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Long-term exposure of 2450 MHz electromagnetic radiation induces stress and anxiety like behavior in rats

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ABSTRACT

Long term exposure of electromagnetic radiations (EMR) from cell phones and Wi-Fi hold greater propensity to cause anxiety disorders. However, the studies investigating the effects of repeated exposure of EMR are limited. Therefore, we investigated the effects of repeated exposure of discrete frequencies of EMR in experimental animals. Male rats were exposed to EMR (900, 1800 and 2450 MHz) for 28 (1 h/day) days. Long term exposure of EMR (2450 MHz) induced anxiety like behavior. It deregulated the hypothalamic pituitary adrenal (HPA) axis in rats as observed by increase in plasma corticosterone levels apart from decreased corticotrophin releasing hormone-2 (CRH-2) and Glucocorticoid receptor (GR) expression in amygdala. Further, it impaired mitochondrial function and integrity. The expression of Bcl_2 showed significant decrease while Bax and ratio of Bax: Bcl_2 were increased in the mitochondria and vice versa in cytoplasm indicating altered regulation of apoptosis. EMR exposure caused release of cytochrome-c and expression of caspase-9 ensuing activation of apoptotic cell death. Additional set of experiments performed to estimate the pattern of cell death showed necrotic and apoptotic amygdalar cell death after EMR exposure. Histopathological studies also revealed a significant decrease in neuronal cells in amygdala. The above findings indicate that long-term exposure of EMR radiation (2450 MHz) acts as a stressor and induces anxiety-like behaviors with concomitant pathophysiological changes in EMR subjected rats.

1. Introduction

Exposures to non-ionizing electromagnetic radiations (EMR) from the 3G cell phones and Wi-Fi have become an unavoidable part of human life. Cell phones and their base antennae produce electromagnetic radiations in the range of 900–1800 MHz for GSM communications. Furthermore, wireless local area network systems and 3G mobile phones employ radiations of 2450 MHz (Çiğ and Nazıroğlu, 2015). Such frequency radiations could have deleterious effects on public health. The cell phones, their base antennae and Wi-Fi devices continuously emit electromagnetic radiations having sufficient energy to change the spin states of atoms in molecules (Willard et al., 1988). Continuous exposures with such electromagnetic radiations can therefore lead to alterations in biological molecules leading to various disorders. Therefore, we used 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). Certain very low radiofrequencies however can have therapeutic effects in disorders such as pain whereby they activate opioid receptors (Tennant, 2009). Since, cell phone radiations are sufficiently high and are held close to the head during use, repeated exposures might affect neuronal functions in the brain (Croft et al., 2002). Earlier studies have reported that long-term exposure to EMR enhances the risk of neuropsychological disorders like anxiety (Jing et al., 2012; Pall, 2016). Exposure to EMR-2450 for 45 min activated the neuroendocrine system and secretion of endorphins, enkephalins and dynorphin which further caused activation of HPA axis, which might be one of the reasons for causing anxiety (Lai, 1992; van

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Bodegom et al., 2017). 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). Dejager et al. 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 amygadalar 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 cause 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. The exposure of EMF activates voltage gated calcium channels resulting in calcium influx and production of nitric oxide (NO). Nitric oxide can result in enhanced oxidative stress which can lead to mitochondrial dysfunctions. Further, NO can bind with heme group of cytochrome oxidase in the mitochondria resulting in a decrease in ATP synthesis which can be a possible reason for the mitochondrial dysfunction (Pall, 2018).

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 Bcl2 protein family reside 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 are no reports on mitochondrial dysfunctions associated with EMR linked stress and anxiety.

Therefore, the present study investigated the effects of repeated exposure of discrete frequencies of EMR on stress induced anxiety-like behavior in experimental rats. The levels 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 bcl2, 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.

2. Materials and methods

2.1. 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 animal 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).

2.2. Chemicals

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 reagent were procured from local supplier (Hi-media, Mumbai, India).

2.3. EMR exposure equipment and design

The exposure equipment was designed according to our previous study (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 interconnected waveguide 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.

2.4. Measurement of power density and specific absorption rate (SAR)

The power density calculated by the formulae described in earlier study (Gupta et al., 2018). The average power density was 0.1227 W/m^2. 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/m^2.

The calculation of SAR.

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).

2.5. Experimental design

All the rats were distributed into four different groups of six each. 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 a.m. and 1 p.m. for 1 h for 28 days beginning from D-1. 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 mazes (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). Amygdala was immediately micro-dissected (Paxinos and Ashwell, 2018; Paxinos and Watson, 1998) and stored at −80 °C for further analysis. 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, on the basis of earlier study, the rats were randomly divided into four groups: control, EMR-900, EMR-1800 and EMR-2450 each having six male rats with similar exposure schedule to EMR. Behavioral assessments were performed on day 1, 7, 14, 21 and 28 as described earlier. On D-28, animals were decapitated and immediately the amygdala was micro-dissected and stored at −80 °C for estimation. Histopathological study (n = 3) and flow cytometry for cell death pattern (n = 3) were done from amygdalar tissues.

