

RESEARCH ARTICLE



Expression levels of tam receptors and ligands in the testes of rats exposed to short and middle-term 2100 MHz radiofrequency radiation

Ertan Katirci PhD¹ | Esma Kirimlioglu PhD¹ | Asli O. Oflamaz PhD¹ |
Enis Hidisoglu PhD^{2,3,4} | Alexandra Cernomorcenca MSc¹ |
Piraye Yargıçoğlu PhD² | Sukru Ozen PhD⁵ | Necdet Demir PhD^{1†}

¹Department of Histology and Embryology, Akdeniz University Faculty of Medicine, Antalya, Turkey

²Department of Biophysics, Akdeniz University Faculty of Medicine, Antalya, Turkey

³Department of Biophysics, Faculty of Medicine, Izmir Bakircay University, Izmir, Turkey

⁴Department of Drug Science and Technology, University of Turin, Turin, Italy

⁵Department of Electrical and Electronics Engineering, Akdeniz University Faculty of Engineering, Antalya, Turkey

Correspondence

Esma Kirimlioglu, PhD, Department of Histology and Embryology, Akdeniz University Faculty of Medicine, Antalya, Turkey.
Email: esmakirimlioglu@gmail.com

Present addresses

Ertan Katirci PhD, Department of Histology and Embryology, Ahi Evran University Faculty of Medicine, Antalya, Turkey.

Asli O. Oflamaz PhD, Department of Histology and Embryology, Faculty of Medicine, Bozok University, Yozgat, Turkey.

Enis Hidisoglu PhD, Department of Drug Science and Technology, University of Turin, Turin, Italy.

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Abstract

With advances in technology, the emission of radiofrequency radiation (RFR) into the environment, particularly from mobile devices, has become a growing concern. Tyro 3, Axl, and Mer (TAM) receptors and their ligands are essential for spermatogenesis and testosterone production. RFR has been shown to induce testicular cell apoptosis by causing inflammation and disrupting homeostasis. This study aimed to investigate the role of TAM receptors and ligands in the maintenance of homeostasis and elimination of apoptotic cells in the testes (weeks), short-term sham exposure (sham/1 week), and middle-term sham exposure (sham/10 weeks). Testicular morphology was assessed using hematoxylin-eosin staining, while immunohistochemical staining was performed to assess expression levels of TAM receptors and ligands in the testes of all groups. The results showed that testicular morphology was normal in the control, sham/1 week, and sham/10 weeks groups. However, abnormal processes of spermatogenesis and seminiferous tubule morphology were observed in RFR exposure groups. Cleaved Caspase 3 immunoreactivity showed statistically significant difference in 1 and 10 weeks exposure groups compared to control group. Moreover, there was no significant difference in the immunoreactivity of Tyro 3, Axl, Mer, Gas 6, and Pros 1 between groups. Moreover, Tyro 3 expression in Sertoli cells was statistically significantly increased in RFR exposure groups compared to the control. Taken together, the results suggest that RFR exposure negatively affects TAM signalling, preventing the clearance of apoptotic cells, and this process may lead to infection and

Abbreviations: EGF, epidermal growth factor; Gas 6, growth arrest-specific 6; GLA domain, gamma-carboxy glutamic acid-rich domain; H&E, hematoxylin-eosin; LH, luteinizing hormone; MHz, megahertz; Pros 1, protein S 1; RFR, radiofrequency radiation; RTK, receptor tyrosine kinase; RTK, receptor tyrosine kinase; TAM receptors, Tyro 3 Axl Mer receptors; TLR, toll-like receptor.

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inflammation. As a result, rat testicular morphology and function may be impaired.

KEYWORDS

radiofrequency radiation, rat, TAM receptors and ligands, testis

1 | INTRODUCTION

Radiofrequency radiation (RFR) is a form of energy ubiquitous in our environment and exists in many different forms, including radio waves, microwaves. One of the most important forms of RFR is the radio waves emitted by mobile phones, which are an indispensable part of our daily lives. Mobile phones emit radiofrequencies between 800 and 2200 MHz, which is equivalent to the low frequency of the microwave range (Agarwal et al., 2011). Studies have shown that RFR can have a negative effect on the brain (Hidisoglu et al., 2016; Megha et al., 2012). Moreover, RFR has been reported to increase resting blood pressure, reduce melatonin production, increase DNA chain breaks, chromosomal instability, and increased risk of cancer (Dyche et al., 2012; Phillips et al., 2009; Szmigielski et al., 1998). Recently, many studies have been carried out on the effect of RFR on male infertility (Al-Damegh, 2012; Dasdag et al., 2003; Guo et al., 2019). Studies have shown that RFR causes over-expression of free radicals in the cell and prolongs the clearance process, disrupting the oxidative balance that affects cell physiology (Challis, 2005; La Vignera et al., 2012).

On 31 May 2011, the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) classified radiofrequency electromagnetic fields (RF-EMF) emitted by mobile phones as a 'Possible Human Carcinogen' (Group 2B) (Cancer, 2011). This classification was based on in vitro, in vivo, and epidemiological studies. Some studies suggest that mobile phone use may be risky for both adults and children, while other review groups claim that mobile phone use does not lead to an increased risk for both adults and children (Hardell et al., 2006; International Commission on Non-Ionizing Radiation Protection, 1998; The INTERPHONE Study Group, 2010; Zeni et al., 2012). It is also reported that most of the experiments showing effects have not been independently replicated (International Commission on Non-Ionizing Radiation Protection, 1998). Gurhan and colleagues have shown that weak static magnetic fields (SMFs) can accelerate or inhibit the growth rate of cells and cause changes in ROS levels (Gurhan et al., 2021). A recent study showed that treatment of cancer cells with radiofrequency (RF) fields led to significant changes in mitochondrial activity and that the effects varied depending on the specific frequencies and amplitudes of the RF fields (Gurhan & Barnes, 2023).

Highlights

- Exposure to radiofrequency radiation (RFR) disrupts normal seminiferous tubule morphology.
- RFR exposure in round spermatids may induce apoptosis independent of Tyro 3, Axl, and Mer (TAM) receptors.
- TAM receptors and ligands are expressed in rat testis.

Tyro 3, Axl, and Mer (TAM) receptors and their ligands are unique members of receptor tyrosine kinases (RTK) that was first discovered in the vertebrate nervous system by Lai and Lemke (1991). The extracellular components of TAM receptors consist of two immunoglobulin-like domains and two fibronectin type three domains. The intracellular components of TAM receptors comprise tyrosine kinase domains. The TAM ligands Gas 6 and Pros 1 contain an amino-terminal Gla domain that is γ -carboxylated in the presence of Vitamin K, four EGF-like repeats, and a carboxy-terminal sex hormone-binding globulin-like (SHBG-like) module (Lemke, 2013). When the ligand binds to the receptor (Figure 1), the receptor activates the RTK, and this process launches cytoplasmic signaling pathways that regulate many biological incidents including survival of cells, proliferation, regulation of the innate immune system, and phagocytosis of apoptotic cells (Lemmon & Schlessinger, 2010). Various studies on testes and testicular cells have

