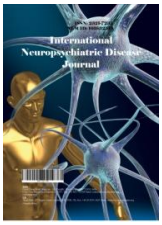

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# A Study of 4G Radiofrequency Radiation effects on Juvenile Wistar Rats Cerebellum and Potential Attenuative Properties of Fish Oil Omega-3 Fat

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

**Introduction:** Technological devices have become a very important part of our everyday life. These electronic devices create an artificial electromagnetic field (EMF) and emit radiofrequency radiations (RFR) which have been reported to have deleterious effects on various tissues in living organisms. Fish oil is a rich source of the n-3 (also known as omega-3) fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The cerebellum plays an important role in motor control and the coordination, precision and timing of movements, as well as in motor learning. This study investigated the effect of radiofrequency radiation on the structures of the cerebellum of adolescent Wistar rats, its effect on pre-pubertal development and the potential attenuative properties of fish oil omega-3 fat.

**Materials and Methods:** Forty (n=40) adolescent Wistar rats of approximately 35 days old were divided into five groups labelled A-E. A 4G RFR-emitting WI-FI device served as the RFR source. The 4G radiofrequency transmitter was installed at a distance of 0.5 meters from the base of the

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cage housing the experimental animals in each group. The fish oil was administered orally to the animals. Rats were sacrificed after 28 days of treatment and their brains harvested. The cerebellar tissues were excised and processed for histological, immunohistochemical and biochemical observations. Neurobehavioural studies including Barnes Maze and beam walk were carried out before sacrifice.

**Results:** 4G radiofrequency radiation caused distortions in the morphology and spatial arrangement of Purkinje cells in the cerebellar tissues. It also induced neuroinflammation as marked by enhanced astrocyte reactions. Also, behavioural aberrations including negative changes in key memory parameters such as increased latency and error poke in Barnes maze assays, and increased movement parameter errors were attributable to FRR effects. Also, aberrations in neurotransmitters activities levels and vital neurochemistry enzymes were observed. The administered Omega-3 fat had an attenuative effect on the affected cortical structures, neurochemistry and selected neurobehavioural parameters.

*Keywords: Cerebellum; radiofrequency radiation; 4G; omega-3 fat; motor functions.*

## 1. INTRODUCTION

Technological devices, especially, electronic ones, now occupy a very important place in human daily activities. However, while making life easier, they may also cause several health problems, since electronic devices emit a surrounding electromagnetic field (EMF) [1,2]. Radiofrequency radiation is non-ionizing radiation, meaning that it has insufficient energy to break chemical bonds or remove electrons (ionization). If radiofrequency radiation is absorbed in large enough amounts by materials containing water, such as food, fluids, and body tissues, it can produce heat. This can lead to burns and tissue damage. Radiofrequency electromagnetic radiation is produced by both natural and artificial sources. Natural sources like the sun, the earth and the ionosphere all emit low-level radiofrequency fields [3]. EMF has been shown to have deleterious effects on various tissues in living organisms [4]. A wide spectrum of electromagnetic waves is emitted from radar equipment, communication devices, mobile phone base stations, high voltage power lines, radio and television transmitters and substations, and particularly from electrical appliances at home, in offices and other electrical systems [5]. Mobile phone use has been shown to exacerbate headaches [6]. Insomnia and significant changes in electroencephalography findings have been also reported. Even low-frequency exposure significantly changes nervous system activity, and modifications may be observed in synaptic plasticity and neurotransmitter release, together with functional changes in hearing perception, balance, learning and memory [7].

In an experiment [8] to determine if prenatal exposure to radiofrequency radiation could lead to impaired memory or behaviour after birth, mice were exposed in-utero to 800-1900 Mhz radiofrequency radiation from cellular telephones. In the study, 161 progeny were given a standard object recognition memory test in three different cohorts. The exposed mice had a significantly lower mean preference index suggesting impairment in memory, compared to the control group.

The richest sources of very-long-chain n-3 fatty acids are oily fish or fish oil supplements. The n-3 fatty acids belong to one of two families of polyunsaturated fatty acids (PUFAs), the other being the n-6 (omega-6) family. The n-3 family is derived from the essential fatty acid, alpha-linolenic acid, and the n-6 family from the essential fatty acid, linoleic acid. The antioxidant and anti-inflammatory properties of fish oil supplements have been reported by Mori et al. [9] in their research to observe the neuroprotective effect of omega-3 fatty in the 6-OHDA model of Parkinson disease. It was reported that fish oil mitigated the loss of SNpc neurons and nerve terminals in the striatum that was caused by 6-OHDA. This protective effect was associated with reductions in the density of iNOS-immunoreactive cells and neuroglia reactivity. These results suggest that the antioxidant and anti-inflammatory properties of fish oil supplementation are closely related to a decrease in dopaminergic damage that is caused by the 6-OHDA model of Parkinson's disease [9].

The cerebellum has an important role in motor control. In particular, it is involved in the coordination, precision and timing of movements, as well as in motor learning. The grey matter is located on the surface of the cerebellum which forms the cerebellar cortex. The white matter is

located underneath the cerebellar cortex and it contains four cerebellar nuclei (the dentate, emboliform, globose, and fastigial nuclei) [10]. The cerebellum contains almost 80% of the total brain neurons [11] and is composed of highly regular arrays of neuronal units, each sharing the same basic cerebellar microcircuitry. Its circuitry is classically viewed to be involved in motor control and motor learning. There is increasing recognition of the cerebellum's role in non-motor cognitive and affective functions [12,13].

