doi: 10.4149/gpb_2017059

Effect of 2.45 GHz microwave radiation on the fertility pattern in male mice

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Abstract. A number of studies have reported that male reproductive organs are susceptible to electromagnetic fields (EMFs). The aim of this study was to explore the effects of microwave radiation exposures on 6–8 weeks old male Swiss albino mice. Mice were divided into two groups: group I – sham-exposed (Control), and group II – microwaves-exposed (MWs). Mice were exposed to 2.45 GHz with power density 0.25 mW/cm^2 and specific absorbtion rate (SAR) 0.09 W/kg. Exposure was given in Plexiglas cages for 2 h/day for 30 days. Results showed that 2.45 GHz microwave radiation resulted in a significant increase ($p < 0.001$) in catalase (CAT), malondialdehyde (MDA), reactive oxygen species (ROS) and decrease ($p < 0.001$) in the levels of glutathione peroxidase (GPx), testosterone and superoxide dismutase (SOD) ($p < 0.05$). Flow cytometer analysis of blood showed the formation of micronuclei in microwave-exposed group. Histopathological changes were also seen in seminiferous tubules of microwave-exposed group as compared to the sham-exposed group. It is concluded that 2.45 GHz microwave radiation exposure causes oxidative stress in testes and it may lead to detrimental and injurious effects on fertility potential of the male reproductive system of Swiss albino mice.

Key words: Microwave radiation — Reactive oxygen species — Testosterone — Micronuclei — Male infertility

Introduction

Reports of declining male fertility have raised interest in assessing the role of environmental and occupational exposure to electromagnetic fields (EMFs) in the aetiology of human infertility. The sources of microwave radiation are mainly from the telecommunication networks, microwave ovens, cell phones, military and domestic applications. Leakage of EMFs radiation from such devices into the environment has posed a severe concern on human health (Khaki et al. 2008; Wang et. al. 2008; Hashem et al. 2009). The male reproductive system is highly vulnerable to physical factors and environmental assaults which may lead to various reproductive disorders like testicular abnormalities, chromosomal aberrations, infertility, testicular cancer and congenital defects in offspring (Havas 2000; Kim et al. 2007). Exposure to EMFs have also shown a significant decrease in sperm parameters seminal vesicle weight, the diameter of the seminiferous tubules, sperm motility, testosterone levels and adverse histological changes in testes (Bahaodini et al. 2015; Shokri et al. 2015; Chauhan et al. 2016). Exposure to microwave radiation promotes histopathological changes in testis, diminishes testosterone levels and it stimulates clastogenic effects in male germ cells (Kumar et al. 2012b; Meena et al. 2013). Exposure to EMF can enhance reactive oxygen species (ROS) that stimulates lipid peroxidation (Kumar et al. 2011; Avendano et al. 2012; Atasoy et al. 2013). An increased level of ROS due to microwave radiation may have an adverse effect on the male reproductive system and may lead to infertility (Kesari et al. 2010, 2011; Nisbet et al. 2012). ROS may also increase the for-

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mation of micronuclei and impaired embryonic development (Kumar et al. 2010, 2011). Micronuclei are used as a sensitive predictor of the clastogenic potential of radiation-induced chromosomal damage (Criswell et al. 1998; Yoshikawa et al. 2000; McNamee et al. 2002). Some previous studies showed that chronic exposure to EMFs induces oxidative stress and reduces the total anti-oxidative capacity of testicular cells by diminishing activity of catalase (CAT) superoxide dismutase (SOD) glutathione peroxidase (GPx) and it also elevates malondialdehyde formation by lipid peroxidation (Kesari and Behari 2010; Kesari et al. 2011). The DNA integrity of male germ cells is extremely important as they pass genetic material to the next generation. Declining sperm quality and increasing infertility patterns over the decades has set the male reproductive organs as most susceptible to electromagnetic radiation. Hence the present study was designed to elucidate the effects and mechanism of microwaves radiations on male reproductive organs.

