2.1.1 `Introduction

Exposure to non-ionizing electromagnetic radiations (EMR) from the 3G cell phones and Wi-Fi has become an unavoidable part of human life. Continuous exposures with such electromagnetic radiations can therefore lead to alterations in biological molecules leading to various disorders. Therefore, we use electromagnetic radiations in the frequency range of 900, 1800, and 2450 MHz which is emitted from mobile phones and Wi-Fi. Previous reports have demonstrated that exposure to radiofrequencies lead to generation of heat, which may lead to excitation of electrons of molecules and alterations in configuration of biological tissues (Challis, 2005). Earlier studies have reported that long-term exposure to EMR enhances the risk of neuropsychological disorders like anxiety (Jinget al., 2012; Pall, 2016). Development of anxiety like behavior in experimental animals critically depends on the duration and frequency of exposure (Shehu et al., 2016). Although, conclusions drawn from behavioral studies are rather ambiguous, some preclinical studies indicate that exposure to high range of EMR may lead to anxiety like behavior (Zhang et al., 2014). However, EMR at lowest frequency (900 MHz) does not cause sufficient impairment to exhibit anxiety like behavior (Júnior et al., 2014). The pathophysiology of EMR-induced anxiety is yet to be explored. In retrospect, the effect of EMR-2450 MHz exposure on the function of HPA axis has also not been extensively studied. Chronic exposure of extremely low frequency of EMR may increase the level of plasma corticosterone in rodents (Mostafa et al., 2002). Previous study suggests that the long term exposure of EMR may act as a stressor in the mice (DeBruyn and DeJager, 1994; Gong et al., 2015). Corticosterone is a functional indicator of stress response in experimental animals (Gong et al., 2015). However, neural mechanisms for the effect of EMR on neurological disorders are yet to be deciphered. Long term exposure of

EMR causes changes in the amygdalar morphology and emotional behavior in rats (Narayanan et al., 2018). Animal models have suggested that the amygdala modulates the consolidation of hippocampal dependent memories through the actions of stress hormones (Phelps, 2004). Further, amygdala can regulate the HPA axis which in turn may lead to anxiety-like behavior (Pawlak et al., 2003). Long-term exposure of EMR modulated the hippocampal function which is responsible for cognitive deficits in experimental rodents (Cohen, 2000). Based on earlier observation that physiological function of specific brain regions can be altered by EMR, we assumed that EMR can act as a stressor and can modulate the amygdalar system leading to anxiety-like symptoms in experimental rats. We exposed experimental rats to discrete frequencies of EMR i.e., 900, 1800, and 2450 MHz and studied the behavioral, biochemical, cellular and molecular changes. Since, humans are chronically exposed to electromagnetic radiations emanating from mobile phones, we exposed the animals 1 h daily for 28 consecutive days to see the effects of long term exposure. The role of central CRH system and GR secretion in inducing negative emotional states and potentiating fear and anxiety like behavior has been well established (Raglan et al., 2017). Clinical and preclinical reports suggest that GR, CRH-2 and corticosterone are involved in maintaining homeostasis and its modulation causes stress induced anxiety in rats (Tinnikov, 1999). There is decrease in expression of CRH-2 and GR in animals showing anxiety-like behavior which further results in decrease in amygdalar volume (Karl et al., 2006). Therefore, we can assume that long term exposure of EMR in rats may modulate CRH-2 and GR expression in amygdala. In addition to above-mentioned pathophysiology of stress-induced anxiety, alterations in mitochondrial complex activities and their dysfunction can lead to altered brain mitochondrial membrane potential which triggers the release of reactive oxygen species (ROS) in rodents

(Adam-Vizi and Starkov, 2010; Hollis et al., 2015). Pro-apoptotic bax, anti-apoptotic bcl_2 protein family resides in the outer membrane of mitochondria (Jarskog et al., 2005). Furthermore, changes in bax protein may lead to opening of mitochondrial transition pores and release of cytochrome-c, which can trigger the intrinsic pathway of apoptosis through activation of caspase-9 ultimately leading to neuronal cell death (Jürgensmeier et al., 1998). Chronic exposure to electromagnetic radiations may cause mitochondrial dysfunction and initiate apoptosis in neurons (Gupta et al., 2018). However, there is no report on mitochondrial dysfunction associated with EMR linked stress and anxiety.

The level of corticosterone, GR and CRH-2 expression were estimated in amygdala as indices of stress response. Furthermore, mitochondrial complex activities, mitochondrial membrane potential, ROS and various proteins like bcl_2, bax, cytochrome-c, caspase-9 and histopathological examination of tissues were done to evaluate the molecular basis of apoptosis in EMR exposed rats. Further, the pattern of cell death to EMR exposure was evaluated by flow cytometric analysis. This study may also provide insights into the pathophysiological mechanisms leading to anxiety-like disorders following chronic exposure to EMR (illustrated in figure 2.1.1).

2.1.2 Hypothesis

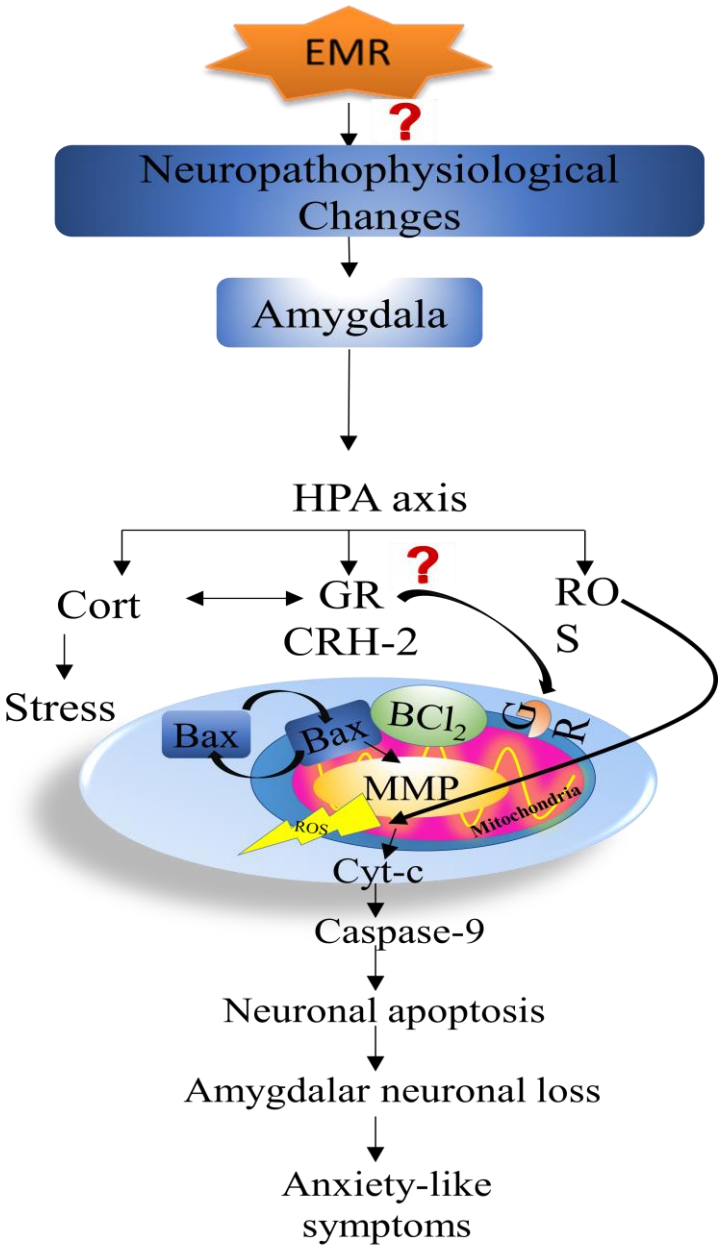


Figure 2.1.1 Proposed hypothesis effect of EMR on stress-anxiety

2.1.3 Materials and Methods

2.1.3.1 Animals

Inbred Charles-foster albino male rats weighing about (180 ± 20 g) were purchased from the central experimental animal facility center, Institute of Medical Sciences, Banaras Hindu University (IMS-BHU). The animals were housed in home cage made up of polypropylene at 25 ± 2 °C temperature and RH 44–56%, light and dark cycle of 12:12 h respectively. The entire animals acclimatized for one week prior to experiments. The food pellets were provided (Paramount pvt.ltd.) and water was allowed *ad libitum*. All the experiments were conducted based on given guidelines (CPCSEA-2010; IMS-BHU; Approval No.: Dean/2015/CAEC/1414).

2.1.3.2 Materials

TMRM (Tetra methyl rhodamine methyl ester; Sigma Aldrich, St.Louis, MO, USA) Corticosterone (C0388, TCI America, Portland, USA), all the antibodies used Abcam Plc. (India) were purchased. All other chemicals and reagents were procured from local supplier.

2.1.3.3 Electromagnetic Radiation Exposure System and Design

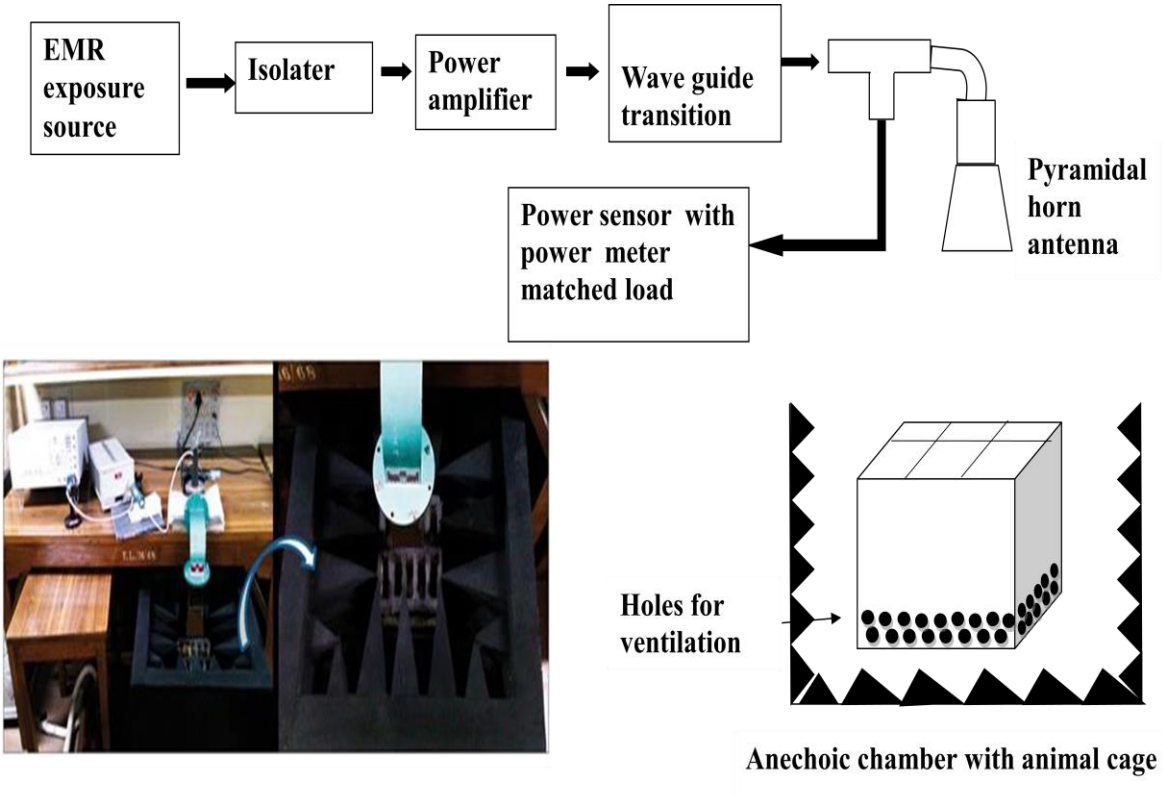


Figure 2.1.2 Exposure system and design

Figure 2.1.2 shows the EMR exposure equipment and design (Gupta et al., 2018). Briefly, the equipment includes inbuilt analog signal generator by Agilent Technologies, USA having a frequency range of 100 kHz-20 GHz. The exposure system had an inter connected wave guide transition microwave amplifier (Hewlett Packard) along with a 20 db cross-coupler, E-plane bend and a brass-silver coated pyramidal horn antenna. The maximum output power was 19.8 dB measured by a power meter (Agilent technologies) and then delivered it to the horn antenna. The whole assembly was kept on a wooden table and the generator emitted discrete range of 900, 1800, and 2450 MHz radiofrequency (rf) signals.

