



Rats exposed to 2.45 GHz of non-ionizing radiation exhibit behavioral changes with increased brain expression of apoptotic caspase 3



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ABSTRACT

In recent years there has been a tremendous increase in use of Wi-Fi devices along with mobile phones, globally. Wi-Fi devices make use of 2.4 GHz frequency. The present study evaluated the impact of 2.45 GHz radiation exposure for 4 h/day for 45 days on behavioral and oxidative stress parameters in female Sprague Dawley rats. Behavioral tests of anxiety, learning and memory were started from day 38. Oxidative stress parameters were estimated in brain homogenates after sacrificing the rats on day 45. In morris water maze, elevated plus maze and light dark box test, the 2.45 GHz radiation exposed rats elicited memory decline and anxiety behavior. Exposure decreased activities of super oxide dismutase, catalase and reduced glutathione levels whereas increased levels of brain lipid peroxidation was encountered in the radiation exposed rats, showing compromised anti-oxidant defense. Expression of caspase 3 gene in brain samples were quantified which unraveled notable increase in the apoptotic marker caspase 3 in 2.45 GHz radiation exposed group as compared to sham exposed group. No significant changes were observed in histopathological examinations and brain levels of TNF- α . Analysis of dendritic arborization of neurons showcased reduction in number of dendritic branching and intersections which corresponds to alteration in dendritic structure of neurons, affecting neuronal signaling. The study clearly indicates that exposure of rats to microwave radiation of 2.45 GHz leads to detrimental changes in brain leading to lowering of learning and memory and expression of anxiety behavior in rats along with fall in brain antioxidant enzyme systems.

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1. Introduction

Mobile phones have been a part of everyday life for most people in the world since the late 1990s. There has been an unprecedented growth in the global communication industry in recent years which has resulted in a dramatic increase in the number of wireless devices. It has been suggested that the widespread use of cellular telephones and wireless devices like Wi-Fi may result in increased health risks resulting from brain exposure to electromagnetic radiation (EMR) [1]. Mobile phones and Wi-Fi make use of Non-Ionizing electromagnetic radiation (NI-EMR). Non-ionizing radiation is the term given to radiation in the part of the electromagnetic spectrum

where there is insufficient energy to cause ionization. It includes electric and magnetic fields, radio waves, microwaves, infrared, ultraviolet, and visible radiation. Microwaves with the frequency range of 300 MHz–300 GHz are used in the telecommunication system.

There are several health hazards associated with microwave radiations. One amongst them is the thermal effects of the radiations that lead to localized increase in tissue temperature, which may cause disruption of cell function. The extent of heating depends on the specific absorption rate (SAR), and power density of the electromagnetic waves that are emitted from the radiation emitting devices. Non-thermal effect mainly results from the stress generated in the cells and tissues due to the interaction between the radiation and cells in the body. The non-thermal effects include changes in the activity of Ca²⁺ dependent K⁺ channels, alterations of membrane structure and function, permeability modifications of liposomes and isolated cells. Non-thermal effects of microwave radiation accumulate over time and their harmful effects are more evident after 8–10 years of exposure and as in the initial years

Abbreviation: TNF- α , Tumor necrosis factor- α .

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of exposure body's defense mechanisms and the stress proteins, specifically the heat shock proteins come into play. These situations has prompted many investigations to probe interaction between EMR and neuronal cells, even at intensities not able to produce thermal effects. Studies on the potential health and biological effects of mobile phone radiation have been extensively conducted over the years. Epidemiological as well as *in vivo* and *in vitro* approaches have been applied to examine the potential health and biological effects of mobile phone radiation [2–4]. Furthermore, human studies have been carried out to examine the effects of these radiations on sleep, cognitive functions and behavioral aspects [5,6]. The brain is the organ which gets affected by the use of cell phones as the device is held often in proximity to the head. Also brain is the most sensitive target organ for microwave radiation where mitochondrial injury occurs early and more severely in comparison to other organs. Thus, among other organs the effect of microwave radiation on brain is the most researched area [3,7].

Considerable amount of research has been focused on the behavioral impairments particularly, learning and memory deficits in rats, following exposure to EMR [7]. For this, various behavioral paradigms are utilised. This includes Morris water maze test for spatial memory evaluation, object recognition test to study recognition memory, Elevated plus maze test and light dark test to evaluate anxiety. All these paradigms have been used in the current study. A number of studies have investigated the effects of NI-EMR on spatial memory and place learning tasks in rodents mainly using radial arm mazes or water mazes [8–11]. Despite numerous studies, there is no definitive conclusion till date that high frequency EMR can affect the memory performance. Also there have been no reliable reports on the effect of these radiations on anxiety behavior in rats. The present study was thus an attempt to study the effects of NI-EMR on learning and memory along with the effect on anxiety behavior in rats.

Oxidative stress is a biochemical condition characterised by a relatively high levels of toxic reactive species mainly reactive oxygen species (ROS) and the natural anti-oxidant defence mechanism in body is unable to remove this excess free radicals [12]. Excessive ROS is detrimental to brain energy metabolism. Electromagnetic radiations are known to increase the activity of free radicals in the cells. EMR could initiate a state of oxidative stress in the brain [13]. This may accelerate neuronal degeneration and can cause molecular damage to other cell organelles [14]. The present study attempted to profile the activities of different anti-oxidant enzymes like superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and determine the extent of lipid peroxidation and protein carbonyl levels in the brains of rats exposed to NI-EMR of 2.45 GHz for 4 h/day for 45 days.

Apoptosis plays a crucial role in homeostasis during the development of embryo. It also plays a fundamental role in organogenesis and during the entire life time of multi cellular organisms. Several *in vitro* studies have been carried out in this context and have reported that exposure to non-ionizing electromagnetic radiation can activate the caspase dependent apoptosis pathway. [15]. TNF- α is a pro-inflammatory cytokine and it is released from the cells during inflammation. It is involved in extrinsic apoptosis pathway and ultimately stimulates the effector caspase which is caspase 3 [16]. The present study has therefore focused on the involvement of the apoptotic markers in the brain when exposed to radiofrequency radiation.

The present study was undertaken to investigate and explore the effects of NI-EMR especially the radiation frequency used in Wi-Fi devices, on the brain of rats focussing on some of the parameters of oxidative stress and apoptosis. The 45 day study was designed to shed light on the impact of these radiations on memory performance and behavioral changes.

2. Materials and methods

2.1. Animals

Female Sprague Dawley rats of body weight 180–220 g were used in the study. They were obtained from Glenmark laboratories Ltd., Mumbai, India. The rats were acclimatised to the laboratory environment for a period of one week before handling them. All the animals were housed in the humidity (40–50%) and temperature (25–27 °C) controlled room, in polycarbonate cages. The animals were kept on 12–12-h light-dark cycle and were provided with water and standard rat chow diet. For the animal experimentation protocols, prior approval was obtained from 'Institutional Animal Ethics Committee' (IAEC, CPCSEA-BCP/2015-01/05 dated 6 June 2015), and all studies were performed in accordance with 'Committee for the Purpose of Control and Supervision on Experiments on Animals' (CPCSEA) guidelines.

2.2. Experimental design and exposure system for the rats

Animals for the study were randomized into following two groups:

Group1: Sham exposed group; Rats unexposed to NI-EMR. A total of 6 rats ($n=6$) were placed in the exposure cage during the exposure hours but with the radiation source kept off. Group 2: Rats exposed to NI-EMR of 2.45 GHz ($n=6$). The animals of group 2 were exposed to the 2.45 GHz radiation for 4 h/day for 45 days between 10 am to 6 pm, at a power density of 7.88 W/m².

During the exposure hours, rats in the group were placed in the individual square Plexiglas cage of dimension 15 × 15 × 15 cm. The cage was perforated with sufficient holes of 1 cm diameter for proper ventilation. A monopole antenna was placed at the centre and the six individual cages were placed surrounding it. The rats were positioned in such a way that every animal received equal intensity of radiation (Fig. 1.). The power absorbed by the rat is given by the formula:-Power density × Surface area of the rat × 0.5. Surface area of the rat was calculated to be 0.012 m². Using 0.5 as the reflection coefficient in biological media at 2.45 GHz, the radiated power absorbed by the body of rat was calculated to be 0.04728 W.

