Indian Journal of Experimental Biology
Vol. 50, December 2012, pp. 889-896

Microwave radiation induced oxidative stress, cognitive impairment and inflammation in brain of Fischer rats

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Received 4 June 2012; revised 21 September 2012

Public concerns over possible adverse effects of microwave radiation emitted by mobile phones on health are increasing. To evaluate the intensity of oxidative stress, cognitive impairment and inflammation in brain of Fischer rats exposed to microwave radiation, male Fischer-344 rats were exposed to 900 MHz microwave radiation (SAR = 5.953×10^{-4} W/kg) and 1800 MHz microwave radiation (SAR = 5.835×10^{-4} W/kg) for 30 days (2 h/day). Significant impairment in cognitive function and induction of oxidative stress in brain tissues of microwave exposed rats were observed in comparison with sham exposed groups. Further, significant increase in level of cytokines (IL-6 and TNF-α) was also observed following microwave exposure. Results of the present study indicated that increased oxidative stress due to microwave exposure may contribute to cognitive impairment and inflammation in brain.

Keywords: Cognitive function, Cytokines, Inflammation, Microwave radiation, Oxidative stress

The enormous use of devices like mobile phones, Wi-Fi, microwave ovens etc. in present time has motivated research on possible adverse effects of exposure to microwave radiation on human health. The possible risks and concerns to these radiations are increasing rapidly in the society. Microwave radiation is part of radiofrequency electromagnetic radiation (RF-EMR) with frequency ranging from 300 MHz to 300 GHz (wavelength ranging from 1 mm to 1 m). These non-ionizing radiations have a large frequency band pattern and interact with information structures of living cells—nucleic acids, proteins and membranes. Microwave radiations emitted by mobile phones are mainly non-thermal in nature and are absorbed by skin and other superficial tissues, resulting in insignificant rise of temperature in the brain or other organs of the body[1]. Various non-thermal effects of microwaves have been reported, such as alterations in cognitive function, decrease in cholinergic activity and gene expression alterations in important areas of brain such as cerebellum, hippocampus and cortex in animals[2-5]. The potential risks of microwave radiations to human health, especially with respect to alterations in neurocognitive functions is a serious matter of concern owing to the fact that mobile phones are used in close proximity to brain.

The hippocampus which is an utmost important part of brain, controls behavioural and cognitive functions including spatial and working memory is also affected due to microwave exposure[6-9]. Microwave radiation exposure due to GSM (900/1800 MHz) mobile phones for 4 weeks leads to alterations in spatial memory performance in Wistar rats[10]. Deshmukh *et al.*[11] have also shown that exposure to low level microwave radiation at different mobile frequencies (900, 1800 and 2450 MHz) leads to alterations in HSP 70 level and cognitive impairment in Fischer rats.

Chronic exposure to microwave radiation at 900 MHz emitted from mobile phones is also known to cause oxidative damage in brain of Wistar rats[12]. Many of the studies have reported that exposure to electromagnetic radiation causes production of free radicals in tissues[13,14]. The increased oxidative stress or imbalance in cellular antioxidant defence system have been implicated in increasing number of neurocognitive dysfunctions like epilepsy including stroke, acute damage to brain and hypoxia reperfusion trauma[15,16].

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Although a number of studies on bioeffects of microwave exposure on cognitive dysfunction are reported, the neural mechanisms behind it are still not clearly understood. In light of the above considerations, the present study has been designed to investigate the effects of microwave radiation (at different mobile frequencies) on cognitive function mediated via development of oxidative stress in rat brain.

Materials and Methods

At the beginning of each experiment, all animals used in this study were naïve, they had not been undergone any kind of treatment.

Materials—Reduced glutathione (GSH), 2, 4-dinitrophenylhydrazine (DNPH), 5, 5′-Dithiobis (2-nitrobenzoic acid) (DTNB) and 2-Thiobarbituric acid (TBA) were procured from Sigma-Aldrich company (St. Louis, Mo, USA) and rest of the chemicals were obtained from Qualigens Fine Chemicals, Mumbai, India and were of analytical grade. IL-6 and TNF-α were estimated with commercially available ELISA kits (Koma Biotech Inc., Korea).