2.6. Evaluation of behavioral parameter

2.6.1. 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 during next 5 min. Further, locomotor activity was measured in terms of total arm entries.

2.6.2. Open-field test paradigm

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).

2.6.3. 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 puts it's 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).

2.7. 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, 500 μL of plasma was extracted with 5 mL of dichloromethane (DCM). The dichloromethane extract was evaporated and eluted with 100 μL of the mobile phase (methanol: water) in the ratio (70:30). 20 μL 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).

2.8. Evaluation of mitochondrial membrane potential, complex activities and oxidative stress

2.8.1. Mitochondria isolation

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

2.8.2. 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 then 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 observation were expressed as fluorescence intensity/mg protein (Huang, 2002).

2.8.3. 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 λ) and emission (470 nm λ) (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 $mmol^{-1}$ 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 ml 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.

2.8.4. 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).

2.8.5. 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).

2.9. Western blot analysis

The amygdalar brain region was lysed with lysis buffer (containing optimum amount of protease inhibitor). The concentration of proteins were 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-Bcl_2 (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 antibodies according to the given proteins of interest further study was done by using our previous studies (Gupta et al., 2018).

2.10. 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 6 μm, 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.

2.11. 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, Thermofischer 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 1 μL 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 ScientificCarisbad, 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).

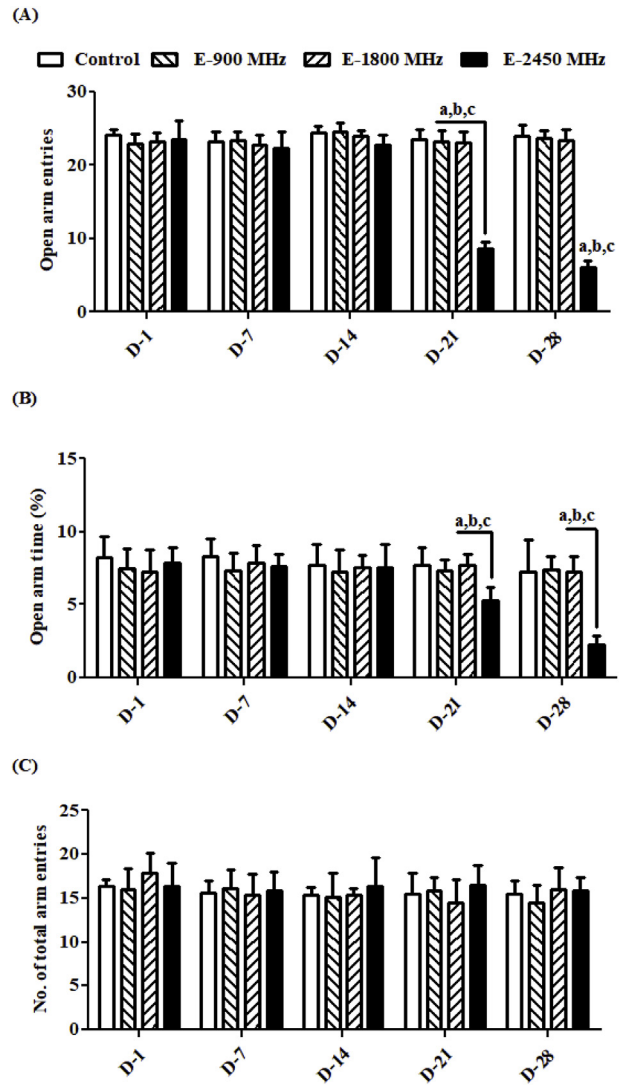

Fig. 1. The effect of EMR (900, 1800 and 2450 MHz) exposure on the **(a)** percentage in open arm entries **(b)** time spent and **(c)** number of total arm entries in EPM test. All results are expressed as mean ± SEM., (n = 6). $^aP < 0.05$, $^bP < 0.05$ and $^cP < 0.05$ compared to control, EMR-900 and EMR-1800 respectively. [Two-way ANOVA followed by Bonferroni post hoc test].

2.11.1. Statistical analysis

Experimental data are expressed as Mean ± standard error of mean (SEM). All the behavioral data were analysed using repeated measures two-way ANOVA followed by Bonferroni post hoc test. The data from molecular studies, plasma corticosterone levels and neuronal count were analysed using one-way analysis of variance (ANOVA) followed by Newman-keuls post hoc test. $P < 0.05$ was considered as statistically significant for all experimental data analysis.

3. Results

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

Elevated plus maze is a main paradigm to estimate the anxiety-like behaviour 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-1(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; $p > 0.05$] (data not shown).

3.2. Long term exposure of EMR caused behavioral changes in open field test (OFT)

Open field test is used to evaluate the locomotor activity in experimental rats. In Fig. 2 , 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, 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 (data not shown).

