Ameliorative effect of *Nigella sativa* and Deferrioxamine against neurotoxicity induced by combination of fluoride and aluminium

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ABSTRACT

The most common source of human exposure to fluoride and aluminium include drinking water and various food items. Both fluoride and aluminium have been reported to induce oxidative stress mediated neurodegeneration. The aim of the present study is to evaluate the protective effect of *Nigella sativa* and deferrioxamine against combination of fluoride aluminium induced toxicity in brain of male Swiss albino mice. Mice were treated intraperitoneally with fluoride and aluminium for one month (thrice a week on alternate days) which resulted in decrease in various antioxidant enzymes like SOD, catalase, GPx, GSH and increase in LPO levels. Neurodegenerative changes were also observed in histology of brain. DNA fragmentation and increased GFAP and Caspase 3 expression was also observed indicating that combination of fluoride and aluminium leads to oxidative stress mediated apoptosis in brain cells. Treatment with *Nigella sativa* and deferrioxamine resulted in amelioration of neurodegeneration induced by fluoride and aluminium indicating their therapeutic potential.

KEY WORDS: Brain, Oxidative stress, Fluoride, Aluminium, Apoptosis, *Nigella sativa*

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INTRODUCTION:

Metals form an important part of earth’s crust. Most of the metals are present in rocks from which they need to be extracted in the form. Metals like iron, copper, zinc, manganese, cobalt and calcium are required for important functions of our body like respiration, circulation and reproduction where they perform catalytic roles. Certain metals are not needed by the body and they form poisonous soluble compounds inside the body. These toxic metals produce adverse effects in the body by interfering with the metabolic processes as they imitate the action of an essential element required in the metabolic process.

Fluorine is distributed widely in nature and is one of the most abundant elements in nature. Fluoride can enter into the body of the animals commonly through drinking water, but there can also be various other sources of fluoride exposure like household and agricultural compounds having fluoride in them, vegetation contaminated with fluoride, emissions from industries or volcanic ash.

Aluminium is another widely distributed metal found on earth. It is used in the production of many products which are used in day-to-day life like cookware, soda cans, aluminium foil, antacids, aspirin, vaccines etc. The body does not need aluminium due to which it accumulates in the kidneys, brain, lungs, liver and even thyroid where it act as a competitor of calcium for absorption and can cause decrease in bone density. Aluminium exposure occurs frequently in some occupations like mining, factory work, and welding.

In today’s world looking at the side effects of synthetic drugs, there is need to find natural products which have therapeutic potential and can serve as an alternative to treat various diseases. One of these products is Nigella sativa (NS) which has powerful healing properties for many diseases. It is a store house of active ingredients like many volatile and non-volatile oils, proteins, carbohydrates, alkaloids, saponins, minerals, phenolic compounds, steroidal compounds etc. It also has a high content of unsaturated fatty acids and various essential oil components. Other main compounds include thymoquinone, P-cymene, Carvacrol, 4-terpineol, T-anethole and Sesquiterpene. But the most active component of NS is thymoquinone and its derivatives. Due to its antioxidant activity, it has the ability to remove free radicals and inhibit lipid peroxidation which becomes the main reason behind most of its ameliorative abilities.

Deferoxamine (DFO) is isolated from the bacterium Streptomyces pilosus and has a binding affinity towards iron and aluminium. It is used in case of iron and aluminum toxicity because it only binds with ferric iron whereas the iron in hemoglobin or cytochromes remains unaffected. Deferoxamine was approved for medical use in the United States in 1968 and it is also approved by WHO as a safe medicine which is required in the health system.
Metals co-exist in the environment and humans are simultaneously exposed to multiple metals which after entering the body interact with each other and form toxic complexes\textsuperscript{11,12}. But only few reports are available discussing the toxic nature of the metal combinations. Lot of research has been done on toxic effects of individual metals however, multimetal exposure is a new area in which lot of work needs to be done and thus this has become an area of interest for researchers around the world.

