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RES ARTICLE

Docosahexaenoic acid ameliorates aluminum induced biochemical and morphological alteration in rat cerebellum

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KEY WORDS

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ABSTRACT

Background: The omega-3 polyunsaturated fatty acids (PUFA), docosahexaenoic acid (DHA) have well-characterized effects on inflammation and oxidative stress and may have neuroprotective effects in a number of neurodegenerative conditions including AD. Brain tissue contains large amounts of polyunsaturated fatty acids, which are particularly vulnerable to free radical injury. **Purpose:** The present study attempts to examine protective effects of docosahexaenoic acid (100 mg/kg body weight) and on aluminum (100 mg/kg b. wt. of $AlCl_3$) mediated oxidative damage in the cerebellum in male albino rats along with the motor and learning ability and morphological changes. **Methods:** Twenty four male Rattusnorigious, Wistar strain rats (weight 220 $\pm$ 10 grams) were randomly divided into four groups (n = 12) viz. Group 1 served as control treated with normal saline, Group 2 treated with 100mg/kg body weight of DHA, Group three treated with 100 mg/kg body weight of $AlCl_3$ and Group four treated with 100mg $AlCl_3$ + 100 mg DHA for 90 days. Dose was directly introduced into the rat pharynx via a feeding cannula to rats for 90 days. Behavioral tests followed by biochemical analysis was performed. **Results:** A significant decrease in the antioxidant status (superoxide dismutase, catalase, glutathione peroxidase and glutathione) and increased lipid peroxide levels and protein carbonyl content in aluminum exposed rats was noted. After DHA supplementation these effects were reversed. Moreover, DHA also significantly ($p<0.05$) prevented aluminum induced dysfunctioning of the motor and learning ability. The light microscopic studies revealed altered Purkinje's neurons and granular layer. These changes were not seen in the DHA treated rats. **Conclusion:** On the basis of our results it may be concluded that Al may be linked with cerebellar degeneration and neuromuscular disorders while DHA helps to prevent these alterations.

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Introduction

The cerebellum is involved in the control of movements, particularly those linked to the voluntary nervous system and movements where timing is an important aspect. It co-ordinates the different muscle groups so that the muscle exerts movements fluently and precisely.[1] Nearly 50% of all neurons of the brain are located in this region, which takes up only 10% of the total brain volume and receives nearly 200 million afferent fibers.[2] It receives continual feedback information about intended movement and actual movements. The cerebellum is often regarded as a stabilizing control system which receives forewarning of each motor impulse. Atrophy of the cerebellum is usually accompanied by different forms of ataxia and an unstable gait. There are a large number of environmental chemical compounds and metals which affect the cerebellum. Among these chemicals, metal, especially aluminum (AL) belong to a group of widespread neurotoxicants. Moreover, AL is abundantly present in the earth's crust. From the environment, it gets access to the human body via the gastrointestinal and the respiratory tracts. AL is a constituent of cooking utensils and medicines such as antacids, deodorants and food additives[3] and this has allowed its easy access into the body. Since, cerebellum is involved in fine coordination and control of voluntary movements and it may be vulnerable to injury, particularly toxic insult in neurodegenerative diseases.[4] AL promotes the formation of amyloid-β protein plaques[5,6] by aggregating tau proteins in Alzheimer's disease.[7]

The omega-3 polyunsaturated fatty acids (PUFA), docosahexaenoic acid (DHA) have well-characterized effects on inflammation and oxidative stress and may have neuroprotective effects in a number of neurodegenerative conditions including AD. Brain tissue contains large amounts of polyunsaturated fatty acids, which are particularly vulnerable to free radical injury. Brain is highly enriched in long chain polyunsaturated fatty acids (PUFAs) particularly DHA which plays an important role in brain structural and biological functions.[8] In view of the aforementioned considerations, the present study was designed to investigate the protective effect of DHA on AL induced altered neuromuscular coordination and motor learning capacity along with oxidative damage in cerebellum as therapeutic agent.

Methods

Chemicals: Nitrobluetetrazolium (NBT), thiobarbituric acid (TBA), phenazinemethosulphate (PMS), nicotinamide adenine dinucleotide (NADH), 5.5′-dithio *bis* 2- nitrobenzoic acid (DTNB), nicotinamide adenine dinucleotide phosphate (NADPH) tricholoroacetic acid (TCA) and reduced glutathione (GSH) were purchased from Sigma Chemical Co., St. Louis, MO, USA and all other reagents used were of high quality and analytical grade.

