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Effects of Radio-Frequency Electromagnetic Radiations (RF-EMR) on Cerebellar Cortex of Albino Rats: - A Light and Electron Microscopic Study

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Abstract: Since the introduction of mobile phones in the late eighties, many studies have raised concerns about the possible adverse effects on health, as a result of the exposure to RF and microwave electromagnetic fields as RF-EMR can penetrate deep into organic tissues and get absorbed producing many biological effects in human body. As brain is involved in very important functions and RF-EMR might have damaging effects on its different parts, the present study was undertaken with an aim to study effects of radio-frequency electromagnetic radiations (RF-EMR) emitted by mobile phones on cerebellar cortex of albino rats under light and electron microscopy and to evaluate such changes after exposure to graded dose of RF-EMR**.** The present study was carried out on twenty four adult albino rats of either sex weighing 180-200 grams each. The animals were divided into four groups: 1 control and 3 experimental and were exposed to RF-EMR via complete missed calls of 45 seconds duration each. Both the experimental and control groups were then sacrificed and cerebellar cortex was isolated for tissue processing. The processed tissues were then studied under light microscope (Hematoxylin & Eosin Staining) and Transmission Electron Microscopy (TEM). Light microscopic findings of the present study showed that cellular size of neuronal cells in purkinje layer of cerebellar cortex of RF-EMR exposed rats decreased along with condensed cytoplasm and nucleus. Electron microscopy showed swollen and vacuolized mitochondria with disordered cristae fewer in number. The rough endoplasmic reticulum also exhibited sacculated distension. From the findings of the present study it appears pertinent that in order to protect the population living around base stations and users of mobile handsets, governments and regulatory bodies adopt safety standards, which translate to limits on exposure levels below a certain value and efforts are underway to harmonize the different standards in existence.

Keywords: Radio-frequency electromagnetic radiations (RF-EMR) MR), albino rats, cerebellar cortex, microwave, electron microscope, Hematoxylin & Eosin Staining.

INTRODUCTION

The development of mobile communications has moved rapidly. In the 1980s, first generation mobile phones, using analogue technology, allowed the transmission of sound only. Digital transmission, and the global system for mobile communication, started in 1991 and include such new developments as data and image transmission. Third generation mobile phones currently in the market offer additional services to the users (such as fax, e-mail and Internet access). For both analogue and digital mobile phones, the signals transmitted and received are in the form of waves in the radio frequency (RF) (analogue) and microwave parts of the electromagnetic spectrum. RFs are non-ionizing radiation with, wavelengths th at range from 3 kHz to 300 MHz, and microwaves range from 300 MHz to 300 GHz1. The frequencies that mobile phones and telecommunication networks use range from 900 MHz to 1.8 GHz and up to 2.1 GHz, although it should be noted that the wavelength of the different types of mobile phones varies. This applies to both mobile phones and their base stations, which send and receive calls [1]. However, concerns about the possible adverse effects on health, as a result of the exposure to RF and microwave electromagnetic fields, have been expressed since the introduction of mobile phones as RF-EMR can penetrate deep into organic tissues and get absorbed producing many biological effects in human body. This ubiquitous exposure to an emerging technology prompted the initiation of large-scale health studies (some started over 20 years ago) in the United States and throughout the world [2].

The cerebellum is a region of the brain that plays an important role in motor control. It may also be involved in some cognitive functions such as attention and language, and in regulating fear and pleasure responses [3]. Its movement-related functions are the most solidly established. The cerebellum does not initiate movement, but it contributes to coordination,

precision, and accurate timing. It receives input from sensory systems of the spinal cord and from other parts of the brain, and integrates these inputs to fine tune motor activity. Cerebellar damage does not cause paralysis, but instead produces disorders in fine movement, equilibrium, posture, and motor learning.

In addition to its direct role in motor control, the cerebellum also is necessary for several types of motor learning, most notably learning to adjust to changes in sensorimotor relationships. Several theoretical models have been developed to explain sensorimotor calibration in terms of synaptic plasticity within the cerebellum. Most of them derive from early models formulated by Marr [4] and Albus [5], which were motivated by the observation that each cerebellar Purkinje cell receives two dramatically different types of input: one type is of thousands of inputs from parallel fibers, each individually very weak; the other is the input from one single climbing fiber, which is, however, so strong that a single climbing fiber action potential will reliably cause a target Purkinje cell to fire a burst of action potentials. The basic concept of the Marr-Albus theory is that the climbing fiber serves as a "teaching signal", which induces a long-lasting change in the strength of synchronously activated parallel fiber inputs. Observations of long-term depression in parallel fiber inputs have provided support for theories of this type, but their validity remains controversial.

