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Original Article

Biological effects of cell-phone radiofrequency waves exposure on fertilization in mice; an *in vivo* and *in vitro* study

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ABSTRACT

Increasing use of cell-phone is one of the most important risk factors for population health. We designed an experimental study aimed at evaluating the effects of cell-phone radiofrequency (RF) waves exposure on fertilization in mice. Two hundred male and female NMRI-mice were used. One hundred males divided in five groups (n = 20) as control and exposed groups. Those irradiated with cell-phone RF in "Standby-mode" 1, 5 and 10 h daily named groups II, III and IV; respectively. Group V irradiated with cell-phone on "Active-mode" one hour daily. After 30 days irradiation, 50 males and 50 females were kept 24 h to assess their embryos. Fifty males were scarified to evaluate both *in vitro* and *in vivo* parameters, and 50 females received PMSG & HCG for both quantitative and qualitative evaluation. Comparing groups III, IV and V with control-group showed significantly decreased in the number of two-cell embryos (p = .000); however, a significant increase was found in the number of dead embryos (p = .000). Furthermore, 5 h daily irradiation significantly decreased grade-A embryos (p = .015); while, it significantly increased grade-B, C and D embryos (p-values = 0.026, 0.007, 0.006; respectively). Moreover, comparing groups IV and V to control-group, significant increase was found in pregnancy duration (p = .005, p = .009; respectively). However, in the mentioned groups a significant decrease was seen in number of newborn mice (p = .001, p = .004; respectively). In conclusion our findings showed that the cell-phone radiation can affect development of embryos as well as the number of newborn and pregnancy duration in NMRI-mouse, which might be a significant cause of reproductive failure.

1. Introduction

Fertility is the ability to have a child and success in reproduction [1]. On the other hand, infertility is the malfunction in reproduction and the problems relating this matter is known as one of the most important issues in couples' life [1,2]. Reports have shown that approximately 35% of the infertility problems are related to men and 40% to women [3]. The most common cause of males' infertility is their inability to produce enough healthy and active sperm [3,4]. In the last few decades, the quality of sperm and its fertility power has had a significant decrease throughout human society [1,4]. This shows that the mentioned quality has been influenced by changes which are rooted in the toxic factors in the medium; such as chemical intoxication and being exposed to a variety of underlying, medical or military radiation [4]. Radio frequency (RF) waves of cell-phones and other electronic equipment, affects the biological system via thermal and non-thermal effects [5–8]. More than 50 studies, that have been investigated the RF effects on different mice, indicated increased frequencies of hypodiploid in mammalian oocytes, fertility loss, impaired spermatogenesis, and reduction of viable embryos in mice [6,8–11]. When the RF waves are absorbed in the body, they contain energy that can produce free radicals. Free radicals can break a chemical bond and become a chain of biological events including damage in the cell membrane of sexual cells [12]. Based on studies, cell-phone radiofrequency (RF) waves have negative effects on sexual

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actions [13]. Reproductive disorders have been reported in both males and females which were caused by oxidative stress, and the effect of reactive oxygen species (ROS) levels on zygotes and on the growth of the fetus has been determined [13,14]. Although ROSs produced by radiation are toxic, intracellular ROS produced in physiological conditions is regulated as essential signal molecules that regulate multiple cellular processes, the ROS spectrum produced in the short run after radiation is similar to metabolic processes [13,15]. However, there is a cellular and physiopathological distribution (single molecules and ROS clusters produced by radiation versus single molecules produced by intrinsic processes) and production time (chronic release of ROS in-production versus instantaneous production during radiation) [15,16]. Oxygen's free radical *in vitro* could affect embryonic development, clinical pregnancy rate and fertility [15]. In both *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) raising the concentration of ROS *in vitro* on the first day showed its association with reduced pregnancy rate [15]. As a result, while the damage from ROS metabolism is randomly distributed in DNA, radiation damage from DNA often occurs in clusters [16]. The purpose of this study was to investigate the effect of 900 MHz cell-phone RF waves on the quality and quantity of the NMRI-mouse embryo (from two-cell to the blastocyst resulting from IVF) as well as impact of the RF on the pregnancy duration and number of the newborn mice.

