

ORIGINAL ARTICLE

Oxidative stress-mediated alterations on sperm parameters in male Wistar rats exposed to 3G mobile phone radiation

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Abstract

In recent years, there has been significant increase in mobile phone users. With this, health concerns associated with the exposure to electromagnetic radiation are also increasing. Continuous exposure to electromagnetic (EM) radiation generated from mobile phone is one of the probable reasons behind increasing male infertility. EM radiations induce oxidative stress that leads to numerous changes in reproductive parameters. With this hypothesis, we studied the effect of 3G mobile phone radiations on the reproductive system of male Wistar rats. Adult rats were divided into two groups: control and radio frequency-exposed. The animals were exposed to 3G mobile phone radiation for 45 days (2 hr/day) in specially designed exposure setup under standard conditions. Various biochemical and physiological parameters such as sperm count, sperm morphology, mitochondrial activity, lipid peroxidation, reactive oxygen species level and histopathological analysis were studied. Histopathological examination revealed a reduction in spermatogenic cells and alterations in sperm membrane. Significant increase in ROS and lipid peroxidation level with simultaneously decrease in sperm count, alterations in sperm tail morphology were observed in the exposed group. In conclusion, exposure to mobile phone radiations induces oxidative stress in male Wistar rats which may lead to alteration in sperm parameters and affects their fertility.

KEYWORDS

3G mobile phone, electromagnetic radiation, male infertility, oxidative stress, sperm parameters

1 | INTRODUCTION

Mobile communication services are the fastest growing field in the telecommunication sector. The ever-increasing use of mobile phones and other microwave gadgets using electromagnetic field (EMF) have become a major concern for human beings due to its adverse effects on human health. Earlier, first generation of mobile network was analogue-based system which has applicability mostly for voice communication. With technological advancement, second-generation mobile network came into existence which uses

digital modulation and time-division multiple access (TDMA) system for better spectral efficiency and early data services. Recently, in a move to high-speed Internet access, highly improved video and fast data transfer, third generation (3G) was introduced. The 3G mobile system is based on Universal Mobile Telecommunications System's (UMTS) with high operation frequency range of 2,110–2,170 MHz (Kesari, Meena, Nirala, Kumar, & Verma, 2014; Mogal, 2012).

Specific absorption rate (SAR) is a standardised unit used to measure the rate of radiofrequency absorption in the body. It is calculated as watt/kg (Agarwal, Singh, Hamada, & Kesari, 2011). Power

levels of mobile phones during the communication in Global System for Mobile Communication (GSM)/UMTS system can be determined by measuring SAR.

The exact mechanism of mobile phone radiation effect on biological system is still unknown. Several studies propose that mobile phone emits radio frequency electromagnetic waves that generate free radicals which ultimately results in oxidative stress (Blettner & Berg, 2000; De Iuliis, Newey, King, & Aitken, 2009). Fenton reaction (iron-mediated process) initiated by mobile phone radiation exposure is reported to cause severe damage to cells via increasing hydroxy free radical formation (Kesari, Kumar, Nirala, Siddiqui, & Behari, 2013). Oxidative stress is redox disequilibrium state where reactive oxygen species (ROS) formation leads to decrease in the cell antioxidant defence capacity and results in DNA damage and lipid peroxidation (Meena et al., 2014).

Male reproductive system is highly sensitive and compartmentalised structure and it requires the integration of intrinsic and extrinsic factors to function properly. ROS have been considered as main causative agent of cell phone EMF damage on spermatozoa (Hamada, Singh, & Agarwal, 2011). Since plasma membranes of spermatozoa are rich in polyunsaturated fatty acids (PUFA), they are highly susceptible to damage caused by ROS leading to lipid peroxidation. Lipid peroxidation alters membrane integrity and motility of spermatozoa (De Iuliis et al., 2009). Radio frequency electromagnetic radiation (RF-EMR) increases ROS generation in mitochondria of human spermatozoa, which decrease its motility and viability and form DNA base adduct (Aitken & Baker, 2006). The EMF-induced ROS adversely affects the activity of antioxidative enzymes such as GPx and SOD. It significantly affects the mitochondrial and nuclear genome integrity and also causes apoptosis, DNA break and alters expression of cytochrome c, Bax etc. (Aitken, Bennetts, Sawyer, Wiklendt, & King, 2005; Kesari et al., 2014; Kesari, Kumar, & Behari, 2011).

