



Mobile phone (1800 MHz) radiation impairs female reproduction in mice, *Mus musculus*, through stress induced inhibition of ovarian and uterine activity

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ABSTRACT

Present study investigated the long-term effects of mobile phone (1800 MHz) radiation in stand-by, dialing and receiving modes on the female reproductive function (ovarian and uterine histo-architecture, and steroidogenesis) and stress responses (oxidative and nitrosative stress). We observed that mobile phone radiation induces significant elevation in ROS, NO, lipid peroxidation, total carbonyl content and serum corticosterone coupled with significant decrease in antioxidant enzymes in hypothalamus, ovary and uterus of mice. Compared to control group, exposed mice exhibited reduced number of developing and mature follicles as well as corpus lutea. Significantly decreased serum levels of pituitary gonadotrophins (LH, FSH), sex steroids (E2 and P4) and expression of SF-1, StAR, P-450scc, 3 β -HSD, 17 β -HSD, cytochrome P-450 aromatase, ER- α and ER- β were observed in all the exposed groups of mice, compared to control. These findings suggest that mobile phone radiation induces oxidative and nitrosative stress, which affects the reproductive performance of female mice.

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1. Introduction

The electronic phone/telephone is one of the greatest inventions created by man for communication in our daily life. After its invention, it has gone through many technological advances. Wireless hand-sets, touchtone phones and most recently the mobile phones and smartphones have all emerged in response to modern life style needs and globalization. During recent years, mobile phone, mobile phone towers and the base stations are widely encountered sources of microwave/radio frequency radiation in our environment. Despite the fact that there are detrimental effects of these radiations on the biological system (cardiovascular, nervous and reproductive), today mobile phone technology has become an integral and unavoidable part of everyday life. Its use will continue to grow as providers proceed to offer wide services and newer as well as better products/models of mobile phones as well as use of these devices for commercial purposes.

The mobile phones (2G and 3G) are microwave (MW) emitting devices. Mobile phones transmit/receive microwave signals to/from base station antennas. Mobile phones use lower frequency

microwave radiation in the range of 900/1800 MHz for 2G and 2100 MHz frequency band for 3G. Risk to the human user persists because our bodies can act as antennas that absorb these waves and convert them into eddy currents [1,2]. Several studies have suggested a relationship between mobile phone use and adverse effects on both male and female reproduction [3–6].

The significance of mobile phone radiation on reproduction is a key element of the debate since number of studies suggested that microwave radiation generated by mobile phone can negatively affect the quality of sperm, egg and embryo, which can potentially impair the reproduction of the humans and animals as well [7–12]. Recent studies suggest that mobile phone radiation can reduce the fertilizing potential of men by decreasing sperm count and altering their morphology [4,13–16]. However, some conflicting reports suggest lesser extent of adverse effects of mobile phone radiation on the reproduction or fertility of humans and rats [17–19]. This contradiction may be attributed to difference in the duration and frequency of mobile phone radiation exposures.

Numerous studies documented the deleterious effects of mobile phone radiation on female reproduction [6,20,21]. One such study reported that mobile phone exposure reduced the number of follicles in the ovaries of pregnant rat [22] and may cause endometrial apoptosis and oxidative stress [23]. Study on 90 women with uncomplicated pregnancies (age 18–33 years) and 30 full-term

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healthy newborn infants show that mobile phone exposure while on dialing mode for 10 min during pregnancy and after birth significantly increased the fetal and neonatal heart rate, and decreased fetal cardiac output [24]. The deleterious effects of low frequency electromagnetic field (EMF) on the ovarian and uterine tissues have also been investigated in several studies where these detrimental radiations have been shown to induce apoptosis in these cells [20,25]. A transmission electron microscopic (TEM) study describing effects of EMFs on female rats showed numerous lipid drops in the luteal and theca cells as well as increased number of macrophages and autophagy vacuoles in some granulosa cells [26]. It has also been shown that EMF exposures lead to the oocyte degeneration [27], increased the number of macrophages in the corpora lutea and growing follicles [20,25]. In addition, it is believed that the destruction of the ovarian cortical tissue, luminal epithelium, glandular epithelium, and stromal cells in the uterus and fallopian tubes through apoptosis is induced by EMF [28].

Repeated short-term exposure to radiofrequency-electromagnetic field (RF-EMF) also affects the course of pregnancy in female mice [29]. Significantly increased incidence of spontaneous abortion and fetal abnormalities in pregnant women is demonstrated following microwave radiation exposure [30]. Birth defects, miscarriages and increased ratio of fetal abnormalities have been documented in pregnant woman using EMF generating occupational monitors/computers [30]. The negative effects of EMF on fetal development are controversial in various animal models [31]. Another study also documented the EMF mediated inhibition in ovulation as well as reduction in the number of corpora lutea in rats [32]. On the other hand, some findings indicate controversial relationship between mobile phone radiation and reproductive performance. Ogawa and his group [33] evaluated the effects of radiofrequency-electromagnetic radiation (RF-EMR) on rat embryogenesis during gestational period and concluded that no adverse effects were observed on any maternal reproductive and embryo toxic parameters and fetuses (such as number of live, dead or resorbed embryos, placental weights, sex ratios, fetus weight; or external, visceral or skeletal abnormalities).

The studies conducted till date are very diverse, highly controversial and report different specific outcomes. The most important outcome would be to investigate the coincidence of infertility with/among heavy mobile phone users compared to those with little or no mobile phone use. Many studies have focused on deleterious effects of mobile phone radiation on male reproduction while the number of studies which demonstrated an adverse impact of mobile phone radiation on female reproduction is limited. Several researchers have already focused on the estrous cycle but the alterations in step-wise ovarian steroidogenic cascades, estrogen-progesterone levels and concurrence in E2 and P4 peaks, which governs the normal cyclicity in rodents, should be considered for future investigation. However, the long term effects of continuous exposure to mobile phone radiation on ovarian steroidogenesis as well as ovarian and uterine activity are not investigated yet. Moreover, it is necessary to conduct more studies concerning potential health effects of mobile phone radiation in different operative modes i.e. stand-by, dialing and receiving modes on female reproductive system following long term exposure. The significance of our study lies in the fact that the dialing (D) and receiving (R) modes are most frequently used by marketing call workers in an organization/industry whereas their mobile phones remain in stand-by (SB) mode less frequently. It is to be noted that mobile phones of general public remain in SB mode for larger fraction of time throughout the day. Hence, present experiment was designed to investigate the effects of long term (120 days) mobile phone exposure/irradiation (1800 MHz) on female reproductive system while handling mobile phone in different operative modes i.e., stand-by, dialing and receiving modes.

In the present study, we have investigated the alterations in stress parameters at the central (hypothalamic) and peripheral tissues (ovary and uterus) as well as monitored the serum levels of LH and FSH (pituitary gonadotrophins), checked the activity and expression pattern of steroidogenic proteins, enzymes in ovary and steroid receptors (ER α and β) in ovary and uterus, serum steroid levels.

2. Materials and methods

2.1. Animals

Twelve weeks old Swiss albino female mice (body weight ~ 30 g) were obtained 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-h photoperiod (12L:12D) and a relative humidity of $50 \pm 20\%$. 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

Randomly selected, 48 female mice showing regular cyclicity and exhibiting at least two consecutive 4–5 days estrous cycle were taken for this study. Before the start of the experiment, all mice of each group (N = 12/group) were housed in a single cage until the synchronization of their estrous cycle (i.e., mice of all 4 groups were in prolonged diestrus stage). The synchronization of estrous cycles in female mice was achieved by group-housing the females in the same cage (four cages, 12 mice per cage), for 10–14 days, prior to the start of our experiment [34]. The group housed female mice, in our study, showed prolonged diestrus phase. The diestrus stage in mice of all the four groups [i.e., sham control (Cont.), dialing (D), receiving (R) and stand-by (SB)] was confirmed by monitoring their vaginal cytology. Two independent experiments were performed and hence a total of 96 mice (24 mice per group) were used in this study. 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 h/day for 120 days) in different operative modes i.e., 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. From D-group mobile phones, only dialing signals (and not speech signals) were sent to the mobile phones of R-group. Dialing signals on the mobile phones of R-group were received so that both D- and R-group animals were in continuous condition of connectivity for $1\frac{1}{2}$ hrs, 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 $1\frac{1}{2}$ hrs. SB-group mice were kept beneath the “switched-on” mobile phones continuously for 3 h. 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. Details of mobile phone exposure and operative modes are compiled below in tabular form.

Groups	Sham Control	Stand-by	Dialing	Receiving
Mobile phone operative mode	“Switched-off”	“Switched-on”	Dialer in continuous condition of connectivity	Receiver in continuous condition of connectivity
Profile	<-----Silent with no vibration----->			
Exposure Duration	<-----3 h/day, continuously for 120 days----->			

Here we have used only one control (sham exposed) group. In our preliminary study, two control groups of mice were kept, one served as cage control and the other served as sham control (control kept under “switched-off” mobile phone). 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 ovarian and uterine histopathophysiology. Hence, to nullify the cage related and other external constraints, only one control group i.e., sham control was taken into account in this study.

Two independent experiments were performed and each experiment was conducted with a total of 48 mice and the data obtained were similar for both the experiments. Hence, the data presented in this study are from a total of 24 mice per group.

2.3. Mobile phone exposure set-up

Exposure set-up was designed in such a way that it contains six separate exposure boxes of equal size mounted on hexagonal strips of wood covered by carbon-impregnated styrofoam microwave absorber to reduce electromagnetic scattering. The dimension of each exposure box is 9.5 cm × 6 cm × 5 cm (length × width × height) accommodating single mouse at a time. The exposure boxes were made up of pine wood material having low dielectric constant to minimize the reflection and standing wave formation. Small circular holes of 0.6 cm diameter were drilled on the walls of each box for air ventilation. To reduce the reflection of MW, each exposure box is provided with MW absorber material (carbon impregnated styrofoam MW absorber) at the base and to minimize the interactions between the mobile phones, MW absorber is also provided in the central space of the hexagonal configuration of the exposure system consisting of six boxes. Further, a combination of pyramidal shaped absorbers is placed in the space between any two exposure boxes to minimize the interaction between/among the mobile phones. Apex of one of the pyramids is almost at the same level as the plane of mobile phone. Base of the absorber structure is at the level of bottom of the exposure box. The upper open part of the exposure box was fabricated to accommodate mobile phone which can be easily slid in and out above the mouse. Mobile phones were kept in the upper portion of each exposure box to irradiate the mouse kept below the mobile phone (Fig. 1). Back side of the mobile phone (and not the display/screen side) faces the animal. The display setting was adjusted in such a way that after 10 s, display used to turn off automatically. Each animal was kept in the separate exposure box of the cage throughout the exposure period. All the exposure boxes and mice were marked so that everyday mice were kept in the same exposure boxes in which they were kept previously to nullify the stress caused due to daily elimination of olfactory traces. Exposure boxes were designed in such a way that during the exposure time mice restrained very comfortably without any restraint stress (confirmed by serum corticosterone level monitoring prior to the start of the experiment). The temperature inside the exposure boxes as well as in the chamber was maintained at $24 \pm 2^\circ\text{C}$ throughout the experiment by circulating air. Mice were exposed for three hours, i.e. from 09:00 to 12:00 daily for 120 days continuously. Details of calculation of SAR values have been described in supplementary material.

Body weight of all the mice was monitored weekly and rectal temperature was measured during first, middle (after 60 days) and last three days of exposure. The mobile phone exposure does not cause any elevation in rectal temperature. The observed average rectal temperatures of the mice are: $36.93 \pm 0.2^\circ\text{C}$ (control group), $36.90 \pm 0.2^\circ\text{C}$ (D-group), $36.72 \pm 0.2^\circ\text{C}$ (R-group) and $36.14 \pm 0.2^\circ\text{C}$ (SB-group) before, and $36.98 \pm 0.2^\circ\text{C}$ (control group), $36.07 \pm 0.2^\circ\text{C}$ (D-group), $36.83 \pm 0.2^\circ\text{C}$ (R-group) and $36.64 \pm 0.2^\circ\text{C}$ (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.