Materials and Methods

Experimental animals

Male Swiss albino mice, 6–8 weeks old, weighing 35.0 ± 3.0 g were used for the present study. Mice were maintained in the animal house as per the norms established by Institutional Animal Ethical Committee (IAEC). The mice were housed in clean Plexiglas cages and maintained under controlled conditions of temperature with constant 12-h light and 12-h dark schedule and provided with standard mice feed (Hindustan Unilever Limited, Delhi, India) and water *ad libitum*. The study was approved by Departmental Ethical Committee (DEC), Department of Zoology, University of Rajasthan.

Exposure chamber

Microwave radiation experimental bench (Fig. 1) was used for the exposure of mice. The bench consisted of a signal generator, isolator, attenuator, frequency meter, horn antenna and a specially designed animal cage. Microwave power was delivered to the isolator by attenuating the power with the tunable probe connected to the frequency meter. The output power was transmitted through the horn antenna to the animal cage. A graphite sheet was used to minimize the reflection of scattered beam. A rectangular box made of Plexiglas was used to house the mice. The box was properly partitioned and well ventilated with holes of 1-cm diameter. The dimensions of the box were made in such a way that animals stay comfortably inside the box. Total 16 mice were divided into two groups: Sham-exposed (n = 8), and microwave-exposed (n = 8) group. The mice were exposed to 2.45 GHz microwave radiation through the horn antenna for 2 h/day for 30 consecutive days.

The bench was set to generate microwave radiation of 2.45 GHz frequency. The power density 0.25 mW/cm^2 was measured by using the formula: $C = P_t \cdot G_t / 4\pi R^2$, where, P_t is transmitting power by the source; G_t is transmitting antenna gain; R is distance between the horn antenna and mid-plane of the exposure cage. The specific absorption rate (SAR) was calculated by using the Durney equation (Durney et al. 1984). The horn antenna horizontally exposed the mice inside the Plexiglas box and power density was recorded at the center of box by a power meter (a peak sensitive device with RF power sensors 6900 series' and 'IFR 6960B RF power meter, made of Aeroflex Inc, Wichita, KS, USA). The whole-body SAR was estimated to be 0.09 W/kg. The horn antenna was kept in H-plane configuration so that electric field of the waves was perpendicular to the ground surface. The electric field in the box was almost uniform because the dimensions

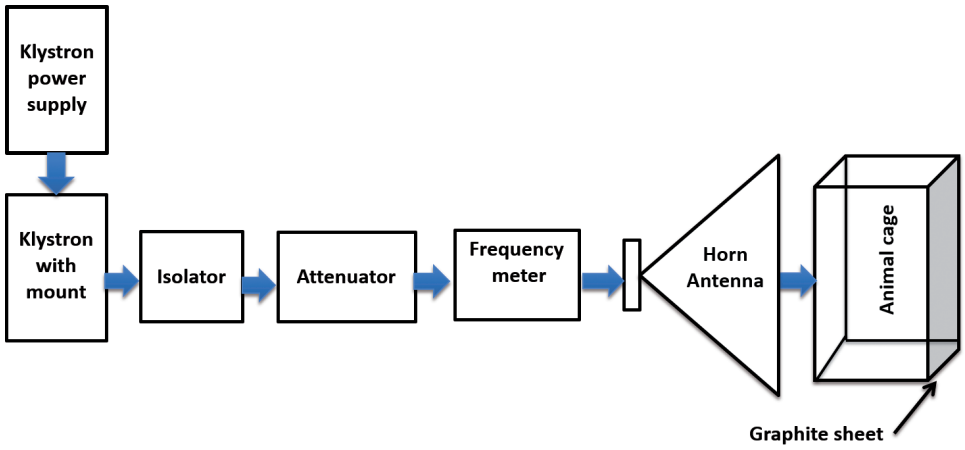

Figure 1. Schematic diagram of 2.45 GHz microwave exposure set-up with animal cage indicating individual animal's position.

of the box were of the order of one wavelength only. Two experimental set-ups were calibrated identically and used for exposure. Every day two mice were housed at a time in the box and the box was placed at the same location facing the horn antenna. No animal blocked the radiation falling on the other animal. The temperature in the chamber was maintained around 25–27°C throughout the experiment. At the end of the exposure period, mice were sacrificed and testes were quickly excised for further studies.

