



Electromagnetic radiation 2450 MHz exposure causes cognition deficit with mitochondrial dysfunction and activation of intrinsic pathway of apoptosis in rats

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Electromagnetic radiation (EMR) can induce or modulate several neurobehavioral disorders. Duration and frequency of exposure of EMR is critical to develop cognitive disorders. Even though EMR-2450 is widely used, its effects on cognition in relation to mitochondrial function and apoptosis would provide better understanding of its pathophysiological effects. Therefore, a comparative study of different frequencies of EMR exposure would give valuable information on effects of discrete frequencies of EMR on cognition. Male rats were exposed to EMR (900, 1800 and 2450 MHz) every day for 1 h for 28 consecutive days. The cognitive behavior in terms of novel arm entries in Y-maze paradigm was evaluated every week after 1 h to last EMR exposure. Animals exposed to EMR-2450 MHz exhibited significant cognitive deficits. EMR-2450 MHz caused loss of mitochondrial function and integrity, an increase in amyloid beta expression. There was release of cytochrome-c and activation of apoptotic factors such as caspase-9 and -3 in the hippocampus. Further, there was decrease in levels of acetylcholine, and increase in activity of acetyl cholinesterase, indicating impairment of cholinergic system. Therefore, exposure of EMR-2450 in rats caused cognitive deficit with related pathophysiological changes in mitochondrial and cholinergic function, and amyloidogenesis.

Keywords. Apoptosis; cognition; electromagnetic radiation – 900, 1800, 2450 MHz; hippocampus; mitochondria

1. Introduction

Mobile phones are now an integral part of modern telecommunications. There is an estimated 6.9 billion subscriptions globally (Brahima 2015). It has been reported that mobile phones like 2G and 3G emit radiofrequency of non-ionizing electromagnetic radiation (EMR) in the range of 869–900 MHz (Code division multiple access; CDMA), 935–960 MHz (Global system for mobile communication; GSM-900), 1805–1880 MHz (GSM-1800) and 2110–2450 MHz (3G and Wi-Fi) (Dogan *et al.* 2012; Naziroglu *et al.* 2012). Since it is widely used by the population in various applications, it may lead to potential irradiation into the environment and associated health hazards to humans. There are reports to suggest that occupational exposure to EMR may increase risk of neurodegenerative diseases (Jiang *et al.* 2013). There is no consensus regarding whether EMR exposure could cause potential detrimental effects in whole animals or isolated

brain regions. The use of the new generation mobile phones has been reported to initiate symptoms like headache, sleep anomalies (Hossmann and Hermann 2003) and cognitive impairment (Levin 2015).

Earlier studies have reported that long term low frequency of EMR exposure altered the cognitive behavior in rats. This leads to a marked hindrance in learning and recall of memory tasks and may cause a further risk of developing Alzheimer's disease (AD) (Dogan *et al.* 2012; Naziroglu *et al.* 2012; Jiang *et al.* 2013). Similarly, recent reports observed that EMR-900 MHz and -1800 MHz may lead to cognitive impairments due to decrease in the level of neurotransmitters (Narayanan *et al.* 2015). However, till date there is no report on the comparative effects of EMR (900, 1800 and 2450 MHz) exposure on the hippocampus. Therefore, the present study was designed to compare the effect of different EMR radiation (900, 1800 and 2450 MHz) on mitochondrial-linked hippocampus-directed cognitive behavior. It has already been shown that EMR-

2.45 GHz at low intensity (50–230 Hz) for short time period (15–120 min daily for 7 days) decreases the level of acetylcholine (ACh) with increase in enzymatic activity of acetylcholinesterase (AChE) in the hippocampus. This could lead to cognitive disorders or memory dysfunction in rats (Lai et al. 1987; Wang and Lai 2000; Afrasiabi et al. 2014).

Mitochondria are essential for typical cognitive functions in the experimental animals (Joshi et al. 2014; Tanaka et al. 2008). Mitochondrial function can regulate synaptic release of acetylcholine (Pochynyuk et al. 2002). It has been shown that mitochondrial dysfunction leads to decrease in acetylcholine release (Lykhmus et al. 2014). Mitochondria impairment leads to production of reactive oxygen species (ROS) (Guo et al. 2013). Several studies report that amyloid beta ($A\beta$) is localized to mitochondrial membranes (Glennner and Wong 1984; Villemagne et al. 2013). Oxidative stress and accumulation of $A\beta$ 1–40 are implicated in the pathogenesis of AD (Chen and Yan 2010; Fu et al. 2016; Zhang et al. 2016). In particular, the $A\beta$ 1–40 has been circumstantially linked to the neurotoxic principle causing cell death in the disease (Neve et al. 1990). Alternatively, deposition of amyloid beta in cases such as AD may alter mitochondrial morphology leading to impaired neurotransmission of ACh, which may ultimately result in synaptic damage and neurodegeneration causes cognitive impairment (Chen and Yan 2010; Pinho et al. 2014). However, there are very few reports on the effects of EMR on mitochondrial function and related physiological effects. Microwave frequency exposure has been reported to alter the hippocampal mitochondrial cristae morphology observed by histopathological analysis (Zhao et al. 2012). Mitochondrial stress and ensuing dysfunction could lead to activation of apoptotic factors leading to cell death (Ott et al. 2007). These apoptotic events are initiated by over activation of caspases-9/3, which has been suggested to be a hallmark in the induction of apoptotic cell death, and may further lead to cognitive impairments (Jänicke et al. 1998). However, the effect of EMR on mitochondrial-linked cell death is yet to be explored (Yang et al. 2015). Therefore, study of effects of EMR on mitochondrial function and apoptosis would provide valuable information to understand the pathophysiological mechanisms of cognitive dysfunction.

Therefore, the present study evaluated the effects of discrete frequencies of EMR on cognitive behavior and associated pathophysiological mechanisms. Mitochondrial integrity, complex enzyme and oxidative stress were evaluated to understand the effects of EMR on mitochondrial function. The expression of $A\beta$ 1–40 was studied to explore the effect of EMR on amyloidogenesis. Further, the effect of EMR on the level of ACh and the activity of AChE were investigated. Furthermore, the mitochondrial mediated apoptosis was assessed by the expression of cytochrome-c, caspase-9 and caspase-3.

2. Materials and methods

2.1 Animals

Inbred Charles–Foster albino male rats (150–180 g) were collected from Institute of Medical Sciences, Banaras Hindu University and were housed at $26\pm 2^\circ\text{C}$, relative humidity 44–56% and light:dark cycle of 12:12 h. Animals were provided with standard rodent pellet diet (Hind liver) and water *ad libitum*. The experiment was conducted in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA-2010; Institute of Medical Sciences, Banaras Hindu University) guidelines (Approval No.: Dean/2015/CAEC/1414).

2.2 Chemicals

AChE, Choline oxidase, horse radish peroxidase, nitroblue tetrazolium (NBT) and Griess reagent were procured from sigma aldrich (St. Louis, MO, USA). Tetramethyl rhodamine methyl ester (TMRM) was purchased from Thermofischer. All antibodies used in this experiment were purchased from Abcam (UK). All other chemicals and reagents of high performance liquid chromatography (HPLC) and analytical grade were procured from Hi-media, Mumbai, India.

