The effect of ethanolic extract of Moringa oleifera leaves on sperm parameters in 4G-cellphone-EMR exposed rats

Sudha Ramalingam, Deepa Somanath

Department of Anatomy, Sri Manakula Vinayagar Medical College and Hospital, Puducherry- 605 107, India

SUMMARY

Cell phone use and infertility are topics of scientific investigation. 2G, 3G and 4G cell phone radiation causes negative effects on spermatogenesis. The aim of this study is to assess the protective efficiency of Moringa oleifera leaves (MOL) on the sperm parameters of Wistar rats against electromagnetic radiation (EMR) emitted from a 4G mobile phone. Male Wistar rats were divided into five groups. Control group (n=3) without mobile phone; Sham group (n=3) with mobile phone in switched-off mode; MOL-2 group (n=6) obtained orally 200 mg ethanol extract of MOL/kg BW/two months; R2 group (n=6) subjected to 4G-EMR for 96 minutes/day/two months; R2+MOL group (n=6) treated with MOL extract while exposed to EMR for two months. After the experimental period, rats were sacrificed to get epididymises. The epididymal fluid was collected to evaluate count, viability, motility and morphology of sperms. The 4G-EMR induced a significant reduction in the count and tail defects of sperms ($P<0.05$) in R2 rats as compared to control, sham and MOL-2 groups. Nevertheless, *MOL* extract significantly inhibited the EMR-caused fall of those variables in R2+MOL group. The R2 group had significantly ($P<0.0001$) lower and higher values concerning the sperm's progressive motility and non-motility rates, the percentages of alive and dead sperms than those in the control, sham, and MOL-2 groups, but the *MOL* extract restored the parameters in R2+MOL as compared to R2 group. The oral administration of 200 mg of ethanolic extract of Moringa leaves protected the sperm parameters of Wistar rats to near normal level from harmful effects of 4G-cell phone-EMR.

Key words: Cell phone – Moringa leaves – Testis – Oxidative stress – Sperm

INTRODUCTION

Infertility is defined as failure to get pregnant after a year of regular, unrestricted sexual copulation, which is a widespread problem with human reproduction ranging from 2.5 to 15 %. It significantly affected 27.5 million of Indian couples, and 40–50% of infertility in those couples is attributable to the male spouse (Agarwal and Durairajanayagam, 2015; Toragall et al., 2019). Male infertility is associated with the use of tobacco, alcohol,

Corresponding author:
Deepa Somanath. Department of Anatomy, Sri Manakula Vinayagar Medical College and Hospital, Puducherry- 605 107, India. Phone:0413-260400. E-mail: deepa.somanath@gmail.com - Orcid: 0000-0002-4732-5406

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pesticides, heavy metals, and radiation, in addition to a few clinical disorders and genetic factors (Oliva et al., 2008).

Smartphones utilize high frequency (850 MHz-2.4 GHz) and emit non-ionizing radiation called radio-frequency electromagnetic radiation (RF-EMR) during its working mode (Agarwal et al., 2008; Kim et al., 2007; Oh et al., 2018). Few human researches has investigated the relationship between cell phone exposure and poor semen quality, which may lower sperm count, motility, viability and normal morphology (Agarwal et al., 2008; Erogul et al., 2006). The rate at which RF-EMR energy is absorbed by human tissues is known as the specific absorption rate (SAR). The amount of SAR absorbed depends on the exposure's frequency, intensity, polarisation, and duration. When conversing on the phone or keeping the phone close to the head or in a pocket of clothing, a greater radiation absorption rate may be seen (Kesari et al., 2018). Health risks by RF-EMR might arise from thermal, non-thermal, or a mixture of both processes (Meo et al., 2011). It may affect gene responsiveness, causes DNA damage or inhibit its repair, and causes oxidative stress (OS) in a non-thermal manner (Belpomme et al., 2018). OS is a condition caused by an imbalance between oxidants and antioxidants, which results in an excessive accumulation of oxidants relative to antioxidants. The relationship between RF-EMR exposure and harmful biological effects is due to the production of reactive oxygen species (ROS) as a result of elevated OS, which undermines the body's natural defences (Avci et al., 2012). Compounds known as bio-antioxidants eliminate, scavenge and resist the formation of ROS or their activity. Among the well-known biological antioxidants, super oxide dismutase (SOD) and its two isozymes, glutathione reductase (GR) and catalase, play a significant role (Turk et al., 2008). The protective properties of plant material including antioxidant chemicals against cell-phone-related RF-EMR tissue damage have been well-documented by academics (Avci et al., 2012; Khaki, 2011; Mailankot et al., 2009). The leaves of the Moringa oleifera (MOL) (drumstick tree), which is an Indian staple food, is a good source of vitamins, minerals, and amino acids. There is literature available regarding the anti-diabetic, anti-hypertensive, anti-inflammatory, antiepileptic and antitumor properties of MOL. A variety of anti-oxidants are identified in MOL such as kaempferol, quercetin, myricetin, vanillin, gallic acid, ellagic acid, ferulic acid, and flavonoids (Stohs and Hartman, 2015). There is a scarcity in the literature investigating MOL as a protector of sperms against the hazardous effects of RF-EMR arising from 4G-cell phone. Hence, this experiment was conducted to determine the possible radio-protective efficacy of ethanolic extract of MOL on sperm parameters in Wistar rats during RF-EMR exposure from 4G-cell phone for duration of two months.