Animals

Twenty four male Rattus norigious, Wistar strain rats (weight 220 $\pm$ 10 grams) were taken from NIMS University animal house. The animals were separately housed in polypropylene cages in a room, which was maintained at a temperature of 22 $\pm$ 2^0C, relative humidity of 50 $\pm$ 10% and 12 h light dark cycles. They were fed with commercial pellet diet and allowed access to water *ad libitum*. The Institutional Animal Ethics Committee approved the study prior to the initiation of the experiment and also approved all experimental protocols.

Treatment

Animals were randomly divided into four groups (n = 12) viz. Group 1 served as control treated with normal saline, Group 2 treated with 100 mg/kg body weight of DHA, Group 3 were treated with 100 mg/kg body weight of $AlCl_3$ and Group 4 treated with 100 mg $AlCl_3$ + 100 mg DHA for 90 days. Dose was directly introduced into the rat pharynx via a feeding cannula to rats for 90 days.

Behavioral studies

The motor activity, behavioral changes, muscle coordination, sensory and motor reflex responses were assessed in control and experimental rats as per the previously described protocol.[9,10] Muscle coordination test (Rota rod): The rotating rod (speed: 5 rotations / min; Total duration of test 2 min) for each control and treated rat were recorded by Rotamex (Techno Electronics, India). The rats were trained to stay for a period of 2 min on rotating rod and only trained rats were included in the study. Motor was measured using Rota Rod for at least 5s and it was rotated at a speed of 10 rpm for 2 consecutive days on third day. The time duration of each rotation speed was also recorded. Active avoidance test: Cognitive behavior was assessed by the number of times the animal escapes, in the series of 10 test trials. The apparatus for this test consists of two chambers separated by a partition. One chamber was lit in which the animals are housed. After 10 sec, the buzzer was set on and another 10 sec, an electric shock at 60 V was given. The animals jumped to the other compartment as soon as the buzzer was set on implying the animal has avoided the test. However, on other hand, the animal's jumps to the other compartment after shock or no jump at all is termed as escapism. A total of 10 trials were given to every animal. To qualify, the animal jumps to avoid at least 8 times out of 10. Passive avoidance test: A test was used to assess the short-term memory which was carried out in both control and experimental rats by Y maze (techno co. 40 cm long x 13 cm height x 10 cm width). Y maze test is a gross test for spatial memory. This test was used to see if the mouse remembers the arm it had just explored and therefore enters in one of the other arms of the maze. Rats were placed at the bottom of the Y (middle arm) maze and are allowed to explore freely in all three arms for an eight-minute session. The first two minutes were meant for habituation and for the last six minutes the alteration between arms was recorded via photo beam breaks. The acquisition time was noted to determine the short-term memory.

Biochemical Analysis

Tissue homogenate preparation

The rats were sacrificed after 90 days of experiment. Their brains were removed and weighed individually. Thereafter, cerebellum was dissected out for biochemical analysis. Ten percent (w/v) homogenate of the cerebellum was prepared using York's homogenizer fitted with Teflon plunger in 0.1 M phosphate buffer (pH 7.1). The whole homogenate was first centrifuged at 2500 × g for 10 minutes in a refrigerated centrifuge. The pellet consisting of nuclear fraction and cell debris was discarded. The supernatant was further centrifuged at 11,000 × g for 15 minutes and mitochondrial fraction was separated. The clear supernatant was further centrifuged at 105,000 × g for 90 minutes and the resultant supernatant was used for determining enzyme activities.

