

RESEARCH ARTICLE

2.45 GHz microwave radiation induced oxidative and nitrosative stress mediated testicular apoptosis: Involvement of a p53 dependent bax-caspase-3 mediated pathway

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Abstract

Deleterious effects of MW radiation on the male reproduction are well studied. Previous reports although suggest that 2.45 GHz MW irradiation induced oxidative and nitrosative stress adversely affects the male reproductive function but the detailed molecular mechanism occurring behind it has yet to be elucidated. The aim of present study was to investigate the underlying detailed pathway of the testicular apoptosis induced by free radical load and redox imbalance due to 2.45 GHz MW radiation exposure and the degree of severity along with the increased exposure duration. Twelve-week old male mice were exposed to 2.45 GHz MW radiation [continuous-wave (CW) with overall average Power density of 0.0248 mW/cm² and overall average whole body SAR value of 0.0146 W/kg] for 2 hr/day over a period of 15, 30, and 60 days. Testicular histology, serum testosterone, ROS, NO, MDA level, activity of antioxidant enzymes, expression of pro-apoptotic proteins (p53 and Bax), anti-apoptotic proteins (Bcl-2 and Bcl-x_L), cytochrome-c, inactive/active caspase-3, and uncleaved PARP-1 were evaluated. Findings suggest that 2.45 GHz MW radiation exposure induced testicular redox imbalance not only leads to enhanced testicular apoptosis via p53 dependent Bax-caspase-3 mediated pathway, but also increases the degree of apoptotic severity in a duration dependent manner.

KEYWORDS

Bax, Bcl-2, microwave radiation, oxidative stress, p53, testicular apoptosis

1 | INTRODUCTION

In the present scenario, the strength of electromagnetic radiations (EMR) especially microwave (MW) radiation has increased manifold in our environment due to large-scale expansion of communication networks such as mobile phones, base stations, WLAN, Wi-Fi, Wi-MAX, etc. MW radiations are categorized as non-ionizing electromagnetic radiation (EMR) since it has very low photon energy which cannot cause molecule ionization when it is absorbed in living organisms. It is absorbed at molecular level and induces non-thermal (include molecular resonance where the molecular bonds are stressed or the cyclic motion of the molecular structure is enhanced or restricted) or thermal effects (where the microwave energy is converted into heat in the living organism).¹

Several studies have demonstrated the negative impact of MW radiation exposure on male reproductive health which may affect male

fertility.^{2–7} Reports are there mentioning the ill effects of short duration exposure to MW radiation on gonadotropin levels (FSH and LH), testicular morphology, histopathology, serum/plasma testosterone levels, sperm counts, and sperm viability.^{8–13} Studies demonstrate that short term exposure to 2.45 GHz MW radiation may lead to testicular apoptosis. It increases caspase-3 activity and number of TUNEL positive cells in the seminiferous tubules of exposed rats.^{6,14,15} 2.45 GHz MW radiation exposure cause damage to seminiferous tubule, decrease Leydig cells number, testosterone concentration, and histone kinase activity, and increase creatine kinase activity^{4,15,16} in rodent testis. Increased expression of different inflammatory markers [viz., calcitonin gene related peptide (CGRP), prostaglandin E2, and interleukin-6 (IL-6)] in the rodent testis and epididymis following the short term exposure to 2.45 GHz MW radiation was also reported.¹⁶

MW exposure to anaesthetized rats and mice results in increased testicular or body temperature which may cause depletion of the

spermatogenic epithelium, abnormal sperm morphology, reduced epididymal sperm count and increased percentage of abnormal sperm, and primary spermatocyte resulting in decreased fertility.^{17,18} Various RF fields exposure at different level (1.7 and 10 mW/m²) from a commercial antenna produced a rapid drop in fertility in mice.¹⁹ MW radiation exposure (2.45 and 9.45 GHz, SAR = 2 W/kg) increased the percentage of abnormal sperm and decreased epididymal sperm count^{6,20} in rats. The adverse effect of 2.45 GHz MW radiation induced oxidative and nitrosative stress has also been reported on the reproductive function of male mice.¹²

Mounting evidence suggest MW radiation as one of the strongest environmental stressor, responsible for inducing oxidative/nitrosative stress by generating free radical havoc load.^{12,21–23} The excessive generation of free radicals species weakens the endogenous defense antioxidant systems and mechanisms, and damages the cellular constituents including lipids, proteins, and nucleic acids.²⁴ It is well established that both oxidative and nitrosative stress has been attributed to affect the fertility status of males²⁵ and are capable of disrupting the steroidogenic capacity of Leydig cell²⁶ as well as the capacity of the germinal epithelium to differentiate into normal spermatozoa.²⁷ Testicular micro-environment has low oxygen tensions despite of that, this tissue remains highly vulnerable to free radical attacks and subsequently undergoes lipid peroxidation (LPO)²⁸ due to high content of polyunsaturated fatty acids (PUFAs). The ROS scavenging enzymes/antioxidant systems (enzymatic and/or non-enzymatic) protect testis against oxidative/nitrosative damage by scavenging the free radicals (ROS/RNS).²⁸ Although, physiological levels of free radicals are essential for fertilization, acrosome reaction, hyperactivation, motility, and capacitation,^{29,30} excessive production of free radicals/pathological condition can be deleterious/toxic for testicular physiology of reproduction.

Free radicals are also implicated in the induction of apoptosis/programmed cell death³¹ and cellular senescence.³² ROS and the cellular redox change can be part of signal transduction pathway during apoptosis.³³ Apoptosis is intrinsic and essential component of normal testicular functions which limit the population of germ cells via preventing the maturation of abnormal/damage germ cells.³⁴ Although, regular apoptosis of the spermatogenic cells is required to maintain proper testicular homeostasis and self-renewal, yet excess cell death can also result in defective spermatogenesis and finally may leading to infertility.³² MW radiation exposure may damage the sperm by producing ROS^{12,35,36} and thus responsible for inducing infertility.^{37,38}

Although, number of studies suggest that MW exposure decreases sperm count, increases oxidative stress and testicular apoptosis,^{3,4,6,15} but no single systematic study is conducted till date to decipher the simultaneous effects of both short and long term 2.45 GHz MW radiation on the underlying detailed mechanism of testicular apoptosis including effects on sperm counts and viability. Based on previous reports, we hypothesize that the apoptotic pathway may actively participating in all the 2.45 GHz MW radiation exposed group of mice testis is p53 mediated Bax dependent and is induced by oxidative stress and/or oxidative/nitrosative stress. Hence, present study was undertaken to explore the underlying molecular mechanism(s) of 2.45 MW

irradiation induced testicular apoptosis and to check the alterations in the expression of pro-apoptotic proteins (p53 and Bax), anti-apoptotic proteins (Bcl-x_L and Bcl-2), apoptotic proteins (Cytochrome c) and executioner caspase (caspase-3), and nuclear DNA repairing enzyme PARP-1. In this study, we demonstrated the detailed sketch of the most plausible apoptotic pathway participating in mouse testis exposed to 2.45 GHz MW radiation for different time durations (15, 30, and 60 days).

2 | MATERIALS AND METHODS

2.1 | Animals

Twelve weeks old, adult, and healthy Swiss albino male mice (body weight ~30 g) were obtained from the extensively inbred colony of Institute of Medical Sciences, Banaras Hindu University maintained in a controlled environment with 12 hr light : dark cycle (LD12:12) and 24 ± 2°C room temperature. Animals were kept under steady-state, received standard commercial pelleted food and water ad libitum. Four animals per cage were housed in standard ventilated polypropylene cages with dry husk as the bedding material. Animals were treated and animal protocols were performed humanely and with regard to alleviation of suffering. All the experimental procedures were approved and performed as per the guidelines of "Institutional Animal Ethical Committee, Institute of Medical Sciences, Banaras Hindu University," (Letter No. Dean/12–13/CAEC/209) and within the framework of the revised animals (Scientific procedures) act of 2002 of the Government of India on Animal welfare. ARRIVE guidelines were followed to report this study.

2.2 | Study design

Randomly selected 40 mice were divided into four groups (one sham control and three experimental, ie, 15 days exposed, 30 days exposed, and 60 days exposed group) of 10 mice each (N = 10). The three experimental groups of mice were exposed to 2.45 GHz (CW) microwave radiation for 2 hr/day from 09:00 to 11:00 hr (30 days), 11:30 to 13:30 hr (60 days), and 14:00 to 16:00 hr (15 days) continuously (Figure 1). The control group mice were subjected to sham exposure for 60 days (to neutralize the cage related and other external constraints). Rectal temperature of all the mice was monitored during the first and the last three days of exposure.

