2.3.1 Introduction

The use of the new generation mobile phones has been reported to symptom like headache, sleep anomalies (Hossmann et al., 2003) and cognitive impairment (Levin ED, 2015).

Earlier studies have reported that long term exposure to EMR altered the cognitive behavior in rats, that lead 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). However, till date there is no report on the comparative consequences of EMR (900, 1800 and 2450 MHz) exposure on hippocampus. Therefore, present study designed to compare 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 for short time period (15-120min for 7 hours) causes decrease in level of acetylcholine (Ach) with increase enzymatic activity of acetyl cholinesterase (AchE) in hippocampus that could lead to cognitive disorders or memory dysfunction in rats (Afrasiabi et al., 2014; Giacobini et al., 2002; Mesulam et al., 1987; Wang et al., 2000).

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 (Aβ) is localized to mitochondrial membranes (Glenner et al., 1984; Villemange et al., 2013). Oxidative stress and accumulation of Aβ1–40 are implicated in the pathogenesis of AD (Chen et al., 2010; Fu et al., 2016; Zhang et al., 2016). In particular, the Aβ1–40 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 et al., 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 (Janicke 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 on mitochondrial function and apoptosis would provide valuable information to understand the pathophysiological mechanisms of cognitive dysfunction with EMR exposure.

Therefore, the present objective evaluates 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 Aβ1–40 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 (in figure 2.3.1).

2.3.1.1 Hypothesis

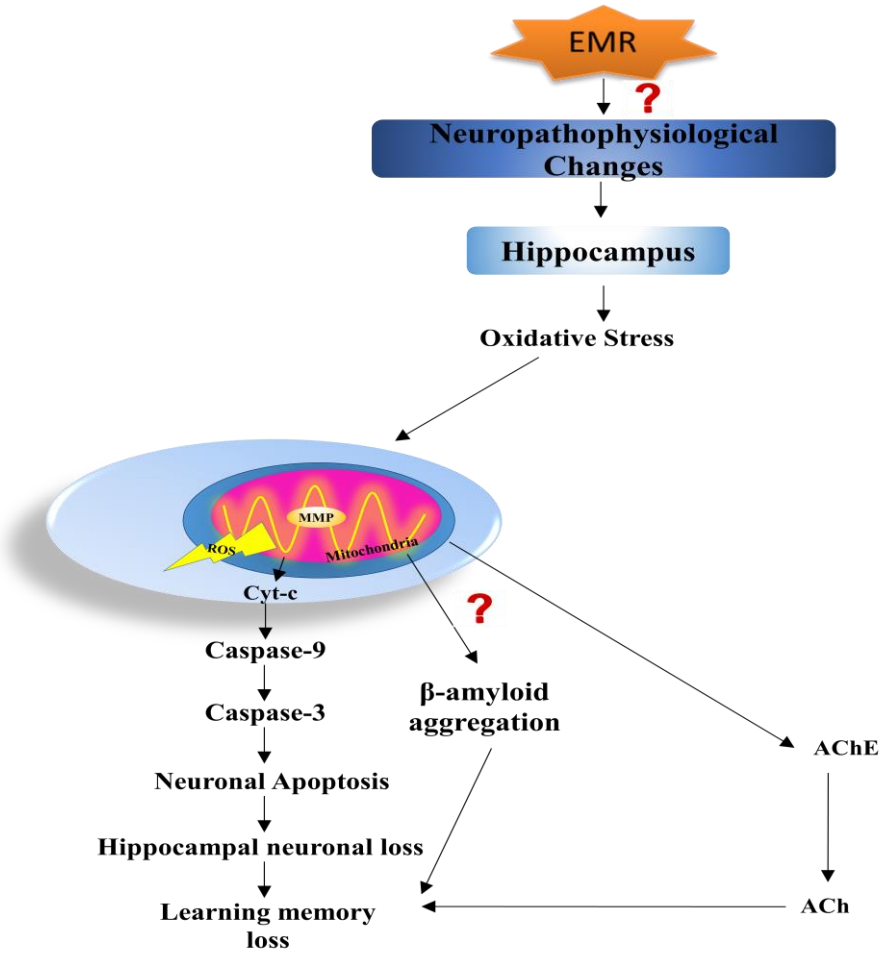


Figure 2.3.1 Proposed hypothesis of Effect EMR on cognitive function

2.3.2 Materials and Methods

2.3.2.1 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 ± 2 °C, relative humidity 44–56% and light:dark cycle of 12:12 hr. 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 guidelines; Approval No.: Dean/2015/CAEC/1414).

2.3.2.2 Materials

Acetylcholine esterase (AchE), Choline oxidase, horse radish peroxidase, Nitro Blue Tetrazolium (NBT), Tetramethyl Rhodamine Methyl Ester (TMRM), and Griess reagent were procured from sigma aldrich (St. Louis, MO, USA). 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.

