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Micronuclei Formation and 8-Hydroxy-2-Deoxyguanosine Enzyme Detection in Ovarian Tissues After Radiofrequency Exposure at 1800 MHz in Adult Sprague–Dawley Rats



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ABSTRACT

Human fertility and its correlation to ovarian function and cytological changes are linked to ever-increasing use of mobile phones. Wireless communications have become a critical topic of concern because of an increasing number of studies in this field with controversial outcomes. The aim of this study was to assess the genotoxic effect of GSM frequency at 1800 MHz on ovarian function. Sixty female Sprague–Dawley rats were distributed over six groups (control group and the exposure groups with whole-body exposure for 2 h/day, 7 days/week for 15, 30 and 60 continuous days). The study investigated the oxidative stress, 8-hydroxy-2-deoxyguanosine enzyme, micronuclei formation and histopathological changes in ovarian tissue. The results showed an induced oxidative stress via an increase in lipid peroxidation and decreased antioxidant enzyme activity. There was also an elevation in the 8-hydroxy-2-deoxyguanosine enzyme and an increased rate of micronuclei formation in ovarian tissues of exposed animals with 60-day exposure compared with control animals. Cytological changes were recorded such as micronuclei formation, vacuolation, degeneration and impaired folliculogenesis. The study suggests that GSM frequency at 1800 MHz was negatively impacted on female reproductive performances mediated by oxidative stress induction and 8-hydroxy-2-deoxyguanosine formation leading to overall impaired ovarian function.

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1. Introduction

Radiofrequency (RF) radiation at 1800 MHz is the worldwide frequency used in mobile phone communication and is characterized by low energy because of the nature of this radiation (non-ionizing radiation). A total of 101 published articles related to the genotoxic effect of RF radiation were reviewed in an analytic study

to investigate the genotoxicity of RF radiation on a biological system, including both *in-vitro* and/or *in-vivo* studies (Ruediger 2009). In this review, 48.58% suggested a genotoxic effect, whereas 41.58% indicated that there is no effect, and 9.9% failed to find any influence on genetic material.

The process of carcinogenesis is mediated by changes in hereditary material in somatic cells, and this is why any agent (chemical and non-chemical) which contributes to alterations of DNA or induces DNA damage could be considered as a carcinogenic agent (Verschaeve *et al.* 2010; Verschaeve and Maes 1998). The International Agency for Research on Cancer in 2011 highlighted at their meeting in France that RF electromagnetic fields

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(EMFs) are possibly carcinogenic to humans based on intensive studies on brain cancer incidence in humans (Baan *et al.* 2011). The risk of cancer development among wireless communication users can be used as an indicator for risk assessment in human studies, especially with modern wireless technologies (Tsybulin *et al.* 2013).

There are different methods used to assess and evaluate the genotoxicity of RF-EMFs in the biological system. For instance, Khalil *et al.* (2012) investigated the presence of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in the urine of male Sprague–Dawley rats. They found that one major form of oxidative DNA damage is 8-oxodG and that the urine level was higher in exposed rats after 1 h of irradiation, suggesting a repair of the DNA lesions leading to 8-oxodG formation. Formation of free radicals such as hydroxyl radical (HO^-), which is a potent oxygen-free radical, negatively affects large molecules in an animal cell (proteins, lipids and DNA) and induces damage. The interaction of HO^- with DNA nucleoside guanine leads to formation of 8-hydroxy-2-deoxyguanosine enzyme (8-OHdG; Valavanidis *et al.* 2009). Formation of reactive oxygen species in liver tissue because of exposure to RF radiation leads to the production of the 8-OHdG enzyme as a result of DNA damage (Güler *et al.* 2012). However, other team researchers found that mobile phone radiation had no effect on the 8-OHdG level in liver tissue, there was an increase in malondialdehyde (MDA) level as an oxidative stress biomarker in exposed animals (Tomruk *et al.* 2010). Karihtala *et al.* (2009) found that free radical activation, especially OH^- plays an important role in oxidative DNA damage in ovarian carcinoma and DNA 8-OHdG enzyme elevation is a potent prognostic factor in ovarian carcinoma. HL-60 leukaemia cells, Rat-1 fibroblasts and WI-38 diploid fibroblasts were irradiated by 50-Hz extremely low frequency (ELF) EMF for up to 3 days to induce DNA damage in cell lines and to assess the 8-OHdG as a biomarker for DNA damage (Wolf *et al.* 2005). The researchers found that the effect of ELF-EMF induced DNA damage in a dose-dependent manner was mediated by the activation of free radical formation, and the study suggests that ELF-EMF leads to cell proliferation and DNA damage in studied cell lines via the reactive oxygen species activity.

