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Evaluation of genotoxic effects in male Wistar rats following microwave exposure

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Wistar rats (70 days old) were exposed for 2 h a day for 45 days continuously at 10 GHz [power density 0.214 mW/cm^2, specific absorption rate (SAR) 0.014 W/kg] and 50 GHz (power density 0.86 μW/cm^2, SAR 8.0 ×10^{-4} W/kg). Micronuclei (MN), reactive oxygen species (ROS), and antioxidant enzymes activity were estimated in the blood cells and serum. These radiations induce micronuclei formation and significant increase in ROS production. Significant changes in the level of serum glutathione peroxidase, superoxide dismutase and catalase were observed in exposed group as compared with control group. It is concluded that microwave exposure can be affective at genetic level. This may be an indication of tumor promotion, which comes through the overproduction of reactive oxygen species.

Keywords: Catalase, Glutathione peroxidase, Micronuclei, Microwave radiation, Reactive oxygen species, Superoxide dismutase

In recent years, the widespread use of electromagnetic field has lead to public concern about its implication on human health. There have been many instances of harmful effects of electromagnetic fields from sources such as power lines, television, mobile phone, laptops, computers, ovens, etc. The electromagnetic fields (EMFs) from the extremely low frequencies (ELF, 30-300 Hz) to radiofrequency and microwaves (100 kHz-300 GHz) are usually known as the non-ionizing radiations. Biological effects resulting from electromagnetic field radiation may depend on dose, which indicate long-term accumulative effects[1,2]. Most studies indicated disruption in carbohydrate, lipid and protein metabolism by electromagnetic fields which may induce changes in enzyme activity and gene expression and cause DNA damage[3]. *In vivo* studies showed an increase in DNA single-strand break[4]. So far numerous studies have shown the genotoxicity of electromagnetic field at frequencies between 900-1800 MHz and greater at 2.45 GHz[5]. If chromosome damage occurs in the cell, a portion of the chromosome (micronucleus) remains in erythrocyte. Erythrocytes do not have nucleus in its mature stage. Micronuclei (MN) are small, nucleus like structures present in the cell. The main reason for micronuclei formation is that chromosomes or fragments have lost their connection to a centromere and thus cannot be properly segregated from the metaphase plate to either of the poles. Thus *in vivo* test is especially relevant in assessing genotoxicity. Therefore, micronuclei in bone marrow or peripheral blood erythrocytes are widely accepted as sensitive predictor of the clastogenic potential of chemical and radiation[6]. *In vivo* and *in vitro* experiments pointed the enhancement of free radical presence after electromagnetic field exposure[7-9]. Excessive production of free radicals specifically reactive oxygen species (ROS), have been reported in wide variety of clinical disorders, environmental stress [such as ultraviolet (UV), heat exposure, electromagnetic field exposure][10-13]. Molecular oxygen in the ground state contain two unpaired electrons known as a triplet state having same spin of electron while reactive oxygen species are chemical species with one unpaired electron derived from molecular oxygen. Excitation of electrons by external stimulus changes the spin of electron resulting in oxidant activity[14]. Free radicals are highly reactive molecules with a very short half life. The balance between production and neutralization of ROS is maintained by concert action of enzymatic and non enzymatic defence systems. ROS levels can increase dramatically, which may cause damage to cell structures and react with various biochemical

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reactions[15]. When unbalanced, it may lead to oxidation of poly-unsaturated fatty acids in lipids, amino acids in proteins and damage to DNA. Cells have a variety of defence mechanisms against the harmful effects of ROS like superoxide dismutase (SOD), vitamin E and C, etc. Cells have their own set of antioxidant defence mechanisms to fight with free radical formation and to overcome the limit of damaging effects. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are the enzymatic defence systems of cells against oxygen radicals. Superoxide is supposed to be first line of defence against the ROS, which catalyses the superoxide anion into hydrogen peroxide. GPx inactivates hydrogen superoxide and organic hydroxides together with GSH and glucose-6-dehydrogenase with reduced glutathione[16]. Recently Kesari and Behari[17], have also shown that microwave radiation may alter the level of antioxidant due to free radicals formations.

