The histological and biochemical analysis of radiofrequency radiation effects on testis tissue of rats and the protective effect of melatonin

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Acknowledgment / Conflict of interest

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The authors declare no conflicts of interest related to this paper.

Informed consent

Ethical approval was taken from Gazi University Local Ethics Committee for Animal Experiments (G.U.ET-22.046).

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Abstract

Background/aim: Due to mainly wireless communication devices, especially mobile phones, there has been a steady increase in the intensity of non-ionizing radiofrequency radiation (RFR). In recent years, increased human health problems raised concerns about whether there was a positive relationship between intense exposure to RFR and public health. The present study aims to investigate the effects of GSM-like RFR exposure in the male reproductive system and the role of melatonin treatment (synergistic, antagonist, or additive).

Materials and methods: Thirty-six male Wistar Albino rats were used and separated into six groups: i. Control; ii. Sham; iii. RFR exposure; iv. Control-melatonin; v. Sham-melatonin; vi. Melatonin + RFR exposure. 2600 MHz RFR exposures were applied to animals with 21.74 V/m Electric (E) field levels for 30 min/day, 5 days/week for 4 weeks. All testicular tissue samples were evaluated under a light microscope about their Hematoxylin-Eosin staining. Biochemical analyses were performed by measuring malondialdehyde, total nitric oxide, glutathione, and glutathione peroxidase levels. We evaluated the combined effects of prolonged RFR exposure and melatonin treatment on ROS-mediated structural changes in testicular tissues.

Results: Results showed that reactive intermediates (malondialdehyde and total nitric oxide) increased significantly with RFR exposure, while the protective effect of melatonin effectively reduced the tissues' radical levels. Histological evaluation showed decreased cell population and connective tissue elements under RFR exposure and induced intense edema in testicular tissues was also distinguished.

Conclusion: Prolonged RFR exposure's structural and functional effects might be ROS-based. Moreover, these adverse effects might be compensated with externally treated supplements. There is a need for new extensive research.

Key words: Radiofrequency, reactive oxygen species, hematoxylin-eosin, testis, rat

1. Introduction

There has been a steady increase in the intensity of radiofrequency radiation (RFR) in the environment due to mobile communication devices. Increased human health problems have raised the concern of whether there is a relationship between human beings' RFR exposure and health. RFR's biological effects depend on some parameters; frequency, polarization, intensity, exposure duration, exposed objects, dielectric properties of exposed material, etc. The interactions of RFR may lead to structural changes in bio-molecules and alterations in the regulatory cascades of biochemical processes [1].

RFR (30 kHz-300 GHz) is involved in the non-ionizing part of the Electromagnetic (EM) spectrum. Because of its insufficient energy for direct ionization of molecules, structural changes in molecules can be observed via the secondary messengers or reactive oxygen/nitrogen intermediates produced by non-ionizing RFR [2]. With the depolarization of the mitochondrial membrane induced by RF exposure, the free reactive oxygen and hydroxyl radicals are formed as the primary sources of degenerative intermediates [3].

Various RF-induced effects on the male reproductive system, mainly based on testicular tissue cells, are available in the literature [4-10]. The fundamental mechanism of RFR on the reproductive organs in man has not been explained yet. Most studies argue that over-produced cellular free radicals after RFR exposure may increase the sensitivity of male reproductive tissues and decrease the quality of sperm capacity by catalyzing redox reactions[11].

The essential impact of exogenous melatonin treatment is expected to be on the electron transport chain as the antioxidant properties. The detoxification of over-produced free radicals by increasing the activities of the antioxidant defense system can have an essential effect on the human body and reproductive systems[12-16]. Melatonin produced by the central nervous and reproductive systems has different impacts on the anterior pituitary gonadotropins, gonadal steroids, and testicular functions [17].

The present study aims to investigate the effects of 2600 MHz GSM-like RFR exposure in the male reproductive system and the role of melatonin treatment (synergistic, antagonist, or additive). 2600 MHz RFR frequency was used to study the 5th generation of mobile system frequency and there are limited studies related to this frequency. GSM-like RFR exposure simulates the RFR exposures of mobile phones. Free radical-mediated structural changes in the primary constituent of the male reproductive and antioxidant defense system were analyzed under combined treatments of melatonin and RFR.

