Effects of Radiofrequency Electromagnetic Wave Exposure from Cellular Phones on the Reproductive Pattern in Male Wistar Rats

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Abstract The present study investigates the effect of free radical formation due to mobile phone exposure and effect on fertility pattern in 70-day-old male Wistar rats (sham exposed and exposed). Exposure took place in Plexiglas cages for 2 h a day for 35 days to mobile phone frequency. The specific absorption rate was estimated to be 0.9 W/kg. An analysis of antioxidant enzymes glutathione peroxidase ($P<0.001$) and superoxide dismutase ($P<0.007$) showed a decrease, while an increase in catalase ($P<0.005$) was observed. Malondialdehyde ($P<0.003$) showed an increase and histone kinase ($P=0.006$) showed a significant decrease in the exposed group. Micronuclei also show a significant decrease ($P<0.002$) in the exposed group. A significant change in sperm cell cycle of G_0–G_1 ($P=0.042$) and G_2/M ($P=0.022$) were recorded. Generation of free radicals was recorded to be significantly increased ($P=0.035$). Our findings on antioxidant, malondialdehyde, histone kinase, micronuclei, and sperm cell cycle are clear indications of an infertility pattern, initiated due to an overproduction of reactive oxygen species. It is concluded that radiofrequency electromagnetic wave from commercially available cell phones might affect the fertilizing potential of spermatozoa.

Keywords Microwave radiation · Histone kinase · Cell cycle · Flow cytometry · Reactive oxygen species · Apoptosis

Introduction

In the last decade or so, concern has aroused about decreasing fecundity and infertility in men [1, 2]. The reasons for this are often linked to various types of environmental and occupational exposure, leading to possible causes of reduced sperm quality [3–5] and

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impact on neurological dysfunction [6, 7]. The effect of mobile phone exposure on male infertility is linked to a decrease in sperm count, affecting motility and structure and causing the DNA strand to break [8, 9]. Radiofrequency radiations affect Leydig cells [10], which are found adjacent to the seminiferous tubules. Moreover, a decrease in the diameter of seminiferous tubule [11, 12], weight of testicular organs (i.e., caput, cauda, corpus) [13], and destruction of Leydig cells due to these radiations are possible indications of male infertility. More recently, several authors [14] have reported that electromagnetic wave affects sperm motility and it is known that a correlation exists between sperm motility and sperm chromatin damage [15]. In an epidemiological study of 361 men [16], it was concluded that the use of cell phones adversely affects the quality of semen by decreasing sperm counts, motility, viability, and morphology, which might contribute to male infertility. Such types of alterations may come through oxidative stress which may also be produced by many environmental factors. These are induced by oxygen and oxygen-derived free radicals commonly known as reactive oxygen species (ROS). This can lead to imbalance in spermatozoa cell cycle, gonadal dysfunctions, poor sperm motility, and change in the level of antioxidant enzymes and histone kinase activity, thereby leading to infertility.

Enzymes play a major role in protecting the cells by removal of free radicals, which are generated by electromagnetic radiations. This may be affected by an overproduction of ROS entering through free radicals formation, which in turn may change the level of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in sperm cells. In addition to this, protein kinases are those that catalyze the phosphorylation of hydroxy amino acids, i.e., serine/threonine protein kinases and tyrosine kinases. Another type of protein kinase, however, has been shown to phosphorylate proteins on the imidazole nitrogen of histidine. The best known groups of these enzymes are the histidine kinases. Phosphorylation by protein kinases is a major signal transduction mechanism used by eukaryotic cells to regulate cell proliferation, gene expression, metabolism, motility, and membrane transport among others. A study of testicular cell suspensions by flow cytometry [17] provides an important clue. The intensity of fluorescence per cell is measured by flow cytometry (FC) and it corresponds to the amount of DNA. The proportion of cells at each intensity represents the proportion of cells at different cell cycle phases viz, apoptosis, G_0–G_1, S, and G_2/M [18]. Interestingly, literature data indicate that cell cycle stimulation following exposure to low frequency electromagnetic fields might promote carcinogenesis [19–21]. Our study at 50 and 10 GHz also shows the changes in cell cycle and a decrease in the level of histone kinase in spermatozoa [22, 23]. Presently, we have also performed micronuclei detection by a flow cytometer. Micronuclei (MN) are small, nucleus-like structures present in the cell. The main reason for micronuclei formation is that chromosomes or fragments have lost their connection to a centromere and thus cannot be properly segregated from the metaphase plate to either of the poles during cell division. Thus, in vivo test is especially relevant in assessing genotoxicity. To find the effect of the broad coverage of the mutagenic and presumably carcinogenic potential of a chemical or radiation, the micronucleus assay is important. Therefore, micronuclei in bone marrow or peripheral blood erythrocytes are widely accepted as a sensitive predictor of the clastogenic potential of chemical and radiation exposure [24].