In the view of fact that cerebellum is involved in important functions and RF-EMR might have damaging effects on it, the present study was planned to be under taken with aim to study effects of radio-frequency electromagnetic radiations (RF-EMR) emitted by mobile phones on cerebrum in albino rats under light and electron microscopy and to evaluate such changes after exposure to graded dose of RF-EMR. The aforementioned light microscopic findings are confirmed under electron microscope.

MATERIAL AND METHODS

The present study was carried out on twenty four adult albino rats of either sex weighing 180-200 grams each. The rats were housed in plastic cages of size 36 cm × 23 cm × 21 cm (three/four rats in each cage) inside a temperature and humidity controlled environment & provided with standard pellet laboratory diet (Lipton India Limited) and water ad-libitum. The animals were weighed, marked and divided into four groups based on the number of calls/day they received. The rats were exposed to RF-EMR by giving complete missed calls of 45 seconds duration each one after the other, everyday for 4 weeks, keeping a GSM (0.9 GHz/1.8 GHz) mobile phone in silent mode (no ring tone & no vibration) in the cage.

Four groups were as under

Control Group

1. CTRL : exposed to NIL calls/day

Experimental Groups

2. E80 : exposed to 80 calls /day
3. E120 : exposed to 120 calls /day
4. E160 : exposed to 160 calls /day

Tissue Processing for Neurohistology

Procurement of the tissue

After proposed experimental duration of 4 weeks, exposure the animals of both the experimental and control groups were sacrificed by giving overdose of diethyl ether vapors and the heart was exposed through the thoracic approach. The needle of the blood transfusion set was introduced into the left ventricle (apex) and a nick was made in the right atrium. After saline wash, Karnovsky fixative was then infused till the body tissues showed signs of fixation.

After a couple of days, the brain was approached through the dorsal aspect of skull. A T-shaped incision was given on the dorsal aspect of the head and the skin was reflected. The skull was cut open at the midline fissure using a pair of scissors. The scissors were lifted up while cutting to avoid any damage to cerebri. Dorsal part of the skull was removed using curved forceps. The brain was removed by releasing it gently from all its attachments from below and sides. Brain was cleaned and washed in tap water. Cerebrum and cerebellum was separated. Both were divided by a median incision into two equal halves. Then each half was sectioned into small pieces of desired size and thickness from different areas of each half.

Tissue Processing

Each tissue was then dehydrated in ascending grades of ethyl alcohol for 30 minutes each separately: 50% Alcohol → 70% alcohol → 90% alcohol → absolute alcohol → xylene I & II (clearing agent) for 1 to 1.5 hrs → impregnation was done using molten wax and xylene mixture (1:1) for 1 hr → 100% wax for 1 hr → embedding in wax, blocked, labeled & stored.

Sectioning was done using rotary microtome at 7 to 10 µm thickness with ribbon formation → 3-4 sections length ribbon selected at 10 section interval with the help of water bath warmed at 50 degree centigrade → the tissue sections were picked up on the egg albumin smeared glass slides. Slides were dried and labeled properly and stored.

Hematoxylin & Eosin Staining

Deparaffinization & Hydration

Slides with paraffin sections were deparaffinized in xylene for 15 min. Hydration done with descending grades of alcohol 5 min. each as under.

Absolute alcohol → 90% alcohol → 70% alcohol → 50% alcohol then distilled water.

Staining

Slides were kept in Hematoxylin for 10 min → Washed with tap water to remove excess hematoxylin → Bluing – section dipped in 1 % HCl for 1 second then washed with tap water for 10 - 20 min. (controlled by repeated checking under light microscope).

Counter staining

The slides were then dipped in Eosin (1%) for 5 min. Sections were then dehydrated by one time dipping in ascending grades of ethyl alcohol → 50% alcohol → 70% alcohol → 90% alcohol → Absolute alcohol → Slide dried in air → Cleared in xylene for 15 min. → Permanent mounting in DPX → Labeled and stored.

