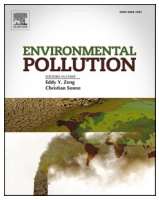
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Detrimental effects of radiofrequency electromagnetic waves emitted by mobile phones on morphokinetics, oxidative stress, and apoptosis in mouse preimplantation embryos☆

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ABSTRACT

Due to the increasing use of smart mobile phones, the impact of radiofrequency electromagnetic radiation (RF-EMR) on reproductive health has become a serious concern. This study investigated the effect of mobile phone RF-EMR with frequency 900–1800 *MHZ* on the mouse embryo morphokinetics and genotoxic effect in laboratory conditions. After ovarian stimulation in mice, the MII oocytes were collected and underwent by in vitro fertilization (IVF) method. The generated zygotes were divided into control and exposed groups. Then, the zygotes with 30 min of exposure to mobile phone RF-EMR, and the control zygotes without exposure, were incubated in the time-lapse for 5 days. The intracellular reactive oxygen species (ROS) level, morphokinetic, embryo viability rate, and Gene expression were evaluated. Exposure of zygotes to RF-EMR by inducing ROS caused a significant decrease in blastocyst viability (87.85 ± 2.86 versus 94.23 ± 2.44), delay in cleavage development (t_3-t_{12}) and also increased the time (in hours) to reach the blastocyst stage (97.44 ± 5.21 versus 92.56 ± 6.7) compared to the control group. A significant increase observed in mRNA levels of *Hsp70* in exposed animals; while Sod gene expression showed a significant down-regulation in this group compared to the controls, respectively. However, there was no significant change in the transcript level of proapoptotic and antiapoptotic genes in embryos of the exposed group compared to the controls. RF-EMR emitted by mobile phone with a frequency of 900–1800 *MHZ*, through inducing the production of ROS and oxidative stress, could negatively affect the growth and development as well as the transcript levels of oxidative stress associated genes in the preimplantation embryos of mice.

1. Introduction

The ever-increasing expansion of using sources which produce electromagnetic radiation (EMR), such as electronic equipment and mass media, such as radio and television transmitters, internet, smart mobile phones have become a serious concern due to the harmful effects on the human biological system (Lopatina et al., 2019; Kıvrak et al., 2017). It is estimated that about 3 billion people worldwide are directly and indirectly exposed to EMR (De Giudici et al., 2021). Non-ionizing radiation caused by mobile phones, which is called radiofrequency electromagnetic radiation (RF-EMR), can target biological systems by creating thermal and non-thermal effects (Yu et al., 2021). During the last decade, epidemiological and laboratory studies have investigated the non-thermal effect of RF-EMR on both male and female reproductive systems (de Seze et al., 1998) and embryo development (Piccinetti et al., 2018). The studies' different results are due to the physical parameters

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of exposure to RF-EMR, such as exposure duration, distance from the production source, frequency, and specific absorption rate (SAR) (Gulati et al., 2020). Mobile communication sources apply their effect on biological structures and functions with non-thermal EMR effects (Blackman, 2009). Also, cell structures such as plasma membrane, mitochondria and DNA are the biological targets of RF-EMR (La Vignera et al., 2012). Cell membranes are shocked after being exposed to RF-EMR and if the intensity of the fields increases beyond the threshold, it causes electroporation, during which water pores are created in the membrane and disrupts the ion balance inside and outside the cell (Ha, 2001). On the other hand, EMR weakens the antioxidant system of the cell by creating oxidative stress conditions (Alchalabi et al., 2015). Following the oxidative stress caused by RF-EMR, reactive oxygen species (ROS) are released from their mitochondria (Panagopoulos, 2011).

Various studies have shown that short-term exposure to EMR leads to an increase in mitochondrial NADH oxidase activity (De Iuliis et al., 2009). These physiological events finally lead to detectable changes such as lipid peroxidation, protein denaturation, and DNA structure damage (Yakymenko et al., 2016), which is associated with changes in the activity of antioxidant enzymes, heat shock proteins (Hsp_s), or cell death (J Hamada et al., 2011). The low-frequency electromagnetic field was evaluated on the differentiation of mouse oocytes and ovarian follicles using electron microscopy, showing a reduced number of differentiated primary follicles and an increased presence of oocytes with heterochromatic and contracted nuclei and vacuolated cytoplasm, in exposed animals (Roshangar et al., 2014). Also, the effect of mobile phone RF-EMR in active antenna mode was assessed on sperm parameters. It was shown that exposed spermatozoa showed lower viability and motility, and a higher percentage of apoptosis and DNA fragmentation compared to the control group (Hassanzadeh-Taheri et al., 2022).

Although several studies have investigated the effect of EMR on different structures, there are no targeted studies about the effect of EMR on the growth and development of early embryos produced by in vitro fertilization (IVF) technology. Evaluating the effects of RF-EMR on the cellular and molecular aspects of embryo are important due to a higher sensitivity to EMR in laboratory conditions than in vivo. During IVF manipulations and incubation, embryo development can be directly exposed to RF-EMR emitted by mobile phones. Therefore, embryos obtained from IVF may be more susceptible to damage caused by EMR compared to in vivo embryos. The environment of embryology laboratories is potentially exposed to mild electromagnetic waves from numerous electrical equipment, such as flow hoods, incubators, refrigerators and heating stages. This study aimed to assess the effect of 900–1800 *MHZ* waves emitted by mobile phones on morpho-kinetic characteristics, survival, and the expression of oxidative stress and apoptotic genes in mouse embryos generated from IVF.

2. Materials and methods

2.1. Chemicals

All materials used for embryological and molecular procedures were obtained from Vitrolife (Kungsbacka, Sweden), Merck (Darmstadt, Germany) and Sigma-Aldrich (St.Louis, USA) companies.

