

1800 MHz mobile phone irradiation induced oxidative and nitrosative stress leads to p53 dependent Bax mediated testicular apoptosis in mice, *Mus musculus*

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Funding information

Indian Council of Medical Research, New Delhi, Grant numbers: 45/2/2012-PHY/BMS, 5/10/FR/13/2010-RHN

Present study was carried out to investigate the effect of long-term mobile phone radiation exposure in different operative modes (Dialing, Receiving, and Stand-by) on immature male mice. Three-week old male mice were exposed to mobile phone (1800 MHz) radiation for 3 hr/day for 120 days in different operative modes. To check the changes/alteration in testicular histoarchitecture and serum testosterone level, HE staining and ELISA was performed respectively. Further, we have checked the redox status (ROS, NO, MDA level, and antioxidant enzymes: SOD, CAT, and GPx) by biochemical estimation, alteration in the expression of pro-apoptotic proteins (p53 and Bax), active executioner caspase-3, full length/uncleaved PARP-1 (DNA repair enzyme), anti-apoptotic proteins (Bcl-2 and Bcl-x_L) in testes by immunofluorescence and cytosolic cytochrome-c by Western blot. Decreased seminiferous tubule diameter, sperm count, and viability along with increased germ cells apoptosis and decreased serum testosterone level, was observed in the testes of all the mobile phone exposed mice compared with control. We also observed that, mobile phone radiation exposure in all the three different operative modes alters the testicular redox status via increasing ROS, NO, and MDA level, and decreasing antioxidant enzymes levels leading to enhanced apoptosis of testicular cells by increasing the expression of pro-apoptotic and apoptotic proteins along with decreasing the expression of anti-apoptotic protein. On the basis of results, it is conclude that long-term mobile phone radiation exposure induced oxidative stress leads to apoptosis of testicular cells and thus impairs testicular function.

KEYWORDS

apoptosis, mobile phone radiation, nitrosative stress, oxidative stress, testis

1 | INTRODUCTION

Mobile phones are one of the most widely used wireless communication device. It uses electromagnetic radiation in the microwave range (in India CDMA and GSM, 900 MHz/1800 MHz; 3G, 2.1 GHz; 4G, 2.3 GHz frequency band) which may be harmful to human health

(WHO, 2014). The radiation caused by mobile phone mast/base station, which usually covers an area of several square kilometers and transmits signals to mobile phones with an electromagnetic field, has an adverse effect on the health of children (Wiaart, Hadjem, Wong, & Bloch, 2008). People living close to 2G and mostly 3G mobile phone masts (base stations) frequently report symptoms of electromagnetic

hypersensitivity such as dizziness, headaches, skin conditions, allergies, and many other problems (Belyaev et al., 2016).

Number of studies demonstrated the negative impact of mobile phone radiation exposure on male reproduction (Agarwal, Deepinder, Sharma, Ranga, & Li, 2008; Agarwal et al., 2009; Fejes et al., 2005; Jurewicz et al., 2014; Kesari & Behari, 2010a, 2010b; Kesari, Kumar, & Behari, 2011; Sepehrimanesh & Davis, 2017). Decrease sperm count, damage sperm cells, reduce seminiferous tubule diameter and testicular weight, and DNA damage are reported in rats exposed to mobile phone radiation (Kumar, Nirala, Behari, & Paulraj, 2014). Mice exposed to 900 MHz radiofrequency electromagnetic radiation (RF-EMR) at a specific absorption rate (SAR) value of approximately 90 mW/kg inside a waveguide for 7 days at 12 hr per day demonstrate a significant genotoxic effect on epididymal spermatozoa but does not have a dramatic impact on male germ cell development (Aitken, Bennetts, Sawyer, Wiklendt, & King, 2005). Rat exposed to 890–915 MHz GSM (global signal module) with whole body SAR value of 0.141 W/kg for 1 month showed decrease in seminiferous tubule diameter (Dasdag et al., 1999) but does not induce apoptosis in testes when exposed for 2 hr per day for 10 months (Dasdag, Akdag, Ulukaya, Uzunlar, & Yegin, 2008). Another study demonstrate that rats exposed to two 3-hr periods of daily cellular phone emissions for 18 weeks, exhibited a significantly higher incidence of sperm cell death along with abnormal clumping of sperm cells than control group rats (Yan et al., 2007). Long term exposure to 900 MHz RF-EMF for a period of 30 days demonstrate a significant decreased in serum testosterone and inhibin B level and significant increase in FSH, LH Serum activin B, and prolactin level (Sepehrimanesh, Saeb, et al., 2014). Mobile phone radiation exposure also increased the number of non-progressive movement spermatozoa as well as DNA fragmentation in sperms (Gorpinchenko, Nikitin, Banyra, & Shulyak, 2014).

Further, mobile phones radiation exposure is also reported detrimental to human fertility (La Vignera, Condorelli, Vicari, D'Agata, & Calogero, 2012). The studies conducted on human male population using mobile phone (GSM) for a period of more than 1–2 years report a decrease in the percentage of sperm cells with normal motility in the semen (Adams, Galloway, Mondal, Esteves, & Mathews, 2014; Liu et al., 2014). Long term exposure (more than 10 years) to mobile phone radiation results in increased percentage of abnormal sperm morphology/structure and decreased percentage of sperm motility (Jurewicz et al., 2014; Wdowiak, Wdowiak, & Wiktor, 2007). Reduce sperm cells motility is also correlated with the duration and/or frequency of using mobile phones. Mobile phone radiation exposure cause a significant decrease in forward sperm movement, total number of motile sperm (Erogul et al., 2006) and mitochondrial membrane potential of the human sperm cells (Falzone et al., 2008). It can reduce the fertilizing potential of men (Agarwal et al., 2008; Fejes et al., 2005; Kilgallon & Simmons, 2005) and may lead to oxidative stress via increasing ROS level and decreasing total antioxidant capacity (TAC) score in human semen (Agarwal et al., 2008, 2009; Falzone, Huyser, Becker, Leszezynski, & Franken, 2011). It has been demonstrated that, the decrease in semen quality in the form of decreased sperm count, motility, viability and normal sperm morphology in men depend on the

duration of daily mobile phone radiation exposure (Boulos & Hassan, 2013). Although, some investigators have reported that the duration of mobile phone usage had no remarkable effect on sperm count and sperm motility (Gutschi, Shamloul, Pummer, & Trummer, 2011; Panagopoulos, 2011; Panagopoulos & Margaritis, 2010; Yildirim et al., 2013).

Recent study conducted on men suggests that mobile phone use might have a negative effect on semen quality parameters (Al-Bayyari, 2017; Zalata, El-Samanoudy, Shaalan, El-Baiomy, & Mostafa, 2015). Decreased sperm motility, sperm linear velocity, linearity index, and acrosin activity along with increased DNA fragmentation was reported in the semen samples following 1 hr mobile phone radiation exposure. However, some study showed that the duration of mobile phone exposure/daily usage of mobile phone does not have significant effect on semen quality (Al-Bayyari, 2017; El-Helaly & Mansour, 2014) but decrease the sperm concentration, volume, viscosity and liquefaction time and increased the number of immotile sperms and sperms with abnormal morphology significantly (Agarwal et al., 2008; Al-Bayyari, 2017). The decrease in semen volume and sperms count is also associated with the distance from telecommunication tower and the time spends on sending or receiving messages. However, Rago et al. (2013) found that long term mobile phone usage results in higher percentage of sperm DNA fragmentation but have no effect on other sperm parameters (density, total count, morphology, progressive motility, apoptosis, mitochondrial membrane potential, chromatin compaction).

Although, number of studies suggest that mobile phone radiation exposure decreases sperm count and seminiferous tubule diameter, increases oxidative stress and cause testicular apoptosis etc. (Kesari and Behari, 2012; Kumar, Kesari, & Behari, 2011; Shokri, Soltani, Kazemi, Sardari, & Mofrad, 2015) but no single systematic study is conducted till date to decipher the effects of long term 1800 MHz mobile phone radiation exposure in different operative modes that is, stand-by, dialing and receiving modes on the testis of immature mice. Hence, present study was undertaken to explore the effect of long term mobile phone (1800 MHz) radiation exposure in different operative modes on immature mice testes and underlying molecular mechanism(s) of mobile phone radiation exposure induced testicular apoptosis.

