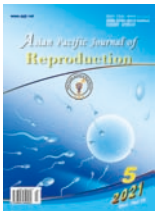

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Ameliorative effect of *Punica granatum* on sperm parameters in rats exposed to mobile radioelectromagnetic radiation

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ABSTRACT

Objective: To study the effect of mobile emitted radio frequency electromagnetic radiation (RF-EMR) on sperm parameters (sperm count, viability, motility, progressivity, and morphology) and ameliorative effect of pomegranate juice in rats.

Methods: Thirty male Sprague Dawley rats were divided into five groups (n=6 in each group). Group Ⅰ was neither exposed to RF-EMR nor given pomegranate juice, group Ⅱ, Ⅲ and Ⅳ were exposed to mobile emitted RF-EMR for 60 min/day for 90 days. After 90-day exposure to RF-EMR, group Ⅲ was supplemented with pomegranate juice for 90 days (1 mL/day) and group Ⅳ was allowed to recover for 90 days without supplementation of pomegranate juice. Group Ⅴ was supplemented with pomegranate juice for 90 days without exposure to RF-EMR. At the end of intervention, epididymal sperm parameters (sperm count, viability, motility, progressivity, and morphology) were measured.

Results: The microscopic examination of sperm parameters such as sperm count, sperm viability, sperm motility, progressivity were significantly decreased in group Ⅱ (the mobile RF-EMR group) compared to group Ⅰ (the control group) (P<0.05). In addition, sperm morphology was also significantly altered (abnormal) in group Ⅱ compared to group Ⅰ (P<0.05). However, the sperm parameters including sperm morphology were significantly altered in group Ⅲ (mobile RF-EMR + pomegranate juice) compared to group Ⅱ (P<0.05). The sperm parameters including sperm morphology were not significantly altered in group Ⅳ (the mobile RF-EMR recovery group) compared to group Ⅱ (P>0.05). The sperm parameters were non-significantly increased in group V (the pomegranate juice group) compared to group Ⅰ (P>0.05).

Conclusions: Mobile RF-EMR exposure reduces the sperm parameters, which, however, can be reversed by treatment with pomegranate juice, indicating that pomegranate juice can be used as a nutritional supplement to improve sperm quality.

KEYWORDS: Mobile radioelectromagnetic radiation; Pomegranate juice; Sperm parameters; Male infertility; Anthocyanin; Spermatocytogenesis; Epididymis; Sperm morphology

1. Introduction

The use of mobile phones has become abundant in recent years throughout the world. According to Agarwal *et al*, 4.8 billion people are said to be using mobile phones worldwide and the usage is continuously increasing from younger to older generations. Mobile phones are operating between 800 to 2 200 MHz frequency bands and emit radiofrequency (RF) electro-magnetic radiation (EMR). The organic effect of RF-EMR release from mobile phones is still a debatable issue[1]. Most of RF-EMR exposure found in the environment is due to commercial radio and TV broadcasting and from mobile phone base stations (telecommunications facilities) and may lead to male infertility. Nowadays, mobile phones are not only used for communication, but also for multipurpose usage like mobile banking, games and social media applications. RF-EMR source in the house includes microwave ovens, cordless telephones, mobile phones, wireless computer networks (laptops),

Significance

Studies have shown that exposure to mobile radio frequency electromagnetic radiation (RF-EMR) reduces the quality of sperm in rats; however, remedies against these changes have not been addressed well. In the study, we show that pomegranate juice significantly improved the sperm quality of RF-EMR exposed rats. It indicates that pomegranate juice can be used as nutritional supplement to improve sperm quality.