2.3.2.3 Electromagnetic Radiation Exposure System and Design (Refer chapter 2.1 Page no. 20)

2.3.2.4 Calculation of Power density and specific absorption rate (SAR) (Refer chapter 2.1 Page no. 21)

2.3.2.5 Experimental design

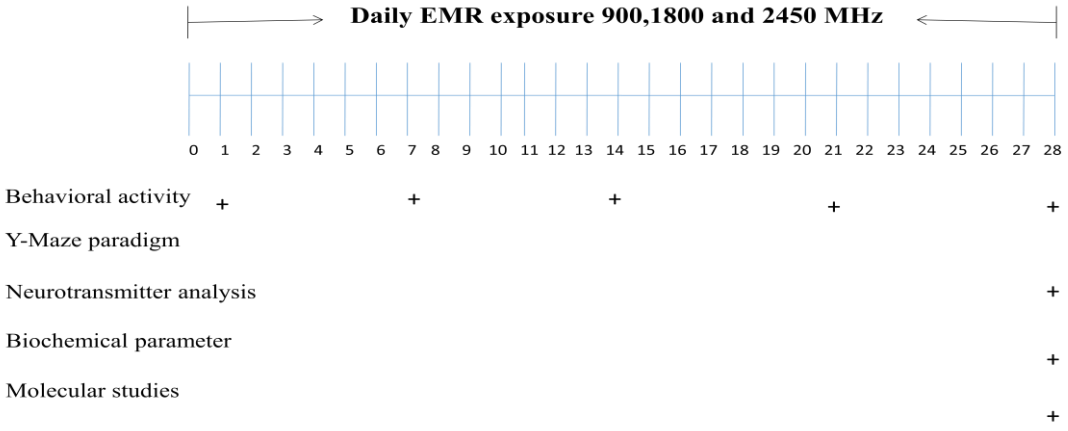


Figure 2.3.2 Schematic representation of the experimental design. '+' denotes experiment performed

Rats were randomly divided into four groups with six animals through G* power analysis software 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 1h from D-1 (day-1) to D-28 (day-28) of experimental schedule illustrates in figure 2.3.2. One hr after EMR exposure on D-1, 7, 14, 21 and 28 of experimental protocol all the animals were subjected to Y-maze paradigm and behavioral observations were recorded and evaluated using ANY- maze^{TM} (version-3.72, USA) video tracking system. Thereafter, animals were killed by cervical dislocation and hippocampus was isolated from brain using the coordinates from rat brain atlas (Paxinos et al., 1986). Hippocampus was identified by dye (Methylene Blue) which was injected into the lateral ventricle of the brain by stereotaxic technique. The rats were stunned and killed by decapitation at various times after the intraventricular injection of

the ink substances, the brain were carefully removed, blotted and chilled. Dissections were performed on an ice-cooled glass plate. The 'hippocampus' was gently separated from the remaining part of the brain.

2.3.2.6 Behavioral parameter assessment

2.3.2.6.1 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 plexiglass 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.

2.3.2.7 Assessment of mitochondrial function, integrity and oxidative stress

2.3.2.7.1 Isolation of mitochondria from rat brain

After the cervical dislocation, the rats were decapitated and brain was dissected out. Mitochondria were isolated from hippocampus brain region by differential centrifugation at 4° C (Pedersen et al., 1978). The Hippocampus was dissected and rinsed in ice-cold saline (isotonic) followed by homogenization in (1:10, w/v) ice-cold extraction buffer (250 mM sucrose, 1 mM EGTA, and 10 mM HEPES–KOH, pH 7.2). After the removal of cell debris by centrifugation at 4000g for 5 min, the supernatant obtained above was again centrifuged at 10000xg for 15 min followed by removal of pellets and discarding the supernatant. Pellets were placed in a medium (1 ml) containing 250 mM sucrose, 0.3 mM EGTA, and 10 mM HEPES–KOH, pH 7.2. The resulting solution thus obtained was centrifuged at 14000xg for 10 min. The pellets obtained were then suspended in a medium (1 ml) containing 250 mM sucrose and 10 mM HEPES–KOH, pH 7.2. The mitochondrial protein content was estimated using the method of (Lowry et al., 1951).

2.3.2.7.2 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.

2.3.2.7.3 Evaluation of mitochondrial membrane potential (MMP)

The MMP was assessed with the help of fluorescent cationic dye TMRM 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 et al., 2002).

2.3.2.7.4 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 nmole NADH oxidized/min/mg protein.

2.3.2.7.5 Assessment of succinate dehydrogenase (Complex-II) activity

The activity of mitochondrial succinate dehydrogenase (SDH) was determined at 570 nm (Sally et al., 1989). Results were reported as micro mole formazan produced/min/mg protein.

