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The Effect of Ethanolic Extract of *Moringa Oleifera* Leaves on 4 G-Cell Phone-EMR-Induced Oxidative Stresses Associated with Altered Sperm Count in Pre-Pubertal Wistar Rats

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ABSTRACT

Introduction

Infertility is defined as the failure to attain pregnancy after a one-year-period of routine non-contracepted sexual intercourse and it has become a global issue of human reproduction. The malefactor is responsible for nearly half of the infertility cases. Currently, scientific studies associate mobile phone usage and male infertility. Earlier studies reported 2G, 3G, and 4G radiations can affect the sperm parameters and few other studies give contradictory opinions. This study was aimed to study the effect of electromagnetic waves from 4G-cell phones and to evaluate the possible radio-curative efficiency of *Moringa oleifera* leaves (*MOL*) on Wistar rats' testis for one month.

Materials and Methods

Four-week-old male Wistar rats were randomly divided into five groups:

Control: no cell phone.

Sham: cell phone in switch off mode.

MOL-1: gavaged with 200mg/kg body weight/day of ethanolic extract of *MOL* for one month.

R1: Exposed to EMR for 96 minutes/day for one month (4 minutes/every half an hour from 8 AM to 8 PM)

R1+MOL: Exposed to EMR for 96 minutes/day (4 minutes/every half an hour from 8 AM to 8 PM) and simultaneously gavaged with 200mg/kg body weight/day of ethanolic extract of *MOL* for one month.

By the end of the experimental period, rats were anesthetized and sacrificed. Blood samples were obtained through the left cardiac ventricle and serum was separated and used for estimation of testosterone and inhibin-B. Then testes were removed along with epididymis through trans-abdominal incision and testes used for the preparation of tissue homogenates for estimation of

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oxidative stress markers. Epididymises were minced to extract the epididymal fluid to evaluate sperm count.

Results

4G-EMR decreased the level of superoxide dismutase (SOD) and increased the level of malondialdehyde (MDA) in testicular tissue in the R1 group. Oxidative stress was accompanied by hormonal disturbances recorded by significant decreases in serum inhibin-B level but an insignificant decrement in serum testosterone level. Besides, R1 group showed an insignificant reduction in the sperm count as compared to the control group. In this study, the ethanolic extract of *MOL* regained most of the parameters to near normal level in R1+MOL.

Conclusion

The oral administration of 200 mg of ethanol extract of *MOL* could revert the hormonal and sperm count variations to near normal level through the antioxidant mechanism of its active components in 4G-cell phone-EMR induced negative alterations in the testis of Wistar rats.

Keywords

Cell phone, moringa leaves, testis, oxidative stress, sperm count

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Introduction

Infertility is described as failing to attain pregnancy after a one-year-period of routine non-contracepted sexual copulation, which makes up a global issue of human reproduction. It ranges from 2.5 percent to 15 percent. [1,2] In India, substantially, it affected 27.5 million couples and in 40%-50% of infertility in the couples is due to male partner.[2] Apart from certain clinical conditions and genetic causes, male infertility is correlated with tobacco and alcohol consumption, pesticides, heavy metals and radiation.[3]

Smartphone uses high frequency (850 MHz-2.4 GHz) and during its operating mode, emit non-ionizing radiation called radio-frequency electromagnetic radiation (RF-EMR).[4, 5, 6] Few human studies have revealed that poor semen quality in men is associated with cell phone exposure, which may reduce sperm count, motility, viability, and normal morphology. [7, 4] The International Agency for Research on Cancer classified RF as 'possibly carcinogenic' to humans and included in the list of environmental pollutants as "electro-pollution". Specific absorption

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rate (SAR) is a rate at which RF-EMR energy is absorbed within human tissues. The frequency, intensity, polarization, and duration of exposure determine the amount of SAR absorbed by human tissue. A higher radiation absorption rate could be observed while talking on the phone, keeping a phone near the head or in trouser's pocket.[8] RF-EMR can cause health hazards through thermal or non-thermal mechanisms or a combination of both.[9] In a non-thermal way, it may induce DNA damage and prevention of its repair, altered gene response and oxidative stress (OS).[10]