## 2. MATERIALS AND METHODS

### 2.1 Animal Treatment and Tissue Processing

Forty (n=40) adolescent male Wistar rats, with an average body weight of 100g, were procured for the study from the institutional animal holding facility and divided into five groups labelled as A, B, C, D and E. The standard procedures for animal use and handling were followed according to Babcock University's Health Research Ethical Committee which approved the project with BUHREC no: 814/18. A 4G LTE MTN mobile wireless fidelity (WI-FI) device designed by the technological company HUAWEI was obtained from Quick Choice telecommunication centre, Sagamu, Ogun state. The fish oil capsules were produced by GAIA pharmaceutical company and procured from MNT pharmacy, Sagamu Ogun State. The 4G MTN mobile WI-FI served as the 4G radio frequency transmitter for the research with SAR of 1.5W/kg. The 4G radiofrequency transmitter was installed at a distance of 0.5 meters from the base of the cage housing the experimental animals in each group. Group A served as control and had no exposure. Group B had daily radiation exposure continually. Group C had exposure to radiation on alternate days. Group D had daily exposure to radiation continually + omega-3 fat supplements. Group E had exposure to radiation on alternate days + omega-3 fat supplements. The rats were sacrificed through cervical dislocation after the treatment period of 28 days and their brains were harvested. Neurobehavioural studies were carried out before sacrifice. The cerebellar tissues were excised and preserved in phosphate-buffered saline for enzymes and neurotransmitter assays. The remaining brain samples were preserved in formal saline for histological and immunohistochemistry demonstrations. The fixed tissues were processed following specific histological and histochemical protocols. Basic tissue processing included dehydration [using a graded concentration of alcohol], clearing [using xylene], impregnation, and embedding [using molten wax]. The tissue samples were sectioned with a rotary microtome (~20 microns). The sections were mounted on glass slides for staining.

**Preparation of fish oil:** A syringe was used to extract 1ml of oil contained in fish oil capsules procured from the pharmacy. 1ml fish oil was mixed with 100ml of olive oil [14] to achieve the appropriate dose for administration. Olive oil that was mixed with the Omega 3 oil was also used as a placebo for the untreated group.

### 2.2 Neurobehavioral Tests

Neurobehavioral tests were carried out at the end of the treatment period to check for the changes in the behaviour of the animals in response to the treatment received. Barnes maze and Beam Walk neurobehavioral assays were employed.

**Barnes maze [15]:** The Barnes maze is a dry, land based behavioural test that was developed by Dr Carol Barnes to assess spatial learning and memory. The animals interacted with the Barnes maze in three phases: habituation (1 day), training (2–4 days in the short training) and probe (1 day). Before starting each experiment, rats were acclimated to the testing room for 1 h. Then rats were placed in individual holding cages where they remained until the end of their testing sessions. On the habituation day, the rats were placed in the centre of the maze underneath a clear 3,500-ml glass beaker for 30s while an appropriately loud noise was played through a sound/buzzer system. Then, the rats were guided slowly by moving the glass beaker, over 10–15s to the target hole that leads to the escape cage. The rats were then given 3 min to independently enter through the target hole into the escape cage or box. If they did not enter on their own during that time, they were guided to the escape box.

During the training phase, measures of primary/secondary latency and primary/secondary error pokes were recorded. On the probe day, 48h after the last training day, the escape cage was removed, rats were placed inside the opaque cylinder in the centre of the maze for 15 s, the buzzer was turned on and the cylinder removed. Each rat was given 2 min to explore the maze, at the end of which, the buzzer was turned off and the mouse was returned to its

holding cage. For these analyses, the maze was divided into quadrants consisting of 5 holes with the target hole in the centre of the target quadrant. The other quadrants going clockwise from the target quadrant were labelled: positive, opposite, and negative. Results in forms of durations that measure specific parameters of learning and memory were recorded and analyzed statistically.

**Beam walk [16]:** The beam walk neurobehavioural test equipment is a 1m long narrow aluminum or wooden beam (1-2cm wide) suspended from its ends with an elevation of 30cm. The procedure was performed to assess locomotor activity and grip strength in rats. The animals were brought into the behavioural test room and the doors were shut. The animals were then gently placed at the centre of the beam, facing one of the ends. The procedure was repeated three times. The behavioural parameters scored were duration, hind limp slips, number of turns and falls.

## 2.3 Histological Techniques

H & E Staining Procedure was done following a standard protocol [17].

Harri's haematoxylin stain was made from a mixture of 1gm of haematoxylin in 10ml ethanol and 20gm of ammonium alum in hot distilled water with 0.5gm mercuric oxide mixture added. The mixture then filtered. Eosin solution was made from yellow eosin 1gm, distilled water 80ml and ethanol 320ml, 0.5% HCl and distilled ammonia water. Mounted tissue sections were deparaffinized, cleared in xylene and hydrated in decreasing grades of alcohol and water and then stained with Haematoxylin for five (5) minutes. The stained tissue sections were washed under running water for five (5) minutes and then differentiated in 1% acid for five (5) minutes. The sections were rewashed with running water slowly till blue again, dipped in ammonia water and rewashed for (5) minutes before they were counterstained in 1% eosin for 10 minutes. Counter stained sections were washed in running water for five (5) minutes and dehydrated in increasing grades of alcohol. Tissues were mounted and coverslips were placed.

**Cresyl Violet (Nissl) Stain [18]:** Reagents used include ninety-five percent (95%) ethanol, seventy percent (70 %) ethanol**,** differentiation fluid (composed of 2 drops of glacial acetic acid in 95% ethanol) and Cresyl fast violet acetate in acetate buffer. The tissue sections, after deparaffinization and rehydration were stained in 0.1% Cresyl fast violet for 15 minutes. Sections were rinsed in distilled water to remove excess stain, washed in 70% alcohol and immersed in differentiation solution for two (2) minutes. Sections were dehydrated in absolute alcohol (3 times), cleared in xylene (2 times) and mounted with a cover slip.

**Luxol fast blue for myelin and myelinated axons [19]:** Reagents used included 0.1gm Luxol fast blue, MBS, 100ml of 95% ethyl alcohol and 0.5ml glacial acetic acid to make 0.1% luxol fast blue solution; 0.1gm Cresyl echt violet, 100 ml distilled water and 10 drops of glacial acetic acid just before use and filtered to make 0.1% Cresyl echt violet solution; 0.05gm Lithium carbonate and 100ml distilled water to make 0.05% Lithium carbonate solution.