Animals—Male Fischer-344 rats (150-200 g body weight) were obtained from central animal house facility and kept under standard conditions of temperature (22 ± 2 ^{o}C) and humidity (40-50%) under alternating 12 h light and dark cycle. They were provided with nutritionally adequate standard diet obtained from Nutrilab, Bangalore, India and water *ad libitum*. Animals were randomly selected and divided into following 3 groups (6 animals in each group): Group I sham exposed—animals not exposed to microwave radiation but kept under same conditions as that of other groups, group II—animals exposed to 900 MHz frequency at an average whole body specific absorption rate (SAR) as 5.953×10^{-4} W/kg and group III—animals exposed to 1800 MHz frequency at an average whole body SAR as 5.835×10^{-4} W/kg. The microwave exposed groups were exposed to 900 MHz and 1800 MHz microwave radiation at power 0.00 dBm (1 mW) in a transverse electromagnetic cell (TEM) cell for 2 h daily, 5 days per week, every day at same time for 30 days. The power density at plane of animal cages was 1.68 W/m^2 (at 900 MHz) and 1.72 W/m^2 (at 1800 MHz). The power received by animals in the chamber was 0.2408 mW. Appropriate permission was taken from Institutional Animal Ethics Committee (IAEC) for animal research, University College of Medical Sciences, Delhi and appropriate care of the animals was undertaken as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India for laboratory animal facilities.

Microwave exposure system, exposure conditions and dosimetry—The gigahertz transverse electromagnetic (GTEM) cell GTE 10 has been designed with the help of Center for Applied Research in Electronics (Microwave laboratory), Indian Institute of Technology, New Delhi and Amitech Electronics Ltd., Sahibabad to estimate biological effects of microwave radiation (Fig. 1). The system provided by the manufacturer (Amitech Electronics Ltd., Sahibabad, Ghaziabad, U.P) was pre-calibrated for various electromagnetic characteristics and uniformity of E-field within the TEM cell. The GTEM cell has been designed for frequencies ranging from 9 KHz to 3.2 GHz, so the same chamber works well for both 900 and 1800 MHz as the present experimental requirements. GTEM cell is a pyramidal tapered, dual terminated section with its outer cell dimension as L: 220 cm × B: 120 cm × H: 80 cm. Microwaves are generated from Microwave Generator SMC 100 (Rohde & Schwarz GmbH & Co, Germany). The microwave source consists of a signal generator operating at a frequency range from 9 KHz to 3.2 GHz, an amplifier, a DC regulator and a power meter. During the exposure rats were restrained in a L: 30 cm × B: 20 cm × H: 15 cm closed box divided into 4 compartments with holes of 1 cm diameter to facilitate easy movement and breathing, kept at a distance of 100 cm from source in quiet zone and uniform field. At a time 6 rats were placed within the device in two such boxes. The microwave chamber is lined with absorbers which minimize the possibility of any reflections. The uniformity of electric field was experimentally checked by means of measurements performed with an E-field probe (Rohde & Schwarz NRV- Z32) inserted into the TEM cell through a slit wall. The TEM cell was placed in a temperature

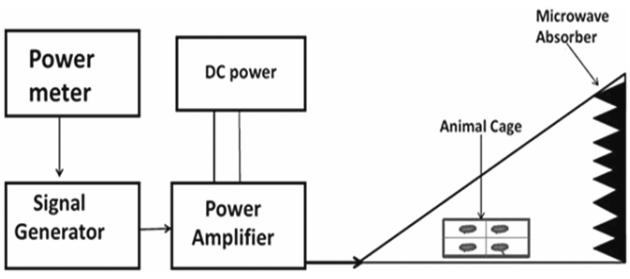

Fig. 1— Diagrammatic view of microwave exposure set up

controlled room under constant lighting conditions. Specific absorption rate (SAR) distribution was calculated by Power Balance Method using the following equation[17]:

$$P_{abs} \text{ per mouse} = 1/n\ (P_{in} - P_{out} - P_{refl})$$

where, P_{abs} = RF power in watt absorbed per animal, n = number of animals within the cell, P_{in}= input power (watt), P_{out}= output power (watt), and P_{refl}= reflected power (Watt).

Microwave radiation exposure procedure— Animals were given whole body exposure to microwave radiation in transverse electromagnetic (TEM) cell for 2 h/day, 5 days per week for 30 days. Body weight of animals was recorded regularly on weekly basis. Electromagnetic field parameters in cages were measured continuously during experimental exposure. After the exposure period, all animals were tested for spatial memory performance using the Elevated plus maze (EPM) and Morris water maze (MWM).