Several workers [25, 26] reported that acute and chronic, continuous or pulsed wave irradiation on animals can produce morphological alterations in biological cells and tissues. It has been pointed out that the use of mobile phones has effects on sperm motility [14, 27, 28], antioxidant enzymes [29], and sperm counts [27]. Electropollution due to this device is increasing exponentially and its biological implications are one of increasing concerns. The

present study has been carried out on antioxidant enzymes, malondialdehyde (MDA), histone kinase, micronuclei, cell cycle, and ROS to investigate the possible adverse effect of mobile phone radiations.

Methodology

Materials

Glutathione peroxidase (catalog no. 703102), catalase (catalog no. 707002), and SOD (catalog no. 706002) antioxidant enzyme kit were purchased from Cayman Chemical Company, Ann Arbor, MI, USA. N, N-diethyl-para-phenylendiamine (DEPPD) sulfate, DL-dithiothreitol (DTT), aprotinin, leupeptin, pepstatin A, phenlymethanesulfonyl fluoride (PMSF), ethylene glycerol bis(2-aminoethyl)-N,N,N′,N′ tetra acetic acid (EGTA), adenosine-5′ triphosphate disodium salt hydrate (ATP), and β-glycerol phosphate disodium salt pentahydrate were purchased from Sigma-Aldrich, Germany, and histone H1 and propidium iodide (PI) were purchased from Upstate, NY, USA and HiMedia, respectively. Ferrous sulfate, hydrogen peroxide, sodium acetate, and other chemicals were obtained locally. P^{32} radioactive-labeled ATP was purchased from BRIT, Hyderabad, India. The rest of the chemicals were purchased from Thomas Baker Chemicals Limited, Marine Drive, Mumbai, India.

Animal Exposure

Male Wistar rats (70 days old and 200±20 g body weight) were obtained from an animal facility of Jawaharlal Nehru University, New Delhi. Animals were divided in two groups: sham exposed ($n=6$) and exposed group ($n=6$). All the experiments were repeated and done in a blind pattern. All animals were housed in an air-conditioned room, where the temperature was maintained at 25–27 °C, with constant humidity (40–50%) and kept on 12/12 h light/dark cycle throughout the experiment. Animals were provided with standard food pellets (Brook Bond India Limited) and water ad libitum.

The protocols for animal experimentation described in this study were approved by the Institutional Animal Ethical Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals. All subsequent animal experiments adhered to the "Guidelines for Animal Experimentation" of the University.

Exposure Chamber

Rats were placed in a Plexiglas cages, fixed with anechoic material which was ventilated with holes of 1 cm diameter. The cages were designed by pasting anaechoic material (radio absorbing material) on the side of each box. Mobile phones were kept on the top of the exposure box housing one animal. The placing of the mobile phone antenna on the top of the animal cage ensured that there was preferential emission towards the animal and there are no scattered radiations around. The frequency of the cell phone was 900 MHz, pulse GSM (global system for mobile) and kept in standby mode. The cell phones were in silent mode without vibration. Maximum and minimum output power were measured falling on the body. Each animal cage was attached with a separate mobile phone. Variation of related power within each animal cage is minimal as discussed earlier [30]. The same experimental methodology was used based on the methods used by Kesari et al. [31] and Narayanan et al. [32].