OS is a state in which excessive accumulation of oxidants occurs, compared to the antioxidants as a result of an imbalance between oxidants and antioxidants. The link between RF-EMR exposure and deleterious biological effects are correlated with the generation of reactive oxygen species (ROS) because of increased OS and thereby compromises bodily protective mechanism.[11] ROS are extremely reactive, free radicals-class oxidizing agents. A free radical is any compound that includes one or more unpaired electrons (may not be from oxygen). Superoxide is the most prevalent ROS with potential consequences in reproductive biology.[12] Lipid peroxidation (LPO) cascade contributes to the development of free radicals and leads to the formation of lipid aldehydes such as acrolein, 4-hydroxynonenal and malondialdehyde (MDA) which is a relevant OS marker. The bio-antioxidants are compounds that dispose of, scavenge, and oppose the development of ROS or their activity. A range of biological and chemical antioxidants that attacks ROS and LPO under inquiry. Super oxide dismutase (SOD) and its two isozymes, glutathione reductase (GR)and catalase play an important part among the well-known biological antioxidants.[13] Many scholars have documented the ameliorative effects of plant material containing antioxidants compounds against cell phones caused RF-EMR tissue damage.[11, 14, 15]

The leaves of the *Moringa oleifera* (*MOL*) (drumstick tree) are a good source of minerals, amino acids, and vitamins, which makes up a part of the Indian food for many centuries. Many kinds of literature available regarding the anti-hypertensive, anti-diabetic, antiepileptic, anti-inflammatory and antitumor properties of *MOL*.[16] A high number of anti-oxidants are identified in *MOL* like kaempferol, quercetin, gallic acid, ellagic acid, ferulic acid, myricetin and vanillin, and flavonoids. [16] There is no study in the literature investigating *MOL* as a testicular protector against the hazardous effects of RF-EMR arising from 4G-cell phone. Hence, this experiment was conducted to study whether exposure to the RF-EMR arising from 4G-cell phone causes OS in testicular tissues of Wistar rats and to determine the possible radio-protective efficacy of ethanolic extract of *MOL*.

Materials and Methods

Animal model

Male Wistar rats (four-week-old) with an average weight of 150-180 gm purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai. They were maintained in Plexiglas cages under the environment having 12 hours/12 hours of light and dark cycle and a temperature of 22–24°C with a commercial balanced diet and tap water ad libitum. This study was approved by the Institutional Animal Ethical Committee (Approval letter No.

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IAEC/2015/02, dated-14/05/2015). The protocols for animal experimentation described in this study were following the guidelines of the Animal Ethical Committee for the Care and Use of Laboratory Animals. After seven days of acclimatization, rats were randomly divided into five groups named: control (n=3), sham (n=3), MOL-1(n=6), R1 (n=6), and R1+MOL (n=6). Control and sham control groups were housed in a similar environment, but each of them in a separate EMR-free room.

Control: no cell phone.

Sham: cell phone in switch off mode.

MOL-1: gavaged with 200mg/kg BW/day of ethanolic extract of *MOL* for one month.

R1: Exposed to EMR for 96 minutes/day for one month (4 minutes/every half an hour from 8 AM to 8 PM)

R1+MOL: Exposed to EMR for 96 minutes/day (4 minutes/every half an hour from 8 AM to 8 PM) and simultaneously gavaged with 200mg/kg body weight/day of ethanolic extract of *MOL* for one month.

Cell phone-EMR Exposure setup

A standard commercial brand of android mobile phones was utilized. They were with a whole-body SAR value of 1.6W/kg and a peak power density of 2W/kg, which were mentioned by the manufacturer. Cornet Electrosmog RF meter was used in the rats' cage to determine the average power density (187.9 mW/m2) from electromagnetic field values. The cell phones were licensed to operate pulse 4G LTE networks over BAND 5 LTE FDD (850 MHz), BAND 3 LTE FDD (1800 MHz), and BAND 40 LTE TDD (2300 MHz). The handset was kept in a box with a wooden floor measuring about 14 x 7 x 5 cm hung from the ceiling of the rat's cage; approaching the center of the cage (an equal distance of 7.6 cm from the bottom and roof of the cage) and movement of the animals were assured.[17] No other cell phone and metal articles were allowed to the experimental room to prevent disturbances in radiation. The mobile phone inside the cage was powered on and kept in an auto answering mode**.** A video call was given from the same brand and model of a separate cell phone.

