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2.45 GHz Microwave Irradiation-Induced Oxidative Stress Affects Implantation or Pregnancy in Mice, *Mus musculus*

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Abstract The present experiment was designed to study the 2.45 GHz low-level microwave (MW) irradiation-induced stress response and its effect on implantation or pregnancy in female mice. Twelve-week-old mice were exposed to MW radiation (continuous wave for 2 h/day for 45 days, frequency 2.45 GHz, power density=0.033549 mW/cm^2, and specific absorption rate=0.023023 W/kg). At the end of a total of 45 days of exposure, mice were sacrificed, implantation sites were monitored, blood was processed to study stress parameters (hemoglobin, RBC and WBC count, and neutrophil/lymphocyte (N/L) ratio), the brain was processed for comet assay, and plasma was used for nitric oxide (NO), progesterone and estradiol estimation. Reactive oxygen species (ROS) and the activities of ROS-scavenging

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enzymes— superoxide dismutase, catalase, and glutathione peroxidase—were determined in the liver, kidney and ovary. We observed that implantation sites were affected significantly in MW-irradiated mice as compared to control. Further, in addition to a significant increase in ROS, hemoglobin ($p<0.001$), RBC and WBC counts ($p<0.001$), N/L ratio ($p<0.01$), DNA damage ($p<0.001$) in brain cells, and plasma estradiol concentration ($p<0.05$), a significant decrease was observed in NO level ($p<0.05$) and antioxidant enzyme activities of MW-exposed mice. Our findings led us to conclude that a low level of MW irradiation-induced oxidative stress not only suppresses implantation, but it may also lead to deformity of the embryo in case pregnancy continues. We also suggest that MW radiation-induced oxidative stress by increasing ROS production in the body may lead to DNA strand breakage in the brain cells and implantation failure/resorption or abnormal pregnancy in mice.

Keywords Microwave radiation · Reactive oxygen species (ROS) · Nitric oxide · Antioxidant enzyme activity · Implantation failure

Introduction

Microwaves (MW) are non-ionizing electromagnetic radiation (EMR) (wavelength ranging from 1 mm to 1 m and frequency between 0.3 and 300 GHz), which unlike ionizing radiation, do not contain sufficient energy to break the bond or chemically change the substances by ionization. In general, non-ionizing radiations are associated with two major potential hazards, i.e., electrical and biological. In recent times, the level of EMR in our environment has increased manifold due to a large-scale expansion of communication networks such as mobile phones, base stations, WLAN, Wi-Fi, Wi-MAX, etc. Radiations emitted from these modern devices are reported to induce various types of biological effects which are of great concern to human health due to its increased use in daily life. MW radiation primarily increases the temperature of the biological system, i.e., thermal effects [1], but its nonthermal effects have also been noted and studied in detail [2–8]. Nonthermal effects occur when the intensity of the MW radiation is sufficiently low so that the amount of energy involved would not significantly increase the temperature of a cell, tissue, or an organism, but may induce some physical or biochemical changes [9]. Prolonged exposure to low intensity 2.45 GHz microwave radiation may affect the cholinergic activity in the rat [2], brain development in mice [10], DNA breakage in rat brain [11], and histone kinase activity in rat [12], which results in neurological problems and reproductive disorders [13–15], in addition to changes in hematopoiesis of pregnant mice [16] and micronucleated erythrocytes in rats [17]. The International Agency for Research on Cancer has also kept radiofrequency electromagnetic fields in the list of factors causing cancer to humans. Some studies performed in this context suggest that people heavily exposed to these radiations are more prone to nonmalignant tumors [18]. It has been reported that mobile phone or cell phone radiation (a type of MW radiation) causes changes in cognitive function [19]. A German study has indicated an increase in cancer around base stations. Mobile phones use electromagnetic radiation in a microwave range (2G—900/1,800 MHz, 3G—2,100 MHz frequency band) which some believe may be harmful to human health. People living close to 2G and mostly 3G mobile phone masts or base stations frequently report symptoms of electromagnetic hypersensitivity such as dizziness, headaches, skin conditions, allergies, and many other problems. Hardell and groups [20, 21] have reported the health implications of mobile phone exposure (800–2,200 MHz). They found that cell phone users had an increased risk of

malignant gliomas and higher rate of acoustic neuromas. The low-level nonthermal MW radiations or mobile phone radiations may propagate through the body and affect the reproductive system.

Nonthermal 2.45 GHz MW radiation may also act as one of the environmental stressors responsible for increasing the production of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and nitric oxide ($NO^{\bullet}$) in the body which in turn are thought to be responsible for various pathological conditions including metabolic disturbances affecting the growth and normal development of an organism. Its production can also be increased in the oocyte microenvironment and uterine horns in response to infection, inflammation, radiation, pollutants, etc. Therefore, increased production of ROS is cytotoxic, while low levels are necessary for the regulation of several key physiological mechanisms. ROS-scavenging enzymes—superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)—combinatorially play a major role in maintaining the intracellular concentration of ROS. SOD, CAT and GPx together with glutathione (GSH) form the first line of defense against ROS. SOD catalyzes superoxide anion ($O_2^{\bullet-}$) to O_2 and H_2O_2, which is then reduced to H_2O by the H_2O_2-scavenging enzymes CAT and GPx [22]. If H_2O_2 is not removed, it will cause oxidative damage to biomolecules or can be converted to more damaging hydroxyl radical ($^{\bullet}OH$) species. Therefore, the elimination of H_2O_2 is critical to the efficacy of SOD in reducing oxidative stress generated by ROS, whereas disruption of the antioxidant SOD leads to congenital anomalies that are associated with increased oxidative DNA damage [23]. This will also result in proliferation-impaired embryonic development and increased morbidity in the offspring [24].

ROS is a well-known factor that disrupts the hormonal communication between the brain, pituitary, and the ovary. Thus, ROS interferes with both the maturation and the ovulation process of an egg along with the movement and implantation of a fertilized egg. High levels of ROS and low antioxidant activity are responsible for decreased steroidogenesis and PCOS condition [25, 26]. Cyclic production of ROS not only decreases ovarian function [27] but also affects the dominant follicle selection [28] and embryonic germ cell proliferation [26]. Studies show that EMR penetrating the living organism may affect the free radical processes/reaction within the cell [29] by altering the cell membrane potential. Nitric oxide (NO), which is a free radical gas and a ubiquitous signaling molecule produced by NO synthases, has been proposed to mediate many physiological functions including smooth muscle relaxation, vasodilation, neuronal signaling, immune response, regulation of embryonic development and implantation [30]. It is a potent relaxant of myometrial smooth muscle, and its upregulation during pregnancy is confirmed by the urinary excretion of cyclic guanosine monophosphate (cGMP) and nitrite–nitrate [31]. NO can promote or inhibit implantation depending on its critical level in the uterus and may also contribute as an antiplatelet agent during implantation [32]. It also stimulates the release and synthesis of GnRH and may play a role in the induction of the steroid-induced LH surge. It is a potential mediator of estrogen action on the hypothalamus and provokes estrogen-stimulated rapid uterine secretory response at the implantation site via cGMP. Under appropriate conditions, NO reacts with $O_2^{\bullet-}$ yielding peroxynitrite ($ONOO^-$), a potent oxidant [33], which further decomposes to form the reactive hydroxyl radical. Moreover, peroxynitrite and its metabolite are capable of producing cytotoxicity by inducing lipid peroxidation, nitrosation of several tyrosine molecules that regulate enzyme function, signal transduction and Na^+ channel inactivation. Nitrosation of tyrosine residues results in the formation of stable adduct nitrotyrosine [34, 35]. The decay of nitrotyrosine causes an increase in the nitrite (NO_2^-)/nitrate (NO_3^-) ratio. The action of NO in a cell depends on its concentration, the

cellular redox state, the abundance of metals, protein thiols and low molecular weight thiols (glutathione) as well as other nucleophile targets [36, 37].