Power Calibration

Exposure was given by mobile phones having a time average specific absorption rate of 0.9 W/kg, as mentioned by the manufacturers. For the purpose of calibration, the emitted power of mobile phones was measured by using a specially designed monopole antenna. The emitted power was measured by a power meter (RF power sensors 6900 series and IFR 6960 B sensors RF power meter), attached to the antenna by SMA connector simulating an actual mobile phone exposure scenario [33]. The maximum average emitted power so measured turned out to be 2 mW. The method of power measurement is the same as described before [31].

Antioxidant Enzyme Estimation

Immediately after exposure, rats were anesthetized by placing them in a glass jar containing cotton dipped in ether. Animals were sacrificed and sperm was collected from the caput and cauda region in cold buffer. The assay was performed with positive control for enzyme estimation.

Sperm Sample Preparation

Sperm sample was added into 5–10 ml of cold buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA and 1 mM DTT) for GPx, 20 mM HEPES buffer (1 mM EGTA, 210 mM mannitol and 70 mM sucrose) for SOD, and 5–10 ml of cold buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) for catalase. All the samples were centrifuged at $10,000 \times g$ for 15 min at 4 °C, the supernatant was collected, and enzyme assay was performed.

Estimation of Glutathione Peroxidase Activity

One hundred twenty microliters of assay buffer and 50 µl of co-substrate mixture (NADPH, glutathione, glutathione reductase) were added into non-enzymatic wells. One hundred microliters of assay buffer, 50 µl of co-substrate mixture, and 20 µl of diluted GPx were added into other wells (control sample). The same amount of assay buffer and co-substrate including 20 µl of sperm sample was added in all the wells. Reaction was initiated by quickly adding 20 µl of cumene hydroperoxide in all the wells. All the samples were mixed formally and incubated for a few seconds. Finally, well plates were placed in a microplate reader spectrophotometer (Spectra Max M_2) and absorbance of the samples was measured at 340 nm.

Estimation of Superoxide Dismutase Activity

Twenty microliters of SOD standard was diluted with 1.98 ml of sample buffer. SOD standard wells were prepared by using 200 µl of the diluted radical detector and 10 µl of diluted standard. Sample wells were prepared by adding 200 µl of the diluted radical detector and 10 µl of sample to the wells. The reaction was initiated by adding 20 µl of diluted xanthine oxidase to all the wells. All the samples were mixed well and incubated for 20 min at room temperature. The sample plate was kept in a microplate reader at room temperature and absorbance was measured at 450 nm.

Estimation of Catalase Activity

One hundred microliters of assay buffer, 30 µl of methanol, and 20 µl of standard were added to the wells, which contained 10 µl of formaldehyde standard (diluted with 9.99 ml

of sample buffer). Control wells were prepared by adding 100 µl of diluted assay buffer, 30 µl of methanol, and 20 µl of diluted CAT. Then, the sample wells were prepared by adding 100 µl of diluted assay buffer, 30 µl of methanol, and 20 µl of sperm samples. The reaction was initiated by adding 20 µl of diluted hydrogen peroxide and incubated for 20 min at room temperature. Thirty microliters of potassium hydroxide was added to terminate the reaction. Thirty microliters of purpled chromogen (4-amino-3-hydrazino-5-mercapto-1,2,4-trizole in 0.5 M hydrochloric acid) was added to each well and thereafter incubated for 10 min at room temperature on a shaker. Ten microliters of potassium periodate was added to each well and incubated for 5 min at room temperature on a shaker, and the absorbance of the samples was measured at 540 nm. The calculation of antioxidant enzymes (GPx, SOD, CAT) was followed as per protocol provided by Cayman Chemicals (USA).

Malondialdehyde Estimation

MDA in sperm (10^6 cells) was measured by monitoring the formation of thiobarbituric acid-reactive substances (TBARS) using the method of Buege and Aust [34]. The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. Briefly, 2 ml of each sample treated with trichloroacetic acid (15% w/v) containing 1 mM EDTA was centrifuged at $1,000 \times g$ for 10 min. The supernatant was heated at 100 °C with an equal volume of TBA (0.7% w/v) for 20 min, and after cooling, the absorbance (532 nm) was measured.

