EFFECT OF MOBILE PHONE RADIOFREQUENCY ON HIPPOCAMPAL CA3 NEURONS

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ABSTRACT

Objective: The purpose of the study is to investigate the effects of mobile phone [MP] radiofrequency electromagnetic fields (RF-EMF) exposure for different durations on dendritic morphology and nerve cell damage in CA3 sub region of Hippocampus in Swiss albino mice.

Materials &Methods: Total 70 Swiss albino mice of both sexes were used in the study. Animals were divided into 10 groups randomly. Five groups (n=6) were used for assessment of neuronal damage by cresyl violet staining. Another five groups (n=8) were used for assessment of dendritic morphology by Golgi- Cox staining. Groups were divided by exposure duration (15, 30, 45 and 60 minutes/ per day for 30 days); age matched unexposed groups served as controls.

Results: Results of the study have shown that there was decrease in the number of viable neurons and dendritic arborization in CA3 sub region of hippocampus in 30, 45 and 60 min exposed groups.

Conclusions: Increased neuronal damage and decreased dendritic arborization of hippocampal CA3 neurons was found with increase in exposure duration of MPRF-EMF.

KEY WORDS: Hippocampus, CA3, Dendritic arborization, Mobile Phone, Radiofrequency.

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INTRODUCTION

Mobile phone [MP] is one of the major inventions which has changed the way of communication in today's world. In 2013, there were almost as many mobile-cellular subscriptions as people in the world, with 6.8 billion mobile-cellular subscriptions [1]. The wide use of MPs raises concerns about the exposure of RF-EMF on the user's health.

Many populations based and experimental studies have shown the effects of RF-EMF on health in particular on the brain. The RF-EMF emitted by MPs held to the ear is absorbed by the brain [2] which could affect brain tissue. Studies on long-term MP use have shown an increased risk for ipsilateral acoustic neuroma and glioma after $\geq$ 10 years cell phone use [3]. While association of tumors and RF- EMF exposure from MP use is a primary concern, other potential RF-EMF related effects on the brain are also of scientific interest. Altered Neurotransmitters levels[4], memory and behaviour[5], permeability of the blood brain barrier (BBB) and neuronal damage[6], increased free radical production and lipid

peroxidation in brain tissue[7] and increase in single strand DNA breaks in the developing brain cells of rats, were reported by exposure of RF-EMF[8].

Hippocampus is an important part of limbic system involved in learning and memory, the cornu ammonis (CA) region; in particular CA3 sub region is concerned with the spatial navigation, learning and memory. Studies have shown that MP RF-EMFs can cause damage to hippocampus. In the present study the effect of MP (800MHz CDMA) RF-EMF exposure on nerve cell damage and dendritic morphology in hippocampal CA3 region after exposure at 15, 30, 45 and 60 minutes durations per day for 30 days was assessed. Aim of the study was to investigate effects of exposure increase.

MATERIALS AND METHODS

Animals: Swiss albino mice (8 weeks old and 30-40 gm. weight) of both sexes were used in the study. Animals were housed in Polypropylene Mice cages of dimensions 29 x 22 x 14 (L x B x H) (cms). The mice were maintained in standard conditions of temperature (23±2ºC), humidity (50±5%) and light (10 and 14 hours of light and dark, respectively). The animals were fed with standard pellet food and water ad libitum. The animal experiments were carried with the prior approval from the Institutional Animal Ethics Committee (Mamata Medical College, Khammam, Andhra Pradesh, India). Animal care and handling was done according to the guidelines issued by Committee for the Purpose of Control and Supervision of Experiments on Animals [CPCSEA], the World Health Organization, Geneva, Switzerland and the Indian National Science Academy, New Delhi, India.

