenging drugs used for therapeutic purposes in different fields were also scrutinized in clinical studies of AD and produced beneficial results: Vitamin E (α-tocopherol), selegeline (also a monoamine oxidase B inhibitor), and G. biloba extract EGb 761.

The earlier studied natural compounds provided positive results against fluoride toxicity, but, no compound is suitable for treating fluorosis completely. In view of this, the present study focused on Abelmoschus moschatus plant, which belongs to Malvaceae family and possesses a number of pharmacologically important chemicals. Lai et al. isolated and identified a number of flavonoids, namely myricetin, myricetin 3-O-beta-D-glucopyranoside, and quercetin from the plant Abelmoschus manihot. Lai et al. isolated a glucuronide from the flowers of A. manihot along with traces of hibifolin. Jain and Bari isolated of Stigmasterol and γ-Sitosterol from the petroleum ether extract of the woody stem of A. manihot. The various parts of the plant, A. moschatus (seeds, leaves, flowers, and some extent roots) is used in Bangladesh by traditional healers, used in the tribal and traditional medicine of India for stomach pain and disorder in Trinidad and Tobago. From the seeds of Ambrette, there are four natural polyphenolic compounds, namely 1-(6-ethyl-3-hydroxypyrpyridin-2-yl) ethanone, 1-(3-hydroxy-5,6-dimethylpyridin-2-yl) ethanone, 1-(3-hydroxy-6-methylpyridin-2-yl) ethanone, and 1-(3-hydroxy-5-methylpyridin-2-yl) ethanone were isolated by Du et al. In our earlier reports, Okra seed extract showed the protective results toward NaF toxicity by reducing ROS production, maintaining NT system, and reversed altered pain and alterations in cell shape, size, Nissl granules, and amyloid plaque formation.

Based on these observations, the present study reports the protective role of A. moschatus seed extract against fluoride induced neuronal disturbances in terms of GABA, aspartate alterations, myelin destruction, and learning abilities.

**MATERIALS AND METHODS**

Wistar rats during developmental periods both pre- and postnatal were used for experimentation, and they were maintained...
in laboratory conditions as per the guidelines of the CPCSEA (CPCSEA No: 383/01/a/CPCSEA) and Institutional Animal Ethical Committee approval was taken for experimentation. Animals were acclimatized for 7 days to light from 6:00 AM to 6:00 PM alternating with 12 h dark and after that doses were started. The animals are housed in stainless steel cages and which were maintained in an air-conditioned room with a temperature at 25 ± 2°C. Rats were allowed to feed on standard chow diet and water ad libitum throughout the experiment. Female and male rats of the same strain are categorized in 2:1 ratio into separate cages for breeding and after confirmed the pregnancy, females were randomized into six groups five subjects for each, and they were treated as:
1. Group I – Control rats (untreated) received normal tap water
2. Group II – Sodium fluoride (NaF) received (20 ppm) rats in their drinking water
3. Group III – NaF (20 ppm) + A. moschatus aqueous seed extract (AMAE) at the rate of 300 mg/kg body weight/rat/day
4. Group IV – NaF (20 ppm) + A. moschatus ethanolic seed extract (AMEE) at the rate of 300 mg/kg body weight/rat/day
5. Group V – AMAE at the rate of 300 mg/kg body weight/rat/day
6. Group VI – AMEE at the rate of 300 mg/kg body weight/rat/day.

The treatment continued for 51 days (prenatal or gestational – 21 days and postnatal 30 days), and the pups from all experimental groups were sacrificed at different age groups such as postnatal day (PND) 1, 7, 14, 21, and 30 and used the brain for all experiments.

Methods

Behavioral parameter

Maze learning test was performed on rats’ pups with age 21 and 30 days. PND 21 and day 30 young rats from all experimental groups were used to perform the maze task.

Maze learning

The maze test was conducted according to the method of Bromley-Brits et al.[38] Maze apparatus (60° × 30° × 15°) is used to study the ability of spatial navigation of animal, in which rats explore novel situations and make decisions based on reward (food) that produce desirable outcome. During the training period, all rats were under starvation for a period of 8–12 h and trials were conducted around 10 A.M.–2 P.M. At the beginning of each trial, animals were placed at the start point of the maze and allowed to explore the maze for 10 min. The training was given to rats for 3 days. On the day of experimentation, rats were allowed into maze task to locate the food which present at the end of the set up and noted the latency time to reach the goal. The data were exposed to statistical analysis and results (i.e., goal reaching time) were expressed in minutes.