2. Materials and methods

Ethical approval was taken for 6 animals per group from Gazi University Local Ethics Committee for Animal Experiments (G.U.ET-22.046). This number of experimental animals is allowed for experiments by the Ethics Committee.

Adult male Wistar Albino rats (12-16 weeks old, weighing 250±30 g) were obtained from the Laboratory Animals Raising and Experimental Research Center (GUDAM) in this

study. Animals were housed under standard conditions (12 h light/12 h dark cycle with 22±2 °C ambient temperature). Tap water and a regular diet were given to animals.

Thirty-six male Wistar Albino rats were separated into six groups (n=6):

Group I (control): Animals maintained regular activity in their cage without application.

Group II (sham): Animals were kept in the exposure setup (30 mins /day for five days for four weeks) with no melatonin or RFR exposure.

Group III (RFR): Animals were exposed to RFR for 30 mins /day for five days for four weeks.

Group IV (Melatonin): Animals were subcutaneously injected with melatonin (10mg/kg/day during a month) and kept usual living place for whole experiment days.

Group V (Sham-Melatonin): Animals were subcutaneously injected with melatonin (10mg/kg per day during a month) and put in the exposure setup with no RFR for 30 mins/day for five days without RFR exposure.

Group VI (Melatonin+RFR): Animals were subcutaneously injected with melatonin (10mg/kg per day during a month) and exposed to RFR for 30 mins /day for five days for four weeks.

The RFR system is described in Figure 1 [6]. This system comprises a vector signal generator (Rohde &Schwarz, Munich, Germany) and a horn antenna (ETS Lingren, Cedar Park, TX). During exposure, rats were placed in plastic cages (34×24×13 cm), three animals in one cage, and 2600 MHz RFR exposures were performed by placing the horn antenna on the plastic cage. For RFR exposures, Electric field levels were measured in the cage and found homogenous, so it was suitable to put 3 animals in the cage for each exposure. External and internal electromagnetic field strength were measured by Narda EMR 300 and its related probe during the exposure, and Electric field levels were measured as;

$E_{external}$: 0.88V/m (outside of the cage)

$E_{internal}$: 21.74 V/m (inside of the cage).

The whole-body SAR values were calculated as 0.616W/kg for a 1 g average and 0.297 W/kg for a 10 g average using the IEEE/IEC 62704-1 method [6].

After the last exposure, rats were anesthetized with intramuscular injections of ketamine (35 mg/kg) and xylazine (5–10 mg/kg) and decapitated. Testis tissues were analyzed both histologically and biochemically.

2.1. Histological Analysis

All testicular tissue samples were first fixed in 10% formaldehyde solution for light microscopic examination. After fixation, tissue samples were placed in cassettes and washed under running water for 24 hours. To remove moisture, tissues were passed through increasing degrees of alcohol (70%, 80%, 90%, 100%). The tissues were then passed through xylol for polishing and embedded in molten paraffin. Hematoxylin-Eosin staining was applied to the 4-5 micron thick sections obtained from the prepared paraffin blocks, and their pictures were taken by evaluating them in the LAS program.

2.2.Biochemical Analysis

The testicular tissues' malondialdehyde (MDA), total nitric oxide (NOx), glutathione (GSH), and glutathione peroxidase (GSH-Px) levels were studied by using a commercial ELISA kit (Shanghai Sunred Biological Technology, Shanghai, China). Samples absorbance was measured at 450 nm. Results were expressed as ng/ml for GSH-Px, nmol/g tissue for MDA, μmol/g tissue for NOx, and mol/g tissue for GSH.

2.3.Statistical analysis

SPSS 20.0 was used for the statistical analyses of the present study. The Kruskal– Wallis (non-parametric) test was applied to evaluate differences among all groups, while the Mann- Whitney test evaluated differences between pairs of groups.

3. Results

3.1.Hematoxylin-Eosin Staining of Testis Tissues

Control and Sham groups were observed with typical structures of the seminiferous tubules of the tissue and the interstitial connective tissue located between these tubules in hematoxylin-Eosin staining of testicular tissue. Spermatogenic cells located in the germinal epithelium of the seminiferous tubule; Spermatocytes (Primary Spermatocytes), which attract attention with their significant appearance in the upper row, and spermatogonia with their tails oriented towards the lumen in the other layers attracted attention. It was observed that the lumen of the seminiferous tubule was densely filled with spermium. Again, among the spermatogenic cells, pyramidal-shaped Sertoli cells extending from basal to apical were observed with their typical structures. Numerous capillaries and interstitial (Leydig) cells in groups were seen in the interstitial space between the tubules with their usual forms **(Figure 2 (a) and (b))**.

