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ORIGINAL ARTICLE

Alterations of the Cerebellar Cortical Structure after Aluminum Chloride Perinatal Exposure in Albino Rats. Does Omega-3 Minimize the Noxious Effect of Aluminum?

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ABSTRACT

Background: Aluminum (AL) is a well-known neurotoxin that impedes more than 200 biologically essential tasks, with effects such as reduced cognitive functions and severe behavioral irregularities. The omega-3 fatty acid is an essential nutrient that may antagonize AL-neurotoxicity. The current study aimed to illustrate the possible protective role of omega-3 on perinatal AL exposure on cerebellar cortical structure in albino pups.

Methods: After mating, 28 pregnant female rats were divided equally into four groups. All treated groups received drugs from day 14 (D14) of gestation until D21 after birth. The pups of each group were divided into subgroups according to the day of sacrifice, either postnatal D14 or D21. Cerebellum specimens were processed for light microscopy, immunohistochemistry, morphometric, and biochemical studies.

Results: There was a significant decrease in weight gain and superoxide dismutase (SOD) levels and a significant increase in the malondialdehyde (MDA) levels compared to the respective groups. The AL-treated groups revealed alteration in the thickness of different cerebellar cortical layers, decrease Purkinje cell count and increased optical density of caspase-3 immunostaining at postnatal D14 and D21.

Conclusions: The cerebellar cortical structure revealed marked alterations in albino pups after exposure of their mother to AL during gestation and lactation periods. Omega-3 played a protective role against AL- toxicity.

Keywords: Aluminum; omega3; developing cerebellum; albino rat offspring.

INTRODUCTION

Aluminum (AL) is considered the third most common element on the earth after oxygen and silicon [1]. AL compounds are broadly used in a wide variety of products from household cookware and storage utensils to water purification mediators, food additives, toothpaste, and pharmaceuticals (such as antacids, vaccines, phosphate binders, anti-diarrhea drugs, and allergy immunotherapy injections)[2]. Antacids have 300–600mg aluminum hydroxide per tablet or capsule. Buffered aspirin may contain 10–20mg of AL per tablet. Vaccines may have small amounts of AL compounds, no more than 0.85mg/dose [3].

Domingo et al. [4] reported that oral exposure of newborns to 100 to 400 mg AlCl3 from their mother from 14 days before birth until weaning interrupted the development of the central nervous system of pups. The brain is a target organ in AL–toxicity for many reasons. First, AL crosses the blood-brain barrier (BBB) and then accumulates in the cortex through varying the physiological ligands present at these barriers [5]. Second, the brain has low glutathione (GSH) content, high oxygen turnover, and low mitotic rate, as well as a low antioxidant concentration [6].

Other studies revealed that pups are the most vulnerable to aluminum toxicity than adult rats in the lactation period due to the peak in synaptic plasticity during this period. In humans, the maximum development of neural tissues occurs during the prenatal period; however, in rats, it occurs mostly in the postnatal period [7]. Exposure to high levels of AL has been shown to lead to

increased concentrations of AL in degenerating neurons and neurofibrillary degeneration. Therefore, aluminum has been implicated in several neurodegenerative disorders, including Alzheimer's disease, dementia, and Parkinson's disease [8]. Shamasundar et al. [9] declared that aluminum disturbs the pro-oxidant/antioxidant balance of tissues by producing free radicals and inhibiting the activity and expression of the antioxidant enzymes catalase (CAT), GSH reductase (GR), and GSH peroxidase (GPx); at the same time, AL induces superoxide dismutase (SOD) activity and gene expression. Cells have various mechanisms to fight oxidative stress and repair damaged macromolecules. The primary defense is offered by enzymatic antioxidants that scavenge reactive oxygen species. The levels of the most important antioxidant enzymes, SOD, CAT, and GPx, are low in the cerebellum of mothers and their pups[10].

Omega-3 fatty acids are a family of unsaturated fatty acids (α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)), which are essential nutrients that must be acquired from food as humans are unable to synthesize these fatty acids de novo. Omega-3 fatty acids are important in brain function, as well as normal growth and development, and have been associated with many health benefits in some psychiatric and neurological disorders, particularly neurodegenerative diseases such as dementia [11].

Therefore, the current study aimed to illustrate the possible protective role of omega-3 on perinatal AL exposure on cerebellar cortical structure in albino pups.

METHODS

Chemicals

Aluminum chloride ($AlCl_3$): These silvery-white crystals with a molecular weight of 133.34; g/mol were dissolved in distilled water and given at a dose of 50 mg per kg. The Aluminum chloride solution was prepared freshly every day. The dose was taken as follows: 50 mg of its solid crystals were dissolved in 5 ml of distilled water. Each pregnant rat was administrated 1ml of distilled water (contained 10mg of Aluminum chloride) orally via gavage once daily. The $Alcl_3$ was obtained from El-Gomhoria Company for Pharmaceutical & Chemical Industries at Mafareq Moaf el Mansoura street, Zagazig, Sharkia, Egypt. [12]

Omega-3: is available in the form of soft yellow gelatin capsules (LYNAE Omega-3 Fish Oil Dietary Supplement) made by LYNAE company, USA. Each capsule has 1000 mg of fish oil, 180 mg epicosapentaenoic acid (EPA), and120 mg docosahexaenoic (DHA)). Omega-3 was given at a dose of 20 mg per kg dissolved in corn oil. The dose was taken as follows: 20 mg of omega-3 were dissolved in 2.5 ml of corn oil. Each pregnant rat was administrated 0.5ml of corn oil (contained 4mg omega-3) orally via gavage once daily[13].