Ethical approval

This study was approved by the institutional committee for the care and use of laboratory animals at Yazd University of Medical Sciences (IR.SSU.AEC.1401.031). All protocols were implemented in accordance with the guidelines of the National Institutes of Health and the welfare rules for the care of laboratory animals.

2.2. Study design

6-8 week-old female mice (25 ± 2.0 g) and 10–12 week old male mice (25 ± 3.2 g) were obtained from the animal house of Yazd Reproductive Sciences Research Institute. All animals were kept in plastic cages of the same size (20.7 × 26.5 × 14 cm), in a room with regulated temperature (22 ± 2 °C), relative humidity (55 ± 15%), 12 h light-dark cycle with free access to food and water.

Fifty adult NMRI mice (35 females and 15 males) were randomly selected. A total of 726 mature oocytes (Metaphase II) were extracted from mice and randomly divided into 2 groups: control (N = 363) and exposure (N = 363). Then, oocytes were fertilized by the IVF method and zygotes were obtained. Differently to the control group, zygotes from the exposure group were placed in the vicinity of RF-EMR of the LG (GT-S6310) mobile phone with a carrier frequency of 900–1800 *MHZ* and SAR ranged from 0.636 to 1.18 W/kg for 30 min. Finally, the zygotes of both groups were incubated in an incubator equipped with a time-lapse system (TLM; Thermo Fisher Scientific, USA) for 5 days. On the 5th day, the blastocysts were subjected to morpho-kinetic, viability rate, and molecular analysis (Fig. 1).

2.3. Sperm and oocyte collection

For sperm collection, male mice were killed by spinal dislocation and underwent surgery under sterile conditions. The epididymal tails were taken out and transferred to a container with human tubal fluid (HTF) heated and equilibrated in an incubator (37 °C, 5% CO_2). Scratches were made on the tails of the epididymis by an insulin needle to allow sperm to escape. Finally, they were incubated (37 °C, 5% CO2) for 1 h to complete the capacitation (Rostamzadeh et al., 2016).

For oocyte collection, female mice were subjected to ovarian stimulation by 10IU i.p. injection of Pregnant Mares Serum Gonadotropin (PMSG) hormone followed by, 48 h later, 10IU of Human Chorionic Gonadotropin (HCG) (Lin et al., 2021). Twelve hrs post-HCG injection, mice were killed by spinal dislocation, and their oviducts were removed and transferred to a pre-warmed HTF medium. Finally, cumulus oophorus masses (COM) containing oocytes were removed by dissecting oviducts under a stereomicroscope (Olympus.SZX16, Japan) and transferred to the HTF medium.

2.4. IVF procedure

Oocytes were transferred to Petri dishes containing 100 μl drops of IVF media covered with equilibrated mineral oil. After sperm evaluation under a phase contrast microscope (Olympus BX5, Japan) to confirm physiological conditions in terms of concentration, motility and morphology, 10 μl of the sperm suspension were transferred to each drop of IVF medium containing COM and incubated for 6 h (37 °C, 5% CO_2). Then, samples were evaluated for successful fertilization, and the loosened granulosa cells around the oocytes were separated with a sterile capillary Pasteur pipette. After washing in a few drops of IVF medium, they were transferred into the final drop. Under the stereo microscope, samples with two pronuclei (2 PN) were considered zygotes. Zygotes were singularly transferred to a time-lapse embryo culture dish (primo vision.vitrolife, Sweden). These containers have a central depression with 9 circular wells, filled with pre-equilibrated embryo culture medium and covered with oil. Finally, zygotes were transferred to the incubator equipped with a time-lapse system (Anbari et al., 2020).

2.5. RF-EMR exposure conditions

A commercial LG (GT-S6310) mobile phone with a carrier frequency of 900–1800 MHZ and SAR 1.8–0.636 W/kg was used to induce the electromagnetic effect in the zygotes of the exposure group. The mobile phone was placed on the mesh screen of the upper tray, at a distance of 10 cm from the culture dish put in the middle tray of the incubator stage (37 °C, 5% CO_2). The mobile phone was placed in conversation mode for 30 min. The placement of the mobile phone was designed in such a way