MATERIALS AND METHODS

Animals

Four-week-old male Wistar rats, weighing approximately 150-180 gm, were used in this study. They were housed in Plexiglas cages with a commercially available balanced diet and tap water ad libitum. They were subjected to light and dark periods of 12 h/12 h and a temperature of 22 to 24°C.

Ethical clearance

The animal experimentation procedures listed in this study were done according to the internationally accepted guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Ethics Committee (Approval letter No. IAEC/2015/02).

Animal grouping

After a week of acclimatization, rats were randomly divided into five groups, and they were maintained in separate rooms without any other external EMR-sources.

Control group (n=3): No cell phone radiation.

Sham group (n=3): Exposed to the cell phone in switch off mode.

MOL-2 group (n=6): Received orally 200 mg of ethanolic extract of *MOL*/kg body weight/day for two months.

R2 group (n=6): Exposed to EMR for 96 minutes/day for two months (4 minutes/every half an hour from 8 AM to 8 PM).

R2+MOL (n=6): Exposed to EMR for 96 minutes/day (4 minutes/every half an hour from 8 AM to 8 PM) and concurrently treated orally with 200 mg of ethanolic extract of *MOL*/kg body weight/day for two months.

Cell phone-EMR System for exposure

A typical commercial brand of Android smartphone with a peak power density of 2W/kg and a whole-body SAR value of 1.6W/kg was employed in this study (as stated by the manufacturer). The Cornet Electrosmog RF meter was used to calculate the average power density of the phones, which was 187.9 mW/m^2, based on the electromagnetic field values of the rat cage's interior. The smartphone was suspended from the rat cage's ceiling to reach the center of the cage while ensuring the free movement of the animals (Narayanan et al., 2009).

MOL collection and preparation of the extract

M. Oleifera leaves that had been verified by a Botanist were collected from a village in Tamil Nadu, India. The leaves were washed with water and air-dried at 44°C for four hours, and then powdered in an electric mixer grinder. The powdered leaves were macerated with 70% ethanol at a ratio of 1:40, w/v, for 72 hours at room temperature to prepare the extract. The extract was filtered using No. 1 Whatman filter paper, and any leftover material was extracted again using the same procedure and solvent until the entire amount of marc was used completely (Vongsak et al., 2013). The resulting crude extract was stored at -4°C for later usage after the solvent was removed using a rotary evaporator.

Collection of semen and sperm evaluation

An intraperitoneal injection of 45 mg/kg of ketamine hydrochloride was given 24 hours after the completion of the experiment to anesthetize the rats, accompanied by sacrifice. Epididymises were obtained by trans-abdominal incision and minced to extract the epididymal fluid to evaluate count, viability, motility and morphology of sperms.

Epididymal fluid was collected from both cauda epididymides, which were cleared from other soft tissues and cut into pieces in a Petri dish containing one ml of physiological saline (0.9% w/v NaCl) and allowed to stay for 15 minutes at room temperature to release all sperms into the saline to make semen sample (Aksu et al., 2015).

Sperm motility

A small drop of semen sample was poured into two droplets of Tris buffer solution which was kept on the slide and mixed on the pre-heated stage of the light microscope. To evaluate the percentage of sperm motility, three random fields were chosen under 400x and the mean of three successive fields was considered as the final score (Sonmez et al., 2005). The percentages of progressively motile (sperms moving faster from one place to another), sluggishly motile (moving sperms at the same place) and non-motile (sperms with the absence of movement) spermatozoa were determined (Luthfi, 2015).