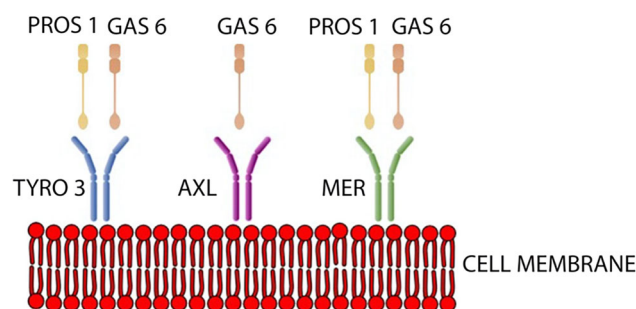


FIGURE 1 Interactions of Tyro 3, Axl, and Mer (TAM) receptors and ligands. While the Gas 6 ligand activates all TAM receptors, Pros 1 activates Tyro 3 and Mer.

reported that TAM receptors and its ligands play a role in suppression of excessive inflammation, apoptosis of apoptotic germ cells, and steroidogenesis (Chen et al., 2009; Lemke & Rothlin, 2008; Rothlin et al., 2007; Xiong et al., 2008). Many studies have revealed that the TAM signal affects the innate immune response and plays an important role in the resolution of inflammation. In study, it has been reported that TAM receptors are expressed in Sertoli cells but not in germ cells, while Axl and Mer are expressed in Leydig cells (Deng et al., 2016). One study has shown that TAM-Gas 6 signaling is fundamental for the removal of apoptotic germ cells by Sertoli cells. Besides the role of Mer in the induction of the phagocytosis pathway, Axl and Tyro 3 contributed to the recognition of apoptotic germ cells by Sertoli cells (Xiong et al., 2008). Since defects in the elimination of apoptotic germ cells can induce endogenous inflammation in the testis and lead to autoimmune orchitis, maintaining the testis in an immune-privileged state is achieved by TAM-mediated removal of apoptotic germ cells by Sertoli cells (Pelletier et al., 2009). TAM receptors prevent inflammation by inhibiting both TLR and TLR-associated cytokine cascades, limiting the duration and intensity of the innate immune response. In a study in TAM^{-/-} mice, it was shown that the phagocytic ability of Sertoli cells was impaired, apoptotic germ cells failed to be eliminated, the inflammatory response induced by TLR in Sertoli cells, proinflammatory cytokines were upregulated, and TAM^{-/-} mice spontaneously developed autoimmune orchitis (Shang et al., 2011; Sun et al., 2010).

RFRs emitted by mobile phones, which are in widespread use today, are known to cause oxidative stress-mediated inflammation. With these details in mind, the rat was exposed to RFR at 2100 MHz for both short-term (1 week) and medium-term (10 weeks) and the effects on testicular tissues were examined. The study examined the expression levels of TAM receptors and ligands in the testicular tissue after both short-term and middle-term RFR exposure.

2 | MATERIALS AND METHODS

2.1 | Animals

Wistar rats were obtained from the Animal Experiment Unit at the University of Akdeniz at the age of 8 weeks and weighed 200–250 g. Wistar rats were traditionally bred. Wistar rats were kept under a 12 h light–dark cycle and were given food and water ad libitum. The study followed the EU Directive 2010/63/EU, which sets forth the guidelines for animal experiments in the European Union, ensuring the protection and welfare of the animals involved. The National Institutes of Health Guide for the Care and Use of Laboratory

Animals (NIH Publications No. 8023, revised 1978) was meticulously followed to ensure the proper care, housing, and experimental procedures for the laboratory animals utilized in this research. The authors hereby confirm that the manuscript reflects the strict adherence to these guidelines and regulations, underscoring the ethical and transparent nature of this study. Moreover, the procedures were reviewed and approved by the Akdeniz University Experimental Animals Local Ethical Committee (AKHADYEK/B.30.2.AKD.0.05.07.00/30).

2.2 | Study design

Wistar rats were randomly divided into five groups of ten rats each: Group 1: Control, Group 2: Short-Term sham (Sham/1 week), Group 3: Short-term 2100 MHz RFR (1 week), Group 4: Middle-Term sham (Sham/10 weeks), Group 5: Middle-term 2100 MHz RFR (10 weeks). Control groups were formed from normal rats that were not exposed to any application. Groups 2 and 4 were kept in plexiglass tubes for 2 h a day without RFR exposure, respectively 1 and 10 weeks. Wistar rats were placed in plexiglass tubes at equal distances around the RFR-producing antenna. Groups 3 and 5 subjects were administered 2100 MHz RFR for 2 h daily, but there were different times between groups, respectively 1 and 10 weeks. Animals were exposed to RFR between 09:00 AM and 11:00 AM. After RFR administered, rats were performed euthanasia by cervical dislocation method by giving high dosage ether and their testes were removed. The removed testicular tissues were immediately placed in bouin fixative.

2.3 | RFR application

A generator (UMTS Simulator 2100 MHz; Everest Company) that produces 2100 MHz RFR, was used. During the exposure, the output power of the generator was fixed at 1.5 W. The generator had a modulation frequency of 217 Hz, a pulse width of 0.577 ms, and a power range of 0–2 W. The rats were confined in plexiglass tubes during the exposure period. These tubes were equipped with ventilation holes to facilitate breathing and to maintain normal body temperature of the rats. In the experiment, detailed measurements and simulations of electromagnetic field exposure of rats were performed. The engineering approach effects of power frequency electromagnetic fields on melatonin and sleep in the rat. Emerg Health Threats Jto the experiment was as follows:

The system was placed on a wooden table, and the antenna of generator was placed at the center of plexiglass carousel to provide equal exposure to the

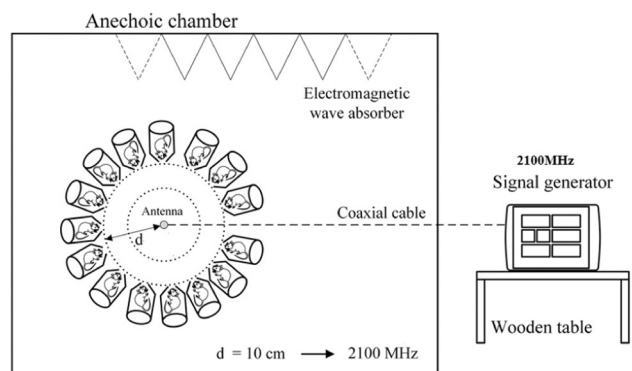


FIGURE 2 Setup of the electromagnetic exposure system.

rats aligned around the antenna (Figure 2). These measurements were performed with a suitable probe EMR300 (Narda). The electric field background level in the shielded room measured between 0.02 and 0.2 V/m. The measured electric field strengths over the rat's head positioned 10 cm away from the antenna were 35.2 V/m for 2.1 GHz RF-EMF when the signal was active (Hidisoglu et al., 2016, 2018). Rats were randomly placed in different positions each day, following a random order to minimise systemic differences that may result from the homogeneity of exposure. This procedure was performed in accordance with similar methodology in other studies (Burkhardt et al., 1997; Fritze et al., 1997; Schönborn et al., 2004).