Cresyl violet staining

Slides with paraffin sections were deparaffinized with xylene for 15 min. Hydration was done with descending grades of alcohol 5 min. Each as under- Absolute alcohol → 90% alcohol → 70% alcohol → 50% alcohol then distilled water. Then slides were stained in 0.1% cresyl violet for 3 minutes. Then slides were rinsed in tap water to remove excess of stain. After that slides were again dehydrated in ascending grades concentration of ethyl alcohol (50%, 70%, 90% and absolute alcohol).Then slides were cleared in xylene for few minutes and afterward mounted with DPX.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Preparation specimen

Fixation: This is the most critical stage in the embedding process. Good results can be obtained with many fixatives, including neutral buffered formalin (NBF), but for optimal preservation of ultrastructural detail, 4% glutaraldehyde in 0.1M phosphate buffer is recommended for most solid tissue specimens. Whatever fixative is used, it is important that the specimen is placed in the fixative as soon as possible, and that there is sufficient fixative relative to the amount of tissue. Keeping above facts in mind the animals were preferably perfused with Karnovsky's fixative to obtain desired fixation for TEM. About 2 mm-thick tissue blocks were cut from all the tissues, under observation, from all groups of the animals, transferred to 0.1M buffer after 4 to 24 hours, and kept in refrigerated at 4° C and subsequently sent for tissue processing to TEM lab, Sophisticated analytical instrument facility, in department of anatomy AIIMS, New Delhi.

Embedding

The fixed tissue was then washed with fresh buffer then post-fixed with osmium tetroxide. The specimens were then dehydrated using a series of ethanol solutions of increasing concentration. When dehydration was complete, they were transferred from 100% ethanol to propylene oxide, then to mixtures of propylene oxide and resin in increasing concentration followed by resin mixture with hardener. Resin infiltration was completed in incubator and allowed to harden along with label to get blocks suitable for storage and cutting.

Sectioning

The hardened blocks were trimmed with a razor blade until the tissue exposed and excess resin was removed from the block face. Semi-thin sections (0.5 µm) for light microscopy were then cut from the block with diamond knives using LKB ultra microtome. Individual sections were mounted on glass slides, heated and stained with Toluidine blue. The sections were examined under light microscopy. These sections helped in selection of appropriate area for electron microscopy. Ultra-thin (75 nm thick) sections were mounted on acetone cleaned 200 mesh copper grids and stained with Uranyl acetate followed by Lead citrate. The stained ultra-thin sections were examined with Morgagni 268D (Fei Electron Optics) Transmission Electron Microscope at SAIF, AIIMS and JEOL JEM-2100 Transmission Electron Microscope in university sophisticated instrument facility (USIF), Aligarh Muslim University, Aligarh. The TEM facility availed did not provide the scale bar on the images.

Thus, tissue blocks, sectioning and staining for TEM was performed at SAIF, AIIMS but viewing of sections was done at SAIF, AIIMS, and USIF, AMU, Aligarh, depending upon the available time slots at these two busy centers.

OBSERVATIONS

Light microscopy

Paraffin sections

Cerebellar cortex is having uniformity in its structure in all parts of cerebellum along with piamater. We can easily identify all three layers of cerebellar cortex- outer molecular, middle Purkinje cell layer and inner most granular layer (Fig-3a). Molecular layer contains a sparse population of neurons, dendritic arborizations, non-myelinated axons and radial fibres of neuroglial cells. Purkinje cell dendritic trees extend towards the surface and spread out in a plane perpendicular to the long axis of the cerebellar folia. Purkinje cell dendrites are flattened.

The Purkinje cell layer contains the large, pear-shaped somata of the Purkinje cells and the smaller somata of Bergmann glia. Clumps of granule cells and occasional Golgi cells penetrate between the Purkinje cell somata. The Purkinje cell layer was formed of single row of large pyriform somata of Purkinje neurons with pale nuclei and prominent nucleoli (Fig-3b). Purkinje cells were surrounded by astrocytes that have pale nuclei and pale cytoplasm. The granular layer was formed of large number of neurons with rounded dark nuclei and scanty cytoplasm, fibers and small

capillaries. In experimental group [Fig. 4(a, b and c)], photomicrographs are from cerebellar cortex. Morphological changes can be well appreciated in these pictures, especially in Purkinje cell layer. These changes are more marked in E-160 (Fig-4c) rats in comparison of E-80 and E-120 rats. Neurons are darkly stained and their size is decreased. Few purkinje cells in E-120 are very small in size and very lightly stained in comparison of other cells. Nucleus is heterochromatic. Blood vessels are also affected by RF-EMR. They are showing signs of hemorrhage and are somewhere congested (Fig-4b).