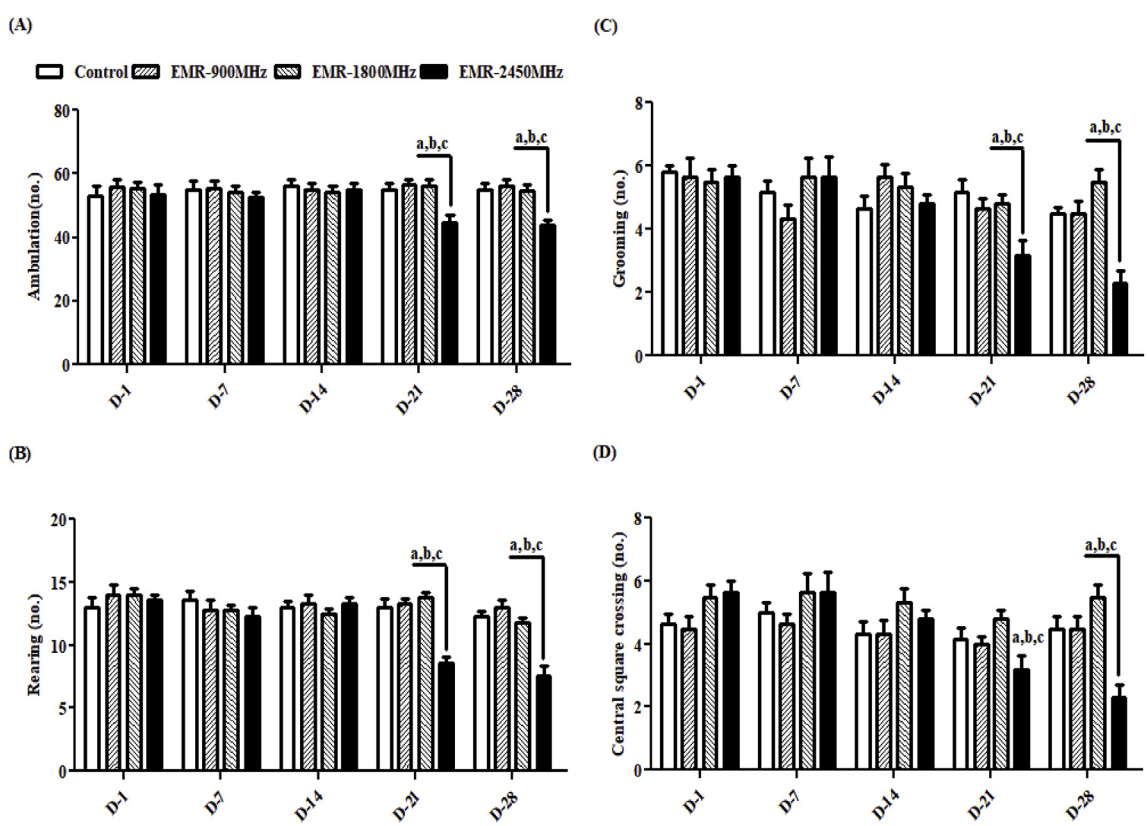

Fig. 2. The effect of EMR (900, 1800, 2450 MHz) exposure on **(a)** ambulation **(b)** rearing **(c)** grooming and **(d)** number of central squares crossed in Open Field Test. All results are expressed as mean ± SEM., (n = 6). $^{a}P < 0.05$, $^{b}P < 0.05$ and $^{c}P < 0.05$ compared to control, EMR-900 and EMR-1800 respectively. [Two-way ANOVA followed by Bonferroni post hoc test].

