Original Article

Comparative Study of the Possible Protective Effects of Omega-3 and Saffron Extract on the Cerebellum of Adult Male Albino Rats Exposed to Cell Phone Electromagnetic Radiations: Histological and Immunohistochemical Study

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ABSTRACT

Background: Quickly changing technologies and dramatic world-wide increase in the uses of radiofrequency electromagnetic radiations (RF-EMRs) emitting cell phones represents a challenge to public health. So, the interest of studying its hazards that could affect human health has been increased. Recently, omega-3 and saffron had attracted a great attention as an antioxidant.
Aim of Work: This study focused on estimation of the neuroprotective effects of omega-3 and saffron against the histological and immunohistochemical changes within the cerebellum of adult male albino rats exposed to the cell phone electromagnetic radiations.
Materials and Methods: Sixty adults male albino rats were used. They were divided into six groups, group I (control group), group II (omega-3 group), group III (saffron group), group IV (cell phone-exposed group), group V (cell phone and omega-3 group) and group VI (cell phone and saffron group). The cerebellum from each animal was cut off and processed for biochemical, histological studies by hematoxylin and eosin (Hx. & E.), toluidine blue (T.B) and electron microscope examination, immunohistochemical studies by glial fibrillary acidic protein (GFAP), caspase-3, myelin basic protein (MBP) and morphometric studies were also, done.
Results: Cell phone exposed group revealed disturbed cerebellar architecture. The molecular layer displayed pyknotic nuclei, perineuronal vacuolation and disruption of myelin sheath around nerve fibers. The Purkinje cells appeared with irregular heterochromatic nuclei, shrunken, and surrounded with empty neuropil. The granular layer appeared with some small, degenerated granule cells. Significant increase in GFAP, caspase-3 and significant decrease in MBP immunoreactivity were also observed. Omega-3 and saffron administrations decreased these adverse effects. Saffron showed higher protection against the histological alterations on the cerebellar cortex exposed to cell phone electromagnetic radiations (EMRs).
Conclusion: Cell phone emitted EMRs induces histological and immunohistochemical changes within the cerebellar tissues of male albino rats and saffron has a better neuroprotective effect than omega 3.

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Key Words: Cerebellum, MBP, omega-3, saffron.

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INTRODUCTION

The twenty-one century is the time of high-level of technology that simplified human life. Many types of telecommunications are used every day. The most common ones are cell or mobile phones, and smart phones[1]. More than half of the world's population at different ages use cell phones. Public interest with the use of these devices have been increased because of expansion of mobile network and multiple services offered by their service providers such as short messaging service, easy and quick electronic payments and sending videos and pictures. So, cell phone has become an essential tool not a luxury device[2].

Cell phones are one of the most prevalent devices releasing radiofrequency electromagnetic radiations (RF-EMRs) at frequencies between 450 and 2700 MHz. Human bodies can act as antennas that absorb these waves emitted from cellular phones, thus RF-EMRs exposure is critical to human health and safety[3]. Mobile phones are held close to the body, especially near the brain, this has given rise to an increasing concern for the effect of mobile phone radiations on human health in general, particularly on nervous system[4].

Previous studies revealed the adverse effects of RF-EMRs on heart, blood pressure and endocrine system. Usage of mobile phones is also, associated with many health problems like neck pain, earache, tinnitus, morning tiredness, headache, fatigue, painful fingers, and eye symptoms[5]. Exposure to RF-EMRs leads to structural neurodegeneration. These structural changes lead to modifications in behavior, memory, and learning. Also, causing toxic psychological impacts as irritability, lack of attention and sleep disturbances. So, the need to search for new effective and safe agents for protection from these changes is inevitable[6].

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Omega-3 fatty acids are essential long chain polyunsaturated fatty acids present in food sources such as fish oils and marine animals[7]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are among the Omega -3 fatty acids. Human should obtain them from food as they are not synthesized in their body[8]. Omega-3 fatty acids are important for growth and reproduction. They are also, essential for the nervous system development, intelligence, and vision[9]. previous studies attributed the beneficial effect of omega-3 fatty acids to their antioxidant, antiapoptotic and neurotrophic properties[10,11].

Saffron, obtained from dried stigmas of Crocus sativus (Iridaceae), is a highly valued spice, usually used in flavoring and coloring food. It is a medicinal plant having many therapeutic effects. It possesses anti-inflammatory, antioxidant, and anti-apoptotic properties due to its active ingredients including crocin, crocetin, picrocrocin and safranal[12].

Saffron extract has been proved to promote learning and memory functions in mice after memory impairment induced by ethanol. Consequently, saffron may be valuable for people with dementia. It has also been suggested that saffron improves the oxidative damage changes due to cerebral ischemia in the rats[13].

So, this study was designed to evaluate the neuroprotective effects of omega-3 and saffron against the histological and immunohistochemical changes within the cerebellum of male albino rats exposed to the cell phone electromagnetic radiations.

MATERIALS AND METHODS

Chemicals

Omega-3: It is available in the form of soft gelatin capsules each contained 300 mg from Fresenius company, Germany. Each capsule was evacuated in 1 ml insulin syringe.

Saffron (stigma of Crocus sativus): It was purchased from local grocery in Shebin el-kom, Menoufia, then prepared in the form of aqueous extract.

Preparation of aqueous extract of saffron

Saffron was prepared in central pharmacological Research Laboratory of Faculty of Medicine, Menoufia University. Stigma is the part of Crocus sativus that is used as herbal medicine. The stigma was dried in air and then grounded to fine powder. Aqueous extract was prepared by adding one gram of saffron stigma powder to 100 ml boiling distilled water, after 2 hours it was homogenized, filtered, and stored in dark bottles immediately until use[14].

Animals

This research was done on sixty adult male albino rats, weighting 170-200g. The rats were housed ten per cage in a clean ventilated room with normal light-dark cycle. Laboratory diet was used for feeding. Handling and treatment of laboratory animals were following guidelines of the animal care committee of the research laboratory of experimental animals at the faculty of medicine, Menoufia University, Egypt.

Experimental design

The rats were divided into six groups at random way, included 10 rats in each group.

Group I (control group): The rats were not exposed to any procedure.

Group II (omega-3 treated group): The rats were given omega-3 at a dose 400 mg/kg/d (equivalent to 0.3 ml), orally by intragastric gavage for 12 weeks[15].

Group III (saffron treated group): The animals were given freshly prepared saffron extract at a dose 40mg/kg/d orally by intragastric gavage. Each animal received 0.7ml saffron extract[16].

Group IV (cell-phone exposed group): The rats were exposed to 900 MHz electromagnetic radiations (EMRs) emitted via ringing mode of cell phone for 2 h/d, 6ds/w for 12 weeks[17].

Group V (cell phone exposed-omega-3treated group): The rats were given omega-3 at a dose 400 mg/kg/d by intra-gastric gavage while receiving 900 MHz EMR exposure as group IV.

Group VI (cell phone exposed –saffron treated group): The rats in this group were given freshly prepared saffron extract at a dose 40 mg/kg/d by intra-gastric gavage while receiving the same EMR exposure.

Cell phone-EMRs exposure

All groups were stayed in polycarbonate cages. Groups IV, V&VI were exposed to EMRs emitted by ringing mode for 2 hours daily from mobile phones, the frequency was 900 MHz (NOKIA 3110; Nokia Mobile Phones Ltd.). The phone was put under the cage at its center with 3 cm distance between the mobile and cage floor.

At the assigned time of scarification, Ketamine (90mg/kg)[18] was injected intraperitonially to anaesthetize the rats from all groups. Then, intra-cardiac perfusion was done by 2.5% glutaraldehyde with 0.1 M phosphate buffer at pH 7.4 for partial fixation of the specimens. From each animal in all groups, the cerebellum was dissected out, then had been cut into right and left halves for biochemical, histological and immunohistochemical studies.

Biochemical study

For determination of malondialdehyde (MDA) oxidant enzyme, cerebellar tissues were homogenized in potassium phosphate buffer solution (50Mm, PH 7.5) via utilizing a potter elvehiem homogenizer to allow 10 % homogenate. Homogenate were centrifuged, supernatant was recovered, placed on ice, and immediately utilized for MDA estimation, MDA is considered indirect indicator for lipid peroxidation, it was measured colorimetrically in homogenate[19].

Histological study

A- Light study

The right half of the cerebellum from each animal was put in 10% neutral-buffered formalin. The specimens were prepared for paraffin blocks, 5 µm thickness sections were cut, then stained with hematoxylin and eosin (Hx. & E.) to exhibit the histological structure[20] and toluidine blue for demonstration of Nissl's granules[21].

B- Transmission electron microscopic study

Small specimens from the left half of the cerebellum from each animal in all groups were rapidly fixed in 3% glutaraldehyde solution and processed for examination using a Joel electron microscope in Alexandria E.M center at Faculty of Medicine, Alexandria University[22].

Immunohistochemical study

Nocturnal incubation of the cerebellar tissues was done with the next primary antibodies.

- **Anti-GFAP (Glial Fibrillary Acidic Protein) antibody:** Mouse monoclonal antibody, 1/100 dilution, Abcam. This stain is considered specific for the intermediate filaments fibrillary acidic protein which is present in astrocytes. Brown coloration of the cytoplasm of astrocytes and their processes was considered positive. Brain tissue was used as a positive control[23].
- **Anti-caspase-3 antibody (a marker of apoptosis):** The primary antibody was the rabbit polyclonal anti-Caspase-3 antibody (1:200 dilution; PA1-26426, Abcam, USA). Cytoplasmic brown coloration was considered positive reaction. Tonsil was used as positive control[24].
- **Anti- Myelin Basic Protein (MBP):** The primary monoclonal antibody was the rabbit- anti MBP (1: 300 dilution; EMD Millipore, Billerica). It is used for identification for non-myelinating and myelinating nerve fibers. Brain was used as positive control[25].

Negative controls were made by primary antibody exclusion.

Finally, counterstaining was performed using Mayer's hematoxylin.

