

Ten gigahertz microwave radiation impairs spatial memory, enzymes activity, and histopathology of developing mice brain

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Abstract For decades, there has been an increasing concern about the potential hazards of non-ionizing electromagnetic fields that are present in the environment and alarming as a major pollutant or electro-pollutant for health risk and neuronal diseases. Therefore, the objective of the present study was to explore the effects of 10 GHz microwave radiation on developing mice brain. Two weeks old mice were selected and divided into two groups (i) sham-exposed and (ii) microwave-exposed groups. Animals were exposed for 2 h/day for 15 consecutive days. After the completion of exposure, within an hour, half of the animals were autopsied immediately and others were allowed to attain 6 weeks of age for the follow-up study. Thereafter results were recorded in terms of various biochemical, behavioral, and histopathological parameters. Body weight result showed significant changes immediately after treatment, whereas non-significant changes were observed in mice attaining 6 weeks of age. Several other endpoints like brain weight, lipid peroxidation, glutathione, protein, catalase, and superoxide dismutase were also found significantly ($p < 0.05$) altered in mice whole brain. These significant differences were found immediately after exposure and also in follow-up on attaining 6 weeks of age in microwave

exposure group. Moreover, statistically significant ($p < 0.001$) effect was investigated in spatial memory of the animals, in learning to locate the position of platform in Morris water maze test. Although in probe trial test, sham-exposed animals spent more time in searching for platform into the target quadrant than in opposite or other quadrants. Significant alteration in histopathological parameters (qualitative and quantitative) was also observed in CA1 region of the hippocampus, cerebral cortex, and ansiform lobule of cerebellum. Results from the present study concludes that the brain of 2 weeks aged mice was very sensitive to microwave exposure as observed immediately after exposure and during follow-up study at 6 weeks of age.

Keywords Microwaves · CA1 region · Hippocampus · Morris water maze · Protein

Introduction

There has been an increasing concern about the adverse effects of radiofrequency electromagnetic field (RF-EMF) and possible health outcomes. There is sufficient number of recent animal studies indicating the causative effect of RF-EMF on brain [1–6]. The evidence is strong enough today to conclude that RF-EMF causes carcinogenic changes (DNA strand break, protein kinases and associated biomarkers) in brain function [7–10] and cognitive impairment [11, 12]. Repacholi et al. [13] report also suggest carcinogenic potential of digital mobile phones exposure and biological effects. In 2011, the International Agency for Research on Cancer (IARC) classified RF-EMF as “possibly carcinogenic to humans” (Group 2B) [14]. It shows a significant link between long-term RF-EMF exposure and cancer risk [15–20]. The X-band, 10 GHz

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microwave exposure was used to investigate the effects on developing mice brain. The rapid expansion of electromagnetic waves with frequencies ranging from 300 MHz to 300 GHz, have been widely used in aeronautical radio-navigation, traffic light crossing detection, space research, satellite broadcasts, communication satellites, meteorology satellites, and especially radar detection [21, 22]. This frequency range is adversely associated with epidemiological studies and impairs the cognitive functions [23] and considered as hazardous for human health [24]. Several studies from our group and others have reported the effect of X-band 10 GHz microwaves radiation on reproduction [25–27], brain and behavioral functions [6, 11, 28–31]. Researchers reported significant changes in DNA strand break, antioxidants, behavioral and cognitive functions, cell cycle checkpoints, protein kinases and many more, which may participate in carcinogenic effects.

The major concern of this study was to observe microwave exposure effects in 2 weeks aged mice developing brain in terms of biochemical, behavioral, and histological alterations. Many researchers have reported the pathological, biochemical, and behavioral changes of central nervous system resulting from microwave exposure [32–36]. The tissue in developing mice brain is more conductive than that of adults as it has higher water content and higher ion concentration. These radiations are potentially strong to penetrate the cranium, and nearly 40% of these can reach deeper into the brain [37, 38], where penetration depth is assumed to be 4–5 cm deep into the brain [39, 40]. Therefore, children have greater absorption of microwaves energy in the brain tissues [41]. Research from our group, including several others has showed the adverse effects of these radiations in rat or mice hippocampus [21, 42]. Any changes in hippocampus impairs long-term potentiation, learning and working memory, decreases neurotransmitter concentrations, reduces synaptic vesicles in number, and results in memory impairment [5, 6, 11, 43–47]. Reactive oxygen species (ROS) is found to be responsible for the cellular damage, tissue injury, biochemical, and histological changes in cells or tissue due to microwave exposures [7, 8, 48–51]. Moreover, ROS is an intrinsic part of the cellular signaling cascades [52], therefore if not scavenged may lead to lipid, protein, and DNA damage [53], which has been investigated in our previous findings [49, 50]. The evidences obtained in this study are also supported by several other findings, where exposure to microwave frequencies showed significant effect on the brain hippocampal region, anxiety-like behavioral and cognitive functions [6, 54, 55]. However, the direct effects of microwave radiation on neurodevelopment and the underlying mechanisms for any such effects are unknown till date. This study will contribute further to explore the underlying mechanism and fill the gap.

Materials and methods

Experimental animals

Two weeks aged Swiss albino mice (postnatal day 14, average weight 8.5 ± 0.25 g) irrespective of gender discrimination were obtained from an animal house facility of University of Rajasthan, Jaipur, India. Animals were divided in two groups: sham-exposed (control) and 10 GHz microwave exposure group. The experiments in the present study were repeated thrice. All animals were housed in an air-conditioned room, where the temperature was maintained at 25 ± 1.5 °C, with constant humidity (40–50%) and kept on 14/10 h light/dark cycle throughout the experiment. The animals were fed on standardized normal diet (Hindustan Unilever Limited, Delhi, India) and water ad libitum. The Institutional Animal Ethical Committee (IAEC) approved the protocols for animal experimentation described in the present study. All subsequent animal experiments were followed as *per* the “Guidelines for Animal Experimentation” of the University.