Experiment I: Effect of microwave exposure on cognitive function

Elevated plus maze (EPM) paradigm testing— Behavioural responses in rodents were measured by elevated plus maze (EPM) test. It consists of two opposite open arms (50×10 cm), crossed with two closed arms of same dimensions with 40 cm high wall. The arms are connected with central square (10 × 10 cm). The animals were trained on EPM one day prior to microwave exposure and acquisition was measured in terms of seconds. Rats were placed individually at one end of an open arm facing away from the central square and allowed to enter either of the closed arms and explore it for 20 sec. The time taken by animal to enter one of the closed arms was recorded as initial transfer latency (ITL). In elevated plus maze the transfer latency (TL) of first day (on 30th day of microwave exposure) indicates acquisition of learning behaviour of animals whereas TL of next day (on 31st day) indicates retention of information or memory. The animal which could not enter the closed arm within 90 sec was gently pushed into one of the closed arms and the ITL was mentioned as 90 sec. Retention of memory after 24 h was evaluated in same manner[18].

Morris Water Maze (MWM) testing—The acquisition and retention of a spatial navigation task was evaluated by Morris water maze. Animals received a training session consisting of four trials in a day, four days prior to exposure in Morris water maze (180 cm diameter × 60 cm in depth) filled with water. The pool was divided into four equal quadrants. An escape platform was hidden 2 cm below the surface of the water in a fixed location in one of four quadrants halfway between the wall and the middle of the pool. The position of the platform was kept constant throughout the experimental trials. The water was made opaque during the task with a non toxic water soluble dye. Each trial consisted of releasing a rat into the water facing the wall of the pool, at one of four starting compass positions (N, S, E, W) so that each position can be explored well. The time to reach the escape platform (latency in seconds) was recorded up to a maximum of 3 min. The animal which could not find the platform up to 3 min was deliberately placed on the platform and allowed to sit for 30 sec. The time taken by a rat to reach the platform on fourth day was recorded and mentioned as escape transfer latency (ETL). Following 24 h after initial acquisition latency, a probe test was done, where there was no platform and each rat was randomly released from any one of the positions and tested for the retention of the acquired memory. During retention the time taken by each rat to locate the target quadrant (quadrant in which platform was placed during training) and time spent in target quadrant for four 15 sec interval over 60 sec was recorded[10].

Experiment II: Effect of microwave exposure on oxidative stress

Sample preparation and tissue homogenate—At the termination of exposure, animals were sacrificed immediately and brain tissues were collected and washed thrice with phosphate buffer saline (PBS) and hippocampus tissue was isolated and subsequently homogenised (10% w/v) in PBS. Homogenate was centrifuged and supernatant was collected for estimation of oxidative stress parameters (malondialdehyde, protein carbonyl and reduced glutathione) and cytokines (IL-6 and TNF-α).

Determination of total brain protein—Total protein content in brain was determined according to Lowry's method using bovine serum albumin as standard[19].

Determination of malondialdehyde (MDA)— The intensity of lipid peroxidation in the rat brain was spectrophotometrically measured based on thiobarbituric (TBA) response products[20]. Absorption was measured at 532 nm. MDA, lipid peroxidation end product concentration was measured per g protein

using the molecular extinction coefficient of MDA (1.56×10^{-5} mol^{-1} cm^{-1}) and was expressed as µmol/g protein.

Determination of protein oxidation—The level of oxidative modification of proteins i.e. carbonyl group concentration was determined spectrophotometrically by standard protocol of Reznick and Packer[21] using 2, 4 dinitrophenylhydrazine (DNPH), reagent often used to test carbonyl groups. Reactive carbonyl concentration was calculated using DNPH molar extinction coefficient (22×10^{3} mol^{-1} cm^{-1}) at 390 nm and expressed in µmol/g protein.

Determination of reduced glutathione (GSH)—The reduced glutathione (GSH) content in rat brain was measured per g protein by the standard protocol of Ellman[22] using 5, 5' dithiobis-2 nitrobenzoic acid (DTNB). In this method, GSH was oxidized by DTNB and then reduced by GSH reductase with NADPH as hydrogen donor. The oxidation of GSH by DTNB was detected by measuring absorbance at 412 nm and GSH content was expressed as µg/g protein.