Wahab (2005) used a micronuclei formation method to evaluate genotoxicity in human peripheral lymphocytes. The author found that ELF-EMF has a clastogenic effect on human chromosomes, which was confirmed by other assessment methods, such as sister chromatid exchange and comet assay. Another study was performed on peripheral blood samples from healthy donors to assess the impact of modulated and unmodulated 2450 MHz RF fields on the induction of micronuclei in human peripheral blood lymphocytes (Reddy *et al.* 2013). They found that modulated and unmodulated 2450 MHz RF exposure did not induce excess micronuclei in human blood lymphocytes, and both types of exposure had the same effect. Amancio *et al.* (2006) irradiated rats during pregnancy to check whether ultra-high frequency EMF from a cellular phone during their embryogenesis induced chromosomal damage in erythrocytes from rat offspring. The study revealed that ultra-high frequency EMF increased the incidence of micronuclei formation in erythrocytes of offspring. Male rats exposed to 10 GHz of microwave radiation for 2 h/day for a continuous 45 days showed formation of the micronuclei body in lymphocytes detected by a flow cytometry technique (Kumar *et al.* 2013). Different techniques were used to assess the genotoxicity of RF-EMF on the biological system with contradictory results.

The objectives of this study was to investigate the occurrence of micronuclei formation in ovarian tissue and detection of 8-OHdG level in ovaries, a biomarker for genotoxicity from mobile phone radiation in an adult female rat model.

2. Materials and Methods

2.1. Animals

The project was approved by the scientific committee of the Fakulti Perubatan Veterinar (FPV) of the University Malaysia Kelantan (UMK) and was performed in accordance with the UMK guidelines for animal experiments (FPV-PGSC-2015). The study used 2-month old Sprague–Dawley rats of an average body weight (b.w.) of 200 g. Oestrus synchronization was performed before starting the experiment. Sixty female Sprague–Dawley rats were distributed over six groups (control group and the exposure groups as whole-body exposure for 2 h/day, 7 days/week for 15, 30 and 60 continuous days). The animals were bred in the laboratory animal research unit of the FPV/UMK. Animals were kept in plastic cages in a light/dark cycle 12–12 h at room temperature $25 \pm 1^\circ\text{C}$ and humidity $60\% \pm 10\%$ (relative humidity) with tap water and standard rat pellets that were provided *ad libitum*.

2.2. RF-EMF setup

RF-EMR exposure was performed as whole-body exposure at a 1800 MHz GSM frequency of a mobile phone using PSG vector signal generators (Agilent Technologies E8267D, 250 KHz–20 GHz, Santa Clara, CA, USA) with an integrated pulse modulation unit. The signal source of the mobile phone antenna was a standard horn antenna (A-INFO Standard Gain Horn Antenna 1.7–2.6 GHz WR430, Beijing, China) at a specific absorption rate (SAR) level of 0.974 W/kg that was calculated using the following equation:

$$\text{SAR} = (\delta/\rho) E^2$$

where E is the magnitude of the electric field 28.156 V/m, δ is conductivity 1.34 S/m and ρ is the mass density of the tissue-equivalent media 1090 kg/m³. The exposure setup was as described by a previous publication (Alchalabi *et al.* 2016; Figure 1).

2.3. Sampling

At the end of the experiment, all rats were weighed and anesthetized with an intraperitoneal injection of ketamine and xylazine combination at a dose 0.1 mL/100 g b.w. at a dose 80 mg/kg b.w. ketamine and 5 mg/kg b.w. xylazine. Blood samples were collected via bleeding from the eye angle (Van Herck *et al.* 1998). Blood samples were collected in plain tubes without anticoagulant for serum preparation, spun at 11,000 rpm for 10 min and the sera was placed in serum tubes, labelled and stored at 20°C until later biochemical analysis.

Ovaries were removed, washed by ice-cold phosphate-buffered saline, and right ovary samples were fixed in 10% neutral buffered formalin for 24 h before starting the process of preparation for histological slides. Specimens were dehydrated, cleared with xylene and processed in tissue blocks. Sections at six microns were prepared, stained with H&E stains and examined under a light microscope to count ovarian follicles. Thirty slides/groups were prepared to count ovarian follicles. Each slide contained three sections from each ovary (Gul *et al.* 2009). Digital histopathology using slide scanner was used to evaluate ovarian tissue slides. The diagnosis of micronuclei foci in ovarian follicles was confirmed by a histologist and pathologist in the FPV/UMK.