2.3 | Exposure set-up

Mice were exposed to 2.45 GHz microwave radiation by using microwave irradiation set-up [consists of Analog Signal Generator microwave source (Model no. E 8257D PSG, manufactured by Agilent Technologies, The United States) 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, pyramidal horn antenna in addition to animal cage kept in a box covered by microwave absorbers] as describe previously²² at overall average power density of 0.0248 mW/cm² and

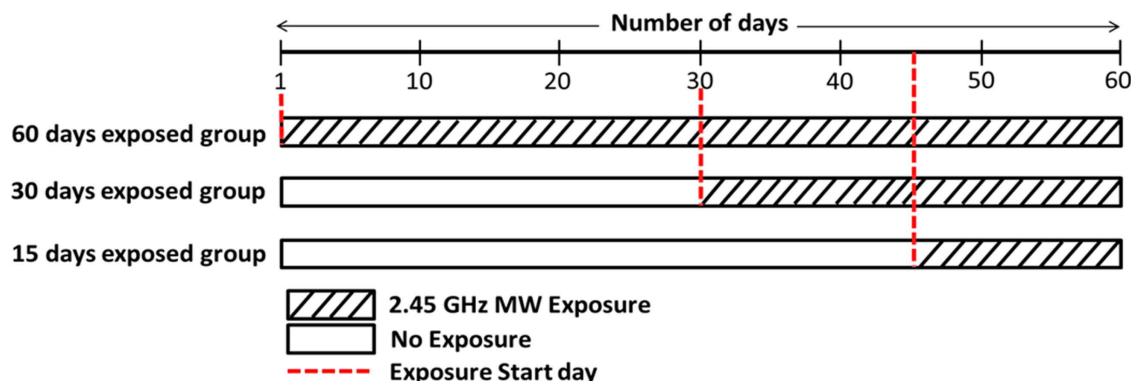


FIGURE 1 Schematic diagram representing the exposure pattern and duration (in days) for all the 2.45 GHz MW radiation exposed mice groups [Color figure can be viewed at wileyonlinelibrary.com]

overall average specific absorption rate (SAR) value of 0.0146 W/kg. The SAR value was estimated for body length parallel to the electric field, as per actual placement of the mouse.^{12,21,22} The details description of microwave generator, animal cage designed for MW exposure, power density, and SAR value calculation has been elaborated in our previous report.²² The exposure at overall average power density of 0.0248 mW/cm² did not cause any elevation in the ambient temperature of animal cage as well as mice rectal temperature. The average observed rectal temperature of the mice were $36.42 \pm 0.2^\circ\text{C}$ (control group) and $36.39 \pm 0.2^\circ\text{C}$ (experimental groups) during first 3 days of exposure and $36.64 \pm 0.2^\circ\text{C}$ (control group) and $36.51 \pm 0.2^\circ\text{C}$ (experimental groups) after the end of exposure.

2.4 | Blood collection and tissues sampling

After, 24 hr of the termination of 15, 30, and 60 days exposure, mice were sacrificed by decapitation. Blood was collected in non-heparinized tubes for serum collection. Testes (10 left and 5 right) were removed and after washing with ice-cold sterile physiological saline solution tissues were stored in -20°C until processed for ROS, NO, and MDA level measurement as well as antioxidant enzymes (SOD, CAT, and GPx) assay and western blot analysis of cytochrome c. Epididymis was removed to study sperm count and sperm viability. Right testes of five mice of each group were fixed in 10% neutral formalin solution for HE staining and immunofluorescence of proteins/enzymes involved in apoptotic pathway such as p53, Bax, Bcl-2, Bcl-x_L, pro-caspase-3, active/cleaved-caspase-3, and full length/uncleaved PARP-1.

2.5 | Histology of testes and seminiferous tubule diameter measurement

Testes fixed in 10% neutral formalin solution for overnight were dehydrated through increasing concentrations of ethanol, and embedded in paraffin prior to sectioning. About 6- μm thick sections of testis were cut by Lieca RM2125 RT rotatory microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany) and processed for routine histology (hematoxylin-eosin staining). The stained and mounted sections were

then viewed under a microscope (Axioskop 2 Plus, Carl Zeiss AG, Oberkochen, Germany) and images were captured with a digital camera.

For seminiferous tubule diameter measurement histological sections of the testis (HE stained) were viewed under a microscope (Axioskop 2 Plus, Carl Zeiss AG, Oberkochen, Germany) and images were captured with a digital camera. Seminiferous tubule diameter was determined in 10 sections per mouse testis by using the image analyzer software Motic Images 2000 version 1.3.

2.6 | Sperm viability and sperm count

Sperm viability was determined by using a supravital staining technique.³⁹ For this, animal were sacrificed by decapitation. Body was dissected, the epididymis was excised and placed in a pre-warmed Petri dish containing 0.5 mL of normal saline (0.9% NaCl) maintained at 37°C . The tissue was minced with scalpel for approximately 1 min. A drop of sperm suspension and a drop of eosin-negrosin stain (1% eosin Y + 5% negrosin, 1:1) were placed on a clear glass slide and mixed thoroughly with the help of a fine glass rod. A portion of the mixture was transferred to a second slide and a thin film was prepared. The slide was then examined under the microscope (400 $\times$), and exactly one hundred spermatozoa (viable and dead) were counted from 10 different areas on the slide. Spermatozoa appearing pinkish (stained) were considered to be dead, while those appearing colorless (unstained) were counted as viable. Sperm viability was calculated in percent by using the following formula:

$$\text{Viability (\%)} = \frac{\text{Number of viable spermatozoa}}{\text{total number of spermatozoa (viable+dead)}} \times 100.$$

Sperm count was performed by using hemocytometer (Fein-Optik, Jena, Germany) with improved Neubauer ruling.³⁹ In brief, sperm suspension was diluted 20-fold with normal saline (0.9% NaCl) in a white blood cell pipette, the sperm suspension was drawn to the 0.5 mark halfway up the stem and saline subsequently to the 11 mark at the top of the bubble chamber. The preparation was mixed thoroughly and then one drop of it was added to both sides of the hemocytometer. The number of spermatozoa was counted in the four corner squares of the hemocytometer under a microscope (400 $\times$). If spermatozoa crossed the lines of the grid, only those at the top and right-hand sides

of the squares were counted. Spermatozoa on both sides of the hemocytometer were counted and the average numbers was recorded. Number of spermatozoa per cauda epididymis was expressed as follows:

$$\begin{aligned}\text{Sperm number} &= \text{Averaged no. of spermatozoa counted (N)} \\ &\times \text{multiplication factor (10,000)} \\ &\times \text{dilution factor (20)} = N \times 10,000 = N \\ &\times 0.2 \times 10^6 \text{ spermatozoa.}\end{aligned}$$

2.7 | Isolation of serum and enzyme linked immunosorbent assay (ELISA) of testosterone

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 was collected as serum and used for testosterone assay. For serum testosterone levels, an enzyme immunoassay was performed using a commercial RH-353-DS-EIA-Steroid-Testosterone kit (DSI, Saronno, Italy) following the manufacturer's protocol. The antiserum used in the assay was specific for testosterone. The cross-reactivity of the assay was 0.056% with progesterone, 0.004% with cortisol, 0.005% with estradiol, 4.8% with dihydrotestosterone, 3.6% with androstenedione, 0.048% with androsterone, 0.004% with cortisone, 0.002% with estriol, and 0.007% with estrone. The analytical sensitivity of the assay was 0.0576 ng/mL. The intra-assay coefficient of variation (CV) was 5.6% whereas the inter-assay CV was 7.1%. The accuracy for this assay was 99%.

2.8 | Tissue homogenization

Testes from each group were separately minced thoroughly. 10% (w/v) homogenate for control and three experimental groups were prepared by homogenizing the tissues in 50 mM phosphate buffer (pH 7.4) for 30 s using a Polytron homogenizer. A small portion of the fresh homogenates was first processed for measuring total reactive oxygen species (ROS) and nitric oxide (NO) level. The remaining homogenates after adding protease inhibitor 0.2 μ M PMSF were centrifuged at 12,000 rpm for 30 min at 4°C. The resultant supernatant was used for measuring malondialdehyde (MDA) level and ROS scavenging enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Total protein concentration was measured by Bradford's method using BSA as standard.

2.9 | Measurement of total ROS level

Total ROS level in fresh homogenate was assessed by using the protocol of Bejma et al.⁴⁰ with slight modification. For this, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a non-polar compound was used as a probe. DCFH-DA after conversion to a polar derivative by intracellular esterase, can rapidly react with ROS to form the highly fluorescent compound dichlorofluorescein. Briefly, homogenate was diluted in PBS to obtain a concentration of 25 μ g tissue protein/mL. The reaction mixture containing diluted homogenate and 10 μ L of DCFH-DA (10 μ M) 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 esterase. After 30 min of further incubation, the conversion of DCFH-DA to the fluorescent product DCF was measured by 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.

2.10 | Estimation of total nitrite and nitrate concentration

The total nitrite and nitrate concentration was measured by the indirect method of Sastry et al.,⁴¹ in testes by using acidic Griess reaction for color development after the reduction of nitrate with copper-cadmium alloy and deproteinization. In brief, the reaction mixture was prepared by adding 100 μ L of sample or standard (KNO_3), 400 μ L 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) in the tubes. The reaction mixture was then incubated at room temperature for 1 hr with thorough shaking. The reaction was stopped by the addition of 100 μ L of 0.35 M sodium hydroxide (NaOH), followed by 400 μ L 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. About 100 μ L aliquots of the clear supernatant were transferred into the wells of a microplate (in quadruplicate) and Griess reagent (50 μ L of 1.0% sulphanilamide prepared in 2.5% orthophosphoric acid and 50 μ L 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.