Plant material and preparation of extract

M. oleifera leaves were harvested and authenticated by a professor of Botany, Annamalai University, Tamil Nadu, India. After a thorough wash, the leaves were air and oven-dried at 44°C for four hours and made into a fine powder. The powdered leaves were macerated with 70% ethanol at the ratio of 1:40, weight/volume for 72 hours at room temperature with occasional shaking. Whatman no1filter paper was used to filter the extract. Then, the rotary evaporator was used to remove the solvent and the final crude extract was stored at - 4°C for future use.[18]

Collection of semen and sperm evaluation

By the end of the experimental period, the rats were anesthetized and sacrificed with intra-peritoneal administration of ketamine hydrochloride. Blood samples were obtained through the left cardiac ventricle and serum was separated and stored in aliquots at -80°C till used for

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estimation of testosterone and inhibin-B. Then testes were removed along with epididymises through the trans-abdominal incision and used for the preparation of tissue homogenates for estimation of OS markers.

Semen samples were collected from both cauda epididymises. They were dissected out and cut into pieces in one ml of physiological saline (0.9% w/v NaCl) in a Petri dish and allowed to stay for 15 minutes at room temperature to release all sperms into the saline to make semen sample.[19]

Sperm count was determined with a hemocytometer. The semen sample was diluted at 1:100 with a fixative/staining solution (a mixture of 5g sodium bicarbonate, 1 ml of 35% formalin and 25 mg eosin per 100 ml of distilled water to make sperm suspension. Approximately, 10µl of diluted sperm suspension was added into each counting chamber and permitted settling down for 5 min for examination under a light microscope at 200x magnification. Sperm cells were counted and expressed in millions/ml.[20]

Serum analysis of testosterone and inhibin-B

The hormones in serum were measured using Enzyme-Linked Immunosorbent Assay (ELISA) kits (Bioassay Technology Laboratory, Shangai, China). The serum testosterone levels were determined according to the manufacturer's instructions. The lower limit of assay detection for testosterone was 10ng／L; the intra-assay coefficient of variation was <8%; the inter-assay coefficient of variation was <10% and the functional sensitivity was 5.25ng／L.

The lower limit of assay detection for inhibin-B was 2ng／L; the intra-assay coefficient of variation was <8%, and the inter-assay coefficient of variation was <10% and the functional sensitivity was 1.03ng／L.

Preparation of tissue homogenates

As per the manufacturer's instructions, a piece of testis was separated weighed and washed with phosphate buffer saline (PBS) to remove excess blood. The tissue was minced and homogenized in phosphate buffer saline (PBS) by a glass tissue homogenizer on ice. The homogenates were filtered and centrifuged at 4ºC (2000-3000 RPM) for about 20 minutes by using a refrigerated centrifuge and then, the supernatant was frozen at -20ºC in aliquots until used for biochemical assays.

Estimation of antioxidant enzymes

The levels of SOD, catalase, GR and MDA were measured by Enzyme-Linked Immunosorbent Assay (ELISA) kits (Bioassay Technology Laboratory, Shangai, China) from tissue supernatant, as per producer's guidelines. All of these enzymes' reactions were terminated by adding an acidic stop solution and absorbances were measured at 450 nm with a microplate reader (Bio-Rad, California, USA).

The measuring range of those enzymes according to the manufacturer was following: for SOD, 0.05–20ng/ml (minimum-maximum), and the functional sensitivity was 0.022ng/ml; for Catalase, 1–300ng/ml, and the functional sensitivity was 0.52 ng/ml; for GR, 0.5–200 ng/ml, and functional sensitivity was 0.24 ng/ml; for MDA, 0.05–10 nmol/ml, and the functional sensitivity

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was 0.01 nmol/ml. All of them had the intra-assay coefficient of variation of <8%; and the inter-assay coefficient of variation of <10%.

Statistical analysis

Using JASP software, differences between obtained values (mean ± SD/SE) were compared by one-way analysis of variance (ANOVA) followed by a post hoc test (Tanhane/Fisher's Least Significant Difference (LSD)). Values were considered being statistically significant at $P < 0.05$.