2.4. Blood collection and tissue sampling

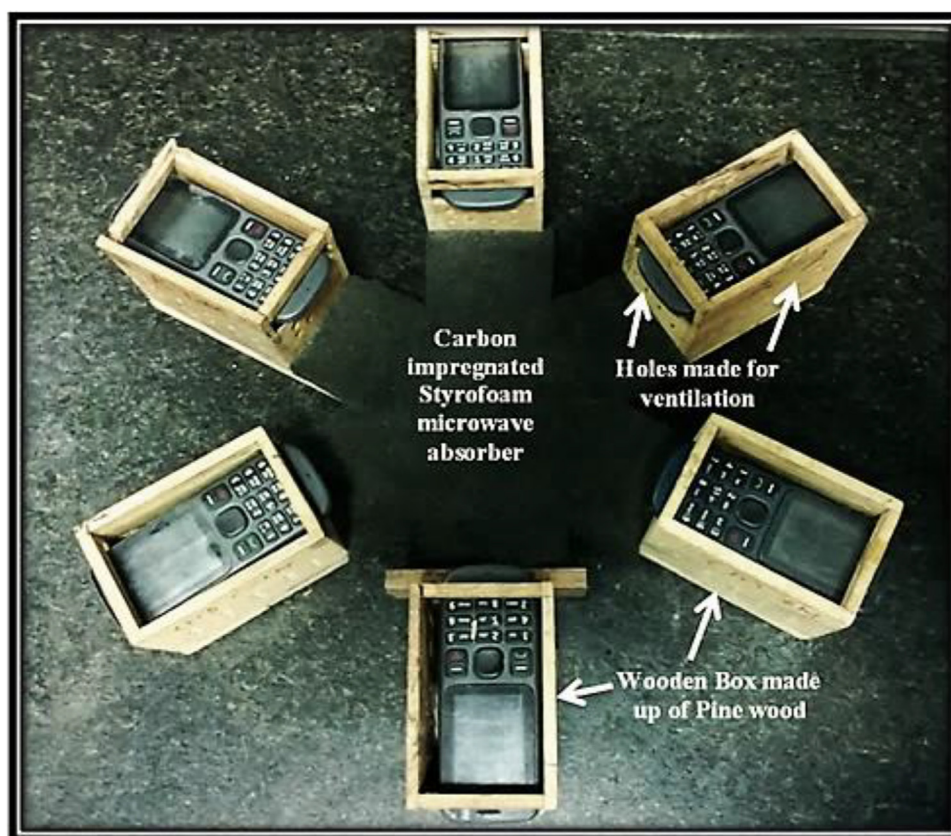
All mice were monitored for vaginal smear cytology for 2 consecutive estrous cycles prior to sacrifice. After the termination of the exposure, mice were weighed and sacrificed at the diestrus stage of estrous cycle by decapitation. Blood was collected in non-heparinized tubes for the evaluation of serum concentration of corticosterone, LH, FSH, E_2 and P_4 and blood smear slides were prepared to determine the neutrophil/lymphocyte ratio (N/L ratio) by the hemocytometer method. Further, hypothalamus of all the 24 mice in each group, uterus and both side ovaries of 12 mice [(N = 12, 6 from Experiment1 (E1) + 6 from Experiment2 (E2))] were used for biochemical estimations. The right ovaries of other 12 mice (N = 12, 6 from E1 and 6 from E2) were used for immunoblot analysis of cytochrome *P-450sc*, and the left ovaries of these 12 mice (N = 12, 6 from E1 and 6 from E2)] and uterus of 12 mice (N = 12, 6 from E1 and 6 from E2)] were processed for Hematoxylin-eosin (HE) staining and immunofluorescence study.

2.4.1. Histology of ovary and uterus

Tissues fixed in 10% neutral formalin solution overnight were processed for routine paraffin embedding. The tissues were dehydrated through increasing concentrations of ethanol and embedded in paraffin prior to sectioning. 6 μm -thick sections of ovaries were cut by Leica RM2125 RT rotatory microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany). HE staining was used for histological evaluation using bright field microscope (Axioskop 2 Plus; Carl Zeiss, Oberkochen, Germany) and the images were captured with HRcam camera (Axioskop 2 Plus; Carl Zeiss).

2.4.2. Counting of follicles number

The total count of healthy follicles (excluding the primordial and atretic follicles), differential count of follicles (i.e., number of different types of follicles excluding primordial follicles) and total count of corpus lutea were done in the ovaries by examining every fifth serial section of each ovary. The developing and mature follicles all with intact oocytes, considered as healthy follicles, were classified in: a) primary follicles, b) secondary follicles, c) antral follicle or Graafian follicles and d) pre-ovulatory follicles [35]. The number of atretic follicles (non-healthy) was also counted. The atretic follicles were characterized by apoptotic bodies/pyknotic cell nuclei in the granulosa cells or degenerating oocytes or changes



Positioning of mobile phone antenna with respect to mouse trunk

Fig 1. Mobile phone exposure setup and the position of mobile phones during exposure. Mice were exposed to non-thermal microwave radiation for 3 h/day continuously for 120 days by using mobile phones.

in the oocytes morphology (e.g. deformed shape, vacuolation, loss of nuclear membrane and/or fragmentation) [36].

2.5. Isolation of serum for the ELISA of corticosterone, LH, FSH, 17 β -estradiol and progesterone

Blood samples were kept at 4 °C for 30 min and then centrifuged at 4000 rpm/1252.16g at 4 °C for 15 min. The supernatant was collected as serum and processed for Corticosterone, LH, FSH, Estrogen (17 β -estradiol) and Progesterone assay by using commercial ELISA kits, according to the manufacturer's instructions.

For serum Corticosterone levels, an enzyme immunoassay was performed using a commercial DRG Corticosterone enzyme immunoassay Kit [DRG® Corticosterone (Rat/Mouse) Elisa (EIA-5186), IBL International GmbH, Hamburg, Germany] following the manufacturer's protocol. The antiserum used in the assay was specific for corticosterone. The cross-reactivity of the assay was 100% with Corticosterone, 0.2% with Aldosterone, 0.3% with Cortisol, 2.4% with 11-Deoxycorticosterone, but cross-reactivity with other related steroids/steroid derivatives is not detectable. The analytical

sensitivity of the assay was 4.1 ng ml⁻¹. The intra-assay coefficient of variation (CV) was <3% whereas the inter-assay CV was <5%.

For serum LH and FSH levels, an enzyme immunoassay was performed using a commercial mouse LH (Luteinizing Hormone) ELISA kit [Cat.No: MBS041300, MyBioSource, Inc., San Diego, USA] and mouse Follicle Stimulating Hormone (FSH) ELISA kit [Cat.No: MBS727159, MyBioSource, Inc., San Diego, USA] following the manufacturer's protocol.

For serum 17 β -estradiol levels, an enzyme immunoassay was performed using a commercial DRG Estradiol enzyme immunoassay Kit [DRG® Estradiol ELISA (EIA-2693), IBL International GmbH, Hamburg, Germany] following the manufacturer's protocol. The antiserum used in the assay was specific for 17 β -estradiol. The cross-reactivity of the assay was 100% with 17 β -estradiol, 0.05% with estriol, 0.2% with estrone and 0% with other related steroids/steroid derivatives. The analytical sensitivity of the assay was 9.714 pg ml⁻¹. The intra-assay coefficient of variation (CV) was <3% whereas the inter-assay CV was <7%.

For serum progesterone levels, an enzyme immunoassay was performed using a commercial Progesterone Enzyme Immunoassay Kit [Progesterone ELISA (RE52231), IBL International GmbH,

Hamburg, Germany] following the manufacturer's protocol. The antiserum used in the assay was specific for progesterone. The cross-reactivity of the assay was 100% with progesterone, 0.3% with 17α OH progesterone, <0.10% with estriol, <0.10% with 17β -estradiol, <0.10% with testosterone, 1.10% with 11-desoxycorticosterone, <0.02% with DHEA-S, <0.02% with Cortisol, 0.20% with corticosterone, 0.35% with pregnenolone, <0.10% with cortisone and 0.10% with 11-Desoxycortisol. The analytical sensitivity of the assay was 0.045 ng ml^{-1} . The intra-assay coefficient of variation (CV) was <6% whereas the inter-assay CV was <5%.

2.6. Tissue homogenization for biochemical estimations and immunoblot analysis

10% homogenate of hypothalamus and uterus for each animal was prepared separately. For ovary, 10% homogenate was prepared by pooling the four ovaries (2 right and 2 left ovaries) from randomly selected two mice and a total of 12 mice were used for the sampling of each group. 10% (w/v) homogenate for both control and experimental (D, R and SB) 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 for both control and experimental (D, R and SB) groups after adding protease inhibitor ($0.2 \mu\text{M}$ PMSF) were centrifuged at 12,000 rpm/11269.44g 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)] and total carbonyl content.

Right side ovaries of 6 mice from each experiment ($N=12$, 6 from E1 and 6 from E2) were pooled and sonicated thoroughly and processed for immunoblot analysis. 10% (w/v) homogenate was prepared for both control and experimental (D, R and SB) groups in a protein extraction buffer containing 50 mM Tris-HCl (pH 7.6), 0.25 M sucrose, 150 mM NaCl, 1 mM EDTA (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, leupeptin ($1 \mu\text{g/ml}$), aprotinin ($2 \mu\text{g/ml}$) and phenylmethylsulfonyl fluoride ($100 \mu\text{g/ml}$) (PMSF), 10 mM DTT, using a Polytron homogenizer and kept on ice for 20 min. After centrifugation at 12,000 rpm/11269.44g for 25 min at 4°C , supernatant was collected. Total protein concentration was measured by Bradford's method using bovine serum albumin (BSA) as the standard [37].

2.7. Total ROS measurement

Total ROS generation in fresh homogenate of different tissues was measured as described previously [38] with slight modifications. 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, the homogenate was diluted in PBS to obtain a concentration of $25 \mu\text{g}$ tissue protein/ml. The reaction mixture containing diluted homogenate and $10 \mu\text{l}$ of DCFH-DA ($10 \mu\text{M}$) 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.

2.8. Measurement of total nitrite and nitrate concentration

The level of Nitric oxide (NO), a reactive free radical which is generally oxidized to NO_x (nitrite- NO_2^- /nitrate- NO_3^-) was measured as described previously [39]. The total nitrite and nitrate concentration was estimated in different tissues (hypothalamus, ovary and uterus) by using acidic Griess reaction for color development after the reduction of nitrate with copper-cadmium alloy and deproteinization. Briefly, reaction mixture containing $100 \mu\text{l}$ of sample or standard (KNO_3), $400 \mu\text{l}$ of carbonate buffer and a small amount (0.15 g) of activated copper-cadmium alloy fillings (washed in buffer and dried on a filter paper) in the tube was incubated at room temperature for 1 hr with thorough shaking. The reaction was stopped by the addition of $100 \mu\text{l}$ of 0.35 M sodium hydroxide (NaOH), followed by $400 \mu\text{l}$ of 120 mM zinc sulphate (ZnSO_4) solution under vortex and allowed to stand for 10 min. The tubes were then centrifuged at 8000 rpm/5008.64g for 10 min $100 \mu\text{l}$ aliquots of the clear supernatant were transferred into the wells of a microplate (in quadruplicate) and Griess reagent ($50 \mu\text{l}$ of 1.0% sulphanilamide prepared in 2.5% orthophosphoric acid and $50 \mu\text{l}$ 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.

2.9. Lipid peroxidation assay

Peroxidative damage to cellular lipid constituents was determined by measuring malondialdehyde (MDA), the end product of lipid peroxidation as described previously [40] with slight modifications. MDA reacts with thiobarbituric acid (TBA) to yield a colored compound. Briefly, to $100 \mu\text{l}$ of tissue homogenate, $50 \mu\text{l}$ of 0.8% butylated hydroxytoluene (BHT), $750 \mu\text{l}$ 0.8% of TBA, $750 \mu\text{l}$ of 20% acetic acid (pH 3.5) and $100 \mu\text{l}$ of 8% SDS were added. The reaction mixture was then incubated at 95°C in water bath for 1 h. After this, the mixture was cooled down by keeping on ice for 15 min and then centrifuged at 2000 rpm/313.04g for 10 min. Absorbance was taken at 532 nm and the result was expressed as nmoles MDA/mg protein.

2.10. Estimation of total carbonyl groups

Hypothalamus, ovary and uterus homogenates of different mobile phone irradiated groups in duplicate aliquots (approximately $500 \mu\text{g}$ of protein) were treated with 10% (w/v) trichloroacetic acid (TCA) [41]. Pellets were collected by high-speed centrifugation and suspended by probe sonication in $200 \mu\text{l}$ of 2 N HCl or $200 \mu\text{l}$ of 10 mM DNPH in 2 N HCl. After incubation for 1 h at room temperature, proteins were precipitated again with TCA. Removal of unreacted DNPH was carried out by washing the pellets once with 1 ml of 10% (w/v) TCA and thrice with 1 ml of ethanol:ethyl acetate ($1:1 \text{ v/v}$). Proteins were dissolved by sonication in 1 ml of 100 mM Tris-HCl buffer, pH 7.5, containing 2% SDS. Insoluble material was removed by centrifugation. The absorbance of the supernatant of the HCl-treated sample was assessed at 360 nm and subtracted from that of the DNPH-treated sample. The amount of carbonyl groups was calculated by using a molar extinction coefficient of $22,000 \text{ cm}^{-1}$ at 360 nm. It is noteworthy that this method is considered by many to be an indicator of total protein carbonylation [42]. However, DNPH also reacts with non-PCO-containing compounds e.g., nucleic acids; [41] that are both present in the tissue samples and precipitated with TCA. These values were considered to be a measure of total carbonyls (TCOs) rather than of protein carbonyl (PCOs) [43].