2.3.2.7.6 Estimation of cytochrome c oxidase (Complex-IV) activity

The activity of complex-IV was assessed as per method (Storrie et al., 1990). The decrease in absorbance was measured at 550 nm for 3 min. Results were expressed as nmole cytochrome c oxidized/min/mg protein (e550 = 19.6 $mmol^{-1}$ cm-1).

2.3.2.7.7 Estimation of F1F0 ATP synthase (Complex-V) activity

Mitochondrial F1F0 synthase was measured according to the method (Griffiths et al., 1974) the inorganic phosphate concentration was measured by the method (Fiske et al., 1925). Results were expressed as n mole ATP hydrolyzed/min/mg protein.

2.3.2.7.8 Estimation of lipid peroxidation (LPO)

Assessment of mitochondrial malonaldehyde (MDA) was measured (Sunderman et al., 1985). The absorbance of sample was measured spectrophotometrically at 532 nm and results were expressed as µmol MDA/mg of protein.

2.3.2.7.9 Assessment of nitric oxide (NO) level

The NO level was estimated by (Green et al., 1982) using Griess reagent (0.1% at 540 nm) and results were expressed as nmoles of NO/mg protein.

2.3.2.7.10 Assay of superoxide dismutase (SOD) (Refer Chapter 2.1 Page no. 26)

2.3.2.7.11 Assay of Catalase activity (Refer Chapter 2.1 Page no. 26)

2.3.2.7.12 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 and 50 µl of 4M potassium acetate was added to adjust the pH to 4.0 followed by centrifugation for 15 min at 4000g (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 et al., 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)

2.3.2.8 Western blotting analysis (Refer Chapter 2.1 Page no. 26, 27)

2.3.2.9 Statistical analysis

All values were expressed as mean±standard error of mean (SEM). 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. The level of statistical significance is often expressed as a p-value between 0 and 1. In this study $p <$

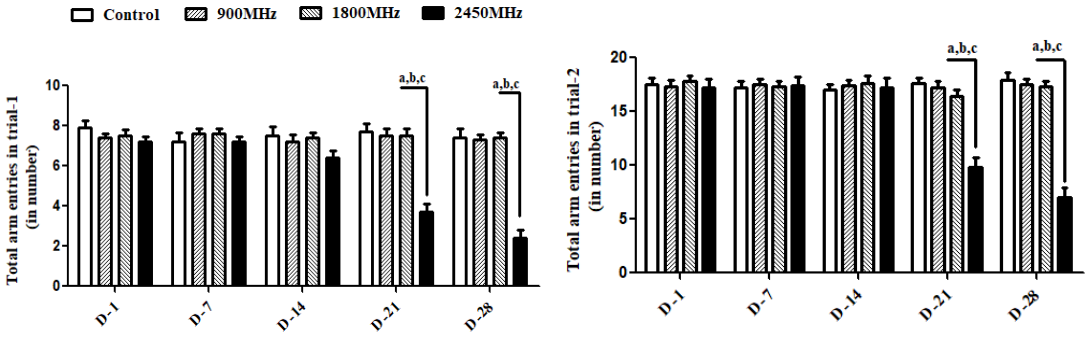
0.05 were considered as statistically significant for all experimental data analysis. The lower the p-value, the greater the chances for rejection of the null hypothesis. Therefore, in our research hypothesis, there is less than a 5% probability of the null hypothesis to be correct. So there are 95% chances that our research hypothesis is true.

Results

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

Fig-2.3.4 depicts the effect of EMR (900, 1800 and 2450 MHz) induced alterations in the exploratory behavior (curiosity) in trial 1 and 2 depicted in fig-2.3.4 (A) and (B) respectively. Fig-2.3.4 (C) 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 [$F(3,100) = 45.60$; $P<0.05$] among groups, time [$F(4,100) = 11.11$; $P<0.05$] and a significant interaction between groups and time [$F(12,100) = 9.982$; $P<0.05$]. Similarly, there was significant differences for curiosity in trial-2 [$F(3,100) = 36.65$; $P<0.05$] among groups, time [$F(4,100) = 13.87$; $P<0.05$] and a significant interaction between groups and time [$F(12,100) = 13.73$; $P<0.05$] respectively. Furthermore, there were significant differences in coping behavior among groups [$F(3,100) = 14.94$; $P < 0.05$], time [$F(4,100) = 3.728$; $P < 0.05$] and an interaction between group and time [$F(12,100) = 3.917$; $P < 0.05$]. Post hoc test revealed that variable stress paradigms on D-1, 7 and 14 did not induce changes in the curiosity both in trial-1 and 2 and in the coping behavior. However, 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 2.3.4 (A) **(B)**



(C)