2 | MATERIALS AND METHODS

2.1 | Animals

For this study we have taken three-weeks old Swiss albino male mice (body weight 10–12 grams) which has completed its weaning period, from the mice colony of our laboratory maintained under strict hygienic and well-ventilated conditions at $24 \pm 2^\circ\text{C}$ with a 12-hr photoperiod (12L:12D) and a relative humidity of $50\% \pm 2\%$. Mice were supplied with standard commercial rodent food pellets and tap water ad libitum. Mice were housed in standard ventilated polypropylene cages with dry husk as the bedding material which changed every other day. Animals were treated and animal protocols were performed humanely and with due regard for alleviation of suffering.

All the experimental procedures were approved and conducted as per the guidelines of "Institutional Animal Ethical Committee, Institute of Science, Banaras Hindu University" and within the framework of the revised animals (Scientific procedures) act of 2002 of the Government of India on Animal welfare.

2.2 | Study design

Forty eight 3 week old Swiss Strain male mice (body weight 10–12 grams) were divided into one sham control (Cont.) and three experimental groups ($N = 12$, for each group). Experimental group mice were exposed to non-thermal (for body as a whole) mobile phone radiation (1800 MHz) by using Nokia 100 (2G, GSM) dual-band mobile phones continuously for four months (3 hr/day for 120 days) in different operative modes that is, dialing (dialing was performed), receiving (dialing signals from D-group was received) and stand-by (mobile phone was kept in just switched on mode) modes as described in our previous study (Shahin, Singh, & Chaturvedi, 2017). From D-group mobile phones, dialing signals (and not speech signals) were sent to the mobile phones of R-group and received so that both D- and R-group animals were in continuous condition of connectivity for 1½ hr, then after a very small break (~10–15 s) again the dialing was initiated from the mobile phones of D-group and signals were received at the mobile phones of R-group and the continuity between the signal transmission and reception is maintained for another 1½ hr. SB-group mice were kept beneath the "switched-on" mobile phones continuously for 3 hr. Before the start of exposure, silent profile with no vibration was set for the mobile phones of all groups. To neutralize the box related and other external constraints, one sham control group was taken into account in each study. The control group mice were subjected to sham exposure in the same mobile phone exposure set-up for same time each day but with the mobile phones in "switched-off" condition study (Shahin et al., 2017).

For this study we have used only one control that is sham control (control kept under "switched-off" mobile phone) group as the cage control and the sham control were concluded similar to each other after assessing all the stress parameters (measurement of ROS, lipid peroxidation, total carbonyl contents, and antioxidant enzymes [SOD, Catalase, GPx]) and testicular histopathophysiology in our preliminary study. Therefore, to neutralize the cage related and other external constraints, only one control group that is sham control was taken into account in this study.

Mice of experimental group were exposed to 1800 MHz mobile phone radiation at Power density of 1.15 W/m^2 and Specific Absorption Rate (SAR) value of 0.05 W/Kg . Detailed design of exposure set-up, exposure cage, Power density, and SAR value calculation (at a point near to testes) has been described in our recent study (Shahin et al., 2017). Rectal temperature of all the mice were measured before and after the exposure during the first, middle (i.e., after 60 days of exposure) and the last 3 days of exposure. The mobile phone exposure does not cause any elevation in rectal temperature. The observed average rectal temperatures of the mice were: $36.51 \pm 0.2^\circ\text{C}$ (control group), $36.55 \pm 0.2^\circ\text{C}$ (D-group), $36.52 \pm 0.2^\circ\text{C}$ (R-group) and $36.64 \pm 0.2^\circ\text{C}$ (SB-group)

before, and $36.67 \pm 0.2^\circ\text{C}$ (control group), $36.73 \pm 0.2^\circ\text{C}$ (D-group), $36.63 \pm 0.2^\circ\text{C}$ (R-group) and $36.79 \pm 0.2^\circ\text{C}$ (SB-group) at the end of exposure period. The mobile phone radiation exposure also did not cause any increase in the ambient temperature of animal cage as documented with the thermometer placed inside the cage. After the exposure, animal exposure cage was cleaned and dried (by using drier) daily.

2.3 | Blood collection and tissues sampling

Twenty-four hour after the termination of 120 days mobile phone radiation exposure, mice were sacrificed by decapitation. Blood was collected in non-heparinized tubes for serum collection. Testes (12 left & 6 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 & GPx) assay and Western blot analysis of cytochrome c. Epididymis was removed to study sperm count and sperm viability. Right testes of six mice of each group were fixed in 10% neutral formalin solution for normal routine histology (haematoxylin-eosin [HE] staining) and immunofluorescence of proteins involved in apoptotic pathway such as p53, Bax, Bcl-2, Bcl- x_L , active/cleaved-Caspase-3, and full length/uncleaved PARP-1.

2.4 | Testes histology and seminiferous tubule diameter measurement

Mice testes fixed in 10% neutral formalin solution for overnight were dehydrated through increasing concentrations of ethanol and embedded in paraffin prior to sectioning. 8- μm -thick sections of testis were cut by Leica RM2125 RT rotatory microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany) and processed for routine histology. 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 testes (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.

2.5 | Sperm count and sperm viability

Sperm count was performed by using hemocytometer (Fein-Optik, Jena, Germany) with improved Neubauer ruling (WHO, 1999). 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:

Sperm number = averaged no. of spermatozoa counted (N) $\times$ multiplication factor (10,000) $\times$ dilution factor (20) = $N \times 20 \times 10,000 = N \times 0.2 \times 10^6$ spermatozoa.

Sperm viability was assessed by using a supra-vital staining technique based on the principle that cells with damaged plasma membrane take up the stain, while viable ones do not (WHO, 1999). 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 approx. 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:

Viability (%) = number of viable spermatozoa/total number of spermatozoa (viable + dead) $\times$ 100.

2.6 | Serum isolation and enzyme-linked immunosorbent assay (ELISA) of testosterone

Blood samples, after keeping 30 min at room temperature, were centrifuged at 4000 rpm at 4 °C for 15 min. The supernatant was collected as serum and used for testosterone estimation. 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⁻¹. 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%.

2.7 | Tissue homogenization

Testes from each group were separately minced thoroughly. 10% (w/v) homogenate for all the 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.

2.7.1 | Measurement of total ROS level

The level of ROS in fresh homogenate was assessed by using the protocol of Bejma, Ramires, and Ji (2000) with slight modification. 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.

2.7.2 | Estimation of total nitrite and nitrate concentration and nitrotyrosine level

The total nitrite and nitrate concentration was measured by the indirect method of Sastry, Moudgal, Mohan, Tyagi, and Rao (2002). In brief, the reaction mixture was prepared by adding 100 μ l of sample or standard (KNO₃), 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 sulphate (ZnSO₄) solution under vortex and allowed to stand for 10 min. The tubes were then centrifuged at 8000 rpm for 10 min. 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.

Nitrotyrosine (3-NT) concentration was measured by using the OxiSelect Nitrotyrosine ELISA kit (STA-305, Cell Biolabs, San Diego, CA) following the manufacturer's protocol.

2.7.3 | Lipid peroxidation test

Level of lipid peroxides was measured by estimation of malondialdehyde (MDA), end product of lipid peroxidation by the method of

Ohkawa, Ohishi, and Yagi (1979) with a slight modification. 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 2000 rpm for 10 min. Absorbance was taken at 532 nm and the results was expressed as nmoles MDA/mg protein.

2.7.4 | Antioxidant enzymes assay

Superoxide dismutase

Testicular SOD activity was determined by the method of Das, Samanta, and Chainy, (2000). In brief, the reaction mixture consisted of 100 μ l of sample, 150 mM phosphate buffer (pH7.4), 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. Total SOD value 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.

Catalase

Catalase activity in the testicular cells was assayed according to Aebi, 1984. 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 H_2O_2 and the decrease in absorbance was measured at 240 nm for 2–3 min. One unit of catalase represents the decrease of 1 μ M of H_2O_2 per minute. The molar extinction coefficient for H_2O_2 is $43.6 \text{ M}^{-1} \text{ cm}^{-1}$.

Glutathione peroxidase

GPx activity was assayed as described by Mantha, Prasad, Kalra, and Prasad (1993). 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 1 U 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 H_2O_2 . 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 ($6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) for NADPH.