The main objective of our study is the analysis of toxicity induced by intraperitoneal treatment of combination of Flouride and aluminium in brain in terms of level of oxidative stress, DNA damage and apoptosis and possible reversal of toxicity by treatment with NS, DFO (chelating agent) alone and in combination.

MATERIALS AND METHOD:

Experimental animal: Male Swiss albino mice weighing around 25-30 g were used. The animals were kept in IIS (deemed to be university) animal house approved by CPCSEA (Registration No: 1689/PO/a/13/CPCSEA). They were maintained on natural light and dark cycle and were given free access to food and water. The animals were kept in the animal house for 15 days before the start of the experiment so that they can adjust to the surrounding conditions. Each group had minimum six animals at the time of autopsy.

Treatment agents and their dose:

20% of LD50 dose was used\textsuperscript{13}.

- Sodium fluoride + Aluminium chloride (F-AL): 5mg/kg b.w. + 50 mg/kg b.w.
- \textit{Nigella sativa} extract (NS): 200 mg/ kg b.w.\textsuperscript{14}. The ethanolic extract was prepared according to WHO protocol CG-04\textsuperscript{15}.
- Deferrioxamine (DFO): 100 mg/kg b.w.\textsuperscript{16}

Mode of administration of dose: The dose was administered intraperitoneally using syringe.

Treatment groups:

Four groups were formed which were treated intraperitoneally for one month (thrice a week on alternate days).

GROUP I: Control receiving distilled water
GROUP II: NaF+AlCl\textsubscript{3}
GROUP III: NaF+AlCl\textsubscript{3}+NS
GROUP IV: NaF+AlCl\textsubscript{3}+ DFO
GROUP V: NaF+AlCl\textsubscript{3} + NS+ DFO
Sample preparation:

At the end of the every dosing period, animals were killed by cervical dislocation and the brain was then gently removed on an ice-chilled glass plate and was homogenised separately in phosphate buffer saline (PBS). The homogenate was centrifuged at 10,000 rpm in cold centrifuge and the supernatant was used for biochemical analysis.

Biochemical parameters:

Catalase: Estimation of Catalase activity was done by method of Luck, 1974. The activity of enzyme is expressed as micromole of $H_2O_2$ decomposed /min/mg of protein using molar extinction coefficient of $H_2O_2$ as 0.036mM$^{-1}$cm$^{-1}$.

Superoxide dismutase (SOD): Estimation of SOD was done by method Kono et al, 1978. The enzyme activity was expressed as units /mg protein where one unit of enzyme is defined as the amount of enzyme inhibiting rate of reaction by 50%.

Glutathione peroxidise (GPx): GPx activity was measured using a modification of the method of Paglia and Valentine 1967. The activity of enzyme is expressed as nmole NADPH oxidised /minute/mg of protein.

Lipid peroxidation (LPO): LPO levels were measured using the conventional method of Beuge and Aust 1978. The concentration of MDA is calculated using extinction coefficient of MDA-TBA complex which is $1.56 \times 105$ M$^{-1}$ cm$^{-1}$ and the results are expressed as nanomoles MDA/mg protein.

Reduced glutathione(GSH): GSH levels were estimated by method of Moron et al, 1979 and calculations were performed using standard graph. Results are expressed as nanomole / mg protein.

Protein: Total Protein was estimated by method of Lowry et al, 1951. The result were expressed as mg of protein/g of tissue.

Histology of brain:

The control and experimental animals were sacrificed by cervical dislocation for each experimental group. Brain was dissected out and washed in normal saline and was fixed in 10% formalin. Then the brain was dehydrated through various alcohol gradients and was embedded in paraffin wax. Slides were prepared using haematoxylin and eosin staining.