2.3 Electromagnetic radiation exposure system and design

The electromagnetic radiation exposure system and design are shown schematically in figure 1. The animals were kept in a wooden cage having dimension of $46\times 30\times 40$ cm having sufficient free space for propagation of EMR for maintaining low dielectric constant (1.25). Hence, the wooden cage was divided into 6 compartments with 2 rows of 3 compartments with dimension $15\times 14.5\times 40$ cm, having ventilation and restrainer (to keep animal immobile) facility during the exposure of EMR. The compartment was designed in such a way which facilitated the propagation of radiation parallel to the electric field. The upper surface and the base of the cage were wrapped with lining of carbon sheets impregnated with Styrofoam absorber in order to minimize the reflections of electromagnetic radiation. Thereafter, the wooden cage was placed and fixed to an anechoic chamber of dimension $65\times 65\times 60$ cm, impregnated with Styrofoam radiation absorber (to reduce the reflection of radiation towards horn antenna). Then walls of the anechoic chamber were surrounded by cones and foam to shield the back reflections same as reported by (Holloway et al. 2002). The horn antenna (Scientific Instrument Co. Ltd. SB-771, India) was kept in such a manner that it received uninterrupted the radiations from the source. The

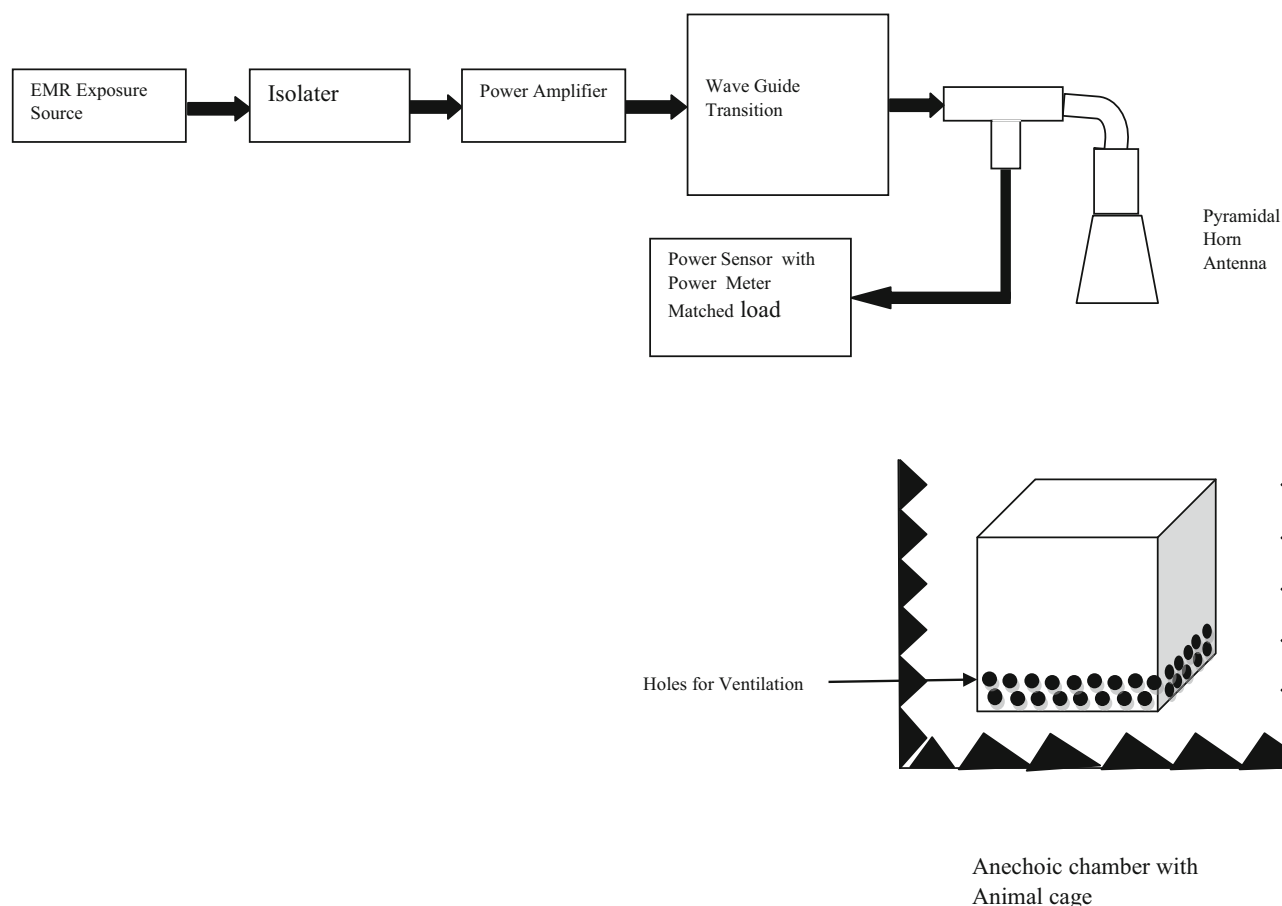


Figure 1. Schematic representation of the EMR exposure set-up and the position of animal cages during exposure. Experimental rats were exposed to 900, 1800 and 2450 MHz EMR exposure radiation for 1 h/day for each group continuously for 28 days by using EMR source and pyramidal horn antenna.

temperature of anechoic chamber was kept constant $25 \pm 2^\circ\text{C}$ throughout the exposure period.

The horn antenna was connected to the generator, which emitted continuous radiofrequency (RF) signals of 900, 1800 and 2450 MHz. The repetition time, frequency and amplitude was continuously monitored by the Analog Signal Generator (Model No. MXG N5183A manufactured by Agilent Technologies, USA) covering the frequency range from 100 KHz to 20 GHz. The exposure system also has a microwave amplifier (Model no. 8349B Hewlett Packard Co) connected with waveguide transition, 20 dB cross coupler, E-plane bend and pyramidal horn antenna (made of brass and coated with silver). The set-up also has a power meter with power sensor (Model No. 836A manufactured by Agilent technologies) to measure the maximum output power. This was measured to be 19.9 dBm which was delivered to the horn antenna and amplifier. The power meter has a range from 30 μW to 3 W (Model no. 8481H, Hewlett

Packard Co.). The antenna used has neck and opening aperture dimensions 7.2 cm $\times$ 3.2 cm and 9.0 $\times$ 5.0 cm respectively while its axial length from the neck is 10 cm. Comparison method was used to measure the gain of the horn antenna (Gt), which was found to be 1.965, 2.862 and 4.8657 (for 900, 1800 and 2450 MHz). The distance between the pyramidal horn and the mid plane of body of the animal was estimated by far field formulae ($R \geq 2D^2/\lambda$) to be 25 cm. The rats were kept inside the cage and positioned at distance of 8, 11 and 15 cm from the antenna for exposure to 900, 1800 and 2450 MHz respectively to maintain the required power density, which is described in the following section. The cross-sectional dimensions of the animal cage in E- and H-planes (electric and magnetic field planes, respectively) were designed on the basis of $E=6.798$, 6.742 and 6.754 V/m and $H=0.01803$, 0.01806 and 0.018.8 A/m for 900, 1800 and 2450 MHz fields in corresponding planes of horn (Balanis 1997; Fontollet 1996; Langrange *et al.*

1999). The power input to the horn antenna and the reflections were measured by using a 20 dB cross-coupler, power sensor and a meter of range 30 μ W to 3 W (Model no. 8481H, Hewlett Packard Co., USA). The power that was transmitted (P_t) to the antenna was estimated by subtracting the reflected power with input power, which was found to be 50.19, 65.15 and 71.43 mW for 900, 1800 and 2450 MHz, respectively.

