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RESEARCH ARTICLE

Effect of mobile phone radiation on oxidative stress, inflammatory response, and contextual fear memory in Wistar rat

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Abstract

In the present lifestyle, we are continuously exposed to radiofrequency electromagnetic field (RF-EMF) radiation generated mainly by mobile phones (MP). Among other organs, our brain and hippocampus in specific, is the region where effect of any environmental perturbation is most pronounced. So, this study was aimed to examine changes in major parameters (oxidative stress, level of pro-inflammatory cytokines (PICs), hypothalamic-pituitary-adrenal (HPA) axis hormones, and contextual fear conditioning) which are linked to hippocampus directly or indirectly, upon exposure to mobile phone radiofrequency electromagnetic field (MP-RF-EMF) radiation. Exposure was performed on young adult male Wistar rats for 16 weeks continuously (2 h/day) with MP-RF-EMF radiation having frequency, power density, and specific absorption rate (SAR) of 1966.1 MHz, 4.0 mW/cm^2, and 0.36 W/kg, respectively. Another set of animals kept in similar conditions without any radiation exposure serves as control. Towards the end of exposure period, animals were tested for fear memory and then euthanized to measure hippocampal oxidative stress, level of circulatory PICs, and stress hormones. We observed significant increase in hippocampal oxidative stress ($p < 0.05$) and elevated level of circulatory PICs viz. IL-1beta ($p < 0.01$), IL-6 ($p < 0.05$), and TNF-alpha ($p < 0.001$) in experimental animals upon exposure to MP-RF-EMF radiation. Adrenal gland weight ($p < 0.001$) and level of stress hormones viz. adrenocorticotropic hormone (ACTH) ($p < 0.01$) and corticosterone (CORT) ($p < 0.05$) were also found to increase significantly in MP-RF-EMF radiation-exposed animals as compared with control. However, alteration in contextual fear memory was not significant enough. In conclusion, current study shows that chronic exposure to MP-RF-EMF radiation emitted from mobile phones may induce oxidative stress, inflammatory response, and HPA axis deregulation. However, changes in hippocampal functionality depend on the complex interplay of several opposing factors that got affected upon MP-RF-EMF exposure.

Keywords Mobile phone · Radiofrequency electromagnetic field · Hippocampus · Oxidative stress · Hypothalamic-pituitary-adrenal axis · Pro-inflammatory cytokines · Contextual fear conditioning

Introduction

Ever increasing use of mobile phones has brought a vast majority of population under the exposure of radiofrequency electromagnetic field (RF-EMF) radiation emitted from these devices, either intentionally or unintentionally. In major portion of the recent decade, mobile communication has been based on the 3G technology which uses Universal Mobile Telecommunications Systems (UMTS) and operates within the frequency band of 1900–2100 MHz (Kesari et al. 2014). Though the intensity of RF-EMF radiation emitted by cell phones are much below the limiting specific absorption rate (SAR) value of 0.08 W/kg for whole body exposure (ICNIRP 1998) to cause any thermal effect, scientific literature in recent times have shown that long-term exposure to these radiation can exert its adverse effect by non-thermal means (Foster and Glaser 2007). Reactive oxygen species (ROS)-mediated changes are the most convincing mechanisms proposed to

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explain non-thermal effects specifically in the case of brain (Yakymenko et al. 2016). In the brain also, hippocampus is the region which is most sensitive to stress including mobile phone radiofrequency electromagnetic field (MP-RF-EMF) exposure, as it plays a major role in regulation of hypothalamic-pituitary-adrenal (HPA) axis through glucocorticoid receptors (Sapolsky et al. 1984), involved in the immune modulation (Burton et al. 2011; Cibelli et al. 2010) and controls important stress-related behavioral and cognitive functions (Eyre et al. 2003; Lemaire et al. 2000). Any of the environmental perturbation including MP-RF-EMF exposure primarily affects HPA axis activity, and altered levels of circulatory glucocorticoids are the primary evidence of HPA axis deregulation (Goosens and Sapolsky 2007). However, reports regarding this are contradictory. Few studies suggest that long-term MP-RF-EMF radiation exposure may elevate the plasma corticosterone (CORT) level (Marino et al. 2001; Mostafa et al. 2002). While, others showed no change in basal level of adrenocorticotropic hormone (ACTH) and CORT upon exposure to MP-RF-EMF radiation (Prochnow et al. 2011). Moreover, elevated glucocorticoids have a role in immune modulation and production of inflammatory cytokines (Elenkov and Chrousos 1999; Tracey 2002). There are several studies regarding direct involvement of MP-RF-EMF exposure as well, in modulating immune response (Doyon and Johansson 2017). One such study observed significant alteration in levels of pro-inflammatory cytokines (PICs) (IFN-gamma, TNF-alpha, IL-6, and IL-2) upon long-term exposure (60 days, 2 h/day, 5 days/week) to different microwave frequencies (Megha et al. 2015), while another study demonstrated that acute MP-RF-EMF exposure cause an increased production of cytokine IL-1beta in the olfactory bulb of middle-aged rats (Bouji et al. 2012). When these PICs reach brain, crossing the blood-brain barrier, they set localized cytokine production by activating microglial cells (Wilson et al. 2013). One of the consequences of elevated level of PICs in the brain is increased generation of ROS and nitric oxide (NO) (Hu et al. 1998; Mokuno et al. 1994). There are several findings which have shown that PICs (IFN-gamma, TNF-alpha, and IL-1beta) increases ROS in microglial cells as well as in various other cell types (Brigelius-Flohé et al. 2004; Kaur et al. 2004; Mendes et al. 2003; Yang et al. 2007). There are a number of studies, which have reported MP-RF-EMF exposure-induced oxidative stress in brain at various SAR values (Avci et al. 2012; Chen et al. 2011; Shehu et al. 2016). In in vitro studies also, one among several such reports has shown that Rat1 and HeLa cells exposed to 875 MHz frequency at 0.07 mW/cm^2 power density generates ROS in extracellular environment by activating plasma membrane nicotinamide adenine dinucleotide (NADH) oxidases (Friedman et al. 2007).