The early detection of oxidative imbalance by simple and reliable methods is important. In this communication we report the assay which is able to trace the early state of oxidative stress. The present study is designed to investigate the effect of electromagnetic field exposure on micronuclei formation and antioxidant enzymatic system by overproduction of reactive oxygen species formation in adult male rats.

Materials and Methods

Materials— *N*, *N*-diethyl-*para*-phenylendiamine (DEPPD) sulphate and acridine orange (AO) were purchased from Sigma Chemicals (St. Louis, MO, USA). Ferrous sulfate, Hydrogen peroxide, sodium acetate and other chemicals were obtained locally. The glutathione peroxidase (GPx, catalogue No. 703102), catalase (CAT, catalogue No. 707002) and superoxide dismutase (SOD, catalogue No. 706002) antioxidant enzyme kits were purchased from the Cayman Chemical Company, Ann Arbor, MI, USA.

Animals exposure—Male Wistar rats (70 days old and 190 ± 20 g body weight) were obtained from animal facility of Jawaharlal Nehru University, New Delhi. These were divided into following four groups of 6 each, 10 GHz sham exposed, 10 GHz exposed, 50 GHz sham exposed and 50 GHz exposed group. A blind study was conducted by repeating all experiments. All animals were housed in an air conditioned room, where the temperature was maintained at 25°C with constant humidity (40-50%). Air conditioner was constantly used to maintain the environmental parameters. The animals were provided with standard food pallets (prepared by Tetragon Chemie Pvt. Ltd, Bangalore) and water *ad libitum*. The protocols for animal experimentation described in this study were approved previously by the Institutional Animal Ethical Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). All subsequent animal experiments adhered to the 'Guidelines for Animal Experimentation' of the University.

Exposure chamber—Rats were placed in a Plexiglas cage which was well ventilated with holes of 1 cm diameter. The dimension of exposure cage was made in such a way that animal moved freely. Chamber is lined with radio frequency (RF) absorbing material (attenuation, 40 db) to minimize the possibility of any reflections. At far field distance from horn antenna, it was found that the field is homogeneous in vertical plane of midline of the beam. Rats were exposed with 50 GHz continuous source through the antenna, 2 hr a day for 45 days (Fig. 1). The power density at receiving end was measured (0.86 μW /cm^2) and the nominal specific absorption rate (SAR) value was calculated (8.0×10^{-4} W/kg). In other similar setup at 10 GHz, animal were exposed 2 h a day for 45 days. The power density (0.214 mW/cm^2) and specific absorption rate was 0.014 W/kg. The output power was measured by the power sensor (model no. 6934) and power meter (model no. IFR-6960B, IRF System Inc, Longacres House, UK). The experiments were organized at 10 and 50GHz exposure in two different setups. The animals faced E filed orientation parallel to the exposure cage. Every day the cage was placed in

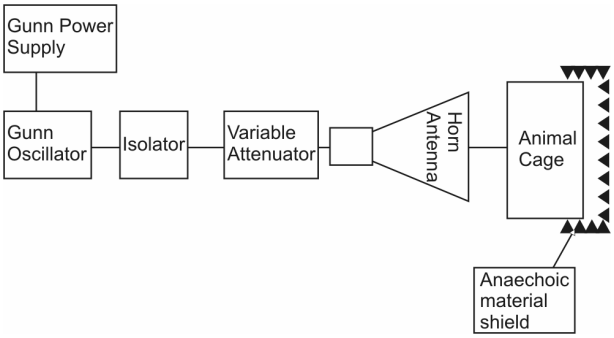

Fig. 1—Microwave anaechoic exposure set-up indicating animal position (10 GHz/ 50 GHz). The same set was used earlier[4,18,19].

same position facing the horn antenna. The same number of rats position was filled randomly reshuffled. The same setup for exposure has been used earlier[4,18,19].

Sample preparation—After the last exposure, rats were sacrificed with overdose of anaesthesia (ketamine/xylazine cocktail) injection. The blood sample was collected by cardiac puncture. Blood samples for micronuclei were kept at 4°C. For ROS and antioxidant, blood samples were allowed to clot at room temperature for one hour. After clotting, blood was centrifuged at 300 *g* for 10 min and transferred immediately to deep freezer.