2.4 Calculation of power density and specific absorption rate

The power density obtained by using the following formula: Power Density = $P_t G_t / 4\pi R^2$, where P_t is the power transmitted into the cage, G_t is the gain of the horn and R is the distance between the horn aperture and mid plane of the rat in the cage. The animals after being exposed to 900, 1800 and 2450 MHz EMR, the average power density was found to be 0.1227 W/m². While the overall value of whole body average SAR was found to be approximately, 0.0227, 0.030 and 0.0616 W/kg for 900, 1800 and 2450 MHz respectively. The measurement of SAR value was dependent upon arrangement of rat in animal cage (Paxinos and Watson 1986; Gandhi 1990; Repacholi et al. 1997). Experimental rats ($n=6$) were subjected to continuous modulated electromagnetic radiation exposure of 900, 1800 and 2450 MHz (for modulation: modulating signal 217 Hz, modulation index 0.1%), for daily 1 h for each group for 28 days between 10.00 AM to 1.00 PM. Control group was placed in anechoic chamber without any exposure. The average rectal temperatures of rat were observed to be 33.71 ± 0.56 (control), 35.3 ± 0.63 (EMR-900 MHz), 35.48 ± 0.67 (EMR-1800 MHz) and 36.96 ± 0.55 (EMR-2450 MHz) before and 33.91 ± 0.82 (control), 35.5 ± 0.51 (EMR-900 MHz), 35.66 ± 0.60 (EMR-1800 MHz) and 37 ± 0.78 (EMR-2450 MHz) just after completion of exposure. These changes were not statistically significant as analysed by Two-way ANOVA.

2.5 Experimental design

The rats were randomly divided into four groups with six animals each, namely: (i) control (animals not exposed to EMR radiation but kept under same conditions as that of other groups), (ii) animals irradiated at EMR-900, (iii) animals irradiated at EMR-1800 and (iv) animals irradiated at EMR-2450 MHz. The animals were exposed to EMR of 900, 1800 and 2450 MHz frequencies between 10 am to 2 pm for 1 h from D-1 (day-1) to D-28 (day-28) of experimental schedule. One hour after EMR exposure on D-1, 7, 14, 21 and 28 of experimental protocol all the animals were subjected to Y-maze paradigm. All the behavioral observations

were recorded and evaluated using ANY-mazeTM (version-3.72, USA) video tracking system. On D-28 day of EMR exposure immediately after behavioral experiment, the rat brains were dissected out immediately after decapitation. The hippocampal regions were quickly isolated using the coordinates from rat brain atlas (Paxinos and Watson 1986) over ice (4°C) for further molecular studies.

2.6 Assessment of behavioral performance

2.6.1 Evaluation of spatial recognition memory in Y-maze test:

In the Y-maze paradigm, general exploratory attitude (curiosity), spatial recognition memory and anxiety-like behavior were assessed as the total number of entries in all arms (15 min for trial 1 and 5 min for trial 2). The percentage of entries in known and novel arms for the 5 min period of trial 2 (for the general exploratory attitude), the percentage ratio of time spent in novel arm to time spent in all the arms (spatial recognition memory) and at the center of the apparatus during trial 2 were taken into count to assess the anxiety-like behavior (Krishnamurthy et al. 2013). The three identical arms (50 cm long, 16 cm wide and 32 cm high) of Y-maze at 120 angles to each other, radiating out from a central point were used to assess the above behavioral tasks. Visual cues were made from colored construction paper and laboratory glassware was placed around the perimeter of the maze and above the top of the black Plexiglas sides. These cues were not repeated for each test to maintain novelty to the animals. The floor of the maze was covered with animal bedding. The Y-maze novel arm was blocked and rats were allowed to visit the other two arms of the maze for 15 min. Four hours after the first phase, the novel arm was unblocked and animals had free access to all three arms for 5 min. The number of entries in each arm was recorded for a 5-min period. The dependent variables such as the total number of entries in all arms (for the trial 1 and 2), the percentage of entries in known and novel arms for the 5 min period of trial 2 and the percentage ratio of time spent in novel arm to time spent in all the arms and at the center of the apparatus during trial 2 were measured. The total number of entries in the trial 1 and 2 is a sign of general exploration attitude (curiosity) and the percentage of entries in known versus novel arms in trial 2 was appraised as a measure of arm discrimination (spatial recognition memory). Coping strategy or behavior to novel environment was assessed by the percentage of time spent in the novel arm to time spent in all arms and at the center of the apparatus during trial 2. An increase in anxiety-like behavior was confirmed by decrease in the coping behavior to novel environment (Poimenova et al. 2010). An arm entry was counted when the head and two front paws were inside the arm, and duration of an arm visit was ended when the head and two front paws were outside the arm again.

2.7 Assessment of mitochondrial function, integrity and oxidative stress

2.7.1 Isolation of mitochondria from rat brain: The mitochondria were isolated from hippocampus by following standard protocol (Pedersen *et al.* 1978). The mitochondrial protein content was estimated using the method of Lowry *et al.* (1951).

2.7.2 Estimation of mitochondrial function: The (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; MTT) reduction was used to assess the mitochondrial function by estimating formazan formed at 595 nm (Kamboj *et al.* 2008). Results were expressed as mg formazan formed/min/mg protein.

2.7.3 Evaluation of mitochondrial membrane potential (MMP): The MMP was assessed with the help of fluorescent cationic dye tetramethylrhodamine methyl ester (TMRM; Catalogue number Thermo fischer T668) to assess the mitochondrial integrity. The peak fluorescence intensity was recorded around 570 ± 5 nm. The results were expressed as fluorescence intensity value/mg protein (Hitachi, F-2500, Japan) (Huang 2002).

2.7.4 Estimation of NADH dehydrogenase (complex-I) activity: Activity of NADH dehydrogenase was evaluated at excitation and emission wavelength of 350 nm and 470 nm respectively (Shapiro *et al.* 1979). Results were expressed as nano-mole NADH oxidized/min/mg protein.

2.7.5 Assessment of succinate dehydrogenase (complex-II) activity: The activity of mitochondrial succinate dehydrogenase (SDH) was determined at 570 nm (Sally and Margaret 1989). Results were reported as micro-mole formazan produced/min/mg protein.

2.7.6 Estimation of cytochrome c oxidase (complex-IV) activity: The activity of complex-IV was assessed as per method (Storrie and Madden 1990). The decrease in absorbance was measured at 550 nm for 3 min. Results were expressed as nmole cytochrome c oxidized/min/mg protein ($\epsilon_{550} = 19.6 \text{ mmol}^{-1} \text{ cm}^{-1}$).

2.7.7 Estimation of F1F0 ATP synthase (complex-V) activity: Mitochondrial F1F0 synthase was measured according to the method of Griffiths and Houghton (1974) the inorganic phosphate concentration was measured by the method of Fiske and Subbarao (1925). Results were expressed as n mole ATP hydrolysed/min/mg protein.

2.7.8 Estimation of lipid peroxidation: Assessment of mitochondrial malonaldehyde (MDA) was measured (Sundermann *et al.* 1985). The absorbance of sample was

measured spectrophotometrically at 532 nm and results were expressed as $\mu\text{mol MDA/mg}$ of protein (Ohkawa *et al.* 1979).

2.7.9 Assessment of nitric oxide level: The NO level was estimated by following standard protocol (Green *et al.* 1982) using Griess reagent (0.1% at 540 nm) and results were expressed as nano-moles of NO/mg protein.