All these factors (oxidative stress, cytokine response, and HPA axis deregulation) are known to have a role in modulation of hippocampus-dependent fear-related memories viz., contextual fear memory, emotional memory, passive avoidance behavior, etc. (Boitard et al. 2014; Shehu et al. 2016), owing to the existence of a rich network of both glucocorticoid and cytokine receptors within the hippocampus (Burton et al. 2011; Sapolsky et al. 1984; Ziegler and Herman 2002). Though the reports regarding direct influence of MP-RF-EMF exposure on contextual fear memory retrieval are very few, a large number of studies have shown contrasting effect of cell phone frequency exposure on other hippocampus-dependent behavioral parameters. Kumlin et al. (2007) reported enhanced spatial learning upon exposure to 900 MHz frequency for 5 weeks (2 h/day, 5 days/week), whereas Saikhedkar et al. (2014) have found that exposure to 900 MHz for 15 days (4 h/day) induces learning and memory deficits as well as hippocampal neuronal degeneration in rats. There are numerous reports showing MP-RF-EMR radiation-induced learning and memory impairment (Bouji et al. 2012; Hao et al. 2013; Li et al. 2016; Narayanan et al. 2015; Ntzouni et al. 2011; Paulraj and Behari 2004; Petitdant et al. 2016), whereas several other have shown either positive or no effect on memory upon MP-RF-EMF exposure (Arendash et al. 2010; Dubreuil et al. 2003; Koivisto et al. 2000; Maier et al. 2004; Nittby et al. 2008).

All these studies indicate that effect of MP-RF-EMF exposure could possibly be similar to other environmental stressors and can induce generalized pattern of alteration in the hippocampus. However, none of the previous studies investigated effect of chronic exposure to MP-RF-EMF radiation on all major parameters linked to hippocampal functionality in a concerted way. In this regard, the objective of this study was to simultaneously determine whether long-term (16 weeks) exposure to MP-RF-EMF radiation induces hippocampal oxidative stress, inflammatory response, and HPA axis deregulation and affects contextual fear memory in Wistar rats.

Material and methods

Animals

Six-week-old male Wistar rats (165–185 g) were obtained from the Central Laboratory Animal Resources, Jawaharlal Nehru University-New Delhi and housed in groups of two animals per cage in an experimental animal house. Ambient environment was maintained at 25 °C room temperature and 12:12 h day/night cycle. Food and water were provided *ad libitum*. Experimental animals were randomly assigned to two independent groups, one control and another exposed with six animals in each group. Animals of both groups were habituated to exposure cage for 1 week prior to start of exposure to avoid procedure-related stress. Body weight of animals were taken every month and also at the end of the exposure

period. Prior approval was taken (IAEC; Code no. 28/2014) for all experimental procedures performed in this study from the institutional animal ethical committee of Jawaharlal Nehru University, New Delhi, India.

RF exposure procedure

Animals were exposed to MP-RF-EMF radiation in a specially designed exposure setup (Fig. 1), with slight modifications from our previous study (Gautam et al. 2018). Briefly, rats were placed in a plexiglass cage individually with cages itself placed in an anechoic chamber (150 cm l × 115 cm w × 130 cm h) to rule out the possibility of interference by ambient electric field. Each plexiglass cage was ventilated with holes of 1 mm diameter and spacious enough to keep the animal stress free while being exposed. Individual cages were separated from each other by radar-absorbing material to minimize interference due to radiation from adjacent phones. Exposure was performed using a Samsung mobile phone operating in UMTS (1915 MHz) band kept horizontally on plexiglas cage at the receiving end of video calling, with audio muted. Since the distance between mobile phone and rat (3 cm) is less than the wave length of emitted frequency (15.26 cm) and mobile phones are considered a near-field source (Lauer et al. 2013), exposure in the present study meets the criteria for near field exposure. Timing of the exposure was 9:00 to 11:00 am daily for 16 weeks (2 h/day). Control rats were kept exactly in the same condition but mobile phone in switched off mode. Near-field receiving frequency and emitted power was measured in both video-calling mode and switched off condition of mobile phone at different positions inside the cage using a monopole probe attach to a spectrum analyzer (N9912A, FieldFox, Agilent technology, USA). Receiving frequency and power density inside the cage (video calling mode) were 1966.1 MHz and 4.0 mW/cm^2, respectively. Ambient power density (switched off mode) comes out to be 0.00001 mW/cm^2. SAR value was calculated using mathematical expression of average SAR for incident power density of 1 mW/cm^2 over the whole frequency range of interest for a prolate spheroidal model animal connected to a ground plane by a resistive connection (Durney et al. 1979). In this case, Wistar rats were considered prolate spheroid with length and width of animals taken as semi-major and semi-minor axes, respectively. The calculated average whole body SAR value was 0.36 W/kg.

Contextual fear conditioning

Animals in both control ($n = 6$) and exposed ($n = 6$) groups were subjected to contextual fear conditioning test on 106th day of exposure for 5 days of fear conditioning protocol. Exposure protocol was followed as earlier during this period. Behavioral experiments for all the animals were always performed between 3:30 and 4:30 pm to avoid diurnal rhythm effect. Contextual fear conditioning test was performed according to protocol described earlier (Pawlyk et al. 2005) with slight modification. Briefly, on the first day of experiment, rats were kept in shock chamber for 30 min with 20 Lux light, exhaust (on), and husk without fragrance constitute the context (context 1). On the 2nd and 3rd day, rats were conditioned in the same chamber in the same context (context 1) for 5 min. On the 4th day, rats were trained in the same chamber for 5 min with different context (context 2), viz. 80 Lux light, exhaust (off), and husk with sandalwood fragrance (0.6 ml)