Animal exposure

Plexiglas was used to fabricate an animal exposure cage of which the dimensions were $4.5 \times 4.5 \times 9$ cm with fixed holes (1 cm diameter). This was designed so that animals could be lightly restrained in a fixed position and with proper ventilation. Exposure was carried out in an anechoic chamber. Two weeks aged mice were mainly divided into two groups: (i) sham-exposed and (ii) microwave-exposed groups. Animals in exposure group were exposed with 10 GHz microwave radiation for 2 h/day for 15 consecutive days as shown in Fig. 1. After completion of microwaves and sham exposure, 12 mice from each group were autopsied immediately within one hour after completion of exposure on last day. These were used for biochemical ($n = 6$) and histological ($n = 6$) studies for each group. The remaining 18 mice from each group were allowed to attain 6 weeks of age for the follow-up study. In the follow-up experiment, 6 animals from each group were used separately for histological, biochemical, and behavioral (Morris water maze) studies. For histology, whole brain was fixed in Bouin’s fluid. The overall experimental plan is depicted in Fig. 2.

All animals were kept in such position, where the head of animals faced the horn antenna. The horn antenna was kept in H (Magnetic field) plane configuration, where electric field was perpendicular to the ground surface. Field was almost uniform because the dimension of the cage was of the order of wavelength. The maximum power density 0.25 mW/cm^2 was recorded at the near field distance from

Fig. 1 Schematic diagram of 10 GHz microwave exposure setup with animal cage indicating individual animal's position

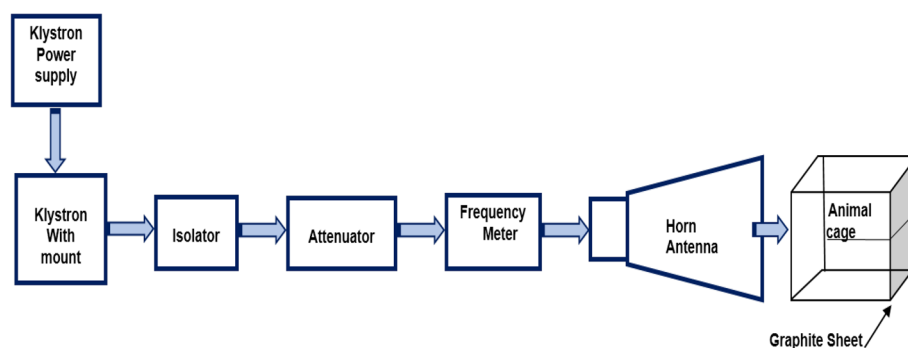
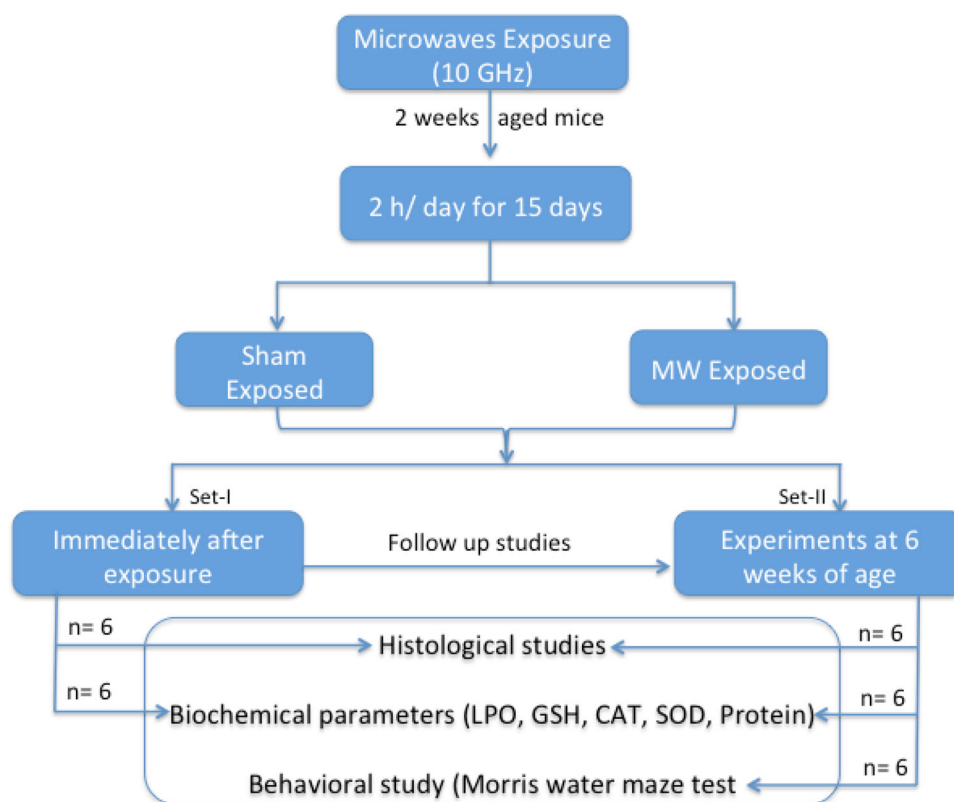


Fig. 2 An overview of experimental plan showing two sets of experiments: Set 1—2 weeks aged mice grouped into sham and microwave exposure for 2 h per day for 15 consecutive days and thereafter performed the endpoints within an hour after exposure. Set 2—follow-up study were done when mice attained 6 weeks of age



the horn antenna. A power meter measured the emitted power of microwaves, which was a peak sensitive device (RF power sensors 6900 series and IFR 6960B RF power meter; Aeroflex, Inc., Wichita, Kansas, USA). The whole body specific absorption rate (SAR) was estimated to be 0.1790 W/kg following the Durney et al. [56] method. Similar experiment with same number of sham-exposed animals was performed without energizing the microwave exposure system. Similar exposure setup was opted earlier by Sharma et al. [6, 11].