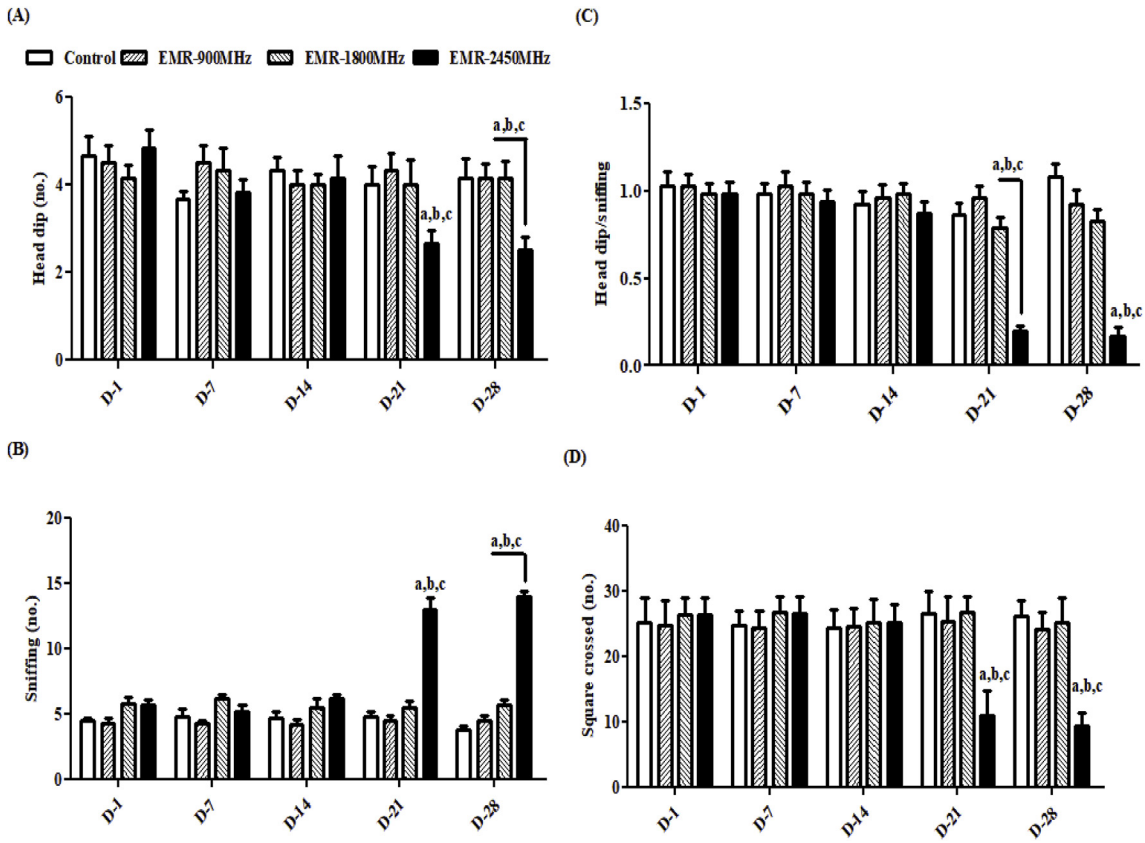

Fig. 3. The effect of EMR (900, 1800, 2450 MHz) exposed rats on **(a)** Head dip **(b)** sniffing **(c)** and number of squares crossed in the hole board test. All results are expressed as mean ± SEM., (n = 6) $^{a}P < 0.05$, $^{b}P < 0.05$ and $^{c}P < 0.05$ compared to control, EMR-900 and EMR-1800 respectively. [Two-way ANOVA followed by Bonferroni post hoc test].

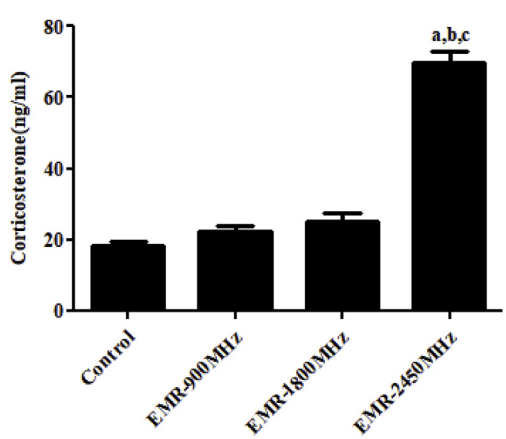

Fig. 4. Effect of EMR (900, 1800, and 2450 MHz) on the level of plasma corticosterone in the amygdalar brain tissue on day 28th. All results are expressed as mean ± SEM., (n = 6). $^{a}P < 0.05$, $^{b}P < 0.05$ and $^{c}P < 0.05$ compared to control, EMR-900 and EMR-1800 respectively. [One-way ANOVA followed by Student–Newman–Keuls post hoc test].

3.3. 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 (Fig. 3). 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, 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 (data not shown).

3.4. EMR -2450 MHz increased plasma corticosterone levels in experimental animals

Corticosterone is the major stress-regulating hormone. Fig-4 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,

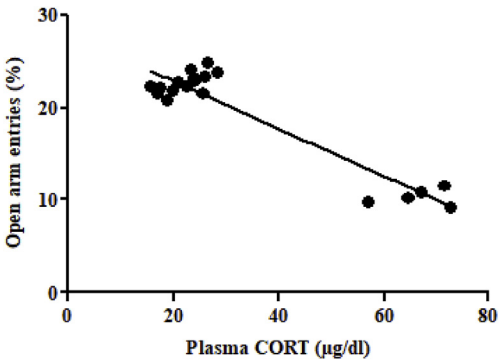

Fig. 5. Effect of 2450 MHz on Pearson correlation between plasma corticosterone and percentage arm entries on 28th days All results are expressed as 28th days data of both experiment, (n = 6). [Pearson correlation test].

20) = 98.42; $p < 0.05$] among groups. Post hoc analysis by Newmans Keuls showed that EMR (2450 MHz) exposure significantly increased the level of basal plasma corticosterone compared to rest of groups.

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

Fig. 5 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.