The slides were placed in 2 changes of xylene, 3 minutes each, then placed in 2 changes of 100% ethanol, 3 minutes each, after which, they were then placed in 95% ethanol for 3 minutes. They were also placed in 75% ethanol for 3 minutes, then rinsed in 2 changes of distilled water, 3 minutes each. The slides were placed in luxol fast blue solution in a plastic Coplin jar and microwaved at the lowest power setting for 1 minute. Care was taken to avoid boiling the solution. The slides were allowed to remain in the hot solution for an additional 20-30 minutes, then rinsed in tap water, 2 times, 3 minutes each, after which, they were briefly rinsed in 3 dips of distilled water. The sections were differentiated in 0.05% lithium carbonate, 5-10 dips, and differentiation was continued in 70% ethanol, 5-10 dips, after which they were rinsed in 2 changes of distilled water, 2 minutes each. Thereafter, sections were counterstained in Cresyl violet solution after which they were dehydrated in 4 changes of 100% ethanol, 2 minutes each. The slides were cleared in 3 changes of xylene, 3 minutes each, after differentiation. They were checked under the microscope and mounted.

**Immunohistochemistry:** Glial acidic fibrillary protein (GFAP) [20] standard method was used. Tissues were deparaffinised using standard techniques and pre-treated with citrate buffer at pH 6.0 and high heat epitope retrieval techniques. Blocking reagent was added to the tissue specimens and incubated in an enclosed chamber for 5 minutes. Specimens were gently rinsed with buffer for a minimum of 15 seconds while holding the slides at a 45° angle.

The end of the slides was tapped onto a paper towel to remove excess buffer. A dilution of the primary antibody was applied over the entire tissue specimen, incubated in an enclosed chamber at room temperature for 60 minutes and specimens were rinsed. Biotinylated secondary antibody was applied to the tissue specimens, incubated in an enclosed chamber for 10 minutes and specimens were rinsed. The streptavidin-HRP solution was applied to the tissue specimens, incubated in an enclosed chamber for 10 minutes and rinsed. DAB (chromogen reagent) was applied to the tissue specimens and incubated in an enclosed chamber for 10 minutes and specimens were rinsed again. Tissue specimens were counterstained with haematoxylin solution and incubated in an enclosed chamber for 1 minute, after which specimens were rinsed. Tissue slides were placed directly into a container filled with deionized water and dehydrated through graded series of alcohols, immersed in xylene before xylene-based mounting media was applied and coverslips were applied for permanent mounting.

## 2.4 Neurotransmitters Essay

Neurotransmitters were assayed from a portion of the cerebellar cortex that was excised and then homogenized. The homogenates were centrifuged, and the supernatants were decanted and used for the neurotransmitter tests. The neurotransmitters that were assayed for in this study were Gamma Amino Butyric Acid (GABA), Glutamate, Serotonin and Dopamine.

**Gamma Amino Butyric Acid (GABA)[21]:** Tissues were first rinsed in ice-cold phosphate buffer saline (0.01 mol/L, pH 7.0-7.2), weighed, minced into small pieces and homogenized in 5-10ml of PBS using a glass homogenizer on ice. The homogenates were centrifuged for 20 minutes at 3000G and supernatants were collected and assayed for GABA. All reagents, samples and standards were prepared. 50µL standard was added to each supernatant, then 50µL of prepared detection reagent A was added immediately. They were shaken and properly mixed, incubated for one hour at 37°C, aspirated and washed five times. 90µL substrate solution was added to each and then incubated at 15-25 minutes at 37°C.50µL of stop solution was added and reading was done immediately at 450nm.

**Glutamate [22]:** 10mL of the 1.0 M glutamate standard was diluted with 900 µL of the glutamate assay buffer and it was used to prepare a 1mM standard solution, 0, 2, 4, 6, 8. 10 µL of the 1mM standard solution was added into a 96 well plate, it was used to generate 0 (blank) and 2, 4, 6, 8 and 10 nmol/well standards. Glutamate assay buffer was then added to each well and the volume was brought to 50µL. Samples were centrifuged at 13,000 x g for 10 minutes to remove all forms of insoluble materials. Samples (10-50 µL) were directly added to the wells. The proteins, fat or solids/particulates were filtered through a 10 kDa MWCO spin filter. This was to prevent enzyme interference with the assay. The samples were brought to a final volume of 50µL with glutamate assay buffer. The reaction mixes were set up. 100µL of the appropriate reaction mix was added to each well. Mixing was done by incubating the reaction for 30 minutes at 37°C. The light was prevented from touching the plates during incubation. The absorbance was measured at 450 nm ($A_{450}$).

**Dopamine [23]:** This assay employed the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to dopamine was pre-coated onto a microplate. A competitive inhibition reaction was launched between biotin-labelled dopamine and unlabeled dopamine (standards and samples) with the pre-coated antibody specific to dopamine. The unbound conjugate was washed off after incubation. Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. After adding the substrate solution, the intensity of colour developed was reverse proportional to the concentration of dopamine in the sample. All reagents, samples and standards were well prepared and50µL standard was added to each well. 50µL prepared Detection Reagent A was added immediately and mixed by shaking. Incubation was done for 1 hour at 37$^{o}$C, aspirated and washed 3 times.100µL of prepared Detection Reagent B was added, incubated for 30 minutes at 37$^{o}$C, aspirated and washed 5 times.90µL substrate solution was added and incubated for 10-20 minutes at 37$^{o}$C**.** 50µL of stop solution was added and absorbance was read immediately at 450nm.