Experimental design: Total number of animals used for study was 70 of which 56 were experimental mice exposed to MPRF-EMF and 14 unexposed animals served as control mice. Experimental mice were exposed to MPRF-EMF for four different time durations 15, 30, 45 and 60 minutes for 30 days. Each exposure time duration group were divided into two groups. In one group (n=6) neuronal damage was assessed by scoring the number of viable neurons in cresyl violet stained CA3 sub region of hippocampus. In another group (n=8) dendritic morphology was

assessed by scoring the dendritic intersections and branching points of apical and basal dendrites of hippocampal CA3 pyramidal neurons. Age matched unexposed animals served as normal controls [NC].

Mobile phone: 800MHz CDMA MPs were used to expose the animals to MP RF-EMF. The phones used in this study were Huawei C2806 model.

Cresyl violet stating: The animals were deeply anesthetized with ether and perfused transcardially with 0.9% saline and 10% formalin. The brain from each animal was dissected out and kept in 10% formalin for 3 days post fixation. Paraffin blocks were made and 5 µm sections were cut in the dorsal hippocampus using rotary microtome. Sections were mounted onto air dried gelatinized slides and labeled. Slides were stained with cresyl violet. In each section, CA3 region of hippocampus of 200 µm length was selected and the number of viable neurons was counted under light microscope (40x). Densely stained, distorted cells with pyknotic cells were excluded in the scoring. Twenty five sections per animal were used for counting viable neurons of CA3 region[9].

Golgi- Cox staining: The Animals of control group and MPRF-EMF exposed four groups were euthanized subsequent to 30 days of exposure by decapitation. The Golgi-Cox staining was done as detailed previously [10]. Briefly, brains were removed and about 5 to 6 mm coronal pieces of brain with hippocampus were prepared. These coronal pieces were put on glass wool or gauge in clean bottles and covered with the Golgi-Cox solution and left at room temperature and in the dark. After 2 days, the Golgi-Cox solution was changed. Tissues were left immersed in the solution for 10 weeks. Sledge microtome sections of 120 µm thicknesses were cut (as many serial sections as possible in the tissue), dehydrated, cleared and mounted with Distrin plasticizer xylene mounting media.

Camera Lucida tracing: From each mice 8–10 hippocampal CA3 neurons were traced at 400X magnification using a camera lucida attached to an Olympus microscope, and their dendritic branching points and dendritic intersections were quantified. Both right and left side hippocampal CA3 neurons were used. Neurons

with minimal overlap of dendrites, heavily impregnated with silver nitrate and without truncate dendrites, were selected for tracing.

Quantification of dendritic branching points and dendritic intersections: The concentric circle method of Sholl DA (1956) was used for dendritic quantification[11]. Five concentric circles on a transparent sheet with a radial distance of 20 μm between them were used for dendritic quantification (dendritic branching points and intersections). The sheet was placed on a neuron tracing such that the center of the cell body of the neuron coincided with the center of the concentric circles. The number of branching points between the two concentric circles, i.e. within each successive 20 μm concentric zone (ring), was counted. The dendritic intersection is the point where a dendrite intersects the given concentric circle [Figure 1].

The dendritic intersections at each concentric circle were counted. Both branching points and intersections were counted up to a radial distance of 100 μm from the center of the soma. Mean number of dendritic branching points in each concentric zone and number of dendritic intersections at each concentric circle, were calculated. This method of scoring was applied for both apical and basal dendritic quantification. The apical dendrite of pyramidal neuron in CA3 region is a single pole extending from the apex of soma. It divides into 2–3 main branches from which the secondary and tertiary branches arise. The basal dendrites of pyramidal neurons arise from several places along the base of the soma, and these repeatedly branch producing a dense tuft [Figure 1]

Fig. 1: Diagram showing a hippocampal CA3 neuron and the scheme of dendritic quantification.
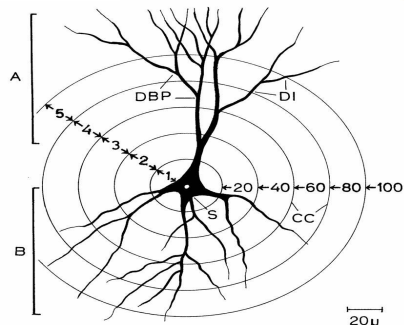
A – Apical dendrites, B – Basal dendrites, DBP – Dendritic branching points, DI – Dendritic intersections, S – Soma, CC – Concentric circles, and 1 to 5 – Concentric zones.