Antioxidant markers

PND 1, 7, 14, 21, and 30 rats from all experimental groups were used for assess oxidative stress markers.

GSHPx

Glutathione peroxidase was estimated by the method of Rotruck et al.[39]

SOD

SOD assay was carried out by the method of Marklund and Marklund.[40] This method is based on the ability of the enzyme to inhibit oxygen dependent auto-oxidation of pyrogallol. The rate of auto-oxidation is measured by noting the increase in absorbance at 420 nm.

Neurotransmitters

Neurotransmitters are assessed from PND 1, 7, 14, 21, and 30 rats of all experimental groups.

Aspartate

Aspartate was assessed by the modified method of Murai et al.[41]

GABA

GABA was assessed by the modified method of Ippolito and Piwnica.[42]

Histological studies

Histological studies were carried out from PND 1, 7, 14, 21, and 30 rats of all experimental groups.

H and E stain

H and E staining was performed by the method of Leeson et al.[43] and observed under Lawrence digital microscope.

Luxol fast blue (LFB) stain

The LFB stain is preferably used for staining myelin sheath and Nissl substance and observed under Lawrence digital microscope.[44]

Statistical Analysis

One-way analysis of variance (one-way ANOVA) to compare the means between the groups and t-test was used to determine the statistical differences between groups. Results were represented as the mean ± standard error of the mean. Significant of the data is \( P < 0.001 \).

RESULTS

Maze Learning

The increased latency time was observed in the maze in fluoride-treated group with respect to control and protective
compound-treated rats [Figure 1]. The percent of change in time latencies in NaF treated rats as compared to control rats is 39.70% and 34.74% after 21 and 30 days. The time latencies reduced in NaF+AMAE about 24.05% and 16.40% after 21 and 30, respectively, and in NaF+AMEE about 22.38% and 14.28% in after 21 and 30 days, respectively. The percent of change in AMAE and AMEE from control is 7.13% and 5.14% in PND 21 and −3.28% and 4.05% in PND 30 (P < 0.01), respectively.

GSH-Px

The GSH-Px activity in NaF treated rats was increased progressively with age, and its percent of change from control was about 23.28%, 23.28%, 26.92%, 24.05%, and 30.12% in PND 1, 7, 14, 21, and 30, respectively. GSH-Px activity significantly (P < 0.001) reversed in NaF+AMAE and NaF+AMEE treated groups and it was about 4.22% and 7.04% in PND 1, 8.21% and 6.84% in PND 7, 6.41% and 5.12% in PND 14, 7.59% and 6.32% in PND 21, and 4.81% and 3.61% in PND 30 rats. AMAE and AMEE treated rats showed the normal enzyme activity as compared to the control group [Figure 2].

SOD

In NaF intoxicated rats, the decreased activity of SOD was found with respect to control rats. The percentage of decrease in its activity in NaF is about −30.50%, −20.27%, −23.80%, −21.73%, and −19.04% from PND 1–30, respectively. Its activity was reverted in AMAE and AMEE administered rats toward fluoride received rats. The reverted activity of SOD in NaF+AMAE and NaF+AMEE from day 1 to day 30 by −11.86% and −15.25%, −12.16% and −13.51%, −7.14% and −9.52%, −5.43% and −7.60%, and −9.52% and −10.47% correspondingly with compared to control. The results showed that the enzyme activity was more prominently decreased in PND 1, 7, and 14 [Figure 3].

Aspartate

Aspartate levels were increased [Figure 4] in the brain tissue of NaF intoxicated rats, and it was about −42.85% in day 1, −37.50% in day 7, −44.44% in day 14, −45.45% in day 21, and −41.66% in day 30 when compared to respective age control rats. On administration of AMAE and AMEE against NaF toxicity, its levels were reversed. The percent of reversal observed in NaF+AMAE treated rats is about −28.57%, −12.50%, −11.11%, −18.18%, and −16.16% in PND 1, 7, 14, 21, and 30, respectively, and in NaF+AMEE treated rats is by −28.57%, −12.50%, −11.11%, −18.18%, and −16.66% in PND 1, 7, 14, 21, and 30 correspondingly. Ethanolic extract of seeds provides better results over aqueous extract.

GABA

γ-aminobutyric acid levels were increased [Figure 5] in the brain tissue of NaF intoxicated rats compared to the control group. The percent of the increase in GABA levels is 53.84%, 43.47%, 22.58%, 18.42%, and 12.24% from PND 1 to PND 30 correspondingly. A. moschatus extract treatment along with NaF reverted the GABA levels. In NaF+AMAE received rats, it was about 15.38%, 12.90%, 8.16%, 10.00%, and 7.89% from PND 1 to 30, respectively. NaF+AMEE treated rats showed that the percent of reversal is about 10.00%, 10.52%, 7.69%, 9.67%, and 6.12% from day 1 to day 30, respectively. AMEE showed more protection than AMAE.