3.6. 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. 6 shows the consequences 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 signifcant decrease in MMP in

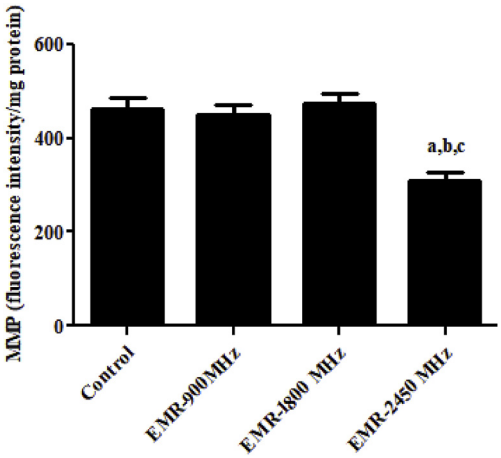

Fig. 6. The effect of various ranges of EMR (900, 1800, 2450 MHz) on MMP in amygdalar brain tissues. All results are expressed as mean ± SEM., (n = 3). $^aP < 0.05$, $^bP < 0.05$ and $^cP < 0.05$ compared to control, EMR-900 and EMR-1800 respectively. [One-way ANOVA followed by Student–Newman–Keuls post hoc test].

EMR-2450 exposed rats compared to control.

3.7. Effect of EMR-2450MHz on mitochondrial complex activities

Table 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 by Newman Keuls test showed that EMR (2450 MHz) caused decrease in amygdalar mitochondrial complex activities compared to control.

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

Fig. 7(A and B) illustrates the effect of EMR-900, 1800 and 2450 MHz on catalase and SOD activities in amygdala. There was a 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 control.

3.9. 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. 8. 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.

3.10. Effect of EMR-2450 MHz on expression of cytoplasmic bax, Bcl_2 and their ratio in amygdalar tissue of brain

Fig. 9 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.

3.11. 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. 10 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.

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

S.No.	Groups	Complex-I activity (nmolNADH oxidized/min/mg/protein)	Complex IIactivity (μ molformazan /min/mg/protein)	Complex-IV activity (nmol cytochrome c oxidized/min/mg/protein)	Complex V activity ATP hydrolysed protein
1	Control	6.12 ± 0.15	0.37 ± 0.07	1.44 ± 0.09	11.86 ± 1.33
2	EMR 900	6.29 ± 0.35	0.38 ± 0.03	1.41 ± 0.11	11.73 ± 1.10
3	EMR1800	5.96 ± 0.27	0.34 ± 0.01	1.28 ± 0.08	9.78 ± 1.16
4	EMR2450	3.57 ± 0.56[a,b,c]	0.11 ± 0.03 [a,b,c]	0.52 ± 0.09[a,b,c]	4.50 ± 0.53[a,b,c]

Values are Mean ± SEM (n = 3).
[a] $p < 0.05$ compared to control.
[b] $p < 0.05$ compared to EMR-900 group.
[c] $p < 0.05$ compared to EMR-1800 group compared to EMR-2450 group [one-way ANOVA followed by Student–Newman–Keuls test].

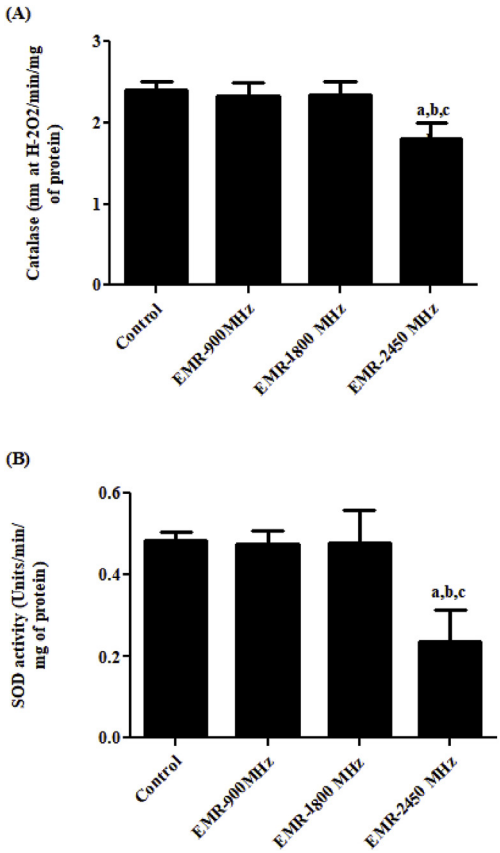

Fig. 7. Shows the effect of EMR (900, 1800 and 2450 MHz) exposed alterations in the activities of (A) Catalase and (B) SOD in the amygdalar brain tissues. All results are expressed as mean ± SEM, (n = 3). [a]P < 0.05, [b]P < 0.05 and [c]P < 0.05 compared to control, EMR-900 and EMR-1800 respectively. [One-way ANOVA followed by Student–Newman–Keuls post hoc test].

3.12. 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 and triggers caspase-9 in rats. Fig. 11(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 comapred with the control rats.