2.4. Biochemical analysis

For oxidative stress assessment, kits were purchased from Cusabio Biotech Co., Ltd. (Wuhan University Science, Wuhan, China) and Abcam (Cambridge Science Park, Cambridge, UK) for biochemical analysis. MDA was assessed as a lipid peroxidation biomarker by using a Lipid Peroxidation (MDA) Assay Kit (ab119870, Abcam, UK) according to the manufacturer's

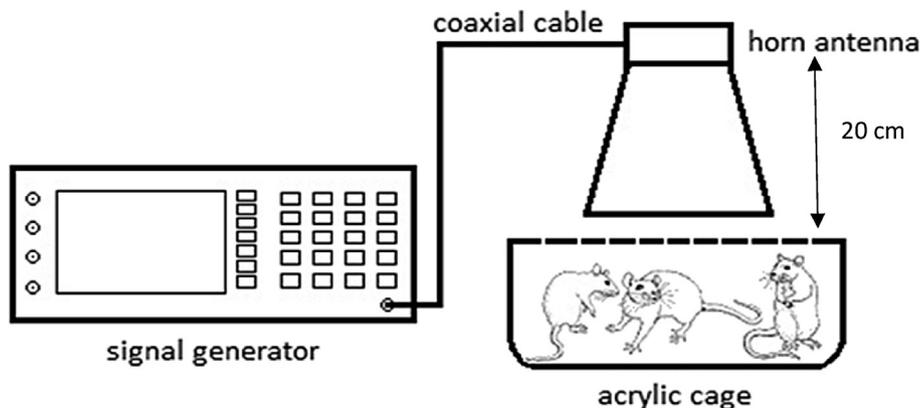


Figure 1. Diagram showing the RF-EMF exposure setup. RF-EMF = radiofrequency electromagnetic field.

instructions. Glutathione peroxidase (GSH-Px) activity and melatonin (MT) concentration in serum were estimated by using a Rat GSH-Px ELISA Kit and Rat MT ELISA Kit (CUSABIO Rat GSH-Px ELISA Kit, CSB-E12146r, CUSABIO MT ELISA Kit, CSB-E13433r, Wuhan University Science, Wuhan, Hubei Province 430223, P.R. China), respectively. Procedures were performed according to the manufacturer's instructions and estimation was performed. An enzyme-linked immunosorbent assay (ELISA) 8-OHdG ELISA Kit (Cloud-Clone Corp., CAT# CEA660Ge, Houston, TX, USA) was used to assess oxidative DNA damage enzyme in serum and was performed according to the manufacturer's instructions.

2.5. Micronuclei evaluation in ovarian sections

Digital images were analysed with free CaseViewer software provided by 3DHISTECH at their website. Each sample section had triplicate readings by a FPV/UMK pathologist to confirm micronuclei occurrence. For each sample, 10 sections were examined. The micronuclei percentage was calculated within each group and then used in the statistical analysis.

2.6. Statistical analysis

Data were expressed as mean \pm standard error and statistical analysis was performed using one-way analysis of variance. Then, the mean differences between experimental groups were checked by a *post hoc* multiple comparisons least significant difference (LSD) test. Pearson's χ^2 test was used to test the significance of the positive micronuclei rate (%) between the control and experimental groups. A p value < 0.05 was considered to be statistically significant against control.

3. Results

1 Effect of 1800 MHz GSM frequency on the serum level of oxidative DNA damage biomarker 8-OHdG and other oxidative stress parameters.

Figure 2 shows that long-term exposure to GSM signals at a 1800 MHz frequency for 30 and 60 days had a significant increase in the serum level of 8-OHdG enzyme compared with their control groups ($p = 0.01$ and 0.03 , respectively). Oxidative DNA damage enzyme 8-OHdG level significantly increases after 30 and 60 days of exposure in comparison with 15-day exposure ($p = 0.005$ and 0.01 , respectively). However, 8-OHdG serum level after 15 days of exposure to GSM frequency remains constant with respect to its control group ($p = 0.623$).

Ovarian MDA was elevated significantly ($p = 0.001$) in both exposure groups 30 and 60 days, whereas the MDA tissue level for the 15-day exposure group ($p = 0.09$) remained constant compared with their control groups. Furthermore, the LSD value among exposure groups shows that 30, and 60 days, was significantly higher than 15 days ($p = 0.01$ and 0.03 , respectively; **Figure 3**).

The GSH-Px level in ovarian tissue decreased significantly in both 30- and 60-day exposure at a p value of 0.021 and 0.014 , respectively, compared with their control groups. Exposure to RF-EMF for 15 days leads to a non-significant lower of GSH-Px tissue level compared with its control group ($p = 0.579$). Differences between mean values of exposure groups 30 and 60 days did not show any significant changes compared with the 15-day exposure group ($p = 0.912$ and 0.934 , respectively; **Figure 4**).