2.7.10 Assay of superoxide dismutase: Mitochondrial superoxide dismutase (SOD) was estimated by the reduction of NBT in the presence of NADH, Phenazine Methosulphate at 560 nm (Kakkar *et al.* 1984). One unit of the enzyme was represented as 50% inhibition of NBT reduction/minute/mg of protein of assay conditions. The absorbance of sample was measured at 560 nm using *n*-butanol as blank. The results were expressed as units [U] of SOD activity/mg of protein.

2.7.11 Assay of catalase activity: Decomposition of H_2O_2 in the presence of catalase was determined at 240 nm for 3 min at 30 s interval by the method of Beers and Sizer (1952). Results were expressed as units [U] of Catalase activity/mg of protein.

2.8 Evaluation of cholinergic system

2.8.1 Preparation of the samples and Estimation of acetylcholine using spectrofluorometer: The hippocampus tissue was homogenized and kept in the polypropylene tubes for 15 min after which 50 μL of 4 M potassium acetate was added to adjust the pH to 4.0 followed by centrifugation for 15 min at 4000 g (Muthuraju *et al.* 2009). The Ach level was measured by using amplex red kit (Molecular Probes Inc. USA) in a hippocampus as per the protocol (Zoukhri and Kublin 2001). The AChE activity was estimated using the Amplex red AChE assay kit (Molecular Probes Inc. USA) in terms of fluorescence with the help of spectrofluorometer at 530 nm excitation wavelength and 590 nm emission wavelength and the protein content was measured by Lowry method (Lowry *et al.* 1951).

2.9 Western blotting analysis

For Western blot analysis, the hippocampus was lysed in buffer containing complete protease inhibitor cocktail. Protein concentrations were determined according to Bradford's method (Bradford 1976). A standard plot was generated using bovine serum albumin. An aliquot of each sample were electrophoresed in 10% SDS-PAGE gels for A β , cytochrome-c, caspase-9 and active caspase-3 proteins, transferred to polyvinylidene fluoride membranes and

probed with specific antibodies. The membrane was incubated overnight with, rabbit anti-beta Amyloid (1:500, 87 kDa; ab62658; Abcam plc., India), rabbit anti-cytochrome-c (1:500, 15 kDa; ab13575; Abcam plc., India), rabbit anti- Caspase-9 (1:200, 45 kDa; ab47537; Abcam plc., India) and rabbit active anti-caspase-3 (1:1000, 32 kDa; ab90437; Abcam plc., India) polyclonal primary antibodies. After detection with the desired antibodies against the proteins of interest the membrane was stripped with stripping buffer (mercaptoethanol, Tris-Hcl and SDS) for 30 min at room temperature and re-probed overnight with rabbit anti β -actin (1:500, 42 kDa; ab93027; Abcam plc., India) polyclonal primary antibody to confirm equal loading of protein. Further, membrane was probed with corresponding secondary antibodies. Immunoreactive band of proteins were detected by chemiluminescence using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, USA). Quantification of the results was performed by densitometric scan of films. The immunoreactive area was determined by densitometric analysis using Biovis gel documentation software.

2.10 Statistical analysis

All values were expressed as mean $\pm$ standard error of mean (S.E.M.). Two-way ANOVA followed by Bonferroni *post hoc* test was performed to estimate total arm entries in trials 1 and 2 and percentage entries into known and novel arm in trial-2 in Y-maze test. Arm discrimination behavior between known and novel arm was assessed by using Two-way ANOVA with Bonferroni test in Y-maze paradigm. All other statistical analysis of data was performed by using One-way ANOVA with Newman-Keuls *post hoc* test. $P < 0.05$ was considered as significant.

3. Results

3.1 EMR attenuated the spatial recognition memory in Y-maze paradigm

Figure 2 depicts the effect of EMR (900, 1800 and 2450 MHz) induced alterations in the exploratory behavior (curiosity) in trial 1 and 2 depicted in figure 2A and B respectively. Figure 2C shows the coping behavior to novel arm (anxiety-like behavior) in Y-maze test. Repeated measure two-way ANOVA revealed significant differences for curiosity in trial-1 [$F(3,100)=45.60$; $P < 0.0001$] among groups, time [$F(4,100)=11.11$; $P < 0.0001$] and a significant interaction between groups and time [$F(12,100)=9.982$; $P < 0.0001$]. Similarly, there was significant differences for curiosity in trial-2 [$F(3,100)=36.65$; $P < 0.0001$] among groups, time [$F(4,100)=13.87$; $P < 0.0001$] and a significant

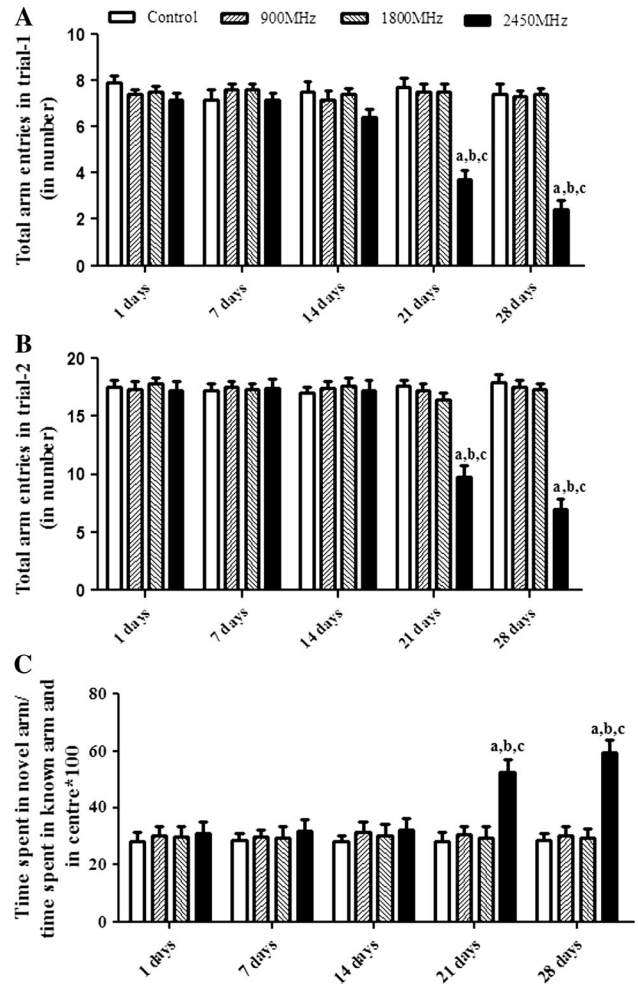


Figure 2. The effect of EMR (900, 1800, and 2450 MHz) exposure ensuing alterations in the total arm entries in trials 1 and 2 (curiosity; **A**), total novel arm entries (spatial recognition memory; **B**), and coping behavior to novel arm (anxiety-like behavior; **C**) in Y-maze paradigm. All results are expressed as mean $\pm$ SEM, (n=6). ^a $P < 0.05$ compared to control, ^b $P < 0.05$ compared to EMR-900 and ^c $P < 0.05$ compared to EMR-1800. Repeated measure two-way ANOVA followed by Bonferroni test for curiosity analysis and percentage entries into known and novel arm.

interaction between groups and time [$F(12,100)=13.73$; $P < 0.0001$] respectively. Furthermore, there were significant differences in coping behavior among groups [$F(3,100)=14.94$; $P < 0.0001$], time [$F(4,100)=3.728$; $P < 0.0072$] and an interaction between group and time [$F(12,100)=3.917$; $P < 0.0001$]. Post hoc analysis using Bonferroni test revealed that the effect of EMR 900 and 1800 MHz did not change the curiosity both in trial-1 and 2 and, in the coping behavior ($P > 0.05$). Whereas EMR (2450 MHz) significantly decreased the curiosity and increased coping behavior on D-21 and this effect was observed up to D-28 compared to vehicle administered rats.

