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Alleviative effect of quercetin on rat testicular against 2600 MHz electromagnetic field

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► ***Original article***

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ABSTRACT

Background: The goal of this study was to analyze the prophylactic effects of quercetin (Qu) against testes damage induced by 2600 MHz electromagnetic field (EMF) in rats. ***Materials and Methods:*** Thirty-two male rats (Wistar-Albino) were indiscriminately separated into four groups which were named: Control, Sham-exposed, EMF-exposed (EMF, 1 h day^{-1} for 30 days), and EMF + Qu (100 mg/kg/daily) groups. After 30 days, the rats were sacrificed, testicular tissues were taken, and routine procedures were performed for histological and immunohistochemical evaluations. ***Results:*** When the testicular tissue of the control group was evaluated histopathologically, it was concluded that structures such as Leydig cells and seminiferous tubules were in normal condition and there was no change. No bleeding or inflammation was observed in the general structure of testicular tissues. Irregularities in spermatogenic cell configuration and shedding in the seminiferous epithelium were observed in the EMF and EMF + Qu groups. Findings close to our control group were detected in the sham-exposed and EMF + Qu groups. In addition, tumor necrosis factor-α (TNF-α) immune reactions in were EMF+ Qu group was decreased compared to the EMF group. ***Conclusion:*** In histopathological and immunohistochemical evaluation, it has been proven that EMF causes damage to testicular tissue. However, Qu demonstrated partial ameliorative effects on the pathological findings of the current study.

INTRODUCTION

Nowadays with the progress of communication technology and the development of communication tools, the electromagnetic field (EMF) level in our environment has increased [1]. Wi-Fi modems, microwave ovens and especially smart mobile phones that we use frequently in our daily lives emit EMF. EMF, which we are exposed to without noticing, harms our bodies by causing various changes. The deterministic effects of EMF were determined to be related to exposure time and dose [2, 3]. Many studies reveal the possible harmful effects of EMF on the reproductive system. They stated that exposure to EMF can induce infertility and structural, and functional damage to testicles in rats [4-7].

Processes such as oxidative stress, which initiate diseases that may occur in case of damage in our body, can be prevented thanks to antioxidants. At the same time are substances contributing to the prevention or slowing of cellular damage due to free radicals even if antioxidants are present in low concentrations [8, 9]. Antioxidants exert their effects by suppressing free radical formation by breaking chain reactions or chelating metal catalysts, reducing hydrogen peroxide, and quenching superoxide and singlet oxygen [10]. Simple phenolic acids, xanthones, lignans, simple phenols, flavonoids, and stilbenes are defined in the antioxidant class. The differences between each other arise according to the bonding structure of the phenol rings and the number of rings [11].

Flavonoids are polyphenolic compounds that exhibit high antioxidant capacity and are commonly found in fruits, vegetables, and other food products [12]. Quercetin (Qu), also known as 3,3′,4′,5,7-pentahydroxyflavone, is the most abundant bioflavonoid with potent antioxidant activities [13]. Quercetin is known to possess a series of therapeutic effects such as potent antioxidant activity, anti-inflammatory, induction of apoptosis, antimicrobial, modulation of the cell cycle, anti-mutagenesis, and anticancer activities [14-17]. In addition, Qu inhibits free radical-forming lipoxygenase, histamine release, protein kinase C, and is very effective in preventing diseases that may occur in our body by scavenging free radicals and preventing protein oxidation [18, 19].

The testes are sensitive organs to oxidative stress, inflammation, hyperthermia, and radiation. Qu has a

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curative effect on some pathological conditions such as disruption of reproductive hormone function, irregularity in testicular apoptosis, formation of inflammation, and deterioration in sperm function. In addition, it was determined that Qu is an antioxidant that greatly inhibits the formation of reactive oxygen species (ROS), and eliminates testicular toxicity. Thanks to the radical scavenging property of Qu, it has been determined that it can change the signal transmission of apoptosis caused by oxidative stress, prevent inflammation and increase sperm quality [16, 20, 21, 22]. Therefore, Qu might inhibit the male reproductive toxicity of EMF.

There is insufficient studies about the possible detrimental effects of the 2600 MHz EMF on testicular tissue, and the application of a treatment such as an antioxidant application that can eliminate these effects. The aim of determining whether Qu has a protective role against testicular damage as a result of EMF exposure, especially in the rat model, increases the importance of the study.