Flow Cytometry: Micronucleus Assay

The blood sample was collected by cardiac puncture. Blood samples were washed by adding 5 ml of phosphate-buffered saline (PBS) and centrifuged at $300 \times g$ for 5 min. The pellet was resuspended in PBS. Five milliliters of fixative [Sorensen buffer A: 0.05 M KH_2PO_4; Sorensen buffer B: 0.05 M $Na_2HPO_4 \cdot 2H_2O$, pH 6.8, 30 µg/ml SDS; and 1% glutaraldehyde (v/v)] was added to 15 ml conical tube containing 100 µl of blood samples. Samples were vortex vigorously and remained in the fixative for 5 min. Thereafter, the samples were centrifuged at $300 \times g$ for 5 min and the pellets were resuspended in 0.5 ml of PBS. Solution A was prepared by adding 0.1 ml Triton X-100, 8 ml 1.0 N HCl, 0.877 g NaCl, and distilled water to a final volume of 100 ml according to Criswell et al. [24]. Solution B contained 37 ml 0.1 M anhydrous citric acid, 63 ml 0.2 M Na_2HPO_4 (pH 6.0), 0.877 g NaCl, 34 mg EDTA disodium salt, and 0.6 ml acridine orange (1 mg/ml). Both the solutions were chilled on ice prior to use. Fixed samples were pipetted into a centrifuge tube, followed by the addition of 400 µl of solution A and 1.2 ml of solution B. Samples were mixed by vortexing and allowed to stain on ice for 30 min in the dark. After staining, samples were centrifuged at $300 \times g$ for 5 min. The supernatant was carefully aspirated so that the cellular pellet was undisturbed. One milliliter of PBS was added to the pellet, resuspended, and mixed by vortexing.

Samples were analyzed on FACS Calibur (Becton & Dickinson) equipped with a 15-mW argon laser. All the events were recorded on forward angle scatter (FALS, linear scale, related to cell size), side scatter (SSC, log scale, related to cellular complexity), DNA fluorescence (FL 1 log, green fluorescence, 530 nm), and RNA fluorescence (FL 4 log, red fluorescence, 675 nm). Analysis of micronucleus events was passed through the polychromatic erythrocyte (PCE) and normochromatic erythrocyte (NCE) population. Toxicity at the stem cell level can be detected as a decrease in the ratio of PCE/NCE. PCE/

NCE ratio was determined by staining blood samples with acridine orange which excited at 530 and 675 nm of wavelengths. NCE does not contain DNA or RNA and hence least fluorescent population. The PCE contains only RNA content and occurs in the middle of the histograms. TNC presents in the extreme upper right of the histograms, containing RNA and DNA content.

Histone Kinase (H1) Assay

Testicular cells were collected in 40 volumes of ice-cold medium containing 10 mM Tris–HCl, pH 7.4, 2 mM EDTA, 1 mM DTT, and protease inhibitors like aprotinin, leupeptin, pepstatin A (each 10 μl/ml), and 0.1 mM (PMSF). The homogenate was centrifuged at $1,000 \times g$ for 5 min at 4 °C. Thereafter, the supernatant was centrifuged at $12,000 \times g$ for 10 min at 4 °C. The pellet was taken and homogenized with ice-cold 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT and stored on ice at 4 °C. Protein concentration was measured by Lowry's method [35]. An assay mixture was prepared by mixing 3 μl of $\gamma[^{32}P]$ ATP (spec. activity 3,000 Ci/mmol), 45 μl of 4 mM ATP, and 222 μl of extraction buffer [80 mM b-glycerophosphate (pH 7.3), 20 mM EGTA, 15 mM $MgCl_2$] and the aliquots were stored at −20 °C. One-millimolar DTT and protease inhibitors (1 mM PMSF, 10 μg/ml each of aprotinin, pepstatin, and leupeptin) and 30 μl of 20 mg/ml histone H1 were added to aliquots just prior to use. Twenty micrograms of each sample protein to be assayed was pipetted into labeled micro-well plate at 4 °C and mixed well by pipetting up and down several times. Each reaction was continued for 15 min of incubation by warming the micro-well plate to 37 °C in water bath. Reaction was stopped by transferring 10 μl of the reaction mixture to phosphocellulose paper pre-cut into 1.5-cm squares. The mixture was allowed to stand for a few seconds to dry and then dipped into a beaker containing 150 mM H_3PO_4 overnight. Filter papers were washed in 150 mM H_3PO_4 for 3×15 min at 20 °C. Filter papers were transferred into scintillation vials containing 5 ml of scintillation fluid. The level of emitted radiations was counted (counts per minute) by using a Hewlett Packard scintillation counter.