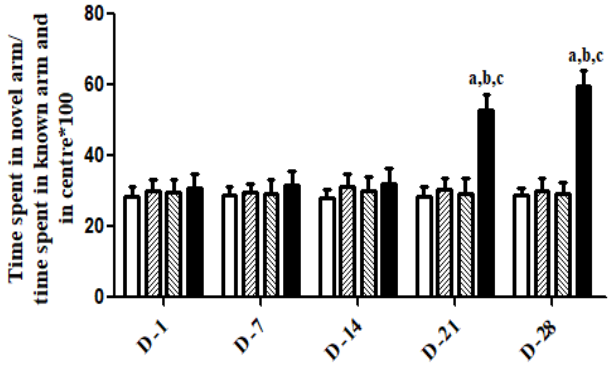


Figure 2.3.4 Effect of EMR (900, 1800, and 2450 MHz) exposure ensuing alterations in the total arm entries in trials 1 and 2 (curiosity; A), total novel arm entries (spatial recognition memory) (B), and coping behavior to novel arm (anxiety-like behavior; C) in Y-maze paradigm. All results are expressed as mean ± 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.

2.3.3.2 EMR attenuated the arm discrimination behaviour in Y-maze paradigm

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 fig-2.3.5. Two way ANOVA showed significant differences in arm discrimination behavior during

trial-2 among groups on D-1 [F(3,40) = 1.497; P<0.05], D-7 [F(3,40) = 6.025; P<0.05], D-14 [F(3,40) = 4.486; P<0.05], D-21 [F(3,40) = 19.39; P<0.05] and D-28 [F(3,40) = 59; P<0.05 respectively]. Significant effect for known and novel arms entries on D-1 [F (1, 40) = 31.68; P<0.05], D-7 [F (1, 40) = 34.92; P<0.05], D-14 [F (1, 40) = 25.53; P<0.05], D-21 [F (1, 40) = 13.53; P<0.05] and D-28 [F(1,40) = 3.944; P<0.05 respectively]. There was significant interaction between group and total arm entries on D-1 [F(3,40) = 0.1068; P<0.05], D-7 [F(3,40) = 2.589; P<0.05], D-14 [F(3,40) = 1.830; P<0.05], D-21 [F(3,40) = 8.396; P<0.05] and D-28 [F(3,40) = 0.7988; P<0.05 respectively] in Y-maze paradigms. Post hoc analysis revealed that EMR-2450 MHz exposed experimental animals showed cognitive impairments in terms of 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.