MATERIALS AND METHODS

Animal Model

All experimental studies and treatment of rats were utmost attention was paid to Animal Research: Reporting in Vivo Experiments (ARRIVE) 2.0 guidelines at all stages of the experiment, and the experimental work was authorized by the Animal Experiments Local Ethical Committee of Süleyman Demirel University (registration number: 06/04 and date of registration: 05.05.2016). Throughout the experiment, animals were used under the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals, prepared by Süleyman Demirel University (Isparta, Türkiye). A total of 32 male adult rats were used. Rats in all groups weighed 250-300 g on average. All the animals were kept in separate cages according to the groups, in rooms with a special ventilation system at 22-24°C, relative humidity of 45 -60%. The lighting was adjusted to be 12 hours light and 12 hours dark. Rats were fed a standard rat pellet food (Korkuteli Yem, Korkuteli-Türkiye) during the experiment and provided with water ad libitum.

Study Groups

This experimental study consisted of four groups with eight rats randomly:

Group I: Control- the rats were not exposed to the electromagnetic field and were fed with a standard pellet feed for 4 weeks. They were kept in a cage far away from the electromagnetic field.

Group II: Sham-exposed group- the rats were fed with a standard diet (pellet feed) for 4 weeks. The rats in the sham-exposed group were kept in a far environment from the electromagnetic field putting in the same system at the same time and for the same duration. These group rats were administered 1 ml of drinking water by oral gavage.

Group III: EMF-exposed group- the rats were fed for 4 weeks with standard rat pellet feed. They were subjected to a 2600 MHz electromagnetic field from the same distance 60 minutes a day, every day for 4 weeks. As in the sham-exposed group, rats in this group were also given 1 ml of drinking water by oral gavage.

Group IV: 2600 MHz electromagnetic field exposed + Quercetin (chemical purity ≥ 95%, HPLC, Sigma-Aldrich, USA) group- the rats were fed for 4 weeks with standard rat pellet feed. They were subjected to a 2600 MHz electromagnetic field from the same distance 60 minutes a day, every day for 4 weeks. These group rats were administered 100 mg/kg Quercetin (3,3',4',5,7-pentahydroxyflavone) by oral gavage [19, 23].

Exposure system and design

We used a 2600 MHz center frequency (2550-2650 MHz tuned) RF generator (Set Elektronik A. S. Sakarya, Türkiye) capable of supplying 1 W RMS (Root Mean Squared) power to a 50 Ohm output load was used as an EMF source. This device has a 50 Ohm impedance monopole antenna and it placed in the middle of the setup. The form of the electromagnetic wave can be adjusted on the test transmitter as pulsed/continuous. In addition, the power can be set to 0.1 W - 1 W. Thus, the intensity of the intended EMF can be generated in the near or far area of the antenna. Experiments were carried out in an electromagnetically isolated room with 80 dB shielding efficiency at operating frequency in Experimental Animals Laboratory of Süleyman Demirel University so that the conditions of the experimental environment did not change. The room was not entered with a mobile phone or any device that could affect the environment. The rats were placed in plastic tubes with a diameter of 5.5 cm and a length of 12 cm. The system was arranged so that the rats placed in the tube would be equidistant from the monopole antenna. Figure 1 shows the carousel experimental setup.

The EMF intensity and the electric field was measured using the Electromagnetic Field Meter (EXTECH Instruments Corporation, USA). The output power of the RF generator was increased until the nearest electric field to the target tissue (rat testis) in the experimental setup was 10 V/m [24]. When the electric field was 10 V/m, the transmitter output power was fixed. SAR (Specific Absorption Rate) value on the testis is calculated. The E-field intensity in a sufficiently large slab (such that it can be considered infinite in all three dimensions) exposed to a plane wave of known intensity can be calculated (Equation 1.)

$$E_z = E_0 e^{-\alpha z} \quad (1)$$

Where; E_o is the tangential E-field intensity at the

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interface between air and the dielectric slab, E_z is the field intensity along the direction of wave propagation, z is the distance from the interface, and α is the attenuation constant. This method is suitable at relatively high frequencies (above 2 GHz) so that the size of the slab is easily manageable. Also, a relatively high power source is needed to provide sufficient power density [25] as ca;culated using equation 2.

$$\alpha = \frac{2\pi f}{c}(\varepsilon_r/2)^{1/2}\{[1+(\sigma/2\pi f\varepsilon_r\varepsilon_0)^2]^{1/2}-1\}^{1/2} \quad \text{(Neper/m)} \qquad (2)$$

Where; f is the frequency, c is the velocity of light in vacuum, ϵ_r is the relative dielectric constant, σ is the conductivity of the dielectric slab, and ϵ_o is the permittivity of free space.