Fig. 1 Image of exposure setup showing Wistar rats exposed to mobile phone radiofrequency electromagnetic field (MP-RF-EMF) radiation. AC, anechoic chamber; RAM, radar absorbing material; EF, exhaust fan; MP, mobile phone; PC, plexiglass cage

spread evenly. Rats were handled and brought to the training room by a different person and different route to avoid contextual reminders. After placing rats in the chamber, three electric foot shocks of 800 μA for duration of 2 s each was delivered at the interval of 1 min through grid floor. First shock was given at 2 min, second at 3.2 min, and third at 4.4 min after placing the rat in the shock chamber. Foot shock was given using a software-controlled protocol (freeze frame). On the 5th day, rats were placed in the same shock chamber with the same context as in training (context2) in the absence of shock for a 5-min context test. Freezing response (percentage freezing time with respect to total time) was recorded for each experimental session by the same software (Freeze Frame, Coulbourn Inc., USA) laced with a CCTV camera (WV-BP334, Panasonic, USA). Freezing response during 2nd and 3rd day was taken as baselines 1 and 2, respectively. Increased percentage freezing on the testing day with respect to baseline day was taken as a measure of learning.

Sample preparation for biochemical and immunological assays

Animals were euthanized after 1 day of contextual testing and just after the last exposure to avoid effect of behavioral experiment. Sodium thiopentone was used as anesthetic. Immediately after euthanizing the animals, blood was collected through cardiac puncture and further brain and adrenal gland were dissected out and weighed accurately. The hippocampus region was separated using the method of Glowinski and Iversen (1966) and weighed. Separated hippocampal tissue was homogenized in a Teflon/glass homogenizer (RU56, Remi, India) with addition of 1:10 (*w*/*v*) 0.1 M PBS (10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, and 1.8 mM KH_2PO_4, pH 7.4) supplemented with 1 mM EDTA. Supernatant was recovered by centrifugation for 10 min at 10,000 rpm, 4 °C (5418 R, Eppendorf, Germany). For serum separation, blood collected in the Eppendorf tubes were made to stand undisturbed for 30 min at room temperature and then centrifuged at 4000 rpm for 20 min at 4 °C. All the samples were stored at − 80 °C until experimental analysis. Chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemicals, USA.

Oxidative stress markers

ROS estimation

The amount of oxygen-centered free radicals in the hippocampus was measured spectro photometrically using *N*,*N*′-diethyl-1,4-phenylenediamine (DEPPD) according to the protocol described earlier (Hayashi et al. 2007). A calibration curve for the hydrogen peroxide (H_2O_2) standard was prepared from optical density graph. ROS levels in the hippocampal tissue homogenate was estimated and expressed in terms of H_2O_2 equivalents (1 unit = 1.0 mg H_2O_2/L).

Lipid peroxidation assay

The level of lipid peroxidation in the rat hippocampus was estimated according to the method described by Ohkawa et al. (1979) with minor modifications. Amount of malondialdehyde (MDA) (a byproduct of lipid peroxidation) in the hippocampal tissue was estimated through thiobarbituric acid (TBA) reaction and termed as thiobarbituric acid reactive substances (TBARS). Quantification of TBARS in hippocampal tissue homogenate was done spectrophotometrically (UV-Vis spectrophotometer, Shimadzu Corp., Japan) at 532 nm using molar extinction coefficient of MDA (1.56×10^{-5} nmol/cm) and presented as nanomoles malondialdehyde equivalent per milligram of protein.

Protein carbonyl estimation

Protein carbonyl content, a measure of total oxidized proteins, in rat hippocampus was determined spectrophotometrically using 2,4 dinitrophenyl hydrazine (DNPH) as chromogenic agent. Concentration of carbonyl derivatives were calculated using molar extinction coefficient of DNPH at 370 nm (22×10^3 mol/cm) and expressed in the unit of nanomoles per milligram of protein (Dubey et al. 1996).

Ferric reducing ability of plasma assay

Ferric reducing ability of plasma (FRAP) assay is a method to estimate total antioxidant capacity of biological samples. In the present study, FRAP assay was performed using protocol of Benzie and Strain (1996) with some minor modifications. A calibration curve was prepared from Fe^{2+} solution of known concentration. The total antioxidant capacity of the hippocampus tissue homogenate was calculated with respect to reducing capacity of Fe^{2+} solution at 593 nm and presented as micromole equivalents of Fe^{2+} per gram of tissue.

Estimation of PICs

PICs (IL-1beta, IL-6, and TNF-alpha) were measured in blood serum using enzyme-linked immunosorbent assay kits (Thermo Fisher Scientific, USA; IL-1beta: KMC0011, IL-6: ER3IL6, IFN-gama: ESS0020 and TNF-alpha: KMC 3011) according to the method specified by manufacturer. Concentration of cytokines were expressed in unit of picograms per milliliter (pg/ml) of serum.

Estimation of CORT and ACTH

CORT and ACTH estimation was done according to basics given earlier (Melmed et al. 2011). Briefly, basal serum ACTH was determined by electrochemiluminescence immunoassay "ECLIA" using Elecsys and Cobas immunoassay analyzers (Roche diagnostics). Assay sensitivity was 2 pg/ml. Basal serum CORT level was also determined by ECLIA. Measuring range of Elecsys and Cobas immunoassay analyzers for serum CORT was 0.036–63.4 μg/dl.

Statistical analysis

Statistical analysis of all the experimental data was done using Graphpad Prism software version 6.1. Two-tailed unpaired *t* test was applied to test statistical significance of data. Results were expressed as mean ± standard error of the mean (SEM). Data for percentage increase in body weight was analyzed using one-way ANOVA followed by Bonferroni's post hock test.

Results

Body and brain weight

Time-dependent increase in body weight (expressed as percentage increase as compared with initial weight) was not getting affected in MP-RF-EMF-exposed animals as compared with the control after 1st, 2nd, 3rd, and fourth month of exposure (Fig. 2a). Change in brain coefficient (weight as percent of body weight) was not significant as well (Fig. 2b).