Experiment III: Effect of microwave exposure on inflammation

IL-6 and TNF-α estimation—IL-6 and TNF-α were estimated with commercially available ELISA kits by following instructions given by the manufacturer.

Statistical analysis—The present report was designed as blind study for statistical analysis. Values were expressed as mean±SD. Statistical analysis was performed with SPSS (version 17.0). Significance of differences among groups was determined by one way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Statistical significance was accepted at $P < 0.05$.

Results

Effect on cognitive function—Effects of microwave exposure on cognitive performance at power level of 0.00 dBm (1mW) and frequencies 900 MHz and 1800 MHz at average whole body specific absorption rate (SARs) as 5.953×10^{-4} W/kg and 5.835×10^{-4} W/kg respectively for 30 days on cognitive performance were estimated by using elevated plus maze (EPM) and Morris water maze (MWM) tests (Experiment I).

During elevated plus maze test significant alterations in transfer latency (TL) of 30th day as well of 31st day were observed in microwave exposed groups when compared with sham exposed groups (Fig. 2). Animals exposed to microwave radiation at above mentioned frequencies for 30 days showed significant reduction ($P< 0.05$) in time taken to enter the closed arm of EPM on 30th as well as 31st day of microwave exposure in comparison with non-exposed animals indicating learning and memory impairment.

Another test (MWM) was also performed to estimate spatial memory performance in rats exposed to microwave radiation. Exposure to microwave radiation at 900 MHz and 1800 MHz for 30 days produced significant alterations on escape transfer latency (ETL) in comparison to sham exposed group ($P< 0.05$). It was also observed that during the probe trials (with platform removed) microwave exposed rats took longer time to locate the place where platform was kept (Fig. 3). The latency to reach the target quadrant was significantly longer ($P< 0.05$) in microwave exposed group and the time spent in the target quadrant was significantly shorter ($P< 0.05$) when compared to the sham exposed group.

Although, the effects of microwave radiation exposure on cognitive function were significant in microwave exposed groups (900 MHz and 1800 MHz) in comparison with sham exposed group but the results were found insignificant when comparison was done between microwave exposed groups.

Effect on oxidative stress—In microwave exposed groups (900 MHz and 1800 MHz), 30 days of exposure lead to significant ($P< 0.05$) increase in the level of MDA in brain, a marker of lipid peroxidation as compared to sham exposed group (Experiment II) (Fig. 4a).

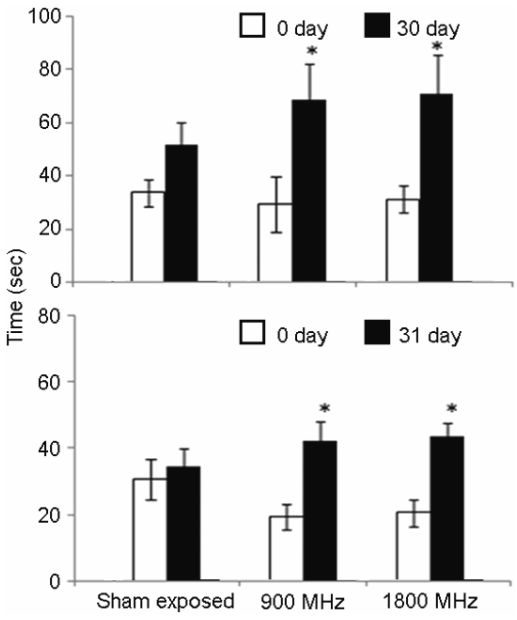

Fig. 2—Transfer latency (TL) of rats during elevated plus maze test (a) Acquisition and (b) retention. *P values < 0.05.