Results

Reproductive hormones

Serum testosterone and inhibin-B levels of rats in all groups were presented in Table. There was no significant difference among groups.

Radiation induced insignificant reduction in the serum testosterone level but significant reduction in the serum inhibin-B level in R1-rats as compared to control and sham groups. *MOL* extract insignificantly increased the levels of hormones in R1+MOL but not to the levels of control, sham and MOL-1 groups.

Biomarkers of oxidative stress in the testis of rat

The testicular activity of the SOD was significantly ($p < 0.05$) decreased in cell phone radiation-exposed rats in the R1 group as compared to sham and MOL-1groups. There were no significant differences observed among other groups. Radiation also caused a significant increase in the level of testicular malondialdehyde (MDA) in the irradiated animals in the R1 group relative to the control, sham and MOL-1 groups. The ethanolic extract of *MOL* significantly reduced the level of MDA in R1+MOL as compared to the R1 group but SOD was not significantly changed. None of the treatment groups showed altered levels of intra-testicular catalase and GR (table).

Epididymal sperm count

There was no much effect of RF-EMR (4G) on the sperm count in R1 rats but a decrease was observed without any statistical significance as compared to control group but differed significantly from MOL group-1. Nevertheless, *MOL* extract brought back the sperm density in R1+MOL nearly like that of the control group (Figure).

Discussion

Electromagnetic waves penetrate the tissues and induce ROS either directly or indirectly, which depends on the power density of EMR apparatus and distance from the apparatus.[8] The testis is the highly susceptible organ for EMR which pervades 4-5 cm deeper into the tissues.[21] Besides, type, mode, dose, duration of exposure and undeveloped testis determine the negative effects of EMR.[22] ROS are usually generated during the metabolic process in living cells including the spermatozoa but excessive production leads to cell mediated-defensive mechanism by using endogenous antioxidants.[13] Such overproduction of ROS can affect the functional ability of sperm by causing OS.[8]

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In this investigation, 4G-EMR induced the OS by decreasing the antioxidant activity of SOD and increasing the level of MDA in testicular tissue in the R1 group. OS was accompanied by hormonal variations; there was a significant reduction in serum inhibin-B level but insignificant decrement in serum testosterone level. However, the EMR-group showed an insignificant reduction in the sperm count.

The results of the present study are well correlated with the results of Ozguner et al (2005) who reported that acute exposure to radiofrequency fields of 900 MHz from mobile phones decreased the SOD activity and enhanced the lipid peroxidation.[23] The diameter of the seminiferous tubules and the mean height of its epithelium were significantly decreased in the EMR group compared to the control group. Okechukvu et al (2018) documented that one month-cell phone radiation produced an insignificant decrease in the serum testosterone level; likewise, exposure to 900 MHz RF-EMR for four hours/one month can induce lower testosterone and inhibin-B levels in rats, which are similar to the results of R1 group in this study.[24, 25] Lower serum inhibin-B may decrease the activity of Sertoli cell which is ultimately required for the sperm production and release and thus, can lead to infertility.

The intracellular molecules, especially polyunsaturated fatty acids, and transmembrane proteins are the major targets of ROS. Hence, ROS readily reacts with them, producing oxidation of these molecules with the by-product of peroxides, alcohol and lipidic aldehydes leading to increased cell membrane permeability. Thus, causing oxidative disintegration of unsaturated fatty acids in membranes of cells.[13] Unsaturated fatty acids present in sperm plasma membrane are highly vulnerable to MDA leading to LPO and thereby disrupting the membrane; subsequently, the rapid loss of intracellular ATP and thus causing axonemal damage, decreased sperm viability; [26, 13] which might be the reason to cause decreased sperm count in the R1 group of this study. Gautam et al (2018) also concluded that 3G mobile phone radiations on the testes of male Wistar rats can induce the formation of free radicals and LPO with a decrease in sperm count, alterations in membrane integrity of spermatozoa.[27]