DNA extraction:

Small amount of brain tissue was finely chopped with a sterile scalpel blade and was transferred to micro centrifuge tube. TNES buffer (600 µl) and 35 µl Proteinase-K (20 mg/ml) was
added to the tube. The samples were incubated overnight (or 5-24 hours) at 50°C. NaCl (6 M) was added and then the tubes were centrifuged at 12-14,000 rpm for 5-10 minutes at room temperature. Supernatant was transferred to a new microfuge tube to which an equal volume of cold 100 % ethanol was added. The contents of the tube were gently mixed by inverting the tube until white DNA precipitates out in the solution. The sample was then centrifuged at 12-14,000 rpm for 10-20 minutes at 4°C. DNA forms a pellet and the supernatant was removed without disturbing the DNA pellet. DNA pellet was washed with 70 % ethanol. After washing, 70 % ethanol was completely removed by pipeting. The DNA sample was left in air for 10-30 min to dry. After drying, the DNA was re-suspended in 100 µl of Tris-EDTA. Nanodrop spectrophotometer was used to quantify DNA and also to check its purity. The absorbance ratio was used to estimate sample purity. Only samples having ratio of 260 nm/280 nm= ~1.8 were used for electrophoresis to visualize DNA fragmentation (laddering), characteristic of apoptosis and then photographs were also taken.

**Immunohistochemistry:**

Formalin fixed, paraffin embedded tissue section were taken on the polylysine coated slides, and were kept in 0.3% Peroxidase solution for 5 minutes. Slides were then transferred to Tris buffer (2-3 minutes). For antigen retrieval the slides were a kept in citrate buffer (retrieval box) and then were washed with tris buffer. Background snipper was applied for 5 minutes. Primary antibody (GFAP and Caspase 3) was applied followed by washing with Tris Buffer twice. Secondary antibody was applied and were again washed with tris buffer. Incubation with DAB was done for visualization followed by counter staining with hematoxylin.
RESULTS:

Figure 1: The levels of various oxidative stress parameters in mice brain exposed to combination of fluoride-aluminium, NS and DFO for one month. Values are mean ± SD (n=6). Values with symbol *, ** Significant at 5% (P<0.05) and 1% (P<0.01) respectively.

# indicate the group which shows most significant level of ameliorative ability against fluoride-aluminium induced neurotoxicity.
Effect of NS and DFO treatment on fluoride- aluminium combination induced changes in various oxidative stress parameters (figure 1):

When mice were treated with combination of fluoride and aluminium intraperitoneally for one month, it resulted in a significant decrease (P<0.01) in levels of protein, catalase, GPx, SOD, GSH and a significant increase in LPO levels as compared to the control group. Simultaneous administration of NS along with fluoride and aluminium combination resulted in a significant increase (P<0.01) in the levels of protein, catalase, GPx, SOD, GSH and a significant decrease in LPO levels as compared to the fluoride and aluminium combination group. Simultaneous administration of DFO along with fluoride and aluminium combination resulted in a significant increase (P<0.01) in the levels of catalase, GPx, SOD, GSH and and protein (P<0.05). A significant decrease (P<0.01) in LPO levels was also observed as compared to the fluoride and aluminium combination group. The amelioration produced by NS was more significant than DFO. But when combination of NS and DFO was given along with fluoride-aluminium treatment the increase in levels of protein, catalase, GPx, SOD, GSH and decrease in LPO levels was most significant when compared to the groups in which NS and DFO were given individually along with fluoride and aluminium combination. Our results indicate that combination of NS and DFO has maximum ameliorative ability against fluoride-aluminium induced oxidative stress in brain.