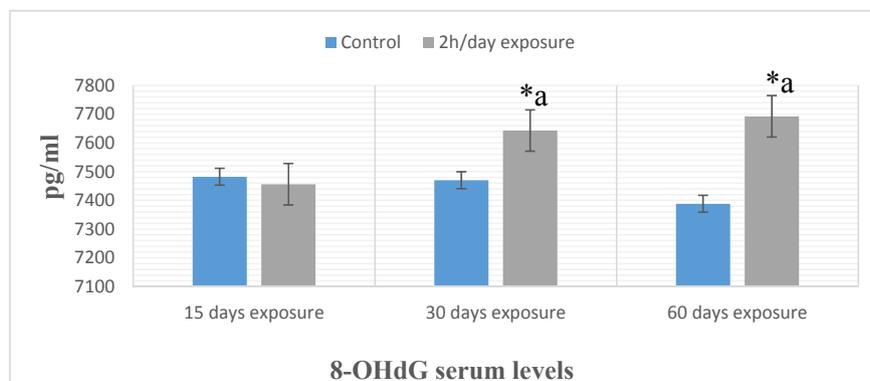


Figure 2. Effect of 1800 MHz GSM frequency on a serum 8-OHdG enzyme level. *Indicates p value < 0.05 , letter mean differences among exposure groups. 8-OHdG = 8-hydroxy-2-deoxyguanosine.

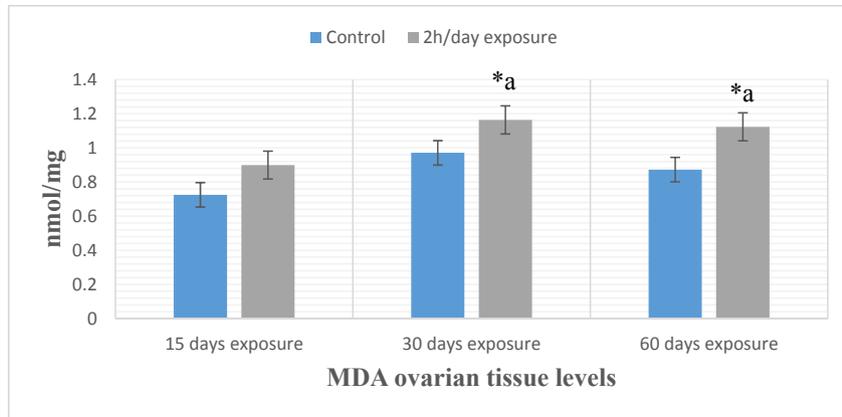


Figure 3. Effect of GSM-like signal at 1800 MHz frequency on ovarian MDA level. *Indicates p value < 0.05, letter mean differences among exposure groups. MDA = malondialdehyde.

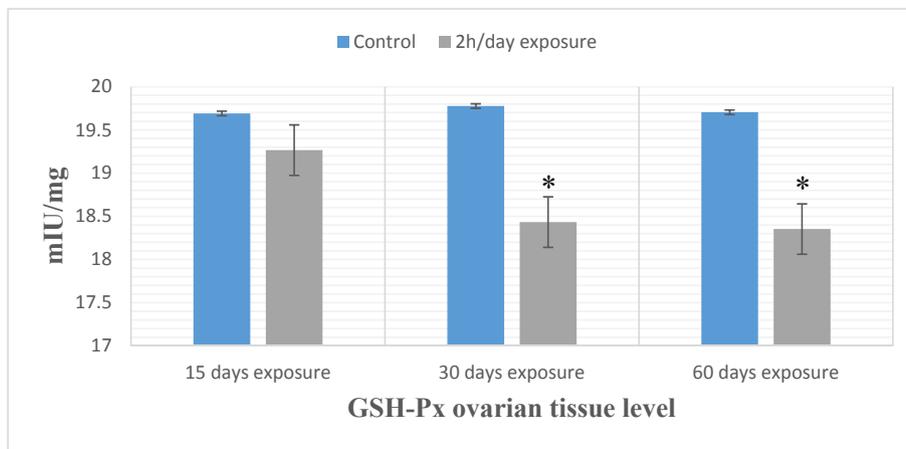


Figure 4. Effect of GSM-like signal at 1800 MHz frequency on ovarian GSH-Px level. *Indicates p value < 0.05.

Analysis of variance of mean serum concentration of MT in the experimental groups for 30- and 60-day exposure showed a significant decrease at a p value 0.029 and 0.001, respectively, compared with their control groups. Long-term exposure to RF-EMF for 15 days had a non-significant decrease in serum concentration in comparison with its control group ($p = 0.134$). However, 60-day exposure exhibited a significant decrease in serum level of MT in comparison with the 15-day exposure group (Figure 5).