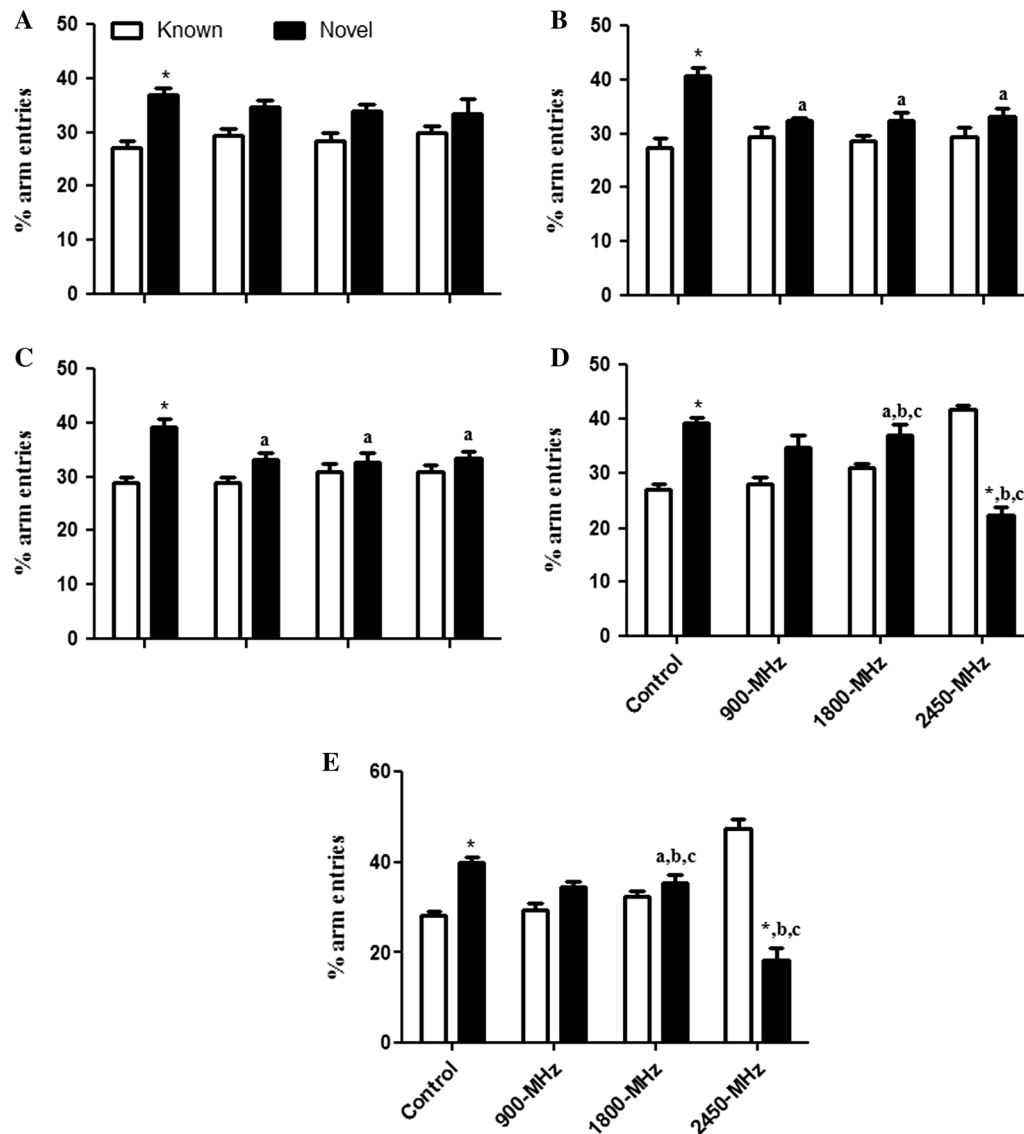


Figure 3. The effect of EMR (900, 1800, and 2450 MHz) exposure ensuing alterations in arm discrimination behavior during Y-maze test paradigm on D-1 (A), D-7 (B), D-14 (C), D-21 (D) and D-28 (E) of the experimental schedule. All values are expressed as mean±SEM, (n=6). *P<0.05 compared to corresponding known arm entries (two-way ANOVA followed by Bonferroni test). ^aP<0.05 compared to control, ^bP<0.05 compared to EMR-900 and ^cP<0.05 compared to EMR-1800 (repeated measure two-way ANOVA followed by Bonferroni test).

EMR (900, 1800 and 2450 MHz)-induced alterations in the spatial recognition memory on D-1 (A), D-7 (B), D-14 (C), D-21 (D) and D-28 (E) are depicted in figure 3. Two way ANOVA showed significant differences in arm discrimination behavior during trial-2 among groups on D-1 [F(3,40)=1.497; P<0.2299], D-7 [F(3,40)=6.025; P<0.0017], D-14 [F(3,40)=4.486; P<0.0083], D-21 [F(3,40)=19.39; P<0.0001] and D-28 [F(3,40)=59; P<0.0001 respectively]. Significant effect for known and novel arms entries on D-1 [F(1, 40)=31.68; P<0.0001], D-7 [F(1,

40)=34.92; P<0.0001], D-14 [F(1, 40)=25.53; P<0.0001], D-21 [F(1, 40)=13.53; P<0.0007] and D-28 [F(1,40)=3.944; P<0.0439 respectively]. There was significant interaction between group and total arm entries on D-1 [F(3,40)=0.1068; P<0.9546], D-7 [F(3,40)=2.589; P<0.0663], D-14 [F(3,40)=1.830; P<0.1571], D-21 [F(3,40)=8.396; P<0.0002] and D-28 [F(3,40)=0.7988; P<0.05019 respectively] in Y-maze paradigms. Post hoc analysis revealed that EMR-2450 MHz exposed experimental animals showed cognitive impairments in terms of

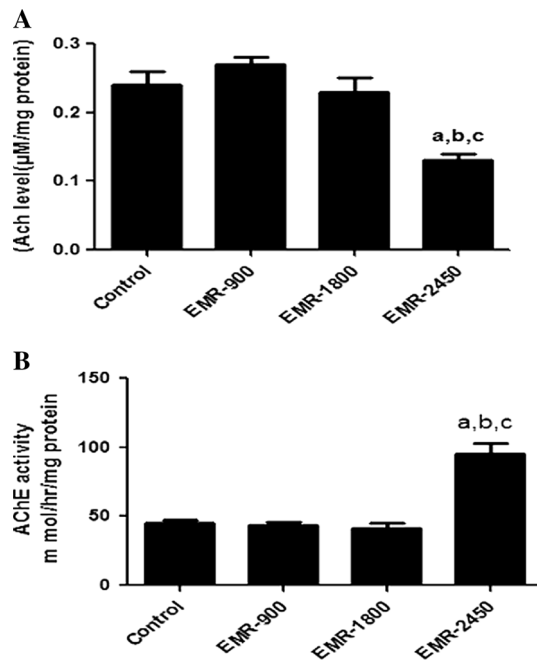


Figure 4. Effect of EMR (900, 1800 and 2450 MHz) induced changes in the level of Ach (A) and activity of AChE (B) in HIP. All values are Mean±SEM. ^aP<0.05 compared to control, ^bP<0.05 compared to EMR-900 and ^cP<0.05 compared to EMR-1800 (one-way ANOVA followed by Student–Newman–Keuls test).

decrease in novel arm entries on D-21 and this effect was persisted up to D-28. However, EMR (900, 1800 MHz) did not show significant changes in novel arm entries on all days of experimental protocol compared to control rodents.

