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Research Article

Effects of radio-frequency electromagnetic radiations (RF-EMR) on cerebral cortex of albino rats-a light and electron microscopic study

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Abstract:

Background: 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. 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 cerebrum in albino rats under light and electron microscopy and to evaluate such changes after exposure to graded dose of RF-EMR

Material and methods: 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 cerebral cortex was isolated for tissue processing. The processed tissues were then studied under light microscope (Hematoxylin & Eosin Staining) and Transmission Electron Microscopy (TEM).

Results: Light microscopic findings of the present study showed that cellular size of neuronal cells in pyramidal layer of cerebral cortex, neurons of hippocampus and some granular layers in cerebral cortex of RF-EMR exposed rats decreased in compare to control groups. Individual cells could be seen with condensed cytoplasm and nucleus. Electron microscopic findings revealed individual shrunken cells with condensed cytoplasm and nucleus.

Conclusion: 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.

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 that 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] (WHO, 2006). 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] (Boice and Tarone, 2011).

The cerebrum refers to the part of the brain comprising the cerebral cortex (of the two cerebral hemispheres, as well as several sub cortical structures, including the hippocampus, basal ganglia, and olfactory bulb. In humans, the cerebrum is the superior-most region of the central nervous system (CNS). With the assistance of the cerebellum, the cerebrum controls all voluntary actions in the body. In humans, the cerebrum surrounds the older parts of the brain. The limbic, olfactory, and motor systems project fibers from the cerebrum to the brainstem and spinal cord. Cognitive and volitive systems project fibers from the cerebrum to the thalamus and to specific regions of the midbrain. The neural networks of the cerebrum facilitate complex behaviors such as social interactions, thought, judgement, learning, working memory, and in humans, speech and language. The cerebrum directs the conscious or volitional motor functions of the body. These functions originate within the primary motor cortex and other frontal lobe motor areas where actions are planned. Upper motor neurons in the primary motor cortex send their axons to the brainstem and spinal cord to synapse on the lower motor neurons, which innervate the muscles. Damage to motor areas of cortex can lead to certain types of motor neuron disease. This kind of damage results in loss of muscular power and precision rather than total paralysis. It functions as the center of sensory perception, memory, thoughts and judgment; the cerebrum also functions as the center of voluntary motor activity. The primary sensory areas of the cerebral cortex receive and process visual, auditory, somatosensory, gustatory, and olfactory information. Together with association cortical areas, these brain regions synthesize sensory information into our perceptions of the world around us.

In the view of fact that brain is involved in very important functions and RF-EMR might have damaging effects on its different parts, 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

1. 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.

2. 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.

3. Hematoxylin & Eosin Staining

a. 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.

b. 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).

c. 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)

a. 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.

b. 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.

c. 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

A) Paraffin sections

This study was performed to determine the effect of radio-frequency electromagnetic radiations (RF-EMR) on cerebrum in albino rats under light microscope.

In cresyl violet and H&E-stained sections, the cerebral cortex of the control group is found to be covered by piamatter containing blood vessels. Six layers are identified in the cerebral cortex; outer molecular layer, external granular layer, external pyramidal layer, inner granular layer, inner pyramidal, and the polymorphic layer (Fig. 1a). The molecular layer is thick (Fig. 1b) and contained dense plexus of nerve fibers with few cells. Whereas, the external granular and external pyramidal contain numerous granular cells and pyramidal cells. While the internal granular and internal pyramidal shows few granular cells and pyramidal cells (Fig. 1c). The pyramidal cell has multipolar shape with basophilic cytoplasm and large, rounded vesicular nucleus. Granular cells could be seen with large open face nuclei, prominent nucleolus and little cytoplasm.