The RFR exposure was performed in a shielded room to avoid interference with other potential sources of radiofrequencies. The source output powers at the frequency used were chosen considering the power values emitted by mobile phones. Electric field background levels ranged from 0.02 to 0.2 V/m, while magnetic field background levels were measured between 0.01 and 0.03 μ T using a Hioki 3470 Magnetic Field Tester (Hioki E. E. Corp.). Additionally, this examination recognized static magnetic fields in the range of 0.03–0.02 μ T, well below the range expected for normal cells, ensuring that the exposure was within safe limits. Dosimetry simulations were performed using commercial software CST Microwave Studio (3DEXPERIENCE®; Dassault Systemes). This software is based on the finite integration technique (FIT) introduced by Weiland (1977). The simulation model includes a rat model consisting of voxels with a resolution of 1.827 mm $\times$ 1.827 mm $\times$ 2.015 mm. The interaction between the incident electromagnetic wave and the biological tissue is determined by the dielectric properties of the tissues of interest (Abdilla et al., 2013; Gabriel et al., 1996). The electrical properties are input to the software to perform the simulation. At 2100 MHz, the average whole-body SAR value was calculated as 0.16 W/kg. The mean SAR value for the testes was

0.0347 W/kg. In addition, the rectal temperatures of the Wistar rat were measured before and after all experimental sessions and it was observed that RFR exposure did not cause any increase in rectal temperature.

2.4 | Tissue processing

Dissected testis tissues were placed into bouin fixative for 3–4 h at room temperature. Testis tissues were trimmed fixed tissues into appropriate sizes and shapes. After passing through the alcohol series (70%, 80%, 90%, and 100%), the tissues were embedded in paraffin. Paraffin blocks were kept at room temperature until sectioning.

2.5 | Hematoxylin and eosin (H&E) staining

Testicular tissues were sectioned at 5 μ m using a microtome for histologic analysis. The sections were stained with H&E and photographed under a light microscope (Figure 3a). The histologic analysis was performed according to the Johnsen scoring system (Johnsen, 1970) (Table 1).

2.6 | Immunohistochemistry

Serial sections were collected on SuperFrost Plus slides. After the deparaffinization, slides were transferred to 0.01 M citrate buffer (pH = 6) and subsequently heated in a microwave oven for 3 $\times$ 5 min at 700 W for antigen-retrieval. After cooling at room temperature for 20 min, the sections were washed three times with phosphate-buffered saline (PBS). Three percent hydrogen peroxide in methanol was used to block endogenous peroxidase activity for 20 min. After washing steps, nonspecific binding sites were blocked with ultra V block (Lab Vision) for 7 min at room temperature. Following nonspecific binding blocking; sections were incubated overnight at +4°C temperature in a moist chamber using primary antibodies that are Cleaved Caspase 3 (9664, 1/500 dilution; Cell Signaling), Tyro 3 (ab109231, 1/400 dilution; Abcam), Axl (af854, 1/100 dilution; R&D), Mer (af591, 1/400 dilution; R&D), Gas 6 (af986, 1/300 dilution; R&D), and Pros 1 (af4036, 1/100 dilution; R&D). Boost detection reagent (8114; Cell Signaling) were used to sections according to the instructions of the manufacturer. These were washed with PBS and stained with 3,3'-Diaminobenzidine (DAB) (8059; Cell Signaling). The sections were counterstained with Mayer's hemalum (Merck) and mounted with entellan (Merck). Negative controls were performed by substitution of the primary antibody with PBS.

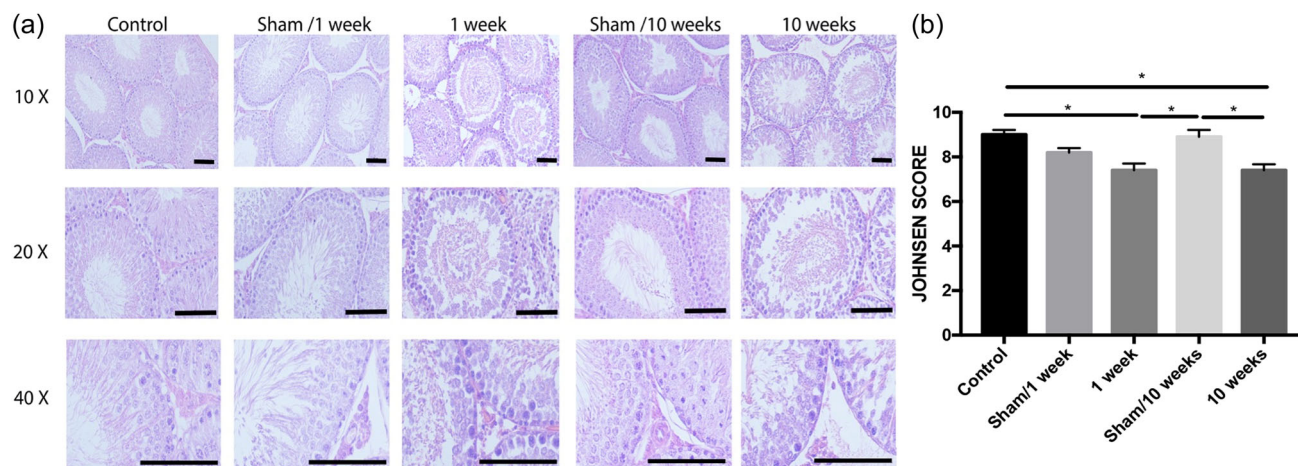


FIGURE 3 (a) Hematoxylin and eosin staining. 1 week radiofrequency radiation (RFR) exposure = 1 week; 10 weeks RFR exposure = 10 weeks. (b) Johnsen score (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$). Scale bar, 50 μ m.

TABLE 1 Qualitative assessments of fertility and the spermatogenesis process according to Johnson's scoring scale.

Scores	Features of seminiferous tubules
1	Germ and Sertoli cells can't be seen. Tubules are atrophic.
2	There are no germ cells, only Sertoli cell can be seen.
3	There are no primary spermatocytes. Just spermatogonia can be seen.
4	Very few primary spermatocytes can be seen.
5	There is no sperm and round spermatid. A large number of primary spermatocytes can be seen.
6	A few round spermatids can be seen.
7	There is no sperm; however, a large number of round spermatids are visible.
8	Sperm count is very low.
9	There is a large number of sperm but round sperm can't be seen and the lumen has no regular contour.
10	Full spermatogenesis, lots of sperm that are regularly rounded in the edge of lumen.

Developmental stages of seminal epithelium in cross-sections of testicles were identified according to the criteria previously described (Leblond & Clermont, 1952). This description of the cycle of the seminal epithelium can serve as a basis for immunohistochemical staining of rat testis. The stages for the seminiferous tubules were summarized as Stages I–V, Stages VI–VIII, and Stages IX–XIV according to the classification used in previous toxicity study (Creasy, 1997).

Immunohistochemical H-score assessments of Tyro 3, Axl, Mer, Gas 6, Pros 1, and Cleaved Caspase 3 between experimental groups were performed in agreement with previous study (Spector & Godlman, 2006). Spermatogonia, spermatocytes, round spermatids, elongated spermatids, sperm cells, Sertoli cells, Leydig cells, and endothelial cells in rat testis were evaluated. All cells were scored according to five different areas per testis section. H-score evaluations were designed

according to the percentage of positively stained cells and differences in signal intensities: for the percentage of positively stained cells, 0 = absent; weak expression = 10%–30%; moderate expression = 40%–60%; and high expression = 70%–100%; and for signal intensities, 0 = absent; 1 = weak signal; 2 = moderate signal, and 3 = high signal. H-score assessments = percentage of positively stained cell count $\times$ signal intensities. The range of possible score was from 0 to 300. Triple-blind studies were conducted.