Lipid peroxidation (LPO) assay

Lipid peroxidation was measured by the method of Buege and Aust [57]. The tissue was homogenized in buffer

containing 1.15% potassium chloride (KCl) at a 1:10 (w/v) ratio to whole homogenate. The tissue homogenate (0.8 ml) was mixed with 1.2 ml of trichloroacetic acid (TCA) (15% w/v), thiobarbituric acid (TBA) (0.375% w/v) and hydrochloric acid (HCl) (0.25 N) solutions (Himedia, Mumbai, Maharashtra, India), prepared in a 1:1:1 ratio. The mixture was heated in a boiling water bath for 30 min. Samples were centrifuged at 1000 g for 10 min. After centrifugation, the absorbance was recorded at 532 nm by using Ultra Violet-Vis double beam spectrophotometer (Double Beam Spectrophotometer 2203, Systronics, Ahmedabad, Gujarat, India). A standard curve was prepared using tetramethoxy-propane (TMP: purchased from Himedia, Mumbai, Maharashtra, India). After comparison with a standard curve, the LPO level was expressed in nmol/gm tissue.

Glutathione (GSH) assay

The reduced GSH level was determined in the brain by the method of Moron et al. [58]. Tissue samples were homogenized in the sodium phosphate–EDTA (Ethylenediaminetetraacetic acid) buffer (Himedia, Mumbai, Maharashtra, India). Thereafter, 0.6 ml of DTNB reagent (5,5'-dithiobis-(2-nitrobenzoic Acid), Himedia, Mumbai, Maharashtra, India) was added into it. In addition, the yellow-colored complex was developed by the reaction of GSH and DTNB, and then the optical density was measured at 412 nm by using a UV–vis spectrophotometer (Double Beam Spectrophotometer 2203, Systronics, Ahmedabad, Gujarat, India). The results were expressed in nmol GSH/100 mg of tissue.

Superoxide dismutase (SOD) assay

SOD was measured by following the method of Marklund and Marklund [59]. The tissue was homogenized in NaCl (sodium chloride, purchased from Himedia, Mumbai, Maharashtra, India) solution at a 1:10 (w/v) ratio to whole homogenate. Then 0.1 ml of supernatant was taken for the estimation. Further, this supernatant was added in 2.8 ml tris buffer, and then 0.1 ml of pyragallol was mixed in this solution. The absorbance was measured at 420 nm, and absorbance per 2 min was recorded. Calculations were done, and SOD level was reported as $\mu\text{mole/mg}$ tissue.

Catalase assay

Catalase enzyme was measured by the method of Aebi et al. [60]. The tissue was homogenized in phosphate buffer at a 1:10 (w/v) ratio to whole homogenate. The homogenate was centrifuged at 10,000 rpm for 10 min. Thereafter, 0.1 ml of supernatant was mixed with 1 ml of phosphate buffer saline (PBS: Sigma-Aldrich, St. Louis, MO, USA) and 0.4 ml of hydrogen peroxide (H_2O_2). Absorbance was measured at 420 nm. Results were expressed in nmol/ml catalase.

Protein estimation

The protein concentration was estimated following the method of Bradford [61]. The procedure was based on an interaction of dye, coomassie brilliant blue with proteins. The absorbance of unbound dye was maximum at 465 nm (nm). After an interaction of dye with protein, it turns blue and their absorbance maxima were displaced to be 595 nm. Thus, from the absorbance at 595 nm, the amount of protein in a sample was estimated in mg/gm tissue. In brief, 10% homogenate of each excised brain was prepared in 0.85 M NaCl solution. Thereafter, 0.1 ml of the sample

was taken for the Bradford assay. The volume in the test tube was adjusted to 1 ml with phosphate buffer saline (PBS with pH 7.4). Five milliliters of Bradford reagent (Sigma-aldrich, St. Louis, MO, USA) was added to the test tube and the contents were mixed by vortexing. The sample absorbance was measured after 2 min at 595 nm in spectrophotometer (Double Beam Spectrophotometer 2203, Systronics, Ahmedabad, Gujarat, India). For calibration, blank was prepared from 0.1 ml of the phosphate buffer saline (pH 7.4) and 5 ml of Bradford reagent. The amount of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples.

Morris water maze (MWM) test

Morris water maze test was carried out by following the method of Fragopoulou et al. [62]. MWM test was performed, when the mice of each experimental group attained the age of 6 weeks. MWM test was undertaken in water filled circular pool (85 cm in diameter), with constant temperature ($23 \pm 1^\circ\text{C}$) and humidity. The surface of movable escape platform made up of clear Plexiglas (8 cm $\times$ 10 cm) was submerged 1 cm below the water surface. The extra maze visual cues including signs on the walls, as well as the organization of experimental room, were also maintained. Mice were brought into the MWM room, 1 h before the trial for habituation. They were trained to find a submerged escape platform, which was located in a fixed position relative to the extra maze visual cues, during four consecutive daily sessions. Each session consisted of four trials. Four different equally spaced starting positions around the perimeter of the pool were used in a fixed order. Each animal was released in the water from the sides of pool from these starting positions. Each trial had a maximum duration of 60 s (seconds). If mice did not find the platform within these 60 s, it was manually placed on the platform. At the end of each trial, the animals were allowed to remain on the platform for 20 s and then returned to their home cage and left there to rest for 15 min before the beginning of the next trial. Two hours after the last training trial (the fourth trial of the fourth day), the animals were subjected to a memory probe trial during which the mice were allowed to swim for 60 s in the absence of the training platform. All mice started from the same position, opposite to the target quadrant (the quadrant where the escape platform had been positioned). The escape latency (in seconds), i.e., the time taken to escape on to the submerged platform, were recorded for each training trial. The average time spent in target quadrant and the other quadrants during the 60 s were recorded during probe trial. For the training trials, escape latency was

averaged per mouse for each daily session in order to calculate the average escape latency per day (mean values).

Histopathological study of brain

After completion of the experiment, brain was excised and fixed in Bouin's fluid. It was dehydrated and embedded in paraffin blocks. 5 µm thick transverse sections of this paraffin embedded tissue were cut. The transverse sections of tissues were passed through a graded series of alcohol and stained in Eosin and Harris Haematoxylin stain [63], then used for various qualitative and quantitative histopathological studies. Three different slides per animals were prepared for further analysis. Qualitative studies were done in CA1 region of hippocampus and ansiform lobule of cerebellum. Quantitative studies include, counting of Purkinje cells number, thickness of molecular layer and granular layer of cerebellum and cerebral cortex. Morphological changes in cerebral cortical cell and hippocampal pyramidal cell regions were visualized under light microscope (400×). Width or thickness of molecular layer, granular layer, and cerebral cortex were recorded using oculometer, which was calibrated with a standard stage micrometer. Counting of Purkinje cell (mm-1) number was carried out manually in six different animals. Randomly selected areas (200 × 200 µm²) from three different slides of the ansiform lobule of cerebellum per animal were used for the study.