**Serotonin [21]:** This assay is used for the quantitative determination of serotonin in platelets, serum, plasma and urine.20µl of samples was pipetted into glass test tubes and100µl of diluted assay buffer was pipetted into each tube and vortexed.25µl of Acylation Reagent was pipetted into each tube and the

tubes were vortexed immediately. Tubes were covered and incubated for 15 minutes at 37$^{o}$C in a water bath.2mL of diluted assay buffer was pipetted into each tube and vortexed. All tubes were centrifuged for 10 minutes at 1500g.50µlL of each sample and standard were pipetted into the respective wells of the microliter plate**.** 50µl of serotonin biotin and serotonin antiserum was also pipetted into each well. Plates were covered with adhesive foil and incubated for 90 minutes at RT 18-25$^{o}$C on an orbital shaker (500rpm). The adhesive foils were removed, and incubation solutions were properly discarded. The excess solution was removed by tapping with the inverted plate on a paper towel.150µl of freshly prepared enzyme conjugate was pipetted into each well, plates were **c**overed with adhesive foil and incubated for 90 minutes at RT 18-25$^{o}$C on an orbital shaker (500rpm).200µl of PNPP Substrate Solution was pipetted into each well and incubated for 60 minutes at RT 18-25$^{o}$C on an orbital shaker (500rpm)**.** 50µl of PNPP Stop solution was pipetted into each well to stop the Substrate reaction. Contents were briefly mixed by shaking the plate**.** Optical density was measured with a photometer at 405nm (reference wavelength: 600-650nm) within 60 minutes after pipetting the Stop solution.

## 2.5 Enzyme Assays

**Lactate dehydrogenase:** Lactate Dehydrogenase is an oxidoreductase that catalyzes the interconversion of lactate and pyruvate. Lactate dehydrogenase is most often measured to evaluate the presence of tissue or cell damage. The non-radioactive colourimetric lactate dehydrogenase assay is based on the reduction of the tetrazolium salt MTT in an NADH-coupled enzymatic reaction to a reduced form of MTT which exhibits an absorption maximum at 565nm. The intensity of the purple colour formed is directly proportional to the enzyme activity [24,25]. The plate reader was turned on to allow the light source to warm up, and the absorbance wavelength was set to 340 nm. Kit reagents were warmed to room temperature for 30 minutes and the reagent mix was reconstituted by adding exactly 27 mL of deionized water to the LDH reagent mix powder. Reagents were mixed by swirling the bottle 10 times. Contents were allowed to dissolve for 10 minutes at room temperature.

For the assay procedure, six clean microcentrifuge tubes were labelled 1, 2, 3, 4, 5 and 6 (Neg). Contents of Standard vial were dissolved in 920L of standard dilution buffer, properly mixed and 150 L of dissolved standard was transferred to tube 1. Standard was serially diluted by adding the appropriate volumes of standard and standard dilution buffer. 5µL of each sample or was added to microplate wells. 250µL reconstituted LDH reagent mix was also added to the wells. The absorbance of the wells was measured at 340 nm (= initial reading) and exactly 5 minutes later, the absorbance was again read. The initial absorbance was subtracted from the 5 min absorbance for each of the samples. Average of the values were taken to obtain the average absorbance increase in 5 minutes for each sample. The average 5 min absorbance increase was multiplied by 2,187 (the conversion factor) to obtain LDH activity (IU/L), [26].

**Cytochrome-C-Oxidase assay [27]:** The absorption of cytochrome C at 550 nm would change with its oxidation state. This property was the basis for the assay. Cytochrome C was reduced with dithiothreitol and then reoxidized by the cytochrome C oxidase. The difference in extinction coefficients (De mM) between reduced and oxidized cytochrome C was 21.84 at 550 nm. The oxidation of cytochrome C by cytochrome C oxidase is a biphasic reaction with a fast initial burst of activity followed by a slower reaction rate. In this essay, the initial reaction rate was measured during the first 45 s of the reaction. The total volume of the reaction was 1.1 mL. Spectrophotometer settings following the decrease in absorption at 550 nm at room temperature (25°C) using a kinetic program were 5 s delay, 10 s interval, and 6 readings. The instrument was set up before starting any reaction. The wavelength set was considered critical and could deviate by no more than 2 nm. No signal was observed with a deviation of 10 nm [27].

The assay procedure included the addition of 0.95 mL of 1 × assay buffer to a cuvette and zeros the spectrophotometer. Also, a suitable volume of enzyme solution or mitochondrial suspension was added to the cuvette, bringing the reaction volume to 1.05 mL with 1 × enzyme dilution buffer. Mixing was by inversion. This was followed by starting the reaction by the addition of 50 mL of ferrocytochrome C substrate solution and mixing by inversion. The A550/min was read immediately due to the rapid reaction rate of this enzyme. Background values were expected between 0.001 and 0.005 A550/min. The activity of the sample was calculated [27].

**Succinate Dehydrogenase [28]:** Succinate Dehydrogenase (SDH) (EC 1.3.5.1) or succinate-coenzyme Q reductase (SQR) is an enzyme complex, which is bound to the inner mitochondrial membrane. SDH participates in both the citric acid cycle and electron transport chain. SDH oxidizes succinate to fumarate and transfers the electrons to ubiquinone. SDH deficiency in humans leads to a variety of phenotypes including Leigh syndrome, a neurometabolic disorder, tumour formation, and myopathy.

For the procedure, tissues were rapidly homogenized (10 mg) or cells (1 x 106) with 100µl ice-cold SDH assay buffer, kept on ice for 10 min, centrifuged at 10,000 x g for 5 min and the supernatants were transferred to fresh tubes. 5-50µl samples were added per well & the volumes adjusted to 50µl with SDH Assay Buffer. Mitochondria were isolated from fresh tissues to check SDH activity, 5-50µl isolated mitochondria were added per well, the volumes were adjusted to 50µl/well with SDH assay buffer. 10-20µl of SDH positive control was taken into the wells for the SDH positive control and the final volumes were adjusted to 50µl with SDH assay buffer.

## 3. RESULTS

### 3.1 Histology and Immunohistochemistry (Figs. 1-4)

The results of the study presented are photomicrograph sections of the cerebellar cortices (x40 & x400) as demonstrated using H & E (Fig. 1), Cresyl fast violet (Fig. 2), Luxol fast Blue technique (Fig. 3) and GFAP (Fig. 4).

Histomorphological presentations of the cerebellar cortices across the treated groups showed the various cell layers of the cerebellum as visible. There were no significant cortical morphological changes between the control and experimental groups, but the Purkinje cells in Group B although preserved, appear morphologically distorted and disrupted in terms of their typical single layer organization within the cortex. There was a relative reduction in the number as well as the size of cells of Purkinje layer in group B and C as well as alterations in the morphological integrity of the cells of molecular and granular layers. There was the observable loss of neurons and glial cells across the treated groups, ranging from mild in D and E to severe in B and C.