Morphometrical study and Statistical analysis

Mean number of Purkinje cells/mm² and mean thickness of granular layer in µm in all studied groups were measured in Hx. & E.-stained sections. While mean area percentage of GFAP and mean color intensity of caspase-3 and MBP were measured in immune-stained sections, using the objective lens of magnification X40. From various sections of each group, 10 non overlapping microscopic fields were measured, utilizing interactive measuring menu of image analyzer (Lecia Qwin 500 image analyzer computer system, England) in anatomy department, Faculty of Medicine, Menoufia University.

The morphometric results were analyzed and compared by student's t-test. The data were tabulated as mean ± SD and analyzed utilizing statistical package for the social science software (version 17.0, Chicago, Illinois, USA). The *p-value* was utilized to test the significant change in-between the experimental groups. Significance was considered at $P<0.05$[26].

RESULTS

Biochemical results

Data in (Table 1) revealed that group IV (cell phone exposed group) exhibited a highly significant increase ($P<0.001$) in the mean value of MDA when compared with the control group. However, group V (cell phone exposed and omega-3 treated group) showed a significant increase in this parameter ($P<0.05$) in comparison with control animals and group IV. While group VI (cell phone exposed and saffron treated group) displayed a non-significant change in comparison with control animals and showed a significant change when compared with group V ($P<0.05$) (Histogram, 1).

Histological results

A- Light microscopic results

Hx. &E.-stained sections of the cerebellum of control groups (groups I, II&III) revealed normal histological structure. The cerebellum was composed of grey matter and white matter. Grey matter was present on the surface of the cerebellum, forming cerebellar cortex which consisted of three layers, molecular, Purkinje and granular layers. White matter located under the cerebellar cortex and formed mainly of myelinated nerve fibers (Figure 1). The superficial molecular layer formed mainly of fibers with scattered small stellate and basket cells. The Purkinje layer was formed of one layer of Purkinje cells. The cells appeared rounded or pyriform in shape with extended apical arborizing dendrites. They had central vesicular rounded nuclei with prominent nucleoli. Bergmann astrocytes with pale cytoplasm and pale nuclei surrounding the Purkinje cells were observed. The deep granular layer contained small, packed granules cells with cerebellar islands between them (Figure 2).

Sections of the cerebellum of group IV (cell phone exposed group) exhibited disturbed cerebellar architecture. The molecular layer displayed pyknotic nuclei with perineuronal vacuolation (Figures 3,4). While Some Purkinje cells appeared shrunken and surrounded with empty neuropil. some of them showed karyolitic changes (Figure 3). They were irregularly arranged (Figure 4), remanent of Purkinje neuron was observed within granular layer (Figures 4,5). The granular layer appeared with clumped degenerated granule cells (Figures 3,4). Moreover, apparent decrease in the Purkinje cells number and granular layer thickness were observed (Figure 5).

Sections of group V (cell phone exposed and omega-3 treated group) showed normal appearance of molecular

and granular layers, pyriform shaped Purkinje cells appeared with arborizing dendrites. One Purkinje cell appeared degenerated (Figure 6). While group VI (cell phone exposed and saffron treated group) exhibited nearly normal appearance of molecular, Purkinje and granular layers (Figure 7).

In Toluidine blue-stained cerebellar sections, control groups (groups I, II&III) revealed Purkinje cells studded with Nissl's granules that surround central rounded vesicular nuclei in their cytoplasm (Figure 8). While in group IV (cell phone exposed group), Purkinje cell appeared shrunken and deeply stained (Figure 9). Moreover, group V (cell phone exposed and omega-3 treated group) showed some Purkinje cells with Nissl's granules in their cytoplasm, while, other appeared deeply stained (Figure 10). Group VI (cell phone exposed and saffron treated group) displayed Purkinje cells with Nissl's granules surrounding centrally located vesicular nuclei in their cytoplasm (Figure 11).

B- Transmission electron microscopic results

Ultra-thin sections of the cerebellum of control rats (groups I, II&III) exhibited normal structure, Purkinje neuron had euchromatic nucleus with prominent nucleoli. The cytoplasm contained mitochondria, Golgi apparatus, multiple cisternae of rough endoplasmic reticulum and free ribosomes (Figure 12). The granule cells had central rounded euchromatic nuclei with peripheral clumps of chromatin surrounded with a thin rim of cytoplasm containing mitochondria. Myelinated nerve fibers containing mitochondria were noticed (Figure 13). Moreover, white matter revealed myelinated nerve fibers of varying sizes and myelin thickness contained mitochondria (Figure 14). While Group IV (cell phone exposed group) exhibited structural changes, the Purkinje neuron appeared with an irregular nucleus, the cytoplasm contained vacuoles, degenerated mitochondria, free ribosomes (Figures 15,16), phagophore containing fragments of cytoplasmic organelles, late phagosome and 2ry lysosome (Figure 16). Myelinated nerve fibers contained mitochondria (Figures 15,17) and interstitial vacuolar space were observed (Figure 15). The granules cells displayed irregular shaped heterochromatic nuclei with wide perinuclear space. Other cell had small, rounded nucleus and vacuolated cytoplasm contained degenerated mitochondria (Figure 17). However, nerve fibers with split degenerated myelin sheaths and unusual myelin protrusions into cortical neurons were observed in the white matter (Figure 18). Moreover, group V (cell phone and omega-3 treated group) revealed Purkinje neuron with euchromatic nucleus and well-defined nucleolus, the cytoplasm contained mitochondria, Golgi apparatus and some dilated cisternae of rough endoplasmic reticulum (Figure 19). Granule cell appeared with irregular heterochromatic nucleus among others with nearly normal appearance. Interstitial vacuolar spaces and myelinated nerve fibers were seen (Figure 20). In the white matter, some nerve fibers with focal areas of myelin sheath separation were noticed (Figure 21). While Group VI (cell phone exposed and saffron treated group) revealed preservation of the cerebellar tissue. The Purkinje neuron appeared pyriform in shape with indented euchromatic nucleus. The cytoplasm had Golgi apparatus, mitochondria, cisternae of rough endoplasmic reticulum and phagosome (Figure 22). Normal shaped granule cells with rounded euchromatic nuclei were noticed (Figure 23). In the white matter, the nerve fibers were surrounded with normal myelin sheath and contained neurofilaments and microtubules (Figure 24).

Immunohistochemical results

GFAP immuno-stained cerebellar section of control groups (groups I, II&III) revealed mild positive cytoplasmic immune expression in the astrocytes in the granular layer (Figure 25). In Group IV (cell phone exposed group) displayed strong positive GFAP immune expression for astrocytes in the granular layer compared to control group (Figure 26). While group V (cell phone and omega-3 treated group) revealed moderate GFAP immune-expression (Figure 27). Mild positive immunoreaction was observed in group VI (cell phone exposed and saffron treated group) (Figure 28).

Caspase-3 immuno-stained cerebellar sections of control groups (groups I, II&III) displayed negative immune expression in the cerebellar cortex (Figure 29). In Group IV (cell phone exposed group) strong positive cytoplasmic immunoreactions for caspase-3 in the Purkinje cells and some granule cells were observed (Figure 30). While group V (cell phone and omega-3 treated group) revealed mild positive cytoplasmic immunoreactions for caspase-3 in the Purkinje cells and some granule cells (Figure 31). Moreover, group VI (cell phone exposed and saffron treated group) showed negative immunoreactions (Figure 32).

Regarding MBP immune expression, control groups (groups I, II&III) showed strong MBP positive myelinated nerve fibers in the white matter of cerebellum (Figure 33). While group IV (cell phone exposed group) revealed mild MBP positive staining within the myelinated nerve fibers of white matter (Figure 34). Sections of the cerebellum of group V (cell phone and omega-3 treated group) revealed moderate positive immune-expression (Figure 35), while group VI (cell phone exposed and saffron treated group) exhibited strong positive immune-expression for MBP in the white matter like control groups (Figure 36).

Morphometric results

Data in (Table 2) demonstrated that group IV (cell phone exposed group) exhibited a highly significant decrease ($P<0.001$) in the number of Purkinje cells and thickness of granular layer in comparison with the control animals, while group V (cell phone exposed and omega-3 treated group) revealed a significant decrease ($P<0.05$) in the Purkinje cells number and a non-significant change in the granular layer thickness ($P>0.05$) in comparison with the control group and showed a non-significant change in the Purkinje cell number and a significant change in

the granular layer thickness in comparison with group IV. Moreover, group VI (cell phone exposed and saffron treated group) displayed non-significant changes in these parameters (P>0.05) in comparison with the control group and a highly significant changes when compared with group IV and a significant change in Purkinje cells number and non-significant change in granular cell layer when compared with group V (Histograms, 2a,2b).

Data in (Table 3) showed that group IV (cell phone exposed group) exhibited a highly significant increase (P<0.001) in the mean area % of GFAP and mean values of color intensity of caspase-3 and a highly significant decrease in the values of MBP intensity when compared with the control group. However, group V (cell phone exposed and omega-3 treated group) showed a significant change in these parameters (P<0.05) in comparison with control animals. While revealed a highly significant changes when compared with group IV. Group VI (cell phone exposed and saffron treated group) displayed a non-significant change in these parameters in comparison with control animals and showed a significant change in the mean area % of GFAP and mean values of color intensity of caspase-3 and non-significant change in the MBP intensity when compared with group V (P<0.05) (Histograms, 3a,3b,3c).