2.11 | Lipid peroxidation test

Level of lipid peroxides was measured by estimation of malondialdehyde (MDA), end product of lipid peroxidation by the method of Ohkawa et al.⁴² with a slight modification. MDA reacts with thiobarbituric acid (TBA) to yield a colored compound. Briefly, to 100 μ L of tissue homogenate, 50 μ L of 0.8% butylated hydroxytoluene (BHT), 750 μ L 0.8% of TBA, 750 μ L of 20% acetic acid (pH 3.5), and 100 μ L of 8% SDS were added. The reaction mixture was then incubated at 95°C in water bath for 1 hr. After this, the mixture was cooled down by keeping on ice for 15 min and then centrifuged at 2,000 rpm for 10 min. Absorbance was taken at 532 nm and the results was expressed as nmol MDA/mg protein.

2.12 | Antioxidant enzymes assay

2.12.1 | Superoxide dismutase

The total SOD activity in testes was determined by the method of Das et al.⁴³ The reaction mixture consisted of 100 μ L of sample, 150 mM phosphate buffer (pH 7.4), 20 mM L-methionine, 50 μ M EDTA, 1% Triton X-100, 100 μ M riboflavin, and Greiss reagent. The absorbance

was taken at 543 nm. The value of SOD was calculated in term 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 mg of protein.

2.12.2 | Catalase

Catalase activity was assayed according to Aebi.⁴⁴ Briefly, the reaction mixture contained 1.9 mL of 50 mM phosphate buffer (pH 7.0) and appropriate dilution of tissue supernatant to make a volume 2 mL. Reaction was initiated by addition of 1 mL of freshly prepared 30 mmol H₂O₂ and the decrease in absorbance was measured at 240 nm for 2–3 min. One unit of catalase represents the decrease of 1 μ M of H₂O₂ per minute. The molar extinction coefficient for H₂O₂ is 43.6/M/cm.

2.12.3 | Glutathione peroxidase

GPx activity was assayed as described by Mantha et al.⁴⁵ The reaction mixture (1 mL) contained 50 μ L sample, 398 μ L of 50 mM phosphate buffer (pH 7.0), 2 μ L of 1 mM EDTA, 10 μ L of 1 mM sodium azide, 500 μ L of 0.5 mM NADPH, 40 μ L of 0.2 mM GSH, and 1U glutathione reductase. The resultant mixture was allowed to equilibrate for 1 min at room temperature. After this, the reaction was initiated by addition of 100 mM H₂O₂. The absorbance measured kinetically at 340 nm for 3 min. The GPx activity was expressed as μ mol of oxidized NADPH oxidized to NADP⁺ per min per mg of protein using an extinction coefficient (6.22/mM/cm) for NADPH.

2.13 | Detection of cytochrome c through Western blot analysis

For cytochrome c detection, testicular extracts were prepared as follows. Testes from each group were decapsulated and gently homogenized with 10 strokes in a glass-glass homogenizer containing 10 volumes (w/v) of cold extraction buffer [250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 2 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 20 mM Hepes, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, and 0.57 mM phenylmethylsulfonyl fluoride (PMSF)]. After centrifugation at 14,000g for 15 min, the supernatant was regarded as the cytosolic fraction.

2.13.1 | SDS-PAGE and Western blot

Testicular protein samples (50 μ g) from each group were resolved on 15% SDS-PAGE at constant setting of 50 V for 30 min and then at 100 V for 1 hr, using the Mini-PROTEAN tetra system, BIO-RAD, The United States as mentioned elsewhere.^{46–48} Proteins were transferred from the polyacrylamide gels to PVDF membranes (Millipore, The United States) by electro-blotting for 15 min in a Semi-Dry blotting apparatus (TRANS-BLOT SD, Semi-dry transfer cell, BIO-RAD, The United States) at constant setting of 15 V. Membrane was then blocked by 5% fat free skimmed (dry) milk in Tris buffered saline with 0.1% Tween-20 (TBST) for 2 hr at room temperature. The membrane was then incubated with a rabbit raised polyclonal cytochrome c antibody (H-104) (sc-7159, Santa Cruz Biotechnology, The United States) (dilution 1:500) for 16 hr at 4°C. After washing with TBST, the membranes were incubated with anti-rabbit IgG-HRP conjugate secondary

antibody (dilution 1:2,500) for 1 hr at room temperature. After washing, the immunoreactive protein was visualized by using Super SignalTM West Pico Chemiluminescent Substrate, Thermo Scientific, The United States, Catalog number: 34080. NEX-GEN-Clear BLUE Pre-stained Protein Ladder (Cat. No. PG700-0500PI) were used as standard Pre-stained molecular weight markers. Experiment was repeated thrice with the same result and single representative blot was provided. Equal loading was confirmed with β -actin. The same membranes of primaries were stripped and re-probed with anti- β -actin antibody to normalize for protein loading.

The intensity of the bands was quantitated by gel densitometry using Image J software (Image J 1.48 bundled with 64-bit Java, NIH, The United States). Using the software's tool, integrated density of all the bands was measured. To get the integrated relative density value (IRDV), the integrated density value of each protein band was determined and normalized level of cytochrome c was calculated by dividing the IDV of a protein band by the IDV of the β -actin.

2.14 | Immunofluorescence of full length p53, Bax, Bcl-2, Bcl-x_L, pro-caspase-3, active/cleaved-caspase-3, and full length/uncleaved PARP-1

Immunofluorescence of full length p53 (rabbit polyclonal, sc-6243, F-393, 1:50), Bax (rabbit polyclonal, sc-6236, 1:50), Bcl-2 (mouse monoclonal, sc-7382, 1:50), Bcl-x_L (mouse monoclonal, sc-8392, 1:50), pro-caspase-3 (mouse monoclonal, sc-7272, 1:50), cleaved caspase-3 (rabbit monoclonal, MAB835, R&D Systems, Inc., Minneapolis, MN, 1:100), and uncleaved/full length PARP-1 (mouse monoclonal, sc-8007, 1:50) was carried out on neutral formalin-fixed paraffin-embedded testes sections using single and/or double immuno-labeling/staining method. Immunofluorescence was performed in a two-step procedure as described by Banerjee et al.⁴⁹ Briefly, in the first step, after initial deparaffinization in xylene and rehydration in graded series of alcohol of testis section slides, antigen retrieval was performed using 1 M citrate buffer (pH 6) in a microwave oven at 1,000 W (3–4 min). Testes sections were then treated with blocking solution for 2 hr at room temperature [5% heat inactivated goat serum (HIGS)], and then sections were incubated with aforementioned mouse monoclonal and rabbit monoclonal/polyclonal antibodies for 24 hr in humid chamber. In second step, sections were washed with TBS (3 $\times$ 5 min) and then incubated with donkey-anti-rabbit IgG H and L (conjugated with FITC, Santa Cruz Biotechnology, Santa Cruz), goat anti-rabbit IgG H and L (conjugated with TRITC abcam, ab6718), and goat-anti-mouse IgG (H and L) (conjugated with TRITC (ab6786) or FITC (abcam, ab6785) [Abcam, Cambridge, MA] for 3 hr at RT in dark (dilution 1:200). After incubation, sections were again washed with TBST (3 $\times$ 5 min) and two drops of the fluorescent media (0.5% N-propyl gallate + 20 mM Tris in 90% glycerol + DAPI) was applied on the sections. Coverslips were applied and then sealed with nail polish after ensuring the spread of the mounting media over all the sections without any bubble formation). The sections were viewed under a fluorescent microscope (Axioskop 2 Plus Carl Zeiss, Oberkochen, Germany) and immunofluorescence were captured with HRcam camera (Axioskop 2 Plus Carl Zeiss).

TABLE 1 Mean ($\pm$ SEM) integrated optical density value for each antigen considered as arbitrary unit (a. u.) thresholds

Antibodies	Control	15 days exposed	30 days exposed	60 days exposed
p53	6.35 $\pm$ 0.461 (weak)	18.11 $\pm$ 0.759 (moderate)	27.06 $\pm$ 2.131 (intense)	31.66 $\pm$ 1.79 (intense)
Bcl-x _L	70.71 $\pm$ 2.71 (intense)	40.55 $\pm$ 2.09 (moderate)	24.65 $\pm$ 1.03 (weak)	22.51 $\pm$ 1.97 (weak)
Bax	41.50 $\pm$ 4.02 (weak)	102.34 $\pm$ 4.53 (moderate)	384.03 $\pm$ 19.58 (intense)	705.80 $\pm$ 28.09 (intense)
Bcl-2	479.73 $\pm$ 25.24 (intense)	100.26 $\pm$ 6.29 (moderate)	57.09 $\pm$ 7.01 (weak)	27.56 $\pm$ 1.96 (weak)
Pro-caspase-3	33.88 $\pm$ 0.589 (intense)	5.45 $\pm$ 0.555 (weak)	3.67 $\pm$ 0.413 (weak)	2.23 $\pm$ 0.240 (weak)
Active caspase-3	24.98 $\pm$ 1.30 (weak)	40.44 $\pm$ 2.27 (moderate)	45.49 $\pm$ 1.93 (intense)	59.34 $\pm$ 2.63 (intense)
PARP-1	29.81 $\pm$ 1.41 (intense)	22.22 $\pm$ 0.765 (moderate)	12.13 $\pm$ 1.06 (weak)	3.60 $\pm$ 0.294 (weak)

2.14.1 Image analysis

Image analysis was performed as described elsewhere.⁴⁹ To measure the integrated optical density (IOD), immunofluorescence of testes of all the groups were captured with HRcam camera at the same magnification and stored as TIF files. Each file was imported by Image J software (Image J 1.48 bundled with 64-bit Java, NIH, Bethesda). Using the software's tool, IOD of all the areas showing positive signals in the different regions of the testes sections were measured. The IOD values (relative units) in testes were averaged (four sections per testis and five testes per group) to determine signal density. Average IOD values of testes sections from each group were considered as arbitrary unit thresholds for each antigen and were stated in Table 1 as mean $\pm$ SEM and on the basis of the difference in the level of significance (P value $< .05$), the testes sections IOD were labeled or classified as intense, moderate, or weak.