3.2 Estimation of Ach and AChE levels in EMR-exposed rats

Figure 4 illustrates the effect of EMR (900, 1800 and 2450 MHz)-induced changes in concentration of Ach and AChE activity in hippocampus. One-way ANOVA revealed that there were significant differences in (A) Ach [F (3, 20)=14.8; P<0.0001] and (B) AChE [F (3, 20)=34.6; P<0.0001] among groups. *Post hoc* analysis revealed that EMR (2450 MHz) significantly decreased levels of Ach and increased the AChE levels compared to control rats respectively.

3.3 Quantization of expression of β-Amyloid in hippocampus of EMR-subjected rats

The effect of EMR (900, 1800 and 2450 MHz)-induced changes in the level of β Amyloid in brain tissues of rodents is depicted in figure 5. There were significant differences in β Amyloid [F (3, 8)=9.3; P<0.0050] among groups. *Post*

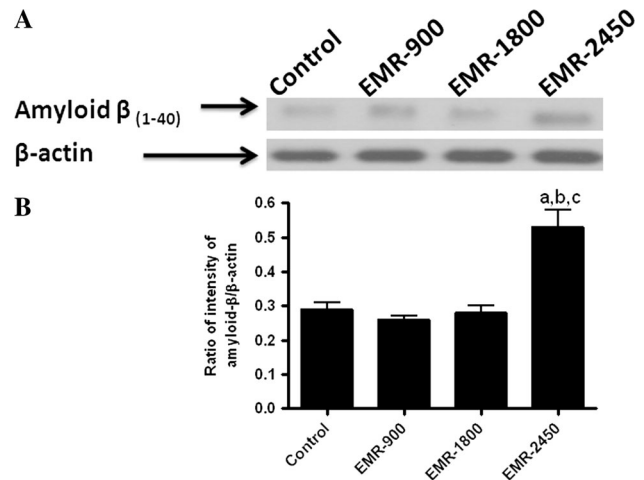


Figure 5. The effect of EMR (900, 1800 and 2450 MHz) exposure induced changes in the level of expression of Aβ₁₋₄₀ in hippocampus. The blots are representative of Aβ₁₋₄₀ (A) in HIP. The results in the histogram are expressed as ratio of relative intensity of levels of protein expression of Aβ₁₋₄₀ to β-Actin (B). All values are Mean±SEM. ^ap<0.05 compared to Hippocampus of respective group, ^bp<0.05 compared to control, ^cp<0.05 compared to EMR-900 and ^dp<0.05 compared to EMR-1800 (one-way ANOVA followed by Student–Newman–Keuls test).

hoc analysis demonstrated that EMR at the frequency of 2450 MHz increased the expression of β Amyloid compared to control rats.

3.4 EMR mitigated the mitochondrial integrity in hippocampus of animals

The effect of discrete range of EMR induced alterations in MMP in EMR subjected rats is depicted in figure 6. One way ANOVA revealed that there were significant differences in MMP [F (3, 20)=36.91; P<0.0001] among groups. *Post hoc* analysis revealed that EMR (2450 MHz) significantly decreased the intensity of TMRM indicating loss of mitochondrial integrity.

3.5 EMR showed the increase in expression of cytoplasmic cytochrome-c, caspase-9 and caspase-3 in hippocampus

Figure 7 depicts the effect of EMR (900, 1800 and 2450 MHz)-induced alterations in the levels of (A) cytochrome-C, (B) caspase-9 and (C) caspase-3 in the hippocampus. There were significant differences in expression of cytochrome-C [F (3, 8)=22.4; P<0.0003], caspase-9 [F (3, 8)=15.8; P<0.0010] and caspase-3 [F (3, 8)=7.9; P<0.0090] among groups. *Post hoc* test revealed that EMR (2450 MHz)

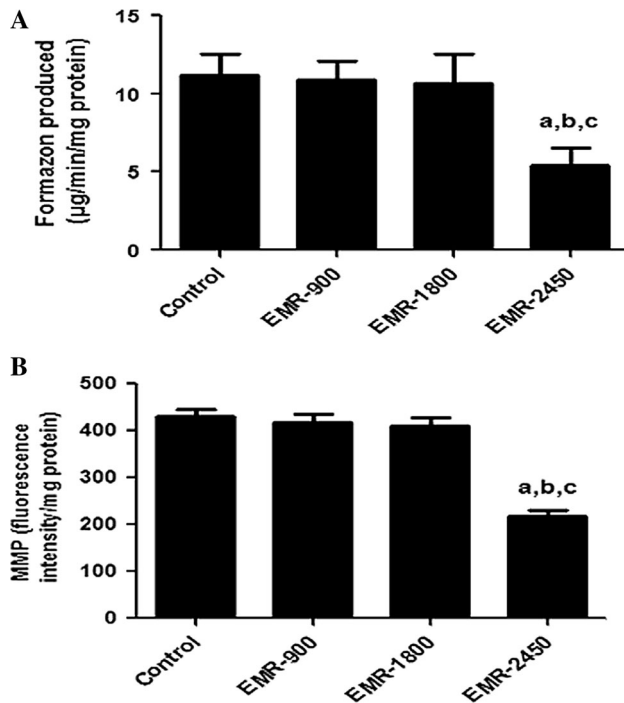


Figure 6. Effect of EMR (900, 1800 and 2450 MHz) exposure on alterations in the mitochondrial function (A) and integrity (B) in hippocampus. All values are Mean $\pm$ SEM. ^aP<0.05 compared to control, ^bP<0.05 compared to EMR-900 and ^cP<0.05 compared to EMR-1800 (one-way ANOVA followed by Student–Newman–Keuls test).

showed significant increase in the expression of cytochrome-C, caspase-9 and caspase-3 compared to control rodents.

3.6 Evaluation of EMR-exposed modulations in mitochondrial enzyme activities

The effect of EMR (900, 1800 and 2450 MHz)-induced changes in the activities of mitochondrial complex-I, II, IV and V in hippocampus is depicted in table 1. One way ANOVA statistical analysis revealed that there were significant differences in mitochondrial complex-I, II, IV, V enzyme activities among groups [F (3, 20)=12.25, P<0.0023], [F (3, 20)=1.521; P<0.02821], [F (3, 20)=4.528; P<0.0389] and [F (3, 20)=4.911; P<0.0223] respectively. Post hoc test illustrated that EMR (2450 MHz) showed the significantly decreased complex-I, II, IV and V activities in hippocampus compared to control animals.