2.8 | Detection of cytochrome c through Western blot analysis

For cytochrome c detection, testicular protein extraction was performed as follows. Testes from each group were decapsulated and gently homogenized with 10 strokes in a glass–glass homogenizer containing 10 vol (w/v) of cold extraction buffer (250 mM sucrose, 10 mM KCl, 1.5 mM MgCl_2 , 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.

2.9 | SDS-PAGE and Western blot

Testicular protein samples (50 μ g) from each group were resolved on 15% SDS-PAGE. Proteins were transferred from the polyacrylamide gels to PVDF membranes (Millipore, Boston, MA) by electro-blotting for 15 min in a Semi-Dry blotting apparatus (TRANS-BLOT SD, Semi-dry transfer cell, BIO-RAD, Hercules, CA) 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, Santa Cruz, CA) (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 1:2500) for 1 hr at room temperature. After washing, the immunoreactive protein was visualized by using Super Signal™ West Pico Chemiluminescent Substrate, Thermo Scientific; 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 (dilution 1:2500) antibody to normalize for protein loading.

The intensity of the bands was quantitated by gel densitometry using ImageJ software (Image J 1.48 bundled with 64-bit Java, NIH, Bethesda, MD). 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.

2.10 | Immunofluorescence of full length p53, bax, bcl-2, Bcl-x_L, active/cleaved-caspase-3x, 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), cleaved caspase-3 (rabbit monoclonal, MAB835, R&D Systems, Inc., Minneapolis, MN, Canada, 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 immunolabeling/staining method. Immunofluorescence was performed in a two-step procedure as described by Banerjee and Chaturvedi (2016). 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 1000 W (3–4 min). Testes sections were then

treated with blocking solution for 2 hr at room temperature (5% HIGS-Heat Inactivated Goat serum) 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 × 5 min) and then incubated with donkey-anti-rabbit IgG H&L (conjugated with FITC, Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-rabbit IgG H&L (conjugated with TRITC abcam, ab6718) and goat-anti-mouse IgG (H&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 × 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).

2.11 | Image analysis

Image analysis was performed as described elsewhere (Banerjee & Chaturvedi, 2016). Briefly, 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 to ImageJ software (ImageJ 1.48 bundled with 64-bit Java, NIH, Bethesda). Using the software's tool, IOD of all the areas showing positive signals in different regions of the testes sections were measured after normalizing to the background. The IOD values (relative units) in testes were averaged (four sections per testis and six 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 ± SEM and on the basis of the difference in the level of significance (*p* value <0.05), the testes sections IOD were labeled or classified as intense, moderate or weak.

2.12 | Statistical analysis

The data for biochemical analysis, non-enzymatic and enzymatic oxidative stress markers and quantitative analysis of immunoblot and

IF plates were presented as mean ± standard error mean (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 post hoc analysis (Tukey's method) was used to determine the significance among four different groups. A *p*-value of <0.05 was considered as significant. Data were analysed using statistical software package Statistical Analysis System (SPSS Statistics 17.0, IBM, Armonk, NY).

3 | RESULTS

3.1 | 1800 MHz mobile phone radiation exposure leads to altered testicular histoarchitecture, decreased seminiferous tubule diameter, sperm count, sperm viability, and serum testosterone level

Mobile phone radiation exposure induce marked alterations in the histoarchitecture of the testes of mice as compared to control group testes in which all the seminiferous tubules showed normal histological features (Figure 1a). As compared to control all the mobile phone exposed groups showed atrophied appearance of seminiferous tubules. Significantly decreased seminiferous tubule diameter and total number of spermatozoa in the lumen of seminiferous tubules as well as depletion of germ cells was observed in the testes of all the exposed mice. Seminiferous tubules epithelium of R- and SB-group mice has a degenerated appearance. Formation of gaps was prominently seen between the peripheral layers of spermatogonial cells in the seminiferous tubules of R- and SB-group mice testes. As compared to control the Leydig cells also presented degenerating appearance in all the mobile phone irradiated mice along with increased interstitial spaces in R- and SB-group mice testes. Moreover, all the mobile phone exposed groups mice testes showed number of germ cells with nuclear pyknosis (Figure 1b). Few giant cells were also observed in the seminiferous tubules of R- and SB-groups mice testes. Moreover, the size and number of interstitial cells/Leydig cells also decrease in all the mobile phone irradiated mice as compared to control (Figure 1b).

The diameter of seminiferous tubule was found to be significantly decreased in all the mobile phone radiation exposed mice testes as compared to the control (Figure 1c). The total number of sperm

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

Antibodies	Control	Dialing	Receiving	Stand-by
p53	3.99 ± 0.216 (weak)	15.68 ± 0.620 (moderate)	24.74 ± 1.495 (intense)	31.74 ± 1.504 (intense)
Bcl-x _L	87.33 ± 0.916 (intense)	32.46 ± 1.441 (moderate)	17.16 ± 1.039 (weak)	13.31 ± 1.014 (weak)
Bax	42.21 ± 1.061 (weak)	126.36 ± 5.285 (moderate)	265.25 ± 13.107 (intense)	326.33 ± 13.276 (intense)
Bcl-2	23.141 ± 0.566 (intense)	8.96 ± 0.541 (moderate)	4.842 ± 0.321 (weak)	3.37 ± 0.229 (weak)
Active Caspase-3	21.19 ± 1.854 (weak)	59.513 ± 1.774 (moderate)	63.34 ± 2.006 (intense)	63.84 ± 2.353 (intense)
PARP-1	24.99 ± 0.864 (intense)	15.71 ± 0.625 (moderate)	13.93 ± 0.683 (weak)	7.85 ± 0.705 (weak)

SEM, standard error of the mean; a. u., arbitrary unit; p53, tumor suppressor protein-53; Bcl-x_L, B-cell lymphoma-extra large; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Active Caspase-3, active activecysteine-aspartic acid proteases-3; PARP-1, poly (ADP) ribose polymerase-1.