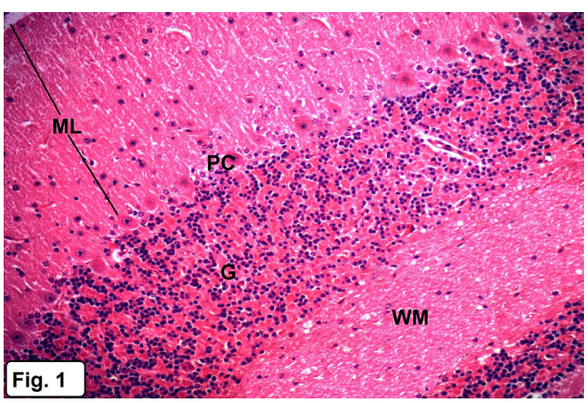


Fig. 1: A photomicrograph of the cerebellum of control group (I) showing cerebellar cortex with its various layers, molecular (ML), Purkinje (PC) and granular (G) layers, as well as white matter (WM) consisting mainly of myelinated axons. (Hx & E X100)

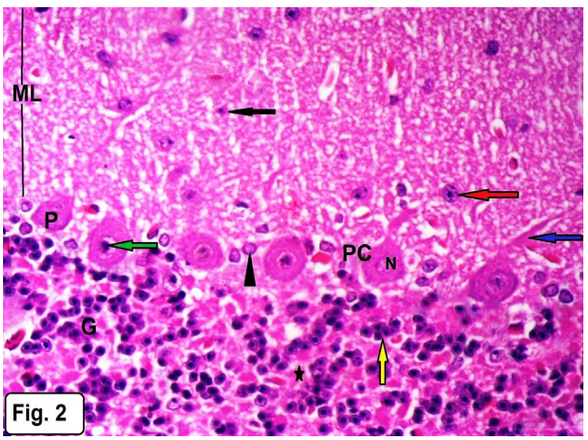


Fig. 2: A Photomicrograph of the cerebellum of control group (I) showing a molecular layer (ML) composed mainly of fibers with scattered small stellate (black arrow) and basket cells (red arrow). Purkinje layer (PC) consists of one layer of Purkinje cells (P), with apical extended arborizing dendrites (blue arrow) and central rounded vesicular nuclei (N) with prominent nucleoli (green arrow). The granular layer (G) comprises small packed granule cells (yellow arrows) with cerebellar islands (star). Note the Bergmann astrocytes (black arrowhead) around the Purkinje cells. (Hx & E X200)

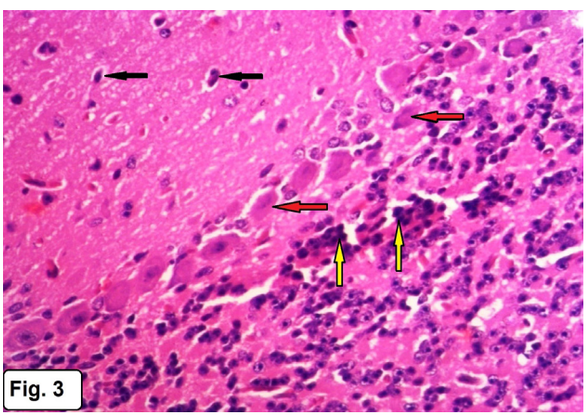


Fig.3: A photomicrograph of the cerebellum of cell phone exposed group (IV) showing a molecular layer with pyknotic nuclei and perineuronal vacuolation (black arrows). Some Purkinje cells tend to have karyolitic nuclear alteration, surrounded by an empty neuropil (red arrows). Some clumped degenerated granule cells (yellow arrows) are located within the granular layer. (Hx & E X200)

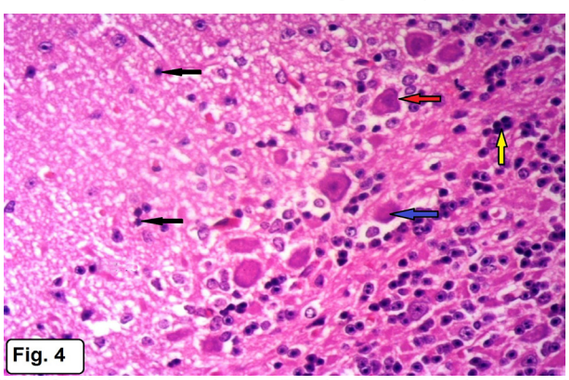


Fig. 4: A photomicrograph of the cerebellum of cell phone exposed group (IV) showing a molecular layer with some pyknotic nuclei (black arrows). The Purkinje cells look irregularly organized, shrunken with empty neuropils (red arrow). The granular layer includes degenerated granule cells (yellow arrow). Notice, the remnant of Purkinje neuron (blue arrow) in the granular layer. (Hx & E X200)

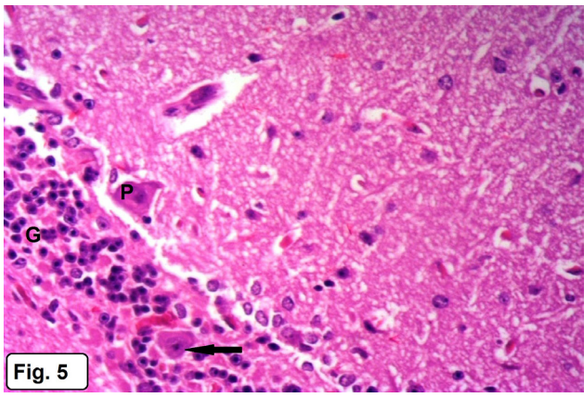


Fig. 5: A photomicrograph of the cerebellum of cell phone-exposed group (IV) showing a decrease in the number of Purkinje cells (P) and the thickness of the granular layer (G). Purkinje neuron is located deep inside the granular layer (black arrow). (Hx & E X200)

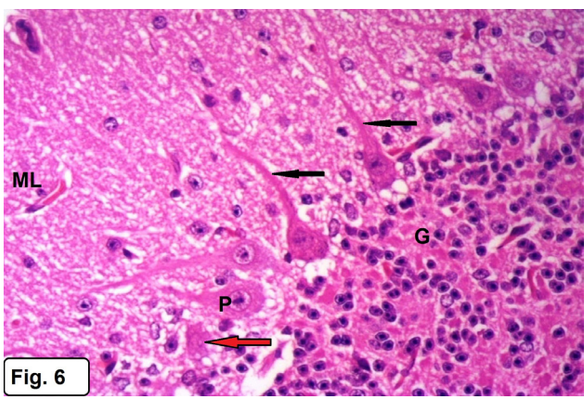


Fig. 6: A photomicrograph of the cerebellum of cell phone exposed and omega-3 treated group (V) shows pyriform Purkinje cells (P) with arborizing dendrites (black arrows). A Purkinje cell seems to be degenerated (red arrow). The molecular (ML) and granular (G) layers have a normal appearance. (Hx & E X200)

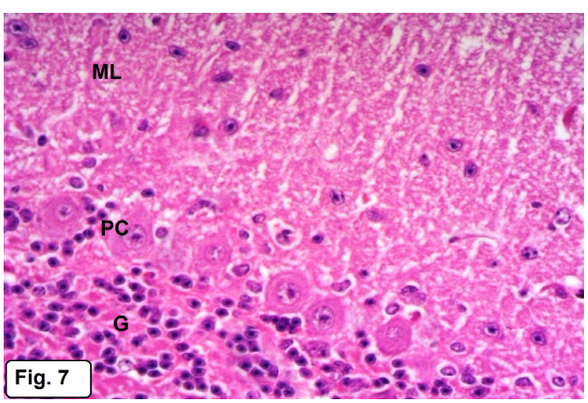


Fig. 7: A photomicrograph of the cerebellum of cell phone exposed and saffron treated group (VI) showing nearly normal appearance of molecular (ML), Purkinje (PC) and granular (G) layers. (Hx &E X200)

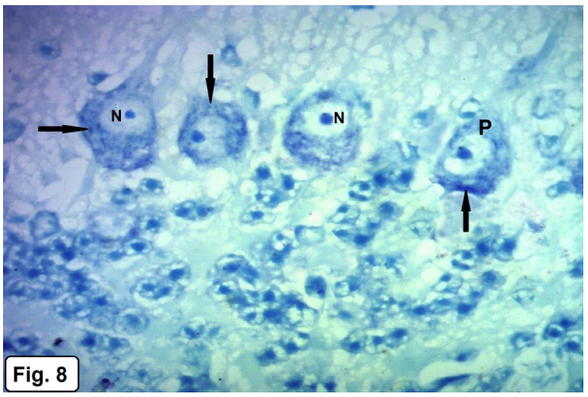


Fig. 8: A photomicrograph of the cerebellum of control group (I) showing Purkinje cells (P) with central rounded vesicular nuclei (N) and Nissl's granules (black arrows) in their cytoplasm. (T.B X400)

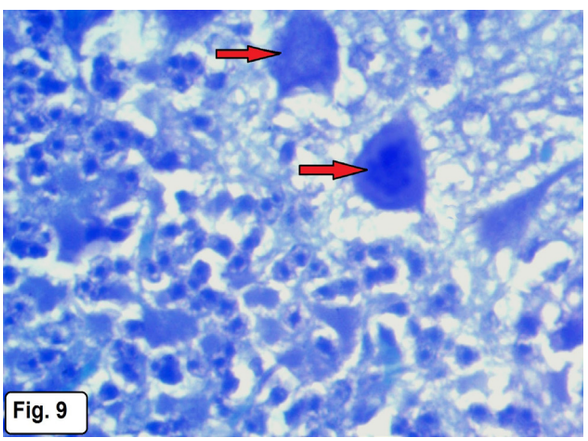


Fig. 9: A photomicrograph of the cerebellum of cell phone exposed group (IV) showing deeply stained Purkinje cells (red arrows). (T.B X400)

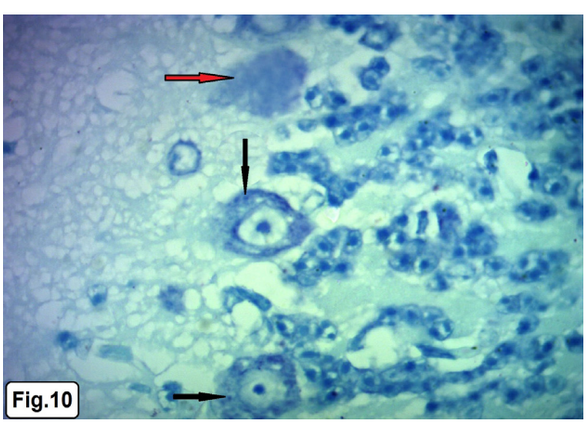


Fig. 10: A photomicrograph of the cerebellum of cell phone exposed and omega-3 treated group(V) showing Purkinje cells with Nissl's granules (black arrows) in their cytoplasm. Others appear deeply stained (red arrow). (T.B X400)