2 Effect of RF-EMF at 1800 MHz GSM frequency on percentage of micronuclei formation.

The Pearson's χ^2 test of micronuclei rate occurrence in ovarian tissue shows that 60-day exposure was significantly higher than its control group with a χ^2 value (11.331) and p value (0.003). However, 15- and 30-day exposure did not show a significant difference in comparison with their control group (Table 1).

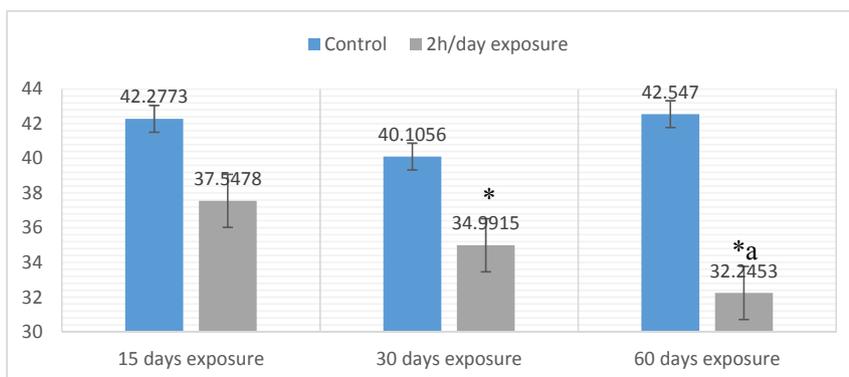


Figure 5. Effect of 1800 MHz GSM-like frequency on serum melatonin level. *Indicates p value < 0.05, letter mean differences among exposure groups.

Table 1. Pearson's χ^2 test of micronuclei formation results between the experimental and control groups

Period	15 d of exposure			30 d of exposure			60 d of exposure			
	Groups	Positive rate (%)	χ^2 value	p Value	Positive rate (%)	χ^2 value	p Value	Positive rate (%)	χ^2 value	p Value
Control	14	1.261	0.532	12	5.577	0.062	14	11.331	0.003	
2 hr/d exposure	19			25			33*			

* Indicates p value < 0.05.

Table 2. Pearson's χ^2 test of micronuclei formation results between two periods of experimental groups

Period	2 hr/d exposure			
	Groups	Positive rate (%)	χ^2 value	p Value
15 d of exposure	19	5.172	0.075	
30 d of exposure	25			
60 d of exposure	33			

A p value < 0.05 was considered to be significant.

Micronuclei formation rate analysis of the experimental groups within the three periods of the study did not show a significant difference and the χ^2 value was 5.172, and the p value was 0.075 (Table 2).

3 Histopathological changes in ovaries associated with RF-EMF exposure.

Ovarian histological sections of chronically exposed animals for 30 and 60 days of exposure to RF-EMF showed in general a decrease of the number of secondary and mature follicles as well as vacuolation in interstitial cells, corpora lutea and ooplasm. The granulosa cell layer showed a separation of granulosa cells with a disrupted, irregular and thin zona pellucida in some mature pre-ovulatory follicles with micronuclei formation in oocytes and close attachments of autophagy granulosa cells to the zona pellucida (Figure 6).

Ovarian sections belonging to the 60-day exposure group showed that some of the mature follicles had separated granulosa cells from the granulosa cell layer and have more than one micronucleus separated from the main nucleus and are enclosed in their own

nuclear membrane. Vacuolation of interstitial cells layer and theca cells was clear in addition to vacuolation in the granulosa cell layer. A separation of granulosa cells from their attachment with each other was also found in some mature ovarian follicles. Furthermore, some of the mature follicles have separated granulosa cells from granulosa cell layer containing more than one micronucleus formation separated from the main nucleus and have their own nuclear membrane with severe apoptosis and apoptotic bodies (Figures 7 and 8).

Luteal cells in ovarian sections of both exposure groups at 30 and 60 days showed cytoplasmic vacuolation of some luteal cells in the corpus luteum with micronucleus formation. Enlargement of vacuolated luteal cells was very clear compared with normal luteal cells (Figure 9).

4. Discussion

The electromagnetic waves emitted from cellular phone devices are considered as non-ionizing radiation and characterized by low energy. Electromagnetic radiation cannot ionize large cellular molecules and do not have the ability to break down DNA directly. However, there might be another pathway by which non-ionizing radiation may affect cellular and physiological functions.