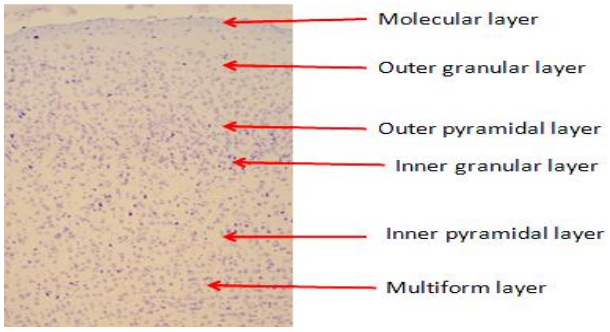

Fig. 1: Representative photomicrograph from the cerebral cortex of control group showing all 6 layers from superficial to deep (The six layers consist of molecular, outer granular, outer pyramidal, inner granular, inner pyramidal and multiform layer. Paraffin sections, Cresyl violet staining, X100.

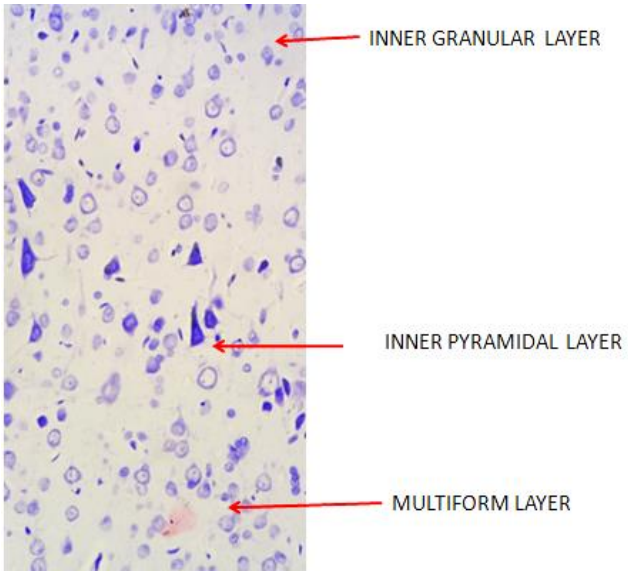

Fig 2: Representative Photomicrograph shows normal study of cerebral cortex in lower three layers in control rats. These layers consist of inner granular, inner pyramidal and multiform layer from superficial to deep. Paraffin section, Cresyl violet staining. Original magnification: X400.

Morphological changes can be well appreciated in these pictures, especially in pyramidal layer. Pyramidal layer is thin comparatively and cells are irregularly arranged. These changes are more marked in E-160 rats in comparison of E-80 and E-120 rats. Cell processes are lost in these affected cells Neurons are darkly stained and their size is decreased in all pictures. Nucleus is heterochromatic. In some pictures nucleus and nucleolus cannot be differentiated separately. In some pictures granule cells are showing effects of RF-EMR (Fig. 2c, 2e and 2g). They are showing shrinkage and nucleus cannot be seen. Blood vessels are also affected by RF-EMR. They are showing signs of hemorrhage and are somewhere congested. Cytoplasm is condensed and minimal in neurons in few photomicrographs (2p). Cell linings and edges cannot be outlined.

Most of the above mentioned changes were also seen in E-120 but less severe as compared to E-160. In E-80 moderate degree of similar changes were seen. When changes were compared among the experimental groups from E-40 to E-160 it was noticed that there is dose-dependent relationship with respect to the dose of exposure (No. of miss calls/day) to RF-EMR.

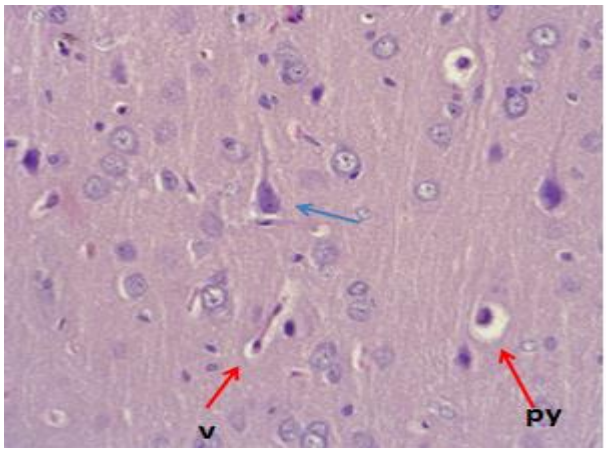

Fig 3: Sample photomicrograph of cerebral cortex from E-80 group showing some pyramidal neurons with normal appearance (blue arrow) with few darkly stained and loosely arranged pyramidal cells (PY). Affected pyramidal cells are small in size with dark heterochromatic nucleus Vessels (v) is congested and showing sign of hemorrhage**.** Paraffin section, H&E, X400