3 | STATISTICAL ANALYSIS

All the data were presented as means $\pm$ SEM. The normality distribution for each type of dataset was assessed and verified by using the Shapiro-Wilk test. For statistical analysis, one way ANOVA followed by Dunnett T3 and Tukey's (for IF plates) post hoc analysis was used to determine the significance among the four different groups. A P -value of $< .05$ was considered as significant. Data were analyzed using statistical software package Statistical Analysis System (SPSS Statistics 17.0, IBM, Armonk, NY).

4 | RESULTS

4.1 | Exposure to 2.45 GHz MW radiation leads to altered testicular histoarchitecture, decreased seminiferous tubule diameter, sperm count, sperm viability, and serum testosterone level

We observed a marked alterations in the histoarchitecture of the testes of all 2.45 GHz microwave irradiated group of mice as compared with control group testes in which all the seminiferous tubules showed

normal histological features. As compared with control, the diameter of seminiferous tubule was found to be significantly reduced in the testes of exposed group of mice. Further, non-uniform degenerative changes were also observed in the seminiferous tubules along with distorted Leydig cells arrangement in the testes of MW exposed mice. Affected seminiferous tubules in the testes of MW irradiated mice shows damaged seminiferous epithelium and depletion of germ cells. The spermatogonial cells of exposed mice have a degenerated appearance in the epithelium along with the formation of gaps between the peripheral layers of spermatogonial cells in some seminiferous tubules (Figure 2A). The diameter of seminiferous tubule was found to be reduced significantly in all the MW exposed mice compared with control. The decrease was highly significant in 60 days exposed mice (Figure 2B).

Duration dependent decrease in the number of spermatozoa and sperm viability was observed in all the MW irradiated group of mice. As the duration of exposure increases the numbers of dead sperms increased significantly in exposed group of mice when checked by eosin-negrosin staining. 30 and 60 days exposed group mice show highly significant decrease in sperm count (Figure 2C) and sperm viability (Figure 2D) than 15 days exposed group as well as control.

The serum testosterone level also decreased significantly in all the MW irradiated groups of mice as compared with the control group in duration dependent manner (Figure 2E).

4.2 | Exposure to 2.45 GHz MW radiation leads to increased testicular total ROS, NO, and MDA level and decreased antioxidant enzymes activities

Duration dependent increment in total ROS, NO, and MDA level was observed in the testes of 2.45 GHz MW exposed group of mice compared with control (Figure 3). Total ROS (Figure 3A), NO (Figure 3B), and MDA (Figure 3C) was significantly high in 60 days exposed group of animals compared with 15 and 30 days exposed groups of animals as well as control.

The activity of ROS scavenging enzymes SOD (Figure 3D), CAT (Figure 3E), and GPx (Figure 3F) showed duration dependent decrease

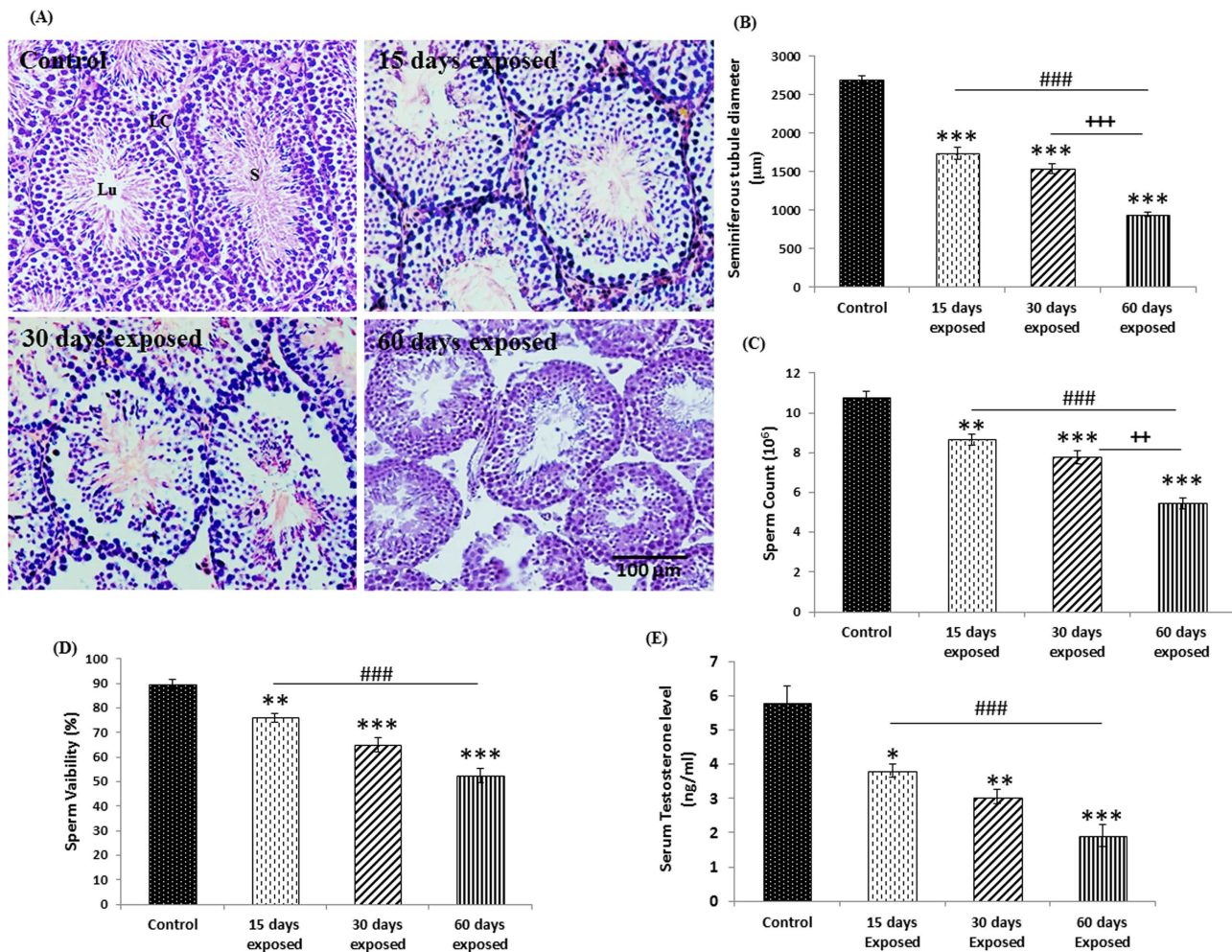


FIGURE 2 (A) Transverse section of testis (H&E staining), (B) seminiferous tubule diameter, (C) sperm count, (D) sperm viability, and (E) serum testosterone level of 2.45 GHz MW irradiated mice. Compared with control, 2.45 GHz MW exposed mice showed significant decrease in the seminiferous tubule diameter and degenerated Leydig cells in the testis. The 30 and 60 days exposed mice testes exhibit degenerated appearance with the formation of gaps between the spermatogonial cells in the seminiferous tubules and decreased number of sperm in the lumen of seminiferous tubule of all the 2.45 GHz MW irradiated mice groups. The viability of sperms as well as serum testosterone level decreased significantly in duration dependent manner in all the exposed group of mice compared with control. The degree of damage is more in 30 and 60 days exposed mice compare to control and 15 days exposed mice. (LC: Leydig cells, Lu: lumen, S: spermatozoa). Data is presented as mean $\pm$ SEM. ** P < .01, *** P < .001 significance of difference from control, ### P < .001 significance of difference from 15 days exposed group, ++ P < .01, +++ P < .001 significance of difference from 30 days exposed group [Color figure can be viewed at wileyonlinelibrary.com]

in the testes of CW 2.45 GHz MW exposed group of mice compared with control. The degree of decrease was more as the duration of exposure has increased.

4.3 | Exposure to 2.45 GHz MW radiation leads to altered expression of p53, Bax, Bcl-x_L, Bcl-2, pro-caspase-3, active-caspase-3, and PARP-1

The expression of p53, Bax, active-caspase-3 in the testes was found to be significantly up-regulated while the expression of Bcl-x_L, Bcl-2, pro-caspase-3, and PARP-1 were significantly down-regulated after 2.45 GHz MW irradiation in duration dependent manner (Figures 4–7). Control group testes showed weak immunoreactivity for p53 in Leydig cells and spermatozoa. 15 days exposed group testes revealed moderate while 30 days exposed group showed strong and 60 days exposed

group testes exhibited intense immunoreactivity for p53 in Leydig cell, spermatogonial cells, and spermatozoa (Figure 4A and B). Intense immunoreactivity for Bcl-x_L was observed in Leydig cells, spermatogonial cells, and spermatozoa of control group mice testes. About 15 days exposed mice testes showed moderate immunoreactivity for Bcl-x_L while 30 and 60 days exposed group mice testes revealed weak immunoreactivity in Leydig and spermatogonial cells only (Figure 4A and C).