2.11. Biochemical estimation of antioxidant enzymes

2.11.1. Superoxide dismutase assay

The total SOD activity in different tissues was assayed as described previously [44]. 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. Absorbance was taken at 543 nm. SOD value was calculated in terms of unit 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/mg of protein.

2.11.2. Catalase assay

Catalase activity in different tissues was measured as described previously [45]. Briefly, the reaction mixture contained 1.9 ml of 50 mM phosphate buffer (pH 7.0) and appropriate dilution of the tissue supernatant to make the volume 2 ml. The reaction was initiated by the addition of 1 ml of freshly prepared 30 mM 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 $43.6\text{ M}^{-1}\text{cm}^{-1}$.

2.11.3. Glutathione peroxidase assay

GPx activity was estimated in different tissues as described previously [46] with slight modification. The reaction mixture (1 ml) containing 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 of glutathione reductase was allowed to equilibrate for 1 min at room temperature. After this, the reaction was initiated by the addition of 100 mM H_2O_2 . The absorbance was 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.

2.12. Biochemical estimation of ovarian steroidogenic enzymes: 3β -HSD and 17β -HSD

To observe the effect on steroidogenic pathway, ovarian 3β -hydroxysteroid dehydrogenase (3β -HSD) and 17β -hydroxysteroid dehydrogenase (17β -HSD) were measured as described previously [47,48] with minor modifications. Ovary was homogenized in 20% spectroscopic grade glycerol containing 5 mM potassium phosphate and 1 mM EDTA and subsequently centrifuged at 10,000g for 30 min at 4°C. The supernatant was used for enzyme assay.

2.12.1. 3β -HSD activity

3β -HSD was measured by mixing 250 μ l of the supernatant with 250 μ l of 100 μ M sodium pyrophosphate buffer, pH 8.9, 10 μ l ethanol containing 30 μ g DHEA (Sigma, St. Louis, USA) and 240 μ l of 25 mg% BSA (Bangalore Genei, India). Enzyme activity was measured after addition of 50 μ l of 0.5 μ M NAD to the mixture in a spectrophotometer at 340 nm against a blank (without NAD). One unit of enzyme activity is the amount causing a change in absorbance of 0.001/min at 340 nm.

2.12.2. 17β -HSD activity

17β -HSD was measured by mixing 250 μ l of the supernatant with 250 μ l of 440 μ M sodium pyrophosphate buffer (pH 10.2), 10 μ l ethanol containing 0.3 μ M testosterone (Sigma, St. Louis, USA) and 240 μ l of 25 mg% BSA. Enzyme activity was measured after addition of 50 μ l of 0.5 μ M NAD to the mixture in a spectrophotometer at 340 nm against a blank (without NAD). One unit of enzyme activity is the amount causing a change in absorbance of 0.001/min at 340 nm.

2.13. SDS-PAGE and western blot analysis

Equal amount of protein (50 μ g) from each group was resolved on 12% SDS–polyacrylamide gel (SDS–PAGE) for 30 min and then at 100 V for one hour, using the Mini-PROTEAN tetra system, BIO-RAD, USA. The resolved proteins were transferred from the polyacrylamide gel to PVDF membrane (Millipore, USA) by electro-blotting for 15 min in a Semi-Dry blotting apparatus (TRANS-BLOT SD, Semi-dry transfer cell, BIO-RAD, USA) 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 1 h at room temperature. The membrane was then incubated with a rabbit raised polyclonal cytochrome P-450scc/CYP 11 A1 (dilution 1:5000) for overnight (16 h) at 4°C. Next day, after washing in TBST thrice for 10 min each, the membrane was incubated with anti-rabbit IgG-HRP conjugate secondary antibody (dilution 1:2500 containing 2% (w/v) fat free skimmed milk) for 1 h at room temperature. The membrane was then washed for three times (15 min each) in TBST and the immunoreactive protein band was visualized by using Super Signal™ West Pico Chemiluminescent Substrate, Thermo Scientific Rockford, IL, USA; Catalog number: 34080. 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, USA). 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 P-450scc was calculated by dividing the IDV of a protein band by the IDV of the β -actin. Data represent the mean $\pm$ SEM of at least 3 independent experiments.

2.14. Immunofluorescence of SF-1, StAR, 3β -HSD, P450arom, ER- α and ER- β in ovary

Immunofluorescences for steroidogenic factor-1 (SF-1/Ad4BP), steroidogenic acute regulatory protein (StAR), 3β -hydroxysteroid dehydrogenase (3β -HSD) and cytochrome P450 aromatase (P450arom/CYP 19 A1) in ovary and, estrogen receptor- α (ER- α) and estrogen receptor- β (ER- β) in both ovary and uterus were carried out on neutral formalin-fixed paraffin-embedded tissue sections using polyclonal antibodies. Immunofluorescence was performed in a two-step procedure as described previously [49]. Briefly, in the first step, after initial deparaffinization in xylene and rehydration in graded series of alcohol of ovary section slides, antigen retrieval was performed using 1 M citrate buffer (pH 6) in a microwave oven at 1000 W (3–4 min). Tissue sections were then treated with blocking solution for 2 h at room temperature (5% HGS- Heat Inactivated Goat serum) and then sections were incubated with rabbit-anti bovine-SF-1 (Ad4BP) polyclonal antibody (1:500), rabbit-StAR antisera (1:1000), rabbit- 3β -HSD antisera (1:500), rabbit-aromatase (P450arom/CYP 19 A1) polyclonal antibody (1:1000), rabbit-ER- α (H-184) (1:200) and mouse-ER- β monoclonal antibody (CFK-E12) (1:100) for 24 h in humid chamber. In second step, sections were washed with TBS (3 $\times$ 5 min) and then incubated with donkey-anti-rabbit IgG H & L (conjugated with FITC, Santa Cruz Biotechnology, Santa Cruz, USA), goat anti-rabbit IgG H & L (conjugated with TRITC abcam, ab6718) and goat-anti-mouse IgG H & L (conjugated with FITC, abcam, ab6785) for 3 h 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) were 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).

2.15. Image analysis

Image analysis was performed as described elsewhere [49]. Briefly, for integrated optical density (IOD) measurement, immunofluorescences of ovary and uterus 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, USA). Using the software's tool, IOD values of all the areas showing positive signals in different regions of the tissues section were measured after normalizing to the background. The IOD values (relative units) were averaged (four middle sections per ovary/uterus and twelve ovary/uterus per group) to determine signal density. Average IOD values of ovary/uterus 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 < 0.05), the IOD values were labeled or classified as intense, moderate or weak.

3. 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 $\pm$ SEM. One-way ANOVA followed by *post hoc* analysis (Dunnett T3) was used to determine the difference in the level of significance among four different groups. p value of < 0.05 was considered as significant. Data were statistically analyzed by using the Statistical Package Statistical Analysis System (SPSS Statistics 17.0, Chicago, USA) software.

4. Results

The body weights of all the mobile phone exposed group mice do not show any significant change compared to control during and after the end of exposure.

4.1. Ovarian histology and follicle number

A marked alteration was evident in the histoarchitecture of the ovary of mobile phone exposed group of mice compared to control group having well developed Graafian follicles or antral follicles. In general, significant degenerative changes in the ovary were observed in all the mobile phone radiations exposed group of mice as compared to control (Fig. 2A). Ovary of control group mice showed number of developing follicles, Graafian follicles, Corpora lutea and few atretic follicles along with stroma interstitial. Total number of healthy follicles (excluding the primordial and atretic follicles) decreased in exposed group compared to control. D-group ovaries showed comparatively less number of developing and Graafian follicles and few corpus lutea. Significant increase in the number of atretic follicles i.e. follicles with vacuolation (due to oocyte degeneration) and apoptotic bodies were observed in D-group. Receiving mode exposure to mice caused a significant decrease in the number of developing and mature follicles with concomitant increase in the number of non-developing/undeveloped and vacuolated follicles. The ovaries of SB-group of mice revealed further significant increase in the number of early stage degenerated follicles with apoptotic bodies i.e. atretic follicles along with fewer number of developing follicles, Graafian follicles and corpus lutea (Fig. 2B i–iii; Table 2). Histologi-

cally also, the effects were more severe in case of R- and SB- groups of animals.

4.2. Histology of uterus

As compared to control, except in D-group, the diameter of the uterus decreased in R-group and SB-group of mice. The thickness of endometrium as well as myometrium also decreased in R- and SB-groups of mice. The degenerative appearance of endometrium and myometrium was also evident in R- and SB-groups of mice compared to D-group as well as organized histoarchitecture of control group (Fig. 2C)

4.3. Neutrophil/Lymphocyte ratio and serum corticosterone level

A significant increase in N/L ratio (Fig. 3A) and corticosterone level (Fig. 3B) was observed in all the mobile phone exposed group of animals compared to control.

4.4. ROS level, total nitrite and nitrate concentration, MDA level and total carbonyl content

A significant elevation in the total ROS production was noted in the hypothalamus, ovaries and uterus of all the exposed groups of mice compared to control. The level of ROS was found significantly high in SB-group of animals compared to D- and R-groups of animals as well as control (Fig. 4A). Similar trend was also noted for total nitrite and nitrate concentration (Fig. 4B), MDA level (Fig. 4C) and total carbonyl content (Fig. 4D) in hypothalamus, ovary and uterus of all the mobile phones exposed groups compared to the control group.

4.5. Antioxidant enzymes activity

SOD, CAT and GPx activity was found to be decreased significantly in the hypothalamus, ovary and uterus of mobile phone radiation exposed group of mice when compared to control (Fig. 5A–C). The D- and R-groups also show a significant decrease compared to control but this reduction is comparatively less than SB-group.

4.6. Activity of ovarian steroidogenic enzymes (3β -HSD and 17β -HSD) and serum LH, FSH, estrogen and progesterone levels

The activity of ovarian steroidogenic enzymes, 3β -hydroxysteroid dehydrogenase (3β -HSD) and 17β -hydroxysteroid dehydrogenase (17β -HSD) decreased significantly in all the mobile phone radiation exposed group of mice (Fig. 6A).

A significantly decreased level of serum LH (Fig. 6B), FSH (Fig. 6C), estrogen (E_2) and progesterone (P_4) was also noted in all the mobile phone exposed mice (Fig. 6D) compared to control. Highly significant reduction in serum LH, FSH, estrogen and progesterone levels were observed in SB- and R-groups of mice compared to control and D-groups ($C > D > R > SB$).

4.7. Western blot analysis of cytochrome P-450scc

Western blot shows down regulation of cytochrome P-450scc expression in the ovaries of mobile phone radiation exposed group compared to control (Fig. 7). Densitometric analysis of western blot of cytochrome P-450scc also showed significantly decreased expression of this protein in the ovaries of mobile phone exposed group compared to control.