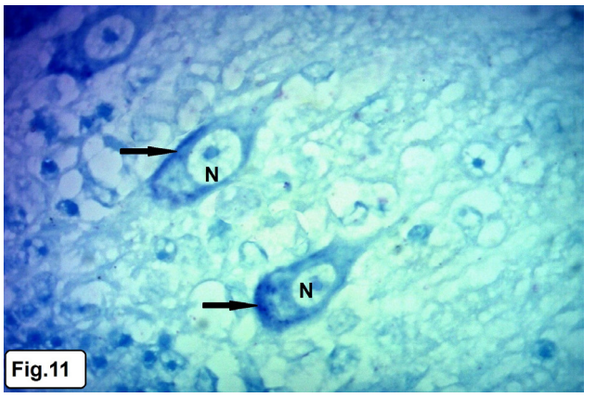


Fig. 11: A photomicrograph of the cerebellum of cell phone and saffron treated group (VI) showing Purkinje cells contain Nissl's granules (black arrows) and centrally located vesicular nuclei (N) in their cytoplasm. (T.B X400)

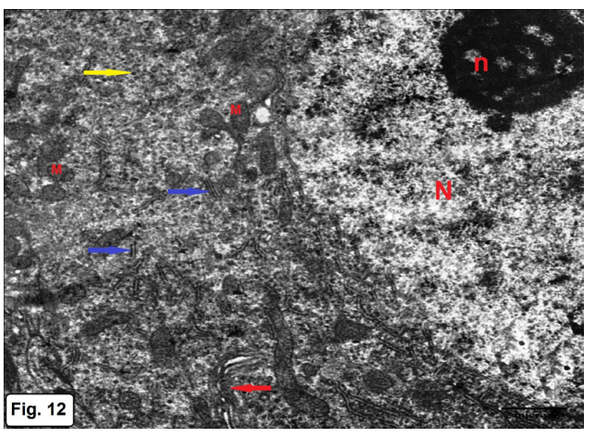


Fig. 12: An electron micrograph of the cerebellum of control group (I) showing Purkinje cell with euchromatic nucleus (N) and well-defined nucleoli (n). The cytoplasm includes mitochondria (M), Golgi apparatus (red arrow), several cisternae of rough endoplasmic reticulum (blue arrows) and free ribosomes (yellow arrow). (TEM X15000)

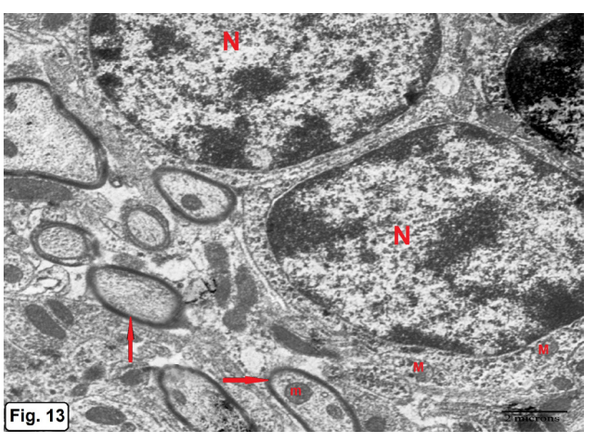


Fig. 13: An electron micrograph of the cerebellum of control group (I) showing granule cells having central rounded euchromatic nuclei (N) surrounded with a thin rim of cytoplasm containing mitochondria (M). Notice, myelinated nerve fibers (red arrows) containing mitochondria (m). (TEM X15000)

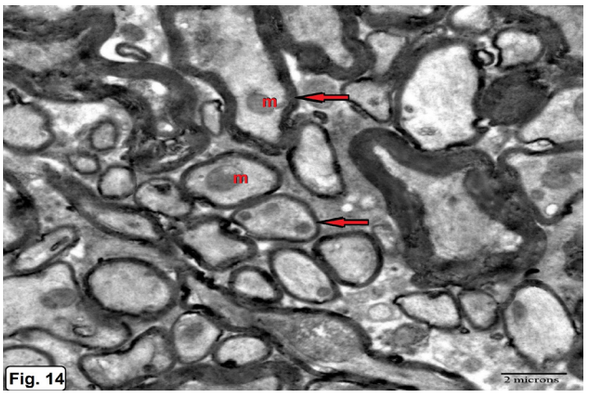


Fig. 14: An electron micrograph of the white matter of cerebellum of control group (I) showing nerve fibers surrounded by normal intact myelin sheath (red arrows) of varying sizes and thickness containing mitochondria (m). (TEM X15000)

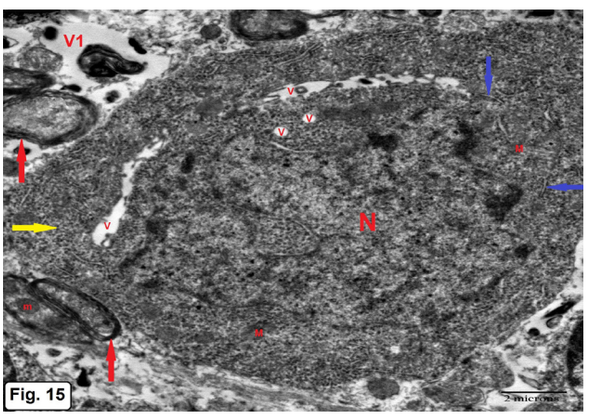


Fig. 15: An electron micrograph of the cerebellum of cell phone exposed group (IV) showing Purkinje cell having an irregular nucleus (N). The cytoplasm contains vacuoles (V), free ribosomes (yellow arrow), degenerated mitochondria (M) and rough endoplasmic reticulum cisternae (blue arrows). Myelinated nerve fibers (red arrows) containing mitochondria (m) and interstitial vacuolar space (V1) are seen. (TEM X15000)

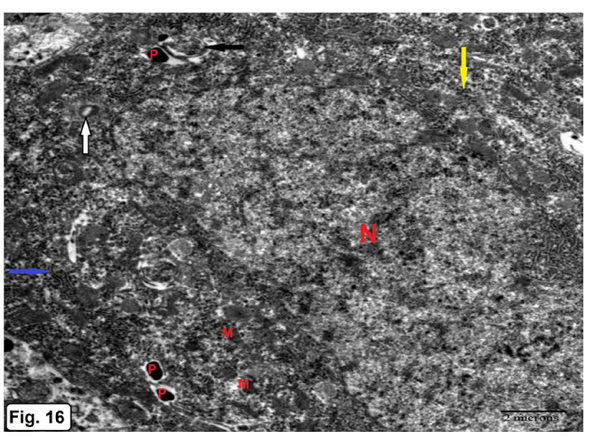


Fig. 16: An electron micrograph of the cerebellum of cell phone exposed group (IV) showing Purkinje cell with an ill-defined degenerated nucleus (N). The cytoplasm has phagophore (black arrow) containing fragments of cytoplasmic organelles, late phagosome (P), 2ry lysosome (white arrow), degenerated mitochondria (M), rough endoplasmic cisternae (blue arrow) and free ribosomes (yellow arrow). (TEM X15000)

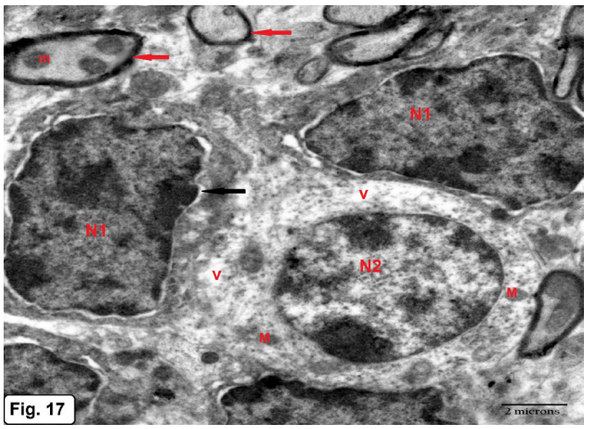


Fig. 17

Fig. 17: An electron micrograph of the cerebellum of cell phone exposed group (IV) showing granule cells having irregularly defined heterochromatic nucleus (N1) with wide perinuclear space (black arrow). Other cells have small, rounded nucleus (N2) and vacuolated cytoplasm (V) containing degenerated mitochondria (M). Myelinated nerve fibers (red arrows) containing mitochondria (m) are observed. (TEM X15000)

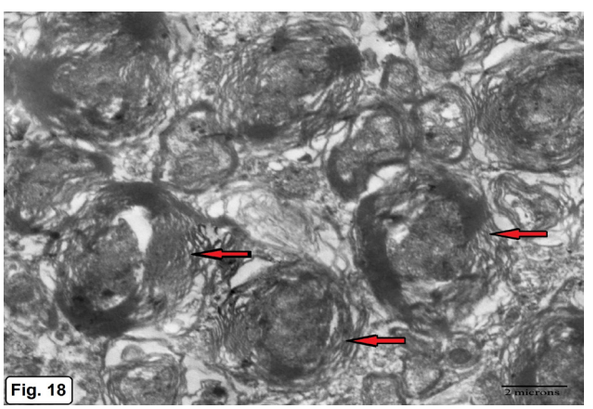


Fig. 18

Fig. 18: An electron micrograph of the white matter of cerebellum of cell phone exposed group (IV) showing split degenerated myelin sheaths with unusual myelin protrusions (red arrows) into cortical neurons. (TEM X15000)