In control group mice testes, weak expression of Bax was observed in Leydig cells while strong expression of Bcl-2 was observed in Leydig cells, spermatogonial cells, and spermatozoa. In 15 days exposed mice testes moderate expression of both Bax and Bcl-2 was observed in Leydig cells only while no immunoreactivity was observed for these antigens in spermatozoa and spermatogonial cells of seminiferous tubules. About 30 days exposed mice testes showed strong immunoreactivity for Bax in Leydig cells, first tier of spermatogonial cells closely

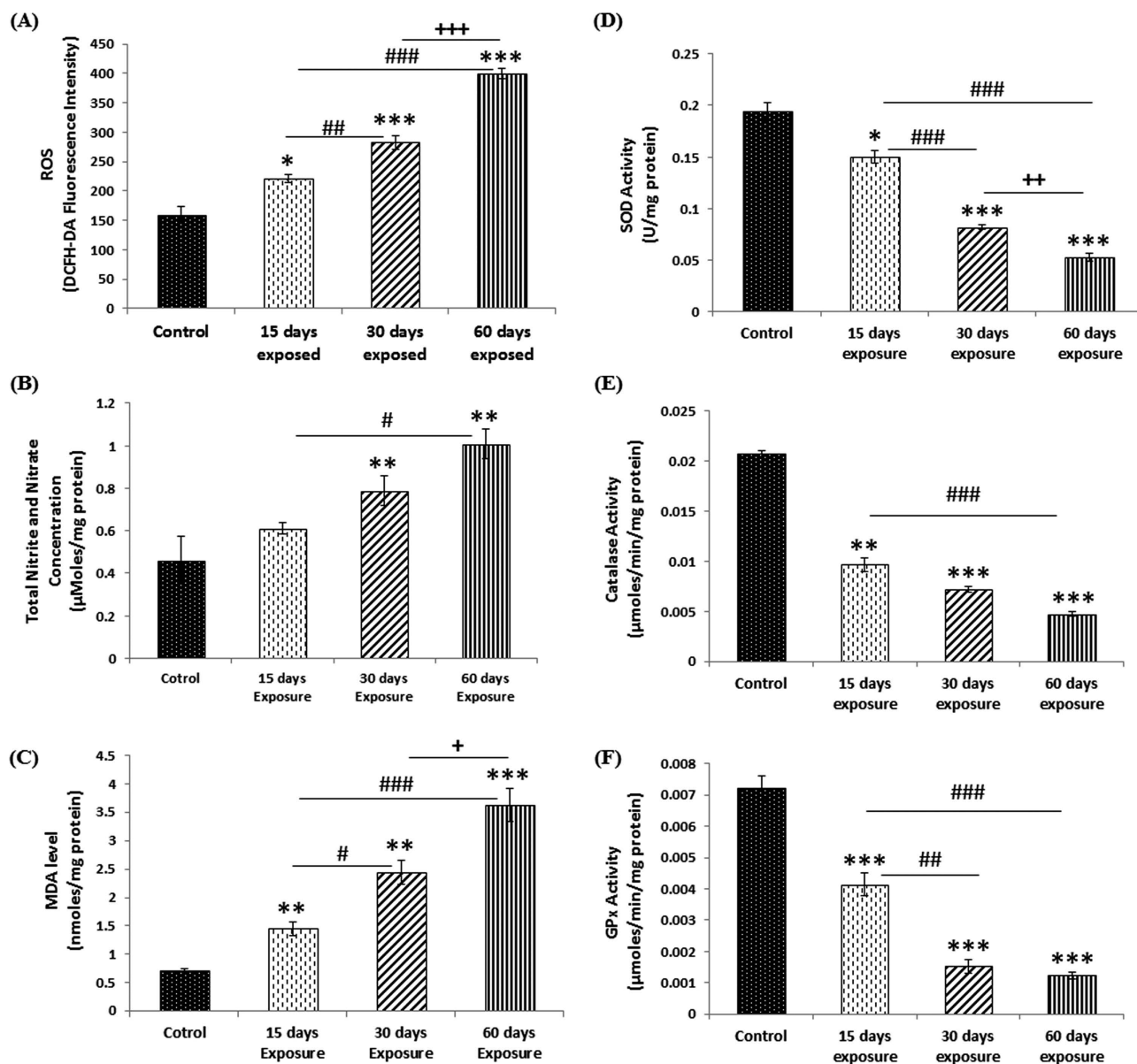


FIGURE 3 Effect of 2.45 GHz MW radiation on (A) ROS, (B) total nitrite and nitrate concentration, (C) MDA level, (D) SOD, (E) CAT, and (F) GPx activity in the testes of mice. Values are expressed as mean $\pm$ SEM. * $P < .05$, ** $P < .01$, *** $P < .001$ significance of difference from control, # $P < .05$, ## $P < .01$, ### $P < .001$ significance of difference from 15 days exposed group, + $P < .05$, ++ $P < .01$, +++ $P < .001$ significance of difference from 30 days exposed group

associated/opposed to tubular epithelium and spermatozoa while the weak expression of Bcl-2 was observed only in Leydig cells. About 60 days exposed group mice testes exhibited intense immunoreactivity for Bax in Leydig cells, spermatogonial cells and spermatozoa while very weak expression of Bcl-2 was observed in Leydig cells compared with their respective controls (Figure 5A–C).

In control group mice testes intense expression of pro-caspase-3 was observed in Leydig cells and spermatozoa while weak expression of active-caspase-3 was observed in Leydig cells and seminiferous tubules (spermatogonial cells and spermatozoa). About 15 days exposed mice testes showed moderate immunoreactivity for pro-caspase-3 in Leydig cells only while moderate immunoreactivity for active-caspase-3 was noted in Leydig cells and seminiferous tubules

(spermatogonial cells and spermatozoa) compared with their respective controls. Contrarily, 30 and 60 days exposed mice testes revealed weak immunoreactivity for pro-caspase-3 in some/few Leydig cells only while strong and intense immunoreactivity for active-caspase-3 in Leydig cells as well as in seminiferous tubules (spermatogonial cells and spermatozoa) compared with their respective controls (Figure 6A–C).

Intense immunoreactivity for uncleaved-PARP-1 was observed in Leydig cells and seminiferous tubules (spermatogonial cells and spermatozoa) of control group mice testes. About 15 days exposed mice testes revealed moderate and 30 and 60 days mice testes revealed weak immunoreactivity for uncleaved-PARP-1 compared with control (Figure 7A and B).