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smart meters, remote controls and burglar alarms[2]. Generally, men carry their mobile phone in their pant pocket and it lies very close to the scrotal sac, and hence they are exposed to an excessive amount of RF-EMR in their daily life that may lead to male infertility[3]. The adverse effects of RF-EMR on heart, blood pressure, brain and endocrine system are widely reported. Usage of mobile phones is also associated with many health issues like neck pain, earache, tinnitus, morning tiredness, headache, fatigue, painful fingers, eye symptoms, restlessness and sleep disturbance[4]. The prevalence of male infertility at the reproductive age has been estimated to be up to 7%-8% and there has been a tendency to increase in recent decades, and infertility issues, like infertility, spermatogenesis disorders and premature abortions have become more prevalent worldwide[5]. With the exception of obstructive causes, idiopathic male infertility originates from decreased sperm parameters with no organic, genetic, or endocrine alterations in the genital tract. With an increase in the rate of male infertility, we should focus on the RF-EMR generated from mobile phones as it has created an increased interest in research due to its effects on human health[6]. It has recently been suggested that the antioxidant effect of alpha lipoic acid may improve sperm parameters[7]. Therefore, 4G mobile RF-EMR studies are needed to properly evaluate the effects of mobile exposure use on male fertility.

Sexual abnormalities increase with etiological factors and ageing, including degenerative diseases and industrialized life cycle with stress. Reactive oxygen species are highly reactive oxidizing agents, belonging to the class of free radicals. Pomegranate fruit is nutrient dense antioxidant rich fruit which has been revered as a symbol of health and fertility. Rich numbers of phenolic compounds are present in pomegranate juice, including punicalagin, isomers, gallic acid, and anthocyanins[8]. Pomegranate juice and dietary sources are a promising source of new therapeutic options. Pomegranate fruit has been used in traditional medicine as a remedy for symptoms like eyesore, scurvy, blood clotting, and diarrhea. The recent interest for this pomegranate fruit is not only because of the pleasant taste, but also due to various medicinal properties such as anti-atherogenic, antiparasitic, antimicrobial, anti-oxidant, anticarcinogenic, anti-inflammatory and cardio-protective factors. These antioxidant molecules are shown to be bioavailable and safe[9]. This study aimed to investigate the effect of 4G mobile RF-EMR exposure use on the Sprague Dawley rats and determine the efficacy of pomegranate juice supplementation in protecting the sperm parameters.

2. Materials and methods

2.1. Animals and groupings

Thirty healthy adult male Sprague Dawley rats (10-14 weeks old, weighing 150-180 g) were procured from Biogen Laboratory Animal Facility, Bangalore, India. The Sprague Dawley rats were housed in polypropylene cages. The experimental rats were maintained under well-ventilated standard laboratory, where the room temperature of (24±2) ℃, with 45%-50% constant humidity and kept in a "12 hours light/12-hour dark" cycle throughout the study. The rats had free access to pellets and water *ad libitum*. Before beginning of the experiment, the rats were allowed to adapt lab condition (12 days). After acclimatization, 30 male Sprague Dawley rats were divided into 5 groups (n=6/group) such as group Ⅰ: the control group, group Ⅱ: the mobile RF-EMR group, group Ⅲ: the mobile RF-EMR + pomegranate juice group, group Ⅳ: the mobile RF-EMR recovery group, and group Ⅴ: the pomegranate juice group. Group Ⅰ was neither exposed to RF-EMR nor given pomegranate juice, group Ⅱ, Ⅲ and Ⅳ were exposed to mobile emitted RF-EMR (800 MHz to 2 400 MHz) for 60 min/day for 90 days[10]. After 90 days of exposure to RF-EMR, the group Ⅲ was supplemented with pomegranate juice for other 90 days (1 mL/day)[8] and group Ⅳ was allowed to recover for 90 days without supplementation of pomegranate juice. Group Ⅴ was supplemented with pomegranate juice for 90 days without exposure to mobile RF-EMR. This period (90 days) was required to determine the effect of pomegranate juice on sperm production because the Sprague Dawley rats needed a time period of 2-3 months for the exact spermatogenic cycle including meiosis, spermiogenesis and spermatocytogenesis. At the end of intervention, epididymal sperm parameters (sperm count, viability, motility, progressivity, and morphology) were measured.