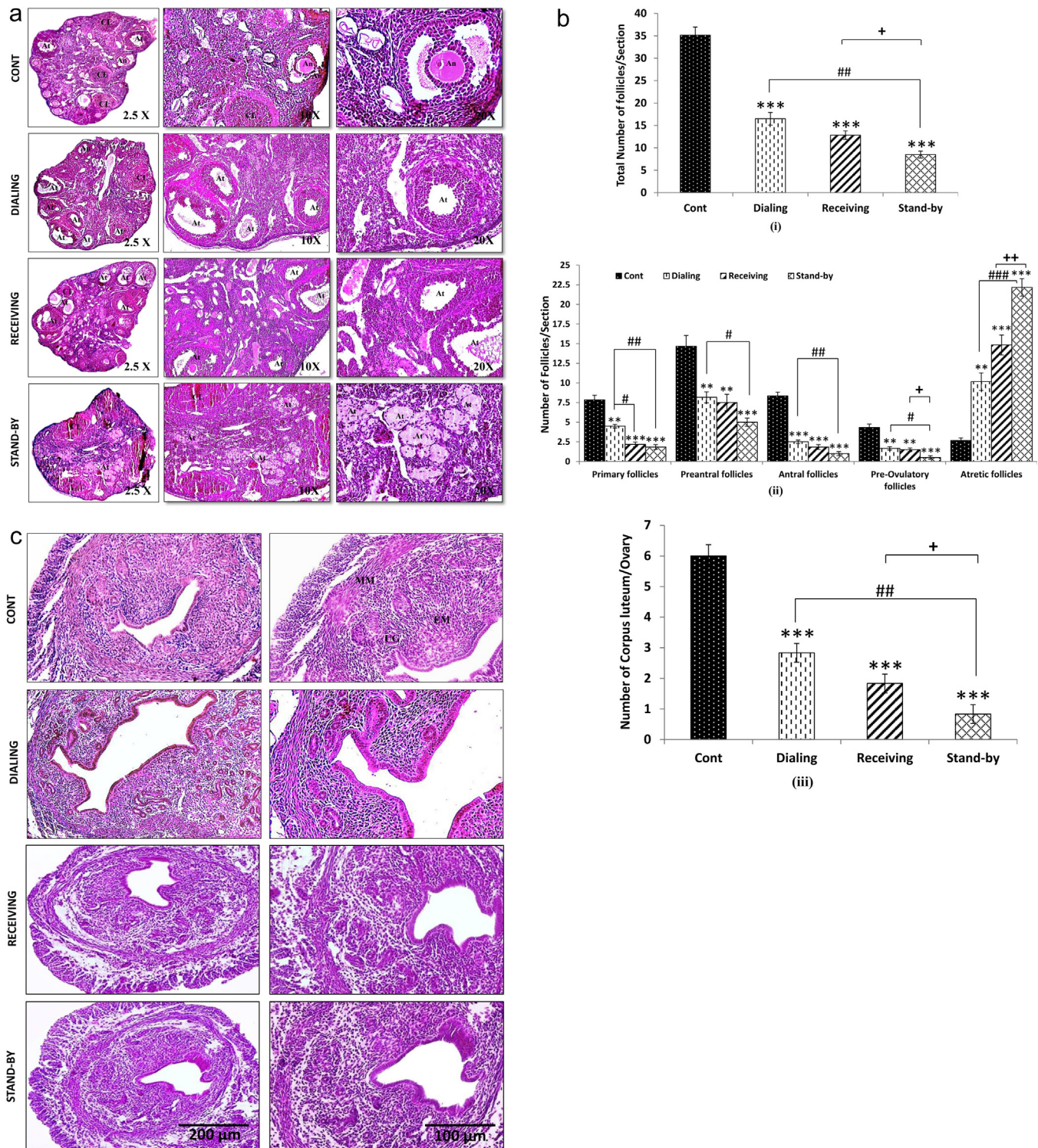


Fig. 2. (A) Transverse Section of ovary of mice exposed to mobile phone radiation in dialing (D-group), receiving (R-group), and stand-by (SB-group) mode: Note significant decrease in the healthy and mature follicles and corpus luteum in mobile phone exposed group of mice compared to control. The ovary of control mice shows healthy antral follicles (An), normal corpus luteum (CL) and a few atretic (At) follicles. D-group ovary shows comparatively less number of developing and antral follicles (An), few corpus luteum and more atretic (At) follicles. Receiving mode exposure to mice caused a decrease in the number of healthy follicles with concomitant increase in the number of non-developing and many atretic follicles. The ovaries of SB-group of mice revealed degenerated appearance with large number of atretic (At) follicles along with fewer numbers of developing follicles. (B) Effect of mobile phone radiation on (i) Total number of follicles (excluding the primordial and atretic follicles), (ii) Number of different types of follicles, and (iii) Total number of corpus luteum of mice. Significant reduction in total number of healthy follicles and corpus luteum was observed in all the mobile phone radiation exposed group of mice compared to control. Values are expressed as mean $\pm$ SEM (N = 12). Significance of difference from control, ** $p < 0.01$, *** $p < 0.001$; significance of difference from D-group # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$; significance of difference from R-group + $p < 0.05$, ++ $p < 0.01$. (C) Transverse Section of uterus of mice exposed to mobile phone radiation in dialing (D-group), receiving (R-group) and stand-by (SB-group) mode: Histologically as well as the diameter of uterine horn of D-group mice was not different from control exhibiting normal and healthy appearance. Diameter of the uterine horn and thickness of both myometrium (MM) as well as endometrium (EM) decreased in R-group and SB-group of mice compared to control. Degenerative appearance of both MM and EM including uterine glands (UG) is also evident in R- and SB-group of mice compared to control.

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

Antibodies	Control	Dialing	Receiving	Stand-by
SF-1 in Ovary	14.23 $\pm$ 0.188 (intense)	1.72 $\pm$ 0.092 (moderate)	0.368 $\pm$ 0.025 (weak)	0.118 $\pm$ 0.006 (weak)
StAR in Ovary	36.17 $\pm$ 0.537 (intense)	3.12 $\pm$ 0.178 (moderate)	1.50 $\pm$ 0.083 (weak)	1.02 $\pm$ 0.073 (weak)
3 β -HSD in Ovary	27.49 $\pm$ 0.620 (intense)	12.43 $\pm$ 1.239 (moderate)	7.39 $\pm$ 0.483 (weak)	5.65 $\pm$ 0.312 (weak)
P-450arom in Ovary	61.13 $\pm$ 1.108 (intense)	13.29 $\pm$ 0.552 (moderate)	7.97 $\pm$ 0.328 (weak)	4.51 $\pm$ 0.295 (weak)
ER- α in Ovary	68.44 $\pm$ 1.226 (intense)	27.43 $\pm$ 0.805 (moderate)	10.29 $\pm$ 0.309 (weak)	0.885 $\pm$ 0.104 (weak)
ER- α in Uterus	83.11 $\pm$ 4.167 (intense)	35.63 $\pm$ 3.224 (moderate)	14.83 $\pm$ 1.109 (weak)	13.71 $\pm$ 0.997 (weak)
ER- β in Ovary	35.84 $\pm$ 1.136 (intense)	11.82 $\pm$ 0.466 (moderate)	4.75 $\pm$ 0.137 (weak)	1.379 $\pm$ 0.145 (weak)
ER- β in Uterus	37.56 $\pm$ 2.604 (intense)	5.74 $\pm$ 0.623 (moderate)	2.37 $\pm$ 0.519 (weak)	2.05 $\pm$ 0.380 (weak)

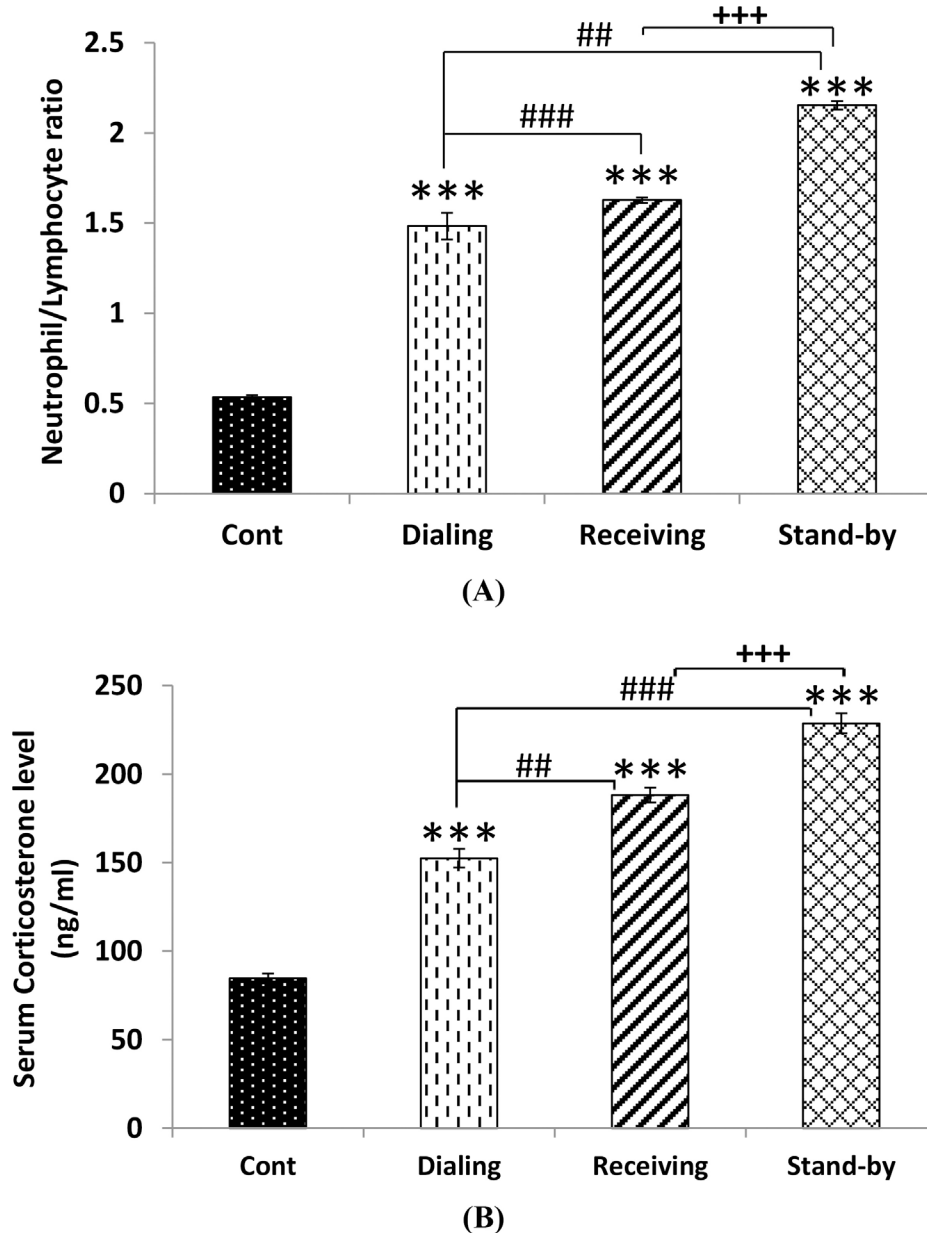


Fig. 3. Effect of mobile phone radiation on the (A) Neutrophil/lymphocyte (N/L) ratio and (B) Serum corticosterone level of different group of mice. Mobile phone exposure significantly increase the neutrophil/lymphocyte (N/L) ratio and serum corticosterone level compared to control. This increase was highly significant in SB-group of mice. Data is presented as mean $\pm$ SEM (N = 24). Significance of difference from control, ***p < 0.001; significance of difference from D-group ##p < 0.01, ###p < 0.001; significance of difference from R-group +++p < 0.001.

4.8. Immunofluorescence of SF-1, StAR, 3 β -HSD, P450arom, ER- α and ER- β

The expression of SF-1, StAR, 3 β -HSD, P450arom in ovary, ER- α and ER- β in the ovary and uterus of all the exposed group of mice

showed a marked decrease as compared to control (Fig. 8). In control group ovaries, intense immunoreactivity for SF-1 (Fig. 8A), StAR (Fig. 8B) and 3 β -HSD (Fig. 8C) was observed in thecal and interstitial regions. D-group showed moderate immunoreactivity for these

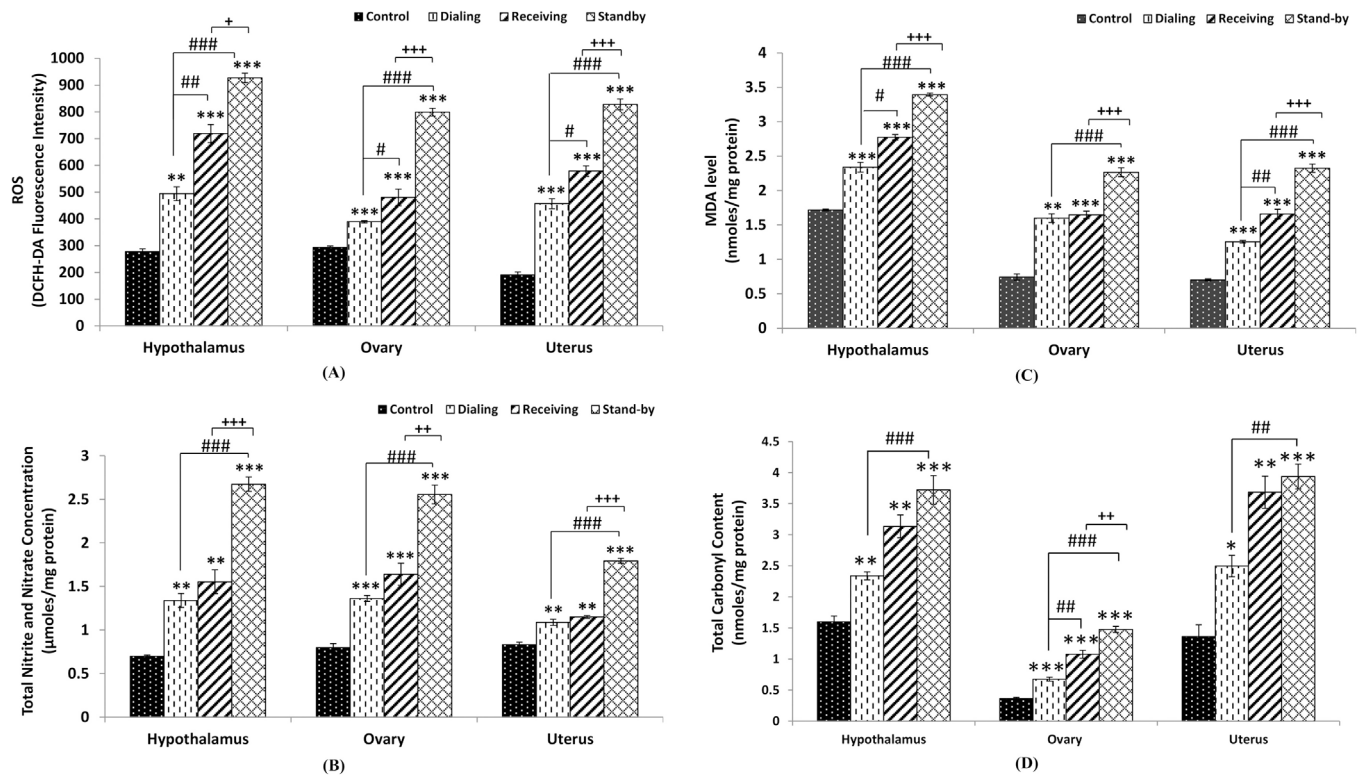


Fig. 4. (A) DCFH-DA fluorescence intensity (measure of ROS), (B) Total nitrite and nitrate concentration, (C) Lipid peroxidation test and (D) Total carbonyl content (oxidative damage to nucleic acids and proteins) in hypothalamus, ovary and uterus of mice exposed to mobile phone radiation in Dialing, Receiving and Stand-by mode. Compared to control, mobile phone exposed mice shows a significant increase in total ROS level, total nitrite and nitrate concentration, malondialdehyde (MDA) level and total carbonyl content in different tissues. The increase was highly significant in case of SB-group of mice. Results are expressed as mean $\pm$ SEM (N = 12). Significance of the difference was determined by using the One-Way ANOVA. ** $p < 0.01$, *** $p < 0.001$ significance of difference from control; significance of difference from D-group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; significance of difference from R-group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 2