The study proved that an EMF at 1800 MHz GSM frequency induces oxidative stress that is mediated by elevation of the lipid peroxidation biomarker MDA level in ovarian tissue of both exposure groups at 30 and 60 days. Furthermore, we found that there is a decrease in GSH-Px enzyme activity and serum MT level, which are potent free radical scavengers. These results are consistent with Gharamaleki *et al.* (2013) who found that ELF-EMF exposure during pregnancy increases oxidative stress and has an effect on

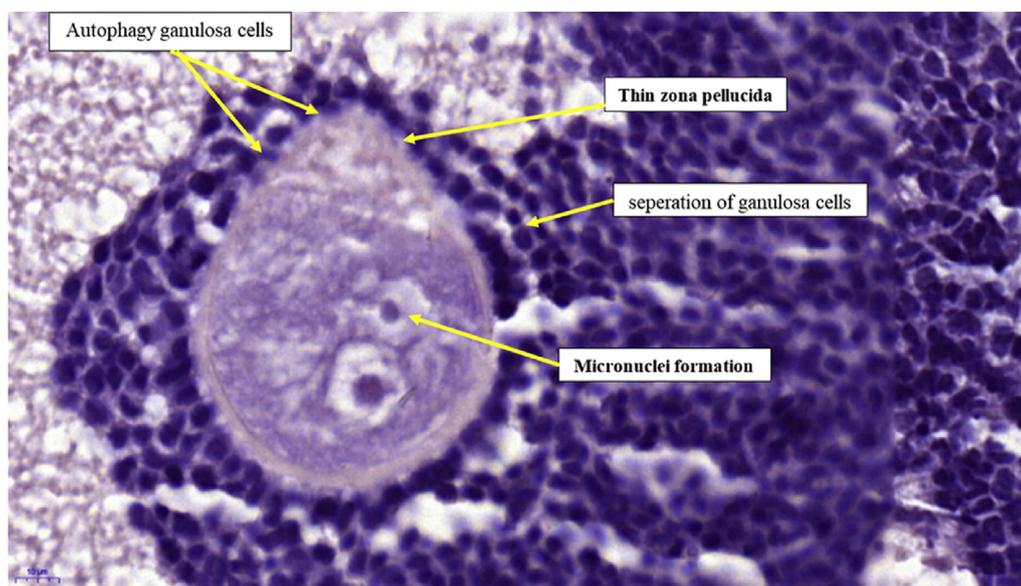


Figure 6. Pre-ovulatory belonging to ovarian section of the 30-day exposure group with follicle micronucleus formation in oocyte and autophagy granulosa cells strongly contact with the oocyte which characterized by a thin zona pellucida. Vacuolation of ooplasm and granulosa cell's layer with a separation of granulosa cells was indicated by arrows. H&E stain 100 \times .

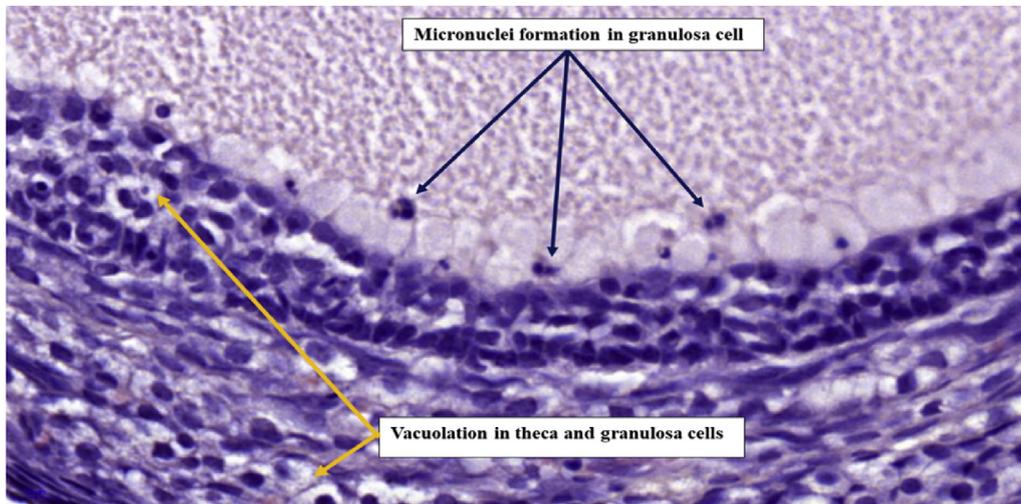


Figure 7. Graafian follicle of exposed female up to 60 days showing vacuolation in granulosa cells layer. Separation of some granulosa cells from their attachment with micronuclei formation. 1000 \times , H&E stains.