2.1.3.4 Calculation of Power density and specific absorption rate of brain region

The power density calculated by the formulae described in earlier study (Gupta et al., 2018). The average power density was 0.1227 W/m2. The whole body SAR values was found in between the 0.025–0.070 W/kg range, representing an average SAR value to be approximately 0.042 W/kg. The value of SAR in head region was found to be 0.131 W/kg (900, 1800 and 2450 MHz) with a value of power density 0.1227 W/m2. The calculation of SAR = 5.94 * average length of animals * power density/Electromagnetic Range in GHz*average wt. of animal; whereas, Avg. length of animal = 17 cm, Avg. wt of animal = 200 g and average length head of animal = 3 cm (Gandhi et al., 1977).

2.1.3.5 Experimental design

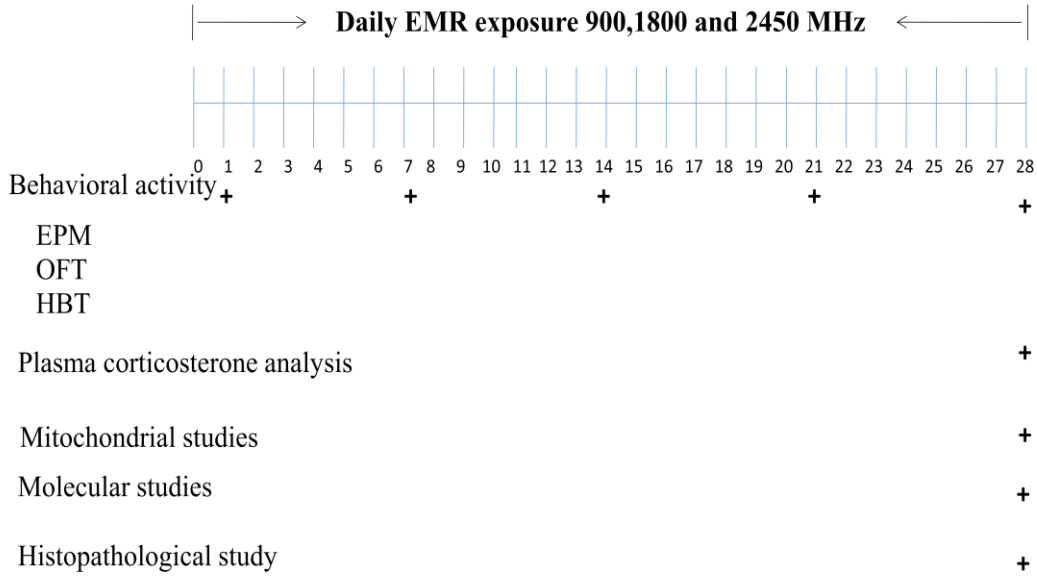


Figure 2.1.3 Schematic representation of the experimental design. '+' denotes experiment performed

All the rats were distributed into four different groups of six each through G* power analysis software. They were designated as control; EMR-900, EMR-1800 and EMR-2450.At the time of exposure, feed and water were not given to experimental rats. The groups, EMR-900 MHz, EMR-1800 MHz and EMR-2450 MHz were continuously exposed to electromagnetic radiations between 10 am to 1 pm for 1 h for 28 days beginning from D-1 as shown in figure 2.1.3. The control animals were kept in an anechoic chamber without EMR for 1h daily from D-1 to D-28 to the same initial stress levels as the experimental group. After 15 min of EMR exposure on D-1 to D-28 at 7-day interval, behavioral assessments were performed. The anxiety-like behavior was evaluated by Elevated Plus Maze (EPM), Open Field Test (OFT) and Hole Board Test (HBT). The behavioral observations were done in the sequential order with a 20 min interval between experiments. The observations were recorded using ANY-maze™ (version-3.72, USA) video tracking system. On D-28, animals were killed by decapitation and blood was collected to estimate the level of plasma corticosterone (n=6). The amygdala was dissected on ice. The brain was placed ventral side up on a glass plate on ice, the brainstem was removed and coronal cuts were made at the optic chiasm and just posterior to the notch on the ventral surface of the brain to remove entorhinal cortex. The remaining block of tissue containing the amygdala was cut from the temporal lobe and stored at −80 °C for further estimation. Out of six from each group, amygdalar tissue (n=3) from three animals were used for protein expression and mitochondrial studies. The other three were used for histology (n=3). To further estimate the nature of cell death and to reconfirm histopathological study in terms of neuronal loss, we planned the second set of experiments. The experimental design was similar to the earlier set of experiments. Briefly, Histopathological study (n=3) and flow cytometry for cell death pattern (n = 3) were done from amygdalar tissue.

2.1.4 Behavioral parameter assessment

2.1.4.1 Anxiety-like behavior in EPM test

EPM is commonly used to evaluate for anxiety-like behavior in experimental animals. This test was performed on D-1, 7, 14, 21 and 28 as per the method of (Pellow et al., 1985). Elevated plus maze was made up of four arms (two open and two enclosed) by a 40 cm high wall, 50 cm long and 10 cm wide. Appearance of plus sign is due to presence of four arms linked with Central Square (10 × 10 cm). The elevated plus maze is kept elevated 50 cm above the flat surface in moderate illuminated behavioral chamber. Experimental rat was kept onto the central square of the plus maze facing an enclosed arm. The measurement of anxiety in terms of percentage of time spent and numbers of arm entries on the open arm were recorded for next 5 min. Further, locomotor activity was measured in terms of total arm entries.

2.1.4.2 Open field test

Locomotor activity was measured in rats using the OFT on D-1, 7, 14, 21 and 28. The apparatus was made up of a square (61 × 61 cm) with high walls (61 × 61 cm). The surface of apparatus was completely colored by white paint except for 6-mm black lines that divided the floor into equal 4 × 4 squares. Experimental rodent was placed on the edge of the test apparatus allowed for 5 min, and the behaviors such as ambulation, rearing, grooming and the duration of central squares crossed were recorded. After the completion of each experiment, surface of wooden apparatus was cleaned by alcohol (Bronstein, 1972; Casarrubea et al., 2009).

2.1.4.3 Hole board test

Hole board test is an another widely accepted method for the measurement of anxiety-like behavior in experimental rats. The hole-board apparatus made up of a wooden box (60 × 60 × 35 cm) with four holes at corners for support to the floor (diameter = 4 cm). The floor of the box was kept 12 cm elevated from the flat surface and divided into 3 × 3 squares (20 × 9 × 20 cm). Experimental rat was kept on the center of apparatus and allowed to freely explore the apparatus up to 5 min. Any change in the emotional state of rat such as anxiety is reflected as a change in its exploratory behavior. Head dip was measured when the animal put its head into one of the holes up to the level of ear. Sniffing was the exploratory behavior of the rat other than outside the hole (Casarrubea et al., 2009; Kong et al., 2006).

2.1.4.4 Plasma corticosterone estimation

The level of plasma corticosterone was estimated using HPLC coupled with an ultraviolet (UV-Vis) detector as described earlier (Woodward and Emery, 1987). Briefly, 500µL of plasma was extracted with 5 ml of dichloromethane (DCM). The dichloromethane extract was evaporated and eluted with 100µL of the mobile phase (methanol:water) in the ratio (70:30). 20µL of the extract was injected into the HPLC system at constant flow rate of 1.2 ml/min. Absorbance of corticosterone was taken at 254 nm using a UV detector (Waters USA). Data collection and handling were carried out by Breeze software (Version 3.2) (Garabadu, Ahmad, & Krishnamurthy, 2015).

2.1.4.5 Evaluation of mitochondrial membrane potential, complex activities and oxidative stress

2.1.4.6 Mitochondria isolation

The amygdalar mitochondria were isolated by using earlier describe standard protocol (Pedersen et al., 1978). The protein content was measured (Lowry et al., 1951).

2.1.4.7 Evaluation of mitochondrial membrane potential (MMP)

The mitochondrial integrity in terms of MMP was measured by using TMRM as a fluorescent cationic dye. The amygdalar mitochondrial sample was taken and dissolved in rhodamine dye, and the intensities were measured by spectrofluorometer using slit no.10 the fluorescence emission (excitation wavelength 535 nm and emission wavelength 580 nm). The peak intensity was 570 nm. The observations were expressed as fluorescence intensity/mg protein (Huang, 2002).

2.1.4.8 Measurement of mitochondrial complex activity (I, II, IV and V)

The assessment of Complex-I activity was measured by the catalytic oxidation of NADH. Oxidation of NADH was determined at excitation (350 nm) and emission (470 nm) (Shapiro et al., 1979), and it is expressed as a nmole of NADH oxidized/min/mg protein. Complex-II (succinate dehydrogenase) activity was determined as the reduction of Nitro Blue Tetrazolium (NBT). The absorbance was taken at 570 nm expressed as a μM formazan produced/min/mg protein (dfz; (Old and Johnson, 1989). The activity of complex-IV (cytochrome c oxidase) was estimated in fraction of mitochondria as earlier described method with some modifications (Storrie and Amadden, 1990). The reduction in absorbance was taken at 550 nm of every minute for 3 times. Results were expressed as nM cytochrome-c oxidized/min/mg protein (e550=19.6 $\mu mol^{-1}cm^{-1}$). F1F0 synthase

(Complex-V) was incubated with mitochondrial suspension with ATPase buffer (Griffiths and Houghton, 1974). Briefly, mitochondrial suspension was incubated in 500 µl of ATPase buffer (50 mM Tris HCl and 5 mM MgCl2, pH 7.5) at 37 °C with 5 mM ATP for 10 min. The reaction was stopped by adding 500 µl of 10% (w/v) trichloroacetic acid. The contents were centrifuged at 3200 g for 15 min and then 500 µL of supernatant was mixed with equal volume of distilled water. Thereafter, the free inorganic phosphate was measured as method explored in (Fiske and Subbarow, 1925). The results of Complex-V were expressed as n mole ATP hydrolysed/min/mg protein.