2. Materials and methods

2.1. Grouping and irradiation

In this experimental study, 200 NMRI mice (100 males and 100 females) aged 6–8 weeks and weight 20–30 g were randomly selected and were purchase from Pasture Institute (Tehran, Iran). The mice were kept in 40 similar wood cages (5 female or 5 male mice in one separate cage) in the animal house of the Shahrekord University of Medical Sciences (Shahrekord, Iran). The animal house had standard conditions of 20 ± 2 °C temperature, brightness/darkness of 12:12 h, and free access to food and water for the mice. The mice were kept one week in the animal house to adapt with the environment. The 100 male mice were randomly divided in five groups (n = 20) as: group I or control group in which the cell-phone was off, and four exposed groups. In the three exposed groups of II, III, and IV the male mice were irradiated with cell-phone RF for 1, 5 and 10 h a day; respectively, while the cell-phone was on "Standby-mode" *i.e.* the cell-phone was ON but no conversation occurred. In the 5th group (group V) the male mice were irradiated with cell-phone RF when it was on "Active Mode" (conversation occurred) one hour daily. The RF irradiation was performed for 30 days. The applied frequency of the waves was 900 MHz irradiated from a Nokia cell-phone (Nokia 1100, Finland). In case of irradiation, the distance between cell-phone and mouse was 10 cm. After that the RF irradiation was finished, the experiment was continued in four parts as follow:

2.1.1. Sperm collecting for IVF

In order to collecting sperm for IVF, fifty male mice (10 mice from control group and 10 mice from each of the four exposed group) were randomly selected. The mice were scarified by cervical dislocation on the day 31 (one day after RF irradiation was finished). In the next step, the mouse skin and peritoneum were opened, the epididymis as well as vas deferens isolated, and transferred into a Petri-dish. The Petri-dish was contained human tubal fluid (HTF) medium which previously reached equilibrium. Afterwards, epididymis and vas deferens were divided into smaller pieces and were kept for bearing capacitating 1–2 h inside a 37 °C incubator with 5% CO_2.

2.1.2. Super ovulation and oocyte collecting

For super ovulation and oocyte collecting in each of the 50 mature female mice the two following hormones were intraperitoneally injected: 10 (IU) PMSG (Sigma Co, USA) and 48 h later 10 (IU) hCG (Sigma Co, USA). Ovulation occurred 10–13 h after the hCG injection. Then, 12 h later, the skin of the injected mouse was sterilized with alcohol and killed by cervical vertebra dislocation. After that, its skin and peritoneum were removed. In the next step, the oocyte and the cells around it (*i.e.* cumulus oophorus) were collected from both sides of fallopian tube and were transferred into a Petri dish containing HTF medium. After washing the droplets of HTF, a total of 100 oocytes were obtained from female mice randomly.

2.1.3. In-vitro fertilization (IVF)

For the IVF 5 μL of the collected sperms were taken with a sampler from those having swimming up movement that were already capacitated and kept in the incubator. We added them to the drops containing 100 oocytes in a way that each ml was including 1×10^6 sperm. Then, it was kept for 5 h inside a 37 °C incubator with 5% CO_2. During this period the sperm nucleus enters the oocyte and the male and female pre-nuclei were detectable using a microscope (SMZ2, Nikon, Tokyo, Japan). About 5 h after adding the sperm, the embryos were transferred into Petri dishes containing 5 drops of KSOM medium (Millipore, Madison, WI, USA). After being washed in the 4 side drops, the embryos were transferred to the 5th drop in the middle of Petri dish. At this time the embryos were free of any impurities To evaluate the quantitative process of IVF, 24 h after the insemination, the number of two-cell (and possibly 4-cell) embryos were counted and recorded under a stereomicroscope. This continued until the embryos reached the blastocyst stage in the following days. Moreover, to assess the qualitative process the two-cell embryos were examined in terms of morphology under a stereomicroscope and according to the Bolton grading scale were grouped into four categories of grades A, B, C and D [17].