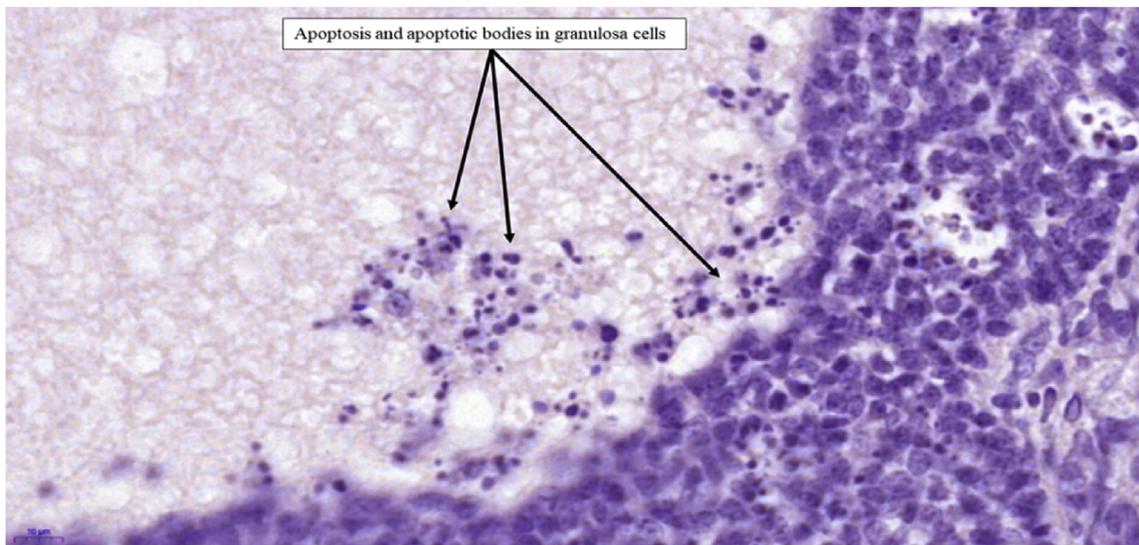


Figure 8. Atretic follicle of exposed female up to 60 days. Severe apoptosis and apoptotic bodies in granulosa cells layer. Separation of granulosa cells from their attachment with the basement membrane theca interna. 1000 \times , H&E stains.

folliculogenesis, which impacts fertility. Furthermore, our results are similar with [Akbari et al. \(2014\)](#) who found that whole-body irradiation with RF-EMF leads to oxidative stress. There is an imbalance between the oxidant and antioxidant system due to increased free radical formation and a reduced scavenging property by MT due to a decrease in its level in the body. Other study results are similar to ours ([Coşkun et al. 2009](#)), indicating that both intermittent and continuous magnetic fields lead to oxidative stress and depend on tissue property, their antioxidant status and responses.

The study results showed that oxidative DNA damage enzyme 8-OHdG was elevated in both exposure groups at 30 and 60 days in comparison with control, and this was in a dose-dependent response. This result is consistent with [Burlaka et al. \(2013\)](#) who found that exposure of quail embryos to low intensity RF-EMR at GSM 900 MHz leads to increased formation of superoxide and nitrogen oxide in embryo cells during the experiment and resulted in an elevation of the 8-oxodG cell level, which is a biomarker for DNA damage. The biomarker 8-OHdG or 8-oxodG has been a crucial biomarker for assessing the effect of endogenous oxidative damage

to DNA and as a factor of initiation and promotion of oncogenesis ([Valavanidis et al. 2009](#)).

These results indicate that there is excessive formation of free radicals that leads to pathologies that can lead to oncogenesis. Furthermore, [Gürler et al. \(2014\)](#) found that whole-body exposure 1 h/day for 30 days increases both plasma and tissue 8-OHdG levels. Another study found that exposure to RF-EMF during pregnancy leads to increased lipid peroxidation levels in different tissues as well as an increased level of the 8-OHdG enzyme in the liver of pregnant rats ([Guler et al. 2010](#)). Furthermore, our data are in agreement with [Da Broi et al. \(2016\)](#) who found that oxidative DNA damage, represented by a higher 8-OHdG follicular level, is possibly related to compromised oocyte quality linked with increased oxidative stress, which is a causative factor for infertility.

Long-term exposure to 1800 MHz GSM frequency radiation leads to histopathological changes in ovarian tissue represented by micronuclei formation, which is another DNA damage biomarker ([Wahab 2005](#)). Our study findings proved that in both exposure groups at 30 and 60 days of exposure leads to an increased rate of