All other reagents that measured oxidative stress enzymes were of high quality and analytic grade.

Animals

Albino rats were the animals of choice for this experimental study because it is easy to handle, and transport, are a formidable breeder and they have a short life span to facilitate the study of different generation in a short duration. Twenty-eight fertile non-pregnant female albino rats (weighing between 180-200 gm) and fourteen fertile adult males (weighing between 180-200 gm). The rats were obtained from the animal house, of the Faculty of Medicine, Zagazig University and were housed under controlled laboratory conditions with a 12-h dark and 12-h light cycle. Animals were fed a standard rodent pellet and allowed free access to food and water. Before the experiment, rats were acclimatized to the experimental conditions for one week. All the procedures performed on rats were approved and conducted following the ZU-IACUC committee (Approval No. ZU-IACUC/3/F/14/2018). Adult females were housed with adult non-treated males at a ratio of 2:1 in each cage. Then vaginal smears were taken from the adult females the next day to detect the occurrence of pregnancy. The day on which sperm were found in the vaginal smear was considered day one of gestation. All animal experiments comply with the ARRIVE guidelines and should be carried out following the U.K. Animals.

Experimental design

Twenty-eight pregnant female rats were separated, and divided into 4 groups (each group containing 7 pregnant rats) as follows:

Group I (Control group): is divided randomly into 2 control groups: a) Control group I-A: Each pregnant rat was administrated 1ml of distilled water (the solvent of AL) orally once daily from day14 of pregnancy until birth. b) Control group I-B: Each pregnant rat was administered 0.5ml of corn oil (the solvent of Omega-3) orally as a single daily dose from day14 of pregnancy until birth. Then continued to receive it until D21 after birth.

Group II (Omega-3-treated group): Omega-3 was given at a dose of 20 mg per kg dissolved in corn oil. 20 mg of omega-3 were dissolved in 2.5 ml of corn oil. Each pregnant rat was administrated 0.5ml of corn oil (contained 4mg omega-3/rat) orally as a single daily dose [13] from day14 of pregnancy until birth. Then continued to receive it until D21 after birth.

Group III (AL-treated group): AL was given at a

dose of 50 mg per kg. The dose was taken as follows: 50 mg of its solid crystals were dissolved in 5 ml of distilled water. Each pregnant rat was administrated 1ml of distilled water (contained 10mg Aluminum chloride/rat) orally via gavage once [14] from day14 of pregnancy until birth. Then continued to receive it until D21 after birth.

Group IV (AL + omega-3-treated group): the pregnant females received omega-3 as a protective agent and AL by gavage at the same doses mentioned before from day14 of pregnancy until birth. Then continued to receive it until D21 after birth.

At birth, each mother was housed with her pups for lactation in a large cage in a ventilated room at a constant temperature (25°C) under a 12:12-h light/ dark cycle. Four of their offspring were taken at D14. The other four offspring were taken at D21 in each group. At the end of the experiment and during overnight fasting. The pups were anesthetized with sodium pentobarbital (60 mg /kg BW) [15].

Bodyweight and cerebellar weight measurements

Just before the pups were sacrificed, a careful record of body weight was measured in all groups at birth. The average weight of pups used in this study at D14 was (26.3 -28.1 gm) and at D21were (30.1 -32gm). After sacrifice, the cerebellum was removed and washed with normal saline, and all extraneous material was removed. The cerebellar weight was then recorded, and the results were expressed in grams (gm).

Assay of antioxidant and oxidative stress biomarkers in the cerebellar cortex homogenate

For evaluation of oxidative status, the cerebellar cortex homogenates were prepared at the clinical pathology department, Faculty of Veterinary Medicine, Zagazig University. The following parameters were measured: SOD and MDA values.

Measurement of Superoxide Dismutase(SOD) in the cerebellar homogenate

SOD expression was assayed according to the method described by Kakkar et al.[16]. The results were expressed in U/g.

Measurement of malondialdehyde (MDA) value in the cerebellar homogenate

Malondialdehyde is a degradation product of peroxidized lipid. It was measured according to the method described by Ohkawa et al.[17] . The results were expressed in nmol/g.

Tissue preparation for histopathological and immunohistochemical analyses

The cerebellar specimens were fixed in Bouin's solution for 3 days. After fixation, the cerebellar specimens were dehydrated in ascending grades of ethanol, cleared in benzene, embedded in paraffin, and sectioned to obtain paraffin sections (5-7 µm thick). Some paraffin sections were deparaffinized and hydrated, followed by staining with hematoxylin and eosin (H&E), for histopathological examination [18].

Immunohistochemistry was performed using primary antibodies against caspase-3 (apopain, SCA-1, Yama, and CPP32 US Biological, USA), and the brown reaction product in antigen-containing cells with a blue-stained background was assessed [19]. The specimens were prepared at the histology department, Faculty of Medicine, Menofia University. The stained slides were examined by a LEICA DM500 light microscope at the Anatomy Department, Faculty of Medicine, Zagazig University, Egypt.

Morphometric analysis

In all groups, the sections underwent H&E staining, and the average thickness of the cerebellar layers was done by taking 10 different thicknesses for each folium at magnification X 100. The ten slides of ten offspring albino rats were taken at the mid-sagittal sections of all groups. Then the five central folia were selected on each slide to measure the thickness of the different cerebellar layers. The Purkinje cell count was measured at a magnification of X 400, and the optical density of the immunohistochemical staining for caspase-3 was measured at a magnification of X 400. All sections were examined and photographed using a Leica DM500, (German) photomicroscope. Image analysis and morphometric measures were performed using Image J (FIJI) software.