Total reactive oxygen species assay— According to Hayashi *et al.*[20] 5 µl of serum was added to 140 µl of pre warmed (40°C) 0.1*M* sodium acetate buffer (*p*H 4.8) in 96-wells (microtiter plate). Mixed solution (100 µl) of DEPPD (100 µg/ml DEPPD was dissolved in 0.1*M* sodium acetate buffer, *p*H 4.8) and ferrous sulfate [ferrous sulfate (4.37 µ*M*) was dissolved in 0.1*M* sodium acetate buffer, *p*H 4.8] at a ratio of 1:25 was added in each well to initiate reaction. Thereafter, microtiter plate was incubated at 37°C for 5 min. Absorbance was measured using a Spectra Max M_2 spectrophotometer plate reader, at 505 nm. ROS levels in serum were calculated from the calibration curve of H_2O_2 and expressed as equivalent to levels of hydrogen peroxide (1 unit = 1.0 mg H_2O_2/l). Calibration curve for the standard solution was obtained by calculating slopes from optical density graph.

Flow cytometry: Micronucleus assay— Blood samples were washed by adding 5 ml of PBS and centrifuged at 300 *g* for 5 min. The pellet was resuspended in PBS. 5 ml of fixative [Sorensen buffer A: 0.05 *M* KH_2PO_4, Sorensen buffer B: 0.05 *M* $Na_2HPO_4.2H_2O$, *p*H 6.8, 30µg/ml SDS, and 1% glutaraldehyde (v/v)] was added to 15 ml conical tube containing 100µl of blood samples. Samples were vortex vigorously and remained in the fixative for 5 min. Thereafter samples were centrifuged at 300 *g* for 5 min and pellets were resuspended in 0.5 ml of PBS. Solution A was prepared by adding 0.1 ml Triton X-100, 8 ml 1.0 *N* HCl, 0.877 g NaCl and distilled water to a final volume of 100 ml according to Criswell *et al*[21]. Solution B contained 37 ml 0.1 *M* anhydrous citric acid, 63 ml 0.2 *M* Na_2HPO_4 (*p*H 6.0), 0.877 g NaCl, 34 mg EDTA disodium salt and 0.6 ml acridine orange (1 mg/ml). Both the solutions were chilled on ice prior to use. Fixed samples were pipetted into a centrifuge tube, followed by the addition of 400 µl solution A and 1.2 ml solution B. Samples were mixed by vortexing and allowed to stain on ice for 30 min in the dark. After staining, samples were centrifuged at 300 *g* for 5 min. The supernatant was carefully aspirated, so that the cellular pellet was undisturbed. PBS (1 ml) was added to the pellet, resuspended and mixed by vortexing. Samples were analysed on FACSCalibur (Becton & Dickinson) equipped with a 15 mW argon laser. All the events were recorded on forward angle scatter (FALS, linear scale, related to cell size), side scatter (SSC, log scale, related to cellular complexity), DNA fluorescence (FL 1 log, green fluorescence, 530 nm), and RNA fluorescence (FL 4 log, red fluorescence, 675 nm).

Sample preparation for antioxidant assay—Serum was added to the cold buffer (50 m*M* tris HCl, *p*H 7.5, 5 m*M* EDTA and 1 m*M* DTT) for GPx, 20 m*M* HEPES buffer (1 m*M* EGTA, 210 m*M* mannitol and 70 m*M* sucrose) for SOD and cold buffer (50 m*M* potassium phosphate, *p*H 7.0, containing 1 m*M* EDTA) for catalase. All the samples were centrifuged at 10,000 *g* for 15 min at 4°C, supernatant was collected and enzyme assay was performed according to supplier kit.

Estimation of glutathione peroxidase activity— Assay buffer (120 µl) and 50 µl co-substrate mixture was added in non-enzymatic wells. Assay buffer (100 µl), 50 µl co-substrate mixture and 20 µl diluted GPx was added in other wells as control sample. Same amount of assay buffer and co-substrate including 20 µl serum sample in place of GPx were added in all the wells. Immediately reaction was initiated by adding 20 µl cumene hydroperoxide to all the wells being used. Finally wells plate was placed in micro-plate reader spectrophotometer and absorbance of the samples were taken at 340 nm.