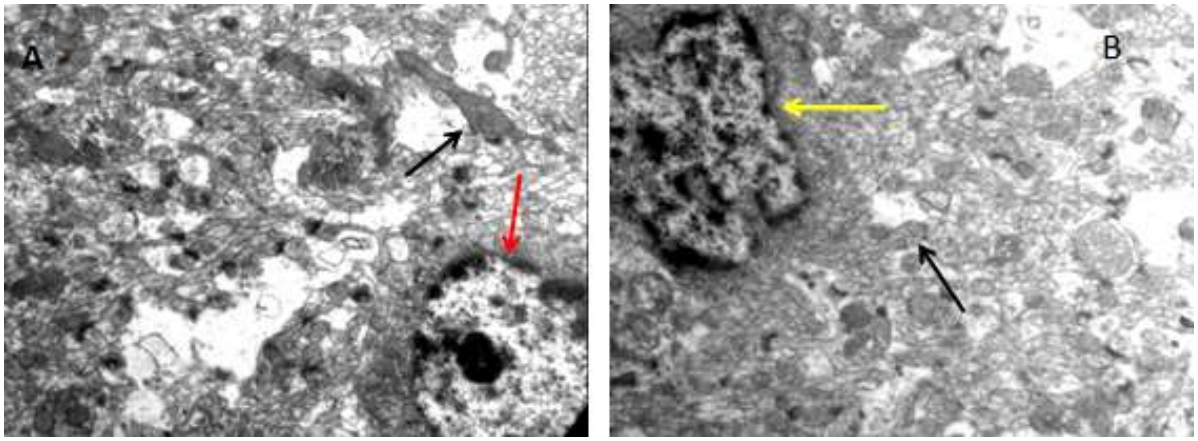

Fig-13a: Sample electronmicrograph of cerebellar cortex is from control rats. Red arrow in picture A is showing a granule cell along with normal neuropil. Nucleus is intact along with prominent nucleolus. Multiple synaptic vesicles can be observed in both pictures. In picture B we can see normal glial cell.

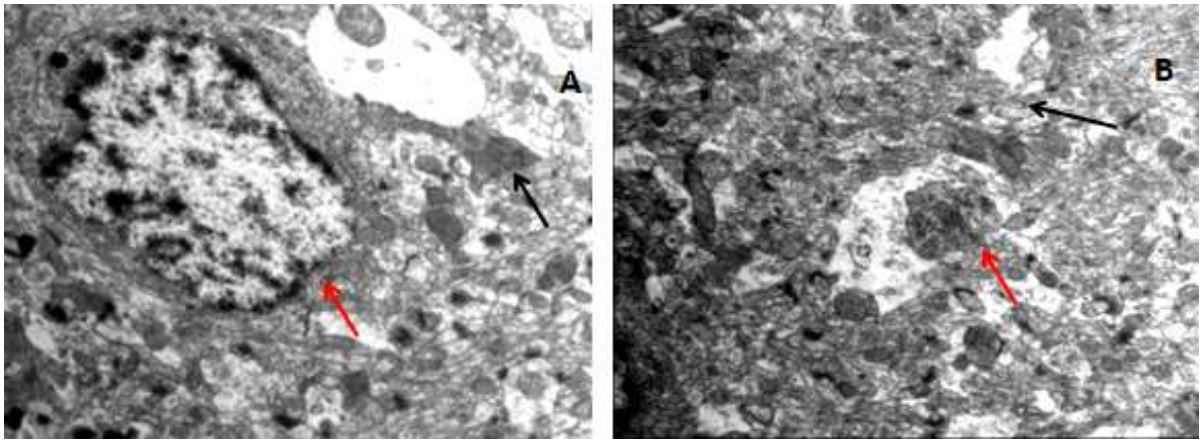

Fig-13b: Sample electronmicrograph of cerebellar cortex is from E-160 rats. In picture A, we can appreciate a cell with swollen nucleus and hazy nuclear envelope, and no nucleolus. In picture B, rarefaction of neuropil can be seen with reduced number of synapses.

In control group Fig-13a is of cerebellar cortex. Red arrow in picture A is showing a granule cell along with normal neuropil. Nucleus is intact along with prominent nucleolus. Multiple synaptic vesicles can be observed in both pictures. In picture B we can see normal glial cell. Fig-13b is of cerebellar cortex is from E-160 rats. In picture B, we can appreciate a cell with swollen nucleus and hazy nuclear envelope, and no nucleolus. In picture B, rarefaction of neuropil can be seen with reduced number of synaptic vesicles.