2.7 | Statistical analysis

The experimental data's means, standard deviations, and medians were calculated using descriptive statistics. Data are presented as mean $\pm$ SEM. The quantitative data were subjected to normality tests by the Shapiro–Wilk normality test. Data were compared

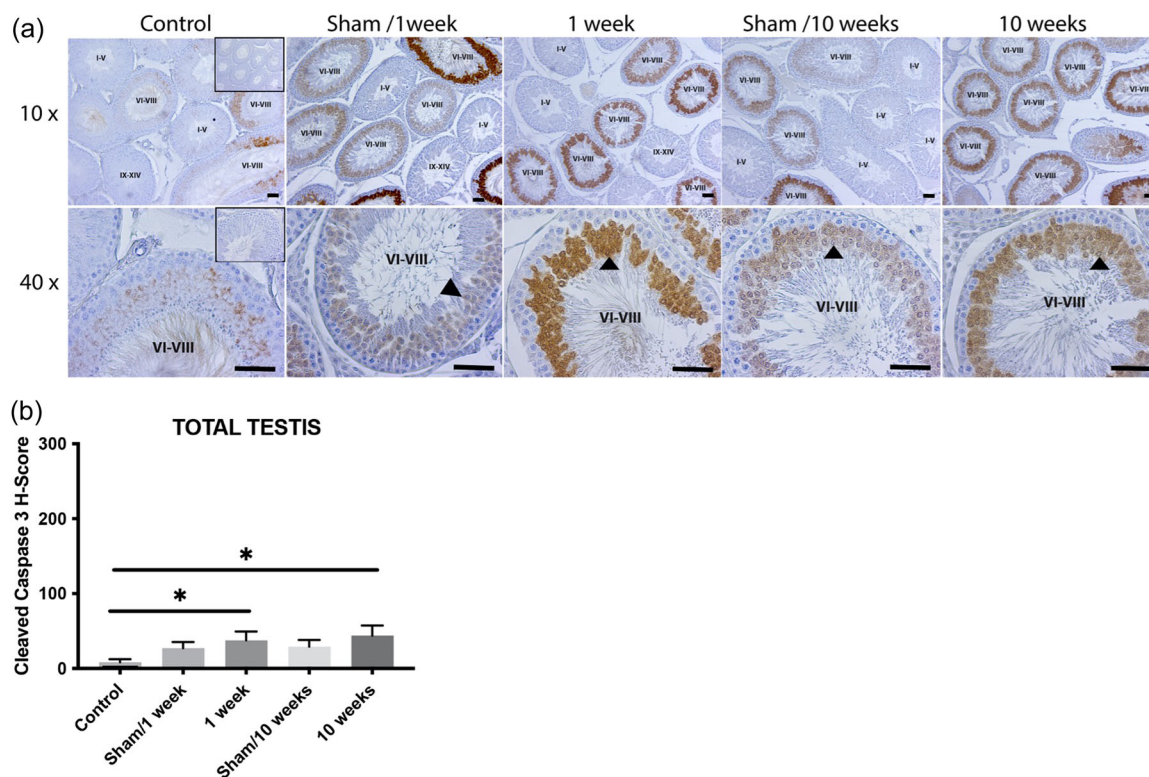


FIGURE 4 (a) Cleaved caspase 3 immunohistochemical staining. Black arrowheads are round spermatids. (b) Total testis Cleaved Caspase 3 H-score. Negative staining is shown in the small squares in "a." The stages of the seminiferous tubules were labeled. Scale bar, 50 µm.

using a one-way analysis of variance followed by a HolmSidak post hoc test for the data passing the normality test, otherwise the Kruskal–Wallis test was followed by Dunn's test (GraphPad Prism 9). $p < 0.05$ between different groups were considered statistically significant.

3 | RESULTS

3.1 | RFR exposure induces changes in normal seminiferous tubule morphology

To evaluate testis morphology, we stained with H&E to slides. When the testicular sections of the control, sham/1 week, and sham/10 weeks groups were examined, the interstitial area containing Leydig cells and the germinal epithelium containing spermatogenic line cells and Sertoli cells had normal morphology. A reduction in seminiferous epithelial thickness, as well as premature release of immature germ cells into the seminiferous tubule lumen were observed in the 1 and 10 weeks groups (Figure 3a,b). RFR exposure was shown to disrupt the normal structure of the seminiferous tubules and interstitial tissue. It was concluded that RFR exposure may cause apoptosis.

3.2 | RFR exposure in round spermatids may induce apoptosis independent of TAM receptors

To reveal the apoptotic cells and apoptosis levels in the rat testis, we examined the testis expression of Cleaved Caspase 3. Cleaved Caspase 3 stained intensely in round spermatids especially 1 and 10 weeks groups (Figure 4a,b and Supporting Information S1: Figure S1). The staining patterns of Cleaved Caspase 3 were detected in the VI–VIII tubule stages of seminiferous tubules. Significant differences were observed between control and RFR groups in terms of Cleaved Caspase-3 immunolocalization patterns. Cleaved Caspase-3 immunostaining is higher in the sham groups compared to the control group. This increase may be attributed to experimental stress induced by transport and placement of rats in the experimental setup. Moreover, there weren't expressions of Tyro 3, Mer, Gas 6, and Pros 1 in round spermatids (Supporting Information S1: Figures S2, S4, S5, S6). Axl expression in round spermatids was similar between groups (Supporting Information S1: Figure S3). These results show that RFR exposure in round spermatids may cause apoptosis independent of TAM receptors.