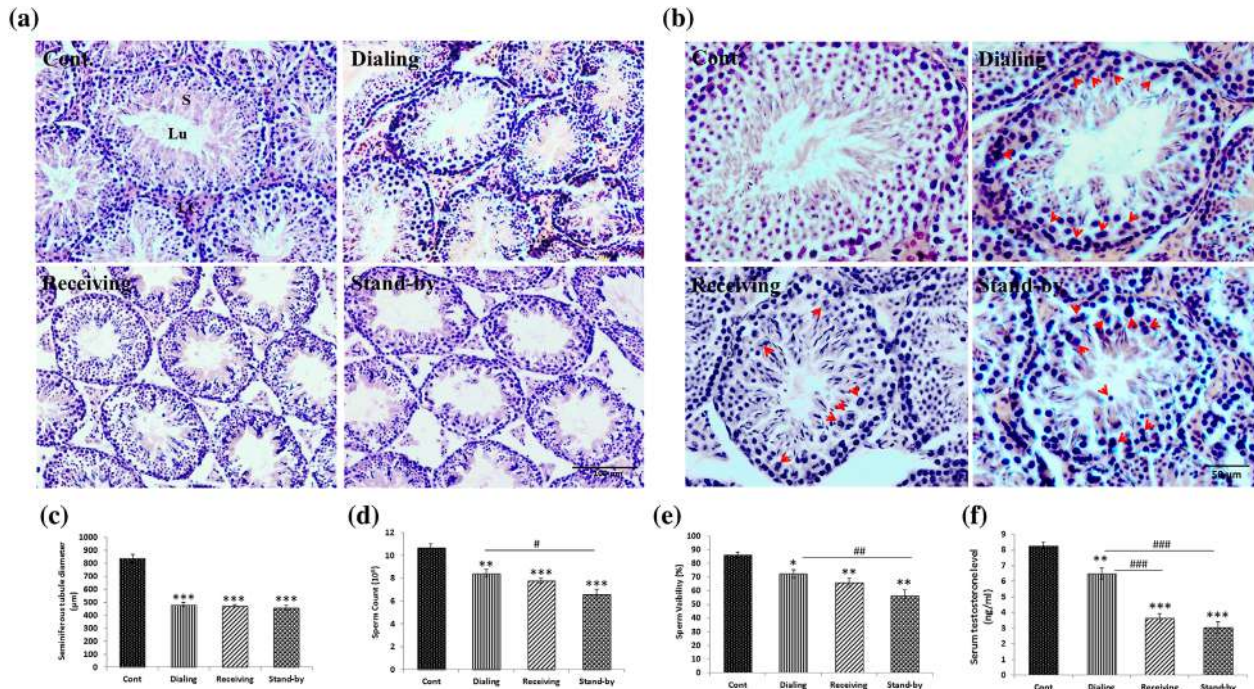


FIGURE 1 (a and b) Transverse Section of testis (H&E staining) (c) Seminiferous tubule diameter, (d) Sperm count, (e) Sperm viability, and (f) Serum testosterone level of mobile phone-irradiated mice. Note significant decrease in the seminiferous tubule diameter and total number of spermatozoa in the lumen as well as depletion of germ cells in the seminiferous tubules of exposed mice testes compared to control. Seminiferous tubules of all the mobile phone exposed groups showed atrophied appearance. R- and SB-group mice have a degenerated appearance in the epithelium of seminiferous tubules. Formation of gaps is also seen between the peripheral layers of spermatogonial cells in the seminiferous tubules of R- and SB-group mice testes. All the mobile phone exposed group mice testes showed nuclear pyknosis along with few giant cells in R- and SB-group. As compared to control, the size and number of interstitial cells/Leydig cells also decrease in all the mobile phone irradiated mice. Sperm viability as well as serum testosterone level decreased significantly in all the mobile phone exposed group of mice compared to control. (LC, Leydig cells; Lu, lumen; S, spermatozoa). Data is presented as mean $\pm$ SEM (for a–c: $n = 6$; for d–f: $n = 12$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significance of difference from control; # $p < 0.05$, # $p < 0.01$, ### $p < 0.001$ significance of difference from D-group

(Figure 1d) and the number of viable sperm (Figure 1e) also decreased significantly in all the mobile phone irradiated groups of mice. The degree of decrease was more in R- and SB-group compared to D-group as well as control group mice.

The concentration of serum testosterone was also found to be significantly decreased in all the mobile phone irradiated groups of mice as compared to the control group. R- and SB-group showed highly significant decrease than D-group (Figure 1f).

3.2 | 1800 MHz mobile phone radiation exposure leads to increased testicular total ROS, NO and MDA level and decreased antioxidant enzymes activities

The total ROS, NO, and MDA level increased significantly in the testis of all the mobile phone radiation exposed group of mice compared to control (Figure 2). Significant increase in ROS (Figure 2a), NO (Figure 2b), 3-NT (Figure 2c), and MDA (Figure 2d) level was observed in the testes of R- and SB-group of mice compared to D-group as well as control.

The activity of ROS scavenging enzymes: SOD (Figure 2e), CAT (Figure 2f), and GPx (Figure 2g) decreased significantly in the testes of

mobile phone exposed group of mice compared to control. The degree of decrease was more in R- and SB-group compared to D-group.

3.3 | 1800 MHz mobile phone radiation exposure leads to altered expression of p53, bax, Bcl-x_L, bcl-2, active-caspase-3, and PARP-1

In all the mobile phone irradiated group mice testes, in general, the expression of p53, Bax, active-caspase-3 was significantly up-regulated while the expression of Bcl-x_L, Bcl-2, and uncleaved PARP-1 were significantly down-regulated in Leydig cells and seminiferous tubules (spermatogonial cells and spermatozoa) (Figures 3–6). Control group testes showed weak immunoreactivity for p53 in Leydig cells and spermatozoa and intense immunoreactivity for Bcl-x_L in Leydig cells, spermatogonial cells and spermatozoa. D-group showed moderately increased immunoreactivity for p53 and moderately decreased for Bcl-x_L in Leydig cells, spermatogonial cells and spermatozoa compared to their respective controls. R- and SB-group revealed intense immunostaining for p53 and weaker immunostaining for Bcl-x_L in Leydig cells, spermatogonial cells and spermatozoa compared to their respective controls as well as D-group (Figure 3a–c).

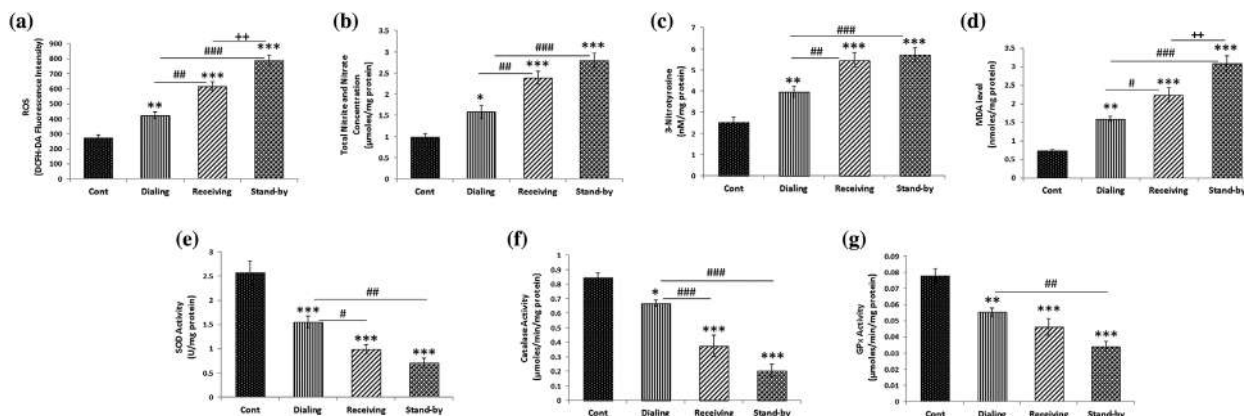


FIGURE 2 Effect of mobile phone radiation on (a) ROS (DCFH-DA fluorescence intensity), (b) Total nitrite and nitrate concentration, (c) 3-Nitrotyrosine, (d) MDA level (measure of Lipid peroxidation), (e) SOD, (f) CAT, and (g) GPx activity in the testes of mice. Values are expressed as mean $\pm$ SEM ($n = 12$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ significance of difference from control; $#p < 0.05$, $##p < 0.01$, $###p < 0.001$ significance of difference from D-group; $++p < 0.01$ significance of difference from R-group

In control group mice testes, very weak expression of Bax was observed in Leydig cells while strong expression of Bcl-2 was observed in Leydig cells, some spermatogonial cells and spermatozoa. In D-group mice testes, increased expression of Bax (in both Leydig cells and seminiferous tubules) and decreased expression of Bcl-2 (only in Leydig cells) was observed compared to their respective controls. Further, while R- and SB-group mice testes showed intense immunoreactivity for Bax in Leydig cells, spermatogonial cells and spermatozoa, very weak expression of Bcl-2 was observed and that too only in Leydig cells (Figure 4a–c).

Compared to the expression of active-caspase-3 observed in Leydig cells and seminiferous tubules (spermatogonial cells and spermatozoa), D-group mice testes showed moderately increased immunoreactivity while R- and SB-group mice testes showed intense immunoreactivity (Figures 5a and 5b).

Compared to control group mice, D-group mice testis showed decreased immunostaining for uncleaved-PARP-1 in Leydig cells and seminiferous tubules (spermatogonial cells and spermatozoa) which reduced further in R-group mice testis. SB-group mice testis revealed significantly weak immunoreactivity for uncleaved-PARP-1 in Leydig cells and seminiferous tubules (spermatogonial cells and spermatozoa) compared to control as well as D- and R-group mice testes (Figures 6a and 6b).

3.4 | Cytoplasmic cytochrome c level

The expression of cytochrome c was found to be increased significantly in the testes of all the mobile phone exposed mice as compared to control (Figure 7).