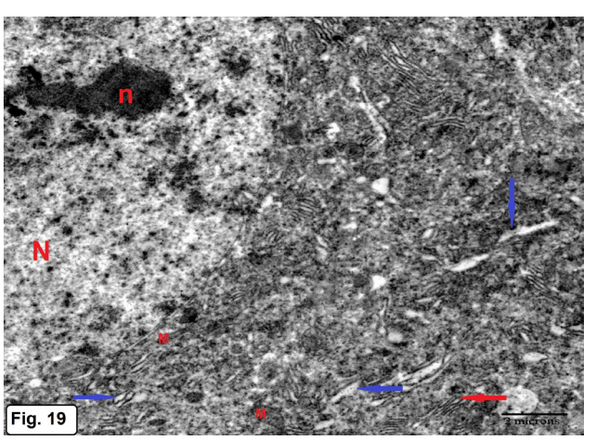


Fig. 19

Fig. 19: An electron micrograph of the cerebellum of cell phone exposed and omega-3 group(V) showing Purkinje cell has euchromatic nucleus (N) with a well-defined nucleolus(n). The cytoplasm includes mitochondria (M), Golgi apparatus (red arrow) and some dilated endoplasmic reticulum cisternae (blue arrows). (TEM X15000)

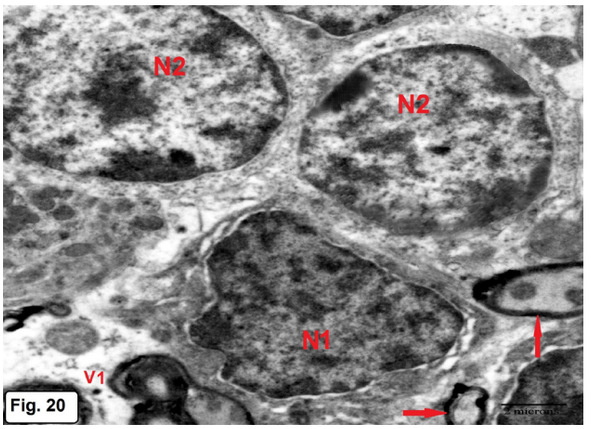


Fig. 20

Fig. 20: An electron micrograph of the cerebellum of cell phone exposed and omega 3 treated group (V) showing granule cell with irregular heterochromatic nucleus (N1). Other granule cells appear normal with rounded euchromatic nuclei (N2). Notice, the presence of interstitial vacuolar spaces (V1) and myelinated nerve fibers (red arrows). (TEM X15000)

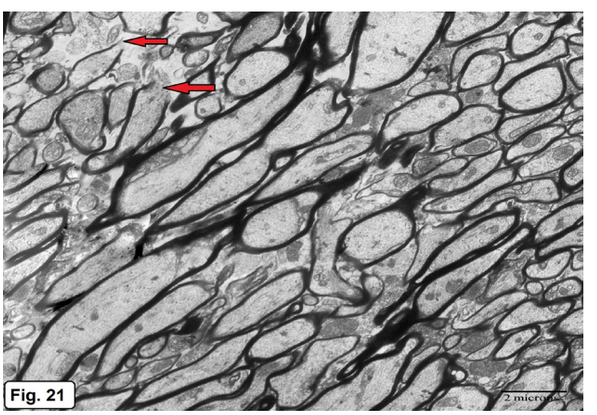


Fig. 21

Fig. 21: An electron micrograph of the white matter of cerebellum of cell phone exposed and omega-3 treated group (V) showing myelinated axons. Some nerve fibers appear with focal areas of myelin sheath separation (red arrows). (TEM X15000)

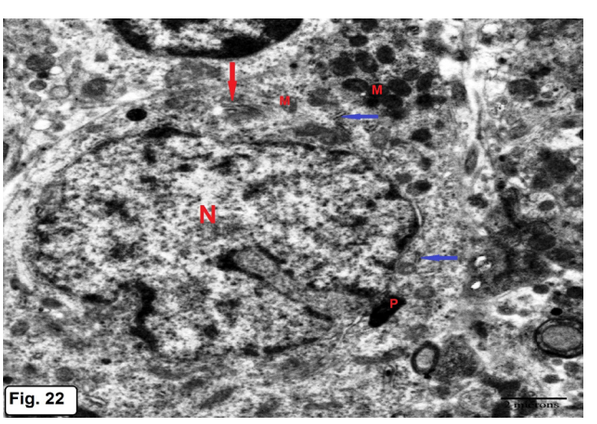


Fig. 22

Fig. 22: An electron micrograph of the cerebellum of cell phone exposed and saffron treated group (VI) showing pyriform shaped Purkinje cell with indented euchromatic nucleus (N). The cytoplasm contains Golgi apparatus (red arrow), mitochondria (M) and cisternae of rough endoplasmic reticulum (blue arrows). Notice, the presence of phagosome (P). (TEM X15000)

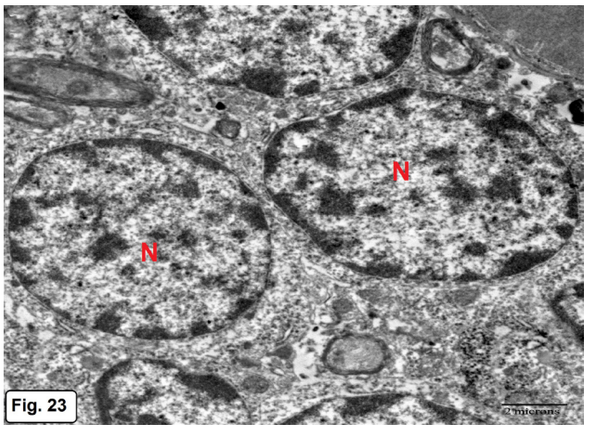


Fig. 23: An electron micrograph of the cerebellum of cell phone exposed and saffron treated group (VI) showing nearly normal granule cells with rounded nuclei (N). (TEM X15000)

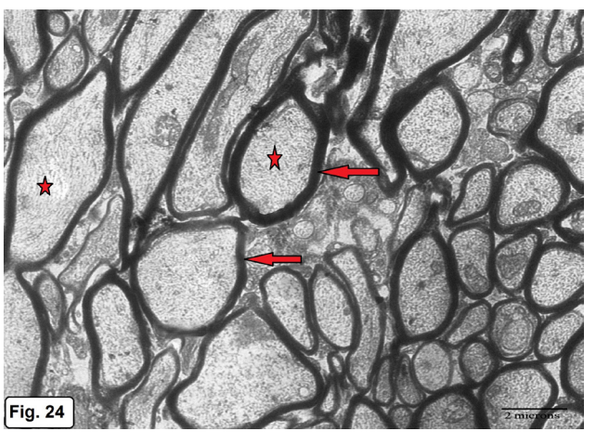


Fig. 24: An electron micrograph of the white matter of cerebellum of cell phone exposed and saffron treated group (VI) showing nerve fibers having normal myelin sheath (red arrows). Microtubules and neurofilaments (red stars) can be identified. (TEM X15000)

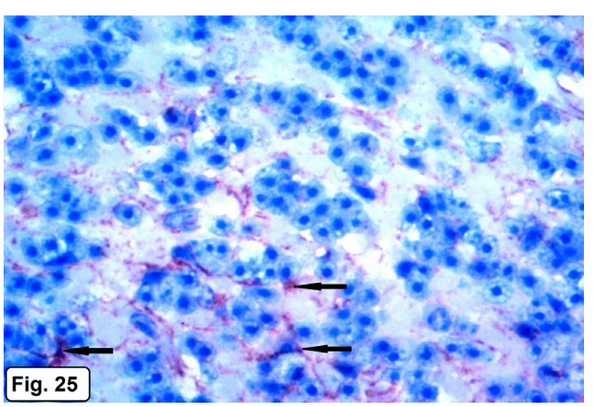


Fig. 25: A photomicrograph of the cerebellum of control group (I) showing mild positive cytoplasmic GFAP immune expression for astrocytes (black arrows) in the granular layer. (GFAP X400)

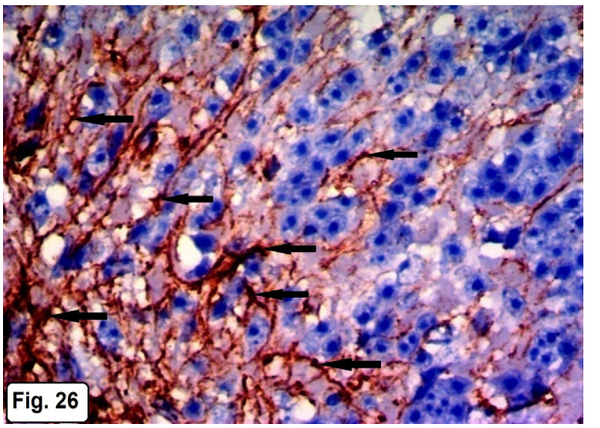


Fig. 26: A photomicrograph of the cerebellum of cell phone exposed group (IV) showing strong positive cytoplasmic GFAP immune expression for astrocytes (black arrows) in the granular layer. (GFAP X400)

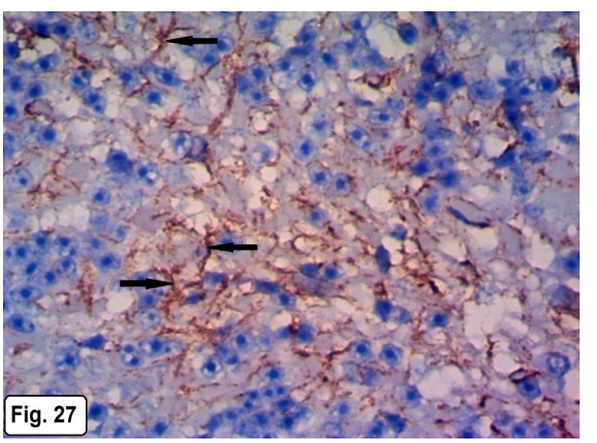


Fig. 27: A photomicrograph of the cerebellum of cell phone exposed and omega-3 treated group (V) showing moderate positive cytoplasmic GFAP immune expression for astrocytes (black arrows). (GFAP X400)