Cell Cycle Estimation

One hundred microliters of testicular spermatozoa (1×10^6 cells/ml) were taken and fixed in 1 ml ice-cold 70% ethanol. After the completion of overnight incubation period at 4 °C, the sample was centrifuged at $1,000 \times g$ for 10 min at 4°C, and finally, the supernatant was decanted. One hundred microliters of RNAase (100 units) was added to the pellet and pipetted well thereafter. The samples were incubated at room temperature for 20 min. Finally, the samples were stained with 50 μl (25 μg/ml) PI in the dark and analyzed within half an hour after staining with PI.

Total Reactive Oxygen Species Assay

Five microliters of semen (collected from the caput and cauda region of 1×10^6 cells/ml) was added to 140 μl of prewarmed (40 °C) 0.1 M sodium acetate buffer (pH 4.8) in 96 wells (microtiter plate). One hundred microliters of the mixed solution of DEPPD (100 μg/ml DEPPD was dissolved in 0.1 M sodium acetate buffer, pH 4.8) and ferrous sulfate [ferrous sulfate (4.37 μM) was dissolved in 0.1 M sodium acetate buffer, pH 4.8] at a ratio

of 1:25 was added in each well to initiate reaction. Thereafter, the microtiter plate was incubated at 37°C for 5 min. Absorbance was measured using a Spectra Max M_2 spectrophotometer plate reader at 505 nm. ROS levels in semen were calculated from the calibration curve of H_2O_2 and expressed as equivalent to levels of hydrogen peroxide (1 unit=1.0 mg H_2O_2/l) [36].

Results

Antioxidant Enzymatic Activity

Compared with the control group (4.13±0.19), those exposed to the microwave showed a significant decrease (2.38±0.09 nmol/min/ml; $P<0.001$) in GPx activity. A significant decrease in SOD activity (150.19±6.49 U/ml; $P<0.007$) as compared to control group (198.78±7.53) was also observed. However, the exposed group of animals showed a significant increase in CAT activity (9.81±1.6 nmol/min/ml; $P<0.005$) as compared to the control group (6.86±0.76 nmol/min/ml).

Malonyldialdehyde

MDA in sperm was measured by monitoring the formation of TBARS. The result shows a significant ($P<0.003$) increase in the mobile phone-exposed group (0.16±0.01) as compared with the control ones (0.08±0.01).

Flow Cytometric Determination of Micronuclei

PCE/NCE ratio of percentage gated value was determined by flow cytometry. The ratio of PCE/NCE in the mobile phone-exposed group (0.67±0.15) was significantly lower ($P<0.002$) as compared with the sham-exposed group (1.36±0.07) (Table 1).

Histone Kinase (H1)

The activity of histone kinase in sperm shows a significant decline ($P=0.006$) in the electromagnetic field-exposed group (3,659.08±1,399.40 p^{32} counts/mg protein) as compared to the sham exposed group (5,374.91±1,366.91 p^{32} counts/mg protein). The decline in the level of histone kinase also indicates that the level of G_2/M phase has decreased (Table 1).

Table 1 Comparative statistical analysis of mobile phone exposure effect on micronuclei formation in peripheral blood and histone kinase activity in rat sperm showing significant changes in the exposed group as compared to the sham-exposed group ($P<0.05$)

	Micronuclei (PCE/NCE)	Histone kinase (H1)
Control (mean ± SD)	(1.36±0.07)	(5,374.91±1,366.91 p^{32} counts/mg protein)
Exposed (mean ± SD)	(0.67±0.15)	(3,659.08±1,399.40 p^{32} counts/mg protein)
P value (Student's t test)	($P<0.002$)	($P=0.006$)