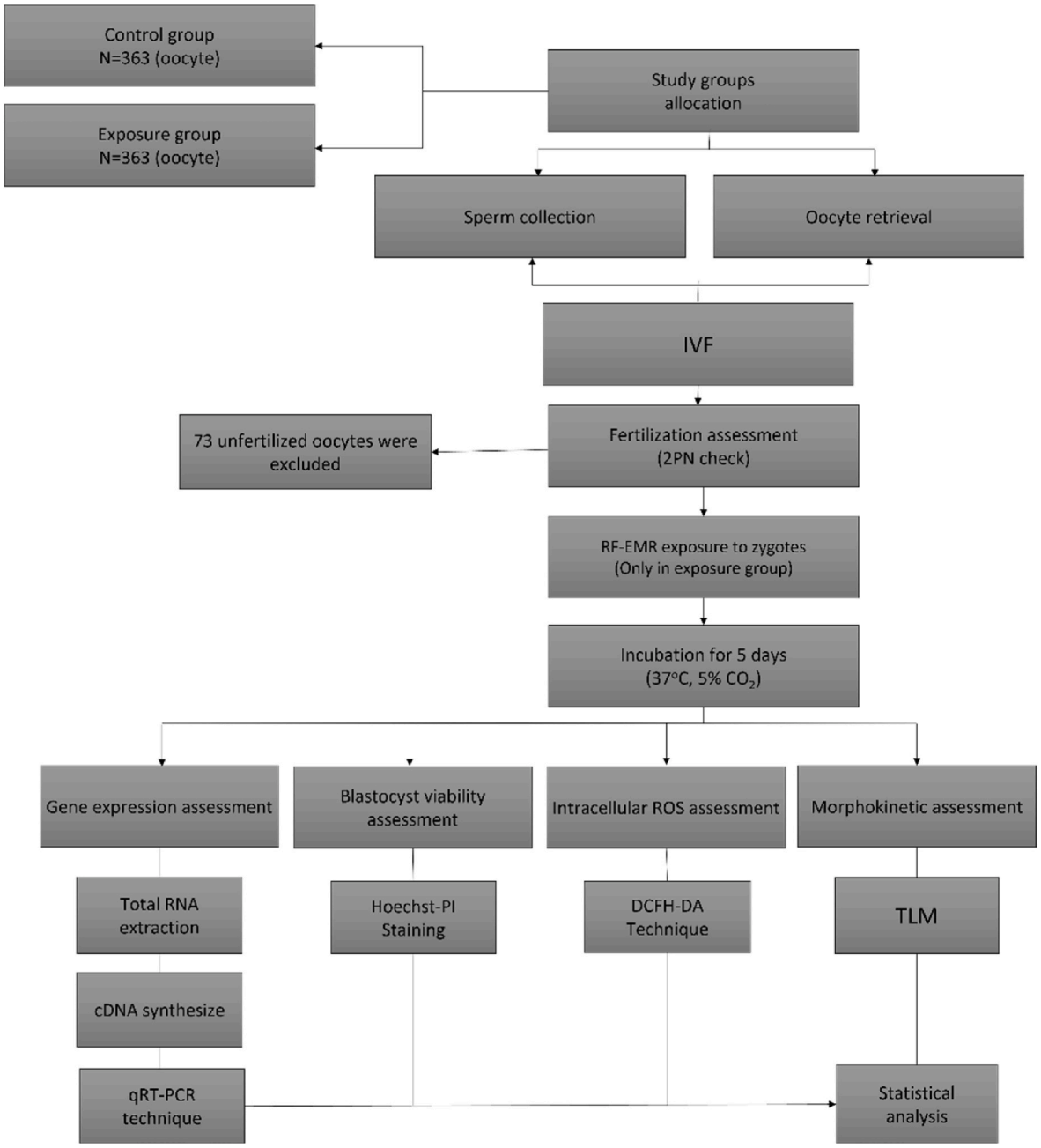


Fig. 1. Experiment design chart. Oocyte and sperm samples were collected and fertilized by IVF. The fertilized zygotes in the exposure group were exposed to mobile phone RF-EMR for 30 min. Embryos of both groups were subjected to the determination of intracellular ROS level, viability, morpho-kinetic, and expression of oxidative stress and apoptotic genes. IVF, Invitro fertilization; PN, pronuclei; RF-EMR, radiofrequency electromagnetic; ROS, reactive oxygen species; TLM, time laps; DCFH-DA, Dichlorohydrofluorescein diacetate; PI, Propidium iodide; qRT-PCR, Quantitative Real -Time Polymerase Chain Reaction.

that during the conversation, the waves spread in all directions in the incubator (Safian et al., 2016) (Fig. 2) .

2.6. Intracellular ROS level evaluation

Dichlorohydrofluorescein diacetate (DCFH-DA) with green fluorescence was used to detect intracellular ROS. To evaluate this parameter, Thirty blastocysts were selected from each group. Blastocyst samples were placed in a dark room in a mixture of 10 μM DCFH-DA and medium 2 (M2) and were incubated (37 °C, 20 min). Next, samples were washed with phosphate-buffered saline (PBS) impregnated with 1 mg/ml polyvinyl alcohol (PVA) and then placed on a clean glass slide in 10 μl drops of PBS-PVA. Blastocysts were evaluated using an epifluorescence microscope (Olympus BX5, Japan) with stimulation (450–490 nm) and emission (515–565 nm) filters (Ren et al., 2015).

2.7. Blastocyst viability

Propidium iodide (PI) and Bisbenzimide (Hoechst 33258) staining were used to evaluate blastocyst cell viability (HOSSEINI et al., 2007). According to the protocol, samples were first immersed in PBS. Next, 300 μg/ml of PI dye solution and 5 μg/ml of Hoechst dye solution were added to the samples and incubated for 30 min at room temperature. Samples were then washed 3 times in pre-warmed PBS to remove extra dyes. Samples were fixed using 2.5% glutaraldehyde, and washed with PBS. Stained blastocysts were placed in a drop of glycerol placed on a

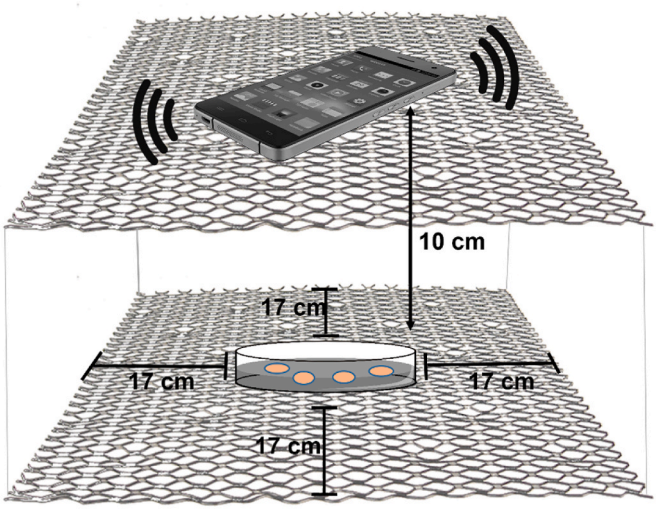


Fig. 2. The design of mobile phone RF-EMR exposure in conversation mode on the zygotes of the exposure group in the incubator (37 °C, 5%CO_2, 98% humidity). The dish with zygotes was placed on the middle tray of the incubator and in the center of the mesh stage. The mobile phone in conversation mode was placed on the upper tray with a distance of 10 cm in the middle of the mesh stage for 30 min.

lame and then covered with a slide. Under an epifluorescence microscope (Olympus BX5, Japan) with stimulation (350–461 nm) and emission (535–617 nm) filters, red cells were considered dead and blue cells as alive.