Estimation of superoxide dismutase activity—SOD (20 µl) standard was diluted with 1.98 ml sample buffer. SOD standard wells prepared by using 200 µl of the diluted radical detector and 10 µl of diluted standard. Sample wells were also prepared by adding 200 µl of the diluted radical detector and 10 µl of sample to the wells. The reaction was initiated by adding 20 µl of diluted xanthine oxidase to all the wells. The sample plate was kept in micro-plate reader and temperature as well as absorbance was taken at 450 nm.

Estimation of catalase activity—Assay buffer (100 µl), 30 µl of methanol and 20 µl of standard was added to wells, which contained 10 µl of formaldehyde and 9.99 ml of sample buffer and formaldehyde wells were prepared. Control wells were prepared by adding 100 µl of diluted assay buffer, 30 µl of methanol and 20 µl of diluted CAT. The sample wells were prepared by adding 100 µl of diluted assay buffer, 30 µl of methanol and 20 µl of samples. The reaction was initiated by adding 20 µl of diluted hydrogen peroxide to all the wells. 30 µl of potassium hydroxide was added to terminate the reaction. 30 µl of purpald (chromogen) was added to each wells and there after incubated for 10 min at room temperature on a shaker. 10 µl of potassium periodate was added to each wells, incubated for 5 min at room temperature on shaker and the absorbance of samples were taken at 540 nm.

Data collection and statistical analysis—Analysis of ROS was done by taking absorbance at 505 nm. The ROS concentration in serum of electromagnetic field exposed group was analysed from the graph of H_2O_2 standard and expressed as equivalent to levelsof H_2O_2 (1 Unit=1.0 mg H_2O_2/l). Analysis of micronucleus events were passed through the Poly-chromatic Erythrocyte (PCE) and Normochromatic Erythrocytes (NCE) population. Toxicity at the stem cell level can be detected as a decrease in ratio of PCE/NCE. PCE/NCE ratio was determined by staining blood samples with acridine orange which excite at 530 and 675 nm of wavelengths. NCE does not contain DNA or RNA and hence least fluorescent population. The PCE contain only RNA content and occur in the middle of the histograms. TNC present in the extreme upper right of the histograms, containing RNA and DNA content. Moreover GPx, SOD and CAT activity were measured at 340, 450 and 540 nm respectively. The statistical analysis was done for the samples (control and experimental) for all case. The obtained results were presented as mean ± SE. Statistical analysis of the data was done by one-way ANOVA (Excel). A value of $P < 0.05$ was considered to be significant.

Results

Reactive oxygen species—Absorbance of serum samples was taken at 30 seconds intervals at 505 nm by spectrophotometric plate reader. ROS was calculated from standard hydrogen peroxide solution. It was observed that an optical density of each solution increased with time. ROS level was expressed as one unit equivalent to 1 mg/l H_2O_2. The mean among the 10 GHz exposed group, 27.38 ± 6.30 units was significantly higher ($P < 0.03$) than that of sham exposed group, 19.78 ± 4.00. In 50 GHz exposed group (45.96±19.07), ROS was significantly higher ($P < 0.04$) as compared to sham exposed (27.29 ± 6.61).

Flow cytometric determination of micronuclei—PCE/NCE ratio of percentage gated value was determined by flow cytometry. The ratio of PCE/NCE in 10 GHz exposed group (1.25±0.20) was significantly lower ($P < 0.05$) as compared with sham exposed group (1.90±0.23). The mean value of 50 GHz exposed groups (0.93 ± 0.06) was observed significantly lower ($p < 0.05$) as compared with sham exposed group (1.55 ± 0.23).

Antioxidant enzyme activity—The activity of GPx, SOD and CAT were presented in Fig. 2.