Prolonged chronic exposure to electromagnetic radiation at higher frequency showed DNA damage in rat's brain. Changes in calcium ion efflux, ornithine decarboxylase (ODC) activity and calcium-dependent protein kinase activity were reported (Paulraj & Behari, 2002, 2006). Studies also report histopathological changes in parotid gland of rats when exposed to 2100 MHz radiofrequency radiation (Aydogan et al., 2015). Similarly, chronic exposure of chick embryo liver to 3G mobile phone radiation leads to various structural changes and DNA damage (D'Silva, Swer, Anbalagan, & Rajesh, 2017).

Previous studies report that long-term exposure to radiofrequency waves has deleterious effect on organs and can also lead to infertility. The increasing instances of infertility have been related to the increasing usage of mobile phones. Generation of ROS and changes in redox equilibrium may be the major factors behind infertility. Based on this hypothesis, our study was aimed at determining the effect of 3G mobile phone radiation on reproductive system of male Wistar rats through studying the changes in the sperm viability, count morphology, mitochondrial activity, ROS and lipid peroxidation level, histopathological analysis and

membrane alteration induced by exposure to 3G mobile phone radiations.

2 | MATERIALS AND METHODS

2.1 | Animal exposure

Adult Wistar male rats (10-week-old) were used for the study. The rats were divided into two groups: control and exposed (eight rats in each). Rats were exposed to 3G mobile phone radiation for 45 days (2 hr/day) in a specially designed Plexiglas rectangular box. All animals were housed in standard conditions, where temperature (25–27°C) and humidity (40%–50%) were maintained with 12-hr day–night cycle. Institutional Animal Ethics Committee (IAEC, Code No. 15 /2013) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) gave the approval of the animals for the study. Standard food pellets and water ad libitum were provided to the animals. All the experiments were performed as per the guideline given by the IAEC, Jawaharlal Nehru University, New Delhi, India.

2.2 | Power and SAR measurement in experimental setup

At first, the electric field in the anechoic chamber was measured without any GSM/UMTS phone switched on and the ambient power level was recorded as 0.1 V/m (Balanis, 1999). In this experiment, electric field measurements were made in video voice calling mode. The near electric field measurement was made at different locations between Wistar rat and mobile phone in the UMTS band (1915 MHz). Mobile phone was fully charged and kept horizontally on the plexi glass cage, and electric field values were recorded. Specially designed monopole probe was used for calibration and measurement of emitted power. This monopole probe was positioned to measure output power of the mobile phone and recorded by spectrum analyser (Field fox Model No -N9912A, Agilent Technologies USA), carried out inside the cage at different positions. Specific absorption rate (SAR) of 3G mobile phone was measured using Wistar rat having average weight of 200 gm in rectangular cage. The estimated SAR value was 0.26 W/kg (Durney, Massoudi, & Iskander, 1986).

2.3 | Organ weight

After 45 days of exposure, the rats were anaesthetised by anaesthetic sodium thiopentone and sacrificed, and testis was excised out, cleared of adhesive tissue and weighed accurately.

2.4 | Sperm parameters

2.4.1 | Epididymal sperm count

Caudal region of epididymis was excised out after the exposure and minced into 3 ml of Ham's F12 solution, later incubated for 15 min at

37°C and allowed the spermatozoa to disperse. Counting of spermatozoa was done by hemocytometer (Lu, Huang, & Lü, 2010).

2.4.2 | Sperm morphology

Sperm suspension was filtered through 80-µm nylon mesh, stained with 0.5% eosin, and smear was prepared on glass slides. The smear was dried, fixed in 70% methanol and mounted under a coverslip with Paramount mounting medium. Morphological evaluation was done under light microscope at 400× magnification (Narayana, D'Souza, & Seetharama Rao, 2002). Normal sperm cells have hook-shaped heads and thin tail with uniform thickness, whereas sperm cells having aberrant structures include cells devoid of the hook-shaped head; nondescript shapes; bent and other anomalies. Around 300 spermatozoa per animal were evaluated, and percentage of abnormal sperm cell was calculated.

2.4.3 | Sperm viability

Viability of spermatozoa was evaluated by staining with eosin Y (Lu et al., 2010). In brief, 10 µl of sperm suspension was mixed with 0.5% eosin Y (w/v) solution on a microscope slide and left for 30 s. Slides were viewed at 400× under light microscope, and viability percentages were calculated. Spermatozoa with white or light pink head were considered as live and with dark pink head as dead.