2.2. Mobile RF-EMR exposure technique

The 4G mobile phone (Vivo 1803 mobile phone) with a specific absorption rate of 0.53 Watt/kg was hung down from the center of the rat roof cage at a distance 5 cm between cage floor and mobile phone, and during the exposure period food and water was available *ad libitum*. Radiation that was emitted during the exposure was quantified by radiation frequency meter (Meco-GRF-EMR) which was kept at the periphery. The control rats were placed in a separate room where there was no exposure of mobile RF-EMR[10].

2.3. Plant material and authentication

Pomegranate fruit (bhagwa variety) was collected from local market in Kolar. The pomegranate fruit material (botanical name: *Punica granatum* L.; voucher number: 0320) was authenticated by Dr. Madhava Chetty K, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupathi-517502, Andhra Pradesh, India.

2.4. Pomegranate juice preparation

Fresh and healthy pomegranate fruit was used in this study. Pomegranate fruit was washed under tap water and cut into four pieces and manually peeled in aseptic conditions. Pomegranate fruit juice was obtained by using mixer blender (Bajaj GX 11 750W),

filtrated with a funnel and juice was immediately poured into dark-bottle wrapped with aluminum foil[11]. Pomegranate juice was given to the mobile RF-EMR + Pomegranate juice group (group Ⅲ) and the pomegranate juice group (group Ⅴ) through oral gavage needle 1 mL/day[8].

At the end of intervention, the rats were euthanized with ketamine (50 mg/kg body weight, *i.p.*). The epididymides were harvested *via* center incisions in the scrotal sac. Immediately, cauda epididymides were dissected and placed in a clean small petri dish.

2.5. Sperm counts

A 1 cm incision was done at the epididymal cauda by using sterilized blade and all the semen was squeezed by using forceps into petri dish, containing 5 mL of phosphate buffered saline. The glass petri dishes were closed and incubated at 34 ℃ for 8-10 min to allow for sperm swim-up. The epididymal sperm was transferred to "Neubauer Hemocytometer". The total sperm count was counted by using a light microscope (Labomed LX 200 LED) at 40× magnification[12]. Sperm count = count in 5 squares × dilution factor 400×10^6/mL[13].

2.6. Sperm motility

Caudal epididymis was mixed with few drops of normal saline. One drop sample was placed on a clean slide and covered with a cover slip. The 200 sperms were examined at 40× magnification. The motile and non-motile sperm number was counted in 10 random fields and motility was expressed as a ratio of number of motile sperms to the total number of sperms[12].

2.7. Sperm viability

One drop of semen sample was placed on a clean glass slide and one drop of 0.5% eosin solution was added. After 2 min, the slides were observed under a light microscope (40× objective). Percentage of viable sperm (color less) and non-viable sperm (colored) was expressed. The relative sperm viability was calculated, total "viable sperms" (unstained) by the total "viable sperms" (stained and unstained) × 100[14] .

2.8. Sperm morphology

One drop of sperm suspension was placed on a glass slide, a thin smear was done and few drops of 95% ethanol and smear were allowed to air dry overnight. The slides were stained with Papanicolaou staining technique. The slide was observed under microscope at 400× magnification, to detect the sperm morphological abnormalities which were as follows: detached head, pyriform head, coiled tail, and multiple abnormalities. For each slide, the % of abnormal sperms was scored from at least 10 fields and 200 sperms[15,16].

2.9. Sperm progressivity

The sperm progressivity was determined by subjecting grading system (grades): 4 or A: Excellent forward directional movement; 3 or B: Good forward direction movement; 2 or C: Fair forward directional movement; 1 or D: Poor forward directional movement, as described by the World Health Organization (2005)[17].