4 | DISCUSSION

In this study we have demonstrated that 1800 MHz mobile phone radiation exposure induced oxidative and nitrosative stress not only

affects the testicular morphology and spermatogenesis but may also lead to testicular apoptosis via p53 dependent Bax mediated pathway in immature mice. Experimental finding clearly elucidate that, 1800 MHz mobile phone radiation exposure in different operative modes for 3 hr/day results in havoc generation of free radical and suppresses the antioxidant defense system (reduction in the activity of SOD, Catalase, and GPx) which subsequently induced the oxidative and nitrosative stress in the testis and results in histological alterations. It affects the testicular morphology/histoarchitecture by reducing the diameter of seminiferous tubules, sperm count and sperm viability, depletion of germ cells, degeneration of seminiferous epithelium, peripheral gaps formation by the detachment of spermatogonial layer from the tunica propria in some tubules, germ cells with nuclear pyknosis and few giant cells in some seminiferous tubules. All these histopathological changes in the testes suggest that exposure to mobile phone radiation results in the disruption/impairment of spermatogenesis. In addition, these results also suggest that long term exposure to 1800 MHz mobile phone radiation increase germ cell apoptosis. Reduced sperm count and sperm viability and increase germ cell apoptosis following 1800 MHz mobile phone radiation exposure might have occurred as a consequence of increased free radical load (Chandra, Samali, & Orrenius, 2000; Rao & Shaha, 2000) and reduced testosterone concentrations, as optimum level of testosterone is essential for the maintenance of normal spermatogenesis, sperm counts and viability (Sharpe et al., 1992, 1998). These observations are consistent with the studies of Sepehrmanesh and group in which they showed that 900 MHz RF-EMF radiation exposure not only decreased antioxidant enzymes activity and induced non-thermal stress in testicular tissue but also up-regulate the expression of that proteins which are related to reproductive damage and carcinogenic risk (Sepehrmanesh, Kazemipour, Saeb, & Nazifi, 2014, Sepehrmanesh, Nazifi, Saeb, & Kazemipour, 2016, Sepehrmanesh, Kazemipour, Saeb, Nazifi, & Davis, 2017).

The increased ROS and NO level in the testes of mobile phone irradiated mice may lead to severe oxidative damage by causing

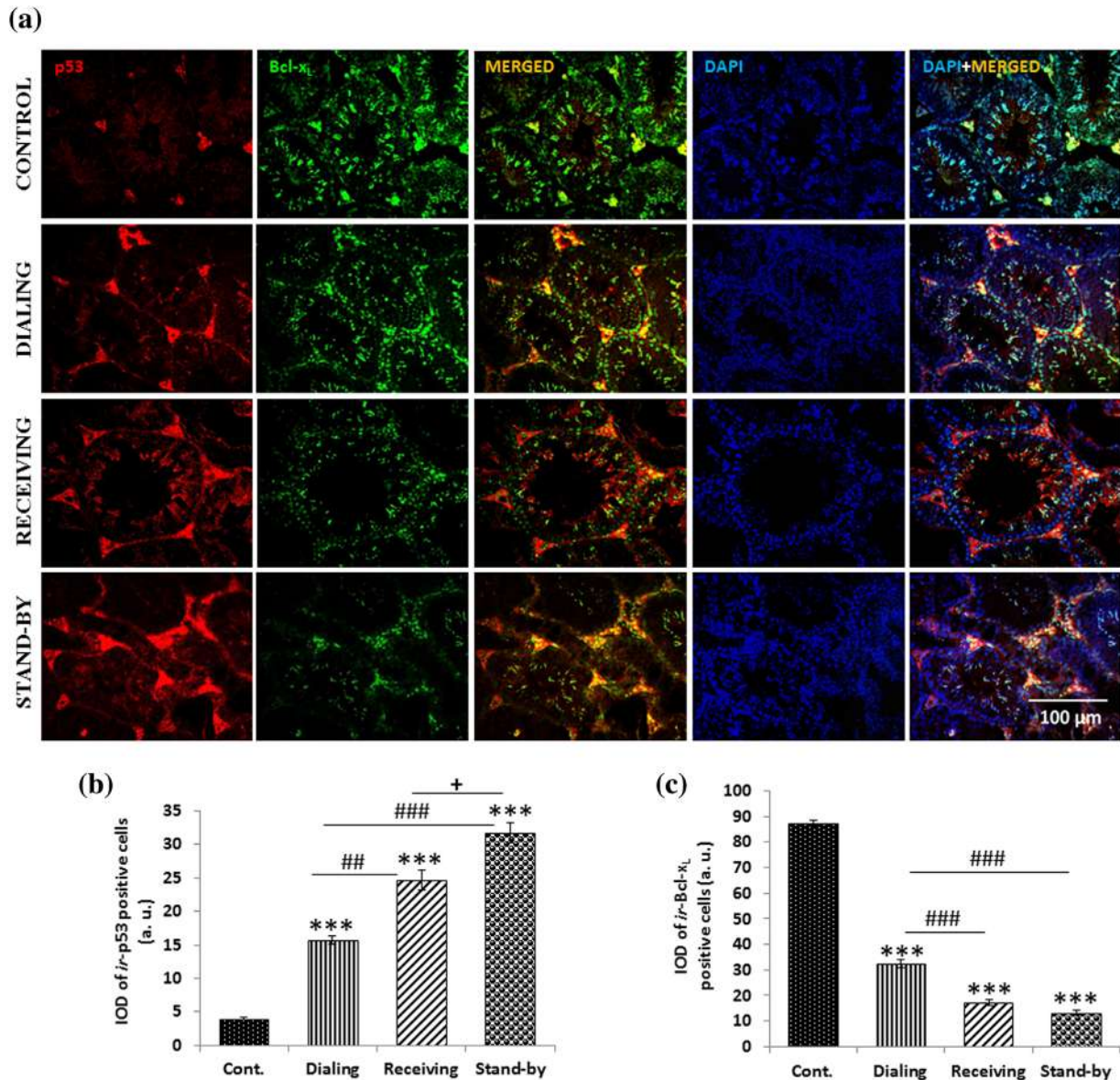


FIGURE 3 Immunofluorescence showing the co-expression of p53 and Bcl-xL (a) and the integrated optical density (IOD) (b and c) of p53 and Bcl-xL in the testes of mobile phone irradiated mice: Control group testis shows weak immunoreactivity for p53 in Leydig cells and spermatozoa and intense immunoreactivity for Bcl-xL in Leydig cells, spermatogonial cells and spermatozoa. D-group shows moderately increased immunoreactivity for p53 and decreased immunoreactivity for Bcl-xL compared to control. R- and SB-group show intense immunostaining for p53 and weak immunostaining for Bcl-xL compared to control as well as D-group. Values are expressed as mean $\pm$ SEM ($n = 6$). *** $p < 0.001$ significance of difference from control; # $p < 0.01$ ## $p < 0.001$ significance of difference from D-group; + $p < 0.05$ significance of difference from R-group

peroxidation of lipids and forming carbonyls (Aitken & Baker, 2006). Our findings also suggest that highly increased ROS and NO level in all the mobile phone exposed mice testis may lead to the formation of peroxynitrite which can further react with tyrosine residues to form nitro-tyrosine. Free radicals generated after mobile radiation 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 of Leydig cells and spermatozoa. Studies showed that, ROS generated during normal testicular function plays an important role in regulating

the function of the testis (Agarwal, Nallella, Allamaneni, & Said, 2004; Griveau & Le Lannou, 1997). Low amount of ROS, generated itself by spermatozoa, participate in the signal transduction mechanism that promotes sperm capacitation/acrosome reaction and acquisition of sperm-fertilizing ability (de Lamirande & Gagnon, 1995; Gagnon, Iwasaki, De Lamirande, & Kovalski, 1991). Although, the physiological levels of ROS are important for both spermatogenesis and steroidogenesis but the overproduction of free radicals (ROS and NO) in the testes of mobile phone radiation exposed mice not only induced oxidative and nitrosative stress but also impaired the two important