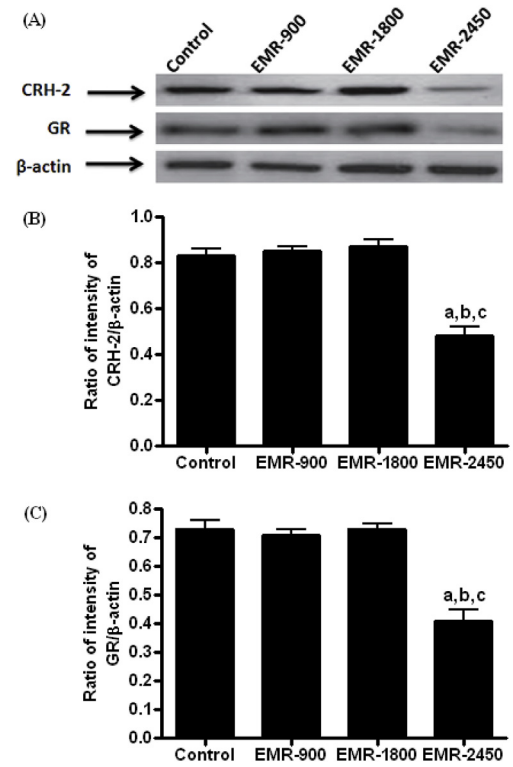

Fig. 8. The effect of EMR (900, 1800 and 2450 MHz) on amygdalar expression of CRH-2 and GR. All values are Mean ± SEM; (n = 3). [a]$p < 0.05$ compared to control, [b]$p < 0.05$ compared to EMR-900 group and [c]$p < 0.05$ compared to EMR-1800 group [one-way ANOVA followed by Student–Newman–Keuls test].

3.13. 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. 12. 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. 13).

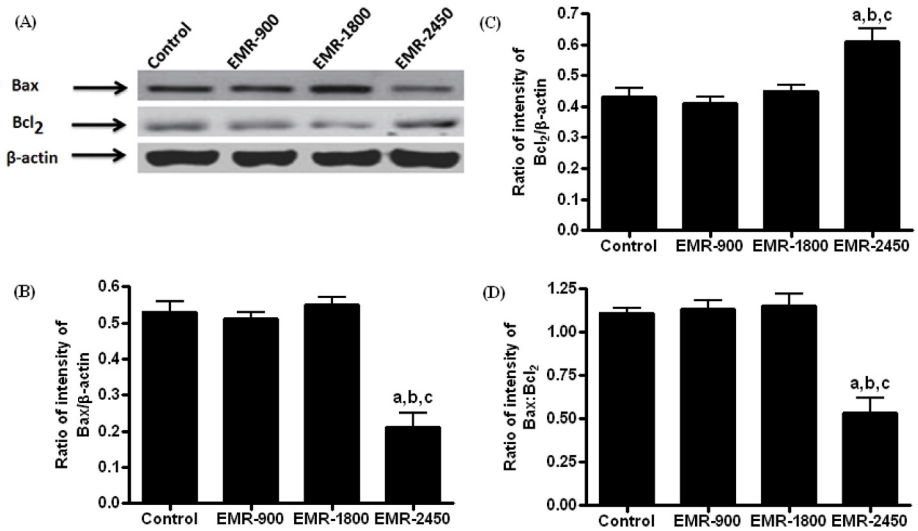

Fig. 9. EMR (900, 1800 and 2450 MHz) exposed alterations in the levels of cytoplasmic **(A)** Bax **(B)** bcl_2 and **(C)** Bax: bcl_2 in amygdala. All values are Mean ± SEM; (n = 3). $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to EMR-900 group and $^{c}p < 0.05$ compared to EMR-1800 group [one-way ANOVA followed by Student–Newman–Keuls test].

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

Fig. 14 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. 15 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).

4. 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 anxiogenic-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