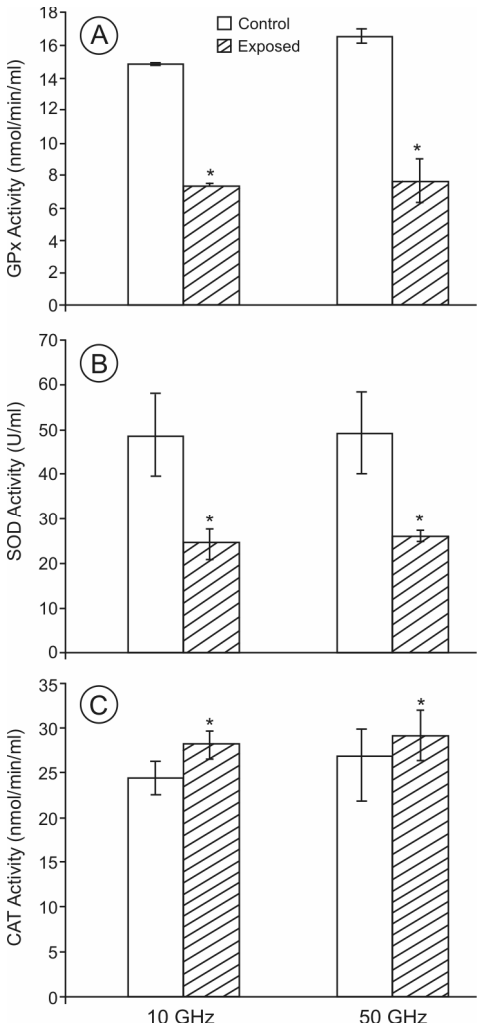

Fig. 2—Activity of antioxidant enzyme (A) glutathione peroxidase (GPx), (B) superoxide dismutase (SOD) and (C) catalase (CAT) in serum of 10 and 50 GHz exposed rats.

Discussion

There has been a long standing debate about the effect of electromagnetic field on biological systems and its mechanism of interaction. In several studies, electromagnetic field exposures causes the overproduction of free radical formation and effects such as genotoxicity and oxidative stress[13,15,22-24]. The present studies have shown alterations in different oxidative stress parameters after electromagnetic field exposure. In present study, a significant increase in ROS level of exposed group as compared with sham exposed ones was observed. Oxygen which is necessary for all aerobic living system, is a substrate for many biological reactions in the body, but it may undergo electron transfer reactions which generate highly reactive oxygen free radicals. An increased ROS production can damage macromolecules (lipid, protein, DNA) and also responsible for heart disease, cancer, arthritis, Alzheimer's disease, and accelerate general ageing[25]. Regoli *et al.*[26] pointed out that hydroxyl radicals, cause more oxidative damage to a wide variety of cellular targets, including nucleic acid. If hydroxyl radicals are generated close to DNA, they can attack the base pair and cause mutations. Electromagnetic field cause DNA single and double strand break[27,4]. The singlet oxygen formed due to electromagnetic field exposure take part in various biochemical reactions and forms the superoxide, hydroxyl radical, alkoxy and peroxy radicals. Free radicals have short life span, however in such time, they may cause damage to the cells. Such alkoxy and peroxy radicals react with the *N*, *N*-diethylparaphenylenediamine, leading to the development of colour correlated with free radicals[28]. Report suggests that electromagnetic field increase the concentration of free radicals which may enhance the probability of damage to the biological system[29-31,8]. Damage to body occurs when intracellular antioxidant mechanism are overwhelmed by ROS[30]. GSH, CAT and SOD protect cells against ROS. In the present study, decreased activities of the key antioxidants, GSH and SOD were found in the serum of rats exposed to the 10 and 50 GHz radiations. SOD plays a key role in defence mechanism against free radical activity. Normally, SOD enzyme works in parallel with GSH, playing an important role in the reduction of hydrogen in the presence of GSH forming GSSG (glutathione disulfide) and protects cell proteins and cell membranes against oxidative damage[32]. In biological system, the mechanisms of tissue damages are thought to involve reactive oxygen species (ROS) produced due to exposure of electromagnetic fields in surrounding environments. Moustafa *et al.*[33] have found a decrease in plasma SOD activity in humans. A decrease in activity of SOD was related to accumulation of superoxide amino radicals in cells. The detoxification is mainly done by SOD. Hydrogen superoxide, a product of SOD activity, is also a strong inhibitor of SOD enzyme[16]. The charge of detoxification is taken by CAT enzyme, which leads to increase its activity. The increased activities of catalase indicate that catalase activity is increased to compensate the overproduction of reactive oxygen species which overproduced in serum by electromagnetic field. Zwirska-Korczala *et al.*[34] also reported a decrease in activity of SOD, GPx and increase activity of CAT. Decreased activity of GPx in exposed group may be due to decrease in its formation, which requires NADPH and GR[33,35]. Normally GPx enzyme works in parallel with SOD[36]. Free radicals are produced continuously and detoxified by SOD, glutathione (GSH), and catalase (CAT). With excessive free-radical production and the resulting consumption of antioxidants, endogenous defense mechanisms become insufficient. The decreased activities of both SOD and GSH in the serum exposed to electromagnetic radiation indicate the highly reduced capacity to scavenge hydrogen peroxide produced in blood in response to acute stress. Micronucleus is also induced by radiation exposure. The interaction of radiation with chromosome result not only in the formation of dicentric chromosome but also acentric fragments. Since acentric fragments do not have a centromere though they are not pulled towards the daughter nuclei at the time of nuclear division. These acentric fragments are left in the cytoplasm which appears as micronuclei. The degree of micronuclei provides an estimation of radiation exposure effects. Darzynkiewicz *et al.*[37,38] successfully used acridine orange to simultaneously measure DNA and RNA in proliferating cell lines of normal or tumor origin. The PCE/NCE ratio is used as genotoxicity detector in micronuclei test, decreased in electromagnetic field exposed group as compared to sham exposed group. Zotti-Martelli *et al.*[39] reported the micronuclei formation in human lymphocytes after an acute exposure to 2.45 and 7.7 GHz RF fields. It is suggested that micronuclei formations occur due to