Reactive oxygen species (ROS)

ROS was measured by the method of Lee et al. (2006). Briefly, 50 mg of the whole testis was homogenized in 1 ml of PBS (0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, 1.37 M NaCl, 2.7 mM KCl, pH 7.4; (Sigma-Aldrich, St. Louis, MO, USA) and filtered by passing through 100 µm pore size strainer and centrifuged at 100 × *g* for 10 min. The pellet was re-suspended in 1 ml of PBS and the cells were treated with 10 µl of 2'7'-dichlofluorescein diacetate (3.3 µM; DCFH-DA; Molecular probes, Eugene, OR, USA). The cells were incubated at room temperature (RT) for 10 minutes in dark and then centrifuged at 100 × *g* for 10 min. The pellet was washed in 1 ml of PBS. Thereafter cells were examined by flow cytometry (Guava technologies, CA, USA). The results were expressed as (U/l).

Malondialdehyde (MDA)

Following the method of Buege and Aust (1978), the amount of MDA in sperm cells (10^6 cells) was measured by monitoring the formation of thiobarbituric acid reactive substances (TBARS). The opted method was based on spectrophotometric measurement of the colour generated by the reaction of thiobarbituric acid (TBA; Sigma-Aldrich, St. Louis, MO, USA) with MDA. 2 ml of each sample treated with trichloroacetic acid (15% w/v) containing 1 mM EDTA was centrifuged at 1,000 × *g* for 10 min. The supernatant was boiled at 100°C in the water bath with an equal volume of TBA (0.7% w/v) for 20 min, and after cooling, samples were centrifuged at 1,000 × *g* for 10 minutes. After centrifugation, the absorbance was recorded at 532 nm by using Ultra Violet-Vis double beam spectrophotometer (Double Beam Spectrophotometer 2203, Systronics, Ahmedabad, Gujarat, India). A standard curve was prepared by using tetramethoxy-propane (TMP) (purchased from Himedia, Mumbai, Maharashtra, India). After comparison with a standard curve, the MDA level was expressed in nmol/10^6 sperm.

Glutathione peroxidase (GPx)

The EIA kit (catalogue No. 703102 of Cayman Chemicals Company Ann Arbor, Michigan, USA) was used for the measurement of GPx. The procedure of measurement for the assay was followed as *per* manufacturer manual. The wells plates were placed in micro-plate spectrophotometer reader (BioTek Instruments Winooski, Vermont, USA) and the absorbance of the samples was taken at 340 nm. The data were expressed in nmol/min/ml.

Superoxide dismutase (SOD)

Superoxide dismutase EIA kit (Catalogue No. 706002 of Cayman Chemical Company Ann Arbor, Michigan, USA) was used. The procedure of measurement for the assay was followed as *per* manufacturer manual. The sample plates were kept in micro-plate spectrophotometer reader and absorbance was taken at 450 nm. The data were expressed in U/ml.

Catalase (CAT)

Catalase EIA kit (catalogue No. 707002 of Cayman Chemical Company Ann Arbor, Michigan, USA) was used. The procedure of measurement for the assay was followed as per manufacturer manual. The absorbance was measured at 540 nm by micro-plate reader spectrophotometer. The data were expressed in nmol/min/ml.