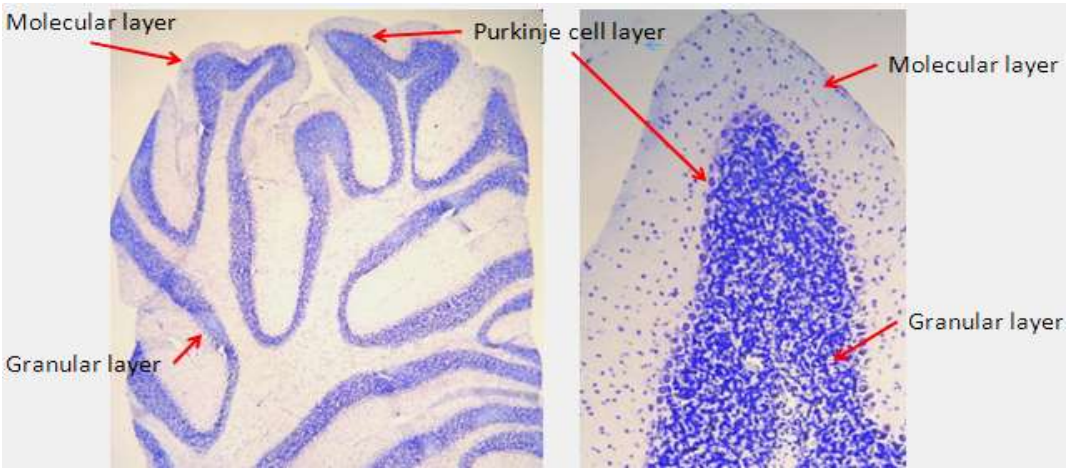

Fig-3a: Representative Photomicrograph shows normal study from coronal sections of cerebellar cortex of control group at low magnification. Molecular layer, Purkinje cell layer and granular layer can be appreciated. Paraffin sections, cresyl violet staining, X40 and X100.

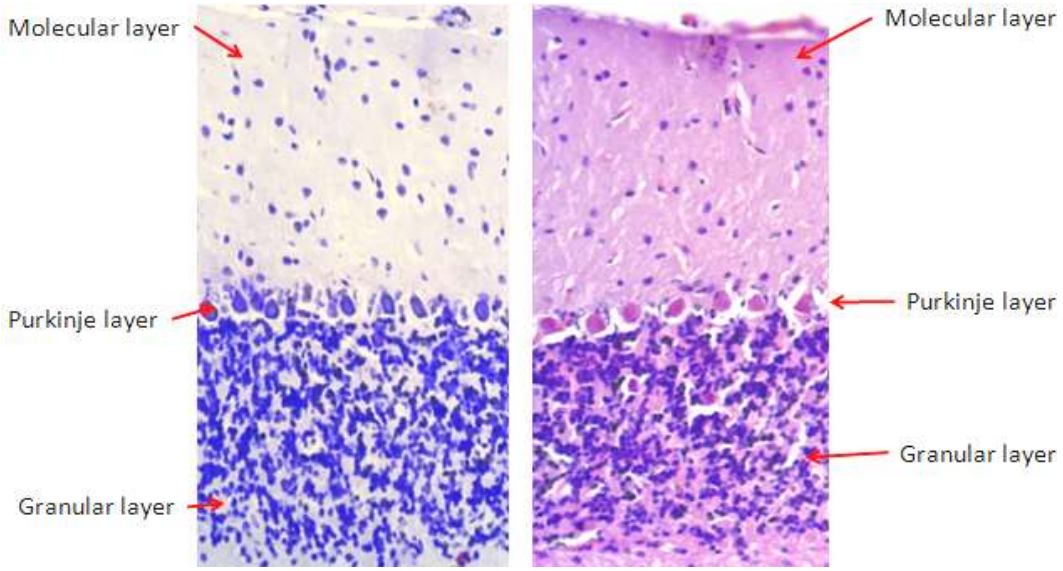

Fig-3b: Representative photomicrographs of cerebellar cortex showing normal morphology in control group at high magnification. We can see molecular, Purkinje cell layer and granular layer. Paraffin sections, Cresyl violet and H&E stanning, X400