Statistical analysis:

Data were statistically analyzed for descriptive measurements including arithmetic mean and standard deviation using SPSS program version 23. The one-way ANOVA test [20] was performed for comparison between the different groups (i.e. control, Omega 3, AlCl3, and Omega 3+AlCl3) and followed by a post hoc test using the Duncan multiple range (DMR) test for comparisons between means of groups.

RESULTS

Body and cerebellar weight

Table (1) showed that all studied factors (i.e. groups and times) significantly affected the body weight and cerebellar weight of postnatal pups (D14, D21) ($p < 0.05$), Regarding the body weight, the control group recorded the highest bodyweight of rat (26.74 ± 1.09 and 31.50 ± 0.94 gram). After 14 and 21 days, respectively from rat birth Al. The chloride group gained the lowest body weight among rats (15.93 ± 3.20 and 25.37 ± 0.82). While co-administration of omega 3 with Al Chloride gained weight (25.10 ± 1.03 and 29.56 ± 0.58) respectively when compared to Al Chloride treated- group at postnatal day 14 and 21. The

cerebellar weight was significantly affected by all studied factors (i.e. groups and times). However, control group recorded the highest cerebellar weight (0.29 ± 0.01 and 0.34 ± 0.02gram). Al. The chloride group, gained the lowest cerebellar weight (0.19 ± 0.02and 0.23 ± 0.02) after 14 and 21 days of birth, respectively. While Al Chloride+ omega 3-treated group increase its cerebellar weight (0.28 ± 0.02 and 0.31 ± 0.01) respectively when compared to Al Chloride treated- group at postnatal days 14 and 21.

Evaluation of antioxidant and oxidative stress biomarkers

Table (2), showed that all studied groups significantly affected the SOD and MDA values of postnatal pups. Al Chloride treated- group recorded the lowest SOD value on postnatal day 14 and 21 (1.82 ± 0.03 and 1.52 ± 0.30) respectively. While co-administration of omega 3 with Al Chloride revealed an increase of SOD value (1.54 ± 0.05 and3.08 ± 0.09) respectively when compared to Al Chloride treated- group at postnatal day 14 and 21. The MDA value showed marked elevation (19.83 ± 0.57 and 12.52 ± 1.86) in Al. chloride-treated group when compared to the control group respectively. While Al Chloride +omega 3-treated group revealed a decrease of the MDA value (12.07 ± 0.50 and12.01 ± 0.72) respectively when compared to Al Chloride treated- group at postnatal days 14 and 21.

Histopathological and immunohistochemical analyses: Light microscopy examination

I. Cerebellar sections at postnatal D14

D14 control group: the general architecture of the cerebellum in this group was divided into several well-defined short broad folia separated by short deep narrow sulci or fissures. Each folium consisted of the cerebellar cortex and a less defined white matter core that was well differentiated from the internal granular layer. The cerebellar cortex at this age was formed of four layers that were arranged from superficial to deep as follows: the outer external granular (EG) layer (covering the folia surfaces and lining the sulci), molecular (M) layer, Purkinje cell (P) layer and internal granular (IG) layer (Fig. 1a). The IG layer was clearly-differentiated and packed with condensed and darkly- stained granule cells. The Purkinje cells were arranged in a single row between the M and IG layers. These cells were pyriform in shape. The network of Purkinje cell dendrites (forming arborizations) originated from the upper end of cell bodies and accumulated in the M layer. The M layer showed a high intensity of vertically migrating spindle-shaped cells, reflecting a high migration rate at this stage. The external granular layer is composed of darkly-stained granule cells (Fig. 1b).

D14 omega-3-treated group: this group exhibited nearly normal histological structure in H&E stained sections.

D14 AL-treated group: an examination of H&E stained sections revealed some convoluted cerebellar folia. The IG layer was clearly visible in certain areas with cracks extending into the layer and deeply penetrating it. The white matter was ill-defined (Fig. 1c). The EG layer revealed disorganized cellularity and the M layer exhibited vacuolation. The Purkinje layer revealed Purkinje cells loss and darkly stained cells. The IG layer exhibited granule cells ((Fig. 1 d).

D14 AL + omega-3-treated group: an examination of H&E stained sections revealed improvement in the histological pattern. Each folium consisted of cerebellar cortex layers, a well-differentiated white matter core, and sulci were widened (Fig. 1 e). The M layer was vacuolated. The Purkinje layer showed intact cells with dendrites, but some of them reveal dark-stained nuclei. The EG layer appeared organized to some extent and the IG layer showed granule cells (Fig. 1f).

II. Cerebellar sections at postnatal D21

D21 control group: well-defined cerebellar folia were separated by even deeper sulci. The cerebellar cortex layers (M, P, and IG) were observed, and white matter was well demarcated from the IG layer. The EG layer disappeared due to the migration of its cells towards the IG layer (Fig. 2 a). The M layer was still contained migrating cells, and Purkinje cells arranged in one layer as pear-shaped cells with frequently-large nuclei and dendrites in apical position extending in the M layer. The IG layer is packed with rounded and deeply stained cells (Fig. 2 b).

D21 omega3-treated group: this group exhibited nearly normal histological structure in H&E-stained sections.