Oxidative stress markers

The levels of ROS in the hippocampus of mobile phone radiation-exposed animals were significantly higher ($p < 0.001$) as compared with the control animals (Fig. 3a). The ROS level was increased by 80% in the hippocampus of exposed group as compared with the control ones. Similarly, the level of lipid peroxides, a measure of oxidative damage to lipids, showed a significant increase in the hippocampus of exposed groups ($p < 0.05$) as compared with the control (Fig. 3b). Lipid peroxidation level was increased by 24% in the hippocampus of exposed group as compared with the control ones. Chronic exposure to MP-RF-EMF radiation also resulted in high level of oxidized protein in the hippocampus ($p < 0.01$) (Fig. 3c). The protein carbonyl content in the hippocampus of exposed group was increased by 37% of the control. Total antioxidant capacity of the hippocampus was also decreased significantly ($p < 0.05$) in exposed group as compared with the control group (Fig. 3d).

Level of PICs

To check whether chronic exposure to mobile phone radiation generates any innate immune response as in the case of other common stressors, levels of PICs such as IL-1beta, IL-6, and TNF-alpha were measured in serum. Levels of all these three PICs were found to be significantly upregulated in exposed animals as compared with the control ones (Fig. 4a–c).

Level of stress hormones (ACTH and CORT) and adrenal coefficient

The increased adrenal coefficient or adrenal hypertrophy was known to be an indicator of HPA axis deregulation, and it is

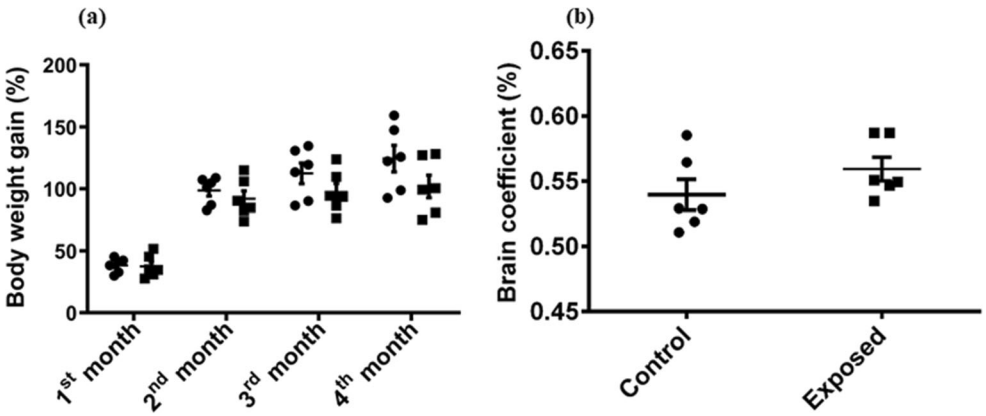


Fig. 2 Exposure to MP-RF-EMF radiation of 1966.1 MHz frequency for 16 weeks (2 h/day) does not significantly alter body weight and brain weight in the experimental animals ($n = 6$/group). **a** Body weight gain, expressed as percentage increase in weight with respect to initial weight after 1st, 2nd, 3rd, and 4th month of exposure and **b** brain weight, expressed as percentage of body weight also called as brain coefficient. Read out of individual data points from the control (circles) and exposed (squares) experimental groups are presented. One-way ANOVA followed by Bonferroni's post hock test and unpaired *t* test was used to calculate *p* values. Data represented as mean ± standard error of the mean (SEM)