Data Analysis

Data was expressed as mean ± SD. The statistical significance between the treatments was evaluated by One-way ANOVA and with Bonforroni's post-hoc test using GraphPAD InStat 2.01, Software, USA.

RESULTS

Assessment of neuronal damage in CA3 of Hippocampus (Figs. 2 and 3): The arrangement of hippocampal CA3 neurons of control group was trim and dense and the Nissl substance in cytoplasm was clearly discernible [Figure 2]. There was no significant difference in the mean number of viable CA3 neurons in 15 minutes MP RF-EMF exposed group (78.41± 2.999) compared to the control group (79.3±0.6867).
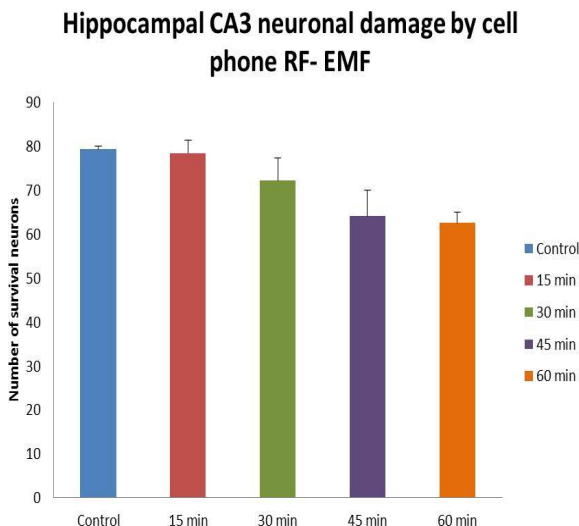
However, the number of viable CA3 neurons in 30, 45 and 60 minutes exposed group were sparse and the Nissl substance was decreasing or dissolving [Figure 2]. Histopathological changes characterized by densely stained, distorted cells with pyknotic appearance observed in CA3 region of the hippocampus of 30, 45 and 60 minutes exposed groups. The number of hippocampal CA3 neurons of 30 minutes exposure group was 72.13 ± 5.178 (P<0.05), 45 minutes group was 64.01 ± 5.922 and 60 minutes group was 62.49 ± 2.517 (P<0.001) which were significantly less than that of control group (79.3±0.6867) [Figures 2 &3]. The results of this study have shown that with increased duration of exposure to MP RF-EMF there is increased damage to hippocampal CA3 neurons.

Fig. 2: Photomicrographs showing hippocampal CA3 neurons of mice brains (40x magnification).
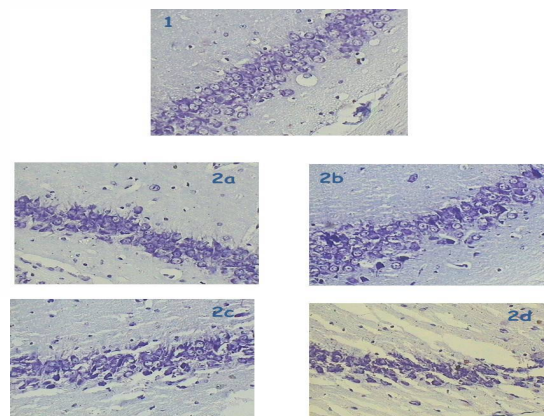
C - Control; MPRF exposed groups: 2a - 15 min, 2b - 30 min, 2c - 45 min and 2d - 60 min. (Note the degenerative changes in 30, 45 and 60 min exposed groups.)

Fig. 3: Histogram showing number of surviving neurons in CA3 region of Hippocampus in mice exposed to MPRF and unexposed control mice. Data is expressed as mean± SD. (NC vs 30 min, * P<0.05; NC vs 45 min & 60 Min, ** P<0.001.