2.1.4. In-vivo assessment

In this part of the study in order to *in vivo* assessment from 40 of the remained exposed mice (group II, III, IV, V) and 10 non exposed mice (control group), one mouse was randomly selected. The mouse was kept together with one of the 50 remained females in a cage for 24 h (overnight) separately. In the next morning, to make sure of the mating, presence of plaques in the female mice's vagina, it was examined and they were recorded as positive if so. Nineteen days later, if the birth had occurred, the most important *in vivo* factors of reproduction characteristics (*i.e.* pregnancy duration and the number of newborns) were registered for each mouse.

2.2. Statistical analysis

In order to analysis the data we applied the Kolmogorov-Smirnov test, analysis of variance (ANOVA) followed by Tukey's-test. SPSS software (Version 18; SPSS Inc., Chicago, USA) was used for the statistical analysis. The p-values are two-sided at a significance level of ≤ 0.05.

3. Results

3.1. Quantitative findings of IVF

Results of our quantitative evaluation are presented in Table 1. As Table 1 shows one day after the IVF there was a significant decrease in the number of two-cell embryos for groups III, IV and V (comparing to the control group); while there was an increase

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Table 1
Quantitative data of cleavage divisions of the embryos after IVF. Data were represented in means and standard deviation (Mean ± SD).

Embryo types; on 1 & 4 days after IVF		Groups				
		Control (I)	1 h standby (II)	5 h standby (III)	10 h standby (IV)	1 h active (V)
1 day	Two-cell	67.5 ± 0.58	65.4 ± 0.63	63.2 ± 0.55*,#	60.8 ± 0.44*,#,$	62.2 ± 0.46*,#
	Dead	5.5 ± 0.54	6.8 ± 0.44	8.9 ± 0.26*,#	10.6 ± 0.4*,#,$	10.3 ± 0.33*,#
4 days	Two-cell	16.4 ± 0.45	15.5 ± 0.47	13.6 ± 0.49	11.2 ± 0.32*,#	12.9 ± 0.52*
	Dead	10.7 ± 0.36	11.9 ± 0.4	13.5 ± 0.34	17.1 ± 0.37*,#	14.9 ± 0.34*
	Blastocyst	64.3 ± 0.78	61.8 ± 0.46	58.5 ± 0.42*,#	54.6 ± 0.54*,#,$	56.5 ± 0.9*,#

* Significantly different from group I ($p < .05$).
# Significantly different from group II ($p < .05$).
$ Significantly different from group III ($p < .05$).

in the number of dead embryos (p-values $\leq .001$). Moreover, comparing groups III and V with group II, we saw higher decrease in the number of two-cell embryos ($p = .046$ and $p = .000$; respectively). Additionally, in groups III and V the dead embryos had also a significant increase compared to the group II ($p = .025$ and $p = .000$; respectively). Furthermore, comparison between group V and II revealed a meaningful decrease in the two-cell embryos ($p = .001$) and significant increase for the dead embryos ($p = .000$). Moreover, increasing the radiation time from 5 h (group III) to 10 h (group IV) caused a significant decrease in the two-cell embryos and increase in the dead embryos ($p = .024$ and $p = .01$, respectively). There were no significant effects in other groups (Table 1 and Fig. 1). Quantitative results revealed that the evolutionary development to the blastocyst formation on day 4 in the groups exposed to RF has had major changes. The results demonstrated that radiation on the mice in groups III, IV and V significantly reduced the number of blastocysts (in comparison to the control group) ($p = .000$ for all). Results also revealed that the number of embryos that developed to the blastocyst stage in groups III, IV and V significantly decreased (compared to group II) ($p = .000$, p