Sperm count

Sperm count was determined with a hemocytometer (Bahmanzadeh et al., 2008).

Abnormalities of spermatozoa

The semen-smeared slides were stained with eosin-nigrosin and studied under a light microscope at a magnification of 400-1000x. 400 spermatozoa were assessed on each slide and abnormalities of spermatozoa were categorized into head, neck and tail defects and given in percentage (%) (Kesari and Behari, 2012).

Alive and dead spermatozoa

Previously stained slides intended for the evaluation of abnormal spermatozoa were used to determine the percentage of live and dead sperm. The slides were examined under a light microscope at 400x magnification and 200 spermatozoa were counted from each sample. They were listed as alive (unstained head) and dead (stained head) as per the eosin staining state of the sperm head (Aksu et al., 2015).

Statistical analysis

Differences between obtained values were compared by one-way analysis of variance (ANOVA),

followed by a post hoc test (Tanhane/LSD). Kruskal Wallis test was applied for the continuous type of data, but not following normal distribution and Mann Whitney U test was used to identify the difference between the groups using JASP statistical software (University of Amsterdam, Netherland). Values were represented as mean±SE and they were considered to be statistically significant at $P < 0.05$.

RESULTS

Epididymal sperm count

Short-term 4G-EMR induced a significant reduction in the sperm count in R2 rats as compared to control, sham and MOL-2 groups. Nevertheless, *MOL* extract inhibited the EMR-caused fall of sperm density in R2+MOL but not to the level seen in control, sham and MOL-2 groups (Fig. 1).

Motility pattern of sperm

The R2 group had significantly ($P<0.0001$) lower and higher values concerning the sperm progressive motility and non-motility rates than those in the control, sham, and MOL-2 groups. The *MOL* extract effectively restored these parameters from radiation in R2+MOL, when compared to R2 group. About the percentage of sluggish sperm motility, no significant differences were seen between groups (Fig. 2).

Sperm viability

The percentages of alive and dead sperms in the R2 group were significantly lower ($P<0.05$) and higher than in control, sham, and MOL-2 groups respectively. In comparison with the R2 group, these parameters were kept at a normal level in R2+MOL but the rate of dead sperms was significantly lower than control and sham groups (Fig. 3).

Sperm morphology

Cell phone-EMR caused significantly more sperm head defects in R2 group as compared to the MOL-2 group only. But no statistically signifi-