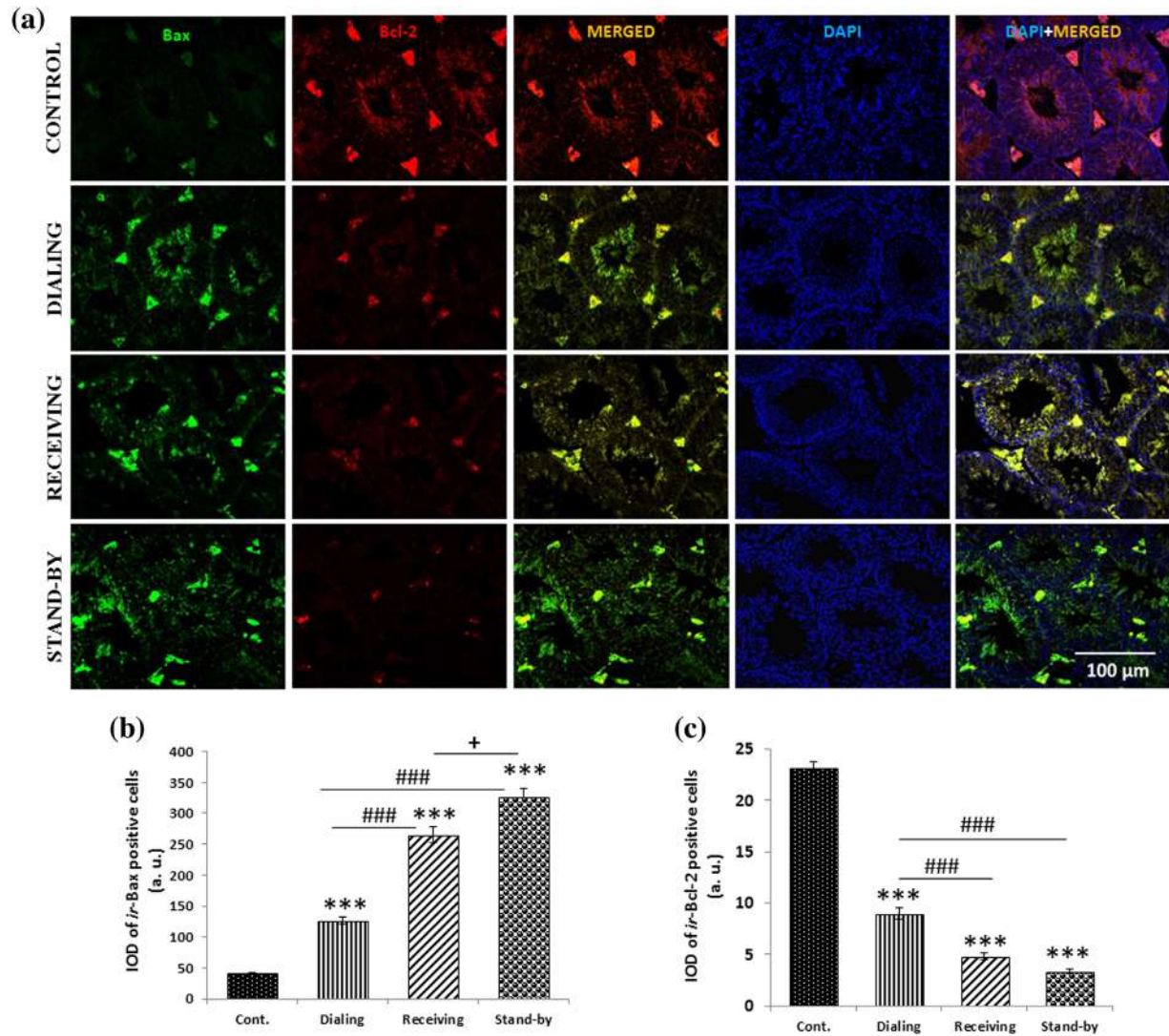


FIGURE 4 Immunofluorescence showing the co-expression of Bax and Bcl-2 (a) and the integrated optical density (IOD) (ba dn c) of Bax and Bcl-2 in the testes of mobile phone irradiated mice: Compared to the control group mice testis, showing weak expression of Bax in Leydig cells only, the D-group mice testis exhibits increased expression in Leydig cells and within seminiferous tubules. Bax immunoreactivity increased further in R- and SB-group mice testes in Leydig cells as well as in spermatogonial cells and spermatozoa. On the other hand, compared to *ir*-Bcl-2 observed in Leydig cells, some spermatogonial cells and spermatozoa of the control mice testis, weak expression of Bcl-2 is observed in the Leydig cells of D-group mice testis. Note further decrease that is very weak expression of Bcl-2 which is restricted only in Leydig cells of R- and SB-group of mice. Values are expressed as mean $\pm$ SEM ($n = 6$). *** $p < 0.001$ significance of difference from control; ### $p < 0.001$ significance of difference from D-group; + $p < 0.05$ significance of difference from R-group

function of testes that is spermatogenesis and steroidogenesis and thus results in decrease sperm count, sperm viability, and testosterone level.

Moreover, our study also demonstrates that the increased free radical load in the testes of 1800 MHz mobile phone irradiated mice results in testicular apoptosis as evident by histological changes (germ cells with nuclear pyknosis, presence of few giant cells in the seminiferous tubules and depletion of germ cells), up-regulation of pro-apoptotic proteins (p53 and Bax), apoptotic proteins (cytochrome c), active executioner caspase-3, and down-regulation of anti-apoptotic proteins (Bcl-x_L and Bcl-2) and nuclear DNA repairing enzyme PARP-1. Mobile phone radiation exposure induced oxidative

and nitrosative stress may activate the p-53 for the trans-regulation of pro-apoptotic (Bcl-2-associated X protein [Bax]) and anti-apoptotic (Bcl-x_L and Bcl-2) proteins. p53 which is a tumor-suppressor protein has a major role in the cellular response to various stresses including DNA damage (Meek, 2009). In absence of cellular stress it is maintained at low steady-state levels (Aylon & Oren, 2007) while in response to stress it becomes activated to transactivate a large number of genes/proteins involved in cell cycle arrest or apoptosis (Aylon & Oren, 2007). p53 can either cause the trans-activation of Bax or trans-suppression of Bcl-2 and Bcl-x_L to mediate apoptosis. The activated p53 stimulates the expression of pro-apoptotic Bax, which in turn binds with and inactivate

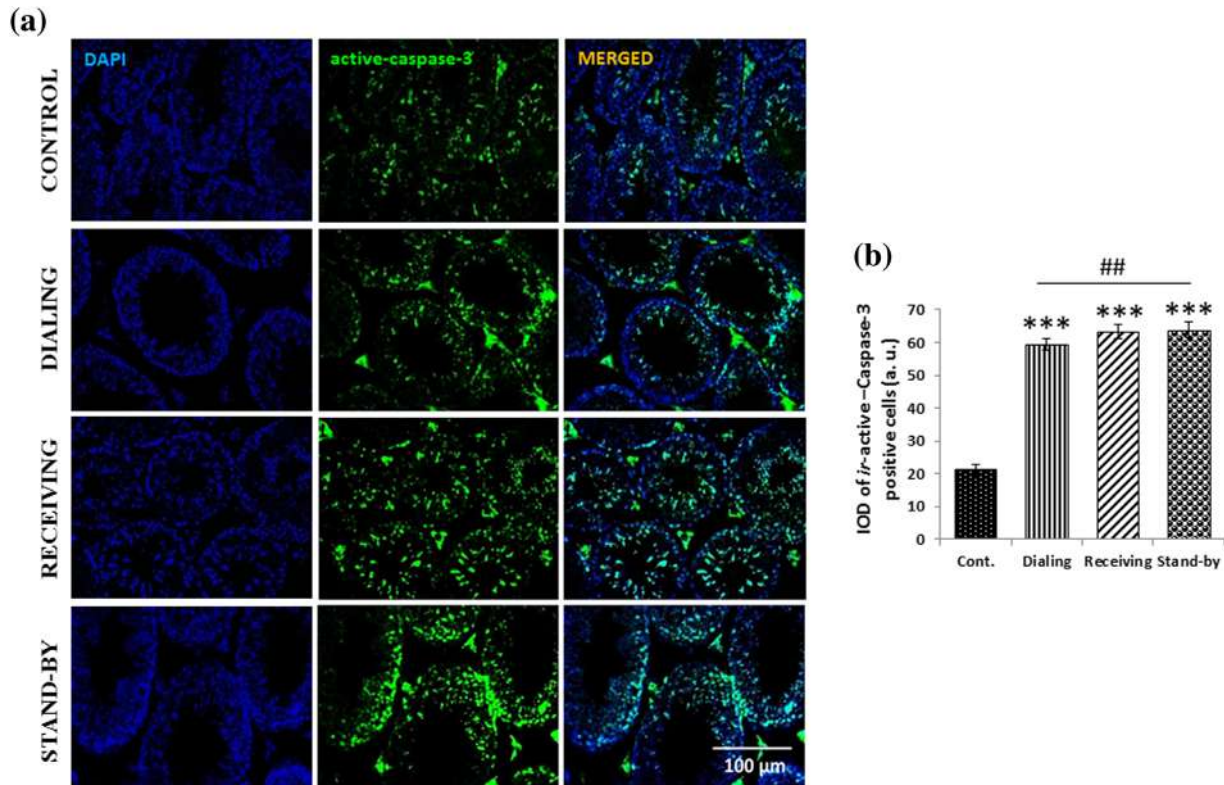


FIGURE 5 Immunofluorescence and the integrated optical density (IOD) of active-caspase-3 in the testes of mobile phone irradiated mice: Note the expression of immunoreactive active-caspase-3 in the Leydig cells and seminiferous tubules (spermatogonial cells and spermatozoa) of control mice testis. D-group mice testis shows moderately increased immunoreactivity for active-Caspase-3 in Leydig cells and seminiferous tubules compared to control, while R- and SB-group mice testes exhibit intense immunoreactivity. Values are expressed as mean $\pm$ SEM ($n = 6$). *** $p < 0.001$ significance of difference from control; # $p < 0.01$ significance of difference from D-group