A mild disruption in relative distribution of myelin in Group B was observed that might suggest a mild effect of the radiofrequency exposure in the group. There is however no evidence of extensive demyelination of nerve cells in the cerebellar cortices. Relative abundant expression of GFAP in Group B and C was observed, indicating enhanced astrocyte reaction in response to the exposure. However, there was reduced astrocyte expression in group D, an indication that the administered agent had ameliorative effects.

### 3.2 Neurochemical Changes (Figs. 5,6)

The results of the study presented (below) are bar charts showing mean values of .ure 6). There was a relative alteration in neurotransmitters activities across the treated groups and a significant increase in the activities of GABA and dopamine in Group D, attributable to continual exposure to radiofrequency radiation. This would suggest that continual exposure to radiofrequency radiation could cause significant alterations in neurotransmitters activities in the cerebellum. However, enzyme activities/activity levels were relatively increased, with a significant increase observed in succinate dehydrogenase (Group B and C).

### 3.3 Neurobehavioral Changes (Figs. 7,8)

Distance covered and foot slips were used as a measure of locomotion/balance and motor activity, while latency was used as a marker of memory quality. Continual exposure of 4G radiofrequency radiation caused impairment of memory, locomotion and motor activity (Group B), however, no fall was recorded across all groups during the neurobehavioral scoring.

## 4. DISCUSSION

### 4.1 Exposure to Radiofrequency Radiation Caused Extensive Disruptions of Purkinje Cells

This study considered the organisation of the cerebellar cortex layers (molecular, Purkinje and granular) which were well represented. Cortical morphological disruptions across the experimental groups were mild when compared to the Control Group. The Purkinje cells (Group B), although preserved, appeared morphologically distorted and disrupted in terms of their typical single layer organization within the cortex. Cells of the Purkinje layer (Group D and E) appeared to be better preserved, though

some of the cells were in clusters and some appeared granulated, while some were missing. A slight reduction in number, as well as sizes of Purkinje cells in group B and C, was observed. This distortion also included loss of integrity of the cells of the molecular and granular layers. Significant distortions of neuronal and glial cells across the treated groups were observed, ranging from mild (D and E) to severe (B and C). This aligns with previous research findings [29] that reported a significant decrease in the total number of Purkinje cells in the group exposed to EMF compared to the control group.

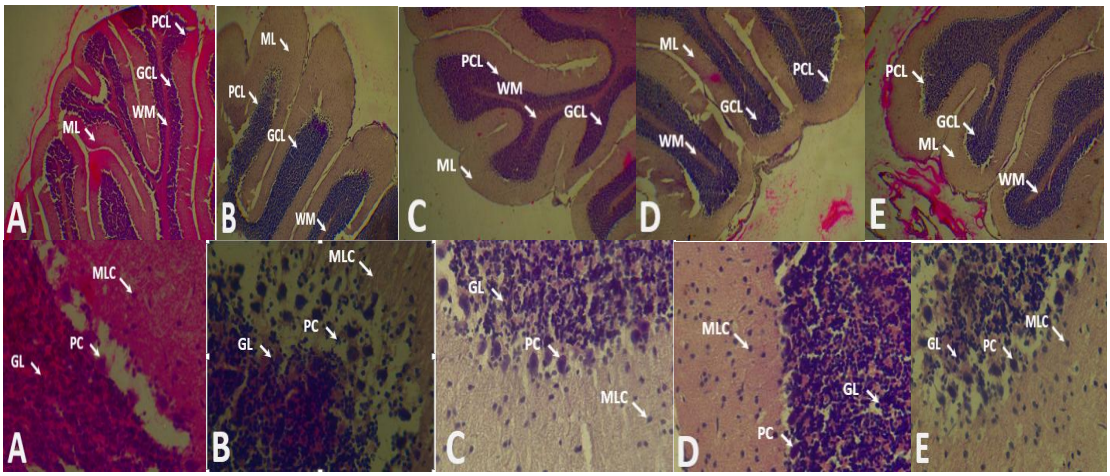



**Fig. 1. Photomicrographs of the cerebellar cortices of rats in Groups A-E, demonstrating the cerebellar cortices stained with the haematoxylin and eosin staining technique [H&E A-E, X40& X400]. There was a relative distortion in the arrangement, as well as the size or shape of cells of the Purkinje layer in group B and C. There was the observable change the distribution of neurons and glial cells across the treated groups, ranging from mild in D and E to severe in B and C**

***Legend:*** *Molecular layer [ML], Purkinje Cell Layer [PCL], inner Granular Cell Layer [GCL] and the White Mater [WM]*

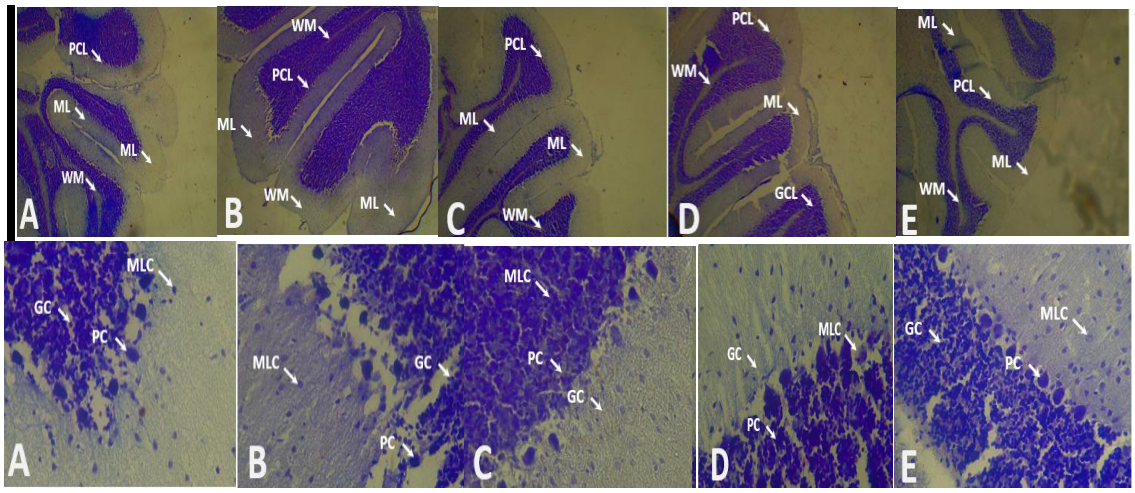



**Fig. 2. Photomicrographs of the cerebellar cortices demonstrating the cells and their Nissl bodies as measures of neuronal activities and functional integrity, stained with Cresyl Fast Violet [CFV A-E, X40 &X400]. There was an observable reduction in the cell number, expression of Nissl bodies and abnormal changes in cell sizes and morphologies in the Purkinje layer across all the treated group most especially group B and C**