Control Melatonin and Sham Melatonin groups were evaluated, and the general structure was identical to the Control and Sham Control groups. Unlike the control groups, it was noted that there were relatively more spermatocytes in some seminiferous tubules in the melatonin-administered groups and the presence of reasonably dense spermium in the lumens of some tubules. This finding was interpreted as melatonin administration may have increased sperm production. In the Sham Control group examinations, it was also noted that some capillaries in the interstitial area were dilated **(Figure 3 (a) and (b)).**

In the RFR group, it was noted that the seminiferous tubules did not show any shape disorder but the presence of intense edema in the germinal epithelium of the seminiferous tubule in most tubules. It was noted that the standard impression and organization of the epithelium disappeared in the tubules, where edema was intense, and some spermatogonia preserved their basal positions. Apart from this, other cells of the spermatogenic series came together out of their regular arrangement and emptied into the lumen. It was clearly distinguished that the intercellular connections were disrupted in the tubules. Again, it was noted that spermium in

the lumens of some tubules was relatively reduced compared to other groups. Intensive vacuolization was observed in the examinations performed in the interstitial area of this group. Again, in this area, it was observed that the cell population and connective tissue elements decreased relatively compared to other groups and gained a hyalinized appearance **(Figure 4).**

The hematoxylin-Eosin staining of testicular tissue belonging to the **RFR and Melatonin group** was described as the most striking finding. The general appearance exhibited a structure similar to the Control and Melatonin groups. On the other hand, it was observed that impaired connections between edema and spermatogenic series cells continued to be kept in some tubules. Again, edema was observed in the interstitial area in some regions. However, it was noted that the cell population in this area increased relatively compared to the RFR group **(Figure 5).**

3.2.Tissues' pro-oxidant and antioxidant levels

The oxidative stress results obtained from all examined testicular tissues are given in **Figure 6.** There was a significant increase in the level of lipid peroxidation with the effect of RFR exposure (7,37± 0,70) compared to control (4,58 ± 0,58) and sham-exposed groups (4,40±0,75) ($p<0,05$).

External melatonin treatment decreased the level of lipid peroxidation's end product significantly for the RFR+melatonin group (5,34 ±0,55) concerning the RFR group (7,37± 0,70) ($p<0,05$).

Increased testicular nitric oxide levels were found in the RFR group (16,50±1,10) concerning control (10,30 ± 1,00) and sham-exposed groups (10,10±0,98)($p<0,05$).

Melatonin treatment decreased the level of testicular nitric oxide for the RFR+melatonin group (12,53±1,34) concerning the RFR group (16,50±1,10) ($p<0,05$).

Testicular glutathione level decreased significantly in the RFR group (49,28±2,36) concerning control (62,00± 1,82) and sham-exposed groups (62,42±2,47)($p<0,05$).

However, melatonin treatment increased the level of testicular glutathione for the RFR+melatonin group (59,22±1,45) concerning the RFR group (49,28±2,36) ($p<0,05$).

Testicular antioxidant levels decreased significantly in the RFR group (5,5±0,84) concerning control (12,56± 0,65) and sham-exposed groups (11,97±1,25)($p<0,05$).

Melatonin treatment increased the testicular glutathione level for the RFR+melatonin group (9,23±0,69) concerning the RFR group (5,5±0,84) ($p<0,05$).

4. Discussion

The present study investigated the possible histological and biochemical effects of RFR exposure in the male reproductive system and the role of melatonin. Results showed no structural disruption in male rats exposed to RFR in the seminiferous tubules. However, intense edema was observed in the germinal epithelium of the seminiferous tubule. The epithelium's usual structural and functional properties disappeared in the tubules with intense edema. It was observed that some spermatogonium protected their locations in basal, but other cells that accumulated out of their regular structure moved into the lumen. Therefore, the intercellular connections in the tubules were distinguished. Additionally, intensive vacuolization was observed in the interstitial area of the RFR group. Cell population and connective tissue elements decreased relatively and gained a hyalinized appearance. An intense decrease was observed in the RFR groups' seminiferous epithelial thickness measurements. Also, the sperm cells in the lumens of some tubules were considerably reduced under RFR.