Electromagnetic radiation has been linked to a variety of adverse human health outcomes including leukemia, brain tumors, genotoxic effects (DNA damage and micronucleation), neurological effects and neurodegenerative diseases, immune system deregulation, allergic and inflammatory responses, breast cancer in men and women, miscarriage and some cardiovascular effects. A number of studies have focused on microwave-induced biological responses including oxidative stress [38, 39] due to overproduction of ROS, but so far, there is no comprehensive study describing the effects of 2.45 GHz low-level MW radiation on reproduction with special reference to implantation. However, it has been reported that MW-irradiated female mouse shows differential sensitivity during the successive stages of the estrous cycle with greater radiosensitivity during estrus than during diestrus [40]. This study also indicated that the pregnant mouse exposed to MW radiation with an average absorbed dose ranging from 3 to 8 cal/g body weight at a temperature–humidity index of 71.6 shows evidence of teratogenesis, particularly in the form of a brain hernia or exencephaly in addition to gross anomalies including hemorrhage, resorptions, stunting and even fetal death. Further, studies related to EMR radiations revealed that exposure to 2.45 GHz continuous wave (CW) microwave affects embryo-fetal development [41], uteroplacental blood flow, progesterone and PGF2 levels [42]. But some investigators have reported that 2.45 GHzMW radiation exposure at different power densities had no remarkable effect on pregnancy or the development of the preimplantation embryo [43–45].

In view of the available reports, it is evident that high ROS may affect ovarian development/function adversely, and MW exposure at higher specific absorption rate (SAR) value may induce adverse embryonic growth if any. However, in our preliminary published study [46], we found that 2.45 GHz low-level CW microwave radiation affects embryo implantation sites and caused DNA damage in the brain cell of mice, but yet no information is available regarding the mechanism of action of low-level MW radiation on implantation/pregnancy especially at lower SAR value. Hence, on the basis of our previous study and available reports, the present experiment was designed to examine whether or not the 2.45 GHz low-level MW irradiation results in oxidative stress by altering ROS and ROS-scavenging enzymes could affect the implantation or pregnancy in mice.

Materials and Methods

Animals and Microwave Irradiation

Twelve-week-old female mice (Parkes strain) were obtained from the mice colony of our laboratory maintained under a light/dark cycle (LD12:12). Animals were supplied with food (standard rodent food pellets supplied by Pashu Aahar Kendra, Varanasi, India) and tap water ad libitum. Twelve randomly selected mice were divided into two groups (control and experimental) of six mice each (N=6). Experimental animals were exposed to 2.45 GHz CW low-level microwave radiation (2 h/day, from 09:00 to 11:00 hours for 45 days) as described below.

Twenty days after MW exposure (pre-mating phase), mice were numbered and mating was set by keeping two females with one male for 5 days. During this mating phase, females were separated from the male only during 2 h of the MW exposure period and then kept back in the regular cage with the same male to avoid Bruce effect. After 5 days, pregnant females were not kept with the males but MW exposure was continued as above for another 20 days

(post-mating phase). The control animals were subjected to sham exposure with similar pre-mating/mating/post-mating condition. The rectal temperature of both groups was measured before and after the exposure during the first and last 3 days of the pre-mating phase and then finally during the last 3 days of the experiment or post-mating phase. The experiment was conducted in accordance with institutional practice and within the framework of the revised Animals (Scientific procedures) Act of 2002 of the Government of India on Animal Welfare. The whole experiment was repeated again to confirm the results.

Exposure Setup

The Analog Signal Generator microwave source (Model no. E 8257D PSG, manufactured by Agilent Technologies, USA) covering a frequency range from 250 kHz to 20 GHz, a coaxial attenuator, microwave amplifier (model no. 8349B Hewlett Packard Co., USA), coaxial to waveguide transition, 20 dB cross coupler and E-plane bend were used to deliver microwave power to pyramidal horn antenna. The maximum output power delivered to the horn antenna from the amplifier as measured by the power sensor (Model no. 836A, manufactured by Agilent Technologies) and power meter (Model no. 8481H, Hewlett Packard Co., USA) having a range from 30 μW to 3 W was 19.8 dBm. A silver-polished brass conductor was used to fabricate the pyramidal horn antenna. The throat and mouth dimensions of the antenna are 7.2 cm×3.2 cm and 9.0 cm×5.0 cm, respectively. The axial length of the antenna from the throat is 10.0 cm. The gain of the horn antenna (G_t) was measured by the comparison method and is found to be 4.0657 dB. The distance between the pyramidal horn and the midplane of the body of the animal (assuming the body length to remain horizontal during exposure) was estimated through far field criterion ($R \geq 2D^2/\lambda$) to be 25 cm. The cross-sectional dimensions of the animal cage in E- and H-planes (electric and magnetic field planes, respectively) were designed on the basis of 1.24 and 1.69 dB beam widths in corresponding planes of the horn, respectively. For this purpose, the horn radiation patterns were also measured at 2.45 GHz in E- and H-planes [47].

The dimensions of the animal cage are 19.2 cm×17.6 cm×15 cm. The cage was designed in such a way that it contains six compartments of equal size (Fig. 1). The dimensions of each compartment are 6 cm×8 cm×15 cm accommodating a single mouse each at a time. The cage and its partitions were made up of pine wood having very low dielectric constant. Small circular holes of 1 cm diameter were drilled on the side and the partition walls of the cage for air ventilation. The upper portion of the cage remained open to keep the animals aerated during exposure. The upper portion of the side and partition walls and the base of the cage were covered by carbon-impregnated styrofoam microwave absorber to reduce electromagnetic scattering. Each animal was kept in a separate compartment of the cage throughout the exposure period. The partitions were made in such a way that mice remain restrained in the cage during the period of microwave irradiation and were exposed parallel to the E field. The animal cage was placed on the microwave absorber and also covered from all four sides by microwave absorbers to minimize scattering/reflections. The cage was also tied to the absorber unit to avoid any change in the position of the cage with reference to the horn antenna. The temperature in the chamber was maintained at 25–27 °C throughout the experiment by circulating air. Mice were exposed for 2 h, i.e., from 09:00 to 11:00 daily for 45 days continuously. The 20-dB cross coupler and power meter with power sensor were used to measure the power input to the antenna and reflections from it. Power transmitted from the antenna was estimated by subtracting the power reflected from the antenna when it faces towards free space from the measured input power. This way, power transmitted by the

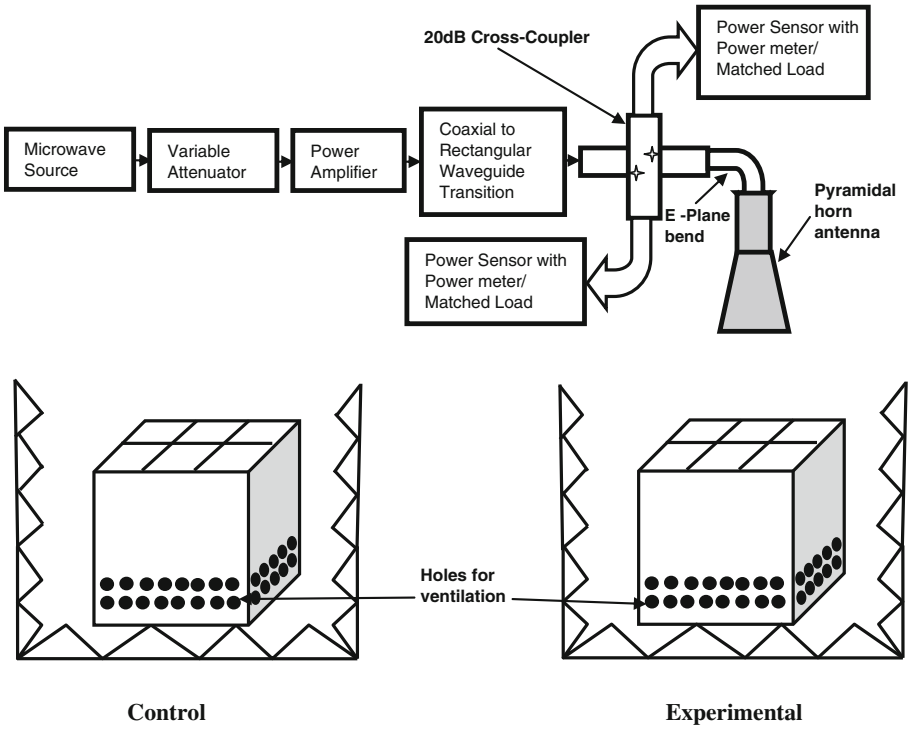


Fig. 1 Diagrammatic representation of the microwave exposure setup and the position of animal cages during microwave exposure. Mice were exposed to 2.45 GHz low-level MW radiation for 2 h/day continuously for 45 days by using microwave source and pyramidal horn antenna

antenna was estimated which is equal to 64.776 mW. The power density was computed using the following formula:

$$\text{Power Density} = \frac{P_t G_t}{4\pi R^2}$$

where P_t is the power transmitted into the cage, G_t is the gain of the horn, and R is the distance between the horn aperture and midplane of the body of the mouse in the exposure cage. The experimental group of mice was exposed to 2.45 GHz microwave radiation at a power density of 0.033549 mW/cm^2 and SAR of 0.023023 W/kg, which is comparatively much lower in context with the SAR of other earlier studies. The SAR was estimated for body length parallel to the electric field, as per actual placement of mice [47]. The 0.033549-mW/cm^2 exposure does not cause any elevation in rectal temperature. The average rectal temperatures of the mice observed were 35.81±0.2 °C (control group) and 35.94±0.2 °C (experimental group) before and 35.91±0.2 °C (control group) and 36.05±0.2 °C (experimental group) just after the termination of exposure.