2.1.4.9 Assay of catalase activity

Decomposition of hydrogen peroxide in the presence of catalase with respect to time follows the catalase peroxide reaction. The absorbance of this reaction was measured at 240 nm and results were expressed as a decomposition of single unit of hydrogen peroxide/min/mg of protein (Beers and Sizer, 1952).

2.1.4.10 Estimation of superoxide dismutase (SOD) activity

The estimation of SOD was evaluated by the reduction of NBT in the presence of phenazine metho sulphate and NADH. The reduction of NBT was measured at 560 nm per minute/mg of protein using n-butanol as blank (Kakkar et al., 1984).

2.1.4.11 Western blot analysis

The amygdalar brain region was lysed with lysis buffer (containing optimum amount of protease inhibitor). The concentration of proteins was assessed (Bradford, 1976). An aliquot of each sample was electrophoresized on 10% SDS-PAGE gel system. Mitochondrial Bax, Bcl-2, GR and CRH-2 and cytoplasmic Bax, Cytochrome-C, caspase-9 were measured by transferring the protein into polyvinylidene fluoride

membranes after overnight incubation with rabbit anti-Bax (1:500,21 kDa; ab53154) and rabbit anti-Bcl2(1:100, 26 kDa ab59348), rabbit anti-GR (1:500, 95 kDa), rabbit anti-CRH-2 (1:500, 29 kDa), rabbit anti Cytochrome-C (1:100, 15 kDa; ab90529), rabbit anti caspase-9 (1:500,45 kDa; ab25758) polyclonal primary antibodies. After detection of the desired proteins, according to the given proteins of interest further study was done by using our previous studies (Gupta et al., 2018).

2.1.4.12 Histopathological studies

Histopathological studies were performed by collecting the amygdalar sample from the EMR exposed rat brain of all the groups, fixed in Bouin's fluid, dehydrated in graded ethanol series, cleared in benzene and embedded in paraffin. Tissues were sectioned at 6μm, and the sections were then stained with Periodic Acid Schiff (PAS) and counterstained with hematoxylin. The obtained sections were finally visualized and photographed using a Leica DFC 290 (Leica Microsystems Ltd., Wetzlar, Germany) at 25× magnification (Mishra and Singh,2009; Srivastava et al., 2017). For evaluation of neuronal cells after electromagnetic radiation (EMR) exposure, ten fields of amygdalar region were randomly selected from sections in each rat. All the neurons were manually counted and the percentage change was calculated.

2.1.4.13 Flow cytometry analysis for measurement of pattern of cell apoptosis

Amygdalar tissue samples were taken and single cell suspension prepared in cold PBS buffer, and the final concentrations were adjusted to 5×10^{6}cells/ml through automated cell counter (Life Sciences Countess II FS, Invitrogen, Thermo fischer scientific, USA). Further, 1×10^{6} cells/ml of suspension were centrifuged at 300×g for 5 min at 4 °C and washed with 1 ml cold PBS thrice. The pellet so formed was then resuspended in 100 μL of annexin binding buffer and incubated with 5 μL fluorescein isothiocyanate

(FITC) conjugated annexin-V and 1µL of propidium iodide (PI) from working solution as per the instructions of the protocol for 15 min at room temperature in the dark condition. Flow cytometric assay was performed using the eBioscience™ Annexin-V Apoptosis Detection Kit FITC and propidium iodide (PI) staining method (Invitrogen by Thermo Fisher Scientific Carisbad, CA-92008). Fluorescence was measured using a FACScan flow cytometer (BD FACS Calibur™, BD Biosciences, San Jose, Calif., CA, USA), equipped with an argon-ion laser tuned at the excitation of FITC (494/518 nm, at FL-1 channel) and PI (536/617 nm, at FL-2 channel). Flow cytometry data were acquired for 10,000 cells/sample, and data analysis was performed using Flowjo software (Samaiya et al., 2016, 2018).

2.1.4.14 Statistical analysis

Experimental data are expressed as Mean ± standard error of mean (SEM). All the behavioral data were analyzed using repeated measures two-way ANOVA followed by Bonferroni post hoc test. The data from molecular studies, plasma corticosterone levels and neuronal count were analyzed using one-way analysis of variance (ANOVA) followed by 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 (Berger & Sellke, 1987).

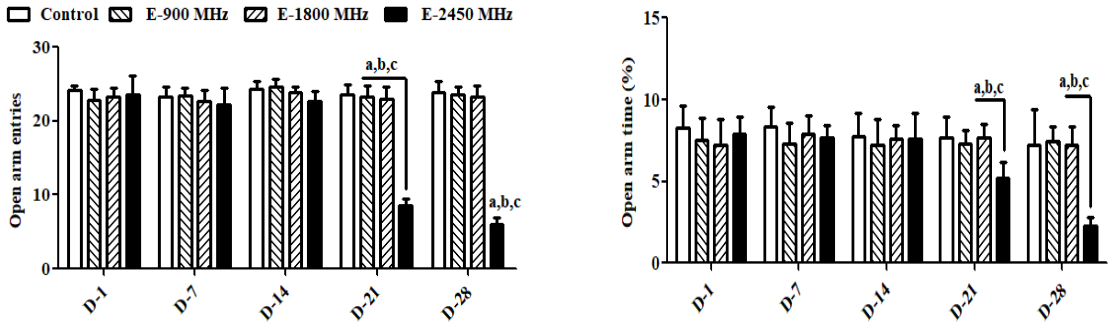
Results

2.1.5.1 Repeated exposure of EMR changes arm entries in elevated plus maze paradigm

Elevated plus maze is a main paradigm to estimate the anxiety-like behavior in laboratory animals. Apparatus utilizes the normal exploratory behavior in rats to evaluate levels of anxiety, with rats having anxiety like symptoms being less willing to explore open arms in the maze. Fig-2.1.4 (A, B and C) illustrates that percentage arm entries, total time spent in arms and total number of arm entries in terms of exploratory behavior changes in EPM due to exposure of EMR-900,1800 and 2450 MHz. Statistical analysis by repeated measures two-way ANOVA showed significant differences among groups for arm entries, total time spent in arms and non-significant changes in the total number of arm entries [$F(3,100) = 41.33$; $p < 0.05$], [$F(3,100) = 13.28$; $p < 0.05$], [$F(3,100) = 0.08$; $p > 0.05$] respectively, time [$F(4,100) = 13.79$; $p < 0.05$], [$F(4,100) = 7.25$; $p < 0.05$], [$F(4,100) = 0.2028$; $p > 0.05$] respectively, and an interaction between groups and time [$F(12,100) = 16.25$; $p < 0.05$], [$F(12,100) = 4.108$; $p < 0.05$], [$F(12,100) = 0.102$; $p > 0.05$]. Post hoc test revealed that on Day 1, 7, 14, 21 and 28 of experimental protocol EMR-900, 1800 MHZ did not change arm entries, time spent and exploratory behavior. However, EMR (2450 MHz) significantly attenuated the percentage arm entries and total time spent in arms from D-21 to D-28. There was no significant difference between total arm entries in EPM on D-21 to D-28 when compared with the control, EMR-900 and 1800 MHz group. Similarly, in second set of experiment, EMR-2450MHz exposed animals showed decrease in percentage arm entries, total time spent in arms and no change in total number of arm entries in EPM among the groups [$F(3,100) = 38.43$; $p < 0.05$], [$F(3,100) = 5.83$; $p < 0.05$], [$F(3,100) = 0.44$; $p > 0.05$] respectively, time [$F(4,100) = 15.49$; $p < 0.05$], [$F(4,100) = 3.87$; p

< 0.05], [F (4,100) = 0.71; p > 0.05] respectively, and an interaction between groups and time [F (12,100) = 10.71; p < 0.05], [F (12,100) = 3.404; p < 0.05], [F (12,100) = 0.85; p > 0.05].

Figure 2.1.4 (A) **(B)**



(C)

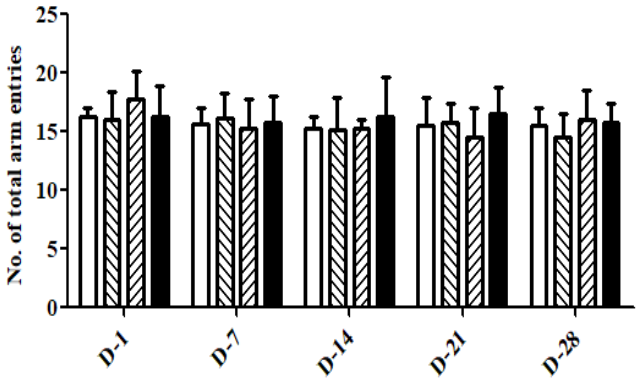


Figure: 2.1.4 Bar graph shows the changes in the percentage of open arm entries **(A)** time spent **(B)** and number of total arm entries **(C)** in elevate plus maze test. All values are expressed as mean±SEM., (n=6). [a]p<0.05 compared to control [b]p<0.05 compared to EMR-900 and [c]p<0.05 compared to EMR-1800; [x]p<0.05 compared to D-1, [y]p<0.05 compared to D-7 and [z]p<0.05 compared to D-14 [Two-way ANOVA followed by Bonferroni test].

2.1.5.2 Long term exposure of EMR caused behavioral changes in Open Field Test (OFT)

OFT is used to evaluate the locomotor activity in experimental rats. In Fig. 2.1.5.1, panels A, B, C and D depict the effect of EMR- 900, 1800 and 2450 MHz exposure on ambulation, rearing, grooming and number of central squares crossed respectively in OFT. Repeated measures two-way ANOVA showed significant differences among groups for ambulation, rearing, grooming and number of central squares crossed [$F(3,100) = 11.60$; $p < 0.05$], [$F(3,100) = 10.69$; $p < 0.05$], [$F(3,100) = 3.254$; $p < 0.05$], [$F(3,100) = 10.05$; $p < 0.05$] respectively, time [$F(4,100) = 2.275$; $p < 0.05$], [$F(4,100) = 5.095$; $p < 0.05$], [$F(4,100) = 5.498$; $p < 0.05$], [$F(4,100) = 4.853$; $p < 0.05$] respectively and an interaction between groups and time [$F(12,100) = 3.169$; $p < 0.05$], [$F(12,100) = 3.084$; $p < 0.05$], [$F(12,100) = 8.057$; $p < 0.05$], [$F(12,100) = 8.716$; $p < 0.05$] respectively. Post hoc analysis demonstrated that on D-1, 7, 14, 21 and 28, EMR-900, 1800 MHz did not change ambulation, rearing, grooming and number of central squares crossed in OFT. However, EMR (2450 MHz) induced significant decrease in ambulation, rearing, grooming and number of central squares crossed in OFT from D-21 to D-28 of experimental schedule when compared with other groups. Similarly, in second set of experiment illustrated in fig. 2.1.5.2, EMR 2450 MHz exposed rodents showed decrease in ambulation, rearing, grooming and number of central squares crossed during OFT [$F(3,100) = 10.58$; $p < 0.05$], [$F(3,100) = 12.80$; $p < 0.05$], [$F(3,100) = 7.46$; $p < 0.05$], [$F(3,100) = 3.11$; $p < 0.05$] respectively, time [$F(4,100) = 3.26$; $p < 0.05$], [$F(4,100) = 4.06$; $p < 0.05$], [$F(4,100) = 6.46$; $p < 0.05$], [$F(4,100) = 6.15$; $p < 0.05$] respectively and an interaction between groups and time [$F(12,100) = 2.72$; $p < 0.05$], [$F(12,100) = 1.98$; $p < 0.05$], [$F(12,100) = 1.80$; $p < 0.05$], [$F(12,100) = 4.78$; $p < 0.05$] respectively.