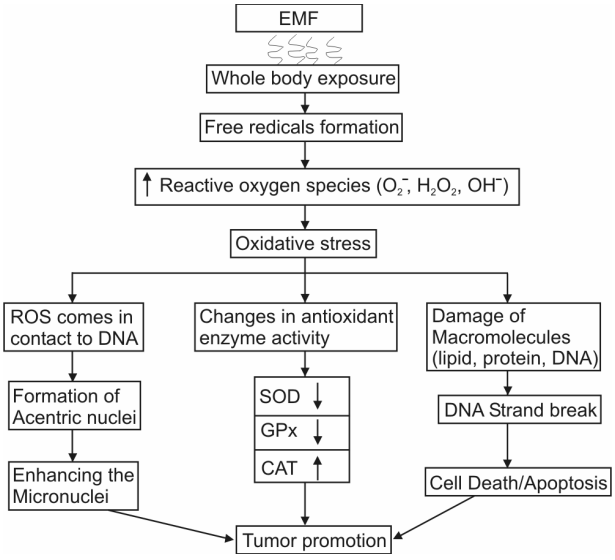

Fig. 3—Flow chart depicting the mechanism of microwave exposure and the cause of tumour promotion.

the redox reactions[40-42]. The present study showed that ROS act as mediators of electromagnetic field induced micronucleus formation and decision in activity of defence mechanism of cells (antioxidant systems). The correlation between micronuclei and cancer is well studied[43-45] and recent analyses also indicate that electromagnetic field correlate well with human cancer risk by inducing ROS production, which overwhelm the defence mechanism of cell and produce micronuclei.

Conclusion

It may be concluded that microwave radiation may produce effect on micronuclei formation, depletion in activity of defence mechanism of cell by overproduction of reactive oxygen species. The effects reported are non thermal.

- Over production of reactive oxygen species are more at 50 GHz as compared to 10 GHz microwave exposure. This is suggestive of the preference for occurrence of macromolecular resonance at 50 GHz.
- Induction of micronuclei in erythropoietic cells due to DNA and ROS interaction.
- ROS overproduction may cause depletion in the activity of SOD, GPx enzymes, and increase in CAT activity.

Results indicate that the 50 GHz microwave radiations are more effective as compared to 10 GHz frequency. The cycle of events has been summarized in Fig. 3.

Authors contribution

Sanjay Kumar and Kavindra Kr. Kesari have equally contributed in experimentation. J. Behari has critical contribution in supervision, drafting article, revision and final approval. All the authors read and approved the final manuscript.

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