2.4.4 | Hypo-osmotic swelling test

Sperm viability and membrane integrity were estimated via hypo-osmotic swelling (HOS) test (Jeyendran, Ven, Perez-Pelaez, Crabo, & Zaneveld, 1984). Briefly, 100 µl of sperm sample was mixed with 1 ml warm hypo-osmotic solution (0.735 g of sodium citrate dihydrate and 1.351 g of D-fructose in 100 ml of MilliQ water) and kept at 37°C for 45 min. After that, 10 µl of suspension was taken and spermatozoa with coiled tails (viable) and noncoiled tails (nonviable) were examined using light microscope (400×). A total of 100 of spermatozoa were observed from each animal.

2.4.5 | Sperm mitochondrial activity

Sperm mitochondrial activity was determined by using 3,3-diaminobenzidine (DAB) which gets oxidised and the polymerised DAB gets deposited at cytochrome c complex (Blumer et al., 2008). Various dilutions (1:1–1:3) of sperm sample were prepared in DAB (1 mg/ml) in PBS solution. The diluted solutions were kept in dark, in a water bath for incubation at 37°C (1 hr). Smears of sperm samples on microscope slides were prepared and kept for air-drying. Fixation of smears was done for 10 min in 10% formaldehyde and then air-dried. A phase contrast optical microscope was used for counting around 200 sperm cells. Classification of cells was done viz. class I (staining of 100% of midpiece took place), class II (staining of >50% of the midpiece took place), class III (staining of <50% of the midpiece took place) and class IV (no staining of midpiece region).

2.5 | Sperm ROS level

The ROS level in sperm cells was measured as per the defined protocol by Hsu, Liu, Hsu, Chen, and Guo (1998). In brief, sperm samples were washed with 300 µl of PBS and mixed with 100 µl 30 mM FeSO₄ and 50 µl of luminol, which gives chemiluminescent signal. This signal was detected by luminescent counter, and ROS level was measured as chemiluminescence counts: 10⁶ spermatozoa per second.

2.6 | Lipid peroxidation

Lipid peroxidation was estimated by the method of Placer, Cushman, and Johnson (1966), with slight modifications. To describe briefly, test sample and stock solution (15%, w/v trichloroacetic acid in 0.25 N HCl and 0.375%, w/v thiobarbituric acid in 0.25 N HCl) were mixed in the ratio of 1:2. Assay solution was incubated for 15 min at 95°C (water bath). Sample was cooled and centrifuged at 200 g for 10 min. The supernatant was removed and optical density was taken at 532 nm against blank. The MDA level was measured as nmol MDA per milligram protein, and protein content was measured via Bradford assay.

2.7 | Transmission electron microscopy of spermatozoa

Sperm samples were fixed in Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde) and washed with cacodylate buffer. Post-fixation of sample was done in 1% osmium tetroxide and then dehydrated through an ethanol graded series and embedded in epoxy resin. Uranyl acetate and lead citrate were used for staining the ultrathin sections, later examined under Jeol Electron Microscope (JEM 2100F) at 120 kV (Gaharwar & Paulraj, 2015).

2.8 | Scanning electron microscope analysis of spermatozoa

The procedure for sperm examination using (SEM) was identical to the method previously reported (Sainath, Meena, Supriya, Reddy, & Reddy, 2011). To describe briefly, washing of spermatozoa was done using phosphate buffer of 0.1 M and pH 7.4. After that, the spermatozoa were centrifuged at 300 g for 5 min at room temperature and pellet was collected. Preparation of a thin smear of sperm pellets was done on a clean microscope slide. After air-drying and mounting the slide on SEM stub, it was spluttered with gold particles and scrutinised under SEM. (Carl Zeiss EVO 40) at 20 kV.

2.9 | Histopathology of testis

After exposure, rats were sacrificed under anaesthetic condition and testis was removed. Testis was washed with PBS and thereafter fixation in formalin was done and finally inserted in paraffin. Sections of

a few micrometres were cut, and haematoxylin-eosin was used for staining these sections. The histopathological slides were observed under light microscope at 400× magnification (Meena et al., 2014).

2.10 | Statistical analysis

Mean $\pm$ standard error of means (SEM) was calculated. Statistical analysis was performed by Student's *t* test, and ANOVA was done by using GraphPad Prism 5.03. The *p* values <0.05 were considered as significant and *p* values <0.01 were highly significant.

3 | RESULTS

3.1 | Organ weight

The weight of testis and epididymis of the exposed and control groups was measured. It was found that the weight of the testis changed significantly in the exposed group but no changes were observed in epididymis weight of the control group (Table 1).