Figure 2.1.5.1

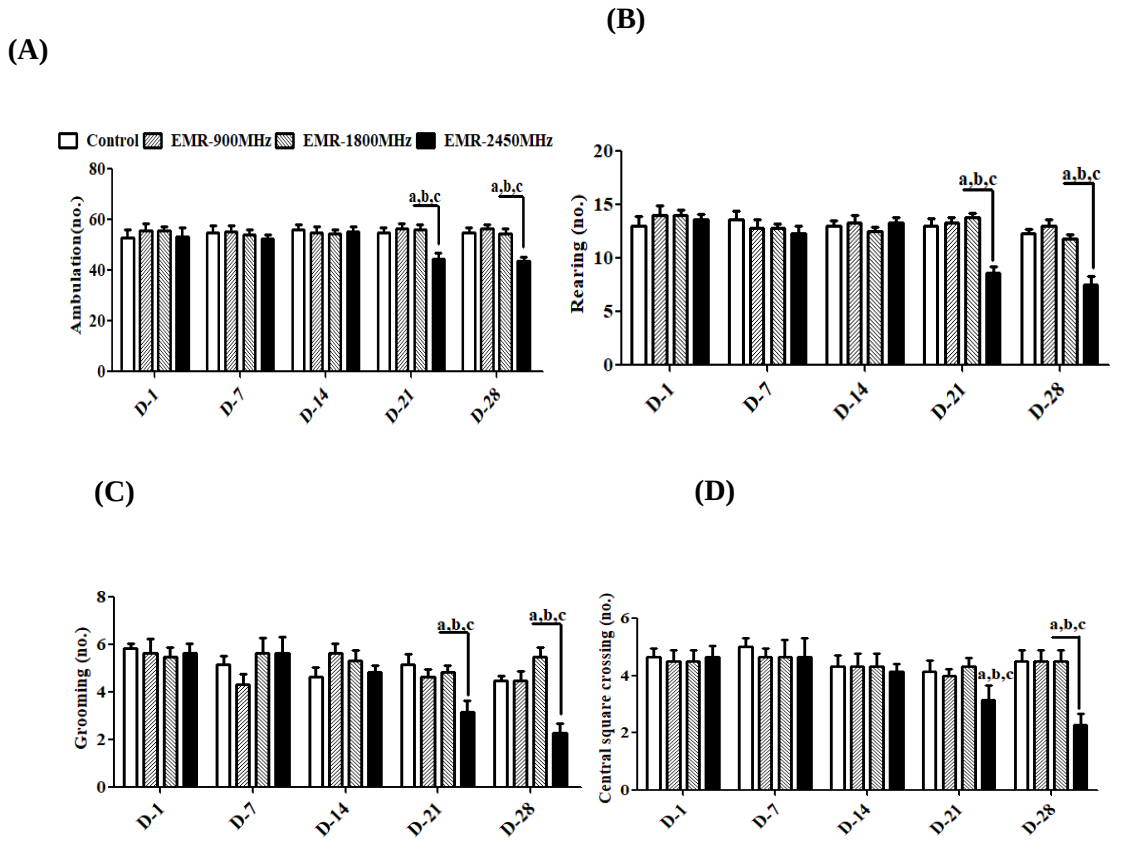


Figure 2.1.5.2

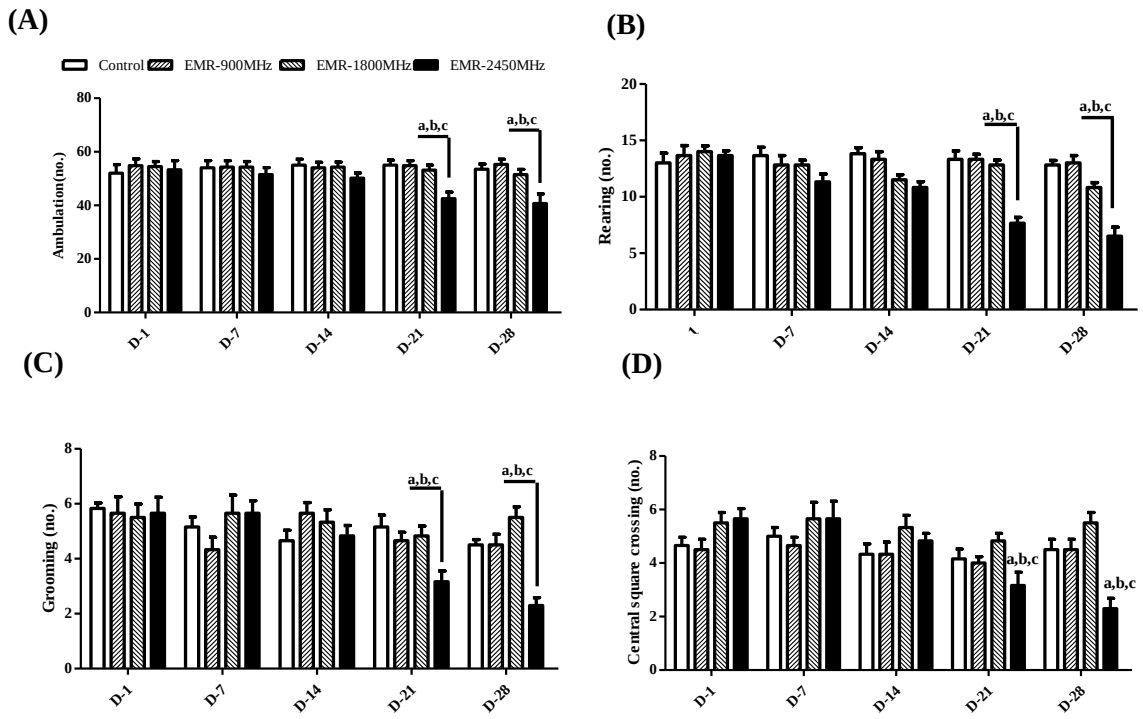


Figure: 2.1.5.1 and 2.1.5.2 Shows changes in **(A)** ambulation **(B)** rearing **(C)** grooming and **(D)** number of central square crossing in open field test. All values are expressed as mean±SEM., (n=6). $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to EMR-900 and $^{c}p<0.05$ compared to EMR-1800; $^{x}p<0.05$ compared to D-1, $^{y}p<0.05$ compared to D-7 and $^{z}p<0.05$ compared to D-14 [Two-way ANOVA followed by Bonferroni test].

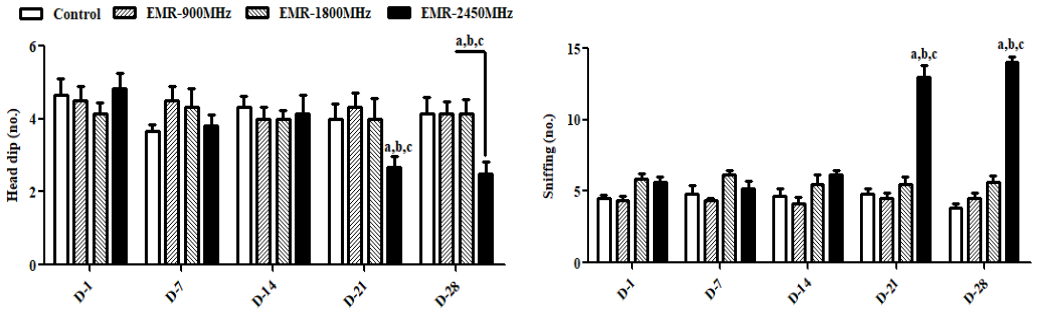
2.1.5.3 Repeated exposure of EMR exhibited anxiety-like behavior in Hole Board Test (HBT)

In hole board test, (A) Head dip, (B) sniffing (C) Head dip/sniffing and (D) number of squares crossed are parameters to evaluate anxiety like behavior in EMR-900, 1800, and 2450 MHz exposed rats (Figure 2.1.6.1). Statistical analysis by repeated measures two-way ANOVA depicted significant differences among groups in head dip, sniffing, head dip/sniffing and number of squares crossed [$F(3,100) = 5.502$; $p < 0.05$], [$F(3,100) = 103.6$; $p < 0.05$], [$F(3,100) = 29.39$; $p < 0.05$] [$F(3,100) = 4.57$; $p < 0.05$] respectively, time [$F(4,100) = 3.69$; $p < 0.05$], [$F(4,100) = 20.67$; $p < 0.05$], [$F(4,100) = 16.75$; $p < 0.05$] [$F(4,100) = 1.76$; $p < 0.05$] respectively and an interaction between groups and time [$F(12,100) = 0.87$; $p < 0.05$], [$F(12,100) = 24.19$; $p < 0.05$], [$F(12,100) = 7.94$; $p < 0.05$] [$F(12,100) = 2.256$; $p < 0.05$] respectively. Post hoc analysis demonstrated that on D-1, 7, 14, 21 and 28, EMR-900 and 1800 MHz did not alter the number of head dip, sniffing, head dip/sniffing and number of squares crossed in HBT. However, EMR (2450 MHz) decreased the head dip, sniffing, head dip/sniffing and number of squares crossed in HBT on D-21 to D-28 of experimental schedule when compared with control and EMR-900 and 1800 MHz exposed rodents. Similarly, in second set of this experiment showed in fig. 2.1.6.2, exposure of EMR-2450MHz decreased head dips, sniffing, head dips/sniffing and number of squares crossed during HBT [$F(3,100) = 7.90$; $p < 0.05$], [$F(3,100) = 86.12$; $p < 0.05$], [$F(3,100) = 32.12$; $p <$

0.05] [F (3,100) = 5.64; p < 0.05] respectively, time [F (4,100) = 5.99; p < 0.05], [F (4,100) = 34.22;p < 0.05], [F (4,100) = 15.; p < 0.05] [F (4,100) = 2.86; p < 0.05] respectively and an interaction between groups and time [F(12,100) = 2.066; p < 0.05], [F (12,100) = 25.83; p < 0.05], [F(12,100) = 7.19; p < 0.05] [F (12,100) = 2.00; p < 0.05] respectively.