2.8. Gene expression profile

Eighty blastocysts in each group were used to evaluate the expression of oxidative stress and apoptotic genes. The selection criterion of blastocysts was similar based on morphology and grade. Total RNA extraction was performed for all samples using the Roch kit with Trizol reagent according to the manufacturer's instructions. Nanodrop spectrophotometer (PhotoBiometer, Eppendorf, Germany) was used to evaluate the quantity and purity of RNA, and their values were reported as 260 nm and 260/280 ratio, respectively. In the next step, RNA was transcribed into cDNA using the Roche kit according to the manufacturer's instructions and stored at 20 °C. For evaluation of the relative expression level of genes (*Bax, Bcl2, Sod, Hsp70*), the Quantitative Real -Time Polymerase Chain Reaction (qRT-PCR) method was used with the help of SYBR master mix green. The Beta-2-Microglobulin (*B2M)* gene was considered as a reference gene and specific primers for each gene were used according to Table 1. The qRT-PCR was performed under the following conditions.

Table 1
Primer sequences.

Gene	Primer sequence (5′-3′)	Sequence amplified	Product Length(bp)
HSP70	F-CATCATCAGTGGGCTGTACCA R-GTGCCCAAGCAGCTATCAAG	NM_010478.2	184
SOD1	F-GGGGAAGCATGGCGATGAAAG R-CTGCACTGGTACAGCCTTGTGTA	NM_011434.2	192
BAX	F- AAACTGGTGCTCAAGGCCC R- GGTCCCGAAGTAGGAGAGGA	NM_001411994.1	147
BCL-2	F-GACTGAGTACCTGAACCGGC R-AGTTCCACAAAGGCATCCCAG	NM_009741.5	72
B2m	F-CCTTCAGCAAGGACTGGTCT R-GATCACATGTCTCGATCCCAG	NM_009735.3	139

Abbreviations: F: Forward, R: Reverse, *HSP70:* heat shock protein*70, SOD1: Superoxide dismutase, BAX: BCL-2-like protein 4, BCL-2: B-cell lymphoma 2, B2m: Beta-2-Microglobulin.*

First, at 95 °C for 10 min, then 40 amplification cycles at 95 °C for 15 s, 60 °C for 20 s and finally 72 °C for 20 s (Taebi et al., 2020). After performing the main qRT-PCR reactions, a melting step was performed to confirm the purity of the reactions. The results of the relative expression of each gene were calculated by a $2^{-\Delta\Delta ct}$ method.

2.9. Time-laps embryo monitoring

To check the quality of embryonic development, the viewer software (vitrolife) was used with the ability to image in seven focal levels every 10 min (Heydari et al., 2021). The morphokinetic of the embryos was evaluated using the following markers: PN fading time (tPN_f), 2-cell to 12-cell division time (t_2-t_{12}), time of starting compaction (early compaction), time of finishing compaction (full compaction), time of blastocoel cavity formation (cavitation), time of blastocyst formation (early blastocyst), time of full blastocyst formation, duration of the second cell cycle ($CC_2 = t_4$-t_2), duration of the third cell cycle ($CC_3 = t_8$-t_4), duration of transition from two cells to four cells ($S_2 = t_4$-t_3) and duration of transition from four cells to eight cells ($S_3 = t_8$-t_5).

In addition, abnormal cleavage patterns were evaluated as qualitative variables, which included the following: 1) Direct Cleavage (DC): division of a blastomere in less than 5 h into 3 blastomeres; 2) Reverse Cleavage (RC): fusion of two daughter blastomeres after complete separation or unsuccessful cytokinesis; 3) uneven blastomeres: the presence of blastomeres with a size difference of more than 33% in two-cell stage.

Finally, the rate of embryo arrest and embryo morphology were investigated based on the main features of embryo grading, including cell number, blastomere size, and fragmentation rate: Grade A: Embryo with even blastomeres and no fragmentation, Grade B: Embryo with few even blastomeres and fragmentation rate up to 10%, Grade C: Embryo with uneven blastomeres and fragmentation rate of 10–50%, Grade D: Embryo with uneven blastomeres and fragmentation rate above 50%. Grades A and B were considered as good quality and grades C and D were considered as poor quality embryos (Halvaei et al., 2011).

2.10. Statistical analysis

Statistical analysis was performed using SPSS version 20 software (SPSS Inc., Chicago, IL). The Kolmogorov-Smirno test was used to check the normality of the data. Mann-Whitney and non-dependent t-tests were used to express significant differences between groups of quantitative variables, and Fisher's Exact and Chi-Square tests were used for qualitative variables. The results for variables with normal distribution were reported as mean ± standard deviation (SD) and for variables with non-normal distribution as median ± interquartile range (IQR). A P-value less than 0.05 was considered significant.

3. Results

3.1. RF-EMR exposure on intracellular ROS level and blastocyst viability

Quantification of intracellular ROS levels was done with the mean fluorescence intensity of dichlorofluorescein using Image J 1.410 (National Institutes of Health, Bethesda, MD, USA) software from fluorescent images recorded from blastocysts of both groups with an epifluorescence microscope. Intracellular ROS levels in embryos of the control and exposure groups showed a significant increase in the latter (101.81 ± 8.00 *vs.* 124.51 ± 12.79, P = 0.0001). A decrease in the number of live blastocyst cells in the exposure group was observed when compared to the control (55.20 ± 7.81 *vs.* 44.26 ± 4.93, P = 0.0001) (Table 2, Fig. 3).

Table 2
Effect of RF-EMR on intracellular ROS level and blastocysts live and dead cells counts.

Variables	Control	Exposure	P-value
intracellular ROS level	101.81 ± 8.00	124.51 ± 12.79	**0.0001**
Live cells	55.20 ± 7.81	44.26 ± 4.93	**0.0001**
Dead cells	3.40 ± 1.42	6.16 ± 1.66	**0.0001**
Viability rate	94.23 ± 2.44	87.85 ± 2.86	**0.0001**

The comparison of the results between the two groups was performed by using an independent *t*-test. Data were presented as mean ± SD. $P < 0.05$ (**bold** values) was considered significant. ROS, reactive oxygen species.