D21 AL-treated group: H&E staining revealed 3 layers of the cerebellar cortex (M, P, and IG). The M layer was separated from the IG layer in certain sites with tissue loss. The wide core of white matter deeply penetrated the folia and was shapely demarcated from the IG layer (Fig. 2c). At higher magnification, the M layer appeared hazy and vacuolated with loss of the dendritic arborizations of Purkinje cells. Distortion of Purkinje cells was observed as a complete loss of nuclear material. The IG layer exhibited granule cells (Fig. 2 d).

D21 AL + omega 3- treated group: an examination of H&E-stained sections revealed that the three cerebellar layers (M, P, and IG) with a nearly normal histological structure; however, a narrow separation between cortical layers still appeared. White matter was noticed (Fig. 2e). There was a

positive effect of omega-3 in minimizing the toxic effect of AL on the three cortical layers. The M layer retained its normal structure except for some vacuolation. Some Purkinje cells exhibited apparently normal nuclear & cytoplasmic profiles; however, others were still small with darkly stained nuclei. Finally, the IG layer contained granule cells (Fig. 2 f).

Immunohistochemical analyses

Caspase-3 immunohistochemically-stained sections from the pups in the postnatal D14 and D21 of control groups showed a negative cytoplasmic immunoreaction in most of the granule and Purkinje cells but there was weak positive immunoreaction in the cytoplasm of some Purkinje and granule cells. Compared with the control group, the AL-treated groups at postnatal D14, and D21 showed a strong positive cytoplasmic immunoreaction of granule and Purkinje cells. In contrast, the AL+ omega-3-treated groups at D14, and D21 showed a weak positive immunoreaction in the cytoplasm of some Purkinje and granule cells. While some Purkinje cells showed a negative immunoreaction (Fig. 3 a-f).

Regarding quantitative morphometric results, the highest optical density of caspase-3 immunoreactivity at postnatal D14 and D21 (0.45± 0.04 and 0.65±0.03, respectively) was recorded in the AL-treated group. In contrast, the AL+omega-3 treated group exhibited a significant decrease in the optical density at postnatal D14 and D21 (0.35± 0.01 and 0.46 ± 0.03, respectively) when compared with the AL-treated groups (Table 3).

Quantitative morphometric results

I. Thickness of cerebellar layers

In postnatal D14 pups, the control group exhibited the thickest (30.67 ±5.064 um) EG layer. The AL and AL + omega-3-treated groups showed significantly decreased EG layer thickness (11.09 ±2.42 and 11.65 ± 0.8305 um, respectively) when compared to the control group (Tab.4).

Moreover, the AL-treated group in postnatal D14 pups exhibited a significant increase in the thickness of M and IG layers (58.93±7.336 and 71.87± 8.93um, respectively). Co-administration of omega-3 with AL revealed a significant decrease in the thickness of these layers (41.22 ±5.213 and 62.08 ±8.05um, respectively) when compared with the AL-treated group, as shown in (Fig. 4 and 5).

In postnatal D21 pups, the EG layer, was absent (Tab.4). Moreover, AL-treated pups exhibited a significant decrease in the thickness of M and IG layers (65.17 ±10.65 and 67.39 ±9.93 um, respectively) when compared to the control group. However, co-administration of omega-3 with AL increased the thickness of the IG layers (84.87 ±5.962 um), while the M layer showed a non-significant difference when compared to the AL-treated group as presented in (Fig. 4 and 5).

II. Purkinje cell count:

The Purkinje cell number/mm2 in pups was significantly affected by treatment. The AL-treated group exhibited the lowest Purkinje cell number at postnatal D14 and D21 (183.9±52.3 and 116.6 ± 56.73, respectively). However, co-administration of omega-3 with AL improved Purkinje cell count and minimized the toxic effect of AL by increasing Purkinje cell numbers at postnatal D14 and D21(305 ±50.74 and 269.1±81.37, respectively) when compared to AL-treated group, as presented in (Fig. 6.).

Table 1: Bodyweight, and cerebellar weight in treated and control groups of postnatal pups (P14, P21).

Groups [G]	Time [T]	Body Weight [gm] ±	Cerebellar Weight [gm]
Control	14	26.74 ± 1.09	0.29 ± 0.01
	21	31.50 ± 0.94	0.34 ± 0.02
Omega 3	14	25.32 ± 1.64	0.28 ± 0.01
	21	31.18 ± 0.97	0.33 ± 0.02
Al. Chloride	14	15.93 ± 3.20	0.19 ± 0.02
	21	25.37 ± 0.82	0.23 ± 0.02
Omega 3 + Al. Chloride	14	25.10 ± 1.03	0.28 ± 0.02
	21	29.56 ± 0.58	0.31 ± 0.01
P value	[G]	0.000	0.000
	[T]	0.000	0.000
	[G]×[T]	0.003	0.644ns

Table 2: Statistical comparisons between mean values of the MDA and the SOD in different studied groups After 14 and 21 days from rat birth using ANOVA (analysis of variance) test.

Groups	Time	SOD	MDA
Control	14	3.71 ± 0.19	10.96 ± 0.29
	21	1.54 ± 0.32	11.07 ± 0.64
Omega 3	14	3.74 ± 0.19	11.00 ± 0.16

Groups	Time	SOD	MDA
	21	1.29 ± 0.30	11.47 ± 0.45
Al. Chloride	14	1.82 ± 0.03	19.83 ± 0.57
	21	1.52 ± 0.30	12.52 ± 1.86
Omega 3 + Al. Chloride	14	1.54 ± 0.05	12.07 ± 0.50
	21	3.08 ± 0.09	12.01 ± 0.72
P value	[G]	0.000	0.000
	[T]	0.000	0.000
	[G]×[T]	0.000	0.000

SD: Standard deviation; **: highly significant (p<0.001). n=5/ group

Table (3): Means and standard deviations of the number of the optical density of caspase-3 immunoreactivity of the D14 and D 21 old offspring. It also shows a one-way ANOVA statistical analysis between groups. (i.e. control, Omega 3, AL andAL+omega-3) and followed by a post hoc test using the Duncan multiple range (DMR) test for comparisons between means of groups.