In the literature, there are many studies related to RFR and the male reproductive system [18-19]. Most are related to the number of spermatozoa, the speed and motility of sperm cells, testis tissues, and the quality of semen. Unlike these studies, the combined effect of RFR and melatonin was investigated in the present study. With melatonin, RFR-induced reactive intermediates were evaluated by measuring tissues' oxidant and antioxidant levels.

The prolonged duration of men's mobile phone exposure changes the main sperm parameters, resulting in male infertility associated with damage to the testicular tissues. This significant consequence mentioned in the study of Agarwal et al. [18] can be clarified by three interaction mechanisms proposed for EM fields: the sensitivity of male reproductive organs to RF exposure, tissue heating associated with RF absorption, and the combined effects of both RFR sensitivity and heating [18]. Studies on the impact of RFR at different frequencies and exposure duration on male animals revealed a decrease in the count of spermatozoa [19].

In contrast to these findings, Lee et al. showed no adverse effect of long-term exposure to 845 MHz RFR on the count of sperm cells [20]. Tas and his colleagues also found no differences in testicular tissues and the quality of semen of male rats long-term exposed to RFR [22].

The histological figures of this study showed similarities with the literature findings. No structural changes in whole testicular tissues, but disruption in intercellular connection and intense edema were necessary for this study. It can also be concluded that the exogenous melatonin treatment reduced the adverse impacts of RFR. In our previous study, increased brain tissues' oxidant levels and decreased antioxidant levels were determined [6]. We discussed the effect of exogenous melatonin treatment on diminished brain tissues' oxidative stress. Moreover, the exogenous melatonin treatment eliminated apoptotic cell formation and structural degeneration after RFR exposure [6].

In another study, the rats' testicular tissues were exposed to Wi-fi radiation for one hour/day for a month. There was no significant difference in seminiferous tubule diameters and the number of pycnotic and eukaryotic cells. Still, a decrease in Leydig cells and an increase in apoptosis were detected [22].

Several studies have shown the disruption of the seminiferous tubules' basal membrane, intense edema, and apoptotic cell formation in the spermatogonium cells under RFR exposure [23-24].

The studies mentioned an inverse ratio between RFR exposure and sperm concentration, motility, and normal morphology parameters. The boost effect of RFR on oxidative stress in semen can be evaluated by acting on plasma membrane enzymes. It is also clear that these adverse effects are related to the duration of RFR exposure and the greater risk for subjects exposed to long-term RFR.

In the literature, many studies have shown that oxidative damage on different tissues is induced by RFR exposure. Various factors can affect this oxidative damage: the exposure duration, frequency, power, polarization, and types of RFR sources, exposed materials' dielectric properties such as conductivity and resistivity of tissues under different frequencies, and the dimensions of the exposed object.

The present study investigated reactive intermediates mediated oxidative stress and structural disruption in testicular tissues under GSM-like RFR signals. Additionally, the effects of exogenous antioxidant supplements on oxidative stress induced by RFR exposure were analyzed by treating melatonin. The melatonin treatment eliminated the adverse effects of RFR exposure. Despite oxidative damage in testicular tissue and melatonin, increased antioxidant levels showed structural deterioration caused by RFR exposure.

The findings in the present study would help many researchers evaluate the interaction mechanism between RFR exposure and the male reproductive system. New studies are needed to show the possible effects of different exposure plans with different frequencies, and duration, and there is a need for quantitative data or measurements related to sperm counts, cell numbers, dilated capillary size, etc. These data will be supportive of the visual observations and interpretations.