H and E stain

The cells of control rats’ brain tissue are with round regular shape and size and are represented by the yellow color arrow

![Figure 1](image-url): Latency time of maze test shown in rats exposed to fluoride and treated with *Abelmoschus moschatensis* extract. Results were presented as the mean ± standard error of the mean (n = 5). *For P < 0.01; **for P < 0.05, ****for P < 0.005
mark. In NaF alone fed rats, the cell with irregular shape and size was observed and they were represented by black color arrow mark. In NaF+AMAE and NaF+AMEE treated some of the cells with irregular shape and size. AMAE and AMEE treated groups showed the normal cells comparable to control. PND 14–30 day rats treated with NaF showed obvious alterations in their shape and size than PND 1 and PND 7 rats of fluoride exposed [Figure 6].

**Figure 2:** Protective effects of *Abelmoschus moschatus* extract on glutathione peroxidase activity in brain tissue of rats exposed to NaF. Results were presented as the mean ± standard error of the mean (n = 5). Significant of the data is $P < 0.001$. Units: Glutathione peroxidase activity was expressed as µg/mg protein.

**Figure 3:** Protective effects of *Abelmoschus moschatus* extract on superoxide dismutase activity in brain tissue of rats exposed to NaF. Results were represented as the mean ± standard error of the mean (n = 5). Significant of the data is $P < 0.001$. Units: Superoxide dismutase activity is expressed as units/mg protein.
LFB Stain

Rats exposed to NaF during pre- and early post-natal periods showed the destructing myelin sheath and cells undergoing apoptosis. The NaF+AMAE, NaF+AMEE treated rats showed the reverted myelin degeneration, and only AMAE and AMEE exposed rats displayed normal myelin sheath as compared with control rats. The myelin sheath degeneration

**Figure 4:** Protective effects of *Abelmoschus moschatus* extract on aspartate levels in brain tissue of rats exposed to NaF. Results were presented as the mean ± standard error of the mean (n = 5). Significant of the data is P < 0.001. Units: Aspartate levels were expressed as µ mole of aspartate/g weight of tissue

**Figure 5:** Protective effects of *Abelmoschus moschatus* extract on Gamma-aminobutyric acid (GABA) levels in brain tissue of rats exposed to NaF. Results were presented as the mean ± standard error of the mean (n = 5). Significant of the data is P < 0.001. Units: GABA levels were expressed as µ mole/g weight of tissue
was clearly seen in PND 14–30 rats. The reason for this may be a long duration of exposure to fluoride [Figure 7].

**DISCUSSION**

The present study is undertaken to evaluate the consequences of high levels of fluoride exposure during pregnancy and lactation on neurodegeneration in young rats’ developing brain whose mothers exposed to fluoride (20 ppm) during pregnancy and on different PNDs (lactation periods) and simultaneous protective treatment of *A. moschatus* seed extract. The fetus is with poorly formed protective mechanisms including blood-brain barrier against xenobiotics that circulate in the maternal blood and the placenta also does not block the entry of numerous environmental toxicants.
from the maternal circulation to fetal circulation. Mullenix et al. observed that the various behavioral alterations and which were common to weaning and adult exposure and were different from those after prenatal exposures. In the present study, the NaF fed rats showed decreased learning ability and spatial navigation compared to control rats. The similar results were observed by Gao et al., they found the decreased capacity of learning and memory of rats which exposed to high fluoride levels. AMAE and AMEE treated rats showed increased learning ability than fluoride alone treated rats.

The fluoride-exposed rats resulted in reduced activity of SOD when compared to control and NaF+AMAE and NaF+AMEE treated groups. Fluoride has been reported as one of the well-known inhibitors of SOD activity. Fluoride exposure during prenatal and early postpartum periods in rats showed significant decrease in SOD activity in their neural tissues with respect to the control group and the similar results were observed by Nabavi et al., in which they exposed the rats to 600 ppm of fluoride for a week through their drinking water. The decreased SOD activity in sodium fluoride administered rats result in an extra superoxide anion accumulation in rat’s neural tissue. SOD activity was reversed in NaF+AMAE and NaF+AMEE treated rats when compared to fluoride-exposed rats. Present results reported that the increased GSH-Px activity in 20 ppm fluoride received rats when compared to control group of rats. AMAE and AMEE treated rats along with sodium fluoride showed the reverted GSH-Px activity. AMAE and AMEE alone treated rats do not showed any significant differences in the activity of GSH-Px, and results were similar to control rats.