An aliquot of frontal cortex of the rat brain homogenate was used for the assay of enzymatic antioxidants. The superoxide dismutase (SOD EC 1: 15.1.1) activity was determined from its ability to inhibit the reduction of NBT in presence of PMS according to the method of McChord and Fridovich.[11] The reaction was monitored spectrophotometrically at 560 nm. The SOD activity was expressed as U/mg protein (1 unit is the amount of enzyme that inhibit the reduction of NBT by one half in above reaction mixture). Catalase (CAT, EC 1.11.1.6) activity was assayed as per the method of Aebi et al[12] using hydrogen peroxide as substrate; the decomposition of H_2O_2 was followed at 240 nm on spectrophotometer. The CAT activity was expressed as U/mg protein. The glutathione peroxidase (GSHPx, EC 1.11.1.0) was assayed by the method of Pagila and Valentine[13] using GSH, NADPH and H_2O_2 as reactants. The oxidation of GSH into GSSG was measured in terms of oxidation of NADPH to $NADP^+$ and assayed as decrease in the absorbance of reaction mixture at 340 nm on spectrophotometer. The activity of GSHPx was expressed as n moles of NADPH oxidized/min/mg protein. Glutathione reductase (EC.1.6.4.2, GR) activity was assayed by the method of Hazelton and Lang.[14] Activity of GR was expressed as nmoles of NADPH oxidised/min/mg protein of cell extract. Reduced glutathione was measured in deprotonized supernatent from various brain regions. Tissue homogenate was deproteinated with tetrachloroacetic acid, centrifuged and supernatant was used for the estimation of reduced glutathione (GSH) by the use of Ellman reagent (5, 5' dithiobis (2-nitro benzoic acid). The optical density of the pale colour was measured on the spectrophotometer on 412 nm. An appropriate standard (pure GSH) was run simultaneously. The level of GSH was expressed as μg/g tissue.[15]

Light microscopy

For the evaluation of histopathological changes in cerebellum, small sections of the tissue were fixed in buffered formalin solution for 5 to 6 days. After fixing the tissues, they were thoroughly washed under running water and dehydrated in graded ethanol. Then the tissue pieces were cleared in benzene and finally embedded in paraffin (60ºC) for 6 hrs for proper impregnation of wax. The blocks were made in paraffin and 8-10 mm thick sections were cut for histological changes in control and experimental groups with hemotoxylin/eosin staining.

Statistical Analysis

Experimental data was summarized as Mean ± SE. Groups were compared together by one way analysis of variance followed by Student Newman-Keuls post hoc test. The acceptance level of significance was $p<0.05$. InStat (version 3) was used for analysis of data.

Results

Behavioral profiles

Behavioral profiles namely SMA, rota rod, passive and active avoidance has been presented in Figure -2. SMA was found to insignificantly ($p>0.05$) reduced in Al treated rats. The muscle in-coordination (Rota Rod) was reduced significantly ($p<0.05$) in Al treated rats. On the other hand altered passive and active avoidance was also observed. These changes were found to be markedly ($p<0.05$) reversed following the co-administration of DHA.

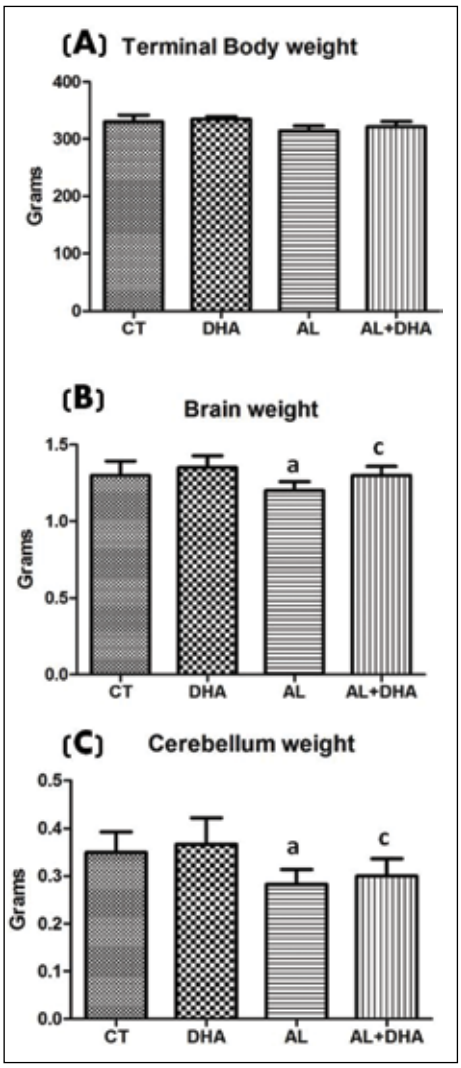

Fig. 1: Weight profiles body, brain and cerebellum weight. Values are expressed as mean ± SEM for six animals in each group. The superscripts relate significant ($p<0.05$; one way ANOVA) comparison between control and Al treated (a), Al treated and DHA (c).