Groups	Optical density (AU)	
	14 day	21 day
Control	0.06 ± 0.01 c	0.07 ± 0.01 c
Omega 3	0.08 ± 0.01 c	0.08 ± 0.01 c
AL	0.45 ± 0.04 a	0.65 ± 0.03 a
AL + Omega 3	0.35 ± 0.01 b	0.46 ± 0.03 b

AU= absorbance/unit length

Table (4): Means and standard deviations of External granular layer thickness (µm)of the D14 and D 21 old offspring. It also shows a one-way ANOVA statistical analysis between groups. (i.e. control, Omega 3, AL andAL+omega-3) and followed by a post hoc test using the Duncan multiple range (DMR) test for comparisons between means of groups.

Groups	External granular layer thickness (µm)	
	14 day	21 day
Control	30.67 ± 5.06 a	
Omega 3	26.66 ± 5.03 b	
AL	11.09 ± 2.42 c	
AL + Omega 3	11.65 ± 0.83 c	

Note: The means followed by the same letter in each column are not significantly different from each other at the 5-percent probability level.

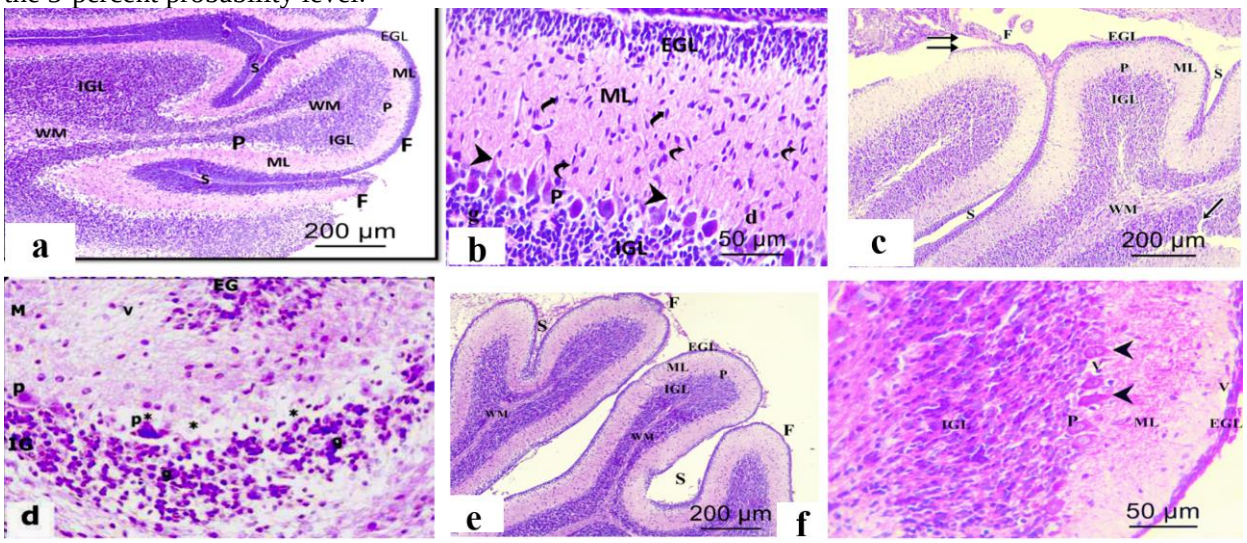

Figure 1: [a-f]: H&E staining of cerebellar sections from postnatal D14 albino rats. (a) A photomicrograph of a cerebellar cortex of control albino rat, 14th day postnatal exhibiting all cortical layers external granular layer

(EGL), molecular layer (ML), Purkinje cells (P), and internal granular layer (IGL). (b) A photomicrograph of a cerebellar cortex of a control albino rat,14th day postnatal shows the internal granular layer (IGL) is clearly differentiated and packed with condensed and darkly-stained granule cells (g). The Molecular layer (ML) is well developed including scattered migrating cells (curved arrow) and a network of dendrites (arrowheads) of Purkinje cells. The Purkinje cells (P) are arranged in a single row between ML and IGL. These cells are pyriform in shape with definite nuclei. The external granular layer (EGL) is composed of darkly-stained granule cells. (c) A photomicrograph of a cerebellar cortex of an $AlCl_3$- exposed rat, 14 days postnatal shows some convoluted cerebellar folds (F) with the presence of certain detachment (double arrow) in the pial surface. (d) The EG layer revealed disorganized cellularity and the M layer exhibited vacuolation. (e) A photomicrograph of a cerebellar cortex of $AlCl_3$- exposed rat treated with omega-3, 14th day postnatal (H & E, scale bar: 200µm) shows The cerebellar folia (F) are separated by well-defined and deeper sulci (S). (f) A photomicrograph of a cerebellar cortex of $AlCl_3$- exposed rat treated with omega-3, 14th day postnatal (H & E, scale bar: 50µm) shows a marked improvement in the histological pattern of the three cortical layers.