Conductivity (S/m) and relative permeability values were calculated according to the formulas given in Equation 3., Equation 4., Equation 5. and Equation 6. using the measured e' and e'' values in the İstanbul Technical University.

Relative dielectric permittivity;

$$\varepsilon_r = \varepsilon' - j\varepsilon'' \qquad (3)$$

$$\varepsilon'' = \frac{\sigma}{\omega\varepsilon_0} \qquad (4)$$

$$\varepsilon = \varepsilon_0\,\varepsilon_r \text{ (Farad/meter)} \qquad (5)$$

ε; dielectric permittivity of any material.

$$\text{Loss tangent} = \frac{\text{Conduction Current}}{\text{Displacement Current}} \qquad (6)$$

The SAR value was calculated by using equation 7.

$$\text{Specific Absorption Rate (SAR)} = E^2\sigma/\rho \qquad (7)$$

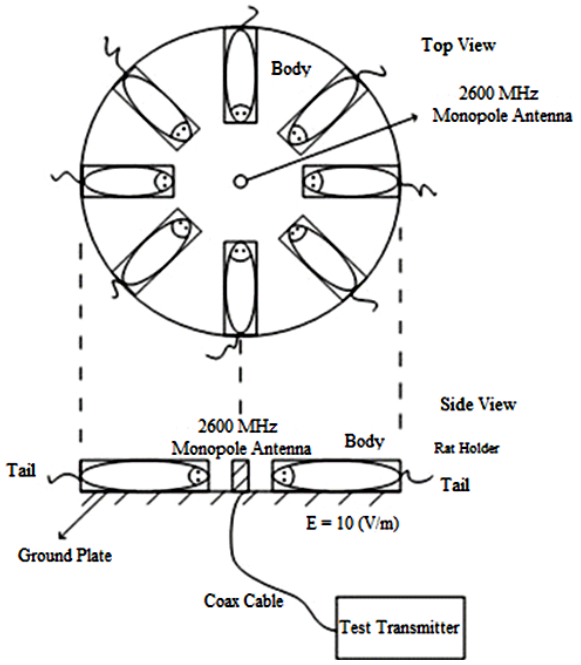

Figure 1. Schematic view of the 2600 MHz exposure setup.

The dielectric properties of testicular tissue samples were measured at İstanbul Technical University, Bioelectromagnetics Laboratory of the Department of Electronics and Communication Engineering. According to the measured ϵ' and ϵ'' values; ϵ'= (11,45).(ϵ_0) and ϵ''= (8,14).(ϵ_0) Calculated dielectric properties of testis tissue at 2600 MHz; conductivity (S/m) = 1,18 and relative permeability= 11,53

With the software developed in the MATLAB (Mathworks, Natick MA, USA) program at Süleyman Demirel University Department of Electrical-Electronics Engineering, the average SAR values induced in the tissue were calculated by using the Finite Difference (FDTD) method. Equation 1. and Equation 2. were automatically calculated in the program. By entering f=2600 MHz, E=10 V/m, ϵ_r=11,53, σ=1,18 S/m, ρ=1 kg/m3, The SAR value was found to be 44 mW/kg according to the Equation 7.

Anesthesia and tissue sampling

After 30 days, the rats were sacrificed under anesthesia with 90 mg/kg ketamine (Richter Pharma AG, Austria) and 10 mg/kg xylazine (Alfasan, Holland). After the testicular tissues were taken quickly, they were fixed in 10% buffered formaldehyde solution for immunohistochemical and histopathological evaluation.

Histopathological examinations

After 4 days of fixation in 10% neutral formalin solution, testicular tissue samples were routinely studied. 4-5 μ sections were taken from paraffin blocks of paraffin-embedded tissues. Tissue sections were stained with hematoxylin (Sigma-Aldrich, St. Louis, Mo.)-eosin (Thermo Fisher Scientific, Cheshire, UK) (H&E) and evaluated under a light microscope (Leica DM 500, Wetzler, Germany) [26]. Damage to the testicular tissues was evaluated with H&E staining. Histopathological changes were scored in a blinded manner. Five different areas from each section were selected and semi-quantitatively scored (0: none, +1: slight, +2: moderate, +3: severe).