Body, brain and cerebellum weight

There is insignificant ($p>0.05$) change in terminal body weight which was observed between groups (Fig-1A) while, the brain and cerebellum were found to be reduced in Al treated group as compared to controls. The co-administration with DHA was found to be increased ($p<0.05$) brain and cerebellum weight (Fig-1B and 1C) when compared with Al rats.

Biochemical study

The profile of oxidative stress markers are presented in Figure-3. Lipid peroxide levels and protein carbonyl content was increased significantly ($p<0.05$) in Al treated rats when compared with their respective controls. The co-administration of DHA showed significant ($p<0.05$) reversible changes when compared with the Al treated rats. On the other hand, the activity of antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase was found to reduced markedly ($p<0.05$) in Al

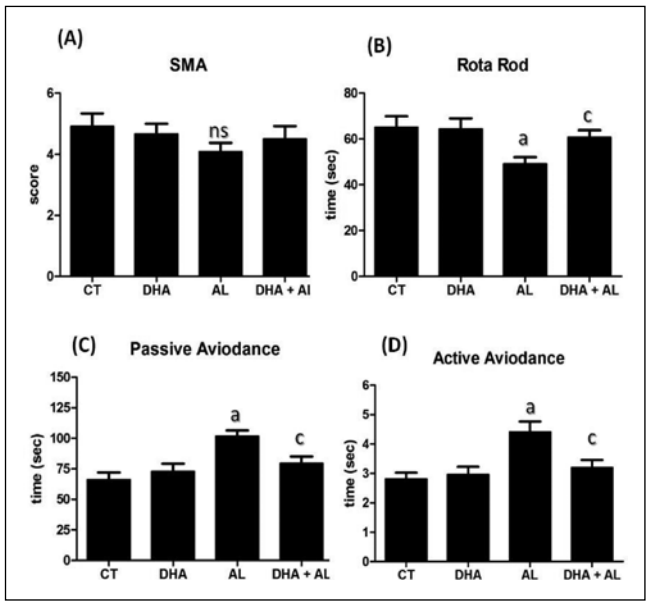

Fig. 2: Behavioral profiles (SMA, Rota Rod, Passive and Active avoidance) activity. Values are expressed as mean ± SEM for six animals in each group. The superscripts relate significant ($p<0.05$; one way ANOVA) comparison between control and Al treated (a), Al treated and DHA (c).

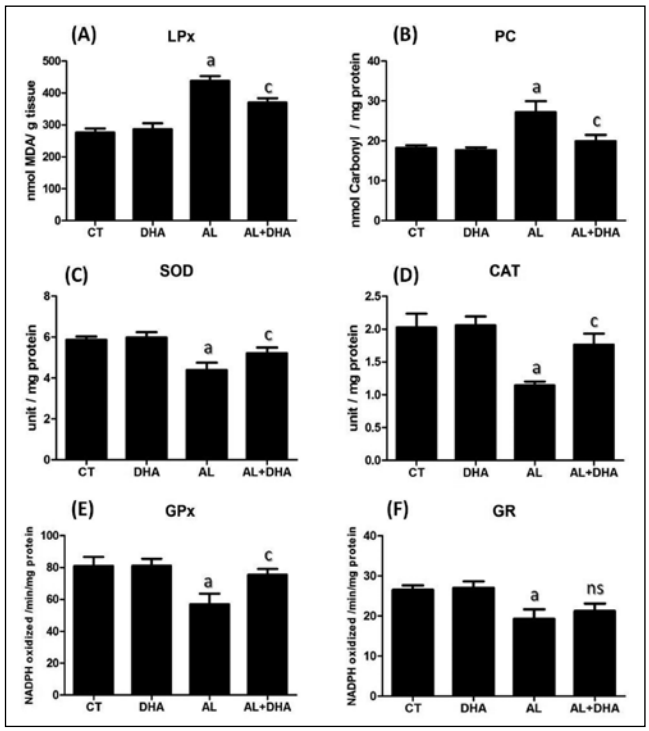

Fig. 3: Levels of Protein carbonyl content (PC), lipid peroxide levels (LPx) and activity of superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GSHPx) and Glutathione reductase in control and aluminum treated group. The results are expressed as Mean ± SEM in six rat of each group. Superscripts relate significant ($p<0.05$) comparison with Control (a), Al treated (b) and AL+ DHA treated (c).

treated rats as compared with controls but these were increased significantly ($p<0.05$) in co-administered with DHA groups when compared with Al treated groups. The glutathione content was also found to be increased significantly in DHA treated group as compared with Al treated rats.