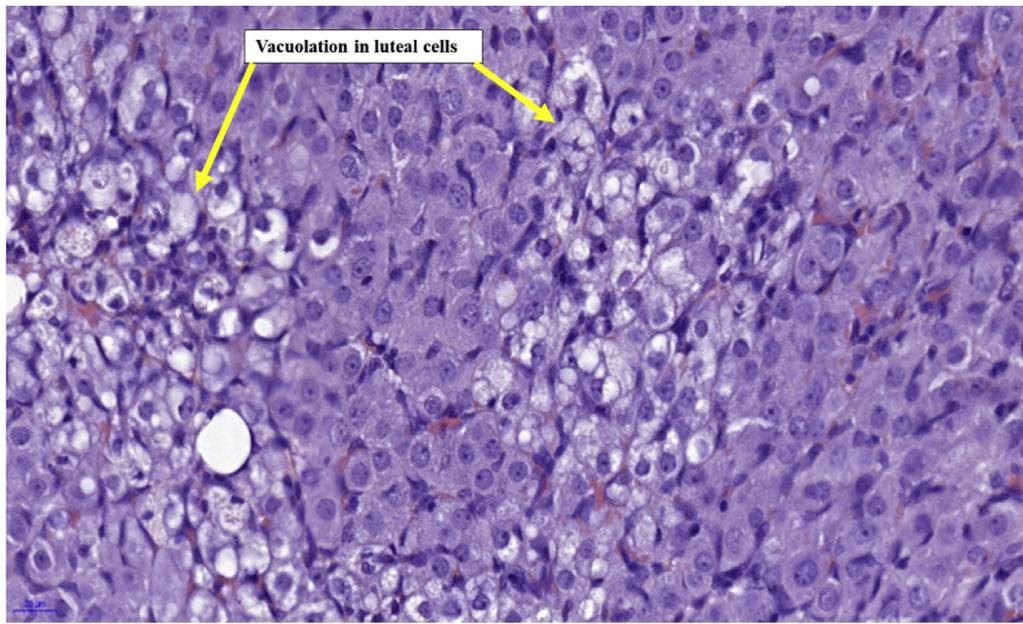


Figure 9. Corpus luteum of exposed female up to 60 days showing hypertrophy of steroidogenic cells. Vacuolation in some luteal cells. 400 \times , H&E stains.

micronuclei formation in different cells of ovarian tissue. In agreement with our results, other studies have reported micronuclei induction in blood cells as a result of exposure to different EMFs (Zotti-Martelli *et al.* 2000; Trosic *et al.* 2004; Zotti-Martelli *et al.* 2005). Several authors concluded that different frequencies have an ability to induce DNA damage; nonetheless, whether this damage is dependent or not on an increase in cellular free radicals production remains as an issue to be better understood. Ferreira *et al.* (2006), in agreement with our findings found that an EMF is able to induce a genotoxic response in haematopoietic tissue during embryogenesis, which is mediated by micronuclei formation through an unknown mechanism.

For other pathological changes in ovarian tissues, for instance impaired folliculogenesis, vacuolation in interstitial cells and granulosa cell layer of mature follicles and apoptosis, we are in agreement with other researchers. Gul *et al.* (2009) found that microwaves emitted from cellular phones negatively affect folliculogenesis through a decrease in the number of ovarian follicles. Furthermore, Roshangar *et al.* (2014) found that *in-utero* low-frequency EMF exposure for 4 h/day had a negative effect on neonatal mice through pathological changes represented by a breakdown of oocyte nests with an irregular arrangement. There is also reduced development of primordial follicles and heterochromatic, shrunken oocyte nuclei and vacuolated cytoplasm. Other findings in agreement with our results by Arash Khaki *et al.* (2013) indicate that exposure to EMFs for 40 days results in an increased rate of apoptosis and presence of fibrosis in ovarian tissue.

The induction of oxidative stress, elevation of 8-OHdG and increased rate of micronuclei formation as a biomarker of DNA damage, in addition to many pathological changes, all play an important role in female infertility. Our findings proved an important point that an imbalance in the oxidative and/or antioxidant system plays an important role in folliculogenesis and the development of ovaries that is mediated by a decreased level of MT. MT plays a role in the activation of insulin-like growth hormone-1 (IGF-1; Jale Oner *et al.* 2009). Dundar *et al.* (2009) indicated that prenatal and post-natal long-term exposure to extremely low-frequency radiation leads to a significant decrease in serum IGF-1 levels during pre-natal exposure. This plays a very important role

in pre-natal and post-natal growth. RF-EMF reduces the IGF-1 levels because it might be linked to the effect of RF-EMF on target organs. Furthermore, it might be related to malabsorption associated with RF exposure, growth hormone level and nutritional status.

The current results suggest that long-term exposure to RF-EMF at the 1800 MHz GSM frequency negatively affects folliculogenesis, which leads to impaired fertility mediated by the induction of oxidative stress. Furthermore, there is DNA damage represented by an activation of micronuclei formation and an increase in the oxidative DNA damage biomarker 8-OHdG enzyme level in serum.

Conflict of interest

Authors declare no conflict of interest.

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