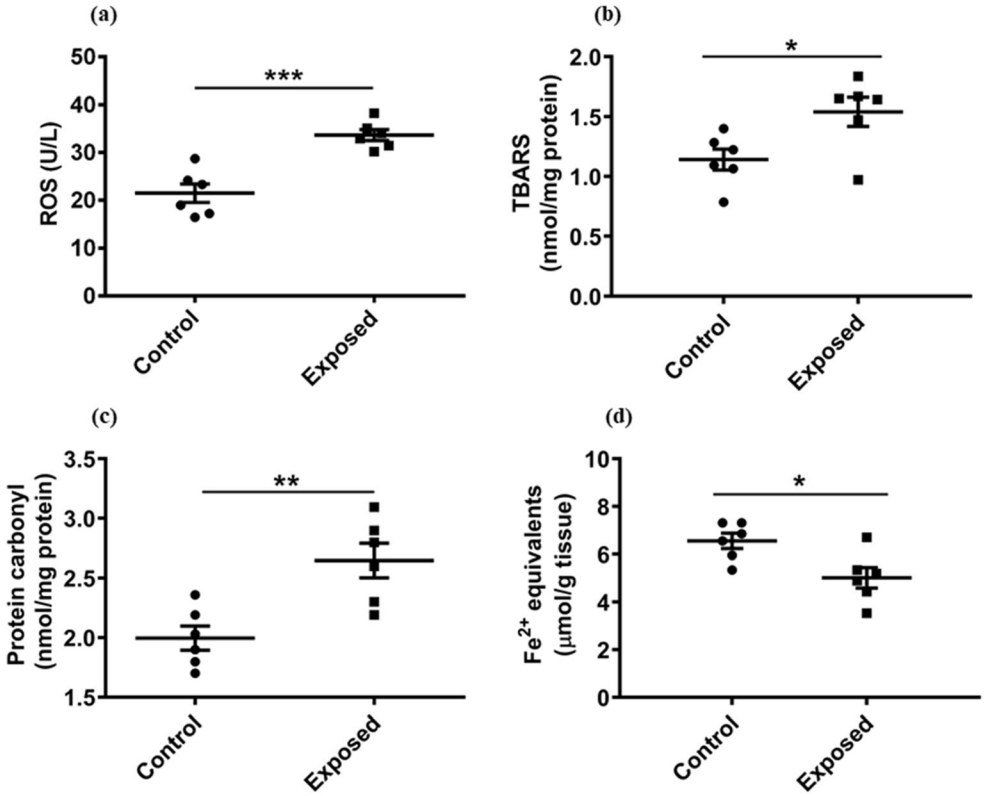


Fig. 3 Exposure to MP-RF-EMF radiation of 1966.1 MHz frequency for 16 weeks (2 h/day) significantly increases oxidative stress in the hippocampus of the experimental animals ($n = 6$/group). **a** Reactive oxygen species (ROS) level, expressed in units (1 unit is equal to 1 mg/L H_2O_2.); **b** lipid peroxidation, presented in terms of nanomoles of TBARS per milligram of protein; **c** protein carbonyl (nmol/mg protein) content, a measure of total oxidized proteins shows significant increase in the hippocampus of the exposed group as compared with the control. **d** Ferric reducing ability of plasma (FRAP) values (μmol of FeSO4 equivalents/g tissue), a measure of total antioxidant capacity shows a significant decrease in the hippocampus of the exposed group as compared with the control. Data presented as mean ± standard error of the mean (SEM). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$—un-paired t test and p values

further manifested in disturbed balance of stress hormones in the body. In this study, animals in the exposure group showed adrenal hypertrophy ($p < 0.001$) as well as very high levels of stress hormones ACTH ($p < 0.01$) and CORT ($p < 0.05$) as compared with the control (Fig. 5a–c). The adrenal coefficient in the exposed group was increased about 1.6-fold as compared with the control ones, indicating a chronic deregulation of HPA axis activity. Similarly, the level of ACTH and CORT was increased by 58 and 20% of control, respectively, indicating a MP-RF-EMF radiation-induced endocrine response.

Contextual fear conditioning

Contextual fear learning is a form of Pavlovian conditioning and a hippocampus-dependent task. Freezing was calculated as percentage time out of a total 5-min observation period for context test, during which the rat shows no movement except breathing movement. Extent of freezing was taken as a measure of context fear memory. Results showed that exposure to mobile phone radiation for a long period caused about 33% decrease in percentage freezing on the testing day in exposed animals as compared with the control ones, but changes were not statistically significant (Fig. 6). No significant decrease in percentage freezing was observed in the exposed group at base line and training day as well. Result showed a very huge difference in freezing values within group which is reflected by a very high standard deviation. Decrease in percentage freezing is a measure of contextual fear memory impairment.

Discussion

This study was designed to see effect of MP-RF-EMF radiation exposure (2 h/day, 16 weeks) at a SAR value of 0.36 W/kg on several parameters (hippocampal oxidative stress, PICs, HPA axis activity, and contextual fear memory) that generally get influenced by common environmental perturbations. An overall representation of experimental protocol, outcomes, and possible explanation in a schematic form is given in Fig. 7. This study shows that there is a significant increase in hippocampal ROS concentration in the exposed group as compared with the control and downstream consequences of increased ROS-like lipid peroxidation, protein oxidation, and antioxidant level were also altered significantly in exposed

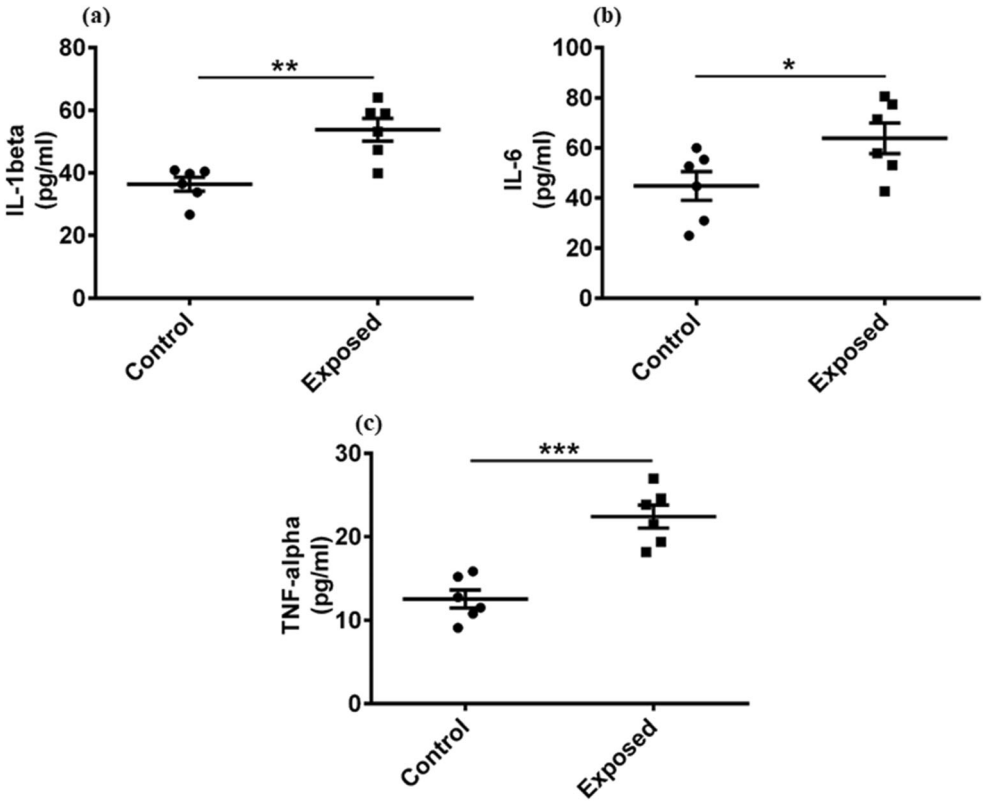


Fig. 4 Exposure to MP-RF-EMF radiation of 1966.1 MHz frequency for 16 weeks (2 h/day) significantly increases pro-inflammatory cytokine level (PICs). **a** IL-1beta (pg/ml), **b** IL-6 (pg/ml), and **c** TNF-alpha (pg/ml) in serum of the experimental animals ($n = 6$/group). Data presented as mean ± standard error of the mean (SEM). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$—un-paired t test and p values