The radiation exposure system was fabricated in the Antenna lab of Electrical Engineering Department of IIT Bombay, Powai. A Printed Circuit Board (PCB) consisting of Voltage Controlled Oscillator (VCO) and amplification stages produced 24 dBm (about 0.252 W) of microwave energy at 2.45 GHz of frequency. Output was measured using spectrum analyser. A printed monopole antenna with a gain of 1 dB to radiate at 2.45 GHz was designed using IE3D simulation tool. The generator was thereafter terminated with this antenna to radiate at designated frequency.

2.3. Behavioral studies

Four different behavioral paradigms were used in the study namely, Elevated Plus Maze test (EPM), Light Dark Box test (LDT), Object Recognition Task (ORT) and Morris Water Maze test (MWM). Rats from each group were subjected to all the four behavioral paradigms.

2.3.1. EPM

The EPM consisted of two open arms (40 × 40 cm) and two closed arm of similar dimension with a wall of 40 cm height. The maze was elevated 50 cm above the ground and was placed in a quiet room. Rats were placed individually in the centre of the maze facing the open arm. The following parameters were calculated:

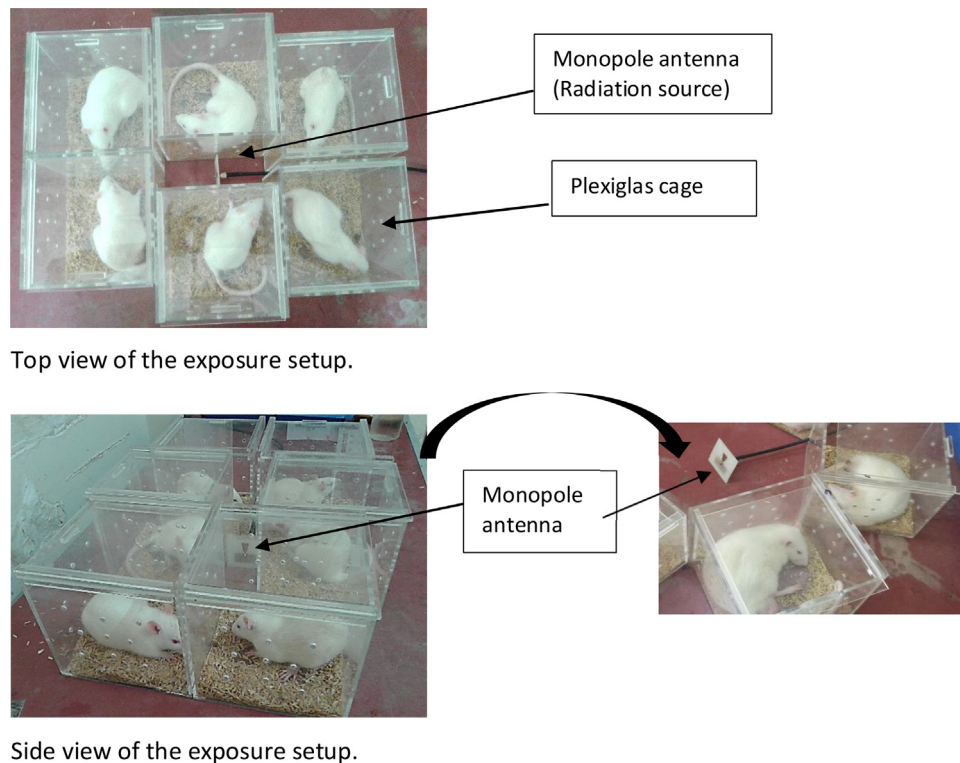


Fig 1. The 2.45 GHz radiation exposure system used in the study. The monopole antenna placed at the centre surrounded by the rats residing in the Plexiglas cage. The rats were exposed to NI-EMR of 2.45 GHz frequency for 4 h/day for 45 days at a power density of 7.88 W/m^2 .

number of closed arm entries, number of rearing and percentage of open arm entries [17].

2.3.2. LDT

The light dark box consisted of two compartments, a dark and a light compartment, both of same dimensions $43 \times 30 \times 30 \text{ cm}$. The two compartments were connected to each other by a $10 \times 10 \text{ cm}$ door. The dark compartment was enclosed so that no light entered it while the light compartment was illuminated. Each animal was placed in the light compartment at the beginning of the test and the behavior of the animal in the box was recorded over a period of 5 min using a video camera. Percentage time spent in the light compartment; number of rearing was determined [18].

2.3.3. ORT

The ORT box consisted of an open black box of dimension $45 \times 45 \times 30 \text{ cm}$. The test lasted for three days. On the first day (habituation phase), the rats were habituated to the empty apparatus for a period of 5 min. On the second day (acquisition phase) two identical objects were placed in the box and the rat was allowed to explore the box for 5 min. On the third day (test session), two identical objects that were placed in the acquisition phase, were kept in the apparatus and each rat was allowed to explore the two objects (Test 1). After an hour, one of the identical objects was replaced by a novel object different in colour, shape and texture from the familiar object and the rats were again allowed to explore the two objects (Test 2). The time spent by the rats in exploring the novel and the familiar objects in Test 2 of the test session was determined using smart video software. Discrimination Index (DI) was calculated which represents the difference in exploration time expressed as a proportion of the total time spent exploring the two objects in test 2 of the test session [19–21].

2.3.4. MWM

The MWM test was performed to assess the spatial learning and memory of animals. The MWM is a swimming-based model where the animal learned to escape on to a hidden platform. The test apparatus was a circular pool made of plastic (150 cm in diameter, 45 cm in height), filled to the depth of 30 cm with clear water at $25 \pm 1^\circ \text{C}$. The water was made opaque with milk. An escape platform (10 cm diameter) was located at the centre of the circular pool. The test lasted for three days and was divided into two phases. Training phase (spatial acquisition phase): The training consisted of two consecutive days with 4 trials per day. The rats were trained to swim freely in the pool of water on day 1 and day 2. Rats were placed gently into the water with its nose pointing towards the wall of the tank. Each time the trials were initiated from different positions. The rats learned to swim in the pool and to find the hidden platform within a period of 60 s. After finding the platform it was allowed to remain there for 15 s. Rats failing to find the hidden platform in 60 s was placed on the platform for 15 s. On both the days of training, the platform was kept 1 cm above the water level in the pool. In between the trials the rats were dried and placed in their home cages for 5 min. Testing phase (memory retention phase): On the third day the platform was lowered 3 cm below the water level. The test lasted for 30 s. The rat was released gently into the water facing the wall of the tank. The time it takes to reach the platform was noted using a stop watch. The escape latency represents the time taken by the rats to find the hidden platform and it is considered as a measure of the acquisition of spatial navigation abilities [22].

2.4. Collection of brain tissue

After the exposure period i.e., after 45 days, the animals were sacrificed using CO_2 chamber. The rats were perfused using 0.9% saline and their brains were dissected out and sectioned. One half (hemisphere) of the brains from each rat was fixed in 10% formalin

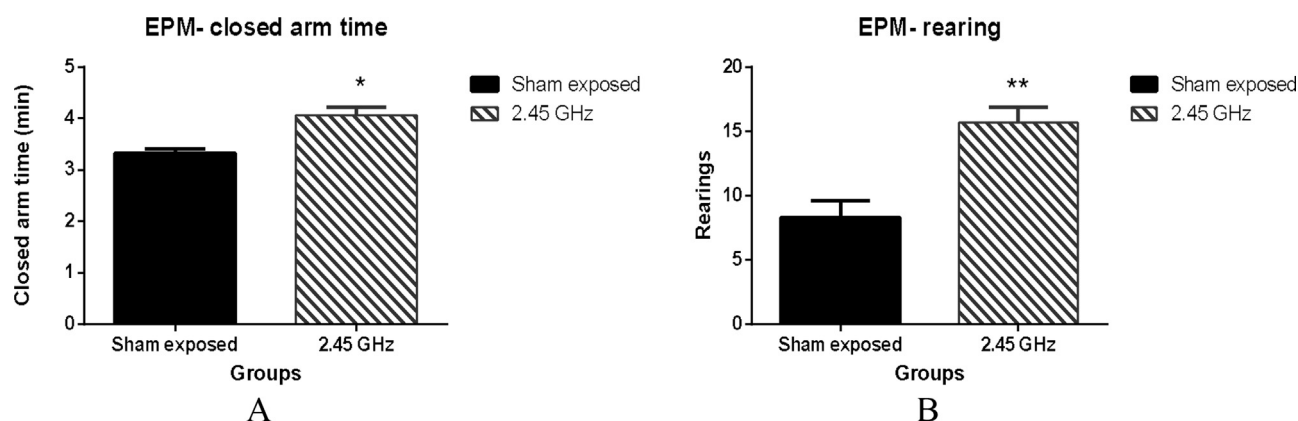


Fig. 2. Effect on 2.45 GHz radiation on anxiety behaviour in EPM test. 2A: time spent in the closed arm, 2B: Number of rearing; A significant difference was found in the number of rearing and the total time spent in the closed arm. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to NI-EMR of 2.45 GHz; Statistical analysis by Wilcoxon Rank-sum test; Values are expressed as mean $\pm$ SEM (n = 6); significantly different from sham exposed group (* $p < 0.05$); significantly different from sham exposed group (** $p < 0.01$).