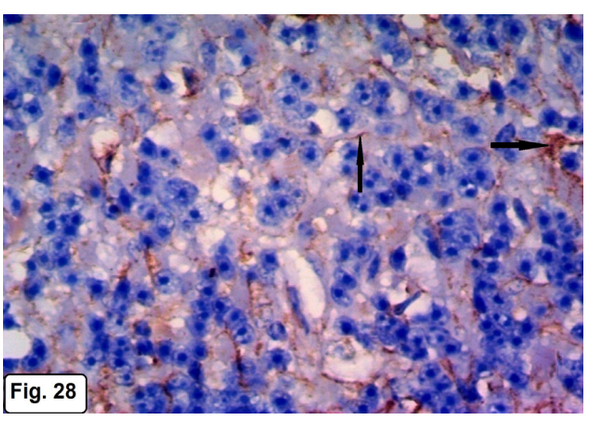


Fig. 28: A photomicrograph of the cerebellum of cell phone exposed and saffron treated group (VI) showing mild positive cytoplasmic GFAP immune expression for astrocytes (black arrows). (GFAP X400)

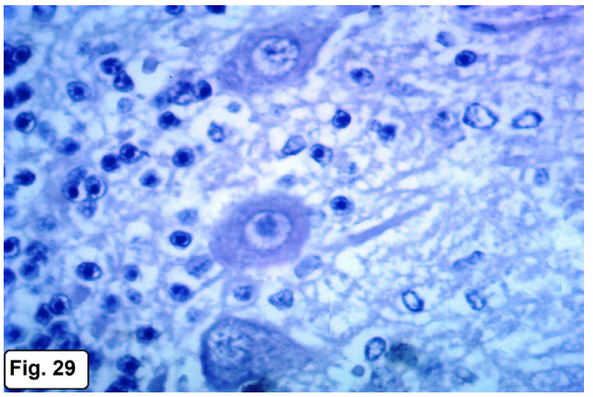


Fig. 29: A photomicrograph of cerebellum of control group (I) showing negative immunoreaction for caspase 3. (Caspase-3 X400)

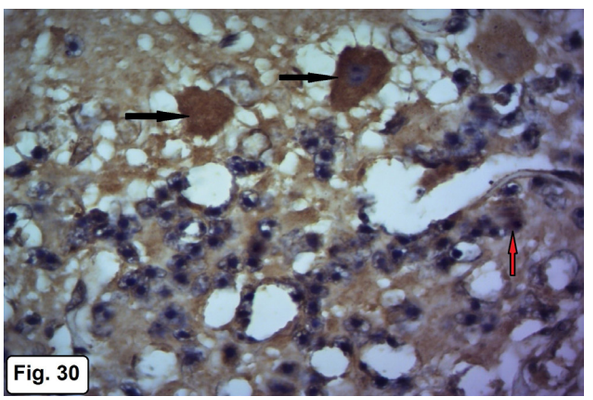


Fig. 30: A photomicrograph of the cerebellum of cell phone exposed group (IV) showing strong positive cytoplasmic immunoreaction for caspase-3 in the Purkinje cells (black arrows) and some granule cells (red arrow). (Caspase-3 X400)

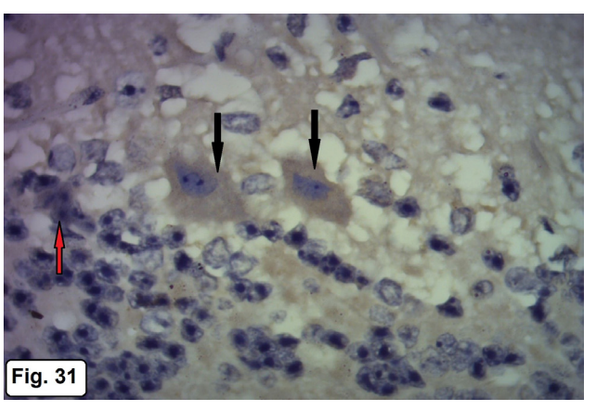


Fig. 31: A photomicrograph of the cerebellum of cell phone exposed and omega-3 treated group (V) showing mild positive cytoplasmic immunoreaction for caspase-3 in the Purkinje cells (black arrows) and some granule cells (red arrow). (Caspase-3 X400)

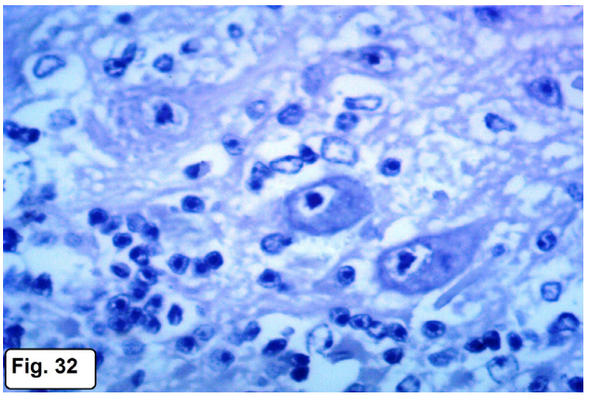


Fig. 32: A photomicrograph of the cerebellum of cell phone exposed and saffron treated group (VI) showing negative immunoreaction for caspase 3. (Caspase-3 X400)

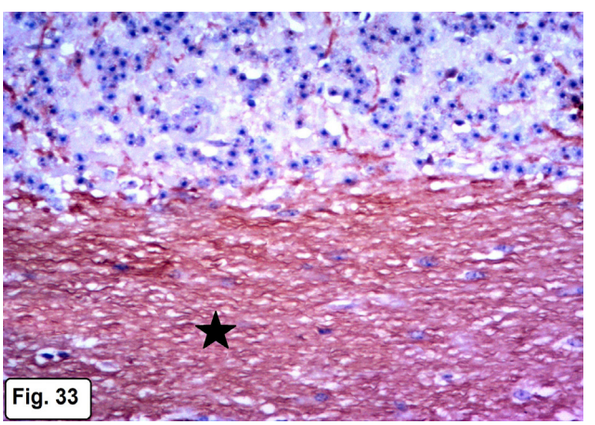


Fig. 33: A photomicrograph of the cerebellum of control group (I) showing strong positive MBP immunostained myelinated fibers (black star) in the white matter. (MBP X200)

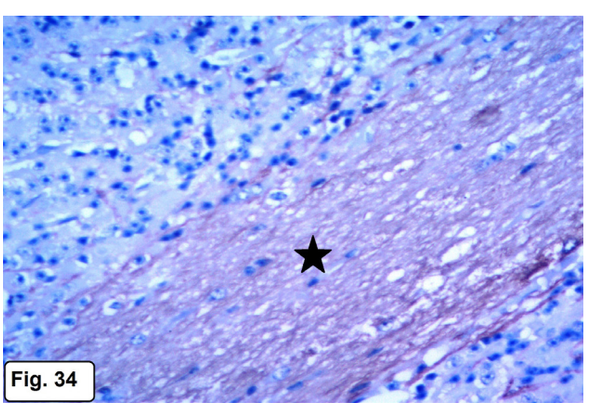


Fig. 34: A photomicrograph of the cerebellum of cell phone exposed group (IV) showing mild positive MBP immunostained myelinated nerve fibers (black star) in the white matter. (MBP X200)

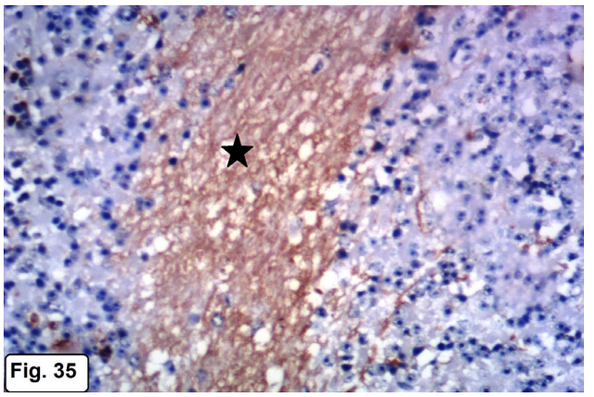


Fig. 35: A photomicrograph of the cerebellum of cell phone exposed and omega-3 treated group (V) showing moderate positive MBP immunostained myelinated nerve fibers (black star) in the white matter. (MBP X200)

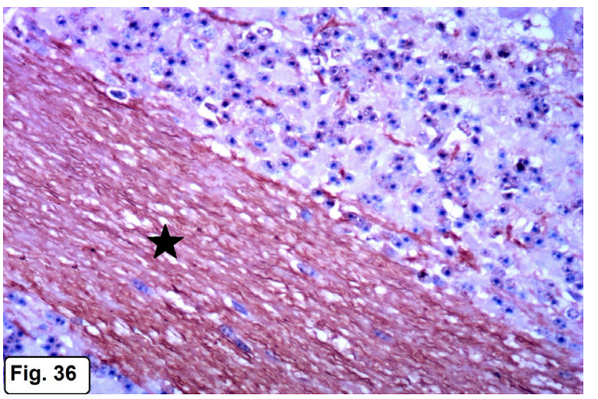


Fig. 36: A photomicrograph of the cerebellum of cell phone exposed and saffron treated group (V) showing strong positive immune expression for MBP (black star) within the white matter. (MBP X200)

Table 1: Mean levels of MDA in the cerebellar tissue in different experimental groups

	Group I M±SD	Group IV M±SD	Group V M±SD	Group VI M±SD	
MDA µmol/g	17±0.9	24.6±0.9	20.5±3.6	17.4±1.1	P1=0.000 P2=0.009 P3=0.917 P4=0.002 P5=0.000 P6=0.020

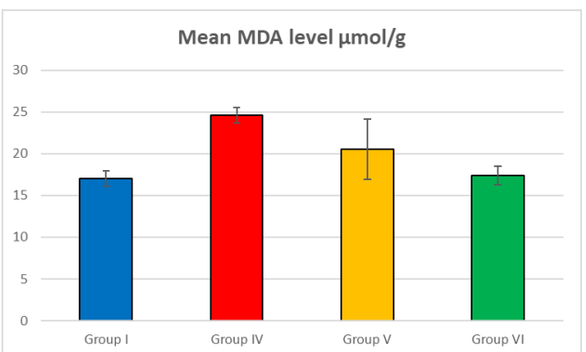
P1 compare group IV with group I (highly significant)
P2 compare group V with group I
P3 compare group VI with group I
P4 compare group V with group IV
P5 compare group VI with group IV
P6 compare group VI with group V