Microscopic study

Figure 4 depicts photomicrographs of the H & E stained section of the cerebellum of control and experimental rats. The control section of the cerebellum (Fig, 4A & 4B) showed normal histological features with well-organized three cortical layers. The superficial molecular layer (M) is occupied mostly by axons and dendrites, middle monolayer of Purkinje cells (P), the dense layer of granular cells (G) and the white matter in the center of folium are clearly visualized (40× and 100× respectively). Al treatment for 90 days was enough to disrupt the normal arrangement of three layers. Large spaces in between Purkinje's cell layer and molecular layer or granular layer were seen (Fig. 4C and 4D). The section obtained from Al + DHA treated rats (fig, 4G and 4H) exhibited well maintained architecture resembling control rats. There were no apparent degenerative changes in any of the three layers (40X and 100X respectively). The histopathological observation revealed that the Purkinje cells in the cerebellum were the most affected cell population and there was an increase in the number of granular cells but a decrease in the number of Purkinje cells and molecular cells.

Discussion

In the present study, we evaluated Al induced cerebellum toxicity and modulation by docosahexaenoic acid. The morphological and biochemical assessment was carried out and their associations with motor and learning ability were tested. Primarily, we observed altered neuromuscular coordination and reduced learning response in Al treated rats after 90 days of treatment. The effect of Al on body weight gain, food intake and feed efficiency was progressively increased during the experimental period. The final body weight of intoxicated rats (Fig.1A) with Al was insignificantly reduced than that of the healthy normal group. The brain and cerebellum weight was found to be reduced significantly. The obtained results are in agreement with the findings of our previous study.[16] It is suggestive that loss of brain and cerebellum indicates that Al induces neurotoxicity and it may be due to loss of lipid, protein and other biomolecules.

Several neurological manifestations have already been attributed to Al administration in humans, including memory loss, tremors, jerky movements, loss of curiosity, ataxia, myoclonic jerk and convulsions.[17] Furthermore, we observed that co-administration of Al alongwith DHA to rats significantly reversed their behavioral changes.

The present study shows that high Al administration potentiates oxidative stress and cause lipid peroxidation and protein carbonylation in the cerebellum. The initiation of antioxidative defense enzymes to oppose the oxidative stress was reported in this study. Study findings revealed that the activities of SOD, catalase and GPx were decreased with increasing lipid peroxidation and protein oxidation events in the cerebellum. These finding are consistent with several other studies which reported significant decrease in antioxidant enzymes in the cerebellum.[17-19] This may be due to Fe mediated production of ROS. Brain contains large amount of polyunsaturated fatty acids; they can react with free radicals and undergo peroxidation.[20,21] However, Al in biological system do not have any direct pro-oxidant properties but it potentiates Fe to promote the formation of ROS and enhance peroxidative damage to lipids and proteins.[22] It is reported that Fe and Al bind to transferrin