Collection of Blood and Tissue Samples

After the termination of 45 days exposure, mice were sacrificed by decapitation and blood was collected in heparinized tubes to study hematological parameters. Brain was dissected out immediately after decapitation and processed for comet assay. Liver, kidney, and ovary

were removed and, after washing with ice-cold sterile physiological saline solution, processed for ROS measurement and antioxidant assay.

Implantation Sites

Sacrificed animals were dissected for visualization of the implantation sites and distribution of embryos in the uterine horns.

Hematology

Blood was used to determine the total number of erythrocytes, leucocytes, and neutrophil/lymphocyte ratio (N/L ratio) by the hemocytometer method. The hemoglobin content in the blood was measured by a hemometer (Sahli's method).

Comet Assay

For comet assay, brain tissue was processed according to the method of Hartmann et al. [48] as described by Patel et al. [49] for a single-cell suspension preparation. Briefly, brain was placed in 1 ml chilled mincing solution (Hank's balanced salt solution, with 20 mM EDTA and 10 % DMSO) in a Petri dish and chopped into small pieces with a pair of scissors. The pieces were allowed to settle and the supernatant containing the single cell suspension was taken. Normal melting agarose (NMA, 1.0 %) was prepared in Milli-Q water, microwaved, and kept at 60 °C. Conventional glass slides were then dipped into molten NMA up to two thirds of their frosted end, the lower surface was wiped clean and the slides were left to dry. On this first layer, 80 μl of diluted sample (100 μl cell suspension mixed with 100 μl of 1 % low melting point agarose, LMA) was added to form the second layer. A cover slip (size 24 mm×60 mm) was placed gently to evenly spread the cells in the agarose. The slides were kept on ice for 5 min to allow the gel to solidify. The cover slips were removed and a third layer of 0.5 % LMA (90 μl) was added onto the slide, and after placing the cover slip, the slide was placed over ice for 10 min. Finally, the cover slips were removed and the slides immersed in freshly prepared chilled lysing solution containing 2.5 M NaCl, 100 mM EDTA, and 10 mM Tris (pH 10) with 10 % DMSO and 1 % Triton X-100 added just before use. The slides remained in the lysing solution overnight at 4 °C, followed by electrophoresis according to the method of Singh et al. [50]. The slides were placed in a horizontal gel electrophoresis tank side by side with agarose ends nearest to the anode. Fresh and chilled electrophoresis buffer (1 mM Na_2 EDTA and 300 mM NaOH, pH>13) was poured into the tank to a level of approximately 2.5 mm above the slides. The slides were left in this solution for 25 min to allow DNA unwinding and expression of alkali-labile sites as DNA strand breaks. Electrophoresis was conducted at 24 V (0.7 V/cm) and a current of 330 mA using a power supply (Electra Comet III from Techno Source India Pvt. Ltd., Mumbai, India) for 30 min at 4 °C. All these steps were performed under dimmed light, and the electrophoresis tank was covered with black paper to avoid additional DNA damage due to stray light. After electrophoresis, the slides were drained and placed horizontally in a tray. Tris buffer (0.4 M; pH 7.5) was added dropwise and left for 5 min to neutralize excess alkali. Neutralizing of slides was repeated thrice. Each slide was stained with 75 μl EtBr (20 μg/ml) for 5 min and dipped in chilled distilled water to wash off excess EtBr and the cover slip placed over it. Slides were placed in a dark humidified chamber to prevent drying of the gel. The slides were scored within 24 h, using an image analysis system (Kinetic Imaging, Liverpool, UK) attached to a fluorescence microscope (Leica, Germany) equipped with appropriate filters

(N2.1, excitation wavelength of 515–560 nm and emission wavelength of 590 nm). The microscope was connected to a computer through a charge-coupled device camera to transport images to a software (Komet 5.0) for analysis. The final magnification was ×400. The comet parameters recorded were Olive tail moment (OTM, arbitrary units), tail DNA (percent), and tail length (migration of the DNA from the nucleus, micrometer). Images from 100 cells (50 from each replicate slide) were analyzed as per the in vivo guidelines [48].

Plasma Isolation and Tissue Homogenization

Blood samples were kept at room temperature for 30 min and then centrifuged at 4,000 rpm at 4 °C for 15 min. The supernatant collected as plasma was used for NO, progesterone, and estradiol estimation. Liver, kidney, and ovary were homogenized in 50 mM phosphate buffer (pH7.4) containing protease inhibitor and 0.2 μM PMSF for 30 s using a Polytron homogenizer. A small portion of the fresh homogenates was first processed for measuring total ROS and the remaining homogenates were centrifuged at 12,000 rpm for 30 min at 4 °C. The resultant supernatant was used for determining antioxidant enzyme activity both biochemically and by native gel. Total protein concentration was measured by Lowry's method using BSA as standard.

Biochemical Estimation of Total Nitrite and Nitrate Concentrations in Plasma

Production of nitric oxide (NO), a reactive free radical generally oxidized to NO_x (nitrite—NO_2^-/nitrate—NO_3^-), is measured by the indirect method of Sastry et al. [51] by measuring total nitrite and nitrate concentrations in plasma using acidic Griess reaction for color development after the reduction of nitrate with copper–cadmium alloy and deproteinization. In brief, in 100 μl of sample or standard (KNO_3), 400 μl of carbonate buffer followed by a small amount (0.15 g) of activated copper–cadmium alloy fillings (washed in buffer and dried on a filter paper) was added in tubes and then incubated at room temperature for 1 h with thorough shaking. The reaction was stopped by the addition of 100 μl of 0.35 M sodium hydroxide (NaOH), followed by 400 μl of 120 mM zinc sulfate ($ZnSO_4$) solution under vortex and allowed to stand for 10 min. The tubes were then centrifuged at 8,000 rpm for 10 min. One-hundred-microliter aliquots of the clear supernatant were transferred into the wells of a microplate (in quadruplicate), and Griess reagent (50 μl of 1.0 % sulfanilamide prepared in 2.5 % orthophosphoric acid and 50 μl of 0.1 %*N*-naphthylethylenediamine prepared in distilled water) was added to it with gentle mixing. After 10 min, the absorbance was measured at 545 nm against a blank (containing the same concentrations of ingredients but no biological sample) in a microplate reader (MS5605A, ECIL, Hyderabad, India) using the path check option.

Enzyme Immunoassay for Progesterone (P_4) and Estradiol (E_2)

Steroids in the plasma were measured directly by using commercial ELISA kits (P_4-Biotran Diagnostics Inc., Hemet, CA, USA and E_2-Labour Diagnostika Nord GmbH & Co. KG, Nordhorn) according to the manufacturer's instructions.

Total ROS Measurement

ROS production was determined in fresh tissue homogenates by using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) as a probe, according to Bejma et al. [52] with a slight

modification. DCFH-DA is a nonpolar compound that, after conversion to a polar derivative by intracellular esterases, can rapidly react with ROS to form the highly fluorescent compound dichlorofluorescein. Briefly, the homogenate was diluted in PBS to obtain a concentration of 25 μg tissue protein/ml. The reaction mixture containing diluted homogenate and 10 μl of DCFH-DA (10 μM) was incubated for 15 min at room temperature to allow the DCFH-DA to be incorporated into any membrane-bound vesicles and the diacetate group cleaved by esterases. After 30 min of further incubation, the conversion of DCFH-DA to the fluorescent product DCF was measured using a spectrofluorimeter with excitation at 484 nm and emission at 530 nm. Background fluorescence (conversion of DCFH-DA in the absence of homogenate) was corrected by the inclusion of parallel blanks.