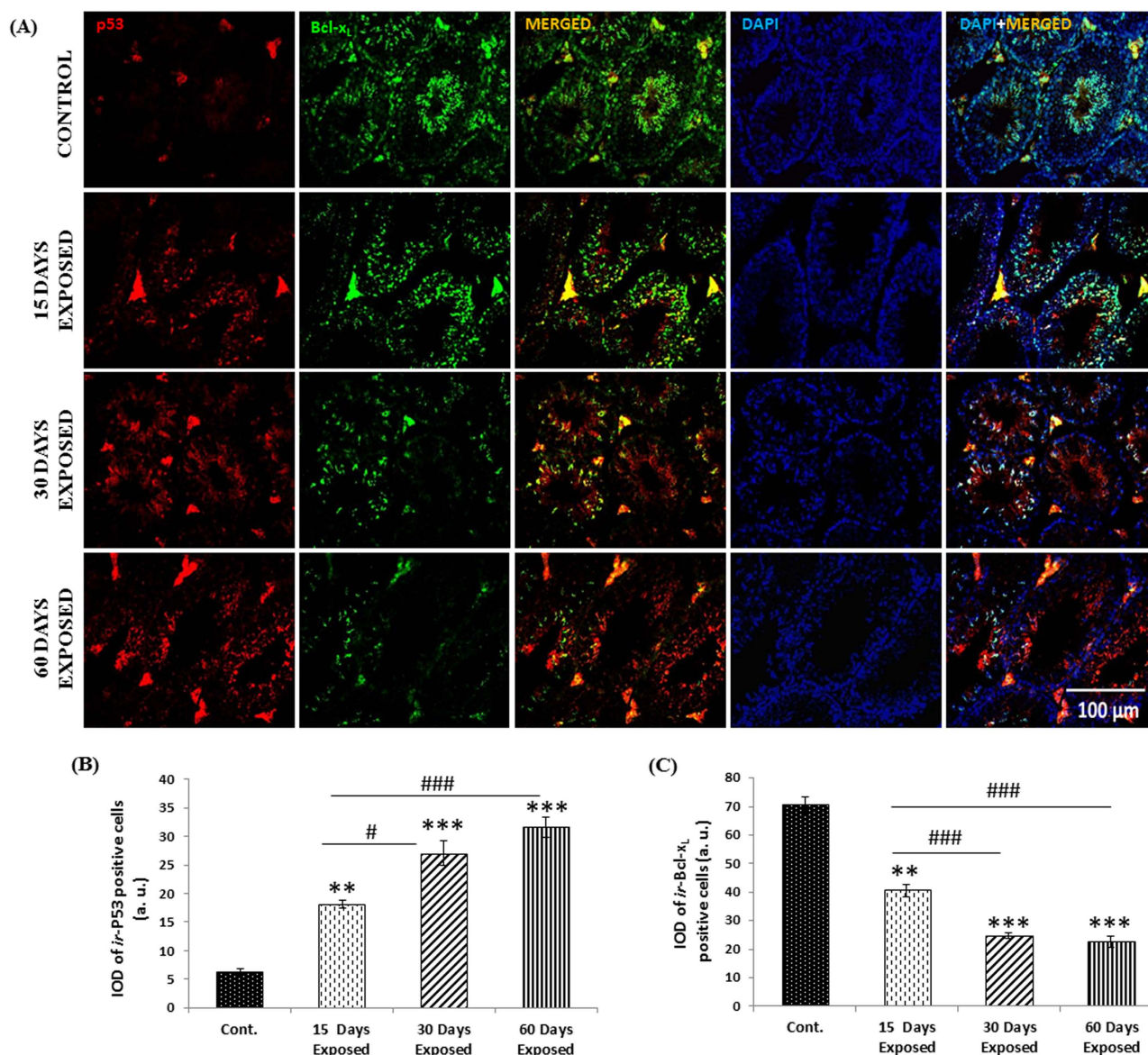


FIGURE 4 Immunofluorescence showing the co-expression of p53 and Bcl-x_L (A) and the integrated optical density (IOD) (B and C) of p53 and Bcl-x_L in the testes of 2.45 GHz MW exposed mice: Control group testis shows weak immunoreactivity for p53 in Leydig cells and spermatozoa which revealed moderate intensity in 15 days exposed group while 30 and 60 days exposed group testes exhibit intense immunoreactivity for p53 in Leydig cells, spermatogonial cells, and spermatozoa as well. Contrarily, intense immunoreactivity for Bcl-x_L is observed in Leydig cells, spermatogonial cells and spermatozoa of control group mice testes. The 15 days exposed mice testes shows moderate immunoreactivity in Leydig and spermatogonial cells as well as in spermatozoa which decreased remarkably and gradually in 30 days and 60 days exposed group mice testes. Values are expressed as mean $\pm$ SEM. ** $P < .01$, *** $P < .001$ significance of difference from control, # $P < .05$, ### $P < .001$ significance of difference from 15 days exposed group [Color figure can be viewed at wileyonlinelibrary.com]

4.4 | Cytoplasmic cytochrome c level

The expression of cytochrome c was found to be increased significantly in duration dependent manner in the testes of all 2.45 GHz MW exposed mice as compared with control (Figure 8).

5 | DISCUSSION

In the present study we have demonstrated that 2.45 GHz MW radiation altered redox status induced testicular apoptosis via p53

dependent Bax-caspase-3 mediated pathway. Exposure of 2.45 GHz MW radiation for 2 hr/day for 15, 30, and 60 days showed duration-dependent increment in free radical load which subsequently induce the oxidative and nitrosative stress at the testicular level and weaken the antioxidant defense system (reduction in the activity of SOD, Catalase, and GPx). The increased free radical load as a consequence of increased ROS and NO generation may lead to severe testicular oxidative damage by causing peroxidation of lipids and forming carbonyls.²⁸ 2.45 GHz MW irradiation induced oxidative and nitrosative stress may leads to histopathological changes in testes (reduced seminiferous

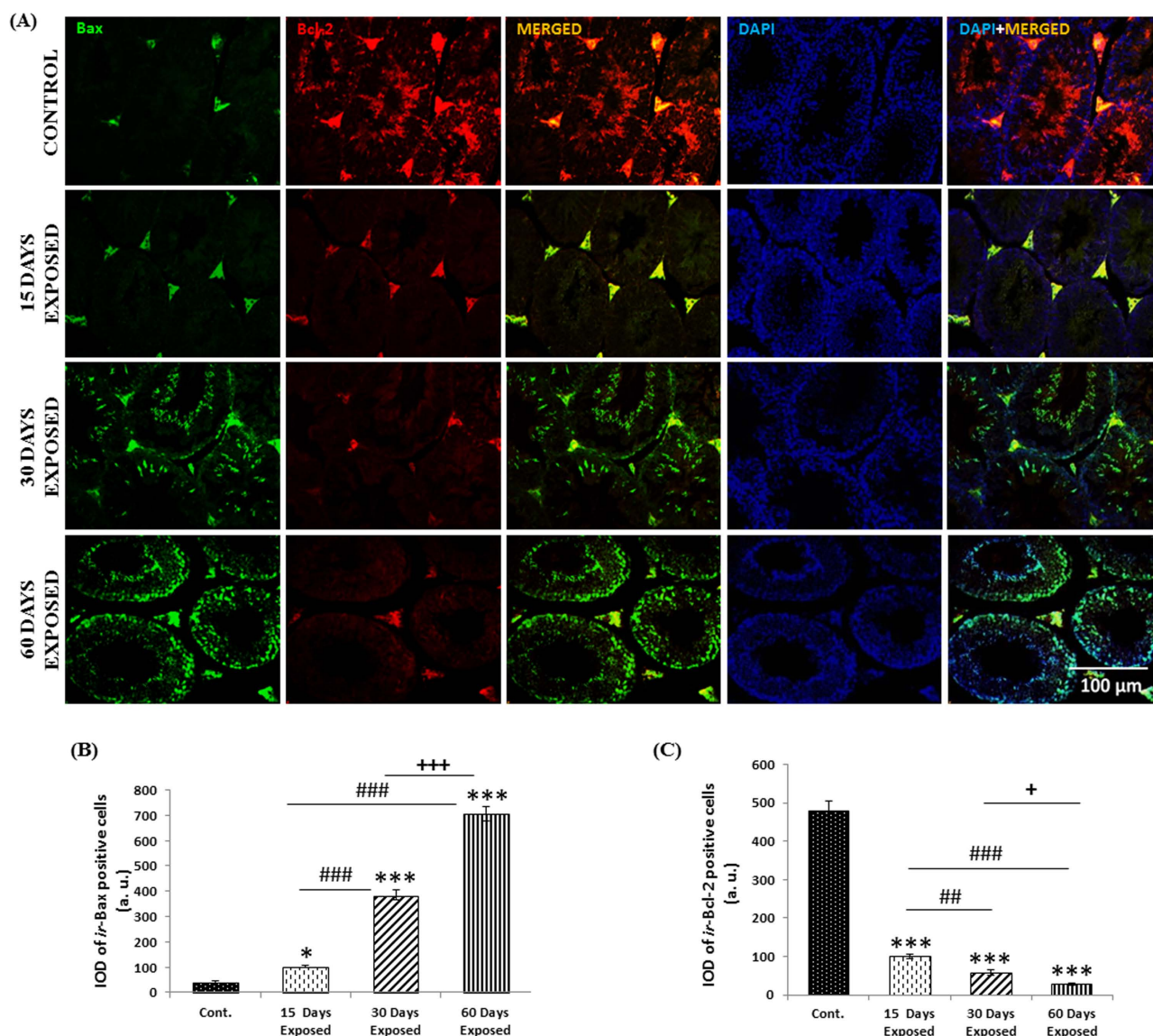


FIGURE 5 Immunofluorescence showing the co-expression of Bax and Bcl-2 (A) and the integrated optical density (IOD) (B and C) of Bax and Bcl-2 in the testes of 2.45 GHz MW exposed mice: In control group mice testis, weak expression of Bax is observed in Leydig cells while strong expression of Bcl-2 is evident in Leydig cells, spermatogonial cells and spermatozoa. In 15 days exposed group, moderate expression of both Bax and Bcl-2 is observed in Leydig cells only compared with their respective controls. 30 days exposed mice testis shows strong immunoreactivity for Bax in Leydig cells, first tier of spermatogonial cells closely associated/opposed to tubular epithelium and in spermatozoa while the weak expression of Bcl-2 is observed only in Leydig cells. 60 days exposed group mice testis shows intense immunoreactivity for Bax in Leydig cells, spermatogonial cells, and spermatozoa while very weak expression of Bcl-2 is evident in Leydig cells only. Values are expressed as mean $\pm$ SEM. * $P < .05$, *** $P < .001$ significance of difference from control, ## $P < .01$, ### $P < .001$ significance of difference from 15 days exposed group, + $P < .05$, +++ $P < .001$ significance of difference from 30 days exposed group [Color figure can be viewed at wileyonlinelibrary.com]

tubules diameter, sperm count, and sperm viability, degeneration of seminiferous epithelium, and in some tubules peripheral gaps formation by the detachment of spermatogonial layer from the tunica propria). These observed histopathological changes were similar to those in our previous report.¹² All these changes in the testicular morphology revealed the disruption/impairment of spermatogenesis. Germ cell degeneration and reduction in sperm counts and viability following 2.45 GHz MW might have occurred as a consequence of reduced testosterone concentrations, as optimum level of testosterone is essential

for the maintenance of normal spermatogenesis, sperm counts, and viability.^{50,51}

Moreover, the increased peroxidation of lipids in 2.45 GHz MW irradiated mice may also cause significant decrease in sperm count and sperm viability. The elevated ROS and NO along with these LPO derivatives may severely affect the spermatogenesis as well as steroidogenesis. It was reported that ROS are also generated during normal testicular function which plays an important role in regulating the function of the testis.^{29,30} Free radicals generated after MW radiation