Table 2: Mean number of Purkinje cells/mm² and mean thickness of granular layer in µm in all studied groups

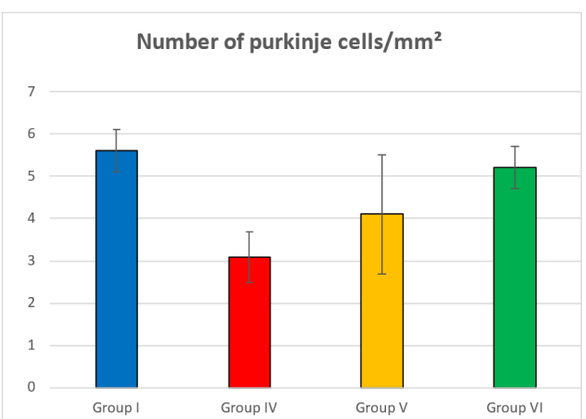
	Group I M±SD	Group IV M±SD	Group V M±SD	Group VI M±SD	
Purkinje cells/mm²	5.6±0.5	3.1±0.6	4.1±1.4	5.2±0.5	P1=0.000 P2=0.008 P3=0.084 P4=0.06 P5=0.000 P6=0.041
Granular layer thickness	136.4±2.3	124.2±3.6	132.6±5.6	134.4±4.7	P1=0.000 P2=0.065 P3=0.239 P4=0.001 P5=0.000 P6=0.462

Table 3: Mean area percentage of GFAP and mean color intensity of caspase-3 and MBP in all studied groups

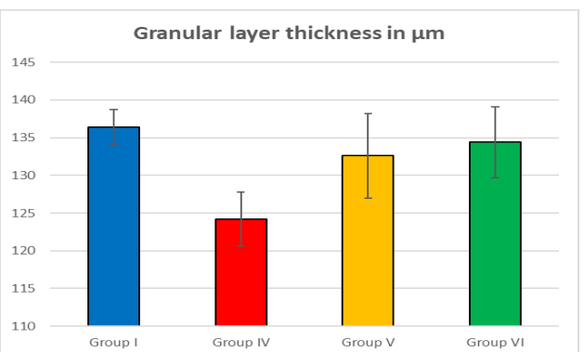
	Group I M±SD	Group IV M±SD	Group V M±SD	Group VI M±SD	
Mean area percentage of GFAP	9±0.6	26.7±2.7	15.1±5.6	9.4±0.9	P1=0.000 P2=0.003 P3=0.284 P4=0.000 P5=0.000 P6=0.005
Mean color intensity of caspase 3	1.7±0.3	8.5±0.6	2.6±1	1.6±0.4	P1=0.000 P2=0.021 P3=0.443 P4=0.000 P5=0.000 P6=0.011
Mean color intensity of MBP	1.4±0.2	0.7±0.2	1.1±0.3	1.2±0.3	P1=0.000 P2=0.010 P3=0.120 P4=0.000 P5=0.000 P6=0.251



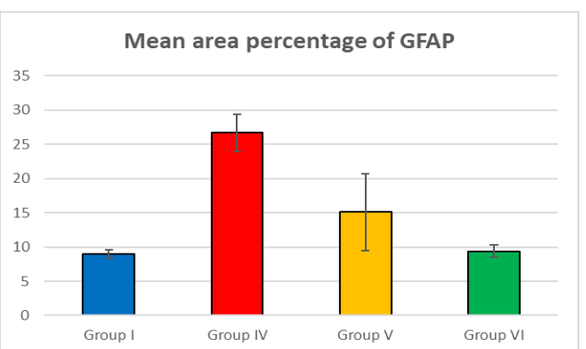
Histogram 1: Mean values of MDA level in different groups.



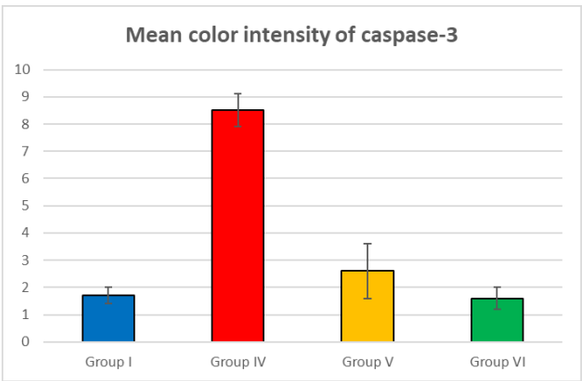
Histogram 2a: Mean number of Purkinje cells in all experimental groups



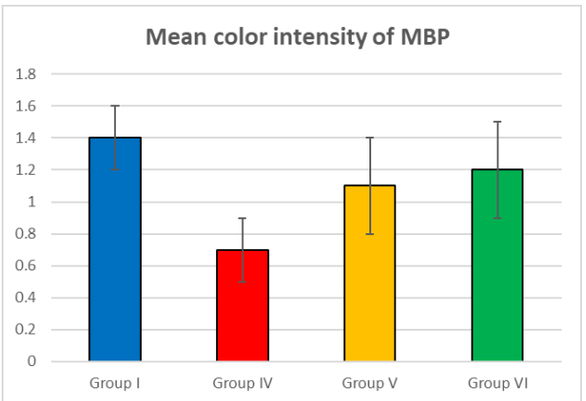
Histogram 2b: Mean values of granular layer thickness in all experimental groups.



Histogram 3a: mean values of area percentage of GFAP in all experimental groups.



Histogram 3b: Mean values of color intensity of caspase-3 in all experimental groups.



Histogram 3c: Mean values of color intensity of MBP in all experimental groups.

DISCUSSION

Cell phone devices have become essential components of daily life. So, its long-term use has recently increased attention regarding the toxic effect of EMRs on the biosystems of human body. These effects may impact the central nervous system (CNS) more than other vital organs due to close exposure or immediate contact with the head during mobile phone usage[27].

The cerebellum is an important organ of the central nervous system participating in cognitive-sensory, motor functions and language[28]. Thus, its affection might lead to obvious neurological problems.

So, this study was designed to assess the histological, immunohistochemical and morphometrical changes within the cerebellum of adult male albino rats exposed to the cell phone EMRs and to compare between the possible neuroprotective effects of omega-3 and saffron in reducing these changes. Global System for Mobile Communications (GSM) standard operating in 900 MHz frequency is the most widely used standard. Therefore, 900 MHz wave frequency was chosen in this study.

In this study, cerebellar sections from adult male albino rats exposed to cell phone EMRs revealed distorted cerebellar architecture. The molecular layer had pyknotic nuclei with perineuronal vacuolation. While Purkinje cells were irregularly arranged, shrunken and their cytoplasm appeared vacuolated having degenerated mitochondria, phagophore and late phagosome. Remanent of Purkinje neuron was observed within granular layer. The cells of granular layer had small darkly stained nuclei and vacuolated cytoplasm. The white matter showed nerve fibers with split degenerated myelin sheaths. Moreover, decrease in the Purkinje cells number and granular layer thickness were observed and confirmed by morphometrical results. These recorded changes indicating cerebellar neuronal degeneration following exposure to repeated doses of radiofrequency electromagnetic radiation (RF-EMR). These results were in harmony with[29,30] who stated that exposure to EMRs emitted from mobile phone may induce morphological changes within the structure of brain, hippocampus and cerebellum including pyramidal neurons shrinkage, with oedema, and vacuolation of neurons. Decreased number of Purkinje cells was also recorded.

Cytoplasmic vacuolation observed in this current study within the molecular, Purkinje and granular cell layers may be due to marked disturbances in lipid inclusions within their cytoplasm. Other researchers[31] suggested that vacuolation might be due to increased reactive oxygen species (ROS) production attacking the cell membrane causing alteration in its permeability. Also, ROS formed following exposure to EMR increases the lipid peroxidation that is considered as an autocatalytic mechanism leading to destruction of cellular membrane and cell death.

Nuclear changes in the form of pyknosis (irreversible condensation of chromatin) occurring with the cells of the cerebellum indicate programmed cell death or apoptosis. This is might be due to damage of DNA in the form of base, sugar lesions, or strand breaks. This damage might be due to production of ROS on exposure to RF-EMRs as previously reported by[32].

Disturbed linear organization of Purkinje cells with observation of remanent of Purkinje neuron within granular layer could be explained by previous scientists who documented that prolonged neuronal insult might result in adaptive mechanism in the form of Purkinje cell crowding in other areas as a trial to reestablish synapsis with other nerve cells to achieve their functions[33].

These hazardous effects occurring on exposure to RF-EMRs might be due to the cell membrane perforations altering calcium (Ca) metabolism. These modifications allow adenosine triphosphate (ATP), and calcium (Ca), to pass out of the cell. Ca is important for activation of protein kinase C (PKC) which in turn, control cell proliferation, protein synthesis and cell death. Also, RF-EMRs activates lysosomes within the cell resulting in cell apoptosis[34]. This was noticed in this study by detection of phagosome within the cytoplasm of Purkinje neurons exposed to RF-EMRs by electron microscopic examination.

EMRs released from mobile phones biochemically increase the levels of Nitric oxide (NO), Malondialdehyde (MDA), Xanthine oxidase (XO), and adenosine deaminase (ADA) activities resulting in oxidative damage in rat brain. These formed ROS are continuously destructed by superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px)[35,36]. On exposure to cell phone EMRs, ROS overproduction, detoxification systems inactivation, and antioxidants consumption occurs resulting in disturbance of endogenous antioxidant defenses with failure of antioxidants renewal in tissues. This could be enforced in the present study by detection of highest level of MDA in cerebellar tissues of group IV exposed to EMRs as compared with control group. High oxygen consumption rate, elevated polyunsaturated fatty acids levels, and low endogenous antioxidative enzymes content within the brain tissues render it more liable to oxidative stress[37].