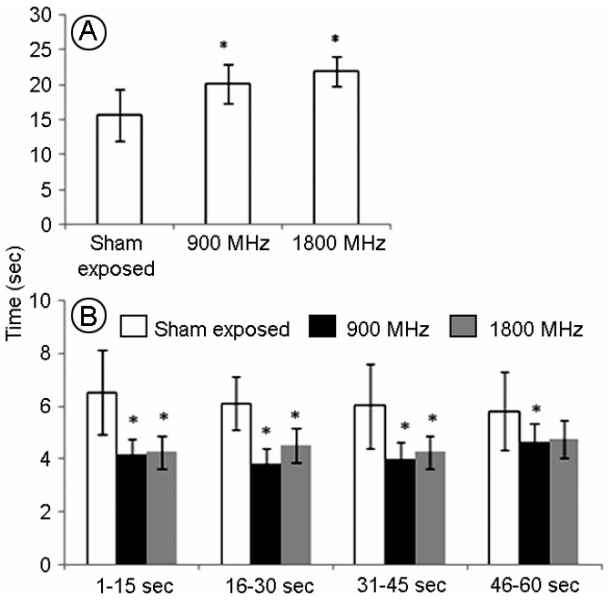

Fig. 3—(a) Escape latency time (ELT) of rats during Morris water maze test to locate hidden platform, (b) time spent in Q-4 (target quadrant). *P values < 0.05.

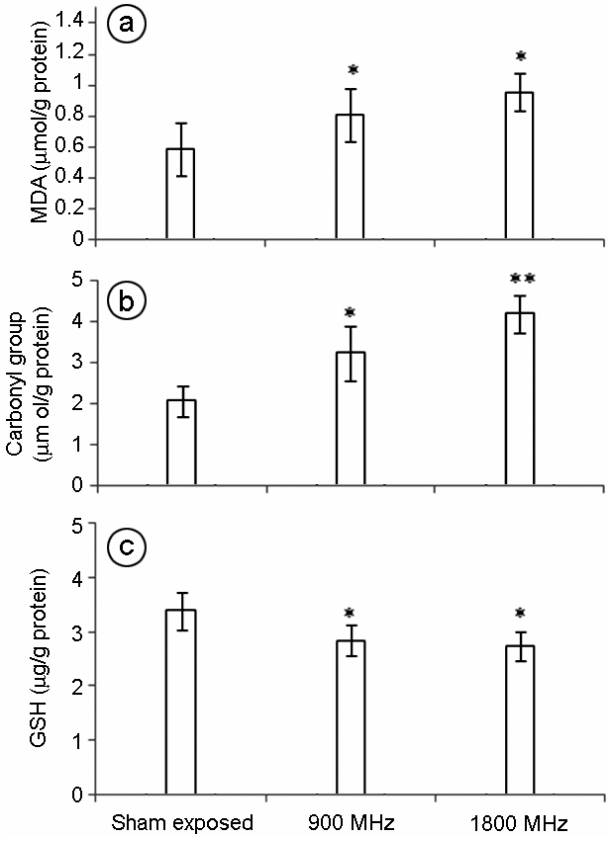

Fig. 4—Effect of microwave radiation exposure on (a) lipid peroxidation (µmol/g protein) (b) protein oxidation (µmol/g protein) and (c) reduced glutathione (GSH) (µg/g) (*P values < 0.05). compared to the sham exposed group, **compared between 900 MHz and 1800 MHz exposed groups

Microwave exposure (900 MHz and 1800 MHz) also produced a significant (P< 0.05) increase in carbonyl content, an index for oxidative modification of proteins in brain tissue of exposed rats in comparison with non-exposed groups (Fig. 4b). The carbonyl content in brain was also found increased significantly in 1800 MHz exposed groups in comparison with 900 MHz group (P< 0.05).

In addition to this exposure to microwave radiation at frequencies 900 and 1800 MHz for 30 days produced a significant (P< 0.05) decrease in reduced glutathione content in brain (Fig. 4c).

Except the level of protein carbonyl, the alterations in levels of MDA and reduced glutathione were found insignificant among microwave exposed groups (900 MHz and 1800 MHz) when compared with each other.

Effect on Inflammation—Microwave exposure at mobile frequencies (900 and 1800 MHz) for 30 days produced a significant increase in level of IL-6 (Experiment III) (Fig. 5a). Also a significant (P< 0.05) increase was observed in level of TNF-α content, a pro-inflammatory marker in microwave exposed groups (Fig. 5b). The results of bioeffects of microwave radiation on levels of these