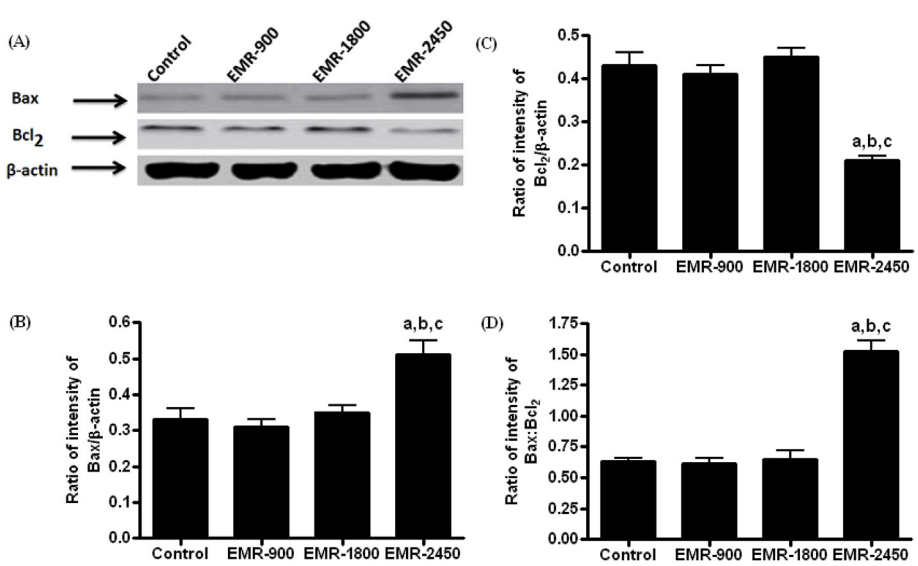

Fig. 10. Effect of EMR (900, 1800 and 2450 MHz) exposed animals on the levels of mitochondrial (A) Bax (B) bcl_2 and (C) Bax: bcl_2 in amygdala. All values are Mean ± SEM; (n = 3). $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to EMR-900 group and $^{c}p < 0.05$ compared to EMR-1800 group [one-way ANOVA followed by Student–Newman–Keuls test].

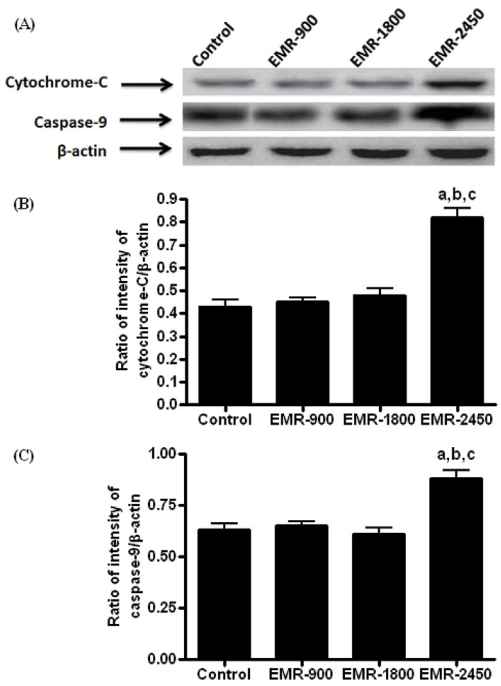

Fig. 11. The effect of EMR (900, 1800 and 2450 MHz) on the expression of (A) cytochrome-C and (B) caspase-9 in amygdalar brain tissues. All values are Mean ± SEM; (n = 3). $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to EMR-900 group and $^{c}p < 0.05$ compared to EMR-1800 group [one-way ANOVA followed by Student–Newman–Keuls test].

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 unpredictable 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

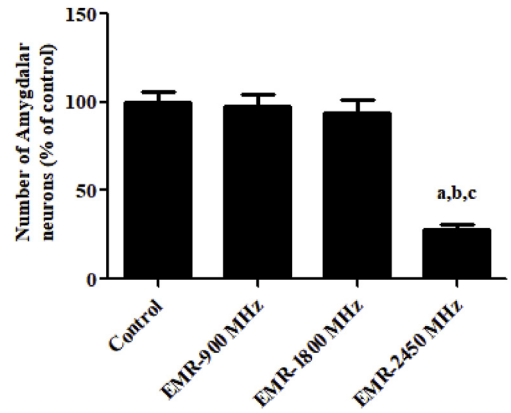

Fig. 13. EMR (900, 1800, 2450 MHz) exposure in rats shows changes in neuronal cells of amygdalar brain tissues (a) Control (b) EMR-900MHz (c) EMR-1800 MHz (d) EMR-2450MHz (n = 3). All values are Mean ± SEM; (n = 3). $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to EMR-900 group and $^{c}p < 0.05$ compared to EMR-1800 group [one-way ANOVA followed by Student–Newman Keuls test].

reports, where 9.417 GHz (two hour for 14 days) *in utero* exposure caused reduction in central square crossings in the OFT by mice (Zhang et al., 2014). The hole board test (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; Boissier et al., 1964). This makes hole board test a good method to measure the anxiety like state. We observed that EMR-2450MHz, but not 900 and 1800 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