HISTOLOGY:

Cerebellum (figure 2):

When mice was intraperitoneally treated with fluoride and aluminium (thrice a week on alternate days) for 30 days resulted in vacoulation and constriction of Purkinje cells (shown by the arrow) as compared to the control mice which showed normal appearance of perkinje cells ((figure N1). Pyknosis is also seen in purkinje cells with loss of Nissl substance from neuroplasm. There is a decrease in the density in the Purkinje cell layer, degeneration of nuclear contents (indicated by arrow) and chromatolysis is also observed in the granule cells (figure A). But when NS was simultaneously given along with fluoride and aluminium resulted in an increase in the density in the Purkinje cell layer, normal appearance of nucleus and dendrites of purkinje neurons (shown by arrow). The granule cells also appear to be normal (figure B). Combination of fluoride, aluminium and DFO lead to slight vacuolation around purkinje cells (indicated by arrow). Degeneration of dendrites of purkinje cells is also observed but the granule cells appear normal (figure C). When mice were treated with combination of fluoride, aluminium, NS and DFO, the purkinje cells appeared normal with a central nucleus and long dendrites (shown by arrow). The granule cells also appear to be normal with a round heterochromatic nucleus (figure D).
**Cerebrum (figure 3):**

Intraperitoneal treatment of mice for 30 days with fluoride and aluminium resulted in constriction of pyramidal cells and shrinkage of nucleus (indicated by arrow). Disorganisation of dendrites of pyramidal cells occurs is also observed. Astrocytes increase in size and number indicating gliosis. Pericellular edema is visible and capillaries appear dilated (figure A) as compared to the control group which normal appearance of pyramidal cells (figure N2). Fluoride, aluminium and NS administered simultaneously resulted in an increase in the density of the pyramidal cells. Nucleus and dendrites of pyramidal neurons also appear normal (indicated by arrow). Decrease in cellular edema in neuropil is seen. Capillaries also appear normal (figure B). When DFO was given along with fluoride and aluminium, it leads to slight vacoulation and edema in neuropil. But the pyramidal cells appear normal (shown by arrow) (figure C). Combination of fluoride, aluminium, NS and DFO resulted in normal histology of Pyramidal cells and astrocytes. Nucleus and dendrites of pyramidal neurons also appear normal (shown by arrow). Decrease in cellular edema in neuropil is also seen. The capillaries and neurofibrillar network also appear normal(figure D).

**FIGURE 2:** Showing T.S. Cerebellum (I.P. treatment) : (N1) Control (A) F + AL (B) F + AL + NS (C) F + AL + DFO (D) F + AL + NS + DFO
DNA FRAGMENTATION ANALYSIS:

Qualitative analysis of DNA fragmentation was done by Agarose gel electrophoresis. DNA equivalent to 4 µg was loaded for each sample. Lane C1 have DNA from brain of control mice which were given distilled water intraperitoneally for 30 days. The genomic DNA from lane C1 forms an intact band and no smearing is seen. Lane 1 has DNA from mice brain which were intraperitoneally injected with F-Al combination for 30 days shows high damage to genomic DNA indicated by small size of the smeared DNA fragments .Lane 2, 3 and 4 has DNA from mice brain which were intraperitoneally injected with F-Al combination for 30 days along with NS extract (Lane 2), DFO (Lane 3) and both NS extract and DFO (Lane 4). Lane 2, 3 and 4 shows only mild smearing indicating less fragmentation of DNA. These results suggest that both NS extract and DFO have
ameliorative abilities against F-Al induced DNA damage but their combination works best against F-Al induced DNA fragmentation as lane 4 DNA showed least smearing of DNA and an intact band of genomic DNA.

**GLIA FIBRILARY ACIDIC PROTEIN (GFAP) AND CASPASE 3 IMMUNOHISTOCHEMISTRY (FIGURE 5):**

Mice intraperitoneally treated with combination of F-AL for 30 days resulted in decrease in expression of reactive astrocytes (Figure A) in as compared to the control mice which showed relatively lower expression of GFAP positive cells (figure N1). But simultaneous administration of *Nigella sativa* along with F-AL (figure B) leads to an increase in GFAP positive cells and astrocyte reactivity. GFAP positive astrocytes are seen when DFO is given with F-AL combination (figure C) indicating that DFO alone can moderately activate astrocytes to compensate for the damage caused by F-AL combination. But when both NS and DFO are given simultaneously with F-AL combination to mice (figure D), there is increase in highly reactive astrocytes indicating that both NS and DFO are efficient in reducing neuroinflammation induced by F-AL combination by increasing the number of reactive astrocytes.