Fig. 4: Photomicrographs (1A, 2A, 3A, 4A, 5A) and Camera lucida tracings (1B, 2B, 3B, 4B and 5B) of Golgi – Cox stained hippocampal CA3 neurons (40x magnification) from control mice (1A, 1B)and mice exposed to MPRF for 30 days for different durations; 15 min(2A, 2B), 30 min (3A, 3B), 45 min (4A, 4B) and 60 min (5a, 5B). A significant decrease in dendritic arborization in 30, 45 and 60 min exposed groups is demonstrated.
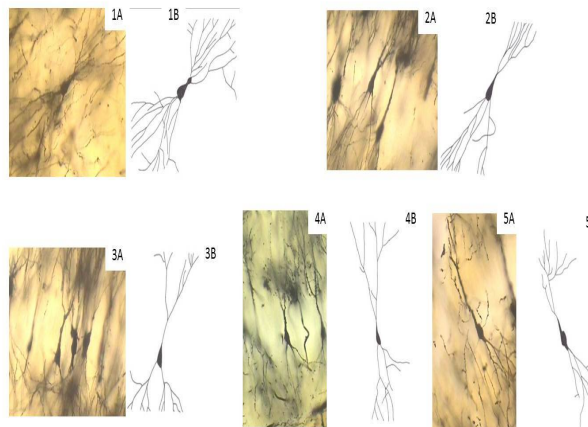

Fig. 5: Apical dendritic intersections of hippocampal CA3 neurons in mice exposed to different durations (15, 30, 45 & 60 Min per day for 30 days) of MP RF and control (NC) mice. NC vs. 30 Min: # p<0.05, ## p<0.01, ### p<0.001; NC vs. 45Min: * p<0.05, ** p<0.01, *** p<0.001; NC vs. 60 Min: $ p<0.05, $$ p<0.01, $$$ p<0.001.

Assessment of dendritic morphology of hippocampal CA3 neurons Apical dendritic intersections [Figures 4 and 5]:
There was no significant change in the dendritic intersections at any of the concentric circles in 15 minutes group when compared to control group.