Biochemical Estimation of Antioxidant Enzymes

Superoxide Dismutase Assay

The method of Das et al. [53] was used for measuring total SOD activity in liver, kidney, and ovary. The reaction mixture consisted of 100 μl of sample, 150 mM phosphate buffer (pH7.4), 20 mML-methionine, 50 μM EDTA, 1 % Triton X-100, 100 μM riboflavin, and Greiss reagent. The absorbance was taken at 543 nm. The value of SOD was calculated in terms of units defined as the amount of SOD that inhibits the reduction of nitroblue tetrazolium (NBT) by 50 %. The final results were expressed as unit of SOD per milligram of protein.

Catalase Assay

Catalase activity in the liver, kidney and ovary was assayed according to Aebi [54]. Briefly, the reaction mixture contained 1.9 ml of 50 mM phosphate buffer (pH7.0) and appropriate dilution of the tissue supernatant to make the volume 2 ml. The reaction was initiated by the addition of 1 ml of freshly prepared 30 mM H_2O_2 and the decrease in absorbance was measured at 240 nm for 2–3 min. One unit of catalase represents the decrease of 1 μM of H_2O_2 per minute. The molar extinction coefficient for H_2O_2 is 43.6 $M^{-1}cm^{-1}$.

Glutathione Peroxidase Assay

GPx activity was assayed in the liver, kidney and ovary as described by Mantha et al. [55]. The reaction mixture (1 ml) contained 50 μl sample, 398 μl of 50 mM phosphate buffer (pH7.0), 2 μl of 1 mM EDTA, 10 μl of 1 mM sodium azide, 500 μl of 0.5 mM NADPH, 40 μl of 0.2 mM GSH and 1 U of glutathione reductase. The reaction mixture was allowed to equilibrate for 1 min at room temperature. After this, the reaction was initiated by the addition of 100 mM H_2O_2. The absorbance was measured kinetically at 340 nm for 3 min. The GPx activity was expressed as micromole of oxidized NADPH oxidized to $NADP^+$ per minute per milligram of protein using an extinction coefficient (6.22 $mM^{-1}cm^{-1}$) for NADPH.

Assay of Antioxidant Enzymes by Native PAGE

SOD, CAT and GPx activities were also determined by nondenaturing polyacrylamide gel electrophoresis (native PAGE). SOD assay was performed by using 12 % native gel slab in Tris-glycine buffer (pH8.3) at a constant voltage of 100 V. In the case of liver—20 μg,

kidney—30 μg and for ovary—30 μg of protein were loaded in each lane. After electrophoresis, the gel was subjected to substrate-specific staining of SOD bands as described by Beauchamp and Fridovich [56]. The staining mixture consisted of 2.5 mM NBT, 28 μM riboflavin and 28 mM TEMED. After 30 min of incubation in the dark, the gel was exposed to a fluorescent light to develop achromatic bands against a dark blue background corresponding to SOD protein in the gel.

For catalase, a sample containing 20 μg proteins for liver, kidney and ovary was electrophoresed on 7.5 % native PAGE. Catalase-specific bands were developed according to Sun et al. [57]. Briefly, gels were soaked for 10 min in 0.003 % H_2O_2 and then incubated in a staining mixture consisting of 1 % potassium ferricyanide and 1 % ferric chloride. Achromatic bands of catalase appeared against a blue–green background.

The active level of GPx was determined by the method of Lin et al. [58]. After 10 % nondenaturing PAGE containing 30 μg protein for liver, 40 μg for kidney and 40 μg for ovary, the gels were incubated in a staining mixture composed of 50 mM Tris–Cl buffer (pH7.9), 3 mM GSH, 0.004 % H_2O_2, 1.2 mM NBT and 1.6 mM PMS for 45 min. Achromatic bands corresponding to GPx activity appeared against a violet–blue background.

Enzyme activity staining after native PAGE was repeated at least five times and the intensity of the bands in all the cases was quantitated by gel densitometry using ImageJ v. 1.36 software (NIH, USA).

Statistical Analysis

Statistical analysis was performed after pooling the data of both experiments by using the SPSS software. Student's t test was used to compare between the means of the different parameters of the control and experimental groups. The null hypotheses were rejected at $p \leq 0.05$.

Results

Implantation Site

The implantation sites in the 2.45 GHz low-level microwave-irradiated mice were affected significantly and exhibited some abnormalities such as unequal/asymmetrical distribution of embryos in the two uterine horns (Fig. 2b(7)) and reduced inter-embryo spaces (Fig. 2b(8)). Further, unlike the sham control, in which a normal number (six to ten) of pups were born, no pups were born in MW-exposed mice which showed increased follicular size and some traces of implantation in the uterine horns (Fig. 2b(5, 7, 8)). However, in one mouse, only a single embryo was found in unilateral uterine position (Fig. 2b(6)) which appeared to be bigger in size than those of the control.

Hematology

It was found that after long-term MW radiation exposure, hemoglobin content (Fig. 3a) and total number of erythrocytes (RBC) (Fig. 3b) and leucocytes (WBC) (Fig. 3c) increased significantly ($p<0.001$) in the exposed group of mice as compared to control. Further, a

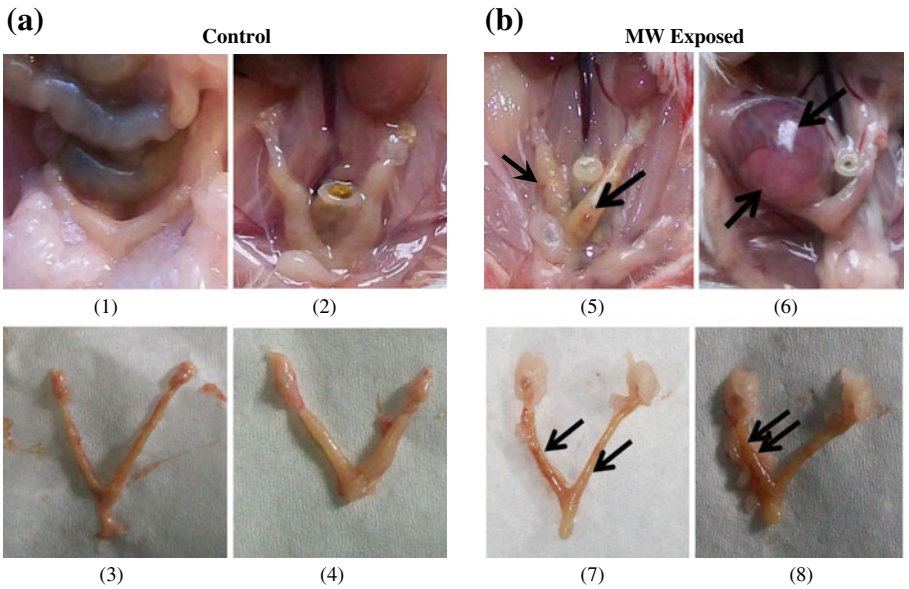


Fig. 2 **a** Uterine horns of control mice in vivo (*1* and *2*) and in vitro (*3* and *4*) after giving birth to pups. **b** Uterine horns of MW-exposed mice in vivo (*5* and *6*) and in vitro (*7* and *8*). Unlike the control, no pups were born in the exposed group of mice; however, some traces of implantation as a *dark red spot* (*arrows*) can be seen (*5*, *7*, and *8*) except in one mouse where only a single embryo (*6*) was found in unilateral uterine position

significant increase in N/L ratio ($p<0.01$, Fig. 3d) was also observed in the irradiated group of animals.

Comet Assay

We have performed alkaline comet assay to illustrate that prolonged exposure to 2.45 GHz microwave radiation caused DNA strand breaks in brain cells of mice. Microwave-exposed mice showed a significant increase ($p<0.001$) in OTM (5.3±0.2), tail DNA (16.1 %) and tail length (26 μm) in the brain cells when compared to control brain cells (Fig. 4).

Total Nitrite and Nitrate Concentrations

In the microwave-irradiated group of mice, a significant decrease in total nitrite and nitrate concentrations was observed in plasma when compared to the control group of animals ($p<0.05$, Fig. 5). This result indirectly shows that the level of NO in female mice decreased after microwave exposure.

Enzyme Immunoassay

We observed an elevation in plasma P_4 and E_2 concentrations (Fig. 6a and b, respectively) in the microwave-irradiated group of mice as compared to the control group. However, a significant increase was observed only in the E_2 level of the microwave-exposed mice ($p<0.05$), but the increase in P_4 concentration was not significant.