Figure 2.3.5

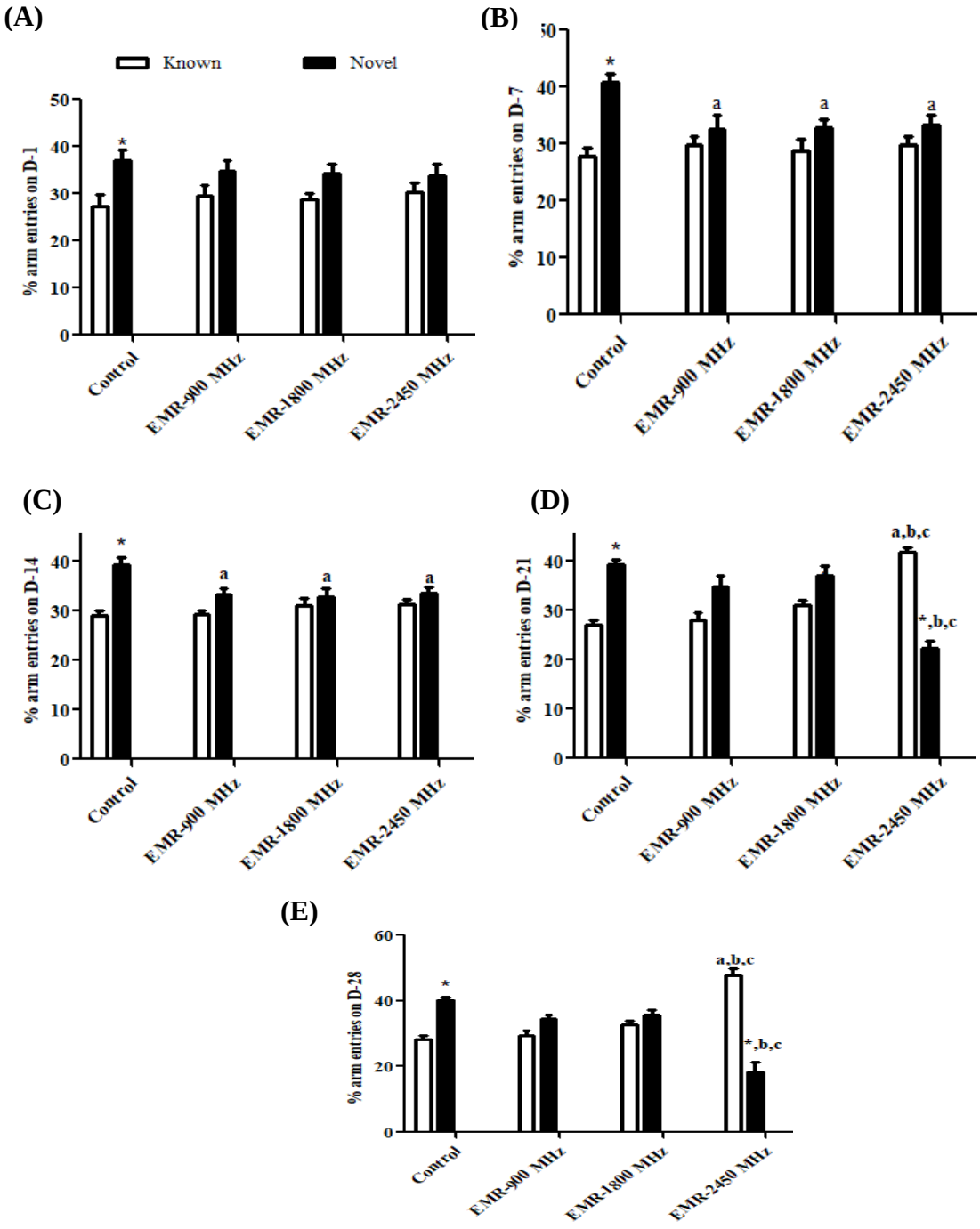


Figure: 2.3.5 Shows alterations in arm discrimination behavior during Y-maze test paradigm on D-1 (A), D-7 (B), D-14 (C), D-21 (D) and D-28 (E) of the experimental schedule. All values are mean ± SEM, (n=6). *p<0.05 compared to corresponding

known arm entries; [a]p<0.05 compared to control, [b]p<0.05 compared to EMR-900 and [c]p<0.05 compared to EMR-1800 [Two-way ANOVA followed by Bonferroni test].

2.3.3.3 Estimation of Ach and AchE levels in EMR exposed rats

Fig-2.3.6 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<0.05] and (B) AchE [F (3, 20) =34.6; P<0.05] 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.

Figure 2.3.6 (A) **(B)**

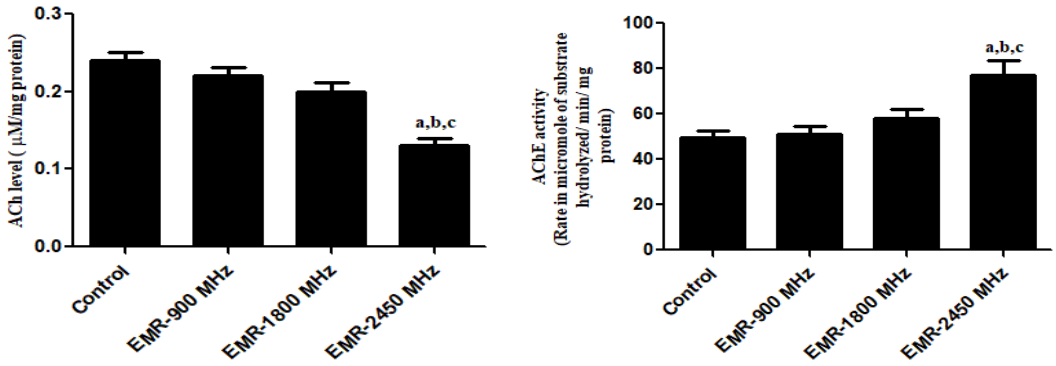


Figure 2.3.6 Shows changes in (A) Ach level and (B) AchE activity in hippocampus. All values are mean ± SEM. [a]p<0.05 compared to control, [b]p<0.05 compared to EMR-900 and [c]p<0.05 compared to EMR-1800 [One-way ANOVA followed by Student–Newman Keuls test]

2.3.3.4 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 Fig-2.3.7. There were significant differences in β Amyloid [$F(3, 8) = 9.3$; $P<0.05$] among groups. Post hoc analysis demonstrated that EMR at the frequency of 2450 MHZ increased the expression of β Amyloid compared to control rats.