However 30, 45 and 60 minutes exposure groups showed significant decrease in dendritic intersections at 20, 40, 60 and 80 μ concentric circles (20 μ concentric circle: 6.78 ± 1.14 in control group vs. 4.89 ± 0.79 in 30 minutes group, P<0.01, 4.38 ± 0.81 in 45 minutes group, P<0.001 and 4.11 ± 0.75 in 60 minutes group, P<0.001, 40 μ concentric circle: 8.12 ± 1.34 in control group vs. 6.12 ± 1.03 in 30 minutes group, P<0.05, 5.46 ± 0.76 in 45 minutes group, P<0.001 and 5.23 ± 0.94 in 60 minutes group, P<0.001, 60 μ concentric circle: 11.16 ± 0.86 in control group vs. 7.82 ± 0.73 in 30 minutes group, P<0.001, 6.23 ± 1.36 in 45 minutes group, P<0.001 and 6.11 ± 0.92 in 60 minutes group, P<0.001, 80 μ concentric circle: 13.26 ± 1.03 in control group vs. 10.57 ± 1.37 in 30 minutes group, P<0.01, 9.34 ± 1.09 in 45 minutes group, P<0.001 and 8.17 ± 2.14 in 60 minutes group, P<0.001). In addition, 45 and 60 minutes groups also showed decreased dendritic intersections in 100 μ concentric circle (10.15 ± 1.03 in control group vs. 7.35 ± 1.07 in 45 minutes group, P<0.01 and 6.22 ± 1.39 in 60 minutes group, P<0.001).
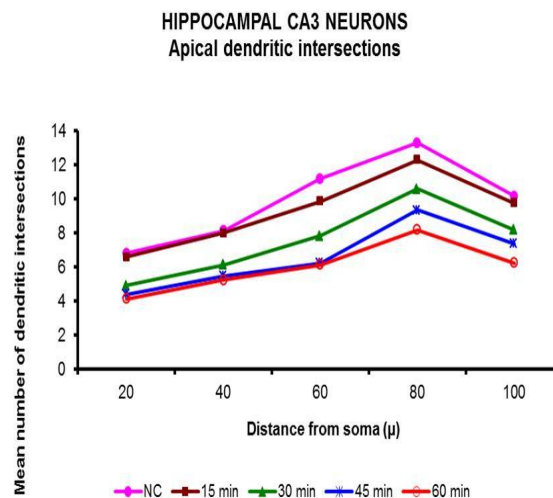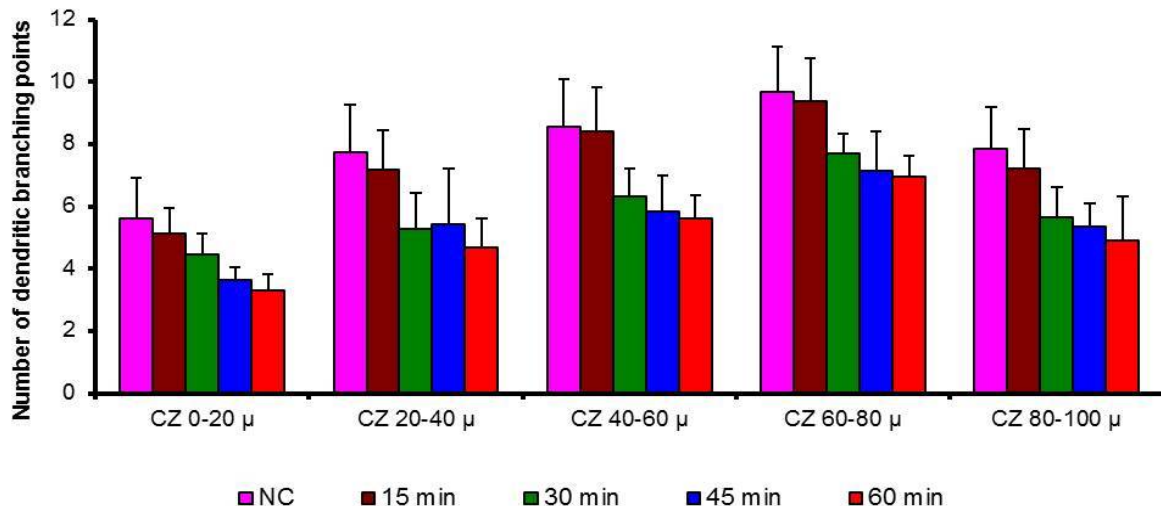
Fig. 6: Apical dendritic branching points of hippocampal CA3 neurons in mice exposed to different durations (15, 30, 45 & 60 Min per day for 30 days) of MP RF and normal control (NC)mice at different concentric zones (CZ) and total number of branching point. Each value represents the mean+ Standard deviation of 8–10 neurons from each mouse.
NC vs.30 Min: # p<0.05, ## p<0.01, ### p<0.001; NC vs. 45Min: * p<0.05, ** p<0.01, *** p<0.001; NC vs. 60 Min: $ p<0.05, $$ p<0.01, $$$ p<0.001.

HIPPOCAMPAL CA3 NEURONS
Apical dendritic branching points

Apical dendritic branching points [Figure 4 and 6]: No significant change was observed in the dendritic branching points at any of the concentric zones in 15 minutes group when compared to control animals.