2.10. Statistical analysis

The statistical analysis was carried out by using the SPSS (version 20; SPSS Inc) software program. Normal distribution of the data was presented as mean±standard deviation (mean±SD). One-way analysis of variance and Bonferroni's *post–hoc* test were used to determine the significance among the multiple comparisons of 4G mobile RF-EMR and pomegranate juice treatment groups. $P<0.05$ was considered statistically significant.

2.11. Ethics statement

The study was approved by Institutional Animal Ethical Committee (IAEC /PHARMA/SDUMC/2018/12a), SDUAHER Tamaka, Kolar, India. The experiments were performed at Central Animal House Laboratory, Sri Devaraj Urs Medical College, Tamaka, Kolar, India.

3. Results

3.1. Effect of mobile emitted RF–EMR and ameliorative effect of pomegranate juice on sperm count

The sperm count was significantly decreased in the mobile RF-EMR group compared to the control and pomegranate juice groups (P both<0.05). However, the sperm count was significantly increased in the mobile RF-EMR + pomegranate juice group compared to the mobile RF-EMR group ($P<0.05$). The sperm count was not significantly increased in the mobile RF-EMR recovery group compared to the mobile RF-EMR group ($P>0.05$). The sperm count was increased in the pomegranate juice group compared to the control group; however, it was not statistically significant ($P>0.05$) (Table 1).

Table 1. Effects of 4G mobile emitted RF-EMR and ameliorative effect of pomegranate juice on sperm parameters.

Parameters	Control	Mobile RF-EMR	Mobile RF-EMR+Pomegranate juice	Mobile RF-EMR recovery	Pomegranate juice
Sperm count($\times 10^6$)	91.80±7.14	74.14±4.21[a]	87.10±2.01[b]	67.43±10.12[a]	93.15±8.01
Sperm motility (%)	92.16±1.72	49.66±2.94[a]	81.40±2.30[b]	69.28±3.11[ab]	91.28±9.25
Sperm viability (%)	90.66±1.21	69.00±2.44[a]	92.40±2.50[b]	54.18±2.48[ab]	87.83±2.31
Sperm progressivity	A	C	B	C	A

Data are expressed as mean±SD. n=6 in each group. One-way analysis of variance and Bonferroni's *post-hoc* test are used to determine the significance among the multiple comparisons of 4G mobile RF-EMR and pomegranate juice treatment groups. Sperm progressivity is graded as A: Excellent, B: Good, C: Fair, D: Poor, as described by the World Health Organization (2005). P<0.05 is considered statistically significant. a: compared to the control group, P<0.05; b: compared to the mobile RF-EMR group, P<0.05. RF-EMR: radio frequency electromagnetic radiation.

Table 2. Effects of 4G mobile emitted RF-EMR and ameliorative effect of pomegranate juice on sperm morphology (percentage of abnormality).

Sperm abnormalities (%)	Control	Mobile RF-EMR	Mobile RF-EMR+Pomegranate juice	Mobile RF-EMR recovery	Pomegranate juice
Detached head	4.40±0.35	8.90±0.22[a]	5.60±0.59[b]	8.80±0.25	4.70±0.56
Pyriform head	4.30±0.12	7.70±0.14[a]	5.80±0.25[b]	7.50±0.20	3.90±0.09
Coiled tail	5.50±0.43	7.10±1.27[a]	5.90±0.40	7.80±1.99	5.20±0.31
Bent tail	4.80±0.15	7.20±2.09[a]	5.10±0.29[b]	8.90±1.25	4.60±0.33

Data are expressed as mean±SD. n=6 in each group. One-way analysis of variance and Bonferroni's *post-hoc* test are used to determine the significance among the multiple comparisons of 4G mobile RF-EMR and pomegranate juice treatment groups. P<0.05 is considered statistically significant. a: compared to the control group, P<0.05; b: compared to the mobile RF-EMR group, P<0.05. RF-EMR: radio frequency electromagnetic radiation.