The statistical variation in between was analyzed by Student's t test

Cell Cycle Analysis

Samples were analyzed with a FACS Calibur (Becton & Dickinson) FC with an argon-ion laser that produced 15 mW at 488 nm. The percentages of testicular sperm cells in each cell division phase (G_0–G_1, S, and G_2/M) were estimated from data obtained from the FL2-A channel. The exposed group shows a significant increase ($P=0.005$) in apoptosis as compared to the sham exposed group. A significant decrease ($P=0.042$) was observed in the G_0/G_1 phase in the exposed group as compared to the control ones. However, the S phase shows nonsignificant changes in the exposed group. A statistically significant decrease ($P=0.022$) was observed in the G_2/M phase of the exposed group as compared to the sham-exposed group. These are indicated in Fig. 1 and data are presented in Table 2.

Reactive Oxygen Species

Absorbance of semen samples was measured at 505 nm by a spectrophotometric plate reader. The reading was measured at 30-s intervals. ROS of the mobile phone-exposed group was estimated from standard hydrogen peroxide solution (10 different concentrations) coupled with blank. Optical density of each solution increased with time. The level of ROS was expressed as 1 U equivalent to 1 mg/l H_2O_2. The calibration curve for the standard solution was obtained by calculating slopes from optical density (absorbance increase at 505 nm/min×1,000). The mean value of the mobile phone-exposed group (58.25±10.36 mg/l) was significantly higher ($P=0.035$) than that of the sham-exposed group (41.78±12.93 mg/l).

Statistical Analysis

The statistical variation in between the exposed and sham-exposed (control) groups was analyzed by Student's t test. Difference is significant with (P two tail) at 0.05 level of significance.
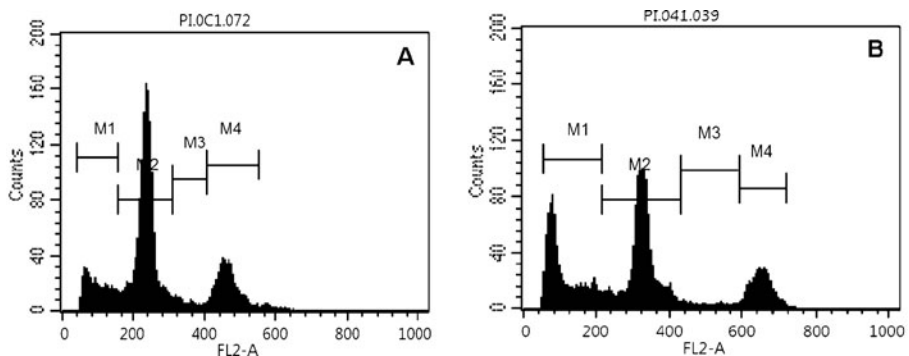
Fig. 1 Flow cytometry analysis in testicular sperm to analyze spermatogenesis cycle. **a, b** The histogram of sham-exposed and exposed groups, respectively. M1 indicates the apoptosis phase, M2 indicates the G_0–G_1 phase, M3 indicates the S phase, and M4 indicates the G_2/M phase. The statistical variation in between was analyzed by Student's t test

Table 2 Comparative statistical analysis of cell cycle (apoptosis, G_0–G_1, S, and G_2/M phase) using a flow cytometer

	Apoptosis (M1)	S (M3)	G_0–G_1 (M2)	G_2/M (M4)
Control (mean ± SD)	18.63±7.96	58.01±8.20	4.12±0.58	18.84±3.05
Exposed (mean ± SD)	31.90±4.84	49.58±3.47	3.26±1.64	15.11±1.41
P value	$P=0.005$	$P=0.042$	Nonsignificant	$P=0.022$

A significant result considered as $P<0.05$. The statistical variation in between was analyzed by Student's t test