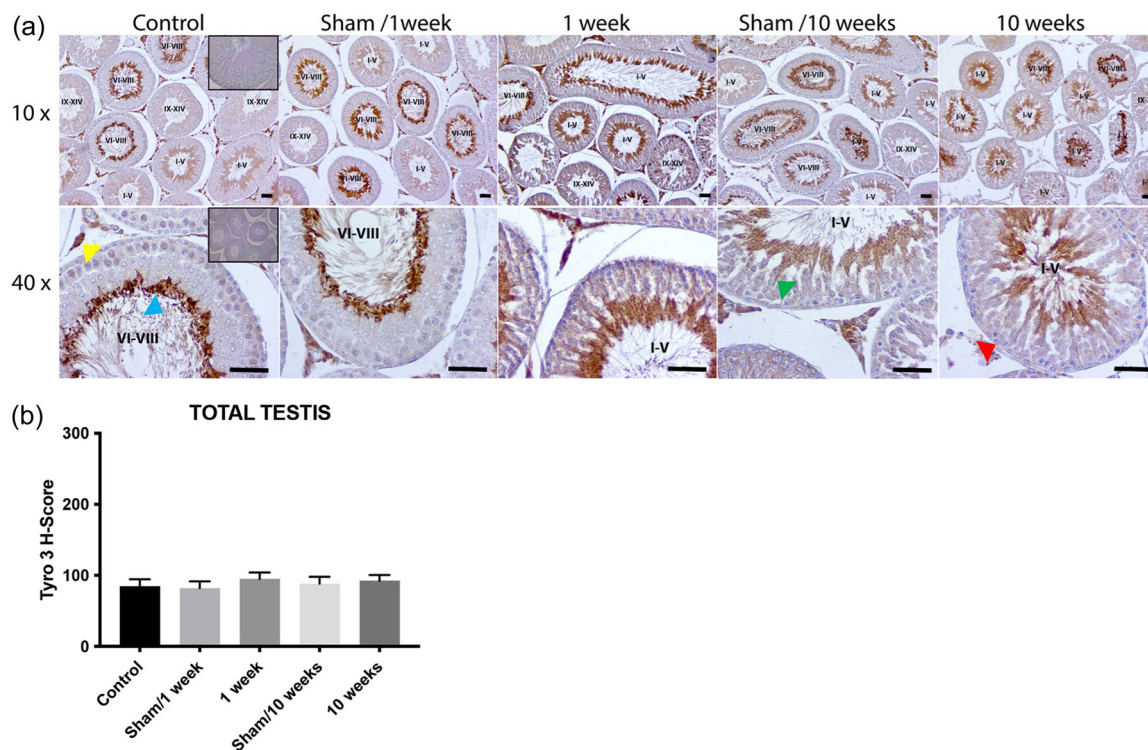


FIGURE 5 (a) Tyro 3 immunohistochemical staining. Red arrowhead shows Sertoli cells, blue arrowhead is sperm cells, green arrowhead is elongated spermatids, and yellow arrowhead is Leydig cells. (b) Total testis Tyro 3 H-score. Negative staining is shown in the small squares in "a." The stages of the seminiferous tubules were labeled. Scale bar, 50 μ m.

3.3 | TAM receptors and ligands are expressed in rat testis

To reveal the roles of TAM receptors in the rat testis, we examined the testis expression of TAM RTK and their ligands. According to our immunohistochemistry results; we showed that the staining pattern of Tyro 3 was intensely observed in the I–V tubule stages in RFR groups compared to other groups. The staining pattern of Tyro 3 was intensely observed in all groups at tubular stages VI–VIII. Tyro 3 was expressed in Sertoli cells and Leydig cells. Also, Tyro 3 staining was more intense in sperm cells compared with cells of other spermatogenic series in the same seminiferous tubule. In spermatogonia, Tyro 3 expression was not seen (Figure 5a and Supporting Information S1: Figure S2). There was no significant difference in the immunoreactivity of Tyro 3 between the experimental groups ($p > 0.05$) (Figure 5b).

Axl immunostainings of Sertoli and Leydig cells were poor. In addition, it was noted that the Axl staining of elongated spermatids in the same seminiferous tubule was quite intense compared to other spermatogenic cells. Axl expression was not seen in spermatogonia (Figure 6a and Supporting Information S1: Figure S3). There was no significant difference in

the immunoreactivity of Axl between the experimental groups ($p > 0.05$) (Figure 6b).

Mer immunostainings of Leydig cells and elongated spermatid were poor. Additionally, the staining intensity of Sertoli cells in the same seminiferous tubule was considerably higher than Leydig cells and elongated spermatids (Figure 7a and Supporting Information S1: Figure S4). There was no significant difference in the immunoreactivity of Mer between the experimental groups ($p > 0.05$) (Figure 7b).

Our results showed that Gas 6, a ligand of TAM receptors, stained extensively in Leydig cells. Also, Gas 6 immunostaining of the elongated spermatid and sperm cells were quite intensified in the same seminiferous tubule (Figure 8a and Supporting Information S1: Figure S5). There was no significant difference in the immunoreactivity of Gas 6 between the experimental groups ($p > 0.05$) (Figure 8b).

Pros 1, which is another ligand of TAM receptors was stained intensely in Leydig cells and also, sperm cells and elongated spermatids were stained more intensively according to the seminiferous tubule phase differences. Pros 1 expression was not seen in spermatogonia (Figure 9a and Supporting Information S1: Figure S6). In addition, there was no significant difference in the immunoreactivity of Pros 1 between the experimental groups ($p > 0.05$) (Figure 9b).

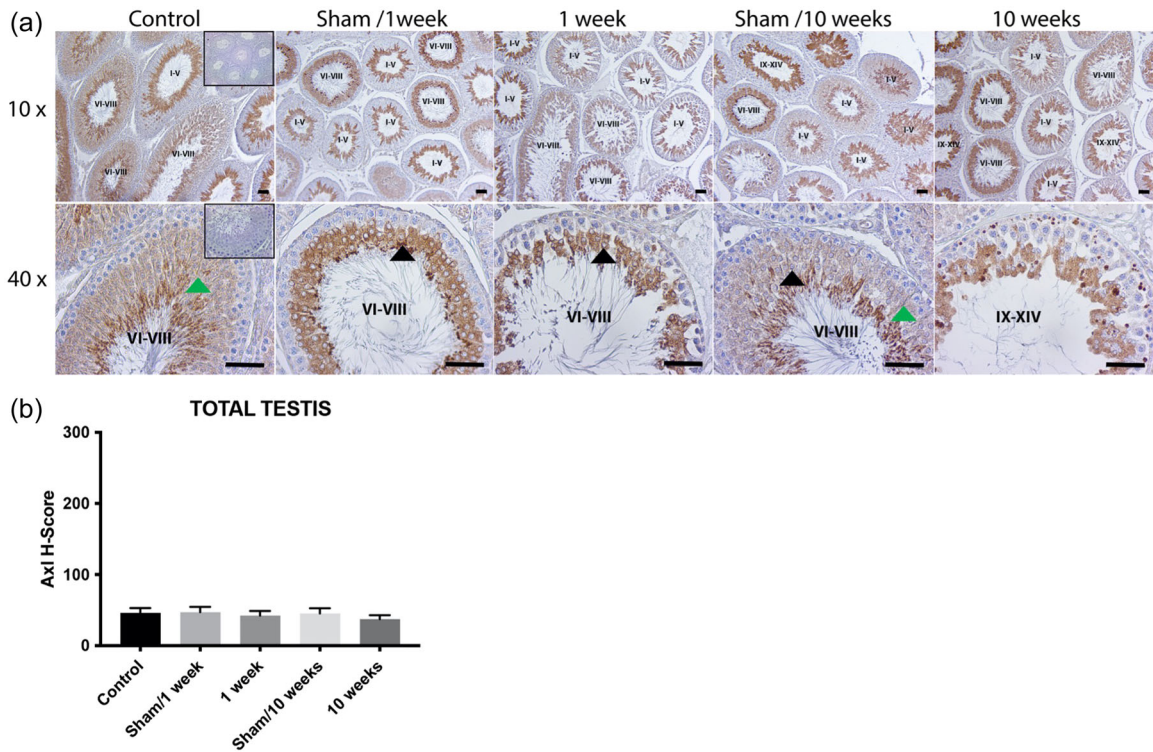


FIGURE 6 (a) Axl immunohistochemical staining. Green arrowheads are elongated spermatids, and black arrowheads are round spermatids. (b) Total testis Axl *H*-score. Negative staining is shown in the small squares in "a." The stages of the seminiferous tubules were labeled. Scale bar, 50 μ m.