Figure 2.1.6.1

(A) **(B)**



(C)

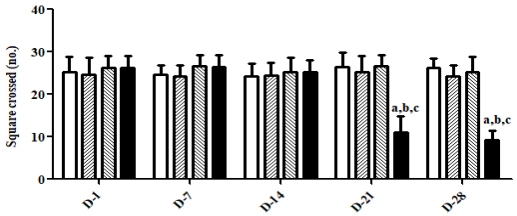


Figure 2.1.6.2

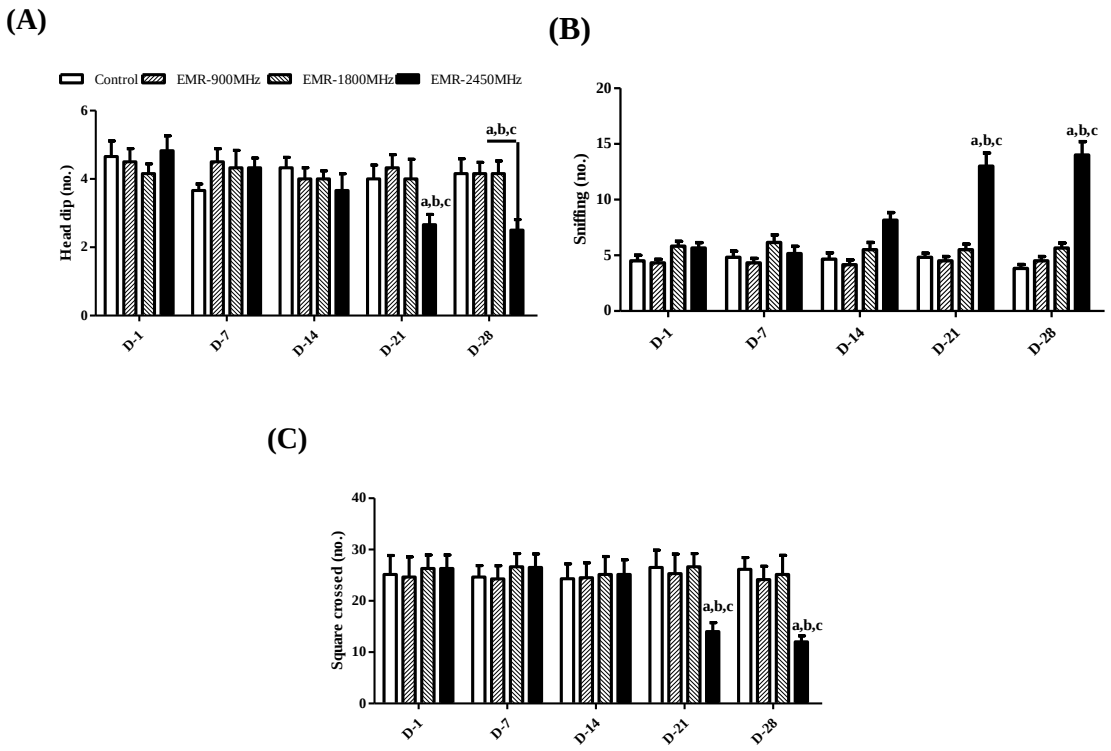


Figure 2.1.6.1 and 2.1.6.2 Represents changes in (**A**) Head dip (**B**) sniffing (**C**) and number of square crossed in hole board test. All values are expressed as mean±SEM, (n=6) $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to EMR-900 and $^{c}p<0.05$ compared to EMR-1800; $^{x}p<0.05$ compared to D-1, $^{y}p<0.05$ compared to D-7 and $^{z}p<0.05$ compared to D-14 [Repeated measure of Two-way ANOVA followed by Bonferroni test].

2.1.5.4 EMR-2450 MHz increased plasma corticosterone levels in experimental animals

Corticosterone is the major stress regulating hormone. Fig-2.1.7 depicts the changes in the level of plasma corticosterone in EMR- 900, 1800 and 2450 MHz exposed rats. Statistical analysis by one-way ANOVA showed significant difference in plasma corticosterone level [$F(3, 20) = 98.42$; $p < 0.05$] among groups. Post hoc analysis by Newman's Keuls showed that EMR (2450 MHz) exposure significantly increased the level of basal plasma corticosterone compared to rest of groups.

Figure 2.1.7

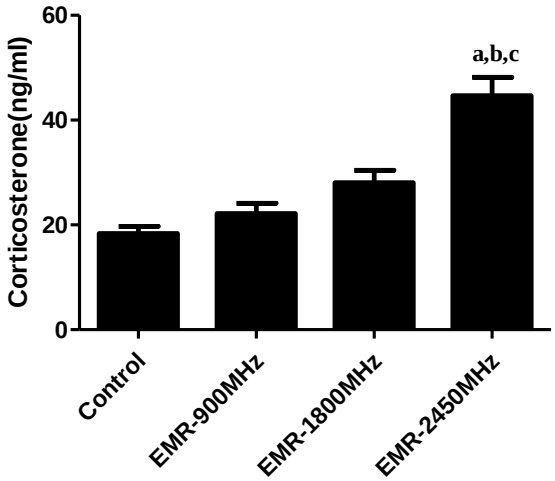


Figure 2.1.7 Shows level of corticosterone in the plasma. All values are expressed as mean±SEM, (n=6). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to EMR-900 and [c]$p<0.05$ compared to EMR-1800 [One-way ANOVA followed by Student–Newman Keuls test].

2.1.5.5 Correlation between plasma corticosterone and percentage arm entries in EMR subjected rats

Fig. 2.1.8 shows the statistical correlation between percentage of open arm entries in EPM and plasma corticosterone. There was a negative correlation between percentage

arm entries and plasma corticosterone (Pearson r =−0.9387 and r^2= 0.8796) in EMR-2450 MHz exposed rats. However, no correlation between plasma corticosterone and percentage arm entries was observed in control rats and in 900 and 1800 MHz-exposed groups.

Figure 2.1.8

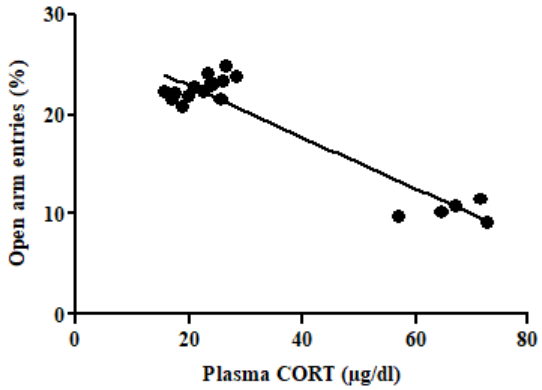


Figure 2.1.8 Effect of 2450MHz on pearson correlation between plasma corticosterone and percentage arm entries on 28th days. All results are expressed as 28th days data of both experiment (n=6) [Pearson correlation test].

2.1.5.6 EMR-2450 MHz decreased the mitochondrial membrane potential (MMP) in amygdalar region

The function of mitochondrial membrane potential is to maintain the physiology of the respiratory chain system leading to generation of ATP. Significant changes in the level of MMP results in attenuation of cellular energy with subsequent cell death. Fig. 2.1.9 shows the con-sequences of discrete range of EMR-900, 1800, and 2450 MHz exposure on MMP. One-way ANOVA showed that EMR-2450 MHz causes significant decrease in the level of MMP among groups [F (3, 8) = 11.94; $p < 0.05$]. Post hoc analysis showed significant decrease in MMP in EMR-2450 exposed rats compared to control.

Figure 2.1.9

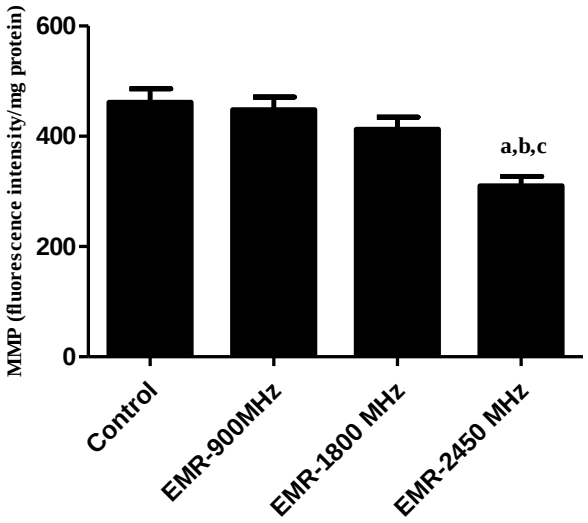


Figure 2.1.9 Shows alteration in MMP in amygdalar brain tissues. All values are expressed as mean±SEM., (n=4). [a]p<0.05 compared to control, [b]p<0.05 compared to EMR-900 and [c]p<0.05 compared to EMR-1800 [One-way ANOVA followed by Student–Newman Keuls test].

2.1.5.7 Effect of EMR-2450MHz on mitochondrial complex activities

Table 2.1.1 illustrates the mitochondrial complex activities (I, II, IV and V) in amygdalar tissue due to exposure to EMR. Statistical analysis by one-way ANOVA showed significant changes in complex activities (I, II, IV and V) among groups [$F(3, 8) = 12.68$, $p < 0.05$], [$F(3, 8) = 7.31$; $p < 0.05$], [$F(3, 8) = 22.23$; $p < 0.05$] and [$F(3, 8) = 11.30$; $p < 0.05$] respectively. Post hoc analysis showed that EMR (2450 MHz) caused decrease in amygdalar mitochondrial complex activities compared to control.

Table 2.1.1

S.No	Groups	Complex-I Activity (nM NADH 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	6.12±.146	0.373±.066	1.44±.087	11.86±1.327
2	EMR-900MHz	6.29±.347	0.383±.0302	1.41±.107	12.05±1.257
3	EMR-1800MHz	5.96±.269	0.336±.0116	1.28±.078	9.780±1.664
4	EMR-2450MHz	3.57±.553^{a,b,c}	0.116±.0302 ^{a,b,c}	0.516±.095^{a,b,c}	4.5±.526^{a,b,c}

Table: 2.1.1 shows mitochondrial complex activities in amygdala. 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].

2.1.5.8 EMR-2450 MHz decreased the catalase and superoxide dismutase activities in rats

Fig. 2.1.10 (A and B) illustrates the effect of EMR-900, 1800, and 2450 MHz on catalase and SOD activities in amygdala. There were significant differences in the levels of catalase and SOD among groups (A) catalase [$F (3, 8) = 4.24$; $p < 0.05$], (B) SOD [$F (3, 8) = 5.20$; $p < 0.05$]. Post hoc analysis demonstrated that EMR-2450 MHz causes significant decrease in catalase and SOD activities as compared to control.

Figure 2.1.10 (A) **(B)**

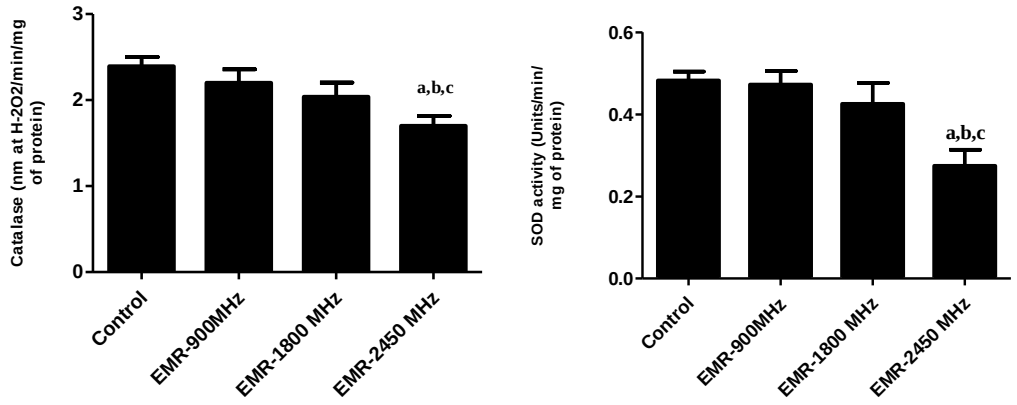


Figure: 2.1.10 shows the levels of (A) catalase and (B) SOD in the amygdalar brain tissues. All values are expressed as mean±SEM, (n=4). [a]p<0.05 compared to control, [b]p<0.05 compared to EMR-900 and [c]p<0.05 compared to EMR-1800 [One-way ANOVA followed by Student–Newman Keuls test].