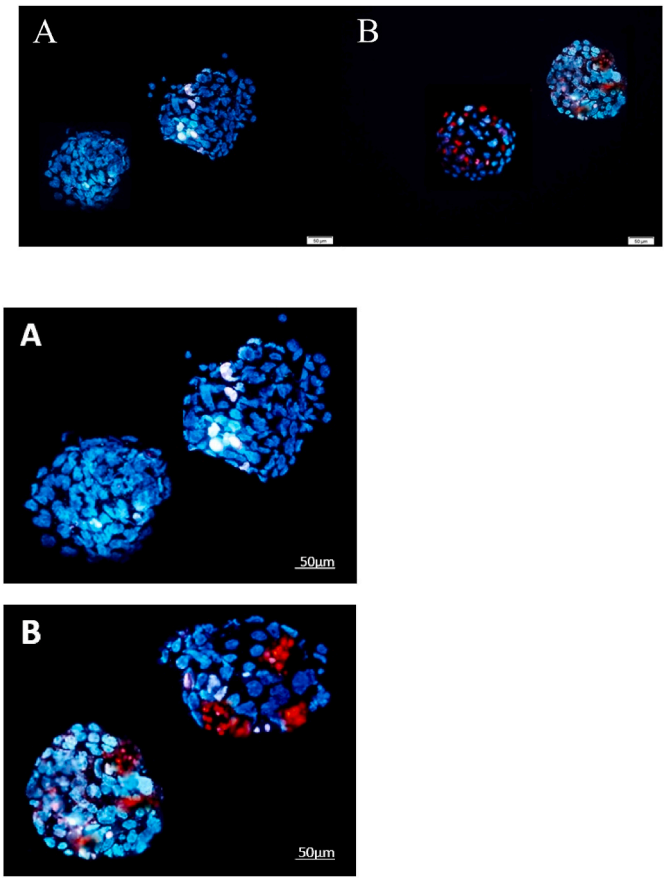


Fig. 3. Blastocyst cell viability (day 5) obtained from Hoechst and PI staining using an epifluorescence microscope with an excitation filter of 350–461 nm and emission of 535–617 nm. A) Control group. Absence of necrotic and deformed cells (blue blastomeres); B) Exposure group. Presence of necrotic cells with deformity and changes in the size and density of blastocyst cells (red blastomeres). Scale bars; 50 μm.

3.2. RF-EMR exposure on gene expression profile

According to the results of qRT-PCR, the transcript levels of *Sod*, an effective gene in antioxidant enzyme activity, significantly decreased compared to controls, while *Hsp70* gene, an effective gene in temperature response, showed a significant increase respect to controls ($P \leq 0.05$). Differently, no significant changes were observed in the mRNA levels of proapoptotic *Bax* and antiapoptotic *Bcl2* genes in the exposure group compared to controls ($P \geq 0.05$) (Fig. 4).

3.3. RF-EMR exposure on embryo development

The average time required in hours to reach a certain growth time point (t_3-t_{12}), showed in the exposed group a delay in terms of embryo growth and division, compared to the control ($P \leq 0.05$). In addition, the time to reach the full blastocyst stage was significantly longer in the group exposed to RF-EMR (97.44 ± 5.21 h), compared to the control group (92.566 ± 6.7 h) ($P \leq 0.05$). On the other hand, no significant differences were observed in the calculated variables of CC_2, CC_3, S_2, and S_3 between the two groups ($P \geq 0.05$) (Fig. 5).

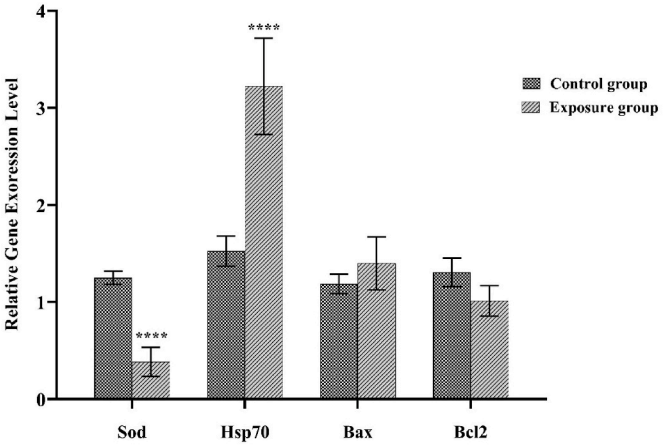


Fig. 4. The effect of RF-EMR on the relative expression of target genes. The data were expressed with an independent *t*-test and Mann-Whitney *U* test for normal and non-normal variables with mean ± SD and median ± IQR/2, respectively. $P < 0.05$ was considered a significant value (****$P = 0.0001$).

The results of the qualitative variables using time-lapse showed that, among the abnormal cleavage patterns, the RC frequency was significantly higher in the exposure embryos ($P = 0.02$). The rates of Direct cleavage (DC) and Multinucleation (MN) in both groups were, differently, reported as 0%. The frequencies of the presence of fragments (63% compared to 25.9%) and vacuoles (51.9% compared to 25.9%) in the embryos of the exposure group showed a significant increase compared to the control ($P \leq 0.05$) (Table 3, Fig. 6).