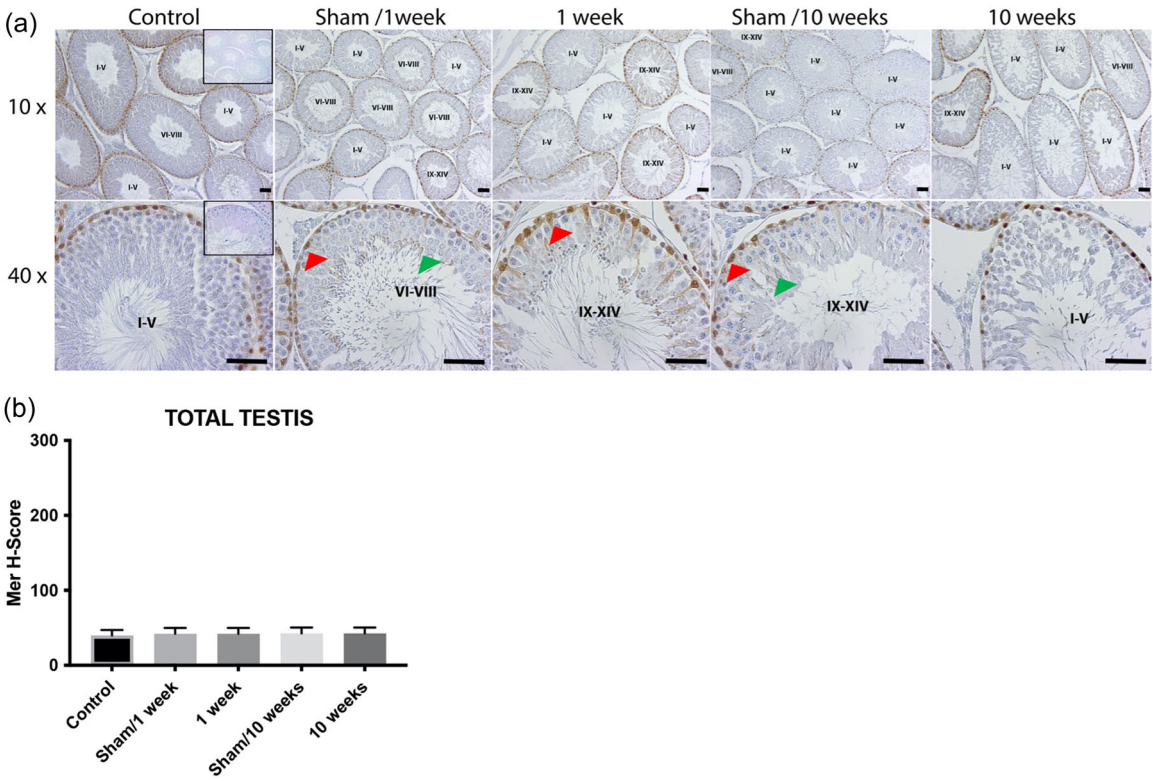


FIGURE 7 (a) Mer immunohistochemical staining. Red arrowheads show Sertoli cells, and green arrowheads are elongated spermatids. (b) Total testis Mer *H*-score. Negative staining is shown in the small squares in "a." The stages of the seminiferous tubules were labeled. Scale bar, 50 μ m.

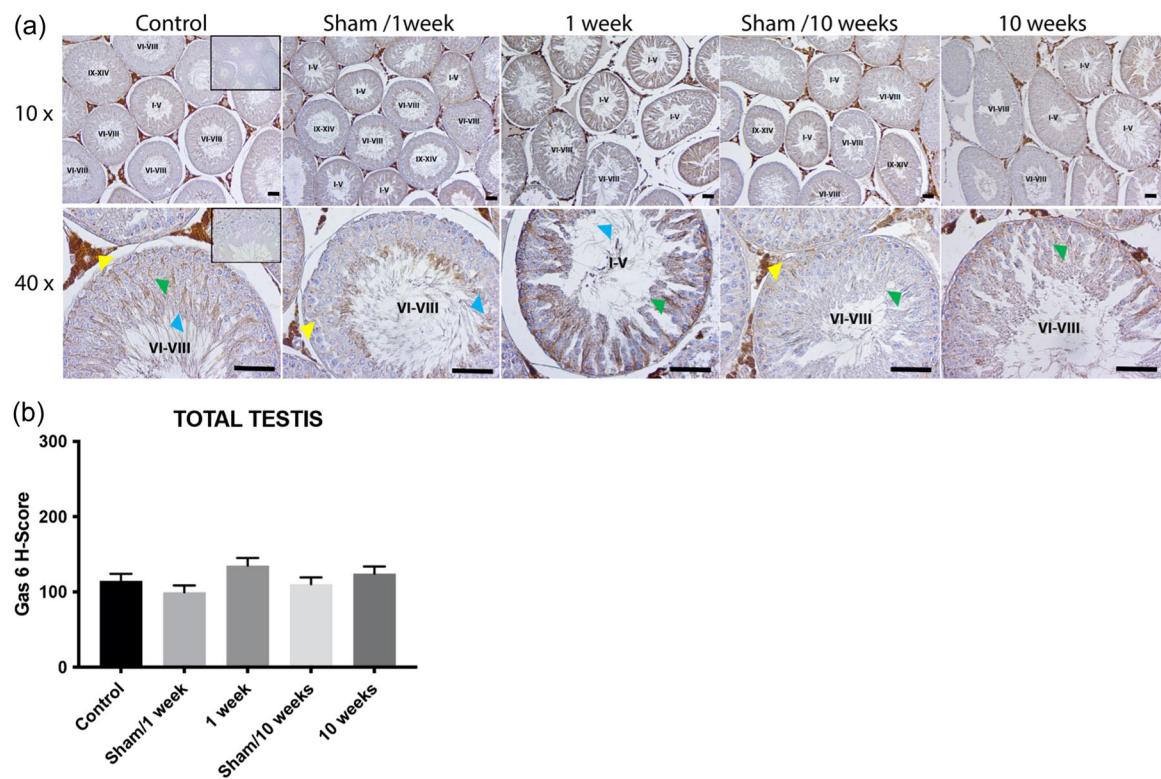


FIGURE 8 (a) Gas 6 immunohistochemical staining. Blue arrowheads are sperm cells, green arrowheads are elongated spermatids, and yellow arrowheads are Leydig cells. (b) Total testis Gas 6 *H*-score. Negative staining is shown in the small squares in “a.” The stages of the seminiferous tubules were labeled. Scale bar, 50 μ m.