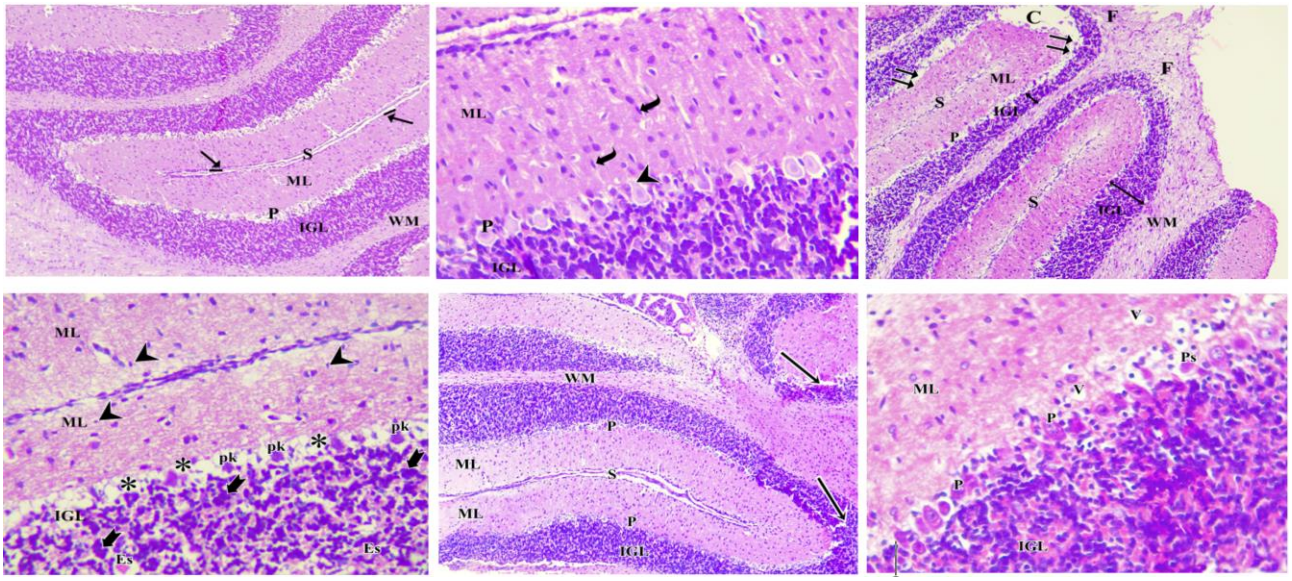

Figure 2[a-f]: H&E staining of cerebellar sections from postnatal D21 albino rats. (a) A photomicrograph of a cerebellar cortex of control albino rat, 21th day postnatal demonstrating well-defined cerebellar folia (F) separated by more deeper sulci (S). (b). A photomicrograph of a cerebellar cortex of control albino rat, 21th day postnatal showing the 3 layers of the cerebellar cortex. (c) A photomicrograph of a cerebellar cortex of an $AlCl_3$- exposed rat, 21th day postnatal showing the 3 layers of cerebellar cortex highly-affected with $AlCl_3$ intoxication. (d). A photomicrograph of a cerebellar cortex of an $AlCl_3$- exposed rat, 21th day postnatal. (e) A photomicrograph of a cerebellar cortex of $AlCl_3$- exposed rat treated with omega-3, 21th day postnatal showing a nearly normal histological profile for the three cortical layers of the cerebellum; the Purkinje cells (P), and the molecular layer ML. The internal granular layer (IGL) shows extending cracks (Arrows) The white matter (WM) is clearly-distinguished from the internal granular layer (IGL). (f). A photomicrograph of a cerebellar cortex of $AlCl_3$- exposed rat treated with omega-3, 21th day postnatal showing an ameliorative effort for omega-3 in the three cortical layers where the vacuolation and apoptotic cells are disappeared from the molecular layer (ML).