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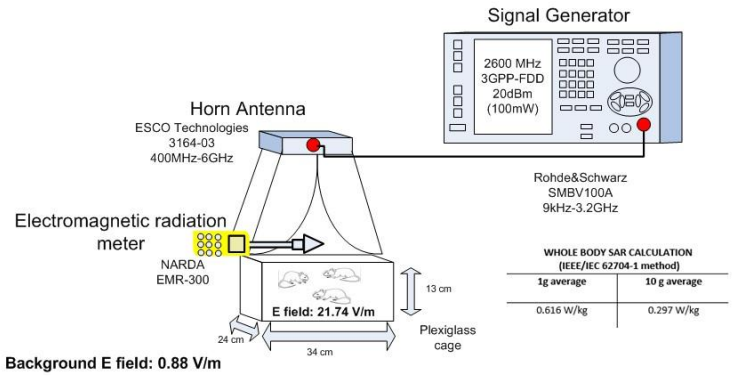
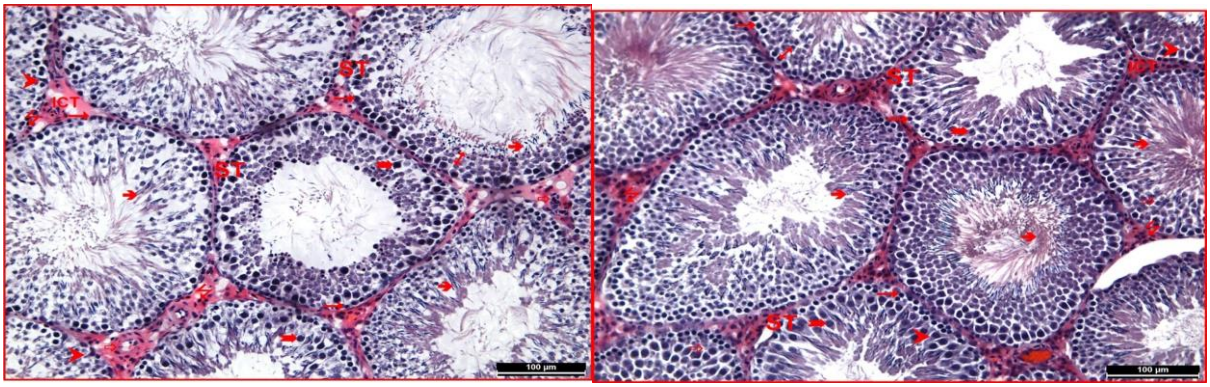
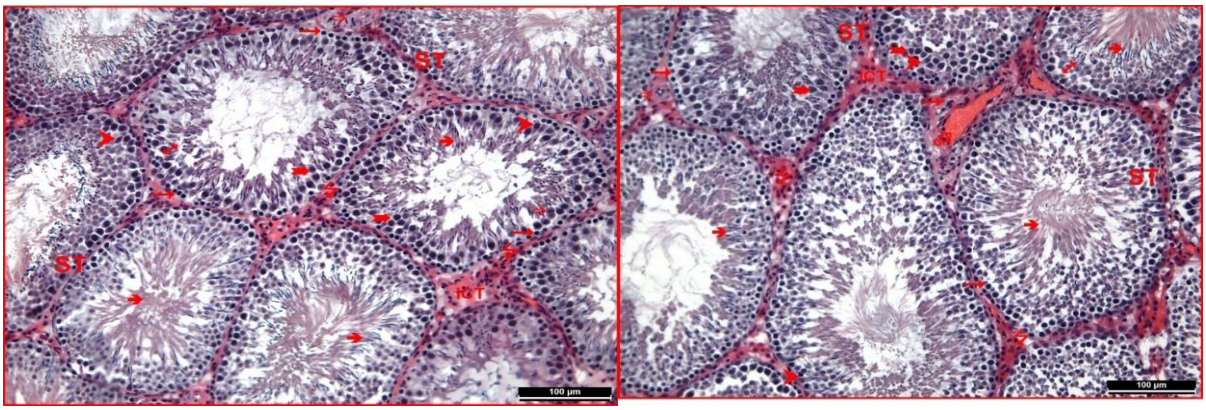


Figure 1. 2600 MHz RFR exposure set up.



(a) (b)

Figure 2. (a) In the testicular tissue of the control group, seminiferous tubule (ST), interstitial connective tissue (ICT), the germinal epithelium(↔), spermatogonium (→), primary spermatocyte (➡), spermium (➔) Sertoli cell (➤), capillaries (⇨) ve Leydig cells (↯) is monitored (Hematoxylin-Eosin x200). **(b)** In the testicular tissue of the Sham group seminiferous tubule (ST), interstitial connective tissue (ICT), the germinal epithelium(↔), spermatogonium(→), primary spermatocyte (➡), spermium(➔), Sertoli cell(➤), capillaries(⇨)and Leydig cells(↯) is monitored (Hematoxylin-Eosin x200).