Testosterone

Testosterone enzyme immunoassay (EIA) kit (catalogue No.582701 of Cayman Chemical Company Ann Arbor, Michigan, USA) was used to perform serum testosterone. For this, 50 ml of testosterone standard, 50 ml of testosterone AChE tracer and 50 ml of testosterone antiserum were added to the wells containing 100 ml of EIA buffer. The sensitivity of the assay was 6 pg/ml. The optical density was measured at 405–420 nm by spectrophotometer (BioTek Instruments Winooski, Vermont, USA). The data were expressed in ng/ml.

Micronucleus measurement

Micronucleus assay was used to detect DNA damage and clastogenic effects of electromagnetic fields by providing a rapid ratio of PCE/NCE (polychromatic erythrocyte/normochromatic erythrocyte) percentage gated values. Micronucleus assay was performed following the method of Criswell et al. (1998) and Kumar et al. (2012b). Briefly, 1 ml blood sample was added to 5 ml of phosphate-buffered saline (PBS) and centrifuged at 300 × *g* for 5 min. The pellet was re-suspended in 1 ml of PBS then 100 µl of sample was mixed with 5 ml of fixative Sorensen buffer A (0.05 M KH_2PO_4), Sorensen buffer B (0.05 M $Na_2HPO_4.2H_2O$, pH 6.8, 30 µg/ml SDS) and 1% glutaraldehyde (v/v) (Fisher scientific chemicals, Mumbai, India). Samples were vortexed vigorously and kept in the fixative for 5 min at room temperature. Thereafter, the samples were centrifuged at 300 × *g* for 5 min and the pellets were re-suspended in 0.5 ml of PBS.

Thereafter 400 µl of cold solution A (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) and 1.2 ml of cold solution B (37 ml 0.1 M anhydrous citric acid, 63 ml 0.2 M Na_2HPO_4 (pH 6.0), 0.877 g NaCl, 34 mg EDTA disodium salt, and 0.6 ml acridine orange (1 mg/ml)) were added to the resuspended pellets. Samples were mixed by vortexing and kept on ice for 30 min in dark for the stain to appear. After staining, samples were centrifuged at 300 × *g* for 5 min. The supernatant was carefully removed without disturbing cellular pellet. 1 ml of PBS was added to the pellet, resuspended, and mixed by vortexing. Thereafter samples were analyzed by Flow-cytometer. 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 (FL1-H log, green fluorescence, 530 nm), and RNA fluorescence (FL3-H log, red fluorescence, 675 nm). Analysis of micronucleus events were passed through the polychromatic erythrocyte (PCE) and normochromatic erythrocyte (NCE) population. Toxicity was detected as a decrease in the ratio of PCE/NCE.

Histopathology of testis

Testes were dissected from animals and fixed in bouin's fixative. Samples were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Slides were examined by light microscopy (40×10 magnification) to observe histopathological changes.

Statistical analysis

Data were presented as the mean ± standard deviation (SD). Student *t*-test (two-tailed) was applied to determine significant differences between sham-exposed and microwave-exposed groups; $p < 0.05$ was considered as significant difference.

Results

The microwave-exposed group showed a statistically significant ($p < 0.001$) increase in the levels of ROS (Fig. 2A) and significant ($p < 0.001$) decrease in the levels of testosterone (Fig. 2B) in comparison to the sham-exposed group (Fig. 2, Table 1). Statistically significant ($p < 0.001$) increase in the levels of MDA (Fig. 3A) and CAT (Fig. 3B) was measured in the microwave-exposed group in comparison to the sham-exposed group. However, statistically significant decrease ($p < 0.001$) in the levels of GPx (Fig. 3C) and SOD ($p < 0.05$) (Fig. 3D) was measured in microwave-exposed group in comparison to the sham-exposed ones. The measurement of micronuclei formation was recorded by flow cytometer (Fig. 4A,B), where the rapid ratio of PCE/NCE percentage