2.1.5.9 Quantification of CRH-2 and GR receptors in amygdala

Corticotropin releasing hormone (CRH) synchronizes different stress responses. CRH evokes behaviors normally associated with stress and anxiety in rats. Decrease in the level of CRH-2 causes anxiety like symptoms in experimental animals. EMR (900, 1800 and 2450 MHz) exposed amygdalar tissues showed modulations in the levels of CRH-2 and GR as shown in Fig. 2.1.11. Statistical analysis showed that significant differences in CRH-2 and GR among groups [$F(3, 8) = 31.3$; $p < 0.05$] and [$F(3, 8) = 29.9$; $p < 0.05$]. Post hoc analysis demonstrated that EMR-2450 MHz decreased the expression of CRH-2 and GR compared to control, 900 MHz and 1800 MHz exposed rats.

Figure 2.1.11

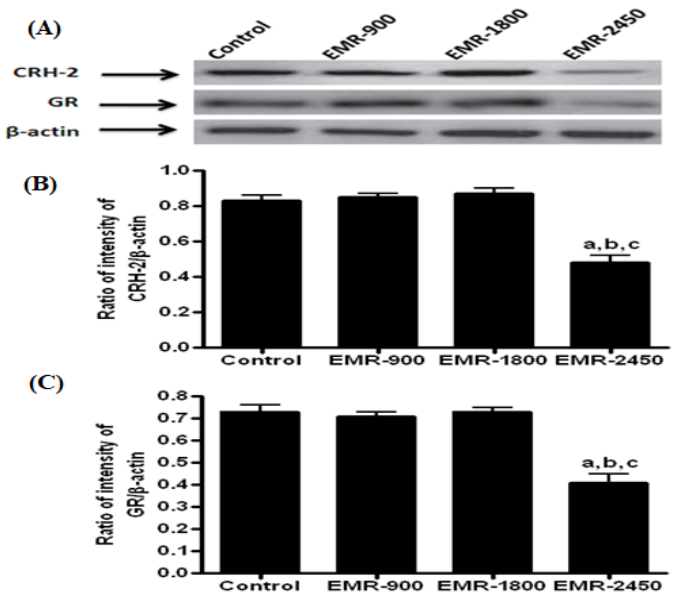


Figure: 2.1.11 (A) Shows histogram of CRH-2, GR and β-actin, (B) represents the ratio of CRH-2/ β-actin and (C) represents the ratio of GR/ β-actin. All values are mean ± SEM. $^{a}p<$ 0.05 compared to control, $^{b}p<$ 0.05 compared to EMR-900 group and $^{c}p<$ 0.05 compared to EMR-1800 group [One-way ANOVA followed by Student–Newman Keuls test].

2.1.5.10 Effect of EMR-2450 MHz on expression of cytoplasmic Bax, Bcl_2 and their ratio in amygdalar tissue of brain

Fig. 2.1.12 reveals the consequence of EMR- 900, 1800 and 2450 MHz exposure on expression of (A) Bax (B) Bcl_2 and (C) Bax: Bcl_2 ratio. Statistical analysis by one-way ANOVA showed significant changes among groups for expression of cytoplasmic Bax [$F(3, 8) = 31.3$; $p < 0.05$], Bcl_2 [$F(3, 8) = 10.1$; $p < 0.05$] and their ratio Bax: Bcl_2 [$F(3, 8) = 22$; $p < 0.05$]. Post hoc test showed that EMR-2450 MHz caused significant decrease in the expression of cytoplasmic Bax. However, EMR (2450 MHz) increased the expression of Bcl_2 and showed decreased in their Bax: Bcl_2 ratio compared with control.

Figure 2.1.12

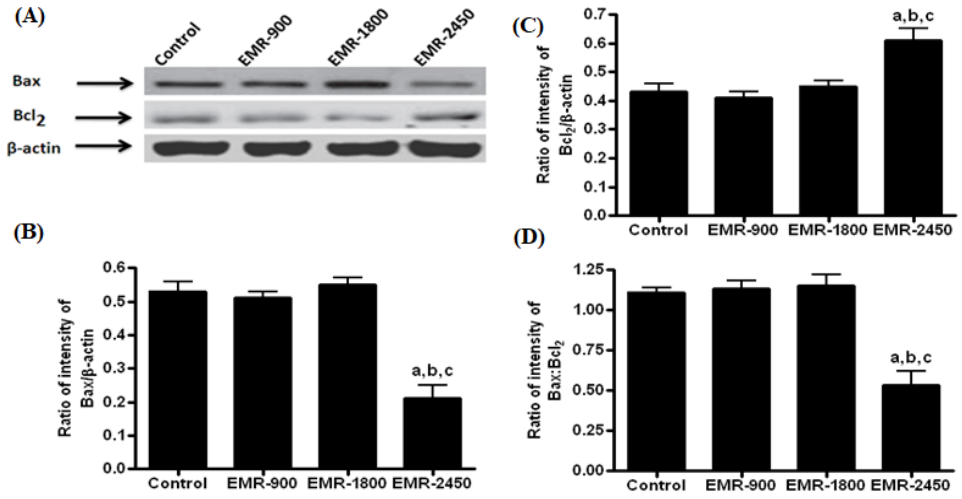


Figure 2.1.12 Shows (A) histogram of Bax, Bcl_2 and β-actin (B) ratio of intensity of Bax/ β-actin (C) ratio of intensity of Bcl_2/ β-actin (D) ratio of intensity of Bax/ Bcl_2 in amygdalar cytoplasm. All values are mean ± SEM. $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to EMR-900 group and $^{c}p< 0.05$ compared to EMR-1800 group [One-way ANOVA followed by Student–Newman Keuls test].

2.1.5.11 EMR-2450 MHz modulated the expression of mitochondrial Bax, Bcl_2 and their ratio in amygdalar tissue in brain

Outer mitochondrial membrane (OMM) is the primary site of action for apoptosis. The OMM proteins like Bcl_2 and Bax are responsible for maintenance of apoptosis. The loss of OMM integrity results in the conformational changes of Bax as well as Bcl_2 which eventually activates apoptosis. Fig. 2.1.13 demonstrates the effect of EMR- 900, 1800 and 2450 MHz exposed changes in the level of (A) Bax (B) Bcl_2 and their ratio (C) Bax: Bcl2. One-way ANOVA analysis indicated significant differences among groups for expression of Bax [$F(3, 8) = 10.4$; $p < 0.05$], Bcl_2 [$F(3, 8) = 27.5$; $p < 0.05$] and their ratio [$F(3, 8) = 48.4$; $p < 0.05$]. Post hoc analysis indicated that EMR (2450 MHz)

significantly decreased the expression of Bcl_2. Furthermore, EMR-2450 significantly increased the levels of Bax and their ratio Bax: Bcl_2 compared to control.

Figure 2.1.13

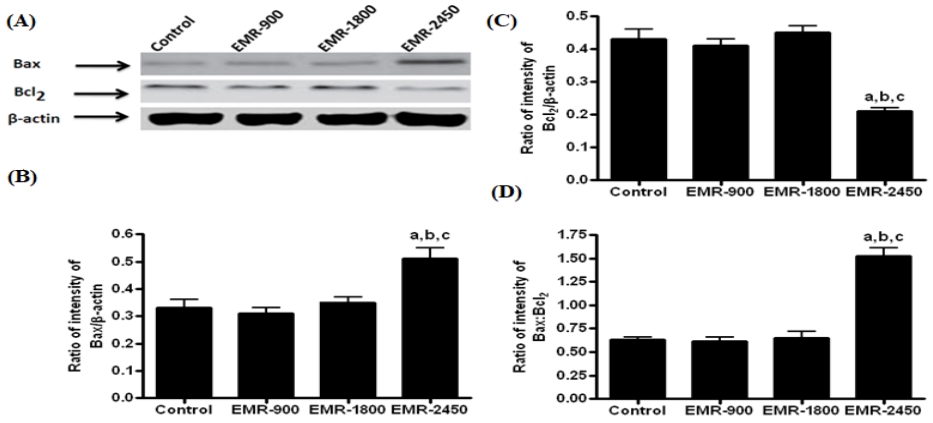


Figure: **2.1.13** Shows (A) histogram of Bax, Bcl_2 and β-actin (B) ratio of intensity of Bax/ β-actin (C) ratio of intensity of Bcl_2/ β-actin (D) ratio of intensity of Bax/ Bcl_2 in amygdalar mitochondria. All values are mean ± SEM. $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to EMR-900 group and $^{c}p< 0.05$ compared to EMR-1800 group [One-way ANOVA followed by Student–Newman Keuls test].

2.1.5.12 EMR-2450 MHz enhanced the expression of apoptotic protein in amygdala

Chronic cellular stress leads to the opening of outer mitochondrial membrane which release cytochrome C trigger caspase-9 in rats. Fig. 2.1.14 (A and B) shows the effect of EMR- 900, 1800 and 2450 MHz on protein expression of (A) Cytochrome-C and (B) caspase-9 in amygdalar tissue. Statistical analysis indicated that EMR-2450 MHz causes significant increase in expression of Cytochrome-C [$F (3, 8) = 35.8$; $p < 0.05$] and caspase-9 [$F (3, 8) = 16.7$; $p < 0.05$] among groups. EMR- 2450 MHz significantly increased the expression of Cytochrome-C and caspase-9 compared with the control

rats.

Figure 2.1.14

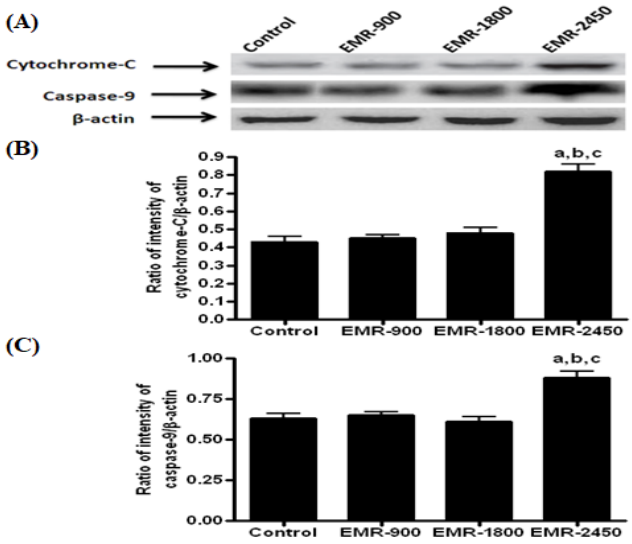


Figure: **2.1.14** Shows (A) histogram of cytochrome-c, caspase-9 and β-actin (B) ratio of intensity of cytochrome-c/ β-actin (C) ratio of intensity of caspase-9/ β-actin in amygdalar tissues. All values are mean ± SEM. $^{a}p< 0.05$ compared to control, $^{b}p< 0.05$ compared to EMR-900 group and $^{c}p< 0.05$ compared to EMR-1800 group [One-way ANOVA followed by Student–Newman Keuls test].