When mice were treated with combination of F-AL for 30 days, high level of expression of caspase 3 positive cells is observed (figure E) as compared to the control group (figure N2). But simultaneous administration of *Nigella sativa* along with F-AL (figure F) leads slight decrease in caspase 3 positive cells. Decrease in caspase 3 expression is seen when DFO is intraperitoneally given with F-AL (figure G) indicating that DFO alone can moderately reduce the toxicity caused by F-AL combination. But when both *Nigella sativa* and DFO are given simultaneously with F-AL intraperitoneally to mice (figure H), there is a decrease in expression of highly reactive brain cells expressing caspase 3 indicating that both NS and DFO are efficient in reducing neuroinflammation and toxicity induced by F-AL combination.
DISCUSSION:

Oxidative stress has been reported to be one of the possible cause of neural degeneration. Brain is prone to oxidative stress due to presence of high levels of polyunsaturated fatty acids, relatively low antioxidant capacity, presence of redox metal ions like iron and copper and high oxygen utilization. Antioxidants contribute to the organism’s defense through different mechanisms including ROS scavenging, increasing the expression and function of endogenous antioxidants and inhibiting the activity of ROS-generating enzymes. Free radicals along with ROS cause damage to lipids, proteins, enzymes, carbohydrates and DNA in cells and tissues. The undesirable oxidation produced due to ROS finally leads to cell death induced by DNA fragmentation and lipid peroxidation.
Aim of our study was to study the effect of NS and DFO combination against neurotoxicity induced by fluoride and aluminium combination. Our results of F-AL neurotoxicity are in agreement with Kaur et al (2009) who studied the effect of aluminium fluoride combination in rats and observed that there was an increase in LPO levels and decrease in SOD activity in different regions of brain like the cerebrum, cerebellum, and medulla oblongata. Our results are also in accordance with Akinrinade et al (2013) who concluded that aluminium and fluoride combination cause an increase in brain LPO and ROS formation. They also cause an increase in glia activation and inflammatory response. Their results indicated a close relationship between oxidative stress, neuronal inflammation and degeneration in aluminium fluoride induced neurotoxicity. The synergistic action of fluoride and aluminium was supported by Varner et al (1998) who observed that binding of aluminium with fluoride may increase aluminium bioavailability to humans from drinking water. Glynn et al (1999) also reported that inside body of rats aluminium forms soluble complexes with citrate and fluoride, such as aluminium citrate(97%) and aluminium fluoride (60%).

Simultaneous administration of Nigella sativa with Fluoride and aluminium combination proved to be very helpful in reducing the neurotoxicity produced. NS treatment produced significant increase in protein, GPx, SOD,GSH levels which were reduced due to fluoride and aluminium combination treatment. LPO levels were also reduced indicating antioxidant property of Nigella sativa. From the experimental and clinical studies performed on Nigella sativa, it seems that most of its pharmacological actions are due to its antioxidant activity, which is mainly due to its ability to scavenge free radicals and / or inhibit lipid peroxidation. NS is rich in tocopherols and thymoquinone, carvacrol, thymol, cymene, t-anethole and 4-terpineol are the major antioxidants of NS essential oil. Our results are in accordance with Akhtar et al, (2013) who observed that pre-treatment with NS extracts also prevented elevation of thiobarbituric acid reactive substance (TBARS) and reduction in glutathione and antioxidant enzymes, viz. superoxide dismutase (SOD) and catalase (CAT) following middle cerebral artery-occluded (MCAO). In another study, chloroform and petroleum ether extract of NS showed antioxidant, free radical scavenging, and anti-inflammatory properties.