The mean diameter of the seminiferous tubules and the thickness of the germinal epithelium were measured for quantitative analysis. Ten sections from each group were evaluated, with ten seminiferous tubules in each section. Results were obtained using a calibrated OLIESIA Soft Imaging System GmbH System (version 3.2 Japan) and a ×40 magnifications.

Immunohistochemistry examinations

The streptavidin-biotin peroxidase method was used for immunohistochemical analysis. Sections of 5 micron thickness taken from testicular tissue were placed on polylysine slides. After, immunostaining of the samples with the primary antibody was performed. All antibodies were purchased from ScyTek (Utah, USA) and used in 1/100 dilution.

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Testes tissues were immunostained by Tumor necrosis factor (TNF-α antibody (ScyTek, USA)) according to the manufacturer's instructions. Five different sections were examined in each sample and scored between 0 and 3 according to the staining intensity (0: No, +1: Mild, +2: Moderate, +3: Severe) [27]. The percentage of positive cells by staining was evaluated as 100 cells for each group (x40).

Statistical analysis

Kolmogorov-Smirnov and Shapiro-Wilk tests were applied to assess whether the groups show a normal distribution. In the comparison between the groups, the Kruskal-Wallis test was used for the groups in which at least one or both of the groups were not normally distributed, and the Independent-Samples T Test was applied for the comparison of the normally distributed groups. All reviews done with the IBM SPSS Statistics 29.0 (The International Business Machines Corporation, Armonk, New York). $p<0.05$ was noted significant.

RESULTS

Histopathology findings

According to the histological evaluation results; When the seminiferous tubules, Leydig cells, Tunica albuginea, and interstitial tissues in the control group were examined, they were found to be in a normal structure. No bleeding or inflammation was observed in the testicular tissue. Sertoli cells and spermatogonia were in normal configuration (figure 2A). Irregularities in spermatogenic cell configuration and shedding in the seminiferous epithelium were observed in the EMF and EMF+Qu groups (figure 2C; D and E; F). Findings close to our control group were detected in the sham-exposed and EMF+Qu groups (figure 2B and E; F). In these groups, the number and location of spermatogenic cell configuration and shedding in the seminiferous epithelium were determined more regularly.

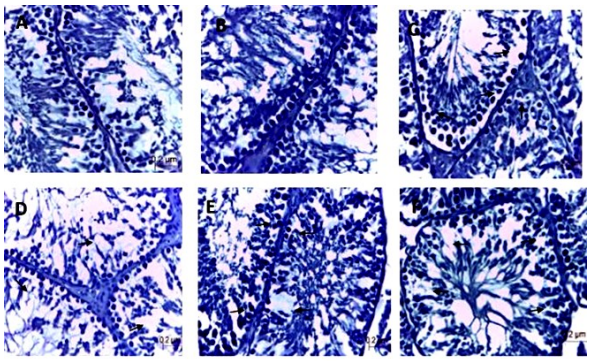

Figure 2. Light microscopic images of testes tissue sections from rats. **(A)** Seminiferous tubules, Leydig cells, Tunica albuginea, and interstitial tissues in the control group were found to be in normal structure, **(B)** Findings close to our control group were detected in the sham-exposed group, **(C and D)** In the EMF group, shedding in the seminiferous epithelium (arrow), **(E and F)** In the EMF+Qu group irregularities in spermatogenic cell configuration and shedding in the seminiferous epithelium (arrow), H&E, ×40.

The highest score in testicular damage was in the EMF (++) and EMF+Qu (++) groups, followed by the sham-exposed (+) group. When the control group and EMF group were compared, the difference was significant ($p<0.05$). While this significant difference was due to the EMF group (1.333±0.516), no considerable difference was observed between the EMF + Qu (1.00±0.632) and sham-exposed (0.833±0.408) groups and the Control (0.500±0.548) group (table 1).

Table 1. Statistical analysis results of histological damage and morphological measurements in testes tissue.