2.1.5.13 Histopathology

Hematoxylin binds to nucleic acid and imparts blue color, whereas eosin binds to cytoplasmic membranes and imparts red or pink color. The histopathological alterations in the amygdalar tissue are represented in Fig. 2.1.15 (A). EMR 2450 MHz caused decrease in number of neuronal cells and structural changes in amygdalar tissue indicating neurodegeneration. However, EMR 900 and 1800 MHz did not change the number of nuclei and structural changes in amygdala suggesting there was no neuronal damage. However, a remarkable decrease (73%) of neuronal cells was observed in the EMR-2450 MHz compared to control group. There was a significant difference in

number of neuronal cells between EMR-2450 MHz and control group as measured by One-way ANOVA [F (3, 8) = 34.62; p < 0.05] (Fig.2.1.15 B).

Figure 2.1.15

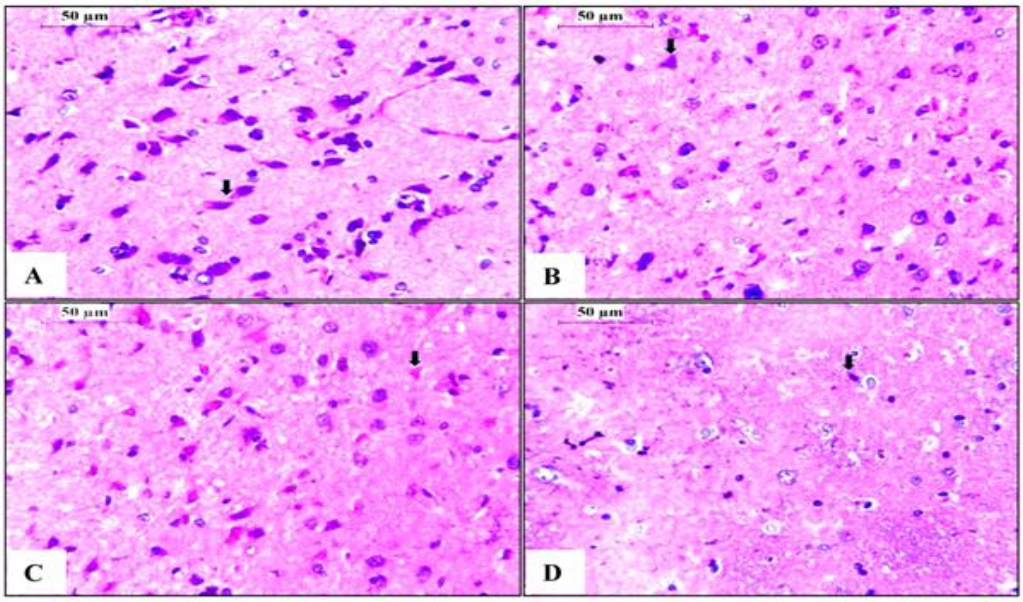


Figure: 2.1.15 (A) Shows changes in histopathology of amygdalar brain tissues (A) Control (B) EMR-900MHz (C) EMR-1800 MHz (D) EMR-2450 MHz having the value of magnification was 25x.

Figure 2.1.15 (B)

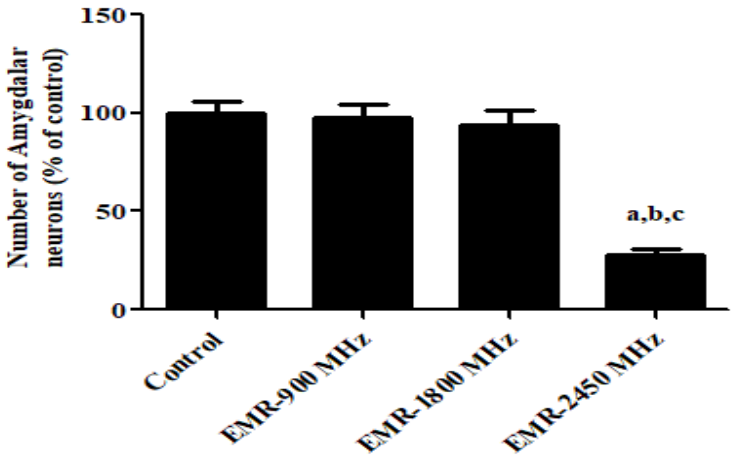
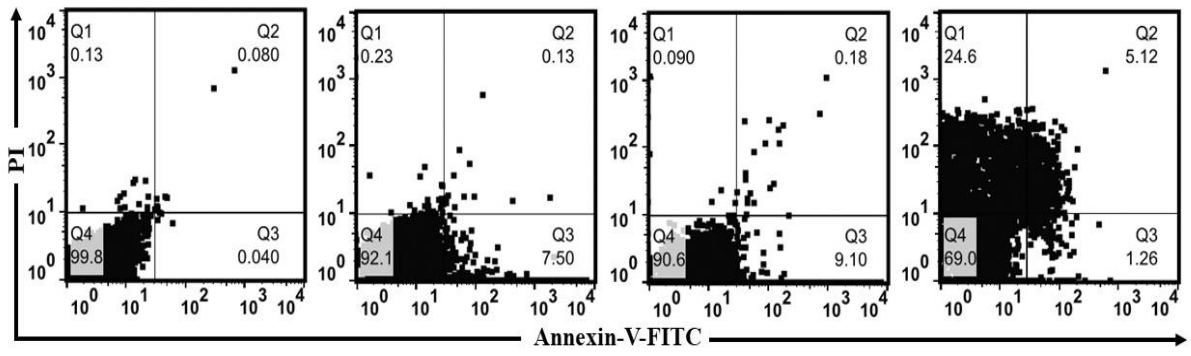


Figure 2.1.15 (B) Quantification value of neuronal damages. All values are mean ± SEM. [a]p< 0.05 compared to control, [b]p< 0.05 compared to EMR-900 group and [c]p< 0.05 compared to EMR-1800 group [One-way ANOVA followed by Student–Newman Keuls test].

2.1.5.14 Effect of EMR (900, 1800 and 2450 MHz) on the pattern of apoptotic and necrotic cells in amygdala

Fig. 2.1.16 represents the flow cytometric analysis. Quadrants are labeled as Q1, Q2, Q3 and Q4 which denotes necrosis, late apoptosis, early apoptosis and live cells respectively. Further, Fig. 2.1.16 shows the percentage of necrosis and apoptosis in amygdala in response to exposure with EMR (900, 1800 and 2450 MHz) on day 28. 24.6% of the amygdalar cells exposed to EMR-2450 MHz entered into necrosis (Q1) while 5.12% of total cell population entered into late apoptosis (Q2). However, EMR- 900 and 1800 MHz exposed cells mainly entered into early apoptosis (Q3).

Figure 2.1.16 (A)



(B)

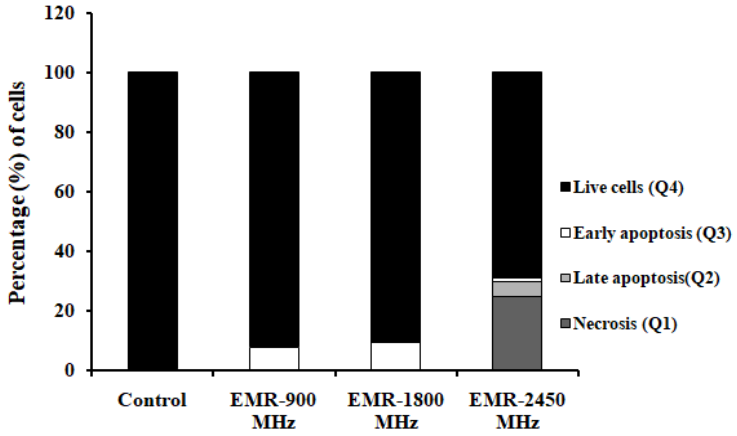


Figure: 2.1.16 (A) Represents the flow cytometric analysis. Quadrants are labeled as Q1, Q2, Q3 and Q4 which denote necrosis, late apoptosis, early apoptosis and live cells respectively, **(B)** shows the percentage of necrosis and apoptosis in amygdala in response to exposure with EMR (900, 1800 and 2450 MHz).

2.1.6 Discussion

In this study, we have shown that rats sub-chronically exposed to EMR at a frequency of 2450 MHz exhibited anxiety-like symptoms. Repeated EMR-2450 MHz exposure caused stress as observed by an increase in corticosterone levels, while the expression of CRH-2 and GR expression were significantly reduced in amygdala. EMR exposed animals showed alterations in the mitochondrial function and integrity. It also induced apoptotic factors leading to decrease of neuronal cells in the amygdalar region.

Various behavioral studies were done to assess the anxiety like symptoms in rats. The innate avoidance behavior in terms of locomotor activity was assessed using EPM, OFT and HBT (Garabadu and Krishnamurthy, 2014). EPM is commonly used for the

assessment of neurobiological disorder such as anxiety like behavior (Garabadu and Krishnamurthy, 2014). In the present study EMR-2450 MHz, but not 900 and 1800 MHz exposure significantly decreased the percentage of open arm entries as well as the time spent exploring the open arms. The decrease in open arm entry was observed only on D-21 and D-28. These results indicate that EMR-2450 MHz caused anxiety-like activity in experimental rats after long term exposure. Previous study has reported that EMR-2450 MHz (one day for 45 min) did not cause alterations in EPM for anxiety like behavior, which was observed with our protocol (Cosquer et al., 2005). However, in the present study, 28 days' exposure to EMR-2450 MHz in the present study, showed the development of anxiety like behavior in rats. The OFT is a widely used paradigm for the simultaneous assessment of ambulation, exploration and anxiety. The number of line crosses and the frequency of rearing are used as a measure of ambulatory activity and exploration (Walsh and Cummins, 1976). A decrease in the frequency of these behaviors are generally used as a measure of lower exploratory behavior, however they are also indicative of an anxiety like state within the animal (Smolinsky et al., 2009). EMR-2450 MHz exposed rats showed anxiety like behavior in terms of reduced ambulation and rearing in the OFT. However, there was no effect in rats exposed to EMR-900 and 1800 MHz. Grooming, a complex innate behavior is sensitive to stress conditions both in humans as well as rats. Rats exposed to various stressors display an increase in the amount of time spent grooming, and an impaired pattern of grooming behavior (Kalueff et al., 2016). EMR-900 and 1800 exposed rats did not have any effect on the grooming behavior. However, EMR-2450 exposed rats showed a decrease in grooming behavior. The anxiogenic effect of repeated exposure of EMR-2450 was seen in terms of decrease in the rearing, and number of line crosses, but not in terms of self-grooming. This finding is similar to earlier report on chronic