Our results of histological alterations observed due to combination of fluoride and aluminium are in accordance with Akinrinade et al (2013) who reported that fluoride and aluminium combination resulted in like appearance of vacuolar spaces, cellular fragmentation and ghost-like appearance of neuronal cells in rat brain, while the cells of the control group appeared intact with regular cell morphology and intact cellular integrity. Nalagoni et al (2016) also reported that treatment with combination of aluminum and fluoride for 2 months causes enlarged cells,
neurofibrillary tangles, and vacuolar spaces in the cerebral cortex and suggested that the histological alteration observed may be a result of Oxidative stress caused by aluminium and fluoride combination\textsuperscript{32}. The use of NS extract to compensate for the decreased antioxidant status of brain cells due to fluoride –aluminium toxicity only has little ameliorative action as shown by our histology results. The seed extract was not able to completely avoid the toxic changes induced by the metal combination but was surely able to reduce the degeneration of brain cells. Ameliorative ability shown by DFO against fluoride and aluminium combination induced changes in histology of brain cells is comparatively less than NS but the combination of both agents (NS and DFO) produces most protective effect. These results of histology correlate with results of biochemical parameters obtained in our study. NS comparatively showed more potential than DFO to increase the activity of various antioxidant enzymes. Since NS is rich in various antioxidants, it decreases the oxidative stress induced by fluoride and aluminium combination. DFO being a chelator of aluminium helps in reducing the toxicity produced by removing aluminium in either in feaces or urine. Both these agents decrease oxidative stress which indirectly reduces the histopathological alterations produced due to fluoride and aluminium combination.

Our results of DNA fragmentation are in accordance with Ohyashiki \textit{et al} (2002) who reported that AlCl\textsubscript{3} treatment induce chromatin condensation and DNA ladder formation in PC12 cells\textsuperscript{33}. Aluminium is also reported to cause oxidation of nucleic acids, including the oxidation of DNA and the breaking of α-helix. The accumulation of many such changes in the DNA, detected by repair mechanisms, leads to the activation of the apoptosis pathway\textsuperscript{34}. The DNA damaging ability of aluminium is also observed by Walton \textit{et al} (2006)\textsuperscript{35} who reported that Al is centrally localized in the nuclear region compared to other intracellular organelles which indicate its DNA damaging potential. Further Lima \textit{et al} (2007) and Mohan \textit{et al} (2007) observed that aluminium treatment induces gaps and breaks in the chromosomes with higher frequency\textsuperscript{36, 37}. Tsubouchi \textit{et al} (2001) also found Al induces DNA strand breaks in PCD12 cells by the generation of reactive oxygen species (ROS), thus leading to apoptosis\textsuperscript{38}. Li \textit{et al} (1987) reported that Fluorine being a negatively charged ion has strong affinity for uracil and amide bonds by the interaction with –NH. Fl also induce lipid peroxidation which can further lead to generation of free radicals\textsuperscript{39}. Theses free radicals can damage DNA by decreasing the activity of DNA polymerase thus affecting DNA replication or repair mechanism\textsuperscript{40}.