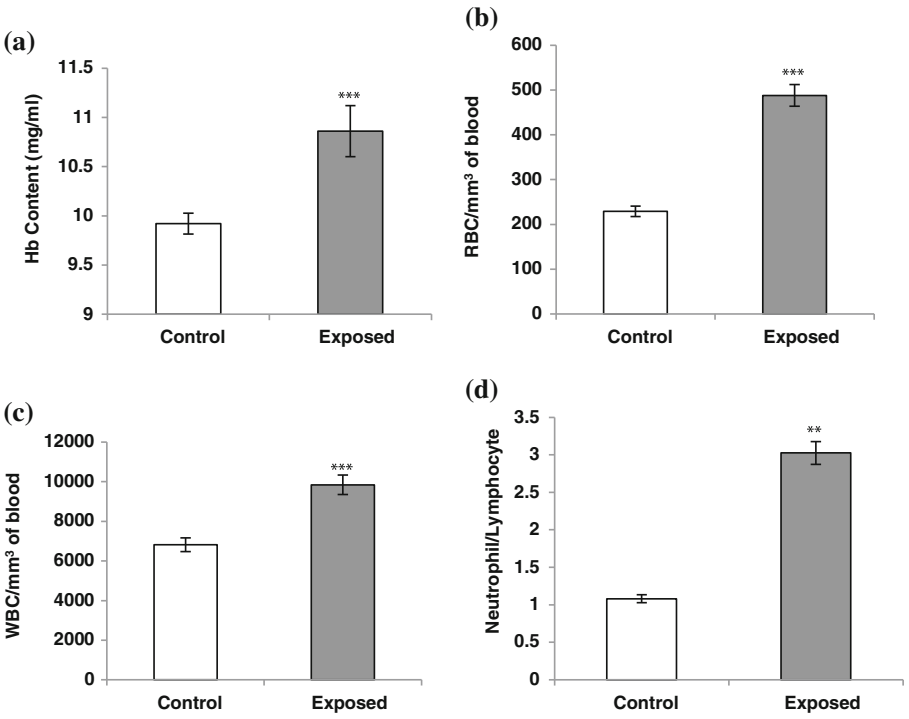


Fig. 3 Changes in **a** hemoglobin (Hb) content, **b** red blood cell (RBC) counts, **c** white blood cell (WBC) counts, and **d** neutrophil/lymphocyte (N/L) ratio after 2.45 GHz microwave irradiation in mice. Results are expressed as mean ± standard deviations (n=12). Significant difference was determined by using Student's t test. **p<0.01 and ***p<0.001, significant difference from thr control group

ROS Level

Total ROS was measured with a DCFH-DA probe to assess the overall oxidative status in liver, kidney and ovary homogenates. An elevated ROS level was observed in the microwave-exposed group of mice as compared to control (Fig. 7). The microwave-irradiated group showed a significant increase in fluorescence intensity of the liver (p<0.001), kidney (p<0.01), and ovary (p<0.01).

Antioxidant Enzyme Activity

We have checked whether the nonthermal microwave exposure-induced oxidative stress alters the activity of ROS-scavenging enzymes: SOD, CAT and GPx. Antioxidant enzyme activities were determined biochemically (spectrophotometrically) as well as by performing native gel of the liver, kidney and ovary. The results are shown in Table 1 and in Fig. 8a, b.

Superoxide Dismutase Activity

In the exposed group of mice, we observed a significant decrease in the SOD activities of the liver, kidney and ovary as compared to the control (Table 1, Fig. 8a(a), b(a)). SOD activity was found to be highly decreased in the liver (p<0.001) of irradiated mice than the kidney (p<0.01) and ovary (p<0.01).

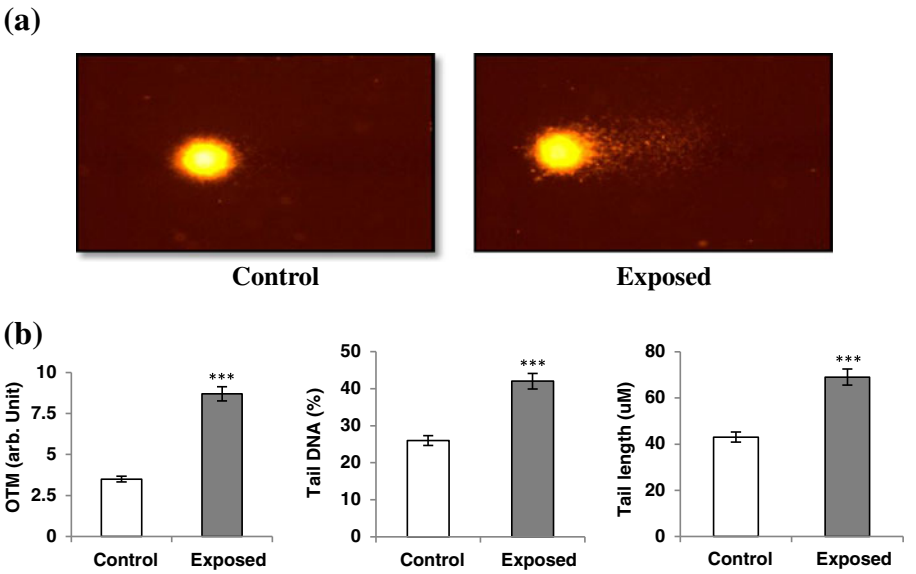


Fig. 4 DNA damage in the brain cells of control and 2.45 GHzMW-irradiated mice was monitored by comet assay (*upper panels*, **a**) and its quantitative measurement (*lower panels*, **b**). Results were statistically analyzed by performing Student's *t* test and are expressed as mean ± standard deviations ($n=12$). ***$p<0.001$, significant difference from the control group

Catalase Activity

CAT activities were found to be highly decreased in all the tissues of microwave-irradiated mice when compared to the control group. This decrease was highly significant, i.e., $p<0.001$ in the liver, kidney and ovary (Table 1, Fig. 8a(b), b(b)).

Glutathione Peroxidase Activity

GPx also shows a similar pattern as SOD and CAT. GPx activity was also found to be significantly decreased ($p<0.001$) in all the tissues of microwave-exposed mice as compared to the control mice (Table 1, Fig. 8a(c), b(c, d)).

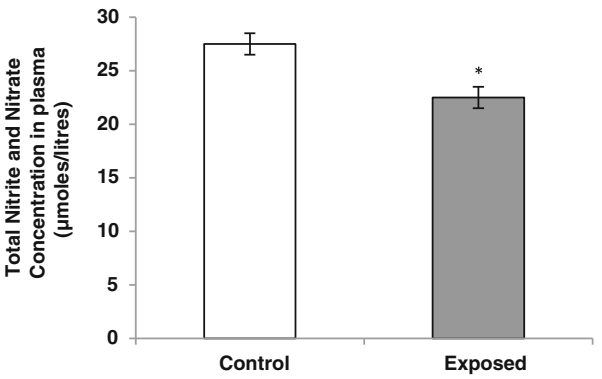


Fig. 5 Total nitrite and nitrate concentrations in the plasma of MW-exposed mice were determined to measure NO level indirectly. A significant decrease in total nitrite and nitrate concentrations was observed in the irradiated group of mice ($n=12$). Values are expressed as mean ± standard deviations. *$p<0.05$, significant difference from the control group