un-predictable stress reduced the number of grid crossings as well as rearing and grooming behavior in rats (Sestakova et al., 2013). Further, continuous exposure of EMR-2450 MHz showed decrease in central square crossing in OFT. Our results are in agreement with previous reports, where 9.417 GHz (two hours for 14 days) *in utero* exposure caused reduction in central square crossings in the OFT by mice (Zhang et al., 2014). The HBT is an experimental paradigm used to examine the anxiety like behavior in rodents. In HBT animals are placed on a square board provided with a number of holes and the animal is allowed to freely explore the board (File and Wardill, 1975). The number of head dips, edge sniffs and their ratio head dips/sniffing are used as parameters to assess anxiety like behavior in rodents (Casarrubea et al., 2009). The number of head dips is inversely proportional to anxiety state (Bilkei-Gorzo and Gyertyan, 1996; Boissieret al., 1964). This makes hole board test a good method to measure the anxiety like state. We observed that EMR-2450 MHz, but not 900 and1800 MHz exposure decreased the number of head dips, increased the edge sniffing and decrease their ratio head dip/sniffing behavior in rodents indicating anxiety like behavior. Locomotor activity is evaluated by the number of square crossings in the HBT which was significantly reduced and was comparable to decrease in locomotor activity observed in OFT. The above behavioral studies indicated the development of an anxiety-like behavior in EMR-2450 chronically exposed rats. However, a study has reported that single exposure to EMR-2450 (45 min) can activate neurons and up-regulate opoid and benzodiazepine receptors leading to an acute positive effect in anxiety (Lai, 1992). The contradiction to our results may be due to the difference in duration of the exposure employed in the study. In another study, repeated exposure to EMR-2450 MHz (24 h for 1 year) caused neurodegeneration in the brain (Dasdag et al., 2015). Chronic exposure to EMR increased baseline plasma

corticosterone levels, which is precursor of an organism's response to stress, anxiety and may be important for adaptogenic activity (Mostafa, Mostafa, & Ennaceur, 2002). Previous studies have reported that EMR 5 HZ exposed to rodent for 14 days caused hypercortisolism (Mahdavi et al., 2014). Correlation analysis showed negative relation between plasma corticosterone and percentage arm entries in the elevated plus maze paradigm of rats exposed to EMR 2450 MHz. Stress like condition with increase in corticosterone can induce anxiety-like symptoms (Ishikawa et al., 1992). There was no difference in corticosterone level in control rats subjected to behavioral analyses. Therefore, the plasma corticosterone was increased in rats owing to repeated exposure of EMR and not due to the behavioral tests.

Therefore, we can assume that continuous exposure with EMR 2450 MHZ induces stress-induced anxiety-like disorder in rats. CRH-2 is a part of the extra hypothalamic axis for stress induced anxiety. CRH-2 terminals and receptors are present in the amygdala and therefore it is considered as an important neuroanatomical area modulating anxiety (Coste et al., 2000; Jankord and Herman, 2008). Hence, any alteration in the density of CRH-2 receptors in amygdala exhibited stress induced anxiety like behavioral changes in the mice (Coste et al., 2000). We observed that continuous exposure to EMR-2450 MHz but not 900 and 1800 MHz showed a decrease in the expression of CRH-2 in rats, which could be accountable for the stress induced anxiety-like behavior. GRs are generally expressed in the amygdalar region (Schulkinet al., 2005) and can mediate changes in the HPA axis. The function of GRs is to play an important role in stress, emotion and fear processing in the amygdala (de Quervain et al., 2017). We here report that repeated exposure to EMR-2450 MHz radiation decreased the expression of GR, but EMR-900 and 1800 MHz did not change

the expression of GR in the amygdala of experimental rats. This might lead to stress induced anxiety like behavior in EMR-2450 MHz exposed rats. Previous reports have suggested that loss of mitochondrial function can cause basolateral amygdala dysfunction, which might lead to stress induced anxiety-like symptoms in experimental rats (Hollis et al., 2015).

Mitochondria, being the principal site for energy metabolism also largely generate oxidative radicals (Borutaite et al., 2013). Stress induced anxiety in rat caused functional changes like reducing complex activities and generation of reactive oxygen species in mitochondria (Hollis et al., 2015). In this study, EMR-2450 MHz, but not 900 and 1800 MHz reduced mitochondrial complex activities in the amygdala. EMR-2450 MHz caused a significant decrease in the levels of catalase and superoxide dismutase resulting in increase in the levels of hydrogen peroxide and decrease in the breakdown of O^{2-} (superoxide radical) in the amygdala. In contrast, there were no changes in the level of catalase and SOD in EMR-900 and 1800 MHz exposed rats. Previous experimental study suggested that chronic exposure to EMR-2450 MHz decreased the levels of catalase and SOD in hippocampus (Gupta et al., 2018; Hidisoglu et al., 2016). However, there were no significant change in oxidative stress markers in EMR-900 and 1800 exposed rodents. Hence, EMR-2450 MHz leads to oxidative stress and damage the amygdalar tissues in comparison to EMR-900 and 1800 MHz. Mitochondrial membrane potential (MMP) plays an important role in maintaining the cellular bioenergetics (Ott et al., 2007). In our study, repeated exposure to EMR-2450 MHz caused significant reduction in the MMP, whereas no change was observed with EMR-900 and1800 MHz. Previous studies have reported that exposure to microwave radiations (0.3 GHz–300 GHz) interfered with oxidative

phosphorylation in neurons that lead to alteration of the energy metabolism in the different brain regions (Hao et al., 2015). Therefore, chronic exposure of EMR-2450 MHz showed significant reduction in the level of MMP and hampered mitochondrial complex activities that lead to destruction of mitochondrial integrity as well as function. The intrinsic apoptotic pathway of mitochondria is synchronized by Bcl_2 (anti-apoptotic) and Bax (pro-apoptotic) proteins which regulate integrity of the mitochondria (Youle and Strasser, 2008). Dynamic equilibrium between cytosol and mitochondria are maintained by apoptotic factor Bax (mainly located in the cytoplasm). The changeover pattern of Bax causes alterations resulting in cell death (Dewson, 2015; Infante et al., 2013). In the present study, EMR-2450 MHz exposure significantly decreased the expression of cytosolic Bax, increased cytosolic expression of Bcl_2 and decreased the ratio of cytosolic Bax/ Bcl_2. In addition to this mitochondrial Bax expression was increased, while mitochondrial Bcl_2 expression decreased, and therefore increase in mitochondrial Bax/Bcl_2 ratio was observed. Previous reports have shown that continuous exposure with EMR-1950 MHz for 48 h caused significant increase in the level of Bax and decrease in Bcl_2 in mitochondria of astrocytes and induced apoptosis (Liu et al., 2012). It is noteworthy that there was increased mitochondrial Bax/Bcl_2 ratio indicating a decrease of mitochondrial integrity in proportion to the duration and frequency of exposure in the brain. Bax translocates to mitochondria indicating a decrease in mitochondrial membrane potential due to enhanced permeability of mitochondrial membrane that leads to mitochondrial swelling and the opening of mitochondrial transition pores (Monaco et al., 2015; Ow et al., 2008). Alterations in mitochondria transition pore lead to the release of cytochrome-c from the outer membrane of mitochondria to cytoplasm thereby activating caspase-9 (Vitagliano et al., 2013).

Current data demonstrated increase in expression of cytochrome-c and caspase-9 following exposure of EMR-2450MHz, both of which can lead to activation of intrinsic pathway of apoptosis. This apoptosis was confirmed by flow cytometric analysis. EMR-900 and 1800 exposed cells showed positive affinity to annexin-V in quadrant 3 and negative affinity to PI suggesting that cells were mostly in the early stage apoptosis. However, EMR-2450 MHz exposed cell exhibited both late stage apoptosis and necrosis as seen by higher affinity to PI in quadrant 1. Therefore, it is evident that necrosis and apoptosis could be important factors for altered function in amygdala of animals during prolonged EMR exposure. The decrease in neuronal cells due to EMR exposure was re-confirmed by histopathological studies. EMR 900 and 1800 MHz exposed rats had intact amygdalar structure and no significant decrease in neurons. However, EMR-2450 MHz exposure decreased the number of nuclei and visible cytostructural changes due to decrease in neuronal cells (73%). Therefore, chronic exposure of EMR-2450 MHz leads to neurodegeneration of amygdalar tissues. However, animals with damaged amygdala are generally more docile and less anxious (Anglada-Figueroa and Quirk, 2005). However, it should be noted that neural correlates of anxiety are highly complicated involving several brain regions.

Changes in the morphology of amygdala can in turn also change the functionality of other related brain regions (such as hippocampus) necessary for regulating anxiety behavior (Roozendaal et al., 2009). Apart from morphological evaluation the functioning of amygdala can also be affected by mitochondrial deregulation along with neuronal apoptosis, and may aggravate anxiety-like behavior (Khalifeh et al., 2017). This might be one of the possible explanations for the increase in anxiety observed in EMR-2450 MHz exposed rats.

2.1.6.1 Summary

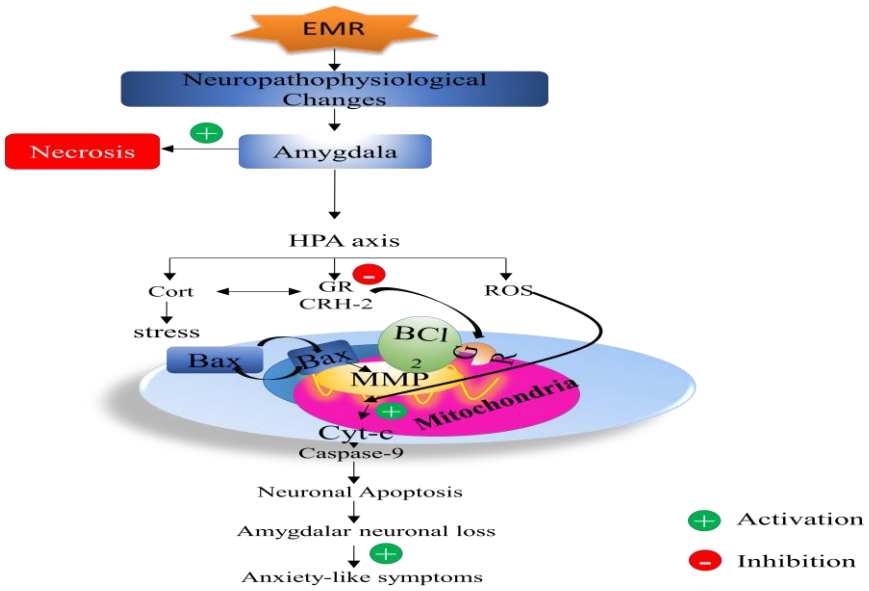


Figure 2.1.17 Summary of Hypothesis

Figure 2.1.17 shows that long term exposure to EMR-2450 MHz observed behavioral abnormalities and the neuronal changes. EMR at 2450 MHz decreased expression of GRs, CRH-2 and increased corticosterone level suggesting the direct effect on HPA axis. EMR 2450 MHz decreased the mitochondrial integrity, complex enzyme activities and increased oxidative stress in amygdala indicating mitochondrial stress. Moreover, altered expression of cytoplasmic and mitochondrial Bax and Bcl2 proteins indicates that there is a change in the turnover of the proapoptotic Bax between the mitochondrial outer membrane and cytoplasm which can result in apoptosis. Furthermore, an increased level of cytochrome-c in cytoplasm activates proapoptotic caspase-9 and subsequent cell death by apoptosis. Cell death resulted in change in the intact structure of amygdala as observed from histopathological studies. Therefore, long term exposure of EMR-2450 MHz may lead to development of stress induced anxiety-like behavior in experimental rats.