Increased free radicals from fluoride initiate oxidative stress pathway which leads to damage in the neuronal cell membranes, destroy them and in turn leads to the release and subsequent extracellular accumulation of glutamate, contributing to excitotoxicity and further promoting ROS generation. These effects of oxidative stress on amino acid accumulation may be due to membrane lipid peroxidation, which may impair the GABA transporter, leading to a decrease of GABA accumulation, and/or it may increase the release and extracellular accumulation of aspartate and glutamate. Other mechanisms that could be involved in the inhibition of glutamate uptake by ROS may be the direct oxidation of the transporter sulfhydryl groups or the impairment of Na+K+ATPase activity. In agreement with these results, the present data showed increased levels of aspartate in NaF treated rats with compared to control rats, and its levels were reverted on the administration of AMAE and AMEE toward fluoride toxicity. In contrary to the Duarte et al., GABA levels were increased in NaF treated rats, and it was reverted on AMAE and AMEE treatment. Glutamate, aspartate is excitatory amino acid NTs and GABA also acts as an excitatory NT in developing the brain. In our earlier report, NaF received rats showed the increased levels of glutamate and in this report, the increased levels of GABA and aspartate were observed in NaF treated rats. These three NTs distributed throughout the CNS and play a very crucial role in cognition, synaptogenesis, memory, etc. Free radicals from fluoride lead to excitotoxicity which, in turn, triggered the release of glutamate, GABA, and aspartate from presynaptic terminals into extracellular space with consequent overstimulation their respective receptors. As a consequence, disturbance in GABAergic, aspartatergic and glutamatergic systems may lead to many psychological and neurodegenerative diseases.

The myelin sheath is a specialized multilayered structure and surrounds selected axons in CNS. Biochemically, myelin is formed of roughly 70–85% lipid, with a high content of cholesterol and only 15–30% of protein, especially with myelin-specific proteins. Myelin degeneration occurs in CNS on chemical toxicity, traumatic brain injury, and demyelinating diseases. Demyelination may induce undesirable inflammation and cell death. Myelin-associated proteins are released after the destruction of the intact myelin sheath. After destruction of the intact myelin sheath, myelin basic protein (MBP) also dissociates from the plasma membrane and acts in a free, membrane-unbound manner in the extracellular matrix.

In the present study, the NaF treated rats showed myelin destruction as compared to control rats. In NaF+AMAE and NaF+AMEE rats, the normal myelin sheath was found. Histological studies indicate myelin destruction, which is correlated with changes in MBP secondary to membrane damage and axonal degeneration on the exposure of fluoride. The histopathological investigations in rat exposure to fluoride seemed thickening and disappearance of dendrites, swelling of mitochondria, dilation of the endoplasmic reticulum in neurons, and diminished hippocampal synaptic interface structure and the changes in the structure of synaptic interface could necessarily affect the neuronal transmission. These findings indicate that high consumption of fluoride resulted in structural and functional damages of the CNS and is associated with CNS dysfunction.

In summary, developing brain is more prone to fluoride toxicity due to poorly formed protective mechanisms such as blood-brain barrier and antioxidant defense. F mediates the generation of superoxide anion (O$_2^-$) and the supplementary production of hydrogen peroxide, peroxynitrite, and hydroxyl radicals. Exposure to NaF during pregnancy and lactation has resulted in a wide range of changes, including histopathological changes. This resulted in alterations in the levels of neurotransmitters and as consequence of behavioral alterations. A. moschatus treatment resulted in a reversal of the alterations resulted in fluoride exposure.

**CONCLUSION**

Fluoride produces excess free radicals which alter the antioxidant status of the brain. These free radicals oxidize...
membrane lipids and thus destruct the myelin sheath of neural cells. These changes lead to neuronal loss, alters in the levels of neurotransmitters, and finally, behavioral alterations. *A. moschatus* possess antioxidants principally quercetin, rutin, catechin, epicatechin, and procyanidin and quercetin derivatives which quench free radicals and maintained lipid membranes of the cell. Thus, the normal myelin sheath was maintained in AMAE and AMEE treated rats against fluoride. The ethanol extract has shown better efficacy than aqueous extract. Yet, further studies are needed to know the exact mechanism of components neuroprotective effects of *A. moschatus* seed extract.

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