solution (Neutral Buffered Formalin) for histopathological examination including Hematoxylin and Eosin (H&E) staining and Golgi cox staining. The other half (hemisphere) of the brains from each rat was washed twice with ice cold saline solution, labelled and stored at -80°C in aluminium foil, until processing, and was used for biochemical estimation including TNF- α and for determining caspase 3 gene expression [23].

2.5. Preparation of brain tissue homogenate

Homogenisation of brain tissue was performed within 24 h of sacrifice. The brain tissues were placed in a glass homogenizer tube (20 ml capacity) after cutting them into small pieces. Ten volumes of chilled Tris-HCL (50 mM, pH 7.4) were added during homogenization. Tris-HCL was added in parts during the process of homogenisation. The crude homogenate obtained was centrifuged at 4°C at 5000 rpm for 5 min in a cooling centrifuge. The supernatant was used for the estimation of brain total proteins, catalase and SOD activities, and nitrite, LPO, GSH and protein carbonyl levels [13,23].

2.6. Biochemical investigations

To evaluate the biological effects of electromagnetic radiation of 2.45 GHz on brain, the extent of lipid peroxidation (LPO), nitrite, reduced glutathione (GSH), carbonylated protein levels and the activities of catalase (CAT), superoxide dismutase (SOD) were determined on brain tissue homogenates.

2.6.1. Estimation of brain tissue lipid peroxidation

The extent of lipid peroxidation was determined spectrophotometrically as thiobarbituric acid reactive substances (TBARS). TBARS are expressed in terms of malondialdehyde (MDA) equivalents. MDA level was determined by a method based on reaction with thiobarbituric acid (TBA). 2 ml of 10% (w/v) trichloroacetic acid was added to 0.1 ml of the homogenate. And the mixture was kept in ice bath for a period of 20 min. The mixture was centrifuged to separate the precipitate. Clear supernatant was separated. 2 ml of the clear supernatant of sample was added to 2 ml of freshly prepared TBA. The resulting mixture was incubated at 95°C for 60 min. The mixture was then cooled under tap water and centrifuged at 4000 rpm for about 10 min. Absorbance of the supernatant was taken at 530 nm. Extent of lipid peroxidation was expressed as μmoles of MDA formed per mg protein [23].

2.6.2. Estimation of brain tissue reduced glutathione levels

The GSH content in the brain homogenate was measured by the method of Jollow et. al., [25] in which 0.5 ml of tissue supernatant was mixed with 0.5 ml of 4% sulphosalicylic acid and incubated at 4°C for one hour. Centrifugation was done at 3000 rpm at 4°C for 15 min. To 27 μl of supernatant obtained after centrifugation, 145 μl of 0.1 M (pH 7.4) PBS and 27 μl of 100 mM 5, 5'-dithiobis-(2-nitrobenzoic acid) or Ellman's reagent was added. Absorbance was read immediately at 412 nm. GSH levels were expressed as micromoles of DTNB conjugate formed/mg protein [25].

2.6.3. Estimation of brain tissue nitrite levels

The measurement of nitrite levels in the brain tissue homogenate was based on Griess reaction. For this, Griess reagent was prepared by adding 0.01 g *N*-(1-naphthyl) ethylene diamine dihydrochloride (NEDA), 0.1 g sulphanilamide and 250 μl of phosphoric acid was mixed and volume was made up to 10 ml. Equal volumes of supernatant (500 μl) and Griess reagent (500 μl) were mixed, the mixture was incubated for 10 min at 37°C and the absorbance was read at 540 nm spectrophotometrically. Nitrite levels were expressed as $\mu\text{g}/\text{mg}$ protein [24].

2.6.4. Estimation of brain catalase activity

For the estimation of catalase activity in brain tissue homogenate, 2.9 ml of 10 mM Hydrogen peroxide (H_2O_2) was added to 0.1 ml of homogenate. 10 mM H_2O_2 was prepared by adding 23 μl of 30% Hydrogen peroxide which was diluted with phosphate buffer (50 mM, pH 7.0) to 20 ml. The change in absorbance was recorded spectrophotometrically at 240 nm for 3 min against blank in which 100 μl of distilled water was added by replacing tissue homogenate. The results were expressed as μmole H_2O_2 consumed/min/mg protein [29,30].

2.6.5. Estimation of brain tissue superoxide dismutase activity

For the measurement of SOD activity, 2 ml of 0.05 M carbonate buffer (pH 10.7), 0.5 ml of 0.1 mM EDTA and 50 ml of tissue homogenate were taken in a glass tube. The reaction was initiated by adding 0.5 ml of 3×10^{-4} M epinephrine. The absorbance was measured immediately at 480 nm for 3 min against blank in which no tissue homogenate but 2.5 ml of carbonate buffer was used. Values were reported as units of SOD per mg protein [28].

2.6.6. Estimation of brain tissue protein carbonyl levels

Protein carbonyl levels were measured using 2, 4-dinitrophenylhydrazine method. This method is based on the spectrophotometric detection of protein hydrazones following

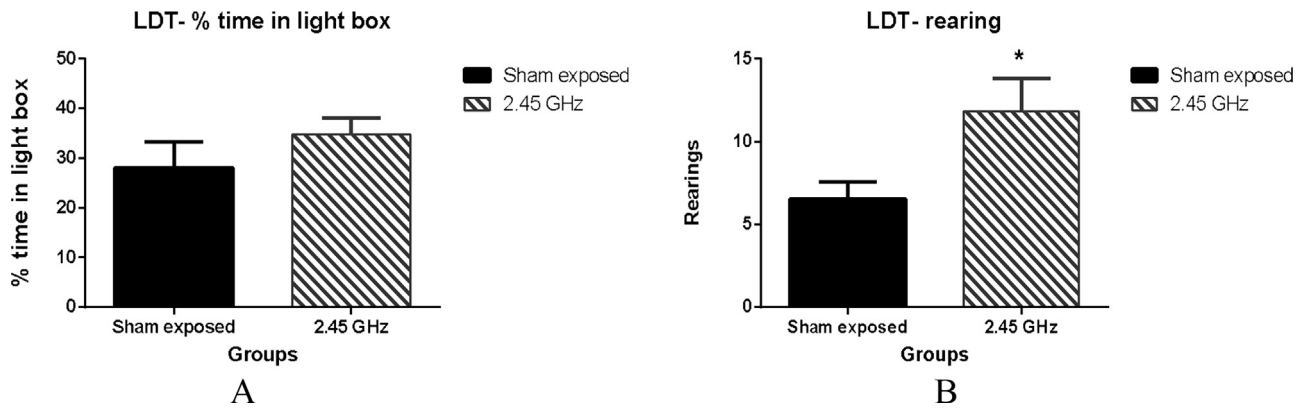


Fig. 3. Effect of 2.45 GHz on anxiety behaviour in LDT. Fig. 3A represents the percentage of time spent in the light compartment (box) and Fig. 3B represents the number of rearing in the light compartment, of sham exposed and 2.45 GHz exposed group in light dark box test. The number of rearing in the 2.45 GHz group were significantly higher as compared to the sham exposed group. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to NI-EMR of 2.45 GHz; Statistical analysis by Wilcoxon rank-sum test; Values are expressed as mean $\pm$ SEM (n = 6); significantly different from sham exposed group (* p < 0.05).