4. Discussion

This study investigated the effect of exposure to 900–1800 *MHZ* waves emitted by mobile phones on intracellular ROS levels, viability, morphokinetics, and the expression of oxidative stress and apoptotic genes in IVF embryos. To the best of our knowledge, this is the first study to comprehensively assess the effect of mobile phone RF-EMR on embryos, simultaneously examining both cellular and molecular levels. Exposure to RF-EMR has been reported to induce the production of ROS in mammalian cells, mediated by the stimulation of membrane and mitochondrial NADH oxidase (Friedman et al., 2007). This phenomenon has been observed in various cell types, as highlighted by previous research conducted by Grundler et al., who reported an increase in free radical production in the endometrium as a result of RF-EMR emitted by mobile phones (Grundler et al., 1992). The amount of cytoplasmic and mitochondrial ROS increases with the elevation of SAR which induces more damage to the cell (De Iuliis et al., 2009). In this study, exposure to 900–1800 *MHZ* waves led to a notable increase in intracellular ROS levels. These findings are consistent with the existing body of research that has demonstrated the ability of RF-EMR to induce oxidative stress and subsequent ROS accumulation. The observed elevation in intracellular ROS levels is likely attributed to the leakage of electrons from the mitochondrial electron transport chain, as has been reported in previous studies (Koppers et al., 2008). These results further strengthen the understanding of the effects of RF-EMR on cells and tissues. Moreover, similar studies conducted on different cell types and tissues have reported comparable outcomes, indicating a consistent association between RF-EMR within the frequency range of mobile phones and increased ROS production. These studies have also identified various

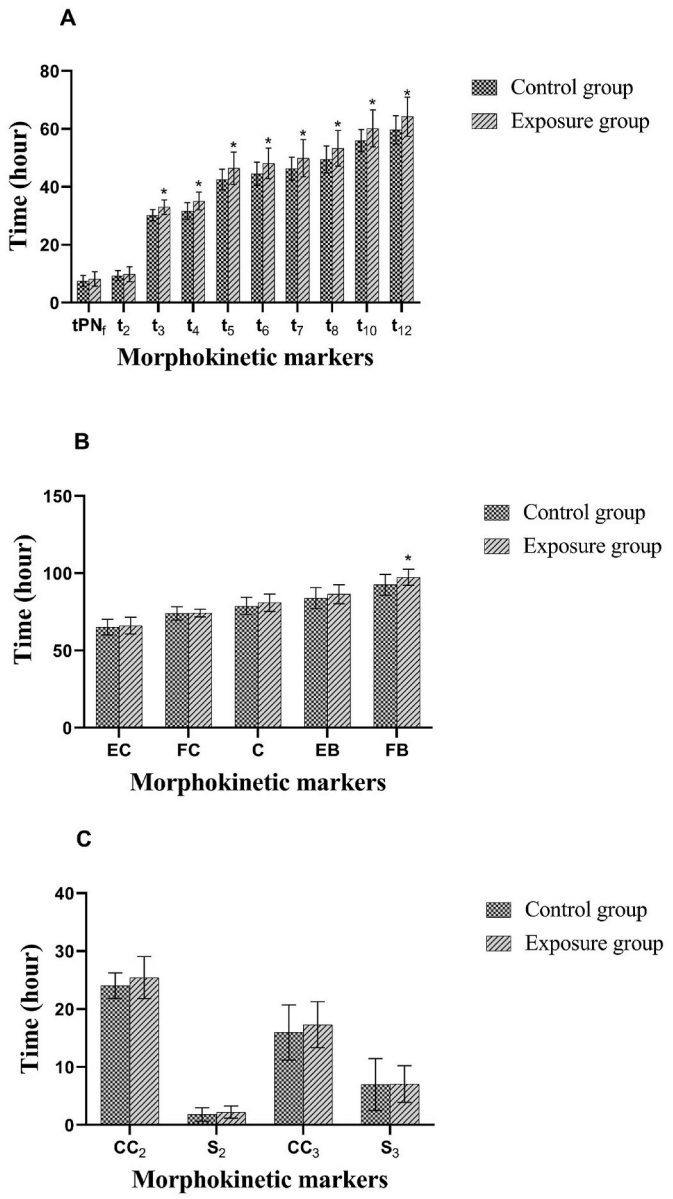


Fig. 5. The effect of RF-EMR on embryo morphokinetic markers in hours. A) Stages of embryo development from the disappearance of PN (tPN_f) to t_{12}; B) Stages of embryo development from early compaction to full blastocyst; C) Calculated variables in embryo development, duration of the second cell cycle ($CC_2 = t_4\text{-}t_2$), duration of the third cell cycle ($CC_3 = t_8\text{-}t_4$), duration of transition from two cells to four cells ($S_2 = t_4\text{-}t_3$) and duration of transition from four cells to eight cells ($S_3 = t_8\text{-}t_5$). EC, early compaction; FC, full compaction; C, cavitation; EB, early blastocyst; FB, full blastocyst. The data were expressed with an independent *t*-test and Mann-Whitney *U* test for normal and non-normal variables with mean ± SD and median ± IQR/2, respectively. *P < 0.05 was considered significant.

detrimental effects, including decreased viability, DNA fragmentation, and alterations in the diameter of seminiferous tubules and the average height of the germinal epithelium (De Iuliis et al., 2009; Ozguner et al., 2006). In a nutshell, our findings contribute to the growing body of evidence regarding the potential impact of mobile phone RF-EMR on cellular and molecular levels in IVF embryos. The observed increase in intracellular ROS levels underscores the occurrence of oxidative stress due to RF-EMR exposure. The consistency of these findings with previous studies reinforces the notion that RF-EMR within the frequency range of mobile phones can disrupt cellular homeostasis, leading to ROS accumulation and various detrimental effects. Further investigations are

Table 3
The effect of RF-EMR on embryo cleavage abnormalities in terms of frequency and percentage.