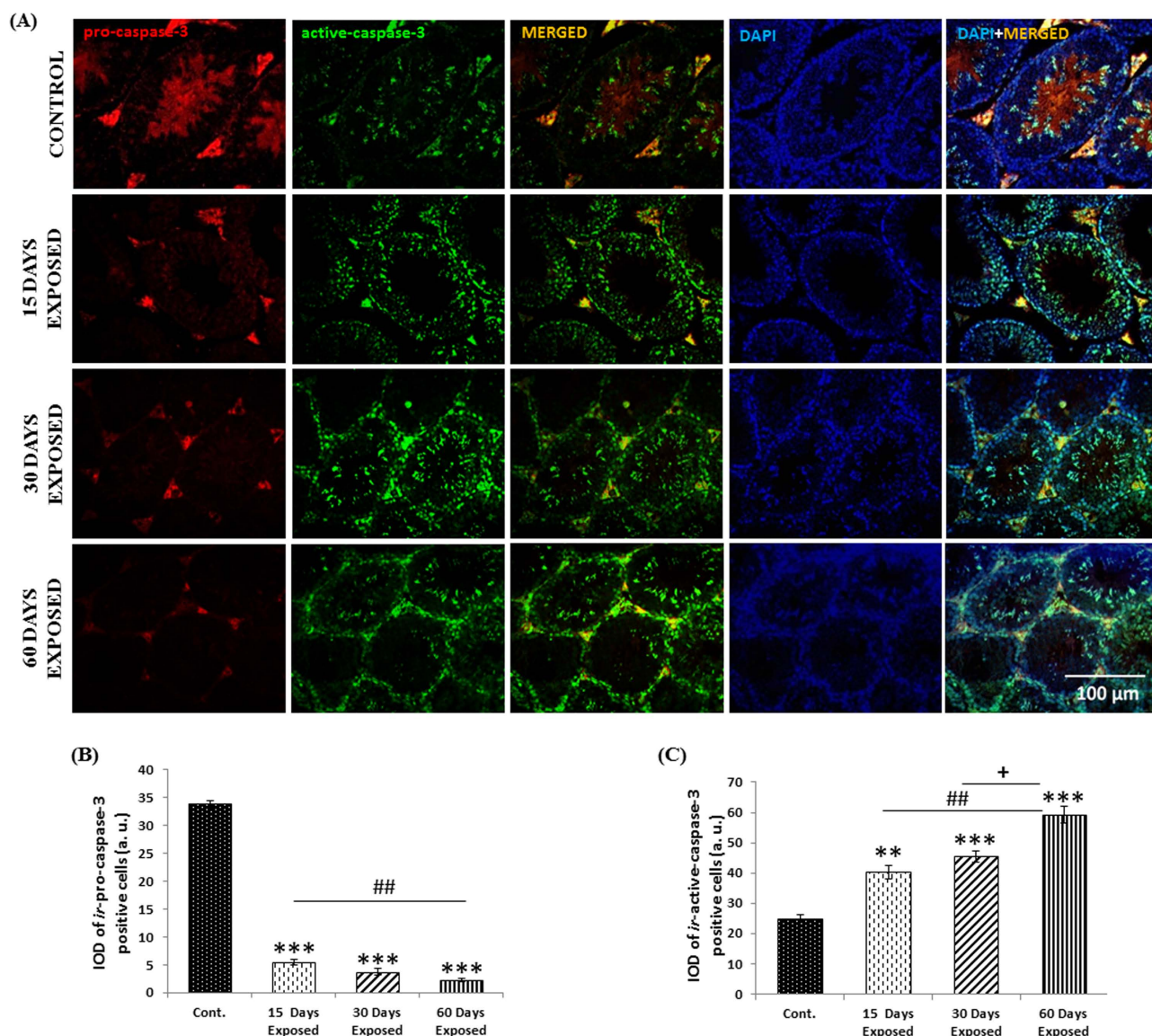


FIGURE 6 Immunofluorescence showing the co-expression of pro-caspase-3 and active-caspase-3 (A) and the integrated optical density (IOD) (B and C) of pro-caspase-3 and active-caspase-3 in the testes of 2.45 GHz MW exposed mice: In control mice testis, expression of pro-caspase-3 is observed in Leydig cells and spermatozoa while *ir*-active-caspase-3 is evident in Leydig cells and seminiferous tubules (spermatogonial cells and spermatozoa). 15 days exposed mice testis shows decreased immunoreactivity for pro-caspase-3 in Leydig cells only while moderately increased immunoreactivity for active-caspase-3 is seen in Leydig cells and seminiferous tubules (spermatogonial cells and spermatozoa) compared with their respective controls. The 30 and 60 days exposed mice testes revealed further weak immunoreactivity for pro-caspase-3 in few Leydig cells only while strong and intense immunoreactivity for active-caspase-3 is evident in different cells (Leydig cells, spermatogonial cells, and spermatozoa) compared with their respective controls. Values are expressed as mean $\pm$ SEM. $^{**}P < .01$, $^{***}P < .001$ significance of difference from control, $^{##}P < .01$ significance of difference from 15 days exposed group, $^{+}P < .05$ significance of difference from 30 days exposed group [Color figure can be viewed at wileyonlinelibrary.com]

exposure may react with the lipid laden steroidogenic Leydig cells and polyunsaturated fatty acid (PUFA) rich spermatozoa and result in peroxidation of the lipid moieties, which finally may lead to destruction in Leydig cells and spermatozoa. Spermatozoa itself also generate low amount of endogenous ROS which participate in the signal transduction mechanism that promotes sperm capacitation/acrosome reaction and acquisition of sperm-fertilizing ability.^{52,53} Thus, although physiological levels of ROS are important for both spermatogenesis and steroidogenesis, an excessive production of ROS resulting from 2.45 GHz

MW radiation appears to have deleterious effects on testicular function.¹²

The increased oxidative and nitrosative stress not only alters the redox status of testes but also may activate the p53 for the trans-regulation of cell survival/death determining proteins.⁵⁴ The expression level of p53 and Bax in the testes of all the 2.45 GHz MW-exposed groups of mice were correlated and increased significantly in a duration dependent manner along with the duration of the exposure. The significantly increased expression of cytosolic cytochrome c and active

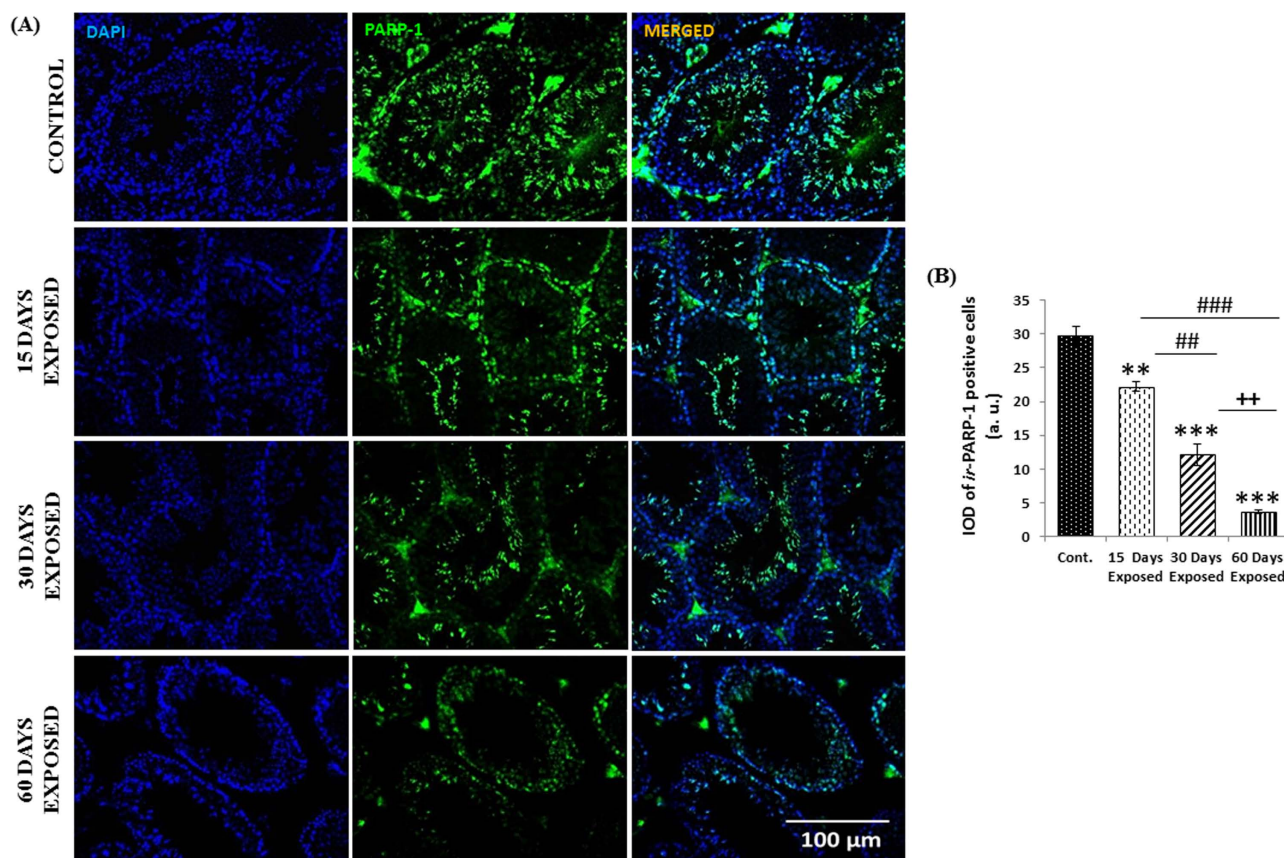


FIGURE 7 Immunofluorescence (A) and the integrated optical density (IOD) (B) of full-length uncleaved PARP-1 in the testes of 2.45 GHz MW exposed mice: Note that control group shows strong immunoreactivity for uncleaved/full-length PARP-1 in Leydig cells, spermatogonial cells as well as inner germ cell stages of the seminiferous tubules. The 15 days exposed mice testis shows moderate while 30 and 60 days exposed mice testis shows weak immunoreactivity compared with control. Values are expressed as mean $\pm$ SEM. ** $P < .01$, *** $P < .001$ significance of difference from control, ## $P < .01$, ### $P < .001$ significance of difference from 15 days exposed group, ++ $P < .001$ significance of difference from 30 days exposed group [Color figure can be viewed at wileyonlinelibrary.com]