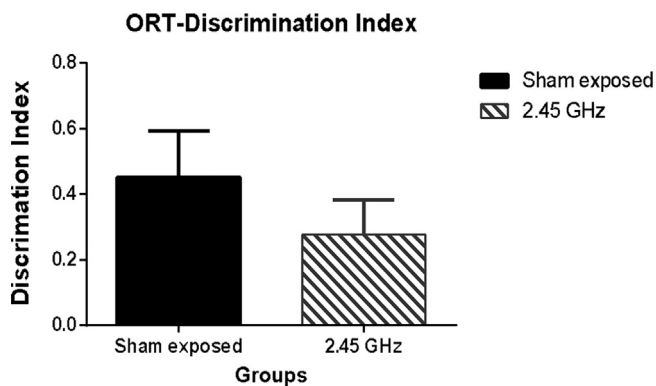


Fig. 4. Effect of 2.45 GHz radiation on memory performance in ORT. No significant difference was found in the discrimination indices of 2.45 GHz group as compared to sham exposed group. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to NI-EMR of 2.45 GHz; Statistical analysis by Wilcoxon rank-sum test; Values are expressed as mean $\pm$ SEM (n = 6).

reaction with 2, 4-dinitrophenylhydrazine. Results were expressed as nmoles of carbonyls/mg of protein. The protein levels in the final pellets were quantified by reading the absorption at 280 nm. The amount of proteins was calculated from bovine serum albumin standard treated in the same way [16,26,27].

2.6.7. Estimation of brain total protein

Total protein in brain homogenates were measured by the method of Hartree [31] using Bovine Serum Albumin as standard.

2.6.8. Estimation of brain TNF- α levels

TNF- α level in rat brain homogenate were quantified using commercially available Rat TNF- α ELISA kit (R & D Biosystems, Quantikine) as per manufacturer's instructions. The concentration was expressed as pg/ μ l. [16]

2.6.9. Quantitative real-time reverse transcription polymerase chain reaction

The frozen brain tissue were cut into small pieces and powdered by grinding the frozen tissue fragments into pre-chilled mortar and pestle. Occasionally liquid nitrogen (N_2) was added during grinding to prevent thawing of the tissue. Approximately, 70–80 mg of the tissue sample was added to 175 μ l of RNA Lysis buffer and RNA was extracted according to manufacturer's protocol (SV Total RNA Isolation System Promega). The yield of total RNA obtained was determined by Nanodrop spectrophotometer (NanoDrop ND-1000, Montchanin, DE, USA). The concentration of RNA was estimated by spectrophotometry from the relative absorbances at 260 and 280 nm (i.e., A260/A280).

The polymerase chain reaction (PCR) reaction was performed using the following primer sequences; the housekeeping gene,

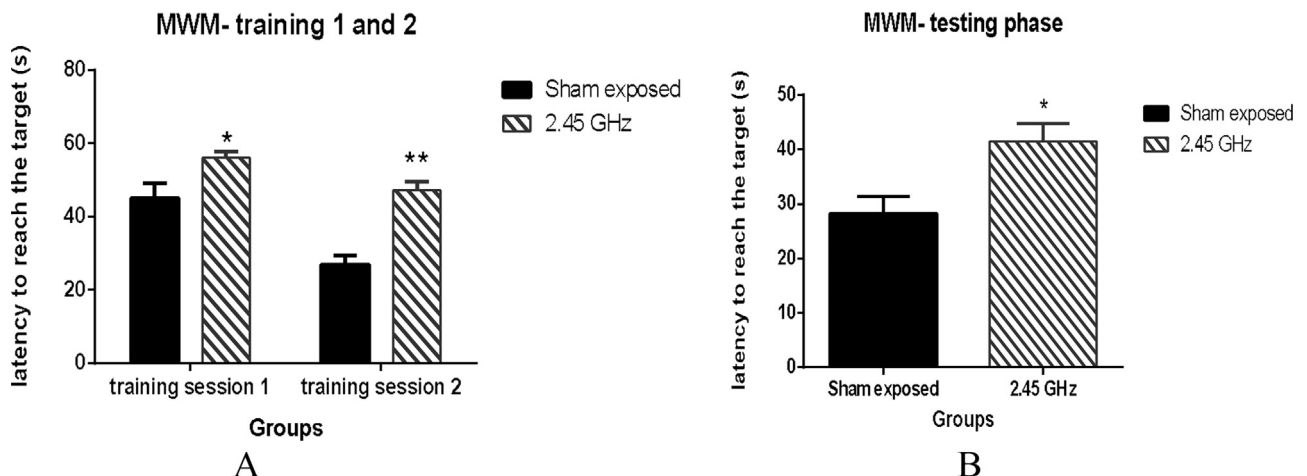


Fig. 5. Effect of 2.45 GHz radiation on memory performance in MWM. A significant increase was found in the latency time to reach the target in both, the training phase as well as the testing phase. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to NI-EMR of 2.45 GHz; Statistical analysis by Wilcoxon rank-sum test; Values are expressed as mean $\pm$ SEM (n = 6); significantly different from sham exposed group (* p < 0.05); significantly different from sham exposed group (** p < 0.01).