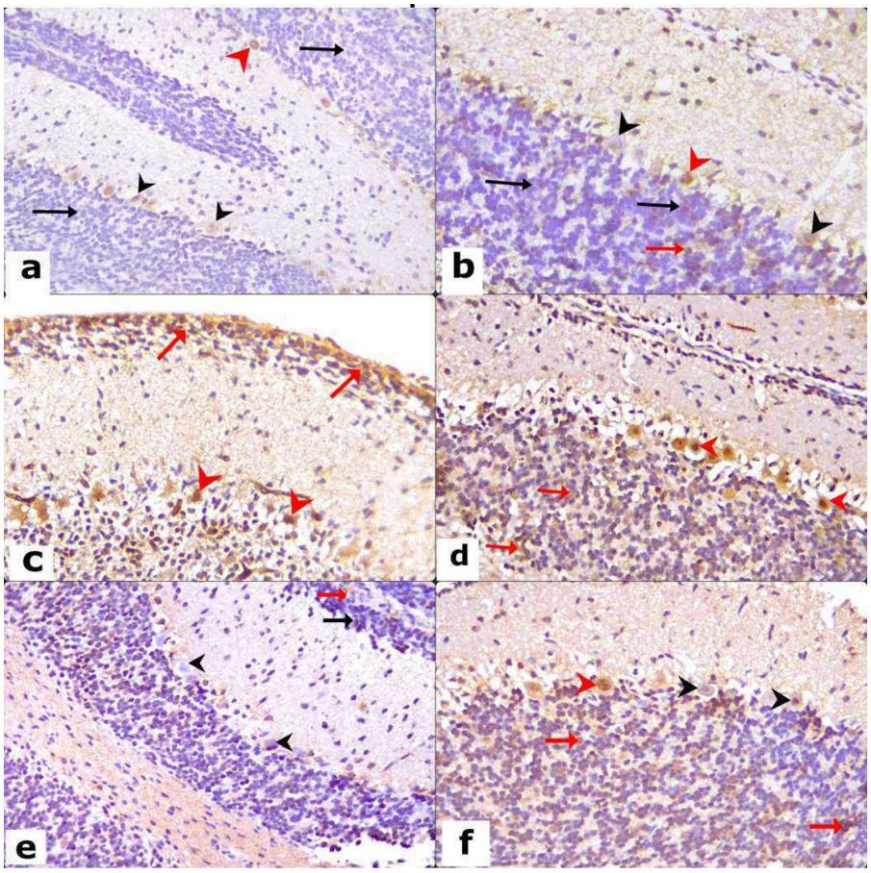

Figure 3: Photomicrographs of immunoreactive staining for caspase-3 in the cerebellar cortex of albino rats. showed a negative cytoplasmic immunoreaction in most of the granule and Purkinje cells but there were weak positive immunoreaction in the cytoplasm of some Purkinje and granule cells. Compared with the control group, the AL-treated groups at postnatal D14, and D21 showed a strong positive cytoplasmic immunoreaction of granule and Purkinje cells.

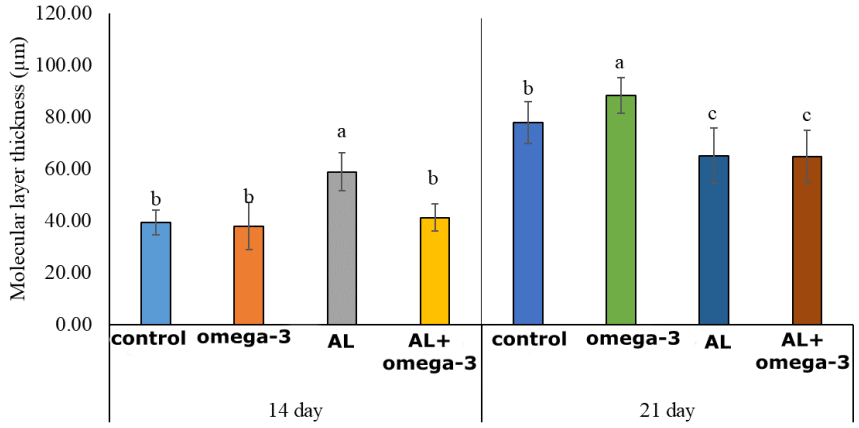

Figure 4: Comparison between different studied groups regarding the molecular layer thickness of postnatal pups (D14, D21) in control and other treated groups.

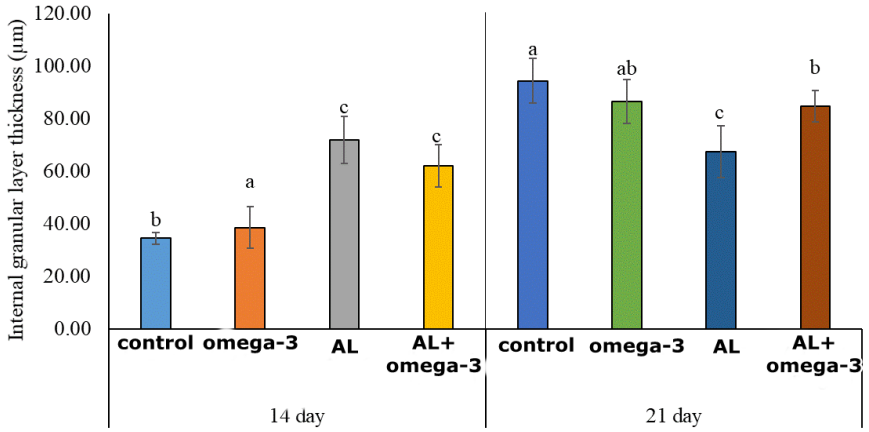

Figure 5: Comparison between different studied groups regarding the internal granular layer thickness of postnatal pups (D14, D21) in control and other treated groups.

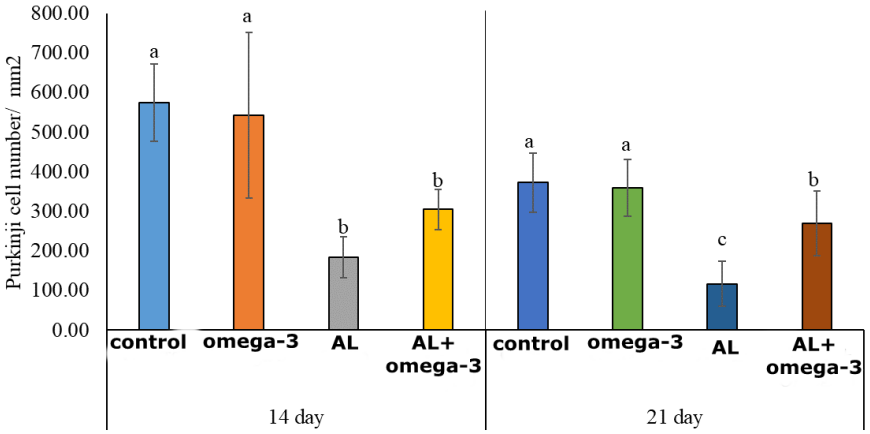

Figure 6: Comparison between different studied groups regarding the Purkinje cell number/mm2 of postnatal pups (D14, D21) in control and other treated groups.