In our study combination of fluoride and aluminium initially increases the GFAP immunoreactivity which could be explained by the fact that astrocytes play an important role in the formation of the blood-brain barrier and fighting against oxidative stress\textsuperscript{41}. Yu \textit{et al} (2015) found that astrocytes are more resistant to oxidative stress mediated apoptosis as they have low production of
reactive oxygen species (ROS) due to high buffering ability against ROS induced toxicity\textsuperscript{42}. However inspite of high GFAP expression, brain cells also showed moderate level of caspase 3 expression which can be explained by the finding that although caspase-3 is involved in the cleavage of cytoskeletal proteins including vimentin\textsuperscript{43} and GFAP in astrocytes indicating cell death\textsuperscript{44}. But cleaved GFAP and vimentin have also been found to co-localize with activated caspase-3 in non-apoptotic astrocytes\textsuperscript{45} suggesting that caspase activation alone is not sufficient for cytoskeletal remodeling but may contribute to astrogliosis\textsuperscript{46}. The decrease in GFAP reactivity and increase in caspase 3 expression after intraperitoneal treatment of mice with combination of fluoride and aluminium for one month can be explained by the ability of F-AL combination to activate caspases and induce apoptosis of astrocytes\textsuperscript{47}. Our results are similar to the findings of Varner et al (1998) who observed that chronic administration of aluminium fluoride and sodium fluoride in the drinking water of rats resulted in distinct morphological alterations in the brain\textsuperscript{27} and leads to the formation of abnormal connections between nerve fibres by interfering with the metabolism of the cytoskeleton in the nerve cells\textsuperscript{48}.

The exact mechanisms of neurotoxic actions induced by the interaction of fluoride and aluminium have still not been clearly established. But study conducted by Miles et al, 2002\textsuperscript{49} and Blaylock et al , 2004 reported that fluoro-aluminium complexes formed due to the interaction of F and AL mimic phosphate groups in biological systems\textsuperscript{50}. One of the possible mechanism of fluoride induced toxicity is excitotoxicity which involves the accumulation of acidic amino acids like cysteine, cysteine sulfenic acid and neurotransmitters like glutamate and aspartate in synaptic cleft. If these excitatory amino acids are not removed from synaptic cleft they cause prolonged stimulation and neuronal destruction by both apoptosis and necrosis\textsuperscript{51}. Due to this excitotoxicity free radicals and lipid peroxidation products are generated to damage dendrites and synaptic connections leading to neuronal destruction\textsuperscript{52}. Thus even if fluoride does not directly trigger excitotoxicity, it can cause it indirectly by production of free radicals and lipid peroxidation products. Ghribi et al,2001 reported about the important role played by endoplasmic reticulum (ER) in regulating aluminium induced neurotoxicity. The ER is the major storage location for calcium and contains members of the Bcl-2 family of proteins, Bcl-2 and Bcl-XL. The stress induced by aluminium in the ER has also been shown to result in a specific type of apoptosis mediated by caspase-12 and is independent of mitochondrial-targeted apoptotic signals\textsuperscript{53}. Blaylock,2012 also highlighted the aluminium activates glial cells leading to immunoexcitotoxicity, impairs a number of energy related enzymes, promotes brain inflammation, oxidative damage, reduces the levels of brain antioxidants (i.e., glutathione) and disturbs calcium homeostasis, thus confirming its role in neurodegeneration\textsuperscript{54}. The exact mechanism of aluminium toxicity to cells still remains unclear.
Simultaneous administration of NS with combination of fluoride and aluminium results in gradual increase in GFAP expression and decrease in caspase 3 immunoreactivity which might be due the ameliorative activity of NS to reduce the oxidative stress and induce astrogliosis to repair the damaged CNS and maintain the integrity of blood brain barrier (BBB). Administration of NS and DFO individually and simultaneously with F-AL combination causes an increase in astrocyte reactivity which indicates that NS and DFO individually have moderate ability to activate astrocytes to repair the damaged CNS but together they can more efficiently decrease the toxic effects of F-Al combination by chelating aluminium from body and acting against the induced oxidative stress and neurodegenerative changes by activating cells like astrocytes.

**CONCLUSION:**

Fluoride and aluminium combination was administered intraperitoneally for one month and NS and DFO, both alone and in combination were also given simultaneously through the same route. Our findings revealed that both NS and DFO resulted in a significant increase in various antioxidant enzymes and decrease in LPO levels showing its ameliorative ability against fluoride and aluminium induced toxicity. But NS caused more significant increase in antioxidant enzymes as compared to DFO. However combination of NS and DFO had the maximum ameliorative ability against fluoride and aluminium induced neurotoxicity.

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