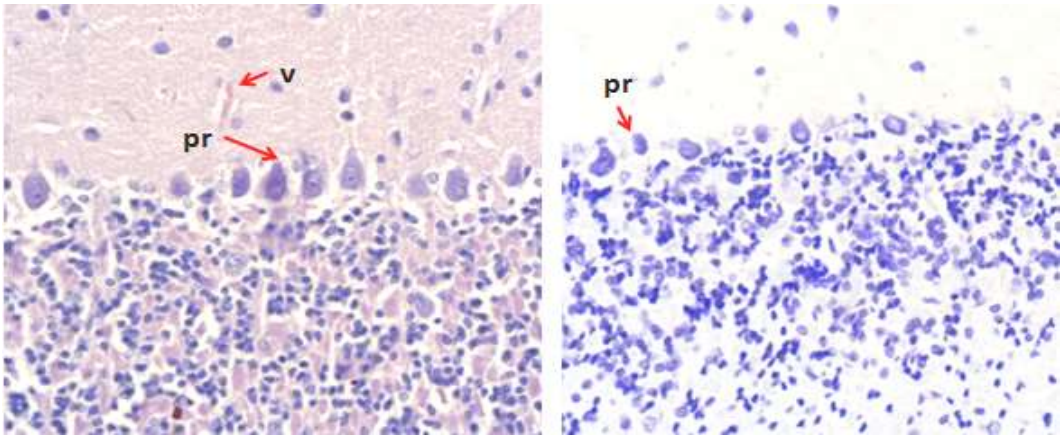

Fig-4a: Representative Photomicrograph of cerebellar cortex from E-80 rat is showing some abnormality in morphology of purkinje cells (Pr). They are slightly dark stained, less dense and irregularly arranged. Nucleus is heterochromatic. Vessel (v) is showing congestion and hemorrhage. H&E and cresyl violet staining, X400

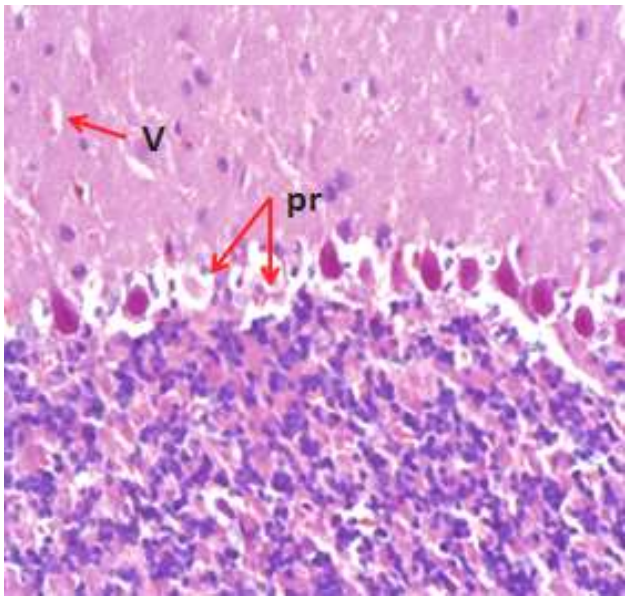

Fig-4b: Representative Photomicrograph of cerebellar cortex from E-120 rats is showing decreased size and density of purkinje cells (pr). They are very lightly stained. Nucleus and nucleolus cannot be differentiated as nucleus is quite heterochromatic. Some vessels (v) are hemorrhagic. Paraffin section, H&E, X400

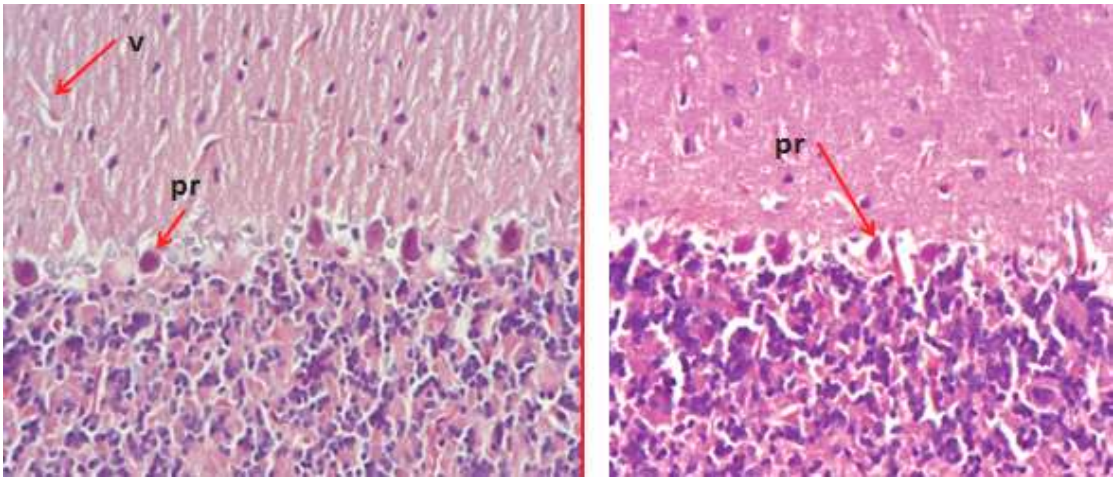

Fig-4c: Representative Photomicrographs of cerebellar cortex from E-160 rats are showing very darkly stained Purkinje cell (pr). Purkinje layer is not uniformly dense. Cytoplasm is very condensed and nucleus is heterochromatic. H&E, X400

DISCUSSION

The present study was planned to evaluate the effects of EMF. The study was planned and executed carefully so that brain is not unduly disturbed by other factors (sound or vibration) during the course of study. Therefore, in this study whatever morphological changes have been observed, they are to be considered to be purely due to EMF (Keeping a GSM (0.9 GHz/1.8 GHz; mobile phone in silent mode - no ring tone & no vibration).