Statistical analysis

Balanced factorial design was used in MWM test; factorial ANOVA was performed with microwave (MW) (or sham MW) and Day (1–4) as fixed factors and replicate as random factor. The general linear model procedure of SPSS (SPSS Inc., Chicago, Illinois, USA) was used for the analysis. Using Prism-GraphPad, the graph was plotted. A *p* value of less than 0.05 was considered statistically significant. For all the biochemical parameters, the difference between microwave-exposed and sham-exposed groups was tested for significance using one-way ANOVA.

Results

Body and brain weight

Healthy body weight is an important indicator for overall health and development, which can help to prevent and control many diseases. Body weight was measured in both groups immediately after microwave exposure and at the age of 6 weeks for the follow-up study. A significant reduction in body weight gain (7.74%) was observed

immediately after microwave exposure by comparing with sham-exposed group (Table 1). No significant changes in body weight were observed in follow-up group.

On the other hand, statistically significant ($p < 0.05$) differences were measured in the weight of mice brain of both sets, immediately after exposure and also on attaining 6 weeks of age compared to sham-exposed groups (Table 1).

Lipid peroxidation (LPO)

The measurement of brain LPO level was performed to assess the quantitative changes in the exposed group compared to those of the control one. The level of LPO in whole brain found to be increased in exposed group ($p < 0.001$) in comparison to sham-exposed group. Microwave exposure resulted in significant increase in LPO level after 15 days of exposure. Exposure initiated at 2 weeks of age led to 32.25 and 30.56% increase as estimated immediately and on attaining 6 weeks of age respectively (Table 2). No recovery was evident in the follow-up study at 6 weeks of age.

Glutathione (GSH)

The increased level of GSH was measured with increasing age of sham-exposed mice. Statistically significant decrease ($p < 0.001$) in the level of brain GSH was measured in microwave-exposed group in comparison with sham-exposed mice. Microwave exposure initiated at the age of 2 weeks for 15 days led to 28.17 and 38.99% decrease in glutathione level as estimated immediately after 15 days of exposure and in follow-up study conducted at 6 weeks of age, respectively (Table 2).

Superoxide dismutase (SOD)

The increased level of SOD was measured in sham-exposed mice to cope up with the increased oxidative stress in the cells. It works parallel with GSH. Statistically significant decrease ($p < 0.001$) in the level of SOD was measured in microwave-exposed group by comparing with sham-exposed group. The decreased level of SOD by 31.48% was estimated immediately after completion of exposure for 15 days. The follow-up study on attaining 6 weeks of age showed a small reversal of the activity as depicted by 29.33% decrease (Table 2).

Catalase (CAT)

The increased level of CAT was estimated in sham-exposed group with the increasing age of mice. However, statistically significant increase ($p < 0.001$) in the level of

Table 1 Variations in body weight $\pm$ SD and brain weight $\pm$ SD of 2 weeks aged mice exposed to 10 GHz microwaves 2 h/day for 15 consecutive postnatal days

Morphometric changes	Groups (exposure 2 h/day)	Immediately after completion of 15 days exposure	Follow-up study at 6 weeks of age
Body weight after treatment days (gm) $\pm$ SD	Sham	11.83 $\pm$ 0.51	19.66 $\pm$ 0.51
	Microwaves exposed	10.91 $\pm$ 0.37* (7.74%)	19.5 $\pm$ 0.44 (0.84%)
Brain weight (mg) $\pm$ SD	Sham	337.5 $\pm$ 4.32	409.66 $\pm$ 1.50
	Microwaves exposed	307 $\pm$ 5.72** (4.25%)	403.66 $\pm$ 1.21** (1.46%)

The statistically significant level for the MW effects are given in the table: * $p < 0.01$; ** $p < 0.001$

Table 2 Variations in various biochemical parameters in whole brain of Swiss albino mice aged 2 weeks exposed to 10 GHz microwaves 2 h/day for 15 consecutive postnatal days during the experimental period and follow-up study at 6 weeks

Biochemical parameters	Groups (exposure 2 h/day)	Immediately after completion of 15 days exposure	Follow-up study at 6 weeks of age
LPO levels (nmol/gm tissue)	Sham	72.07 $\pm$ 1.30	96.52 $\pm$ 0.68
	Microwaves exposed	95.32 $\pm$ 1.78** (32.25%)	126.03 $\pm$ 0.97** (30.56%)
GSH (nmol/100 mg)	Sham	10.52 $\pm$ 0.53	17.60 $\pm$ 0.94
	Microwaves exposed	7.56 $\pm$ 0.27** (28.17%)	10.73 $\pm$ 0.50** (38.99%)
Catalase (nmol/ml)	Sham	5.75 $\pm$ 0.07	7.01 $\pm$ 0.13
	Microwaves exposed	7.57 $\pm$ 0.04** (31.75%)	10.71 $\pm$ 0.33** (52.76%)
SOD (μ mol/mg tissue)	Sham	1.65 $\pm$ 0.04	2.02 $\pm$ 0.09
	Microwaves exposed	1.13 $\pm$ 0.07** (31.48%)	1.43 $\pm$ 0.05** (29.33%)
Protein levels (mg/gm of tissue)	Sham	106.84 $\pm$ 0.87	116.44 $\pm$ 1.66
	Microwaves exposed	103.34 $\pm$ 0.85** (3.27%)	92.97 $\pm$ 1.22** (20.15%)

The statistically significant level for the MW effects are given in table: ** $p < 0.001$

CAT was measured in exposed group immediately after treatment. It resulted in an increase of 31.75% as estimated immediately after exposure. The follow-up study at the age of 6 weeks showed an increase of 52.76% compared with sham-exposed group (Table 2).