3.2 | Sperm parameters

The effect of mobile phone radiation exposure on sperm parameters is shown in Table 2. The result shows significant alteration in sperm count, viability and HOS tail-coiled spermatozoa in the exposed group as compared to the control ones (Figure 1). Meanwhile, decreased percentage of tail-coiled spermatozoa measured by hypo-osmotic swelling test indicates that membrane integrity of the spermatozoa deteriorated in mobile phone radiation-exposed groups. The ultrastructural images showing the breakage in plasma membrane of midpiece region of sperm cells exposed to mobile phone radiation support the deterioration of membrane integrity (Figure 2). The spermatozoa from both the control and exposed group rats showed hook-shaped head when observed under scanning electron microscope (Figure 3). Morphological analysis also showed no abnormalities in the head of spermatozoa in the exposed group of rats.

3.3 | Histopathology of testis

Histopathological micrograph of testis in the control group rats shows uniform-sized seminiferous tubules with a well-defined lining of spermatogenic cells resting on a thin basement membrane (Figure 4), while that of the exposed group indicates the

TABLE 1 Effect of 3G mobile phone exposure on sperm parameters

Organ weight	Control	Exposed
Epididymis	0.57 $\pm$ 0.01	0.55 $\pm$ 0.01
Testis	1.60 $\pm$ 0.01	1.544 $\pm$ 0.01 ^a

Note. Values are mean $\pm$ SEM, *n* = 8 and

^a*p* < 0.05.

variation in size of seminiferous tubules, vacuolar degeneration, disruption of germinal layer and reduced number of spermatogenic cells.

3.4 | Generation of ROS

The luminol probe is used to measure ROS and is nonspecific, reacting with a variety of ROS, such as H₂O₂, O₂⁻ and OH⁻ free radicals (Hsu, Liu, Hsu, Chen, & Guo, 1997). ROS level in spermatozoa of the exposed group showed significant elevation as compared to the control group. The results indicate that RF-EMF exposure induced free radical formation, which may cause oxidative stress in reproductive organ (Figure 5). Previous studies also suggested that RF-EMF may induce ROS production in reproductive system (De Iuliis et al., 2009).

3.5 | Lipid peroxidation

Elevated MDA shows lipid peroxidation in sperm membrane lipids. The RF-EMF-exposed group showed a significant rise in MDA as compared to the control group (Figure 6). Sperm plasma membrane is rich in PUFA and it plays a major role in membrane functioning and constitution. PUFA is also one of the main targets in the lipid peroxidation process (Lenzi, Picardo, Gandini, & Dondero, 1996).

3.6 | Sperm mitochondrial activity

The imbalance between ROS generation and antioxidant protection results in oxidative stress that impairs mitochondrial membrane potential (MMP) and cytochrome *c* cytochrome *c* can be used. Impairment of cytochrome *c* complex results in reduction of ATP formation and affects sperm motility. Our results showed that the overall mitochondrial activity of the spermatozoa was reduced in the exposed group. There was a significant decrease in spermatozoa with total active mitochondria (class I) while increase in spermatozoa with partially active mitochondria (class II and class III) and total

TABLE 2 Effect of 3G mobile phone exposure on sperm parameters

Sperm parameters	Control	Exposed
Sperm count	62.38 $\pm$ 1.90	54.79 $\pm$ 1.74
Sperm viability	70.13 $\pm$ 1.26	63.63 $\pm$ 1.80 ^a
HOS test	67.25 $\pm$ 3.69	60.63 $\pm$ 5.23 ^a
Head morphology		
Amorphous	0.08	0.17
Hook less	0.33	0.58
Banana	0.17	0.08
Bent head	0.04	0.08
Total abnormality	0.62	0.92

Note. Values are mean $\pm$ SEM, *n* = 8 and

^a*p* < 0.05.