Figure 2.3.7

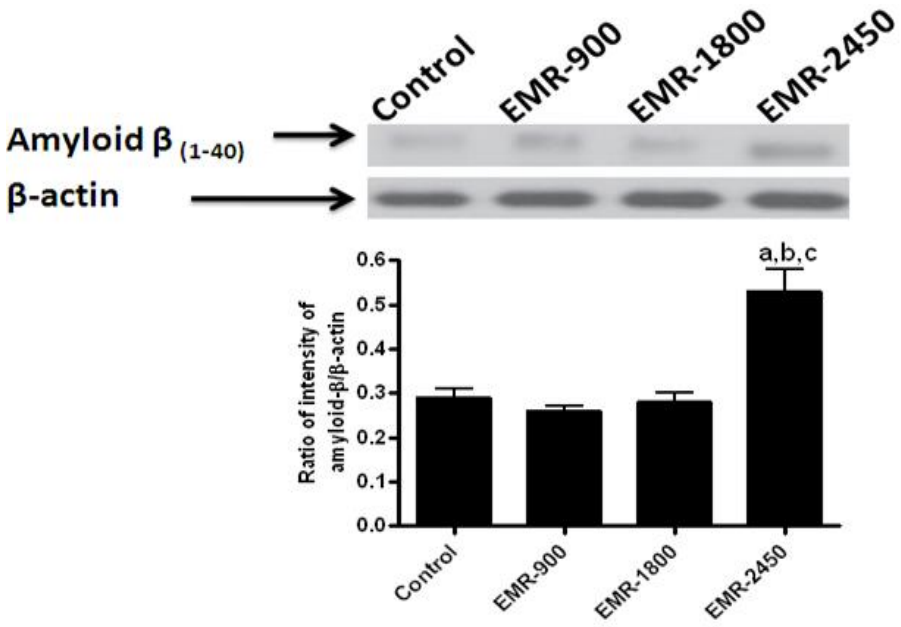


Figure 2.3.7 the blots and ratio of relative intensity of level of protein expression of Aβ1–40 to β-Actin in hippocampus. All values are mean ± SEM. [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to EMR-900 and [c]$p<0.05$ compared to EMR-1800 [One-way ANOVA followed by Student–Newman Keuls test].

2.3.3.5 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 Fig-2.3.8. One-way ANOVA revealed that there were significant differences in MMP [F (3, 20) =36.91; P<0.05] among groups. Post hoc analysis revealed that EMR (2450 MHz) significantly decreased the intensity of TMRM indicating loss of mitochondrial integrity.

Figure 2.3.8

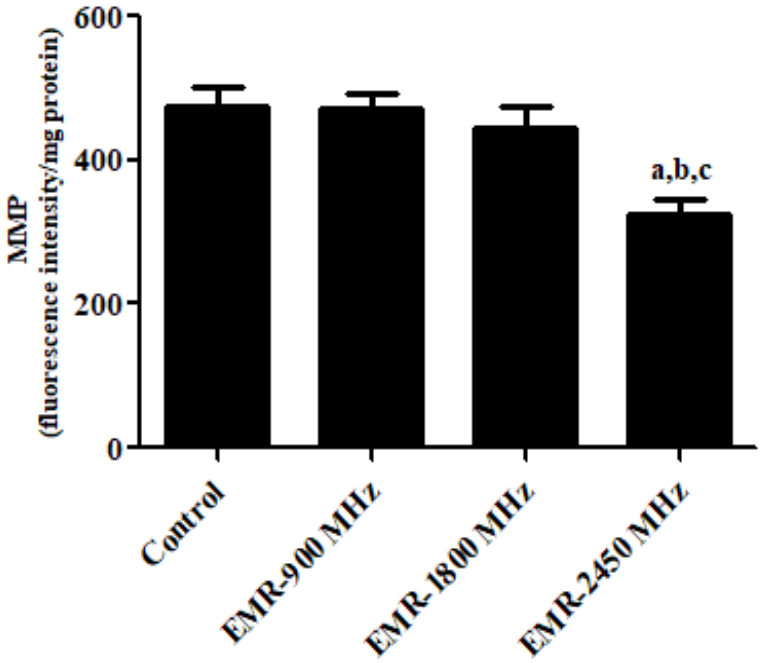


Figure 2.3.8 the effect of EMR induced alterations in MMP in EMR exposed rats. All values are mean ± SEM. [a]p<0.05 compared to control, [b]p<0.05 compared to EMR-900 and [c]p<0.05 compared to EMR-1800 [One-way ANOVA followed by Student–Newman Keuls test].

2.3.3.6 EMR showed the increase in expression of cytoplasmic Cytochrome-C, Caspase-9 and Caspase-3 in hippocampus

Fig-2.3.9 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 hippocampus. There were significant differences in expression of Cytochrome-C [$F(3, 8) = 22.4$; $P<0.05$], caspase-9 [$F(3, 8) = 15.8$; $P<0.05$] and caspase-3 [$F(3, 8) = 7.9$; $P<0.05$] among groups. Post hoc test revealed that EMR (2450 MHz) showed significant increase in the expression of cytochrome-c, caspase-9 and caspase-3 compared to control rodents.