Mean ($\pm$ SEM) value of total number of follicles, types of follicles and total number of corpus luteum.

Experimental groups	Control	Dialing	Receiving	Stand-by
Total number of follicles/section (excluding atretic follicles)	35.17 $\pm$ 1.797	16.50 $\pm$ 1.408	12.84 $\pm$ 0.946	8.50 $\pm$ 0.764
Types of follicles/section				
Primary follicles	7.83 $\pm$ 0.601	4.50 $\pm$ 0.224	2.17 $\pm$ 0.307	1.833 $\pm$ 0.307
Preantral follicles	14.67 $\pm$ 1.382	8.17 $\pm$ 0.703	7.50 $\pm$ 1.056	5.00 $\pm$ 0.516
Antral follicles	8.34 $\pm$ 0.494	2.5 $\pm$ 0.224	1.84 $\pm$ 0.307	1.00 $\pm$ 0.307
Pre-ovulatory follicles	4.34 $\pm$ 0.422	1.67 $\pm$ 0.211	1.50 $\pm$ 0.224	0.50 $\pm$ 0.223
Atretic follicles	2.67 $\pm$ 0.334	10.18 $\pm$ 1.108	14.840 $\pm$ 1.276	22.170 $\pm$ 1.108
Total number of corpus luteum/ovary	6.00 $\pm$ 0.365	2.84 $\pm$ 0.307	1.84 $\pm$ 0.307	0.834 $\pm$ 0.308

proteins and enzymes in thecal and interstitial region while R- and SB-groups showed very weak immunoreactivity respectively in these regions of the ovary compared to control.

The expression of P450arom was found to be significantly decreased in the ovary of all mobile phone radiation exposed group as compared to the control group. Intense immunoreactivity for P450arom was observed in granulosa cells, thecal and interstitial regions of control group. D-group showed moderate immunoreactivity for P450arom in thecal and interstitial regions of the ovary while only few granulosa cells showed weak immunoreactivity. R- and SB-groups exhibited very weak immunoreactivity for P450arom in granulosa cells, thecal and interstitial regions of the ovary compared to control group (Fig. 8D).

Similar trend of immunoreactivity was observed for ER- α in the ovary of control and exposed groups of mice (Fig. 8E i). Intense immunoreactivity for ER- α was observed in thecal and interstitial regions of ovary of control group as compared to D-, R- and SB-groups of mice. D- and R-groups showed moderate immunostaining while SB-group showed very weak or almost no

immunostaining for ER- α in thecal and interstitial regions of ovary (Fig. 8E i).

The number of ER- β positive granulosa cells was found to be significantly decreased in the ovary of mobile phone radiation exposed group as compared to the control. The number of ER- β positive granulosa cells is more in control group ovary. D-group ovary reveals less number of ER- β positive granulosa cells while in R- and SB-groups ovaries, only few granulosa cells showed very weak immunoreactivity for ER- β (Fig. 8F i).

Control group mice uterus showed intense immunostaining for ER- α (Fig. 8E ii) and ER- β (Fig. 8F ii) in luminal epithelium (LE), epithelium lining of uterine glands (UG) and endometrial stroma (ES) region. D-group showed moderate while R- and SB-groups showed weak immunostaining for ER- α and ER- β in luminal epithelium and epithelium lining of uterine glands. Very weak or no immunoreactivity was observed in the endometrial stroma of all the exposed groups of uterus (Fig. 8E ii and F ii).

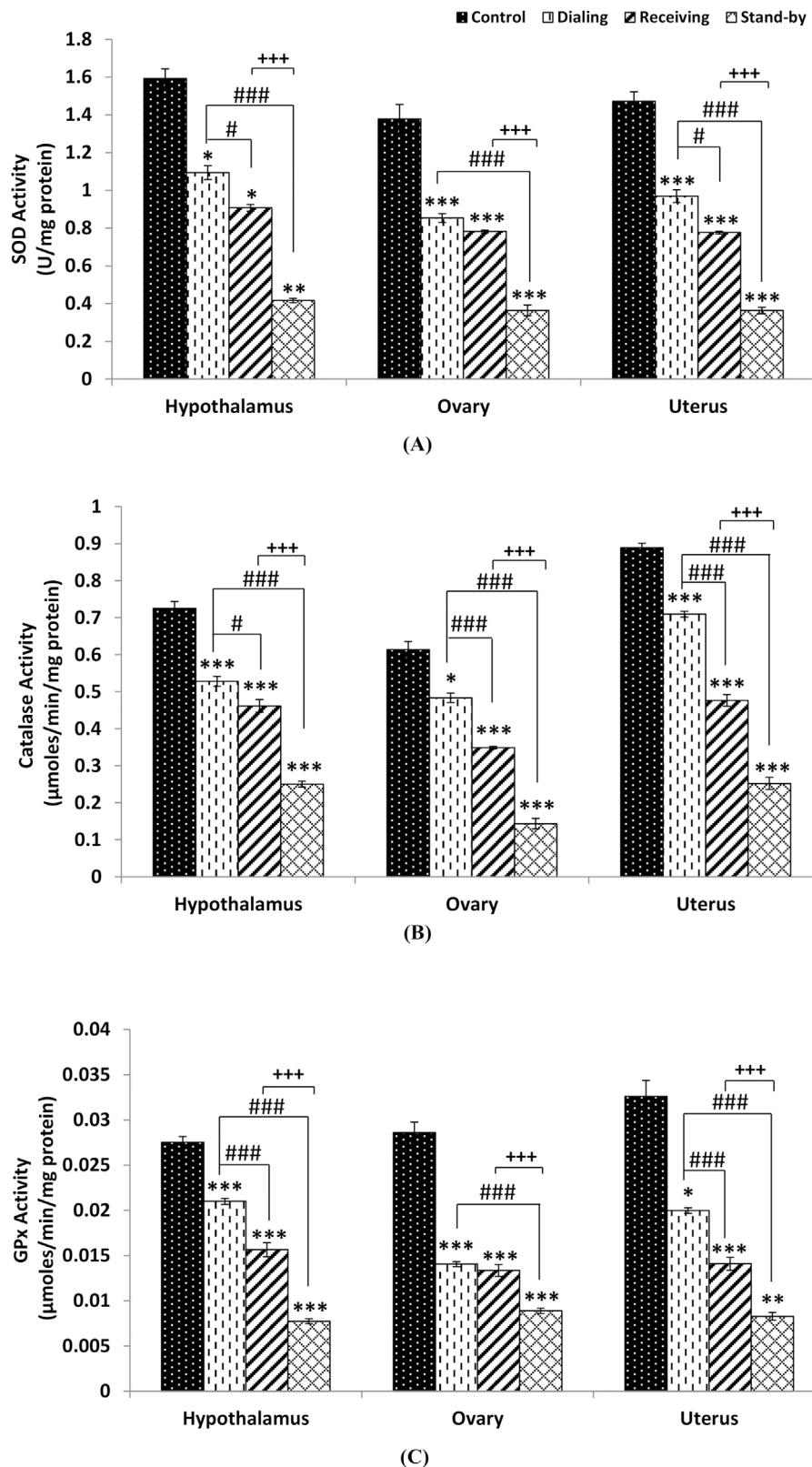


Fig. 5. Changes in Antioxidant enzymes activities: (A) SOD, (B) CAT and (C) GPx in ovary of mobile phone exposed group. Mobile phone exposed mice show significant decrease in all the antioxidant enzymes compared to control. Values are expressed as mean $\pm$ SEM (N = 12). Significance of difference from control * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; significance of difference from D-group # $p < 0.05$, ### $p < 0.001$; significance of difference from R-group +++ $p < 0.001$.

5. Discussion

This study demonstrate the deleterious effects of long-term 1800 MHz mobile phone radiation exposure in different operative

modes i.e. dialing (D), receiving (R) and stand-by (SB) modes on female reproduction. Present experimental findings clearly elucidate that mobile phone radiation has a negative impact on female

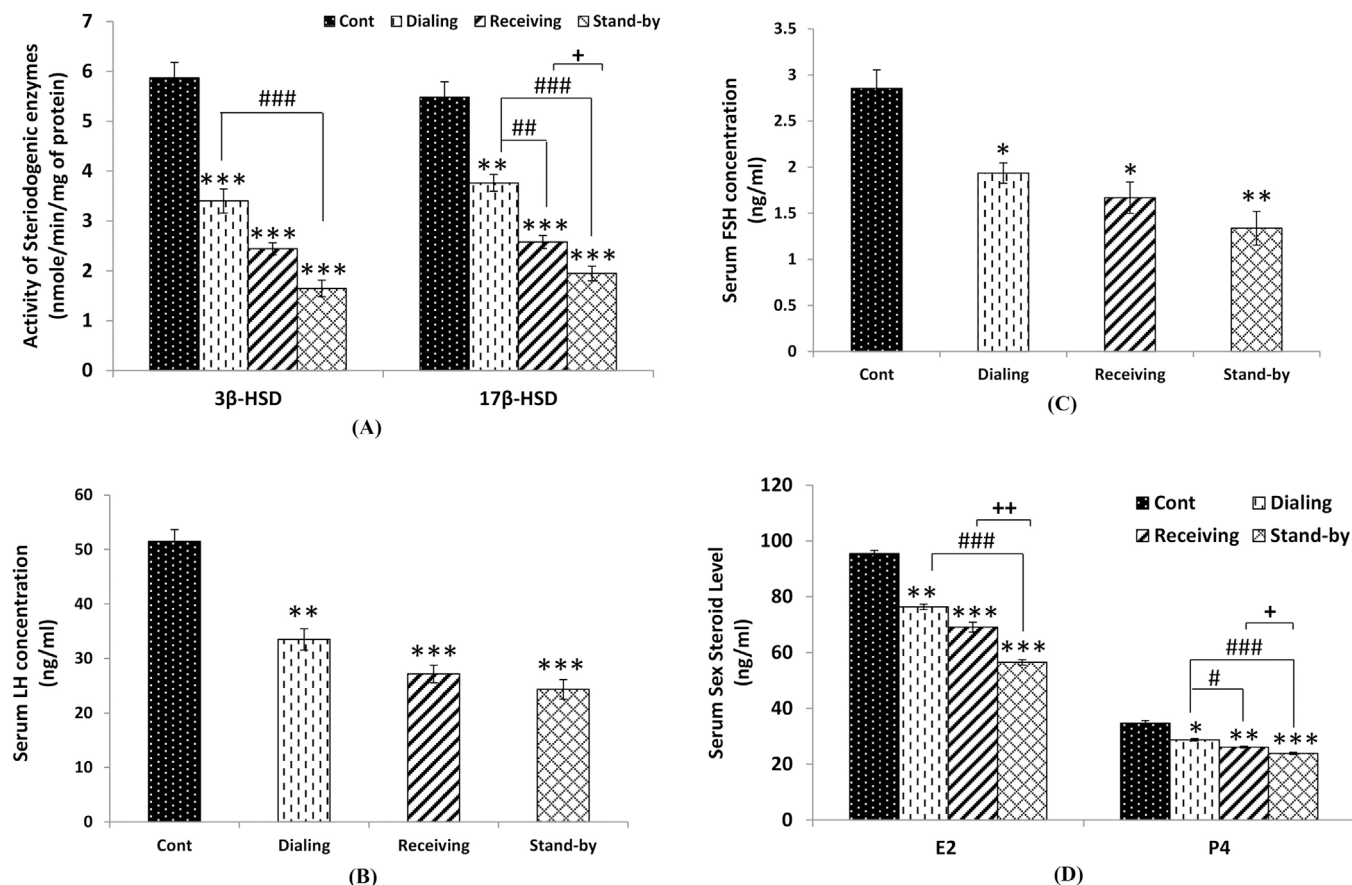


Fig. 6. Effect of mobile phone radiation on (A) ovarian steroidogenic enzymes: 3β-HSD & 17β-HSD (B) Serum LH, (C) Serum FSH and (D) Serum Estrogen & Progesterone concentration. Highly significant decrease was observed in 3β-HSD & 17β-HSD activity, the key enzymes in steroidogenesis pathway. Serum LH, FSH, E₂ and P₄ level was also found to be significantly reduced in mobile phone exposed group compared to control. Highly significant decreased was observed in SB-group. Values are expressed as mean ± SEM (for A: N = 12; for B, C & D: N = 24). Significance of difference from control, *p < 0.05, **p < 0.01, ***p < 0.001; significance of difference from D-group #p < 0.05, ##p < 0.01, ###p < 0.001; significance of difference from R-group +p < 0.05, ++p < 0.01.