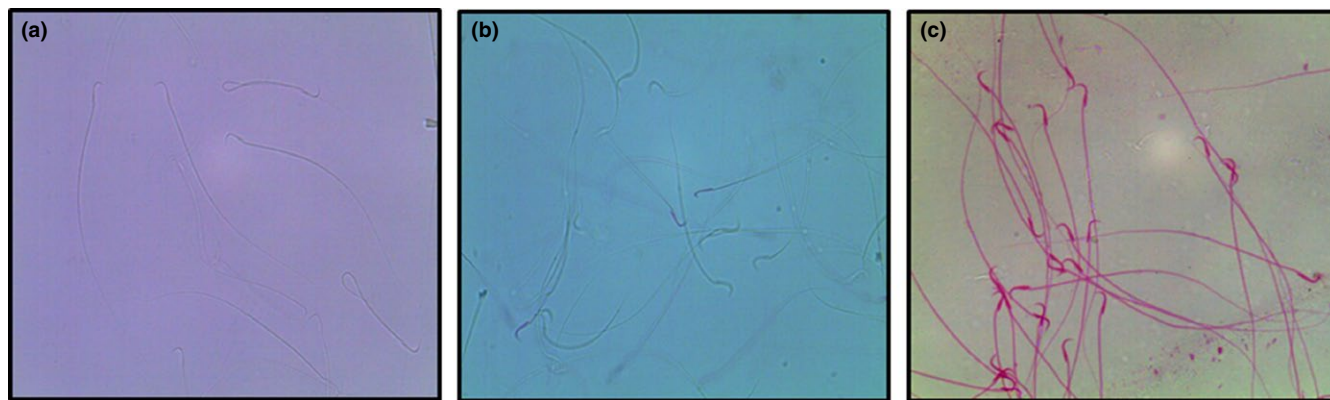


FIGURE 1 (a) Hypo-osmotic swelling test, (b) sperm viability test (eosin staining) and (c) sperm morphology

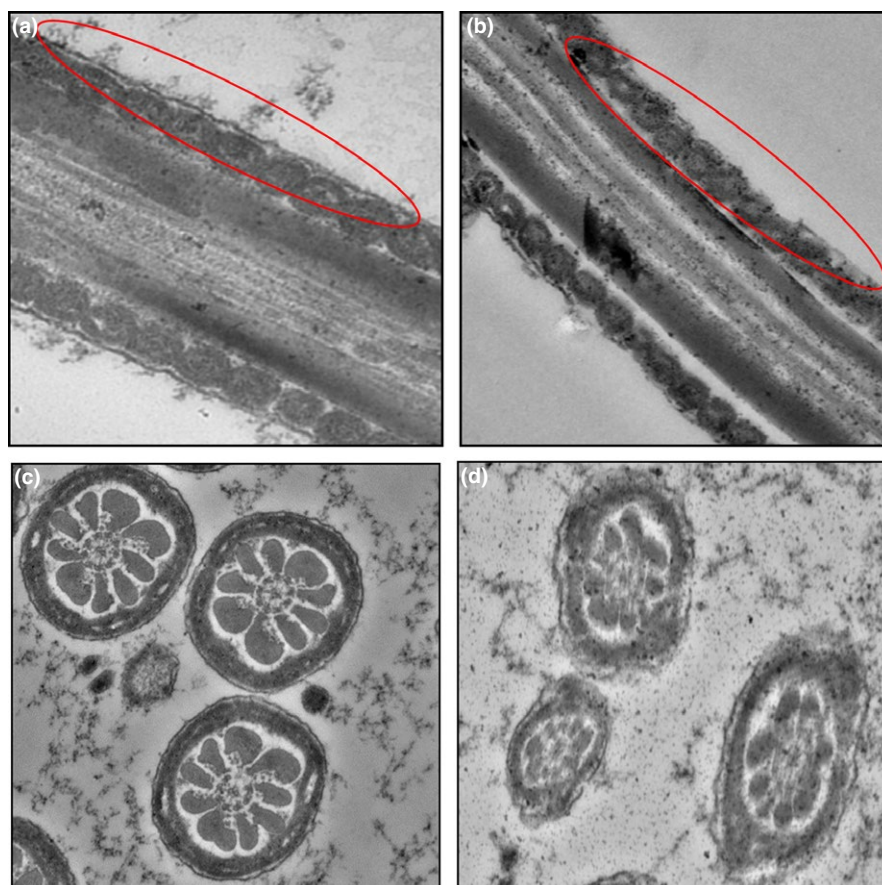


FIGURE 2 Effect of exposure on sperm tail (midpiece; 10,000 $\times$). (a & c) The control group showed normal (intact) membrane. (b & d) The exposed group showed alterations in tail membrane

inactive mitochondria (class IV) as observed in the RF-EMR-exposed group of rats as compared to the control group (Figures 7 and 8). RF-EMR can damage sperm functioning via mechanisms leading to leakage of electrons from the mitochondria and further results in oxidative stress.