3.2. Effect of mobile emitted RF-EMR and ameliorative effect of pomegranate juice on sperm motility

The sperm motility was significantly decreased in the mobile RF-EMR group compared to the control and pomegranate juice groups (P both<0.05). However, the sperm motility was significantly increased in the mobile RF-EMR + pomegranate juice group compared to the mobile RF-EMR group (P<0.05). The sperm motility was significantly increased in the mobile RF-EMR recovery compared to the mobile RF-EMR group (P<0.05); however, it did not reach to the control and RF-EMR + pomegranate juice group levels. The sperm motility was not significantly increased in the pomegranate juice group compared to the control group (P>0.05) (Table 1).

3.3. Effect of mobile emitted RF-EMR and ameliorative effect of pomegranate juice on sperm viability

The sperm viability was significantly decreased in the mobile RF-EMR group compared to the control and pomegranate juice groups (P both <0.05). However, the sperm viability was significantly increased in the mobile RF-EMR + pomegranate juice group compared to the mobile RF-EMR group rats (P<0.05). The sperm viability was significantly decreased in the mobile RF-EMR recovery group compared to the mobile RF-EMR group (P<0.05). The sperm viability was not significantly different between the pomegranate juice group and the control group (P>0.05) (Table 1).

3.4. Effect of mobile emitted RF-EMR and ameliorative effect of pomegranate juice on sperm progressivity

The sperm progressivity was remarkably declined in the mobile RF-EMR group (grade C) compared to the control group (grade A) and the pomegranate juice group (grade A). However, the sperm progressivity was increased in the mobile RF-EMR + pomegranate juice group (grade B) compared to the mobile RF-EMR group (grade C). The sperm progressivity was not increased in the mobile RF-EMR recovery group (grade C) compared to mobile RF-EMR group (grade C) (Table 1).

3.5. Effect of mobile emitted RF-EMR and ameliorative effect of pomegranate juice on abnormality sperm morphology

The abnormality of sperm morphology (detached head, pyriform head, coiled tail, and bent tail) was significantly increased in the mobile RF-EMR group compared to the control and pomegranate juice groups (P both<0.05). However, the abnormality of sperm morphology was significantly decreased in the mobile RF-EMR + pomegranate juice group compared to the mobile RF-EMR group (P<0.05). The abnormality of sperm morphology was comparable between the mobile RF-EMR recovery group and the mobile RF-EMR group. The abnormality of sperm morphology was decreased in the pomegranate juice group compared to the control group; however, it was not statistically significant (P>0.05) (Table 2).

4. Discussion

The mobile phones have become a part of daily life. In view of COVID-19 pandemic, the usage of smart phones was increased in adults including adolescents[18]. The usage of 4G mobile phones has been accompanied by parallel increase of the RF-EMR density in environment. On the other hand, the male infertility has increased in recent years[19,20]. However, the epidemiological cause for male infertility has not been understood clearly. Hence, we were interested to study the effect of long term exposure of 4G mobile phone-emitted RF-EMR on male reproduction and efficacy of pomegranate juice on RF-EMR induced male infertility.

Our current study results showed that the chronic exposure [90 days (1 hour/day)] to RF-EMR emitted by 4G mobile leads to decrease of the sperm count, viability, motility, and progressivity in rat semen. In addition, RF-EMR exposure also increased the abnormality of sperm morphology in rat semen. It indicates the chronic exposure of RF-EMR emitted by 4G mobile causes male infertility in rats. Jong *et al* reported that the 30-day exposure (18 hours/day) to the 4G mobile emitted RF-EMR leads to decrease of spermatogonia, germ cells and leydig cell count in rats[21]. Shokri *et al* reported that seven hours/day for 30 days exposure to the 2.45 GHz Wi-Fi EMR (high frequency radiation) induced a decrease in sperm parameters and increase of apoptosis positive cells and increased the caspase 3 activity in seminiferous tubules in rats[22]. Tas M *et al* reported that long-term exposure (3 hours/day for 365 days) of 900 MHz RF radiation altered male reproductive parameters in rats[23].