However 30, 45 and 60 minutes groups showed significant decrease in the dendritic branching points in 20-40 μ, 40-60 μ, 60-80 μ and 80-100 μ concentric zones (20-40 μ concentric zone: 7.72 ± 1.53 in control group vs. 5.28 ± 1.17 in 30 min, $P<0.05$, 5.42 ± 1.81 in 45 minutes group, $P<0.001$ and 4.67 ± 0.94 in 60 minutes group, $P<0.001$, 40-60 μ concentric zone: 8.54 ± 1.56 in control group vs. 6.32 ± 0.91 in 30 minutes group, $P<0.01$, 5.82 ± 1.18 in 45 minutes group, $P<0.001$ and 5.61 ± 0.75 in 60 minutes group, $P<0.001$, 60-80 μ concentric zone: 9.67 ± 1.45 in control group vs. 7.71 ± 0.64 in 30 minutes group, $P<0.01$, 7.14 ± 1.27 in 45 minutes group, $P<0.001$ and 6.94 ± 0.67 in 60 minutes group, $P<0.001$, 80-100 μ concentric zone: 7.84 ± 1.34 in control group vs. 5.65 ± 0.98, $P<0.01$, 5.34 ± 0.76 in 45 minutes group, $P<0.01$ and 4.92 ± 1.41 in 60 minutes group, $P<0.001$). In addition 45 min and 60 minutes groups showed decreased dendritic branching points in 0-20 μ concentric zone (5.61 ± 1.32 in control group vs. 3.63 ± 0.43 in 45 minutes group, $P<0.001$ and 3.32 ± 0.51 in 60 minutes group, $P<0.001$)

Basal dendritic intersections [Figures 4 and 7]: There were no significant changes in the dendritic intersections at any of the concentric circles in 15 minutes group when compared with the control group.

However 30, 45 and 60 minutes groups showed significant decrease in the dendritic intersections at 40, 60, 80 and 100 μ concentric circles (40 μ concentric circle: 9.71 ± 0.78 in control group vs. 7.14 ± 1.28 in 30 minutes group, $P<0.001$, 6.38 ± 0.84 in 45 minutes group, $P<0.001$ and 6.18 ± 0.78 in 60 minutes group, $P<0.001$, 60 μ concentric circle: 11.23 ± 1.35 in control group vs. 7.91 ± 0.88 in 30 minutes group, $P<0.001$, 6.75 ± 0.91 in 45 minutes group, $P<0.001$ and 6.39 ± 1.32 in 60 minutes group, $P<0.001$, 80 μ concentric circle: 10.48 ± 0.83 in control group 5.32 ± 1.22 in 30 minutes group, $P<0.001$, 4.61 ± 1.23 in 45 minutes group, $P<0.001$ and 4.56 ± 0.91 in 60 minutes group, $P<0.001$, 100 μ concentric circle: 6. 86 ± 1.42 in control group vs. 4.13 ± 1.31 in 30 minutes group, $P<0.01$, 3.14 ± 0.82 in 45 minutes group, $P<0.001$ and 2.98 ± 0.75 in 60 minutes group, $P<0.001$).