4 | DISCUSSION

The current study aimed to investigate the effect of 3G mobile phone radiation induced reproductive alterations in male Wistar

rats. Stressful life and occupational exposure to a variety of chemicals have been reported as some of the causes behind increasing male reproductive disorders (Kourouma et al., 2014). In addition to the lifestyle and constant exposures to toxic chemicals, radio frequency electromagnetic field also causes reproductive disorders (Agarwal et al., 2011). Radiations from mobile phone are a source of contamination in the environment. Previously, it has been shown that mobile phone radiation affects sperm parameters in addition to causing oxidative stress in the body and consequently leading to various diseases (Ghanbari, Mortazavi, Khavanin, & Khazaei, 2013). Mobile phone radiation has an adverse effect on testicular function

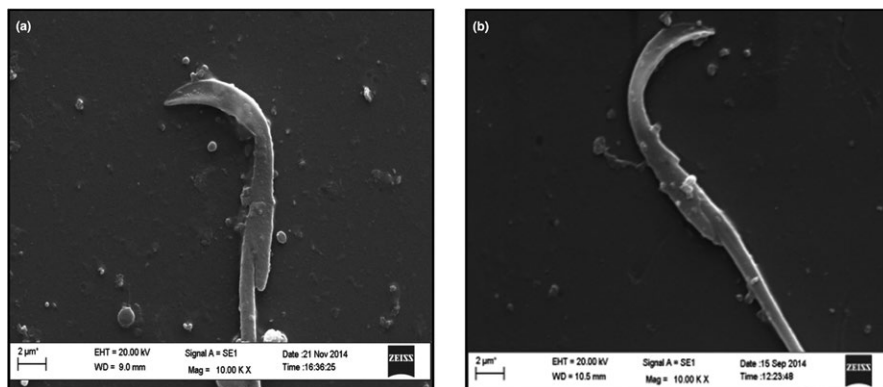


FIGURE 3 SEM images showing morphology of spermatozoa from (a) control and (b) exposed rats

by increasing oxidative stress and disrupting testicular antioxidant status (Al-Damegh, 2012).

In the present study, we observed that animals in the exposed group showed increased ROS production which may be associated with a reduction in number of spermatogenic cells and bringing about variation in seminiferous tubules. Significant changes in sperm count, viability, membrane integrity (HOS test) were also observed. In accordance with previous studies, our results show that poor sperm quality is caused due to oxidative stress as a result of ROS generation which may finally lead to infertility (Panagopoulos, Karabarbounis, & Margaritis, 2002). Maturation and storage of spermatozoa takes place in epididymis. Epididymis spermatozoa are vulnerable to damage from ROS due to lack of cytoplasm and high presence of PUFA in sperm plasma membrane composition (Sharma, Pasqualotto, Nelson, Thomas, & Agarwal, 1999).

Exposure to RF-EMR increases ROS level in mitochondria, which leads to a decrease in the motility, viability and ultimately leading to

DNA fragmentation (De Iuliis et al., 2009). Our study is in coherence, as we observed a significant decrease in the mitochondrial activity in the exposed group as compared to the control group. Further, in the exposed group of animals, an increase in lipid peroxidation was seen, while ultrastructure analysis of sperm cells showed breakage in plasma membrane of midpiece region. Exposure to GSM cellular phone induces oxidative damage in rats by enhancing lipid peroxidation activity (Dasdag, Bilgin, Akdag, Celik, & Aksen, 2008; Mailankot, Kunnath, Jayalekshmi, Koduru, & Valsalan, 2009). Similarly, exposure to cellular phone radiations induced sperm death and cell clumping in exposed set of rats (Yan et al., 2007).

Similar, results were also obtained in human spermatozoa exposed to radiations. Literature suggests that ROS generating capacity and peroxidation of unsaturated fatty acids in the sperm plasma membrane of human spermatozoa plays a vital role in male infertility (Sharma & Agarwal, 1996). An increased lipid peroxidation found in the exposed group validated the previous studies. EMF exposure also alters the gonadal function, reproductive endocrine hormones,