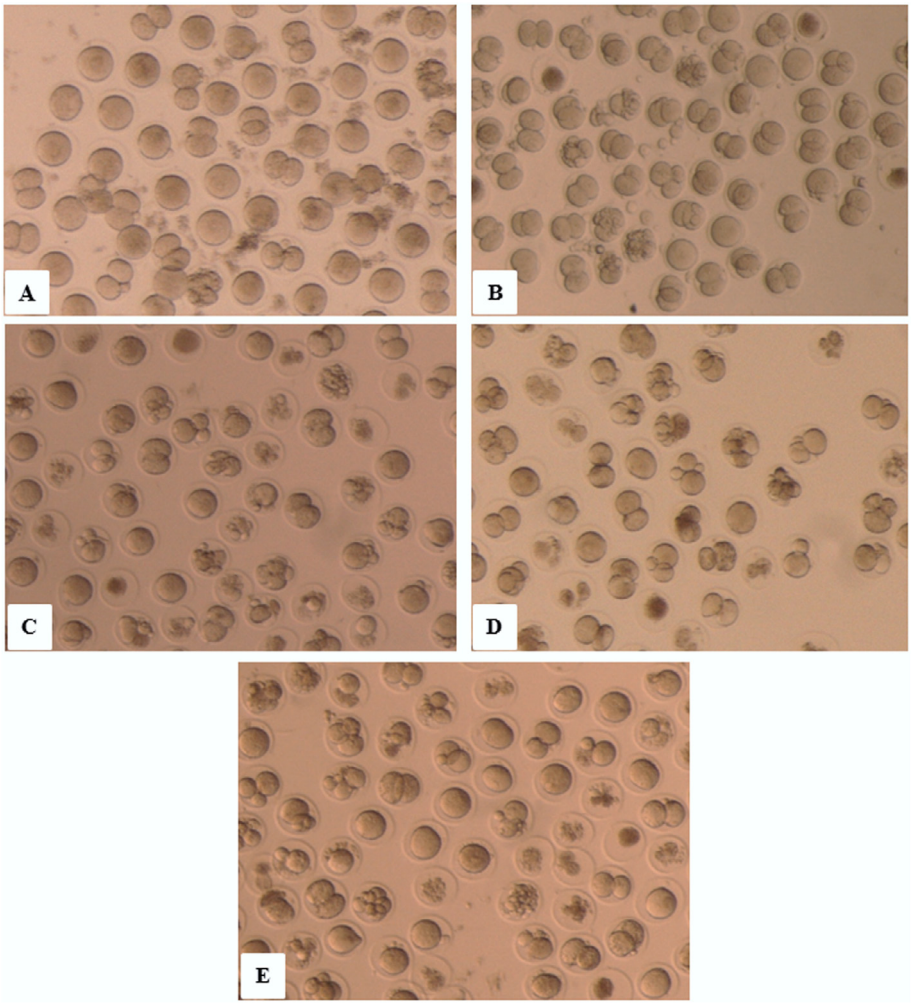

Fig. 1. Stereomicroscope images of the fetus in KSOM medium at the first day after IVF. Part A exhibits embryos of the control groups that most of them entered into the two-cell (and some of them to 4-cell) stage without any fragmentation and disturbance. Images B, C, D, and E illustrate embryos of exposed groups that some are caught in the two-cell stage, others suffer from fragmentation, dark cytoplasm, destruction of the zona pellucida and death. In the exposed groups there was a significant increased rate for the embryonic mortality and also decrease in the number of two-cell embryos, compared to the control group; especially in group IV changes are severe (image D).

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= .000 and p = .029; respectively). Furthermore, increase in the time of radiation from 5 h (group III) to 10 h (group IV) on Standby-mode showed significantly decreased in the number of blastocysts (p = .018).

3.2. Qualitative finding of IVF

Results of our qualitative evaluation showed that radiation for 5 h per day (group III) significantly decreased number of grade A embryos, compared to the control group (p = .015); and have significantly increased the embryos grade B, C and D (p = .026, p = .007 and p = .006; respectively) (Table 2 and Fig. 2). Results also demonstrated that increasing the irradiation time to 10 h per day (group IV) caused high decrease in embryos Grade A and impressive increase in embryos Grade B, C and D compared to the group I (p = .000) and group II (p = .000). Moreover, results demonstrated that RF waves in group IV cause significant decrease in embryos Grade A (p = .002) and significant increase in embryos Grade B, C and D compared to the group III (p = .029, p = .026 and p = .038; respectively). Additionally, radiation for 1 h on active mode (group

Table 2
The results of two-cell embryos on the scale of Bolton for the present study groups. Data were represented in means and standard deviation (Mean ± SD).