Figure 2.3.9

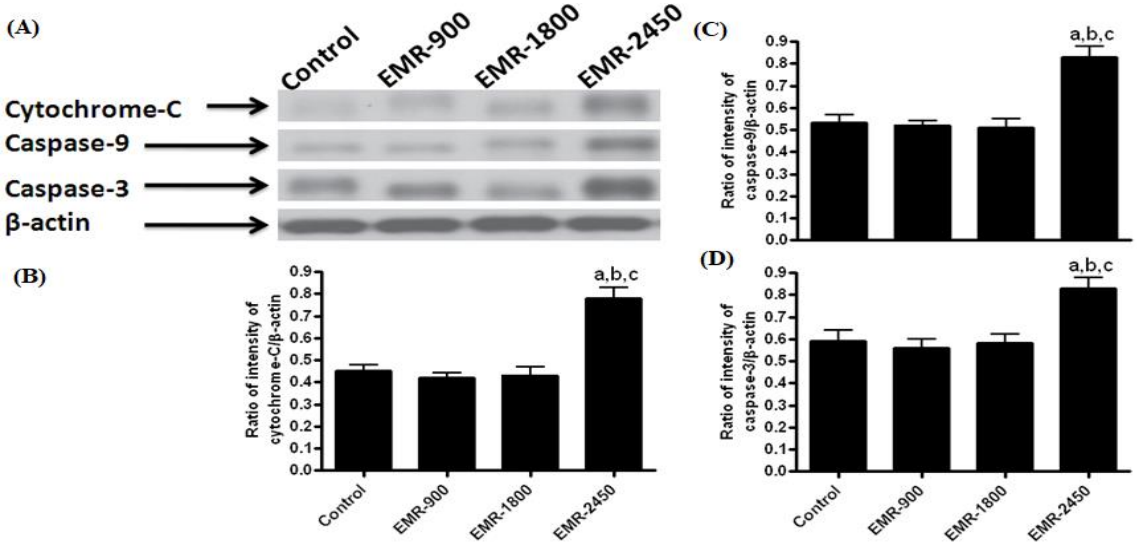


Figure 2.3.9 Shows (A) histogram of cyto-c, caspase-9, caspase-3 and β-actin (B) ratio of intensity of cyto-c/ β-actin (C) ratio of intensity of caspase-9/ β-actin (D) ratio of intensity of caspase-3/ β-actin in hippocampus tissues. All values are mean ± SEM. $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to EMR-900 group and $^{c}p< 0.05$ compared to EMR-1800 group [One-way ANOVA followed by Student–Newman Keuls test].

2.3.3.7 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-2.3.1. One-way ANOVA statistical analysis revealed that there were significant differences in mitochondrial Complex-I, II, IV, V enzyme activities among groups [$F(3, 20) = 12.25$, $P < 0.05$], [$F(3, 20) = 1.521$; $P<0.05$], [$F(3, 20) = 4.528$; $P<0.05$] and [$F(3, 20) = 4.911$; $P<0.05$] 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.

Table 2.3.1

S.No	Groups	Complex-I activity(nMNADH oxidized/min/mg/protein)	Complex-II activity(µM formazan/min/mg/protein)	Complex-IV activity (nM cytochrome c oxidized/min/mg/protein)	Complex V Activity (nM ATP hydrolysed/mg protein)
1	Control	5.36±.27	0.49±.11	0.81±.08	12.41±.52
2	EMR-900MHz	5.21±.42	0.46±.07	0.76±.05	12.19±.57
3	EMR1800MHz	5.14±.19	0.42±.09	0.72±.08	12.05±.47
4	EMR2450MHz	3.10±.30^{a,b,c}	0.26±.05 ^{a,b,c}	0.47±.07^{a,b,c}	8.86±1.09^{a,b,c}

Table 2.3.1 Effect of EMR on mitochondrial Complex-I, II, IV and V activities of hippocampus in rats. All values are mean ± SEM. $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to EMR-900 group and $^{c}p< 0.05$ compared to EMR-1800 group [One-way ANOVA followed by Student–Newman Keuls test].

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

Table-2.3.2 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<0.05], NO [F (3, 20) =3.341; P<0.05], SOD [F (3, 20) =3.588; P<0.05] and catalase [F (3, 20) = 4.085; P<0.05] 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.

Table 2.3.2

S.No	Groups	LPO level (µM MDA/mg protein)	Nitrite level (nM NO/mg protein	Catalase level (nM H_2O_2/min/mg of protein)	SOD activity (Units/min/ mg of protein)
1	Control	0.78±.15	1.24±.85	0.42±.10	0.61±.0.09
2	EMR-900MHz	0.75±.17	1.18±.71	0.38±.13	0.54±.11
3	EMR-1800MHz	0.71±.12	1.14±.77	0.36±.11	0.51±.07
4	EMR-2450MHz	2.09±.19^{a,b,c}	1.85±.66^{a,b,c}	0.15±.021^{a,b,c}	0.25±0.05^{a,b,c}

Table 2.3.2 Shows the levels of (A) LPO, (B) NO, (C) catalase and (D) SOD in the hippocampus tissues. All values are mean ± SEM. $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to EMR-900 group and $^{c}p< 0.05$ compared to EMR-1800 group [One-way ANOVA followed by Student–Newman Keuls test].