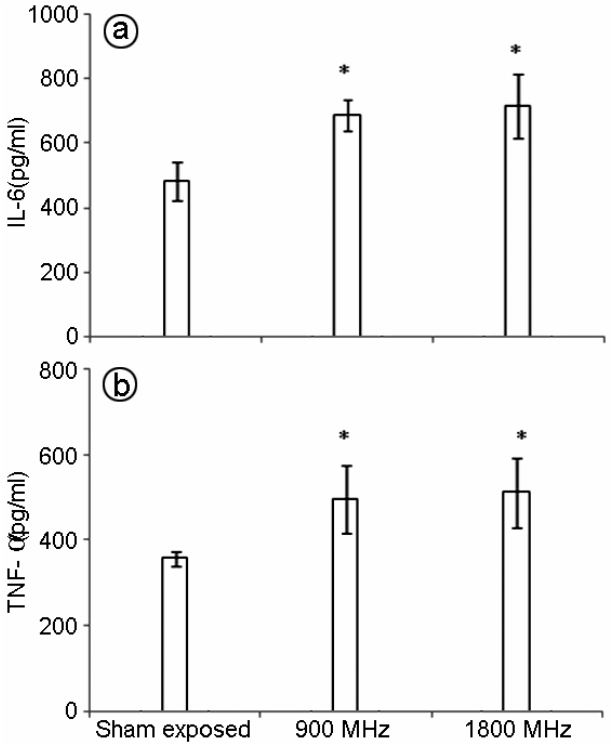

Fig.5—Effect of microwave radiation exposure on (a) level of pro- inflammatory cytokine IL-6 (pg/mL), (b) level of TNF-α (pg/mL) in rat brain. *P values < 0.05.

pro-inflammatory cytokines were not found significant in microwave exposed groups (900 and 1800 MHz) in comparison with each other.

Discussion

The present study is a part of our efforts to recognize the bioeffects of microwave radiation exposure due to excessive usage of devices like mobile phones, Wi-Fi devices, microwave ovens etc. on human health. Various studies on possible adverse effects of exposure to microwave radiation on human health provide contradictory results[23,24].

This study provides three important findings related to effects of microwave exposure at frequencies 900 and 1800 MHz and power 0.00 dBm for 30 days.

Firstly, it was shown that exposure to low level (0.00 dBm) microwave radiation at above mentioned frequencies for 30 days leads to significant alterations in spatial learning and memory functions in rats (Experiment I). Exposure to these radiations at SAR levels (5.953×10^{-4} W/kg and 5.835×10^{-4} W/kg) even far below the limit of 2 W/kg for possible exposure to head as per International Commission on Non-Ionizing Radiation Protection (ICNIRP) guidelines caused neurocognitive dysfunction in rats[25]. The SAR values in the present study were low but and not too different from each other due to the reason that frequencies chosen (900 MHz and 1800 MHz) were also close to each other. Inspite of same input power, the two SAR values differ due to the differences in values of power levels (i.e. $P_{in,}$ $P_{out,}$ and P_{refl}) used in equation to calculate the SAR are different at 900 MHz and 1800 MHz. The results of tests used to estimate cognitive function indicate increased anxiety level and impairment in learning and memory in response to microwave radiation exposure (at 900 and 1800 MHz) for 30 days. The present results are in support with the study of Narayanan *et al.*[10] where effects of microwave radiation emitted from GSM (900/1800 MHz) mobile phones for 4 weeks have been reported on cognitive function in Wistar rats. In another study by Lai *et al.*[26] it has been shown that microwave exposure at 2.45 GHz alters the spatial working memory assessed using radial arm maze. Recently Deshmukh *et al.*[11] have shown that low level microwave radiation (power level 0 dBm, frequencies— 900, 1800 and 2450 MHz) leads to cognitive impairment by alternative pathway suggesting role of heat shock protein (HSP 70).

Although a number of studies have been reported on effects of microwave exposure on cognitive function but to the best of our knowledge none of the studies have shown effects of these radiations on cognitive function at different mobile frequencies (lower range-900 MHz and higher range— 1800 MHz). Moreover, the neural mechanism behind cognitive impairment caused due to microwave radiation is still unclear. Thus the present study was carried out in order to bridge the gaps between knowledge related to bioeffects of microwave radiation on cognitive function and to understand the possible causes behind microwave induced cognitive impairment.