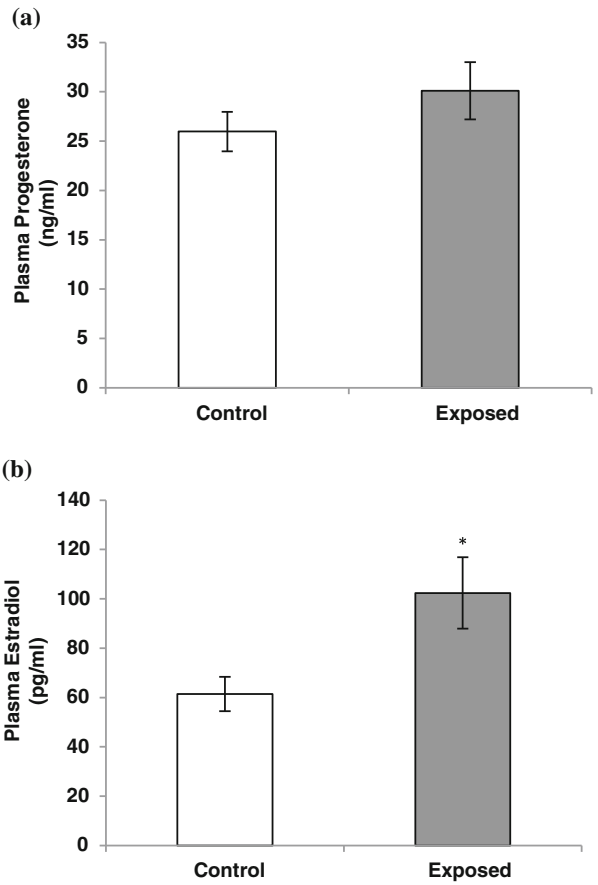


Fig. 6 Effect of 2.45 GHz MW irradiation on circulating **a** progesterone and **b** estradiol concentrations. No significant increase was observed in P_4 concentration while this increase was significant for E_2. Results were statistically analyzed by Student's *t* test. Values are expressed as mean ± standard deviations ($n=12$).*$p<0.05$, significant difference from the control group

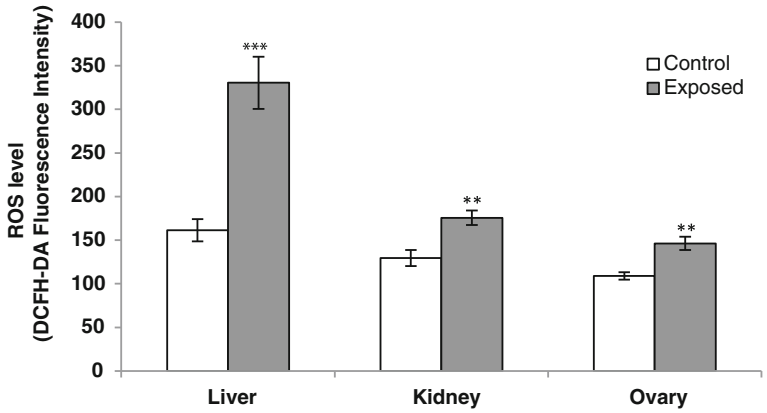


Fig. 7 Elevation of DCFH-DA fluorescence (measure of ROS) was observed in the liver, kidney and ovary of MW-irradiated mice as compared to the control group. A highly significant increase was observed in all the tissues. Results are expressed as mean ± standard deviations ($n=12$). Significant difference was determined by using Student's *t* test. **$p<0.01$ and ***$p<0.001$, significant difference from the control group

Table 1 Comparative statistical analysis of changes in antioxidant enzyme activities: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in 2.45 GHz microwave-irradiated mice. Values are expressed as mean ± standard deviation (n=12)

		SOD (U/mg protein)	CAT (μmol/min/mg protein)	GPx (μmol/min/mg protein)
Liver	Control	1.8482±0.0881	0.1743±0.0042	0.0253±0.0031
	Exposed	0.7661±0.0880	0.0795±0.0050	0.0144±0.0013
Kidney	Control	0.8931±0.0431	0.1237±0.0074	0.0081±0.00039
	Exposed	0.6838±0.0169	0.0386±0.0034	0.0058±0.00031
Ovary	Control	0.7642±0.0384	0.1137±0.0109	0.0067±0.00036
	Exposed	0.4938±0.0264	0.0217±0.0056	0.0026±0.00046

Discussion

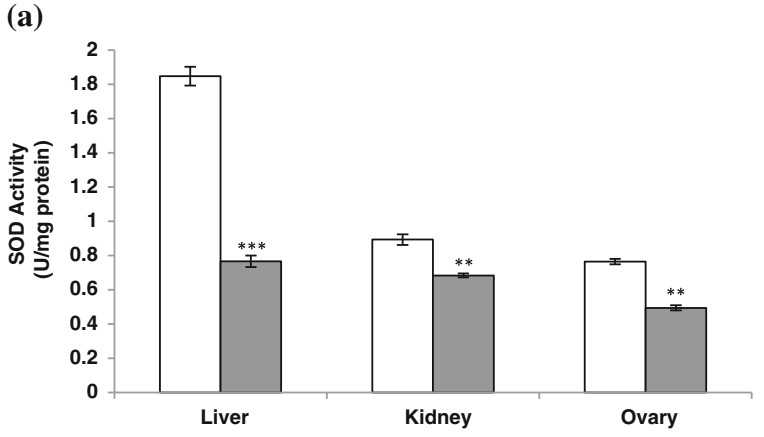
The present study clearly demonstrates for the first time that low-level 2.45 GHz MW radiation is able to affect implantation/pregnancy via inducing oxidative stress. Our study elucidated that in MW-exposed mice, implantation sites were affected significantly when compared to control. This is consistent with our previous study and other investigations on mice exposed to 2.45 GHz (CW) at a power density of 30 mW/cm^2 have shown decreased implantation sites per litter and reduction in fetal weight during 1–6 days of gestation. Exposure of mice at the same power density but 6–15 days of gestation resulted in a slight but significant increase in the percentage of malformed fetuses, predominantly with cleft palate [41]. We also observed some abnormalities such as unequal/asymmetrical distribution of embryos in the two uterine horns and reduced inter-embryo spaces in the exposed group of mice. No pups were born in the exposed group unlike the control, although some traces of embryos were observed in the uterine horns. However, in one mouse, only a single embryo of abnormally increased size and deformed was found in unilateral uterine position. This could be due to resorption of embryo during early pregnancy as a consequence of MW radiation-induced oxidative stress which also exerts adverse effects on birth outcome. It was found that exposure to an electromagnetic field during pregnancy was linked to an increased risk of miscarriage [59, 60]. Pregnant women and children are exposed to this low-level MW radiation (especially by microwaves, mobile phones, and Wi-Fi signals) in the same degree as the general public. A study on human females also suggests that pregnant physiotherapists, who are exposed to microwaves and shortwave as a result of their occupation, had an increased risk of miscarriage. A total of 1,753 pregnancies involving first trimester miscarriages were matched to 1,753 control pregnancies. This revealed a 7 % but nonsignificant rise in miscarriages associated with shortwave exposure and a significant 28 % increase in first trimester miscarriages for those exposed to microwaves, including a highly significant dose–response relationship [61]. However, our results contradict with the reports of Sambucci et al. and Jensh et al. [44, 45] which had shown no remarkable effect of 2.45 GHz MW radiation exposure on pregnancy or the development of preimplantation embryo. We can only speculate on the explanation of our results that the resorption of the embryo might have occurred due to MW radiation-induced oxidative stress.

Oxidative stress is a pronounced prooxidant state, caused by the excessive production of free radicals or from the weakening of the antioxidant defense system. Our experimental data clearly show that exposure to MW radiation led to the generation of ROS. Our results support the earlier study which suggests that extremely low frequency magnetic field as a potent activator of macrophages produces a large amount of ROS [62]. An imbalance

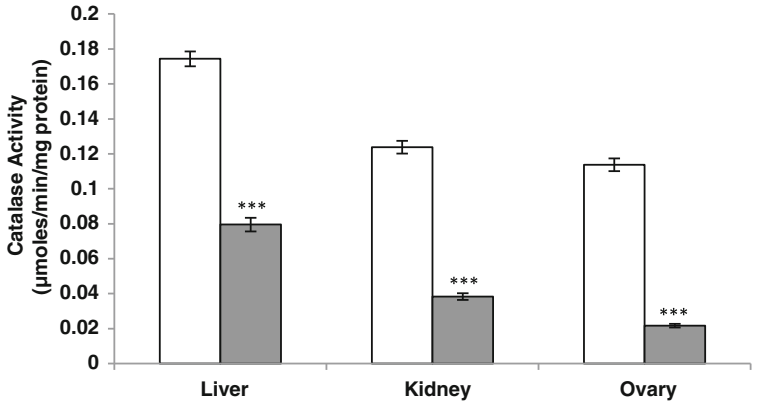
Fig. 8 **a** Decreases in antioxidant enzyme activities of *a* SOD (*upper panel*), *b* CAT (*middle panel*), and *c* GPx (*lower panel*) were observed in the liver, kidney, and ovary of female mice after 2.45 GHzMW irradiation. Data were statistically analyzed by Student's *t* test. Values are mean ± standard deviations (n=12). **p<0.01 and ***p<0.001, significant difference from the control group. **b** Changes in antioxidant enzyme activities of SOD (*a*), CAT (*b*), and GPx (*c*, *d*) in the liver, kidney, and ovary of 2.45 GHzMW-irradiated mice were demonstrated by native gel and enzyme activity staining. The intensity of bands in all the cases was quantitated by gel densitometry using ImageJ v. 1.36 software (NIH). Significant difference was determined by using Student's *t* test. Values are expressed as mean ± standard deviations. **p<0.01 and ***p<0.001, significant difference from the control group