Fig. 7: Basal dendritic intersections of hippocampal CA3 neurons in mice exposed to different durations (15, 30, 45 & 60 Min per day for 30 days) of MP RF and normal control (NC). mice NC vs.30 Min: # $p<0.05$, ## $p<0.01$, ### $p<0.001$; NC vs. 45Min: * $p<0.05$, ** $p<0.01$, *** $p<0.001$; NC vs. 60 Min:$ $p<0.05$, $$ $p<0.01$, $$$ $p<0.001$.
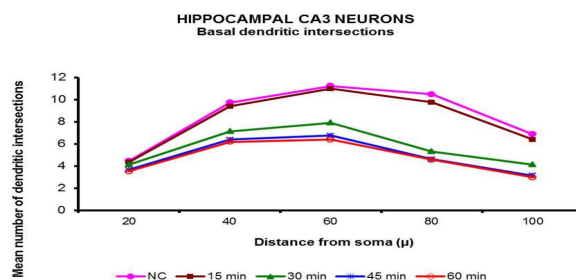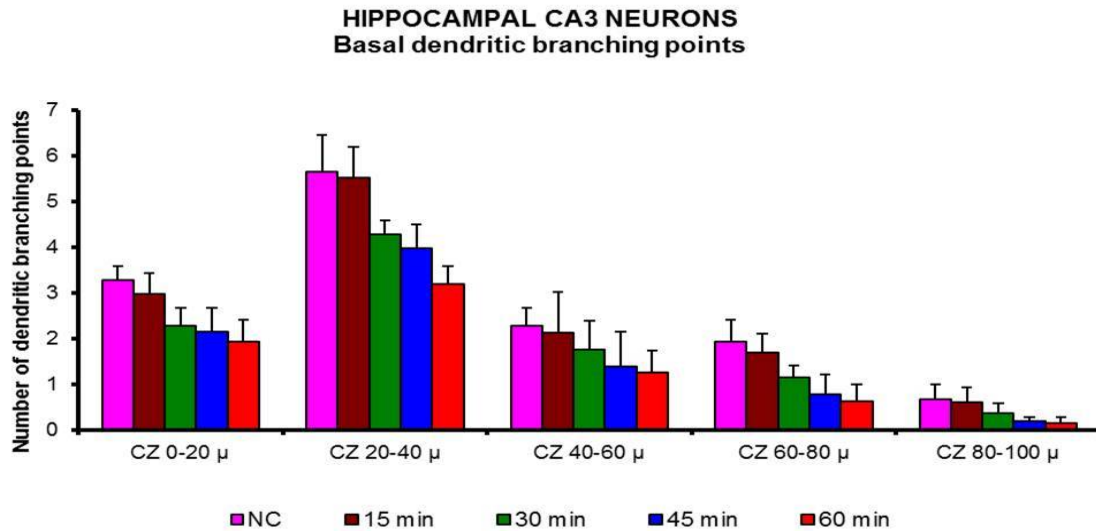
Fig. 8: Basal dendritic branching points of hippocampal CA3 neurons in mice exposed to different durations (15, 30, 45 & 60 Min per day for 30 days) of MP RF and normal control (NC) mice at different concentric zones (CZ) and total number of branching points. Each value represents the mean+ Standard deviation of 8–10 neurons from each mouse. NC vs. 30 Min: # p<0.05, ## p<0.01, ### p<0.001; NC vs. 45Min: * p<0.05, ** p<0.01, *** p<0.001; NC vs. 60 Min: $ p<0.05, $$ p<0.01, $$$ p<0.001.

HIPPOCAMPAL CA3 NEURONS
Basal dendritic branching points

Basal dendritic branching points [Figures 4 and 8]: No significant changes were observed in the dendritic branching points at any of the concentric zones in 15 minutes group.

However 30, 45 and 60 minutes groups showed significant decrease in the dendritic branching points in concentric zone 0-20 µ (3.28 ± 0.30 in control group vs. 2.27 ± 0.40 in 30 minutes, P<0.001, 2.15 ± 0.51 in 45 minutes group, P<0.001 and 1.93 ± 0.47 in 60 minutes group, P<0.001), concentric zone 20-40 µ (5.65 ± 0.80 in control group vs. 4.28 ± 0.30 in 30 minutes, P<0.001, 3.97 ± 0.53 in 45 minutes group, P<0.001 and 3.18 ± 0.64 in 60 minutes group, P<0.001), concentric zone 60-80 µ (1.93 ± 0.47 in control group vs. 1.15 ± 0.26 in 30 minutes, P<0.01, 0.78 ± 0.43 in 45 minutes group, P<0.001 and 0.63 ± 0.37 in 60 minutes group, P<0.001). In addition 60 minutes group alone showed decreased dendritic branching points in concentric zone 40-60 µ (2.27 ± 0.40 in control group vs. 1.26 ± 0.48 in 60 minutes, P<0.05) and both 45 minutes and 60 minutes groups showed decreased dendritic branching points in concentric zone 80-100 µ (0.66 ± 0.34 in control group vs. 0.18 ± 0.09 in 45 minutes group, P<0.01 and 0.15 ± 0.13 in 60 minutes group, P<0.01).

DISCUSSION

Possible effects of RF-EMFs on hippocampal neuronal morphology are important due to the fact that the hippocampus is the part of the brain that controls important behavioural and cognitive functions, including spatial learning and working memory[12-20]. The hippocampus is a critical integrative center involved in the regulation of exploratory activities and for incorporating spatial information[21]. The CA3 region forms an essential link in the hippocampal trisynaptic circuit and is implicated as substrate for learning and memory, and CA3 field appears to be the major focus of integration of intrahippocampal activity [22, 23].