***Legend:*** *Molecular layer [ML], Purkinje Cell Layer [PCL], inner Granular Cell Layer [GCL] and the White Mater [WM]*

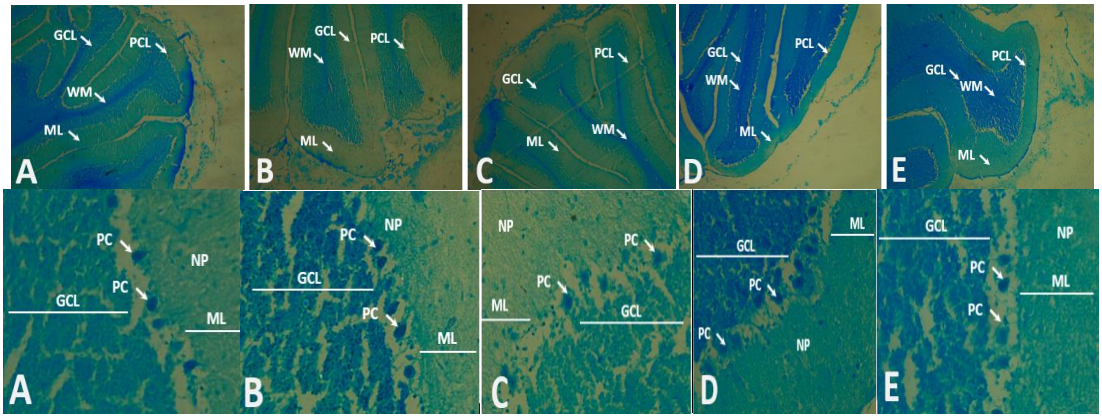



**Fig. 3. Photomicrographs of the cerebellar cortex demonstrating myelin in the cortex and fibres in the subcortical layer that forms the core of the folds, stained with Luxol Fast Blue [LFB A-E, X40& X400]. There was no extensive observable demyelination or localized disruptions in myelin distribution in the myelin sheath integrity across all the treated groups. However, relative disruptions of myelin distribution in Group B were observed that might suggest a mild effect of the exposure in the group**

***Legend:*** *Molecular layer [ML], Purkinje Cell Layer [PCL], inner Granular Cell Layer [GCL] and the White Mater [WM]*

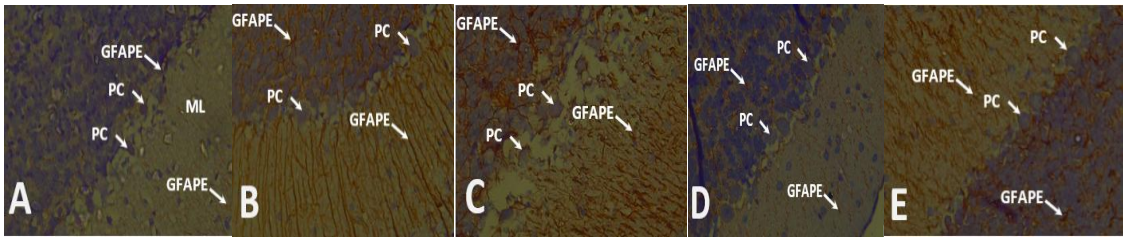
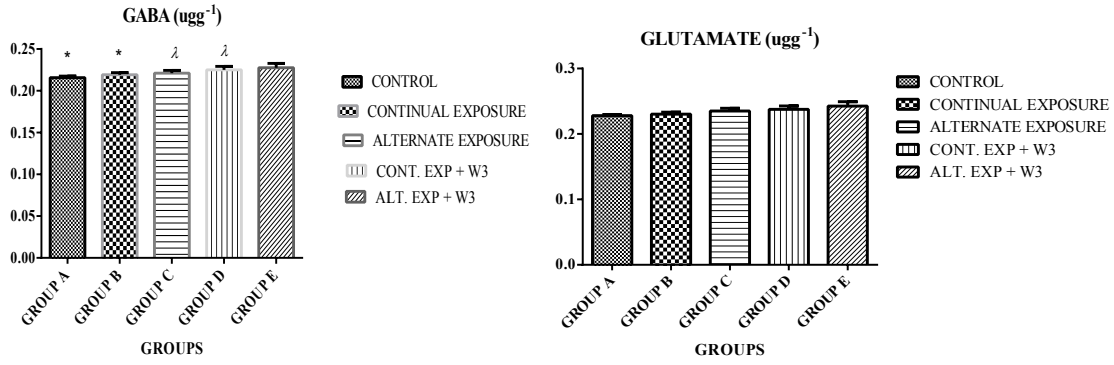



**Fig. 4. Photomicrographs of the cerebellar cortex demonstrating the expression of the glial fibrillary acidic protein [GFAP] in the cerebellar cortical layers, [GFAP, X400]. Relative abundant expression of GFAP in Group B and C indicate enhanced astrocyte reaction in response to the exposure**