between the production and manifestation of ROS, i.e., extensive production of peroxides and free radicals, causes toxic effects and damages all components of a cell including proteins, lipids and DNA. We also observed a highly significant DNA damage in the brain cells of mice after low-level MW exposure which is supported by our previous study and also by Lai and Singh [63]. This could be due to the elevated ROS load, providing nucleophilic SN2 attack on the sugar moiety of the nucleotide residues producing apurinic or apyrimidinic sites, causing single- or double-stranded DNA breaks. ROS specially hydroxyl ions ($^{\bullet}OH$) generated close to DNA attack the base pair and may cause mutation [64]. Increased DNA damage consequently affects the embryonic development and could increase the chances of abnormal embryo or may also result in resorption. We have also observed an alteration in total protein content of liver, kidney and ovary but this alteration was insignificant.

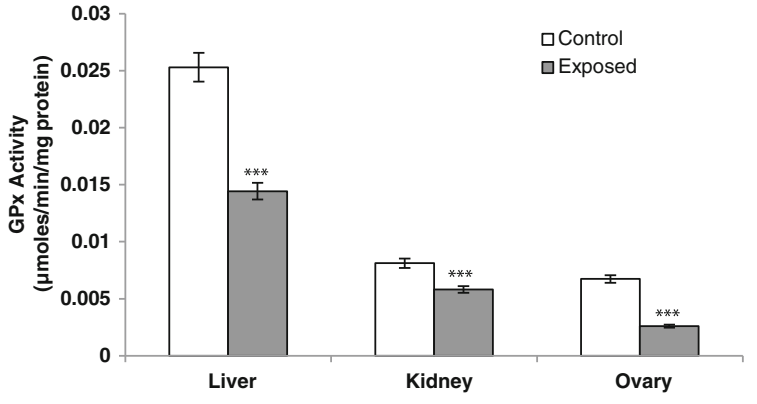
ROS plays a role in the modulation of an entire spectrum of physiological reproductive functions such as oocyte maturation, ovarian steroidogenesis, corpus luteal functions, luteolysis, fertilization, embryo development and pregnancy [65]. To ensure physiological levels of ROS, oocytes and granulosa cells in all follicular stages as well as follicular fluid are well endowed with the major antioxidant and detoxifying enzymes [66, 67]. During the estrous cycle, ovarian follicles, corpus luteum (CL) and the uterus exhibit rapid cellular proliferation, growth and development to ensure a hormonal environment suitable for early embryonic development and the establishment of pregnancy. Estradiol produced by the developing ovarian follicles interacts with progesterone produced by the CL to induce uterine receptivity for embryo implantation. Normally, during and just after pregnancy, the level of estradiol remains low due to the inhibitory effect of progesterone and prolactin. But in case of the MW-irradiated group of animals, we found a significant increase in estradiol level. We also observed an insignificant increase in progesterone level which might be due to the presence of functional corpus lutea in the ovary of MW-exposed mice. This suggests the possible effect of MW radiation-induced oxidative stress as a consequence of elevated ROS on steroidogenesis. We also suggest that the elevated estradiol level affects the secretion of progesterone during the gestation period, and thus, it could not maintain the pregnancy after implantation. ROS is also implicated in the induction of apoptosis [68] and cellular senescence [69]. It may affect the dynamic processes of gamete maturation, its transportation through the reproductive tract, and fertilization and thus, consequently affect the integrity, viability, and function of gametes and embryos. Further, the subsequent development and implantation of the preimplantation embryo get affected by ROS. We observed a significant increase in hemoglobin content as well as RBC and WBC counts in female mice exposed to low-level MW radiation. We also found a significant increase in N/L ratio which is an indicator of a hematologic response to inflammatory stimuli and stress. All these changes might have occurred in response to increased ROS production after weakening of the antioxidant defense system. It is also well-established that an oxidative stress condition affects the whole body physiology of the animal including its blood profile and blood cell number.


(a)
SOD Activity (U/mg protein)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
**
**
Liver
Kidney
Ovary

(a)


Catalase Activity (µmoles/min/mg protein)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
***
***
***
Liver
Kidney
Ovary

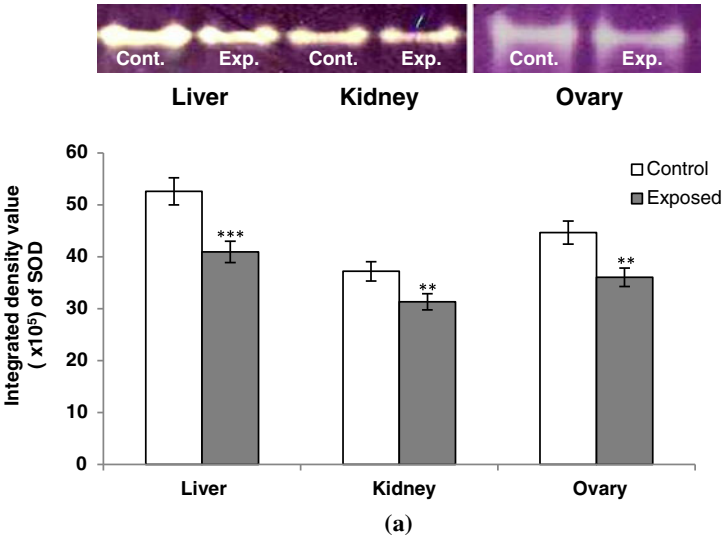
(b)


GPx Activity (µmoles/min/mg protein)
0.03
0.025
0.02
0.015
0.01
0.005
0
Control
Exposed
***
***
***
Liver
Kidney
Ovary

(c)

(b)

1. Superoxide dismutase activity



2. Catalase activity

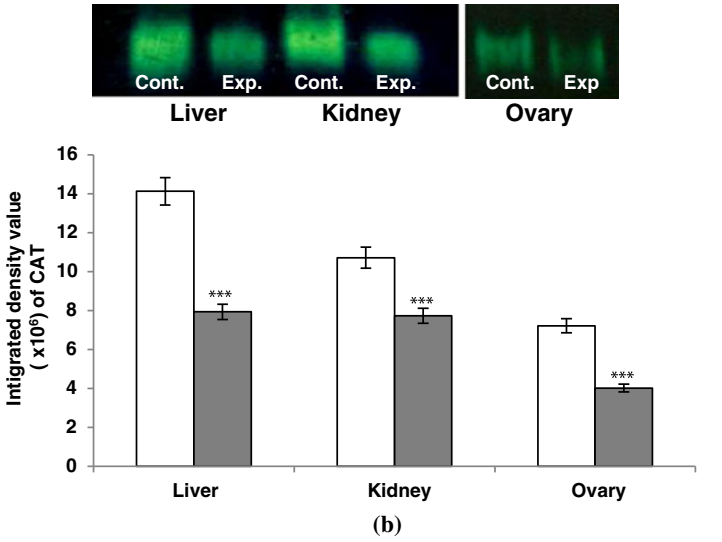


Fig. 8 (continued)

Any alteration in ROS level directly indicates changes in antioxidant enzyme activities; therefore, we next examined ROS-scavenging enzymes SOD, CAT and GPx activities responsible for inducing oxidative stress. MW-irradiated mice show a significant decrease in the antioxidant enzyme activities in the liver, kidney and ovary. This decreased antioxidant activity results in response to elevated ROS level, which causes oxidative damage, although we found that the liver is much more affected than the kidney and ovary which may