anti-apoptotic factors Bcl- x_L and Bcl-2 resulting in a sustained increase in the Bax/Bcl-2 and Bax/ Bcl- x_L ratio (Ho & Benchimol, 2003; Laptenko & Prives, 2006). The increased Bax/Bcl-2 and Bax/ Bcl- x_L ratio promotes apoptosis (Laptenko & Prives, 2006). The activated Bax mediates the mitochondrial inner membrane leakage and helps in the release of apoptogenic proteins, such as cytochrome c (Liu, Kim, Yang, Jemmerson, & Wang, 1996).

We suggest that 1800 MHz mobile phone radiation exposure induced oxidative and nitrosative stress may leads to testicular apoptosis via p53 dependent Bax mediated pathway. Exposure to 1800 MHz mobile phone radiation results in havoc generation of free radical in the testes which may lead to DNA strand breaks and thus up-regulate the expression of p-53, which consequently increased the expression of Bax and decreased the expression of Bcl-2 and Bcl- x_L . On the other hand ROS may also increase the expression of Bax and decreased Bcl-2 and Bcl- x_L expression. Therefore, both p53 and ROS cumulatively increased the Bax/ Bcl-2 and Bax/ Bcl- x_L ratio in all the mobile phone exposed group in mode dependent manner and promote testicular apoptosis. Activation and upregulation of Bax will subsequently permeabilize the outer mitochondrial membrane to release cytochrome c. Further, 1800 MHz mobile phone radiation induced elevated ROS may also cause the oxidation of the mitochondrial pores, disrupt the mitochondrial membrane potential ($\Delta\psi_m$) and release cytochrome c.

The significantly up-regulated level of cytosolic cytochrome c in the testis of all the 1800 MHz mobile phone irradiated mice may lead to the formation of apoptosomes and activation of caspase cascade (Mishra, Pal, & Shaha, 2006). Release of cytochrome c from mitochondria in the testicular cells of mobile phone irradiated mice initiates the other apoptotic changes including activation of caspase-3 (Rao & Shaha, 2000; Mishra & Shaha, 2005; Mishra et al., 2006). The increased Bax/Bcl-2 ratio and cytochrome c level in the mobile phone exposed mice testis results in proteolytic cleavage of pro-caspase-3 (initiator caspase) into active caspase-3 (effector caspase). The active caspase-3 cleaves its substrate poly (ADP) ribose polymerase-1 (PARP-1) that is nuclear protein/DNA repair enzyme and thus subsequently stalls the DNA repair which ultimately leads to apoptosis. It has been showed that during apoptosis, DNA repair would be a futile attempt due to the protease mediated cleavage of PARP-1 into fragments (Lazebnik, Kaufmann, Desnoyers, Poirier, & Earnshaw, 1994). Therefore, the significantly increased expression of active-caspase-3 and decreased level of uncleaved PARP-1 in all the mobile phone irradiated group mice testis clearly suggest the increased degree of apoptotic induction in operative mode dependent manner.

Above all, we also observed that spermatozoa are more susceptible to 1800 MHz mobile phone radiation. We observed that in all the mobile phone exposed groups of mice, the process of

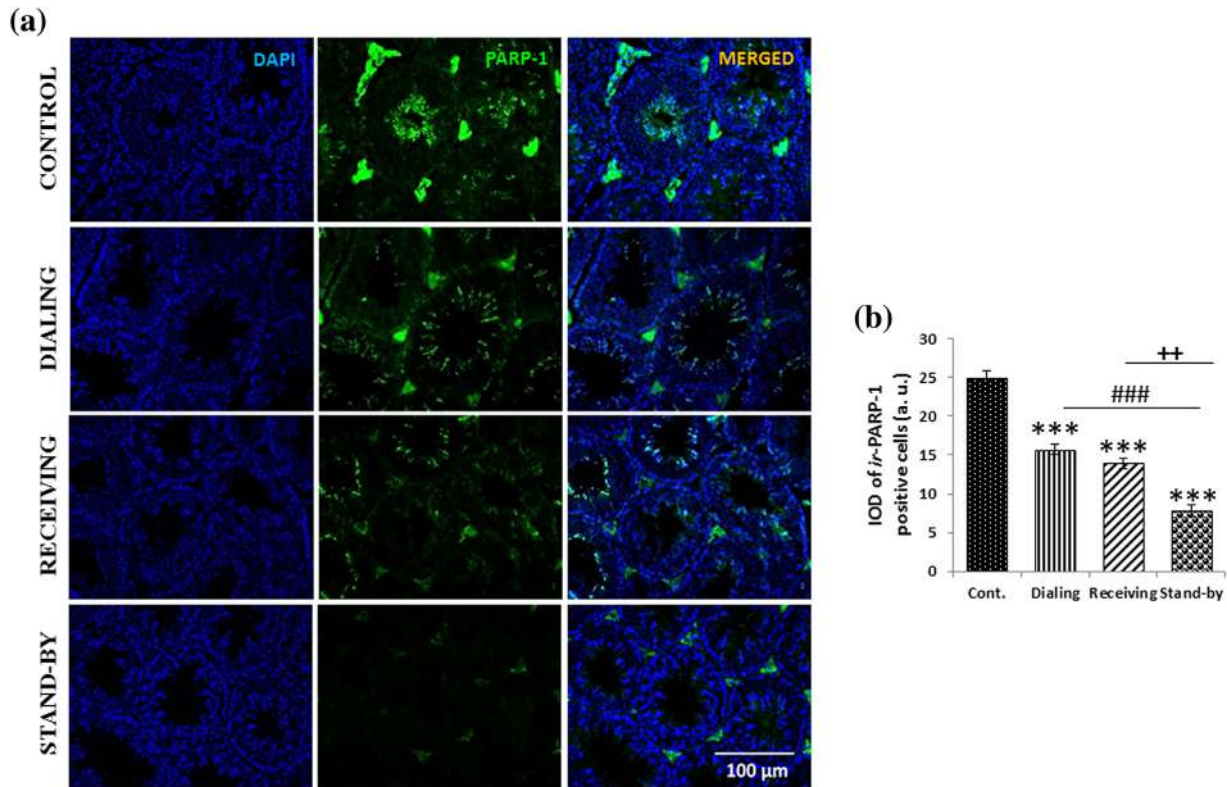


FIGURE 6 Immunofluorescence (a) and the integrated optical density (IOD) (b) of full-length uncleaved PARP-1 in the testes of mobile phone irradiated mice: Note immunoreactivity for uncleaved-PARP-1 in Leydig cells and seminiferous tubules (spermatogonial cells and spermatozoa) of control group mice testis. D-group mice testis shows moderate decrease while R- and SB-group mice testes reveals further decrease that is weak immunoreactivity for uncleaved-PARP-1 compared to control. Values are expressed as mean $\pm$ SEM ($n = 6$). *** $p < 0.001$ significance of difference from control; ## $p < 0.001$ significance of difference from D-group; ++ $p < 0.01$ significance of difference from R-group

apoptosis is initiated first in the sperms as the depletion of anti-apoptotic proteins and accumulation of pro-apoptotic and apoptotic proteins were found to initially begin in sperms and then gradually also seen in the spermatogonial cells and Leydig cells of testes. We also found that R- and SB-groups sperms are more susceptible than D-group sperms.