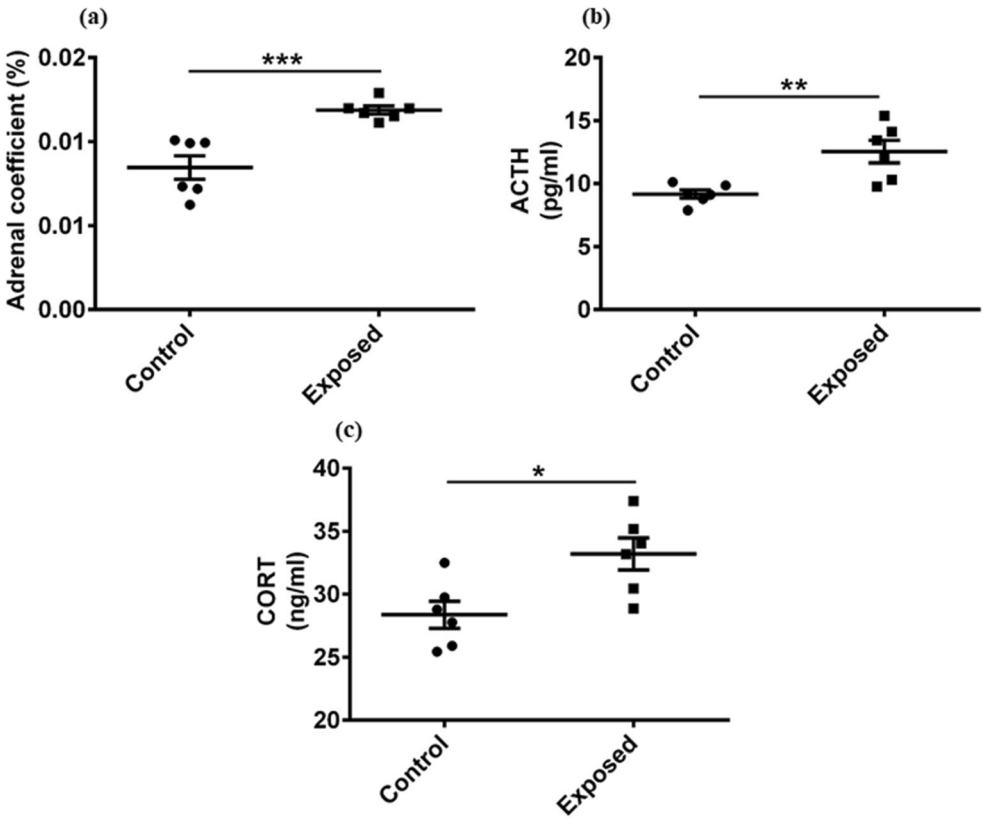


Fig. 5 Exposure to MP-RF-EMF radiation of 1966.1 MHz frequency for 16 weeks (2 h/day) causes adrenal hypertrophy and elevates basal level of stress hormones in experimental animals ($n = 6$/group). **a** Weight of adrenal gland (expressed as % of body weight also called adrenal coefficient), **b** serum level of adrenocorticotropic (ACTH) hormone (pg/ml), and **c** corticosterone (CORT) hormone (ng/ml) was significantly increased in the exposed group as compared with the control. Data presented as mean ± standard error of the mean (SEM). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$—un-paired t test and p values

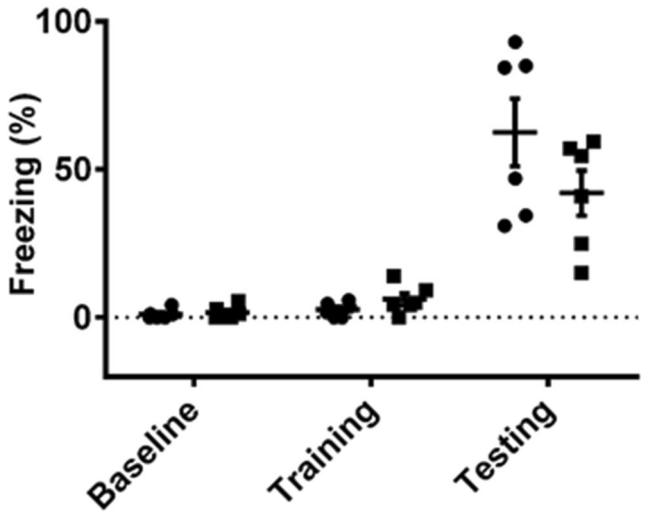


Fig. 6 Exposure to MP-RF-EMF radiation of 1966.1 MHz frequency for 16 weeks (2 h/day) did not cause significant change in contextual fear memory of the experimental animals ($n = 6$/group). Contextual fear conditioning was measured in terms of percentage freezing during base line (day 3), training (day 4), and testing (day 5) in the control and exposed groups. Results were calculated as percent time the rat expressed freezing behavior during the 5-min observation period for context. Read out of individual data points from the control (circles) and exposed (squares) experimental groups are presented. Unpaired *t* test was used to calculate *p* values. Data represented as mean ± standard error of the mean (SEM)

group. These results suggest that exposure to MP-RF-EMF radiation for a long time makes hippocampal redox environment more oxidizing. Our findings are more or less in line with previous studies which have invariably shown MP-RF-EMF radiation-induced oxidative stress in the brain (Chen et al. 2011; Ertilav et al. 2018), owing to its polyunsaturated fatty acid-rich composition and high metabolic rate (Bilgici et al. 2013; Özmen et al. 2007). However, level and extent of changes in oxidative stress parameters are specific to dose and duration of exposure and experimental condition. Megha et al. (2015) reported an increase in oxidative stress in the brain of male Fischer rat as we shift towards higher frequency (900–2450 MHz) and SAR values (0.59–0.66 W/kg).