3. Glutathione peroxidase activity

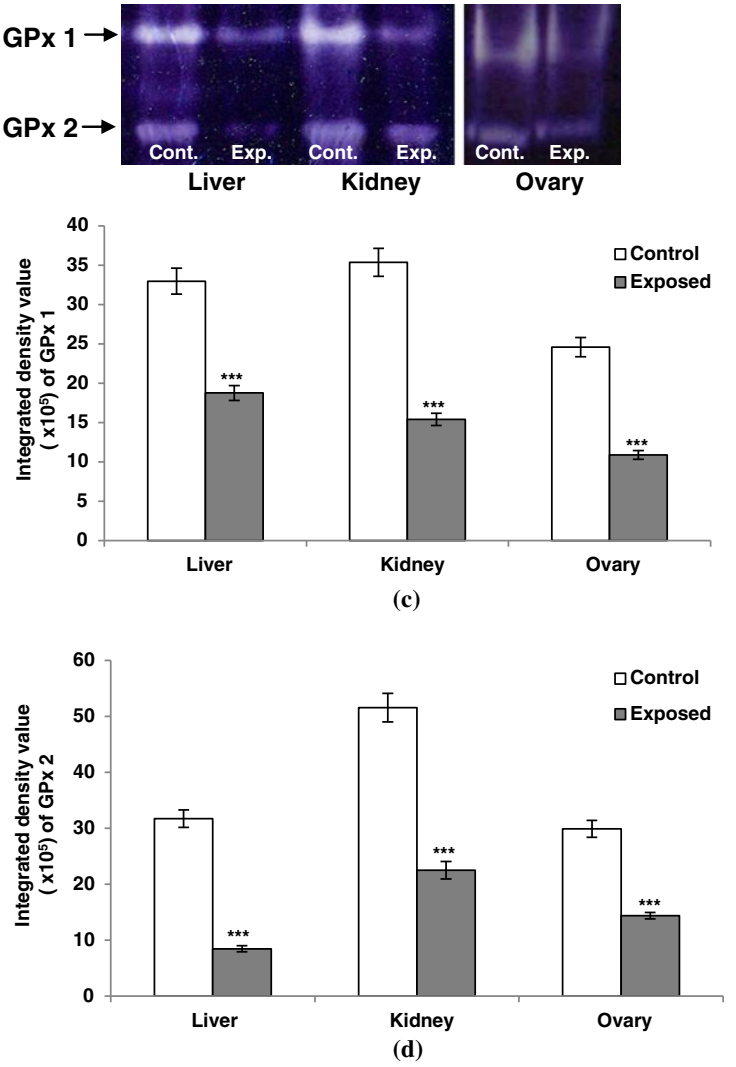


Fig. 8 (continued)

be quite possible because the liver is the main detoxifying organ that metabolized MW-induced toxicity, i.e., ROS. Further, we also observed that CAT activity consistently decreased in all the tissues than SOD and GPx. CAT plays a major role in protecting the cell against oxidative damage by catalyzing the decomposition of H_2O_2 to water and molecular oxygen. Decreased CAT activity may compromise the overall antioxidant enzyme defense system. It is also well known that GPx is not an efficient H_2O_2 decomposer as compared to CAT, and hence, a high level of H_2O_2 is observed in CAT-depleted cells than GPx-sufficient cells. Therefore, the compromised CAT activities may significantly impair the capacity of the antioxidant enzyme defense system. CAT and GPx are well-known H_2O_2 scavengers which serve to protect cells from the toxic effects of H_2O_2. Low-level MW exposure decreased

SOD activity significantly which could enhance the production of $O_2^{\bullet-}$. Further, $O_2^{\bullet-}$ dismutase by SOD undergoes nonenzymatic reaction to form H_2O_2, a precursor of the more toxic hydroxyl radical. Since the activities of these enzymes were significantly decreased in irradiated mice, the level of H_2O_2 increased. This increased H_2O_2 production may be responsible for elevated embryo fragmentation, suggesting that ROS may be implicated in the induction of apoptosis in embryos [70].

We also found a significant decrease in NO level in the exposed group of mice. A decrease in NO level may result in cell toxicity by increasing free radical load since it can also act as a free radical scavenger and inactivate $O_2^{\bullet-}$, thereby preventing cell toxicity [71]. NO causes vasoconstriction within the vasculature which may result in hypertension. Normally, low oxygen tension is required to enhance embryo development to the blastocyst stage and to increase their total cell counts by reducing the proportion of apoptotic cells [72, 73]. This enhanced embryo development is thought to be responsible for improved embryo metabolism [74, 75]. Reduced NO level also affects the pregnancy/implantation by affecting the embryonic growth and development. However, it has been reported that during normal pregnancy, NO production increases with advancing gestation which returns to its normal level within 12 weeks after delivery [76]. The increased nitric oxide synthesis may be partially responsible for regulating the vascular adaptation of pregnancy while reduction of nitric oxide production in rats results in a significant intrauterine growth retardation [77]. NO regulates the uterine and fetoplacental blood flow and could be involved in uterine quiescence prior to parturition.

The results of our present study clearly demonstrate that MW irradiation results in the accumulation of havoc-free radicals in different organs and thus induced oxidative stress which is in accordance with the earlier published studies [38, 39]. Elevated free radicals not only disturb the normal physiology of the body but also exert an adverse effect on the pregnancy or implantation in female mice. There are several studies which demonstrate that the imbalance in free radicals and antioxidant enzymes responsible for causing oxidative stress influences the timing and maintenance of viable pregnancy. A review by Gray and Becker [78] also suggests that the delayed conception and the early pregnancy loss may share a common etiology, possibly through events or exposures prior to or during implantation and embryogenesis. Our results demonstrate that MW irradiation is responsible for generating extensive free radical load due to a significant reduction in antioxidant activities and NO production which could possibly cause the aging and exhaustion of the ovary and increased the contractile activity of the uterus. The accumulation of damage exerted by increased ROS levels is claimed to be involved in ovarian aging [79] and hormone alteration. The inadequate hormone production has been proposed as a cause of embryonic losses in humans [80] and ruminants [81]. ROS is among the most important physiological inducers of cellular injury associated with aging [82]. Oocyte aging and deteriorating oocyte quality known to occur with advancing age and many pathological conditions contribute significantly to reproductive failure. Miscarriage during the establishment of cellular and biochemical interactions between the uterine endometrium and the developing post-implanting conceptus is associated with increased oxidative stress [83].

Therefore, on the basis of our observations, we propose that the resorption of the embryo/fetus in MW-irradiated mice might have occurred due to altered estradiol and progesterone levels, increased cellular fragmentation, and gross abnormal morphology which may result from oxidative damage to cytoskeletal fibers. This could be due to increased ROS load caused by the reduction in NO level and antioxidant enzyme activities, responsible for oocyte aging, hormonal changes, DNA damage, and protein expression. There is evidence supporting the concept that oxidative damage to cytoskeletal fibers may result in abnormal

embryo morphology. In contrast to the surface of vital blastomeres, which is organized into short, regular microvilli, the surface of cellular fragments exhibits irregularly shaped blebs and protrusions [84]. Cytoskeletal alterations associated with the formation of surface blebs are characteristic signals of oxidative cell injury. It is also well-established that the elevated ROS levels generate damage to cells/embryos through increased lipid peroxidation [85, 86] and protein oxidation and induce DNA strand breaks [30, 87].

In spite of the number of reports indicating radiation-induced oxidative stress and its effects on implantation/pregnancy, the precise mechanism on how MW radiation induced these effects is still unknown. However, we suggest that all these effects of MW radiation at low SAR value might have been caused due to resonant absorption of microwave by the animal body. Also, there is a possibility of molecular resonance absorption of energy by genetic material. The energy can be absorbed over time involving many photons. Therefore, the molecule can absorb the energy of not just one photon but many photons. This energy may be smaller compared with the total thermal energy already present in the molecule, but more than enough to affect its performance, especially if it is all in one oscillation mode [88, 89].

On the basis of our experimental findings, we conclude that 2.45 GHz MW radiation, a nonthermal electromagnetic radiation, acts as a strong environmental stressor responsible for inducing oxidative stress. It increases ROS load, hemoglobin, RBC and WBC count, N/L ratio as well as P_4 and E_2 concentrations. Decreased NO and antioxidant enzyme activity (SOD, CAT and GPx) observed in the 2.45 GHz low-level MW-irradiated mice may be correlated with increased ROS which adversely affects the implantation or pregnancy in mice. Enhancement of ROS, altered antioxidant activities and sex steroid level responsible for oocyte aging and deteriorated oocyte/embryo lead to implantation failure and/or resorption. Further, MW radiation-induced oxidative stress may also affect the timing of implantation, maintenance of viable pregnancy, and prenatal development. However, further studies are required to understand the exact mechanism and site of action of low-level MW radiation and its effect on implantation/pregnancy via oxidative damage.

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