Cleavage abnormalities	Control (%)	Exposure (%)	OR (95%CI)	P-value
Reverse cleavage	3(11.1)	11(40.7)	0.182 (0.044–0.756)	**0.02**
Uneven blastomers	4(14.8)	9(33.3)	0.348 (0.092–1.31)	0.20
Fragmentation	7(25.9)	17(63)	0.206 (0.064–0.658)	**0.00**
Vacuoles	7(25.9)	14(51.9)	0.325 (0.103–1.02)	**0.05**
Arrest	5(18.5)	16(59.3)	0.156 (0.045–0.539)	**0.00**

The data were expressed with the Pearson Chi-Square Test and Fisher Exact Test for normal and non-normal variables, respectively. OR, odds ratio; CI, confidence interval. P ≤ 0.05 (**bold** values) was considered significant.

needed to elucidate the underlying mechanisms and establish comprehensive guidelines to mitigate the potential adverse effects of RF-EMR exposure, particularly in embryonic environments. The cellular effect of RF-EMR primarily occur at the cell membrane level. Upon exposure to RF-EMR, the cell membrane absorbs alternating currents generated by the EMR, resulting in the generation of eddy currents. magnetic current alteration subsequently leads to changes in the cell membrane structure. Calcium and magnesium cations play a role in reducing bilayer separation and enhancing membrane stability by binding to the phospholipid structures of the cell membrane (Agarwal et al., 2011). Specifically, RF-EMR induces the release of calcium ions from the membrane. replacing them with potassium ions. As potassium ions cannot adequately maintain membrane structure, the membrane becomes weakened and unstable (Goldsworthy, 2007). Consequently, membrane instability can facilitate pore formation and disrupt ionic balance, affecting cell viability (Ha, 2001). Moreover, the destabilized membrane becomes more susceptible to lipid peroxidation caused by ROS (Agarwal et al., 2009).

In light of these cellular effects, the reduced viability of embryo cells observed in the present study can be attributed to the alteration in membrane permeability induced by RF-EMR. This change led to the penetration of PI and Hoescht dyes into the cells. The decrease in cell viability observed in our study was attributed to necrosis rather than apoptosis. During the process of necrosis, as indicated by our samples, cell damage led to a loss of membrane integrity, decreased membrane stability, and increased permeability.In contrast, during apoptosis, the membrane remains intact, preventing the entry of PI into the cell (HOSSEINI et al., 2007). Our findings align with previous research by Safian et al. (2016), who exposed 2-cell mouse embryos to 900–1800 *MHZ* mobile phone waves and observed a significant decrease in blastocyst cell viability compared to the control group (Safian et al., 2016). Additionally, Gautam et al. (2022) exposed male mice to 1915 *MHZ* radiation for 2 h daily over a 45-day period, with a SAR of 0.26 W/kg. They demonstrated that these waves caused changes in the sperm membrane integrity, mitochondrial activity, and a decrease in viability (Gautam et al., 2022). We also evaluated the effect of mobile phone RF-EMR on gene expression in the exposed group embryos. As previously mentioned, the oxidative stress induced by mobile phone EMR has been linked to increased lipid peroxidation. This oxidative stress is associated with a decrease in the activity of antioxidant enzymes, such as superoxide dismutase (*SOD*), glutathione peroxidase (*GSH-PX*), and catalase (*CAT*). This reduction in antioxidant activity contributes to a decrease in the overall antioxidant defense capacity (Kesari et al., 2011; Kumar et al., 2011).

Several studies have demonstrated a decrease in the expression levels of SOD and GSH-PX genes following exposure to RF-EMR in various cells and tissues. For instance, Jonwal et al. (2018) reported a significant decrease in testicular levels of *SOD* and GSH-PX genes in male rats

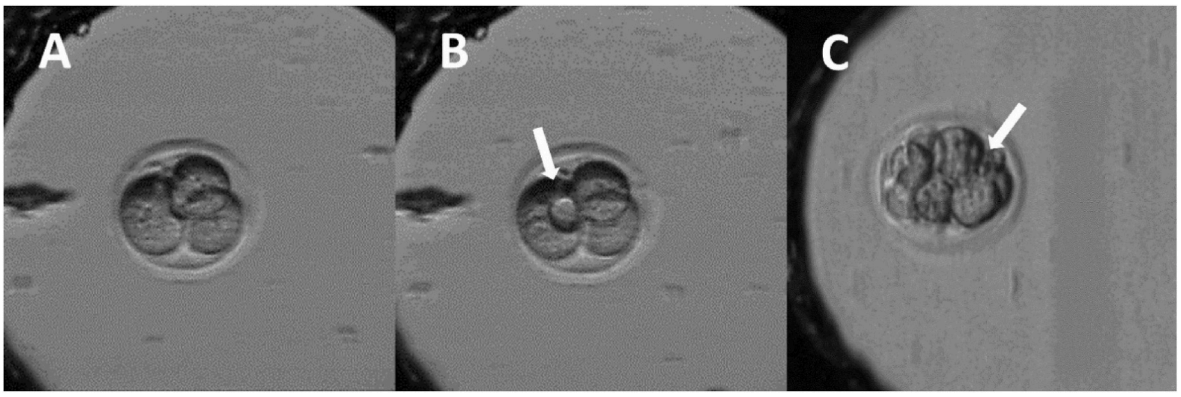


Fig. 6. Cleavage embryo abnormalities. A) Embryo with normal cleavage development; B) Embryo with abnormal cleavage pattern, Arrow indicates the presence of vacuoles in blastomeres; C) Embryo with abnormal cleavage pattern, Arrow indicates the presence of fragmentation in blastomeres.

exposed to 2.45 GHz EMR for 2 h daily over a 30-day period (Jonwal et al., 2018). Furthermore, numerous studies have shown that RF-EMR exposure reduces the activity of *SOD* and *GSH-PX* in different cell types (Balci et al., 2007; Ribeiro et al., 2007).

Our results are consistent with these findings, as we observed a lower relative expression of *Sod* gene in embryos exposed to RF-EMR compared to the control group. This decrease in expression confirms the increased production of ROS in the exposed group (Alvarez et al., 1987). However, it is important to note that the study by Wang et al. (2022) reported no decrease in the relative expression of the *Sod* gene in melanogaster flies exposed to 3.5 *GHZ* electromagnetic waves (Wang et al., 2022). The contrasting findings could be attributed to the absence of significant ROS production in their study, thereby maintaining the antioxidant system. It should be noted that the non-thermal effects of RF-EMR depend on different biological and physical parameters that may differ between studies (Belyaev, 2010; Cancer IAfRo, 2013).