Table 1. Variations in the endpoints after 2.45 GHz microwave exposure

Biochemical parameters	Sham-exposed	Microwave-exposed
ROS (U/l)	14.7 ± 0.83	27.23 ± 1.64**
MDA (nmol/10^6 sperm)	0.11 ± 0.03	0.19 ± 0.05**
GPx (nmol/min/ml)	11.67 ± 0.91	4.73 ± 0.68**
SOD (U/ml)	42.56 ± 3.96	18.85 ± 1.78*
CAT (nmol/min/ml)	21.43 ± 1.67	45.85 ± 2.97**
Testosterone (ng/ml)	4.02 ± 0.31	1.95 ± 0.49**
Micronuclei formation	1.56 ± 0.22	0.73 ± 0.04**

Data are presented as the mean ± SD; * $p < 0.05$ (significant), ** $p < 0.001$ (highly significant) *vs.* sham-exposed group. ROS, reactive oxygen species; MDA, malondialdehyde; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase.

gated value of 2.45 GHz exposed group showed a statistically significant ($p < 0.001$) decrease in comparison to the sham-exposed group. (Fig. 4, Table 1).

Results observed for the histopathology of testis showed abnormal spermatogenic cycle, irregular epithelial lining, detachment between the adjacent seminiferous tubules, occlusion of the lumen of seminiferous tubules, large vacuoles and condensed nuclei in some cells of microwave-exposed group (Fig. 5B) in comparison to the sham-exposed group (Fig. 5A).

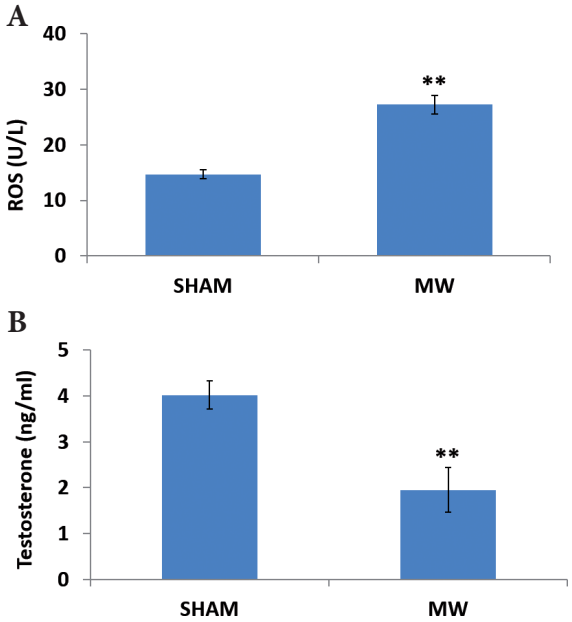

Figure 2. The effects of 2.45 GHz microwave radiation on the levels of reactive oxygen species (ROS, **A**) and testosterone (**B**) in testis of Swiss albino mice. The values are means ± SD; ** $p < 0.001$ *vs.* sham-exposed group. MW, microwave-exposed group.