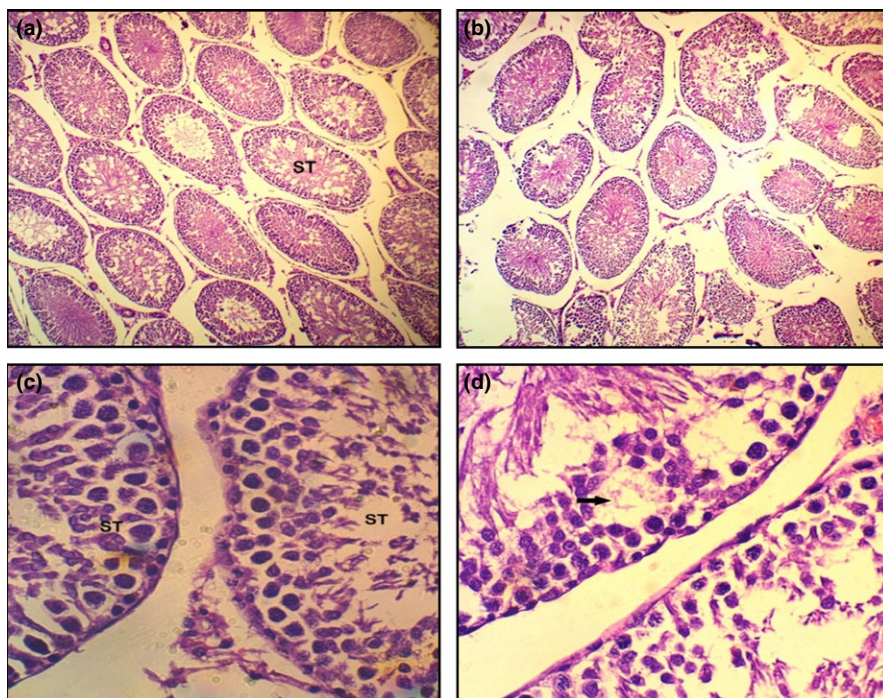


FIGURE 4 Histopathology of testis by HE staining from the exposed group (b & d) showing variation in size of seminiferous tubules and reduced number of spermatogenic cells as compared to the control group (a & c)

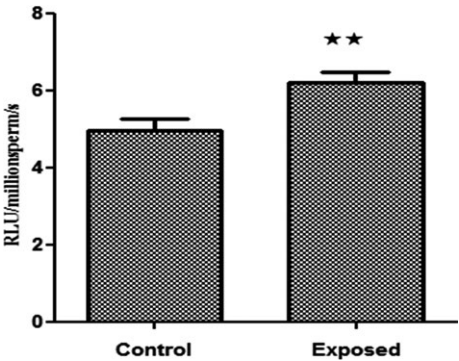


FIGURE 5 ROS levels were assayed using luminol; exposed group showed a significant increase in ROS level: ***p* < 0.01

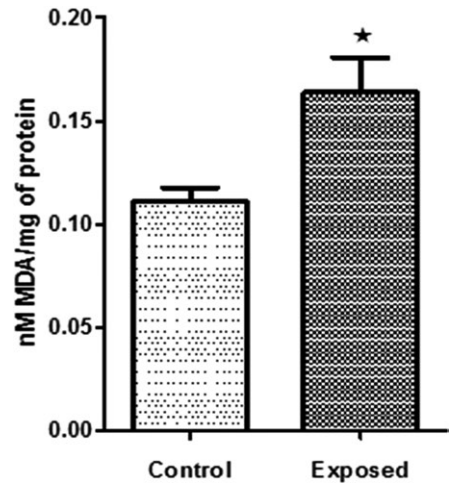


FIGURE 6 The concentration of malondialdehyde (MDA) in rat spermatozoa; exposed rats showed an increase in MDA level in spermatozoa as compared to the control group. **p* ≤ 0.05

pregnancy, embryonic development and foetal development depending upon the frequency, duration of exposure and strength of exposure (Gye & Park, 2012). A study on men semen reported that the use of cell phone leads to decrease in sperm count, viability, motility and normal morphology of spermatozoa thereby resulting in declination of semen quality. They further stated that these changes in sperm parameters are dependent on daily exposure to the radiation (Agarwal, Deepinder, Sharma, Ranga, & Li, 2008).

Our results suggested significant changes in sperm parameters which validates the previous research on animal and human spermatozoa exposed to radiations. This thus confirms our hypothesis that the mobile phone radiation induces oxidative damage and may cause harmful reproductive changes which in turn can affect male fertility. Constant exposure to 3G mobile phone radiations resulted in increased ROS and lipid peroxidation level with simultaneous decrease in sperm mitochondrial activity. Electromagnetic radiations emitted from mobile phones generate mitochondrial ROS. The generated superoxide radicals and electrophilic aldehydes form adducts by interacting with the proteins that act as the integral components of the mitochondrial electron transport chain (Aitken, Jones, & Robertson, 2012). This chain of reaction goes on till equilibrium is reached, wherein an intrinsic apoptotic cascade gets initiated. The initiation of the cascade leads to deleterious changes in the spermatozoa such as loss of sperm motility and mitochondrial membrane potential, activation of caspases, initiation of oxidative DNA damage, DNA fragmentation and loss of vitality.