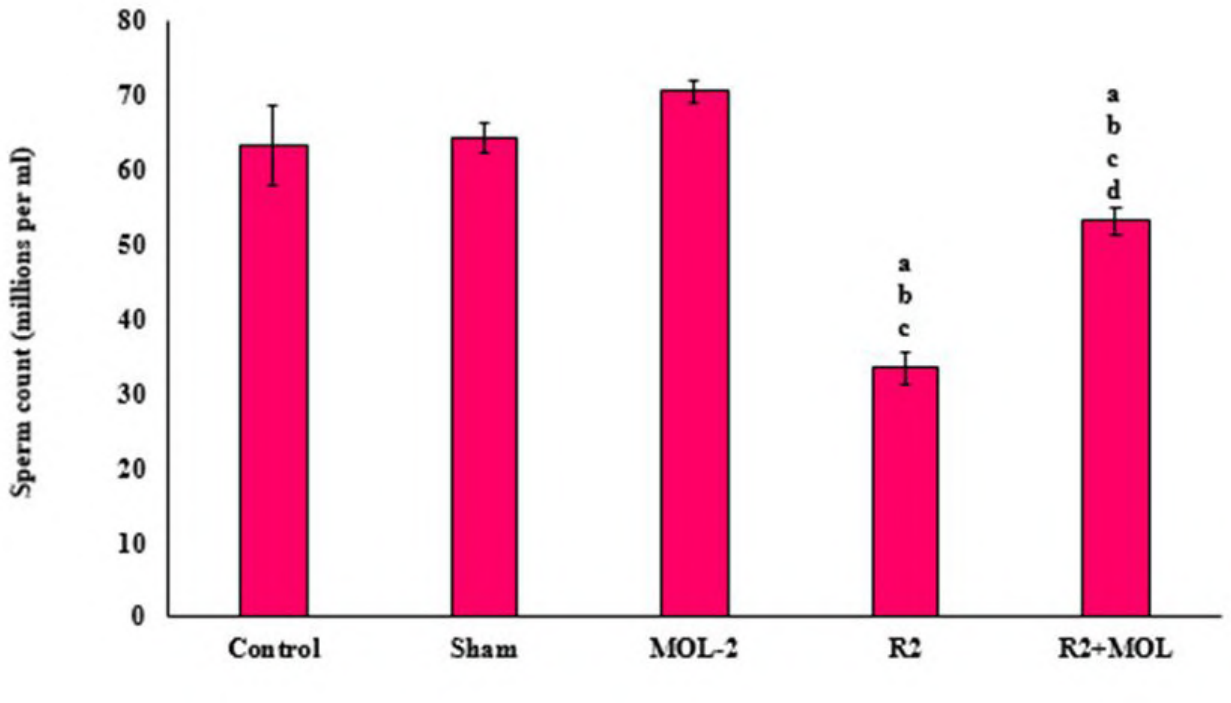


Fig. 1.- Effect of ethanolic extract of MOL on epididymal sperm count in the 4G-mobile phone-induced-EMR exposed rats. Control group-not exposed to 4G-cell phone for two-months, sham group-exposed to 4G-cell phone in switch off mode for two-months (n=3), MOL-2 group-not irradiated and treated with ethanolic extract of MOL 200 mg/kg for two-months (n=6), R2 group-exposed to 4G-EMR for two-months (n=6) and R2+MOL group-treated with ethanolic extract of MOL 200 mg/kg during 4G-EMR exposure for two-months (n=6). Values are expressed as mean ± SE in each group. P values are by one-way ANOVA with LSD/Tanhane test. Different superscript letters show significant differences between groups ($P<0.05$). a- different from the control group; b- different from the sham group; c- different from the MOL-2 group; d- different from the R2 group.