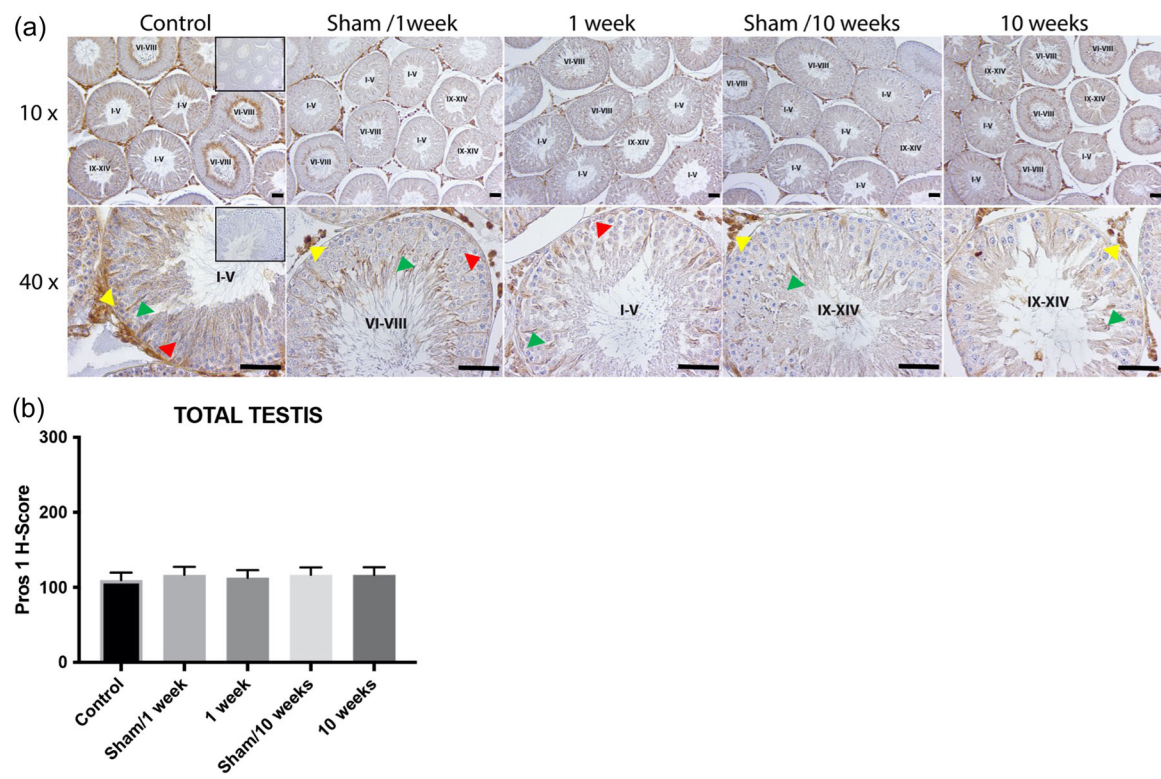


FIGURE 9 (a) Pros 1 immunohistochemical staining. Red arrowheads show Sertoli cells, green arrowheads are elongated spermatids, and yellow arrowheads are Leydig cells. (b) Total testis Pros 1 *H*-score. Negative staining is shown in the small squares in “a.” The stages of the seminiferous tubules were labeled. Scale bar, 50 μ m.

4 | DISCUSSION

RFR emitted by technological devices may have some adverse effects on human health. Recent studies have shown that RFR exposure affects the male reproductive system (Adams et al., 2014; Gorpichenko et al., 2014; Gutschi et al., 2011; Gye & Park, 2012; Qi et al., 2015). The most radiosensitive organ reported is the male testis with the germinal epithelium, including the spermatogonia, being more sensitive to radiation exposure than other cells (Fischbein et al., 1997; Xu et al., 2008). Our study revealed significant impacts of short-term and mid-term RFR exposure on testicular morphology, observing abnormal spermatogenesis processes and seminiferous tubule morphology. RFR exposure resulted in decreased seminiferous epithelial thickness, premature release of immature germ cells, disruption of seminiferous tubule structure, and an increase in apoptotic cells. Our study is compatible with the studies in the literature (Ozguner et al., 2005; Saygin et al., 2011).

Many animal experiments have shown that RFR caused oxidative stress and apoptosis in various tissues (Oktem et al., 2005; Oral et al., 2006; Ozguner et al., 2006). Our study indicated elevated expression of Cleaved Caspase 3 in RFR-exposed groups, suggesting a potential link between the observed testicular morphological disorders and increased apoptosis (Figure 3). Moreover, If these increased apoptotic cells are not eliminated, they cause many diseases, including infection and inflammation (Yang et al., 2015). TAM receptors and ligands were found expressed in Sertoli and Leydig cells, playing a crucial role in maintaining testicular homeostasis by eliminating apoptotic germ cells and supporting immune tolerance.

Apoptotic germ cells and cytoplasmic portions of elongated spermatids are eliminated from Sertoli cells via phagocytosis. These phagocytic processes result in the removal of autoantigen, thereby preventing apoptotic cells. Besides, the usage of degenerated substrates provides an energy source. These phagocytic events depend on TAM receptors and ligands. Qingxian et al. showed that the loss of all TAM receptors impaired phagocytic events of Sertoli cells and thereby caused the accumulation of apoptotic germ cells. As a result, loss of all TAM receptors causes orchitis, an autoimmune disease that causes loss of male fertility (Qingxian et al., 2010). Another study showed that mRNAs of Tyro 3, Mer, and Axl are abundantly expressed in Sertoli cells (Lu et al., 1999). These receptors play a redundant role in taking up apoptotic cells, and male mice lacking all three receptors are infertile (Lemke, 2019; Rothlin et al., 2015; Yanagihashi et al., 2017). These results indicate that apoptotic germ cells exposing phosphatidylserine (PtdSer) by the actions of Xk-related protein 8 (Xkr8) are engulfed by Sertoli cells via the TAM receptor system (Yamashita

et al., 2020). According to our study, RFR exposure affected the expressions of Tyro 3 and Gas 6 in Sertoli cells (Figures 5, 8, and 9). The results suggest that some signaling pathways mediated by Tyro 3, Gas 6, and Pros 1 may be activated by RFR exposure in Sertoli cells. Tyro 3 may be responsible for clearing increasing apoptotic cells after both short-term and long-term RFR exposure. In addition, Tyro 3 may regulate the abnormal process of spermatogenesis after RFR exposure. Sertoli cells are crucial for normal spermatogenesis. Tyro 3 may accomplish these tasks by interacting with Gas 6 for short-term RFR exposure and interacting with Pros 1 for long-term RFR exposure. Significant differences were observed between control and RFR groups with respect to the immune localization pattern of Cleaved Caspase-3. Furthermore, there was no expression of Tyro 3, Mer, Gas 6, and Pros 1 in round spermatids (Supporting Information S1: Figures S2, S4, S5, S6). Axl expression in round spermatids was similar between groups (Supporting Information S1: Figure S3). These results demonstrate that RFR exposure causes TAM receptor-independent apoptosis in round spermatids.

Leydig cells are found in the interstitial area of the testicles. They produce testosterone hormone in the presence of the luteinizing hormone (LH). Leydig cells are one of the targets of RFR-induced damage to the testicle. The studies have shown that RFR exposure disrupts the steroidogenic capacity of Leydig cells (Lin et al., 2019; Qin et al., 2018). Moreover, low testosterone levels led to increased apoptosis of germ cells, disrupting spermatogenesis (Forgacs et al., 1998; Ozguner et al., 2005; Saygin et al., 2011). According to our results, the expressions of Tyro 3, Gas 6, and Pros 1 were the highest in the Leydig cells under both normal and abnormal conditions. This result indicates that TAM receptors and ligands may play the role in Leydig cell functions.

5 | CONCLUSIONS

TAM receptors and ligands may play an important role in the spermatogenesis processes and may affect the functions of Leydig cells and Sertoli cells. In the present study, we have reported that RFR exposure impair the morphology of rat testis. However, the impact on testicular function remains uncertain and requires further investigation. RFR exposure may negatively affect TAM signaling and enhances the accumulation of apoptotic cells.