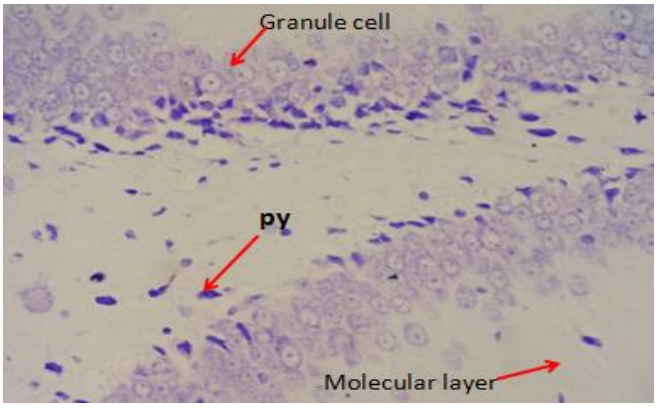

Fig 4: Sample photomicrograph of dentate gyrus of hippocampus from E-80 group is showing darkly stained small sized and heterochromatic pyramidal cells (py). Granular layer and molecular layer can also be appreciated. Paraffin section, cresyl violet, X400

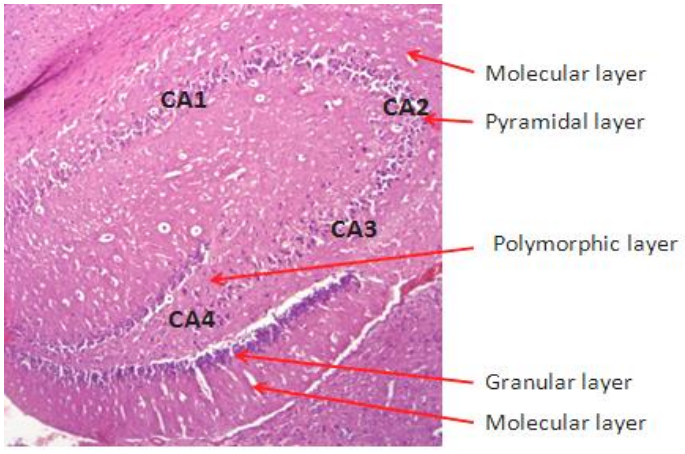

Fig.5: Representative Photomicrograph is showing different layers in the different parts of hippocampus from coronal section of cerebral hemisphere of E-120 rats. The three layers of this primitive cortex consist of molecular layer (stratum molecular), pyramidal layer (stratum pyramidale) and

polymorphic layer (stratum multiforme). Neurons are showing shrinkage and karyopyknosis**.** Paraffin section, H&E, X400

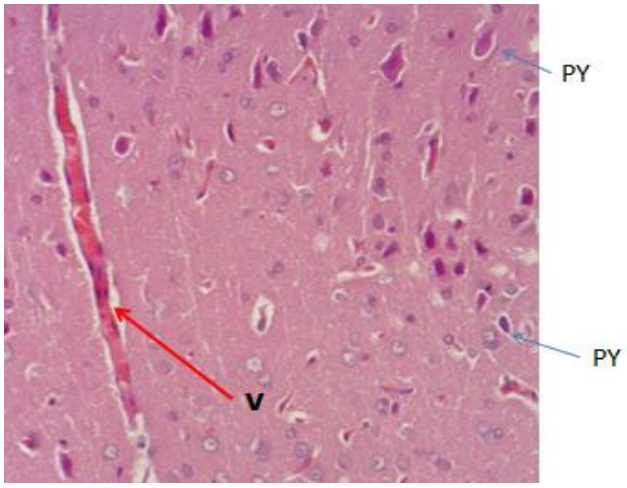

Fig. 6: Representative Photomicrograph of cerebral cortex is of E-160 rats is showing very darkly stained and heterochromatic Pyramidal cells due to effects of RF-EMR. Nucleus and nucleolus cannot be marked out. Cells are of smaller size as well. A vessel can be seen very congested. Paraffin section, H&E, X400