Groups	Control	Sham-exposed	EMF	EMF+Qu
Histopathological scores	0.50± 0.548*/**	0.833± 0.408*/**	1.333± 0.516*	1.00± 0.632*/**
Epithelial thickness (μM)	90.2± 18.5*/**	87.3± 10.1*/**	48.6± 6.4*/**	74.4± 4.1*/**
Seminiferous Tubule (μM)	336.3± 40.9*/**	313.7± 28.2*/**	169± 55.3*	225.2± 17.9*/**

* The difference between the EMF and the control group was considerable, $p<0.05$. ** The difference between the control-sham-exposed group and EMF-EMF+Qu group wasn't considerable, $p>0.05$

Parameters showing sperm production (the diameter of seminiferous tubule and the thickness of germinal epithelial) are given in table 1. Epithelial thickness decreased in the 2600 MHz EMF-exposed group compared to the control, sham-exposed, and EMF+Qu groups ($p<0.05$). The mean seminiferous tubule diameter in rats exposed to EMF decreased compared to other groups ($p<0.05$).

Immunohistochemical findings

The TNF-α immunoreactions in the testes in the EMF group was increased (figure 3). Statistical analyses of immunohistochemical scores are shown in table 2. The density of apoptotic cells between the control and experimental groups was scored semi-quantitatively (0: No, +1: Slight, +2: Moderate, +3: Severe). Apoptotic cell density in the EMF (++) and EMF + Qu (+) groups was found slightly higher than the control group. Apoptotic cell number in the sham-exposed (+) and EMF + Qu (+) groups was declined to the EMF group.

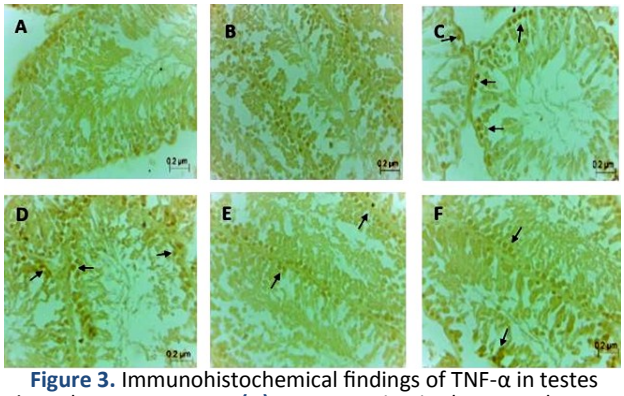

Figure 3. Immunohistochemical findings of TNF-α in testes tissue between groups. **(A)** No expression in the control group. **(B)** Sham-exposed group **(C and D)** EMF groups, apoptotic cells in testes (arrow) **(E and F)** Decrease in expression in EMF+Qu group Streptavidin biotin peroxidase method, ×40.

Table 2 presents the apoptotic cell count and statistical analysis. When the apoptotic cell numbers were compared, the EMF group (1.50±0.548) came first, followed by the EMF+Qu group (0.833±0.752). Difference between the Sham-exposed group (0.667±0.516), the EMF+Qu group (0.833 ± 0.752),

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and the control group (0.333±0.516) was insignificant (Table 3). When the number of apoptotic cells in the groups was evaluated, the difference was not significant (p>0.05).

Table 2. Statistical analysis results of immunohistochemical scoring

Groups	Control	Sham-exposed	EMF	EMF+Qu
TNF-α	0.333 ± 0.516*/**	0.667 ± 0.516*/**	1.50 ± 0.548*	0.833 ± 0.752*/**

There is a significant difference between the control group and the EMF group, p<0.05 ** There is no significant difference between the control group-sham-exposed group and EMF group-EMF+Qu group, p>0.05.

DISCUSSION

There is insufficient number of studies in the literature which examine the effect of Qu on the damage caused by EMF in testicular tissue. In this study, it was shown that EMF can cause damage on testicular tissue, and Qu may have a weak ameliorative effect against this damage.

Many studies have reported that the impacts of EMF on humans depend on the field strength, distance from the source intensity, and duration of exposure [28, 29, 30]. One of the most pronounced effects of EMF on health is the heating of human tissues, that is, the thermal effect. In exposure to EMF, some of the radiation is absorbed by our body and some is reflected. SAR is defined as the energy absorbed by the human body following continuous exposure to EMF fields. The energy absorbed by the human body is measured by SAR, which is the power absorbed per unit mass (Watt/kg) [31, 32, 33]. According to the International Commission on Non-Ionizing Radiation Protection (ICNIRP) guidelines, the maximum SAR limit for a mobile phone is 2 W/kg [34].