In contrast, studies reported that RF-EMR exposure did not decrease the sperm count in the experimental group compared to the control animals[24,25]. In contrast, our study reported that chronic exposure [90 days (1 hour/day)] to RF-EMR emitted by 4G mobile leads to decrease of the sperm count, viability, motility, and progressivity in rat semen including abnormality of sperm morphology. It might be due to the increase of oxidative stress in rat testis exposure to the RF-EMR emitted by 4G mobile phone. Damegh *et al* stated that the 1 hour/day for 14 days exposure to the RF-EMR increases the oxidative stress in testis of rats[26].

The direct biological effects of mobile RF-EMR are divided into thermal effects by the RF-EMR energy absorption, stimulation function by the induced electric current and athermic action by the long term RF-EMR exposure. No clinical studies have evaluated the effect of mobile RF-EMR and co-administration of pomegranate juice on the sperm parameters, but some epidemiologic studies have shown the mobile phone use has negative effects of sperm parameters[27]. *Punica granatum* has protective effects due to its ingredients like phenolic acids[28], estrogenic flavonoids[29], tannins[30,31], and conjugated fatty acids[32]. These compounds are found in substantial amounts in the seed oil, peels and juice of the *Punica granatum* fruits[33].

Plant products such as leaves, seeds and fruits have natural antioxidants. Pomegranate juice has an ancient history of being used in several diseased conditions[34]. Pomegranate juice is polyphenol rich juice with high antioxidant capacity. Pomegranate has protective effects on cell due to its active ingredients like phenolic acids, tannins, estrogenic flavonoids and conjugated fatty acids[35]. Moreover, several studies have shown that the "pharmacological effects of flavonoids" are related to their antioxidant activity, which can be due to their ability to scavenge OH and O_2, to chelate metal ions and to exert a synergistic effect of other anti-oxidant metabolites. Our results in conjunction with the others mentioned above, suggest that flavonoids could constitute one of the active components of pomegranate[36,37].

In addition, the abnormality of sperm morphology in rat semen was also decreased in the RF-EMR exposed rats supplemented with pomegranate juice compared to RF-EMR group rats. Turk *et al*[8] reported that 7-week supplementation of pomegranate juice increases the sperm quality, antioxidant capacity and decreases the abnormality of sperm in rats. Yassien *et al* observed that 52 days of pomegranate juice supplementation increased the structural integrity of testis and testosterone levels in old rats compared to age-matched rats[38]. Mohsen *et al* stated that pomegranate juice supplementation increases the sperm count, progressive sperm motility, testosterone levels and epithelial diameters of the seminiferous tubule of the exposed male mice[39]. In line with reported studies, the current study reported that 90 days supplementation of pomegranate juice increases the sperm parameters and decreases the sperm abnormality in rats exposed to mobile RF-EMR. This could be due to the rich antioxidant capacity of pomegranate juice, serving as protective mechanism against the oxidative free radicles generated by the mobile RF-EMR.

The limitation in the present study was that we did not analyze the oxidative status of testis and also the testosterone levels in rat, which can give us a better understanding of the role of pomegranate juice on reduced sperm parameters induced by the mobile emitted RF-EMR.

In conclusion, the study results indicate that the mobile RF-EMR exposure reduces the sperm parameters and increases the sperm abnormality. However, the pomegranate juice supplementation reverses the 4G mobile RF-EMR induced damage on sperm quality in rats. It indicates that the pomegranate juice can be used as a nutritional supplement to improve the sperm quality.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Authors' contributions

The design of the study was done by Anjaneyababu Naik Banavath. The literature search was done and the experiment was conducted by Anjaneyababu Naik Banavath. The data analysis as well as "drafting of the manuscript" was done by Anjaneyababu Naik Banavath. The editing and reviewing of the draft manuscript as well as revising the manuscript for intellectual content were done by Anjaneyababu Naik Banavath and Sridevi Nangali Srinivasa.

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