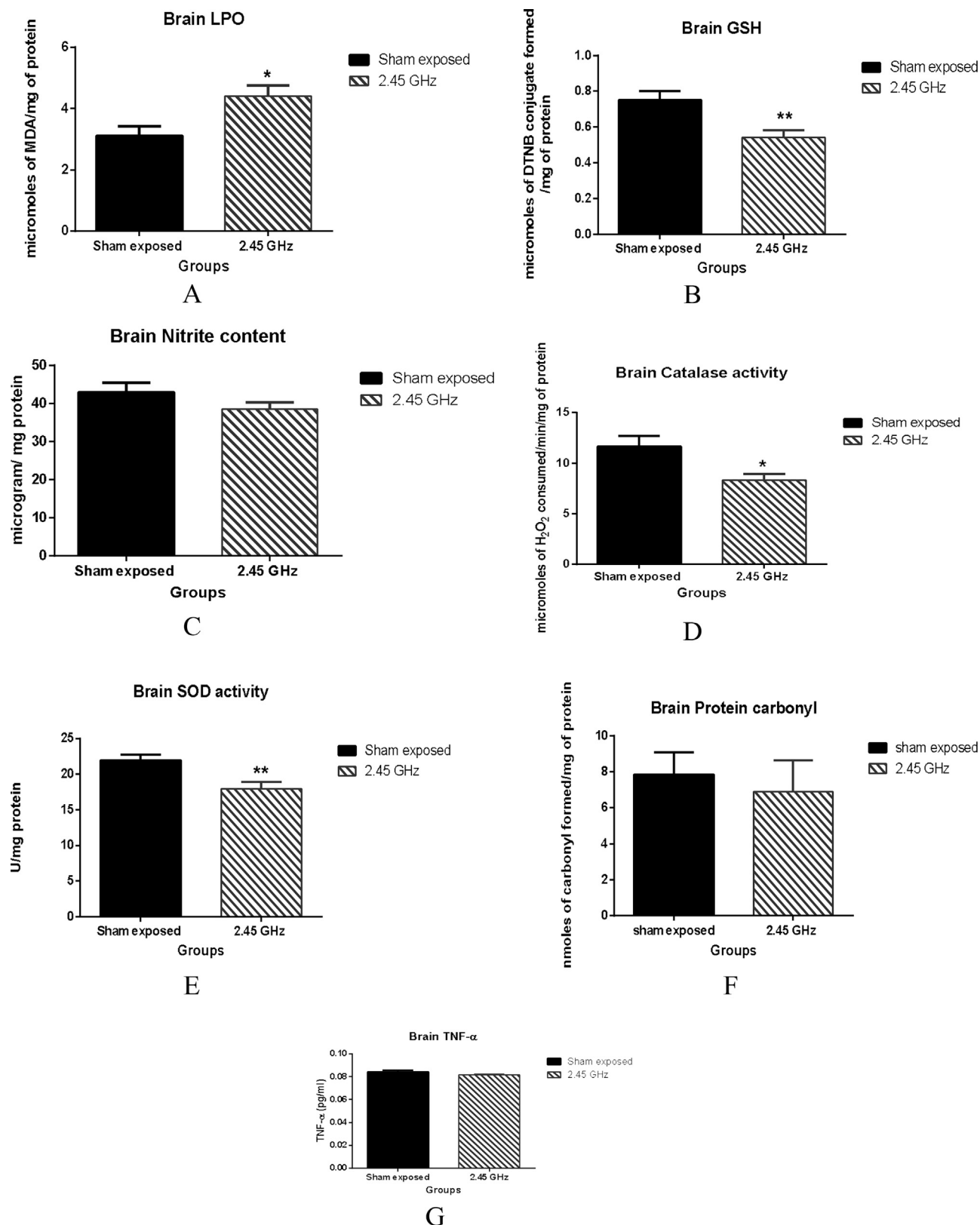


Fig. 6. A) Effect of 2.45 GHz radiation on MDA levels in rat brain homogenate. Levels of MDA were significantly higher in the rat brain homogenates of 2.45 GHz group than the sham exposed group. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to the NI-EMR of 2.45 GHz; Statistical analysis by student's *t*-test; Values are expressed as mean $\pm$ SEM (*n* = 6); significantly different from sham exposed group (**p* < 0.05). B) Effect of 2.45 GHz radiation on GSH levels in rat brain homogenate. Micromoles of DTNB conjugates formed were significantly lower in 2.45 GHz exposed group than the sham exposed group. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to the NI-EMR of 2.45 GHz; Statistical analysis by student's *t*-test; Values are expressed as mean $\pm$ SEM (*n* = 6); significantly different from sham exposed group (***p* < 0.01). C) Effect of 2.45 GHz radiation on Nitrite content in rat brain homogenate. No significant difference was found in the nitrite content between both the groups. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to the NI-EMR of 2.45 GHz Statistical analysis by student's *t*-test; Values are expressed as mean $\pm$ SEM (*n* = 6). D) Effect of 2.45 GHz radiation on Catalase activity in rat brain homogenate. Catalase activity significantly reduced in the 2.45 GHz group in comparison to sham exposed group. Sham exposed: not exposed

glyceraldehyde 3-phosphate dehydrogenase (GAPDH-Forward 5'-TGACCACTGCTTAGC-3', Reverse 3'-GCCCCACGGCCATCA-5'), was used as an internal control for determining gene expression and Caspase 3 gene (Forward 5'-GACTGCGGTATTGAGACAG-3', Reverse 3'-CGAGTGAGGATGTGCATGA-5'). One-step quantitative Real-Time RT-PCR was performed using One Step GoTaq® qPCR Master Mix (Promega) on 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The Real-Time PCR reactions (20 µl) contained 0.4 µM forward primer, 0.4 µM reverse primer, 90 ng/µl of sample mRNA, 10 µl of Master Mix (1X) and 0.4 µl of RT Mix (1X), 0.31 µl of Supplemental CXR Reference Dye and nuclease free water. The reaction conditions were as follows: initial 2 min (50 °C), denaturation at 95 °C (10 min), followed by 50 cycles of denaturation at 95 °C (15 s), annealing at 60 °C (30 s) and extension at 60 °C (30 s). The results were expressed as fold change of the threshold cycle (Ct) relative to control group using $2^{-\Delta\Delta C_t}$ method [16].

2.6.10. Histopathological examination and analysis of dendritic arborization

The sagittally sectioned brain hemispheres were fixed in 10% Neutral buffered Formalin (NBF) solution. They were dehydrated using ascending strengths of ethanol. The specimens were then cleared in xylene and embedded in paraffin wax in metal moulds for another 24 h. 5 µm thick sections were cut from the wax blocks using a microtome. The sections were fixed on clean glass slides and stained with Hematoxylin and Eosin (H&E). The stained slides were observed under the microscope and imaging was done at a magnification of 100 X (10 X objective and 10 X eyepiece).

To study the changes in the dendritic arborization of neurons in the brain the camera lucida neuronal tracing was carried out on Golgi cox stained brain sections. Concentric circle method of Sholl DA, 1953 [32] was adopted for dendritic quantification. Neurons were traced on a transparent sheet, under 400× (10 neurons from each animal) using a camera lucida attached to a monocular microscope. In order to be selected for analysis, the Golgi impregnated neurons must satisfy following criteria: 1. Presence of dendrites that are untruncated. 2. Consistent and dark impregnation along the entire extent of dendrites. 3. The dendrites should be isolated from neighbouring neurons to avoid interference. The neurons that satisfy these criteria were selected and dendritic morphology at a radial distance of 100 µm from the centre of the soma was examined and studied.

2.7. Statistical analysis

All results are presented as mean ± SEM. Statistical analysis of behavioral parameters were analysed using Wilcoxon Rank Sum test (Mann Whitney's *U* test) while the data of biochemical tests and dendritic arborization were analysed using student's *t*-test. Differences were considered significant at **p* < 0.05, ***p* < 0.01 and *****p* < 0.0001 respectively as compared to sham exposed group. All statistical analysis was performed using Graphpad Prism 6 software for windows (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. Effect of microwave radiation on behavioral parameters

In EPM test, the 2.45 GHz radiation exposed rats spent considerably more time in the closed arm of plus maze (Fig. 2A) with a notable increase in number of rearing (Fig. 2B). Similarly, the rats exposed to 2.45 GHz showed an increased number of rearing in the light box in LDT test, as compared to sham exposed group (Fig. 3B). These findings were consistent with the anxiety behavior observed in rats. In MWM test, the rats from 2.45 GHz radiation exposed group took more time to reach the target platform in training as well as in testing phase, than the sham exposed group (Fig. 5). This directs towards the impairment in spatial memory of the radiation exposed rats. However no significant difference was seen in time exploring the novel and familiar objects in ORT (Fig. 4) and percentage of time spent in the light box in LDT (Fig. 3A) in 2.45 GHz exposed group.

3.2. Effect of microwave radiation on biochemical parameters in brain

Fig. 6(A–F) represents the changes in antioxidant parameters in rat brains. A significant increase was observed in the levels of MDA (Fig. 6A) as indicator of lipid peroxidation in 2.45 GHz radiation exposed rats (4.40 ± 0.35 µmol/mg protein) as compared to sham exposed rats (3.12 ± 0.29 µmol/mg protein). Reduced glutathione (GSH) protects the cells from free radicals and oxidative insults. In the current study, there was a significant decrease (Fig. 6B) in the levels of GSH in 2.45 GHz (0.54 ± 0.03 µmol/mg protein) exposed rats in contrast to sham exposed rats (0.75 ± 0.05 µmol/mg protein). Catalase scavenges the hydrogen peroxide and decreases its levels. The exposure of rats to 2.45 GHz radiation led to a significant drop (Fig. 6D) in catalase activity in the 2.45 GHz exposed rat brains (8.32 ± 0.59 µmol/mg protein) when compared to sham exposed rats (11.63 ± 1.04 µmol/mg protein). The SOD, dismutase's O₂ anions and decrease in its activity directly relates to augmentation in oxidative stress. The decrease in its activity in the 2.45 GHz radiation exposed rats (17.93 ± 0.94 U/mg protein) as compared to sham exposed rats (21.97 ± 0.78 U/mg protein) testifies the oxidative stress inducing effects of microwave radiations (Fig. 6E). No significant changes were observed in protein carbonyl levels (6.90 ± 1.73 nmol/mg protein, 7.85 ± 1.21 nmol/mg protein in 2.45 GHz radiation exposed and sham exposed rats respectively) (Fig. 6F) and nitrite content (38.53 ± 1.79 µg/mg protein, 42.95 ± 2.54 µg/mg protein in 2.45 GHz radiation exposed and sham exposed rats respectively) (Fig. 6C).