According to studies, EMF leads to the structural changes in the appearance of interstitial edema, and the spermatogonial cells lining seminiferous tubules, deterioration of the structures of the seminiferous tubules, vacuolation of the germinal epithelium, the start of the formation of spermatid giant cells. In addition, as a result of EMF exposure, vacuolation in the germinal epithelium, formation of spermatid giant cells and severe occlusion of intertubular blood vessels have been demonstrated [3, 5, 35, 36, 37, 38]. Histopathological abnormalities were irregularities in spermatogenic cell configuration and shedding in the seminiferous epithelium in the EMF group revealed histopathological lesion formation in this study. Administration of Qu in the EMF + Qu caused a slight ameliorative in structural abnormalities compared to the EMF group.

Studies on this topic have reported that EMF causes apoptosis by increasing the formation of ROS. Apoptosis, programmed cell death, activates intrinsic (ROS increase, and oxidative stress generation), and extrinsic (mediated by FASL, TNF-α and the like) pathways [39, 40]. TNF-α, a pleiotropic cytokine, also known as a potent modulator of apoptotic cell death, is made by TNF-α germ cells in testicular tissue. It is one of the testicular paracrine factors that regulate spermatogenesis [41, 42].

In immunohistochemical evaluation, TNF-α was applied to detect apoptosis in testicular tissue exposed to EMF. Previous studies have shown that EMF affects TNF-α and gene expression. Similarly, positive staining was observed in spermatozoa in the EMF exposure group in this study [43, 44] Oxidative damage can be seen in DNA structure due to excessive ROS production [45]. Studies determined that EMF causes oxidative stress in testicular tissue [46- 49]. Therefore, a change in TNF-α expression may occur as a result of the emergence of ROS and damage to the oxidative balance [50]. In a study, Khoshbakht *et al.* showed that 2100 MHz EMF application caused apoptosis in rat testis tissue [6]. It was reported in another study that EMF exposure during embryonic development and adolescence caused apoptosis in testicular tissue [49]. We also found that EMF slightly increased the number of positively stained apoptotic cells compared to the EMF+Qu group. This probably was due to the administration of Quercetin at less than the recommended daily dose (200-1200 mg) [51].

Long-term exposure to EMF disrupts the physiological electrical currents of the cells, causing an increase in temperature. With the increase in temperature, the formation of free radicals increases and the antioxidant defense systems of the tissues change. This causes oxidative stress by affecting biological systems [52, 53]. Quercetin can protect cells suffering from oxidative stress by preventing cell death [54]. At the same time, Qu is an excellent free radical scavenging antioxidant [55]. There are studies stating that Qu may has a toxic effects depending on the dose [56]. The toxic effect of Qu was probably associated with the formation of possible toxic products upon oxidation of Qu during its ROS scavenging activity [51]. *In vivo* studies have determined that low doses and long-term administration of quercetin do not have a protective effect on the male reproductive system [57, 58, 59]. In this study, it was observed that the use of Qu alleviated the damage of EMF on testicular tissue through its antioxidant effect.

According to this study, 2600 MHz EMF has been shown to cause a detrimental effect on testicular tissue. As it is known, the occurrence of apoptotic cell death and inflammation processes in EMF exposure, it has been concluded that infertility may occur as a result of these processes. Qu exhibited partial protection against 2600 MHz EMF-induced testicular damage. Considering the duration of the use of mobile phones in our daily life, it is recommended to conduct studies in which the application time and the number of subjects are increased to better examine

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the effects on testicular tissue.

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Conflict of interest: The authors declare that there is no conflict of interest.

Ethical considerations: Experimental procedure was approved by the Animal Experiments Local Ethical Committee of Süleyman Demirel University, Isparta, Türkiye(registration number: 06/04 and date of registration: 05.05.2016).

Author contributions: IPK did this experiment; IPK, and NS took tissues at the end of the experiment; IPK, OC, and SC made electromagnetic field setup, electromagnetic measurements, and calculations; NS and MS applied the histopathological and immunohistochemical examination of the study; OC Supervised, directed and managed the study; IPK, OC, NS, MS and SC Final approved of the version to be published.

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