Protein estimation

A significant decrease ($p < 0.001$) in the level of protein was estimated in all microwave-exposed group. Also, statistically significant ($p < 0.001$) reduction of 3.27% was measured immediately after microwave exposure. Decreased level by 20.15% of protein was estimated in the

follow-up study at 6 weeks of age on comparing with sham-exposed group (Table 2).

Morris water maze test

Morris water maze test (MWM) was performed when mice attained 6 weeks of age after 15 consecutive days of exposure from 2 weeks of age to assess the alteration in spatial learning and memory of adult mice. A significant difference was recorded between sham-exposed and microwave-exposed group of mice with respect to escape time during the training trials. Both group of animals improved their water maze acquisition performance, i.e., decreased the latency to find the platform from the first to

the last day of the experiment (day 4). However, comparison of response curves between these groups indicated that microwave exposure significantly affects spatial memory of the animals in learning to locate the position of platform. They took significantly longer time to locate and land on the platform (sham vs exposed) (Fig. 3).

The strength of long-term spatial memory was tested using a probe, i.e., free swimming without platform, after 2 h of the last training trial (the fourth trial of the fourth day). When tested in the probe trial, sham-exposed animals spent more time searching for the platform in the target quadrant than in opposite or other quadrants. Statistically significant ($p < 0.01$) difference was observed in exposed group of mice and days (1–4) compared to sham-exposed group (Fig. 3).

Qualitative histological studies

In the present study, histopathological changes were studied in hippocampus immediately after 15 days of microwave exposure and on attaining 6 weeks of age for the follow-up study. Results obtained from sham-exposed group showed normal cytoarchitecture (4A, 4C, 4E). However, after 15 days of microwave exposure (2 h/day), resulted in depopulation and reduction in density of pyramidal cells with edema, loss in connective tissue and neurons exhibited pyknosis and hyperchromatism in the exposed groups (Fig. 4b). Also degenerated pyramidal cells were observed (Fig. 4d). Neuronal degeneration in cerebral cortex was also observed in form of darker neurons as observed immediately after exposure compared

with sham-exposed group. An observable increase in glial cell population was also noticed (Fig. 4f).

Follow-up study of all the mice was done to study the histopathological alterations on attaining 6 weeks of age after postnatal exposure, starting at the age of 2 weeks. Normal cytoarchitecture of cerebellar folia was observed in sham-exposed groups of mice on attaining 6 weeks of age (Fig. 5a). Disintegration of granular layer with loss and necrosis of purkinje cells were evident in microwaves exposed mice (Fig. 5b) along with reduction in pyramidal cell density on attaining 6 weeks of age. Normal cytoarchitecture of pyramidal cell layer was evident in all sham-exposed mice (Fig. 5c). Reduction in pyramidal cell density and elongated with necrotic cells was evident in mice after 15 days of exposure initiated at 2 weeks of age (Fig. 5d). Normal cytoarchitecture of cerebral cortex region of 6 weeks aged mice showed normal distribution of neuronal and glial cells as depicted in sham-exposed groups (Fig. 5e). Higher neuronal degeneration was observed in cortical cells of microwaves-exposed mice (Fig. 5f).

Quantitative histological studies

Quantitative studies were done to estimate the number of purkinje cells in ansiform lobule of the cerebellum. Thickness of molecular layer and granular layer was also measured and found significant decrease after microwave exposure for 15 days and on attaining 6 weeks of age. Significant decrease in number of purkinje cells were also observed after 10 GHz microwave exposure in both 15 days and in follow-up at 6 weeks of age groups (Table 3).