***Legend:*** *Molecular layer [ML], Purkinje Cell Layer [PCL] and inner Granular Cell Layer [GCL]*

## Neurotransmitters



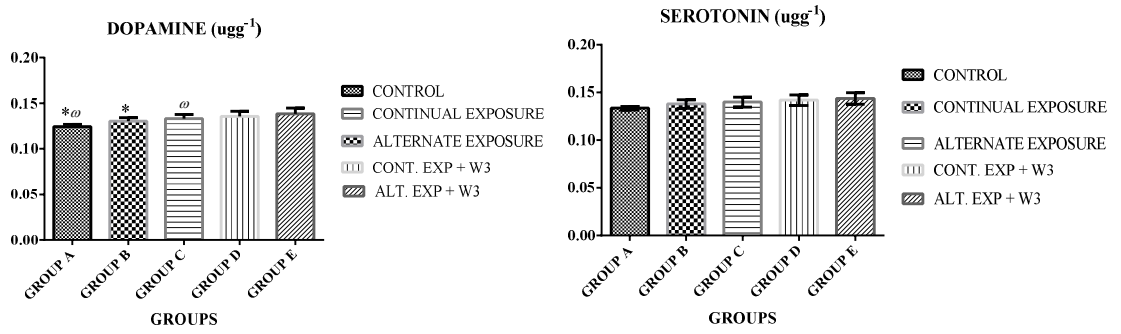



**Fig. 5. Bar chart, showing neurotransmitter levels in treated and control groups after 28 days of treatment. There was no significant alteration in the levels of the neurotransmitters' activities across the experimental groups, except for GABA and dopamine that were significantly expressed in the Groups B, C and D and the Groups B and C respectively, when compared to the control (P≤ 0.05)**

## Enzyme Activities

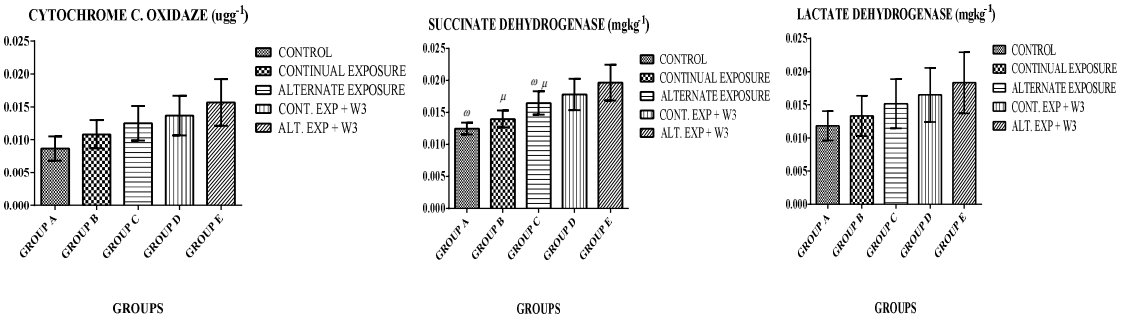
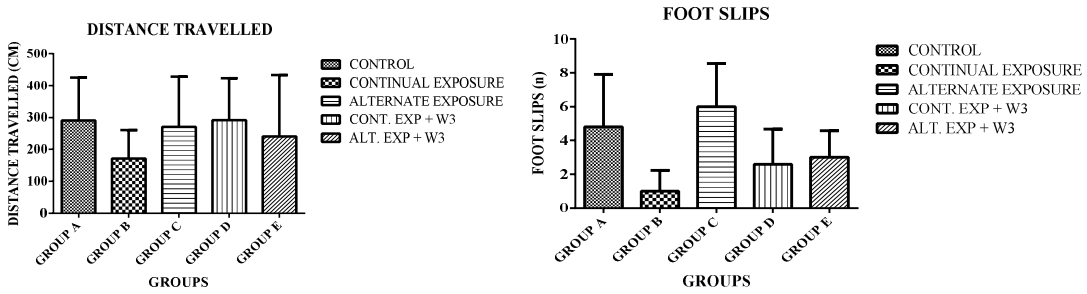



**Fig. 6. Bar chart, showing activity levels of enzymes in treated and control groups after 28 days of treatment. There was no significant difference in the activities of cytochrome C oxidase and lactate dehydrogenase across the groups (p<0.05). The activities of succinate dehydrogenase in group A and B was significantly (p<0.05) lower than that of group C. [$\omega$ = significant difference between group A and group C; $\mu$ = significant difference between group B and group C]**

## Neurobehavioral Study

## Beam Walk



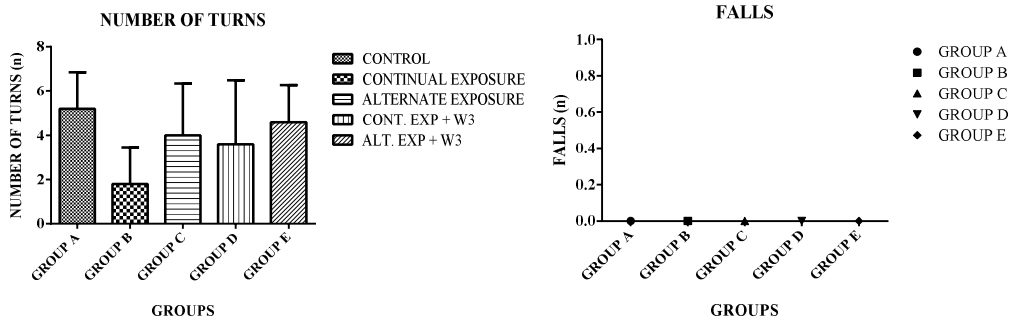



**Fig. 7. Bar chart, showing the distance covered and the number of foot slips as a measure of locomotion and motor activity. A high incidence rate of foot slips was recorded in Group C, though there was no significant statistical difference in the distance travelled by the animals while on the beam, across the groups. For distance covered, there was a relative increase in group D when compared to group B which shows that fish oil omega-3 fat had a neuroprotective or ameliorative effect against the 4G radiofrequency radiation**