3.3. Effect of microwave radiation on TNF-α levels

TNF-α is a cell signaling protein (cytokine), involved in inflammation. Analysis of TNF-α levels in the brain of the rats by ELISA, revealed no inflammatory changes in the brain of 2.45 GHz radiation exposed rats (0.082 ± 0.00067 pg/mL) when compared to sham exposed rats (0.084 ± 0.001 pg/mL) (Fig. 6G).

to NI-EMR; 2.45 GHz: exposed to the NI-EMR of 2.45 GHz Statistical analysis by student's *t*-test; Values are expressed as mean ± SEM (n=6); significantly different from sham exposed group (**p* < 0.05). E) Effect of 2.45 GHz radiation on SOD activity in rat brain homogenate. SOD activity significantly reduced in the 2.45 GHz exposed group in comparison to sham exposed group. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to the NI-EMR of 2.45 GHz; Statistical analysis by student's *t*-test; Values are expressed as mean ± SEM (n=6); significantly different from sham exposed group (***p* < 0.01). F) Effect of 2.45 GHz radiation on carbonylated protein levels in rat brain homogenate. No significant difference in the protein carbonyl content between both the groups. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to the NI-EMR of 2.45 GHz Statistical analysis by student's *t*-test; Values are expressed as mean ± SEM (n=6). G) Effect of 2.45 GHz radiation on TNF-α level in rat brain homogenate. Statistical analysis by student's *t*-test revealed no significant difference in the brain TNF-α level of rats exposed to 2.45 GHz group when compared to sham exposed group. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to the NI-EMR of 2.45 GHz.

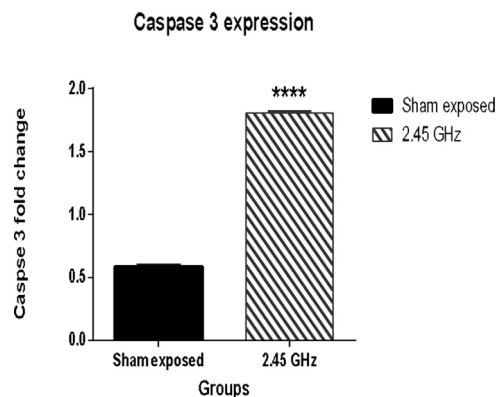


Fig. 7. Effect of 2.45 GHz radiation on Relative gene expression of caspase 3 in rat brain. A significant increase in caspase 3 gene expression was found in the 2.45 GHz group as compared to sham exposed group. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to the NI-EMR of 2.45 GHz; Statistical analysis by student's *t*-test; Values are expressed as mean ± SEM (*n* = 6); significantly different from sham exposed group (*****p* < 0.0001).

3.4. Quantitative real-time reverse transcription polymerase chain reaction

Fold change was calculated after normalization of GAPDH mRNA expression. Statistical test revealed an increase in expression of caspase 3 gene in the brain of 2.45 GHz radiation exposed rats (1.80 ± 0.0065) as compared to sham exposed rats (0.58 ± 0.0068) (Fig. 7).

3.5. Histopathological examination and analysis of dendritic arborization

Gross examination of sagittal sections of rat brain hippocampi and cerebral cortices by H&E staining did not reveal any histopathological changes in 2.45 GHz radiation exposed group when compared to sham exposed group in the current study (Fig. 8).

Fig. 9 represents the microscopic image and the corresponding tracing of Golgi impregnated neurons from 2.45 GHz and sham exposed rats. Fig. 10 represents the effect of 2.45 GHz radiation on dendritic branch points and dendritic intersections. Analysis of dendritic arborization by Golgi-cox staining showed a significant reduction in overall dendritic branching (in the concentric zones of 10–20, 20–30, 30–40, 40–50 and 60–70 μ m) and dendritic intersections (at a distance of 20, 30, 40, 50 and 70 μ m) in 2.45 GHz exposed group as compared to sham exposed group which indicates alteration in the arbor structure of dendrites.

4. Discussion

The investigations focused on estimating the impact of non-ionizing electromagnetic radiations (NI-EMR) of frequency 2.45 GHz for a period of 4 h/day for 45 days in rats on brain biochemical parameters, caspase 3 gene expression and on cognition and behavior. This study was expected to generate preclinical evidences on possible adverse impact of non-ionizing radiations of frequencies used in mobile phone and Wi-Fi transmissions in humans.

Spatial and associative memory is checked through different learning and memory paradigms. Ammari et al., [33] have reported that subchronic and chronic head-only exposure of rats to GSM 900 MHz signal (45 min, SAR 1.5 W/kg or 15 min, SAR 6 W/kg) did not induce spatial memory deficit in rats in the radial arm maze test. Dubreuil et al. [34] did not find any effect on spatial memory in rats subjected to head-only exposure to 900 MHz GSM signals. Likewise, a 10-day whole-body exposure to 900 MHz microwave radiation did not cause any deficit in the performance of mice in

a spatial learning task. Timo Kumlin et al., [17] found that 5 week exposure to a 900 MHz frequency signal (2 h/day, 5 days a week, SAR 3 W/kg) had no evident effect on the response of young rats in open field, the plus-maze, and acoustic startle tests. Cosquer et al., [35] indicated that anxiety responses were not changed in a maze test of rats by 2.45 GHz EMR exposure. Narayanan et al., [22] placed a mobile phone in silent but vibratory mode beneath a cage containing young adult rats. Each day for 4 weeks, these animals were exposed to the fields associated with 50 missed calls and then their spatial learning capabilities were tested using a water maze. Significant differences in behavior were seen. Exposed animals initially took far longer to locate the escape platform during acquisition trials and, although their latencies improved, they remained slower than controls. The current study revealed significant changes in the behavioral paradigms of LDT, EPM and MWM in non-ionizing radiation exposed rats thus leading us to conclude that exposure to non-ionizing radiation of 2.45 GHz frequency can elicit decline in learning and memory performance (cognitive decline) and cause anxiety like behavior in rats. One of our earlier study reported anxiety like behavior in rats exposed to 900 MHz radiation which is in line with the present findings [36].

The human body is equipped with a complete arsenal of defenses against environmental and internal hazards. Exposure of cells and tissues to Electromagnetic Radiation (EMR) leads to production of Reactive Oxygen Species (ROS) such as superoxide anion, hydroxyl radicals and hydrogen peroxide [37]. The concentrations of ROS intermediates are kept under strict control by the activity of a complex antioxidant defense system. The uncontrolled production of ROS can lead to a situation known as “oxidative stress” [33,38–40]. The ROS can attack many different biomolecules such as nucleic acid, proteins, lipids and even cell structure causing oxidative damage. EMR may disturb ROS metabolism by increasing their production or by decreasing antioxidant enzyme activity. Organs such as brain and muscle contain a high amount of water [41,42]. Microwave exposure results in dielectric heating. Dielectric heating occurs when an alternating electromagnetic field is applied to an imperfect dielectric material [1,43]. Absorption of EMR and dielectric heating occurs more in organs and tissues which contain high percentage of water while less heating occurs in tissues with less water content. Heating is more efficient in materials with a high conductivity at microwave frequencies typically 915 MHz or 2.45 GHz. Wireless radiation can have different effects on health, and one of the possible actions could result from effects on the water content of our body because water molecules are strongly heated at microwave frequencies around 2.45 GHz. Tissue water can absorb much of this wireless radiation. When water, which is present in about 80% of cells, is exposed to EMR, it splits with formation of ROS that contributes to cellular radiation injury [1,15,43,44]. Our earlier studies have reported changes in the brain biochemical parameters of rats exposed to non-ionizing radiations [36,45]. In the present study, a notable decrease was found in the rat brain levels of GSH. A significant decrease was also found in the catalase and SOD activities. There was also a marked increase in brain lipid peroxidation as indicated by enhanced MDA levels.

Brain is the organ which is composed of high content of lipids. As a result, the cells of the brain are more liable to oxidation. Lipid peroxidation, a type of oxidative degeneration of polyunsaturated fatty acids, has been linked with altered membrane structure and enzyme inactivation, is an indication of damage of the neurons. Increase in lipid peroxides such as TBARS and lipid hydroperoxides (LOOH) result in increased oxidative damage. SOD, CAT and GSH are first line cellular defenses against oxidative injury. Their activities and levels were found to be significantly decreased in 2.45 GHz group. These results are in line with earlier reported studies [33,40]. Their levels may be lowered due to enhanced lipid peroxidation indicating enhanced oxidative stress in brain.