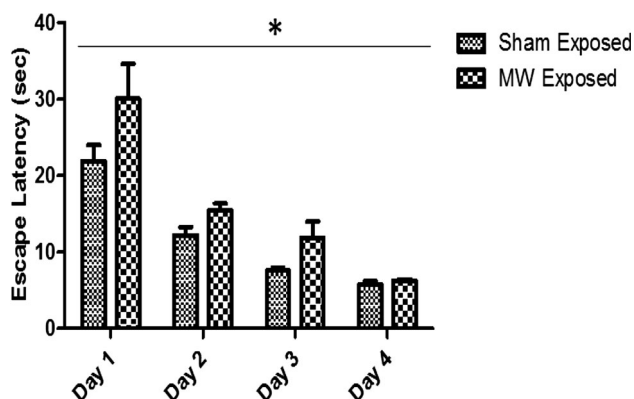


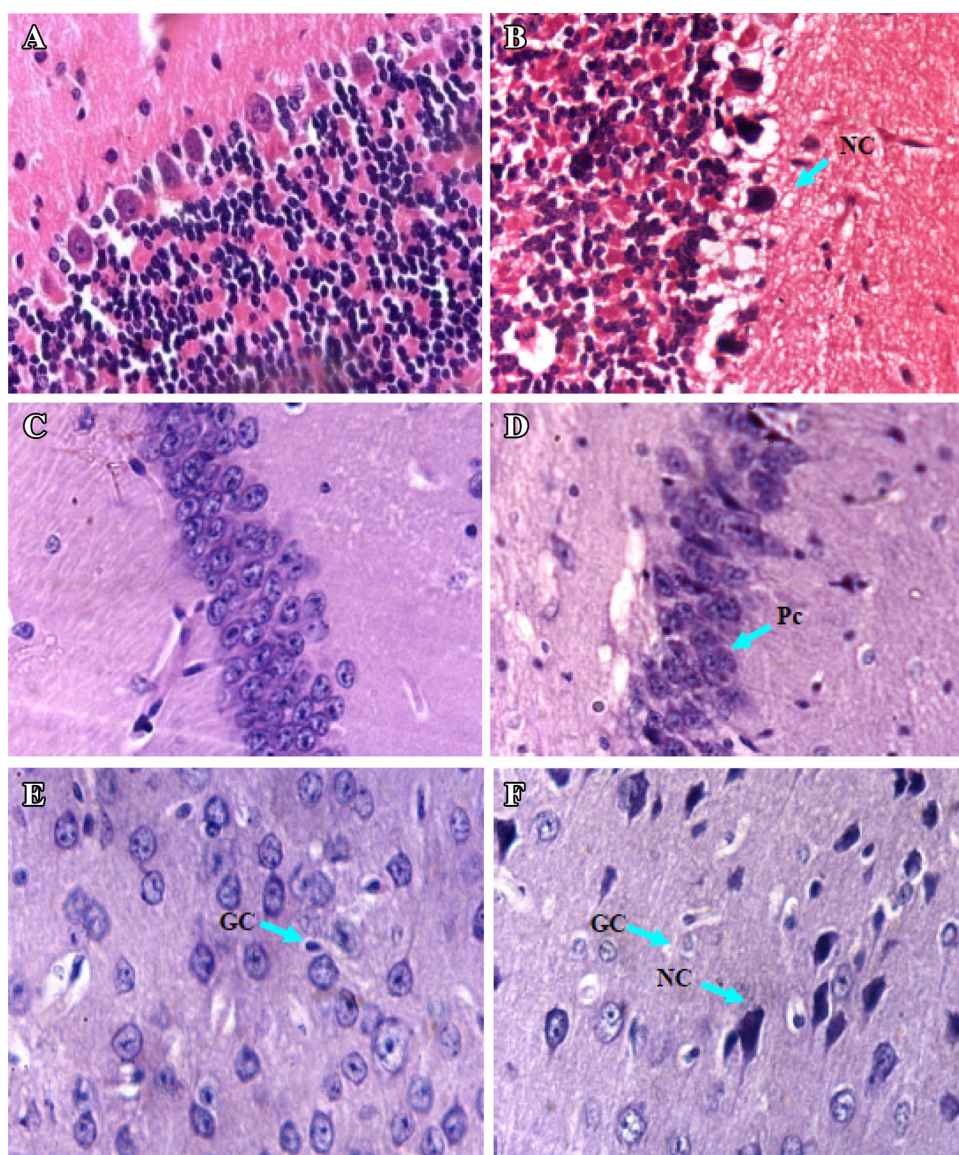
Fig. 3 Morris water maze test shows average escape latency (time to reach the platform after release into the water) of 6 weeks aged Swiss albino mice during the 4-day training sessions. Mice were exposed for 2 h per day for 15 consecutive postnatal days (PND) at 2 weeks of age. Factorial ANOVA was used for statistical testing; statistically significant effects for the two factors (MF and Days) are reported below. MWM test: MW $p < 0.01$; Days (1–4) $p < 0.001$. The level of significance for the MW effects is given in the figure: * $p < 0.01$; ** $p < 0.001$

Discussion

The present study provides several important findings related to behavioral, biochemical and pathophysiology of 10 GHz microwave radiations and its effects on developing mice brain. The investigated findings in this study are associated with oxidative damage via antioxidative parameters, after microwave exposure of 2 weeks aged mice and their follow-up till 6 weeks of age. In this study, significant changes were observed in histopathology of CA1 region of hippocampus. It was demonstrated that microwaves induce oxidative damage biochemically as evident by decreased levels of protein and changes in antioxidant enzymes. Then finally, the Morris water maze (MWM) test was performed to observe alterations in spatial memory and learning on attaining 6 weeks of age. Our study are in corroboration with Stead et al. [64], who reported that the suckling period (early postnatal period) is

Fig. 4 Histopathological changes in cerebellum, pyramidal cells, and cerebral cortex after exposure to 10 GHz microwaves for 15 days (2 h/day) when exposure was initiated from 2 weeks of age.

a Sham—normal cytoarchitecture of cerebellar folia with regular arrangement of purkinje cells. **b** Microwaves exposure—reduced purkinje cells density with necrotic cells (NC), migration of cells into granular layer, disturbance of cytoarchitecture, and loss of connective tissue. **c** Sham exposure—normal cytoarchitecture of pyramidal cells. **d** microwave exposure—reduced density with pyknosis (Pc) and depopulation of pyramidal cells. **e** Sham exposure—normal cytoarchitecture of cerebral cortex. **f** microwave exposure—reduced number of glial cells (GC) and necrotic neuronal cells (NC) in cerebral cortex. Images were observed at $\times 400$



a crucial phase for mouse CNS development. The exposure during the brain developmental stage (1–3 weeks) is associated with delay in the development and maturation of CNS. Stringari et al. [65] found that developing stage of brain was extremely sensitive to exposure of microwaves. Several other studies also reported that developing brain is particularly vulnerable to oxidative damage due to various oxidizing factors [66, 67]. This may also impair the neuroanatomical and neurochemical activity by affecting enzymes, receptors, and membrane transport, which supposed to be associated with water maze performance.

The significant changes were observed in the brain development of 2 weeks aged mice after microwave exposure. This might be because the first 2 weeks of postpartum period are critical for regular neural development. Mouse pups display rapid behavioral changes during

this time period [64]. Therefore, it is reasonable to suggest that postnatal day 14 to postnatal day 21 represents a critical phase, where 10 GHz radiation can potentially cause an alteration in oxidative or antioxidative status of whole brain of mice. The present study investigates the delayed effect of microwave exposure in 6 weeks aged mice during follow-up period. In the follow-up study, significant effects were observed by estimating several antioxidant enzymes, histological and behavioral parameters. The results of present study showed significant increase in the level of brain LPO and catalase, whereas decrease in the level of GSH, SOD, and protein, as observed immediately after 15 days of microwave exposure. More interestingly, an increasing trend in the level of brain LPO, catalase, GSH, and SOD and also a decreasing trend in protein level was recorded on attaining 6 weeks of

Fig. 5 Histopathological changes in cerebellum, pyramidal cells, and cerebral cortex in follow-up studies at 6 weeks of age **a** Sham exposure—normal cytoarchitecture of purkinje cell layer. **b** Microwave exposure—more reduction in purkinje cell density with elongated and necrotic cells. **c** Sham exposure—normal cytoarchitecture of pyramidal cell layer. **d** Microwave exposure—more reduction in pyramidal cell density with elongated and necrotic cells (NC). **e** Sham exposure—normal cytoarchitecture of cerebral cortex cells layer. **f** Microwave exposure—increased neuronal degeneration (ND). Images were observed at $\times 400$