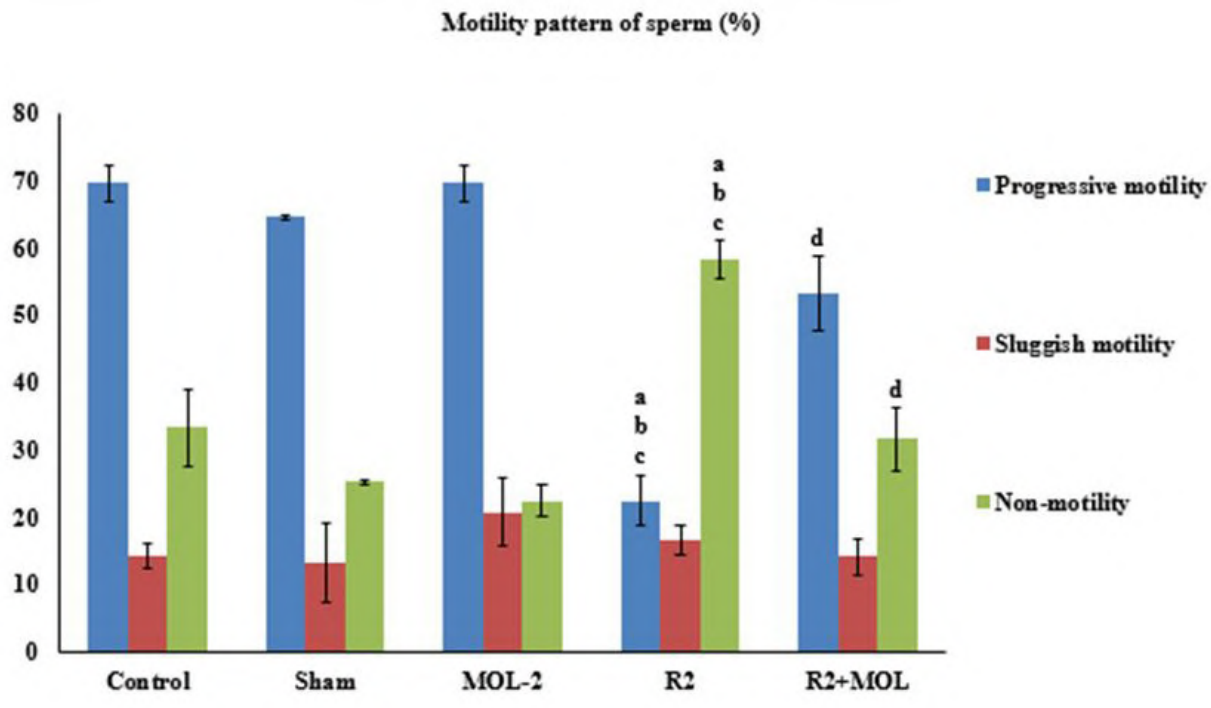
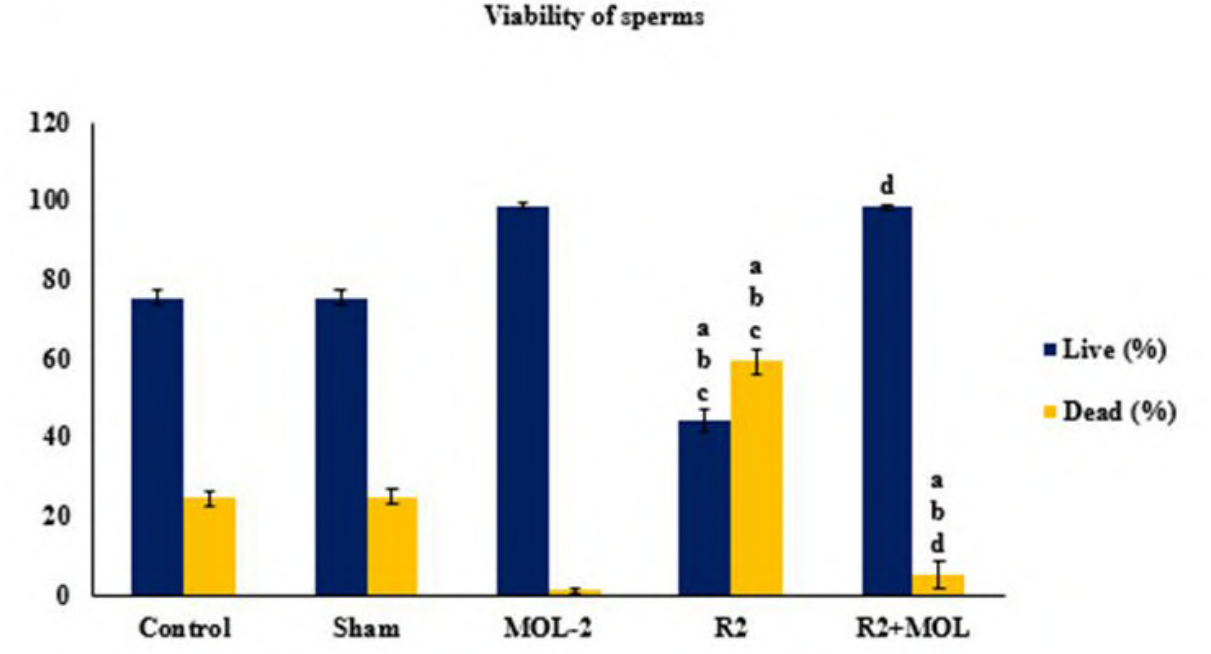


Fig. 2.- Effect of ethanolic extract of MOL on sperm motility pattern in the 4G-mobile phone-induced-EMR exposed rats. Control group-not exposed to 4G-cell phone for two-months, sham group-exposed to 4G-cell phone in switch off mode for two-months (n=3), MOL-2 group-not irradiated and treated with ethanolic extract of MOL 200 mg/kg for two-months (n=6), R2 group-exposed to 4G-EMR for two-months (n=6) and R2+MOL group-treated with ethanolic extract of MOL 200 mg/kg during 4G-EMR exposure for two-months (n=6). Values are expressed as mean ± SE in each group. P values are by one-way ANOVA with LSD/Tanhane test. Different superscript letters show significant differences between groups (P<0.05). a- different from the control group; b- different from the sham group; c- different from the MOL-2 group; d- different from the R2 group.