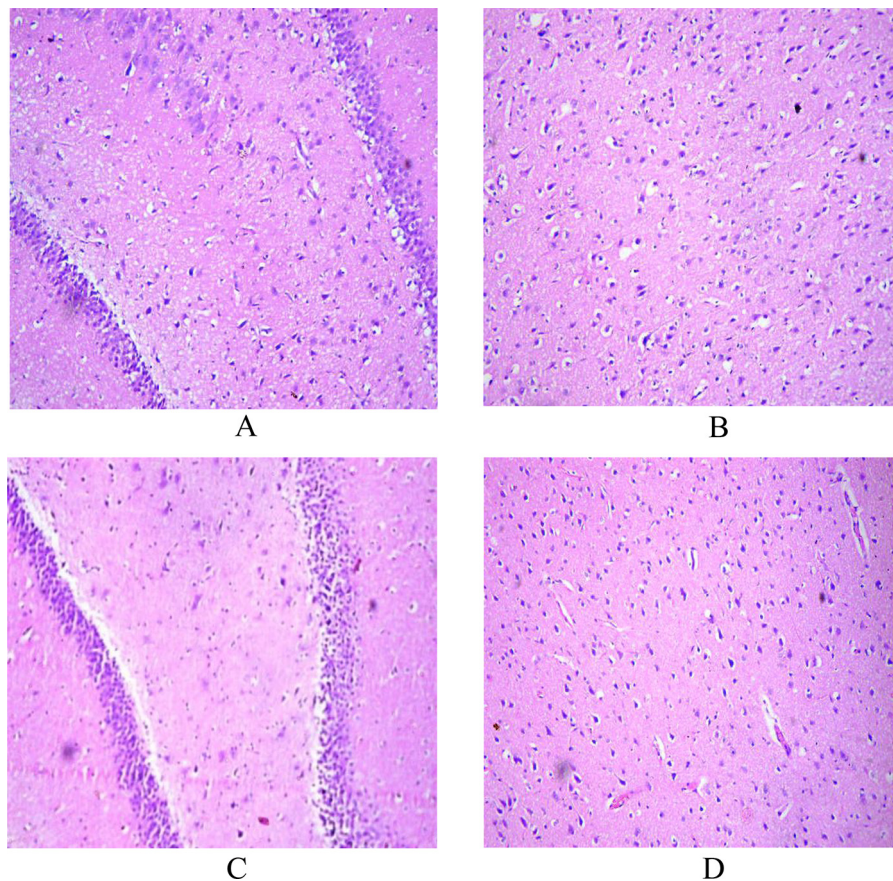


Fig. 8. Represents the photomicrographs of Hematoxylin and eosin stained hippocampal and cerebral cortex region from sham exposed and 2.45 GHz group respectively. No histopathological changes were observed in the micrographs showing brain sections of 2.45 GHz exposed group.

Superoxide dismutase (SOD) belongs to the group of metalloenzymes and forms the first line of defense by catalyzing the dismutation of superoxide radical to O_2 and H_2O_2 in all aerobic organisms undergoing oxidative stress and is located in almost all subcellular compartments that generate activated oxygen. The enzyme activity is increased when the superoxide radicals react with nitric oxide (NO) to form peroxynitrite, a strong oxidizing agent that can lead to depletion of thiol groups, damage to DNA, and nitration of proteins. The enzyme levels are also elevated during superoxide radical participation in formation of hydroxyl radicals [12,46].

Catalase (CAT) is an abundant tetrameric heme-containing enzyme that catalyzes the dismutation of two molecules of H_2O_2 into water and oxygen and plays a very important role in combating oxidative stress in brain. It has high specificity for H_2O_2 , but weak activity against organic peroxides. H_2O_2 production occurs majorly at peroxisome site. H_2O_2 generated in this organelle during β -oxidation of fatty acids provokes CAT scavenging activity. H_2O_2 has been implicated in many stress conditions. When cells are stressed for energy and are rapidly generating H_2O_2 through catabolic processes, H_2O_2 is degraded by CAT in an energy efficient manner [12,33,46].

Reduced glutathione (GSH) occurs in relatively high concentrations (1–10 mM) in all cells but the brain levels are comparatively lower. Specific mitochondrial transporters are involved in transportation of almost 15% of GSH to mitochondria. GSH molecule has cysteine protein which is most essential functional component, being responsible for supplying active thiol groups that are involved in directly reacting with ROS or establishing reversible bonds with protein thiol groups. Thus, its direct antioxidant func-

tion by reacting with superoxide radicals, peroxy radicals and singlet oxygen followed by the formation of oxidized GSH and other disulfides thus inactivating the free radicals. Glutathione plays an important role in the regulation of variety of cell functions and in cell protection against oxidative injury. In this investigation, a significant decrease was found in the levels of GSH which is indicative of their increased usage to combat oxidative stress in the brain due to exposure to non-ionizing radiations [12,46].

Superoxide radicals generated at site of damage modulate SOD and CAT activities resulting in lowered activities of these enzymes and accumulation of superoxide anions. In this study, the activities of antiperoxidative enzymes (SOD and CAT) were significantly decreased in the brain tissues of 2.45 GHz group as compared to sham exposed group. Reduction in the activities of these antiperoxidative enzymes may be because of the increased generation of reactive oxygen radicals, such as hydrogen peroxide and superoxide, which in turn leads to the inhibition of activities of these enzymes [47].

Cells regulate intracellular Ca^{2+} levels lightly and over Ca^{2+} entry can lead to inappropriate activation of processes that operate normally at low levels, causing metabolic derangements and eventual cell death [48]. For example, excessive elevations in intracellular Ca^{2+} through transient receptor potential (TRP) and voltage gated calcium channels (VGCC) may activate antioxidant degradation, induce formation of reactive oxygen species (ROS). It may also disrupt normal mitochondrial function leading to oxidative stress and bioenergetic failure. The rise in Ca^{2+} stimulates the release of superoxide radicals via activation of NADPH oxidase and MPAK. The superoxide radical is dismutated to hydrogen peroxide (H_2O_2) in the presence of superoxide dismutase (SOD). Then it is detoxified

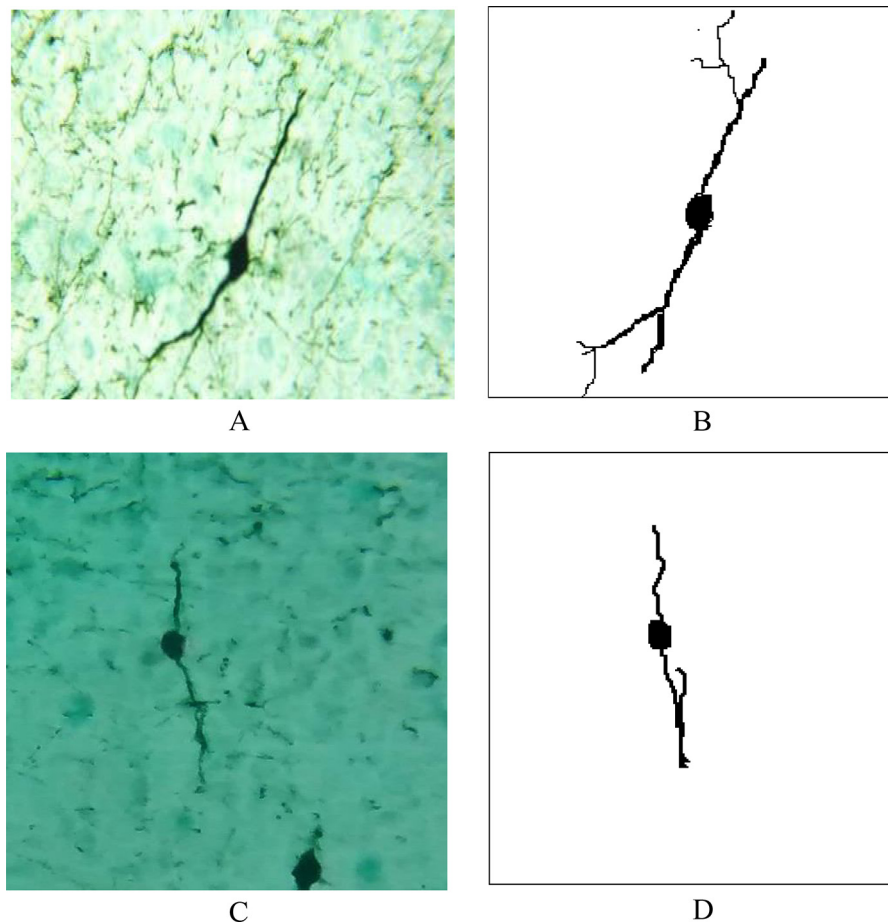


Fig. 9. Representative photomicrographs and camera lucida tracings of Golgi-Cox stained neurons from sham exposed (A, B) and 2.45 GHz radiation exposed (C, D) rats. (Drawn at 400x magnification).