reproductive system. Outcome of the study demonstrates that long-term mobile phone irradiation causes alteration in ovarian and uterine morphology, histoarchitecture and activity. Mobile phone irradiated mice ovary revealed less number of developing and mature follicles with few corpus lutea and increased number of atretic/degenerative follicles. Although marked changes were observed in all the three experimental groups, the effects were more pronounced and severe in cases of R- and SB-groups of mice.

Mobile phone irradiation leads to the generation of free radical (ROS & NO) in hypothalamus, ovary and uterus. These free radicals become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any nearby molecule causing a cascade of chain reactions resulting in cellular damage [50,51]. The three major types of ROS are: superoxide (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radical (•OH). These free radicals, being highly reactive, oxidize the cellular proteins/enzymes. Oxidative damage of proteins/enzymes (protein oxidation) as well as the lipids (lipid peroxidation) may affect the steroidogenic pathway and thus may inhibit the synthesis of ovarian steroids from cholesterol [51]. It is well established fact that ROS are double-edged sword as they serve as key signal molecules in various physiological processes but also have negative impact on the ovarian and uterine activity/functional status [52–56].

Mobile phone radiation induced stressed condition in all the experimental groups is further strengthened with the results of serum corticosterone and N/L ratio. The increased N/L ratio and serum corticosterone in the experimental D-, R- and SB-groups compared to sham control demonstrate the increased stress level

in the mice kept in different operational modes. Since NO level is also elevated significantly in the mice of all the exposed groups, it seems that, increased stress level in these mice may be due to the cumulative effect of both oxidative and nitrosative stresses.

From this study, it is clear that increased free radical load is a consequence of mobile phone radiation induced depletion in the activity of antioxidant enzymes: SOD, CAT and GPx. Thus the spontaneous increased cytotoxicity in hypothalamus, ovary and uterus may be due to elevated levels of superoxide (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radical (•OH), nitric oxide (NO•) etc. In ovary, steroidogenic cytochrome P450 enzymes are also sources of ROS [57,58]. This cumulative ROS may lead to the imbalance in the redox status of ovarian steroidogenic cells and generate oxidative stress.

These findings are consistent with our previous study, which demonstrated that 2.45 GHz (CW) MW irradiation is responsible for generating hormonal imbalance by producing extensive free radical load as evident from increased ROS/RNS and reduction in antioxidant enzyme activities in mice [59–61]. Studies from different groups also suggest that low frequency MW radiation exposure is responsible for inducing oxidative/nitrosative stress by the excessive production of free radicals [62] and might impair mammalian female reproductive potentiality by reducing the capacity of the follicles to reach a developmental stage that is an essential pre-requisite for reproductive success [63]. Low frequency MW radiation may affect both oocyte differentiation and folliculogenesis and may result in reduced fertility, by decreasing ovarian reservoir [25]. Although, some researchers do not observe

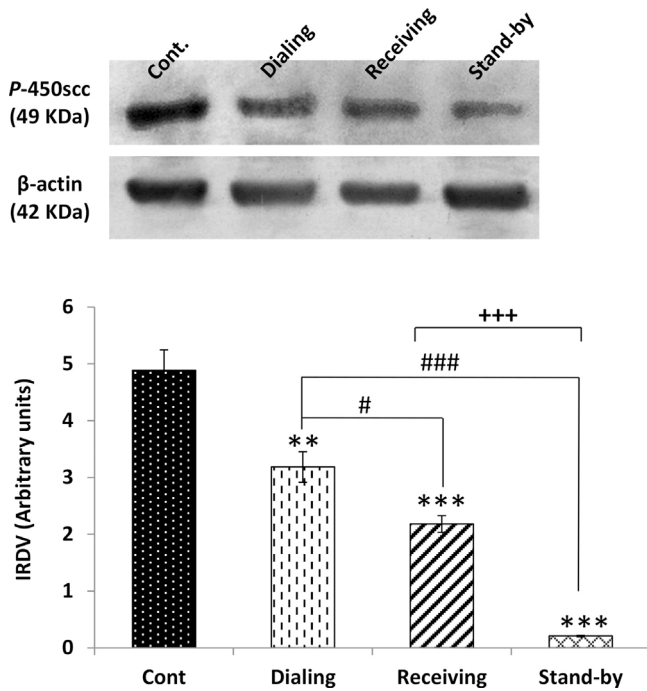


Fig. 7. Western blot analysis of cytochrome P-450scc protein in the ovaries of mice exposed to mobile phone radiation in Dialing, Receiving and Stand-by mode. IRDV = integrated relative density value. Values are expressed as mean $\pm$ SEM (N = 12). Significance of difference from control ** $p < 0.001$, *** $p < 0.001$; significance of difference from D-group # $p < 0.05$, ### $p < 0.001$; significance of difference from R-group +++ $p < 0.001$.

any alteration in sex steroid hormone level and suggest that exposure of male or female mice to low frequency microwave field had no adverse effects on fertility and reproduction in mice [64].

Mobile phone exposed mice also exhibited markedly reduced expression of SF-1, StAR and cytochrome P-450scc protein and 3β -HSD, 17β -HSD and P450arom enzymes in the ovary. The decrease in these proteins and enzymes in the ovaries of all the mobile phone irradiated groups suggest that the steroidogenic pathway gets disturbed and thus results in low level of estrogen and progesterone in all the exposed groups of mice. Estrogen also plays an intraovarian role in regulating follicle development and function [65–67]. Decrease in estrogen level results in blockage of follicle development at the antral stage, absence of corpora lutea, as well as decrease in the primordial and primary follicle numbers [68,69].

Estrogen signals act through estrogen receptors (ERs), which are members of the nuclear receptor family. Two major forms of ERs have been identified, ER- α [70–72] and ER- β [73–75] and both are expressed in the mouse ovary. ER- β is the most abundant form of ER and is expressed predominantly in the granulosa cells, whereas ER- α is expressed mostly in the theca and interstitial cells [76,77]. Estrogen may promote the proliferation, differentiation, follicle formation and maturation through its β receptor. Estrogen receptor α , β or compound knock-out mice shows various defects in follicle development/immature follicles and/or ovulation [78–80]. Our results show that the expression of both ERs i.e. ER- α and ER- β decreases significantly in both ovary and uterus of mice exposed to mobile phone radiation. Further, it was observed that decrease in the expression of both these estrogen receptors is more pronounced in both the gonad (ovary) as well as accessory sex organ (uterus) of R- and SB-groups of mice compared to the dialing group. Since estrogen and the estrogen receptors are required for proliferation and differentiation of granulosa cells and maturation of dominant follicle and progesterone is required for ovulation [81–83], it may be suggested that mobile phone radiation induced oxidative and

nitrosative stress may also be responsible for inhibition of follicular development, oocyte maturation and ovulation. These observations led us to suggest that the significant reduction in the SF-1, StAR, steroidogenic enzymes (cytochrome P-450scc, 3β -HSD, 17β -HSD, P450arom), estrogen and ERs (α and β) along with decreased progesterone affects folliculogenesis and hence results in decreased number of developing and mature follicles as well as corpus lutea in different mobile phone radiation exposed groups.

From the present study, it is found that mobile phone radiation exposure increases free radical load in hypothalamus, ovary and uterus which in turn increases the cellular oxidative and nitrosative stress. Thus, significantly increased oxidative and nitrosative stress induces severe oxidative damage at the hypothalamic level and may impose negative impact on hypothalamic reproductive neuropeptides. Significant reduction in serum LH and FSH was also observed in all the mobile phone exposed groups, confirming the deleterious effects of long term mobile phone radiation on pituitary gonadotrophs also. Hence, mobile phone radiation exposure induced oxidative and nitrosative stress reduces the activity of the crucial steroidogenic enzymes and subsequently inhibits the production of sex steroids (E_2 and P_4) through down regulating the expression of SF-1, StAR, 3β -HSD, 17β -HSD and P450arom in ovary, ER- α and ER- β in ovary and uterus.

In adults, ovarian steroidogenesis, folliculogenesis (development and maturation) and selection of the pre-ovulatory follicle as well as uterine structural and functional status are fully gonadotropin dependent. Obviously the hypothalamus and pituitary in combination secrete several hormones as well as signaling molecules which regulate the folliculogenesis through combinatorial endocrine interactions of HPG axis. Mobile phone radiation induced free radical load (ROS/RNS) at hypothalamic as well as at gonadal level may disturb the ovarian steroidogenic pathway and thus alter the hormonal milieu and folliculogenesis significantly in ovary as well as the structural and functional status of ovary and uterus. Increased oxidative/nitrosative stress in the hypothalamus, the important regulatory component of HPG axis, leading to deleterious effect on reproductive performance of female mice is further enhanced by elevated intraovarian ROS and NO levels.

Based on adverse ovarian and uterus histoarchitecture, it is evident that the 1800 MHz mobile phone irradiation induced free radical load not only affects the ovarian and uterine activity as well as steroidogenesis at the molecular level but its deleterious consequences have also been reflected in the ovarian and uterine histomorphology of all the exposed groups. Thus, it is clear from our study that mobile phone radiation induced oxidative and nitrosative stress, both at the central (hypothalamus) and peripheral (ovary and uterus) ends/levels, may have a negative effect on female reproduction [ovarian (steroidogenesis and folliculogenesis) and uterine activity]. We also suggest that the exposure to mobile phone radiation in stand-by mode is more deleterious than receiving and dialing modes (SB > R > D), although the power density is higher in dialing and receiving modes than stand-by mode. This difference i.e., variation in the degree of effect can be explained on the basis of nature and fluctuations of pulses, pings and connectivity duration in different operational modes. Continuous pulses are maintained between mobile phone antenna and base station (D-mode) and from base station to mobile phone antenna (R-mode) when mobile phones are operated in dialing and receiving modes respectively. In D- and R-modes, continuous condition of connectivity was maintained for 1½ hrs, then after a very small break of ~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 hrs. Therefore, in D- and R-modes, a fixed number of information packets (pings) will be exchanged between the mobile phone antenna and base station and a fixed