(a) **(b)**

Figure 3. (a) In the control melatonin group testicular tissue, seminiferous tubule (ST), interstitial connective tissue (ICT), the germinal epithelium (↔), spermatogonium (→), primary

spermatocyte(➡), spermium(➔), Sertoli cell (➤),capillaries(⇨)and Leydig cells(↯) is monitored (Hematoxylin-Eosin x200). **(b)** Sham Melatonin group in testicular tissue, seminiferous tubule (ST), interstitial connective tissue (ICT), the germinal epithelium(↔), spermatogonium(→), primary spermatocyte(➡), spermium (➔), Sertoli cell (➤),capillaries (⇨),and Leydig cells(↯) are observed (Hematoxylin-Eosin x200).

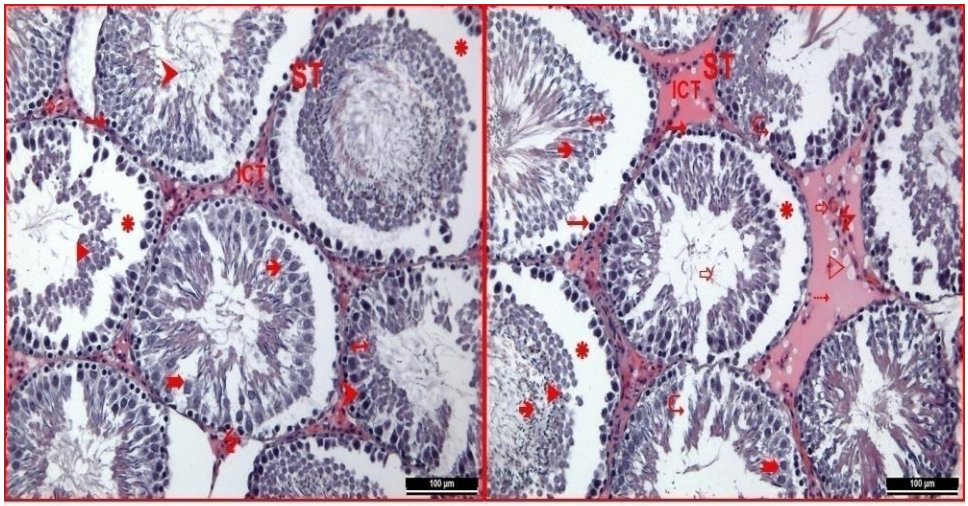


Figure 4. In the RFR group testicular tissue, seminiferous tubule (ST), interstitial connective tissue (ICT), germinal epithelium(↔), spermatogonium(→), primary spermatocyte (➡), spermium(➔), Sertoli cell (➤), capillaries(⇨), dilated capillaries(⇪), Leydig cells(↯), edema(✱), cells of the spermatogenic series emptied towards the lumen(▶), regions where intercellular connections are broken(↪), vacuolization(▷)and hyalinization(⇢)are observed (Hematoxylin-Eosin x200).