Secondly, it was observed that microwave radiation at frequencies 900 and 1800 MHz, SAR levels (5.953×10^{-4} W/kg and 5.835×10^{-4} W/kg respectively) and power 0.00 dBm for 30 days caused oxidative stress in rat brain biochemically by increasing levels of MDA, a marker of lipid peroxidation (Fig. 4a) (Experiment II). This observation suggests that microwave radiation at above mentioned frequencies can induce oxidative stress as indicated by increased level of MDA in brain. Similar findings have been reported by Sokolovic *et al.*[12] where it has been shown that exposure to 900 MHz microwave radiation at SAR 0.043-0.135 W/kg can induce brain damage by increasing lipid peroxidation. In another study reported by Ilhan *et al.*[27] it has been shown that exposure to microwave radiation at 900 MHz leads to increase in MDA level in rat brain tissue. In addition to increased MDA level it was also observed in the present study that microwave radiation exposure under the present experimental conditions caused significant increase in protein carbonyl content, an index of oxidative modification of proteins (Fig. 4b). Microwave radiations could be possible sources of generation of free radicals which are known to cause oxidative stress[13,14]. Proteins are known to be susceptible to attack of free radicals due to amino acid residues present in them. Thus it suggests that free radicals produced as a result of microwave radiation might have caused oxidation of proteins in brain. The present results are in support with the findings of Sokolovic *et al.*[12] where it has been reported that exposure to 900 MHz microwave radiation at SAR 0.043-0.135 W/kg causes protein oxidation in rat brain. Besides these two oxidative stress parameters the level of reduced glutathione (GSH), the most important antioxidant in brain was also estimated

(Fig. 4c). Exposure to microwave radiation at 900 and 1800 MHz for 30 days caused significant reduction in level of GSH. GSH protects cells from reactive singlet oxygen, hydrogen radical and superoxide radical damage by reacting with them. Abnormalities in glutathione scavenging system have been associated with several psychiatric and neurological processes[28]. Thus the present observation suggests that microwave radiation exposure causes decrease in scavenging ability of reduced glutathione. In a similar study by Moussa *et al.*[29] it has been reported that whole body microwave radiation exposure to rats at 3.5 GHz leads to reduction in reduced glutathione.

The free radicals generated due to microwave radiation exposure stimulate T-helper cells that are important during the beginning of an immune response. Stimulated T-helper cells may secrete pro-inflammatory cytokines like IL-6, TNF-α, IL-2 etc. Thus the induced oxidative stress may also lead to inflammatory imbalances in whole brain or in isolated regions that control specific functions related to learning and memory.

Exposure to these radiation lead to inflammation in brain tissues of exposed rats as evidenced by significantly increased levels of pro-inflammatory cytokines namely, IL-6 and TNF-α (Figs 5a and b) (Experiment III). Thus it is suggested from the observations that microwave radiation induced free radicals might have lead to inflammation in brain of exposed rats. Similar findings have been reported by Wu *et al.*[30] where increased levels of TNF-α, IL-1β and IL-6 were observed in sertoli cells after exposure to microwave radiation. In another study by Yang *et al.*[31] it has been reported that exposure to electromagnetic fields (EMFs) activate cultured microglial cells to produce TNF-α through signal transduction.

In summary, the present results suggest that nervous system due to being vulnerable to attack of free radicals because of its low cellular turn over, poor antioxidant defence system and high metabolic rate is an easy target of microwave radiation emitted by mobile phones. Oxidative stress caused due to increased production of reactive oxygen species (ROS) or deterioration of antioxidant system has been closely linked to *in vivo* neuronal degeneration, as well as in stroke, trauma, and seizures[32]. Thus the present findings support the hypothesis that induced oxidative stress in response to microwave radiation could be one of the common causative factors in derangement of cognitive function and inflammatory imbalances.

Interestingly, similar observations were noted in animals exposed to both frequencies due to the reason that frequencies (900 MHz and 1800 MHz) used in the present study are quiet close to each other in terms of their biological effects.

Conclusion

Based on the present findings it is concluded that there is a probable role of microwave radiation exposure induced production of free radicals in development of inflammatory imbalances and oxidative damage in brain as indicated by impairment in spatial learning and memory. Further studies on mechanisms leading to altered cognitive function following microwave exposure with reference to neurotransmitters are undergoing.

Acknowledgement

Authors are thankful to Indian Council of Medical Research (ICMR), New Delhi for providing the microwave exposure facility and supporting the study. One of the authors, Kanu Megha is grateful to Department of Science and Technology, Govt. of India for providing DST/INSPIRE Fellowship support.

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