The present study also suggested that chronic exposure to MP-RF-EMF radiation triggered an early immune response as there was an increase in the level of PICs viz. IL-1beta, TNF-

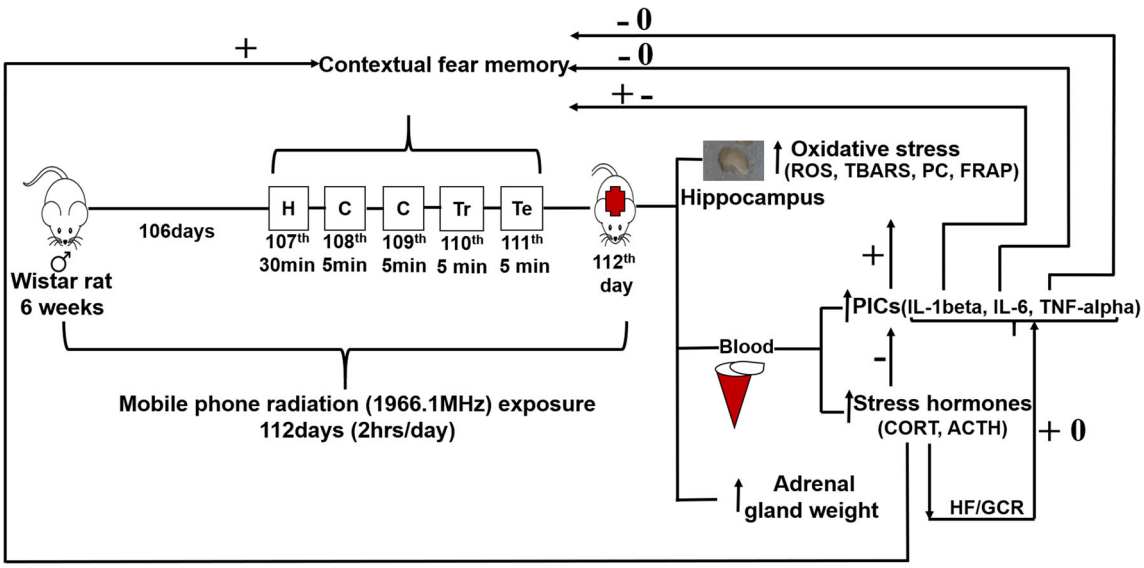


Fig. 7 An overall schematic representation of complete experimental plan, outcomes, and possible explanation. H, habituation; C, conditioning; Tr, training; Te, testing; "+" positively impact; "–" negatively impact; 0, no impact; ROS, reactive oxygen species; TBARS, thiobarbituricacid reactive substance; PC, protein carbonyl; FRAP, ferric reducing ability of plasma; HF, HPA axis fatigue; GCR, glucocorticoid receptor resistance. Upward arrow depicts significant increase in mentioned parameters in the exposure group as compared with the control

alpha, and IL-6 in the exposure group as compared with the control one. These cytokines are markers of innate immune response and released by activated immune cells like macrophages, fibroblast, and endothelial cells immediately after any environmental stress (Silverman et al. 2005). In line with this fact and our current findings which demonstrated significant increase in PICs upon MP-RF-EMF exposure, there are several in vitro as well as in vivo studies which have shown microglial activation and increased release of IL-1beta, IL-6, and TNF-alpha upon exposure to 2450, 1800, and 900 MHz radiation (Megha et al. 2015; Yang et al. 2010). There was another report which found differential activation and release of PICs from astrocytes and microglial cells when exposed to 1800 MHz MP-RF-EMF radiation at SAR value of 2 W/kg for different time periods (Lu et al. 2014). However, some other studies have not shown any significant alteration in level of PICs after exposure to 900 and 1950 MHz with SAR value ranging from 0.2 to 2 W/kg (Hirose et al. 2010; Thorlin et al. 2006). These conflicting results might be due to the differences in the RF signal characteristics like frequency, power density, modulation, and exposure duration. Like in one study where no remarkable change in the production of TNF-alpha, IL-1beta, and IL-6 were found, exposure was performed with 1950 MHz, W-CDMA RF field at different SAR values (0.2, 0.8, and 2 W/kg) for a duration of 2 h (Hirose et al. 2010), while in another such report, 8 h exposure in an intermittent mode was given using GSM basic 1950 MHz radiation at SAR value of 1 mW/g (Tuschl et al. 2006). However, in our case, continuous exposure of 2 h/day was given for 16 weeks at SAR value of 0.36 W/kg. So, the MP-RF-EMF radiation exposure affects cytokine production differentially, depending on duration and characteristics of exposure.

One of the evident outcomes of elevated level of PICs (TNF-alpha and IL-1beta) is induction of ROS and NO generation in various types of cells including microglial and astrocytic cells of the central nervous system (Corda et al. 2001; Hu et al. 1998; Mendes et al. 2003; Yang et al. 2007). It has also been demonstrated that 2.45 GHz RF-EMF radiation exposure induces increased release of both NO and TNF-alpha in microglial N9 cells (Yang et al. 2010). Similarly, there is another study showing differential release of TNF-alpha IL-1beta, IL-6, and NO in astrocyte and microglial cells upon exposure to 1800 MHz radiation (Lu et al. 2014). Furthermore, our present study and several other studies have demonstrated that MP-RF-EMF radiation induces oxidative stress in the brain. So, in this context, it is reasonable to say that early immune response after mobile phone radiation exposure might have facilitated the oxidative stress in the hippocampus.