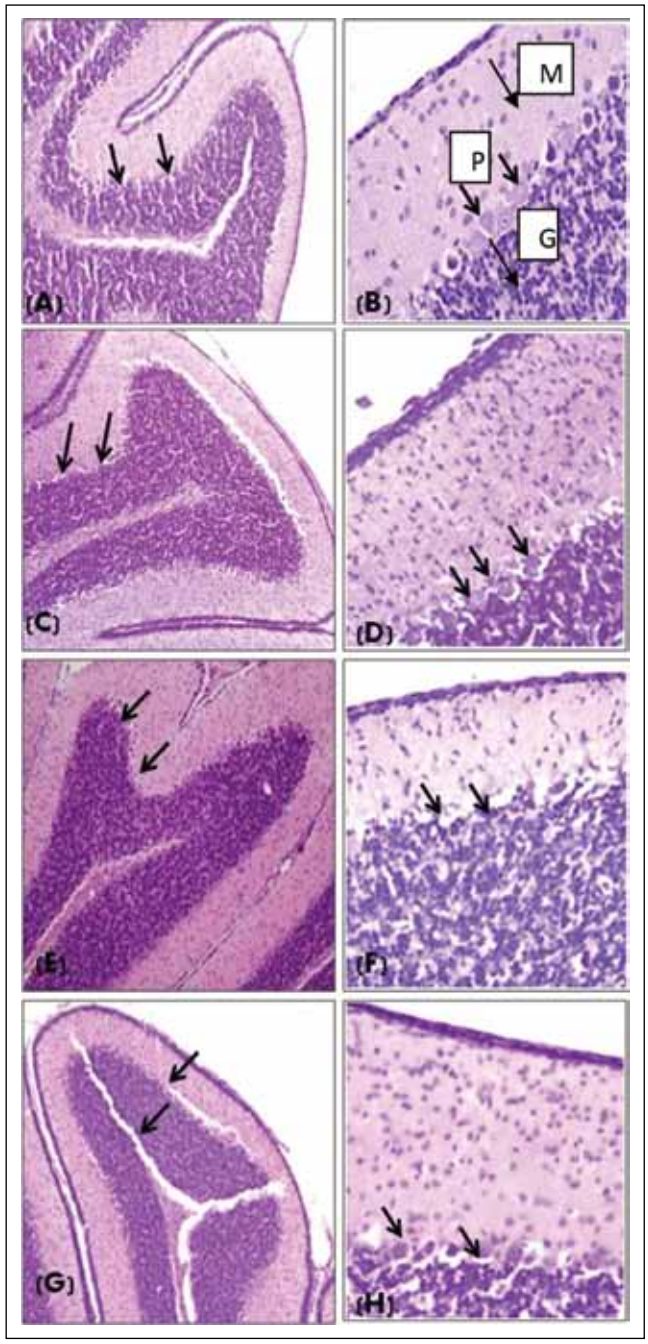

Fig. 4: Light photomicrograph of H & E stained section of rat cerebellum. (A & B) Representative section from the control group shows three cortical layers namely molecular (M), purkinje cell layer (P) and granular cell layer (G). Well architecture layer of the cerebellum of DHA treated rats (C & D). Photograph (E & F) shows damaged purkinje cells of Al treated rats. Section of cerebellum (G & H) shows maintained architecture of three layers of the DHA + Al treated rats.

receptor before crossing the blood brain barrier via transferrin-mediated endocytosis and enter into the brain.[23] Earlier it has been reported that the increased lipid peroxidation and protein oxidation in Al neurotoxicity may be due to the accumulation of excess iron and it may further lead to an increase in Fe catalyzed Fenton reaction resulting in generation of more reactive oxygen species.[16]

Results of our histopathological studies (H&E) have revealed that Al effects the architectural integrity of the cerebellar cell layers in Al treated rats. The alteration of Purkinje's and granular cell layers of the cerebellum, as reported here may be responsible for the associated functional changes.[24] The results of our studies suggest that the alterations in Purkinje's and granular cells of the cerebellum which could have directly contributed to neuromuscular alterations and performance activity of the brain.

Effect of DHA on Aluminum induced changes in cerebellum

In the present study we observed reversal of changes in Al induced behavioral alteration after co administration of DHA. It is possible that DHA exerts neuroprotective effect on synapse. This help nerve cells to release neurotransmitters. Brain cells whose membranes are rich in DHA, therefore, communicate more quickly with each other. Several mechanisms have been proposed to explain DHA's action as a cellular antioxidant in certain experimental conditions: regulation of the level of reactive oxygen species due to its chemical structure, modulation of membrane properties, or activation of cellular antioxidant enzymes.[25,26] DHA is now recognized as a physiologically-essential nutrient in the brain. Mechanism of action of DHA in the nervous system including its modulators effect on the activity ion channels, are thought to underlie its role in supporting electrical signaling and brain functioning including learning and memory.

On the basis of these results, it may be concluded that DHA may prove efficacious in ameliorating the aluminum-induced alterations in neuromuscular and cognitive behavior and neurochemical changes of rat cerebellum. Moreover, our results also showed that DHA significantly recovers endogenous antioxidants (i. e., CAT, SOD, GPx, and GSH) which protects neurons against ROS. DHA possess certain additional antioxidative and neuroprotective properties therefore, it may be used as supplement for the treatment of neurodegenerative disorders. However, there is a need for further in depth studies to confirm these findings.

This article complies with International Committee of Medical Journal Editor's uniform requirements for manuscript.

Competing interests: None

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