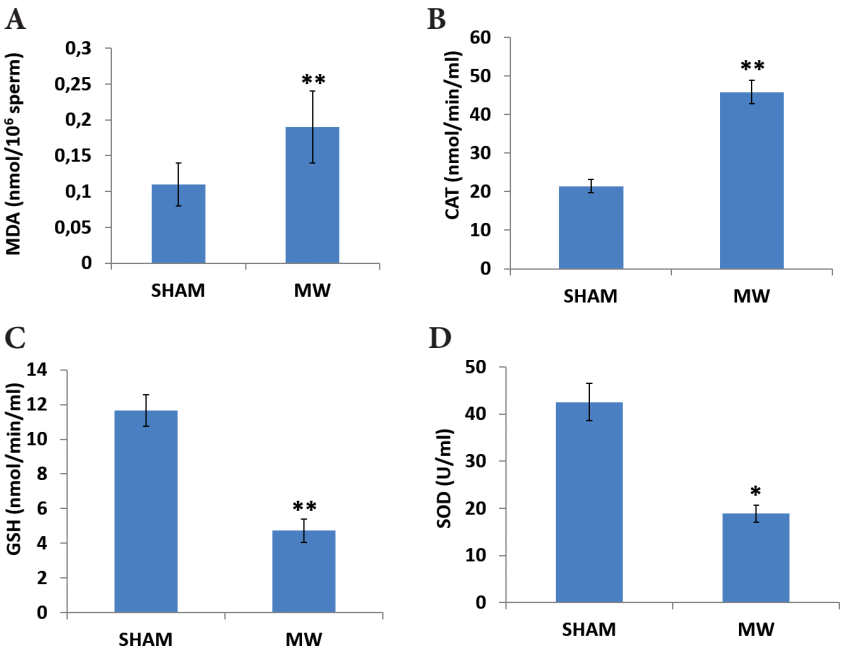

Figure 3. The effects of 2.45 GHz microwave radiation on malondialdehyde (MDA, **A**), catalase (CAT, **B**), glutathione peroxidase (GPx, **C**) and superoxide dismutase (SOD, **D**) levels in testis of Swiss albino mice. The values are in means ± SD; ** $p < 0.001$, * $p < 0.05$ *vs.* sham-exposed group. MW, microwave-exposed group.

Discussion

The present investigations provide several important findings related to biochemical, pathophysiology and clastogenic effects of 2.45 GHz microwave radiation on the male reproductive system of mice. The findings in this study are associated with significant changes in histopathology and oxidative damage in testes through altered anti-oxidative parameters after microwave exposure of 30 days (2 h/day). The present study revealed that microwaves induce oxidative damage biochemically as evident by decreased levels of GPx, SOD and testosterone and increased levels of MDA and CAT. Our

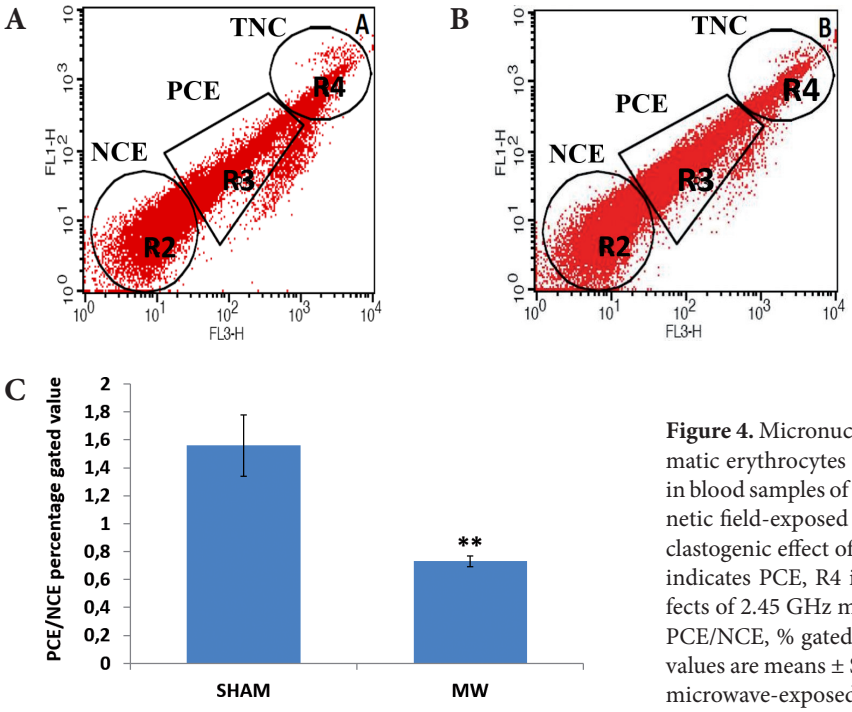

Figure 4. Micronucleus test results showing the ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE/NCE) in blood samples of mice from sham-exposed (**A**) and electromagnetic field-exposed (**B**). PCE/NCE was calculated to find out the clastogenic effect of electromagnetic fields. R2 indicates NCE, R3 indicates PCE, R4 indicates total nucleated cells (TNC). The effects of 2.45 GHz microwave radiation on micronuclei formation PCE/NCE, % gated value (**C**) in blood of Swiss albino mice. The values are means ± SD; ** $p < 0.001$ *vs.* sham-exposed group. MW, microwave-exposed group.