caspase-3 was also observed in the testes of all the 2.45 GHz MW exposed groups of mice. The expression of Bcl-2, Bcl- χ_L , and pro-caspase-3 reduced significantly along with the exposure duration in a time dependent manner. Increased oxidative and nitrosative stress related free radical generation may lead to DNA strand breaks which may upregulate the p53 expression in all the 2.45 GHz MW-exposed groups of mice testis. The increased ROS may also induce apoptosis by regulating the phosphorylation and ubiquitination of Bcl-2 family proteins, resulting in increased pro-apoptotic protein levels and decreased anti-apoptotic protein expression. ROS suppresses the Bcl-2 expression, but on the other hand increases the Bax.⁵⁵

P53 controls various cell-cycle checkpoints by activating or inhibiting genes involved in cell cycle, apoptosis, and DNA repair.⁵⁶ Bax may also play an important role in cell cycle regulation and passage through cell cycle check point corridors.⁵⁷ To mediate apoptosis, p53 can either cause the trans-activation of Bax or trans-suppression of Bcl-2. The activated p53 stimulates the expression of pro-apoptotic Bax, which in turn binds with and inactivate Bcl-2 and thus increases Bax/Bcl-2 ratio.⁵⁸ P-53 can also trigger apoptosis by transcriptional repression of anti-apoptotic factors Bcl- χ_L and Bcl-2.⁵⁹ Study suggest that the action of p53, being a transcription factor, is mediated by upregulating the

transcription of pro-apoptotic proteins, and repressing the transcription of cell survival proteins.⁶⁰ It is also reported that at constitutively high levels of Bax, p53 may mediate apoptosis through a transcriptionally independent pathway. P-53 can free Bax for apoptotic induction by antagonizing the Bcl-2 proteins.⁵⁸ As a result of stimulated Bax expression, the skewed Bax/Bcl2 promotes apoptosis.⁵⁵ Activated Bax mediates the mitochondrial inner membrane leakage and helps in the release of apoptogenic proteins, such as cytochrome c.⁶¹

The significantly increased level of cytosolic cytochrome c in the testes of all MW irradiated mice may lead to the formation of apoptosomes and activation of caspase cascade.^{62,63} MW radiation induced elevated ROS may also cause the oxidation of the mitochondrial pore, disrupt the mitochondrial membrane potential ($\Delta\psi_m$) and release cytochrome c. Cytochrome c released from the mitochondria in the testicular cells of MW irradiated mice initiates the activation of a chain of caspases, thereby initiating other apoptotic changes including activation of caspase-3.⁶²⁻⁶⁵ The increased Bax/Bcl-2 ratio and cytochrome c level in 2.45 GHz exposed mice testes leads to proteolytic cleavage of pro-caspase-3 (initiator caspase) into active caspase-3 (effector caspase). The active caspase-3 may cleave its substrate, that is, nuclear protein/DNA repair enzyme poly (ADP) ribose polymerase-1 (PARP-1).

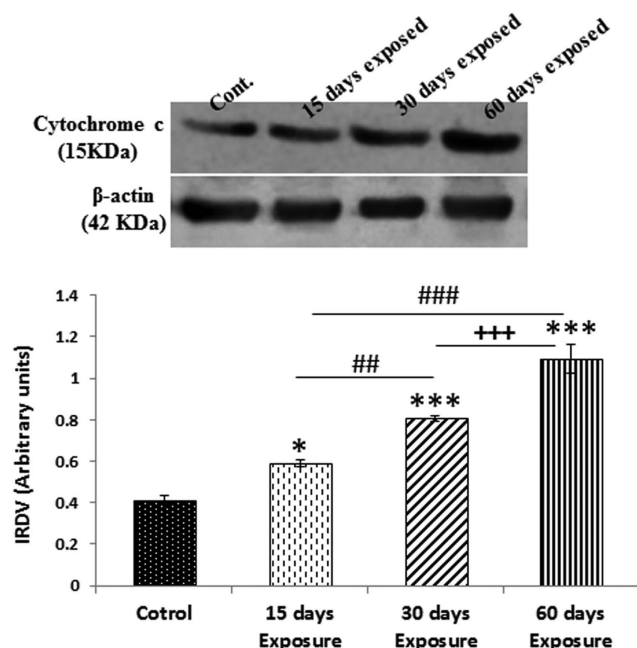


FIGURE 8 Western blot analysis of cytochrome-c protein in the testes of mice exposed to 2.45 GHz MW radiation for 15, 30, and 60 days. β -actin expression was used as loading control. IRDV, integrated relative density value. Values are expressed as mean $\pm$ SEM. * P < .05, *** P < .001 significance of difference from control, ## P < .01, ### P < .001 significance of difference from 15 days exposed group, +++ P < .001 significance of difference from 30 days exposed group

During apoptosis, the repairing of the damaged DNA would be a futile attempt due to the protease mediated cleavage of PARP-1 into fragments.⁶⁶ Therefore, the significantly increased expression of active-caspase-3 and decreased level of pro-caspase-3 and uncleaved PARP-1 in all MW irradiated group mice testes clearly suggest the increased degree of apoptotic induction in a dose dependent manner with the duration of exposure.

Further, one important observation of our study was that the alterations in testicular expression pattern of pro-apoptotic, apoptotic, and anti-apoptotic proteins found to be initiated first in sperms and gradually also seen in the spermatogonial cells and Leydig cells of all the exposed groups of mice. The degree of depletion of anti-apoptotic proteins and accumulation of pro-apoptotic and apoptotic proteins was also found to begin in sperms and the same pattern was eventually observed in the spermatogonial cells and Leydig cells of the MW irradiated mice testes. Therefore, it is suggested that spermatozoa are more susceptible to MW radiation.

Overall, our findings suggest that MW irradiation induced oxidative and nitrosative stress mediated generation of free radical load results in testicular apoptosis. ROS stimulate the expression of Bax or mediates the transactivation the Bax. ROS can also induce p53 mediated Bax activation through creating DNA breaks. Increased expression of Bax coupled with reduced expression of Bcl-2 and Bcl-x_L may cause the permeabilization of the mitochondrial outer membrane and consequently release the cytochrome-c (apoptotic protein) from the mitochondria. This subsequently serves in the activation of caspase-3 which

cleaves the nuclear DNA repair enzyme PARP-1 and ultimately leads to testicular somatic as well as germ cell apoptosis which may impedes the germ cells or sperms production and may results in reduction in sperm count which may lead to infertility like condition.

6 | CONCLUSION

In summary, we conclude that 2.45 GHz MW radiation induced formation and excessive accumulation of free radicals in the testicular tissue provoked the testicular apoptotic pathway. The significantly increased expression of pro-apoptotic (p53 and Bax) and apoptotic protein (cytochrome-c) and active executioner caspase (active caspase-3), along with decreased expression of anti-apoptotic proteins (Bcl-2 and Bcl-x_L), inactive executioner caspase-3 (pro-caspase-3), and DNA repairing enzyme (uncleaved PARP-1) were observed in all the exposed groups of mice testes. The degree of severity was observed in the mice groups exposed to 2.45 GHz MW radiation for 30 and 60 days. Based on these findings it is concluded that, 2.45 GHz MW radiation induced apoptosis may also leads to infertility like condition. We also suggest that as the duration of 2.45 GHz MW radiation increases, the severity of the testicular damage also increases.

Overall, findings from this study have highlighted the apoptotic pathway involved in testicular somatic and/or germ cell death in MW irradiated mice. However, further studies are required to elucidate the (i) interdependent cross-talks in between different testicular apoptotic pathways and their degree of concurrence in MW irradiated testes and (ii) upstream signaling cascades/pathways involved in the initiation of apoptosis. Hence, in future studies, p-53 null and other upstream knockout mice models as well as the anti-oxidants/free radical scavengers as therapeutic agents can be used to monitor the suppression of the induction of apoptosis, that is, amelioration of the adverse impact of MW radiation on reproductive health.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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