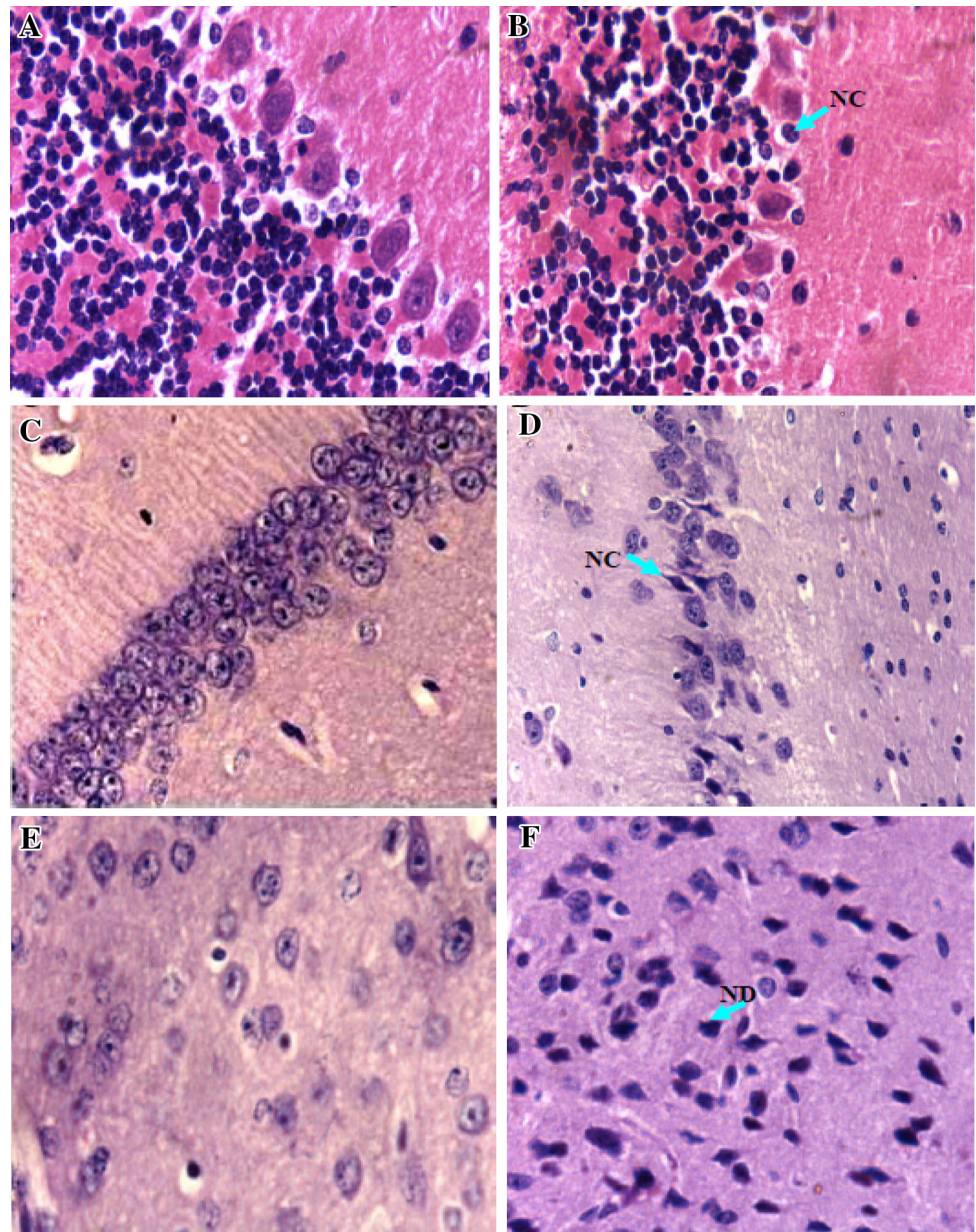


Table 3 Variations in thickness of granular layer (μm) $\pm$ SD and molecular layer (μm) $\pm$ SD in cerebellum and number of purkinje cells $\pm$ SD in cerebellum of Swiss albino mice aged 2 weeks exposed

to 10 GHz microwaves 2 h/day for 15 consecutive postnatal days during the experimental period

Parameter	Groups (exposure 2 h/day)	Immediately after completion of 15 days exposure	Follow-up study at 6 weeks of age
Thickness of granular layer (μm) $\pm$ SD	Sham	80.4 $\pm$ 1.7	91.7 $\pm$ 0.87
	Microwaves exposed	78.2 $\pm$ 1.6**	88.34 $\pm$ 1.4**
Thickness of molecular layer (μm) $\pm$ SD	Sham	103.4 $\pm$ 1.7	106.2 $\pm$ 1.05
	Microwaves exposed	97 $\pm$ 2.8**	102.3 $\pm$ 2.1**
Number of purkinje cells $\pm$ SD	Sham	24.8 $\pm$ 1.2	25.1 $\pm$ 1.6
	Microwaves exposed	15.8 $\pm$ 1.8**	16.2 $\pm$ 1.6**

The statistically significant level for the MW effects are given in the table: ** $p < 0.001$

age during follow-up experiments by comparing with levels observed immediately after exposure. Antioxidants play an important role in distress of cells or in other words, to protect the cells from oxidative damage. Cellular antioxidants like SOD, GSH, catalase, and LPO are an important marker of free radical generation. Adequate level of cellular antioxidants maintains the free radicals scavenging potential in brain. Several studies from our group investigated significant changes in the level of SOD, GPx, CAT, and LPO in various organs exposed to different frequencies of microwaves [7–9, 11, 49, 68, 69].

In the present study, decreased level of protein was measured in the mice whole brain on attaining 6 weeks of age after 15 days of microwave exposures (Table 1). A loss in concentration of protein was estimated immediately after exposure recorded lesser degree compared to 6 weeks aged mice. This can be defined on the basis of the expression of various heat shock proteins immediately after exposure, which declines as cell recover from the stress. This declined level in microwave exposure group in the follow-up studies may indicate the irreversible loss of many important proteins due to oxidative stress during brain development.