3.7 Assessment of the mitochondrial oxidative and nitrosative stress markers in EMR-induced animals

Table 2 shows the effect of EMR (900, 1800 and 2450 MHz)-induced alterations in the levels of (A) LPO, (B) NO, (C) SOD and (D) Catalase in the brain tissues. There were significant differences in LPO [F (3, 20)=17.74; P<0.0007], NO [F (3, 20)=3.341; P<0.013], SOD [F (3,

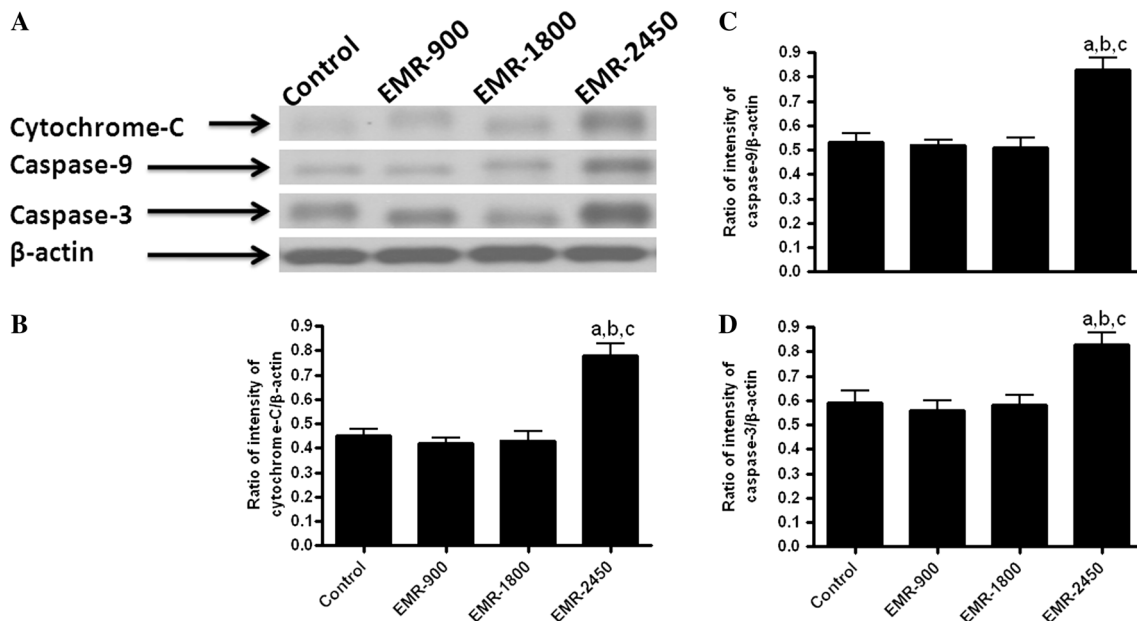


Figure 7. The effect of EMR (900, 1800 and 2450 MHz) exposure on modifications in the level of expression of cytochrome-c, caspase-3 and -9 in the hippocampus. The blots are representative of cytochrome-c, caspase-3 and -9 expressions (A). The results in the histogram are expressed as ratio of relative intensity of levels of protein expression of cytochrome-c, caspase-3 and -9 in the hippocampus to that of β-actin (B). All values are Mean $\pm$ SEM. ^aP<0.05 compared to control, ^bP<0.05 compared to EMR-900 and ^cP<0.05 compared to EMR-1800 (one-way ANOVA followed by Student–Newman–Keuls test).

Table 1. The effect of EMR (900, 1800 and 2450 MHz) exposure on changes in the level of mitochondrial enzyme activities in the hippocampus

S. No.	Groups	Complex-I activity (nmolNADH oxidized/min/mg/protein)	Complex-II activity (μ molformazan/ min/mg/protein)	Complex-IV activity (nmol cytochrome c oxidized/min/mg/protein)	Complex-V activity ATP hydrolysed protein
1	Control	5.36 $\pm$.27	0.49 $\pm$.11	0.81 $\pm$.08	12.41 $\pm$.52
2	EMR900	5.21 $\pm$.42	0.46 $\pm$.07	0.76 $\pm$.05	12.19 $\pm$.57
3	EMR1800	5.14 $\pm$.19	0.42 $\pm$.09	0.72 $\pm$.08	12.05 $\pm$.47
4	EMR2450	3.10 $\pm$.30 ^{a,b,c}	0.26 $\pm$.05 ^{a,b,c}	0.47 $\pm$.07 ^{a,b,c}	8.86 $\pm$ 1.09 ^{a,b,c}

Values are Mean $\pm$ SEM.

^a p<0.05 compared to control, ^bp<0.05 compared to EMR-900 group and ^cp<0.05 compared to EMR-1800 group (one-way ANOVA followed by Student–Newman–Keuls test).

Table 2. The effect of EMR (900, 1800 and 2450 MHz) exposure on changes in the level of mitochondrial oxidative and nitrosative stress markers in hippocampus

S. No.	Groups	LPO level (μ M MDA/mg protein)	Brain nitrite level (nM NO/mg protein)	Catalase level (nm at H ₂ O ₂ /min/mg of protein)	SOD activity (units/min/mg of protein)
1	Control	0.78 $\pm$.15	1.24 $\pm$.85	0.42 $\pm$.10	0.61 $\pm$.09
2	EMR-900	0.75 $\pm$.17	1.18 $\pm$.71	0.38 $\pm$.13	0.54 $\pm$.11
3	EMR1800	0.71 $\pm$.12	1.14 $\pm$.77	0.36 $\pm$.11	0.51 $\pm$.07
4	EMR2450	2.09 $\pm$.19 ^{a,b,c}	1.85 $\pm$.66 ^{a,b,c}	0.15 $\pm$.21 ^{a,b,c}	0.25 $\pm$ 0.05 ^{a,b,c}

Values are Mean $\pm$ SEM.

^a p<0.05 compared to control, ^bp<0.05 compared to EMR-900 group and ^cp<0.05 compared to EMR-1800 group (one-way ANOVA followed by Student–Newman–Keuls test).

20)=3.588; P<0.044] and catalase [F (3, 20)=4.085; P<0.0252] among groups. Post hoc test revealed that the concentration of MDA and NO significantly increased by highest frequency of EMR compared to control animals. Furthermore, EMR (2450 MHz) modulated the activities of SOD and catalase in hippocampus of rodents.

4. Discussion

In the present study, experimental animals exposed to EMR at frequency of 2450 MHz exhibited cognitive dysfunction. For the first time we report that the cognitive deficit was associated with hippocampal mitochondrial dysfunction and amyloidogenesis. In contrast, EMR-900 and EMR-1800 did not show any effect on cognitive function.

EMR-2450 exposure significantly induced cognitive deficit in rats. Y-maze paradigm is commonly used for the assessment of cognitive impairments (McEwen *et al.* 1997). Y-Maze accentuates spatial recognition memory, visuospatial tasks in addition to hippocampus dependent tasks (Cai *et al.* 2013). In the present study, we found that EMR-2450-subjected rats showed abnormalities in number of behavioral indices in the Y-maze paradigm like spatial recognition memory (increase in percentage of entries into known arm)

and coping behavior (anxiety-like behavior) to novel environment (decrease in time spent in novel arm to time spent in all arms and in the center) on D-21 and D-28. However, EMR-900 and EMR-1800 exposed rats did not show significant differences in novel arm entries on D-21 and D-28 indicating the fact that these frequencies did not induce any significant cognitive deficits. Earlier study (Choi and Choi 2016) reported that long term (10 weeks) exposure to electromagnetic radiation (smartphones) may induce delayed hyperactivity like behavior without affecting spatial working memory through Y-maze in brain. This task allows the simultaneous assessment of hyperactivity independent of spatial memory and it exploits the natural inclination of rats to investigate their environment. The differences in observations may be due to the use of continuous frequency exposure whereas the other study had pulsed exposure. Previous studies have reported biological differences with pulsed and continuous EMR exposure. Pulsed microwaves alter not only the EEG but also regional cerebral blood flow, and continuous exposure of EMR causes alterations in brain physiology (Huber *et al.* 2005).