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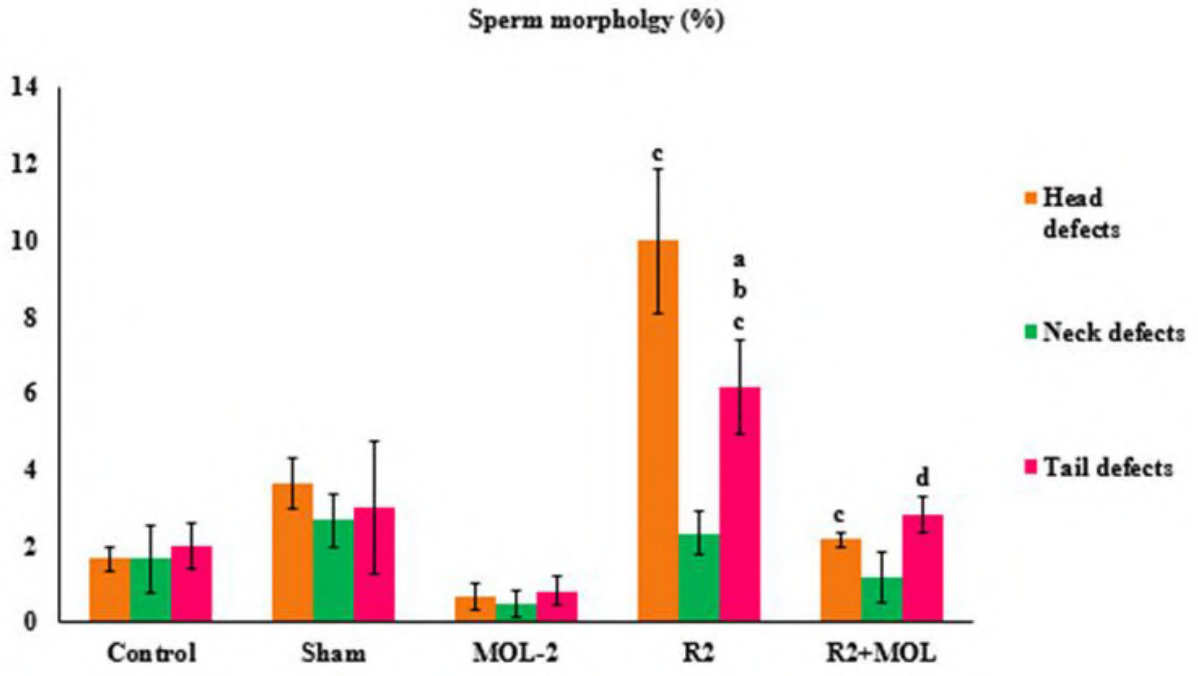

Fig. 4.- Effect of ethanolic extract of MOL on sperm morphology in the 4G-mobile phone-induced-EMR exposed rats. Control group-not exposed to 4G-cell phone for two-months, sham group-exposed to 4G-cell phone in switch off mode for two-months (n=3), MOL-2 group-not irradiated and treated with ethanolic extract of MOL 200 mg/kg for two-months (n=6), R2 group-exposed to 4G-EMR for two-months (n=6) and R2+MOL group-treated with ethanolic extract of MOL 200 mg/kg during 4G-EMR exposure for two-months (n=6). Values are expressed as mean ± SE in each group. P values are by one-way ANOVA with LSD/Tanhane test. Different superscript letters show significant differences between groups ($P<0.05$). a- different from the control group; b- different from the sham group; c- different from the MOL-1 group; d- different from the R2 group.

cant change was observed in the sperm neck defects among the groups. High-frequency radiation significantly elevated the percentage of sperm tail defects in R2 group as compared to the control, sham and MOL-2 groups. The simultaneous treatment with MOL significantly protected the sperms from such defects in R2+MOL group from radiation as compared to the R2 group (Fig. 4).

DISCUSSION

Depending on the power density of the EMR apparatus and the distance from the apparatus, electromagnetic waves can pass through the tissues and promote the synthesis of ROS either directly or indirectly (Kesari et al., 2018). The metabolic cycle of all living cells including spermatozoa produce ROS normally. However, when it builds up too much, the body's own antioxidants help the cell to defend itself (Turk et al., 2008). The OS may be activated by such enhanced ROS production, which could affect the functioning of sperm (Kesari et al., 2018). In this study, the 4G-cell-phone-EMR significantly decreased the sperm count, progressive motility and viability rates but increased the sperm head and neck defects in the radiation group. On the other hand, the ethanolic extract of MOL nearly normalized all the affected values.