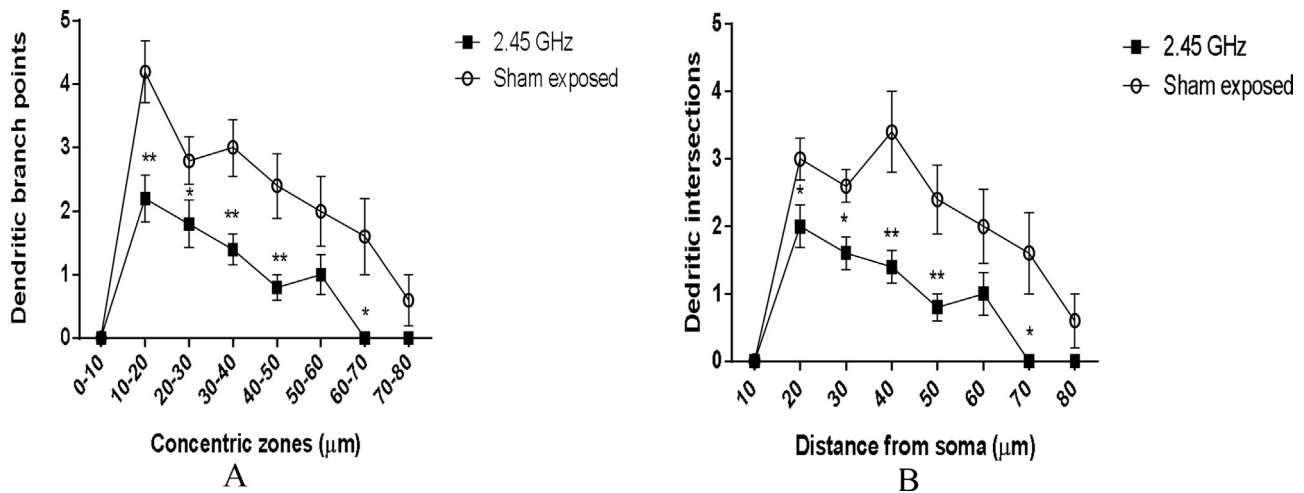


Fig. 10. Effect of 2.45 GHz radiation on dendritic branching (A) and dendritic intersections (B) of neurons of cerebral cortex. Statistical analysis revealed a significant decrease in the number of dendritic branching points in the concentric zones of 20–30 and 60–70 μm (* $p < 0.05$) and also in the concentric zones of 10–20, 30–40, 40–50 μm (** $p < 0.01$). No significant difference was found in other concentric zones. There was a significant decrease in the number of dendritic intersection studied at a distance of 20, 30 and 70 μm (* $p < 0.05$) from the soma and also at a distance of 40 and 50 μm (** $p < 0.01$), in the 2.45 GHz group. Statistical tests revealed no significant difference between the groups in the dendritic intersections studied at other concentric circles. Sham exposed: not exposed to NI-EMR; 2.45 GHz: exposed to the NI-EMR of 2.45 GHz; Statistical analysis by student's *t*-test; Values are expressed as mean ± SEM ($n = 6$); significantly different from sham exposed group at * $p < 0.05$; significantly different from sham exposed group at ** $p < 0.01$.

to water by catalase and glutathione peroxidase (GSH-Px) antioxidant enzymes. Antioxidants regulate EMR induced Ca^{2+} influx into the cytosol by inhibition of ROS and regulation of TRP and VGCC.

EMR such as mobile phones and wireless induces also apoptosis, caspase enzyme activation and DNA damage through increase of ROS production [1,15,44].

Paulraj and Behari, [49] indicated that chronic exposures to 2.45 GHz may affect brain growth and development. This is because of the decrease in the PKC activity in the hippocampus. An increase in the glial cell population in the exposed group was also detected. Our study indicated that exposure to non-ionizing radiation leads to fall in brain anti-oxidant defense rendering it more susceptible to ROS induced stress and eventual damage.

To determine if the exposure of rats to non-ionizing electromagnetic radiation led to inflammation in the brain, the pro-inflammatory cytokine, brain TNF- α levels were measured. There were no changes observed in the brain levels of TNF- α in 2.45 GHz exposed rats. While a study carried out by Tarek K. Motawi et. al., [16], indicated a marked increase in the levels of brain TNF- α in rats exposed to a radiation frequency of 900 MHz. Thus we deduced that the extent of non-ionizing radiation exposure in the present study did not elicit inflammatory changes in the brain. Chronic studies should be undertaken to substantiate the safety of these non-ionizing radiations.

The process of apoptosis or programmed cell death is characterized by a variety of genetic markers. There are basically two types of apoptotic pathways- the extrinsic pathway also termed as death receptor pathway which involves binding of death ligands such as TNF- α to the cell surface receptors and the activation of caspase 8 and eventually caspase 3 activation. The second pathway is the intrinsic or the mitochondrial pathway which is activated in cases of stress conditions triggering apoptosis. This pathway involves Bcl-2/Bax, caspase 9 and eventually activation of caspase 3, which is the effector caspase. In the present study the expression of caspase 3 gene was studied. An upregulation of caspase 3 gene was detected in the rats exposed to 2.45 GHz radiation which suggests that exposure to non-ionizing radiation can disturb the apoptotic machinery in the brain. The current study however did not show any significant difference in the TNF- α levels in the brain, which is involved in the extrinsic apoptosis pathway while an upregulation of caspase 3 was evident. This points toward the involvement of intrinsic pathway of apoptosis.

Masuda et. al., [50] indicated that there were no morphological changes observed in Wistar rats exposed to a pulsed radiation frequency of 915 MHz. The current study corroborated that claim as we have not detected any changes in the brains of rats exposed to radiation of 2.45 GHz frequency. One of the reason for lack of any histopathological changes can be attributed to sub-chronic exposure of radiation used in the study. However, chronic exposure to microwave radiation can lead to damaging effect to brain cells. Dendritic arborization tracing was carried out to analyze the dendritic branching and the dendritic intersections of the neurons in these rat brains. Neurons are specialized computational compartments that integrate and regulate the propagation of information. The principle language used by neurons to communicate with each other is the action potential. Dendrites differ from axons in many important respects, both morphologically and functionally. Dendrites have specialized structures including spines, which are the main excitatory synaptic sites that are not found in axons. Unlike axons, dendrites have tapering processes such that distal branches have smaller diameters than proximal ones. An astonishing diversity of dendritic arbor structures exists among neurons of different and similar classes. The shape, size, and complexity of dendritic trees can modulate action potential propagation and influence the intrinsic firing pattern of a neuron. Action potential propagation is strongly influenced by (a) the number of branching points, (b) the rate of increase in dendritic membrane area, and (c) the relationship between the diameter of parent and daughter dendrites at branch points. In the present study the number of dendritic intersections and the number of branch points in the brains of 2.45 GHz radiation exposed rats were significantly reduced when compared to sham exposed rats. These findings indicate that the branching and

extension of dendrites are stunted in the brains of rats exposed to non-ionizing radiation of frequency 2.45 GHz.

5. Conclusion

The study was an attempt to draw attention towards the adverse effects of non-ionizing electromagnetic radiations (NI-EMR) in the frequency that is used widely in the field of telecommunication. Many studies have captured the impact of the 900 MHz and 1800 MHz frequencies, however the frequency of radiation in Wi-Fi range has not been much explored. Along with the use of cell phones there is a growing concern with the use of Wi-Fi devices which continuously emit radiations in the frequency of 2.4 GHz. Hence we thought it prudent to investigate the impact of radiation of the frequency of 2.45 GHz. It can be concluded that the exposure to non-ionizing radiation of 2.45 GHz caused detrimental changes in rat brain leading to learning and memory decline and expression of anxiety behavior along with fall in brain antioxidants. The exposure triggered the gene expression of caspase 3 which plays a major role in the apoptotic pathway. The chronic impact of non-ionizing radiation needs to be thoroughly evaluated in humans so that combative steps can be taken.

Conflict of interest

The author(s) declared that no conflicts of interest exist with respect to the authorship and/or publication of this article.

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