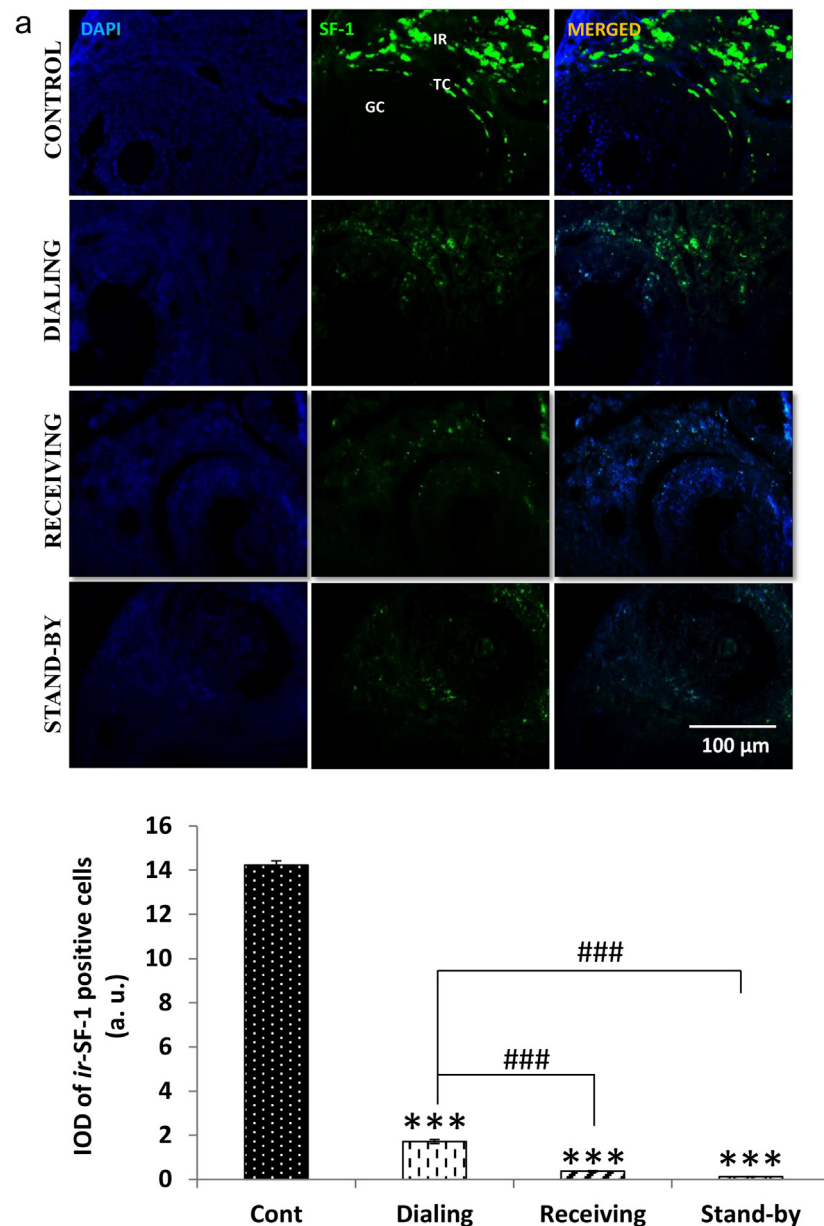


Fig. 8. (A) Immunofluorescence (Upper panel) and the integrated optical density (IOD) (Lower panel) of SF-1 in the ovary of mice exposed to mobile phone radiation in Dialing, Receiving and Stand-by mode: In control group ovary, intense immunoreactivity for SF-1 is observed in thecal and interstitial region. D-group shows moderate immunoreactivity for SF-1 in thecal and interstitial region of the ovary. R-group and SB-group mice shows very weak immunoreactivity for SF-1 in thecal and interstitial region of the ovary compared to control. (GC-granulosa cells, TC-theca cells, IR-interstitial region). Values are expressed as mean $\pm$ SEM (N = 12). Significance of difference from control *** p < 0.001; significance of difference from D-group *** p < 0.001. (B) Immunofluorescence (Upper panel) and the integrated optical density (IOD) (Lower panel) of StAR in the ovary of mice exposed to mobile phone radiation in Dialing, Receiving and Stand-by mode: In control group ovary, intense immunoreactivity for StAR is observed in thecal and interstitial region (but not in granulosa cells). D-group mice show moderate immunoreactivity for StAR in thecal and interstitial region of the ovary. R-group and SB-group mice exhibit very weak immunoreactivity for StAR in thecal and interstitial region of the ovary compared to control. (GC-granulosa cells, TC-theca cells, IR-interstitial region). Values are expressed as mean $\pm$ SEM (N = 12). Significance of difference from control *** p < 0.001; significance of difference from D-group *** p < 0.001; significance of difference from R-group ** p < 0.01. (C) Immunofluorescence (Upper panel) and the integrated optical density (IOD) (Lower panel) of 3 β -HSD in the ovary of mice exposed to mobile phone radiation in Dialing, Receiving and Stand-by mode: In control group ovary, intense immunoreactivity for 3 β -HSD is observed in thecal and interstitial region only but not in the granulosa cells. D-group shows moderate immunoreactivity for 3 β -HSD in thecal and interstitial region of the ovary. R-group and SB-group exhibit weak immunoreactivity for 3 β -HSD in thecal and interstitial region of the ovary compared to control. (GC-granulosa cells, TC-theca cells, IR-interstitial region). Values are expressed as mean $\pm$ SEM (N = 12). Significance of difference from control *** p < 0.001; significance of difference from D-group * p < 0.05, ** p < 0.01; significance of difference from R-group * p < 0.05. (D) Immunofluorescence (Upper panel) and the integrated optical density (IOD) (Lower panel) of cytochrome P450 aromatase (P450arom) in the ovary of mice exposed to mobile phone radiation in Dialing, Receiving and Stand-by mode: In control group ovary, intense immunoreactivity for P450arom is observed in granulosa cells, thecal cells and interstitial region. D-group shows moderate immunoreactivity for P450arom in thecal and interstitial region of the ovary while only few granulosa cells show weak immunoreactivity. R-group and SB-group exhibit very weak immunoreactivity for P450arom in granulosa cells, thecal cells and interstitial region of the ovary compared to control. (GC-granulosa cells, TC-theca cells, IR-interstitial region). Values are expressed as mean $\pm$ SEM (N = 12). Significance of difference from control *** p < 0.001; significance of difference from D-group *** p < 0.001; significance of difference from R-group *** p < 0.001. (E) (i) Immunofluorescence (Upper panel) and the integrated optical density (IOD) (Lower panel) of Estrogen receptor- α (ER- α) in the ovary of mice exposed to mobile phone radiation in Dialing, Receiving and Stand-by mode: Control group ovary shows intense immunoreactivity for ER- α in thecal and interstitial region. D-group shows moderate immunoreactivity for ER- α in thecal and interstitial region of the ovary. R-group exhibits weak immunoreactivity for ER- α in thecal and interstitial region of the ovary compared to control which decreases further remarkably in SB-group mice ovary. (GC-granulosa cells, TC-theca cells, IR-interstitial region). Values are expressed as mean $\pm$ SEM (N = 12). Significance of difference from control *** p < 0.001; significance of difference from D-group *** p < 0.001; significance of difference from R-group *** p < 0.001. (E) (ii) Immunofluorescence

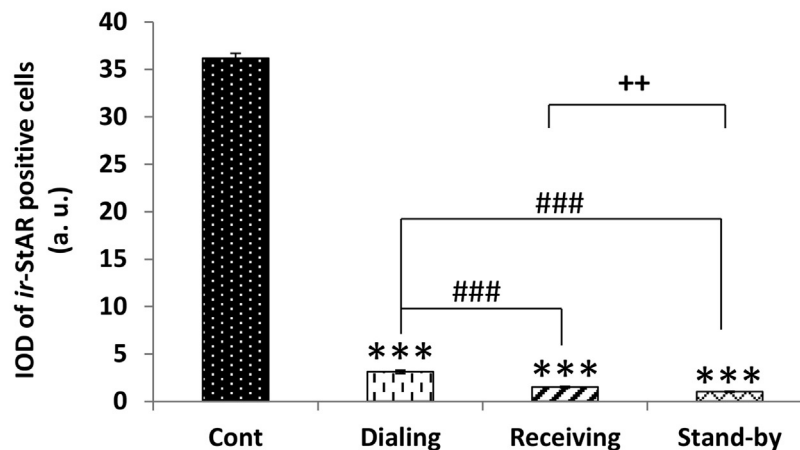
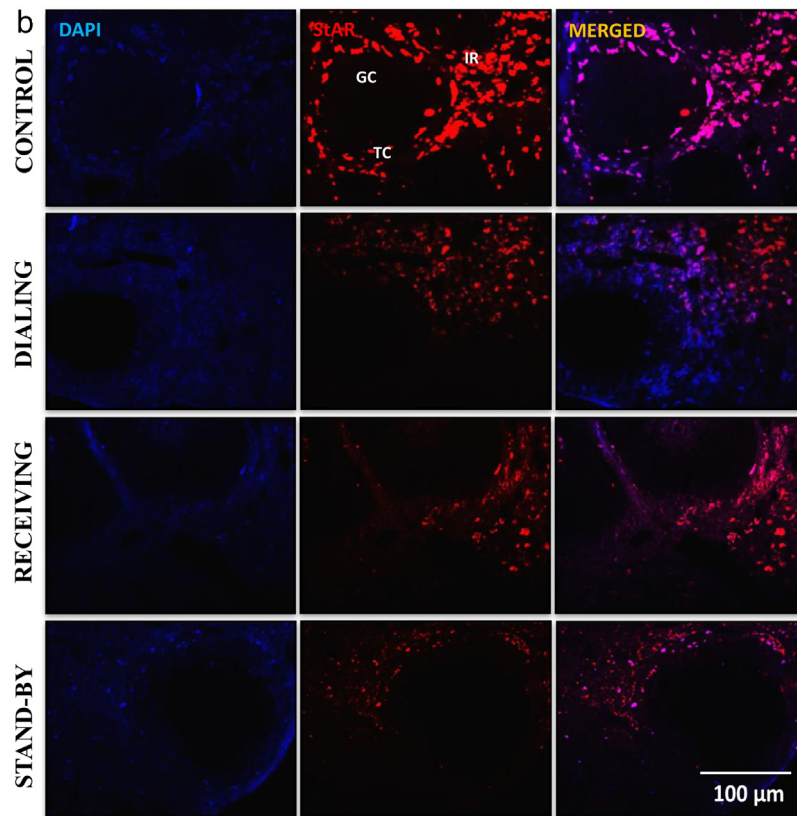


Fig. 8. (Continued)

(Upper panel) and the integrated optical density (IOD) (Lower panel) of Estrogen receptor- α (ER- α) in the uterus of mice exposed to mobile phone radiation in Dialing, Receiving and Stand-by mode: Control group mice uterus shows intense immunostaining for ER- α in luminal epithelium (LE), epithelium lining of uterine glands (UG) and endometrial stroma (ES) region. D-group shows moderate, R-group shows weak and SB-group shows very weak and less immunostaining for ER- α in luminal epithelium and epithelium lining of uterine glands. Very weak or no immunoreactivity was observed in the endometrial stroma of all the exposed group uterus. Values are expressed as mean $\pm$ SEM (N = 12). Significance of difference from control ***p < 0.001; significance of difference from D-group ##p < 0.01. (F) (i) Immunofluorescence (Upper panel) and the integrated optical density (IOD) (Lower panel) of Estrogen receptor- β (ER- β) in the ovary of mice exposed to mobile phone radiation in Dialing, Receiving and Stand-by mode: Control group ovary shows many ER- β positive granulosa cells while no immunoreactivity is seen in the thecal cells and interstitial region. D-group shows less number of ER- β positive granulosa cells in the ovary. R-group and SB-group shows very weak immunoreactivity for ER- β . The average numbers of ER- β positive granulosa cells are very less in R- and SB-groups of ovary compared to control. (GC-granulosa cells, TC-theca cells, IR-interstitial region). Values are expressed as mean $\pm$ SEM (N = 12). Significance of difference from control ***p < 0.001; significance of difference from D-group ###p < 0.001; significance of difference from R-group ***p < 0.001. (F) (ii) Immunofluorescence (Upper panel) and the integrated optical density (IOD) (Lower panel) of Estrogen receptor (ER)- β in the uterus of mice exposed to mobile phone radiation in Dialing, Receiving and Stand-by mode: Control group mice uterus shows intense immunostaining for ER- β in luminal epithelium (LE), epithelium lining of uterine glands (UG) and endometrial stroma (ES) region. D-group shows moderate while R- and SB-group exhibit weak immunostaining for ER- β in luminal epithelium and epithelium lining of uterine glands. Very weak or no immunoreactivity was observed in the endometrial stroma of all the exposed group uterus. Values are expressed as mean $\pm$ SEM (N = 12). Significance of difference from control ***p < 0.001; significance of difference from D-group #p < 0.05, ##p < 0.01.