The results of our study demonstrated hippocampal CA3 neuronal damage by the exposure of mice to commercially available MP RF-EMF for extended durations of 30, 45, and 60 min. This decrease in number of cells observed in the present study may be due to effect on neurogenesis[24]. In recent past, several studies have demonstrated that degenerative changes of different tissues induced by MP RF-EMF. Neuronal damage may also be due to RF- EMF induced oxidative stress[25]. Where, reactive oxygen species (ROS) generated by external field application may interact with cellular biomolecules, such as lipids, proteins, and DNA, leading to modification and damage[26]. It was reported that 2 hour exposure to cell phone RF-EMF emissions can up-regulate elements of apoptotic pathways in culture cells derived from the brain [27].

RF-EMF mediated ROS formation can lead to heat shock protein (HSP) phosphorylation, which can alter the secretion of growth factors. This, in turn, can increase the permeability of the BBB [28, 29]. RF- EMF induced BBB leakage resulted in albumin extravasation and albumin uptake into neurons [30]. Leakage of BBB by RF – EMF shown to result in increase of dark, shrunken neurons, that is neuronal damage in hippocampus [6, 30, 31]. Exposure to 900 MHz EMF has shown to decrease in neuron number and cause neuronal damage in the cortex, cerebellum, hippocampus, and basal ganglia in animals [8, 30, 32-35]. The hippocampus, especially the granule cells of the dentate gyrus [24] and the cells of CA areas [36, 37] are selectively vulnerable to RF exposure.

Along with RF- EMF induced neuronal damage, RF- EMF induced changes in dendritic arborization (dendritic length and branching points) of hippocampal CA3 neurons was also studied in the present study. The apical dendrites of hippocampal CA3 pyramidal neurons receive mossy fiber inputs, at the proximal segment, entorhinal fibers at the molecular layer, septo-hippocampal fibers at the middle and terminal dendritic regions and noradrenergic fibers as well as other associational, commissural and collateral inputs at the middle segment (stratum radiatum) [15, 38, 39]. The basal dendrites receive inputs from collateral branches of mossy fibers [15]. The hippocampus is highly susceptible to various endogenous and exogenous insults, which are known to cause neuroanatomical changes in the hippocampus, such as atrophy of apical dendrites of CA3 pyramidal neurons. In the present study, assay of neuroanatomical changes that is dendritic arborization changes induced by MPRF-EMF revealed that there was no significant change in dendritic arborization in apical and basal dendritic branches of CA3 pyramidal neurons of hippocampus in unexposed control and 15 minutes exposed groups. However, there was a significant decrease in apical and basal dendritic branches in CA3 pyramidal neurons in 30 Min, 45 min & 60 minutes MPRF-EMF exposed groups compared to control group. Significant increase in dendritic atrophy was found as the exposure duration increased. These results indicate that long term exposure to may cause dendritic atrophy.

Chronic restraint stress induced apical dendritic atrophy in CA3 pyramidal neurons of the hippocampus was associated with impairment in the spatial memory task in rats [40, 41]. The possible mechanisms underlying RF- EMF induced dendritic atrophy might be due to various changes in neurochemical substances. Experiments with RF-EMF were shown to induce changes in Neurochemical functions. Decreased NMDA receptors in cortex [42] and hippocampus [43] were found on exposure to RF- EMF. Exposure to extremely low frequency magnetic fields changes $Ca2+$ signaling events and N-methyl-D-aspartate receptor activity in the rat hippocampus.[44] Changes in the expression of calcium binding proteins (CaBP) such as calbindin D28-k (CB) and calretinin (CR) were found in the hippocampus of mice exposed to RF- EMF.[45] NMDA receptors play a crucial role in spatial memory[46]; regulate dendritic development[47-49].

CONCLUSION

Results of present study indicated that there was increase in hippocampal CA3 neuronal damage and dendritic atrophy with increase in duration of exposure, which might have an effect on the cognitive function and behavioural performance following long term MPRF-EMF exposure.

Conflicts of Interests: None

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