Hsp70 acts as a biomarker and plays a crucial role in cellular protection under stress conditions. An imbalance in Hsp70 levels can induce cellular stress (SuNadeem et al., 2020). *Hsp70* acts as a chaperone, binding to damaged proteins to facilitate their removal (Othman et al., 2021). Recent studies have shown that exposure to EMR can upregulate theexpression of proteins such as *Hsp70* (Blank and Goodman, 2008), which acts as a protective response against waves-induced stress (Ohtani et al., 2016). In our study, we found a significant increase in the relative expression of the *Hsp70* gene in blastocysts of the exposed group. This upregulation suggests that the environmental stress caused by RF-EMR triggers a heat shock response, leading to an increase in the expression of this gene. Similarly, Wang et al. (2021,2022) reported a significant increase in *Hsp70* expression in the larvae of melanogaster flies exposed to 3.5 GHz EMR at intensities of 0.1, 1, and 10 W/m2 (Wang et al., 2022; Wang et al., 2021). In a study conducted by Weisbrot et al. (2003). It was also demonstrated that exposure of Drosophila to RF-EMR at frequencies ranging from 900 to 1900 *MHZ* with SAR of 1.4 W/kg resulted in an increase in *Hsp70* level. In addition, they yielded that RF-EMR emitted by the mobile phones can stimulate cellular function by upregulating *Hsp70* level (Weisbrot et al., 2003). Other studies have also reported similar results, highlighting the role of ROS in cell death and alterations in the apoptotic signaling cascade induced by exposure to ionizing radiation from EMR (Desai et al., 2009). Contrary to these findings, our study revealed that exposure of embryos to RF-EMR did not significantly change the relative expression of pro-apoptotic and anti-apoptotic genes. Similarly, investigation into the effects of different mobile phone frequency channels on lymphocytes indicated no induction of apoptosis (Gulati et al., 2020). It is worth noting that while EMR-induced stress damage may potentially influence these apoptotic pathways, it is plausible to argue that the duration of RF-EMR exposure in our study, along with the associated stress, may not have been sufficient to elicit significant changes in the relative expression of these genes. Previous reports have suggested that the expression of apoptotic genes may occur as a result of chronic exposure to EMR (Shokri et al., 2015; Liu et al., 2015).

The results of our study indicated a delay in the development of cleavage embryos in the t_3 to t_{12} stages in the group exposed to RF-EMR compared to the controls. In addition, the time to reach the full blastocyst stage in the exposure group occurred over a longer period. Although some studies have shown this delay in development could be attributed to disruption of the centrioles of the division spindle or chromosomal changes anda delay in DNA replication (Tabibnejad et al., 2019; Prados et al., 2012). According to these findings, oxidative stress created in laboratory conditions may have influenced the development of the embryo by affecting the structure of centrioles or DNA. Similarly, Luo et al. (2006) conducted research on rat embryos exposed to ELF-EMF 50 *HZ* and observed a significant delay in cleavage divisions at the two- and eight-cell stages. This is supported by previous studies that showed exposure to magnetic fields induced DNA damage in embryos and resulted in significant delays in cleavage divisions (Luo et al., 2006). In addition, the delay in the growth of embryos observed in our study is consistent with previous findings that reported a decrease in the survival rate of embryos to the final stages of development after exposure to ELF-EMF (Dal Canto et al., 2012). Beraldi et al. (2003) conducted a study observing the effects of 50 Hz ELF-EMF exposure on mouse zygotes obtained through IVF. The results indicated a significant decrease in the survival rate of embryos progressing to the final stages of development following exposure to the electromagnetic field (Beraldi et al., 2003). The qualitative data from our study showed that the morphology of embryos in the RF-EMR exposed group was of lower quality compared to the control group.However, there was no significant difference in the percentage of embryos with good quality between the two groups. This finding is in accordance with the results a study done by Safian et al. (2016).

We also observed a higher percentage of abnormal RC cleavage pattern in embryos of the exposed group. The abnormal pattern can lead to embryo mosaicism and aneuploidy (Somfai et al., 2010),which may contribute to embryo arrest and failure to reach full blastocyst stage. Therefore, one possible reason for embryo arrest in the exposed group is the higher percentage of RC. Furthermore, we found a significant presence of fragmentation and vacuoles in cleavage embryos exposed to RF-EMR. These disorders can affect the dynamics of embryo growth. As mentioned earlier, the size and number of vacuoles can affect the ability of cleavage embryos growth to reach the blastocyst stage (Knez et al., 2013). Taken together, our findings suggest that exposure to RF-EMR can negatively affect the morphology and development of embryos. The presence of abnormal RC cleavage pattern, fragmentation, and vacuoles in the exposed group may contribute to delays in development and decreased embryo quality. These results highlight the potential detrimental effects of RF-EMR on embryo growth and support the need

for further research in this area.

5. Conclusion

The exposure of zygotes to the 900–1800 *MHZ* mobile phone was associated with a developmental delay of cleavage embryos, by increasing the time to reach the full blastocyst stage. Embryo arrest, an increased presence of vacuoles and blastomere fragmentation of cleavage embryos, the occurrence of RC, were also observed in exposed embryos. These findings were associated with changes in the expression of genes connected with oxidative stress. As an environmental stress factor, RF-EMR increased embryonic ROS levels and altered the cytoplasmic structure and developmental behavior of the embryo. We strongly recommend assessing the ultrastructural characterization of the embryos exposed to mobile phone waves.

Authors' statement

All the authors of this study declare that they have read the paper and confirm that it has not been reviewed and published by any other scientific authority or journal.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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