results are in the line of earlier findings (Shang et al. 2004; Kesari et al. 2010; Kumar et al. 2012a, 2012b; Bahaodini et al. 2015). Antioxidants play an important role to protect the cells from oxidative damage. Cellular antioxidants like SOD, GSH, Catalase and LPO are important markers of free radical generation. An adequate level of cellular antioxidants maintains the free radicals scavenging potential in testes. The authors observed an increase in CAT activity, which suggests high levels of H_2O_2 in the testicular cells (Condell et al. 1993). Free radicals are produced continuously and detoxified by SOD, GPx and CAT. With excessive free radical production and the resulting consumption of antioxidants, endogenous defense mechanisms become insufficient. This leads to decreased activity of SOD and GPx in response to electromagnetic field exposure (Amara et al. 2006). In the present study, significant changes were also observed in testosterone levels after microwave exposure. These changes might be because of overproduction of ROS and interaction between EMFs radiation and testicular cells. The ROS-induced interaction between EMF and testicular cells affect the functioning of the testes adversely. The inadequate polarization of cellular membrane is responsible for the process of various abnormalities of testosterone synthesis and secretion which may impair spermatogenesis and ultimately become a cause of infertility (Meo et al. 2010). ROS and free radicals are generated in the cells by energy transfer or by electron transfer reactions induced by EMFs exposure. During this process, a highly reactive singlet oxygen atom is formed which results in the sequential reduction to various molecules of the cell such as superoxide, hydrogen peroxide, and hydroxyl radicals (Georgiou 2010). EMFs affect spermatogenesis and testicular function through defects in the male germ line, decreased sperm count, narrower seminiferous tubules and reduction in intrascrotal testosterone levels (Dasdag et al. 1999). In agreement with the present study, EMFs exposure resulted in deceleration of spermatogenesis, disorders in germinal cell distribution, reduction in germ cell population and decrease in epithelium thickness and diameter of seminiferous tubules (Dasdag et al. 1999, 2003; Ozguner et al. 2005; Aydin et al. 2007; Rajaei et al. 2009; Khayyat 2011). The light microscopic examination of the testes sections in exposed group revealed abnormal spermatogenic cycle, irregular epithelial lining, detachment between the adjacent seminiferous tubules, occlusion of the lumen of seminiferous tubules and large vacuoles and condensed nuclei in some cells (Fig. 5B) in comparison to the control mice testes with normal spermatogenic cycle, germ cells and Sertoli cells (Fig. 5A). Therefore, it may be suggested that abnormalities in testicular morphology may be due to susceptible nature of testes towards microwave radiation. Increased level of LPO induces cellular injury in the spermatozoa due to leakage of sperm membrane fluidity (Aitken et al. 1994). Since mammalian sperm membranes contain a high level of unsaturated fatty acids and are also sensitive to oxygen-induced damage mediated by lipid peroxidation and free water-induced oxygen (Russo et al. 2006). De Iuliis et al. (2009) found that radiofrequency exposure enhances