**Barnes Maze**

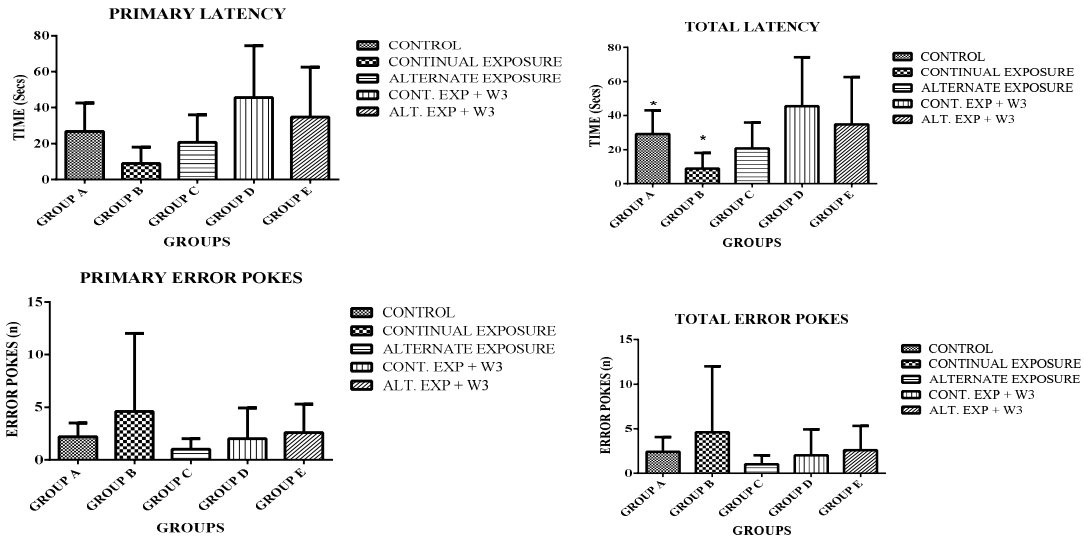



**Fig. 8. Bar chart, showing memory levels and motor control in treated and control groups after 28 days of treatment. There was generally no statistically significant difference in most parameters across groups, however, group B was relatively low when compared with group A which depicts memory impairment. Also, total latency was significantly lower in this group compared to other groups including the control, however, this was accompanied by increased errors**

## 4.2 Fish Oil Omega-3 Fat Supplement Attenuates Alterations Caused by 4G Radiofrequency Radiation

Observable alterations in Nissl substance in Groups B and C which was considered a measure of neuronal activities and functional integrity. The observed alteration would indicate that the functional integrity of certain cells was compromised. This might further imply functional alterations that might possibly result in functional aberrations and death in certain cells. There were also alteration in size and shape of the Purkinje cells across all the treated groups (especially Groups B and C). This agrees with another previous study [30], that reported a wide

variety of changes in brain chemistry and structure which included a decreased number of Purkinje cells and structural alterations in the hypothalamic region after exposure of animals to high-intensity radiofrequency fields. In the current study, there were no alterations observed within the Purkinje layer in Group E, which suggests that the fish oil omega-3 supplement was able to attenuate the effect of 4G radiofrequency radiation.

There was no extensive observable demyelination or localized disruptions in myelin distribution in the myelin sheath integrity across all the treated groups. However, there was a mild reduction and relative disruptions of myelin in Group B that might suggest a mild effect of the exposure in the group. From these observations, it can be said that radiofrequency radiation at the dose and durations employed, only mildly affected cortical myelin distribution when exposure was daily and consistent without intervention. There is, however, no evidence of extensive demyelination of nerve cells in the cerebellar cortices. This observation agrees with the study [30] that reported changes in brain chemistry and structure after exposure of animals to radiofrequency fields.

The glial fibrillary acidic protein immunohistochemical method was used to demonstrate astrocytes in the tissues and to observe possible astrocyte reaction in response to the exposure. Relative abundant expression of GFAP in Group B and C indicated enhanced astrocyte reaction, in response to the exposure. This also being supported by another previous [31]. This result further showed that 4G radiofrequency radiation had observable effects, to which the tissues responded in manners that were characteristic of a radiation assault. There was a reduction in astrocyte expression in Group D, an indication that the administered agent had ameliorative effects.

### 4.3 Continual Exposure to Radiation Caused Alterations in Neurochemical Activities

There were relative alterations in neurotransmitters activities across the treated groups and, in particular, a significant increase in the activities of GABA and Dopamine (Group D) that would be attributable to continual exposure to radiofrequency radiation. This would suggest that continual exposure to radiofrequency radiation could cause significant alterations in neurotransmitters activities in the cerebellum. Also, enzyme activities were relatively altered, with a significant increase observed in succinate dehydrogenase (Group B and C). This would further suggest that that both continuous and alternate exposures to radiofrequency radiation could still significantly increase enzyme activities in the cerebellum.

### 4.4 Memory, Locomotion and Motor Activity Impairment is Attributable to 4G Radiofrequency Radiation

Continual exposure of 4G radiofrequency radiation caused impairment of memory, locomotion and motor activities, especially when there was no intervention (Group B). This had been earlier reported from another study [32]. A high incidence rate of foot slips was also recorded in Group C, which is indicative of ataxia. However, in Group D, an increase was observed in locomotion and distance travelled, while in the Group E, there was a reduction in the frequency of foot slips, which would suggest that the intervention (fish oil omega-3 fat) had a neuroprotective effect against the 4G radiofrequency radiation. No fall was recorded across all groups during the neurobehavioral scoring. This could suggest that the effects from the exposure of the radiofrequency radiation was not severe enough to cause the animals to fall off the beam, suggesting that the exposure effects could not have caused severe ataxia or pathological impartments in balance.

## 5. CONCLUSION AND RECOMMENDATIONS

Exposure to 4G radiofrequency radiation produced observable immunohistochemical and histological changes in the cerebellar tissue and caused behavioural changes in terms of impairment of motor functions and locomotion. The fish oil omega-3 fat also provided observable neuroprotective functions against the effect of radiofrequency radiation. Further research should be done on the mechanisms of the neuroprotective properties of the omega-3 fish fat as it might protect the brain against other similar forms of neurological assaults.

## CONSENT

It's not applicable.

## ETHICAL APPROVAL

This study was carried out according to the guidelines of National Research Council Guide

and in accordance with the principles of Good Laboratory procedure (GLP) following approval of the Institutional Ethical Committee on the Use and Care of Animals.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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