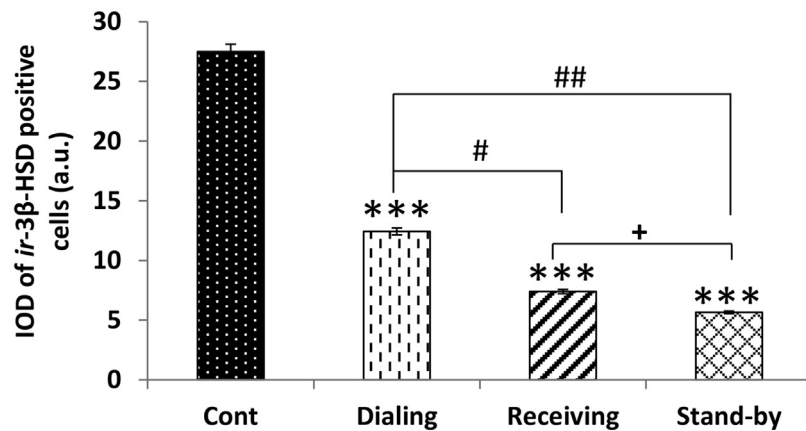
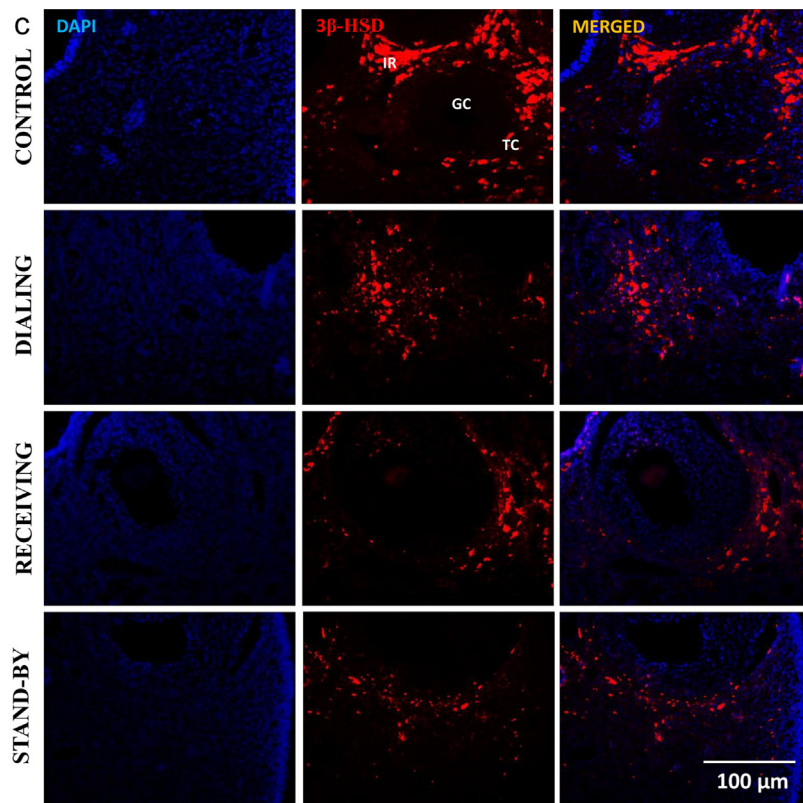


Fig. 8. (Continued)

number of information packets (pings) will be exchanged between the mobile phone antenna and base station. Therefore, in both D- and R-modes, consistency is there in power transmission and hence power density. While in the SB-mode, pulses are discrete in the form of transients from base stations to stand-by mobile phone antenna for small time duration (i.e., communication between the mobile phone and base station is minimal, typically a few seconds in every 30 min) to maintain the connectivity or in search of the signals from the nearby base stations or mobile phone towers, hence the bandwidth of the signal increases. In the SB-mode, the pings may also vary depending upon the number of users, availability of the connections with the base station and the distance as well as orientation of the mobile phone. Therefore, in SB mode the effect may be due to wider bandwidth of the signal which may cause different frequency dependent electrochemical effects/responses as compared to those in D- and R-modes (having relatively smaller signal bandwidth).

Further, the differences between the biological effects of the D- and R-modes can be explained on the basis of a) difference in frequencies used for dialing and receiving, i.e., separate frequencies used in D- and R-modes, b) difference in the number of operating channels and c) difference in the band-width. Transmission and reception of signals occur at different frequencies, down link frequency being higher than uplink frequency. Therefore in receiving mode, the frequency is higher as compared to dialing mode. Downlink channels (from base station to mobile phone) operated at higher frequency, need relatively higher power for transmission to take care of attenuation in the troposphere. Relatively higher transmitted power at higher frequency in R-mode might be causing more deleterious effects. It is to be noted that the penetration depth is almost same in both the cases i.e., for up-link and down-link frequencies.

The theoretical aspects of different operational modes (technical points) and experimental findings [central (hypothalamic) and

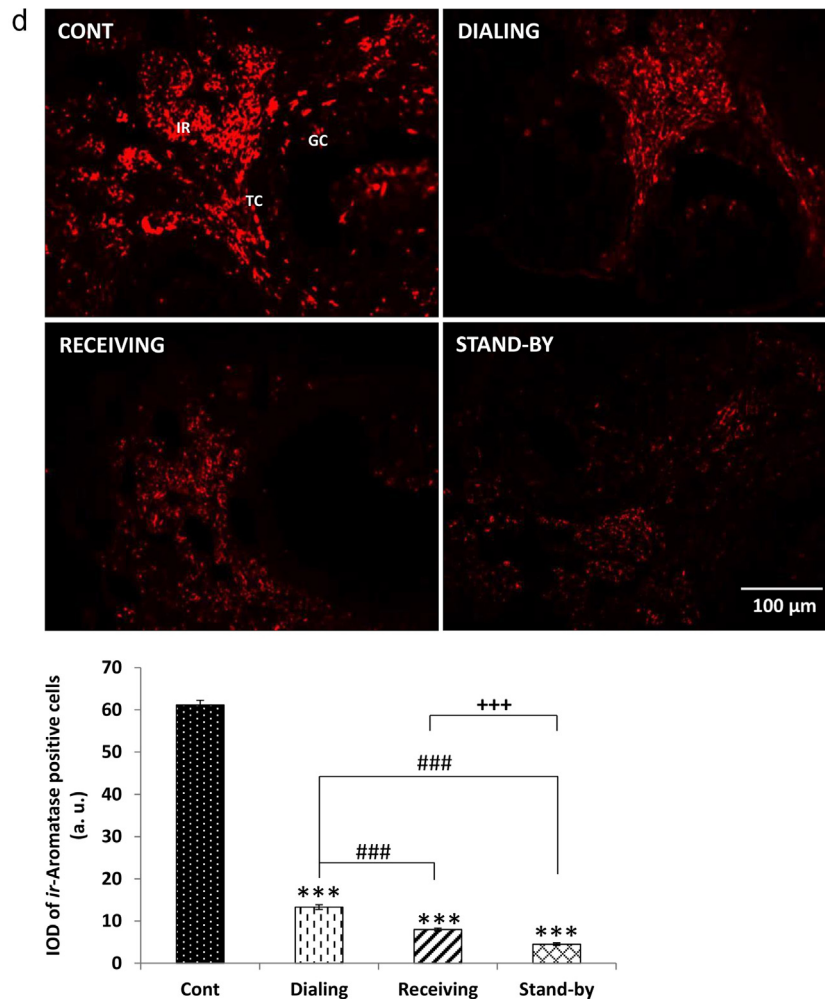


Fig. 8. (Continued)

peripheral (ovarian and uterine) responses in mouse] cumulatively indicate that the degree of severity in detrimental/deleterious biological effects of mobile phones is in the order of SB > R > D (due to fluctuations in pulses, pings, user density and the connection availability).

Further, the observed effects of mobile phone (1800 MHz) may be due to the possible microthermal effects which are in consonance with the observations of our previous report [60]. The mouse body is composed of multiple structural layers due to which reflections of low-level MWs at the interfaces of these layers occur which may generate regional micro-thermal hot-spots in the region of animals' reproductive organs of interest that may induce observed microthermal effects in different tissues.

6. Conclusion

Overall, our study clearly elucidates that the long-term 1800 MHz mobile phone exposure impairs female reproductive system possibly via inducing both oxidative and nitrosative stress. Our study also suggests that mobile phone exposure produces deleterious effect on hypothalamus, ovary and uterus, and thus affects the ovarian and uterine activity and histoarchitecture adversely. Mobile phone radiation may result in ovarian and uterine dysfunction by increasing ROS and RNS production and disturbing antioxidant status. Oxidative and nitrosative stress created at the hypothalamus and peripheral level (ovary and uterus) as a consequence of long-term mobile phone exposure may severely reduce

both steroidogenesis and folliculogenesis in the ovary as well as the structural and functional status of the uterus. These results led us to conclude that chronic exposure to long-term mobile phone radiation may severely affect the ovarian and uterine activity of female mice and thus may lead to infertility. The effects were more pronounced/deleterious in stand-by and receiving conditions. Further, the results of this study performed on the rodent model, *Mus musculus*, may not be extrapolated to human being as the SAR value delivered to human at the ovary or uterus end will be much less than (and not comparable to) the value for the rodent at the desired site due to greater depth of the site from the skin surface of human being, assuming large body size ratio between two type of subjects, i.e., human being and rodent. However, if the human being is exposed to mobile phone radiation over longer duration, there may be the possibility of the radiation producing similar effect on human female reproductive system, on the assumption, that the total energy absorbed in the two cases is of same order of magnitude. Therefore, we anticipate that, these findings will improve our understanding of the etiology of female infertility due to heavy mobile phone usage. The rise in female infertility problems may be, at least in part, due to a contribution from mobile phone radiation exposure to females. Hence, we anticipate that the outcome of the present study will not only contribute in framing of proper guidelines for safer use of mobile phone, which is an unavoidable device of present life style but may also assist in deciding the threshold limits to minimize adverse effects of the long term exposure to mobile phone radiations for females. However, further investiga-

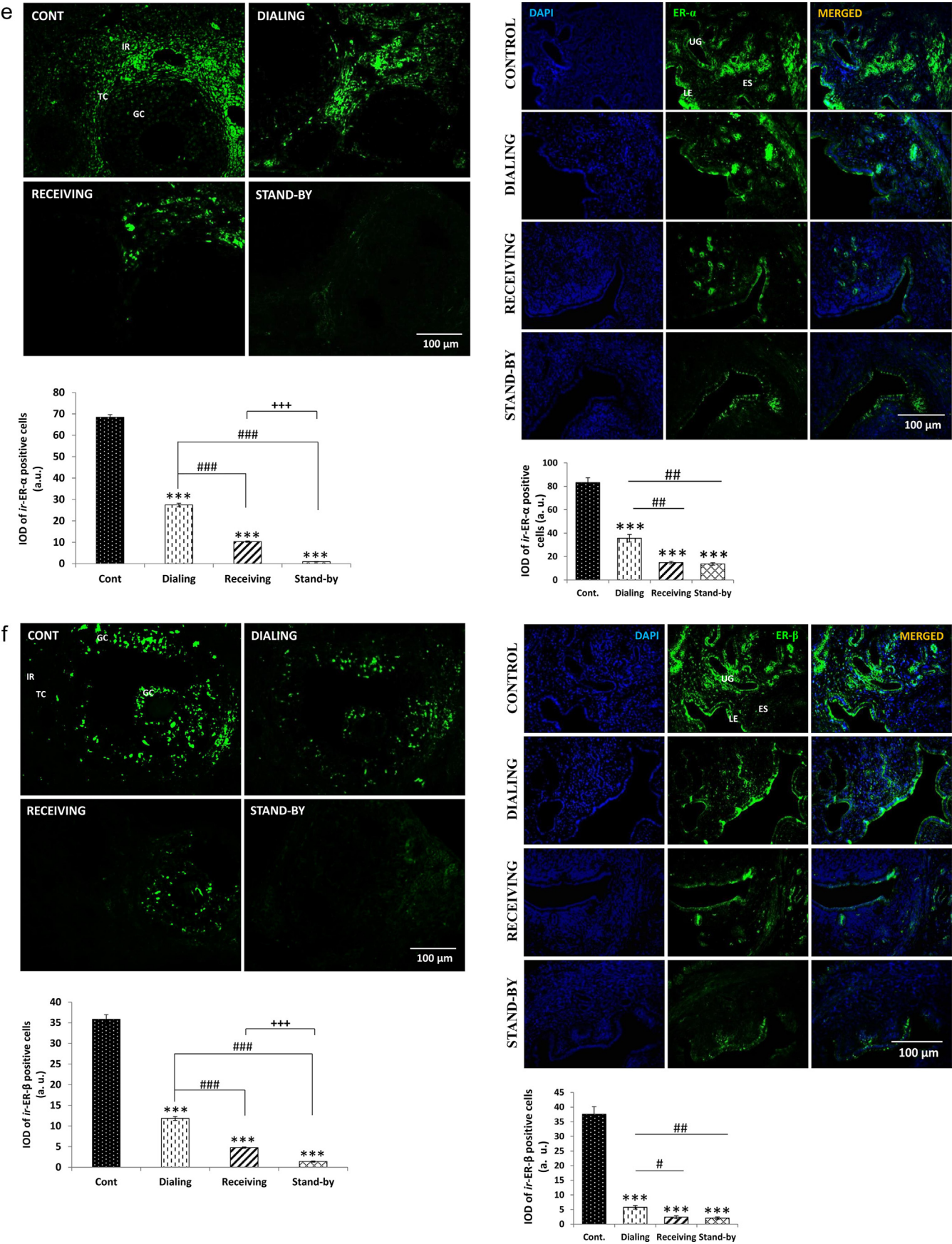


Fig. 8. (Continued)

tion is required in humans and non-human primates to determine whether the risks are similar and to establish safe exposure limits.

Conflict 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.reprotox.2017.08.001>.

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