Groups	Cell embryo grades			
	A	B	C	D
Control (I)	59.8 ± 0.82	16 ± 0.93	8.3 ± 0.33	13.9 ± 0.27
1 h standby (II)	56.7 ± 0.47	16.9 ± 0.52	9.7 ± 0.26	14.7 ± 0.21
5 h standby (III)	55.6 ± 0.58*	17.3 ± 0.3*	10.4 ± 0.4*	15.5 ± 0.26*
10 h standby (IV)	49.4 ± 0.87*,#,$	19.6 ± 0.22*,#,$	13.3 ± 0.63*,#	16.8 ± 0.32*,#,$
1 h active (V)	52.7 ± 0.55*,#	18.7 ± 0.3*,#	12.3 ± 0.33*,#	16.5 ± 0.42*,#

* Significantly different from group I (p < .05).
\# Significantly different from group II (p < .05).
$ Significantly different from group III (p < .05).

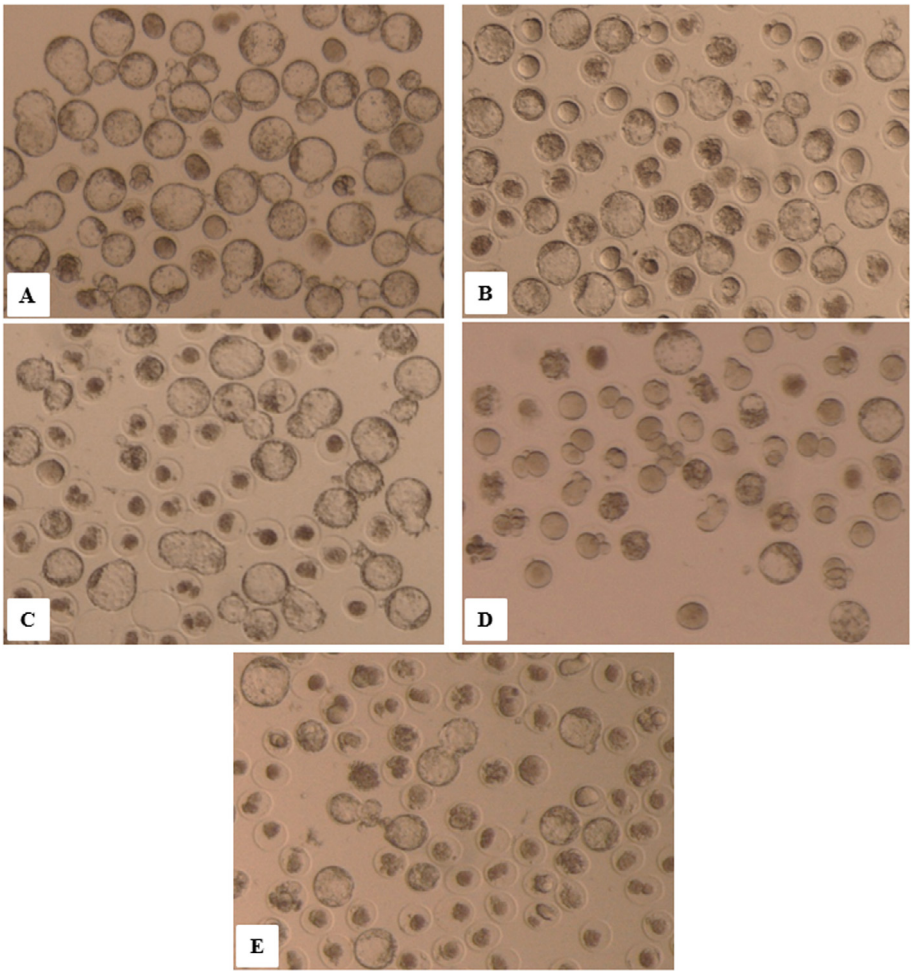

Fig. 2. Stereomicroscope images of the embryos blastocyst in KSOM medium on the fourth day after IVF. Part A (control group): In this figure one can see that a great number of embryos have entered the blastocyst stage and there is no morphological abnormalities in the inner mass of the cell, cytoplasm or even in the cell wall. Parts B, C, D and E (from exposed groups): one can conclude from these figures that almost half of the embryos are stuck in the one-cell or two-cell stage. Some of the fetuses suffer from fragmentation, vacuolization and even death. Cell size decreased in most of the embryos that left over at the two-cell stage. The appearance of the blastomers is abnormal and their cytoplasm is dark which led to reduction in the number of embryos developed into the blast stage. This was more considerable in group IV (image D).