Another possible cause for the changes within the cerebellar tissues exposed to cell phone EMRs is disturbance of blood brain barrier following exposure to RF-EMRs. The blood-brain barrier is the first line of defense mechanism for CNS protecting it from environmental toxic substances. Permeability of this barrier is essential for maintenance of neural environment stability. Experimental studies have reported that disturbed permeability of blood-brain barrier following exposure to RF-EMRs, leads to passage of undesirable molecules into the CNS with impairment of its functions[38].

In the present study, the cytoplasm of Purkinje cells in toluidine blue stained sections of cerebellum of group IV exposed to EMRs revealed that Purkinje cells appeared shrunken and deeply stained as compared to control group with apparent reduction in their Nissl's granules content. This result was in harmony with[39] who documented the reduction in Nissl's granules content within the cytoplasm of cells of hippocampus following repeated exposure to EMRs. This process is known as chromatolysis that was explained by[40] as disintegration of the basophilic Nissl bodies. The reaction occurs in the neuronal cytoplasm following metabolic or traumatic injuries. This is simultaneously occurring with nuclear migration to the periphery of soma, resulting in reduction of RNA level, thereby affecting their metabolic activity.

These observed histological and ultrastructural changes, were confirmed by the applied immunohistochemical staining. In the present study, GFAP stained cerebellar sections of group IV revealed multiple astrocytes with positive immunoreaction. This was confirmed statistically as significant increase in mean area percentage of GFAP positive astrocytes as compared to the control group. This finding was supported by[41] who detected multiple GFAP positive astrocytes in all layers of cerebellar cortex of rats treated with cisplatin. This positive reaction could be due to astrocytes proliferation and hypertrophy with increase expression of their cytoskeletal GFAP that occurs because of any mechanical, chemical, or degenerative insult to the brain leading to astrogliosis as previously stated by[42].

Astrogliosis occurred as a compensatory reaction to cerebellar injury that occurs due to oxidative stress and production of excessive ROS aiming to reducing neuronal death. Some researchers also, proposed that following brain injury, astrocytes might act as stem cell initiating repair process. Also, they produce neurotrophic factors which are important for survival of neurons[43,44].

Obvious strong caspase-3 immunoreactions observed within the Purkinje and some granule cells of cerebellar sections of Group IV exposed to RF-EMRs and confirmed statistically by highly significant increase in its mean color intensity as compared with the control group. Some authors agree with this result[45]. This result clarifies that caspase-3 activation plays a central role in the mechanism of EMRs-related cerebellar damage. This is could be due to the harmful effects of electromagnetic radiations in impairing DNA repair capacity resulting in apoptosis within the brain cells[46].

Regarding MBP immune expression, cerebellar sections of group IV exposed to EMRs revealed marked decrease in the intensity of MBP immune expression within the myelinated fibers in the white matter. This result was confirmed morphometrically by significant decrease in its mean color intensity as compared with control group. Some researchers agree with this result[47] who reported that traumatic brain injury causes structural damage of myelin sheaths surrounding the axons, initiating MBP degradation resulting in myelin sheath instability and demyelination.

MBP is the main structural protein in the central nervous system (CNS). It stabilizes the myelin sheath structure surrounding nerve fibers via binding to the cytoplasmic surfaces of cell membrane. The absence of MBP promotes a neuroinflammatory environment[48].

Some improvement of the cerebellar sections of group V exposed to cell phone and treated with omega-3 was noticed. Normal appearance of molecular, Purkinje and granular layers was shown. However, some Purkinje cells appeared degenerated with dilatation of rough endoplasmic reticulum. interstitial vacuolar spaces were also shown. In the white matter, some nerve fibers with focal areas of myelin sheath separation were noticed. These results were confirmed morphometrically by revealing a significant decrease in the Purkinje cells number and a non-significant change in the granular layer thickness in comparison with the control group. These results agreed with[27] who stated that omega 3 had a neuroprotective effect against cellular damages occurred within the cells of hippocampus and cerebellum of rat model exposed to EMRs.

Omega-3 polyunsaturated essential fatty acids (ω3-PUFA) are found in large amounts in fish oil. It contains eicosapentaenoicacid (EPA) and docosahexaenoic acid (DHA) playing a major role in brain development, maintaining its functions[49].

EPA is an essential component within the nerve cells for the synthesis of leukotriene-5, prostaglandin-3, and tromboxane-3. These substances

are useful in inflammation and mental conditions such as schizophrenia[50]. DHA is an essential fatty acid present in highest concentrations within the neuronal and glial cell membrane phospholipids. It is considered a potent neuroprotective agent, improving neuronal survival, and slowing down neurodegeneration[51,52]. Its presence is valuable for preservation of cell integrity once homeostasis is threatened[53]. DHA also produces neuroprotectin-D1, which is an effective anti-inflammatory mediator, having anti-apoptotic, anti-oxidative properties. Thus, increase neuronal longevity[54].

In the present study, cerebellar sections of group VI exposed to cell phone and treated with saffron displayed marked histological, immunohistochemical and morphometrical improvement. Similarly,[55] reported the protective effects of saffron in ocular pathologies, especially neurodegenerative diseases of retina.

Saffron was used in traditional herbal medicine in treating several human health conditions, such as dysentery, measles, hepatomegaly, urological infections, cough, stomach disorders because of its hypolipidemic, anti-cancer, anti-apoptotic, and oxygenation enhancement agent increasing oxygen transport as previously reported by[56].

Saffron is safe possessing no significant toxicity in therapeutic doses. It has pharmacological effects on nervous system as it can permeate the blood brain barrier[57]. Saffron has also recently been tested in management of Alzheimer's disease, and other neurodegenerative brain disorders[58]. In recent years, the potential modulatory role of various phytonutrients is assumed to be an effective agent for neuroprotection[59].

It has been proved that saffron and its constituents exerts its neuroprotective effects mostly via antioxidative stress mostly due to their role in scavenging ROS, mainly the superoxide anions, and so may protect cells against oxidative stress. Also, it has been reported that saffron decrease lipid peroxidation following oxidative damage in rats. This might be proved by detection of decreased level of malondialdehyde (MDA) which is an indicator for lipid peroxidation induced by ROS[60].

From the foregoing, it is concluded that repeated exposure to cell phone emitted electromagnetic radiations induces marked biochemical, histological, immunohistochemical, and morphometrical changes within the cerebellar tissues of adult male albino rats. These changes could be ameliorated with the use of omega 3 and saffron as neuroprotective agents. Marked improvement was noticed with the use of saffron. So, we recommend use of saffron as neuroprotective. More further studies on human are required for its clinical application.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة مقارنة للتأثيرات الوقائية المحتملة لأوميجا ٣ ومستخلص الزعفران على مخيخ ذكور الفئران البيضاء البالغة المعرضة للإشعاعات الكهرومغناطيسية للهاتف الخلوي: دراسة هستولوجية وهستوكيميائية مناعية

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تعد التغيرات التكنولوجية السريعة والزيادة الهائلة في استخدامات الهواتف المحمولة التي ينبعث منها الاشعاعات الكهرومغناطيسية في جميع أنحاء العالم تحديًا للصحة العامة. لذلك زاد الاهتمام بدراسة أخطار هذه الاشعاعات التي يمكن أن تؤثر على صحة الإنسان في الآونة الأخيرة. وقد حظي أوميجا ٣ والزعفران باهتمام كبير كمضادات للأكسدة.

الهدف من البحث: ركزت هذه الدراسة على تقدير التأثيرات الوقائية لأوميجا ٣ والزعفران على المخيخ المعرض للإشعاعات الكهرومغناطيسية للهاتف الخلوي

مواد وطرق البحث: تم استخدام ستين من ذكور الجرذان البيضاء وتقسيمهم إلى ست مجموعات. المجموعة الأولى (المجموعة الضابطة)، المجموعة الثانية (مجموعة أوميجا ٣)، المجموعة الثالثة (مجموعة الزعفران)، المجموعة الرابعة (المجموعة المعرضة للهاتف الخلوي)، المجموعة الخامسة (المجموعة المعرضة للهاتف الخلوي وأوميجا ٣). والمجموعة السادسة (المجموعة المعرضة للهاتف الخلوي ومجموعة الزعفران). تم استئصال المخيخ من كل حيوان ودراسته بصبغة الهيماتوكسيلين والايوسين والتوليدين بلو وكذلك تم تحضير العينات للدراسة بالمجهر الإلكتروني. وتم عمل دراسة كيميائية وكيميائية مناعية بصبغات البروتين الليفي الحامضي ولكسباس ٣ وكذلك لبروتين الميلين القاعدي. وأيضا تم عمل دراسة مورفومترية.

النتائج: أظهرت المجموعة المعرضة للهواتف المحمولة خلل في تركيب المخيخ. وظهرت انوية الطبقة الجزيئية منكمشة وداكنة كما تكونت الفجوات حول خلاياها العصبية وظهر اضطراب في غشاء الميلين حول اليافها العصبية. وقد ظهرتطبقة خلايا البركينجى منكمشة ومحاطة بخيوط عصبية فارغة وبدت أنويتها داكنة وغير منتظمة. وقد ظهرت بعض الخلايا الصغيرة الضامرة في الطبقة الحبيبية. كما لوحظ وجود زيادة في التفاعل المناعي للبروتين الليفي الحامضي ولكسباس ٣ ونقصان في التفاعل المناعي لبروتين الميلين القاعدي. كما أوضحت النتائج ان تناول أوميجا ٣ والزعفران أدى الى التقليل من هذه الآثار الضارة. وأظهر الزعفران حماية أفضل ضد التغيرات الهستولوجية التي حدثت بالمخيخ الناجمة عن التعرض للإشعاعات الكهرومغناطيسية للهواتف الخلوية

الخلاصة: يؤكد البحث ان التعرض للإشعاعات الكهرومغناطيسية للهواتف الخلوية يؤدى الى تلف في مخيخ ذكور الفئران البيضاء البالغة وأن الزعفران له دور وقائي أفضل من أوميجا ٣