2.3.4 Discussion

In the present study, experimental animals exposed to EMR at frequency of 2450 MHz exhibited cognitive dysfunction. We for the first time 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 MHz 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 MHz 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 MHz 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 et al., 2016) reported that long term (10 weeks) exposure to electromagnetic radiation (smart phones) 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 agreement 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 2450MHz (Wang et al., 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 et al., 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).

It has been reported that loss of mitochondrial function could cause hippocampal synaptic dysfunction that leads to memory deficits (Santini et al., 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 Aβ 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. 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 and1800 MHz. It is interesting to note that marked decrease of fluorescence intensity was observed reflecting compromised mitochondrial membrane integrity. 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 Aβ is observed in AD along with cognitive deficits (Pozueta et al., 2013). The cellular concentration of Aβ is kept within a precise range by balancing its synthesis and degradation (Pinho et al., 2014). It has also been suggested that Aβ is found in the several compartments of the cell including mitochondria (Villmange et al., 2013). In the current study, EMR-2450 MHz shows increase in expression of Aβ 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 *Aβ* in rats (Suleyman et al., 2012). In summary, 28 days exposure of EMR-2450 MHz induced expression of hippocampal Aβ which may be one of the reasons for EMR-induced cognitive deficits in rats.

Aβ 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 et al., 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 hippocampus. It has been reported that EMR 2450 MHz exposure leads to neurodegeneration of cholinergic neurons followed by increase in the activity of AChE with decrease in level of Ach (Mesulam 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 et al., 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.

2.3.4.1 Summary

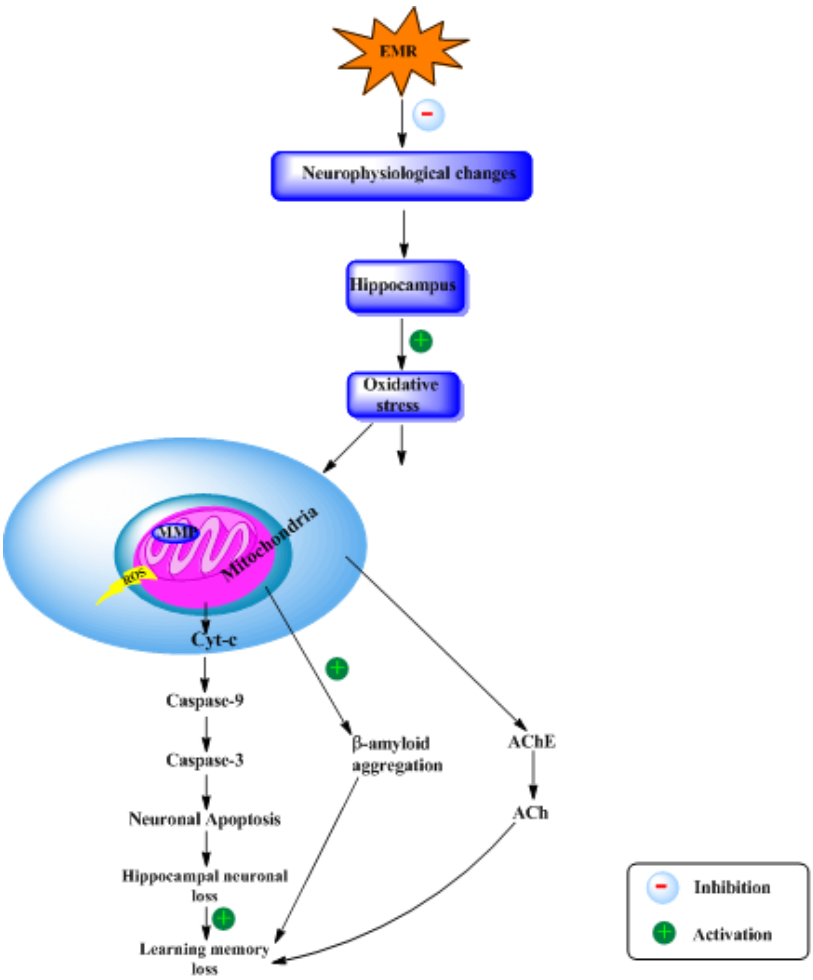


Figure: 2.3.10 Summary of hypothesis

Figure 2.3.10 shows that 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 of mitochondrial linked apoptosis. Furthermore, exposure with EMR-2450 increased expression of hippocampal Aβ and decreased cholinergic neurotransmission in hippocampus which are considered to be important factors for development of cognitive dysfunction.