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Table 3
The quantitative comparison of *in vivo* parameters including pregnancy duration and number of the newborn mice in the studied groups. Data were represented in means and standard deviation (Mean ± SD).

In vivo parameters	Groups				
	Control (I)	1 h standby (II)	5 h standby (III)	10 h standby (IV)	1 h active (V)
Pregnancy duration (days)	19.20 ± 0.13	19.40 ± 0.16	19.80 ± 0.32	20.80 ± 0.44[*,#]	20.70 ± 0.33[*,#]
Number of newborn mice	10.80 ± 0.29	10.30 ± 0.26	9.50 ± 0.26	9.20 ± 0.13[*,#]	9.40 ± 0.30[*]

[*] Significantly different from group I ($p < .05$).
[#] Significantly different from group II ($p < .05$).

V) compared to the control group cause significant decrease in embryos grade A and significant increase in embryos grade B, C and D ($p = .000$). Furthermore, results revealed that RF radiation for 1 h on active mode (group V) compared to that of 1 h Standby-mode (group II) has more destructive effects, in a way that it caused a great reduction of embryos grade A ($p = .001$) and impressive increase in embryos grade B, C and D ($p = .009$, $p = .001$ and $p = .002$; respectively).

3.3. In vivo findings

3.3.1. Pregnancy duration

Our evaluation for *in vivo* research is summarized in Table 3. As Table 3 revealed comparison to the control group, mice irradiated 10 h daily in Standby-mode (group IV) had significant increases in pregnancy duration ($p = .005$). Similarly, mice exposed 1 h daily in active mode (group V) significantly had a longer pregnancy duration ($p = .009$). Moreover, the results showed that increasing of the exposure time from 1 h per day to 10 h per day (group II vs. group IV) caused a significant difference in the pregnancy duration ($p = .017$). Furthermore, when the cell-phone changed from off mode to active mode, a significant delay was seen in pregnancy duration ($p = .031$). There was no significant difference between the other groups (Table 3).

3.3.2. Number of newborn mice

As Table 3 illustrates RF radiation on the mice of group IV and V lead to a significant decrease in the number of newborn mice compared to the control group ($p = .001$ and $p = .004$; respectively). The results also demonstrated that the increase of the exposure time from 1 h per day (group II) to 10 h per day (group IV) in the Standby-mode caused a significant difference in the number of the newborns ($p = .034$). There was no significant difference among the other groups.