Similar to this study set-up, a recent experiment used an EMR device to create an EMR at a frequency of 2.104 GHz (4G LTE), such EMR had been given to rats for four weeks. Results showed significantly lower Leydig cell count, germ cell counts and Johnsen biopsy score.[6] Kesari et al (2011) documented that cellular phone-induced RF-EMR can affect the fertilizing competency of spermatozoa in rats by decreasing the activities of SOD, glutathione peroxidase, histone kinase and increasing the level of MDA.[26] Likewise, Kesari et al (2010) revealed a significantly declined level of protein kinase C and sperm count along with the higher number of apoptotic cells in testes of adult rats exposed to phone radiation for 5 weeks.[28] Furthermore, Mailankot et al (2009) documented that rats exposed to RF-EMR with a frequency of 0.9-1.8 GHz, transmitted by an active GSM cell phone for four weeks displayed decreased sperm motility with elevated MDA levels and decreased glutathione content in testis and epididymis.[15]
In contrast to the findings of the current study, few authors reported that short term exposure to the mobile phone with a carrier frequency of 890- 915 MHz failed to cause alteration in the

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microscopic structure of testes, sperm morphology and its count and concentration of MDA.[29, 30, 31]

Leaves of M. oleifera are a rich source of natural antioxidants since it possesses polyphenols and flavonoids which protect organism and cell from oxidative DNA damage associated with aging, cancer and degenerative diseases.[32] In this study, the ethanolic extract of *MOL* regained all the parameters to near normal level (as that of the control group) in R1+MOL. Administration of *MOL* did not cause any significant change in the activity of antioxidant enzymes and MDA levels and sperm count in MOL-1 group.

The antioxidant activity of phytomolecules of *MOL* was demonstrated in human serum albumin protein from OH -mediated oxidative damage. It is proposed that phytomolecules in *MOL* such as phenols and flavonoids might scavenge free radicals or chelate transitional metal ions leading to the conversion of radical to non-radical or inhibition of Fenton-like reaction. They are potential electron donors to scavenge free radicals or they transform Fe3+–Fe2+. Such a reducing capacity of *MOL* on making Fe3+–Fe2+ is greater than α-tocopherol. The inhibition of lipid peroxidation could be caused by trapping free radicals or the chelation of Fe2. [32]

The findings of this study in irradiated rats treated with *MOL* came in accordance with Nayak et al (2015) who suggested prior administration of ethanolic extract of *MOL* may have potential benefit in reversing the loss of male gonadal function to the normal level by decreasing MDA level and increasing the activities of SOD and catalase in case of chemotherapy with cyclophosphamide.[33] He added that the phytochemicals and secondary metabolites in *MOL* possess free radical scavenging ability. Similar results were showed by Zahran et al (2015) concurrent administration of *MOL* extract played a prophylactic role against Equigan-induced testicular damage.[34] Simultaneous oral administration of 50mg/BW *MOL* protected the testis against hydroxyurea caused pathological alteration in the morphologic, spermatogenic and oxidative status.[35] Furthermore, Bassey et al (2013) reported that the antioxidant properties of *MOL* are comparable to vitamin C, which exhibited protective and reversibility effects on alcohol-induced testicular toxicities in pre-pubertal Wistar rats.[36]

Earlier studies have shown that *MOL* extract has a radioprotective effect. Rao et al (2001) revealed *MOL* has a significant radioprotective effect on the chromosomes of the bone marrow of mice exposed to whole-body gamma radiation.[37] Sinha et al (2011) demonstrated pre-treatment with *MOL* protected the liver against γ radiation-induced damage by increasing the level of SOD, Catalase and glutathione.[38] A previous experiment revealed the testicular protective effects of *MOL* from mobile phone triggered alteration. Bin-Meferij et al (2015) filed evidence that 900 MHz-EMR treated rats can show microscopical testicular derangements with degenerated spermatozoa and these effects can be regained with the synchronous treatment of 200 mg/kg aqueous extract of *MOL* along with EMR; which support the results of the present study.[39]

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Conclusion

Short-term exposure to 4G-EMR-induced mobile phone radiation caused an insignificant reduction in sperm count and a significant reduction in serum inhibin-B level. The occurrence of this effect may be through the OS mechanism which was characterized by a reduction in the activity of SOD and accumulation of MDA. The ethanolic extract of *MOL* could retrieve the sperm count and serum inhibin-B to near-normal level in treated rats by the antioxidant activity of its phytochemicals through reduction of OS. Thus, *MOL* can protect male gonadal function from RF-EMR and it must be taken as a part of a regular diet to avoid such hazards.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Nil

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. Sudha Ramalingam and X. Chandra Philip designed the study; Sudha Ramalingam performed the research; Sudha Ramalingam and X.Chandra Philip analyzed the data; Sudha Ramalingam wrote the paper; and Sudha Ramalingam, X. Chandra Philip and K.V.P. Suriyakumari revised and finalized the manuscript for submission.