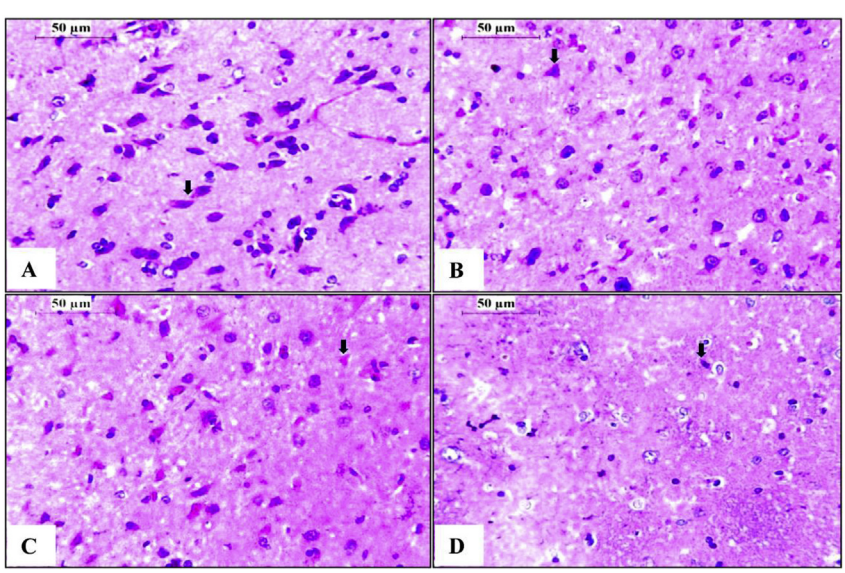

Fig. 12. The effect of EMR (900, 1800, 2450 MHz) exposed rats on histopathology of amygdalar brain tissues (a) Control, (b) EMR-900MHz, (c) EMR-1800 MHz and (d) EMR-2450MHz (n = 3). There is no significant difference between control and EMR-900, 1800 groups. EMR-2450 exposed rats shows a decrease in amygdalar neuronal cells (arrow). Original magnification (A, B, C and D) ×250.

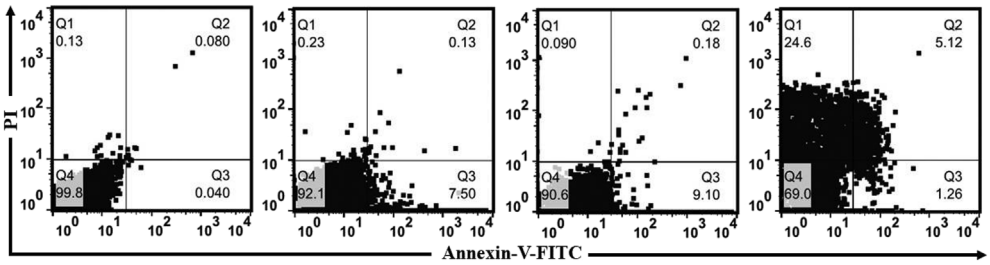

Fig. 14. Flow cytogram of EMR-induced cell death in the amygdala of brain assessed by flow cytometric analysis using annexin-V/PI. Speckle plots represent (a) control, (b) EMR-900 MHz, (c) EMR-1800 MHz and (d) EMR-2450 MHz. Each cytogram is divided into four quadrants: Upper left quadrant (Q1) shows annexin-V^{-ve} and PI^{+ve} cells. Upper right quadrant (Q2) represents both annexin V- FITC^{+ve} and PI^{+ve}. Lower right quadrant (Q3) shows annexin V-FITC^{+ve} and PI^{-ve}. Lower left quadrant (Q4) represents cells annexin-V^{-ve} and PI^{-ve}. Whereas, intensity of green fluorescence emerging from cell bound annexin V- FITC is indicated on the x-axis, intensity of red fluorescence by PI-stained cells is indicated on the y-axis.

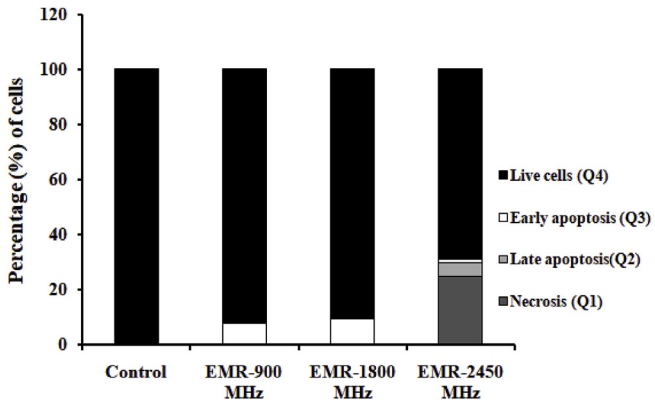

Fig. 15. Amygdalar cell death pattern of EMR (900, 1800 and 2450 MHz) exposed rats.

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-2450 MHz increased baseline plasma corticosterone levels, which is precursor of an organism's response to stress, anxiety and may be important for adaptogenic activity (Muruganandam et al., 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). 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 900,1800 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 (Schulkin et 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 and 1800 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 Bcl2 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 exposed rats. Further studies in this regard may throw light on the 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 Bcl_2 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 pro-apoptotic 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-2450MHz may lead to development of stress induced anxiety-like behavior in experimental animals.

Author's contribution

SK & SKG planned the study. SKP and SKS have done histopathology and interpretation of the result. Munendra Singh Tomar performed FACS experimental work and compiled data. MKM designed the model for EMR exposure system. SKG was responsible for data acquisition, interpretation of results and writing the manuscript. SK checked the manuscript and takes overall responsibility for publication.

Conflicts of interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.04.001.

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