4. Discussion

Based on the findings of the present study it seems that the cell-phone radiation causes significant changes on both quantity and quality of the *in vitro* embryos as well as pregnancy duration and newborn mice of the *in vivo* conditions. In 2010, Rajaei et al. investigated the effects of extremely low frequency electromagnetic fields (ELF-EMF) on the fertility and height of the epithelial cells of the fallopian and endometrial tube of NMRI-mice in the pre-implantation phase [18]. They have irradiated 40 female mice at a frequency of 50 Hz and 0.5 mT intensity, 4 h daily, 6 days per week for 2 weeks [18]. The results of their study showed that the number of blastocysts in the exposed group was significantly reduced compared to the control group. Studies show that RF waves have non-thermal effects that can be due to free radical production [19,20]. Free radicals, in turn, can cause trigger phosphorylation and activation of certain messenger proteins such as histone kinases and creatine kinases [15,21]. Consequently, the rate of ROS production increases significantly and activates the Caspase-3 pathway in the sperm cell; causing apoptotic death during spermatogenesis or sperm maturation period and failure the fertility [21]. Results of the present study showed that the number of two-cell embryos and newborn mice decreased after cell-phone RF radiation; while, the number of dead embryos increased. These results are similar to those of Bayat et al., Kesari et al. and Fatehi et al. [21–23]. The human body has a defense system against free radicals called the antioxidant system [17,20]. An imbalance between the amount of produced free radical and the antioxidant capacity results in oxidative stress [15,21]. The most important free radicals in human semen include radical anion superoxide, hydrogen peroxide and radical hydroxyl [24]. These free radicals are typically produced by metabolism of oxygen [20,21,25]. Although, under physiological conditions, low levels of active oxygen species are essential for normal sperm function such as capacity, acrosome response, mobility, fertility, and fertilization; excessive amounts of ROS can seriously damage sperms [15,26]. Non-thermal effect of RF is one of the reasons for ROS production [26]. During capacitating process of intracellular calcium levels, ROS activity and tyrosine kinase enzyme increases; which lead to an increase in cyclic adenosine monophosphate (cAMP) [17,22]. The increase in cAMP facilitates the activation of sperms during which their mobility also increases [23]. Only sperms that have a capacity-building process are prone to excessive activation and acrosome reaction, which ultimately leads to fertility [19,24]. Active oxygen species are involved in the interaction between the sperm and the oocyte, in a way that the phenomenon of lipid peroxidation caused by small amounts of ROS leads to a change in the sperm membrane and facilitates sperm-oocytes binding [21]. With these evidences, the excessive amount of ROS results in failing of the binding of sperm-oocytes, and ultimately fertilization or fertility occurs or that it is incomplete and leads to fertility failure [25,26]. This factor can justify a reduction in the number of newborns *in vivo* and also a reduction in the number of two-cell embryos in the *in vitro* after IVF in the present study [27,28]. On the other hand, sperm DNA can be damaged during the oxidative stress process [23,28]. Bases and phosphodiester bonds in DNA are susceptible to peroxidation damage caused by free radicals, which can lead to abnormalities such as switching bases, producing free bases, removing bases, crossing joints, and chromosomal rearrangements [28,29]. The apoptosis process can help eliminate abnormal production of cells and prevent their excessive production [27]. Free radicals have the ability to stimulate the onset of apoptotic process reactions [15,28]. Damaged DNA can accelerate the process of apoptosis, which finally leads to a reduction in the number of sperms, loss of testis weight and possibly the inability to survive and survive until different stages of evolution, such as blastocyst stage [23,29]. These were also observed in the present study. Kesari et al. investigated the effect of microwave waves at the frequency of 50 Hz (2 h daily for 45 days) on the reproductive system of male Wistar rat [30]. They reported significant decrease in the antioxidant enzymes of superoxide dismutase, glutathione peroxidase, and activity of histone kinase; while they found increase in catalase, which enzymatic changes result in catastrophe cascade as well as apoptosis and finally failure in fertility

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and reproduction [30]. They believed that these events are an important indicator of infertility in male mice. We believe according to the results of Kesari et al. our mentioned findings can be due to apoptosis induced by waves in the sexual cells and genital organs. Bernabò et al. irradiated wild pigs with 50 Hz electromagnetic fields 12 h daily for 6 days [31]. They reported that the irradiation caused acrosome degradation, decreased acrosome reaction capacity and fertilization process, as well as reduction in sperm fertility. In the present study, we found reduction in the number of sperms and two-cell embryos in the exposed groups. This could be due to the effects of cell-phone RF waves. The RF waves probably impair the acrosome, resulted in DNA damage; consequently induce death of the sperm. Furthermore, cell-phone waves affect on the structure and morphology of the mice sperm resulted reduction of the capacity of the sperm in the fertilization process. These induced the reduction in the number of fertilized oocyte and two-cell embryos. We know that electromagnetic waves increase the amount of oxygen free radicals in the body of animals. These free radicals disturb the spermatogenesis process and changes in the cell membrane of the sperm, which causes changes in the capacity and acrosome response of the sperm cell. This process causes disorder in the process of binding the sperm to the oocyte. An incomplete finding leads to failure of normal fertility or laboratory one.

5. Conclusion

The results of this study indicate that cell-phone RF waves decreases the quantity of two cells embryos as well as embryos with grade-A quality at the developmental process; while it increases the fragmentation of IVF-derived cells as well as grade-C and D cells in the NMRI-mouse. Cell-phone RF waves also reduces the number of newborn mice, where it increases the pregnancy duration which result in fertility failure in NMRI-mouse.

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Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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