Thus, the exposure of Wistar rat to 3G mobile phone radiation leads to considerable changes in fertility parameters, brought about by a cycle of process initiated by oxidative stress, suggesting our hypothesis to be true. However, complete elucidation of the mechanism via which radiation includes the generation of ROS is still

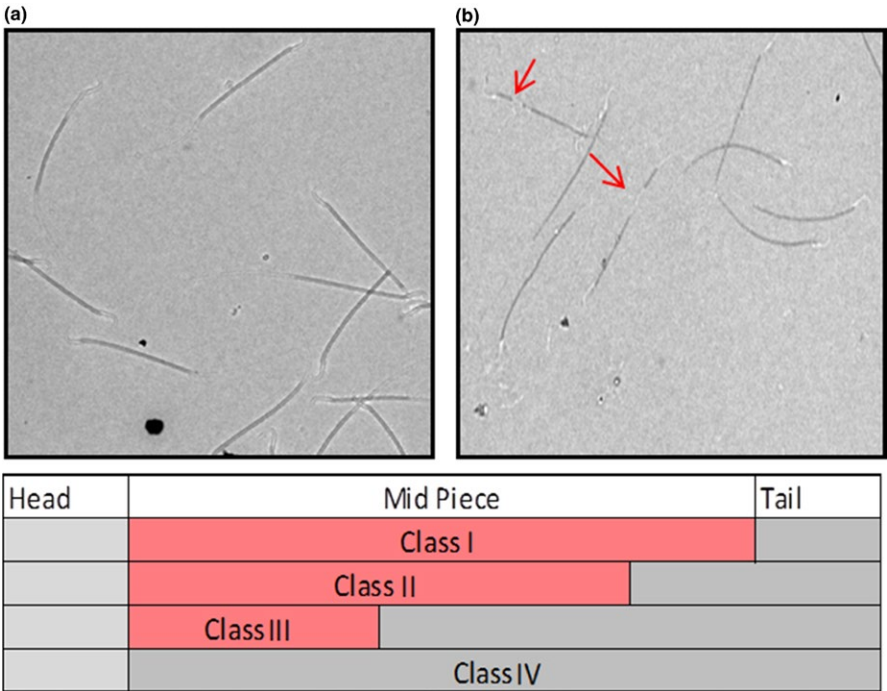


FIGURE 7 Mitochondrial function test by DAB staining of spermatozoa upon radiation exposure. (a) Control (b) Exposed. Class I (100% of the midpiece stained), class II (>50% of the midpiece stained), class III (<50% midpiece stained) and class IV (absence of staining in the midpiece)

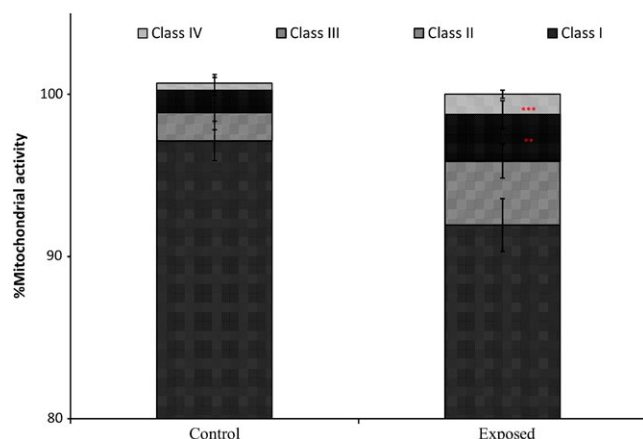


FIGURE 8 Sperm mitochondrial activity in male rats of the control and exposed groups. Significance at *** $p \leq 0.001$ and ** $p \leq 0.01$

unknown and needs to be addressed. Further, to correlate the obtained results with the human populations, long-term exposure to radiation is required because of species variation in terms of size, shape, age and cellular morphology (D'Silva et al., 2017).

5 | CONCLUSION

The present study shows that RF-EMR emitted from mobile phone affects the male reproductive system via increasing oxidative stress, which alters testicular structure and changes the sperm parameters such as sperm count, morphology, viability and membrane integrity in the midpiece and tail of sperm cells. However, no significant changes were found in sperm head morphology. In addition to this, an increase in ROS level was the main cause of elevated lipid peroxidation, sperm plasma membrane damage and alteration in mitochondrial activity.

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