Altogether, on the basis of our findings we suggest that long term exposure to 1800 MHz mobile phone radiation induced oxidative and nitrosative stress not only cause the testicular damage (reduced seminiferous tubule diameter and degeneration of spermatogonial cells & Leydig cells) but may also lead to testicular apoptosis via p53 dependent Bax mediated pathway. This will result in hypo-spermatogenesis and decreased steroidogenesis as evident by decreased sperm count, sperm viability and testosterone level. All these changes will affect the testicular physiology as well as testicular functions and thus reduce the fertilizing potential of mice which may lead to infertility like condition. Further, we also observed that long term exposure of mobile phone (1800 MHz) radiation in receiving and stand-by modes causes more deleterious effects than dialing mode (SB- > R- > D-mode). This study also provides essential information on cascades of reaction/mechanism of action regarding mobile phone radiation induced testicular apoptosis in rodent system.

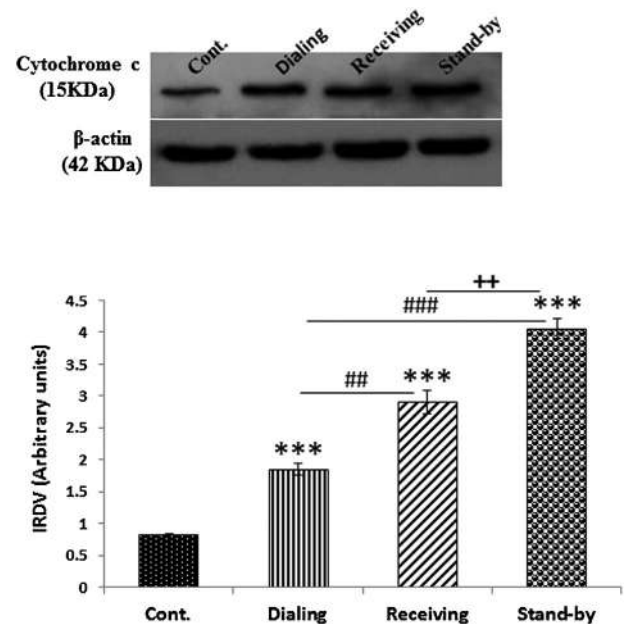


FIGURE 7 Western blot analysis of cytochrome-c protein in the testes of mobile phone exposed mice. β -actin expression was used as loading control. IRDV, integrated relative density value. Values are expressed as mean $\pm$ SEM ($n = 12$). *** $p < 0.001$ significance of difference from control; # $p < 0.01$, ## $p < 0.001$ significance of difference from D-group; ++ $p < 0.01$ significance of difference from R-group

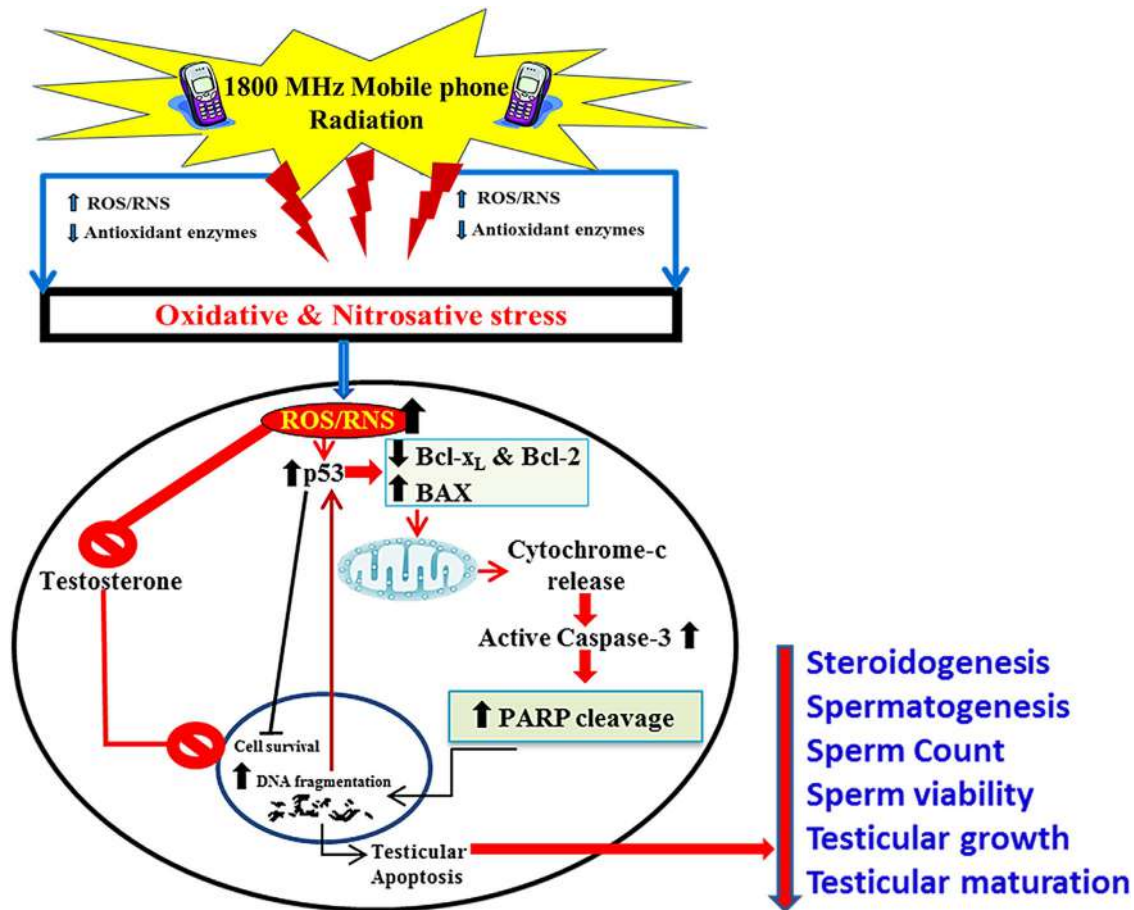


FIGURE 8 Schematic showing the 1800 MHz mobile phone radiation induced testicular apoptosis mediated via oxidative/nitrosative stress induced p53 dependent/independent Bax mediated pathways

5 | CONCLUSION

Our findings, in brief, suggest that the long term 1800 MHz mobile phone irradiation induced oxidative and nitrosative stress mediated havoc generation of free radical results in decreased seminiferous tubule diameter, sperm count, sperm viability and testosterone level. Prolong exposure to 1800 MHz mobile phone radiation also results in depletion of spermatogonial cells and Leydig cells. We also suggest that the elevated ROS and NO level in the testes of all the mobile phone irradiated groups provoked the testicular apoptotic pathway. ROS can either directly stimulate the expression of Bax or can induce p-53 mediate Bax activation. Increased Bax expression coupled with reduced Bcl-2 and Bcl-x_L expression may cause the permeabilization of the mitochondrial outer membrane and consequently release the cytochrome-c from the mitochondria. This will subsequently serves in the activation of caspase-3 which cleaves the nuclear DNA repair enzyme PARP-1 and ultimately leads to testicular apoptosis (Figure 8). Based on the results of present findings it is concluded that, mobile phone radiation induced oxidative and nitrosative stress will the affect the testicular physiology and testicular functions (spermatogenesis and steroidogenesis) as well as cause the apoptosis of testicular cell and thus may also leads to infertility like condition. Present study also suggests that the degree of severity is more in R- and SB-groups of

mice. We also suggest that as the duration of mobile phone radiation increases, the severity of the testicular damage also increases.

ACKNOWLEDGMENT

This work was funded by a research grant (5/10/FR/13/2010-RHN) from the Indian Council of Medical Research, New Delhi, India to CMC and ICMR Senior Research Fellowship (45/2/2012-PHY/BMS) to Ms. Saba Shahin.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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How to cite this article: Shahin S, Singh SP, Chaturvedi CM. 1800 MHz mobile phone irradiation induced oxidative and nitrosative stress leads to p53 dependent Bax mediated testicular apoptosis in mice, *Mus musculus*. *J Cell Physiol*. 2018;233:7253–7267. <https://doi.org/10.1002/jcp.26558>