Discussion

Macleod [37] was the first who reported that ROS has harmful effects on sperm. It is now generally accepted that overproduction of ROS in sperm is associated with infertility [38–43]. The electromagnetic field exposure emitted from various gadgets (mobile phones and microwave ovens) leads to the generation of ROS [23, 44] which may also alter enzyme activities. It is proposed [45] that moderate levels of ROS can induce an increase in antioxidant enzymes. ROS is able to damage many biomolecules, including DNA, enzyme, lipids, and protein. Lai and Singh [46] reported that free radical generation in a microwave-exposed body may cause biological damage like DNA strand breaks in rat brain cells when exposed to 2.45 GHz of continuous and pulsed radiofrequency (RF) radiation for 2 h per day. Earlier from our laboratory, DNA single and double strand breaks in brain cells have also been reported due to microwave exposure [6, 47, 48]. In light of these findings, it is suggestive that mobile phone radiation may lead to oxidative stress due to overproduction of ROS in human semen [49]. Contrary to this, Dasdag et al. [50] failed to report any adverse effect of cell phone exposure on sperm count, morphology, and histological structure of testis in rats. Some other observations suggest that there is no effect of RF radiation exposure in DNA strand break on mammalian somatic cells [51, 52].

In support of available data and to confirm their pathological implications, we have measured the activities of antioxidant enzymes (SOD, CAT, GPx), MDA, histone kinase, micronuclei, and cell cycle in rat sperm cells. An overproduction in ROS level coupled with these parameters appears to confirm the relationship between mobile phone radiation and infertility. The present study suggests an enhancement of free radicals by these radiations, which may increase lipid peroxidation and change the antioxidative activities of sperm cells leading to oxidative damage. The outcome of oxidative damage induced by electromagnetic fields will therefore depend on various factors, including the oxidative status of the cell, capability of endogenous antioxidant enzymes and processes to counteract free radical buildup, availability of exogenous antioxidants, iron homeostasis (a balance of iron influx, storage, and use), the parameters of exposure (e.g., intensity and duration of exposure and possibly the wave shape), and whether the oxidative damage is cumulative.

A decrease in the level of SOD activity suggests an increase in the generation of reactive superoxide ions as also reported by Alvarez et al. [53]. The decrease in GPx activity might have been due to an excessive production of free radicals. Although GPx is a relatively stable enzyme, it can be inactivated under conditions of severe oxidation stress. CAT activity is enhanced when H_2O_2 levels are particularly high [54]. The extent of microwave damage to the membrane was monitored by measuring the amount of thiobarbituric acid-reactive material (malondialdehyde) produced when polyunsaturated fatty acids in the

membrane undergo peroxidation and the amount of solute leakage from cells. The MDA levels of all experimental groups increased significantly as compared to the control ones. This occurs due to generation of charge imbalance in unsaturated fatty acids that affect membrane structure and properties under microwave radiation which enhances the production of free radical formation due to its trigger action. Its interaction with the membrane may affect its overall integrity. Mammalian sperm membranes contain highly unsaturated fatty acids and are also sensitive to oxygen-induced damage mediated by lipid peroxidation and free water-induced oxygen [55]. The current study primarily confirms an increase of ROS production occurring due to mobile phone exposure. Our result shows that mobile phone exposure increases the concentration of MDA in the testis which is associated with decrease/increase of antioxidant enzyme activity due to oxidative stress induced by reactive oxygen species [56]. Electromagnetic field exposure may thus increase free radical formation in cells as has also been proposed by other workers [56, 57]. Our present findings viz significant decrease in histone kinase (H1), an increase in micronuclei level, alteration in cell cycle, and antioxidant enzyme levels are suggestive that these are affected due to mobile phone radiation exposures.