Light microscopic findings of the present study showed that cellular size of neuronal cells in purkinje layer of cerebellar cortex of RF-EMR exposed rats decreased in compare to control groups. Individual cells could be seen with condensed cytoplasm and nucleus. These changes in size could be due to effects of EMF on the genetic material. Such effects have been reported in various studies after exposure to RFR [6, 7]. Recently, several studies have reported cytogenetic changes in brain cells by RFR, and these results could have important indication on the health effects of RFR. Singh *et al*., reported significant decreases in poly-ADP-ribosylation, a process involved in chromatin functions, in the brain of rats after sixty days of exposure to 2450-MHz RFR (1 mW/cm2) [8]. Sarkar *et al*., reported changes in DNA sequences in mouse brain cells after exposure to RFR (1 mW/cm2, 2 hr/day for 120, 150, and 200 days) [9].

In the present study we observed that there are marked histological changes in purkinje layer of cerebellar cortex. Morphological changes can also be observed in few cells of granular layer of cerebellar

cortex. These findings are in agreement with study of Azmy and Abdallah [10]. They had shown, most of Purkinje neurons in group (II) were shrunken, deeply stained, surrounded by perineuronal spaces and arranged in more than one row. They appeared distorted with different ultrastructural features due to effect of RF-EMR. Some of granular neurons had deeply stained nuclei. Purkinje layer of group (III) showed disarrangement with few darkly stained shrunken Purkinje neurons were dispersed among numerous lightly stained ones. Few affected granular neurons were observed. Though no obvious behavioral changes were observed among these rats .That may be because of some compensatory physiological processes going on within these rats body.

It has been recently reported by Lai and Singh [11] an increase in DNA double strand breaks in brain cells of rats after acute exposure to RFR. Double strand breaks, if not properly repaired, is known to lead to cell death. Indeed, it has been observed that there is an increase in apoptosis (scheduled cell death) in cells exposed to RFR.

Free radicals also play an important role in aging processes which have been ascribed to be a consequence of accumulated oxidative damage to body tissues [12], and involvement of free radicals in neurodegenerative diseases, such as Alzheimer's, Huntington, and Parkinson, has also been suggested [13]. Furthermore, the effect of free radicals could depend on the nutritional status of an individual, e.g. availability of dietary antioxidants, consumption of alcohol and amount of food consumption. Various life conditions, such as psychological stress [14] and strenuous physical exercise [15], have been shown to increase oxidative stress and enhance the effect of free radicals in the body. Thus, one can also speculate that some individuals may be more susceptible to the effects of RFR exposure because of increase production of free radical. These findings indicate that exposure to EMF has a detrimental effects on the neurons of cerebral and cerebellar cortex.

In this study, the vessels appear congested and showed signs of hemorrhage with an enlarged perivascular space. This finding is in partial agreement with other studies like those of Finnie *et al*., [16] that showed RFR increases blood-brain barrier permeability. At the molecular level EMF produces biological stress and free radical, which can make the susceptible animal population prone to increase permeability of BBB, congenital malformation, tissue and cell damage or death [17] and free radical**s** can cause oxidative stress at the cellular level, interfering with protein synthesis. These elements also play an important role in acute inflammation, endothelial destruction, resulting in tissue edema. It has been postulated that EMF-exposure produces high levels of oxidative stress as a result of its effect on the immune response [18] and long-term exposure to EMF may be linked to even higher levels of oxidative stress**.**

The blood-brain barrier (BBB) protects the neural tissue from variations in blood composition and toxins. Elsewhere in the body the extracellular concentrations of hormones, amino acids and potassium undergo frequent fluctuations, especially after meals, exercise or stressful times. Since many of these molecules regulate neuronal excitability, a similar change in the composition of interstitial fluid in the CNS can lead to uncontrolled brain activity. The endothelial cells forming the blood-brain barrier are highly specialized to allow precise control over the substances that enter or leave the brain. The BBB in adults consists of a complex cellular system of a highly specialized basal membrane, a large number of pericytes embedded in the basal membrane and astrocytic end feet. Whereas the endothelial cells form the barrier proper, the interaction with adjacent cells seems to be required for the development of the barrier. The brain endothelial cells differ from endothelial cells from other organs in two important ways. First, continuous tight junctions are present between brain endothelial cells. These tight junctions prevent par acellular movement of molecules. Second, there are no detectable Tran's endothelial pathways such as intracellular vesicles. These properties of brain endothelial cells provide a barrier between the blood and the brain.