Increased free radicals are produced during the developmental period, especially during the period of myelination and synaptogenesis (approximately 10–14 postnatal days) in the mouse brain [65]. Therefore, age-dependent increase in the level of free radical formation or decline in the cellular repair mechanism could lead to the cellular abnormalities and results in an increased concentration of oxidized proteins [70]. Khan and Black [71] have also reported such changes in protein and enzymes level in the mouse brain during the early postnatal period. Our previous findings report that microwave exposures increases the free radicals and produce reactive oxygen species in brain and affect the antioxidant enzymes level [49, 50].

Spatial learning and memory deficits observed in this study was an evidence of adverse effects produced by age-dependent microwave exposure at the time of initiation (Fig. 4). The increased latency and distance to reach the platform in Morris water maze test may be due to alterations in the hippocampal synaptic plasticity as also reported by Kamal et al. [72]. This synaptic activity is regulated by the cholinergic system that acts synergistically with glutamatergic transmission [73]. In general, the cholinergic system plays an important role in hippocampal-dependent learning and memory and the cells possess several varieties of long-lasting synaptic plasticity. Liu et al. [74] reported that the early-onset decrease in learning and memory is linked to a significant increase in oxidative stress-related parameters, such as lipid peroxidation or protein oxidation. Such findings support the hypothesis that microwaves-induced oxidative stress contributes to age-related

impairment in learning and memory. Additionally, Sakhnini et al. [75, 76] also reported that EMF exposure causes a deficit in motor coordination in the prenatal mice. Authors reported that prenatal and neonatal exposure to EMF significantly affects the animal's rate of learning, when compared with sham-exposed one. In our previous findings, decrements in the ability of mice to learn the spatial memory task was observed in adult mice (6–8 weeks) exposed to 10 GHz microwave radiations. This has been correlated to the lowered protein synthesis during this stage of translation, which stabilizes long-term memory [11].

The growth of mice brain is a definitive postnatal event. It evolves a complex network of neurons, axons, dendrites, glial cells, and synaptic junctions during this growth period. This may subsequently result in the complex development of physiological and biochemical function of the brain. Although it can be further correlated to altered spatial memory and learning as also evident in this study. Therefore, histopathological changes observed in CA1 region in the present study are in a line with the results of Miranda et al. [77], where they investigated that a functional hippocampus is required for the acquisition of spatial tasks in the Morris water maze test. Transient synaptic changes were observed in CA1 region after water maze training, involving a downregulation proportion of the symmetric synapses, although the total number of synapses counted remains stable. This synaptic remodeling was observed in the spatial and non-spatial versions of the MWM shortly after training, suggesting an association of the hippocampus in the acquisition of non-spatial tasks with a time-limited role in spatial memory consolidation. Therefore, the current model of memory consolidation assumes that the storage of long-term memory (LTM) is associated with gene expression, new protein synthesis, and synaptic remodeling [78, 79].

The results presented here reveals that 2 weeks aged mice are very sensitive to 10 GHz microwave radiations in terms of biochemical parameters and histological architectural disturbances in CA1 region of hippocampus (Fig. 5a, b). The CA1 region also appears to be involved in retrieval effect after longer time delays. Although rats, which had lesions in the CA1 region, had no difficulty in encoding new information, they were impaired in retrieval after 24-h interval [80, 81]. Evidence reviewed elsewhere [82] indicates that the CA1 region makes a special contribution to the temporal aspects of memory, including associations over a delay period, sequence memory, and order memory. The CA1 network is thought to play an important role in retrieval of information to the neocortex. The cortical projection to CA1 region is sufficient for place recognition. Also, the Schaffer pathway is necessary for the rapid learning and recall of a spatial location [83, 84].

We report in this paper a reduced density of pyramidal neuronal cells and degeneration of cerebral cortical neurons in the exposed group of animals. Salford et al. [85] found significant evidence for neuronal damage in the cortex, hippocampus, and basal ganglia also abnormal neurons (dark neurons) in the rats exposed to RF EMF. The pyramidal neurons play a key role in memory, attention, language, and consciousness in humans. Therefore, any such changes occurring in humans due to microwave exposure could be detrimental in terms of memory and cognitive functions [12, 86], as also reported in this study. Degeneration of the cerebellum, particularly the purkinje cells was observed in our present study as a result of microwave exposure. Purkinje cells are responsible for motor coordination and control of the motor functions in humans. In the present study, thickness of granular layer and molecular layer were significantly decreased after microwaves exposure initiated at 2 weeks of age for 15 consecutive days. Sharma [87] reported that granular layer was more radiosensitive compared to molecular layer. Kaplan et al. [23] investigated the effect of EMF on the purkinje cells in the cerebellum of 4-week-old rats. Authors reported degeneration of purkinje cells with nuclear swelling and irregular boundaries of the nuclei. Some purkinje cells had pyknotic nuclei and dark cytoplasm suggesting cell death in this group. Although present study reveals the loss in connective tissue, neurons exhibited pyknosis and hyperchromatism. Therefore, it can be suggested that the reduction in number of purkinje cells may be due to the radiosensitive nature of the cells. Several researchers have reported a significant decrease in the number of purkinje cells of rat cerebellum after microwave exposure [88–90]. Results of this study are also in support of the fact that first 2 weeks postpartum would be critical for continued neural development, because mouse pups display rapid behavioral changes during that time [64]. Therefore, neuroanatomical and functional development of the mouse hippocampus extends up to 2 weeks. Thus, 10 GHz microwaves exposure for 15 days starting from 2 weeks of age causes cytoarchitectural damage as assessed immediately and on attaining 6 weeks of age.

Conclusions

The results from the present study are consistent with our previous findings and suggest that the decreased protein levels and changes in MWM test and histological parameters associated with microwave-induced oxidative damages. It was concluded that 2 weeks aged mice are highly sensitive after microwave exposure in terms of morphometric, biochemical, behavioral, and histopathological parameters. Lastly, this concludes the damages caused by

EMF during early postnatal age also possess long-term effects as observed in follow-up studies.

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Compliance with ethical standards

Conflict of interest Authors have no conflict of interest.

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