Humans are exposed to RFR at a variable rate on a daily basis. Cell phones are the main source of RFR exposure, further complicating personal exposure times. In this study, cleaved caspase 3, TAM receptors and ligands were evaluated in rat testicular tissue exposed to 2100 MHz RFR. This study highlights the

importance of radiofrequency exposure in male infertility. It provides a model for RFR reproductive toxicity studies. In order to better study the toxicity potential of RFR in the male reproductive system, it is necessary to study the intrauterine development and possibly live/healthy birth rates of the fetuses produced by mating RFR-exposed male mice with healthy female mice. In addition, long-term animal studies are urgently needed to investigate the potential health effects of frequencies used by upcoming 5G on the male reproductive system.

AUTHOR CONTRIBUTIONS

Ertan Katirci performed hematoxylin-eosin staining and immunohistochemistry, also scored the results of the immunohistochemistry, analyzed them statistically, created all figures, and table, and drafted the manuscript. Aslı O. Oflamaz worked with Esma Kirimlioglu to obtain and prepare biological samples, tissue processing, and their cutting. Alexandra Cernomorcenco worked together with Ertan Katirci on immunohistochemistry scoring. Sukru Ozen, Enis Hidisoglu, and Piraye Yargicoglu contributed to establishing the study design for RFR exposure. Necdet Demir provided BAP (TSA-2015-790) funding to Akdeniz University and assisted with data evaluation. Esma Kirimlioglu formed the hypothesis of the project, obtained and prepared biological samples, and assisted with both drafting the manuscript and data interpretation and evaluation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The authors have respected the ethical principles of research. The procedures were reviewed and approved by the Akdeniz University Experimental Animals Local Ethical Committee (AKHADYEK/B.30.2.AKD.0.05.07.00/30).

ORCID

Ertan Katirci  <http://orcid.org/0000-0002-3781-9162>

Enis Hidisoglu  <https://orcid.org/0000-0002-1729-1209>

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AUTHOR BIOGRAPHIES



Dr. Ertan Katircil is a Research Assistant at the Department of Histology and Embryology in the Faculty of Medicine at Ahi Evran University. He has completed his Master's and doctoral degrees in Histology and Embryology from Akdeniz University. Dr. Katircil's research interests are focused on reproductive immunology, morphology, and medicinal plants. He is a passionate scientist who aims to contribute

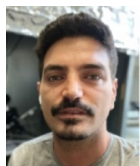
to the knowledge of various aspects of histology, embryology, and their practical applications in medical science. Additionally, he desires to become a successful academic mentor in his field.



Dr. Esma Kirimlioglu completed her Master of Science degree in Child Health and Diseases and Immunology, followed by a PhD in Histology and Embryology at Akdeniz University, Turkey. She is currently a lecturer at the university where she specializes in Histology, and Embryology. Dr. Kirimlioglu's extensive research portfolio includes areas such as stem cell techniques, molecular biology, experimental animal handling, and reproductive health issues like pregnancy and infertility. She has developed expertise in a variety of laboratory techniques, such as immunohistochemical staining, ELISA, qPCR, and both transmission and scanning electron microscopy. Kirimlioglu's research has particularly focused on TAM receptor tyrosine kinases and their role in recurrent pregnancy loss, a topic on which she has conducted significant studies. Additionally, her work extends to the effects of catecholestradiol on endometrial cell survival, emphasizing her broad interest in cellular responses and signaling pathways in reproductive health. Dr. Kirimlioglu is actively involved in research and education. She delivers lectures on Histology and Embryology, with a focus on immunological and molecular mechanisms related to recurrent pregnancy loss. She's involved in national and university research projects and serves as a consultant, which bolsters her academic contributions.

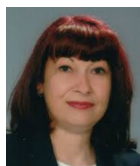


Dr. Asli O. Oflamaz received her master's and PhD degrees from Akdeniz University Faculty of Medicine, Department of Histology and Embryology. He works as an Assistant Professor in Histology and Embryology at Yozgat Bozok University Faculty of Medicine. His research focuses on examining the unfolded protein response in the ovarian tissues of diabetic mice and the usability of these inducers in new treatments through dpp4 inhibitors, developing new treatment mechanisms in neurological diseases such as MS and epilepsy, and investigating the health benefits of natural compounds such as apilarnil and propolis. His research publications cover a wide range of topics, including the potential therapeutic effects of natural substances and cellular mechanisms on health and disease. Oflamaz's research has had a significant impact on medical science, advancing both theoretical and practical knowledge in his field.



Dr. Enis Hidisoglu started his academic career as a research assistant at Akdeniz University, Department of Biophysics, where he gained valuable experience teaching activities at various educational levels. During his tenure at Akdeniz University, he continued and completed his master's degree at Akdeniz University. In 2015, Prof. His master's thesis in Biophysics, under the supervision of Dr. Piraye Judgeglu, on the investigation of the effects of high-frequency electromagnetic fields on brain activity, demonstrated his early commitment to neuroscience. Currently working as an Assistant Professor at the Department of Pharmaceutical Science and Technology at the University of Turin, Enis Hidisoglu's research focuses on in vivo and in vitro electrophysiological studies. His work aims to uncover the complex roles of neuronal ion channels and internal calcium stores in governing excitable and synaptic functions in physiological and pathological conditions. "His research focuses specifically on neurodegenerative disorders such as Alzheimer's disease and aims to contribute to the understanding of these complex conditions and potential treatment pathways."

Alexandra Cernomorcenco received his Master's degree in Histology and Embryology from Akdeniz University in 2023



Prof. Dr. Piraye Yargicoglu is a retired faculty member from Akdeniz University who specializes in Biophysics and Neuroscience. She investigated the effects of certain substances on neurodegenerative diseases and the neurological effects of environmental toxins. Dr. Yargicoglu's work involved using techniques such as visual evoked potentials to understand the brain's response to these factors, making significant contributions to neurohealth.



Prof. Dr. Sukru Ozen graduated from Yildiz Technical University, Faculty of Engineering, in 1992 as an Electrical Engineer. He completed his master's degree at Süleyman Demirel University, Institute of Science, Department of

Electronics and Communication in 2000 and his PhD at Sakarya University, Institute of Science, Department of Electrical-Electronics in May 2003. In 2004, he was appointed as a lecturer at Akdeniz University, Faculty of Engineering, Department of Electrical and Electronics Engineering. He carried out the establishment of studies for the electrical and electronic engineering department, served as the head of the department between 2006 and 2018, and became an associate professor in 2010 and a professor in 2016. His research interests include Electromagnetic Fields and Waves, Antennas and Propagation, Electromagnetic Interference and Compatibility, Bio-Electromagnetics, Numerical Methods, Electrical Safety, and Energy.



Prof. Dr. Necdet Demir was a retired academic from the Faculty of Medicine at Akdeniz University who made significant contributions to medical science, particularly in the fields of reproductive health and cellular histology. He earned his Master's and doctoral degrees in Histology and Embryology from Akdeniz University in 1989 and 1996, respectively. Prof. Demir's research focused on endoplasmic reticulum stress and its implications in pregnancy and developmental biology, examining the effects of environmental and genetic factors. His involvement in supervising doctoral theses and participation in international conferences underscores his impact in his field. It is with great sadness that we inform you that Prof. Demir passed away in 2022.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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