Moreover, inflammatory cytokines are involved in the increased release of HPA axis hormones including CORT (Besedovsky and del Rey 1996), which is quite evitable as there is a rich network of glucocorticoid and cytokine receptors at each level of HPA axis (Silverman et al. 2005). In this study, we also found an enhanced release of PICs upon MP-RF-EMF exposure. In this context, we measured adrenal coefficient and circulatory level of CORT and ACTH to determine HPA axis activity. Our finding suggests an adrenal hypertrophy as well as significant increase in level of CORT and ACTH upon chronic exposure to MP-RF-EMF radiation. However, reports regarding MP-RF-EMF exposure-induced dysregulation of HPA axis are very few and contradictory. In one such study, 2-h exposure of rats to UMTS at SAR value of 10 W/kg caused an increase in plasma level of CORT and ACTH (Prochnow et al. 2011). However, Jin et al. (2012) reported no effect on serum ACTH level even after combined exposure to CDMA and WCDMA for 8 weeks at whole-body SAR of 4 W/kg. Similarly, another report found that chronic exposure to extremely low-frequency EMF causes no change in basal serum ACTH and CORT level; however, there is an elevation in the level of proopiomelanocortin (POMC) mRNA which is the precursor of ACTH, in the pituitary gland (Szemerszky et al. 2010). Further, glucocorticoids (ACTH and CORT) are known to downregulate further production of PICs due to its immune suppressant effect (Tracey 2002). However, in our investigation, we found significant upregulation of both PICs and glucocorticoids in the exposure group. These two findings seem contradictory due to the simple notion that chronic stress exerts its immune response through direct effect of elevated glucocorticoids (Cole 2008), but long-term and consistent exposure to any environmental stressor may cause HPA axis fatigue (Webster et al. 2002) and glucocorticoid receptor resistance (GCR), a state of decreased sensitivity of immune cells to glucocorticoids (Cohen et al. 2012). Lack of glucocorticoid regulation may lead to increased intensity and duration of immune response. These observations are thus in support of our results where a significant increase in the level of both glucocorticoids and PICs was observed on long-term exposure to MP-RF-EMF (16 weeks, 2 h/day).

Moreover, there is sufficient evidence to show the role of PICs in modulation of hippocampus-dependent cognitive functions (Marin and Kipnis 2013; Yirmiya and Goshen 2011), like fear and anxiety, depression, anhedonia (an inability to experience pleasure), impaired sleep, anorexia, and reduced locomotor activity (Capuron and Miller 2004; Dantzer 2004). Similarly, glucocorticoids also have complex effect on cognitive functions especially fear-related memory viz. contextual fear memory retrieval, consolidation, and extinction (de Quervain et al. 2017; Roozendaal 2002). Out of these, contextual fear memory retrieval is largely controlled by the hippocampus (Holland and Bouton 1999; Sandi et al. 2001). So, pertaining to these facts and our findings in the present study, it is of interest to study effect of mobile phone radiation exposure on hippocampal-dependent contextual fear memory retrieval. We found no impairment of contextual fear memory,

upon exposure to MP-RF-EMF radiation. Though there is no such study accessing effect of MP-RF-EMF exposure on contextual fear memory process, studies on several other behavioral parameters after RF-EMF exposure have shown contradictory results so far. One such study on young male Wistar rats found poor learning ability, spatial memory deficit, and poor memory retention in animals exposed to 900 MHz frequency for 28 days (1 h/day) at SAR value of 1.15 W/kg (Narayanan et al. 2018). Another report found that exposure of 6-week-old Sprague Dawley rats to the SAR value of 6 W/kg causes long-term memory impairment, but no effect was observed at lower SAR values (Barthélémy et al. 2016). In contrast with these, Othman et al. (2017) reported that rats exposed to 2.4 GHz signal for 20 consecutive days (2 h/day) showed increased anxiety but no effect on spatial learning and memory. There are as many numbers of studies showing impairing effect of MP-RF-EMF (Bouji et al. 2012; Hao et al. 2013; Li et al. 2016; Narayanan et al. 2015; Petitdant et al. 2016) as showing either positive or negligible effect (Arendash et al. 2010; Maier et al. 2004; Nittby et al. 2008).

Our results suggest no significant impairment in fear memory retrieval upon MP-RF-EMF exposure, which can be explained by the fact that different inflammatory cytokines modulate learning and memory in a different way. Small increases (1 ng) of IL-1beta enhances passive avoidance, context fear conditioning (Goshen et al. 2009), and spatial memory (Song et al. 2003; Yirmiya et al. 2002). However, chronic overexpression of IL-1beta in the hippocampus impairs the same (Hein et al. 2010; Moore et al. 2009). In contrast, TNF-alpha and IL-6 are not involved in normal learning and memory functions but overexpression of both cause broad memory impairments (Donzis and Tronson 2014). Similarly, glucocorticoids also have contrasting effects on fear memory retrieval, where CORT injection just after training in a contextual fear testing or enhanced level of CORT due to sudden exposure to any aversive stimuli impairs the retrieval of contextual fear-conditioned memory, acquired 24 h earlier but enhances consolidation of the same (de Quervain et al. 2017). However, repeated exposure to a stressor (chronic stress)-induced increase in basal level of glucocorticoids potentiate contextual fear memory retrieval both at intermediate freezing (0.4 mA) and high freezing (1 mA) shock intensity (Conrad et al. 1999; Sandi et al. 2001). Therefore, unaltered memory in present study might be due to complex interplay of opposing effects exerted by glucocorticoids and PICs. Further, as reported in one of the few studies, exposure of rats to 916 MHz (10 W/m^2) RF-EMF has an influence on learning and memory in a period during exposure. However, exposure for longer durations caused an adaptive response (Hao et al. 2013). So, in the present case, it might be possible that adaptive response generated in animals due to long-term exposure to MP-RF-EMF (2 h/day, 16 weeks) is the reason behind unaffected fear memory.

Conclusion

The present study shows that long-term exposure to mobile phone radiation indeed have an impact on immunity, hippocampal oxidative stress, and HPA axis activity. However, functional consequence in terms of memory impairment is not significant. This might be due to the diverse and mutually opposing impact of different parameters which are altered upon MP-RF-EMF radiation exposure on hippocampal functionality. Though these results cannot be extrapolated to humans directly, it gives an idea about health consequences of long-term exposure to MP-RF-EMF radiation, and more elaborate study looking in to mechanism of interaction between MP-RF-EMF radiation and biological matter is needed to establish the fact.

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