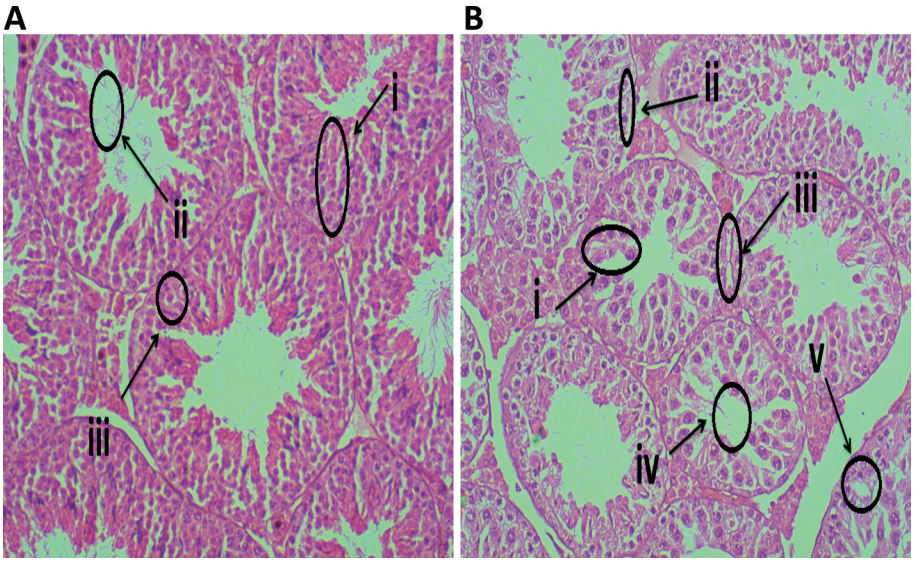

Figure 5. The light microscope examination of transverse section of sham-exposed mice testis stained with HE stain (**A**) shows normal spermatogenic cycle (i), germ cells (ii), sertoli cells (iii) (40×10 magnification). The light microscope examination of transverse section of mice testis exposed to 2.45 GHz and stained with HE stain **(B)** shows abnormal spermatogenic cycle (i), irregular epithelial lining (ii), detachment between the adjacent seminiferous tubules (iii), occlusion of the lumen of seminiferous tubules (iv), large vacuoles and condensed nuclei in some cells (v) (40×10 magnification).

mitochondrial ROS around spermatozoa, which reduces both their motility and vitality and promotes DNA fragmentation. EMFs exposure induces oxidative stress, which may lead to DNA damage and micronuclei formation. Our study is in corroboration with the findings of earlier studies (Aweda et al. 2002; Kesari et al. 2010). The main reason for micronuclei formation is that the fragments, which are lost during cell division, cannot be segregated from the metaphase plate to either of the poles. Our results are in support of the fact that microwave radiation may be a causative factor for male infertility because it affects anti-oxidants activities and causes histopathological changes in testes. Hence it may lead to DNA damage and micronuclei formation, which may further be a cause of male infertility.

Conclusion

We conclude that microwaves at 2.45 GHz frequency adversely affect the testicular functions and reproductive patterns by induction of oxidative stress-mediated cellular toxicity. The present study concludes that EMFs may lead to elevated oxidative stress, formation of micronuclei, decrease in testosterone levels, compromised antioxidant activities and increase in MDA levels. Histological changes in testicular tissues also revealed that microwave exposure has causative effect on fertility patterns. Results from this study also revealed important implications of microwave exposure on the male reproductive health. As a consequence of increase in free radicals and ROS, microwaves-induced oxidative stress resulted in increased ROS level, which indicates the possibility of infertility. The available evidence indicates that a very limited research has been reported on protective measures against microwave radiation induced adverse effects. This further worsens the problem of EMFs generated health problems including increased rates of male infertility. Therefore, further studies are needed to elaborate the molecular mechanism of cellular level damage in testes exposed to microwave radiation and the protective measures against adverse effects of microwave radiation should also be explored.

Acknowledgement. The authors are thankful to Department of Physics, University of Rajasthan, Jaipur for providing microwave exposure facility.

Conflict of interest. The authors report no conflict of interest.

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Received: June 25, 2017
Final version accepted: November 22, 2017