Intracellular molecules, notably polyunsaturated fatty acids and transmembrane proteins, are the main targets of ROS. In order to oxidize these molecules with the byproducts of peroxides, lipid aldehydes and alcohol, ROS combines spontaneously with them. Increased permeability results from this, and encourages the oxidative destruction of unsaturated fatty acids in cell membranes (Turk et al., 2008). This disruption can lead to axonemal damage and decreased sperm viability, which may be the cause of the increased sperm mortality rate and decreased sperm count in the R2 group of this study (Turk et al., 2008; Kesari and Behari, 2012). Further, Gautam et al. (2019) concluded that 3G cell phone radiation on male Wistar rats' testes could provoke the development of free radicals causing reduction in sperm count, changes in sperm membrane integrity.

The results of this study are supported by Salama et al. (2010) showed that 12 week-exposure to the GSM handset can result in a significantly low sperm count at the 8th week and more declined sperm motility after 10 weeks in the phone group. Similarly, Abd El Rahman et al. (2014) documented 950 MHz for 2 months could provoke oxidative stress with a significant increase in the level of TBARS, advanced oxidation protein products and carbon monoxide associated with a significant decrease in activities of antioxidant enzymes and GSH content. He added that OS will be accompanied by reproductive hormonal disturbances.

Few studies documented that mobile phone duration for a longer period did not lead to male infertility. Lee et al. (2010) concluded that rats treated with RF-EMR of 848.5 MHz with 2.0 W/kg SAR value for the period of 45 minutes twice a day with the interval of 15 minutes for 12 weeks could not produce harmful effects on male infertility, because EMR did not alter the sperm counts, the testicular and epididymal concentration of MDA. He also reported that CDMA (SAR-2.0 W/kg) and WCDMA (SAR-2.0 W/kg) RF signals failed to produce changes in similar parameters (Lee et al., 2012). Similarly, Ribeiro et al. (2007) argued that rats exposed to RF-EMR emitted by a cellular phone using GSM at the frequency rate of 1835–1850 MHZ for 1 h/d for 11 weeks did not show alteration in testicular and epididymal weight and sperm count. The negative changes in the variables of the present study can be due to the high-frequency induced-EMR and longer duration of exposure/day.

The leaves of drumstick tree are an abundant source of natural antioxidants, as they contain polyphenols and flavonoids that safeguard organisms and cells from oxidative DNA damage involved in aging, cancer and degenerative diseases (Nayak et al., 2016). In this research, the ethanolic extract of MOL recovered all parameters to a near-normal level in R2+MOL (as in the control group). The group with only MOL intervention showed significantly enhanced and decreased viability and abnormal morphology of sperms respectively as compared to the control and sham groups; created negligible discrepancies in the remaining sperm variables.

The antioxidant potential of MOL-Phyto molecules was shown from OH-mediated oxidative damage in human serum albumin protein. It is claimed that phytomolecules of MOL like phenols and flavonoids may hunt free radicals or chelate transitional metal ions resulting in inhibition of Fenton-like reaction or transformation of radical to non-radical (Singh et al., 2009). The results of the present experiments in irradiated animals administered with MOL are in agreement with Nayak et al. (2015), who indicated that previous administration of MOL ethanolic extract could restore the depletion of male gonadal function to normal levels in the case of cyclophosphamide chemotherapy. He continued that perhaps the MOL-phytochemicals and secondary metabolites possess a marked ability to scavenge free radicals. Similar patterns have been shown by Zahran et al. (2015), who conclude that simultaneous administration of MOL extract performed a preventative role against testicular harm caused by Equigan. It can significantly increase the sperm count, germ cell count, testicular superoxide dismutase and total protein in the crypt orchid rats (Afolabi et al., 2013). Bin-Meferij and El-Kott (2015) provided evidence that 900 MHz-EMR treatment can cause microscopic testicular abnormalities with deteriorated spermatozoa in animals, and these effects can be reversed by administering 200 mg/kg of MOL aqueous extract concurrently with EMR; these findings are consistent with the findings of the current investigation.

In conclusion, in this study, short-term exposure to 4G-mobile phone radiation caused a significant change in the sperm count and other sperm parameters. The ethanolic extract of MOL could save the variables from the harmful effects of 4G-EMR. Such protective ability of MOL could be due to antioxidant activity of its phytochemicals.

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Authors' contributions

All the authors contributed significantly to the intellectual content, collection and analysis of data and the reviewing of final version of the work.

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