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Variables	Control	Sham	MOL-1	R1	R1+MOL
Testosterone (ng/L)	20.101 ±1.182	19.850 ±4.934	20.433 ± 3.306	17.167 ±1.798	18.517±1.063
Inhibin-B (ng/L)	0.191±0.004	0.187± 0.008	0.1943 ±0.004	0.169±0.012[a,b]	0.177±0.005[a,b, c]
SOD (ng/ml)	82.615±15.781	82.400 ±5.120	82.767 ±2.246	63.383±4.728 [b, c]	70.100±9.611
Catalase (ng/ml)	81.717±10.625	81.367 ±10.028	81.967 ±5.554	73.183±19.393	75.517±8.544
GR (ng/ml)	65.767±3.824	65.550 ±2.519	66.200 ±2.802	64.083±3.460	64.717±3.168
MDA (nmol/ml)	62.350±3.952	62.833 ±1.855	61.733 ±10.62	115.083± 25.24 [a,b,c]	104.200±11.451 [a,b,d]

Figure:

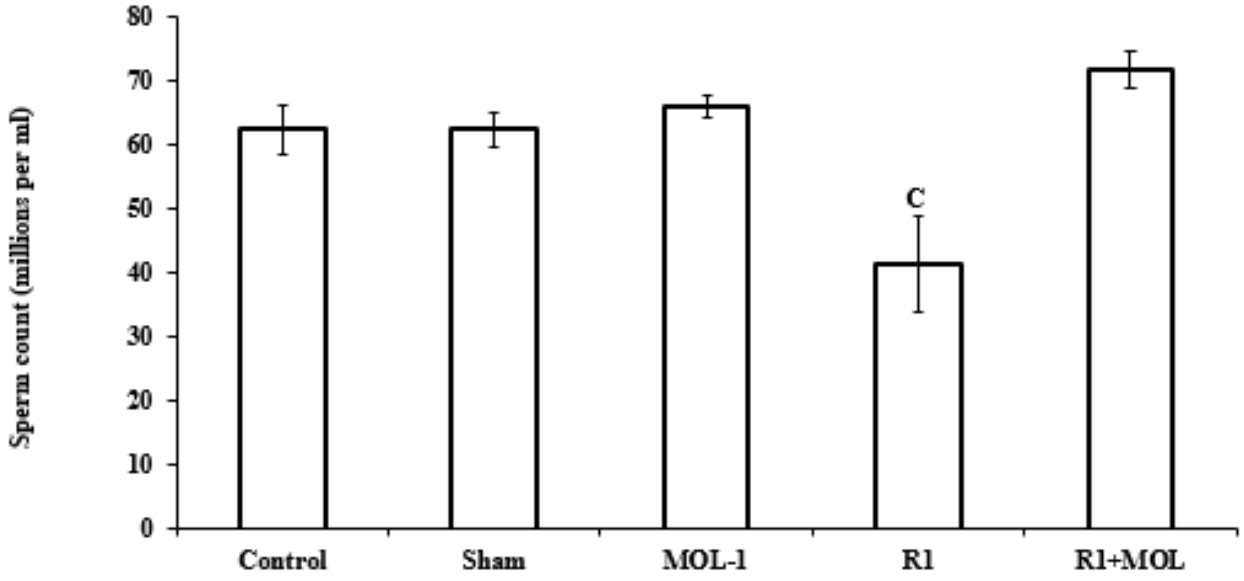

Table legend:

Table: Effect of ethanolic extract of *MOL* on the serum hormone levels, SOD, catalase, GR and MDA levels of EMR-treated rats.

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Table Foot note:

Values are expressed as mean ±SD. 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 R1 group.

Figure legends:

Figure:

Effect of ethanolic extract of MOL on epididymal sperm count of 4G-EMR-exposed Wistar rats. Values are expressed as mean±SE. Superscript letter 'c' shows R1 significantly different from the MOL-1 group.