In general, initiation of mitosis (M phase) requires a protein kinase complex (MPF) consisting of a catalytic subunit (Cdc_2 protein kinase) [58, 59] and a regulatory subunit (cyclin B). Assessment of the catalytic activity of a specific protein kinase plays an important role in elucidating signal transduction pathways. Our results are in agreement with the findings that the activity of histone H1 kinase is closely related to the G_2/M transition during the cell cycle [60]. We observed a decrease in histone H1 kinase
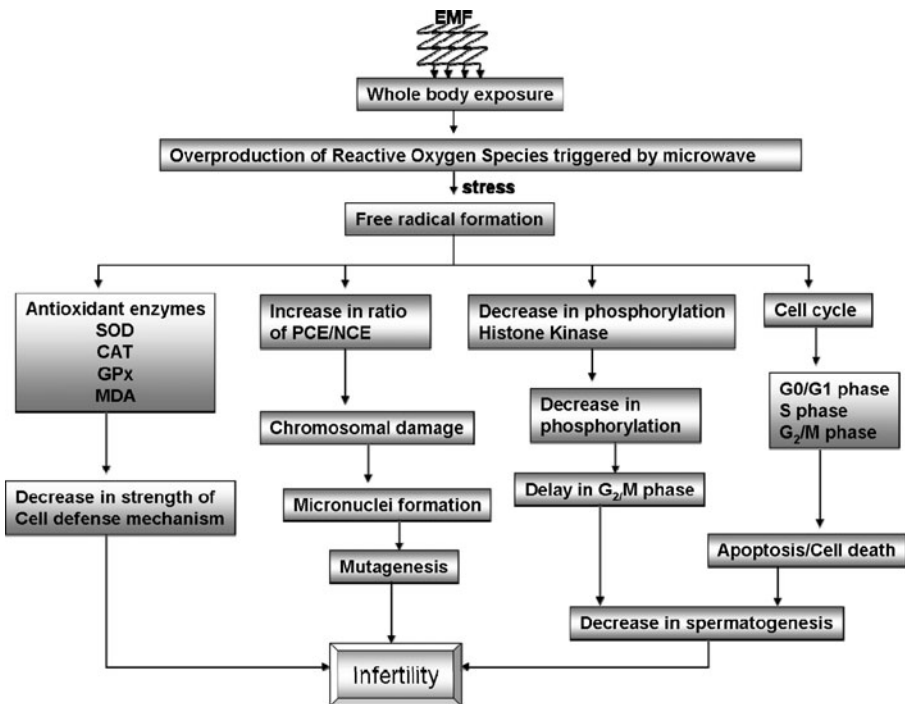
Fig. 2 Summary of the possible sequence of events in mobile phone frequencies' interaction with the reproductive system. The statistical variation in between was analyzed by Student's t test

activity was realized just before the entry of differentiating cells into the M phase, suggesting a universal role of Cdc_2/Cdk_2 (cell division cycle/cyclin-dependent kinase) kinase to make the G_2/M transition [61]. The study also confirms that microwave affects cAMP-independent protein kinases. In continuation of histone kinase and cell cycle analysis, micronucleus assay has been widely used to measure genotoxicity in an in vivo study. During proliferation, the cells continue to divide and cause chromosomal damage such as breaks and exchanges, which eventually lead to formation of micronuclei. The increase in the frequency of micronucleated PCE in the experimental group is an indication of induced chromosomal damage. MN formation occurred with the loss of chromosome fragments due to microwave radiation. We found a significant increase of MN levels in cultures irradiated at mobile phone frequency after 35 days of exposure. Recently, Kumar et al. [23, 44] have also shown the causative effect by an increase in micronuclei and ROS at different frequency levels. The phenomenon of micronuclei shows that during RBC formation, erythroblasts expel their nucleus and may also damage the chromosome in the cytoplasm of young erythrocyte (in the form of micronuclei). Due to their relatively small size, the radiofrequency-induced MN is likely to arise via a clastogenic effect. These results are consistent with the findings on the influx of micronuclei into the peripheral circulation after mobile phone exposure. In support to this, Garaj-Vrhovac et al. [62] reported a significant increase in micronuclei frequency following treatment of human lymphocytes with radiation in the microwave range.

Studies on reproductive pattern are reported by Kim et al. [63] suggesting that long-term exposure to EMF has adverse effects on the proliferation and differentiation of spermatogonia and this may be important in understanding the pathogenesis of EMF-induced male infertility. Lee et al. [64] reported that EMF may induce cell death (apoptosis) in several in vivo studies mostly on mice and rats. Our findings are in support of Agrawal et al. [16] and Kesari et al. [32] who suggested that cell phones adversely affected the quality of semen by decreasing the sperm count, motility, viability, and morphology and an increase in apoptosis, which might contribute to male infertility.

Conclusion

We suggest that a reduction in GPx and SOD activity and an increase in CAT activity observed in our study are linked to an overproduction of ROS under microwave field exposure. Our findings on histone kinase, micronuclei, sperm cell cycle, and antioxidant enzymes point towards the possibility of infertility. A proposed hypothesis of the cycle of events is summarized in Fig. 2.

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