Ultrastructure (TEM)

Most of the neurones in the CNS are either clustered into nuclei, columns or layers, or dispersed within grey matter. Neurones of the PNS are confined to ganglia. Irrespective of location, neurones share many general features. Neurones exhibit great variability in their size (cell bodies range from 5 to 100 μm diameter) and shapes. Their surface areas are extensive because most neurones display numerous narrow branched cell processes. They usually have a rounded or polygonal cell body (perikaryon or soma). This is a central mass of cytoplasm which encloses a nucleus and gives off long, branched extensions, with which most intercellular contacts are made

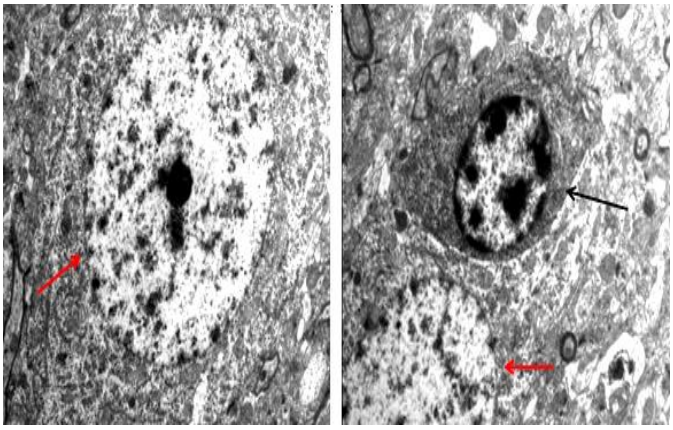

Fig.7 Sample electronmicrograph of cerebral cortex is showing neuron and neuroglia with neuropil in control rats. Neuron (red arrow) exhibits a large round euchromatic nucleus with a distinct nucleolus and nuclear envelope, and numerous rough endoplasmic reticulums. In second picture we can see a neuroglia (Black arrow) with a small, round and pachychromatic nucleus. High magnification

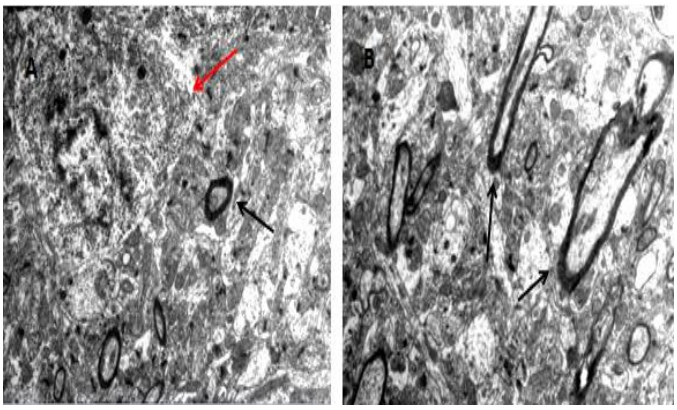

Fig. 8 Sample electronmicrograph of cerebral cortex is showing neuron (red arrow) and neuronal processes (black arrow) in E-160. Individual affected cell with no cellular margin can be seen. Neither intact nucleus nor nucleolus can be observed. Neuropil is showing rarefaction. Processes are thin and elongated. Medium magnification.