When low-intensity RFR was studied, generally, no significant effect on the blood-brain-barrier was observed. For example, Gruenau *et al*., [19] reported no significant change on the penetration of 14C-sucrose into the brain of rats after 30 min of exposure to pulse or continuous-wave 2800-MHz RFR of various intensities (1-15 mW/cm2 for the pulsed radiation, 10 and 40 mW/cm2 for the continuous-wave radiation). Ward *et al*., [20] irradiated rats with 2450-MHz RFR for 30 min at different power densities (0-30 mW/cm2, SAR 0-6 W/kg) and studied entry of 3H-inulin and 14C-sucrose into different areas of the brain. They also reported no significant increase in penetration of both compounds into the brain due to RFR exposure; but they reported an increase in 14C-sucrose entry into the hypothalamus when the ambient temperature of exposure was at 40 degree centigrade.

This increase in permeability was suggested to be due to the hyperthermia induced in the animals exposed in high ambient temperature. In a further study, Ward and Ali [21] exposed rats to 1700-MHz continuous-wave or pulsed RFR for 30 min with the radiation concentrated at the head of the animal (SAR 0.1 W/kg). They reported no significant change in permeability into the brain of 3H-inulin and 14C-sucrose after the exposure. Williams *et al*., [22] carried out a series of experiments to study the effect of RFR exposure on blood-brain-barrier permeability to

hydrophilic molecules in unrestrained, conscious rats. The effects of exposure to continuous-wave 2450-MHz RFR at 20 or 65 mW/cm2 (SAR 4 or 13 W/kg) for 30, 90, or 180 min were compared with those of ambient heating (42 degree centigrade)-induced hyperthermia and urea infusion, on sodium fluorescein, horseradish peroxidase, and 14C-sucrose permeability into different areas of the brain. They concluded that RFR did not significantly affect the penetration of the tracers into the brain.

Electron microscopic findings of the present study revealed among the cells of different parts of brain from RF-EMR-exposed rats, individual shrunken cells could be seen with condensed cytoplasm and nucleus. The mitochondria which were swollen and vacuolized, and the cristae were disordered and fewer in number. The rough endoplasmic reticulum also exhibited sacculated distension. This finding was in accordance with the study of sanders *et al*., [23].

He studied the components of the mitochondrial electron transport system that generates high energy molecules for cellular functions. The compounds nicotinamide adenosine dinucleotide (NAD), adenosine triphosphate (ATP), and creatine phosphate (CP) were measured in the cerebral cortex of rats exposed to RFR. In one study, sanders *et al*., [24] exposed the head of rats to 591-MHz continuous-wave RFR at 5.0 or 13.8 mW/cm^2 for 0.5-5 min (local SAR at the cortex of the brain was estimated to be between 0.026 and 0.16 W/kg per mW/cm^2). A decrease in concentrations of ATP and CP and an increase in NADH were observed in the cerebral cortex. These changes were found at both power densities of exposure. Furthermore, the researchers reported no significant change in cerebral cortical temperature at these power densities. They concluded that the radiation decreased the activity of the mitochondrial electron transport system.

Radio based licensing procedures have been established in the majority of urban spaces regulated either at municipal/county, provincial/state or national level. Mobile telephone service providers are, in many regions, required to obtain construction licenses, provide certification of antenna emission levels and assure compliance to ICNIRP standards and/or to other environmental legislation. Many governmental bodies also require that competing telecommunication companies try to achieve sharing of towers so as to decrease environmental and cosmetic impact. This issue is an influential factor of rejection of installation of new antennas and towers in communities. The safety standards in the U.S. are set by the Federal Communications Commission (FCC). The FCC has based its standards primarily on those standards established by the Institute of Electrical and Electronics Engineers (IEEE), specifically Subcommittee 4 of the "International Committee on Electromagnetic Safety". In response to public and governmental concern, WHO established the International Electromagnetic Fields (EMF) Project in 1996 to assess the scientific evidence of possible adverse health effects from electromagnetic field. WHO will conduct a formal risk assessment of all studied health outcomes from radiofrequency field exposure by 2016. In addition, and as noted above, the International Agency for Research on Cancer (IARC), a WHO specialized agency, has reviewed the carcinogenic potential of radiofrequency fields, as from mobile phones in May 2011.

WHO also identifies and promotes research priorities for radiofrequency fields and health to fill gaps in knowledge through its research agendas.

WHO develops public information materials and promotes dialogue among scientists, governments, industry and the public to raise the level of understanding about potential adverse health risks of mobile phones.

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