DISCUSSION

The cerebellum is known as a "neuronal machine" or as the "little brain" and coordinates many behaviors, such as motor behavior, eye movement, cognition, and respiration [21].

AL is naturally present in compound forms, such as $AlCl_3$ and sulfate, as a result of its reactivity and has injurious effects on human health. AL is capable of inducing marked changes in behavior and biochemical parameters by inhibiting antioxidant enzyme activities, thereby inducing oxidative damage [15].

In the current study, AL-induced neurotoxic changes in rat pups were exhibited by the alterations in biochemical values, cerebellar cortical tissue architecture, and morphological parameters. The present experimental results revealed a significant reduction ($P<0.01$) in the mean body and cerebellum weights at postnatal D14 and D21 after AL exposure. These results are in agreement with another study, which reported that AL interrupted protein synthesis and hormonal status, leading to a decrease in weight gain. Their results and the present results may be due to oxidative stress caused by AL leading to an increase in the peroxidation of lipids thus initiating a reduction in brain weight [22].

In the present study, co-administration of omega-3 with AL increased body and cerebellar weight gain at postnatal D14 and D 21 compared to the AL-treated group. Previous literature also supports these findings [15]. These results explained as omega-3-fatty acids have a pro-survival role in the cerebellum, and propose its prophylactic potential against developing brain damage[23].

The current results indicated that oxidative stress was the main pathological mechanism by which AL induced cerebellar toxicity. Specifically, AL-treated groups at postnatal D14 and D21 exhibited significantly lower SOD levels and higher MDA levels than the respective control groups. Previous literature found that AL initiates lipid peroxidation and affects the brain antioxidant defense system. Consequently, when free radicals surpass antioxidant defense capacity, oxidative stress may result in cellular dysfunction, membrane degradation, and apoptosis [24].

The current study showed that AL exposure caused histopathological changes in different layers of the cerebellar cortex. Interestingly, the M layer had

marked vacuolation, especially at postnatal D21. No previous studies reported the presence of such spaces in the cerebellum after AL exposure; however, Nehru et al. [25] found that the M layer had prominent perineuronal spaces around both basket and stellate cells.

Loss of Purkinje neurons and other cells in the P layer that were highly damaged with pericellular vacuolization at postnatal D14 and D21. This finding was in line with Atici et al. [26] who reported that chronic use of morphine led to the loss of the pyriform shape of Purkinje cells, darkly stained cytoplasmic inclusions, and many empty spaces around the cells with degenerated nerve fibers between. In addition, Bhalla and Dhawan [22] noticed that animals exposed to AL showed a disorganized Purkinje cell layer with Purkinje cell damage.

The current histopathological results in the omega-3 treated group revealed that the three cerebellar layers exhibited a nearly normal histological structure. Omega-3 was found to have a positive effect, minimizing the toxic effect of AL in the three cortical layers at postnatal D14 and D21. However, a few cells were still apoptotic or exhibited pericellular vacuolation or gliosis in the IG layer. This suggestion coincided with the study of Trabelsi et al. [27] who emphasized the pro-survival role of omega-3-fatty acids in the cerebellum and proposed that omega-3 has a prophylactic potential against brain damage.

The morphometric results of this study indicated that AL treatment during pregnancy and during lactation caused various changes in cerebellar cortex layer thickness at different postnatal ages. At postnatal D14, the AL and AL+ omega 3-treated groups revealed a decrease in the thickness of the EG layer compared to the control group. These findings were in agreement with the previous literature reported by the study of Sinha et al. [23], who also found that the EG layer was thin at D7 due to chronic pre-and postnatal acrylamide administration and attributed this to a delay in the proliferation and differentiation of these cells.

The AL-treated group at postnatal D14 exhibited the thickest M and IG layers. Our findings were in agreement with the study of Allam et al. [28], who reported an increase in the thickness of the M and granular layers in morphine-treated mice. The AL-treated group at postnatal D21 exhibited the lowest thickness of the M and IG layers. Also, Golalipour and Ghafari [29] showed that exposure of the mother to $AlCl_3$ for a longer time resulted in poorly differentiated Purkinje cell bodies in the cerebellum of the pups. This effect may be explained by the retardation of the migration of granular cells from the EG layer to the M and IG layers.

In this study, caspase-3 immunohistochemistry revealed that AL treatment resulted in apoptotic cell death in the P layer. These results were emphasized by Purkinje cell count and optical density of caspase-3 immunoreactivity that revealed the fewest Purkinje cells and the highest optical density in AL-treated groups at postnatal D 14 and D21. These results were in agreement with [30] who examined neuronal cell death after monosodium glutamate exposure and suggested that cell death in response to neurotoxins might activate an apoptotic death pathway in brain cells.

Finally, a morphometric analysis of the Purkinje cell number revealed that co-administration of omega-3 with AL increased the Purkinje cell number and decrease the optical density of caspase-3 immunoreactivity at postnatal D14 and D21 compared to the AL-treated group. These observations were close to the findings reported by Eweka and Om'Iniabohs [31] who detected marked improvement of structural alterations in rat pup brains after co-administration of cod liver oil(CLO).

CONCLUSION

Prenatal and postnatal AL exposure results in alterations in the cerebellar cortical structure in albino rat pups through oxidative damage. Co-administration of omega-3 with AL minimizes the harmful effects. This research recommends avoiding exposure of pregnant females and their babies to drugs, chemicals, and pollutants that affect prenatal and postnatal development of the central nervous system.

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