The nucleus in **cerebral** cortical neurons is characteristically large, round and euchromatic, which contains at least one prominent nucleolus (Fig, 12a). These are features typical of all cells engaged in substantial levels of protein synthesis. The cytoplasm contains many mitochondria and moderate numbers of lysosomes. Golgi complexes are usually close to the nucleus, near the bases of the main dendrites and opposite the axon hillock. Fig. 12b is of cerebral cortex is showing neuron (red arrow) and neuronal processes (black arrow) in E-160. Individual affected cell with no cellular margin can be seen. Neither intact nucleus nor nucleolus can be observed. Neuropil is showing rarefaction. Processes are thin and elongated.

Discussion:

Very fast advancements in the Electromagnetic Field (EMF) technologies and communications have greatly increased the human populations' exposure to EMFs. Many studies have demonstrated that EMF does affect the nervous tissue in one or the other way. Since brain is a very important part of human body which is concerned with monitoring the whole human body. 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 pyramidal layer of cerebral cortex, neurons of hippocampus, dorsal root ganglionic cells and some granular layers in cerebral 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[3,4] (Garaj et al., 1990 and

1991). 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. (1994) 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)[5]. Sarkar et al. (1994) 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)[6]. Lai and Singh (1995) reported an increase in single strand DNA breaks in brain cells of rats after 2 hours of exposure to 2450-MHz RFR (whole body SAR 0.6 and 1.2 W/kg)[7]. Genetic damages to glial cells can result in carcinogenesis. However, since neurons do not undergo mitosis, a more likely consequence of neuronal genetic damage is changes in functions and cell death, which could either lead to or accelerate the development of neurodegenerative diseases. Lai and Singh (1996) have reported an increase in DNA double strand breaks in brain cells of rats after acute exposure to RFR. Double strand breaks, if not probably repaired and 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[8]. This type of response would lead to an inverted-U response function in carcinogenesis and may explain recent reports of increase (Repacholi et al. 1997)[9] and decrease (Adey et al. 1996)[10] on cancer rate of animals exposed to RFR.

In present study, it is observed that there is decrease in a number of Pyramidal cells in experimental group as well as decrease in size of pyramidal cells due to harmful effects of EMF on scheduling death of cells and their apoptosis. These morphologic changes can be because of decreased activity of nucleus and its result is decreasing activity of the cell. These findings are in agreement with other studies which have shown that EMF can decrease the DNA regeneration and affect the cellular genome[11, 12] (Manti and Darco, 2010, Lai and Singh, 2004). This finding can be confirmed with the fact that neurotransmitters, for example, GABA in Purkinje cells decrease as a result of exposure to the EMF.

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 (2013)[13]. 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 behavioural 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[8] (1996) 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.

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.[14] (2006) 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[15] (Soeradi and Tadjudin, 1986) and **free radicals** 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**[16] (Zhitkevich et al., 2001) and long-term exposure to EMF may be linked to even higher levels of **oxidative stress.**

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.[17] (1984).

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[17] (1984) 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.

In a further study by Sanders (1985)[18] the effects of continuous-wave, sinusoidally amplitude-modulated, and pulsed 591-MHz RFR were compared after five minutes of exposure at power densities of 10 and 20 mW/cm2 (SARs at the cerebral cortex were 1.8 and 3.6 W/kg). Different modulation frequencies (4-32 Hz) were used in the amplitude-modulation mode. There was no significant difference in the effect on the NADH level across these modulation

frequencies. Furthermore, pulsed radiations of 250 and 500 pps were compared with power densities ranging from 0.5-13.8 mW/cm2. The 500 pps radiation was found to be significantly more effective in increasing the concentration of NADH in the cerebral cortex than the 250 pps radiation. Since changes in these experiments occurred when the tissue (cerebral cortex) temperature was normal, the authors speculated that they were not due to hyperthermia, but to a direct inhibition of the electron transport functions in the mitochondria by RFR-induced dipole molecular oscillation in divalent metal containing enzymes or electron transport sites.

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. There are many proposed national and international standards, but that of the International Commission for Non-Ionizing Radiation Protection (ICNIRP) is the most respected one, and has been adopted so far by more than 80 countries. For radio stations, ICNIRP proposes two safety levels: one for occupational exposure, another one for the general population. Currently there are efforts underway to harmonize the different standards in existence.

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