In consonant with our results, a report (Dubreuil *et al.* 2003) suggested that continuous GSM (900/1800 MHz) electromagnetic radiations do not alter memory of rat in spatial and non-spatial tasks. In contrast, other studies have

reported learning and memory deficit in rats with the Morris water maze (MWM) using pulsed 2450 MHz (Wang and Lai 2000). The MWM is a memory test, frequently used for demonstrating visuospatial navigation, topographic disorientations and motivational deficits and to examine the facilitations of content dependent behavior and reference memory in rodents (Vorhees and Williams 2006). However, in our study we have shown cognitive deficits using the Y-Maze test. The Y-maze task is dependent on the clinically relevant factors such as the speed of information processing and psychomotor ability, executive functions, learning, memory and general cognitive treatments (Conrad *et al.* 1997).

Earlier, a study has reported that exposure of EMR 900 MHz for 2 h daily over 28 days in rats with SAR average in between 0.52–1.08 W/kg led to reduction of synapses and decreased the postsynaptic density of neuron in the hippocampus CA1 region, resulting spatial learning and reference memory deficit in rats (Li *et al.* 2012). Similarly, it has been shown that exposure of microwave radiation for 6 min (3 times in a week up to 6 weeks) causes shrinkage and loss of dendritic spines in hippocampus lead to cognitive impairments (Zhi *et al.* 2017). However, in our study we did not observe cognitive deficit with EMR 900 MHz. The reason may be that the experimental design and duration of exposure was different. Moreover, it has been shown that loss of mitochondrial function could cause hippocampal synaptic dysfunction that leads to memory deficits (Santini and Turner 2015). Mitochondria are considered as the principal site of the oxidative and nitrosative stress (Borutaite *et al.* 2013). Oxidative stress in addition to alteration in functional changes like reducing complex activities and also facilitates deposition of A β in mitochondria (Diana *et al.* 2008; Zuo *et al.* 2015). In present study EMR-2450 significantly increased the level of LPO and NO resulting in the decrease in the level of antioxidant property causing damage to hippocampus. In contrast, EMR-900 and 1800 MHz did not show any changes in the level of LPO and NO. A previous report suggests that long term exposure of EMR-2100 MHz increases LPO and NO production and decreases the level of catalase and SOD (Hidisoglu *et al.* 2016). In the present study, decrease in level of catalase and SOD activities in the 2450-MHz group indicated excessive level of hydrogen peroxide and less decomposition of superoxide radicals in the hippocampus. However, EMR-900 and 1800 MHz did not show any significant changes in oxidative stress markers. Therefore, EMR-2450 MHz induces oxidative stress in contrast to EMR-900 and 1800 MHz.

Mitochondrial membrane potential (MMP) is a key factor for bioenergetics as it regulates the energy needs of cell (Ott *et al.* 2007). MMP was significantly decreased with long term exposure of EMR-2450 MHz while no significant changes were observed with exposure of EMR-900 and 1800 MHz. It is interesting to note that marked decrease

of fluorescence intensity was observed reflecting compromised mitochondrial membrane integrity. However, *in vitro* study has revealed that EMR-1800 MHz causes neurotoxicity due to mitochondrial DNA toxicity in neuron cultures (Xu *et al.* 2010). This may be due to the compensatory mechanisms stabilizing the mitochondria *in vivo* and also may be due to difference in intensity and duration of EMR exposure. Earlier study reported that exposure of microwave radiation ranging from 300 MHz to 300 GHz causes alteration of brain energy metabolism due to oxidative phosphorylation (Hao *et al.* 2015). Hence, exposure of EMR-2450 MHz causes alteration of MMP and interferes with the complexes-I, II, IV and V activities that predisposes to loss of mitochondrial integrity leading to mitochondrial dysfunction.

Aggregation of A β is observed in AD along with cognitive deficits (Pozueta *et al.* 2013). The cellular concentration of A β is kept within a precise range by balancing its synthesis and degradation (Pinho *et al.* 2014). It has also been suggested that A β is found in the several compartments of the cell including mitochondria (Villemagne *et al.* 2013). In the current study, EMR-2450 MHz shows increase in expression of A β in contrast to the effect of EMR-900 and 1800 MHz in the hippocampus. It has been reported that EMR 900 MHz exposure for ten months induced over expression of A β in rats (Suleyman *et al.* 2012). In summary, 28 day's exposure of EMR-2450 MHz induced expression of hippocampus A β , which may be one of the reasons for EMR-induced cognitive deficits in rats.

A β accumulation in the mitochondria can lead to irregularities in the secretion of neurotransmitters such as acetylcholine. This may ultimately cause synaptic damage followed by neurodegeneration and cognitive deficits (Grill and Cummings 2010). The cholinergic system plays a critical role in performing cognitive tasks (Himmelheber *et al.* 2000). The activity of AChE regulates sustained extracellular ACh release which plays a crucial role in restoring cognitive function (Chen *et al.* 2016). Our results showed that ACh and the activity of AChE were not altered by EMR-900 and EMR-1800 animals. However, EMR-2450 significantly decreased the level of ACh with increase in the enzymatic activity of AChE in the hippocampus. It has been reported that EMR 2450 MHz exposure leads to neurodegeneration of cholinergic neurons followed by decrease in level of ACh and increase in the activity of AChE with (Lai *et al.* 1987). On the basis of neurochemical changes, it can be presumed that EMR-2450 causes alteration in the cholinergic neurotransmission which can lead to cognitive deficits.

The mitochondrial pathway of apoptosis regulated by mitochondrial integrity may lead to mitochondrial swelling and opening of mitochondrial transition pore (Webster 2012). Apoptosis is an organized, energy-dependent process in which mitochondria plays a pivotal role as regulators of

cell death (Samaiya *et al.* 2016). In the current study, EMR-2450 exposure led to increase in expression of cytochrome-c, indicating opening of mitochondrial transition pore due to loss of mitochondrial integrity. Cytochrome-c activated the proapoptotic factors caspase-9 and caspase-3. However, EMR-900 and EMR-1800 exposed rats did not show significant differences in mitochondrial dysfunction and expression of apoptotic markers. Exposure to mobile phone radiation has been reported to up-regulate apoptotic genes in primary cultures of neurons and astrocytes (Zhao *et al.* 2007). Therefore, exposure to EMR-2450 caused mitochondrial dysfunctions leading to leakage of cytochrome-c from the mitochondria and activation of intrinsic pathway of apoptosis. Apoptosis has been reported in cognitive impairment (Man *et al.* 2015) and therefore mitochondrial-linked apoptosis may be one of the major factors involved in EMR induced cognitive changes.

In summary, EMR at 2450 MHz induced cognitive behavioral deficit with concomitant loss in mitochondrial function. Alteration in the activity of mitochondrial complex enzyme systems caused oxidative stress and decrease in MMP, which ultimately lead to loss of mitochondrial integrity. Further, mitochondrial stress as observed from increases in cytochrome-c activated the expression of caspase-9 and caspase-3, indicating mitochondrial-linked apoptosis. Furthermore, exposure with EMR-2450 increased expression of hippocampal A β and decreased cholinergic neurotransmission in the hippocampus, which are considered to be important factors for development of cognitive dysfunction.

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