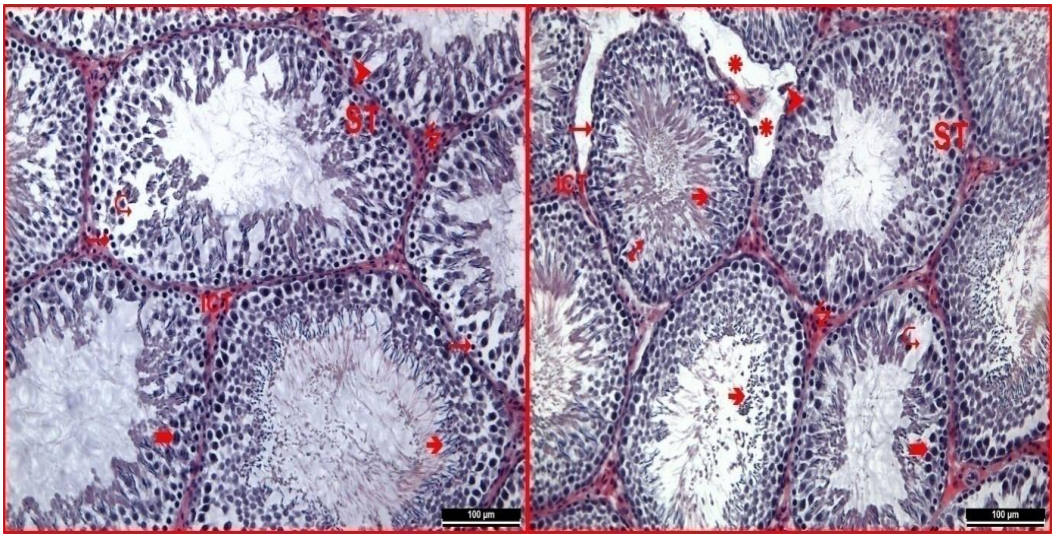
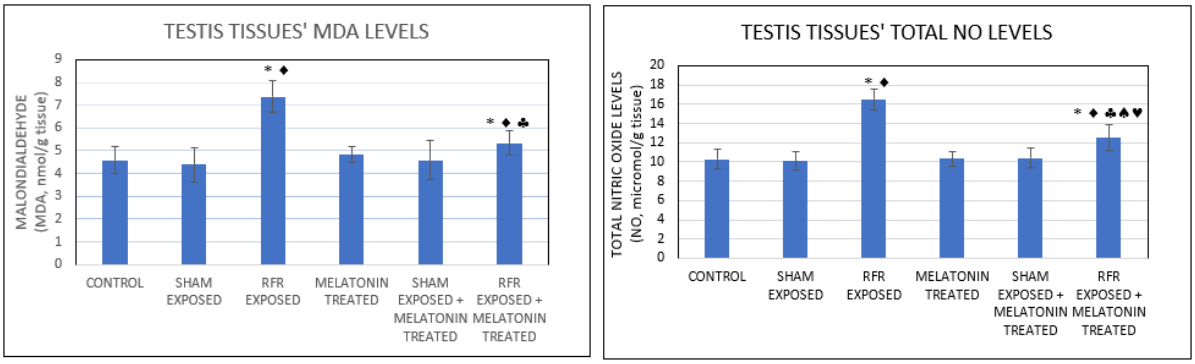
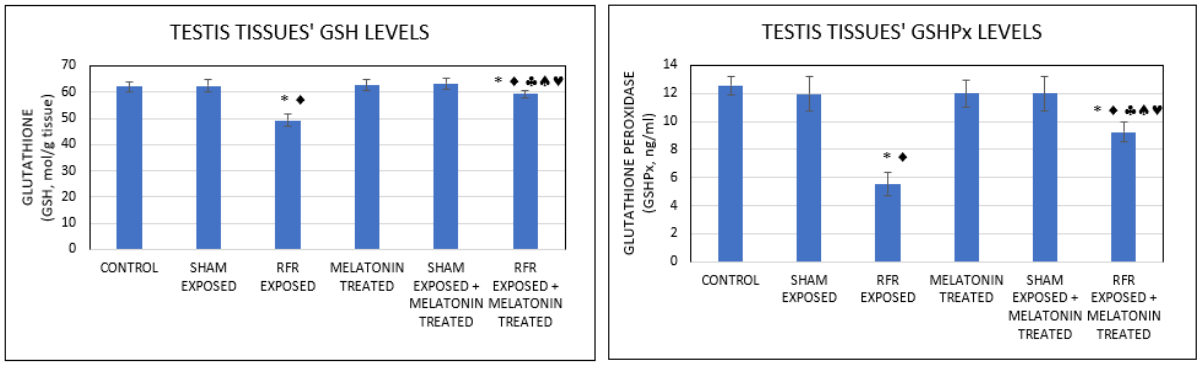


Figure 5. RFR and Melatonin group in testicular tissue, seminiferous tubule (ST), interstitial connective tissue (ICT), germinal epithelium(↔), spermatogonium(→), primary spermatocyte(➡),spermium (➔), Sertoli cell(➤), capillaries(⇨), Leydig cells(↯), edema(✱)and regions where intercellular connections are disrupted (↪)are observed (Hematoxylin-Eosin x200).



(a) (b)



(c) (d)

Figure 7. Testicular tissues' pro-oxidant and antioxidant levels under long-term exposure to 2600 MHz, for 30 mins/day for five days/week and four weeks.

All data are given as mean±SD ($p<0.05$)

Intergroup comparisons

GROUPS vs. CONTROL: ∗

GROUPS vs. SHAM:♦

GROUPS vs. RFR EXPOSED:♣

GROUPS vs. MELATONIN TREATED: ♠

GROUPS vs [SHAM EXPOSED+ MELATONIN TREATED]:♥